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Abstract

Introduction: Bio-C Sealer (BC) and Sealer Plus BC (SPBC) are new ready-to-use bioceramic endodontic sealers. The aim of the study was to evaluate biocompatibility and bioactive potential of BC and SPBC sealers in comparison to AH Plus (AHP) in subcutaneous tissue of rats. Methods: Polyethylene tubes filled with materials and empty tubes, as control group, were implanted in the subcutaneous tissues of rats. After 7, 15, 30 and 60 days, the tubes with connective tissue were removed and inflammatory cells / mm$^2$ (IC) and immunolabeled cells for interleukin-6 (IL-6) were evaluated. Osteocalcin (OC) and von Kossa analysis were also performed. Data were submitted to ANOVA and Tukey tests, with a significance of 5%. Results: At 7 days, SPBC showed lower IC than BC (p <0.05). AHP exhibited greater immunolabeled cells for IL-6 (p <0.05). After 15 days, BC showed lower IC and IL-6 when compared to other materials. At 30 days, SPBC and AHP showed higher values for IC (p <0.05). After 60 days, calcium silicate sealers did not show statistical difference (p>0.05) for IC and IL-6, with values lower than AHP (p <0.05). The materials showed positive structures to von Kossa. BC exhibited osteocalcin labeling in all periods. SPBC showed osteocalcin labeling from 15 to 60 days. AH Plus and the control group did not exhibit osteocalcin labeling. Conclusions: Bio-C Sealer and Sealer Plus BC sealers are biocompatible and present bioactive potential.

Key words: Calcium silicate, biocompatibility, root canal filling material, endodontics.
INTRODUCTION

Premixed calcium silicate-based sealers are developed presenting proper physicochemical and biological properties. EndoSequence BC and TotalFill BC Sealer (FKG Dentaire AS, Switzerland) are endodontic sealers composed of calcium silicates, zirconium oxide, monobasic calcium phosphate, and ready-to-use. These materials have biocompatibility, release calcium ions, present high pH, dimensional stability and radiopacity. Zirconium oxide used as radiopacifier in a calcium silicate-based material stimulate fibroblast proliferation and collagen formation in subcutaneous tissue of rats.

New calcium silicate-based ready-to-use endodontic sealers have been developed. Bio-C Sealer (Angelus, Brazil) is a new sealer composed of tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium oxide, zirconia oxide, silicon oxide, polyethylene glycol, iron oxide. The physico-chemical properties of Bio-C Sealer are different from TotalFill BC and AH Plus sealers, presenting the shortest setting time and highest solubility, but with low dimensional change. When compared to Bio-C Repair, a repair calcium silicate ready-to-use material, Bio-C Sealer presented less cell viability, however, it was better than AH Plus. The Sealer Plus BC (SPBC; MK Life, Brazil) is composed of calcium disilicate, nanoparticulate calcium trisilicate and zirconium oxide. This sealer presents alkaline pH, releases calcium ions and has proper setting time and radiopacity. When compared to AH Plus (Dentsply De Trey GmbH, Konstanz, Germany), Sealer Plus BC showed higher solubility, less radiopacity and higher pH. When compared to MTA Fillapex and AH Plus, Sealer Plus BC showed greater biocompatibility in the subcutaneous of rats.

Biocompatibility and bioactivity are important properties for endodontic sealers, since they maintain contact with the periapical tissues and can influence repair. Both properties are directly related to the composition of the material. It is well reported that calcium silicate sealers promote low cytotoxicity and induce mild/moderate inflammatory reaction. Therefore, the aim of this study was to evaluate the biocompatibility and bioactive potential of two bioceramic sealers, in comparison with AH Plus. The null hypothesis is that the difference between the compositions of the materials would not interfere in the tissue reaction induced by bioceramics or epoxy resin sealers.

MATERIALS AND METHODS
The materials used in this study, their manufacturers, compositions and proportions are included in Table 1.

The research protocol was approved by the Ethical Committee for Animal Research of São Paulo (CEUA, Process number 35/2018). Twenty-four male Holtzman rats (*Rattus norvegicus albinus*) were used and distributed in four groups (n = 6): sealers and control group (empty polyethylene tubes). The materials were inserted in polyethylene tubes implanted in dorsal subcutaneous sites. Four tubes were inserted per animal, one from each group (ISO-10993-6, 2007). The animals were anesthetized with ketamine (80 mg/kg body weight, Virbac do Brasil Indústria e Comércio Ltda., São Paulo, SP, Brazil) and xylazine (4 mg/kg body weight, União Química, São Paulo, SP, Brazil). After 7, 15, 30 and 60 days, the animals were euthanized with anesthetic overdose, the implants with adjacent tissues were removed.

### Histological procedures

The implants and surrounding tissues were removed and immersed for 72 hours in a 4% formaldehyde solution and pH adjusted to 7.2. After fixation, the specimens were dehydrated, diaphanized, immersed in liquid paraffin (60ºC) and embedded in paraffin. Longitudinal sections with 6 μm thickness were obtained. Non-serial sections were stained with hematoxylin and eosin (HE) to estimate the number of inflammatory cells in the capsules and thickness of the capsules adjacent to the materials.

### Numerical density of inflammatory cells

The numerical density of inflammatory cells (IC) was evaluated using a light microscope (BX51, Olympus, Tokyo, Japan) and an image analysis system (Image Pro-Express 6.0, Olympus). In each implant, three H&E-stained sections of the capsule were selected at intervals of at least 100 mm. In each section, a standardized field 0.09 mm\(^2\) of the capsule adjacent to the opening of the tube implanted was analyzed, totaling 0.27 mm\(^2\) per implant. In each area, the total number of IC (neutrophils, lymphocytes, plasma cells, and macrophages) was counted using the image analysis system at 695 magnification. Thus, the number of ICs/mm\(^2\) was obtained for each implant\(^{10-12}\).

### Thickness of capsules
The thickness (in µm) of the capsules adjacent to the implanted tubes was measured. Three images of non-serial sections stained with HE of each specimen were captured. The thickness of the capsules was estimated in the middle portion from its surface adjacent to the material to its limit with the adjacent tissues. After obtaining the values, the average was calculated from the measurements obtained from the three sections for each specimen.

**Immunohistochemical detection of IL-6**

For the detection of IL-6, mouse anti-IL-6 antibody (Abcam, Cambridge Science, UK; code Ab 9324,) was used. The sections were incubated overnight in a humidified chamber with anti-IL-6 antibody. Subsequently, the sections were incubated in Labeled StreptAvidin-Biotin kit (Universal Dako LSAB, Dako Inc., Carpinteria, CA, USA; K0675). After washing, peroxidase activity was revealed by the 3,3′-diaminobenzidine chromogen (ImmPACTTM DAB Vector, Burlingame, CA, United States). The sections were counterstained with Carazzi's hematoxylin. So, the number of IL-6-immunolabeled cells per mm$^2$ of the capsule was counted at 695X using an image analysis system (Image Pro-Express 6.0, Olympus). For each tube implanted, the IL-6 positive cells were counted in a standardized area (0.09 mm$^2$). In each group, the values were divided by the total area, and then, the number of IL-6/mm$^2$ was obtained.$^{10,12}$

**Immunohistochemical detection of osteocalcin**

The sections were incubated with rabbit anti-osteocalcin antibody (1:200; Sigma-Aldrich Co., Saint Louis, Missouri, USA; code SAB1306277). After 16 hours, in a humidified chamber, the sections were incubated in Labeled StreptAvidin-Biotin kit (Universal Dako LSAB, Dako Inc., Carpinteria, CA, USA; K0675). Subsequent to buffer washes, peroxidase activity was revealed by the 3,3′-diaminobenzidine chromogen (ImmPACTTM DAB Vector, Burlingame, CA, United States). The sections were counterstained with Carazzi’s hematoxylin. The number of osteocalcin-immunolabeled cells was performed similarly to IL-6.

**von Kossa reaction and analysis under polarized light**

The sections were immersed in a 5% silver nitrate solution. Posteriorly immersed in a 5% sodium hyposulfite solution. The sections were then stained with picrosirius-red and mounted in resinous medium (Permout®, Fisher Scientific, New Jersey, USA$^{11,12}$). Sections close to those
subjected to von Kossa were deparaffinized, dehydrated. The unstained sections were analyzed under a light microscope equipped with polarization filters (Olympus, BX51).

**Statistical analysis**

The statistical analysis was obtained with the aid of the GraphPad Prism 5 software (Jandel Scientific, Sausalito, CA, USA). The data were evaluated by the two-way ANOVA followed by the Tukey test. The level of significance considered was $p \leq 0.05$.

**Results**

**Numerical density of inflammatory cells**

At 7 days, SPBC showed lower values than BC ($p < 0.05$) (Fig. 1A and 1B). After 15 days, BC showed lower IC than SPBC and AHP (Fig 1E, 1F and 1G). At 30 days, there was no statistical difference between SPBC and AHP for IC ($p > 0.05$) (1J and 1K). At 60-day period, BC and SPBC showed no statistical difference between them ($p > 0.05$). AHP presented the highest IC values when compared to the others ($p < 0.0001$) (1O) (Table 2).

**Thickness of the capsules adjacent to the implants**

The capsules exhibited a moderate inflammatory reaction. At 7 days, BC and SPBC showed no statistical difference ($p > 0.05$). At 15 and 30 days, there was no statistical difference between BC and AHP ($p > 0.05$). SPBC exhibited the highest values. After 60 days, all materials showed reduction in the thickness of the capsules, with no statistical difference between them (Table 2).

**Immunohistochemical detection of IL-6**

At 7 days, the number of immunostained inflammatory cells/mm$^2$ was significantly higher compared to the other periods ($p < 0.05$). AHP exhibited the highest values ($p < 0.0001$) (Fig 2C). At 15 and 30 days, BC exhibited less marking when compared to SPBC ($p < 0.05$). At 60 days, BC, SPBC and control showed the lowest values (Figure 2I, 2J and 2L), with no difference between BC and SPBC ($p > 0.05$). The AHP presented the highest values in all the periods analyzed ($p < 0.0001$) (Table 2).
Immunohistochemical detection of osteocalcin

At 7 days, only BC group (Figure 2E) exhibited positive marking for osteocalcin. At 15 and 30 days, BC and SPBC had immunopositive cells. After 60-day period BC showed higher number of labeled cells compared to SPBC (p <0.05) (Table 2) (Figure 2M and 2N). AH Plus sealer and the control group did not show positive marking in any period (Figure 2G, 2H, 2O and 2P).

von Kossa reaction and analysis under polarized light

BC, SPBC and AHP presented positive structures to von Kossa method in all periods analyzed (Fig 3A, 3B, 3C, 3G, 3H and 3I). The control group did not show positive structures. BC and SPBC showed birefringent structures in all periods spread by the adjacent tissues, and AH Plus presented structures located only on the surface of the capsules. The control group did not exhibit birefringent structures.

Discussion

The Bio-C Sealer was compared with Sealer Plus BC and AH Plus for up to 60 days to assess tissue repair and bioactive potential. The present study demonstrated that Bio-C Sealer and Sealer Plus BC induced a less intense inflammatory process than AH Plus, and exhibited positive marking for osteocalcin. Bio-C sealer presents tricalcium aluminate, calcium oxide, silicon oxide, iron oxide and polyethylene glycol, as dispersant agent, which is different from other bioceramic sealers. Sealer Plus BC presents calcium disilicate, nanoparticulate calcium trisilicate, zirconium oxide, and the dispersant agent is not described. These differences may interfere in the physicochemical properties and also in the biocompatibility and bioactive potential. Therefore, the null hypothesis was rejected, as differences were observed in tissue responses induced by bioceramics and epoxy-based sealers.

At 7 days, all materials showed moderate inflammatory reaction, with presence of plasma cells, neutrophils, macrophages and giant cells close to the sealer particles. The control group exhibited the lowest number of inflammatory cells and a thick capsule. Considering that polyethylene tubes are inert, the inflammatory reaction observed in the control group may be related to the surgical procedure\textsuperscript{11}. Bio-C Sealer and Sealer Plus BC have been biocompatible
over time. However, in the initial periods an inflammatory reaction was observed in the subcutaneous tissues. The setting reaction of these materials promotes the formation of calcium and hydroxyl ions (OH-), and the alkaline pH stimulates the recruitment of inflammatory cells and the production of cytokines. The large number of inflammatory cells observed in the initial periods may be related to the elevated pH observed for bioceramic materials.

IL-6 is a proinflammatory cytokine that may activate and modulate specific cells, playing an important role in inflammatory reaction and in bone resorption. Our findings revealed that sealers promoted significant increase in the number of IL-6 immunolabeled cells in the capsules, especially, after 7 days. However, the increase was greater for AH Plus compared to Bio-C Sealer and Sealer Plus BC in all experimental periods. The gradual and significant reduction in inflammatory cells and IL-6 immunolabeled cells suggests that the capsules showed an intense remodeling process in the initial periods. In addition, the presence of well-oriented collagen fibers bundles in the capsules indicates that initial inflammatory reaction was replaced, after 60 days, by a dense connective tissue. This hypothesis is reinforced by the reduction in the capsule thickness over the evaluated periods.

AH Plus presented the highest inflammatory reaction and IL-6 immunolabeled cells after 60 days. It is possible that the epoxy resin released from AH Plus delays the healing process. It was already demonstrated that bioceramic sealers are less cytotoxicity than AH Plus and that they present biocompatibility after extended periods. Furthermore, when analyzed in the subcutaneous tissue of rats, AH Plus sealer presented greater quantity of IL-6 immunolabeled cells when compared to MTA sealer. A systematic review that investigated the gene expression of various cells in response to different tricalcium silicate cements showed that these materials were related to significantly increased gene expression related to periapical regeneration.

Tricalcium silicate materials are usually related to biocompatibility and osteoblastic differentiation. Organic matrix of mineralized tissues can be evaluated by immunohistochemistry to detect proteins such as osteocalcin, secreted by osteoblasts, identified as marker of mature osteoblasts, and by the von Kossa histochemical technique to evaluate calcium precipitates. SPBC did not show positive marking for osteocalcin at 7 days. SPBC showed immunopositive cells for osteocalcin from 15 to 60 days, and BC presented in all periods. The ability to induce mineral deposition by using immunohistochemical analysis for osteocalcin was demonstrated for the calcium silicate-based cement Bio-C Pulpo (Angelus), a
ready-to-use repair material. López-Gárcia et al. 2019\cite{23} showed that the Bio-C Sealer is able to promote cellular viability, cell survival and cell migration of periodontal ligament stem cells.

In order to identify amorphous calcite deposits, the von Kossa birefringence technique was performed. Bio-C Sealer and Sealer Plus BC presented irregular structures that were positive to von Kossa staining with calcium deposits in the adjacent capsules at 7 days. AH Plus exhibited von Kossa positivity, but did not show positive marking for osteocalcin in any evaluated period. Carvalho et al. 2017\cite{24} showed that bioactivity is not expected for AH Plus.

Calcium silicate sealers release calcium favoring mineralization and differentiation of dental pulp cells\cite{13}. The reaction process of calcium ions and carbon dioxide leads to the formation of calcite crystals, birefringent structures that may lead to the formation of calcified structures\cite{25,26}. Positivity for von Kossa and birefringent to polarized light are usually performed to evaluate the mineralization potential of calcium silicate cements\cite{27,28}. The calcium silicate-based sealers showed birefringent structures in all analyzed periods.

Biocompatibility and bioactivity are important properties as endodontic sealers maintain contact with periapical tissues and may influence tissue repair\cite{3,5,9}. In the present study, Bio-C Sealer and Sealer Plus BC presented biocompatibility since they allow the regression of inflammatory reaction faster than AH Plus. Furthermore, both bioceramic sealers show bioactive potential, exhibiting osteocalcin immunopositive cells and structures that were positive to von Kossa staining in the capsules.

Conclusion

Bio-C Sealer and Sealer Plus BC are biocompatible to be used in close contact with periapical tissue, inducing a mild inflammatory reaction and favoring repair. In addition, both sealers may contribute to the periapical tissue mineralization process, as they demonstrate bioactive potential.

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


Figure legends

Figure 1 - Photomicrographs of the sections showing portions of the capsules adjacent to the opening of the implanted tubes (T) after 7 (A-D), 15 (E-H), 30 (I-L) and 60 (M-P) days of implantation. The capsules show numerous inflammatory cells, mainly lymphocytes, plasma cells and macrophages. In the capsules of the control group (Figure 1D), the inflammatory cells (arrows) have a lower amount, with an evident presence of fibroblasts (Fb). The capsules of the AH Plus and BC group show a greater number of inflammatory cells (Figure 1A and 1C). Figures 1E to 1H present capsules after 15 days with a reduction in the total number of cells. The AH Plus group exhibited a greater number of cells (Figure 1G). At 30 days, the capsules of AH Plus (Figure 1K) and SPBC (1J) exhibit a greater number of inflammatory cells, compared to the other groups, while BC (Figure 1I) already have lower amount of inflammatory cells. The control group (Figure 1L) has fibroblasts arranged between the collagen fibers. Figures 1M-1P show capsules after 60 days. The capsules of BC (Figure 1M) and SPBC (Figure 1N) exhibit mainly fibroblasts (Fb) located between bundles of collagen fibers. The AH Plus sealer capsule (Figure 1O) has more inflammatory cells compared to the other groups. Fb, fibroblasts; BV, blood vessels. Bars: 18 µm.

Figure 2 - Photomicrographs showing portions of the capsules adjacent to the opening of the subcutaneous implanted tubes for 7 (A-D) and 60 (E-H) IL-6 immunopositive cells and 7 days (I-L) 60 (M-P) OC immunopositive cells. The capsules contain several IL-6 immunopositive cells, inflammatory cells (arrows), in the period of 7 days, at 60 days reduced marking is observed in all groups. AH Plus (Figure 2K) with greater quantity. At 7 days, only Bio-C showed marking for OC, at 60 days Bio-C and Sealer Plus BC showed marking for OC. Bars: 18 µm.

Figure 3 - Photomicrographs of sections showing portions of capsule adjacent to the opening of the tubes implanted in the subcutaneous tissue submitted to the von Kossa reaction after 7 (A, B, E, F, I, J) and 60 (C, D, G, H, K, L) days. The BC (Figures 3A and 3G), SPBC (Figures 3B and 3H), AH Plus (Figures 3C and 3I) capsules exhibit positive von Kossa structures (black / brown) and 3D, 3E, 3F, 3J, 3K and 3L compatible birefringent structures. Positive structures for calcium precipitation. Von Kossa and Picrosirius-red. Bars: 18 µm.
## Tables

Table 1 - Endodontic sealers used, manufactures and proportions.

<table>
<thead>
<tr>
<th>Sealers</th>
<th>Composition</th>
<th>Manufacturers</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio C- Sealer (BC)</td>
<td><strong>Tricalcium Silicate, Dicalcium Silicate, Tricalcium Aluminate, Calcium Oxide</strong>, <strong>Zirconia Oxide, Silicon Oxide, Polyethylene Glycol, Iron Oxide</strong></td>
<td>Angelus, Londrina, Brazil</td>
<td>Ready to use</td>
</tr>
<tr>
<td>Sealer Plus BC (SPBC)</td>
<td>Calcium disilicate, nanoparticulate calcium trisilicate, zirconium oxide.</td>
<td>MK Life, Porto Alegre, Brazil</td>
<td>Ready to use</td>
</tr>
<tr>
<td>AH Plus (AHP)</td>
<td><em>Paste A</em>: epoxy bisphenol-A resin and epoxy bisphenol-F, calcium tungstate (CaWO₄), zirconium oxide (ZrO₂), silica, iron oxide. <em>Paste B</em>: dibenzyl-diamine, aminoadamantane, CaWO₄, ZrO₂, silica, silicone.</td>
<td>Dentsply DeTrey GmbH, Konstanz, Germany</td>
<td>1g:1g (paste/paste)</td>
</tr>
</tbody>
</table>
Table 2 - Capsule thickness (µm), number of inflammatory cells (IC) per mm² and number of immunopositive cells for IL-6 and osteocalcin per mm². Bio-C (BC), Sealer Plus BC (SPBC), AH Plus (AHP) and Control (CG) after 7, 15, 30 and 60 days.

<table>
<thead>
<tr>
<th></th>
<th>BC</th>
<th>SPBC</th>
<th>AHP</th>
<th>CG</th>
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<tr>
<td>7 days</td>
<td></td>
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</tr>
<tr>
<td>thickness</td>
<td>285±79</td>
<td>270±97</td>
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<tr>
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<td>775±26</td>
<td>690±40</td>
<td>865±63</td>
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<tr>
<td>IL-6</td>
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<td>451±5</td>
<td>782±8</td>
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<td>-</td>
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<td>15 days</td>
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<td></td>
<td></td>
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<tr>
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<tr>
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<td>46±9</td>
<td>14±3</td>
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</table>

Mean (standard deviation).
The comparison between groups in the same period is indicated by superscript letters on the line. Same letters = no statistically significant difference.
The comparison between intervals in the same group is indicated by numbers superscript in the columns; same numbers = no statistically significant difference.
Tukey’s test (p ≤ 0.05)