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Comparative Evaluation of MTA Sealers Biomineralization Ability: an in Vivo Study

Avaliação Comparativa da Capacidade de Biomineralização de Cimentos MTA: Estudo in Vivo

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Abstract

This research aimed to evaluate the biomineralization induced by different concentrations of MTA Flow® and compare it to MTA Angelus®. Fifteen male Wistar rats received subcutaneous implants containing the materials to be tested (MTA Flow® at putty, thick, and thin consistencies and MTAAngelus®) and empty tubes (control). After 7, 40 and 90 days, the animals were euthanized, and the implants were removed with the surrounding tissue. The presence of biomineralization was analyzed in light microscope by Von Kossa technique. The statistica l differences were considered for $p < 0.05$. Calcification areas were present in all the MTA Flow® and MTA Angelus® groups. In the control group, no mineralized areas were observed. MTA Angelus® and thin-MTA Flow® showed significant reduction in calcification as time went by. A significant increase in areas with calcification, proportional to the exposure time, was observed in putty-MTA Flow® and thick-MTA Flow®. MTA Angelus® and thin-MTA Flow® showed significantly higher calcification than thick-MTA Flow® in the shortest exposure time. Analysis of putty-MTA Flow® showed significantly higher calcification areas than MTA Angelus® and thin-MTA Flow® in the longest exposure time. MTA Flow® stimulated mineralization, which has varied according to the concentration. Besides, in longer periods, MTA Flow® biomineralization performance was higher than MTA Angelus®, especially in highest concentration.

Keywords: Biomineralization. Calcium. Rats, Wistar.

Resumo

Esse estudo teve como objetivo avaliar a biomineralização induzida por diferentes concentrações do MTA Flow® e compará-la ao MTA Angelus®. Quinze ratos Wistar machos receberam implantes subcutâneos contendo os materiais a serem testados (MTA Angelus® e MTA Flow® nas consistências pastosa, espessa e fluida) e tubos vazios (controle). Após 7, 40 e 90 dias, os animais foram eutanasiados e os implantes foram removidos juntamente com o tecido circundante. A presença de biomineralização foi analisada em microscópio de luz pela técnica de Von Kossa. Diferenças estatísticas foram consideradas para valores de $p < 0,05$. Áreas de calcificação estavam presentes em todos os grupos do MTA Flow® e MTA Angelus®. No grupo controle não foram observadas áreas mineralizadas. O MTA Angelus® e o MTA Flow® fluido apresentaram redução significativa na quantidade de calcificação ao longo do tempo. Um aumento significativo na quantidade de áreas calcificadas, proporcional ao tempo de exposição, foi observado no MTA Flow® pastoso e no MTA Flow® espesso. O MTA Angelus® e o MTA Flow® fluido apresentaram calcificação significativamente maior do que o MTA Flow® espesso no menor período de exposição. Análises contento o MTA Flow® pastoso demonstraram áreas de calcificação significativamente maior do que o MTA Angelus® e MTA Flow® fluido no maior tempo de exposição. O MTA Flow® induziu a formação de áreas mineralizadas, que variou de acordo com a concentração do cimento. Em períodos mais longos, o MTA Flow® apresentou desempenho superior ao MTA Angelus®, principalmente quando utilizado na maior concentração.

Palavras-chave: *Biomineralização. Cálcio. Ratos Wistar.*

1 Introduction

Mostendodonticpathologies arecaused bymicroorganisms in the root canal system. Endodontic treatment aims to eliminate and avoid canal reinfection by microorganisms, which eventually may penetrate the physical barrier shaped by sealer[1]. Endodontic sealers are used to complete the filling of root canal, assist local bacterial control and induce mineralized tissue formation[2].

To be considered effective, the sealers must have some specific properties, such as: a) physical: sealing, dimensional and color stability, radiopacity, insolubility when close to tissue fluids, fluidity and ease of insertion; b) chemical: alkaline pH and calcium ions release; and c) biological: antimicrobial property, calcium deposits induction by fibroblasts and biocompatibility[3, 4]. Thus, sealing materials need to attend their functions without damaging the underlying tissues, associated with low toxicity and adequate immune response[5,6]. Mineral trioxide aggregate (MTA) is a powder that consists of tricalcium silicate, tricalcium aluminate, tricalcium oxide, silicate oxide and bismuth oxide[7]. Due to its physicochemical and biological properties, MTA has been used in dentistry since its development in the 1990s, in different clinical situations related to endodontics.

It has been proven that MTA-based cements are effective

in root filling, production of apical barrier, pulp capping and repair of pathological or iatrogenic perforations[3,8]. These properties depend on the cement ability to release hydroxyl and calcium ions, which induces biomineralization[9,10]. Furthermore, these cements are biocompatible, have antimicrobial property and low cytotoxicity[3,5]. However, the MTA consistency is considered a disadvantage that makes it difficult its manipulation and insertion into the root canal[3].

To improve this characteristic, the MTA formulation has been modified. Therefore, the MTA-Flow® cement has recently launched and may be manipulated using different powder-to-gel proportions, being possible to obtain different consistencies. Thus, it is considered an innovation when compared to other MTA-based cements[11].

However, there are still few studies evaluating the MTA Flow® biologic properties. Thus, this study aimed to evaluate the ability of different concentrations of MTA Flow®, compared to MTA Angelus®, to stimulate biomineralization in rats' subcutaneous connective tissue .

2 Material and Methods

This prospective randomized controlled study was performed *in vivo* and was approved by the Ethics Committee on Animal Use (CEUA) of the State University of Maringá (CEUA/UEM - 8523280519).

2.1 Subcutaneous implants

Fifteen male Wistar rats, weighting 250±30 g, were used in this study. The rats were kept under controlled temperature (20 ºC+2 ºC) and humidity (60-70%) environment, under 12/12 hours light/dark cycle, with food and water provided *ad libitum*.

The sample size for the experiment was established based on previous studies[12-14]. The animals were randomly divided into three groups of five animals each, according to histological observation periods of 7, 40 and 90 days.

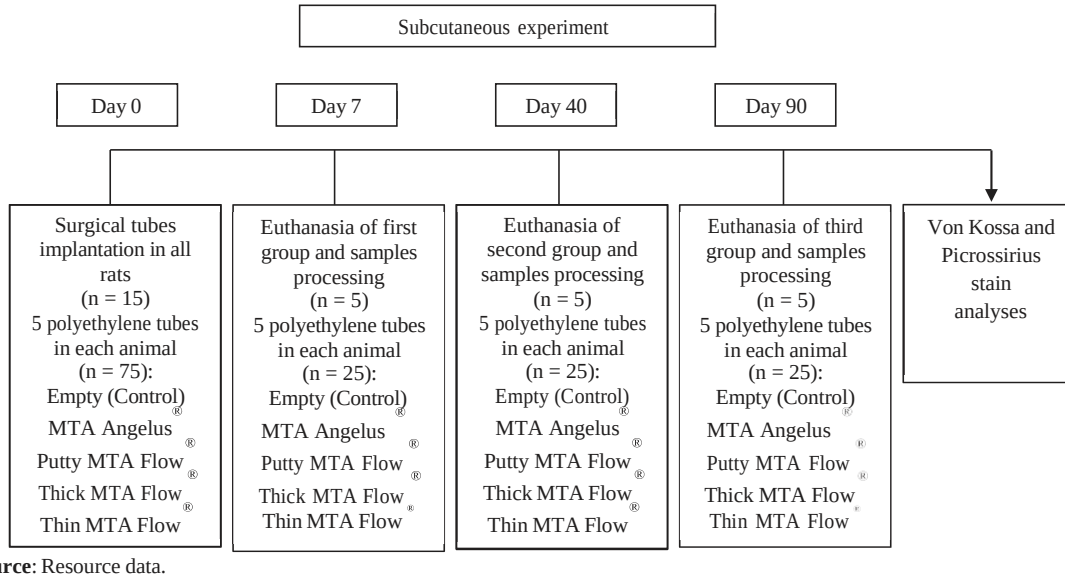
MTA Flow® was manipulated according to the manufacturer's guidelines, in three concentrations: 0.19 g powder/50 µl gel (putty MTA), 0.26 g powder/100 µl gel (thick MTA), and 0.19 g powder/100 µl gel (thin MTA). MTA Angelus® (Angelus, Londrina, Brazil) was utilized according to the manufacturer's concentration of 0.28 g powder/50 µl distilled water.

Five polyethylene tubes with a 1.0 mm internal diameter, 1.6 mm external diameter, and 10.0 mm length (four were filled with cements and one empty tube was negative control) were implanted in each of the fifteen animals, totaling seventy-five tubes.

The animals were anesthetized with Xylazine hydrochloride (30 mg/kg) + Ketamine hydrochloride (240 mg/kg) and 5 incisions of 10.0 mm each were made on their back (two in a cranial position, two in a caudal position, and one in a central position). Polyethylene tubes were implanted in the animals' subcutaneous tissue, then, incision sites were sutured (Nylon 4.0 MT, Shalon Suturas, São Luís de Montes Belos, Goiás - Brazil).

At the end of each experimental period, the animals were euthanized. The tubes and the adjacent subcutaneous connective tissue with a 0.5 cm margin were removed, washed with saline solution and fixed in 10% formalin. After 24 hours, the tissue samples were dehydrated, diaphanized and embedded in paraffin. Semi-serial sections of 6 µm thickness were collected on slides and stained by the Von Kossa with Picrosirius contrast[3,15,16]. Twenty slides from each group (thin, thick and putty-MTA Flow®, MTA Angelus® and control), for each period (7, 40 and 90 days) were analyzed, totaling three hundred slides (Figure 1).

Figure 1 - Experiment protocol of days 0 (tubes implantation), 7, 40 and 90 (euthanasia and histological processing: excess subjacent tissue removal, tubes removal and incorporation of samples in paraffin), followed by histological analysis



Source: Resource data.

Analyses were performed using a light microscope (Motic, USA) with magnification of 100X, 200X and 400X by two qualified and previously calibrated operators.

2.2 Histological analysis

Von Kossa technique was used to observe the mineralized structures in the tissue, which was stained dark. Calcium deposits formation was classified as present or absent. Calcification areas were graded according to Danesh et al.[15] according to the following scores: (0) no calcification; (1) calcification in less than ⅓ of the tube periphery; (2) calcification between ⅓ and ⅔ of the tube periphery; and (3) calcification in more than ⅔ of the tube periphery.

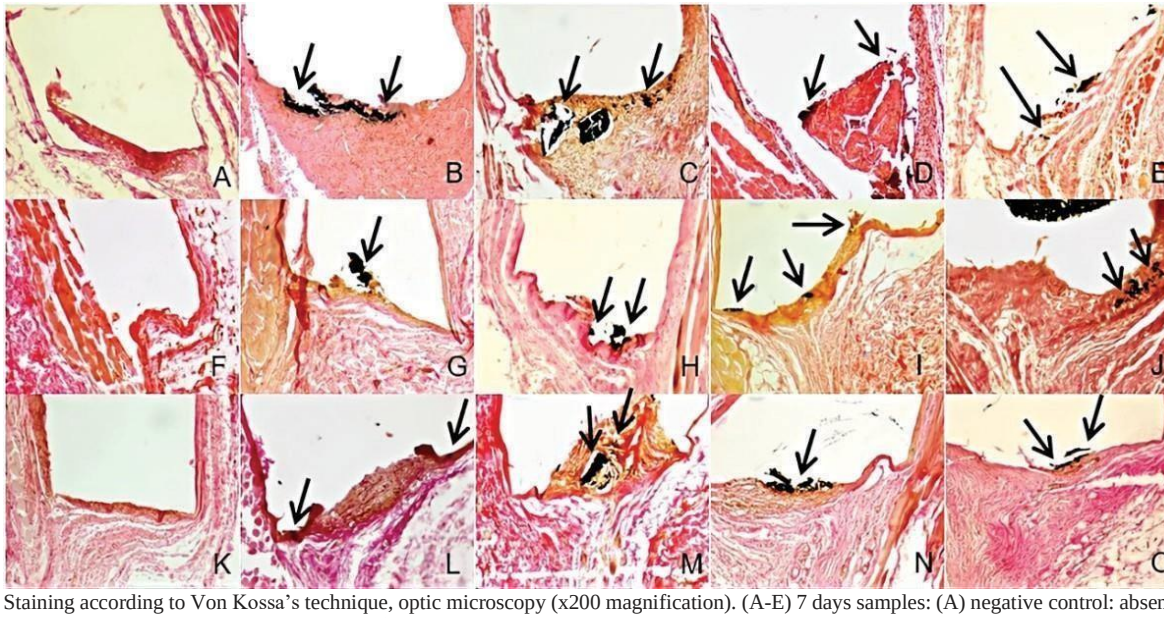
2.3 Statistical analysis

The results were statistically analyzed with Jamovi 1.2.27 software. Kruskal-Wallis test and Steel-Dwass-Critchlow-Fligner test were carried out to compare the data between the groups . The analysis of differences among the three periods for each group, considering the different scores, was performed by nonparametric repeated measures ANOVA (Friedman test). Significance level was defined as $p < 0.05$.

3 Results and Discussion

Biomineralization was evaluated through the Von Kossa method (Figure 2).

Figure 2 - Representative images of biomineralization in experimental groups in all the analyzed periods. The arrows indicate the presence of Von Kossa positive structures



Staining according to Von Kossa's technique, optic microscopy (x200 magnification). (A-E) 7 days samples: (A) negative control: absence of dystrophic calcification; (B) MTA Angelus®, (C) MTA Flow® 0.19 g/50 µl (putty), (D) MTA Flow® 0.26 g/100 µl (thick), I MTA Flow® 0.19 g/100 µl (thin): Von Kossa's positive structures near the tube opening for all the cements with more areas of dystrophic calcification for MTA Angelus®, putty and thin-MTA Flow®. (F-J) 40 days samples: (F) negative control: absence of dystrophic calcification; (G) MTA Angelus®, (H) MTA Flow® 0.19 g/50 µl (putty), (I) MTA Flow® 0.26 g/100 µl (thick), (J) MTA Flow® 0.19 g/100 µl (thin): Von Kossa's positive structures near the tube opening for all the cements with more areas of dystrophic calcification for thick and thin-MTA Flow®. (K-O) 90 days samples: (K) negative control: absence of dystrophic calcification; (L) MTAAngelus®, (M) MTA Flow® 0.19 g/50 µl (putty), (N) MTA Flow® 0.26 g/100 µl (thick), (O) MTA Flow® 0.19 g/100 µl (thin): there was a reduction in areas of dystrophic calcification for MTA Angelus®. Higher level of Von Kossa's positive structures for putty and thick-MTA Flow® were observed.
Source: Resource data.

Concerning MTA Angelus® and the three concentrations of MTAFlow®, biomineralization was observed in all the tested periods. However, differences in dystrophic calcification, positive to Von Kossa's stain were observed as follows:

3.1 Groups

MTA Angelus® group

7 days: 90% of the samples with calcification (20% score 1; 15% score 2; 55% score 3);

40 days: 45% of the samples with calcification (40% score 1; 5% score 2);

90 days: 43.75% of the samples with calcification (18.75% score 1; 25% score 2), with a significant reduction in calcification in relation to 7 and 40 days and 7 and 90 days of exposure, considering the different scores ($p < 0.05$) (Table 1).

Table 1 - Sample percentage of each group categorized according to score (0 to 3) concerning the calcification area in the subcutaneous implants region of 7, 40 and 90 days

Cements	Score (%)				Calcification (%)
	0	1	2	3	
7 days					
Control [a]	100	0	0	0	0
MTA Angelus® 0.28 g/50 µL[a, b, 1]	10	20	15	55	90

Cements	Score (%)				Calcification (%)
	0	1	2	3	
Putty-MTA Flow® 0.19 g/50 µL[a, 2]	33.3	0	0	66.6	66.6
Thick-MTA Flow® 0.26 g/100 µL [b, c ,4]	62.5	12.5	18.75	6.25	37.5
Thin-MTA Flow® 0.19 g/100 µL [a, c, 6]	0	31.25	31.25	37.5	100
40 days					
Control [d]	100	0	0	0	0
MTA Angelus® 0.28 g/50 µL [d, e, 1]	55	40	5	0	45
Putty-MTA Flow® 0.19 g/50 µL [d, 2, 3]	35	45	20	0	65
Thick-MTA Flow® 0.26 g/100 µL [d, 5]	55	25	15	5	45
Thin-MTA Flow® 0.19 g/100 µL [d, e, 6, 7]	0	80	20	0	100
90 days					
Control [f]	100	0	0	0	0
MTA Angelus® 0.28 g/50 µL [g, 1]	56.25	18.75	25	0	43.75
Putty-MTA Flow® 0.19 g/50 µL [f, g, h, 3]	5.5	33.4	33.4	27.7	94.5
Thick-MTA Flow® 0.26 g/100 µL [4, 5]	50	16.66	0	33.34	50
Thin-MTA Flow® 0.19 g/100 µL [f, h, l, 6, 7]	29.4	58.8	11.76	0	70.56

Notes: (0) absence of calcium deposits; (1) ⅓ of tube extension with calcium deposits; (2) ⅔ of tube extension with calcium deposits; (3) more than ⅔ of tube extension with calcium deposits. [(a, b, c, d, e, f, g, h)] Same letters indicate statistical differences among the groups in the same period (Kruskal-Wallis and Steel-Dwass-Critchlow-Fligner), $p < 0.05$. [(1, 2, 3, 4, 5, 6, 7)] Same numbers indicate statistical differences among the periods, nonparametric repeated measures ANOVA (Friedman test), $p < 0.05$.
Source: Resource data.

Putty-MTA Flow® group (0.19 g/50 µL)

7 days: 66.6% of the samples with calcification (66.6% score 3);

40 days: 65% of the samples with calcification (45% score 1; 20% score 2);

90 days: 94.5% of the samples with calcification (33.4% score 1; 33.4% score 2; 27.7% score 3), with a significant increase in dystrophic calcification in relation to 40 and 90 days of exposure, considering the different scores ($p < 0.05$) (Table 1).

Thick-MTA Flow® group (0.26 g/100 µL)

7 days: 37.5% of the samples with calcification (12.5% score 1; 18.75% score 2; 6.25% score 3);

40 days: 45% of the samples with calcification (25% score 1; 15% score 2; 5% score 3);

90 days: 50% of the samples with calcification (16.6% score 1; 33.34% score 3), respectively, with a significant increase in dystrophic calcification in relation to 7 and 90 days of exposure, considering the different scores ($p < 0.05$) (Table 1).

Thin-MTA Flow® group (0.19 g/100 µL)

7 days: 100% of the samples with calcification (31.25% score 1; 31.25% score 2; 37.5% score 3);

40 days: 100% of the samples with calcification (80% score 1; 20% score 2);

90 days: 70.56% of the samples with calcification (58.8% score 1; 11.76% score 2), with a significant reduction in dystrophic calcification in relation to 7 and 90 days and 40 and 90 days of exposure, considering the different scores ($p < 0.05$) (Table 1).

Control

No Von Kossa's positive structure was observed in the tissues around the negative control tubes (empty tube) at any of the tested periods (Table 1), which is similar to the results of the previous reports.[17, 18, 19]

After 7 days of treatment, dystrophic calcification induced by thick-MTA Flow® (0.26 g/100 µL) group was statistically lower than MTA Angelus® and thin-MTA Flow® (0.19 g/100 µL) groups ($p = 0.004$ and $p = 0.006$, respectively). At 40 days, dystrophic calcification also was higher in thin-MTA Flow® (0.19 g/100 µL) group than MTA Angelus® group ($p = 0.001$). There was no statistically significant difference between thin-MTA Flow® (0.19 g/100 µL) group and other MTA Flow® groups in this period. For 90 days samples, dystrophic calcification in putty-MTA Flow® (0.19 g/50 µL) group was statistically higher than MTA Angelus® and thin-MTA Flow® (0.19 g/100 µL) groups ($p = 0.015$ and $p = 0.013$, respectively) (Table 1).

Biomineralization activity stimulated by MTA is related to the release of calcium ions. MTA has in its composition calcium oxide and calcium phosphate. Calcium oxide, in contact with tissue fluids, generates calcium hydroxide, which dissociates into calcium ions (Ca^{++}) and hydroxyl ions (OH^-). The interaction between the ions (Ca^{++}) and the carbon dioxide present in the tissues creates granules of calcium carbonate ($CaCO_3^{--}$), which are responsible for the formation of mineralized areas[16,20,21].

Moreover, a study conducted in 2004 showed that MTA can intensely trigger the activity of alkaline phosphatase enzyme in fibroblasts, which may have assisted in the mineralization process induced by the cements evaluated.[22]

In this study, significant differences were observed between the cements biomineralization capacity in relation to the control in all the tested exposition times: MTA Angelus®, putty- MTA Flow® and thin- MTA Flow® at 7 days of treatment; all cements after 40 days of treatment and putty and thin-MTA Flow® after 90 days of exposure.

In 1997 another research proved that increased biomineralization activity is related to calcium ions concentration (Ca^{++}) available and that the constant formation of mineral precipitation depends on the continued calcium liberation[23].

Previous studies have also shown that physical-chemistry features may affect MTA based sealers biomineralization capacity[17,24]. Some of these features are the particles size, the ability of calcium ions release and the surface´s porosity after the cement mixture. A study compared MTA Angelus® surface features, which has distilled water in its composition, with MTA Plus®, that can be handled with a gel provided by the manufacturer.[25] That study proved that the gel used in MTA Plus® sealer manipulation, promotes better surface material features, reducing porosity, increasing its resistance to washout and reducing the interaction between the sealer and tissue fluids. These data corroborate the results observed in this research, which demonstrate that in longer periods, MTA Flow®, which is diluted in gel, had superior biomineralization performance than that of MTA Angelus®, which is diluted in distilled water, especially in the highest concentration (putty-MTA Flow® [0.19 g/50 µL]).

The compounds added to the gel formulation used in sealers manipulation increase the viscosity of the water that will be mixed to the sealer powder. This fact promotes higher resistance to degradation of cement surface due to the action of external fluids[25]. For MTA Flow®, the variation of powder-to-gel proportions may cause changes in superficial porosity, which changes the interaction between the sealer and the tissue fluids. Thus, the discrepancies in biomineralization capacity observed in different concentrations of MTA Flow® in this study may be related to changes in the sealer surface porosity after handling. These changes in sealer concentration lead to a different tissue response according to the availability and intensity of ions released in tissues.

Comparing the three concentrations of MTA Flow®, data show that putty-MTA Flow® (0.19 g/50 µL) promoted a higher calcium deposits induction than thin-MTA Flow® (0.19 g/100 µL) over 90 days. In the longest exposure time, 27.7% of putty-MTA Flow® samples were in score 3 while none was observed for thin-MTA Flow®. This fact may be related to an increase in the sealer density, which allows gradual and sustained calcium ions liberation, due to the lower interaction between tissue fluids and sealer surface.

Previous studies demonstrate that MTA Angelus®, after handled, shows higher superficial porosity, which provides an increased interaction between sealers and tissue fluids, ensuring an initial spike of calcium ions on the sealer/ tissues surface[18,19]. Therefore, as soon as the contact between the sealer and the tissues starts, there is an intense liberation of calcium ions and local alkalization due to hydroxyl ions presence (OH^-). However, overtime, there is a decrease in the concentration of these ions.[5] All these features may be related to the biomineralization levels reduction observed for MTA Angelus® after 40 days in this study.

Furthermore, the higher levels of biomineralization observed at 7 days for MTA Angelus® are related to the initial spike in ions release, promoted by the interaction between the sealers surface and the tissue fluids.

This study showed that putty-MTA Flow® induced higher biomineralization than MTA Angelus® at 40 and 90 days and thin-MTA Flow® induced higher biomineralization than MTA Angelus® at 40 days. Then, the differences observed between the sealers in this research may be related to higher or lower capacity of calcium oxide dissociation, in calcium ions. These data are in line with previous studies,[15, 21] which indicates that MTA sealers can induce biomineralization.

4 Conclusion

In conclusion, results show that MTA Flow® induces calcium deposits formation in rat subcutaneous tissue, even in different concentrations. Thin-MTA Flow® showed higher calcification than thick-MTA Flow® at 7 days while putty-MTA Flow® showed higher calcification than thin-MTA Flow® at 90 days. MTA Angelus® and thin-MTA Flow® showed significant reduction in calcification as time went by. Instead, a significant increase in dystrophic calcification was observed in putty-MTA Flow® in the longest exposition time. Moreover, data suggest that, in longer periods, MTA Flow® performance is superior to that of MTA Angelus®, especially in the highest concentration (putty-MTA Flow®). Thus, MTA Flow® may be considered an option in terms of calcium silicate material due to its bioactivity and it seems to be a viable alternative to conventional MTA sealers. However, there are just a few studies involving biomineralization provided by this endodontic sealer, and then, additional research is required to confirm the present findings.

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