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Хистолошке реакције ткива на новосинтетисане биоактивне материјале на бази калцијум силикатних система и хидроксиапатита – *in vivo* студија

SUMMARY

Introduction/Objective Developing of the materials, which could be used as biological bone substitutes, is one of the most valuable and active fields of biomaterial research.

Objective The goal was to research the reaction of the tissue on newly synthesized nano-materials with the calcium silicate system (CS) and hydroxyapatite (CS-HA) based, after being implanted into the subcutaneous tissue of a rats and direct pulp capping of the tooth of the rabbits.

Methods The tested materials were implanted in 40 Wistar male rats, sacrificed after 7, 15, 30, 60 days. The direct pulp capping is realized on the teeth of the rabbits. Cavities were prepared on the vestibular surface of the incisors. The animals are sacrificed after 10 and 15 days. The control material was mineral trioxide aggregate (MTA). Histological analysis covered inflammatory reaction cellular components tracking, presence of gigantic cells and necrosis of the tissue.

Results Seven days after implantation the strongest inflammatory response was given by the MTA (3,30±0,48), while CS and CS-HA scored 3,00±0,71. After 60 days the rate of inflammatory reactions dropped, which was the least visible with CS-HA (0,20±0,45). The least visible inflammatory reaction of the rabbit's pulp tissue was spotted with the CS (1,83±0,75), than with the MTA and CS-HA (2.67±1.53, 3.00±0.63).

Conclusion The newly synthesized materials caused a slight reaction of the subcutaneous tissue. CS-HA showed the best tissue tolerance. Nanostructural biomaterials caused a slight to moderate inflammatory reaction of the rabbit`s pulp tissue only in the immediate vicinity of the implanted material.

Keywords: Biocompatibility; calcium silicate system; hydroxyapatite; mineral trioxide aggregate

САЖЕТАК

Увод/Циљ Усавршавање материјала, који би могли да се користе као биолошке замене кости, једна је од најзначајнијих и најактивнијих области истраживања биоматеријала.

Циљ овог рада је био да се испита одговор ткива на новосинтетисане наноматеријале на бази калцијум силикатних система(КС) и хидроксиапатита (КС-ХА) после имплантације у поткожно ткиво пацова и директног прекривања пулпе зуба кунића.

Методе У поткожно ткиво 40 Вистар пацова су имплантирани тестирани материјали, а после 7, 15, 30 и 60 дана зивотиње су жртоване. Директно прекривање пулпе је реализовано на зубима кунића. На вестибуларним површинама секутића прекривани су кавитети. Животиње су жртоване после 10 и 15 дана. Контролни материјал у оба експеримента је био минерални триоксидни агрегат МТА). Хистолошка анализа је обухватила праћење ћелијске компоненте запаљења, присуства гигантских ћелија и некрозе ткива.

Резултати Седам дана после субкутане имплантације најјачи запаљенски одговор дао је МТА (3,30±0,48), док је за КС и КС-ХА он оцењен са 3,00±0,71. После 60 дана дошло је до опадања знакова запаљења, које је било најмање изражено око КС-ХА (0,20±0,45). Најмање изражена запаљенска рекција пулпног ткива кунића уочена је код материјала КС (1,83±0,75), затим код МТА и КС-ХА (2.67±1.53, 3.00±0.63).

Закључак Новосинтетисани материјали су изазвала благу запаљенску реакцију поткожног ткива пацова, а КС-ХА је показао најбољу ткивну толеранцију. Наноструктурни биоматеријали КС и КС-ХА су узроковали благу до умерену запаљенску реакцију пупног ткива кунића само у непосредној близини имплантираног материјала.

Кључне речи: биокомпатибилност; калцијум силикатни систем; хидроксиапатит; минерал триоксид агрегат

INTRODUCTION

Biocompatibility of dental materials and the constraints imposed by their toxicity in contact with dental and other oral tissues are an important segment in the research of the newly synthetized materials. Cytotoxicity of the material can cause inflammatory reaction in contact with the surrounding tissue, significantly affecting the therapy outcomes [1, 2]. Therefore, multiple testing of the synthetized materials is required in order to ensure their reliable application in day-to-day clinical

practice. For many years now the scientific community has been focusing on the need to evaluate the biological properties of new materials at the pre-clinical stage by using various *in vitro* and *in vivo* methods, as these mostly remain in long-term contact with local cells and tissues [3]. In this interaction, the onset of the foreign body reaction usually takes place immediately after the material is implanted (going through the stages of inflammation and healing, and involving a number of different cell types), making the *in vivo* biocompatibility testing one of the most important steps towards their prospective clinical application.

While the evaluation made based on *in vitro* assays may be faster in rendering biological interaction data, the reliability of such data remains questionable compared to the data obtained in more complex *in vivo* conditions. *In vivo* assays are normally carried out on animal models (implantation in subcutaneous, muscle, bone or other tissues), and they precede assays on target animal tissues and human clinical trials [4].

Subcutaneous tissues are tissues of choice for the biocompatibility evaluation of the implanted material. In the opinion of the authors they are found on highly accessible sites, enabling the evaluation of the biological reactions to biomaterials or, in other words, facilitating detection of inflammatory tissue reactions to the agents in the implanted material [3]. Histological examination is the most frequently used method in the research of tissue compatibility and its capability to restrict the inflammatory reaction to the implanted material [5-9].

The direct pulp capping (DPC) is a therapeutic procedure for preserving the dental pulp vitality by covering the exposed pulp injury with materials that will foster reparative dentine formation [10]. The direct capping material plays a key role in the course of this treatment as it comes into direct contact with the pulp tissue. Although calcium hydroxide has been a DCP agent in most frequent and longest use [10-12], the practice of many years has also shown frequent unforeseeable outcomes of this therapy [13, 14]. In the era of regenerative endodontics, new procedures and materials for biological therapy and tooth revitalisation have been introduced, including biomaterials such as calcium phosphate, calcium silicate, and bioactive glass-ceramic cements. These bioactive dental materials are essential for a better and more effective regenerative endodontic treatment [15].

The objective of this study was to investigate the tissue inflammatory response to newly synthetized nanomaterials based on calcium silicates (CS) and hydroxyapatite (CS-HA) in *in vivo* conditions by a) implanting the material in the subcutaneous tissue of rats and b) direct capping of exposed dental pulp of rabbits.

METHOD

Permission for the experimental work on animals was obtained from the Ethical Committee of the Belgrade University School of Dentistry (number 36/5 of April 4, 2012). The experiment was conducted in line with the international standards ISO 7405 and ISO 10993-2 (animal welfare requirements) [16, 17]. The initial step in carrying out this study was an innovative method of bio-

ceramic material synthesis (with nanoscale particles) applied for the first time by V. Jokanovic. The materials used in this study were nanostructured calcium silicates with and without the addition of 40% hydroxyapatite (CS and HA-CS) mixed with distilled water in the 2:1 ratio of powder to water, according to the recommended protocol. The control material was the MTA (ProRoot MTA, Tulsa OK, USA) mixed in the 3:1 ratio, according to the instructions of the manufacturer.

Design of the Subcutaneous Implantation Experiment

Forty male rats (Wistar albino), between 2.5 and 3 months old and weighing on average 350gr each were used. After the animals were anaesthetized, 2-cm-long incisions in the animals' backs were made in the head to tail direction. Using the blunt dissection to the right and the left of the spine, two pockets approximately 15 mm deep were opened and sterile polyethylene tubes with the test materials were implanted using sterile clinical tweezers. Polyethylene tubes 10 mm long and with internal diameter of 1 mm, half-filled with freshly mixed materials (CS, CS-HA, and MTA) were implanted in the subcutaneous tissue. The empty half of the tube was used as the negative control. Each animal received two tube implants. The tube with the test material was inserted on the right side of the spine and the tube with the MTA, on the left. The tubes were positioned so that the material was at all times oriented towards the head and the empty half of the tube, towards the tail. By random selection the animals were divided into two groups of 20 for each tested material. Ten animals (five of each material) were sacrificed in each of the four observation periods - days 7, 15, 30 and 60.

Tissue samples together with polyethylene tubes were fixed in 10% buffered formalin. Then the polyethylene tubes were removed. The tissue was then prepared for light microscopy in a standard way, involving dehydration in a series of ethanol solutions of increasing concentrations; illumination in xylol; and paraffin embedding. Paraffin samples of 4 μm in width were stained in haematoxylin and eosin (HE). Microscopic slides were analysed in an optical microscope (Olympus BX-51, Japan) and micro photographs were taken by a digital camera (CD video camera, PixeLink, connected to 19"Dell PC screen).

In line with international standards (ISO 10993-6. Biological evaluation of medical devices - Part 6: Test for local effect after implantation), local tissue reactions where the materials had been implanted were evaluated. In the histological examination of the prepared samples, the parameters were analysed qualitatively and semi-quantitatively (modified according to Lotfi and Scarparo) [6, 18]: a) Inflammatory response (0 – no inflammation; 1 – minimal (<25 inflammatory cells); 2 – mild (26–50 inflammatory cells); 3 – moderate (51–100 inflammatory cells); 4 – severe (>100 inflammatory cells), b) Vascular congestion (0 – absent; 1 – minimal, 2 – mild, 3 – moderate, 4 – severe, involving blood vessel burst).

Design of the Direct Pulp Capping Experiment

The animal model used in this experimental part of the study were four rabbits (Oryctolagus cuniculus) of both sexes, from different broods, aged about 12 months, average weight 4 kilograms,

on controlled diet and receiving daily care. For the purposes of the surgical procedure, the general dissociative anaesthesia (xylazine, ketamine, acepromazine) was administered. The average duration of anaesthesia was 100 minutes.

The surgical procedure was carried out in aseptic conditions and so as to ensure minimum trauma. Each tooth was cleaned, dried and disinfected (30% hydrogen peroxide and 5% iodine tincture). Class V cavities were then created in the gingival third of vestibular surfaces of incisors by using round, water-cooled diamond burs. A new set of diamond and carbide burs was used for each animal. In the middle of the cavity, pulp was exposed using sterile, round bur. Cavities were gently dried, with no pressure exerted, using sterile cotton wool balls. Freshly mixed material was applied to the exposed pulp. All cavities were closed with glass ionomer cement (GC FUJI VIII, GC Corporation, Tokyo, Japan) as a definitive filling., The material used for direct capping of the exposed pulp was mineral trioxide aggregate (MTA) and it was implanted in the upper right maxillary incisor, while the other three incisors were implanted with calcium silicate cement (CS) and the mixture of calcium silicate cement and hydroxyapatite (CS-HA), in two rabbits respectively. The animals were sacrificed after ten and fifteen days, by intravenous injections. Having removed soft tissues, the teeth in the alveoli were cut with a diamond disc. The samples were fixed in 10% formalin and decalcified. Following the decalcification, the tissue was fixed in semi-enclosed benchtop tissue processor (Leica TP 1020, Germany) and then embedded in paraffin blocks. Serial tissue sections of 5 μm in width (8 from each sample) were cut from the paraffin blocks. The slides were stained in haematoxylin and eosin (HE), 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 x10, x40, x100 and x200. In addition to the software, the pathohistological parameters were assessed qualitatively, semi-quantitatively and quantitatively. Examination of every tooth included the scoring of the following parameters (scoring system 1– 4): a) Pulp inflammatory response: i) intensity (1- no inflammation, 2 - mild, 3 - moderate, 4 – severe, > 25 inflammatory cells), ii) extent of inflammation (1 - no inflammation, 2 – mild, inflammatory cells close to the exposed portion of the pulp, 3 – moderate, inflammatory cells in the area of coronal pulp, 4 – severe, whole coronal pulp is infiltrated or necrotic), iii) general state of the pulp (1 - no inflammation, 2 – with inflammation, 3-abscess, 4- necrosis), b) Other findings in the pulp (gigantic cells, direct capping material particles, presence of microorganisms).

In the statistical analysis, the non-parametric Kruskal-Wallis test with Dunn's *post hoc* test for inter-group comparisons was used. The statistical analysis was made using the Minitab 16 software package (Minitab Inc. State College, PA, USA).

RESULTS

The results of the histological examination of subcutaneous implantation are shown in Table 1 and in Figures 1 - 6.

Table 1. Inflammation scores (mean value and SD).

Material	7 days	15 days	30 days	60 days
CS	3.00±0.71	2.00±0.71	1.60±0.55	0.50±0.58
CS-HA	3.00±0.82	1.60±0.55	1.40±0.55	0.20±0.45
MTA	3.30±0.48	2.30±1.06	1.90±0.88	0.44±0.73
Control	2.50±1.43	1.90±0.74	1.50±0.53	0.67±1.00

In the examined samples 7 days after the implantation of CS and CS-HA a moderate inflammatory reaction was observed (*score* 3) (Figure 1), while the connective tissue with the MTA implant showed somewhat more intensive inflammatory reaction (3.3) with diffuse and focal subcapsular and perivascular inflammatory infiltrates (Figure 2). Inflammatory infiltrate cells included lymphocytes and plasmocytes, while rare granulocytes were observed in only two samples. The connective tissue had a small number of normal-structure blood vessels with signs of moderate congestion, and it could receive scores 2.8 and 2.5 for CS and CS-HA, respectively, and score 2.4 for MTA.

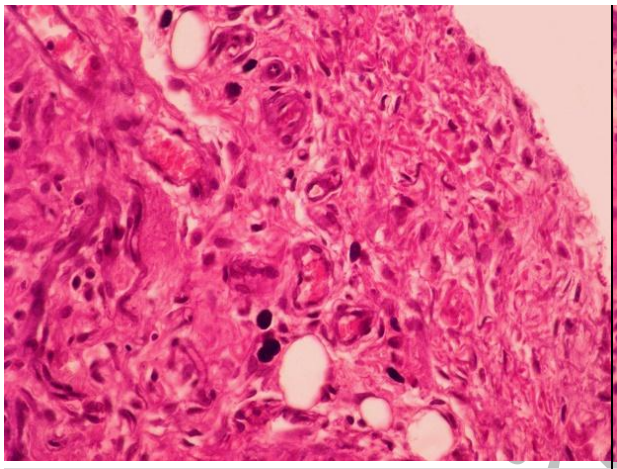

Figure 1. CS implantation after 7 days. Mild inflam-matory reaction is visible, while rare monocytes, lymphocytes and granulocytes can be found in infiltrate. Blood vessels with signs of moderate congestion (HE, ×40).

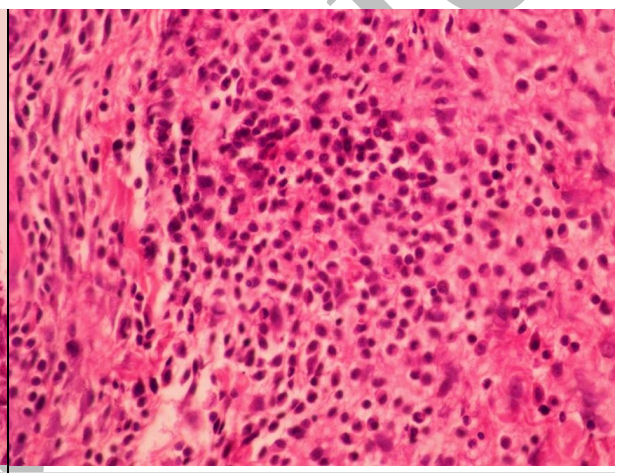

Figure 2. MTA implantation after 7 days. Visible focal and diffuse inflammatory reaction of connective tissue. (HE, ×40).

In the observed slides 15 days after implantation of CS and CS-HA materials, mild inflammation was observed (scores 2 and 1.6, respectively) (Figure 3), while the MTA group displayed mild to moderate inflammation (score 2.3). Blood vessels were of the normal number and with signs of minimal veinal stasis, receiving score 0.8 in the CS-HA group, while the MTA and CS groups displayed minimal and mild congestion, respectively (scores 1.3 and 1.6, respectively).

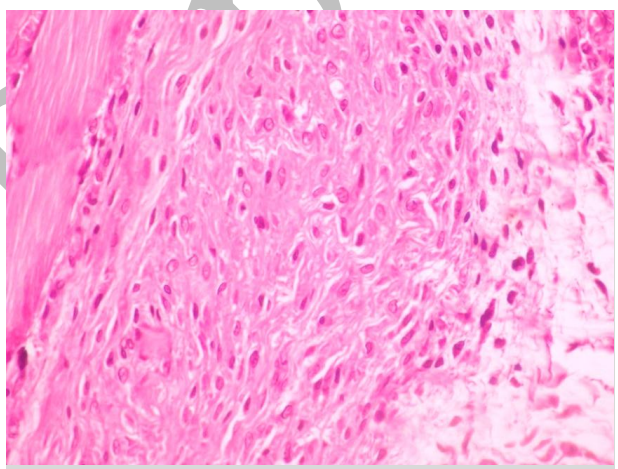

Figure 3. CS implantation after 15 days. Connective tissue of mostly preserved integrity is visible, no vane stasis (score 1.4). Presence of lymphocytes and plasmacytes what confirm chronically inflammatory reaction. (HE, ×40)

In the observed samples 30 days after the implantation, the connective tissue was of normal structure and with minimal number of inflammatory cells in the CS and CS-HA groups

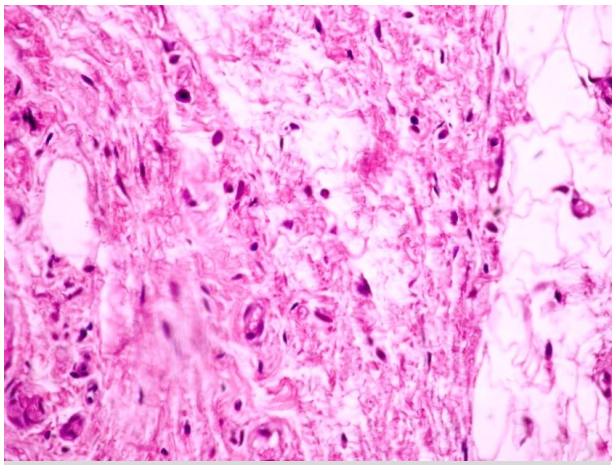

Figure 4. CS implantation after 30 days. Integrity of the connective tissue is visible, with minimal number of inflammatory infiltrate`s cells (score 0.6). (HE, ×40).

(scores 1.6 and 1.4, respectively) (Figure 4). In the MTA group, the surrounding tissue in most samples showed signs of mild inflammation (score 1.9). The connective tissue was with a usual number of blood vessels and no signs of veinal congestion, receiving score 0.6 in the CS and CS-HA groups, and score 0.8 in the MTA group.

In the tested samples, 60 days after the beginning of the experiment loose connective tissue with individual cells of inflammatory infiltrate could be observed (score 0.4 for CS-HA and MTA) (Figure 6) and score 0.5 for CS (Figure 5). The blood vessels of normal structure and in a normal number, with no signs of veinal stasis were observed for all the tested materials (0).

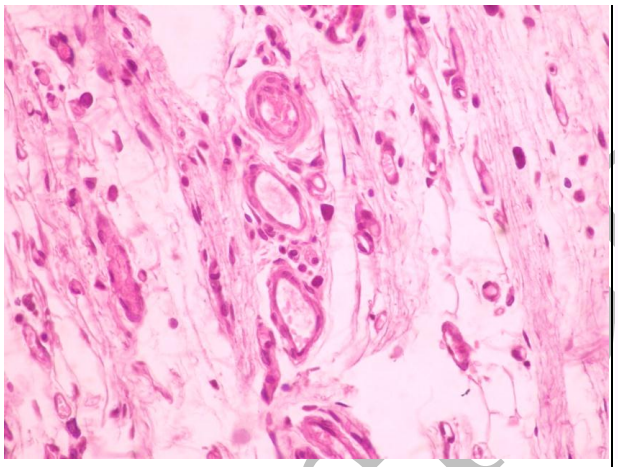

Figure 5. CS implantation after 60 days. Loose connective tissue with preserved integrity is visible. Single cells of inflammatory infiltrate are present (score 0.5) and increased number of new blood vessels, what indicate tissue remodelling (HE, ×40).

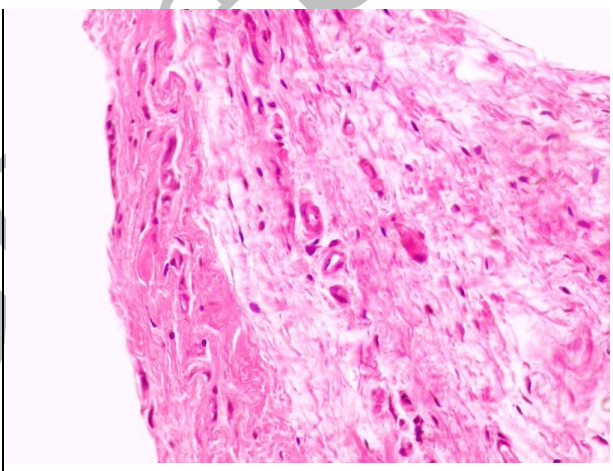

Figure 6. CS-HA implantation after 60 days. Subcapsular connective tissue with preserved integrity (score 0) is visible (HE, ×40).

The histological examination results of the rabbit teeth DPC are shown in Table 2. and Figures 7 - 13.

Table 2. Mean inflammation score values of the tested materials.

Material	Intensity	Extent	General state of the pulp
CS	1.83±0.75	2.17±0.75	1.50±0.55
CS-HA	3.00±0.63	2.17±0.41	2.17±0.41
MTA	2.67±1.53	2.67±1.15	2.67±1.15

The pulp tissue below the CS material showed signs of mild inflammatory reaction (Figure 7). The general state of the pulp suggested a mild inflammatory reaction (score 2). While the difference between the tested materials (CS and CS-HA) was not statistically significant, it was significant between CS and MTA (p=0.040).

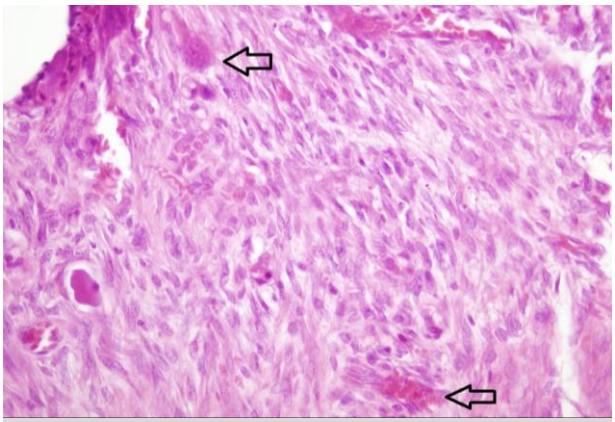

Figure 7. Pulp directly covered with CS. Reactive chronically inflammation with rare mononuclear cells and focally present giant type cells around the foreign body (arrows) (HE, ×400)

All the observed samples where CS-HA was used as the direct capping material showed signs of moderate inflammation (Figure 8). The general state of the pulp inflammation received score 2. A significant number of gigantic cells and scattered particles of the material were observed in the samples (Figure 9), which was statistically significant in the CS (p=0.001) and MTA groups (p=0.033). No bacteria were detected in any of the samples.

All the samples with MTA as the direct capping material showed signs of mild to moderate inflammation**,** with a few inflammatory cells immediately around the site of the exposed pulp. An intensive inflammatory reaction was detected in one sample, where inflammatory cells infiltration throughout the coronal pulp and necrosis were present (Figure 10). The presence of a small number of gigantic cells was detected in two samples; the

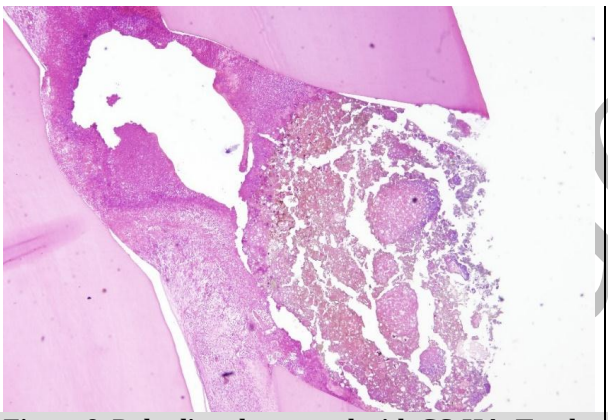

Figure 8. Pulp directly covered with CS-HA. Tooth enamel cavity. Implanted material surrounded with mild to intensive pulp inflammatory reaction with focal necrosis (HE, ×40).

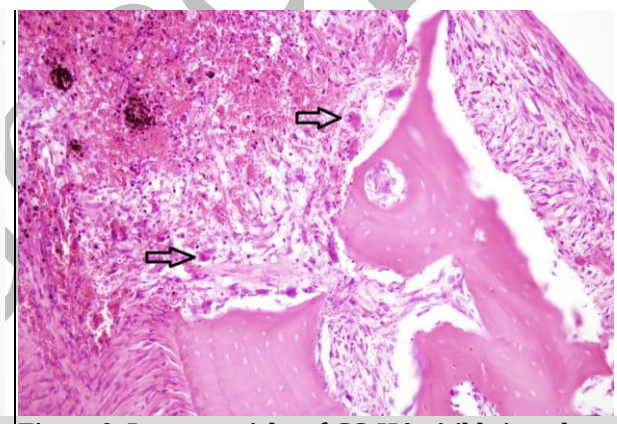

Figure 9. Loose particles of CS-HA visible in pulp tissue surrounded with giant type cells around the foreign body (arrows) (HE, ×200).

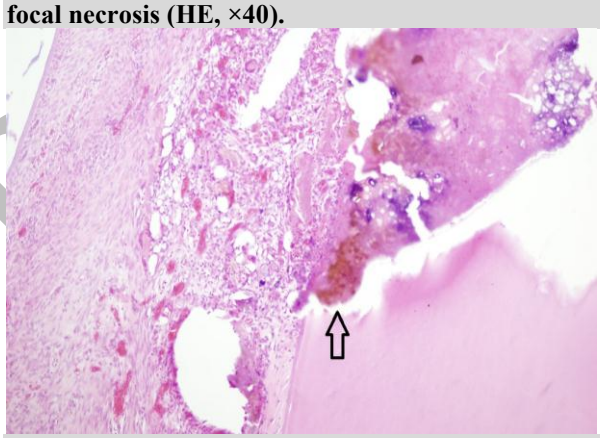

Figure 10. Pulp directly covered with MTA. Necrotic zones spreaded around the MTA particles. Blood vessels show sign of accute hyperemia (HE, ×100).

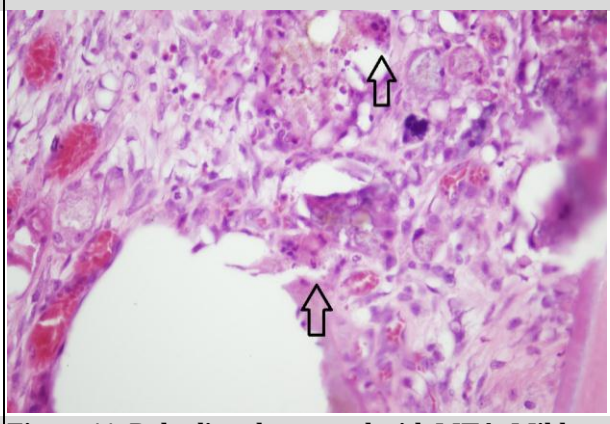

Figure 11. Pulp directly covered with MTA. Mild inflammatory reaction with phagocyted material, a number of foamy hystiocytes and giant type cells around the foreign body (arrows) (HE, ×400).

particles of the phagocytized material were also observed (Figure 11). No bacteria were detected in any of the samples.

DISCUSSION

At present an enormous progress has been achieved in synthesizing a number of new materials used in the clinical practice.

The materials tested in this study were newly synthesized nanomaterials based on tricalcium and dicalcium silicates and hydroxyapatites (CS and CS-HA). Their structure and biological properties were compared with those of mineral trioxide aggregate (MTA), which is the golden standard of tricalcium silicate cements.

Understanding the mechanism of interaction between biological fluids or cells and endodontic materials is key to assessing new materials used in diagnostics and therapy, and to avoiding materials' harmful reactions after their use [19]. Nanoparticles of different size and chemical structure typically get deposited in mitochondria, causing considerable structural damage due to the reactive oxygen species (ROS) synthesis that leads to oxidative stress. Mitochondria are the main locations of ROS production in a cell, which can result in the generation of a hydroxyl radical (OH): one of the most potent oxidising agents in nature. Oxidative stress is the most common cause of cell injury.

Nanostructured calcium silicates, CS and CS-HA (with particles from 117 to 477nm), in the initial *in vitro* assays designed to assess genotoxicity and cytotoxicity showed the absence of harmful cell effects within the parameters of the Comet and the MTT assays [20, 21].

In vitro studies are fundamentally different from *in vivo* ones where proteins, tissue fluids and other factors can reduce the toxic effects of the material [22]. *In vivo* assays render more comprehensive and clinically more relevant information about the tissue response over a protracted time period. The soft tissues' histological reaction to biomaterial has been a long established and frequently used method of biocompatibility assessment [7, 9]. These assays are highly reliable in evaluating the tissue irritation, and the interaction of tissue and biomaterial. Tissue's reaction to an implant is a cumulative pathophysiological consequence of a) the healing of an acute injury sustained due to a surgical wound and the presence of implant, b) possible chronic inflammation, c) the surrounding tissue repair while adapting to the implant.

This experiment monitored short-term, but also long-term, effects of the materials on the tissue, since these data are relevant to the clinical use of the endodontic materials.

Monitoring of a specific response to the foreign body following the material implantation starts with *inflammation*, continuing through the stages of *wound healing* with the involvement of various cell types being specific indicators of the tissue repair stage. With the inflammatory process attenuating, the total number of inflammatory cells decreases while the wound healing process moves towards formation of granulation tissue and *fibrous encapsulation* of the implanted material.

The tissue surrounding the tested bioceramic materials (CS, CS-HA) showed the highest level of inflammation in the first 15 days, with moderate disruption in the connective tissue structures. The connective tissue around the MTA showed signs of the most severe inflammatory reaction with diffuse and focal subcapsular and perivascular infiltrates, which was evaluated as moderate and severe inflammation. Other researchers also reported such a powerful tissue response, observing even coagulative necrosis and dystrophic calcifications [7]. A number of factors caused initially such a severe inflammatory reaction to MTA. High pH values of the freshly mixed material, heat release upon setting, and stimulation of inflammatory cytokines (interleukin 1 and interleukin 6) contribute to such a powerful tissue response to MTA [18, 23, 24, 23].

Tissue reaction to empty tubes (negative control) in this experiment was similar to the findings reported by other researchers [9, 22, 24]. It was most severe on the 7^{th} day and the 15^{th} day with cellular infiltrate dominated by lymphocytes and plasmocytes, which is indicative of a chronic inflammatory reaction. The gigantic cells detected in some sites suggest the tissue's reaction to a foreign body. This reaction could partly be a result of surgical trauma sustained upon implanting the tubes in the tissue. At the end of weeks four and eight, the inflammatory infiltrate cells were disappearing, and the tissue at the point of contact with the tube was encapsulated. This suggests the body's capacity to contain the inflammatory reaction thus preventing further tissue damage, as confirmed in papers by Sumer [5] and Scarparo [6].

Further into the tissue repair process (after 30 and 60 days), significant decrease in the inflammation intensity; disappearance of inflammatory infiltrate cells, but also tissue repair and remodelling were observed in all tested materials. The inflammatory response after four and eight weeks of the experiment can be explained by inducing the release of proinflammatory cytokines by released particles of the hydroxyapatite layer formed on the surface of the bioceramic materials. This also suggests good interaction between the material and the cells from the surrounding tissue, which is a sign of good biocompatibility of the materials.

The pulp tissue response to the implanted material, usually, starts with an acute inflammation, which need not be present in all cases [25]. The topography and chemistry of the surface of newly synthetized materials plays an important role in odontoblast adhesion to biomaterial. It is a known fact that micro- and nano-topography of the surface and adsorbed proteins have direct influence on the cell behaviour and activity, primarily with respect to their adhesion and retention at the point of application [26].

Nanostructured calcium silicate cements show increased osteoblast adhesion, proliferation and differentiation, since the bone itself has nano-structure, and the crystal size and geometry can modify the response of the surrounding tissue. The interaction between the direct capping material and the injured pulp tissue, and the ways in which the healing and repair processes are initiated and developed are still not fully clear. While many hypotheses exist, recent studies have accorded the main role to

growth factors in angiogenesis, mobilisation of progenitor cells, differentiation and, finally, biomaterial-assisted mineralisation [27].

As a consequence of experimental perforation, but also of the initial effect of the tested materials, a mild to moderate inflammatory reaction was detected in all observed teeth samples. The inflammatory infiltrate was in close proximity of the implanted material and was not spreading further into the coronal pulp. The weakest inflammatory reaction, sporadically with total absence of inflammation, was observed in samples where calcium silicate cement (CS) was implanted as the direct capping material, which agrees with the findings of other researchers who find up to 50% samples with no signs of inflammation in the early stage [12, 28]. Moderate inflammation was detected in the CS-HA and MTA samples, which is confirmed by similar experiments where inflammation appeared in more than 62% of the MTA samples after two weeks, with the intensity declining after eight weeks. This initially severe inflammatory response is a result of the pulp tissue coagulative necrosis in contact with the MTA (pH is 9 - 10). This zone has a stimulating effect on the surrounding vital pulp tissue, which initiates a string of healing processes. Due to bio-degradation of the material in contact with the tissue fluids, Ca and P ions are released, creating alkaline environment, which has a favourable effect on adhesion and proliferation of cells involved in the healing processes [12, 29]. Asgary observes that new endodontic cements having similar structure as the MTA, but improved physical and chemical properties – they include the nanomaterials tested – show better pulp response (weaker inflammatory reaction) and thicker dentine bridge then the MTA at the later stage [30].

Biomaterials are normally tested on animals, since they are a model of the environment one can find in humans. Nevertheless, animas are characterised by a huge range of differences with respect to anatomy, biochemistry, physiology, and other. In the absence of confirmation from human clinical trials, it is often difficult to draw a conclusion solely on the basis of animal testing. Testing carried out on live systems invariably leads to experimental variability. The more complex the system (human cells versus microorganism cells) the higher statistical variability of testing results can be expected [3].

CONCLUSION

The results shown in the present *in vivo* study on the animal model have proven that the subcutaneous tissue of rats and the pulp tissue of rabbits have favourable biological response to newly synthetized nanostructured biomaterials (CS and CS-HA). The inflammatory reaction in the subcutaneous tissue was severe only in the initial days after the implantation and its intensity declined as a function of time. In direct pulp capping there was a mild to moderate inflammatory reaction in the close proximity of the implanted material. The tissues showed high tolerance to the implanted materials, which confirms their biocompatibility, as in previous *in vitro* studies.

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