Tissue Response and Immunoexpression of Interleukin 6 Promoted by Tricalcium Silicate-based Repair Materials after Subcutaneous Implantation in Rats

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DOI:
10.1016/j.joen.2017.12.006

Elsevier Editorial System(tm) for Journal of Endodontics

Manuscript Draft

Manuscript Number: JOE 17-441R1

Title: Tissue response and immunoexpression of IL-6 promoted by tricalcium silicate-based repair materials after subcutaneous implantation in rats

Article Type: Basic Research - Biology

Keywords: Tricalcium Silicate; Zirconium Oxide; Biocompatibility; Immunohistochemistry; Interleukin-6.

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Manuscript Region of Origin: Latin & South America

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Tissue response and immunoexpression of IL-6 promoted by tricalcium silicate-based repair materials after subcutaneous implantation in rats

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The authors deny any conflicts of interest related to this study.

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***Statement of Clinical Relevance (max 40 words)**

Statement of Clinical Relevance

- The evaluation of inflammatory response induced by experimental tricalcium silicate cement with 20% zirconium oxide and MTA Plus showed similar subcutaneous reaction in rats, suggesting that they are biocompatible to be used as reparative materials.

HIGHLIGHTS

- Inflammatory response induced by experimental tricalcium silicate cement with 20% zirconium oxide and MTA Plus was evaluated.
- A gradual decrease was observed in the number of the inflammatory and IL-6-immunopositive cells.
- Tricalcium silicate repair materials caused similar subcutaneous and immune responses in rats, suggesting that they are biocompatible.
- The tricalcium silicate cements may be use as reparative materials.

Tissue response and immunoexpression of IL-6 promoted by tricalcium silicate-based repair materials after subcutaneous implantation in rats

Abstract

The aim of the present study was to evaluate the inflammatory response induced by experimental tricalcium silicate cement with 20% zirconium oxide (TSC) and MTA Plus (MTAP) in rat subcutaneous tissues. Polyethylene tubes were filled with TSC (n = 20) and MTAP (n = 20), and implanted in the dorsal subcutaneous tissues of thirty two rats. Empty tubes were used as control (CG; n = 20). After 7, 15, 30 and 60 days, the tubes with connective tissue were removed and the inflammatory cells (IC) and immunolabelled cells for interleukin-6 (IL-6) were counted. Data were statistically analyzed by ANOVA and the Tukey tests ($p \leq 0.05$). An increased number of inflammatory and immunolabelled cells for IL-6 were observed at 7 days. The number of inflammatory cells was higher for TSC and MTAP than the GC ($p<0.001$) at 7 days; after 30 and 60 days, no significant differences were observed among TSC, MTAP and CG ($p=0.955$). The number of immunolabelled cells for IL-6 was similar for TSC, MTAP and CG at all evaluated periods. A gradual and significant decrease was observed in the number of IC and IL-6-immunopositive cells. At 60 days, the capsules adjacent to TSC and MTAP exhibited fibroblasts and bundles of collagen fibers. The TSC and MTAP caused similar subcutaneous reaction in rats, suggesting that they are biocompatible and present similar immune responses.

Key Words: Tricalcium Silicate, Zirconium Oxide, Biocompatibility, Immunohistochemistry, Interleukin-6.

Introduction

Mineral Trioxide Aggregate (MTA) is a tricalcium silicate-based cement, with bismuth oxide (Bi_2O_3) as radiopacifier (1). Tricalcium silicate-based biomaterials have demonstrated proper biological and physicochemical properties (2-5). The replacement of Bi_2O_3 by other radiopacifers has been proposed (6-8), since Bi_2O_3 interferes in the hydration of MTA (9), resulting in an increase in porosity and reduction of material strength (10). Furthermore, bismuth oxide interferes in cellular proliferation (11) and calcium silicate-based materials containing Bi_2O_3 induce inflammatory response in rat subcutaneous tissue (12).

Another disadvantage of some MTA materials is the presence of Portland cement as major component, which may contain heavy metal contamination (13). MTA Plus (MTAP) (Avalon Biomed Inc., Bradenton, FL, USA) is composed of tricalcium silicate and dicalcium silicate added to Bi_2O_3 (14). As a material used in the pulpotomies, MTAP produced calcium hydroxide and did not exhibit dental discoloration (15). Moreover, MTAP promoted low immunoexpression of interleukin-1β and -1α, inflammatory cytokines, and allowed mineralized tissue formation over the pulp tissue of rats (16). Gomes-Cornélio *et al.* demonstrated that MTAP presents biocompatibility and bioactivity in human osteoblast-like cells (17).

A manufactured tricalcium silicate cement [$(CaO)_3.SiO_2$, Mineral Research Processing, Meyzieu, France], associated with zirconium oxide (ZrO_2) as a radiopacifier is used as reparative material (18). The association of 80% pure tricalcium silicate (TSC) and 20% ZrO_2 presents proper properties, such as, solubility, radiopacity, compressive strength, setting time and microhardness (3). Furthermore, this cement in contact with simulated tissue fluid demonstrated bioactivity (3,19). Despite these, until now, there is no other evidence of the biological *in vivo* response of this material reported in the literature.

The aim of the present study was to evaluate the tissue response promoted by TSC and MTAP in rat subcutaneous tissues. Morphological and morphometric analyses and the immunohistochemical reaction for detection of interleukin-6, a pro-inflammatory cytokine, were performed. The null hypothesis was that there would be no difference in tissue reactions caused by TSC and MTAP.

Material and methods

The present research protocol was analyzed and approved by the Ethical Committee for Animal Research of Araraquara Dental School, UNESP, Brazil (Process CEUA No. 33/2014).

In this study thirty two rats were used, distributed into three groups: TSC (Tricalcium silicate, Mineral Research Processing, Meyzieu, France, and 20% ZrO_2, Sigma Chemicals, St Louis, Missouri, USA), MTAP (MTA Plus^{TM} Avalon Biomed Inc., Bradenton, Florida, USA) and CG (Control Group; empty polyethylene tubes). The composition and proportion of the materials evaluated are described in Table 1.

After anesthesia with 80 mg/kg ketamine chloride 10% (Virbac do Brasil Indústria e Comércio Ltda, São Paulo, São Paulo, Brazil) and 8 mg/kg xylazine chloride 2% (União Química Farmacêutica Nacional S/A, São Paulo, São Paulo, Brazil), administered by intraperitoneal route, dorsal skin was shaved and disinfected with 5% iodine solution. A 2 cm incision was made with a nº15 scalpel blade (Fibra Cirúrgica, Joinville, Santa Catarina, Brazil). Afterwards, polyethylene tubes (Embramed Indústria e Comércio Ltda., São Paulo, São Paulo, Brasil), measuring 10 mm long x 1.5 mm in diameter, were filled with TSC or MTAP and immediately implanted into the subcutaneous tissue. The site of the incised skin was sutured with simple stitches using 4-0 silk thread (Ethicon Inc., São José dos Campos, São Paulo, Brazil). Two polythene tubes was used in each animal; 5 animals per group were used in each time point.

After 7, 15, 30 and 60 days of implantation, the animals were euthanized with anesthetic overdose, and the implants with the surrounding tissues were removed. The specimens were immediately immersed in a formaldehyde 4% solution (prepared from paraformaldehyde) buffered with sodium phosphate 0.1 M with pH 7.2, for 48 hours. After fixation, the specimens were dehydrated, treated with the xylene and embedded in paraffin. Longitudinal sections (6 µm thick) were obtained and were stained with hematoxylin and eosin (HE) for morphological analysis and quantification of inflammatory cells in the capsules. Other sections were adhered to slides previously treated with silane 4% (Sigma-Aldrich Co., Saint Louis, Missouri, USA), to perform the immunohistochemical reaction for interleukin-6 (IL-6) detection.

Numerical density of inflammatory cells

The images were obtained with a digital camera (DP-71, Olympus, Tokyo, Japan) attached to a light microscope (BX-51, Olympus, Tokyo, Japan).

The number of inflammatory cells (IC) was quantified using an image analysis system (Image Pro-Express 6.0, Olympus), as previously described (12). The quantitative analysis was performed in all samples or implants; in each implant, three HE-stained sections of the capsule were used at intervals of at least 100 µm. In each section, a standardized field of 0.09 mm^2 of the capsule in close juxtaposition to the opening of implanted tube was captured totalling 0.27 mm^2 per implant. In each field, the number of IC (neutrophils, lymphocytes, plasma cells and macrophages) was computed. In this way, in each implant, the number of IC/mm^2 was obtained. The numerical density of inflammatory cells was measured by two calibrated and blinded examiners.

Immunohistochemical reaction for detection of IL-6

For IL-6 detection, the primary goat antibody anti-IL-6 (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) was used. Deparaffinized sections were immersed in 0.001 M sodium citrate buffer with pH 6.0, and submitted to microwave oven cycles for 20 minutes at 90-94ºC for antigen retrieval. After cooling, the slides were washed in 0.05 M Tris-HCl buffer with pH 7.4, and subsequently immersed in 5% hydrogen peroxide for 20 minutes, for blocking endogenous peroxidase. After washing, the sections were incubated with 2% bovine serum albumin (Sigma-Aldrich Co., Saint Louis, Missouri, USA), for 20 minutes. Sections were incubated overnight in a humid chamber at 4°C with anti-IL-6 antibody, diluted 1:100.

Subsequent to washing in Tris-HCl buffer, the sections were incubated for 40 minutes at room temperature with a multi-link solution containing biotinylated rabbit/mouse/goat antibodies (LSAB, Dako Inc., Carpinteria, California, USA). After washing in Tris-HCl buffer, the sections were incubated with HRP complex (System-HRP, Dako Inc., Carpinteria, California, USA), for 40 minutes at room temperature. Subsequent to washing in buffer, peroxidase activity was revealed by the chromogen 3.3-diaminobenzidine-HCl (DAB, Dako Inc., Carpinteria, California, USA) for 3 minutes. The sections were counterstained with Carazzi's hematoxylin. As negative control, some sections were submitted to all the stages, except incubation with primary antibody; in this step the sections were incubated in non-immune serum.

Numerical Density of cells positive for IL-6

With the purpose of verifying whether there were differences among the groups, the number of IL-6-immunolabeled cells was estimated in the capsules of the 5 implants of each group/time point. As previously described, the Image Pro-Express 6.0 Olympus software was used. The numerical density of IL-6-immunolabeled cells was obtained by two calibrated and blinded examiners. For each animal, the number of immunolabeled cells was quantified at x695, in a standardized field (0.09 mm^2) of the capsule in close juxtaposition to the opening of the implanted tube. Thus, the number of IL-6 immunolabeled/mm^2 per animal was obtained (8,12).

Statistical Analysis

The quantitative data were submitted to statistical analysis by using the Sigma Stat 2.0 program (Jandel Scientific, Sausalito, California, USA). After normality was confirmed, the two-way ANOVA and Tukey tests were used, at a level of significance of 5%.

Results

Morphological and numerical density analysis of inflammatory cells

At 7 days, the capsules in all the groups exhibited several inflammatory cells, mainly lymphocytes and macrophages among the blood vessels (Figs. 1A-1C). At 15 days, the capsules contained fibroblasts and collagen fibers, particularly in TSC and CG. However, inflammatory cells and blood vessels were still clearly observed in the capsules (Figs. 1D and 1F). Occasionally, some multinucleated giant cells were also observed, mainly in the internal portion of the capsules, adjacent to the materials (Fig. 1D). After 30 and 60 days, the connective tissue of the capsules contained several fibroblasts between the collagen fiber bundles; the scarce inflammatory cells were represented by plasma cells, macrophages and mast cells (Figs. 2A-2F).

Quantitative analysis revealed that in all the groups, the number of inflammatory cells was significantly higher at 7 days. After this period, the statistical analysis of each group revealed a gradual and significant reduction in the number of inflammatory cells in the capsules of all groups ($p<0.001$) over time. At 7 days, no significant differences were verified between TSC and MTAP ($p=0.592$), however, these values were statistically higher in comparison with those of the control group ($p<0.001$). In turn, in

the periods of 30 and 60 days, no significant differences were observed among TSC, MTAP and control group (p=0.955) (Table 2).

Immunohistochemical detection of IL-6 and numerical density of immunopositive cells

The sections of capsules submitted to the immunohistochemical reaction for IL-6 showed inflammatory cells (lymphocytes, plasma cells, macrophages and mast cells) and some fibroblasts exhibiting positivity to immunoreaction. Morphological analysis revealed immunolabeled cells in the capsules of all the groups; however, an accentuated immunopositivity was observed in the capsules at 7 days in comparison with the other periods (Figure 3). On the other hand, sections of capsules incubated without the primary antibody (negative control) exhibited no immunopositive cells (data not illustrated).

According to Table 2, the number of IL-6-immunolabeled cells was significantly higher at 7 days than at 15, 30 and 60 days. In all the groups, the number of immunolabeled cells was significantly reduced from 7 to 60 days. However, no statistically significant differences were detected among TSC, MTAP and CG, in any of the periods.

Discussion

Histopathological analysis of the subcutaneous response after different experimental periods evaluate not only the tissue characteristics to irritant potential but also the duration of this effect on tissues (5,20,21).

The present study showed that the TSC and MTAP induced an inflammatory reaction in the subcutaneous tissues. However, this reaction was gradually reduced, leading to the formation of dense connective tissue in the capsules. The reduction in the inflammatory reaction occurred concomitantly with the reduction in the immunoexpression of IL-6 in the capsules adjacent to the materials, indicating that the experimental TSC cement and MTAP are biocompatible materials. The inflammatory reaction initially caused by TSC and MTAP may be related to the hydroxyls (OH^-) released after hydration of the materials, providing an environment with an alkaline pH (3,14,18). The alkaline pH stimulates recruitment of the leukocytes of the blood vessels (8,12,22,23) and therefore, may explain the higher values of inflammatory cells verified in the initial time point.

The tissue reaction observed is a consequence of the host response to substances released by the materials. Therefore, the host cells (inflammatory and resident) produce and release several cytokines and growth factors that play a specific role in the complex cascade of cellular events involved in the tissue response. Among the different cytokines, IL-6 is considered a powerful mediator of the inflammatory process (24).

An association has been observed between the reduction in immunoexpression of IL-6 and a reduction in the inflammatory process in the capsules adjacent to the calcium silicate-based cements implanted in the rat subcutaneous tissues (8,12), indicating that these materials do not have an irritating effect on connective tissue during a prolonged period.

The gradual and significant reduction in the number of immunolabeled cells for IL-6 suggests that the capsules adjacent to the materials implanted undergo an intense remodeling process from 7 to 60 days. Moreover, our results point out that this cytokine may be involved in the response promoted by tricalcium silicate-based materials, because a reduction in both immunolabeled cells for IL-6 and in the number of inflammatory cells was observed. Considering that no significant differences in the IL-6 immunoexpression was observed for TSC and MTAP compared with the control group, it is possible to suggest low irritant potential of the materials to the tissues. In spite of this, materials promoted a greater recruitment of inflammatory cells than the empty tube. Therefore, these materials may release some substances that stimulate the migration and differentiation of cells, such as macrophages and plasma cells, frequently observed in the capsules at 7 days. However, the inflammatory process was replaced by a capsule containing well-developed bundles of collagen fibers between fibroblasts at 30 and 60 days after implantation. This tissue response indicates that the materials are biocompatible.

It has been demonstrated that ZrO_2 added to calcium silicate cement promotes adequate physicochemical properties and a better biologic response than Bi_2O_3 (12). Nevertheless, in the present study, MTAP which presents Bi_2O_3 promoted a subcutaneous tissue response similar to that of TSC. The hypothesis for this finding may be related to the fact that MTAP does not have Portland cement in its composition but instead it has tricalcium silicate (approximately 50%), dicalcium silicate, calcium sulphate and silicon dioxide in addition to Bi_2O_3 (approximately 30%). The substitution of Portland cement by tricalcium silicate and dicalcium silicate allows better control of heavy metals that are found in the MTA (18), favoring the hydration reaction (3).

Conclusion

The TSC and MTAP caused similar subcutaneous implantation reaction in rats, indicating that both present similar immune response. The significant reduction in the inflammatory reaction accompanied by decrease in the IL-6 immunoexpression in the capsules over time indicates that these tricalcium silicate-based materials may have potential as root repair material.

Acknowledgements

The authors thank to Mr. Pedro Sérgio Simões and Mr. Luis Antonio Potenza for kind help and technical assistance.

This study was supported by São Paulo Research Foundation (FAPESP – nº 2015/03437-6 and 2014/13353-1).

The authors deny any conflicts of interest related to this study.

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Figure legends:

Figure 1 – Light micrographs of sections showing portions of capsules adjacent to the opening of the tubes implanted (T) in the subcutaneous tissue after 7 **(Figs. 1A-1C)** and 15 **(Figs. 1D-1F)** days. In Figs. **1A**, **1B** and **1C**, several inflammatory cells (arrows) are observed, mainly, in the portion of the capsule adjacent to the opening of the tubes (T). N, neutrophil; Bv, blood vessels. **Figs. 1D**, **1E** and **1F**, exhibit inflammatory cells (arrows) among blood vessels (Bv) and fibroblasts (Fb). Gc, giant cells. HE. 695x.

Figure 2 – Light micrographs of sections showing portions of capsules adjacent to the opening of the tubes implanted (T) in the subcutaneous tissue after 30 **(Figs. 2A-2C)** and 60 **(Figs. 2D-2F)** days. Typical fibroblasts (Fb) are observed among bundles of collagen fibers (Cf) in the capsules.Few inflammatory cells (arrows) are present, mainly in the capsules of MTAP and control group. Mp, macrophages; P, plasma cells; M, mast cells; Bv, blood vessels. HE. 695x.

Figure 3 – Light micrographs of sections showing portions of capsules adjacent to the opening of the tubes (T) implanted in the subcutaneous tissue after 7 **(Figs. 3A-3C)**, 15 **(Figs. 3D-3F)**, 30 **(Figs. 3G-3I)** and 60 **(Figs. 3J-3L)** days of implantation. Sections were submitted to immunohistochemistry reaction for IL-6 detection and counterstained with haematoxylin. Several immunolabeled cells (arrows; brown/yellow colour), especially, plasma cells, mast cells and some fibroblasts (Fb) are observed in the capsules at 7 and 15 days. At 60 days, scarce cells exhibit immunolabelling for IL-6. Bv, blood vessels. 500x.

Tables:

Table 1 – Materials evaluated and used proportions.

Group	Material	Composition	Proportion (powder/distilled water)
TSC	Cement tricalcium silicate* with 20% ZrO_2**	Tricalcium silicate, Zirconium oxide	1g/ 340 µL
MTAP	White Mineral Trioxide Aggregate***	Tricalcium Silicate, Dicalcium Silicate, Calcium Sulphate, Silica, Bismuth Oxide	1g/ 340 µL

* Mineral Research Processing, Meyzieu, France
** Sigma Chemicals, St Louis, Missouri, USA
*** MTA Plus™ Avalon Biomed Inc., Bradenton, Florida, USA

Table 2 – Numerical density of inflammatory cells (IC) and IL-6 immunolabeled cells in the capsules of TSC, MTAP e control groups at 7, 15, 30 and 60 days.

	TSC	**MTAP**	**control**
7 days			
IC	482.5 (45.9)^{b,1}	448.8 (34.8)^{b,1}	324.0 (29.9)^{a,1}
IL-6	475.5 (16.4)^{a,1}	480.0 (14.4)^{a,1}	464.4 (19.8)^{a,1}
15 days			
IC	185.1 (28.2)^{ab,2}	214.8 (43.6)^{b,2}	154.0 (8.1)^{a,2}
IL-6	186.6 (18.2)^{a,2}	195.5 (12.6)^{a,2}	182.2 (12.6)^{a,2}
30 days			
IC	160.0 (30.3)^{a,2}	135.5 (29.0)^{a,3}	148.8 (8.0)^{a,2}
IL-6	148.8 (18.5)^{a,3}	151.1 (14.9)^{a,3}	144.4 (17.5)^{a,3}
60 days			
IC	71.1 (9.5)^{a,3}	70.3 (16.1)^{a,4}	69.0 (6.5)^{a,3}
IL-6	75.9 (8.9)^{a,4}	75.5 (9.3)^{a,4}	71.1 (9.9)^{a,4}

*mean (standard deviation) The comparison amongst groups ($p<0.05$) is indicated by different superscript (a, b, c and d) in the various lines.
The comparison amongst periods ($p<0.05$) is indicated by number superscripts in the various columns.

Figure 1

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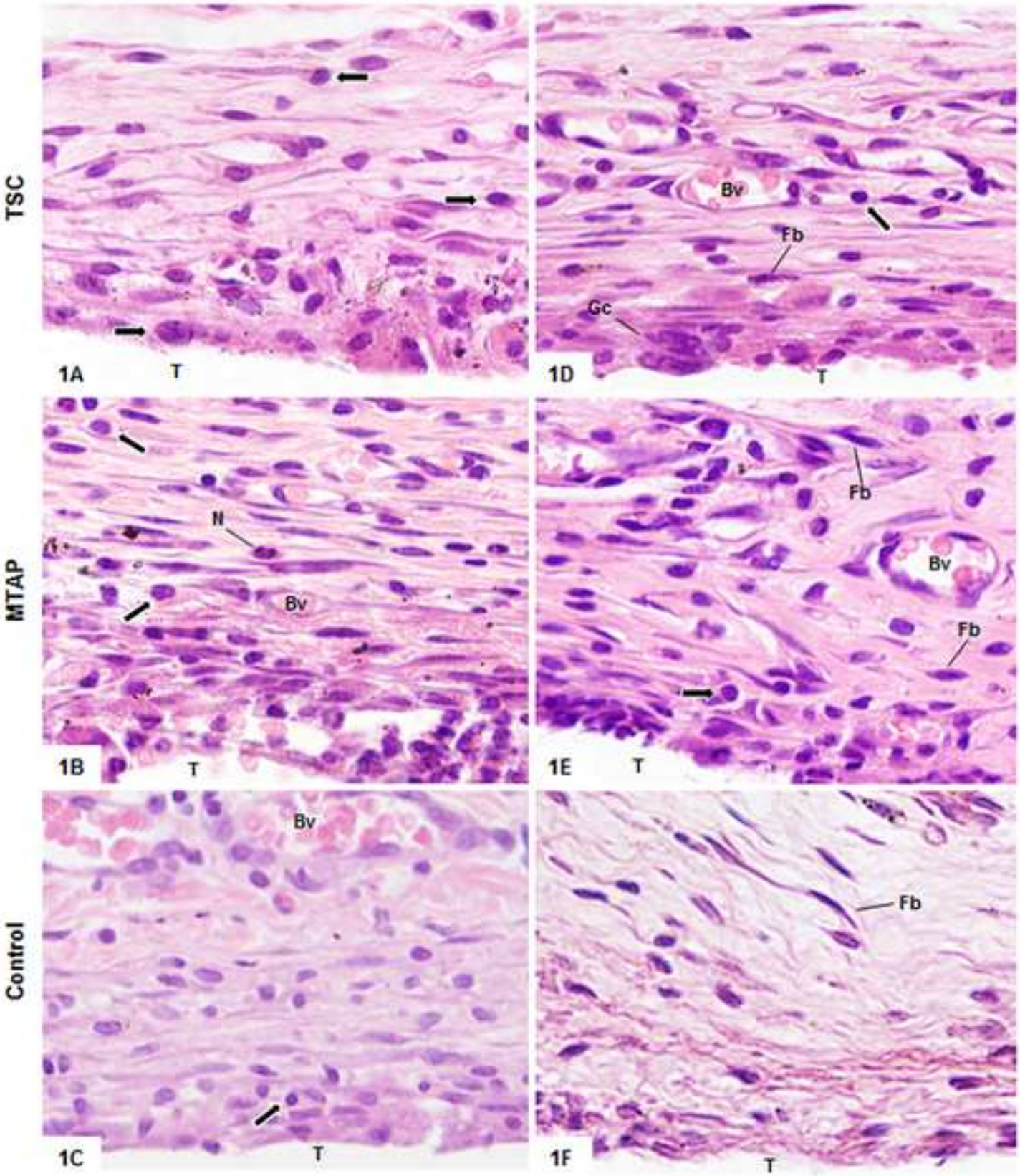

Figure 2

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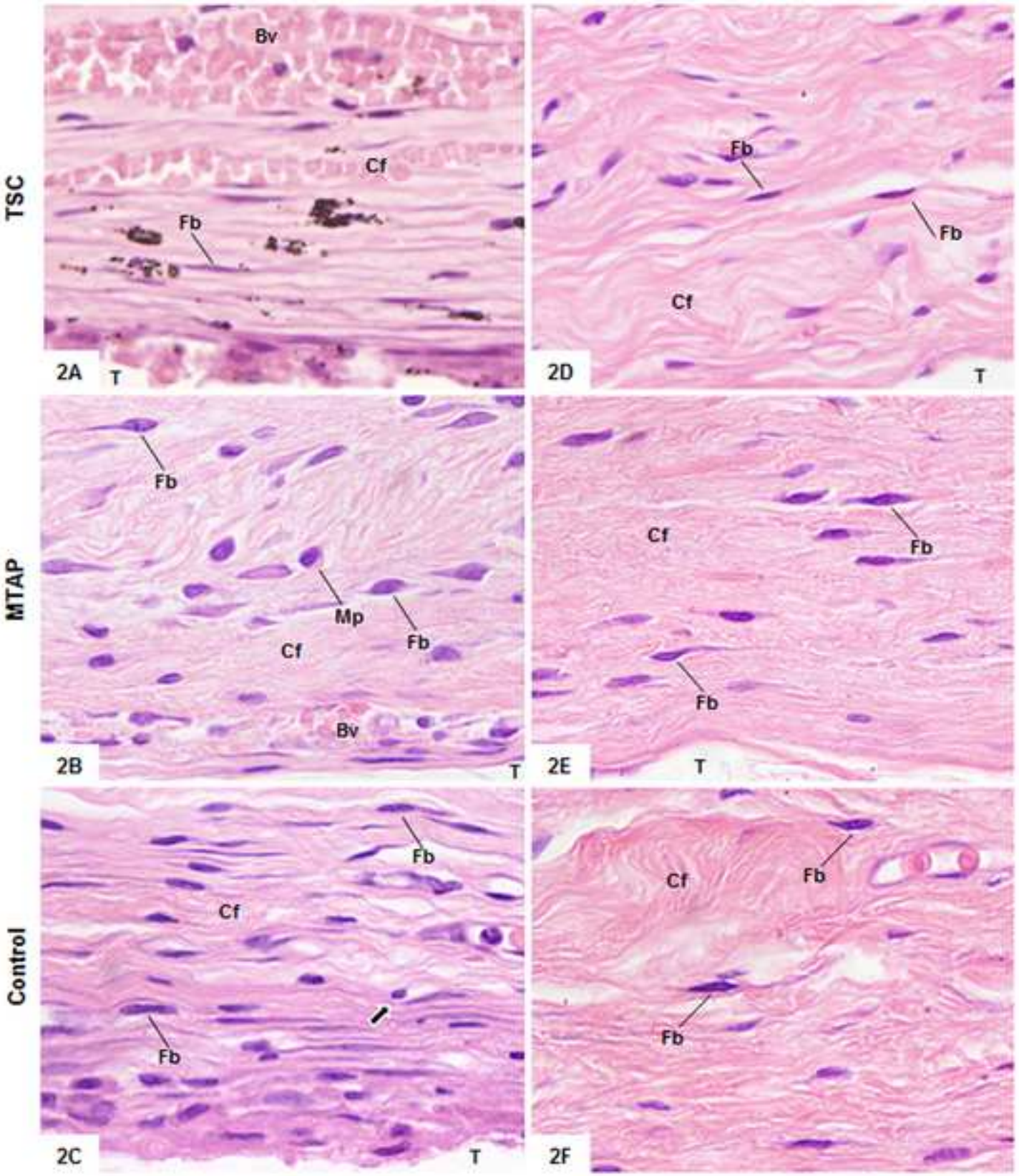

Figure 3

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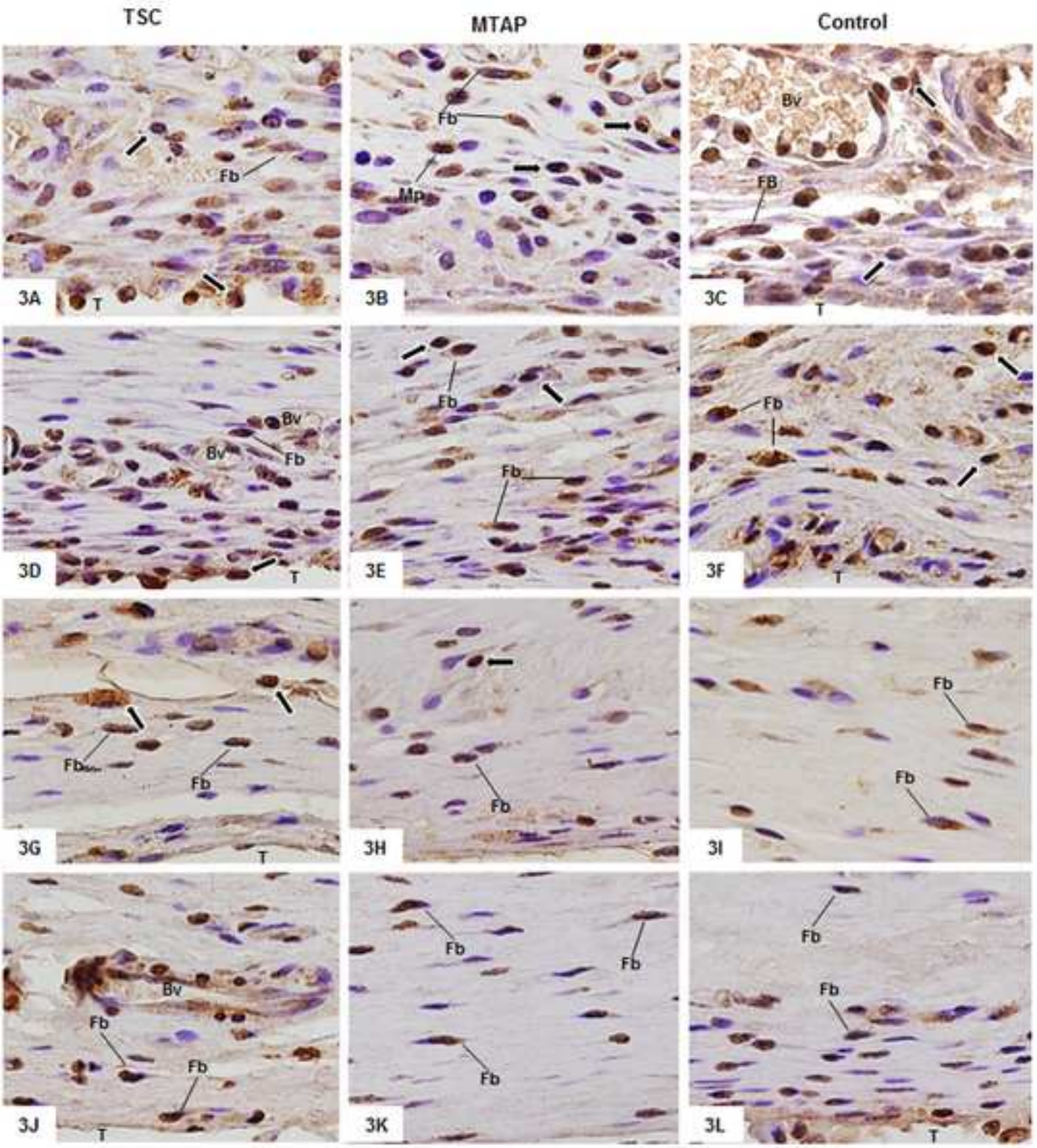

Author's Point-by-Point Response to Reviewers

Reviewers' comments:

Reviewer #1:

Introduction

1. Paragraph 1. Reference 7 not applicable here; join with reference 9 instead?

A: Reference 7 (Camilleri et al., 2011) is related to a study of some characteristics, such as, microstructure and pH, of Portland cement with zirconium oxide. The authors stated that zirconium oxide is inert and can be used as a substitute of bismuth oxide. On the other hand, reference 9 (Camilleri 2007) evaluate the hydration mechanisms of calcium silicate materials with bismuth oxide. That is why we used the reference in the text.

2. Paragraph 2. Line 24. Change to, "as a material used in pulpotomies...". Line 31: move the reference (17) to the end of the sentence.

A: The corrections were performed in the text.

3. Paragraph 3. Line 36. Change to, "as a radiopacifier...". Line 45: change to, "There is no other evidence of the biological in vivo response of this material reported in the literature."

A: The corrections were performed in the text.

4. Paragraph 4. Line 48. Remove "thus"; remove since this method determined...". Line 55 change to " ...there would be no difference..."

A: The corrections were performed in the text.

Material and Methods

1. Paragraph 3. Why not use an unbranded, monofilament suture to decrease inflammatory response.

A: We really appreciate the suggestion and it will considered for futures studies. However, incision was performed in the centre of dorsal subcutaneous and the pockets, where tubes were implanted, were performed at a distance of 10 mm. Based in the histological images, we believe that the inflammatory response caused by the incision and its healing process does not interfere with the tissue reaction of the materials.

2. Immunohistochemical reaction for detection of IL-6: Paragraph 2. Line 60. "In this step the sections were incubated in non-immune...?"

A: We have made the correction.

Discussion.

1. Paragraph 5. Lines 35-42. Consider shortening this into 2-3 sentences instead of one. General rule is that shorter sentences allow for better reading comprehension.

A: We have made the corrections

References.

1. Recommendations as stated above.

A: We have made the corrections.

Legends.

1. Add "tissue" after "subcutaneous".

A: We have made the corrections.

December 05, 2017.

The manuscript title

Tissue response and immunoexpression of IL-6 promoted by tricalcium silicate-based repair materials after subcutaneous implantation in rats

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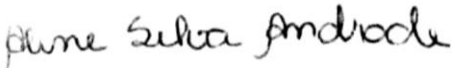

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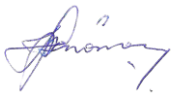

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