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**Biocompatibility of new nanostructural materials based on active silicate systems and hydroxyapatite: *in vitro* and *in vivo study***

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**Abstract**

**Aim** To evaluate *in vitro* cytotoxicity and *in vivo* inflammatory response to new nanostructural materials based on active calcium silicate systems (CS) and hydroxyapatite (HA-CS).

**Methodology** Cytotoxicity of eluates of new nanostructural non-commercial materials CS and HA-CS, and MTA (White MTA, Angelus® Soluções Odontológicas, Londrina, Brazil) as a control, were tested using the MTT assay on MRC-5 cells. Eluates of set materials were tested in 100% and 50% concentrations, 24h, 7 days and 21 days post-elution. The pH values were determined for undiluted eluates of set materials. Polyethylene tubes containing the test materials (CS, HA-CS, MTA) were implanted in subcutaneous tissue of Wistar rats. Histopathological examinations were conducted at 7, 15, 30 and 60 days after the implantation. Data were statistically analyzed using three-way and one-way ANOVA Tukey's post-hoc test as well as Kruskall-Wallis test with Dunn's post-hoc test at $\alpha=0.05$.

**Results** All materials significantly reduced cell viability; especially when undilluted eluates were used ($p<0.001$). After 24h elution cell viability was 10±1.8 %, 49.5±4.2 % and 61±7.4 %, for MTA, CS and HA-CS, respectively. However, CS and HA-CS was significantly less toxic than the control material MTA ($p<0.05$). Cytotoxicity could be at least partially attributed to pH kinetics over time. Dilution of eluates of all tested materials resulted in better cell survival. Histopathological examination indicated similar inflammatory reaction, vascular congestion and connective tissue integrity associated with CS, HA-CS and MTA at each observation period ($p>0.05$). The only significant difference was found for capsule thickness, i.e. thicker capsule was associated with HA-CS compared to MTA at 60 days

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(p=0.0039). HA-CS induced moderately thick capsules (median score 3, score range 2-3) whereas MTA resulted in thin capsule formation (median score 2, score range 1-3).

**Conclusions** Evaluation of cytotoxicity and inflammatory response indicated better biocompatibility of CS and HA-CS, in comparison with MTA (White MTA, Angelus® Soluções Odontológicas, Londrina, Brazil).

## Introduction

The potential use of calcium phosphate cements in endodontic therapy is an active area of research. Hydroxyapatite is one of the most commonly used calcium phosphate materials in medicine and dentistry. Biocompatibility of hydroxyapatite is closely related to its chemical composition, similar to dental and bony tissues. However, inferior mechanical properties limit the use of hydroxyapatite as an endodontic material. Recent studies have focused on new and modified formulations of calcium-phosphate-based biomaterials with improved mechanical and maintained favorable biological properties (Huan & Chang 2009, Khashaba *et al.* 2011, Damas *et al.* 2011, Modareszadeh *et al.* 2012, Chen *et al.* 2013, Hakki *et al.* 2013).

Recently, two non-commercial new materials based on calcium silicates and hydroxyapatite have been synthesized using two combined techniques – a hydrothermal sol-gel method and self-propagating combustion waves. One material is a calcium silicate system of tricalcium and dicalcium silicates (CS) and the other one is a mixture of the calcium silicate system and hydroxyapatite in 1:2 ratio (HA-CS). Barium sulphate was added for radiopacity. Both CS and HA-CS are nanostructural materials containing agglomerates, several micrometers in size, formed by smaller particles, 117-447 nm in size, which contain even smaller building blocks, 20 nm-sized crystallites (Opačić-Galić *et al.* 2013). Particle size affects cement hydration and consequently setting time and final quality of the cement. Smaller particles provide larger surface area available for hydration and speed up setting (Asgary *et al.* 2009). The nanostructure of

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the newly synthesized materials improves particle activity and shortens setting time to 10 minutes (CS) and 15 minutes (HA-CS) (Opačić-Galić *et al.* 2013). Fast setting is a clear clinical advantage whilst cement composition and internal nanostructure are expected to provide biological behavior in vital tissues.

The aim of this study was to evaluate the biocompatibility of new nanostructural materials based on calcium silicates (CS) and hydroxyapatite (HA-CS) by applying an *in vitro* cytotoxicity test, as well as *in vivo* histological responses to the materials. The results were compared with *in vitro* cytotoxicity and *in vivo* biocompatibility of MTA (White MTA, Angelus® Soluções Odontológicas, Londrina, Brazil), used as a control material.

**Materials and Methods**

The study was approved by the Ethical Committee of the School of Dental Medicine, University of Belgrade, Serbia (Protocol No. 36/5, 12/04/2012 and 36/21, 20/06/2013). All experimental procedures were conducted in accordance with ISO 10993-5, ISO 7405 and ISO 10993-2 specifications. CS and HA-CS were synthesized according to the protocol explained previously and mixed with distilled water in a powder-to-liquid ratio of 2:1 (Opačić-Galić *et al.* 2013). MTA (White MTA, Angelus® Soluções Odontológicas, Londrina, Brazil) was used as a control material. The powder-to-liquid ratio for MTA was 3:1 according to manufacturer's instructions.

Preparation of eluates of set materials

Prefabricated polyethylene moulds 5 mm in diameter and 2 mm deep, placed on sterile glass plates, were filled with unset materials CS, HA-CS and MTA, (12 replicas per material). Materials were left to set in an incubator at 37°C for 24h. After setting, the samples were sterilized under UV light (Laminar, BIO-CL-130, EHRET Gmbh&CO.KG, Emmendingen, Germany). Eluates of materials were prepared by immersing each of them in 2 mL of DMEM, previously poured in a separate well of 12 well-

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culture plates (Sarstedt Inc, Newton, NC, USA).The ratio between the surface of the samples and the volume of medium was approximately 71 $mm^2$/mL. Eluation were performed without medium changing in an atmosphere with 5% $CO_2$ at 37°C for 24h, 7 and 21 days, for cytotoxicity testing, and for 24h, 4, 7 and 21 days, for pH analysis. For cytotoxicity testing, undiluted eluates (100% concentration) and dilluted ones (50% concentration) were used, both sterilized by filtration through 0.45 µm Cronus Sterile Syringe filters (SMI-Labhut Ltd, Gloucester, UK).

Cell culture

Human lung fibroblasts MRC-5 (ECACC Nº84101801) were grown in 75 $cm^2$ culture plates (Sarstedt Inc, Newton, NC, USA) in DMEM supplemented with 10% fetal bovine serum, containing 2 mM of L-glutamine and 100 IU/mL penicillin/streptomycin at 37°C in a humidified incubator containing 5% $CO_2$/37°C (MRC Scientific instruments, Holon, Israel). Confluent monolayer of cells, verified under light microscope (Boeko, Hamburg, Germany), were washed with phosphate-buffered saline (PBS), detached with 0.25% trypsin, centrifuged and re-suspended in medium. The cells were then seeded in 96-well plates, ($1x10^4$cells/200µL medium/well) and incubated for 24h to form cell monolayer before its exposure to test materials. All manipulations with cells were done under aseptic conditions (Laminar, BIO-CL-130, EHRET Gmbh&CO.KG, Emmendingen, Germany).

Cytotoxicity test

The cytotoxicity of eluates was determined by MTT reduction assay (Hansen *et al.* 1989) using the 1-(dimethyltiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) as an indicator of cell viability. This colorimetric method is based on reduction of tetrazolium compound into colored formazan product by mitochondrial dehydrogenases in viable and active cells. For each test material, both 100% and 50% eluates were tested in two independent experiments with six wells per treatment point.

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After 20h incubation of cells in eluates of materials prepared in DMEM the medium was removed and cells were washed with PBS. The MTT in final concentration 0.5 mg/mL (Sigma Chemical Co., St Louis, MO, USA) was then added and the plates were incubated for additional 3h. The MTT solution was then carefully removed and the formazan crystals were dissolved in dimethyl-sulphoxyde. Cell viability was determined by measuring the absorbance at 570 nm, using a Micro-plate reading Spectrophotometer (Thermo, Scientific Multiscan FC, Vantaa, Finland). The relative cell viability of the test materials was calculated as follows:

$$\text{Relative cell viability of test material (\%)} = \frac{\text{OD value of test material}}{\text{OD value of negative control *}} \times 100$$

* Untreated cells served as negative control.

pH analysis

pH analysis of undiluted eluates was performed within 24h, 4, 7 and 21 days, using a calibrated pH meter (Cyberscan pH 510, Eutech Instruments Europe B.V, Landsmeer, The Netherlands).

Experimental design for subcutaneous implantation

Forty Wistar albino male rats, a mean weight 350 g, were selected and randomly divided into four groups ($n$ = 10). The animal received intraperitoneal injection of 75 mg/kg ketamine and 5 mg/kg of body weight of diazepam. After shaving the backs of animals, skin was disinfected by iodine tincture. Two incisions on both sides of spinal cord in a head-tail direction, 15 mm in length were created using the scalpel blade. Two pockets, 15 mm deep, were than prepared by blunt dissection. Samples of freshly mixed materials was placed in one half of a sterile polyethylene tube, 10 mm long and with 1 mm inner diameter, while the other half remained empty and served as a negative control. The tubes were implanted subcutaneously in the previously prepared pocket and the incisions were sutured using single resorbable sutures. Each animal received two tubes: on the right side the one filled with the test material (CS or HA-CS), and on the left side the one filled with MTA, used as a control material.

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Animals were sacrificed in groups of ten after 7, 15, 30 and 60 days (5 animal per tested material), using a high dose of anaesthetic. 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 haematoxylin and eosin and analyzed histologically.

A modified criterion described by Lotfi *et al.* (2009) and Scarparo *et al.* (2010) was used for evaluation of tissue reaction. Slides were analyzed qualitatively and semi-quantitively under a light microscope (Olympus BX-51, Tokyo, Japan) at x40, x100 and x400 magnification. The cellular inflammatory component was determined by the presence of neutrophils, lymphocytes, plasmacytes, macrophages and giant cells. Quantitative evaluations of inflammatory cells were made in four separate areas. The inflammatory reactions were categorized as 0 or none (without inflammatory cells), 1 or minimal (inflammatory cells<25), 2 or mild (26-50 inflammatory cells), 3 or moderate (51-100 inflammatory cells), and 4 or severe (more than 100 inflammatory cells). Fibre condenzation were classified according to the following scale: 0 - absence of collagen fibre, 1 - presence of a minimal layer of collagen fibre, 2 - presence of a thin layer of collagen fibre, 3 - presence of a thick layer of collagen fibre and 4 - large capsule.

Statistical analysis

The data for the cytotoxicity of eluates were analyzed statistically using three-way ANOVA for the factors 'material', 'concentration' and 'time'. Interactions between the factors were also tested. Differences for each material across time periods were tested using repeated measures ANOVA. When it was necessary, *i.e.* interaction was significant, one-way ANOVA with Tukey's post-test was used to test differences between materials at each concentration / time.

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For histological analyzes one-way analysis of variance (non-parametric ANOVA, Kruskal–Wallis test) was used to analyze differences between the treatments within each experiment. Dunn's Post Test was used to compare the differences between groups. Statistical analysis was performed in Minitab 16 (Minitab Inc. State College, PA, USA).

## Results

*Cytotoxic potential of nanomaterials*

The results of cytotoxicity of eluates are presented in Fig. 1. Cell viability following exposure to undiluted eluates (100%) of all materials was significantly lower in comparison with untreated control at all time periods ($p<0.001$). There were no significant differences in cell viability following exposure to CS and HA-CS eluates at 24h and 7 days ($p>0.05$). In 21-day eluates, cell viability was greater after treatment with HA-CS than with CS ($p<0.001$). In 100% concentration, MTA eluates resulted in significantly lower cell viability compared to CS and HA-CS eluates at all test periods ($p<0.001$). Cytotoxicity of 100% MTA eluates increased over time and was greater after 21 days elution, compared to previously performed (24h and 7 days) ($p<0.001$). Dilution of all eluates resulted in significantly lower toxicity in comparison to undiluted ones but cell viability was still lower than in the untreated group ($p<0.001$).

The highest pH values of all materials was obtained after 7 days, and virtually no further changes were observed for CS and MTA, whilst HA-CS showed a slight decreasing tendency over time (Fig. 2).

*Inflammatory response to nanomaterials*

The histological micrographs (20 microscope slides were prepared in duplicate for each material/evaluation period) are presented in Fig. 3. The parameters of inflammation response and thickness of formed fibrous capsule are estimated and presented in Table 1.

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Seven days after implantation the intensity of the inflammatory reaction in connective tissue around the implant (Fig. 3A, Table 1) was estimated as moderate for all materials. The histological data obtained for negative controls showed mild inflammatory reaction with destruction of collagen fibres but without necrosis. The increased number of blood vessels indicated formation of granulation tissue and repair. Furthermore, thin fibrous capsules were observed around all materials, as well as around control parts of the tubes.

The micrographs of tissue after 15 days of implantation showed mild inflammation associated with all materials and the negative control (Fig. 3B, Table 1). Connective tissue contained the usual number of blood vessels with minimal signs of venous stasis. The integrity of pericapsular connective tissue was disturbed only minimally, with small numbers of lymphocytes and plasma cells. A thin capsule was formed around the implant tubes while inflammatory cells were not detected in the capsule. The thickness of capsules was most pronounced for HA-CS implants, and the thinnest for the negative control (empty tubes).

Thirty days after implantation the connective tissue exhibited signs of good recovery with minimal numbers of inflammatory cells for all materials, (Fig 3C, Table 1). Furthermore, capsules around all implants were well-developed and no significant differences were observed between them.

At the end of the evaluation period (60 days) a well-developed fibrous capsules were formed around all implants, furthermore the common structure and number of blood vessels was detected, almost without signs of inflammation (Fig 3D, Table 1). The calming of inflammation and the best formed capsule were the most notable for HA-CS implant. The difference in the thickness of the capsule between HA-CS and MTA was significant ($p=0.0039$).

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**Discussion**

An excellent tissue biocompatibility of endodontic cements materials is desirable for clinical use, since these materials come into direct contact with living connective tissues. Ideally, a good endodontic cement material should be biologically compatible and well tolerated, avoiding any possible interference and/or delay of the healing process (Testarelli *et al.* 2012).

In the present study, initial screening of *in vitro* cytotoxicity of two experimental materials and MTA, used as the control material, was performed using the MTT assay, one of the most commonly used tests for evaluation of material toxicity in a cell culture (Damas *et al.* 2011, Gomes Cornelio *et al.* 2011, De Deus *et al.* 2005, Modareszadeh *et al.* 2011). Cytotoxicity of undiluted (100%) and diluted (50%) eluates from set materials was measured at three time intervals to evaluate dependency of material toxicity and concentration and to follow toxicity over time. The use of eluates for cytotoxicity testing is a routine method for *in vitro* evaluation of potential toxic effect of leachable ingredients and by-products. This approach may be considered as a simplified *in vitro* simulation of clinical conditions, since leachable substances may elute out from the material after its *in vivo* application (Modareszadeh *et al.* 2011).

In general, CS and HA-CS eluates had lower cytotoxicity than MTA eluates. Previous studies have shown variable results regarding the peak of cytotoxicity of MTA which spanned from the lack of any effect on cell viability (Damas *et al.* 2011, Modareszadeh *et al.* 2012), to measurable cytotoxic effects after 24h (De Deus *et al.* 2005) and 14 days post-exposure (Modareszadeh *et al.* 2011). Similarly, some authors have reported increased cytotoxicity over time (De Deus *et al.* 2009, Karimjee *et al.* 2006), whilst others found variable cytotoxic effects of different MTA-based commercial materials (Lee *et al.* 2012). Difference in the cell line used, in the protocols for material preparation and setting, in the ratio of sample surface area and volume of the elution medium, in duration of exposure, as well as in the monitored parameters of cell viability, may account for variable results of the aforementioned studies.

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The present pH data indicates different ion elution kinetics, as well as different ion concentrations eluted from the test materials. The highest and most rapid ion release was found for MTA which is in agreement with the findings of Duarte *et al.* (2003), reporting high pH value of MTA eluates prepared in distilled water over 7 days. Formosa *et al.* (2013) reported high pH value of calcium silicate-based cement eluates in simulated tissue fluid after 28 days. Cytotoxicity of the materials could be associated with pH values obtained at the corresponding time point. Cytotoxicity of MTA corresponded well with high pH observed in this study. The dynamics of pH and cytotoxicity of undiluted CS eluates corresponded to each other: they increased over first 7 days and were maintained at high levels up to 21 day. Similarly, undiluted HA-CS eluates exhibited the same relationship between pH values and cytotoxicity: at the 21 days both pH values and cytotoxicity were decreased.

Despite the similar calcium silicate nature of MTA and CS, the greater cytotoxicity of MTA could be related to differences in radiopacifying agents. CS contains barium sulfate, a well-known radiopaque contrast medium used in medicine, whereas MTA contains bismuth oxide (Camilleri & Ford, 2006). Bismuth oxide interferes with MTA hydration, becomes a constituent of calcium silicate hydrate and may elute along with calcium hydroxide from set MTA (Camilleri 2008). Gomes Cornelio *et al.* (2011) reported the effects of radiopacifying agents, including bismuth oxide, on cytotoxicity of Portland cement. They detected that Portland cement alone was not cytotoxic even at high concentrations (100 mg/mL) whereas its cytotoxicity significantly increased after addition of radiopacifying agents.

It needs to be emphasized that nanostructural materials do not have heavy metals in their composition. Furthermore, structures built on three hierarchical levels (agglomerates, particles and crystallites) could be especially promising. Dimensions of agglomerates are not comparable with dimensions of membrane channels, preventing harmful biological effects, whereas their nano-elements (nano-crystallites) provide evident improvements to settings characteristics.

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Lower cytotoxicity of HA-CS compared to CS, and especially to MTA, could be associated with lower pH and the presence of hydroxyapatite, the main ingredient into which calcium silicate is added to improve mechanical properties (Huan & Chang, 2009). Beside lower cytotoxicity found in the present study, HA-CS has previously been related to have lower genotoxic potential in human lymphocytes (Opačić-Galić *et al.* 2013).

The histological response of tissue to the biomaterials is one of the most important criteria for biocompatibility determination, as well as the dynamics of fibrose capsule formation over 60 days, as a good marker of tissue recovery (Lotfi *et al.* 2009, Martinez *et al.* 2009, Yavari *et al.* 2009, Khashaba *et al.* 2011, Silva-Herzog *et al.* 2011, Parirokh *et al.* 2011, Gomes-Filho *et al.* 2012). Histological examination was performed 7, 15, 30, and 60 days after implantation. The regions around CS and HA-CS implants exhibited the most notable inflammatory reaction within 7 days, with moderate disturbance of connective tissue. After that period, the intensity of inflammation induced by CS and HA-CS decreased up to 30$^{th}$ day, and dramatically decreased in subsequent periods, resulting in negligible inflammation at 60$^{th}$ day. The tissue around the part of tube with MTA showed the most evident signs of inflammation, which was characterized as pronounced at 7$^{th}$ day, moderate at the 15$^{th}$ and 30$^{th}$ day and negligible at the 60$^{th}$ day. Similar tissue response was reported by Parirokh *et al.* (2011), who observed coagulation necrosis and dystrophic calcification. High inflammatory reaction induced by MTA could be a consequence of the high pH of mixed MTA and heat released during the binding of MTA. It is known that high pH and heat release can stimulate induction of inflammatory cytokines (interleukins 1 and 6) which contributes to the strong inflammatory response of tissues (Moretton *et al.* 2000, Modaresi *et al.* 2005, Camilleri and Ford 2006, Lotfi *et al.* 2009). With the reduction of inflammation, formation of good organized fibrous capsule was observed for all materials. The best developed capsule was detected for HA-CS, indicating well tolerance of tissue to this material. However, the difference between tissue response obtained for empty part of tubes, used as negative controls, and for tested materials, indicated that MTA, CS, and especially HA-CS, did not significantly contribute to the inflammation reaction. The similar results corresponding

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tissue reaction to the control tubes have already been reported (Vosoughhosseini *et al.* 2008; Yavari *et al.* 2009; Gomes-Filho *et al.* 2012).

CS and especially HA-CS, induced significantly lower cytotoxicity in comparison with MTA, which could be associated with slower ion elution and steady pH values. Both experimental materials exhibited dose-dependent cytotoxicity. The results obtained from histological examination showed that CS, and especially HA-CS, induced significantly less inflammation than MTA, which disappeared 60 days after implantation. Taking into account previously reported data, indicating good mechanical properties, short setting time, and low genotoxicity (Opačić-Galić *et al.* 2013), as well as significantly lower cytotoxicity and inflammation, i.e. better biocompatibility than MTA, both nanostructural biomaterials based on active silicate systems and hydroxyapatite, especially HA-CS, could be candidates for future clinical studies.

**Conclusions**

Evaluation of cytotoxicity and inflammatory response indicated better biocompatibility of CS and HA-CS, in comparison with MTA (White MTA, Angelus® Soluções Odontológicas, Londrina, Brazil)

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**Figure legends**

**Figure 1** Cell viability following exposure to undiluted (100%) and diluted (50%) eluates of set materials. Upper case letters indicate statistical differences between materials at one test period (*e.g.* MTA at 24 h *versus* CS at 24 h *versus* HA-CS at 24 h); groups with the same letter are not significantly different ($p>0.05$). Lower case letters indicate statistical differences within one material at different test periods; groups with the same letter are not significantly different ($p>0.05$). Asterisks indicate statistical differences between undiluted (100%) and diluted (50%) eluates of the same material at the same test period ($p<0.05$).

**Figure 2** Kinetics of pH values of eluates from set materials over 21 days.

Figure 3 The effect of nanomaterials on the inflammation induction 7 (A), 14 (B), 30 (C) and 60 (D) days of exposure. A a) CS: moderate inflammatory reaction with rare appearance of monocytes, lymphocytes and granulocytes in inflammatory infiltrate; gentle, very thin capsule (arrows) around the implant was observed (HE, 400 x). b) HA-CS: connective tissue revealed normal histological structure, without signs of necrosis, infection or inflammation. Connective tissue was composed of normal collagen fibers, fibroblasts and minor number of inflammatory cells, suggesting biocompatibility of applied material (HE, 400 x). c) MTA: pericapsular connective tissue with a marked inflammatory response and prominent venous stasis (VS); gentle, very thin capsule around the implant was observed (HE, 400 x). d) Negative control (empty tube): the integrity of connective tissue (CT) was weakly disrupted, the inflammatory infiltrate was dominated by lymphocytes and plasmacytes (HE, 400x).

B a) CS: mild inflammatory reaction, the connective tissue (CT) is mostly preserved integrity. The presence of lymphocytes and plasma cells indicated the chronic inflammatory reaction (HE, 400x). b) HA-CS: mild inflammatory reaction and well developed capsule (arrows) were observed in HA-CS sample, with perivascular and subcapsular inflamatory infiltrate consisted of lymphocytes and plasma cells in the connective tissue (HE, 400x). c) MTA: moderate inflammation, connective tissue exhibited mildly impaired integrity (CT) (HE, 400x). d) Negative control (empty tube): the integrity of connective

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tissue was weakly disrupted. A number of blood vessels (arrows) were observed in pericapsular connective tissue confirming the neoangiogenesis (HE, 400x).

C a) CS: the connective tissue with well-preserved structures; well-developed capsule around the implant (HE, 100x). b) HA-CS: the pericapsular connective tissue integrity was preserved with no signs of vascular congestion; very well-developed capsule around the implant (HE, 100x). c) MTA: Histological analysis showed presence of well developed, dense collagen capsule (arrow). Histological structure of underlying connective tissue was completely maintained without signs of inflammation. (HE, 100x). d) Negative control (empty tube): Well organized capsule (arrow), composed of parallel and thick collagen fibers was observed, separating implanted tube from surrounding tissue. Underlying connective tissue revealed normal histological organization with minimal inflammatory cells. (HE, 100x).

D a) CS: the integrity of connective tissue (SCT) was well preserved; well-developed capsule (arrows) around the implant (HE, 100x). b) HA-CS: the integrity of connective tissue (SCT) was well preserved; very well-developed capsule (arrows) around the implant (HE, 100x). c) MTA: the connective tissue (SCT) was with the preserved integrity; capsule (arrows) was well-formed but thinner in comparison with CS and especially HA-CS (HE, 100x). d) Negative control (empty tube): the integrity of connective tissue was well preserved; capsule (arrows) was well-developed (HE, 100x).

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**Table 1** Histological analysis of the effect of nanomaterials

| Reaction | CS | | CS-control | | HA-CS | | HA-CS-control | | MTA | | MTA-control | | p-value | Intra-group p-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Score range | Med | Score range | Med | Score range | Med | Score range | Med | Score range | Med | Score range | Med | | |
| **Inflammatory reaction** | | | | | | | | | | | | | | |
| 7 days | 2-4 | 3 | 2-4 | 3 | 2-4 | 3 | 2-4 | 3 | 3-4 | 3 | 1-4 | 3 | 0.637 | |
| 15 days | 1-3 | 2 | 1-4 | 2 | 1-2 | 2 | 1-3 | 2 | 1-4 | 2.5 | 1-3 | 2 | 0.567 | |
| 30 days | 1-2 | 2 | 0-2 | 1 | 1-2 | 1 | 1-2 | 1 | 0-3 | 2 | 1-2 | 1.5 | 0.233 | |
| 60 days | 0-1 | 0.5 | 0-1 | 0.5 | 0-1 | 0 | 0-1 | 0 | 0-2 | 0 | 0-3 | 0 | 0.764 | |
| **Vascular congestion** | | | | | | | | | | | | | | |
| 7 days | 2-3 | 3 | 3-4 | 3 | 0-4 | 3 | 2-4 | 2.5 | 1-3 | **2*** | 0-4 | **3*** | 0.098 | 0.0128* |
| 15 days | 1-3 | 1 | 0-3 | 2 | 0-3 | 0 | 0-3 | 1 | 0-3 | 1.5 | 0-2 | 1 | 0.563 | |
| 30 days | 0-1 | 1 | 0-1 | 0 | 0-1 | 1 | 0 | 0 | 0-2 | **1*** | 0-1 | **0*** | 0.112 | 0.0275* |
| 60 days | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0-1 | 0 | 0-1 | 0 | 0.787 | |
| **Capsule thickness** | | | | | | | | | | | | | | |
| 7 days | 1-3 | 1 | 0-2 | 1 | 0-3 | 2.5 | 1-2 | 1.5 | 0-3 | 2 | 0-3 | 1.5 | 0.377 | |
| 15 days | 2 | 2 | 1-2 | 2 | 1-3 | 2 | 1-2 | 2 | 1-3 | 2 | 1-2 | 2 | 0.265 | |
| 30 days | 1-3 | 2 | 1-3 | 2 | 2-3 | 3 | 1-3 | 2 | 1-3 | 2 | 2-3 | 2 | 0.397 | |
| 60 days | 2-3 | 2 | 1-2 | 2 | 2-3 | **3*,**** | 2-3 | **2*** | 1-3 | **2**** | 2-3 | 2 | 0.05 | 0.0027* 0.0039** |
| **Connective tissue integrity** | | | | | | | | | | | | | | |
| 7 days | 2-3 | 3 | 1-4 | 4 | 2-3 | 2.5 | 1-3 | 2 | 1-4 | 3 | 0-3 | 3 | 0.778 | |
| 15 days | 1-3 | 1 | 0-3 | 2 | 0-3 | 1 | 1-3 | 2 | 0-3 | 2 | 0-3 | 1.5 | 0.963 | |
| 30 days | 0-2 | 1 | 0-2 | 1 | 0-1 | 0 | 0-2 | 1 | 0-2 | 1 | 0-1 | 1 | 0.258 | |
| 60 days | 0 | 0 | 0-1 | 0 | 0 | 0 | 0 | 0 | 0-1 | 0 | 0-1 | 0 | 0.321 | |

Med-Median. Within a row, bolded cells with the same superscript are significantly different and matched with the intragroup p-value with the same superscript.

**Inflammatory reaction**: 0-absent, 1-minimum <25 cells, 2-mild 26-50 cells, 3-moderate 51-100 cells, 4-pronounced >100 cells; **Vascular congestion**: 0-absent, 1-minimum, 2-mild, 3-moderate, 4-pronounced, reaction with firing of blood vessels; **Capsule**: 0-– absent, 1-minimum, 2-thin, 3-moderately thick, 4-thick; **Connective tissue integrity**: 0-complete integrity, 1-minimal disruption, 2-mild disruption, 3-moderate disruption, 4-necrosis.

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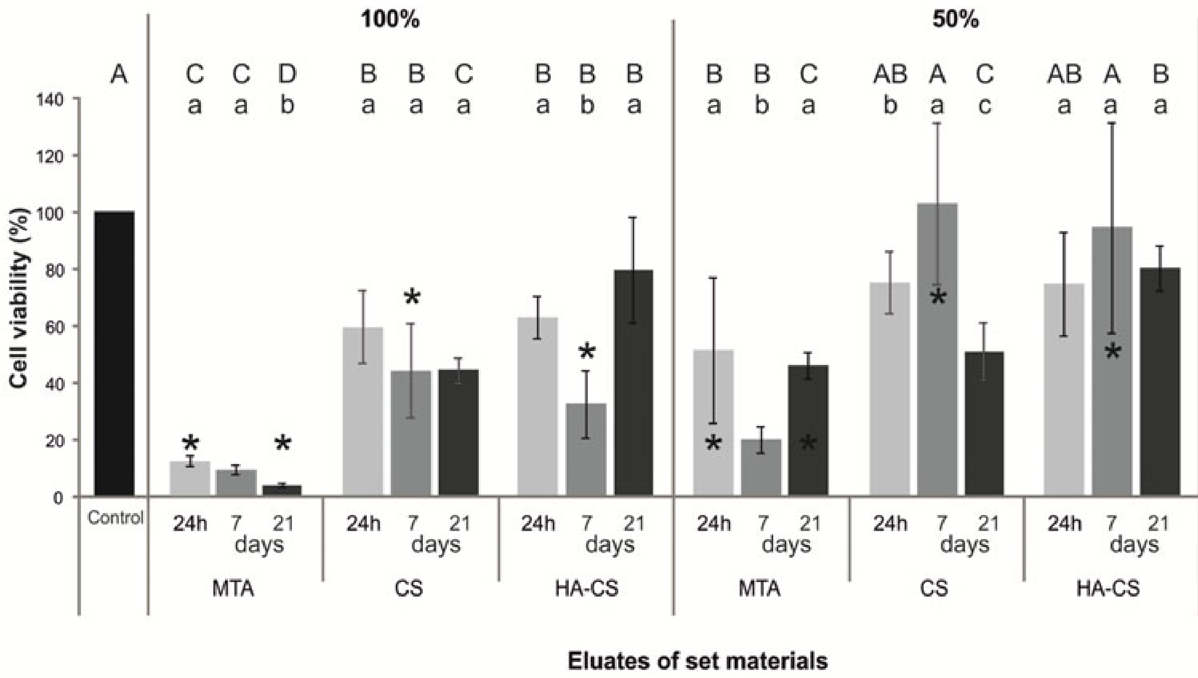


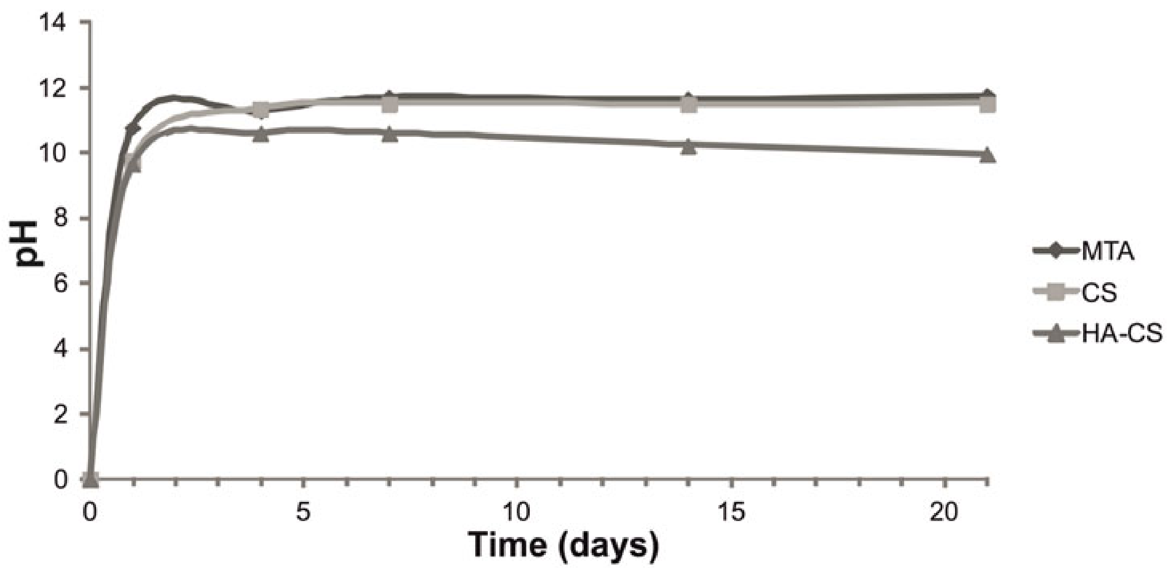


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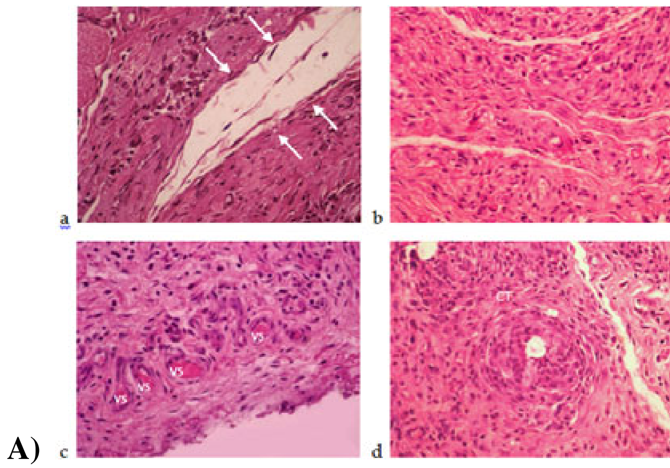


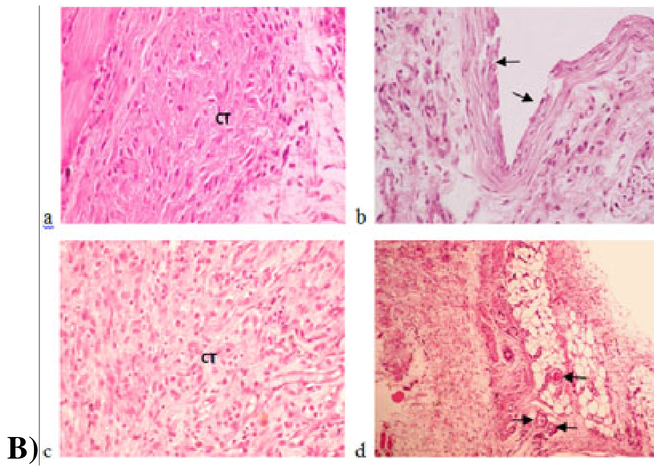


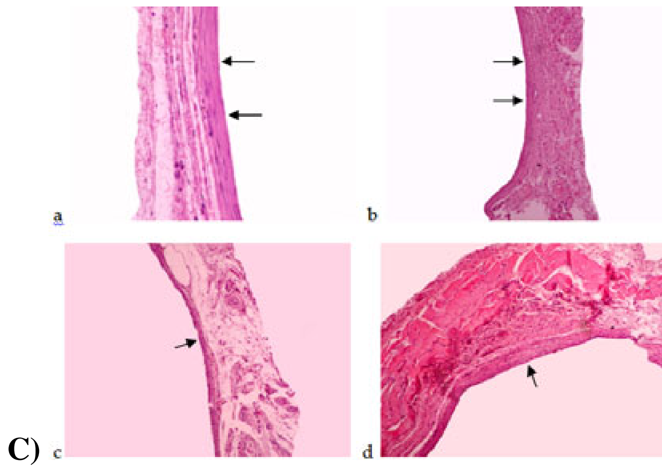


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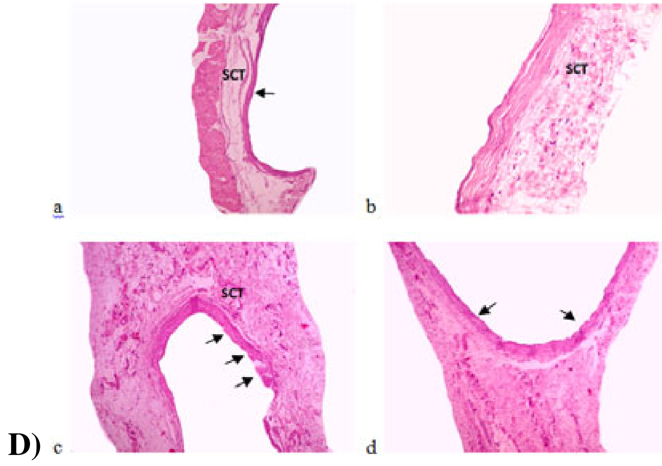


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