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

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

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

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

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

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

INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

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

Animals and Study Design

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

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

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

0 – no inflammation (no inflammation cells),

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

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

3 – strong inflammatory response (125 inflammatory cells).

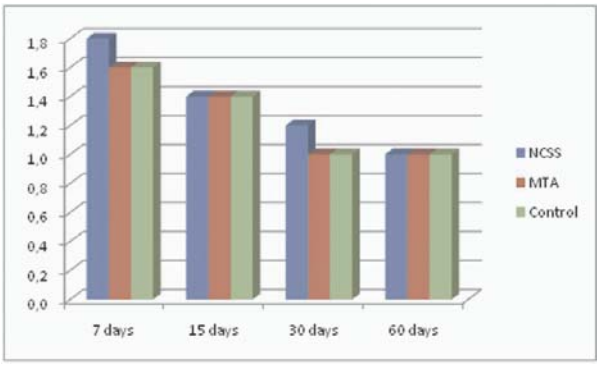
Statistical analysis was performed using one-way ANOVA and Holm-Sidak's test for multiple comparisons.

RESULTS

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

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

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



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

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

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

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

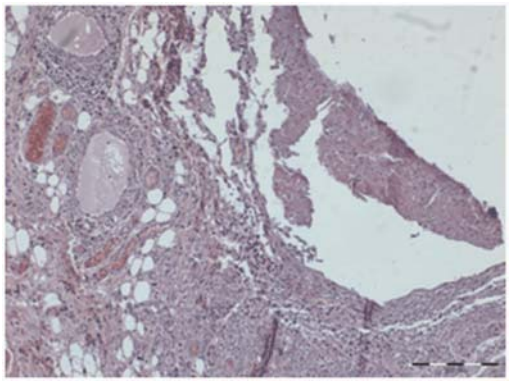


Figure 1. Histological image of the tissue in the control group after 7 days, tissue necrosis, magnification 10×

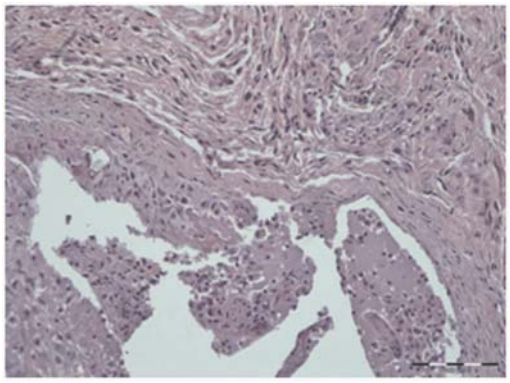


Figure 2 Histological image of the tissue in the MTA group after 7 days, tissue necrosis, magnification 20×

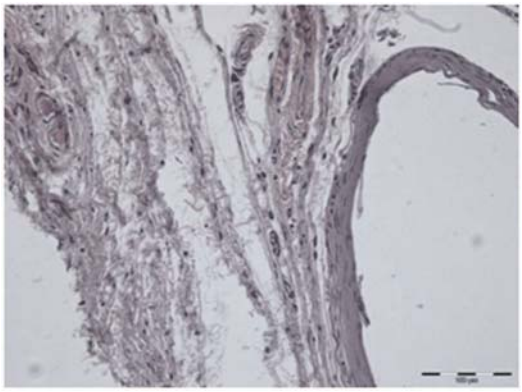


Figure 3. Histological image of the tissue in the NCSS group after 30 days, mild inflammatory reaction (score 1), magnification 200×

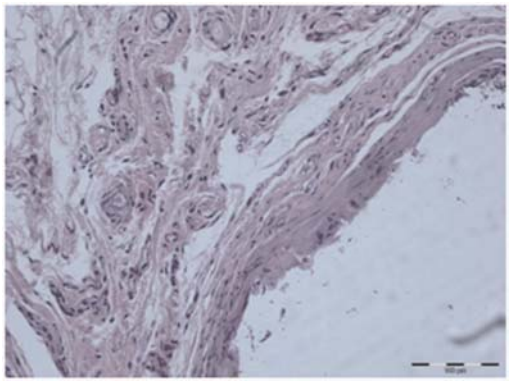


Figure 4. Histological image of the tissue in the MTA group after 30 days, mild inflammatory reaction, (score 1), magnification 200×

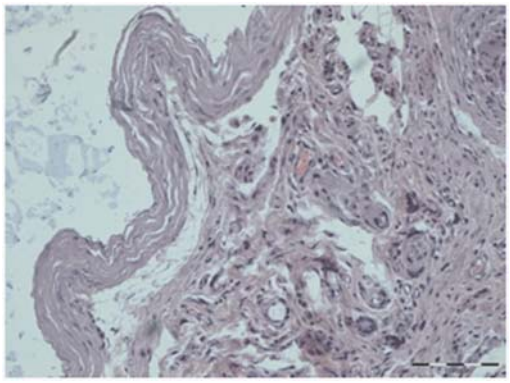


Figure 5. Histological image of the tissue in the control group after 30 days, mild inflammatory reaction, (score 1), magnification 200×

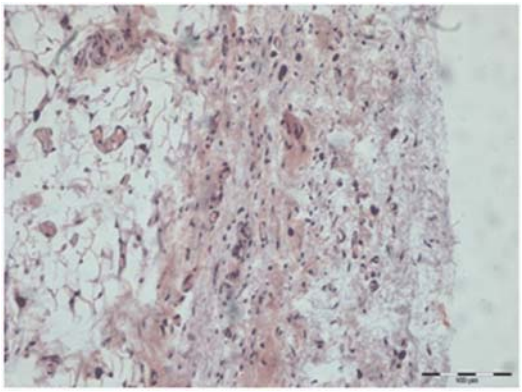


Figure 6. Histological image of the tissue in the NCSS group after 60 days, mild inflammatory reaction, (score 1), magnification 200×

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

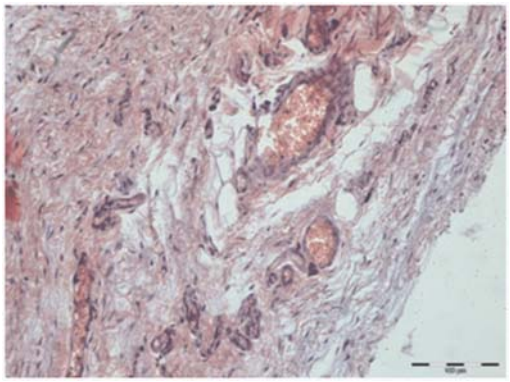


Figure 7. Histological image of the tissue in the MTA group after 60 days, mild inflammatory reaction, (score 1), magnification 200×

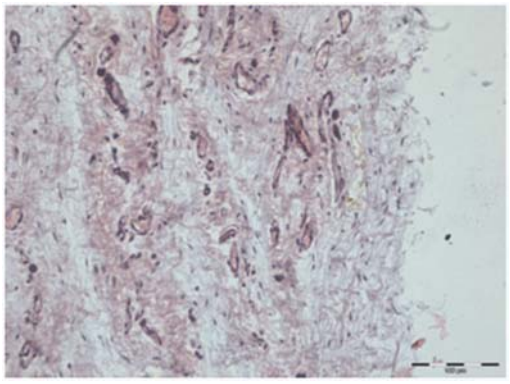


Figure 8. Histological image of the tissue in the control group after 60 days, mild inflammatory reaction, (score 1), magnification 200×

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

DISCUSSION

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

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

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

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

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

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

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

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

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

CONCLUSION

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

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

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SADRŽAJ

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

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

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

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

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