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Histological evaluation of periapical tissue response after implantation of experimental nanostructured calcium aluminate cement – *in vivo* study

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SUMMARY

Introduction ALBO-HA (Vinca, Serbia) is new nanostructured calcium aluminate cement, synthesized as a potential alternative to mineral trioxide aggregate (MTA). The purpose of this study was to compare the periapical tissue response to new nanostructured calcium aluminate cement ALBO-HA with white MTA (MTA Angelus, Londrina, Brasil) as root-filling material into the root canal of sheep's teeth.

Material and methods Sixteen mandibular incisors from two 24-month-old sheep were used. Root canals were prepared and filled with ALBO-HA (group 1) or MTA (group 2) (eight teeth per group in each sheep). After four weeks the animals were sacrificed, teeth with surrounding tissue removed, and histologically processed. The sections were analyzed for determination of scores of the following parameters: periapical inflammatory infiltrate, newly mineralized apically formed tissue, apical periodontal ligament space thickness and resorption of dentin, cementum and bone. Data were analyzed statistically (α=0.05) using Mann-Whitney U test.

Results Slight inflammatory infiltrate was observed in 75.0% and 62.5% of samples in the group 1 and group 2, respectively ($p>0.05$). Partial newly mineralized apically formed tissue was found in 75.0% of samples in the group 1, and 87.5% of samples in group 2 ($p>0.05$). No significant difference was noted for periodontal ligament space thickness ($p>0.05$). Resorption of dentin, cementum or bone was not observed.

Conclusion ALBO-HA and white MTA Angelus had a similar effect on inflammation, newly mineralized apical tissue formation and thickness of periodontal ligament space after root canal filling under the present experimental conditions.

Keywords: calcium aluminate; MTA; sheep; periapical tissue response

INTRODUCTION

In the past two decades, mineral trioxide aggregate (MTA) and calcium silicate materials represent the "gold standard", as materials used for a large number of indications in endodontics. Biocompatibility, bioactivity, and sealing ability of MTA made it ideal for use as a root-end filling material, vital pulp therapy, root resorption, treatment of teeth with incomplete apexification, regenerative endodontic procedures and apical plug. However, some of its negative properties such as long setting times, difficulty with manipulating, low-flow capacity and potential for tooth discoloration. Physical and chemical properties of MTA are affected in an acidic environment, which leads to increasing efforts to create an alternative endodontic cement [1, 2].

Technological progress in recent years has led to synthesis of nanostructured materials for use in endodontics. Using the method of nanomodification of existing commercial calcium silicate cements, some researchers tried to improve their properties. What distinguishes nanomaterials from conventional ones is the greater difference between size and mass, as well as the surface reactivity of the particles. Smaller particles, in addition to having high reactivity, promote hydration of material, which favorably affects its faster bonding and hardening [3]. Saghiri et al. reported that nanomodified MTA showed higher microhardness, lower solubility in acidic and basic media compared to MTA, as well as a positive tissue response in an animal model. Also, the addition of 2% tricalcium aluminate to nano MTA affected the improvement of osteoinductive characteristics [4, 5, 6]. Preliminary studies of recently synthesized nanostructured calcium silicate materials indicated the absence of toxicity, good dentinogenic and osteogenic potential [7, 8, 9].

Calcium aluminate-based biomaterials for use in endodontics have been investigated in the last decade and the available scientific literature provides limited data. Calcium aluminate cement Endobinder (Binderware, São Carlos, SP, Brazil) has shown good biological properties, while physical properties and shear bond strength to root dentin are comparable to other commercial materials based on calcium silicate [10, 11, 12]. *In vivo* studies that investigated the effect of calcium aluminosilicate material

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(Quick-Set) and white MTA found that these materials have a similar effect on dentin and cementum formation as well as periapical tissue healing in dogs after pulpotomy and application in root canals [13]. Recently, a new nanostructured cement based on calcium aluminate (ALBO-HA, Vinča, Serbia) was developed in an attempt to use nanotechnology to synthesize a material with improved mechanical characteristics compared to MTA, without impairing biological properties. The material is a mixture of calcium aluminate in combination with calcium carbonate and barium sulfate for radiopacity. It was synthesized by the innovative sol-gel method and self-propagating combustion waves at high temperature. The manufacturer states that this method of synthesis allows for obtaining a specific nanostructure of particles with high activity and improved bonding time through accelerated hydration. Also, the addition of rheological modifiers to the mixture extended the working time and improved the manipulation ability [14]. Initial trials for ALBO-HA indicated similar biocompatibility to MTA [14, 15].

In addition to biocompatibility, one of the basic requirements for endodontic biomaterials that come into direct contact with vital pulp and periodontal tissue for a long period of time is to elicit a positive response and induce histological repairs. Certain components of the material can act as toxins and damage the cells of the periodontium. As a consequence, tissue damage or a prolonged repair process may occur.

The aim of this study was to evaluate the histological response of the periapical tissue after the application of an experimental nanostructured cement based on calcium aluminate (ALBO-HA) in the root canals of sheep teeth.

MATERIAL AND METHOD

The study was approved by the Ethical Commitete of Faculty of Medicine Foca. Experimental research was conducted in cooperation with Faculty of Veterinary Medicine, University of Belgrade. The study was conducted in accordance with the International standards ISO 7405 and ISO 10993-2 [16, 17]. Sixteen mandibular incisors were treated in two Wirtemberg sheep, aged 24 months, on average 50 kg in weight. During the implementation of the experiment, the sheep were kept and fed at a farm with a controlled diet and daily care. Premedication was done with Xylazine (2% Xylazin, CP, Pharma, Bergdorf, Germany) 0.2 mg/kg. After that the animal was introducted in general anesthesia using Ketamine Hydrochloride 10% 7.5 mg/kg i.v. The surgical procedure was performed in aseptic conditions. The teeth were cleaned with 2% chlorhexidine gluconate and class I cavities were prepared on the oral surfaces of mandibular incisors, using round diamond burs. After trepanation, coronal pulp tissue removed using sterile, round, carbide burs. The radicular pulp was removed with pulp extirpator and root canals were instrumented with K files #15-40 (Dentsply Maillefer, Ballaigues, Switzerland) using Crown-down technique, with irrigation with 1.0 % sodium hypochlorite solution. The apical cementum layer was perforated with the sequential use of a size #15-25 K file, in order to create a standardized apical opening for material contact with periapical tissue. A new set of endodontic instruments were used for each animal. After biomechanical preparation, final irrigation was performed with 5 ml 1.0 % sodium hypochlorite and 17.0% EDTA solution. The root canals were dried by suction and sterile paper points and filled with freshly mixed materials. Experimental, nanostructured cement ALBO-HA, was mixed with distilled water in 2:1. Control material, Mineral trioxide aggregate (White MTA, Angelus ® Soluões odontológicas, Londrina, Brazil) was mixed in a 3:1 powder to water ratio, according to manufacturers' instructions. The ALBO-HA was implanted in the four right mandibular incisors, while MTA was implanted in the four left mandibular incisors of the two animals. Into the root canals, materials were applied with a lentulo spiral and compacted by a hand compactor. All cavities were restored with glass-ionomer cement (GC Fuji VIII, GC Corporation, Tokyo, Japan). Postoperatively, the animals were given an analgesic dose of butorphanol (Butorfanol,10mg/ml, Richter Pharma AG Austria), 0.1 mg/kg body weight subcutaneously, for the next three days. After four weeks, the animals were sacrificed by prolonged general anesthesia with Ketamine i.v. and Potassium Chloride intracardiac.

After the removal of soft tissues and separation of the upper and lower jaw, the treatedteeth were cut with a diamond disk and fixed in 10% formalin. Following the decalcification, the tissue was fixed in semi-enclosed benchtop tissue processor (Leica TP1020, Leica Biosystems, Wetzlar, Germany) and then embedded in paraffin blocks. Serial tissue sections 5 μm thick (eight from each sample) were cut from the paraffin blocks. The slides were stained in haematoxylin and eosin, following the standard procedure. The microscopic slides were examined by optical microscopy, using Olympus Cell-B software package and Olympus 5 microscope at magnifications of ×10, ×40, and ×100. The following parameters for histological evaluation of periapical tissue response were evaluated: A. periapical inflammatory infiltrate: absent, slight, moderate, and severe; B. newly mineralized apical formed tissue: complete, partial, thin layer and absent; C. the apical periodontal ligament thickness: normal, slightly increased, moderately increased, and severely increased; D. cementum resorption: absent and present; E. dentin resorption: absent and present; F. bone tissue resorption: absent and present [18].

The statistical analysis was made using the SPSS 20.0 (IBM Corp., Armonk, NY, USA). Statistical analysis of these histologic parameters was performed using the Mann-Whitney U test with a significance level of P = .05.

RESULTS

The results of the histological analysis are shown in table 1. In 6 samples filled with ALBO-HA, slight inflammation was noted in the area of the apex, while in 2 samples moderate inflammation was observed. slight inflammation

Table 1. Histological analysis of periapical tissue after canal root filling for each material
Tabela 1. Histološka analiza periapikalnog tkiva posle punjenja korenskih kanala za oba materijala

	ALBO-HA n (%)	MTA n (%)	p value
Inflamation Zapaljenje			
Absent Bez zapaljenja	0 (0)	0 (0)	> 0.05
Slight Blago	6 (75)	5 (62.5)	
Moderate Umereno	2 (25)	3 (37.5)	
Severe Teško	0 (0)	0 (0)	
Newly calcified apical formed tissue Novostvoreno kalcifikovano tkivo			
Complete Kompletno	0 (0)	0 (0)	> 0.05
Partial Parcijalno	3 (37.5)	2 (25)	
Thin layer Tanak sloj	3 (37.5)	5 (62.5)	
Absent Odsutno	2 (25)	1 (12.5)	
Periodontal ligament space Širina periodontalnog ligamenta			
Normal Normalna	2 (25)	1 (12.5)	> 0.05
Slightly increased Blago povećana	4 (50)	6 (75)	
Moderately increased Umereno povećana	2 (25)	1 (12.5)	
Severely increased Izrazito povećana	0 (0)	0 (0)	
Dentin resorption Resorpcija dentina			
Absent Odsutna	8 (100)	8 (100)	> 0.05
Present Prisutna	0 (0)	0 (0)	
Cementum resorption Resorpcija cementa			
Absent Odsutna	8 (100)	8 (100)	> 0.05
Present Prisutna	0 (0)	0 (0)	
Bone resorption Resorpcija kosti			
Absent Odsutna	8 (100)	8 (100)	> 0.05
Present Prisutna	0 (0)	0 (0)	

n – number of samples
n – broj uzoraka

was observed in 5 root samples filled with MTA, and moderate inflammation in 3 samples. Partially calcified tissue, as well as a thin layer of calcified tissue, were observed in 3 samples of 1st group, while in the 2nd group of samples, the formation of partially calcified tissue was observed in 2 samples, that is, in 2 samples a thin layer of calcified tissue was observed. The periodontal ligament space thickness was slightly increased in half of the samples of group 1, that is, in 75% of the samples of group 2. Dentin, cementum, and alveolar bone resorptions were not recorded in any sample for both tested materials. Statistical analysis of the obtained data did not reveal any significant difference between the examined groups in relation to the observed parameters.

DISCUSSION

Unlike *in vitro* tests on cell cultures, research conducted on experimental animals provides better data on the biocompatibility of materials, as well as different forms of the organism's biological response to the tested material [19]. In *in vivo* studies proteins, tissue fluids and immune system factors can reduce the toxic effect of the material [20]. The histological reaction of soft tissues to a biomaterial is a frequently used method for assessing biocompatibility as well as tissue irritation caused by the material. Wirtemberg sheep were used as an animal model in this study. The advantage of larger animal models for dental research lies in the fact that they can be more relevant, as surgical procedures can be performed using identical clinical instruments used on human teeth [21]. Examination of the anatomy of sheep teeth showed that the length of the roots, the thickness of the dentin and the diameter of the apical foramen are comparable to the same structures on human teeth. The 2-year age of the animals was chosen due to the fact that at this age a larger number of teeth can be used for research, as well as the diameter of the apical foramen of 1 mm width and more can simulate teeth with an open apex [22]. MTA is characterized by high biocompatibility, osteoinductive properties, slow release of Ca^{2+} ions and alkaline pH, as well as exceptional hydrophilicity, which enables use in clinical conditions in a humid environment [1, 2]. For the reasons stated before, MTA was used, in this study, as a control material.

The results of the study showed that the inflammatory reaction of the periapical tissue after the implementation of the tested materials was assessed as mild in the largest number of samples of both groups and is in accordance with the results of previous research, in which materials of similar chemical composition were used and applied to the root canals of experimental animals. Although there was no statistically significant difference in the intensity of inflammation between the tested materials, calcium aluminate produced a slightly lower inflammatory response. Continuous calcified tissue was not observed, while the formation of a thin layer of discontinuous calcified tissue with foci of vascular fibroblastic proliferation was observed in the majority of samples of examined materials in the area of the root apex. The appearance of tissue formed in this way with mild inflammation in the short time interval of observation used in this research represents a positive result and confirmation that the tested materials have inductive potential. The assumption is that over time there could be further mineralization and the formation of regular continuous mineralized tissue with the disappearance of fibroblastic foci. Both investigated materials

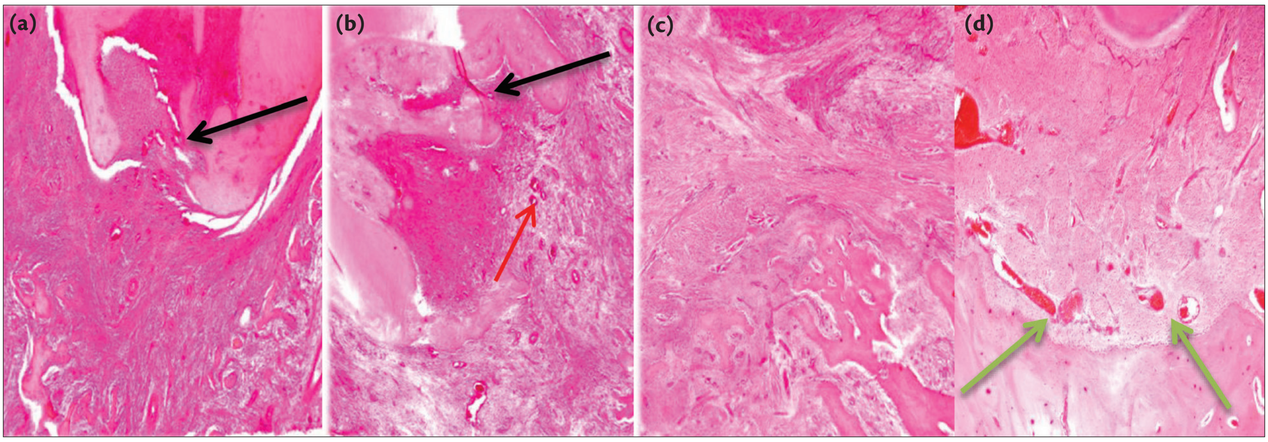

Figure 1. Material ALBO-HA
(a) Discontinuous newly formed calcified tissue of the root apex (black arrow), Mesenchymal cells with osteoblastic differentiation on the periphery of the calcified tissue
(b) thin layer of calcified tissue with connective vascular tissue, Slight inflammatory reaction of root apex (red arrow)
(c) No significant vital reaction of the bone tissue is observed in the area root apex, (d) chronic proliferative inflammation of a moderate intensity with signs of angiogenesis and acute hyperemia of the root apex (green arrows) (HE ×40).
Slika 1. Materijal ALBO-HA
(a) Na preparatu se u predelu apeksa zuba uočava hipercelularno diskontinuirano novostvoreno, kalcifikovano tkivo (crna strelica). U neposrednoj okolini uočavaju se mezenhimalne ćelije sa osteoblastnom diferencijacijom.
(b) Tanak sloj kalcifikovanog tkiva prožetog fokusima vezivno-vaskularnog tkiva. Uočava se blaga inflamatorna reakcija sa elementima hroničnog zapaljenja (crvena strelica).
(c) Ne uočava se značajna vitalna reakcija koštanog tkiva u predelu apeksa zuba.
(d) Hronično proliferativno zapaljenje umerenog intenziteta u predelu apeksa sa znacima neoangiogeneze i akutne hiperemije (zelene strelice) (HE, ×40).

belong to the group of bioactive materials characterized by the release of calcium ions during binding. In the process of hydration of calcium aluminate cement, calcium aluminate (CA) hydrate and aluminium hydroxide hydrate are formed. The release of Ca^{2+} ions is the result of the decomposition of CA hydrate, whose decomposition during the hydration process leads to the release of $Al(OH)_4$ and OH^- ions [14]. The continuous release of these biologically active ions induces the formation of calcified tissue, regulates the proliferation, differentiation and mineralization of cells. Calcium-releasing materials can also induce the proliferation of periodontal fibroblasts osteoblasts and cementoblasts [23, 24]. Due to the biological decomposition of materials in contact with tissue fluids, the released ions create an alkaline environment. On the other hand, in an alkaline environment, the neutralization of lactic acid released from osteoclasts occurs. In this way, the dissolution of the mineral components of dentin is prevented. The alkaline environment leads to the activation of alkaline phosphatase, which plays a significant role in the processes of creating hard dental tissues. Calcium aluminate cement was found to stimulate the expression of RUNX2, alkaline phosphatase, bone sialoprotein and osteopontin which are biochemical markers of mineralization. The RUNX2 gene encodes a transcription factor that plays a key role in the differentiation of pluripotent mesenchymal cells into osteoblasts [25]. Also, it was confirmed that the nanostructured surface of the material affects the behavior and activity of cells in terms of increased osteoblastic adhesion, proliferation and differentiation [26].

The research results for ALBO-HA cannot be directly compared with those from other studies due to the lack of relevant literature, because it is a new material whose application in endodontics is still at the level of preclinical and *in vivo* experiments on animals. However, the induction of mineralized tissue after the application of calcium aluminate-based materials to the dental pulp and root canal of experimental animals is consistent with the results of other studies [13, 27]. Kramer et al. investigated pulp inflammatory reaction, dentin bridge formation in rats after pulpotomy with calcium aluminosilicate cement (Quick-Set). ProRoot MTA and MTA Plus were used as control materials. The inflammatory response of the covered pulp measured by the quantification of inflammatory cytokines, interleukins IL-1α and IL-1β was approximately the same in all three treated materials. Dentin bridges were observed already 30 days after pulpotomy in all materials, while pulp vitality was preserved even after 60 days [28]. In a study by Kohout et al. it was determined that Quick set and white MTA after apicotomy and application to the roots of Beagle dogs had a similar effect on tissue healing promoting regeneration, periodontal ligament formation and cement deposition on the periodontal side of the material [13]. In a more recent study, Walsh et al. investigated the dentinogenic and osteogenic potential of modified variants of the materials used in the previous two mentioned studies in contact with the pulpal and periradicular tissue of Beagle dogs. After an observation period of 90 days, healing of the pulp and periapical tissue with the formation of a dentine bridge and cementum deposition was reported under both materials (Quick-Set 2 and NeoMTA plus) [29].

Calcium aluminate cement synthesized for use in endodontics has shown good results in the available literature in terms of biocompatibility after subcutaneous and intraosseous implementation in experimental animals

[10]. Moraes et al. indicated the possibility of using calcium aluminate as a scaffold and biomembrane in bone regeneration [30]. The biocompatibility of nanostructured calcium aluminate with the same composition as the material used in this research was examined after subcutaneous implementation in the subcutaneous tissue of rats. After observation periods of 7, 15 and 30 days, the mutual relationship between inflammation and the thickness of the fibrous capsule was observed. It was observed that with the reduction of inflammation during the observation period, there is an increase in the thickness of the fibrous capsule around the implemented materials, which indicates a good tissue tolerance to these materials [31].

CONCLUSION

Using nanostructured ALBO-HA and MTA in this study, it was observed that both materials cause a similar inflammatory effect and ability to induce calcified tissue after 30 days on sheep teeth. The favorable biological response of the tissue after the application of the experimental calcium aluminate cement represents a good basis for its further testing in *in vivo* conditions and possible modifications in the composition in order to improve its physical and biological characteristics.

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Histološka analiza reakcije periapikalnog tkiva posle implantacije eksperimentalnog nanostrukturnog cementa na bazi kalcijum-aluminata – *in vivo* studija

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KRATAK SADRŽAJ

Uvod ALBO-HA (Vinča, Srbija) novi je eksperimentalni nanostrukturni cement na bazi kalcijum-aluminata sintetisan kao moguća alternativa komercijalnom mineralnom trioksidnom agregatu (MTA). Cilj ovog rada bio je da se proceni histološka reakcija periapikalnog tkiva posle implantacije nanostrukturnog cementa na bazi kalcijum-aluminata i MTA (MTA Angelus) u kanale korenova zuba ovaca.

Materijal i metode U studiji je korišćeno ukupno 16 mandibularnih sekutića dve ovce starosti oko 24 meseca. Korenski kanali su hemomehanički obrađeni primenom krunično-apeksne tehnike i ispunjeni sa ALBO-HA (grupa 1) i MTA (grupa 2) (po osam zuba za svaki materijal). Posle četiri nedelje životinje su eutanazirane, a zubi sa okolnim tkivom odsečeni i pripremljeni za histološku analizu. Posmatrani su sledeći parametri: intenzitet inflamacije, apikalno novostvoreno kalcifikovano tkivo, širina periodontalnog ligamenta i resorpcija dentina cementa i kosti. Podaci su analizirani statistički ($\alpha = 0,05$) korišćenjem Man–Vitnijevog U testa.

Rezultati Blaga zapaljenska reakcija uočena je kod 75% uzoraka grupe 1, odnosno 62,5% uzoraka grupe 2 ($p > 0,05$). Novostvoreno diskontinuirano kalcifikovano tkivo u predelu apeksa je uočeno kod 75% uzoraka u grupi 1, dok je u drugoj grupi taj procenat iznosio 87,5%. Nije zabeležena značajna razlika za širinu prostora periodontalnog ligamenta između grupa ($p > 0,05$). Resorpcija dentina, cementa ili kosti nije uočena ni u jednom uzorku.

Zaključak ALBO-HA i MTA imali su sličan učinak na pojavu inflamacije, formiranje novog kalcifikovanog apikalnog tkiva i debljinu periodontalnog ligamentnog prostora posle punjenja korenskih kanala zuba ovaca u eksperimentalnim uslovima.

Ključne reči: kalcijum-aluminat; MTA; ovca; reakcija periapikalnog tkiva

UVOD

U protekle dve decenije mineralni trioksidni agregat (MTA) i kalcijum-silikatni materijali predstavljaju „zlatni standard“ kao materijali koji se koriste u velikom broju indikacija u endodonciji. Biokompatibilnost, bioaktivnost i sposobnost zaptivanja čine MTA idealnim za upotrebu kao materijala za punjenje kanala korena, za terapiju vitalne pulpe, lečenje korenskih perforacija i apeksifikaciju. Takođe se koristi tokom regenerativnih endodontskih procedura i kao apikalni čep posle endodontske hirurgije. Međutim, neka od njegovih negativnih svojstava, kao što su dugo vreme stvrdnjavanja, poteškoće tokom manipulacije, mogućnost promene boje zuba, kao i izmena fizičkih i hemijskih karakteristika samog materijala u kiseloj sredini, doveli su do povećane potrebe za stvaranjem alternativnih endodontskih cemenata [1, 2].

Tehnološki napredak poslednjih godina doveo je do sinteze nanostrukturnih materijala za primenu u endodonciji. Metodom nanomodifikacije postojećih komercijalnih kalcijum-silikatnih cemenata neki istraživači su pokušali da poboljšaju njihova svojstva. Ono što razlikuje nanomaterijale u odnosu na konvencionalne jeste veća razlika između veličine i mase, kao i površinska reaktivnost čestica. Čestice manje veličine, pored toga što poseduju veliku reaktivnost, pospešuju i hidrataciju materijala, što povoljno utiče na njegovo brže vezivanje i očvršćavanje [3]. Saghiri i saradnici su pokazali da je nanomodifikovani MTA pokazao veću mikrotvrdoću, manju rastvorljivost u kiseloj i baznoj sredini u odnosu na MTA, kao i pozitivan odgovor tkiva na animalnom modelu. Takođe, dodatak 2% trikalcijum-aluminata u nano MTA uticao je na poboljšanje osteoinduktivnih karakteristika [4, 5, 6]. Preliminarna istraživanja nedavno sintetisanih nanostrukturnih kalcijum-silikatnih materijala ukazala su na odsustvo toksičnosti, dobar dentinogeni i osteogeni potencijal [7, 8, 9].

Biomaterijali na bazi kalcijum-aluminata za primenu u endodonciji se ispituju u poslednjoj deceniji i malo je dostupnih podataka u naučnoj literaturi. Kalcijum-aluminatni cement Endobinder (Binderware, São Carlos, SP, Brazil) pokazao je dobre biološke osobine, dok su fizička svojstva i adhezivnost za dentin komparabilna sa drugim komercijalnim materijalima na bazi kalcijum-silikata [10, 11, 12]. U *in vivo* studijama u kojima je ispitivan efekat kalcijum-aluminosilikatnog materijala (Quick-Set) i belog MTA utvrđeno je da ovi materijali imaju sličan efekat na formiranje dentina i cementa, kao i zarastanje periapikalnog tkiva kod pasa posle pulpotomije i aplikacije u korenske kanale [13]. Nedavno je razvijen novi nanostrukturni cement na bazi kalcijum-aluminata (ALBO-HA, Vinča, Srbija) u pokušaju da se primenom nanotehnologije sintetiše materijal sa poboljšanim mehaničkim karakteristikama u odnosu na MTA, bez narušavanja bioloških osobina. Materijal predstavlja smešu kalcijum-aluminata u kombinaciji sa kalcijum-karbonatom i barijum-sulfatom kao rendgenskim kontrastnim sredstvom. Sintetisan je inovativnom sol-gel metodom i metodom samosagorevajućih talasa na visokoj temperaturi. Proizvođač navodi da ovakav način sinteze omogućava dobijanje specifične nanostrukture čestica visoke aktivnosti, uz poboljšano vreme vezivanja, kroz ubrzanu hidrataciju. Takođe, dodavanje reoloških modifikatora u smešu produžilo je radno vreme i poboljšalo sposobnost manipulacije [14]. Inicijalna ispitivanja za ALBO-HA ukazala su na sličnu biokompatibilnost sa MTA [14, 15].

Pored biokompatibilnosti, jedan od osnovnih zahteva za endodontske biomaterijale koji dolaze u neposredan kontakt sa vitalnim pulpnim i periodontalnim tkivom tokom dužeg perioda je da izazovu pozitivan odgovor i da indukuju histološke reparacije. Pojedine komponente materijala mogu delovati kao toksini i oštetiti ćelije periodoncijuma. Kao posledica toga može doći do oštećenja tkiva ili prolongiranja procesa reparacije.

Cilj ovog rada je bio da se proceni histološki odgovor periapeksnog tkiva posle aplikacije eksperimentalnog nanostrukturnog cementa na bazi kalcijum-aluminata (ALBO-HA) u kanale korena zuba ovaca.

MATERIJAL I METODE

Dozvola za sprovođenje istraživanja dobijena je od Etičkog komiteta Medicinskog fakulteta u Foči. Istraživanje je sprovedeno u saradnji sa Veterinarskim fakultetom Univerziteta u Beogradu. Eksperiment je sproveden u skladu sa međunarodnim standardima ISO 10993-2 i ISO 7405 [16, 17]. U studiju je uključeno 16 kanala korenova donjih sekutića kod dve ovce (ovis aries) rasa Virtemberg, starosti oko 24 meseca i prosečne težine oko 50 kg. Životinje su tokom trajanja eksperimenta bile smeštene u farmskim uslovima, u kontrolisanoj sredini, sa kontrolisanom ishranom uz svakodnevnu profesionalnu negu. Kod životinja je izvršena sedacija ksilazinom (2% Xylazin, CP, Pharma, Bergdorf, Germany) u dozi od 0,2 mg/kg telesne težine. Opšta anestezija postignuta je sa ketamin-hidrohloridom 10% u dozi od 7,5 mg/kg telesne težine, intravenski. Hirurška procedura je sprovedena u aseptičnim uslovima. Zubi su očišćeni 2% hlorheksidin-glukonatom. Okruglim dijamantskim borerom formirani su pristupni kaviteti prve klase na oralnoj površini sekutića. Sterilnim okruglim karbidnim borerom uklonjen je krov pulpe i koronarna pulpa. Korenski deo pulpe je uklonjen pulp-ekstirpatorima, a kanali obilno isprani 1% natrijum-hipohloritom (NaOCl). Kanali korena su zatim prošireni ručnim K turpijama ♯15-40 (Dentsply Maillefer, Ballaigues, Switzerland) krunično apeksnom tehnikom (Crown-down) do apeksnog suženja, uz ispiranje rastvorom 1% NaOCl između instrumenata. Instrumentima ♯15-25 prošireno je apeksno suženje kako bi se obezbedio kontakt materijala sa periodontalnim tkivom. Novi set endodontskih instrumenata je korišćen za svaku životinju. Posle mehaničke obrade finalna irigacija obavljena je sa 5 mL 1% NaOCl i 17% EDTA u trajanju od 1 minut, kanali su posušeni sterilnim papirnim poenima i napunjeni sveže zamešanim materijalima po uputstvu proizvođača. Eksperimentalni nanostrukturni kalcijum-aluminatni cement ALBO-HA je zamešan sa destilovanom vodom u omeru 2 : 1. Kontrolni materijal MTA (White MTA Angelus, Londrina, Brazil) zamešan je u omeru prah–tečnost 3 : 1 prema uputstvu proizvođača. Materijali su u kanale korena unešeni lentulo-spiralom. ALBO-HA je aplikovan u četiri desna mandibularna sekutića (grupa 1), dok je MTA aplikovan u četiri leva mandibularna sekutića (grupa 2) kod obe ovce. Višak materijala je uklonjen na ulazu u korenski kanal, a zatim je pasta komprimovana vertikalnom kondenzacijom pomoću endodontskog plugera. Pristupni kaviteti su zatvoreni svetlosno polimerizujućim glasjonomernim cementom (GC Fuji VIII, GC Corp, Tokyo, Japan). Posle završene hirurške procedure životinje su primale analgetik butorfanol (Butorfanol, 10 mg/ml, Richter Pharma AG Austria) u dozi od 0,1–0,2 mg/kg/tm i.m u naredna tri dana. Posle opservacionog perioda od 30 dana, životinje su eutanazirane produženom anestezijom ketaminom i.v i kalijum-hloridom intrakardijalno.

Posle uklanjanja mekih tkiva zubi su isečeni dijamantskom šajbnom i fiksirani u 10% formalinu i dekalcifikovani. Nakon toga je tkivo fiksirano u kružnom tkivnom procesoru (Leica TP 1020, Germany), a zatim kalupljeno u parafinske blokove. Iz parafinskih kalupa sečeni su serijski tkivni preseci (sa svakog uzorka po osam) debljine 5 μm. Preparati su standardno bojeni hematoksilin-eozin (HE) bojenjem. Mikroskopski preparati su analizirani optičkom mikroskopijom uz primenu programa za morfometriju Software «Cell-B» by Olympus, mikroskopom Olympus 5, na uveličanjima ×10, ×40 i ×100. Kao histološki kriterijumi za procenu reakcije periapeksnog tkiva korišćeni su: A. zapaljenje (bez zapaljenja, blago, umereno, teško); B. novostvoreno kalcifikovano tkivo (kompletno, parcijalno, tanak sloj, bez); C. širina periodontalnog ligamenta (normalna, blago proširen, umereno proširen, izrazito proširen); D. resorpcija dentina (odsutna, prisutna); E. resorpcija cementa (odsutna, prisutna); F. resorpcija kosti (odsutna, prisutna) [18].

Statistička obrada podataka urađena je u programu SPSS 20.0 (IBM Corp., Armonk, NY, USA). Za poređenje razlika u rezultatima histoloških analiza između grupa korišćen je Man–Vitnijev test.

REZULTATI

Rezultati histološke analize prikazani su u Tabeli 1. Kod šest uzoraka ispunjenih sa ALBO-HA zabeležena je blaga inflamacija u predelu apeksa, dok je kod dva uzorka uočeno umereno zapaljenje. Kod korenova ispunjenih sa MTA u pet uzoraka je uočeno blago zapaljenje, a kod tri uzorka umereno zapaljenje. Parcijalno kalcifikovano tkivo, kao i tanak sloj kalcifikovanog tkiva uočeni su u po tri uzorka grupe 1, dok je u grupi 2 kod dva uzorka uočeno formiranje parcijalnog kalcifikovanog tkiva, odnosno kod dva uzorka tanak sloj kalcifikovanog tkiva. Širina periodontalnog ligamenta bila je blago uvećana kod polovine uzoraka grupe 1, odnosno kod 75% uzoraka grupe 2. Resorpcije dentina, cementa i alveolarne kosti nisu zabeležene ni u jednom uzorku za oba ispitivana materijala. Statističkom analizom dobijenih podataka nije uočena značajna razlika između ispitivanih grupa u odnosu na posmatrane parametre.

DISKUSIJA

Za razliku od *in vitro* ispitivanja na ćelijskim kulturama, istraživanja koja se sprovode na eksperimentalnim životinjama daju bolje podatke o biokompatibilnosti materijala, kao i različitim oblicima biološkog odgovora organizma na ispitivani materijal [19]. U *in vivo* studijama proteini, tkivne tečnosti i faktori imunog sistema mogu da smanje toksični efekat materijala [20]. Histološka reakcija mekih tkiva na biomaterijal je često korišćena metoda za procenu biokompatibilnosti, kao i iritacije tkiva od strane materijala. Kao animalni model u ovom istraživanju korišćene su ovce rase Virtemberg. Prednost većih modela životinja za dentalna ispitivanja je u činjenici da mogu biti relevantniji, jer se operativni zahvati mogu sprovesti upotrebom identičnih kliničkih instrumenata koji se koriste na humanim zubima [21].

Ispitivanja anatomije zuba ovaca pokazala su da su dužina korenova, debljina dentina i dijametar apikalnog foramena uporedivi sa istim strukturama na humanim zubima. Dvogodišnja starost životinja je odabrana zbog činjenice da se u ovom uzrastu može koristiti veći broj zuba za istraživanje, kao i da prečnik apikalnog foramena širine od 1 mm i više može simulirati zube sa otvorenim apeksom [22]. MTA odlikuju visoka biokompatibilnost, osteoinduktivna svojstva, sporo oslobađanje jona Ca^{2+} i alkalni pH, zatim izuzetna hidrofilnost, koja omogućava korišćenje u kliničkim uslovima u vlažnoj sredini [1, 2]. Iz navedenih razloga, u ovoj studiji MTA je korišćen kao kontrolni materijal.

Rezultati studije pokazali su da je zapaljenska reakcija periapeksnog tkiva posle implementacije ispitivanih materijala ocenjena kao blaga u najvećem broju uzoraka obe grupe i u skladu je sa rezultatima prethodnih istraživanja, u kojima su korišćeni materijali sličnog hemijskog sastava aplikovani u korenske kanale eksperimentalnih životinja. Iako u pogledu intenziteta inflamacije nije bilo statistički značajne razlike između testiranih materijala, kalcijum-aluminat je proizveo nešto niži inflamatorni odgovor. Kontinuirano kalcifikovano tkivo nije uočeno, dok je u većini uzoraka ispitivanih materijala u predelu apeksa zuba uočeno formiranje tankog sloja diskontinuiranog kalcifikovanog tkiva sa fokusima vaskularne fibroblastne proliferacije. Pojava ovako formiranog tkiva uz blagu inflamaciju u kratkom vremenskom intervalu opservacije koji je korišćen u ovom istraživanju predstavlja pozitivan rezultat i potvrdu da ispitivani materijali poseduju induktivni potencijal. Pretpostavka je da bi tokom vremena moglo doći do dalje mineralizacije i formiranja regularnog kontinuiranog mineralizovanog tkiva uz iščezavanje fibroblastnih fokusa. Oba ispitivana materijala pripadaju grupi bioaktivnih materijala koje karakteriše oslobađanje jona kalcijuma tokom vezivanja. U procesu hidratacije kalcijum-aluminatnog cementa dolazi do stvaranja kalcijum-aluminatnog (CA) hidrata i aluminijum-hidroksid hidrata. Oslobađanje jona Ca^{2+} je rezultat razlaganja CA hidrata, čijom razgradnjom tokom hidratacionog procesa dolazi i do oslobađanja $Al(OH)_4$ i OH^- jona [14]. Kontinuirano oslobađanje ovih bioloških aktivnih jona indukuje formiranje kalcifikovanog tkiva, reguliše proliferaciju, diferencijaciju i mineralizaciju ćelija. Materijali koji oslobađaju kalcijum takođe mogu izazvati proliferaciju parodontalnih fibroblasta osteoblasta i cementoblasta [23, 24]. Zbog biološke razgradnje materijala u kontaktu sa tkivnim tečnostima oslobođeni joni stvaraju alkalno okruženje. S druge strane, u alkalnom okruženju dolazi do neutralizacije mlečne kiseline oslobođene iz osteoklasta. Na taj način je sprečeno rastvaranje mineralnih komponenata dentina. Alkalna sredina dovodi do aktiviranja alkalne fosfataze, koja ima značajnu ulogu u procesima stvaranja tvrdih zubnih tkiva. Za kalcijum-aluminatni cement je utvrđeno da stimuliše ekspresiju RUNX2, alkalne fosfataze, koštanog sijaloproteina i osteopontina, koji predstavljaju biohemijske markere mineralizacije. RUNX2 gen kodira transkripcioni faktor koji ima ključnu ulogu u diferencijaciji pluripotentnih mezenhimalnih ćelija u osteoblaste [25]. Takođe, potvrđeno je da nanostrukturna površina materijala utiče na ponašanje i aktivnost ćelija u smislu povećane osteoblastne adhezije, proliferacije i diferencijacije [26].

Rezultati istraživanja za ALBO-HA ne mogu se izravno uporediti sa onima iz drugih studija zbog nedostatka relevantne literature, jer se radi o novom materijalu čija je primena u endodonciji još na nivou pretkliničkih i *in vivo* eksperimenata na životinjama. Međutim, indukcija mineralizovanog tkiva posle aplikacije materijala u čijoj osnovi se nalazi kalcijum-aluminat na zubnu pulpu i u korenski kanal eksperimentalnih životinja u skladu je sa rezultatima drugih studija [13, 27]. Kramer i saradnici su ispitivali inflamatornu reakciju pulpe, formiranje dentinskog mosta kod pacova posle pulpotomije sa kalcijum-aluminosilikatnim cementom (Quick-Set). Kao kontrolni materijali korišćeni su ProRoot MTA i MTA Plus. Inflamatorni odgovor prekrivene pulpe meren kvantifikacijom inflamatornih citokina, interleukina IL-1α i IL-1β bio je približno isti kod sva tri tretirana materijala. Dentinski mostići uočeni su već nakon 30 dana od pulpotomije kod svih materijala, dok je vitalnost pulpe bila očuvana i nakon 60 dana [28]. Kohout i saradnici su u svojoj studiji utvrdili je da su Quick set i beli MTA posle apikotomije i aplikacije u korenove pasa rase Beagle imali sličan efekat na zarastanje tkiva pospešujući regeneraciju, formiranje periodontalnog ligamenta i depoziciju cementa na periodontalnoj strani materijala [13]. U novijoj studiji Walsh i saradnici su ispitivali dentinogeni i osteogeni potencijal modifikovanih varijanti materijala korišćenih u prethodne dve navedene studije u kontaktu sa pulpnim i periradikularnim tkivom Beagle pasa. Nakon opservacionog perioda od 90 dana, ispod oba materijala (Quick-Set 2 i NeoMTA plus) došlo je do izlečenja pulpe i periapeksnog tkiva formiranjem dentinskog mosta i depozicijom cementa [29].

Kalcijum-aluminatni cement sintetisan za primenu u endodonciji je u dostupnoj literaturi pokazao dobre rezultate u pogledu biokompatibilnosti posle supkutane i intraosealne implementacije kod eksperimentalnih životinja [10]. Moraes i saradnici su ukazali i na mogućnost primene kalcijum-aluminata kao skafolda i biomembrane u koštanoj regeneraciji [30]. Biokompatibilnost nanostrukturnog kalcijum-aluminata identičnog sastava kao i materijal korišćen u ovom istraživanju, ispitivana je posle potkožne implementacije u supkutano tkivo pacova. Nakon opservacionih perioda od 7, 15 i 30 dana posmatran je uzajamni odnos zapaljenja i debljine fibrozne kapsule. Uočeno je da sa smanjenjem zapaljenja tokom perioda opservacije dolazi do porasta debljine fibrozne kapsule oko implementiranih materijala, što ukazuje na dobru toleranciju tkiva na ove materijale [31].

ZAKLJUČAK

Primenom nanostrukturnog ALBO-HA i MTA u ovom istraživanju uočeno je da oba materijala izazivaju sličan inflamatorni efekat i sposobnost indukcije kalcifikovanog tkiva posle 30 dana na zubima ovaca. Povoljan biološki odgovor tkiva posle aplikacije eksperimentalnog kalcijum-aluminatnog cementa predstavlja dobru osnovu za njegova dalja ispitivanja u *in vivo* uslovima i eventualne modifikacije u sastavu u cilju poboljšanja fizičkih i bioloških karakteristika.