

Comparison of the biocompatibility of grey mineral trioxide aggregate and sealapex plus zinc oxide in rat subcutaneous tissue

• **Waldécio Vita** Department of Health, State University of Feira de Santana, Feira de Santana, BA, Brazil • **Mitermayer Galvão Reis** Gonçalves Moniz Research Center, Laboratory of Molecular Biology and Pathology, Oswaldo Cruz Foundation (FIOCRUZ), São Paulo, SP, Brazil • **Théo Araujo-Santos** Center of Biological sciences and Health, Federal University of Western of Bahia • **Ana Maria Carvalho** Gonçalves Moniz Research Center, Laboratory of Molecular Biology and Pathology, Oswaldo Cruz Foundation (FIOCRUZ), São Paulo, SP, Brazil • **Cristina Mota** Gonçalves Moniz Research Center, Laboratory of Molecular Biology and Pathology, Oswaldo Cruz Foundation (FIOCRUZ), São Paulo, SP, Brazil • **Eduardo Antonio Gonçalves Ramos** Gonçalves Moniz Research Center, Laboratory of Molecular Biology and Pathology, Oswaldo Cruz Foundation (FIOCRUZ), São Paulo, SP, Brazil

ABSTRACT | *Objectives:* The aim of this study was to compare the subcutaneous tissue response to grey mineral trioxide aggregate and white Sealapex plus zinc oxide. *Methods:* Polyethylene tubes filled with the tested materials were implanted in the connective tissue of rats. Control animals received empty tubes. Tissue samples were collected after 7, 60, and 90 days and stained with hematoxylin-eosin, picosirius-fast green, and von Kossa stain for morphological analysis. The connective tissue response to the implanted materials was evaluated descriptively and semi-quantitatively by scoring the degree of inflammation, granulation tissue formation, fibrosis, and calcification. *Results:* Examinations of the grey mineral trioxide aggregate group over time revealed more intense inflammation at 7 days than at 60 days ($P < .05$). In the Sealapex plus zinc oxide group, granulation tissue was more abundant at 7 days than at 60 days ($P < .05$). Regarding calcification, von Kossa-positive granules were observed in the grey mineral trioxide aggregate and Sealapex plus zinc oxide groups at all time points studied. In the Sealapex plus zinc oxide group, calcification was more apparent at 60 days than at 7 days ($P < .05$). *Relevance:* This study demonstrates that all tested materials result in similar tissue reactions.

DESCRIPTORS | Biocompatibility Testing; Endodontics; Dental materials; Retrograde obturation.

RESUMO | **Comparação da biocompatibilidade do agregado de trióxido mineral cinza e sealapex acrescido de óxido de zinco em tecido subcutâneos de ratos** • *Objetivos:* O objetivo deste estudo foi comparar a biocompatibilidade do agregado trióxido mineral cinza (GMTA) com o Sealapex acrescido de óxido de zinco (Sealapex/ZnO) em tecidos subcutâneos de ratos. *Métodos:* Tubos de polietileno preenchidos com os materiais testados foram implantados no tecido conjuntivo de ratos. Os animais do grupo controle receberam tubos vazios. Amostras de tecido foram coletadas após 7, 60 e 90 dias e coradas com hematoxilina-eosina, picosirius-fast green e von Kossa para a análise morfológica. A resposta do tecido conjuntivo aos materiais implantados foi avaliada descritiva e semi-quantitativa, marcando o grau de inflamação, formação de tecido de granulação, fibrose e calcificação. *Resultados:* Análise do grupo GMTA, ao longo do tempo, revelou inflamação mais intensa com 7 dias do que com 60 dias ($p < 0,05$). No grupo Sealapex/ZnO, o tecido de granulação foi mais abundante com 7 dias do que com 60 dias ($p < 0,05$). Quanto a grau de calcificação, granulações von Kossa-positivas foram observadas nos grupos GMTA e Sealapex/ZnO em todos os períodos de tempo estudados. No grupo Sealapex/ZnO, a calcificação foi mais aparente com 60 dias do que com 7 dias ($p < 0,05$). *Relevância:* Este estudo demonstra que todos os materiais testados promovem reações teciduais semelhantes.

DESCRITORES | Teste de Biocompatibilidade; Endodontia; Materiais Dentários; Obturação Retrograda.

CORRESPONDING AUTHOR | • **Waldécio Vita** State University of Feira de Santana, Department of Health • **Rua Barão do Rio Branco, 1309** Feira de Santana, BA • **44001-205** E-mail: endovita@gmail.com

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INTRODUCTION

Apicectomy combined with retrograde filling is a surgical endodontic procedure that involves exposure of the area, root tip removal, cavity preparation, and sealing with a retrograde filling material that has adequate physicochemical and biological properties for long-term survival in the oral cavity.^{1,2} An ideal retrograde filling material should seal the pathways of communication between the root canal system and the surrounding tissues.³ Failures in periapical surgery can also be attributed to poor sealing of retrograde cavities, which is characterized by inappropriate contact between filling materials and the dentinal walls.⁴ Because materials used in endodontics are frequently placed in close contact with the periodontium, they also must be biocompatible with host tissues.⁵

Different materials have been used for retrograde filling, including silver amalgam, resin, glass ionomer cement, zinc oxide and eugenol, super EBA, mineral trioxide aggregate (MTA), and Sealapex plus zinc oxide (Sealapex/ZnO).⁶

MTA is indicated mainly for retrograde filling and repair of root perforations.^{7,8} The biocompatibility of MTA has been demonstrated by examining tissue reactions in experimental animals⁵ and its sealing capacity.⁹ Despite its excellent biological properties, MTA has some disadvantages, such as its questionable antimicrobial activity, difficulties concerning its manipulation and insertion, potential tooth discoloration, and high cost.^{8,2} Therefore, other less expensive materials that are more easily manipulated and have good biological and physicochemical properties have been tested.

Sealapex, a calcium hydroxide-based root canal sealer developed in 1984, showed good biological and physical properties.⁷ The use of Sealapex as a retrograde filling material requires the addition of zinc oxide until the cement acquires a putty-like consistency. This type of material is called Sealapex plus zinc oxide.⁶ In addition to its biocompatibility,

Sealapex plus zinc oxide can stimulate the deposition of mineralized tissue in the periapical region.⁶

Reagents

Grey mineral trioxide aggregate (GMTA) was purchased from Angelus Indústria de Produtos Odontológicos S/A (Londrina, Brazil). Sealapex/ZnO was prepared as a mixture of Sealapex (Sybron Corporation, Orange, CA, USA) and zinc oxide (k-Dent, Quimidrol Farm. Brás. Joinville-SC, Brazil). Sealapex/ZnO was manipulated in a 1:1:2 proportion by weight of base paste, catalyzer, and zinc oxide.⁶ Polyethylene tubes (length: 10 mm; inner diameter: 2 mm; outer diameter: 3 mm) were obtained from disposable brushes (Vigodent SA Indústria e Comércio, Rio de Janeiro, Brazil). In addition, all polyethylene tubes were sterilized by autoclaving before use in the experiments.

OBJECTIVE

The objective of this study was to compare the biocompatibility of Sealapex/ZnO and GMTA retrograde filling materials by evaluating the reaction of subcutaneous connective tissue in Wistar rats. The following histological parameters were analyzed: inflammatory reaction, granulation tissue formation, fibrous capsule presence, and mineralization.

MATERIALS AND METHODS

Animals

The study was approved by the Ethics Committee of Centro de Pesquisa Gonçalo Moniz, Fundação Oswaldo Cruz, Brazil (permit number: 023/2009). Male Wistar rats (*Rattus norvegicus*), aged 3 to 6 months and weighing 300 to 450 g, were obtained in the animal house of Centro de Pesquisa Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Bahia, Brazil. The animals were maintained at 24°C under a 12-hour light/dark cycle with free access to food and water.

Implants

The animals were anesthetized by intraperitoneal injection of 50 mg/kg ketamine hydrochloride and 10 mg/kg xylazine hydrochloride. Then 3 transverse incisions were made in the dorsal region of each animal, and polyethylene tubes filled with Sealapex/ZnO (n=6) and GMTA (n=6) were implanted. Animals implanted with empty tubes served as controls (n=6). Each rat received 3 implants according to a previously established protocol. A rotation system of the anatomical sites was employed to rule out possible local tissue interference. The animals were sacrificed after 7, 60, and 90 days, and the areas containing the implants and adjacent tissue were removed in blocks and fixed in 10% buffered formalin. After this, the polyethylene tubes were removed without damaging the tissue in the area to be studied. Each tissue block was dehydrated, cleared, and embedded in paraffin for routine histological processing.

Macroscopic analysis

Serial 5- μ m thick sections were made and stained with hematoxylin-eosin and picosirius-fast green (PIFG) to identify collagen. The von Kossa technique was used to observe mineralized structures in the tissue, which are stained dark in that test. Descriptive and semiquantitative analysis of the connective tissues' response to the cements was performed to evaluate the characteristics of the inflammatory process and of the reparative phenomena. The inflammatory reaction, granulation tissue formation, fibrous capsule presence, and mineralization were observed and classified as absent, mild, moderate, or intense (scores of 0, 1, 2 and 3, respectively).¹⁰⁻¹³ Only 1 examiner (a pathologist) analyzed the histological sections; the examiner was blinded to the group assignments.

Statistical analysis

The results are reported as means and standard deviations and were analyzed using GraphPad

Prism 5.0 (GraphPad Software, San Diego, CA, USA). The nonparametric Kruskal-Wallis test and Dunn's posttest were used to evaluate the tissue-repair kinetics of each cement at 7, 60, and 90 days after implantation. The nonparametric Friedman test was used for comparison among the Sealapex/ZnO, GMTA, and control groups. A $P < 0.05$ was considered to indicate statistical significance in all tests.

RESULTS

The mean of the results obtained at each time point was calculated for each group. After this, scores were attributed to the intensity of tissue reactions – these scores are shown in Table 1.

Descriptive microscopic analysis

Empty tubes: 7 days

We observed mild chronic inflammation around the lumen of the empty polyethylene tubes in the samples. The inflammatory infiltrate consisted of mononuclear cells, macrophages, lymphocytes, and plasma cells (Figure 1A). Mild granulation tissue formation and newly formed vessels were observed in all samples. Fibrosis was mild in 3 samples, moderate in 1, and intense in 1. There were no von Kossa-positive granules near the tube openings in any of the samples (Figure 2A).

Sealapex/ZnO: 7 days

We observed mild chronic inflammation with a predominance of macrophages around the lumen of the tubes in most of the Sealapex/ZnO samples (Figure 1D). In addition, we verified mild to moderate granulation tissue formation and fibrosis in these samples. Four samples exhibited small amounts of von Kossa-positive granules in close proximity to the implants (Figure 2D), and 2 samples did not show any von Kossa staining.

Table 1 | Results of the histomorphological events analyzed in each rat (R) euthanized at each time point.

Rats	Groups	Inflammation			Granulation tissue			Fibrosis			Calcification		
		7 days	60 days	90 days	7 days	60 days	90 days	7 days	60 days	90 days	7 days	60 days	90 days
R1	Control	1	0	0	1	0	1	1	1	2	0	0	0
	Sealapex/ZnO	1	1	1	2	0	1	2	2	1	1	2	1
	GMTA	2	1	1	3	1	1	2	1	2	0	2	1
R2	Control	1	1	1	1	1	1	1	2	1	0	0	0
	Sealapex/ZnO	1	1	1	1	0	1	2	1	1	0	2	1
	GMTA	1	0	1	1	0	0	2	1	2	1	0	2
R3	Control	1	1	1	1	0	1	1	1	2	0	0	0
	Sealapex/ZnO	1	1	1	1	1	1	2	2	1	1	1	2
	GMTA	1	0	1	1	1	1	2	2	2	0	0	3
R4	Control	2	0	1	1	1	1	2	1	2	0	0	0
	Sealapex/ZnO	1	1	1	1	0	1	1	2	1	0	1	2
	GMTA	1	1	1	0	1	1	1	2	2	1	0	0
R5	Control	1	0	1	1	1	1	3	1	3	0	0	0
	Sealapex/ZnO	1	1	1	1	1	1	1	2	1	1	2	2
	GMTA	1	0	1	1	1	1	1	3	1	1	0	1
R6	Control	-	0	1	-	1	1	-	2	2	0	0	0
	Sealapex/ZnO	1	1	-	2	1	-	2	1	-	1	2	-
	GMTA	1	1	1	1	1	1	1	2	2	1	0	1

Groups: negative control; sealapex/ZnO, sealapex plus zinc oxide; GMTA, grey mineral trioxide aggregate.

Score: 0 = absent; 1 = mild; 2 = moderate; 3 = intense. (-): Sample not analyzed due to the absence of the area of interest near the tube opening.

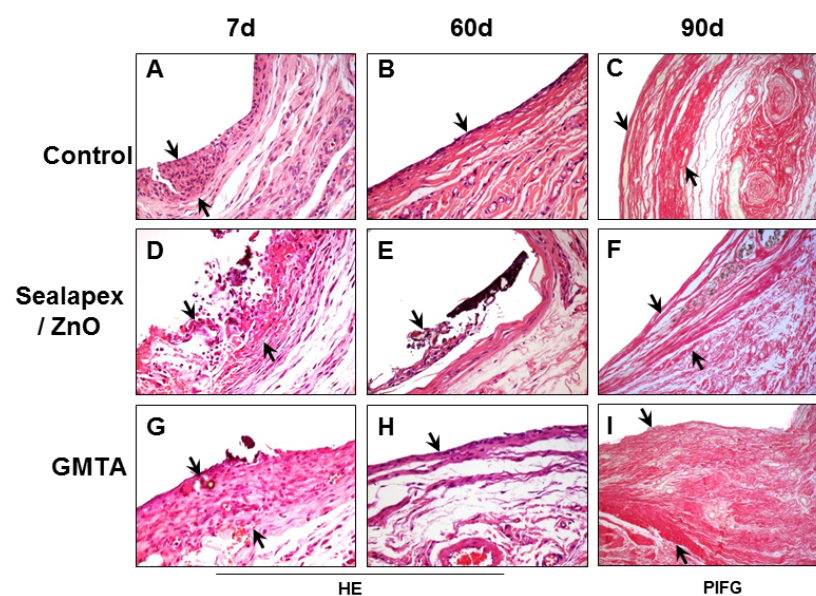


Figure 1 | Kinetics of the inflammatory response and tissue repair induced by retrograde filling materials.

Empty polyethylene tubes (control) and polyethylene tubes filled with Sealapex plus zinc oxide or grey mineral trioxide aggregate were implanted subcutaneously in the dorsal connective tissue of rats. A – Mild chronic inflammatory infiltrate and newly formed blood vessels (arrow); hematoxylin-eosin, 400×. B – Absence of inflammation and presence of a small amount of granulation tissue (arrow); hematoxylin-eosin, 400×. C – Fibrous wall showing a moderate collagen matrix (arrow); picrosirius-fast green, 200×. D – Mild chronic inflammatory infiltrate (arrow); hematoxylin-eosin, 400×. E – Mild mononuclear inflammatory infiltrate and a reduced number of vessels (arrow); hematoxylin-eosin, 400×. F – Thin fibrous capsule (arrow); picrosirius-fast green, 200×. G – Mild chronic inflammation (arrow); hematoxylin-eosin, 400×. H – Mild chronic inflammation with a predominance of macrophages (arrow); hematoxylin-eosin, 400×. I – Moderate fibrosis in the wall showing a dense collagen matrix (arrow); picrosirius-fast green, 200×.

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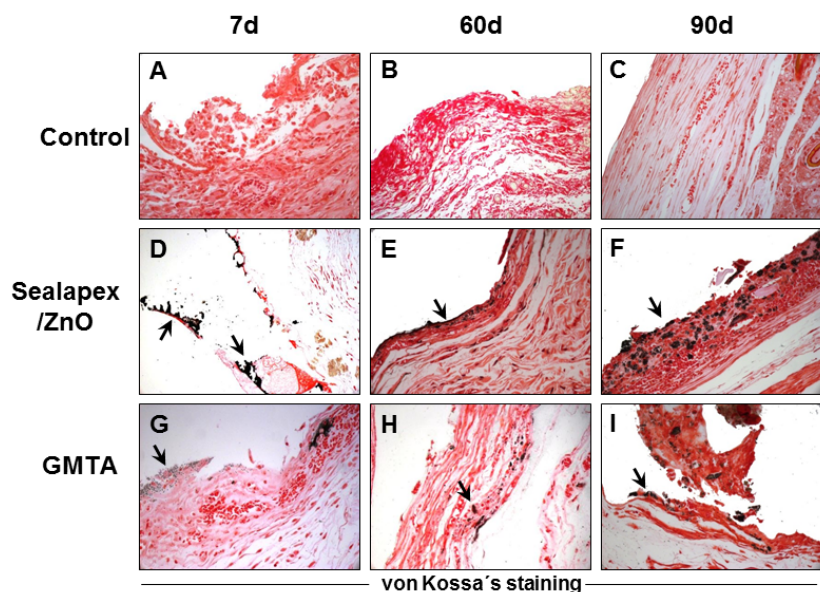


Figure 2 | Kinetics of tissue calcification induced by retrograde filling materials. Photomicrographs of von Kossa-stained samples (200×). Empty polyethylene tubes (control) and polyethylene tubes filled with Sealapex plus zinc oxide or grey mineral trioxide aggregate were implanted subcutaneously in the dorsal connective tissue of rats. A, B, and C – Absence of von Kossa-positive granules. D – Small amount of von Kossa-positive granules (arrow). E and F – Moderate amount of von Kossa-positive granules (arrows). G, H, and I – Small amount of von Kossa-positive granules (arrows).

GMTA: 7 days

We observed mild chronic inflammation near the implants in the MTA group (Figure 1G), as there was a small amount of immature granulation tissue containing newly formed vessels in most of the specimens. All these samples showed mild to moderate fibrosis. Small amounts of von Kossa-positive granules were observed in close proximity to the implants (Figure 2G). Intense von Kossa-positive staining was observed in 1 sample, and von Kossa-positive granules were absent from the other samples.

Empty tubes: 60 days

Inflammation was absent near the lumen of the empty tubes (Figure 1B). Granulation tissue formation was mild in 4 samples and absent from the other 2 (Figure 1B). Mild fibrosis was observed in 4 samples, and moderate fibrosis was observed in 2 samples. No von Kossa-positive granules were found near the tube openings in any of the samples (Figure 2B).

Sealapex/ZnO: 60 days

A mild chronic inflammatory infiltrate consisting of lymphocytes, plasma cells, and macrophages was observed around the tubes in close proximity

to the implants in all 6 samples (Figure 1E). Small amounts of granulation tissue were observed in 3 samples, and no granulation tissue was found in the other 3 samples. Fibrosis was moderate in 4 samples and mild in 2 samples (Figure 1E). Low to moderate amounts of von Kossa-positive granules were observed near the implants (Figure 2E).

GMTA: 60 days

In this group, mild chronic inflammation, characterized by a predominance of macrophages, was observed around the tubes in close proximity to the implants in 3 specimens; the other specimens presented no inflammation (Figure 1H). Fibrosis was found to be denser in this group; it ranged from mild to intense but was moderate in most cases. Von Kossa-positive granules were observed in 2 samples, which were of mild and moderate intensity (Figure 2H).

Empty tubes: 90 days

Mild chronic inflammation and granulation tissue were observed in all samples in this group. Fibrosis was found to be denser in most specimens; it ranged from mild to intense and was moderate in most cases (Figure 1C). No von Kossa-positive granules were found (Figure 2C).

Sealapex/ZnO: 90 days

A sample was not analyzed due to the absence of the area of interest near the tube opening. A mild chronic inflammatory infiltrate (consisting of mononucleated cells, macrophages, lymphocytes, and some plasma cells) was observed around the tubes near the implants in the remaining 5 samples. Small amounts of granulation tissue and mild fibrosis were observed in all samples (Figure 1F). Von Kossa-positive granules were present in all samples, and their intensity was moderate in most cases (Figure 2F).

GMTA: 90 days

Mild chronic inflammation (characterized by a predominance of macrophages and some lymphocytes) was observed in all 6 samples of this group. Macrophages containing stained remnant material in their cytoplasm were also observed. Small amounts of granulation tissue were observed in most of the samples. Moderate fibrosis was also observed in most of the samples (Figure 1I), as were Von Kossa-positive granules with mild intensity (Figure 2I).

Intergroup comparison

No significant differences in inflammation, granulation tissue formation, or fibrosis were observed between the groups. As expected, calcification was greater in the GMTA and Sealapex/ZnO groups than in the control at each time point. However, no differences in calcification were observed between the GMTA and Sealapex/ZnO groups.

Comparison between time points

No significant differences in inflammation were observed over time in the control group or in the Sealapex/ZnO group. In contrast, in the GMTA group, inflammation was significantly more intense at 7 days than at 60 days ($P < .05$). Granulation tissue formation did not differ significantly between

time points in either the control group or GMTA group. However, a significant difference was observed in the Sealapex/ZnO group, with granulation tissue being more abundant at 7 days than at 60 days ($P < .05$). No significant difference in fibrosis was observed over time in any of the 3 groups. The calcification analysis revealed the absence of von Kossa-positive granules in the control group, whereas these granules were observed in the Sealapex/ZnO and GMTA groups at all time points studied. However, there was no significant difference between 7 and 60 days in any group except in the Sealapex/ZnO group, which had a larger amount of von Kossa-positive granules at 60 days ($P < .05$).

DISCUSSION

MTA is the most commonly used material for retrograde fillings and for root perforations fillings;^{3,5,14} however, it presents some disadvantages regarding manipulation and insertion.^{2,3,8} MTA has also been used in endodontic sealer formulations.^{15,16} In the study, the empty tubes used in the control group generated few or no reactions in the subcutaneous tissue, similar to the result previously reported.^{10-12,17} In this study, we verified that the biocompatibility of Sealapex/ZnO is similar to that of GMTA by evaluating the reactions to these materials in the subcutaneous connective tissue of rats. Tubes containing GMTA and Sealapex/ZnO are known to display moderate to intense inflammation few days after implantation.^{1,11,18-20} The high initial pH of the GMTA implants may be responsible for triggering inflammatory cytokines and may exacerbate early tissue inflammation.¹ The high pH in the environment is associated with the constant release of MTA and with the formation of calcium hydroxide.³ In this study, we observed a mild chronic inflammatory infiltrate around GMTA and Sealapex/ZnO with the presence of some foreign-body giant cells 7 days after surgery. Fillapex, another cement-based MTA, also triggered a severe inflammatory

reaction; this may be related to the presence of arsenic-containing compounds or resin.^{15,16} These differences in the inflammation intensity at the implantation site may be related to the examiner's criteria for sample evaluation. However, we did not observe a difference between GMTA and Sealapex/ZnO in the inflammatory infiltrate at the final time point. The observation of a fibrous capsule around the implant indicates that the tissues tolerate the material.²⁰ We observed that GMTA and Sealapex/ZnO induced mild to moderate fibrosis 7 days after implantation. Similar results were reported in other *in vivo* studies.^{11,13,21,22} Moreover, we verified that the GMTA and Sealapex/ZnO groups had denser fibrosis 60 and 90 days after surgery than at earlier time points. Previous studies demonstrated that MTA did not interfere with the natural healing process^{1,12,20}, but that Sealapex/ZnO elicited chronic inflammation followed by moderate fibrosis, a reduction in the number of vessels, and the presence of foreign-body giant cells.²³ However, some studies have shown that MTA induces the formation of new vessels and restoration of microcirculation.⁵ Other studies have shown that MTA induces the formation of mineralized tissue such as dentin and cementum-like tissue; osseous reaction investigations have shown that the bone's response to MTA is relatively mild and has only minor inflammation.^{5,21,24} MTA has no calcium hydroxide in its formulation; mixing the powder with water results in a structure that contains basically calcium oxide and calcium phosphate. The calcium oxide can react with tissue fluids to form calcium hydroxide, which, when in contact with water, can dissociate into calcium ions and hydroxyl.^{21,24}

We observed no differences between the GMTA and Sealapex/ZnO groups at any time point in the study, although the calcification intensity seemed to be higher in the Sealapex/ZnO group than in the GMTA group after 60 days. MTA induces increased calcium ion release and produces an alkaline

pH shortly after implantation, whereas Sealapex increases calcium ion release and pH after a longer period of time.^{25,26} This fact may explain the results of this study, in which an apparent increase in calcification over time was observed in the Sealapex/ZnO group, but a decrease was observed in the GMTA group. The high solubility of Sealapex cement means that it has stronger physicochemical and biological effects than do other calcium hydroxide-based materials, thus promoting biological root-end filling via the formation of mineralized tissue.¹⁷ Similar results have been obtained with MTA^{21,27} and with pure Sealapex.^{12,17,24} Some studies have not reported the formation of calcified structures around implanted MTA despite using the Von Kossa technique.⁵

CONCLUSIONS

Our results showed that the tissue reactions to GMTA and Sealapex/ZnO were very similar. Tissue calcification around these 2 materials was more significant than that in the control group. However, studies that examine marginal infiltration, carcinogenicity, and long-term stability in an *in vivo* environment are needed. Thus, further studies are necessary to better characterize the biological properties of Sealapex/ZnO and to confirm the results observed during clinical treatment.

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