Histological evaluation of tissue reactions to newly synthetized calcium silicate- and hydroxyapatite-based bioactive materials – *In vivo* study

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**SUMMARY**

**Introduction/Objective** Development of materials which could be used as biological bone substitutes is one of the most valuable and active fields of biomaterial research. The goal of the study was to research the reaction of tissue on calcium silicate- (CS) and hydroxyapatite-based (CS-HA) newly synthesized nanomaterials, after being implanted into the subcutaneous tissue of a rat and direct pulp capping of rabbit teeth.

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

**Results** Seven days after the implantation, the strongest inflammatory response was given by the MTA (3.3 ± 0.48), while CS and CS-HA scored 3 ± 0.71. After 60 days, the rate of inflammatory reactions dropped, which was the least visible with CS-HA (0.2 ± 0.45). The least visible inflammatory reaction of the rabbits’ pulp tissue was spotted with the CS (1.83 ± 0.75), than with the MTA and CS-HA (2.67 ± 1.53, 3 ± 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 rabbits’ pulp tissue only in the immediate vicinity of the implanted material.

**Keywords:** biocompatibility; calcium silicate system; hydroxyapatite; mineral trioxide aggregate

**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 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 *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 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 direct pulp capping
agent in most frequent and longest use [10, 11, 12], the prac-
tice of many years has also shown frequent unforeseeable
outcomes of this therapy [13, 14]. In the era of regenerative
dentistry, new procedures and materials for biological
therapy and tooth revitalization have been introduced, in-
cluding biomaterials such as calcium phosphate, calcium
silicate, and bioactive glass-ceramic ceramics. These bioac-
tive dental materials are essential for a better and more ef-
efective regenerative endodontic treatment [15].

The objective of this study was to investigate the tissue
inflammatory response to newly synthesized nanomate-
rials based on calcium silicates (CS) and hydroxyapatite
(CS-HA) in in vivo conditions by a) implanting the ma-
terial into the subcutaneous tissue of rats, and b) direct
capping of exposed dental pulp of rabbits.

METHODS

Permission for the experimental work on animals was
obtained from the Ethical Committee of the Belgrade
University Faculty of Dental Medicine (No. 36/5, 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. Jokanović. The materials used in this
study were nanostructured CS with and without the ad-
dition of 40% HA-CS mixed with distilled water in the 2:1
ratio of powder to water, according to the recommended
protocol. The control material was mineral trioxide aggre-
gate (ProRoot MTA, Dentsply Maillefer, Ballaigues, Swit-
zerland) mixed in a 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 350 g each, were used. Af-
aft the animals were anaesthetized, two-centimeter-long
incisions in the animals’ backs were made in the head-to-
tail direction. Using the blunt dissection to the right and
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 diam-
eter 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 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, il-
nuation in xylol, and paraffin embedding. Paraffin sam-
ple: 4 μm thick were stained in haematoxylin and eosin.
Microscopic slides were analyzed in an optical microscope
(BX-51, Olympus Corporation, Tokyo, Japan) and micro-
photographs were taken by a digital camera (CD video
camera, PixeLink, Gloucester, Ontario, Canada) connected
to a 19” PC screen (Dell Inc., Round Rock, TX, USA) [2].

In line with international standards (ISO 10993-6, Bi-
ological 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 his-
tological examination of the prepared samples, the param-
eters were analyzed qualitatively and semi-quantitatively
(modified according to Scarparo et al. [6] and Lotfi et al.
[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 – moder-
ate; 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, av-
erage weight of 4 kg, on controlled diet and receiving
daily care. For the purposes of the surgical procedure, the
general dissociative anesthesia (xylazine, ketamine, ace-
promazine) was administered. The average duration of
anesthesia was 100 minutes.

The surgical procedure was carried out in aseptic con-
ditions and so as to ensure minimum trauma. Each tooth
was cleaned, dried and disinfected (30% hydrogen perox-
ide and 5% iodine tincture). Class V cavities were then cre-
ated 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 MTA and it was
implanted in the upper right maxillary incisor, while the
other three incisors were implanted with CS and CS-HA,
in two rabbits respectively. The animals were sacrificed
after 10 and 15 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 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, ×100, and ×200. 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.

In the examined samples seven 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.

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 venous stasis, receiving score of 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).

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 (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).

Table 1. Mean value of inflammation scores and standard deviation

<table>
<thead>
<tr>
<th>Material</th>
<th>7 days</th>
<th>15 days</th>
<th>30 days</th>
<th>60 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>3 ± 0.71</td>
<td>2 ± 0.71</td>
<td>1.60 ± 0.55</td>
<td>0.5 ± 0.58</td>
</tr>
<tr>
<td>CS-HA</td>
<td>3 ± 0.82</td>
<td>1.6 ± 0.55</td>
<td>1.40 ± 0.55</td>
<td>0.2 ± 0.45</td>
</tr>
<tr>
<td>MTA</td>
<td>3.3 ± 0.48</td>
<td>2.3 ± 1.06</td>
<td>1.90 ± 0.88</td>
<td>0.44 ± 0.73</td>
</tr>
<tr>
<td>Control</td>
<td>2.5 ± 1.43</td>
<td>1.9 ± 0.74</td>
<td>1.50 ± 0.53</td>
<td>0.67 ± 1.00</td>
</tr>
</tbody>
</table>

CS – calcium silicates; CS-HA – calcium silicate with hydroxyapatite; MTA – mineral trioxide aggregate
The connective tissue was with a usual number of blood vessels and no signs of venous 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 venous stasis observed in any of the tested material (0).

The histological examination results of the rabbit teeth direct pulp capping are shown in Table 2 and Figures 7–11.

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).

All the observed samples where CS-HA was used as the direct capping material showed signs of moderate inflammation (Figure 8).

The blood vessels of normal structure and in a normal number, with no signs of venous stasis observed in any of the tested material (0).

The histological examination results of the rabbit teeth direct pulp capping are shown in Table 2 and Figures 7–11.

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).

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 a score of 2. A significant number of gigantic cells and scattered particles of the material were observed in the samples (Figure 9), which was statistically significant.
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 particles of the phagocytized material were also observed (Figure 11). No bacteria were detected in any of the samples.

DISCUSSION

At present, enormous progress has been achieved in synthesizing a number of new materials used in 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 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 synthesis that leads to oxidative stress. Mitochondria are the main locations of reactive oxygen species production in a cell, which can result in the generation of a hydroxyl radical – one of the most potent oxidizing agents in nature. Oxidative stress is the most common cause of cell injury.

Nanostructured calcium silicates, CS and CS-HA (with particles in the range of 117–477 nm), 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 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 a 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 initially caused 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].

Tissue reaction to empty tubes (negative control) in this experiment is similar to findings reported by other researchers [9, 22, 24]. It was most severe on the seventh and the 15th 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 et al. [5] and Scarparo et al. [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 remodeling 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 needs 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 nanotopography of the surface and adsorbed proteins have direct influence on the cell behavior and activity, primarily with respect to their adhesion and retention at the point of application [26].

Nanostructured CS cements show increased osteoblast adhesion, proliferation, and differentiation, since the bone itself has nanostructure, 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, mobilization of progenitor cells, differentiation, and, finally, biomaterial-assisted mineralization [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 tooth 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 CS cement 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 MTA (pH being 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 favorable effect on adhesion and proliferation of cells involved in the healing processes [12, 29]. Asgary et al. [30] observed that new endodontic cements having similar structure as MTA but improved physical and chemical properties – they include the nanomaterials tested – show better pulp response (weaker inflammatory reaction) and thicker dentine bridge then MTA at a later stage.

Biomaterials are normally tested on animals, since they are a model of the environment one can find in humans. Nevertheless, animas are characterized by a huge range of differences with respect to anatomy, biochemistry, physiology, and other variables. 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 favorable 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.
REFERENCES


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 кунцита.

Методе У поткожно ткиво 40 вистар пацова су имплантирани тестирани материјали, а после 7, 15, 30 и 60 дана животиње су жртвоване. Директно прекривање пулпе је реализовано на зубима кунцита. На вестибуларним површинама секутића препарисани су кавитети, а експонирана пулпа је прекривана тестираним материјалима. Животиње су жртвоване после 10 и 15 дана. Контролни материјал у оба експеримента је био минерални триоксидни агрегат (МТА).

Резултати Седам дана после супкутане 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 реакција пулпног ткива кунцита уочена је код материјала КС (1,83 ± 0,75), затим код МTA и КС-ХА (2,67 ± 1,53, 3,00 ± 0,63).

Закључак Новосинтетисани материјали су изазвали блаху запаљенску реакцију поткожно ткива пацова, а КС-ХА је показао најбољу ткивну толеранцију. Наноструктурни биоматеријали КС и КС-ХА су узроковали благу до умерену запаљенску реакцију пулног ткива кунцита само у непосредној близини имплантираног материјала.

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