Nastavno-naučnom Veću Stomatološkog fakulteta

Univerziteta u Beogradu

Na šestoj sednici Nastavno-naučnog Veća Stomatološkog fakulteta u Beogradu održanoj 23.09.2013. godine, imenovana je Komisija u sastavu:

Prof. dr Đurica Grga, Stomatološki fakukltet, Beograd

Prof. dr Jelena Sopta, Medicinski fakultet, Beograd

Dr sci Dragana Mitić-Ćulafić, Biološki fakultet, Beograd

za ocenu završene doktorske disertacije pod nazivom **„ ISPITIVANJE BIOLOŠKIH I FIZIČKIH SVOJSTAVA NANOSTRUKTURNIH BIOMATERIJALA NA BAZI AKTIVNIH KALCIJUMSILIKATNIH SISTEMA I HIDROKSIAPATITA".**

Kandidat: Mr sci dr Violeta Petrović

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Imenovana komisija je proučila tekst doktorske disertacije i Nastavno-naučnom Veću Stomatološkog fakulteta u Beogradu podnosi sledeći

IZVEŠTAJ

A. Prikaz sadržaja doktorske disertacije

Doktorska disertacija dr Violete Petrović pod nazivom ISPITIVANJE BIOLOŠKIH I FIZIČKIH SVOJSTAVA NANOSTRUKTURNIH

BIOMATERIJALA NA BAZI AKTIVNIH KALCIJUMSILIKATNIH SISTEMA I HIDROKSIAPATITA je napisana na 159 strana i sadrži 8 tabela, 13 garfikona, 31 sliku i 207 referenci iz savremene, značajne naučne literature. Tekst disertacije uključuje: sažetak na srpskom i engleskom jeziku, uvod, pregled literature, radne hipoteze, ciljeve istraživanja, materijal i metode, rezultate, diskusiju, zaključke i literaturu.

U **Uvodu** je predstavljena naučna problematika ove teze koja se odnosi na osnovne karakteristike aktuelnih biomaterijala koji se preporučuju za primenu u savremenoj endodonskoj terapiji zuba. Pored niza prednosti ukazano je na određena negativna svojstva biomaterijala koja otežavaju njihovu kliničku primenu. Ukratko su predstavljena dva nova nanostrukturna biomaterijala sintetisana inovativnom tehnologijom sa ciljem dobijanja materijala superiornih osobina u odnosu na dostupne komercijalne biomaterijale sličnog hemijskog sastava.

U **Pregledu literature** su navedena dosadašnja istraživanja o hemijskom sastavu, vezivanju, kao i fizičkim i biološkim svojstvima cemenata na bazi kalcijum silikata i kalcijum fosfata. Ukazano je na negativne karakteristike kalcijum silikatnih i kalcijum fosfatnih cemenata i pregledno su opisani dosadašnji pokušaji da se svojstva materijala unaprede. Navedena su takođe aktuelna ispitivanja mogućnosti primene biomaterijala u svojstvu silera za obturaciju kanala korena. Predstavljenja su i dosadašnja saznanja o osnovnim svojstvima nanostrukturnih materijala i njihovom ponašanju unutar živog sistema.

Novosintetisani materijali na bazi kalcijumsilikatnih sistema (CS) i mešavine hidroksiapatita i kalcijumsilikatnih sisitema (HA-CS) predstavljeni su sa aspekta sinteze, sastava i strukture. Ukazano je na tehnologiju koja je primenjena u sintezi materijala, a kojom su dobijeni nanostrukturni materijali izražene aktivnosti čestica koja rezultira brzim vezivanjem materijala, značajno kraćim u odnosu na aktuelne kalcijum silikatne cemente. Imajući u vidu da su materijali dobijeni inovativnom tehnologijom, postavljena je radna hipoteza ove doktorske disertacije da su fizička i biološka svojstva nanostrukturnih materijala na bazi kalcijumsilikatnih sisitema i mešavine hidroksiapatita i kalcijumsilikatnih sistema

komparabilna sa komercijalnim cementima odnosno silerima, sličnog hemijskog sastava od kojih imaju značajno kraće vreme vezivanja.

Ciljevi istraživanja su jasno i precizno definisani: Ispitati rastvorljivost, poroznost, marginalnu mikropropustljivost i jačinu veze sa dentinom novosintetisanih nanostrukturnih biomaterijala CS i HA-CS u odnosu na komercijalni kalcijum silikatni cement; Ispitati rastvorljivost, poroznost i jačinu veze sa dentinom kanala korena silera CS i HA-CS u odnosu na konvencionalni siler na bazi kalcijum hidroksida; Ispitati citotoksičnost materijala CS i HA-CS i silera CS i HA-CS u *in vitro* uslovima u kulturi ćelija; Ispitati biokompatibilnost i bioinduktivnost materijala CS i HA-CS u *in vivo* uslovima na animalnom modelu kunića.

U poglavlju **Materijal i metode,** u delu koji se odnosi na ispitivanje fizičkih svojstava materijala i silera CS i HA-CS, detaljno je opisana priprema uzoraka i svi eksperimentalni postupci. Jasno je opisan postupak ispitivanja rastvorljivosti i poroznosti primenom standardnog testa, merenjem promena u masi materijala posle čuvanja u veštačkom tkivnom fluidu. Opisan je postupak ispitivanja marginalne mikropropustljivosti primenom testa pasivnog prodora boje, nakon aplikacije materijala u eksperimentalno preparisane interradiksne furkacije ekstrahovanih zuba. Detaljno je opisan postupak određivanja jačine veze materijala i silera sa dentinom kanala korena, primenom testa smicanja.

U delu koji se odnosi na ispitivanje bioloških svojstava materijala detaljno je opisana priprema rastvora i izlužaka materijala, kao i sam postupak izvođenja MTT testa, primenjenog za utvrđivanje citotoksičnosti testiranih materijala u *in vitro* uslovima. U delu koji opisuje studije na životinjama jasno je opisana operativna procedura implantacije testiranih materijala u kanale korena kunića. Objašnjena je procedura pripreme uzoraka za histološku analizu tkiva i precizno su definisani kriterijumi za procenu dobijenih rezultata. U statističkoj obradi podataka, navedeni su racionalno upotrebljeni statistički testovi.

Rezultati su prikazani u dva odvojena poglavlja koja se odnose na rezultate ispitivanja fizičkih svojstava odnosno rezultate ispitivanja bioloških svojstava testiranih materijala. U prvom poglavlju su opisani rezultati ispitivanja

rastvorljivosti i poroznosti, marginalne mikropropustljivosti i jačine veze testiranih materijala i silera sa dentinom kanala korena. U drugom poglavlju su opisani rezultati ispitivanja bioloških svojstava materijala. Rezultati citotoksičnosti prikazani su u korelaciji sa koncentracijom i pH vrednostima materijala, kao i sa trajanjem izluživanja materijala odnosno silera. U rezultatima studije na animalnom modelu detaljno su izloženi analizirani histološki parametri u pogledu inflamacije kao i strukture, kontinuiteta i debljine novoformiranog kalcifikovanog tkiva, nakon implantacije testiranih materijala.

U **Diskusiji** su naučnom analizom dobijenih rezultata objašnjene dobijene vrednosti fizičkih svojstava testiranih materijala i silera. Ukazano je na razlike u fizičkim svojstvima ispitivanih materijala CS i HA-CS. Objašnjene su uočene razlike u fizičkim svojstvima CS i HA-CS silera u odnosu na kontrolni konvencionalni siler. Razmatrana je zavisnost bioloških od fizičkih svojstava materijala. Dat je bliži uvid u zavisnost citotoksičnih efekata od koncentracije ali i sastava, odnosno pH vrednosti materijala. Ukazano je na izrazitu biokompatibilnost, kao i na osteoinduktivni potencijal materijala CS i HA-CS. Dobijeni rezultati su diskutovani u odnosu na nalaze dosadašnjih istraživanja iz ove oblasti.

Na osnovu iznetih i diskutovanih rezultata izvedeni su **Zaključci** koji predstavljaju jasne odgovore na postavljene ciljeve.

Korišćena **Literatura** sadrži spisak od 207 referenci iz savremene i značajne naučne literature, koje su citirane u rukopisu disertacije.

B. Kratak opis postignutih rezultata

Dobijeni rezultati ukazuju na postojanje značajnih razlika u rastvorljivosti, poroznosti, marginalnoj mikropropustljivosti između ispitivanih materijala. Najveća poroznost uočena je kod materijala HA-CS (25.87%). Poroznost materijala CS (9.96%) i kontrolnog materijala MTA (8.86%) bila je slična, i značajno manja u odnosu na HA-CS. Najveća rastvorljivost je izmerena kod materijala HA-CS (0.24 ± 0.03 mg/mm^3), dok je rastvorljivost materijala CS (0.15

± 0.06mg/mm^3) bila značajno manja i slična MTA-u (0.10 ± 0.02 mg/mm^3). Poroznost silera CS (43.3%) i silera HA-CS (41.8%) bila je međusobno slična i značajno veća u odnosu na poroznost kontrolnog silera Acroseal. Nisu uočene značajne razlike u pogledu rastvorljivosti silera CS (16.6 mg/mm^3) i silera HA-CS (18.6 mg/mm^3), ali su oba eksperimentalna silera bila značajno rastvorljivija u odnosu na Acroseal. Najmanji marginalni prodor boje izmeren je kod materijala CS (0.44 ± 0.54 mm), a slične vrednosti su izmerene i kod MTA (0.54 ± 0.76 mm). Marginalni prodor boje kod materijala HA-CS (2.00 ± 0.70 mm) bio je značajno veći u odnosu na materijale CS i MTA. Jačina veze sa dentinom kanala korena materijala CS (3.83 ± 2.86 MPa) i materijala HA-CS (3.22 ± 1.35 MPa) nije se značajno razlikovala u odnosu na MTA (5.23 ± 3.22 MPa). Jačina veze sa dentinom kanala korena, silera CS i HA-CS bila je međusobno slična i značajno manja u odnosu na jačinu veze sa dentinom kontrolnog silera Acroseal. Dobijeni rezultati su takođe ukazali na postojanje značajnih razlika u pogledu bioloških svojstava testiranih materijala. Rezultati testa citotoksičnosti ukazali su na zavisnost toksičnosti CS i MTA od koncentracije materijala u rastvoru. Nerazblaženi i razblaženi izlušci materijala CS i HA-CS pokazali su značajno manju toksičnost u odnosu na MTA u svim testiranim vremenskim intervalima. Posle 21-dnevnog izluživanja, izlušci materijala HA-CS bili su manje toksični od izlužaka materijala CS. Razblaženi izlušci svih testiranih materijala bili su značajno manje toksični u odnosu na nerazblažene izluške. Citotoksičnost silera CS i HA-CS bila je slična toksičnosti osnovnih formulacija materijala. U *in vivo* studiji, na animalnom modelu nisu uočene značajne razlike u pogledu inflamatornog odgovora nakon implanatacije materijala CS i HA-CS, kao ni između eksperimentalnih materijala i MTA. U većini uzoraka, inflamatorna reakcija je ocenjena kao blaga do umerena. Novoformirano kalcifikovano tkivo uočeno je u svim uzorcima svih testiranih materijala, ali je bilo deponovano u većoj količini kod materijala CS (>150μm) i HA-CS (>250 μm) u odnosu na MTA (<150 μm). Najbolje organizovano kalcifikovano tkivo uočeno je nakon implanacije materijala HA-CS.

Celokupni rezultati ove doktorske disertacije ukazuju da su fizička svojstva novosintetisanog nanostrukturnog materijala CS komparabilna sa komercijalnim kalcijum silikatnim cementom, a da su po biološkim svojstvima materijali CS i

HA-CS superiorni u odnosu na MTA. Modifikacije osnovnih formulacija materijala u cilju dobijanja materijala osobina kanalnih silera nisu negativno uticale na biološka svojstva, ali su rezultirale slabijim fizičkim svojstvima eksperimentalnih silera u odnosu na osnovne formulacije materijala kao i u odnosu na konvencionalni kontrolni siler.

C. Uporedna analiza doktorske disertacije sa rezultatima iz literature

Rezultati ove doktorske disertacije, proistekli iz eksperimentalnih *in vitro* i *in vivo* studija, pokazali su da hemijski sastav, struktura i način sinteze materijala utiču na njihova fizička i biološka svojstva. Kod svih materijala ispitivanih u ovom istraživanju uočeno je upijanje tečnosti i posledično uvećanje mase materijala, čime je potvrđena hidrofilna priroda svih testiranih materijala. Najizraženije upijanje tečnosti zabeleženo je posle prvih 24h od potapanja uzoraka u veštački tkivni fluid, a dobijeni rezultati su saglasni sa podacima iz literature (Camilleri i sar. 2008 i Formosa i sar. 2013). Sorpcija tečnosti evidentirana u ovom istraživanju ukazuje na inicijalno poroznu strukturu svih ispitivanih materijala. Poroznost kalcijum silikatnih cemenata poput MTA i njemu sličnih materijala u literaturi je detaljno dokumentovana (Coomaraswamy i sar. 2007, Gandolfi i sar. 2012, Formosa i sar. 2013, Gandolfi i sar. 2014) i pripisuje se specifičnoj strukturi cemenata izgrađenoj od pora i kapilara (Fridland i Rosado 2003). Poroznost materijala CS bila je komparabilna sa MTA-om što bi se moglo objasniti vrlo sličnim hemijskim sastavom ovih materijala. Poroznost HA-CS bila je značajno veća u odnosu na materijale CS i MTA, a dobijeni rezultati su u skladu sa nalazima Vitti i sar. (2013) koji su ispitujući različite formulacije eksperimentalnih kalcijum silikatnih cemenata utvrdili da dodavanje hidroksiapatita kalcijum silikatnom cementu značajno povećava njegovu poroznost. Veća poroznost rezultira većom rastvorljivošću materijala (de Souza i sar. 2013) što je potvrđeno i u ovom istraživanju obzirom da je najveća rastvorljivost uočena kod materijala HA-CS. Sličan hemijski sastav, rezultirao je sličnom rastvorljivošću materijala CS i MTA, a dobijene vrednosti su saglasne sa

rezultatima drugih istraživača (Gandolfi i sar. 2013). Kako je glavna solubilna frakcija kalijum silikatnih materijala kalcijum hidroksid, pretpostavka je da će u kliničkim uslovima aplikovani materijali postati izvor kalcijum hidroksida, odnosno da će vlažna sredina obezbediti konstantnu disocijaciju jona (Fridland i Rosado 2003, Gandolfi i sar. 2012). Ipak, iako veća rastvorljivost rezultira većim oslobađanjem jona, balans između oslobađanja jona i rastvorljivosti je neophodan u cilju očuvanja integriteta materijala, što je naročito važno neposredno nakon aplikacije, a pre potpunog vezivanja materijala (Cavenago i sar 2014). S tim u vezi, značajno kraće vezivanje novosintetisanih materijala u odnosu na MTA, moglo bi sprečiti odnosno umanjiti ispiranje i dezintegraciju materijala.

Rezultati ispitivanja silera CS i HA-CS ukazali su na značajno veću rastvorljivost i poroznost eksperimentalnih biokeramičkih silera u odnosu na konvencionalni siler, Acroseal. Minimalna rastvorljivost Acroseal-a dobijena u ovom istraživanju u skladu je sa nalazima iz literature (Poggio i sar. 2010, Grga i sar. 2011, Azadi i sar. 2012). Razlog minimalne rastvorljivosti Acroseal-a bi mogao bi mogla biti epoksi smola u sastavu silera, koja je poznata po svojoj nerastvorljivosti. Eksprerimentalni sileri CS i HA-CS su pokazali izraženiju rastvorljivost i u odnosu na osnovne formulacije materijala CS i HA-CS te je očigledno da su hemijske modifikacije materijala u cilju unapređenja fluidnosti imale negativan uticaj na rastvorljivost. Pregledom literature, takođe se uočava izrazita rastvorljivost biokeramičkih silera, kako eksperimentalnih tako i komercijalnih, kao i to da su nove formulacije silera rastvorljivije od MTA i drugih sličnih kalcijum silikatnih cemenata (Borges i sar. 2011, Faria-Júnior i sar. 2013, Vitti i sar. 2013).

Kod materijala CS i MTA dobijene su značajno manje vrednosti marginalne mikropropustljivosti u odnosu na HA-CS. Osim prodora boje, u većini uzoraka materijala HA-CS, uočena je i dezintegracija materijala. Obzirom na poroznost i rastvorljivost hidroksiapatita koji čini veći deo materijala HA-CS ovakvi rezultati su očekivani i saglasni sa nalazima iz literature (Tsatsas i sar. 2007, Sanghavi i sar. 2013). Kalcijum silikatni cemente odlikuje kvalitetnije rubno zaptivanje u odnosu na većinu aktuelnih dentalnih materijala (Torabinejad i Parirokh, 2010). Ipak, uočeno je da MTA i slični materijali nemaju sposobnost apsolutnog

hermetičnog zaptivanja (Tsatsas i sar. 2005, De Deus i sar. 2006, 2007, Parirokh i sar. 2009) što je u skladu sa rezultatima ovog istraživanja. Značajno je napomenuti da je u ovom istraživanju osim boje detektovane na spoju materijala i zubnih struktura, uočeno prebojavanje samog materijala, o čemu su pisali i Tobón- Arroyave i sar. (2007), do čega je mogla dovesti sama struktura cemenata koja je mogla dovesti do apsorpcije boje unutar samog materijala.

Izmerena jačina veze eksperimentalnih materijala CS i HA-CS bila je slična jačini veze MTA sa dentinom kanala korena. Vrednosti jačine veze MTA dobijene u ovom istraživanju u skladu su sa podacima iz literature (Formosa i sar. 2013, EL- Ma'aita i sar. 2013). Dobijeni rezultati bi se mogli objasniti sličnim načinom vezivanja testiranih materijala za zubne strukture. Biokeramički materijali ostvaruju vezu sa dentinom depozicijom hidroksiapatita na površini materijala usled postepenog rastvaranja materijala i reakcije oslobođenog kalcijuma sa fosfatima iz tkivnih fluida (Sarkar i sar. 2005). Analizom fraktura nakon dislokacije materijala kod CS i HA-CS uočene su frakture kohezivnog i kombinovanog tipa dok su kod MTA dominirale kombinovane frakture. Dobijeni rezultati su u skladu su sa nalazima Formosa i sar. (2013) i EL- Ma'aita i sar. (2013), koji takođe testirajući različite kalcijum silikatne cementte uočavaju kohezivne i kombinovane frakture u većini uzoraka. Nedavno je demonstrirano da mineralni kristali prodiru i u dentinske tubule doprinoseći boljoj adhezivnosti materijala (Han i Okiji 2013), odnosno da veličina čestica ima značajnu ulogu u vezi materijala sa dentinom . Imajući u vidu da su CS i HA-CS testirani u ovom istraživanju, nanostrukturne prirode, čestice materijala su obzirom na svoju veličinu mogle penetrirati u dentinske tubule, što je moglo rezultirati kohezivnim frakturama unutar samog materijala.

Ispitujući jačinu veze testiranih silera sa dentinom kanala korena kroz dva metodološki različita pristupa, uočeno je da bolja hidratacija silera značajno poboljšava vezu sa dentinom kanala korena. Ipak, u oba eksperimenta, najjača veza je izmerena kod Acroseal-a, dok je veza biokeramičkih silera CS i HA-CS sa dentinom bila značajno slabija. Kod biokeramičkih silera dominirale su kombinovane i kohezivne frakture unutar materijala dok su kod Acroseal-a dominirale adhezivne frakture materijala i kohezivne frakture dentina, koje se

uobičajeno povezuju sa hemijskim sastavom, odnosno polimerizacionom kontrakcijom materijala (Ersahan i sar.i Formosa i sar. 2013) Vrednosti jačine veze za Acroseal komparabilne su sa rezultatima iz literature (Rosa i sar. 2012). Vrednosti jačine veze biokeramičkih silera CS i HA-CS slične su rezultatima drugih istraživanja u kojima su biokeramički sileri takođe pokazali značajno slabiju vezu sa dentinom u odnosu na konvencionalne, a naročito epoksi silere (Ersahan i sar. 2010, Shokouhinejad i sar. 2011, Sagsen i sar. 2011).

Ispitivanje citotoksičnosti rastvora materijala ukazalo je da razblaživanje rastvora materijala CS rezultira skoro linearnim porastom preživljavanja ćelija u kulturi. Slični rezultati dobijeni su i za rastvore MTA, što je u skladu sa nalazima Hakki i sar. (2009) koji su ukazali na zavisnost toksičnosti kalcijum silikatnih cemenata od koncentracije materijala u rastvoru. Suprotno tome, razblaživanje rastvora HA-CS nije dovelo do značajnijih promena u preživljavanju ćelija. Razlog tome bi mogle biti različite inicijalne vrednosti pH rastvora testiranih materijala, a koje su posledica prisustva hidroksiapatita u materijalu HA-CS i posledično nižem pH. Na bazi rezultata citotoksičnosti izlužaka vezanih materijala uočava se da su svi testirani materijali redukovali preživljavanje ćelija u određenoj meri, čime je potvrđeno da se joni mogu izlužiti i iz vezanih materijala (Torabinejad i sar. 1995). Izraženija toksičnost MTA u odnosu na CS, mogla bi se objasniti određenim razlikama u sastavu materijala i posledičnim izluživanjem različitih jona u medijum. CS je čist, laboratorijiski sintetisan cement sa dodatkom barijum sulfata, rendgen kontrasnog sredstva koje se rutinski koristi u medicinskoj praksi. Dok se barijum sulfat u kalcijum silikatnim cementima ponašao kao punioc, bizmut oksid prisutan u MTA, učestvuje u procesu hidratacije MTA, ulazeći u sastav kalcijum silikatnog hidrata (Camilleri 2010), odakle se izlužuje zajedno sa kalcijum hidroksidom, što je moglo usloviti veću toksičnost MTA. Veća toksičnost MTA bi se mogla pripisati i činjenici da su u komercijalnim kalcijum silikatnim cementima detektovani teški metali (Bramante i sar. 2008), sa sadržajem arsena preko granice propisane ISO standardom (Schembri i sar. 2010). Manja toksičnost materijala HA-CS u poređenju sa materijalom CS, i naročito MTA-om, mogla bi se pripisati drugačijem hemijskom sastavu materijala, različitoj kinetici oslobađanja jona i nižim vrednostima pH. Visoko preživljavanje ćelija nezavisno od koncentracije rastvora materijala u

skladu je sa rezultatima istraživanja Huang i Chang-a (2009) koji su dobijene rezultate objasnili činjenicom da kod ovih materijala ne dolazi do značajnijih promena u vrednostima pH, usled potrošnje kalcijum hidroksida za formiranje kalcijum CDHA tokom hidratacije materijala. Obzirom da je preživljavanje ćelija nakon izlaganja silerima CS i HA-CS bilo slično ili veće u odnosu na osnovne formulacije materijala CS i HA-CS, može se zaključiti da dodati modifikatori fluidnosti nisu imali negativan uticaj na toksičnost materijala.

Implantacija eksperimentalnih materijala CS i HA-CS u kanale korena kunića rezultirala je blagom do umerenom zapaljenskom reakcijom, slično reakciji nastaloj nakon aplikacije MTA, što upućuje na dobru toleranciju aplikovanih materijala od strane tkiva domaćina. Dobijeni rezultati su u skladu sa brojnim nalazima iz literature koji upućuju na dobru biokompatibilnost kalcijum silikatnih odnosno kalcijum fosfatnih cemenata (Accorinte i sar. 2008, Zarrabi i sar. 2010, da Silva i sar. 2011). Novostvoreno kalcifikovano tkivo je uočeno nakon aplikacije svih testiranih materijala, čime je potvrđeno da pored biokompatibilnosti testirane materijale odlikuje bioaktivnost i bioinduktivnost. Kontinuirano oslobađanje Ca^{2+} jona, što je svojstveno testiranim materijalima, smatra se ključnim za indukciju formiranja čvrstog tkiva (Gandolfi i sar. 2012). Materijali koji oslobađaju kalcijum indukuju proliferaciju periodontalnih fibroblasta (Bonson i sar. 2004), proliferaciju i diferencijaciju ćelija pulpe (Takita i sar. 2006), osteoblasta i ćelija nalik osteoblastima (Gandolfi i sar. 2008), kao i cementoblasta (Hakki i sar. 2013). Procesi mineralizacije tkiva povezuju se takođe i sa oslobađanjem hidroksilnih jona iz materijala i posledično visokim pH (Sangwan i sar. 2013). Visoke (alkalne) vrednosti pH izmerene su u ovom istraživanju kod svih testiranih materijala. Takođe u sastavu svih materijala nalaze se i Si joni, kojima se takođe pripisuje značajna uloga u mineralizaciji tkiva (Gough i sar. 2004, Pietak i sar. 2007). Aplikacija oba eksperimentalna materijala rezultirala je intezivnijim formiranjem kalcifikovanog tkiva u odnosu na MTA, što može biti posledica samog načina sinteze eksperimentalnih materijala. Materijali su dobijeni sol-gel metodom a prema podacima iz literature takvi materijali su bioaktivniji u odnosu na materijale istog sastava koji su dobijeni drugim metodama (Li i de Groot, 1994). Najbolje organizovano novostvoreno kalcifikovano tkivo uočeno je nakon aplikacije materijala HA-CS. Kako veći deo

ovog materijala čini hidroksiapatit, do ovakvih rezultata su mogli dovesti i fosfatni joni kojih nema u CS-u i MTA-u. Dobijeni rezulati su u skladu sa nalazima Zarrabi i sar. (2010) i Zhang i. sar (2013) koji su takođe uočili izraženiju mineralizaciju tkiva nakon aplikacije materijala na bazi kalcijum silikata i kalcijum fosfata u odnosu na čiste kalcijum silikatne cemente.

D. Objavljeni radovi

Petrović V, Opačić-Galić V, Jokanović V, Jovanović M, Basta-Jovanović G, Živković S. Biocompatibility of a new nanomaterial based on calcium silicate implanted in subcutaneous connective tissue of rats. Acta Veterinaria 2012;62:697-708

Opačić-Galić V, **Petrović V**, Živković S, Jokanović V, Nikolić B, Knežević-Vučković J, Mitić-Ćulafić D. New nanostructural biomaterials based on active silicate systems and hydroxyapatite: characterization and genotoxicity in human peripheral blood lymphocytes. Int Endod J 2013;46:506-16

E. Zaključak (obrazloženje naučnog doprinosa)

Doktorska disertacija „Ispitivanje bioloških i fizičkih svojstava nanostrukturnih biomaterijala na bazi aktivnih kalcijumsilikatnih sistema i hidroksiapatita" dr Violete Petrović predstavlja značajan i originalan naučni doprinos u istraživanjima novih nanostrukturnih biomaterijala i rastvetljavanju problematike vezane za uticaj hemijskog sastava, strukture i načina sinteze na fizička i biološka svojstva biomaterijala. U radu su primenjene savremene eksperimentalne metode za ispitivanje navedenih svojstava. Izabranim naučnim metodom dat je bliži uvid u fizička i biološka svojstva novih nanostrukturnih biomaterijala i ukazano je na njihove prednosti u odnosu na aktuelne biomaterijale.

Ova doktorska disertacija je urađena prema svim principima naučnog i eksperimentalnog istraživanja, sa precizno definisanim ciljevima, originalnim naučnim pristupom, savremenom metodologijom rada, adekvatno prikazanim i diskutovanim rezultatima i jasno uobličenim zaključcima.

Na osnovu svega navedenog, i imajući u vidu dosadašnji naučni rad kandidata, Komisija predlaže Nastavno-naučnom Veću Stomatološkog fakulteta Univerziteta u Beogradu da prihvati pozitivan izveštaj Komisije za ocenu doktorske disertacije dr Violete Petrović i odobri njenu javnu odbranu.

U Beogradu, 30.09.2014.

Članovi Komisije:

_____

Prof. dr Đurica Grga

_____

Prof. dr Jelena Sopta

_____

Dr sci Dragana Mitić-Ćulafić

Na osnovu člana 49. Statuta Stomatološkog fakulteta Univerziteta u Beogradu, Nastavno naučno veće Stomatološkog fakulteta, na II redovnoj sednici u školskoj 2014/15. godini, održanoj 16.12.2014. godine, donelo je sledeću

O D L U K U

Usvaja se pozitivan izveštaj Komisije za ocenu završene doktorske disertacije **dr Violete Petrović**, pod nazivom „ISPITIVANJE BIOLOŠKIH I FIZIČKIH SVOJSTAVA NANOSTRUKTURNIH BIOMATERIJALA NA BAZI AKTIVNIH KALCIJUMSILIKATNIH SISTEMA I HIDROKSIAPATITA“.

Imenovani/a će javno braniti doktorsku disertaciju, ukoliko dobije pozitivno mišljenje Veća naučnih oblasti medicinskih nauka Univerziteta u Beogradu, pred komisijom u sastavu:

1. prof. dr Đurica Grga
2. prof. dr Jelena Sopta, Medicinski fakultet u Beogradu
3. dr sc Dragana Mitić Ćulafić, Biološki fakultet u Beogradu

O b r a z l o ž e nj e

Veće naučnih oblasti medicinskih nauka, na sednici od 03.12.2013. godine, dalo je saglasnost na predlog teme doktorske disertacije dr Violete Petrović, pod nazivom „ISPITIVANJE BIOLOŠKIH I FIZIČKIH SVOJSTAVA NANOSTRUKTURNIH BIOMATERIJALA NA BAZI AKTIVNIH KALCIJUMSILIKATNIH SISTEMA I HIDROKSIAPATITA“.

Imenovani/a je objavio/la dva rada: u časopisu „Acta Veterinaria“, objavio/la je rad pod nazivom: „Biocompatibility of a New Nanomaterial Based on Calcium Silicate Implanted in Subcutaneous Connective Tissue of Rats“ (2012) i u časopisu „International Endodontic Journal“, objavio/la je rad pod nazivom: „New Nanostructural Biomaterials Based on Active Silicate Systems and Hydroxyapatite: Characterization and Geotoxicity in Human Peripheral Blood Lymphocytes“ (2013).

Imajući u vidu napred navedeno, Nastavno naučno veće Stomatološkog fakulteta Univerziteta u Beogradu, rešilo je kao u dispozitivu.

Odluku dostaviti: Imenovanom/oj, Univerzitetu u Beogradu, Odseku za nastavu, Veću, Komisiji (3) i Pisarnici.

Referent kadrovskog odseka
Violeta Rastović

Dekan
Stomatološkog fakulteta

Prof. dr Miroslav Vukadinović

International Endodontic Journal

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New nanostructural biomaterials based on active silicate systems and hydroxyapatite: characterization and genotoxicity in human peripheral blood lymphocytes

V. Opačić-Galić[1], V. Petrović[1], S. Živković[1], V. Jokanović[2], B. Nikolić[3], J. Knežević-Vukčević[3] & D. Mitić-Ćulafić[3]

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Abstract

Opačić-Galić V, Petrović V, Živković S, Jokanović V, Nikolić B, Knežević-Vukčević J, Mitić-Ćulafić D. New nanostructural biomaterials based on active silicate systems and hydroxyapatite: characterization and genotoxicity in human peripheral blood lymphocytes. *International Endodontic Journal*, **46**, 506–516, 2013.

Aim To characterize and investigate the genotoxic effect of a new endodontic cement based on dicalcium- and tricalcium-silicate (CS) with hydroxyapatite (HA) on human lymphocytes.

Methodology Hydrothermal treatment was applied for synthesis of CS and HA. The final mixture HA-CS, with potential to be used in endodontic practice, is composed of CS (34%) and HA (66%). Human lymphocytes were incubated with HA, HA-CS and CS for 1 h, at 37 °C and 5% CO_2. Cell viability was determined using the trypan blue exclusion assay. To evaluate the level of DNA damage comet assay (single cell gel electrophoresis) was performed. For the statistical analysis ANOVA and Duncan's Post Hoc Test were used.

Results The SEM analysis indicated that CS consisted mostly of agglomerates of several micrometers in size, built up from smaller particles, with dimensions between 117 and 477 nm. This is promising because dimensions of agglomerates are not comparable with channels inside the cell membranes, whereas their nano-elements provide evident activity, important for faster setting of these mixtures compared to MTA. Values of DNA damage obtained in the comet assay indicated low genotoxic risk of the new endodontic materials.

Conclusion The significantly improved setting characteristics and low genotoxic risk of the new material support further research.

Keywords: comet assay, dicalcium- and tricalcium-silicate, genotoxicity, hydroxyapatite, lymphocytes.

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Introduction

Ceramics for bone replacement is one of the most active areas of biomaterial research. Biomaterials are biocompatible and bioactive and are intended to interact with biological system to restore function of defective tissue and/or organs in the human body. A material can be considered bioactive if it evokes a positive response from the host; it must be able to elicit a biological response at the interface and induce the formation of a bond between tissue and the material (Theiszová *et al.* 2005, Gandolfi *et al.* 2010). Bioactivity is closely correlated with the ability to exchange information within a biological system; this means that a bioactive material reacts chemically

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with body fluids in a manner compatible with the repair processes of the tissue (Wahl & Czernuszka 2006, Bohner & Lemaitre 2009, Gandolfi *et al.* 2010). One of the main characteristics of bioactive materials is their ability to form a layer similar to apatite on its surface, when they are in contact with tissue fluids. In addition to biocompatibility, the materials for endodontic use must be bio-inducers that protect the health of the pulp or periodontal tissue and stimulate regeneration of tissue mineralization (Bose *et al.* 2002, Braz *et al.* 2006).

For many years, Mineral Trioxide Aggregate (MTA) has been available in two forms, grey (GMTA) and white (WMTA). MTA has been identified as biocompatible, hard tissue inductive and conductive and therefore has been accepted as a gold standard in endodontic practice (Moretton *et al.* 2000, Parirokh & Torabinejad 2010a). Despite its biocompatibility and satisfactory biological properties, difficult clinical manipulation due to its long setting time, as well as its high price, has prompted researchers to design alternative materials. New nanostructure materials based on calcium silicates and hydroxyapatite (HA) with advanced properties of setting time and decreased price have been developed recently (Asgary *et al.* 2008, Takenaka *et al.* 2008, Parirokh & Torabinejad 2010b). The choice of such chemical composition and structural design was made with assumption that their chemical and mineralogical properties should enable biological responses similar to MTA and other biological cements, as well as adequate antimicrobial response in an aqueous environment due to their high pH (11 - 13).

Recently, a new nanodesigned biomaterial has been developed by combination of two techniques/hydrothermal sol-gel and self-propagating combustion waves, providing high activity and short setting time (Jokanović *et al.* 2006, 2008). This material, based on calcium silicates (tricalcium and dicalcium silicate) and HA, are highly active due to their nanostructure, and provide more rapid binding and consequently shorter setting time in comparison to MTA. In addition, these materials have better rheological properties and easy handling (Jokanović *et al.* 2008).

Only a few studies have evaluated the genotoxicity of calcium silicate materials, including MTA (Ribeiro *et al.* 2005a,b, 2006, Camargo *et al.* 2009, Ding *et al.* 2010). Besides, none of these reports included analysis of a range of concentrations and/or several donors. Additionally, the materials developed by Jokanović *et al.* (2006, 2008) have been synthesized for the first time.

The aim of this study was to characterize and investigate the genotoxic effect of a new endodontic cement (HA-CS), based on dicalcium- and tricalcium-silicate (CS) with HA added. To evaluate the magnitude of DNA damage, comet assay (the single cell gel electrophoresis) was performed in human peripheral blood lymphocytes.

Materials and methods

Synthesis and characterization methods of obtained inorganic phases

For synthesis of calcium silicates, $CaCl_2 \times 5H_2O$ (Merck, Germany) and silica sol obtained by hydrothermal treatment were used. The stoichiometric quantities of $CaCl_2 \times 5H_2O$ (42.41 g) and silica sol (15 g of 30% sol solution), corresponding to the ratio tricalcium/dicalcium ($C_3S/\beta\text{-}C_2S$) = 2 : 1, were used to obtain silicate active phase. Al ($C_2H_3O_2$) was added to the mixture to provide the production of small amount (3.01%) of active C_3A phase. Ammonium nitrate (NH_4NO_3), as an oxidation agent, and citric acid ($C_6H_8O_7 \times H_2O$), as a fuel, were added to the mixture to cause a combustion reaction.

The mixture of silica sol and $CaCl_2 \times 5H_2O$ was dried at 80 °C to obtain a gel, and then heated to 150 °C to remove free and weakly bounded water amongst the silica particles. In the next stage, the increase of temperature up to 180 °C led to the ignition of the gel. Afterwards, the gel turned into a foam and a strong self-propagating combustion reaction produced large volume of gases. Black ashes were obtained after auto-ignition. Rapid release of large volumes of the gaseous products during the combustion dissipated the heat and limited the temperature rise. This consequently reduced the possibility of premature local partial sintering amongst the primary particles, which is important for maintenance of the final powder activity. After high-temperature self-propagating thermal treatment, the samples were quickly cooled using copper plates, providing low crystallinity and high reactivity of obtained $\beta\text{-}C_2S$ and C_3S phases. The resulting black powder contained some carbon residues and was further calcined in air at 650 °C for 4 h to obtain the desired products with small crystallite sizes. After thermal treatment, this powder was additionally milled and final silicate phases were obtained. The HA, produced by a hydrothermal method of synthesis (Jokanović *et al.* 2006, 2008), was added to produce

the final mixture (HA-CS), composed of CS (34%) and HA (66%).

Dicalcium- and tricalcium-silicate, with or without addition of HA, were mixed with water (water-to-powder ratio 1 : 2) and compacted using a stainless steel plunger into cylindrical polyethylene containers with diameter of 10 mm and height of 15 mm. Both cements were allowed to set up to 28 days at 37°. Each sample was analysed after 1, 3, 7 and 28 days of setting time. The beginning and the end of the setting time was determined using Vicat needle in agreement with ASTM C191 standard (Hsieh *et al.* 2009). The synthesized products were identified by X-ray diffractometry, XRD (Philips PW 1050, Almelo, the Netherlands), using Ni–filtered Cu-K$\alpha_{1.2}$ radiation. The patterns were registered in the 2θ range 9–67° with a scanning step size of 0.02°. The morphology and the agglomerate size distribution of the milled powders were studied using scanning electron microscopy, SEM (JEOL, JSM-5300, Tokyo, Japan) under a vacuum pressure of 1.33×10^{-3} Pa and voltage of 20 kV. Energy dispersive analysis (EDS) measurements were performed to detect chemical homogeneity of the phases and ratio of Ca and Si in various areas of silicate active phase. In addition, the compressive strength of the materials was analysed using Instron machine model 4204 loading frame (Instrom corp. Norwood, MA, USA). The samples were prepared by polishing with 600 and 1200 grit sandpaper on an automated rotary grinder under gently running tap water and dried with adsorbent paper.

Genotoxic assays

The genotoxic potential of HA was evaluated in human lymphocytes from three donors, whilst the genotoxic potential of CS and HA-CS was evaluated in human peripheral blood lymphocytes from five donors.

Blood sampling

A total number of five young, healthy donors (male, avg. 22 years of age) were used. Smokers, those with history of cancer, previous exposure to radio or chemotherapy and diagnostic X-rays in last 6 months, high alcohol consumption or use of therapeutic drugs were excluded. All blood donors were acquainted with the study and signed the informed consent. The research was reviewed and approved by the ethical board of School of Dental medicine, University of Belgrade.

The lymphocytes were isolated on Ficoll Plaque (Amersham, Uppsala, Sweden) gradients according to the technique described by Fenech (2000) with slight modifications.

Cell viability

Cell viability was measured using the trypan blue exclusion test. A 40 μL of 0.4% trypan blue was mixed with 10 μL of cell suspension for 5 min and spread onto microscopic slide. Non-viable cells appeared blue whilst viable cells were colourless (Freshney 1987).

Cell treatment

The cells were treated with different concentrations of the test materials dissolved in sterile distilled water for 1 h, at 37 °C in 5% CO_2. After treatment, the cells were washed by centrifugation for 5 min at 800 rpm, and re-suspended in PBS buffer**.** Non-treated cells served as negative control, whilst cells exposed to *t*-butyl hydro peroxide (*t*-BOOH, CAS No. 75-91-2, Sigma-Aldrich) were positive control. Cells were exposed to 0.5 mmol L^{-1} *t*-BOOH for 20 min at 4 °C.

Comet assay

The assay was performed as described by Singh *et al.* (1988) and Tice *et al.* (2000). Cell suspension (30 μL) was added to 70 μL of 1% LMP (low melting point) agarose and placed on slides covered with a layer of 1% NMP (normal melting point) agarose. The slides were lysed with 2.5 mol L^{-1} NaOH, 0.1 mol L^{-1} EDTA, 0.01 mol L^{-1} Tris and TritonX-100, pH 10, for 1 h at 4 °C, transferred into electrophoresis solution (300 mmol L^{-1} NaOH, 1 mmol L^{-1} EDTA, pH 13) for 20 min to allow DNA unwinding, and electrophoresed for 20 min at 25 V and 300 mA. Finally, the slides were neutralized with 0.4 mol L^{-1} Tris buffer (pH 7.5), stained with ethidium bromide (5 μg mL^{-1}) and analysed using fluorescence microscope (Leica, DMLS, Vienna, Austria) with an excitation filter of 510–560 nm, barrier filter of 590 nm, at 400× magnification. Image analysis software (Comet Assay IV, Perceptive Instruments, Haverhill, UK) was used for interpretation of the results. Fifty nuclei per experimental point in each of the three independent experiments were analysed, and the percentage of fluorescence in the comet tail was scored as a reflection of DNA damage.

Statistical analysis

For the results of the comet assay, one-way analysis of variance (nonparametric ANOVA, Mann–Whitney *U*-test) was used to analyse differences between the treatments within each experiment. Duncan's Post

Hoc Test was used to compare median values of the percentage of fluorescence in comet tail; $P < 0.05$ was considered as statistically significant.

Results

Materials characterization

XRD analysis

X-ray diffraction patterns for C3S and β-C_2S phases of the given CS system is shown in Fig. 1. The peaks at angles [11.7° plane (202), 23.6° plane (−114), 29.8° plane (1002), 32.5° plane (−114), 32.8° plane (224), 33.4° plane (−424), 34.6° plane (1004), 41.7° plane (−626)] corresponded to C_3S phase, whilst peaks at angles [23.3° plane (012), 29.7° plane (120), 32.3° plane (−121), 33° plane (121), 34.6° plane (103), 34.6° plane (103), 38.9° plane (222), 41.6° plane (031), 43.2° plane (222), 46.9° plane (024), 48° plane (222), 51.9° plane (310), 56.8° plane (−303), 60.2° plane (303) and 62.7° plane (043)] corresponded to β-C_2S phase. Based on the Sherrer equation calculation (measured for highly pronounced planes at 32.1° (plane −121) for β-C_2S and (plane −715) for C_3S, the obtained values of crystallite sizes of these phases were about 19.9 nm.

Scanning electron microscopy and energy dispersive analysis

The structure of CS (Fig. 2) consisted mostly of agglomerates of several micrometers in size, built up from smaller particles, with dimensions between 117 and 477 nm. These particles were preferentially of spherical or ellipsoidal shape, more or less elongated along one direction. EDS (Fig. 3) revealed that the chemical composition of calcium silicates (Ca 22.21, Si 8.22, O 69.7 in atomic%) corresponded to the ratio Ca/Si equal to approximately 2.7 (atomic%). This ratio was obviously close to the CaO/SiO_2 ratio corresponding to $C_3S/\beta\text{-}C_2S = 2 : 1$, showing homogenous distribution of both phases within the sample. Comparing values for the particle sizes by SEM and crystallite sizes obtained from XRD spectra, it was clear that these particles consisted of smaller building elements (crystallites), showing a significant activity of the system.

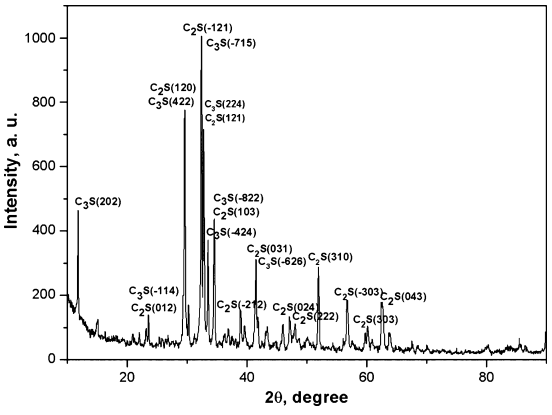

Figure 1 X-ray diffractometry spectra of calcium-silicate phases.

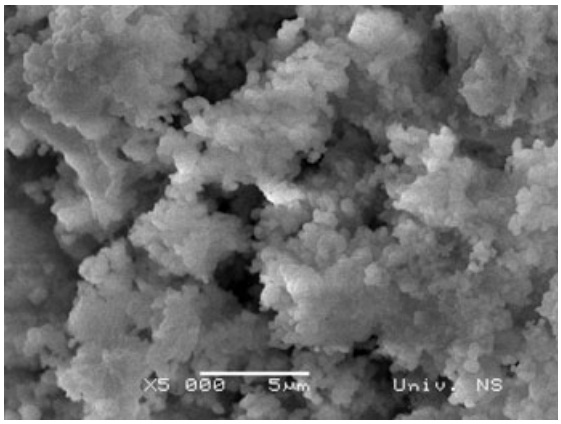

Figure 2 Typical appearance of calcium-silicate agglomerates and particles.

Mechanical strength and setting time

Setting time was measured for CS phases and their mixture with HA (HA-CS). Setting time for CS began 3 min after addition of deionized water and was completed in 10 min. For HA-CS, these values were 5 and 15 min, respectively. Compressive strength for CS was 3.5 MPa (24 h after), 4.4 MPa (3 days after), 4.8 MPa (7 days after) and 5.4 MPa (28 days after), and for HA-CS 2.8 MPa (24 h after), 3.2 MPa (3 days after), 3.5 MPa (7 day after s) and 4.1 MPa (28 days after).

Genotoxic potential

The genotoxic potential of HA, CS and HA-CS was tested by comet assay, performed on human lymphocytes. In preliminary screening with trypan blue assay, no cytotoxic effects of the concentration ranges occurred (data not shown). To correctly assess the genotoxicity, *t*-BOOH was used as a positive control. *t*-BOOH is the latent donor of reactive oxygen species (ROS) that induces oxidative DNA damage, and its

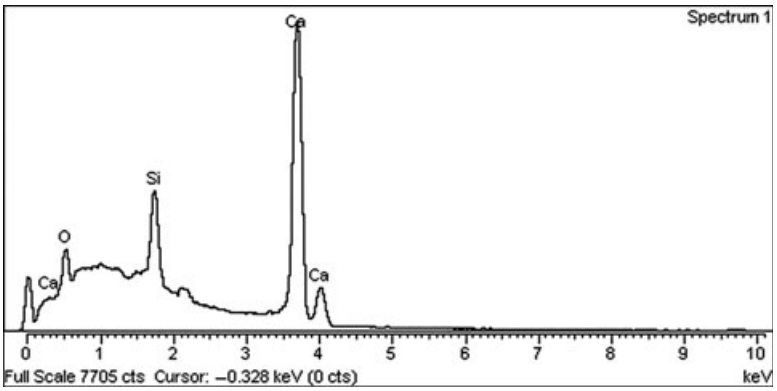

Figure 3 Energy dispersive analysis spectra of calcium-silicate phases.

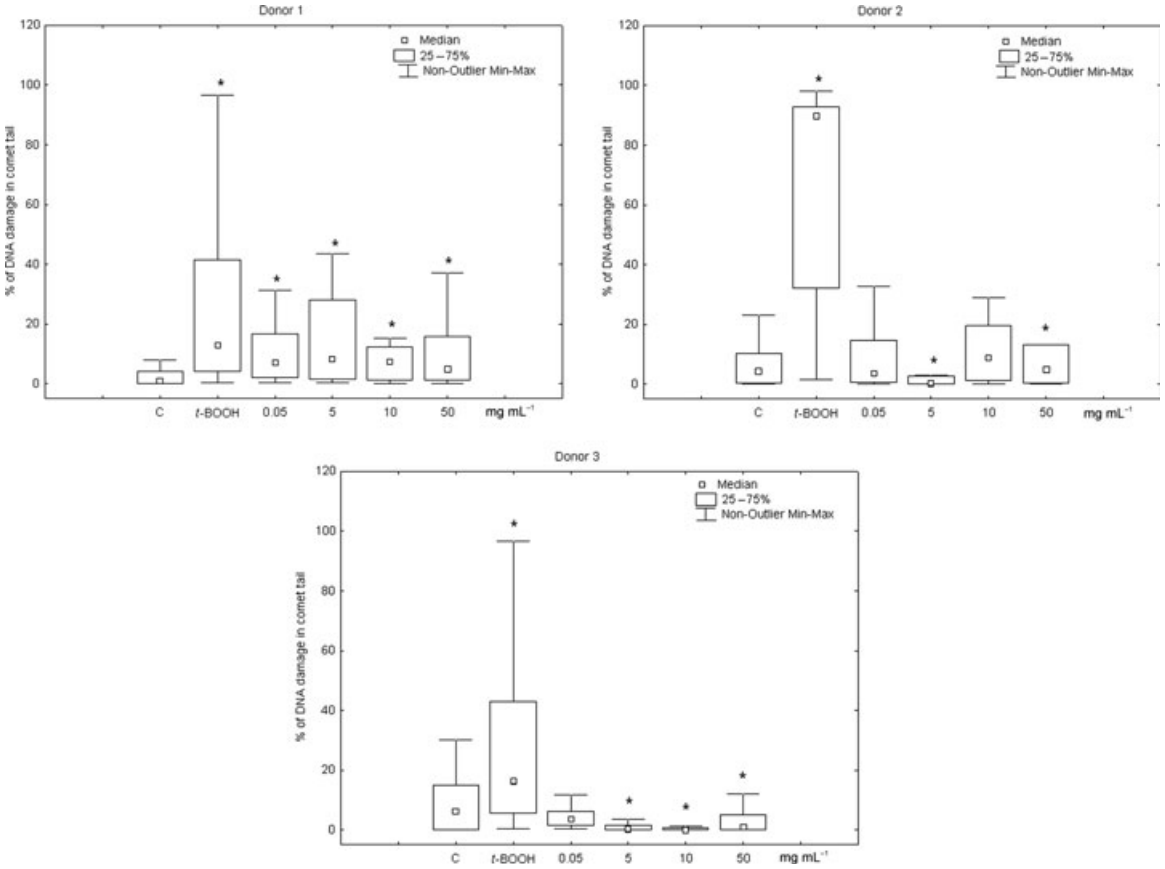

Figure 4 Genotoxic effect of HA in human lymphocytes. The level of DNA strand breaks is expressed as the percentage of DNA in the comet tails. As a positive control, 0.5 mmol L^{-1} t-BOOH was used. Fifty cells were analysed per experimental point in each of the three independent experiments. *Significantly different (amongst median value) from the untreated group, $P < 0.05$.

high genotoxic potential was confirmed (Urios & Blanco 1996).

The genotoxic potential of HA was tested in the concentration range 0.05–50 mg mL^{-1}, using lymphocytes from three donors. The results showed some inconsistency and are presented in Fig. 4. After the treatment of lymphocytes originated from donor 1, HA exhibited statistically significant genotoxicity ($P < 0.05$) at all

tested concentrations in comparison with negative control. However, HA did not induced DNA damage in the two other donors; moreover, the percentage of DNA damage in HA-treated cells was decreased (the donor 2: conc. 5 mg mL^{-1}; the donor 3: conc. 5, 10 and 50 mg mL^{-1}) comparing to control cells.

The genotoxic effect of CS, a newly synthesized material, was tested on lymphocytes from five donors (two donors were added), and the results are presented in Fig. 5. CS had no genotoxic effect on lymphocytes from donor 2, but exhibited strong genotoxic effect in lymphocytes from donor 1 (concentrations 1–10 mg mL^{-1}). On the contrary, CS induced the opposite effects and decreased the percentage of DNA damage in lymphocytes from donors 3–5, but only at highest tested concentrations (5 and 10 mg mL^{-1}).

After the testing of partial components, the genotoxic effect of a corresponding mixture that could be used in endodontic practice (HA-CS) was tested. The results are presented in Fig. 6. The induction of DNA damage under the influence of HA-CS was not observed in lymphocytes obtained from donors 1, 4 and 5. However, HA-CS at all tested concentrations except the lowest (0.01 mg mL^{-1}) induced DNA damage in lymphocytes from donor 2. Interestingly, the highest tested concentration of HA-CS (10 mg mL^{-1}) reduced the percentage of DNA damage in lymphocytes obtained from donor 3.

Discussion

Understanding the mechanisms of interaction between biological fluids or cell constituents with endodontic materials are essential for avoiding premature clearance of material used in diagnosis and therapy, as well as for avoiding adverse reactions to the materials used as implants (Fenoglio *et al.* 2011). To achieve successful regeneration of damaged bone, it is important to synthesize materials that have the qualities biocompatibility, bioactivity and biodegradability. Ideally, endodontic materials should exhibit the same properties as real bone and should also be biocompatible with existing tissue.

HA is one of the most widely used calcium phosphate based bioceramics, which comprise osteoconductive ability. As its structure is similar to bone mineral, it can form direct bonds with bone. HA has several advantages; it is well tolerated and integrated into the host tissue, providing the base for new bone growth (Suwanprateeb *et al.* 2012). It does not contain any protein and does not cause any allergic or immune reaction (Hu *et al.* 2011). However, HA has intrinsically poor mechanical properties (Arun *et al.* 2011), which can be improved by combining it with newly synthesized nanomaterials. To improve its characteristics and produce the new endodontic material, the mixture of HA with CS was formulated. In production of CS a hydrothermal sol-gel method was used along with the method of self-propagating combustion waves, which provide a high activity and a short setting time in the materials. The characterization of HA-CS indicated that the setting time of CS and HA-CS ranged from 10 to 15 min, what is significantly shorter in comparison with the setting time of MTA, which is approximately 4–6 h (Jokanović *et al.* 2008). Structures built on three hierarchical levels (agglomerates, particles and crystallites) may be promising because they are not biologically destructive (dimensions of agglomerates are not comparable with channels inside the cell membranes), whereas their nano-elements (nano-crystallites) provide evident activity, important for fast setting of these mixtures in endodontic therapy.

Biomaterials should not only have good physical qualities, but also be safe. As the tested materials are nanostructured and the penetration of nanoparticles into the cell nucleus correlated with the number of harmful effects on nuclear structure and function (inhibition of replication, transcription and cell proliferation), additional attention was recommended (Chen & Von Mikecz 2005). However, according to the SEM images, particle size between 117 and 477 nm for CS and similar values for the agglomerates of HA had almost negligibly harmful effects on the cell structure.

Furthermore, the assessment of the genotoxic potential of new biomaterials is an imperative. Briefly, the alkaline comet assay is a method used to measure both single and double strand breaks and alkali-labile sites. It is a quick and simple assay, recommended for the evaluation of the genotoxic potential of materials. One reason for the increasing interest in using the method is the low number of cells required to measure DNA lesions. Different laboratories use different end points (i.e.%DNA in tail, tail moment, tail length) when reporting the results, but regardless of the measured endpoint, comet assay is frequently performed method for estimating DNA damage (Collins 2004, Azqueta *et al.* 2009, Gaivao *et al.* 2009, Johansson *et al.* 2010). However, although the comparison of results obtained from *in vitro* and *in vivo* genotoxicity

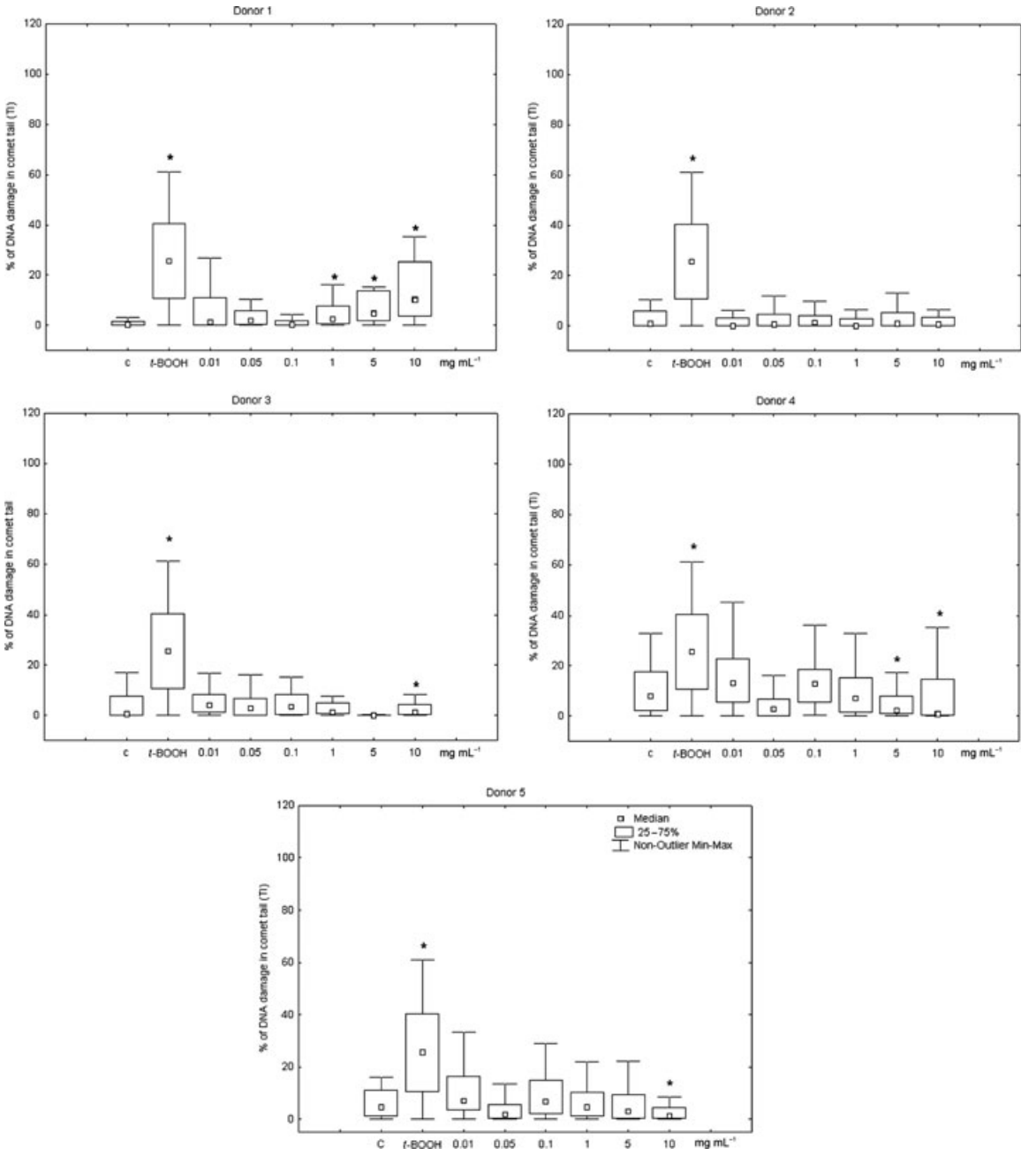

Figure 5 Genotoxic effect of CS in human lymphocytes. The level of DNA strand breaks is expressed as the percentage of DNA in the comet tails. As a positive control, 0.5 mmol L^{-1} t-BOOH was used. Fifty cells were analysed per experimental point in each of the three independent experiments. *Significantly different (amongst median value) from the untreated group, $P < 0.05$.

assays indicates that the comet assay gives relevant information, it is important to emphasize that *in vitro* assays do not take into consideration the complex homoeostatic situation that occurs *in vivo* (Ribeiro 2008, Liuyun *et al.* 2009). The genotoxic effect of CS and HA separately, as well as of corresponding mixture (HA-CS), was evaluated by comet assay with human lymphocytes.

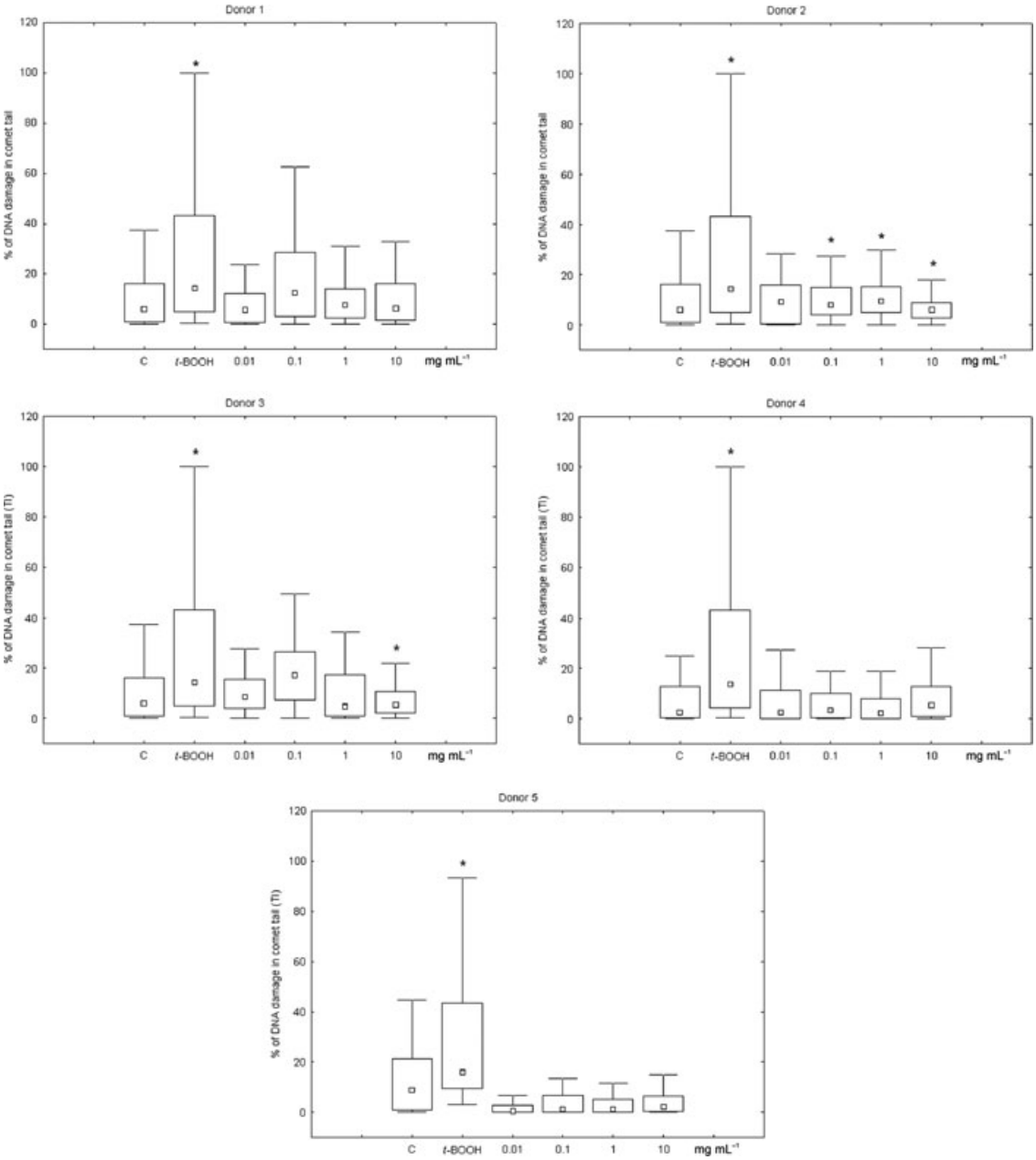

Figure 6 Genotoxic effect of HA-CS in human lymphocytes. The level of DNA strand breaks is expressed as the percentage of DNA in the comet tails. As a positive control 0.5 mmol L^{-1} t-BOOH was used. Fifty cells were analysed per experimental point in each of the three independent experiments. *Significantly different (amongst median value) from the untreated group, $P < 0.05$.

According to the literature HA exhibits strong cytotoxic effects in the murine fibroblast cell line NIH-3T3 (Gomes-Filho *et al.* 2009). It was not mutagenic in bacterial assay (Jantova *et al.* 2008, Hassan & Swaminathan 2011), and not genotoxic in scaffold MG63 and mesenchymal stem cells (MSCs) (Liuyun *et al.* 2009), as well as in leucemia L1210 cells (Jantova *et al.* 2008). However, results from the current study suggested a genotoxic effect of HA, at least under some conditions and for some individuals. It can be assumed that this is due to induction of DNA damage, obtained with HA in lymphocytes from donor 1. On

the contrary, HA decreased the level of DNA damage in lymphocytes from donors 2 and 3, probably due to possible cytotoxic effects. Although no cytotoxic effect was obtained in preliminary study performed with the trypan blue exclusion assay, the possible toxicity obtained in this experiment is not excluded. Moreover, the literature suggests that difference between cell lines and test system and experimental conditions can result in variated cytotoxicity results (Scudiero *et al.* 1988, Gomes-Filho *et al.* 2009, Scelza *et al.* 2012). Furthermore, the decreasing of basal genotoxicity levels in genotoxicity testing is usually interpreted as a toxic effect of test substance.

Diverse sensitivity of cells obtained from different donors could be the result of the different genetic backgrounds of donors, and consequent difference in their DNA repair capacities. The confirmation of different sensitivity of lymphocytes from different donors was also noticed when CS, HA-CS, as well as *t*-BOOH (positive control) were tested. To quantify this difference, a comparison of negative control (untreated cells) by themselves and the same comparison after the *t*-BOOH treatment was conducted (Fig. 7). The difference between individuals and variation within one individual can be expressed as the coefficient of variation (CV). In the current study, the mean CV for non-treated control cells was about 34%, whilst for *t*-BOOH-treated cells it was up to 80.6%; such high values of CV indicated high variability of tested cells. Although there are no statistically significant differences between untreated cells, when the response of different donor cells to *t*-BOOH treatment was compared, significant difference in amounts of induced DNA damage were obtained. Lymphocytes from donor 2 exhibited the highest sensitivity to *t*-BOOH treatment (tail intensity 66%), whilst sensitivity of donor 3 lymphocytes was the lowest one (tail intensity 22%). This difference indicated various inductions of DNA repair enzymes in different donors and is consistent with data reported by Hazra *et al.* (2007), who showed high variability of genes involved in base excision repair of DNA (BER) and nucleotide excision repair of DNA (NER) in lymphocytes from healthy subjects. As *t*-BOOH induces ROS, which cause DNA lesions predominantly repaired via BER pathway (except double strand breaks), the results from the current study probably indicate the difference in BER capacities of donors.

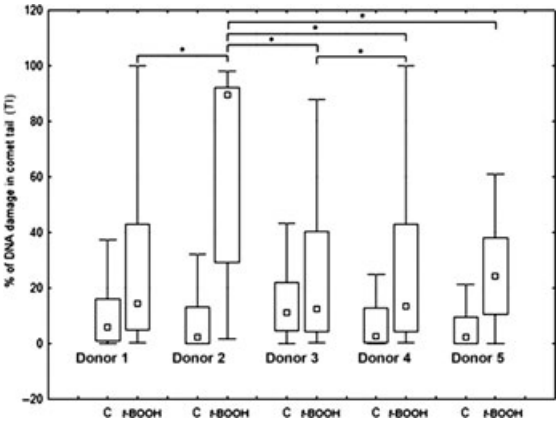

Figure 7 DNA damage induced with *t*-BOOH in lymphocytes obtained from different donors. *Statistically significant difference, $P < 0.05$.

Overall, CS was not genotoxic in the tested concentration range, with the exception of the lymphocytes from donor 1, where high induction of DNA damage was obtained. HA-CS was genotoxic only in lymphocytes acquired from donor 2, whilst no significant increase of DNA damage was obtained in cells from the other four donors. Inconsistency in genotoxic response in cells exposed to different endodontic materials is not surprising; other studies have also shown a wide range of genotoxic responses (Brzović *et al.* 2009, Camargo *et al.* 2009, Zeferino *et al.* 2010, Zhang *et al.* 2010, Kalmodia *et al.* 2011). Therefore, research on gene polymorphism from case–control studies has become a common practice.

More than one concentration of tested materials was used. The appropriate concentration range that should be tested depends on the substance absorption in biological fluids and cells, and its metabolism within the cells. To correctly estimate the values of tested concentration ranges, it is important to note that cell treatment was performed in suspension. Some evidences suggest the higher exposure of suspended cells to the test substance, than cells growing in monolayer (Mitić-Ćulafić *et al.* 2009). Accordingly, it can be assumed that lymphocytes in suspension were more exposed to testing material then cells in dental and periodontal tissues would be, and furthermore, that tested concentrations significantly exceeded the dose that would be absorbed regularly *in vivo*.

Obtained values of DNA damage amplification clearly indicated that HA-CS exhibited lower genotoxic potential as compared with HA and CS separately. As HA has already been in use in dental practice and its genotoxic potential is confirmed as low, it appears that HA-CS would be safe for human application. The

results obtained from the current study are encouraging for further research.

Conclusion

A new endodontic material using a combination of two promising technologies, hydrothermal sol-gel process and self-containing synthesis using combustion waves, was prepared. This material is important because it exhibits significantly improved setting characteristics (several times faster setting) as compared to MTA. Taking into account, the lack of currently available data, the assessment of the potential genotoxicity of new endodontic material is justified. Results obtained by comet assay in human lymphocytes indicated that all materials (HA, CS and HA-CS) had a low genotoxic potential; HA-CS exhibited the lowest genotoxicity. Taking together the improved setting characteristics and results suggesting low genotoxic risk, the new material is considered as a potentially good candidate for further research about possible endodontic use.

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References

Arun M, Silja PK, Mohanan VP (2011) Evaluation of hydroxyapatite-bioglass and hydroxyapatite-ethyl vinyl acetate composite extracts on antioxidant defense mechanism and genotoxicity. *Toxicology Mechanisms and Methods* **21**, 561–6.

Asgary S, Shahabi S, Jafarzadeh T, Amini S, Kheirieh S (2008) The properties of a new endodontic material. *Journal of Endodontics* **34**, 990–3.

Azqueta A, Lorenzo Y, Collins A (2009) *In vitro* comet assay for DNA repair: a warning concerning application to cultured cells. *Mutagenesis* **24**, 379–81.

Bohner M, Lemaitre J (2009) Can bioactivity be tested in vitro with SBF solution? *Biomaterials* **30**, 2175–9.

Bose S, Darsell J, Hosick H, Yang L, Sarkar D, Bandyopadhyay A (2002) Processing and characterization of porous alumina scaffolds. *Journal of Materials Science: Materials in Medicine* **13**, 23–8.

Braz MG, Camargo EA, Salvadori DMF, Marques MEA, Ribeiro DA (2006) Evaluation of genetic damage in human peripheral lymphocytes exposed to mineral trioxide aggregate and Portland cements. *Journal of Oral Rehabilitation* **33**, 234–9.

Brzović V, Miletić I, Zeljezić D *et al.* (2009) In vitro genotoxicity of root canal sealers. *International Endodontic Journal* **42**, 253–63.

Camargo SEA, Camargo CHR, Hiller K-A, Schweikl H, Schmalz G (2009) Cytotoxicity and genotoxicity of pulp capping materials in two cell lines. *International Endodontic Journal* **42**, 227–37.

Chen M, Von Mikecz A (2005) Formation of nucleoplasmic protein aggregates impairs nuclear function in response to SiO_2 nanoparticles. *Experimental Cell Research* **305**, 51–62.

Collins A (2004) The comet assay for DNA damage and repair. *Molecular Biotechnology* **26**, 249–61.

Ding SJ, Kao CT, Chen CL, Shie MY, Huang TH (2010) Evaluation of human osteosarcoma cell line genotoxicity effects of mineral trioxide aggregate and calcium silicate cements. *Journal of Endodontics* **36**, 1158–62.

Fenech M (2000) The in vitro micronucleus technique. *Mutation Research* **455**, 81–95.

Fenoglio I, Fubini B, Ghibaudi EM, Turci F (2011) Multiple aspects of the interaction of biomacromolecules with inorganic surface. *Advanced Drug Delivery Reviews* **63**, 1186–209.

Freshney RI (1987) *Culture of animal cells: a manual of basic technique*, 2nd edn. New York, USA: Alan R. Liss Inc.

Gaivao I, Piasek A, Brevik A, Shaposhnikov S, Collins A (2009) Comet assay –based methods for measuring DNA repair *in vitro*; estimates of inter- and intra-individual variation. *Cell Biology and Toxicology* **25**, 45–52.

Gandolfi GM, Taddei P, Tinti A, Prati C (2010) Apatite-forming ability (bioactivity) of ProRoot MTA. *International Endodontic Journal* **43**, 917–29.

Gomes-Filho JE, Watanabe S, Gomes AC, Faria MD, Lodi CS, Penha Oliviera SH (2009) Evaluation of the effects of endodontic materials on fibroblast viability and cytokine production. *Journal of Endodontics* **35**, 1577–9.

Hassan A, Swaminathan D (2011) An *In vitro* study to evaluate the genotoxicity of value added hydroxyapatite as a bone replacement material. *Sains Malaysiana* **40**, 163–71.

Hazra TK, Das A, Das S, Choudhury S, Kow YW, Roy R (2007) Oxidative DNA damage repair in mammalian cells: a new perspective. *DNA Repair* **6**, 470–80.

Hsieh SC, Teng NC, Lin YC *et al.* (2009) A novel accelerator for improving the handling properties of dental filling materials. *Journal of Endodontics* **35**, 1292–5.

Hu H-t, Xu G-h, Liu J-c *et al.* (2011) Biological security of nano-hydroxyapatite wisker and β-tricalcium phosphate composited bone substitute. *Journal of Clinical Rehabilitative Tissue Engineering Research* **15**, 9575–8.

Jantova S, Theiszova M, Letašiova S, Birošova L, Palou M (2008) *In vitro* effects of fluor- hydroxyapatite, fluorapatite and hydroxyapatite on colony formation, DNA damage and mutagenicity. *Mutation Research* **652**, 139–44.

Johansson C, Møller M, Forchhammer L *et al.* (2010) An EC-VAGy trial on assessment of oxidative damage to DNA measured by the comet assay. *Mutagenesis* **25**, 125–32.

Jokanović V, Izvonar D, Dramićanin M *et al.* (2006) Hydrothermal synthesis and nanostructure of carbonated calcium hydroxyapatite. *Journal of Materials Science: Materials in Medicine* **17**, 539–46.

Jokanović V, Jokanović B, Marković D *et al.* (2008) Kinetics and sintering mechanisms of hydro-thermally obtained hydroxyapatite. *Materials Chemistry and Physics* **111**, 180–5.

Kalmodia S, Sharma V, Pandeu KA, Dhawan A, Basu B (2011) Cytotoxicity and Genotoxicity Property of Hydroxyapatite-Mullite Eluates. *Journal of Biomedical Nanotechnology* **7**, 74–5.

Liuyun J, Yubao L, Chengdong X (2009) Preparation and biological properties of a novel composite scaffold of nano-hydroxyapatite/chitosan/carboxymethyl cellulose for bone tissue engineering. *Journal of Biomedical Science* **16**, 65–74.

Mitić-Ćulafić D, Žegura B, Nikolić B, Vuković-Gačić B, Knežević-Vukčević J, Filipič M (2009) Protective effect of linalool, myrcene and eucalyptol against t-butyl hydroperoxide induced genotoxicity in bacteria and cultured human cells. *Food and Chemical Toxicology* **47**, 260–5.

Moretton TR, Brown CE Jr, Legan JJ, Kafrawy AH (2000) Tissue reactions after subcutaneous and intraosseous implantation of mineral trioxide aggregate and ethoxybenzoic acid cement. *Journal of Biomedical Materials Research Part A* **52**, 528–33.

Parirokh M, Torabinejad M (2010a) Mineral trioxide aggregate: a comprehensive literature review-Part I: chemical, physical, and antibacterial properties. *Journal of Endodontics* **36**, 16–27.

Parirokh M, Torabinejad M (2010b) Mineral trioxide aggregate: a comprehensive literature review-part III: Clinical applications, drawbacks and mechanism of action. *Journal of Endodontics* **36**, 400–13.

Ribeiro DA (2008) Do endodontic compounds induce genetic damage? *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics* **105**, 251–6.

Ribeiro DA, Matsumoto MA, Duarte MAH, Marques MEA, Salvadori DMF (2005a) In vitro biocompatibility tests of two commercial types of mineral trioxide aggregate. *Brazilian Oral Research* **19**, 183–7.

Ribeiro DA, Duarte MAH, Matsumoto MA, Marques MEA, Salvadori DMF (2005b) Biocompatibility in vitro tests of mineral trioxide aggregate and regular and white Portland Cements. *Journal of Endodontics* **31**, 605–7.

Ribeiro DA, Sugui MM, Matsumoto MA, Duarte MAH, Marques MEA, Salvadori DMF (2006) Genotoxicity and cytotoxicity of mineral trioxide aggregate and regular and white Portland cements on Chinese hamster ovary (CHO) cells in vitro. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics* **101**, 258–61.

Scelza MZ, Linghares AB, da Silva LE, Granjeiro JM, Alves GG (2012) A multiparametric assay to compare the cytotoxicity of endodontic sealers with primary human osteoblasts. *International Endodontic Journal* **46**, 12–8.

Scudiero DA, Shoemaker RH, Paull KD *et al.* (1988) Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Research* **48**, 4827–33.

Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research* **175**, 184–91.

Suwanprateeb J, Thammarakcharoen F, Wasoontararat K, Chokevivat W, Phanphiriya P (2012) Preparation and characterization of nanosized silver phosphate loaded hydroxyapatite by single step co-conversion process. *Materilal Science and Engineering* **32**, 2122–8.

Takenaka Y, Iijima M, Kawano S *et al.* (2008) The development of carbonate-containing apatite/collagen composite for osteoconductive apical barrier material. *Journal of Endodontics* **34**, 1096–100.

Theiszová M, Jantová S, Dragúnová J, Grznárová J, Palou M (2005) Comparison the cytotoxicity of hydroxyapatite measured by direct cell counting and MTT test in murine fibroblast NIH-3T3 cells. *Biomedical Papers* **149**, 393–6.

Tice RR, Agurell E, Anderson D *et al.* (2000) Single cell gel comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis* **35**, 206–21.

Urios A, Blanco M (1996) Specifity of spontaneous and *t*-butyl hydroperoxide-induced mutations in ΔoxyR strains of *Escherichia coli* differing with respect to the SOS mutagenesis proficiency and to the MutY and MutM functions. *Mutation Research* **354**, 95–101.

Wahl DA, Czernuszka JT (2006) Collagen-hydroxyapatite composites for hard tissue repair. *European Cells and Materials/ECM* **11**, 43–56.

Zeferino GE, Bueno SEC, Oyama ML, Ribeiro AD (2010) *Ex vivo* assessment of genotoxicity and cytotoxicity in murine fibroblasts exposed to white MTA or white Portland cement with 15% bismuth oxide. *International Endodontic Journal* **43**, 843–8.

Zhang W, Li Z, Peng B (2010) *Ex vivo* cytotoxicity of a new calcium silicate-based canal filling material. *International Endodontic Journal* **43**, 769–74.

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BIOCOMPATIBILITY OF A NEW NANOMATERIAL BASED ON CALCIUM SILICATE IMPLANTED IN SUBCUTANEOUS CONNECTIVE TISSUE OF RATS

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The aim of the study was to investigate rat connective tissue response to a new calcium silicate system 7, 15, 30 and 60 days after implantation.

Twenty Wistar albino male rats received two tubes half-filled with a new calcium silicate system (NCSS) or MTA in subcutaneous tissue. The empty half of the tubes served as controls. Five animals were sacrificed after 7, 15, 30 and 60 days and samples of the subcutaneous tissue around implanted material were submitted to histological analysis. The intensity of inflammation was evaluated based on the number of inflammatory cells present. Statistical analysis was performed using one way ANOVA and Holm Sidak's multiple comparison tests.

Mild to moderate inflammatory reaction was observed after 7, 15 and 30 days around a NCSS while mild inflammatory reaction was detected after 60 days of implantation. In the MTA group, mild to moderate inflammatory reaction was found after 7 and 15 days while mild inflammatory reaction was present after 30 and 60 days. There was no statistically significant difference in the intensity of inflammatory reactions between the tested materials and control groups in any experimental period (ANOVA $p>0.05$). Regarding the intensity of inflammatory reactions at different experimental periods, a statistically significant difference was observed between 7 and 30 days, 7 and 60 days and 15 to 60 days for both materials. For the controls, a statistically significant difference was found between 7 and 60 days and 15 and 60 days of the experiment (Holm Sidak $<p$ 0.001).

Subcutaneous tissue of rats showed good tolerance to a new calcium silicate system. Inflammatory reaction was similar to that caused by MTA.

Key words: biocompatibility, calcium silicate cements, MTA, subcutaneous tissue

INTRODUCTION

Several new obturation materials have been developed recently. In the past 20 years, the greatest attention has been given to mineral trioxide aggregate (MTA) material. Previous studies have demonstrated its biocompatibility, good chemical and physical properties, good sealing ability and antimicrobial effect (Parirokh and Torabinejad 2010a; Torabinejad and Parirokh, 2010). MTA has been recommended for a number of clinical indications, such as direct pulp capping, pulpotomy, iatrogenic perforations, formation of the apical plug in teeth with necrotic pulp and open apex, apexification and closing apex after apical surgery (Pararirokh and Torabinejad, 2010b).

MTA is a mixture of tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium sulfate (gypsum) and bismuth oxide (Camilleri *et al*., 2005). An important property of this material is that it can set in the presence of humidity. However, one of its disadvantages is the setting time longer than 3 hours (Parirokh and Torabinejad, 2010a). There have been attempts to add various accelerators to speed up the setting time of MTA (Kogan *et al*., 2006; Huang *et al*., 2008), however, they were found to adversely affect the mechanical properties of this cement (Oki and Yoshiba, 2009). Therefore, the research aimed to find a material that will have similar physical properties and biocompatibility as MTA, but a shorter setting time is still actual (Tay *et al*., 2007; Takenaka *et al*., 2008; Asgary 2008; Chen *et al*., 2009; Scarparo *et al*., 2010; Gandolfi *et al*., 2011; Lin *et al*., 2011).

A new nano-material based on calcium silicate system was synthesized at the Institute for Nuclear Research - Vinca by V. Jokanović. This material was obtained by combining hydrothermal sol-gel method and the method of self combustion waves. It consists of dicalcium and tricalcium silicate (60%), gypsum (20%) and barium sulfate (20%). The material includes agglomerates of a few micrometers built of 117-477 nm particles. The particles consist of smaller elements- crystallites, 20 nm in size. This material structure composed at three hierarchical levels (agglomerates, particles and crystallites) does not cause biological tissue destruction since the size of agglomerates is not comparable to the pores of cell membranes. On the other hand, particle size is the main factor that affects the degree of cement hydration and consequently its hardness and setting time. Smaller size of particles provides a larger surface area available for hydration speeding up the setting of the material (Asgary *et al*., 2009). In this regard, the new nanostructure of calcium silicate system provides a distinct activity important for fast setting. The setting time of the new material begins 3 minutes after the addition of distilled water and ends after 10 minutes. This characteristic is important for its potential clinical application.

The aim of the study was to investigate rat connective tissue response to a new calcium silicate system 7, 15, 30 and 60 days after implantation.

MATERIALS AND METHODS

The study was approved by the Ethical Committee of the School of Dentistry, University of Belgrade, Serbia (Protocol No. 36/5, 12/04/2012). Twenty Wistar albino male rats, 2-3 months old and the average weight of 350 g were obtained from the kennel of the Faculty of Biology (Belgrade, Serbia). A new calcium silicate system (NCSS) was prepared according to the recipe of V. Jokanović (Institute for Nuclear Research, Vinca, Serbia), mixed with distilled water in 2:1 ratio and compared with MTA (Solucoes Odontológicas Angelus, Londrina, Brazil).

Animals and Study Design

The animals received intraperitoneal injection of 75 mg/kg ketamine and 5 mg/kg of body weight of diazepam. After shaving, the skin on the backs of animals was disinfected by iodine tincture. Two incisions, 15 mm in length, in head-tail direction (one on either side of the spinal cord) were created using the the scalpel blade. Two pockets 15 mm deep were prepared by blunt dissection. Freshly mixed materials were placed in sterile polyethylene tubes 10 mm long and 1 mm inner diameter. Each half of the tubes was filled with the tested material, while the other half was left empty and served as the control. The tubes were implanted subcutaneously and the incisions were sutured using single resorbable sutures. Each animal received two tubes, one filled with MTA (to the left of the spine) and one with a new calcium silicate system (the right of the spine). The animals were housed (two animals in one cage) under standard conditions with controlled diet and professional daily care. Health condition check-ups were performed three times a day during the experiment.

Animals were sacrificed using a large dose of anesthetic in groups of five after 7, 15, 30 and 60 days. After shaving and disinfecting the skin on their backs, the tubes were removed along with surrounding connective tissue. Samples were fixed in 10% formalin solution. The tissue was cut in 4 micrometers thick sections, stained with hematoxylin and eosin and submitted to histological analysis. Quantitative assessment of inflammatory cells (lymphocytes, granulocytes, monocytes and histiocytes) was performed under light microscope OLYMPUS BX 51 (New York, USA) at magnification of 200× and 400× by a trained observer. The average value for each sample was obtained from the sum of cells counted in five fields. For each material the average value of cells was obtained from five animals for each experimental period.

The intensity of inflammation was evaluated based on the following criteria (Lotfi *et al*. 2009):

0 – no inflammation (no inflammation cells),

1 – mild inflammatory response (the number of cells),

2 – moderate inflammatory reaction (the number of cells 25-125),

3 – strong inflammatory response (125 inflammatory cells).

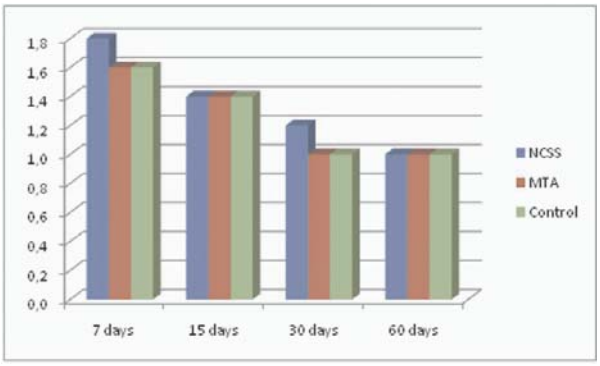
Statistical analysis was performed using one-way ANOVA and Holm-Sidak's test for multiple comparisons.

RESULTS

The results are shown in Table 1, Graph 1 and Figures 1-8.

Table 1. Average inflammatory score and standard deviation for the tested materials and controls after 7, 15, 30 and 60 days of the experiment

	7 days	15 days	30 days	60 days
NCSS	1.80± 0.45	1.40± 0.55	1.20± 0.45	1.00± 0.00
MTA	1.60± 0.55	1.40± 0.55	1.00± 0.00	1.00± 0.00
Control	1.60± 0.55	1.40± 0.55	1.00± 0.00	1.00± 0.00



Graph 1. Average inflammatory score for the tested materials after different experimental periods

After 7 days the mean inflammatory score for the NCSS group was 1.80 ± 0.45 (mild to moderate infiltration of inflammatory cells). The mean inflammatory score for the MTA and the control group was 1.60 ± 0.55, suggesting a mild to moderate inflammatory reaction, as well. There was no statistically significant difference between the tested materials and the control group ($p>0.05$). Both materials showed initial necrosis in the subcutaneous tissue (Figure 1-2).

After the experimental period of 15 days, the mean inflammatory score for each material and the control was 1.40±0.55, which corresponded to mild to moderate inflammatory reaction. There was no statistically significant difference in the intensity of inflammatory reactions between the tested materials and the control group ($p>0.05$).

In the experimental period of 30 days, the mean inflammatory score for the NCSS group was 1.20 ± 0.45, corresponding to mild to moderate inflammatory reaction (Figure 3). The mean inflammatory score for the MTA group and the control group was 1.00 ± 0.00, which corresponded to mild inflammatory reaction (Figures 4-5). Statistical analysis revealed no significant difference within experimental groups and between the tested materials and the control group ($p>0.05$).

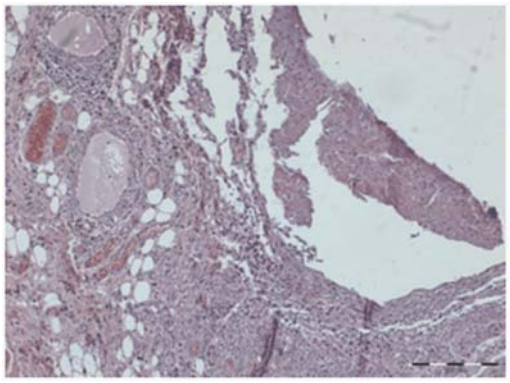


Figure 1. Histological image of the tissue in the control group after 7 days, tissue necrosis, magnification 10×

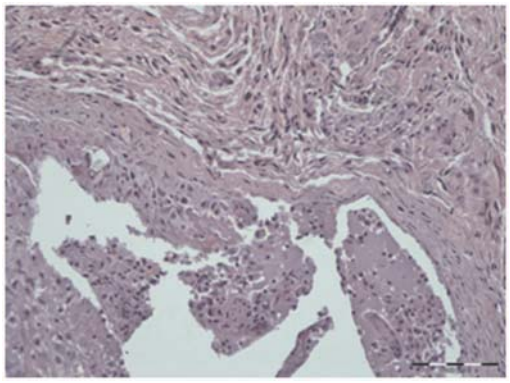


Figure 2 Histological image of the tissue in the MTA group after 7 days, tissue necrosis, magnification 20×

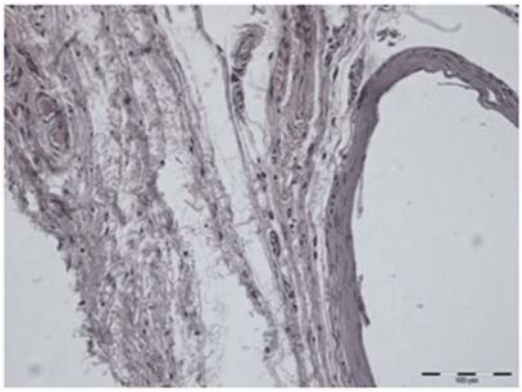


Figure 3. Histological image of the tissue in the NCSS group after 30 days, mild inflammatory reaction (score 1), magnification 200×

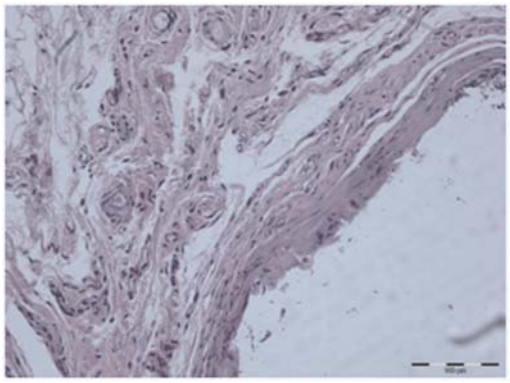


Figure 4. Histological image of the tissue in the MTA group after 30 days, mild inflammatory reaction, (score 1), magnification 200×

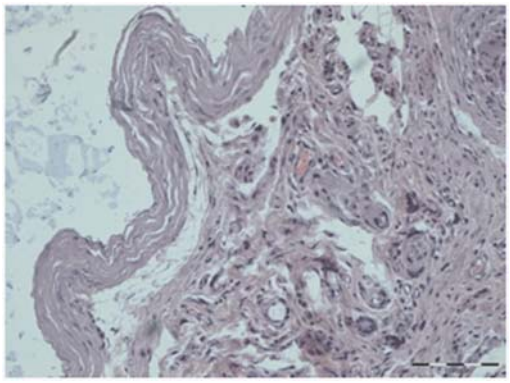


Figure 5. Histological image of the tissue in the control group after 30 days, mild inflammatory reaction, (score 1), magnification 200×

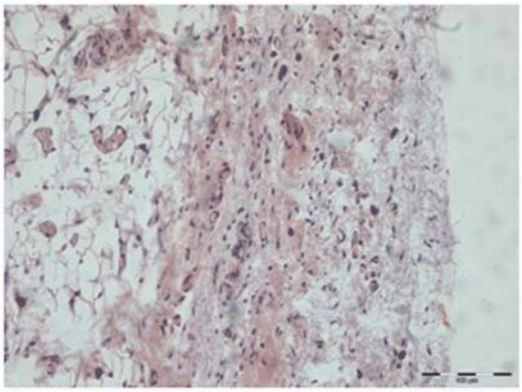


Figure 6. Histological image of the tissue in the NCSS group after 60 days, mild inflammatory reaction, (score 1), magnification 200×

After 60 days, the mean inflammatory score for both materials and the control group was 1.00±0.00 which corresponded to mild inflammatory reaction (Figures 6-8). There was no statistically significant difference between the tested materials and the control group ($p>0.05$).

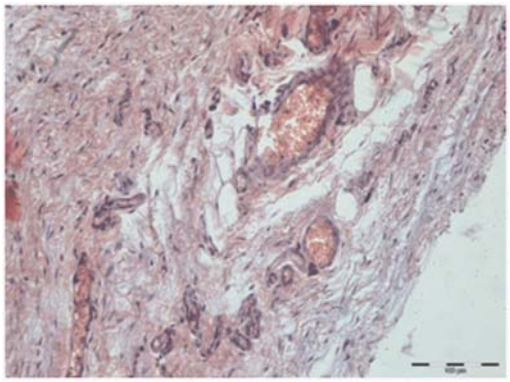


Figure 7. Histological image of the tissue in the MTA group after 60 days, mild inflammatory reaction, (score 1), magnification 200×

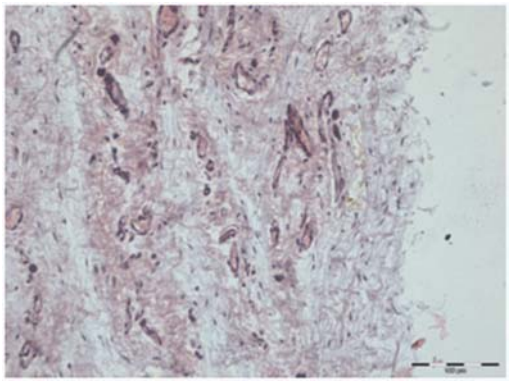


Figure 8. Histological image of the tissue in the control group after 60 days, mild inflammatory reaction, (score 1), magnification 200×

There was statistically significant difference in the intensity of inflammatory reaction between the tested materials and the control group after different experimental periods (ANOVA $p<0.001$). The significant difference in the intensity of inflammation after 7 and 60 and after 15 and 60 days of the experiment was found in the control group (Holm-Sidak's test $p<0.001$), as well as after 7 and 30 days, 7 and 60 days and 15 and 60 days of the experiment for both tested materials (Holm-Sidak's test $p<0.001$).

DISCUSSION

Subcutaneous implantation was performed to assess the biocompatibility of the tested materials. Material implantation in the subcutaneous tissue of small experimental animals and histological evaluation of the surrounding tissue reaction is valid and frequently used as an *in vivo* test. Inert polyethylene tubes stimulate clinical conditions, but also they provide material stabilization in place and a standardized contact area between material and surrounding tissue (Yaltirik *et al*., 2004; de Morais *et al*., 2006; Vosoughhosseini *et al*., 2008; Lotfi *et al*., 2009; Scarparo *et al*., 2010; Khashaba *et al*., 2011; Parirokh *et al*., 2011).

At the experimental periods of 7 and 15 days, a higher number of inflammatory cells in both materials and the control group were present compared to other experimental periods. The presence of an early inflammatory response may not necessarily be associated to the toxicity of materials, but to the surgical trauma after tube implantation that leads to tissue disintegration and consequent infiltration of inflammatory cells. These results are consistent with findings of other authors who investigated the biocompatibility of different formulations and new MTA endodontic cements (Parirokh *et al*., 2011; Khashaba *et al*., 2011).

Given that the effects caused by material implantation after longer periods of time are more significant than initially caused, the intensity of inflammatory reaction was monitored for up to 60 days (Yaltirik *et al.,* 2004; Lotfi *et al*., 2009; Scarparo *et al*., 2010; Paririkh *et al*., 2011). The number of inflammatory cells was significantly lower after 30 and 60 days as compared to 7 or 15 days of the experiment for both materials. Successive reduction in the number of inflammatory cells indicated a good biological potential of the tested materials. These results are consistent with results of other researchers who examined the biocompatibility of MTA (Vassoughosseini *et al.,* 2008; Parirokh *et al*., 2011).

The presence of necrosis in the subcutaneous tissue was recorded after 7 days of implantation for both materials. The presence of necrosis after subcutaneous implantation of MTA was detected by other authors (Yaltirik *et al*., 2004; Parirokh *et al*., 2011). They found necrosis only in tissue samples after 7 days of implantation while in the later experimental periods (15, 30, 60 and 90 days) it was not recorded similarly to the results of the current study. Necrosis, as an early tissue reaction to MTA, has been usually associated to high pH of freshly mixed material. It is known that the products of the reaction between MTA and water are calcium silicate hydrate and calcium hydroxide which explains the high pH value (Torabinejad and Parirokh, 2010).

A fibrous capsule around implanted material was present in both tested materials, as well as the control samples. The capsule was observed in the MTA and in the control group after 7 days whereas in the new calcium silicate cement group after 15 days of the experiment. The presence of fibrous capsule has been considered a favorable result because it indicated the body's ability to limit the inflammatory reaction to the implanted material and prevent further tissue damage (Yaltirik *et al*., 2004; de Morais *et al*., 2006; Scarparo *et al*., 2010; Parirokh *et al*., 2011).

A new calcium silicate system caused tissue reactions very similar to those in the control group or MTA, presenting the biological potential. Mild to moderate inflammatory reactions seen after 7, 15 and 60 days of the experiment were rated as mild in the presence of rare inflammatory cells and an almost normal appearance of the tissue. A similar tissue response to MTA and a new calcium silicate system could be explained by similar composition of these materials. The main ingredients of both, a new cement and MTA are tricalcium and dicalcium silicate. Materials differ by contrast additive. Barium sulfate was added to NCSS. There are conflicting opinions regarding the biocompatibility of bismuth oxide (added to MTA) (Yamamoto *et al*., 1998; Gandolfi *et al*., 2010). Furthermore, bismuth oxide changes the hydration process of MTA and undermines its physical properties. Since barium sulfate did not affect the hydration process of new calcium silicate cement it could replace bismuth oxide in MTA (Camilleri *et al*., 2010).

The results obtained after subcutaneous implantation of MTA and a new calcium silicate system demonstrated their biocompatibility. There was no difference in the intensity of inflammation between the MTA and the control group in any experimental period in the current study. These results are consistent with the findings of Scarparo *et al*. (2010) who also found a similar tissue response after subcutaneous implantation both in MTA and the control group. Vossoughosseini *et al*. (2008) reported the difference in the intensity of inflammation of the examined tissue as the response to two formulations of MTA and the control (empty tube) only after 7 days whereas in other periods (15, 30, 60 and 90 days) the difference in tissue reaction was not found.

Materials containing calcium exhibit good biological properties because of their ability to release calcium ions (Oki and Yoshiba, 2009). Sarcar *et al*. (2005) first reported the physical-chemical basis of MTA biological properties. These authors described that products obtained by MTA hydration were calcium silicate hydrate and calcium hydroxide (high pH of the material), and that the released calcium ions reacted with phosphate groups in the tissue fluids forming hydroxyapatite crystals on the surface of the material (Sarcar *et al*., 2005). Since the ability to release calcium is a common feature of all calcium silicate cements, it can be expected that the same physical-chemical reaction found in MTA will occur when a new calcium silicate system is in contact with tissue fluids.

Bioactivity of a material depends also on the process of its synthesis. The new calcium silicate system tested in this study was produced using new technology, a combination of hydrothermal sol-gel method and the method of self combusting waves. According to the literature, materials obtained by sol-gel processes have better bioactivity compared to the materials of the same composition, but synthesized by other methods (Li and de Groot, 1994). Therefore, good results obtained after subcutaneous implantation of a new material can be partially explained by the specific method of synthesis that favored its bioactivity.

CONCLUSION

A new calcium silicate system implanted in the subcutaneous tissue of rats did not cause significant inflammatory reactions in any of the experimental periods. The effects of a new calcium silicate system were similar to the effects of MTA, as well as the histological response of surrounding tissue.

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REFERENCES

1. *Asgary S, Shahabi S, Jafarzadeh T, Amini S, Kheirieh S*, 2008, The properties of a new endodontic material, *J Endod*, 34, 990-3.
2. *Asgary S, Eghbal MJ, Parirokh M, Ghoddusi J,Kheirieh S,Brink F*, 2009, Comparison of mineral trioxide aggregate s composition with Portland cements and a new endodontic cement, *J Endod*, 35, 243-50.
3. *Chen CC, Ho CC, Chen CHD, Ding SJ*, 2009, Physicochemical properties of calcium silicate cements for endodontic treatment, *J Endod*, 35, 1288-91.
4. *Camilleri J, Montesin FE, Brady K, Sweeney R, Curtis RV, Pitt Ford TR*, 2005, The constitution of mineral trioxide aggregate, *Dent Mat*, 21, 297-303.
5. *Camilleri J*, 2010, Hydratation characteristics of calcium silicate cements with alternative radiopacifiers used as root-end filling materials, *J Endod*, 36, 502-8.
6. *Gandolfi MG, Perut F, Ciapetti G, Mongiorgi R, Prati C*, 2008, New portland cement-based materials for endodontics mixed with articaine solution: A study of cellular response, *J Endod*, 34, 39-44.
7. *Gandolfi MG, Ciapetti G, Perut F, Taddei P, Modena E, Rossi PL et al*., 2009, Biomimetic calcium-silicate cements aged in simulated body solutions. Osteoblast response and analyses of apatite coating, *J Appl Biomater Biomech* 2009, 7, 160-70.
8. *Huang TH, Shie MY, Kao CT, Ding SJ*, 2008, The effect of setting accelerator on properties of mineral trioxide aggregate, *J Endod*, 34, 590-3.
9. *Khashaba RM, Moussa MM, Chutkan NB, Borke JL*, 2011, The response of subcutaneous connective tissue to newly developed calcium phosphate-based root canals sealers, *Int Endo J*, 44, 342-52.
10. *Kogan P, He J, Glickman GN, Watanabe I*, 2006, The effects of various additives on setting properties of MTA, *J Endod*, 32, 569-72.
11. *Li P, de Groot K*, 1994, Better bioactive ceramics through sol-gel process, *J Sol-Gel Sci Technol*, 2, 797-801.
12. *Lotfi M, Vossoughhosseini S, Saghiri MA, Mesgariabbasi M, Ranjkesh B*, 2009, Effect of white mineral trioxide aggregate mixed with disodium hydrogen phosphate on inflammatory cells, *J Endod*, 35, 703-5.

13. *de Morais CAH, Bernardineli N, Garcia RB, Duarte MAH, Guerisoli DMZ*, 2006, Evaluation of tissue response to MTA and Portland cement with iodoform, *Oral Surg Oral Med Oral Path Oral Radiol Endod*, 102, 417-22.
14. *Okiji T, Yoshiba K*, 2009, Reparative dentinogenesis induced by mineral trioxide aggregate: A review from the biological and physicochemical points of view, *Inter J Dent*, 1-12.
15. *Parirokh M, Torabinejad M*, 2010a, Mineral trioxide aggregate: A comprehensive literature review-Part I: Chemical, physical and antibacterial properties, *J Endod*, 36, 16-27.
16. *Parirokh M, Torabinejad M*, 2010b, Mineral trioxide aggregate: A comprehensive literature review-Part III: Clinical applications, drawbacks and mechanism of action, *J Endod*, 36, 400-13.
17. *Parirokh M, Mirsoltani B, Raoof M, Tabrizchi H, Haghdoost AA*, 2011, Comparative study of subcutaneous tissue responses to a novel root-end filling material and white and grey mineral trioxide aggregate, *Int Endod J*, 44, 283-9.
18. *Sarkar K, Caicedo R, Ritwik P, Moiseyeva R, Kawashima I*, 2005, Physicochemical basis of the biologic properties of mineral trioxide aggregate, *J Endod*, 31, 97-100.
19. *Scarparo RK, Haddad D, Acasigua GAX, Fossati ACM, Fachin EVF, Grecca FS*, 2010, Mineral trioxide aggregate-based sealer: Analysis of tissue reactions to a new endodontic material, *J Endod*, 36, 1178-4.
20. *Takenaka Y, Iijima M, Kawano S, Akita Y, Yoshida T, Doi Y et al*, 2008, The development of carbonate-containing apatite/collagen composite for osteoconductive apical barrier material, *J Endod*, 34, 1096-100.
21. *Torabinejad M, Parirokh M*, 2010, Mineral trioxide aggregate: A comprehensive literature review-Part II: Leakage and biocompatibility investigations, *J Endod*, 36, 190-202.
22. *Tay KCY, Loushine BA, Oxford C, Kapur R, Primus CM, Gutmann JL et al*, 2007, In vitro evaluation of a ceramicrete-based root-end filling material, *J Endod*, 33, 1438-43.
23. *Vosoughhosseini S, Lotfi M, Shahi S, Baloo H, Mesgariabbasi M, Saghiri MA et al*, 2008, Influence of white versus gray mineral trioxide aggregate on inflammatory cells, *J Endod*, 34, 715-7.
24. *Yaltirik M, Ozbas H, Bilgic B, Issever H*, 2004, Reactions of connective tissue to mineral trioxide aggregate and amalgam, *J Endod*, 30, 95-9.
25. *Yamamoto A, Honma R, Sumita M*, 1998, Cytotoxity evaluation of 43 metal salts using murine fibroblasts and osteoblastic cells. *J Biomed Mater Res*, 39, 331-40.

ISPITIVANJE BIOKOMPATIBILNOSTI NOVOG NANOSTRUKTURALNOG MATERIJALA NA BAZI KALCIJUM SILIKATNIH SISTEMA IMPLANTIRANJEM U POTKOŽNO TKIVO PACOVA

PETROVIĆ VIOLETA, OPAČIĆ GALIĆ VANJA, JOKANOVIĆ V, JOVANOVIĆ M,
BASTA JOVANOVIĆ GORDANA i ŽIVKOVIĆ S

SADRŽAJ

Cilj ovog rada je bio da se ispita biokompatibilnost novog kalcijum silikatnog sistema nakon *in vivo* implantacije u potkožno tkivo pacova.

Istraživanje je obuhvatilo 20 Wistar albino pacova muškog pola. U svaku životinju implantirane su dve tube do pola ispunjene novim kalcijum silikatnim sistemom (NCSS) odnosno MTA-om. Prazne polovine tuba služile su kao kontrola. Po 5 životinja žrtvovano je nakon 7, 15, 30 i 60 dana, nakon čega su uzorci pot-

kožnog tkiva oko implantiranog materijala pripremljeni za histološku analizu. Intezitet zapaljenske reakcije je procenjivan na osnovu broja prisutnih ćelija zapaljenja. Statistička analiza je urađena ANOVA testom i Holm Sidak-ovim testom višestruke komparacije.

U eksperimentalnim periodima 7, 15 i 30 dana u NCSS grupi je uočena blaga do umerena zapaljenska reakcija, a nakon 60 dana samo blaga zapaljenska reakcija. U MTA grupi, nakon 7 i 15 dana uočena je blaga do umerena zapaljenska reakcija, a nakon 30 i 60 dana blaga zapaljenska reakcija. Nije bilo statistički značajne razlike u intezitetu zapaljenske reakcije između testiranih materijala i kontrolne grupe ni u jednom eksperimentalnom periodu (ANOVA $p>0.05$). Poređenjem inteziteta zapaljenskih reakcija u različitim eksperimentalnim periodima, uočeno je postojanje statistički značajnih razlika kod oba testirana materijala između 7 i 30 dana, 7 i 60 dana kao i između 15 i 60 dana, a kod kontrole između 7 i 60 dana i 15 i 60 dana eksperimenta (Holm Sidak $p<0.001$).

Novi kalcijum silikatni sistem je pokazao biokompatibilno ponašanje. Inflamatorne reakcije potkožnog tkiva bile su slične onima koje je izazvao MTA.

Obrazac 1.

Fakultet STOMATOLOŠKI

UNIVERZITET U BEOGRADU

Stručno veće za medicinske nauke

Broj zahteva

(naziv stručnog veća kome se zahtev upućuje , shodno čl.6 Statuta Univerziteta u Beogradu i čl. 7. st.1 ovog pravilnika)

..god.

(Datum)

ZAHTEV

za davanje saglasnosti na izveštaj o urađenoj doktorskoj disertaciji

Molimo da, shodno članu 68. st.3. Zakona o univerzitetu ("Službeni glasnik RS" br. 20/98), date saglasnost na

izveštaj o urađenoj doktorskoj disertaciji kandidata PETROVIĆ SRBOLJUB VIOLETE
(ime, ime jednog od roditelja i prezime)

KANDIDAT PETROVIĆ SRBOLJUB VIOLETA prijavila je doktorsku disertaciju pod nazivom
(ime, ime jednog od roditelja i prezime)

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Univerzitet je dana 03.12.2013. svojim aktom pod br. 61206-5828/2-2013 dao saglasnost na predlog teme

doktorske disertacije koja je glasila

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Komisija za ocenu i odbranu doktorske disertacije kandidata PETROVIĆ SRBOLJUB VIOLETA
(ime, ime jednog od roditelja i prezime)

obrazovana je na sednici održanoj 23.09.2014. odlukom fakulteta pod br. 3/89

u sastavu:

ime i prezime člana komisije:	zvanje:	naučna oblast:
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JELENA SOPTA	PROFESOR	KLINIČKE STOM. NAUKE
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Nastavno-naučno veće fakulteta prihvatilo je izveštaj Komisije za ocenu i odbranu doktorske disertacije na sednici održanoj dana 09.12.2014.

DEKAN FAKULTETA

Prof. dr Miroslav Vukadinović

Prilog:
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2. **Akt Nastavno-naučnog veća fakulteta o usvajanju izveštaja**
3. **Primedbe date u toku stavljanja izveštaja na uvid javnosti, ukoliko je takvih primedbi bilo.**