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HISTOLOGICAL EVALUATION OF PERIRADICULAR TISSUE INFLAMMATORY REACTIONS AND CALCIFIED TISSUE FORMATIONS AFTER IMPLANTATION OF EXPERIMENTAL CALCIUM SILICATE AND HYDROXYAPATITE BASED NANOSTRUCTURAL CEMENTS INTO ROOT CANALS OF RABBITS TEETH

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The aim of the study was to evaluate inflammatory tissue reactions and the formation of calcified tissue after implantation of experimental nanostructured calcium silicate cement (CS) and hydroxyapatite with calcium silicate cement (HA-CS) into root canals of rabbits' teeth. The study was conducted on four rabbits of the genus Oryctolagus cuniculus. After instrumentation and irrigation, the root canals of the central incisors were dried and filled with CS, HA-CS and control material (MTA Angelus). The animals were sacrificed after 28 days. After histological preparation and hematoxylin-eosin staining, tissue samples were evaluated for the intensity and extension of inflammatory tissue reaction; continuity, morphology and thickness of the newly formed calcified tissue; and presence of giant cells, materials particles and microorganisms. Kruskal Wallis and Dunn's post hoc test were used for data analysis (α=0.05). There were no significant differences in the intensity of inflammatory reactions between CS, HA-CS and MTA control. HA-CS showed significantly better results than MTA and CS with respect to continuity of the newly formed calcified tissue (P=0.003 and P=0.010, respectively). Significant differences in thickness of the calcified tissue existed between CS and MTA (P=0.004) and between HA-CS and MTA (P=0.012). Application of CS and HA-CS resulted in minimal inflammatory tissue response, similar to the MTA control. CS and HA-CS were more efficient than MTA in supporting hard tissue formation. The best organized newly formed calcified tissue was seen after HA-CS application.

Keywords: biocompatibility; calcium silicate; hard tissue formation; hydroxyapatite.

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INTRODUCTION

Calcium silicate cements are superior to most endodontic materials, taking into account their biocompatibility, bioactivity and sealing properties. These cements promote the formation of dentin, cementum, bone and regeneration of the periodontal ligamentum. They are considered to be the materials of choice for numerous clinical indications such as pulp capping, root end closure in apical surgery, treatment of root perforations, apexifications and pulpotomies [1,2]. Good biological properties of calcium silicate cements are related to calcium hydroxide, which is released during the material setting and stimulates the proliferation and differentiation of different cells response for tissue repair [3-5]. The first generation of commercial calcium silicate cements known as MTAs cements, has a long initial setting time (3h), as a result of their chemical composition and hygroscopic nature [6] and contain a trace of heavy metals as a result of the manufacturing by purifying and modification of Portland cement [7]. Also, these cements usually contain bismuth oxide as radiopacifier, which interferes with the hydration processes of cement [8] and may react with dentin collagen resulting in tooth staining [9]. Newer generations of MTAs cements have a shorter initial setting time, as a result of changes in chemical composition and contain non toxic radiopacifiers such as zirconium oxide [10].

Hydroxyapatite, despite biocompatibility and bioconductivity did not find its place in endodontic therapy, mainly because of inappropriate mechanical properties. Combinations of hydroxyapatite with calcium silicate were initially promising, since these composite cements showed improved mechanical properties and bioactivity, compared to pure calcium phosphate cements [11,12].

Technological progress has led to the synthesis of nanostructured materials. Distinct activity of nano particles enhances hydration of nanostructured calcium silicate cements, improving their hardening and setting as well as physical and chemical properties [13,14]. Still, there is concern about the biological behavior of these materials since nano particles are typically deposited in the mitochondria, causing structural cell damage. According to the available literature data, a commercial nanostructured calcium silicate cement with added hydroxyapatite (BioAggregate, Innovative bioceramix, Vancouver, BC, Canada) shows a similar toxicity in cell cultures [15], but lower systemic toxicity [16] compared to commercial microstructural MTAs, which was related to differences in manufacturing and consequently differences in heavy metals content.

Recently, two new nanostructured cements have been developed in an attempt to synthetize materials with good biological properties, short setting time and without heavy metals and bismuth oxide. The first cement (CS) is based on dicalcium and tricalcium silicate and the other (HA-CS) is a mixture of hydroxyapatite with CS, in 2:1 ratio. According to Jokanović et al. [17], CS was synthetized using hydrothermal sol-gel methodology and self-propagating combustion waves. Hydroxyapatite was synthetized by a hydrothermal method. Both materials contain barium sulphate for

radiopacity. Enhanced nanotechnology used in its synthesis resulted in the short setting time of CS and HA-CS (10 minutes and 15 minutes, respectively). Also, addition of rheological modifiers prolonged working time and enhanced handling properties of these materials. Initial *in vitro* testing of CS and HA-CS has shown the absence of toxic effects on human cells in terms of genotoxicity [18]. Implantation of these cements in subcutaneous rat tissue or as pulp capping materials in rabbits' teeth resulted in minimal inflammatory response, confirming their biocompatibility [19, 20].

The aim of this study was to evaluate periradicular tissue inflammatory reactions and the formation of calcified tissue after implantation of CS and HA-CS into root canals of rabbits' teeth.

MATERIALS AND METHODS

Experimental animals

Experiments were conducted at the Faculty of Veterinary Medicine, University of Belgrade. The research protocol was approved by the Ethical Committee of the School of Dental Medicine, University of Belgrade, Serbia (Protocol No.36/21/2013). Four rabbits of the genus *Oryctolagus cuniculus*, aged 12 months and average weight of 4 kg were included in the study. The study was performed in accordance with ISO 10993-2 (Animal welfare requirements) and ISO 7405 [21,22].

Experimental procedure

The animals were kept in standard, individual cages, given *ad libitum* access to standard rabbit feed and water and daily monitored during the experiment. Before the surgical procedure, the animals were introduced into general anesthesia by Xylazine (2% Xylazine, Check Republic) 35 mg/kg body weight and Ketamidor (100% Ketamidor 100 mg/ml, Richter Pharma AG, Austria) 5 mg/kg body weight. The surgical procedure was performed in aseptic conditions. The working field was disinfected with 5% iodine tincture and class I cavities were prepared in the upper and lower central incisors using round diamond burs. Access to the cavity was prepared and coronal pulp tissue removed using sterile, round, carbide burs. After extirpation of the radicular pulp, root canals were instrumented with K files #40 (VDW Gmbh, Germany), and irrigated with 5 ml of saline between each instrument. A new set of endodontic instruments was used for each animal. Then, the canals were dried with paper points and filled with freshly mixed materials. Experimental, nanostructured cements, CS and HA-CS were mixed with distilled water in 2:1 [17]. Control material, Mineral trioxide aggregate (White MTA, Angelus ® Solu□ ões odontológicas, Londrina , Brazil) was mixed in a 3:1 powder to water ratio, according to manufacturers's instructions. The control material was implanted in the right maxillary incisors of all four animals. CS was implanted in the left maxillary incisors and both mandibular incisors of the two animals, and HA-CS was implanted in the left maxillary incisors and both mandibular incisors of the

remaining two animals. Into the root canals, materials were applied with a lentulo spiral and compacted by a hand compactor. Class I cavities were sealed with resin modified cement (GC FUJI VIII, GC Corporation, Tokyo, Japan). Postoperatively, the animals received subcutaneously an analgetic (Butorfanol,10mg/ml, Richter Pharma AG Austria), 0.1 mg/kg body weight, every 8 h for the next three days and an antibiotic (Baytril®, 25mg/ml, KVP Pharma und Veterinär Produkte GmbH), 10 mg/kg body weight, daily for the next five days.

The animals were sacrificed after 28 days, by intravenous injection of 10 ml Pentobarbital solution (Pentobarbital sodium salt 100 mg ml^{-1}, Sigma-Aldrich Chemie GmbH, Steiheim, Germany).

Histological procedure and histological analysis

After the removal of soft tissues and separation of the upper and lower jaw, the treated teeth were cut with a diamond disk and fixed in 10% formalin. After decalcification (8% HCl from 37% (v/v) concentrate and 10% HCOOH from 89% (v/v) concentrate in the PBS during 24h at 37◦C), the tissue was fixed in a semi-enclosed benchtop tissue processor (Leica TP1020, Leica Biosystems, Wetzlar, Germany) and embedded in paraffin blocks. Serial tissue sections (eight per sample) 5μm thick, were cut from the paraffin blocks and stained with haematoxylin eosin (HE) according to standard procedure. The slides were analyzed by optical microscopy (Olympus 5 microscope) using morphometric software package „Cell-B“ (Olympus), at magnification 40x, 100x and 200x, by an experienced pathologist blinded to the types of the tested materials. The histological parameters were analyzed qualitatively (extension of inflammation, general state of the tissue, continuity and morphology of calcified tissue), semi-quantitatively (presence of giant cells, material particles and microorganisms) and quantitatively (inflammation intensity, thickness of calcified tissue). Histomorphometric analysis was carried according to the cellularity and thickness of calcified tissue. Parameters were scored using a 1 - 4 scoring system according to modified criteria of Accorinte et al.[23].

Statistical analysis

The data were analyzed statistically using non-parametric Kruskal-Wallis test and Dunn's post hoc test for inter-group comparison ($\alpha = 0.05$). Non-parametric testing was chosen to compare categorical variables. Statistical analysis was done in Minitab 16 software package (Minitab Inc., State College, PA, USA).

RESULTS

The results of the histological examination are presented in Table 1. and Figures 1-3.

Table 1. Histological analysis for each material according to the scores

	CS		HA-CS		MTA			
	Score		Score		Score		Intragroup p-value	
	range	Med	range	Med	range	Med		
Inflammatory reaction								
Intensity	1-4	2	1-2	1	1-3	2	0.004*	
Extension	2-3	2.5*	1-2	1*	2	2		
General state of the tissue	1-3	2	1	1	1-2	2		
Calcified tissue								
Continuity	2-3	2.5*	1-2	1*#	2-3	3#	0.010*	0.003#
Morfology	1-3	2	1-2	2	2-3	2		
Thickness	1-2	1*	1-2	1.5#	3	3*#	0.004*	0.012#
Other findings								
Giant cells	1-2	2	1	1	1-2	2		
Material particles	2-4	3.5*	1-2	2*	2	2	0.003*	
Microorganisms	1	1	1	1	1	1		

CS-calcium silicates cement; HA-CS-hydroxyapatite with CS; MTA-Mineral trioxide aggregate.
Med-Median. Within a row, cells with the same superscript are significantly different and matched with the intragroup P-value with same superscript ($p<0.05$).

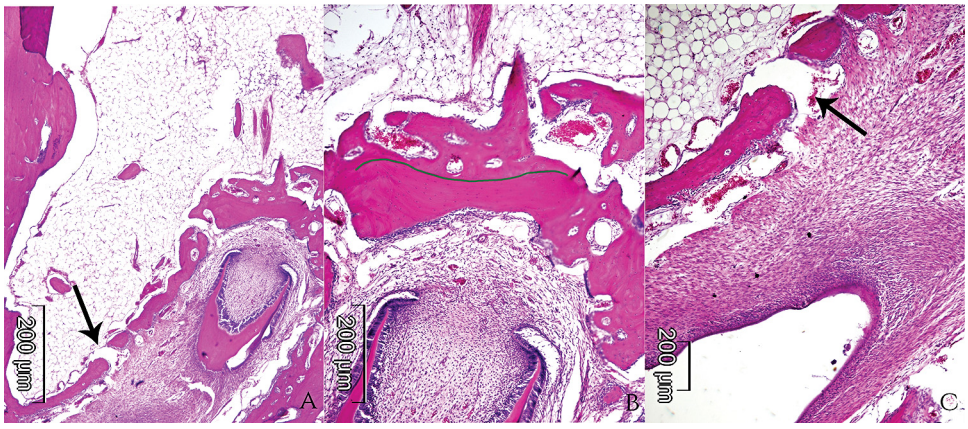

Figure 1. CS. **A**: Photomicrograph of calcified tissue with proliferation of the connective tissue, moderate cellularity with slight macrophage infiltration (HE,40x). **B** and **C**: Details from the previous photomicrography. Discontinuous newly formed calcified tissue (black arrow). Irregular structure of newly formed calcified tissue with a clear border between old and new osteoid (green line). Intensive angiogenesis is presented. Mesenchymal cells with osteoblastic differentation on the perifery of the calcified tissue can be seen (HE,200x).

After implantation of material CS, half of the samples showed no inflammatory reaction (*score* 1), i.e only few inflammatory cells next to the implanted material were detected (Figure 1 A-C). A moderate inflammatory reaction was found in two samples (*score* 3). A severe inflammatory reaction (*score* 4) was observed in one sample, with extension of the inflammatory cells deeper into the tissue (*score* 3) and abscess formation (*score* 3). Material particles were detected in all samples, albeit in a different number (*score 2* - 4). Half of the samples were giant cells - free (*score* 1) and in the other half giant cells were detected in a small number (*score* 2). Microorganisms were not detected in any of the samples.

After implantation of material HA- CS, the tissue was unchanged (*score* 1) in most of the samples (Figure 2 A-C). A mild inflammatory reaction (*score* 2) was observed in two samples with inflammatory cells next to the implanted material (*score* 2). A small number of material particles (*score* 2) was detected in most of the samples. No giant cells or microorganisms was found (*score* 1).

After implantation of MTA, an inflammatory reaction of a different intensity (*score* 2 - 3) was noticed with inflammatory cells localized next to the implanted material (*score 2*) (Figure 3 A-C). A small number of material particles (*score* 2) was detected in all samples. Similarly, a small number of giant cells was found in most of the samples (*score* 2). No microorganisms were found (*score* 1).

Statistical analysis did not show significant differences in the intensity of inflammatory reactions between the tested materials. There were statistically significant differences between CS and HA-CS with respect to the extension of inflammation ($p = 0.004$) and the number of material particles ($p = 0.003$). There were not significant differences among materials tested with respect to giant cells or microorganisms.

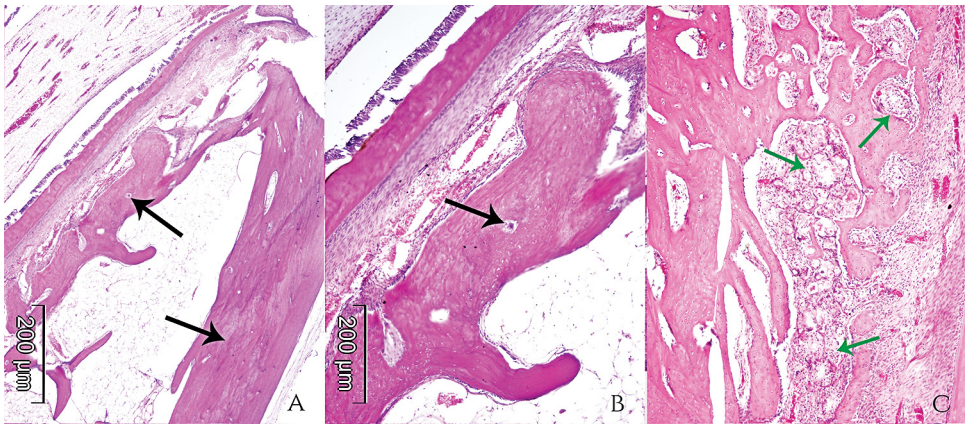

Figure 2. HA-CS. **A**: Continuous calcified tissue with lamellar structure (HE, 40x). **B** and **C**: Details from the previous photomicrography. Calcified tissue with regular mineralization, lamellar structure and slight to moderate cellularity. Viabile osteocyte is presented in newly formed bone (black arows). Particles of the material (green arrows) can be seen as well as chronic proliferative inflammation of a moderate intensity with signs of angiogenesis and acute hyperaemia around them. (HE,200x).

In the samples with CS, newly formed calcified tissue was mostly of an irregular morphology (*score* 2), deposited at a thickness exceeding 250 μm (*score* 1-2) in most of the samples, but discontinuous with foci of fibrovascular tissue (*score* 2-3) (Figure 1 A-C).

In most samples with HA-CS, the implanted material was completely separated from the adjacent tissue by newly formed, regularly structured, calcified continuous tissue (*score* 1). The thickness of newly formed tissue varied between 150 and -250 μm (*score* 1 - 2). Mesenchymal cells with osteoblastic differentiation were observed on the outskirts of the newly calcified tissue (Figure 2 A-C).

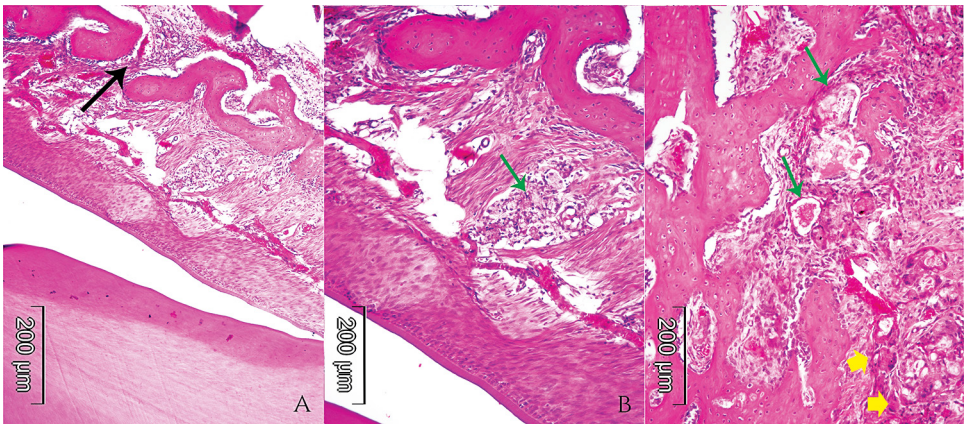

Figure 3. MTA. **A**: Partially discontinuous newly formed calcified tissue permeated with foci of fibrovascular proliferation (black arrow) (HE,40x). **B** and **C**: Details from previous photomicrography. Regular structure of calcified tissue with increased cellularity and many osteocytes in lacunar spaces. Presence of material particles. Scattered particles of material (green arrows) surrounded with multinuclear foreign body giant cells can be seen (yellow arrows) (HE, 200x).

In all of the samples with MTA, newly calcified tissue was deposited in small quantities, up to 150 μm thick (*score* 3). The newly formed calcified tissue was irregularly structured, discontinuous with foci of fibrovascular proliferation (*score* 2-3) (Figure 3 A-C).

HA-CS exhibited significantly better results than MTA and CS with respect to the continuity of the newly formed calcified tissue ($p = 0.03$ and $p = 0.010$, respectively). There were significant differences in thickness of the calcified tissue between CS and MTA ($p = 0.004$) and between HA-CS and MTA ($p=0.012$).

DISCUSSION

In vivo tests enable the evaluation of complex interactions between materials and host tissue. Therefore, such tests, beside biocompatibility, enable the assessment of materials biofunctionality. With respect to ethical principles, actual studies in animal models are conducted on laboratory animals, mainly rodents [24, 25]. In this study,

materials effects were evaluated after their implantation in the root canals of rabbit teeth.

Application of CS and HA-CS in this study resulted in periradicular tissue inflammatory reactions similar to control material (MTA) with respect to intensity. In most of the samples, inflammatory reactions were scored as mild to moderate suggesting good tolerance of the host tissue to the applied materials. These findings are consistent with results of other authors who evaluated biocompatibility of the materials with similar chemical composition [23, 26].

Inflammatory reactions after application of calcium silicate cements are considered to be the result of calcium hydroxide release during material setting. Alkaline pH causes local tissue necrosis with the development of local inflammatory reactions [24]. It is known that calcium silicate cement induces the expression of proinflammatory cytokines (IL-6 and IL-8) also as a result of high pH [27]. Although tissue necrosis is in general, considered to be an initiator of mineralization processes [28], some studies suggested that repair processes could commence even without necrosis or acute inflammation [29]. The amount of released calcium hydroxide from calcium silicate cements decreases over time [30], along with material setting, creating favorable conditions for the start of the repair processes.

Although no statistically significant differences between CS, HA-CS and MTA were found with respect to inflammatory response, tissue conditions in the samples with HA-CS were rated the best. This finding may be due to the composition of this material. HA-CS consists mostly of hydroxyapatite with a lower pH value than CS and MTA, although still alkaline [19]. It was confirmed that lower pH values allow alkaline phosphatase activity, but cause a smaller zone of superficial necrosis compared to highly alkaline materials such as calcium silicate cements [31]. A small number of giant cells in the samples with CS and MTA, or their absence in the samples with HA-CS implicated low activity of tissue hystocytes, and good tissue tolerance to the implanted materials. Still, giant cells would presumably be detected in deeper tissues sections.

Newly calcified tissue was observed in all samples of tested materials. This finding confirms that tested materials have, besides biocompatibility, an inductive potential. The present results are consistent with previous studies which reported the formation of mineralized tissue after application of materials with a similar chemical composition in different clinical indications [26].

All of the tested materials belong to the group of bioactive materials which are characterized by the release of biologically active ions. As mentioned, the major soluble fraction of these cements is calcium hydroxide, released during material setting. Since calcium silicates are slow setting materials, they release calcium hydroxide over several weeks [30]. In contact with tissue and tissue fluids, calcium hydroxide dissolve to calcium and hydroxyl ions. Continuous release of calcium ions from the material is considered to be crucial for the induction of calcified tissue formation. In addition to its role in chemotaxis, calcium regulates cell proliferation, differentiation and mineralization [28].

It was confirmed that calcium releasing materials induce proliferation of periodontal fibroblasts, growth and differentiation of pulp cells, osteoblasts, osteoblast-like cells and cementoblasts [3-5].

The processes of tissue mineralization are also associated with the release of hydroxyl ions. The pH increase results in an increase of alkaline phosphatase (ALP), the expression of growth factors and the formation of calcified nodules. Also, hydroxyl ions neutralize mediators of inflammation and possess antimicrobial activity [28].

The tested materials are composed of Si ions which are known to have a role in material bioactivity [11] and proliferation and differentiation of osteoblast-like cells. High concentrations of Si ions (> 30 ppm) can inhibit the growth of osteoclasts and resorption processes, but can also increase the level of ALP participating in the mineralization of the newly calcified tissue [32].

The thickness and continuity of the newly calcified tissue observed in this study was different and material-dependent. The application of both nanostructured-materials resulted in a thicker layer of calcified tissue compared to MTA. Materials synthetized by the sol-gel method, as CS and HA-CS in the present study have improved bioactivity compared to the same materials obtained by other methods [33]. It is also known that the topography of the material surface, which is related to their chemical composition and structure, affects cell activity, especially their adhesion and viability [34]. It is possible that the nanostructure of CS and HA-CS, which is similar to the nanocrystalline structure of the bone, could be associated with the obtained results.

Additionally, newly formed calcified tissue associated with HA-CS was continuous and without foci of vascularized fibroblast proliferation, which was not the case with MTA and CS. Unlike CS and MTA, HA-CS contains phosphate ions which could be associated with the aforementioned histological finding. More efficient calcified tissues were previously reported after application of calcium silicate cements containing phosphate ions, compared to pure calcium silicate cement [26]. The authors attributed these findings to a greater amount of phosphate ions available for the hydroxyapatite formation. Similarly, Zhang et al. [35] reported pronounced mineralization and odontoblast differentiation of human pulp cells induced by calcium silicate-based materials with added hydroxyapatite (Bioaggregate and iROOT BP Plus) compared to pure calcium silicate cement (MTA). The present and cited findings support the hypothesis that hydroxyapatite-containing materials with high phosphate ion content have a higher potential for tissue mineralization than MTA.

Microorganisms were not detected in any sample of the tested materials. The microbial presence is usually correlated with inappropriate crown restorations and subsequent microleakage [36]. Good sealing properties of GIC used in this study might be the reason for the obtained result. Still, it must be pointed out that microorganisms are difficult to detect with this type of histochemical staining and that they could be removed during tissue preparation for histological analysis.

CONCLUSION

Application of CS and HA-CS resulted in a minimal inflammatory tissue response similar to control MTA. CS and HA-CS were more efficient than MTA in inducing hard tissue formation after implantation in the root canals of rabbit teeth. The best organized newly formed calcified tissue was seen after HA-CS application. The present results serve as a solid foundation for further CS and HA-CS testing.

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Authors' contributions

PV carried out the study, wrote, prepared and formated the manuscript for publication. OGV participated in experimental procedures and analysis of the results. JV synthesized the experimental material. SJ carried out the histological examination. PBB participated in experimental procedures. ŽS gives the idea of experiment, a lead of role in planning the experiment and revised the manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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HISTOLOŠKA ANALIZA ZAPALJENSKIH REAKCIJA U PERIRADIKULARNOM TKIVU I FORMIRANJA KALCIFIKOVANOG TKIVA POSLE IMPLANTACIJE EKSPERIMENTALNIH NANOSTRUKTURNIH CEMENATA NA BAZI KALCIJUM SILIKATA I HIDROKSIAPATITA U KANALE KORENA ZUBA KUNIĆA

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Cilj rada je bio da se ispitaju zapaljenske reakcije u periradikularnom tkivu i formiranje kalcifikovanog tkiva posle implantacije eksperimentalnih, nanostrukturnih cemenata na bazi kalcijum silikata (CS) i mešavine hidroksiapatita i kalcijum silikata (HA-CS) u kanale korena zuba kunića.

Kanali korena centralnih sekutića su posle instrumentacije, ispiranja i sušenja napunjeni materijalima CS, HA-CS i kontrolnim materijalom, mineral trioksid agregatom (MTA). Životinje su žrtvovane posle 28 dana. Posle histološke pripreme, uzorci tkiva su analizirani u pogledu inteziteta i raširenosti zapaljenske reakcije; kontinuiteta, morfologije i debljine novoformiranog kalcifikovanog tkiva; prisustva džinovskih ćelija, čestica materijala i mikroorganizama. Dobijeni rezultati su statistički obrađeni ($\alpha = 0,05$)

Nisu uočene statistički značajne razlike u intezitetu zapaljenske reakcije između CS, HA-CS i MTA. U pogledu kontinuiteta novostvorenog kalcifikovanog tkiva HA-CS je pokazao bolje rezultate u odnosu na MTA i CS ($p=0,003$ i $p=0,010$). Značajne razlike utvrđene su u pogledu debljine kalcifikovanog tkiva između CS i MTA ($p=0,004$), kao i HA-CS i MTA ($p=0,012$).

Aplikacija materijala CS i HA-CS je rezultirala minimalnom zapaljenskom reakcijom tkiva, slično kontrolnom materijalu (MTA). CS i HA-CS su bili efikasniji u pogledu stimulacije formiranja kalcifikovanog tkiva u odnosu na MTA. Najbolje organizovano novoformirano tkivo uočeno je posle aplikacije materijala HA-CS.