

Preliminary Evaluation of BMP-2 Expression and Histological Characteristics During Apexification with Calcium Hydroxide and Mineral Trioxide Aggregate

Karla A. Ham, DDS, David E. Witherspoon, BDS, MS, James L. Gutmann, DDS, S. Ravindranath, BDS, MDS, Tob Chooi Gait, BDS Hons, MSc, FDSRCPS, DRDRCS, FDSRCS, Lynne A. Opperman, PhD

Abstract

Histological characteristics and bone morphogenetic protein-2 (BMP-2) expression were evaluated in apexification procedures using calcium hydroxide [Ca (OH)₂] and mineral trioxide aggregate (MTA). In three of four quadrants of the mouth, monkey (*Macaca fascicularis*) teeth were infected by pulpal exposure to saliva. After 90 days, each group was treated with Ca (OH)₂ or MTA. Infected, untreated teeth were used as negative controls and normally developing teeth were used as positive controls. Areas within the apical region of each sample were scored based upon histological findings and BMP-2 expression. Overall, BMP-2 expression in all areas was similar in the Ca (OH)₂, MTA, and infection/no treatment groups, which tended to be more immunoreactive for BMP-2 than the untreated group. Use of MTA in combination with Ca (OH)₂ may initiate regeneration of the periodontium more quickly than either material used alone, but further studies involving MTA use in apical closure are indicated.

From the Department of Restorative Sciences, and Department of Biomedical Sciences, Baylor College of Dentistry, Texas A&M University System Health Science Center, Dallas, Texas; and the Department of Conservative Dentistry, Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia.

Address request for reprints to Lynne A. Opperman, Ph.D., Texas A&M University System Health Science Center, Baylor College of Dentistry, Department of Biomedical Sciences, P.O. Box 660677, Dallas, TX 75266-0677; E-mail address: lopperman@bcd.tamhsc.edu.

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Dental caries and trauma are the most common challenges to the integrity of a tooth as it matures. Both insults can render the dental pulp nonvital. If the dental pulp is damaged before development of the root length and closure of the apical foramen, normal root development is altered or halted completely. When abnormal development of the root occurs, apexification treatment must be initiated.

Successful apexification depends on the formation of a hard tissue barrier by cells that migrate from the healing periradicular tissues to the apex and differentiate under the influence of specific cellular signals to become cells capable of secreting a cementum, osteocementum or osteodentin organic matrix (1).

During tooth formation and apexogenesis, growth factors, cytokines, transcription factors and morphogenetic target genes establish the distribution, shape and form of cells and tissues via specific signaling pathways and transduction mechanisms (2). Interactions between epithelial and mesenchymal cells regulate both the morphogenesis of the tooth and the differentiation of the dental cells. It is this epithelial-mesenchymal interaction that mediates the terminal differentiation of odontoblasts. Certain growth factors have been identified as epithelial inductive signals and therefore can regulate the differentiation of dental cells in both periodontium morphogenesis and repair (2, 3).

The molecular basis for regeneration of the periodontium is related to the activities of the bone morphogenetic proteins (BMPs), which are members of the transforming growth factor β (TGF- β) superfamily (1). Unlike the TGF- β s, BMP will induce the production of bone when injected into ectopic sites (4). BMPs' multiple effects on inducible cell populations include, (a) acting as mitogens on undifferentiated mesenchymal cells and osteoblast precursors; (b) inducing the expression of the osteoblast phenotype; and (c) acting as chemoattractants for mesenchymal cells and monocytes as well as binding to extra cellular matrix type IV collagen (5).

Several BMPs affect various hard tissues. Dentin formation has been induced in the pulp of dog teeth with BMP-2 and BMP-4 (6). Likewise, BMP-7 has been shown to stimulate dentin repair in monkey teeth, while BMP-2 has been shown to enhance the regeneration of bone, cementum, and periodontal tissue in dogs (7). The fact that BMPs induce cementogenesis and periodontal ligament formation indicates that these proteins may have multiple functions in vivo not limited to cartilage and bone induction (1).

For more than 40 yr, the placement of calcium hydroxide [Ca (OH)₂] within the root canal space has been the standard treatment to stimulate apical closure in a developing tooth with a necrotic pulp (8). Ca (OH)₂ demonstrates antibacterial properties (9), enhances tissue dissolution, and induces bone formation (10). The mechanisms by which Ca (OH)₂ promotes these responses are vague. Studies suggest that a rise in pH induced by Ca (OH)₂ combined with the availability of Ca²⁺ and OH⁻ ions have a stimulating effect on enzymatic pathways and therefore mineralization (11). Also unclear is the interactive role Ca (OH)₂ has with cytokines and growth factors in the stimulation of an osseous response.

A promising but less characterized material that may be used to induce apexification is mineral trioxide aggregate (MTA). MTA is composed of tricalcium silicate, tricalcium aluminate, tricalcium oxide and silicate oxide. Hydration of the powder results in a colloidal gel that solidifies to a hard structure in less than 3 h (12). MTA is

a promising material because of its sealing property, its ability to set up in the presence of blood, its bactericidal effects, and its biocompatibility (13, 14).

Shabahang et al. (1999) examined hard tissue formation and inflammation histomorphologically after treating open apices in dogs with osteogenic protein-1, MTA, and Ca (OH)₂. MTA induced hard tissue formation with the greatest consistency, but the amount of hard tissue formation and inflammation was not statistically different among the three materials (15). Although both Ca (OH)₂ and MTA appear to have the ability to stimulate a hard tissue response, the immunohistochemical characterization of the cellular response and the nature of the hard tissue that is formed have not been determined, nor has the interplay of specific cytokines and growth factors involved in this process.

The purpose of this study was to examine and characterize histologically the expression of BMP-2 in the periradicular tissue after treatment of immature teeth with pulpal necrosis treated with Ca (OH)₂ or MTA. Immature teeth with pulpal necrosis receiving no treatment served as negative controls and immature teeth undergoing normal development served as positive controls.

Materials and Methods

Five *Macaca fascicularis* monkeys, of varying ages under five, were obtained and quarantined for 14 days to insure optimal health. The animals were housed and cared for by The Animal Center of the University of Malaysia, Kuala Lumpur, Malaysia. Within each animal, the central incisor, lateral incisor, first premolar, second premolar, and first molar from each quadrant was used. Each tooth was radiographed using a radiographic jig to confirm the stage of root development. The quadrants were randomly allocated in each animal as follows:

1. Upper right quadrant: noninfected normally developing teeth (positive control).
2. Upper left quadrant: teeth infected and treated with MTA (Pro-Root, Dentsply, Tulsa Dental, OK).
3. Lower left quadrant: teeth infected and treated with Ca (OH)₂ placed within the length of the canal and the access opening sealed with glass ionomer.
4. Lower right quadrant: teeth infected only and the access opening sealed with a cotton pellet and glass ionomer (negative control). A cotton pellet was placed to separate the glass ionomer from the pulp.

Treatments were not randomly allocated or mixed, to prevent any localized effect of infection from control untreated teeth, or from any localized tissue reaction to filling materials in adjacent teeth. All endodontic procedures were performed under general anesthesia using 3 ml of Zoletil 50 (tiletamine hydrochloride plus zolazepam hydrochloride, Virbac Laboratories, France) intramuscularly and 1 ml added each 30 min or as needed.

Infection Phase

The pulp of each tooth was exposed with a high-speed bur. A #10 K file bathed in the animal's saliva and plaque into the canal of each tooth to induce infection. The access openings were then sealed with cotton and glass ionomer.

Treatment Phase

Thirty days after the infection phase, the animals were again sedated. Intraoral anesthesia was achieved by 2% lidocaine with 1:100,000 epinephrine (Astra Pharmaceutical Products, Westborough, MA) was injected into the upper left and lower left quadrants. Each quadrant was isolated with a rubber dam during the procedure. The

access seal was removed from the upper left and lower left quadrants. Working lengths of each canal were determined by placement of a premeasured K-file and a radiograph was exposed. The canals were cleaned and shaped to the working length using .02 and .04 Profile Series 29 (Dentsply, Tulsa Dental) and GT NiTi rotary instruments (Dentsply Tulsa Dental) at 300 RPM. Hand K-files sizes 8–25 and Hedström files (size 25) were also used. Copious 5.25% sodium hypochlorite (NaOCl) (The Clorox Company, Oakland, CA) and RC Prep (Premier, Norristown, PA) were used during instrumentation. Once instrumentation was complete, the canals were irrigated with 10cc of 5.25% NaOCl for a period of 1 min, followed by 10 ml of 17% ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, MO) for 2 min and finally 10 ml of 5.25% NaOCl for 1 min. The canals were dried with 90% alcohol and paper points and treated as follows:

1. Untreated group.
2. Teeth infected and treated with MTA: MTA was mixed with sterile saline according to manufacturer's instructions. MTA was placed the length of the canal using a carrier, pluggers and an ultrasonic until a level 1-mm below the cemento-enamel junction was reached. Placement was confirmed by radiograph. Glass ionomer (Ketac, ESPE, Norristown, PA) was placed directly onto the MTA to seal the access opening.
3. Teeth infected and treated with Ca (OH)₂: Ultracal Ca (OH)₂ (South Jordan, UT) was injected into each canal and pressure applied apically with a cotton pellet. The canal was obturated with Ca (OH)₂ to the level of 1 mm below the level of the cemento-enamel junction.
4. Teeth infected only.

Tissue Removal and Processing

The animals were sacrificed at 90 days post-treatment under general anesthesia achieved with intravenous administration of sodium pentobarbital 33.0 mg/kg. Following overdose and lack of palpable pulse, the carotid artery was perfused with 1.0 to 1.5 liters of 10% phosphate buffered formalin. Block sections of the mandible and maxilla were obtained using a Stryker autopsy saw. All sections were fixed in 10% buffered formalin. Specimens were sectioned into smaller segments containing one tooth and then decoronated and placed in 0.5 M EDTA at 4°C for demineralization.

The block specimens were dehydrated in alcohol and infused with Paraplast Plus Paraffin (Monojet, St. Louis, MO), and serially sectioned at 5.0 to 7.0 μ m. Selected sections in which the apex was visible were used as follows:

1. Three sections for Hematoxylin and Eosin (H&E) procedures in which the one exhibiting the best view of the root end was chosen, and
2. Fifteen sections for monoclonal antibodies to BMP-2, from which one exhibiting the best view of the root end was chosen for descriptive purposes.

Immunohistochemical Staining

Before staining, the sections were deparaffinized in xylene and rehydrated in descending concentrations of alcohol, phosphate-buffered saline (PBS) and methanol with 3% hydrogen peroxide (H₂O₂) in a 4:1 ratio to remove endogenous peroxidase activity. Tissues were incubated for 18 h at 4°C with primary antibody against BMP-2 (Austral Biologicals, San Ramon, CA) diluted 1:100 in PBS and 2% rabbit serum, following a 20 min incubation with a blocking serum (2% rabbit serum in PBS). Thereafter, slides were incubated with the secondary biotinylated antibody for 2.5 h at room temperature, then rinsed and incubated in streptavidin/peroxidase complex working solution. Sections were

TABLE 1. H&E staining for inflammation, internal and external root resorption, and hard tissue formation (mean \pm standard deviation)

Treatment <i>n</i> = 20	Inflammation	Internal root resorption	External root resorption	Hard tissue formation
Ca (OH) ₂	1.50 \pm 1.40	0.00	0.37 \pm 1.39	1.00 \pm .076
MTA	0.61 \pm 0.99	0.04 \pm 0.21	0.13 \pm 0.34	2.80 \pm 1.40
Infection, no treatment	0.94 \pm 1.20	0.00	0.25 \pm 0.58	1.50 \pm 1.40
Untreated	0.10 \pm 0.43	0.00	0.00	0.80 \pm 1.30

n = Number of available samples in each treatment group.

again rinsed and incubated in peroxidase substrate solution (DAB Substrate kit for Peroxidase, Vector Laboratories, Inc., Burlingame, CA) for 7 min. All rinses performed were done for 5 min. Finally, sections were counterstained with hematoxylin, dehydrated, cleared in xylene and mounted using the liquid cover slip mounting medium Acrymount (Statlab Medical Products, Lewisville, TX).

Evaluation of Tissue Sections

All immunohistochemical slides were scored at 40 \times magnification. Three calibrated and trained examiners recorded degree of stain independently. H&E staining was analyzed at 10 \times magnification. Several categories were examined, including inflammation, internal and external root resorption, and hard tissue formation. The data were recorded and entered into a spreadsheet program for analysis (Microsoft Excel). Evaluation of immunoreactivity was scored in cells of the papilla, cementoblasts at the apex, apical hard tissue, cells of the internal dentinal wall of the apex, odontoblasts, and Hertwig's Epithelial Sheath (HERS) as follows:

- 0 = no stain (all blue)
- 1 = light stain
- 2 = moderate stain
- 3 = dark stain

Evaluation of H&E staining was as follows:

Inflammation:

- 0 = none
- 1 = mild
- 2 = moderate
- 3 = severe
- 4 = periradicular abscess

Root Resorption:

- 0 = none
- 1 = mild
- 2 = moderate
- 3 = severe

Hard Tissue Formed (percentage of hard tissue formed):

- 0 = 0%
- 1 = 1–25%
- 2 = 26–50%
- 3 = 51–75%
- 4 = 76–100%

Results

Because of the limited number of samples in which the entire apical portion was present, no statistics were performed on the data other than means and standard deviations. Direct comparisons were also difficult because the exact ages of the animals were unknown. Therefore, all data presented are for descriptive and qualitative analysis.

Initial exposure of the pulp resulted in a sustained root canal infection in all teeth, before root canal treatment. After cleaning and shaping procedures and before sealing, all canals were similarly cleaned using procedures comparable to those used on human patients, and all canals were considered free of bacterial contaminants. At sacrifice, sealants were examined and noted to be intact in all teeth where they were applied.

Histological Evaluation

Inflammation was noted more often in the Ca (OH)₂ group, than in the infection/no treatment group and both of these groups had more inflammation than the MTA or normal development groups (Table 1). Samples with internal root resorption were only found in the MTA group. Samples with external root resorption were found in all but the normal development group, with the Ca (OH)₂ groups having slightly more recorded external root resorption than the infection/no treatment group. Hard tissue formation was highest in the MTA group, while the infection/no treatment group actually showed slightly more hard tissue development than the Ca (OH)₂ group.

Immunohistochemistry

Table 2 contains results of immunoreactivity of some cells involved in hard tissue formation of the tooth. Within the cementoblasts and cells of the papilla and internal dentinal wall, BMP-2 immunoreactivity increased in the presence of infection, then decreased within the treatment groups. The decrease of BMP-2 expression approaches the same level of BMP-2 immunoreactivity seen in the untreated group that represents normal development. Immunoreactivity for apical hard tissue was similar in all four groups. The odontoblasts in the MTA group expressed the least BMP-2, followed by the infection group. The Ca (OH)₂ and untreated groups showed slightly more BMP-2 immunoreactivity within the odontoblastic layer when compared to the other groups.

BMP-2 expression within HERS is detailed in Table 3. The sheath tended to be very lightly immunoreactive for BMP-2, but these numbers are not significant because of the low number of samples in which the sheath was identifiable. The external and internal layers of HERS were most immunoreactive for BMP-2 in the Ca (OH)₂ group, followed by the untreated group. The MTA and infection/no treatment groups express similar immunoreactivity results.

Discussion

Root dentin is formed by odontoblasts. Odontoblasts differentiate as the epithelial cells of the external and internal dental epithelium proliferate from the cervical loop of the dental organ, forming a double layer of cells known as HERS. Once the root sheath forms, the sheath initiates rapid root formation and then becomes fragmented (16). HERS induces the differentiation of odontoblasts, and when dentin is deposited, the sheath disintegrates and brings connective tissue into contact

TABLE 2. BMP-2 immunoreactivity for cells at the papilla, cementoblasts, apical hard tissue, cells of the internal dentinal wall of the apex, and odontoblastic layer

Treatment	Cells at papilla	Cementoblasts at apex	Apical hard tissue	Internal dentinal wall of apex	Odontoblasts
Ca (OH) ₂	2.3 ± 1.3 <i>n</i> = 8	2.3 ± 0.6 <i>n</i> = 25	2.3 ± 1.0 <i>n</i> = 24	2.1 ± 1.3 <i>n</i> = 10	2.2 ± 0.8 <i>n</i> = 8
MTA	2.3 ± 1.1 <i>n</i> = 6	2.2 ± 0.6 <i>n</i> = 27	2.3 ± 0.7 <i>n</i> = 25	2.3 ± 1 <i>n</i> = 8	1.9 ± 1.0 <i>n</i> = 8
Infection, no treatment	3.0 ± 0.0 <i>n</i> = 4	2.6 ± 0.4 <i>n</i> = 20	2.2 ± 0.6 <i>n</i> = 19	2.8 ± 0.4 <i>n</i> = 6	2.0 ± 0.4 <i>n</i> = 3
Untreated	1.7 ± 1.2 <i>n</i> = 7	1.7 ± 0.7 <i>n</i> = 27	2.1 ± 1.0 <i>n</i> = 26	1.6 ± 0.9 <i>n</i> = 9	2.4 ± 0.3 <i>n</i> = 25

n = Number of available samples.

TABLE 3. BMP-2 immunoreactivity for HERS layers

Treatment	HERS-external	HERS-internal
Ca (OH) ₂ <i>n</i> = 4	1.4 ± 1.3	2.0 ± 1.7
MTA <i>n</i> = 6	0.8 ± 1.5	0.5 ± 1.2
Infection-no treatment <i>n</i> = 2	0.8 ± 1.1	0.3 ± 0.4
Untreated <i>n</i> = 3	1.0 ± 1.3	0.8 ± 1.3

n = Number of available samples.

with the dentin. The root sheath is also involved in cementum formation. After the sheath fragments, the dental follicular cells of the area are influenced by the adjacent, newly formed root surface. These cells differentiate into cementoblasts and deposit an organic matrix against the root surface, around the forming ligament fiber bundles. Therefore, the root consists of dentin and cementum (16). Hertwig's sheath is a very important and resistant tissue in the development of an apical barrier. The sheath may remain vital even after complete pulpal necrosis and periradicular tissue changes and it can initiate root growth even after treatment (17).

When hard tissue-producing cells express BMP-2, then hard tissues like bone, cementum, and cartilage are likely being enhanced or regenerated. BMP-2 is a growth factor that has been shown clinically to induce hard tissue formation and to serve as scaffolding for bone regeneration (4). Therefore, the role BMP-2 may play in apexification warrants study.

More inflammation was found in the group treated with Ca (OH)₂ than in the group that was infected and not treated (Table 1). Ca (OH)₂ is known to induce an inflammatory response before promoting healing, which may explain the observations in this study (18). The MTA group showed less inflammation than Ca (OH)₂ and infection groups, and not unexpectedly, more than the untreated group (Fig. 1). Because the standard deviations are high compared to the means, no statistical analysis was performed. However, these trends are consistent, so it is likely that with larger sample sizes, significance could be achieved.

The same disclaimer must be stated regarding incidence of root resorption. The standard deviation on root resorption in all groups was very high compared to the mean, so there was great variability in reported tissue resorption. Internal root resorption in the MTA group was the only exception since that was the only group to show any evidence of internal root resorption. Still, the amount noted was almost negligible, and the number of samples in which resorption was noted was very small.

Examination of hard tissue formation showed that MTA had more hard tissue formed than the other groups, and the infection/no treat-

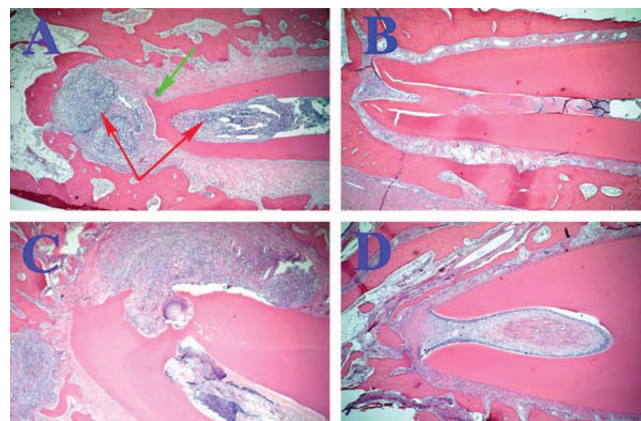


Figure 1. H&E stained samples. (A) Ca (OH)₂ treatment at 2× magnification. Note hard tissue formation (single arrow) despite presence of inflammation (double arrow). (B) MTA treatment at 2× magnification. Note hard tissue in-growth within canal space. (C) Infection, no treatment at 2× magnification. Note high degree of inflammation. (D) Normal development at 2× magnification.

ment group exhibited more hard tissue formation than the Ca (OH)₂ group. These results differ from those of Shabahang and co-workers (1999) who found a difference in the consistency of the hard tissue formed comparing MTA and Ca (OH)₂, but no difference in the amount of hard tissue formed by the two materials (15).

Table 2 shows the rise and fall of BMP-2 levels in the presence of infection when followed by treatment. In both treatment groups, the level approached that of the normally developing group. The BMP-2 levels were highest in the presence of inflammation, implying that endogenous BMP-2 was upregulated as a response to inflammation. Furthermore, BMP-2 levels were reduced in groups treated with either MTA or Ca (OH)₂ (Fig. 2). This result implies that caution should be taken when interpreting data in which growth factors associated with wound healing are elevated.

Immunoreactivity for apical hard tissue was similar in all groups. This was not an indicator of the amount of hard tissue present, only an indicator of the amount of BMP-2 that was bound to the hard tissue. Therefore, the implication was that apical hard tissue is equally immunoreactive for BMP-2 regardless of how the hard tissue was induced or formed.

Odontoblastic expression of BMP-2 was the least in the MTA group, followed by infection/no treatment, Ca (OH)₂, and the untreated groups. In contrast to the findings for the cells of the papilla, cementoblasts, and the internal dentinal wall, the three groups that were infected expressed less BMP-2 within the odontoblastic layer than the untreated group that contained a healthy pulp. This may have been because sam-

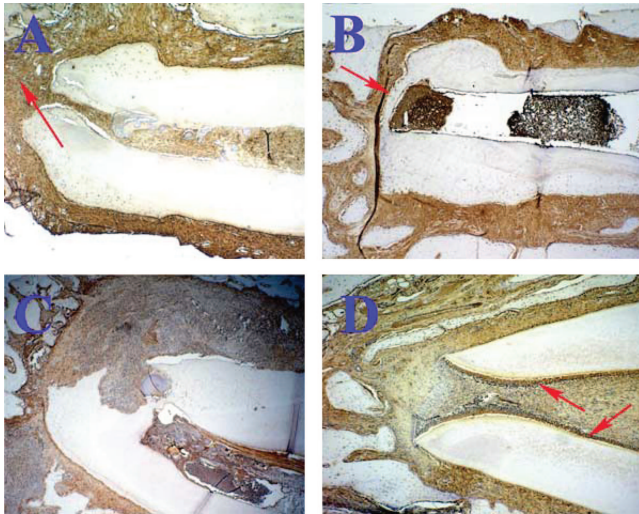


Figure 2. BMP-2 immunoreactivity. (A) $\text{Ca}(\text{OH})_2$ treatment at $4\times$ magnification. Note high degree of inflammation. (B) MTA treatment at $4\times$ magnification. Note hard tissue formation. (C) Infection, no treatment at $4\times$ magnification. Note external root resorption. (D) Normal development at $4\times$ magnification. Note normal odontoblastic cells.

ples that were infected should not have odontoblasts remaining, so if some of these cells survived, then they were not in the process of regenerating and thus did not express BMP-2.

Table 3 describes BMP-2 immunoreactivity within HERS. HERS consists of a double layer of cells. The internal layer was exposed to the pulp while the external layer was exposed to the papilla. The external and internal layers of HERS were most immunoreactive for BMP-2 in the $\text{Ca}(\text{OH})_2$ and untreated groups. In very few cases was the sheath wholly identifiable. Considering the role of HERS, any sample that contained an active HERS expressing BMP-2 was probably undergoing continued root formation. Each treatment group had a high standard deviation when compared to the mean so that definitive interpretation was difficult.

Conclusion

Overall, BMP-2 expression in all areas examined was similar in the $\text{Ca}(\text{OH})_2$, MTA, and infection/no treatment groups, and the exact role that it plays apical barrier formation is not clear. This study indicated that it may be helpful in inducing in immature teeth, and further investigation is war-

ranted. However, caution should be taken when interpreting data in teeth where inflammation was present, as inflammation appeared to be associated with increased BMP-2 levels. MTA used in combination with $\text{Ca}(\text{OH})_2$ may stimulate the regeneration of the periodontium more quickly than these materials used alone, but further studies involving MTA use in apical closure in the absence of induced infection are indicated.

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