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In Vivo Evaluation of Tissue Biocompatibility of Calcium Silicate-based and Epoxy Resin-based Sealers

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Introduction: Calcium silicate-based sealers are an alternative to be used into root canal, mainly to their biological properties. However, some biological parameters need to be determined in an *in vivo* animal research model. So, the aim of the present study was to evaluate *in vivo* the tissue biocompatibility of a calcium silicate-based sealer (EndoSequence BC Sealer) and an epoxy resin-based sealer (AH-Plus). **Materials and Methods:** Polyethylene tubes were filled with freshly mixed sealers and implanted in connective subcutaneous tissue of 25 rats (5/euthanasia day) (*Rattus norvegicus albinus*). Empty tubes were used as controls and no tubes as sham. Histopathological (hematoxylin eosin) and histochemical (Picrosirius red) examinations were conducted at 3, 7, 15, 30 and 60 days (five rats/day) after the implantation procedure (n=5/group). The type/intensity of inflammation and collagenesis was analyzed statistically with Friedman or Kruskal-Wallis/Dunn tests (P<0.05). **Results**: The profile of inflammation induced by AH-Plus (Median=2, Range=2-3) was significantly greater than that by Endosequence BC Sealer (Median=1, Range=1-1) during the 15-day experimentation period (P=0.018). After 30 days, both materials produced similar tissue reaction (P>0.05). AH-Plus and Endosequence BC Sealer (Median=2, Range=1-2) induced a high level of fibrosis after 60-day than control (Median=1, Range=1-1) and sham (Median=0, Range=0-0) groups (P<0.001) of fibrosis based in type I collagen increase (P=0.025 and P=0.021, respectively). Tissue necrosis was not observed and the bioceramic sealer showed significant signs of endocytosed (Median=1, Range=1-1) material after 7 days than other groups (Median=0, Range=0-0) (P<0.05). The calcium silicate-based sealer induced tissue repair faster than the epoxy resin-based sealer tested. However, both materials showed adequate biocompatibility and tolerance by subcutaneous tissues, with few differences in inflammatory profiles, formation of granulation tissue, and collagenesis. **Conclusions**: It may be concluded that calcium silicate-based sealer (EndoSequence BC Sealer) and an epoxy resin-based sealer (AH-Plus) presented suitable biocompatibility.

Keywords: Biocompatible Materials; Calcium Silicate; Root Canal Obturation; Sealers; Subcutaneous Tissue

Introduction

Root canal obturation aims to seal the root canal system as tightly as possible after cleaning and shaping procedures. It has been suggested that inappropriately filled root canals may be an underlying factor for treatment failures [1].

The physicochemical properties of endodontic sealers are constantly studied and are of high importance in sealing protocols and clinical practice. Studying their biological properties such as cytotoxicity, genotoxicity and tissue biocompatibility is also important because it allows the evaluation of the inflammatory effects of such materials after contact with various cell types and tissues, as well as the examination of their mutagenic potential [2-5].

Calcium silicate-based or bioceramic sealers have been introduced in endodontics mainly as root repair cements [6, 7]

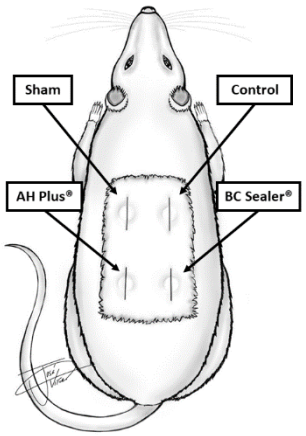

Figure 1. Distribution in experimental groups of endodontic sealers subcutaneously implanted in rats

and root canal sealers [4, 8-11]. According to previous researches, bioceramic materials exhibit alkaline pH, antibacterial activity, radiopacity, and cellular biocompatibility. Thus, the main advantages of bioceramic materials in dental applications are related to their physical, chemical and biological properties. Calcium silicate-based sealers are biocompatible, nontoxic, non-shrinking, and chemically stable within a biological environment [12]. An additional advantage of this material is its ability to form hydroxyapatite and, ultimately, a strong bond between dentin and the filling material during the setting process [10, 11, 13]. Previous studies that evaluated biological properties of bioceramic endodontic sealers reported that these materials present a positive biological behavior with several cells and conjunctive tissue of the periodontal ligament [3, 4, 10, 14-17]. To this day, there are a limited number of studies that evaluate the biocompatibility of calcium silicate-based sealers in subcutaneous tissue and the inflammation, granulation tissue, fibrosis, necrosis, dystrophic calcification, and collagen profile promoted by these endodontic cements.

The aim of the present study was to evaluate the degree of inflammation and collagenesis induced by a calcium silicate-based sealer (EndoSequence BC Sealer, Brasseler USA, Savannah, GA, USA) and an epoxy resin–based sealer (AH-Plus, Dentsply Tulsa Dental, Tulsa, OK, USA) by optical microscopy with hematoxylin and eosin staining and polarized light microscopy with Picrosirius red staining.

Materials and Methods

Ethics, specimen selection and material preparation

The Ethics Research Committee of Christus University, Brazil approved the present study with protocol number 26/2015. After approval by the Ethics Committee on Animal Research, 25 male Wistar rats (*Rattus norvegicus*) were used in this study. Animals (250–280 g; 4- to 6-month-old) were provided access to water and food *ad libitum*, housed individually under a light–dark cycle of 12 h with a temperature of 20–25°C, and weighed weekly. Ethical behaviors were adopted to minimize the suffering of animals in line with the principles of 3 R's (Reduction, Refinement, Replacement).

The animals were randomly ("=random()" command of Microsoft Excel, Microsoft Corp., Redmond, WA, USA) divided into groups based on euthanasia day and a total of 75 polyethylene tubes (Abbott Lab of Brazil, Sao Paulo, SP, Brazil) with internal diameter of 1.0 mm, external diameter of 1.6 mm, and length of 5.0 mm were sterilized and used to dispose of the tested materials. The Endosequence BC Sealer (n=25 tubes) and AH-Plus endodontic sealer (n=25 tubes) were prepared according to the manufacturer's recommendations and inserted into the tubes with a lentulo spiral (Dentsply, Tulsa, OK, USA) under aseptic conditions. Twenty-five polyethylene tubes were empty and used as controls.

Sample size calculation

Based on the study from Santos [18] that showed a higher frequency of intense inflammation for AH-Plus cement (87.5% *vs.* 0.0%) after 8-days of inoculation in subcutaneous tissue of rats, it is necessary to evaluate 5 rats/group to display a 90% power and 95% confidence in order to reject the null hypothesis of this study.

Surgical procedures

The dorsal skin of rats was shaved and received antiseptic treatment with 2% chlorhexidine (Fórmula e Ação, São Paulo, Brazil). Following that, the animals were anesthetized with xylazine (10 mg/kg) and ketamine (90 mg/kg). A total of four 2 cm-long incisions at 3-cm intervals were made in a head to tail orientation with a number 15 Bard-Parker blade (Becton Dickinson, Franklin Lakes, NJ, USA). The incisions were made in four quadrants, 3 cm apart from the edges, horizontally and vertically. After dissection, quadrant I was sutured without tube insertion (Sham group), quadrant II was sutured with insertion of a tube *without* material (control group), and quadrants III and IV were sutured with insertion of a tube with AH-Plus or Endosequence BC Sealer, respectively (Figure 1). The tested materials were prepared at the time of the surgical procedure. The sutures were performed with 4/0 nylon suture thread. All procedures were performed by a single operator with standardized surgical procedures.

After 3, 7, 15, 30, and 60 days from the time of implantation, five animals were euthanized (five per day, total 25 animals) [17] by overdose of pentobarbital, and the tubes and surrounding tissues were removed and fixed in 10% buffered formalin at neutral pH for 24 h and then kept in 70% alcohol.

Histological evaluation

After fixation, the samples were hemisectioned, the tubes were removed, and the cut side was prepared for histological processing. They were dehydrated with alcohol, cleared in xylene, *embedded* in paraffin, and serially sectioned into 4-µm-thick slices. The slices were mounted on slides and stained with hematoxylin and eosin and Picrosirius red staining to view the general tissue structure.

The slides were blindly analyzed by an experienced oral pathologist under a light microscope (DM2000, Leica Microsystems GmbH, Wetzlar, Hesse, Germany) coupled to a digital camera using 1×1 mm reticulum divided into quadrants with an area of 0.25 mm^2. Ten microscopic fields were selected at 400× magnification in which the tissue sample could be observed for analysis of the following parameters: inflammatory infiltrate, granulation tissue and fibrosis. The blinding was performed to avoid the identification of histological slides before the delivery to pathologist.

The inflammatory infiltrate was phenotyped as absent (score 0), predominantly acute (score 1) or predominantly chronic (score 1); and classified according to intensity as absent (score 0), mild (up to 25 cells per microfield, score 1), moderate (between 26 and 125 cells per microfield, score 2), or intense (more than 125 cells per microfield, score 3). Fibrosis and granulation tissue around the implanted material were classified as absent (score 0), thin (score 1) or thick (score 2); and the other histological findings (necrosis, endocytosis, and calcification) were classified as being present (score 1) or absent (score 0) [19].

The slides stained with Picrosirius red were observed under a polarized light microscope and classified as predominantly containing whitish-green birefringence (suggestive of type III collagen), containing similar ratios of whitish-green and reddish-yellow birefringence or containing a predominance of reddish-yellow birefringence (suggestive of type I collagen).

Statistical analysis

Data was showed as median (minimum-maximum) and analyzed with software SPSS for Windows (SPSS version 17.0, SPSS Inc., Chicago, IL, USA) using the Friedman/Dunn for analysis between groups and Kruskal-Wallis to analyze time points. The significance level adopted was $P<0.05$.

Results

Evaluation of inflammation

The inflammatory profile was predominantly acute on the third day of the experimental protocol in all animals ($P=1.000$); it evolved to chronic inflammation on day seven ($P=1.000$). Quadrants I (Sham), II (control), and IV (BC Sealer) displayed a complete regression of the inflammatory infiltrate in the 15-day experimental period ($P<0.001$), but in quadrant III (AH-Plus) this reduction was only partial ($P=0.018$). There was a significant reduction only from day 15 onwards in comparison to day 3 in all groups ($P<0.05$). On day 15, quadrant III implantations presented significantly more instances of score 2 compared to the other groups ($P=0.018$) (Table 1, Figure 2).

Quadrant I, II, and IV implantations presented higher inflammatory infiltrate on day 3 than quadrant III (AH-Plus) ($P=0.006$); and on day 7 quadrant III (AH-Plus) showed higher inflammatory infiltrate than Quadrant I, II, and IV implantations ($P=0.009$). After the 15^{th} day, all groups showed significant reduction in inflammatory infiltration ($P<0.05$) with no differences on 15^{th} ($P=1.000$), 30^{th} ($P=0.549$), and 60^{th} ($P=1.000$) days (Table 1, Figure 2).

Evaluation of granulation tissue and necrosis

There was little granulation tissue on day 3 ($P=1.000$) and 7 ($P=0.081$) in the experimental groups. Quadrant I areas presented a significant increase in the range of fine granulation tissue on day 15, with total remission of this histological finding from day 60 ($P=0.001$) (Table 2, Figure 2).

Quadrant II areas also presented with thin bands of granulation tissue around the tubes on day 3 and 7, with predominantly thick areas of this histological finding on day 15 and total remission on days 30 and 60 ($P<0.001$). Quadrant III areas presented with thick bands of granulation tissue on days 7 and 15, with a significant remission from day 30 ($P<0.001$), and quadrant IV areas presented with thick granulation tissue on days 7 and 15, with maintenance of thin bands on day 30 and absence of this finding on day 60 ($P<0.001$) (Table 2, Figure 2).

Although there were no significant differences in granulation tissue findings on days 3 ($P=1.000$), 7 ($P=0.081$) and 60 ($P=1.000$) between the four groups, on day 15, quadrant I areas only presented thin bands, while the other groups presented thick bands of granulation tissue around the implanted materials ($P=0.011$). Similarly, while quadrant I, II and III areas showed no granulation tissues on day 30, quadrant IV areas maintained thin bands of this histological finding ($P=0.010$) (Table 2, Figure 2).

Low frequency of necrosis was observed in the four quadrants throughout the experimental protocol ($P>0.05$) (Table 2).

Evaluation of fibrosis

No fibrotic patterns were observed on day 3 in any groups ($P=1.000$). There was a significant increase in the frequency of thin fibrotic tissues from day 15 in quadrant I ($P=0.001$) and quadrant II ($P<0.001$). In both groups, only thin bands of fibrotic tissue were observed at the experimental endpoint. Thin bands of fibrotic tissue appeared on day 15 in quadrant III areas and

became significantly thicker from day 30 and 60 (P<0.001). In quadrant IV areas, thin bands of fibrotic tissue were observed on days 15 to 60, with significant thickening from day 15 onwards (P<0.001) (Table 3, Figure 2).

On day 60, thin fibrotic tissue that could already be observed in quadrants III and IV areas was significantly higher than quadrants I and II (P<0.001) (Table 3, Figure 2).

There was no difference in the collagenesis profiles assessed at each timepoint; however, the temporal course of fibrosis was altered throughout the evaluated timepoints. The birefringence profile in the samples of the Sham group was predominantly of whitish-green fibers until day 15, presenting reddish-yellow fibers on days 30 and returning to whitish-green fibers on day 60 (P=0.001). In the control group (P=0.003), AH-Plus group (P=0.025) and Endosequence BC Sealer group (P=0.021), the birefringence profile

of collagen fibers was predominantly whitish-green on days 3 and 7, with the same proportion of whitish-green and reddish-yellow fibers on day 15. From day 30, there was a predominance of reddish-yellow birefringence (Table 3, Figure 3).

Table 1. Inflammation profiles of the subcutaneous tissues of rats grafted with AH-Plus or BC Sealer in sterile polypropylene tubes

	Experimental day					
	3	**7**	**15**	**30**	**60**	***P*-value**
Inflammation profile						
Sham						
Absent	0%	0%	100%*	80%*	100%*	<0.001
Acute	100%*	0%	0%	0%	0%	
Chronic	0%	100%*	0%	20%	0%	
Control						
Absent	0%	0%	100%*	100%*	100%*	<0.001
Acute	100%*	0%	0%	0%	0%	
Chronic	0%	100%*	0%	0%	0%	
AH-Plus						
Absent	0%	0%	40%	80%*	100%*	<0.001
Acute	100%*	0%	0%	0%	0%	
Chronic	0%	100%*	60%*†	20%	0%	
BC Sealer						
Absent	0%	0%	100%*	100%*	100%*	<0.001
Acute	100%*	0%	0%	0%	0%	
Chronic	0%	100%*	0%	0%	0%	
P-value	1.000	1.000	**0.014**	0.528	1.000	
Inflammation intensity						
Sham						
Absent	0%	0%	100%*	80%*	100%*	<0.001
Mild	20%	80%*	0%	0%	0%	
Moderate	80%*	20%	0%	20%	0%	
Intense	0%	0%	0%	0%	0%	
Control						
Absent	0%	0%	100%*	100%*	100%*	<0.001
Mild	0%	60%*	0%	0%	0%	
Moderate	60%*	40%	0%	0%	0%	
Intense	40%*	0%	0%	0%	0%	
AH-Plus						
Absent	0%	0%	40%	80%*	100%*	<0.001
Mild	100%*†	0%	60%*†	20%	0%	
Moderate	0%	80%*†	0%	0%	0%	
Intense	0%	20%	0%	0%	0%	
BC Sealer						
Absent	0%	0%	100%*	100%*	100%*	<0.001
Mild	20%	100%*	0%	0%	0%	
Moderate	80%*	0%	0%	0%	0%	
Intense	0%	0%	0%	0%	0%	
***P*-value**	0.005	0.048	0.014	0.399	1.000	

**P<0.05 intra group analysis; †P<0.05 inter group analysis; chi-square test or Fisher's exact test (percent frequency of n=5/group/day). The inflammatory infiltrate around the implanted material was phenotyped as absent, predominantly acute or predominantly chronic (Bueno et al., 2019). The inflammatory infiltrate around the implanted material was scored as absent, mild (up to 25 cells/microfield), moderate (between 26 and 125 cells/microfield) or intense (more than 125 cells/microfield) (Bueno et al., 2019)*

Discussion

The biocompatibility of endodontic sealers is an important property in view of the possibility of inflammatory reactions caused by the extrusion of these filling materials beyond the apical foramen [3]. The inflammatory process generated may be intense, causing tissue necrosis in the area, and thus, compromising the success of endodontic therapy [2]. The biological properties are hardly dependent on the physicochemical properties [5, 11].

Recently, bioceramic-based materials have been extensively studied because of their excellent biological, physical and chemical properties [4, 11, 20, 21]. Additionally, these materials have the ability to form hydroxyapatite and, ultimately, a bond between dentin and the filling material during the setting process [10, 11, 13].

Biological properties are among the most important aspects of root filling materials. However, there are a limited number of studies evaluating the biocompatibility of the bioceramic root canal sealer EndoSequence BC Sealer, comparing it with the epoxy resin–based sealer AH-Plus [1, 4].

The methodology used in the present study involved the implantation of sealer in the subcutaneous tissue of rats, similar to previous studies recently published [2, 3, 22, 23]. Implantation experiments in which materials come in direct contact with subcutaneous tissue have been widely accepted as appropriate methods for evaluating their biocompatibility since they were initiated by Torneck [23]. The periods of implantation used in this study were consistent with the majority of studies carried out on tissue reactions to materials implanted in subcutaneous connective tissue [2, 14, 15]. A sham group (simulated surgical procedure) and a control group (empty tube) were used. Previous studies that evaluated inflammation of biomaterials in the first days after inoculation show that the surgical procedure generates an inflammatory process, and without a sham group, it is impossible to distinguish whether this inflammation is caused by the material or the surgical procedure [17, 24].

The results of the present study demonstrated that the inflammatory response was predominantly composed of polymorphonuclear cells on day 3 of the experimental protocol in all animals, independent of group, further evolving to chronic inflammation on day 7. Quadrant I and II areas (Sham group and control group, respectively) demonstrated moderate or intense inflammatory infiltrates on day 3. However, after 15 days, no animal presented significant inflammation.

Table 2. Thickness of granulation tissue and frequency of necrosis in the subcutaneous tissues of rats grafted with AH-Plus or BC Sealer in sterile polypropylene tubes

	Experimental Day					
	3	**7**	**15**	**30**	**60**	***P*-value**
Granulation tissue						
Sham						
Absent	100%*	0%	0%	100%*	100%*	<0.001
Thin	0%	100%*	100%*†	0%	0%	
Thick	0%	0%	0%	0%	0%	
Control						
Absent	100%*	0%	0%	100%*	100%*	<0.001
Thin	0%	60%*	0%	0%	0%	
Thick	0%	40%	100%*	0%	0%	
AH-Plus						
Absent	100%*	0%	0%	80%*	100%*	0.004
Thin	0%	40%	40%	20%	0%	
Thick	0%	60%*	60%*	0%	0%	
BC Sealer						
Absent	100%*	0%	0%	0%	100%*	<0.001
Thin	0%	20%	20%	100%*†	0%	
Thick	0%	80%*	80%*	0%	0%	
***P*-value**	1.000	0.070	0.009	0.001	1.000	
Necrosis						
Sham	20%	0%	0%	0%	0%	0.384
Control	0%	0%	0%	0%	0%	1.000
AH-Plus[•]	20%	0%	0%	0%	0%	0.384
BC Sealer[•]	0%	0%	0%	0%	0%	1.000
***P*-value**	0.528	1.000	1.000	1.000	1.000	

**P<0.05 intra group analysis; †P<0.05 inter group analysis; Chi-square test or Fisher's exact test (percent frequency of n=5/group/day). The granulation tissue around the implanted material was classified as absent, thin or thick (Bueno et al., 2019)*

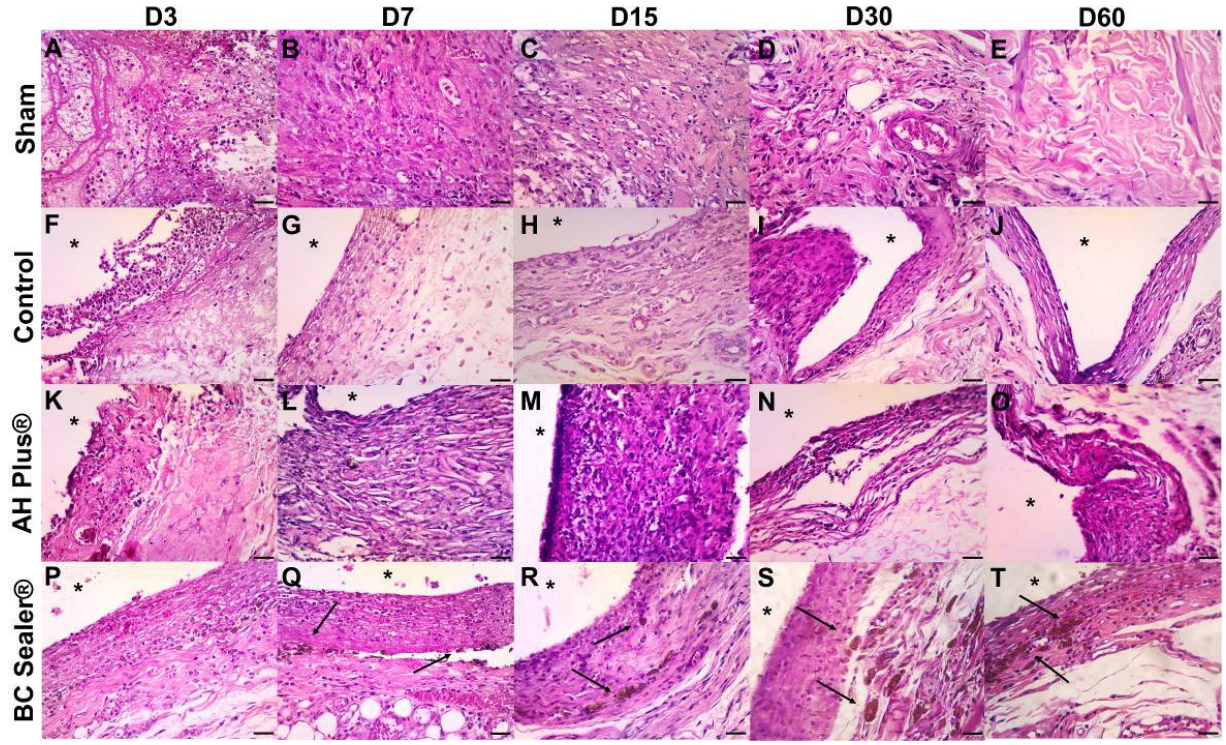

Figure 2. Inflammation profiles, Intensity of inflammation and thickness of granulation tissue of the subcutaneous tissues of rats grafted with AH-Plus or BC Sealer in sterile polypropylene tubes; 400× magnification (scale bar=50 μm)

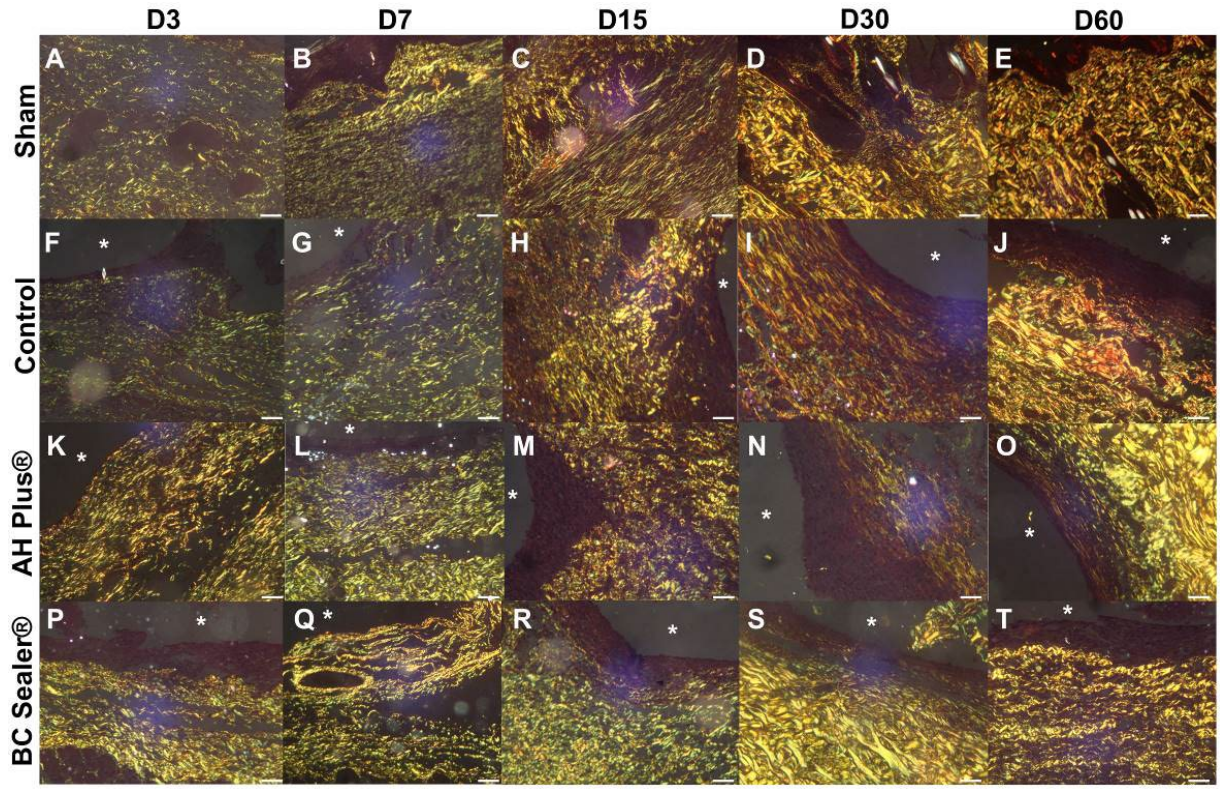

Figure 3. Thickness of fibrotic tissue, phenotype of fibrosis (collagenesis) and endocitiated material in the subcutaneous tissues of rats grafted with AH-Plus or BC Sealer in sterile polypropylene tubes; 400× magnification (scale bar=50 μm)

Table 3. Thickness of fibrotic tissue and collagen profile in the subcutaneous tissues of rats grafted with AH-Plus or BC Sealer in sterile polypropylene tubes

	Experimental Day					
	3	**7**	**15**	**30**	**60**	***P*-value**
Fibrosis						
Sham						
Absent	100%*	20%	0%	0%	0%	<0.001
Thin	0%	80%*†	100%*	100%*	100%*	
Thick	0%	0%	0%	0%	0%	
Control						
Absent	100%*	100%*	0%	0%	0%	<0.001
Thin	0%	0%	100%*	60%*	100%*	
Thick	0%	0%	0%	40%	0%	
AH-Plus						
Absent	100%*	80%*	0%	0%	0%	<0.001
Thin	0%	20%	100%*	40%	0%	
Thick	0%	0%	0%	60%*	100%*	
BC Sealer						
Absent	100%*	40%	%	%	%	<0.001
Thin	0%	60%*†	100%*	40%	%	
Thick	0%	0%	0%	60%*	100%*	
***P*-value**	1.000	***0.040***	1.000	0.172	**<0.001**	
Collagen Profile						
Sham						
>III	100%*	80%*	80%*	0%	0%	0.015
III≅I	0%	20%	20%	80%*	80%*	
>I	0%	0%	0%	20%	20%	
Control						
>III	100%*	100%*	20%	0%	0%	<0.001
III≅I	0%	0%	80%*	40%	0%	
>I	0%	0%	0%	60%*	100%*	
AH-Plus						
>III	100%*	60%*	60%	0%	0%	0.009
III≅I	0%	40%	60%*	40%	40%	
>I	0%	0%	0%	60%*	60%*	
BC Sealer						
>III	100%*	60%*	60%	0%	0%	0.018
III≅I	0%	40%	60%*	40%	40%	
>I	0%	20%	0%	60%*	60%*	
***P*-value**	1.000	0.469	0.308	0.493	0.083	

*$P<0.05$ intra group analysis; †$P<0,05$ inter group analysis; chi-square test or Fisher's exact test (percent frequency of $n=5$/group/day). Fibrosis around the implanted material was classified as absent, thin or thick (Bueno *et al.*, 2019). The phenotype of fibrosis was scored as >III (predominantly containing birefringence, suggestive of type III collagen), III≅I (approximately the same proportion of birefringence of whitish-green and reddish-yellow birefringence) and >I (predominance of reddish-yellow birefringence, suggestive of type I collagen)

The inflammatory responses exhibited by Endosequence BC Sealer and AH-Plus sealer were mild to moderate during the initial periods, with complete remission over time. The findings for AH-Plus are in agreement with those of a recent study that demonstrated a similar inflammatory response [5]. AH-Plus presents excellent physical properties, such as low solubility, dimensional stability, and greater bond strength to dentin than other root canal sealers [11, 13]. However, this epoxy resin–based sealer does not possess bioactive properties or osteogenic potential [12, 13].

AH-Plus promoted a moderate inflammatory reaction on day 7 that decreased over time. This result corroborates previous *in vitro* or *in vivo* studies [14, 25]. Therefore, this root canal sealer can still be considered a biocompatible material.

Endosequence BC Sealer showed a moderate inflammatory reaction on day 3, decreasing to mild inflammation on day 7 and absent on day 15. These findings demonstrated that Endosequence BC Sealer is a biocompatible sealer. The favorable biologic activity of bioceramic sealers may be associated with their alkaline pH, greater release of Ca^{2+} ions and formation of hydroxyapatite, as reported previously [10, 11, 20].

The slight presence of mild inflammatory infiltrate in areas in the AH-Plus group at day 15 and the thin bands of granulation tissue in areas in the Endosequence BC Sealer group at day 30 did not modify the collagenesis profile around the tubes. This is important because the presence of inflammation is directly associated with the release of a series of inflammatory chemical mediators that may increase collagen degradation [26]. Previous studies comparing AH-Plus and BC Sealer showed similar results with AH-Plus exhibiting higher inflammation scores than BC Sealer and although BC Sealer is described to induce more collagen formation than AH-Plus [18], our results showed equal production of fibrous capsule in two cements.

It was also observed that, under polarized light microscopy, the thinner and less organized fibers acquire a whitish-green birefringence (type III), while the thicker and more mature fibers have a reddish-yellow birefringence (type I) [27]. This change in fiber color is attributed to the fact that collagen fibrils organize into distinct patterns of physical aggregates [28, 29]. Type I collagen is found constitutively in bone, and the increase of its synthesis observed in the two endodontic sealers may be of fundamental importance in the repair process of the apical tissues, since inflammatory mediators that modulate both processes of collagenesis and bone mineralization are expressed in parallel, depending on tissue location [30].

Several inflammatory cytokines, such as Transforming growth factor beta (TGF-β), are directly related to the formation of fibrosis in subcutaneous tissue and with the inhibition of osteoclastogenesis, osteoblastic differentiation and deposition of bone mineral matrix in bone tissue [31, 32]. Thus, the process of collagenesis may be a point of intersection in the biological behavior of different tissues.

Regarding the other histological findings in the present study, a low frequency of necrosis was observed in the four quadrants throughout the experimental protocol and no animals showed signs of calcification. Significant presence of endocytosed material was only observed in the Endosequence BC Sealer group after 7 days. When phagocytosis of material is observed along with acute

inflammation, it is usually associated with neutrophil lysis and tissue damage [33]. However, in the presence of chronic inflammation or absence of inflammatory infiltrate, as observed in the Endosequence BC Sealer group, it is assumed that the internalization of the material is performed by macrophages.

Macrophages are the major phagocytic cells that can be activated by inflammatory processes stemming from the implantation procedure of the tubes; they have a series of receptors on their surface, such as mannose receptors, receptors for immunoglobulins and complement system receptors that are responsible for the endocytosis of non-biological material. These cells have little ability to phagocyte polymeric material, such as AH-Plus, and hydrophobic material; this leads to the polarization of these macrophages into proinflammatory cells (M1), which are potent inducers of tissue damage and cell death and were not identified in any groups. When activated by anti-inflammatory pathways (M2), macrophages are associated with angiogenesis and tissue fibrosis [16]. This cell type was observed in the Endosequence BC Sealer group and it can be assumed that the presence of this material is capable of activating both anti-inflammatory and pro-angiogenic pathways partially responsible for tissue repair.

The biocompatibility of dental materials is essential for a good clinical response, because biocompatible endodontic cements generate little evidence of inflammation and stimulate biomineralization [17]. Since extravasation of micrometer amounts can occur in the dental apex, this property is crucial for apical repair. The limitation of this animal study is that the protocols were carried out in subcutaneous tissue of rats, a tissue very different from bone or apex that may not fully replicate human responses. The choice of histological evaluation methods, which may have inherent biases for such subjective analyses, also posed as a limitation. However, the connective tissue is widely used to study the biocompatibility of dental materials as it is also a tissue of mesenchymal origin and this study design is classically used in literature to evaluate biocompatibility in an *in vivo* setting [16, 18, 23, 24]. Therefore, studies with tissues more similar to dental tissues should be performed to observe the impact of this process on the dentin-pulp complex and studies with human scenarios evaluating pain, inflammation and bone formation by image exams are crucial for translation of the data described in this study.

In clinical practice, during non-surgical endodontic therapy, overfilling may occur, leaving endodontic sealer beyond the apical foramen. An endodontic sealer should collaborate with the processes of tissue repair, avoiding undesirable foreign body reactions that may induce or maintain persistent apical periodontitis. In the present research, both sealers showed suitable biological behavior in order to be used in endodontic treatments. Additional clinical studies are necessary to analyze the behavior of this calcium silicate-based sealer and its biological properties and confirm the observed data.

Conclusion

The calcium silicate-based and epoxy resin-based sealers tested showed adequate biocompatibility and tolerance by subcutaneous tissues, and despite AH-Plus showing more inflammation and BC sealer showing more granulation tissue formation, few differences between the two cements were observed after 60 days. Therefore, these endodontic cements are clinically safe, and their extravasation, especially BC Sealer, which showed good endocytosis capacity, can be reabsorbed in a short time.

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Conflict of interest
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GTMC: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing original draft, Writing review & editing. AKM: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, writing original draft, Writing review & editing. LSE: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, writing original draft. ABS: Data curation, Funding acquisition, Investigation. LBD: Data curation, Funding acquisition, Investigation. HCP: Methodology, Formal analysis, Writing review & editing. GG: Writing review & editing. PGBS: Methodology, Statistical analysis, Writing review & editing. All authors contributed to the study and approved the final manuscript

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