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An *in vitro* evaluation of the antibacterial properties of three mineral trioxide aggregate (MTA) against five oral bacteria

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ABSTRACT

Objective: The purpose of this study was to evaluate the antibacterial ability of three MTA (MTA-Angelus, Endocem MTA, and ProRoot MTA) against five typical oral bacteria (*Streptococcus mutans*, *Enterococcus faecalis*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei*, and *Porphyromonas gingivalis*).
Design: For disc diffusion test, each test material was placed into agar plates after inoculation of each bacterial strain. The zones of inhibition of bacterial growth were then measured. Antibacterial broth test was performed by adding the test material into the media. Colony-forming units were counted after incubation with bacteria. The data were analyzed using ANOVA and the Tukey's test.
Results: Disc diffusion test showed that the antibacterial activity against *S. mutans*, *L. rhamnosus*, *L. paracasei*, and *P. gingivalis* ranked in decreasing order of MTA-Angelus > ProRoot MTA > Endocem MTA ($p < 0.05$). An inhibitory effect against *E. faecalis* was only observed in Endocem MTA. Antibacterial broth test showed that the antibacterial activity against all bacteria was Endocem MTA > MTA-Angelus > ProRoot MTA ($p < 0.05$).
Conclusion: Discrepant results were obtained from the disc diffusion and antibacterial broth test, with MTA-Angelus and Endocem MTA being most effective, respectively. Both tests revealed that the most resistant bacteria was *E. faecalis*, which was not susceptible at all, except to Endocem MTA in disc diffusion test.

1. Introduction

Mineral trioxide aggregate (MTA) has become the material of choice in various endodontic procedures such as pulp capping, pulpotomy, apexogenesis/apexification, repair of root resorption and lateral or furcal perforations, and retrograde filling, because of its superior properties including sealability (Torabinejad, Rastegar, Kettering, & Pitt Ford, 1995), biocompatibility (Torabinejad, Hong, Pitt Ford, & Kettering, 1995a), and bioactivity (Enkel et al., 2008). However, the main drawbacks of MTA are difficulty in handling, long setting time, and discoloration potential (Parirokh & Torabinejad, 2010). The main ingredients are tricalcium silicate, dicalcium silicate, and bismuth oxide, with small quantities of iron and aluminum (Ferris & Baumgartner, 2004). Since the introduction of ProRoot MTA (Dentsply, Tulsa, OK, USA) in 1998, novel commercially available MTA-based products including MTA-Angelus (Angelus, Londrina, PR, Brazil) and Endocem MTA (Maruchi, Wonju, Korea) have been developed in an attempt to improve these shortcomings by modifying the composition and/or concentration of each ingredient. Endocem is an MTA-derived pozzolan cement with a lesser setting time (4 min ± 30 s) and similar biocompatibility and osteogenicity compared to conventional MTA (Choi et al., 2013). As the characteristics of a material may change along with composition modification, numerous studies have evaluated the biological and physical properties of these MTA products.

Considering the indispensable role of microorganisms in the development and progress of pulpal and periapical disease as well as the failure of endodontic treatment, the eradication of microorganisms from the root canal system in endodontic treatment and prevention of bacterial ingress to the root canal system during restorative treatment are the key factors in successful clinical outcome (Baumgartner & Falkler, 1991; Fabricius, Dahlen, Ohman,

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& Moller, 1982; Fouad, Zerella, Barry, & Spangberg, 2005; Kakehashi, Stanley, & Fitzgerald, 1965; Moller, Fabricius, Dahlen, Ohman, & Heyden, 1981; Siqueira, Rocas, Souto, de Uzeda, & Colombo, 2000; Sundqvist, 1992). Therefore, an ideal dental material should also possess antibacterial property, but not at the expense of its other biological and physical properties.

There is to date limited information, however, on the comparative antibacterial activity of MTA-based products against some of the predominant clinically relevant bacteria including *Enterococcus faecalis* in endodontic disease, *Porphyromonas gingivalis* in periodontal disease, *Streptococcus mutans* in the initiation of dental caries, *Lactobacillus rhamnosus*, and *Lactobacillus paracasei* in the progression of dental caries.

This study was conducted to compare the antibacterial effects of three MTA products (MTA-Angelus, Endocem MTA, and ProRoot MTA) against these five typical oral bacteria, and thereby to develop a clinical recommendation for their specific use based on their antibacterial activity.

2. Materials and methods

2.1. Compositions of tested cements

The chemical composition of the tested materials (MTA-Angelus, Endocem MTA, and ProRoot MTA) were analyzed by X-ray fluorescence spectrometer (ZSX100e, Rigaku, Akishima, Japan). The powders of each material were pressed into rigid pellets, which were then evaluated twice qualitatively and quantitatively using the fundamental parameter method.

2.2. Disc diffusion test

The antibacterial activity was evaluated using five standard bacterial strains: *S. mutans* (ATCC 25175), *E. faecalis* (ATCC 4082), and *P. gingivalis* (ATCC 33277) obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), and *L. rhamnosus* (KCTC 3237) and *L. paracasei* (KCTC 3165) obtained from Korean Collection for Type Cultures (KCTC, Daejeon, Korea).

S. mutans and *E. faecalis* were cultivated in brain-heart infusion (BHI) (Difco, Detroit, MI, USA) broth, and *L. rhamnosus and L. paracasei* were inoculated in de Man, Rogosa and Sharpe (MRS) (Becton, Dickinson and Co., Flanklin Lakes, NJ, USA) broth aerobically at 37 °C for 1 day. *P. gingivalis* was grown in BHI broth supplemented with Hemin and Vitamin K at 37 °C and incubated anaerobically for 3 days to a concentration of 0.5 McFarland turbidity standard, which corresponds to approximately 3×10^8 colony-forming units (CFU)/mL. A sterile cotton-tipped swab was used to inoculate the bacterial suspension onto the agar plate to achieve a lawn of growth. MRS (Becton) agar was used for the cultivation of *L. rhamnosus and L. paracasei*. For *P. gingivalis*, Brucella Blood Agar plate (Hanil KOMED, Sungnam, Korea) was used. As for *E. faecalis* and *S. mutans*, Muller-Hinton Agar (Hanil KOMED) and Tryptic Soy Agar (Becton) were used, respectively, for cultivation. Thereafter, 4 equidistant wells with a diameter of 3.5 mm and a depth of 4 mm were made in each plate (total 32 wells in 8 plates for each bacterial strain) by removing the agar with a sterile hand tissue punch (Osung, Kimpo, Korea).

The tested MTA were mixed with a sterile spatula on a sterile glass slab according to the manufacturer's instruction. The mixed MTA were placed into the wells on the agar plate using a sterile MTA carrier (Dentsply-Tulsa Dental, Johnson City, TN, USA) and gently pressed into place. All plates were maintained at room temperature for 2 h to allow prediffusion of the test materials and then incubated at 37 °C for 3 days, except the *P. gingivalis* plates, which were incubated for 7 days anaerobically. After incubation, the diameter of the zone of inhibition was measured with a 0.5 mm precision ruler to the nearest millimeter ($n = 32$).

2.3. Antibacterial broth test

Antibacterial activity of the tested MTAs was carried out according to the methods of Clinical Laboratory Standard Institute (CLSI). Each MTA was dissolved in the selective media, as described in disc diffusion test, to a concentration of 10 mg/mL. 180 μL of the specific media for bacteria dispensed in each well of 96-well plate (SPL Life Sciences, Pocheon, Korea). 180 μL of the MTA-dissolved medium was added to the first row of 96-well plate and performed serial 2-fold dilution using a multi-channel micropipette. The bacterial suspensions (20 μL; 1.5×10^5 cells of *P. gingivalis*, 1.0×10^5 cells of the others) were inoculated in each well ($n = 6$). The plate was incubated at 37 °C in an anaerobic condition for *P. gingivalis* for 2 days and in an aerobic condition for *E. faecalis, S. mutans, L. paracasei* and *L. rhamnosus* for 1 day. Broth without MTA materials and Ampicillin (1 μg/mL) were served as controls for comparison. The cultured bacteria in the plate were suspended using up and down with multi-channel micropipette to homogenize them, and the plate was then centrifuged at 500 × g for 5 min to sink MTA particles. The bacteria level in each wells was counted by Petroff-Hausser bacteria counter (Hausser Scientific, Horsham, PA, USA).

2.4. Statistical analysis

The data were analyzed with one-way analysis of variance and the Tukey's honest significant difference post hoc test for multiple comparisons between the antibacterial effects of the three MTA materials against each bacteria tested. The level of significance was established at 5%. Statistical analysis was performed with SPSS software (IBM, Armonk, NY, USA).

3. Results

3.1. Compositions of the cements

The chemical compositions of the cements by X-ray fluorescence spectrometer are shown in Table 1. Main chemical compounds were calcium oxide (lime; CaO), silicon dioxide (silica; SiO_2), and bismuth oxide (Bi_2O_3), accounting for approximately 95% of the total mass of MTA-Angelus and ProRoot MTA, and 83% of Endocem MTA. When compared with MTA-Angelus and ProRoot MTA, the amount of CaO was relatively lower, while aluminum oxide (Al_2O_3), magnesium oxide (MgO), ferric oxide (Fe_2O_3), and

Table 1
Chemical compositions of each MTA material (mass%).

	MTA-Angelus	Endocem MTA	ProRoot MTA
CaO	68.85	54.47	63.06
Bi_2O_3	13.13	14.24	14.46
SiO_2	14.28	14.25	17.06
Al_2O_3	3.1	5.83	1.74
SO_3	0.02	3.32	1.96
MgO	0.34	3.21	0.75
Fe_2O_3	0.03	2.53	0.28
K_2O	–	1.34	0.05
P_2O_5	0.07	0.06	0.28
TiO_2	–	0.28	0.07
F	–	0.21	–
SrO	0.17	0.08	0.09
Na_2O	0.01	0.11	0.12
MnO	–	0.05	0.03
V_2O_5	–	–	0.03
NiO	–	0.01	0.01
Cl	–	0.01	0.01

potassium oxide (K_2O) were present at a much greater concentration in Endocem MTA.

3.2. Disc diffusion test

The mean diameters and standard deviations of the zones of bacterial growth inhibition for each material against each species of bacteria are shown in Table 2 and Fig. 1. The antibacterial activities of the MTA materials tested were found to be bacteria-dependent. Despite differences in the extent of antibacterial activity, all materials showed zones of inhibition of bacterial growth against *L. rhamnosus*, *L. paracasei*, *S. mutans,* and *P. gingivalis*. MTA-Angelus exhibited the largest inhibition zone, followed by ProRoot MTA and Endocem MTA ($p < 0.05$). The growth of *E. faecalis* was affected neither by MTA-Angelus nor by ProRoot MTA, but was only found to be susceptible to Endocem MTA. Among the bacteria tested, the largest inhibition zone was observed in *L. rhamnosus*, followed by *S. mutans*, *L. paracasei*, *P. gingivalis*, and *E. faecalis.*

3.3. Antibacterial broth test

The mean number of CFU/mL for each material against each species of bacteria are present in Table 3 and Fig. 2. Among the tested MTA materials, the antibacterial activity against all the tested bacteria was greatest in Endocem MTA, followed by MTA-Angelus, and ProRoot MTA (in the order of increasing number of CFU/mL) ($p < 0.05$).

4. Discussion

The methods used in the present study for evaluation of antibacterial activity of MTA materials were disc diffusion test and antibacterial broth test. The results of the two tests were not consistent due to the difference in the application of MTA materials. For antibacterial broth test, fresh powder of each MTA material was added to the broth before incubation with bacteria. The growth of bacteria is inhibited if the compositions of the test materials have antibacterial properties, when in contact with the bacteria. The addition of powder, however, substantially impaired the accuracy of bacterial counting. Although there was an increase in the antibacterial activity of all three materials with increasing concentration of the powder, the results shown in this study were obtained when the powder concentration was 12.5 mg/mL, at which clear comparison between materials could be made without having counting problem. Moreover, antibacterial broth test was not suitable for evaluation of MTA materials because powder is mixed with water in clinical setting to a thick-creamy consistency before placement. The results from the disc diffusion test, in which discs of MTA material were prepared by mixing with water according to the manufacturer's instruction, seem more clinically relevant than those from the antibacterial broth test. Therefore, the following discussion on antibacterial activity of test MTA materials against each bacteria is based on the findings from disc diffusion test.

The microorganisms tested in this study were the key bacteria that are frequently associated with certain oral diseases.

It has been well documented that initial carious lesions have a high level of *S. mutans* (a facultative anaerobe), while cavitated lesions have an increased population of both *S. mutans* and Lactobacillus. As the caries advance, such as in deep caries or root caries, the number of *S. mutans* and Lactobacillus, respectively, declines and exponentially increases (Bonecker, Grossman, Cleaton-Jones, & Parak, 2003; Brown, Billings, & Kaster, 1986; Gross et al., 2010). *L. rhamnosus* and *L. paracasei* used in this study belong to the *L. casei* group, which is the predominant *Lactobacillus* species in carious lesions (Byun et al., 2004; Chhour et al., 2005; Marchant, Brailsford, Twomey, Roberts, & Beighton, 2001; Martin, Nadkarni, Jacques, & Hunter, 2002; Munson, Banerjee, Watson, & Wade, 2004). Dental restorative materials used in deep cavities, therefore, should possess antibacterial properties, since the pulp vitality in deep carious lesions could be maintained when residual bacteria or their byproducts are precluded from entering the root canal system or inducing pulp inflammation. High success rates of MTA used for indirect/direct pulp capping, as reported in recent studies (Bogen, Kim, & Bakland, 2008; Hilton, Ferracane, & Mancl, 2013; Mente et al., 2014), could be attributed to the outstanding biological properties including antibacterial activity. All the three MTA materials tested inhibited the growth of caries-associated bacteria, with MTA-Angelus having the greatest effect, followed by ProRoot MTA, and finally Endocem MTA. Despite the fact that ProRoot MTA was also very effective in inhibiting the growth of caries-associated bacteria, either MTA-Angelus or Endocem MTA seem to be the materials of choice for pulp capping in one-visit deep caries treatment, because the shorter setting time of MTA-Angelus (14 (Santos, Araujo, Yukimitu, Barbosa, & Moraes, 2008)–15 min (Angelus, 2015)) and Endocem MTA (4 (Choi et al., 2013)–15 min (Kim, Yang, Kim, & Ko, 2014)) compared to that of ProRoot MTA (140 (Islam, Chng, & Yap, 2006)–318 min (Kim et al., 2014)) enables the placement of permanent restoration in a single visit. This would not only save chair time but also help minimize possible contamination of the cavity, which may occur during the temporization period in a conventional two-visit treatment wherein a permanent restoration is placed over the set MTA in the next visit. However, MTA-Angelus and ProRoot MTA have been shown to cause discoloration at the MTA-dentin interface, while discoloration was not found at the interface between dentin and Endocem MTA (Jang et al., 2013). Therefore, the use of Endocem MTA may be preferable for pulp capping over MTA-Angelus or ProRoot MTA in terms of aesthetic outcome.

The inclusion of *P. gingivalis*, a major periodontopathic bacteria, in the present study was based on the findings that the bacteria is one of the most frequently detected anaerobic microorganisms in subgingival plaque samples from periodontal-endodontic combined lesions and necrotic pulp (Gomes et al., 2004; Socransky, Haffajee, Cugini, & Smith, 1998). In addition, Gomes et al., (2005) performed PCR-based identification of bacteria in teeth with

Table 2
Means and standard deviations of diameters (mm) of antibacterial inhibition zones from disc diffusion test.

Bacteria	MTA			Material-dependent significant differences
	MTA-Angelus	Endocem MTA	ProRoot MTA	
L. rhamnosus	17.58 ± 0.42	11.92 ± 0.62	14.78 ± 0.75	Angelus > ProRoot > Endocem
L. paracasei	14.77 ± 0.54	8.58 ± 1.03	10.63 ± .73	Angelus > ProRoot > Endocem
S. mutans	16.65 ± 0.95	10.15 ± 0.74	14.78 ± .75	Angelus > ProRoot > Endocem
E. faecalis	0	5.08 ± 0.46	0	n/a
P. gingivalis	12.98 ± 0.75	5.71 ± 0.70	7.04 ± 0.90	Angelus > ProRoot > Endocem

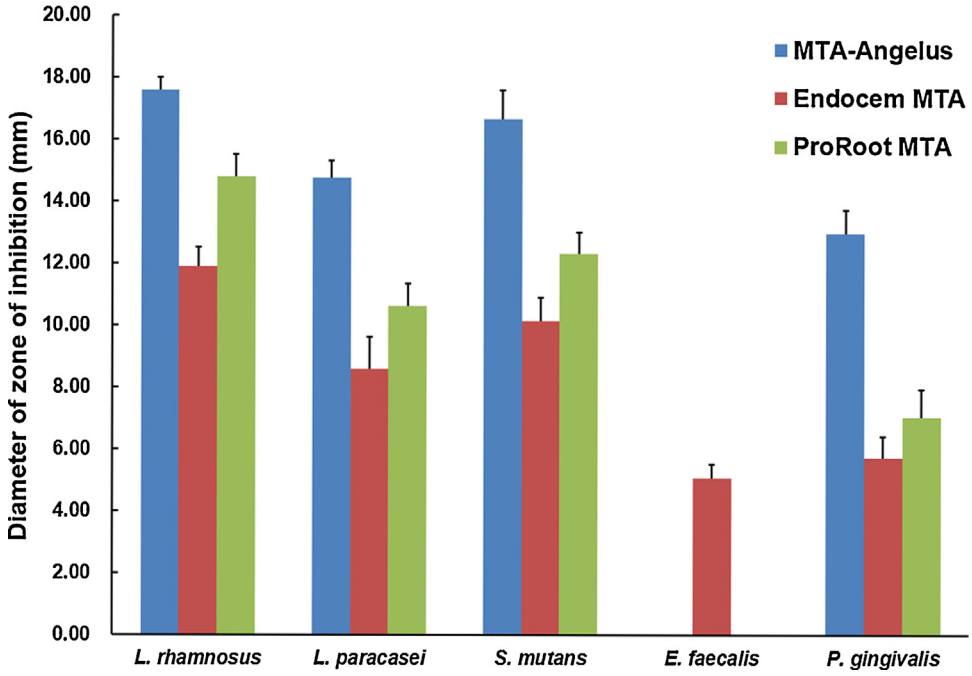

Fig. 1. Results of the disc diffusion test. Mean diameters and standard deviations of zones of bacterial growth inhibition for each material against each species of bacteria.

Table 3
Means and standard deviations of CFU (10^6)/mL from antibacterial broth test.

Bacteria	Control	Ampicillin (1 μg/mL)	MTA (12.5 mg/mL)			Material-dependent significant differences
			MTA-Angelus	Endocem MTA	ProRoot MTA	
L. rhamnosus	12.01 ± 0.81	0.66 ± 0.07	3.24 ± 0.27	2.18 ± 0.23	4.94 ± 0.43	Endocem < Angelus < ProRoot
L. paracasei	11.84 ± 0.65	0.64 ± 0.12	4.76 ± 0.29	2.80 ± 0.33	6.34 ± 10.6	Endocem < Angelus < ProRoot
S. mutans	13.26 ± 1.05	0.75 ± 0.19	4.14 ± 0.36	3.04 ± 0.43	5.28 ± 0.33	Endocem < Angelus < ProRoot
E. faecalis	13.91 ± 0.90	3.09 ± 0.41	9.08 ± 0.32	6.92 ± 0.43	10.03 ± 0.69	Endocem < Angelus < ProRoot
P. gingivalis	10.95 ± 0.87	0.31 ± 0.08	5.09 ± 0.29	4.33 ± 0.31	6.54 ± 0.38	Endocem < Angelus < ProRoot

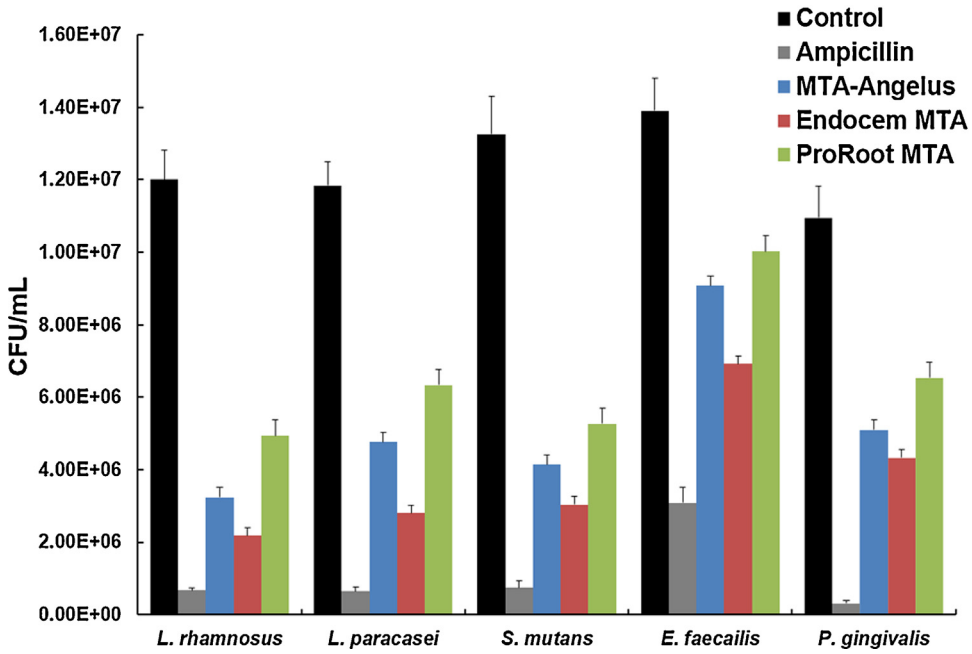

Fig. 2. Results of the antibacterial broth test. Mean colony forming units (CFU)/mL and standard deviations for each material against each species of bacteria.

necrotic pulp, and showed that the most frequently identified species was *P. gingivalis* (38%). Considering the high prevalence of *P. gingivalis* not only in periodontal disease but also in necrotic pulp or combined lesions, sealing the root canal system with a material that possesses antibacterial activity would contribute to a successful outcome. Such clinical situations include those where there is a communication between the periodontium and root canal system, such as in accidental mishaps like apical or lateral/furcation perforation or in inflammatory external resorption. Although *P. gingivalis* appears more resistant to the MTA materials when compared to the caries-associated bacteria, all of the materials tested showed inhibitory effects against *P. gingivalis*, with MTA-Angelus being the most effective.

The antibacterial activity of MTA is derived from tricalcium silicate and dicalcium silicate, which are the main components of MTA. These constituents hydrate to form alkaline calcium silicate gel in a few hours when mixed with water (Camilleri et al., 2005; Dammaschke et al., 2005; Sarkar, Caicedo, Ritwik, Moiseyeva, & Kawashima, 2005; Yoshimine, Ono, & Akamine, 2007). Calcium hydroxide in a silicate matrix releases hydroxide ions, resulting in high alkalinity (Camilleri et al., 2005; Dammaschke et al., 2005), which in turn creates an unfavorable environment for microbial growth. Fridland and Rosado, (2005) reported that MTA was able to maintain a high pH in the range of 11–12 for 78 days by releasing its soluble fraction to an aqueous environment over a long period at a decreasing rate. These features of MTA account for the antibacterial effect against the aforementioned bacteria: *S. mutans*, *L. rhamnosus*, *L. paracasei*, and *P. gingivalis.*

The antibacterial activity against *E. faecalis* was also investigated because it is the most frequently isolated microorganism from infected root canals, especially in recalcitrant infection after endodontic treatment (Stuart, Schwartz, Beeson, & Owatz, 2006; Sundqvist, Figdor, Persson, & Sjogren, 1998). The presence of serine protease and collagen-binding protein (Ace) contribute to the adhesion of *E. faecalis* to the root canal and invasion into the dentinal tubules (Hubble, Hatton, Nallapareddy, Murray, & Gillespie, 2003). McHugh, Zhang, Michalek, and Eleazer, (2004) investigated the pH required to kill *E. faecalis* and found that growth was retarded at pH of 10.5–11.0, whereas the bacteria were not able to survive at a pH greater than 11.5. Previous studies have shown conflicting results as to whether MTA has antibacterial activity against *E. faecalis*. It could be speculated that *E. faecalis* would not survive in the vicinity of MTA due to the high alkalinity (pH 11–12). However, our finding showed that MTA-Angelus and ProRoot MTA failed to inhibit the growth of *E. faecalis*, which was in accord with the results of previous studies (Estrela, Bammann, Estrela, Silva, & Pecora, 2000; Torabinejad, Hong, Pitt Ford, & Kettering, 1995b). On the other hand, MTA has also been shown to be an antibacterial material against *E. faecalis* (Al-Hezaimi, Al-Shalan et al., 2006; Asgary and Kamrani, 2008; Stowe, Sedgley, Stowe, & Fenno, 2004). Thus, the antibacterial action cannot be rationally explained by pH alone. In clinical situations, a desirable high pH after MTA application cannot be maintained due to the buffering capacity of dentin. In addition, *E. faecalis* has a proton pump that helps reduce the intracellular pH level (Stuart et al., 2006). The susceptibility of *E. faecalis* only to Endocem MTA, as found in the present study, could be explained by the difference in the chemical compositions of the materials. Mickel, Sharma, and Chogle, (2003) showed that stannous fluoride inhibited the growth of *E. faecalis* significantly more than calcium hydroxide. The analysis of chemical compositions by X-ray fluorescence spectrometry showed that fluoride was only found in Endocem MTA, albeit low. Further evaluation of the role of fluoride on physical and bioactive properties of MTA-based materials would be of value. Two different colored MTA products were tested, with MTA-Angelus and ProRoot MTA being white-colored MTA (WMTA), while Endocem MTA is a gray-colored MTA (GMTA). Al-Hezaimi, Al-Shalan et al., (2006) and Al-Hezaimi, Naghshbandi, Oglesby, Simon, and Rotstein, (2006) compared the antibacterial effect against *E. faecalis* and *Streptococcus sanguis* and antifungal effect against *Candida albicans* between two versions (WMTA *vs.* GMTA) of ProRoot MTA and revealed that GMTA requires a lower concentration than WMTA to be effective against both bacteria and fungus. Despite the fact that main constituents of the MTA materials were calcium oxide, silicon dioxide, and bismuth oxide, Endocem MTA had a lower concentration of calcium oxide (54%) than MTA-Angelus (69%) and ProRoot MTA (63%). On the other hand, Endocem MTA contained much higher concentrations of aluminum oxide, magnesium oxide, and ferric oxide than WMTAs (MTA-Angelus and ProRoot MTA). Our finding corroborates the results of Asgary, Parirokh, Eghbal, and Brink, (2005) who reported the major compositional differences between WMTA and GMTA. Lower concentrations of aluminum oxide, magnesium oxide, and ferric oxide in WMTA may be a factor accounting for the stronger antibacterial properties of GMTA. Although both GMTA and WMTA are generally recognized as biocompatible, a biocompatibility study demonstrated that WMTA was superior to GMTA in supporting cementoblast and keratinocyte growth (Oviir, Pagoria, Ibarra, & Geurtsen, 2006). The discrepancy between the antibacterial property and biocompatibility implies that there is no single ideal material that possesses the greatest antibacterial properties not at the expense of its biological function, and that could entirely substitute conventional materials.

Within the limitations of this study, the MTA materials (MTA-Angelus, ProRoot MTA, and Endocem MTA) inhibited the growth of *S. mutans*, *L. rhamnosus*, *L. paracasei*, and *P. gingivalis*. Therefore, based on our findings on the antibacterial effect against the bacteria predominantly associated with polymicrobial dental diseases, these MTA materials could be recommended to seal the root canal systems in order to improve the clinical success in both vital and non-vital pulp therapy. For a pulp capping material, provided that the mechanical and biological properties of MTA-Angelus and Endocem MTA fulfill the requirements for clinical success at least comparably to conventional MTA, MTA-Angelus or Endocem MTA can be used to complete the treatment in one visit because of their fast-setting property. However, considering the discoloration effect of ProRoot MTA as well as MTA-Angelus, Endocem MTA appears to be the material of choice for pulp capping. Furthermore, since Endocem MTA seems to be the only inhibitory material against *E. faecalis* among the materials tested, Endocem MTA could be recommended as a retrograde/orthograde root-end or perforation/resorption repair material, especially in cases where infection persists after conventional endodontic treatment.

Conflict of interest

There are no conflicts of interest to declare.

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