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Original Article

The antibacterial activity of mineral trioxide aggregate containing calcium fluoride

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Abstract *Background/purpose:* Because complete microbial elimination of the infected root canal system is nearly impossible to achieve, the use of root canal fillings with antibacterial effects may help to minimize intracanal infections. Our previous study, MTA modified by CaF_2 addition, improved the biocompatibility and mineralization potential of human dental pulp cells without adverse effect on the physical properties. In this study, the antibacterial effect of MTA after CaF_2 addition was evaluated for use as a root canal sealer. The objective of this study was to evaluate the antibacterial activity of MTA after the addition of CaF_2.
Materials and methods: The antibacterial activities of MTA and MTA–CaF_2 mixture against *Enterococcus faecalis* (*E. faecalis*), *Porphyromonas endodontalis* (*P. endodontalis*), and *Porphyromonas gingivalis* (*P. gingivalis*) were investigated with MTA-CaF_2 powder and eluates. The bacterial growth was measured by optical density using a spectrophotometer and the bacterial colony counting.
Results: MTA inhibited the growth of *E. faecalis*, *P. endodontalis*, and *P. gingivalis*, and the addition of CaF_2 improved this antibacterial effect in a concentration dependent manner ($p < 0.05$). *E. faecalis* was more resistant to MTA-CaF_2 than the other groups of bacteria.
Conclusion: The addition of more than 5% CaF_2 to MTA can increase the antibacterial activity.
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Introduction

Considering the indispensable role of microorganisms in the development and progress of pulpal and periapical disease, the eradication of microorganisms from the root canal system and prevention of their invasion is essential for root canal treatment.[1–4] However, because complete microbial elimination of the infected root canal system is nearly impossible to achieve, the use of root canal fillings with antibacterial effects may help to minimize intracanal infections. When MTA is used for root canal fillings, to increase its antibacterial effect will be beneficial clinically.[5,6]

MTA, a calcium silicate-based cement with excellent biological and physical properties,[7] was introduced to dentistry in the mid-1990s.[8,9] It is able to set and seal even in moist and blood-contaminated environments.[10] It is routinely used to induce hard tissue in various procedures, such as root perforation repair, pulp-dentin regeneration, apical barrier formation, pulp capping, pulpotomy, and root-end filling.[9,11] With these excellent properties of calcium silicate-based cement, an endodontic sealer based on the calcium silicate has been introduced.[12]

When calcium silicate based sealer is used for root canal filling, the antibacterial effect will be one of the important properties. Even though there are various calcium silicate-based root canal sealers commercially available on the market, some are still early stages, requiring further laboratory and clinical study, therefore, MTA was used in this study.[12] Previous studies have reported conflicting results about the antibacterial activity of MTA.[13–15] The antibacterial property of MTA is related to the release of calcium hydroxide and the alkalinizing activity.[16]

Since the 1940s, fluoride has been reported to affect dental plaque and oral bacteria,[17] and it has been used for dental caries prevention. It interferes bacterial metabolism by inhibiting glycolytic enzymes.[18,19] CaF_2 is one of the widely used fluoride composites, and it has been studied in medical and dental material science for its antibacterial effect.[20–22]

Our previous study,[23] MTA modified by CaF_2 addition, improved the biocompatibility and mineralization potential of human dental pulp cells (HDPCs) without negatively affecting the physical properties. In this study, the antibacterial effect of MTA after CaF_2 addition was evaluated for use as a root canal sealer.

The objective of this study was to evaluate the antibacterial activity of MTA after the addition of CaF_2.

Materials and methods

The antimicrobial activity of MTA and MTA-CaF_2 mixture against *Enterococcus faecalis*, *Porphyromonas endodontalis*, and *Porphyromonas gingivalis* was investigated according to the method of Clinical Laboratory Standard Institute (CLSI). Because the lower solubility of CaF_2 could show different result,[23] both MTA or MTA-CaF_2 powder and eluate were used.

Preparation of MTA samples

ProRoot MTA (Lot number: 212470; Dentsply Sirona, York, PA, USA) and CaF_2 powder (Sigma–Aldrich, San Jose, CA, USA) were used in this study. MTA powder was mixed with various concentrations of CaF_2 as Table 1.

For the MTA eluate test, the MTA and MTA–CaF_2 mixture (2g) was mixed with sterile distilled water (DW; 5 ml) in 15 ml conical tubes (SPL Lifesciences, Pocheon, Korea). The tubes containing MTA and MTA-CaF_2 were mixed for 30 s using a vortex mixer (Scientific Industries Inc., Bohemia, NY, USA) and centrifuged at 3000×*g* for 5 min using the method in the previous study.[23] The supernatant was transferred to a new tube for the experiment.

Bacteria culture

E. faecalis ATCC 29212, *P. endodontalis* ATCC 35406, and *P. gingivalis* ATCC 33277 were used in this study. *E. faecalis* was cultivated with brain heart infusion (BHI) (BD Biosciences, Franklin Lakes, NJ, USA) broth. *P. endodontalis* and *P. gingivalis* were cultivated in BHI broth supplemented with 0.2 μg/ml of vitamin K (Sigma–Aldrich) and 1 μg/ml of hemin (Sigma–Aldrich). The bacteria were cultured at 37 °C under anaerobic conditions (H_2 5%, CO_2 10%, and N_2 85%).

The cultured bacteria were adjusted to 1.0×10^6 cells/ml for *E. faecalis* and 1.5×10^6 cells/ml for *Porphyromonas* spp, respectively, after measuring the bacterial concentration using a bacterial counting chamber (Marienfeld, Lauda-Konigshöfen, Germany).

Investigation of antibacterial activity with MTA eluate

Suitable media (180 μl) for bacteria were dispensed in each well of a 96-well plate (SPL Lifesciences), and MTA eluate (180 μl) were then added to the wells of the twelfth row. The eluates were two-fold serially diluted using a multichannel micropipette to the second row.

Then, 20 μl of each bacterial suspension was inoculated into the well containing the prepared broth, and the plate was incubated at 37 °C under anaerobic conditions (5% H_2, 10% CO_2, and 85% N_2) for 36 h to culture *E. faecalis* and 48 h to culture *Porphyromonas* spp, respectively. The pure bacterial suspension without MTA eluate was used the control group. Bacterial growth was measured by optical density at 660 nm using a spectrophotometer (Biotek, Winooski, VT, USA).

Investigation of antibacterial activity after direct contact with MTA powder

For investigating the direct antibacterial activity of MTA and MTA-CaF_2, the powder (total weight of 1 g) of MTA and

Table 1 The compositions of each group.

Group	MTA	MTA + 1% CaF_2	MTA + 5% CaF_2	MTA + 10% CaF_2
MTA	1 g	0.99 g	0.95 g	0.9 g
CaF_2	0g	0.01 g	0.05 g	0.1 g

MTA; mineral trioxide aggregate, CaF_2; calcium fluoride.

MTA-CaF_2 was placed into a 15 ml conical tube. BHI broth (4.5 ml) was added, and the bacterial suspension (500 μl) was inoculated into the prepared tube. Then, the tube was incubated at 37 °C with shaking for 10 min.

A 10-fold serial dilution (from 10 to 10^6) of the suspension in the incubated tube was performed using BHI broth. Each dilution of *E. faecalis* was spread on a BHI agar plate. *P. endodontalis* and *P. gingivalis* were spread on a blood agar plate containing hemin and vitamin K. The plates were incubated at 37 °C under anaerobic condition (H_2 5%, CO_2 10%, and N_2 85%) for 36 h and 48 h to culture *E. faecalis* and *Porphyromonas* spp, respectively. The bacterial colonies were counted, and expressed as colony-forming unit (CFU). CFU was calculated according to the following equation.

$$\text{CFU} / \text{ml} = \frac{(\text{No. of bacterial colonies} \times \text{dilution factor})}{\text{volume of culture transferred to plate}}$$

Statistical analysis

SPSS 23.0 (IBM Software, Armonk, NY, USA) was used for statistical analysis. Normality was verified with Shapiro–Wilk tests. A non-parametric Kruskal–Wallis test was carried out and post-hoc analysis was performed with the Mann–Whitney U test. Statistical significance was considered at $p < 0.05$. All data from repeated tests are described as median and interquartile range.

Results

Antibacterial activity with MTA eluate

MTA and MTA–CaF_2 mixture showed inhibitory effects on the growth of all the bacteria. In the *E. faecalis* test, bacterial growth was inhibited from 50 mg/ml. *P. gingivalis* and *P. endodontalis* were inhibited from 1.6 mg/ml.

1) *E. faecalis*

Bacterial growth inhibition began at 50 mg/ml of the MTA-5% CaF_2 and MTA-10% CaF_2 groups, but pure MTA and MTA-1% CaF_2 groups showed inhibition effects only at 200 mg/ml ($p < 0.05$). There was no difference between the pure MTA and MTA-1% CaF_2 groups. The MTA-1% CaF_2 and the MTA-5% CaF_2 groups showed differences at 100 and 200 mg/ml, with greater differences detected at 100 mg/ml ($p < 0.05$). The MTA-5% CaF_2 and MTA-10% CaF_2 groups showed differences at 50 and 100 mg/ml, with greater differences observed at 100 mg/ml ($p < 0.05$). However, there was no difference between 5% and 10% CaF_2 groups at 200 mg/ml (Fig. 1).

2) *P. endodontalis*

P. endodontalis growth was inhibited from 1.6 mg/ml of the MTA-10% CaF_2, while pure MTA showed an effect from 25 mg/ml ($p < 0.05$). Pure MTA and MTA-1% CaF_2 showed no difference except at 25 mg/ml. MTA-1% CaF_2 and 5% CaF_2 showed different effects from 3.2 to 25 mg/ml ($p < 0.05$). Differences between MTA-5% CaF_2 and MTA-10% CaF_2 were detected from 1.6 to 6.25 mg/ml ($p < 0.05$). Differences among the CaF_2 addition groups disappeared after 50 mg/ml. Therefore, after 50 mg/ml, all MTA groups had similar effects on *P. endodontalis* (Fig. 1).

3) *P. gingivalis*

P. gingivalis growth was inhibited from 1.6 mg/ml of the MTA-10% CaF_2 and pure MTA showed an antibacterial effect from 25 mg/ml ($p < 0.05$). Pure MTA and MTA-1% CaF_2 showed no difference. The MTA-1% CaF_2 and the MTA-5% CaF_2 showed a difference from 3.2 mg/ml to 25 mg/ml ($p < 0.05$). The MTA-5% CaF_2 and the MTA-10% CaF_2 showed a different effect from 1.6 mg/ml to 6.25 mg/ml ($p < 0.05$). From 50 mg/ml, all MTA groups show similar effects on *P. gingivalis* (Fig. 1).

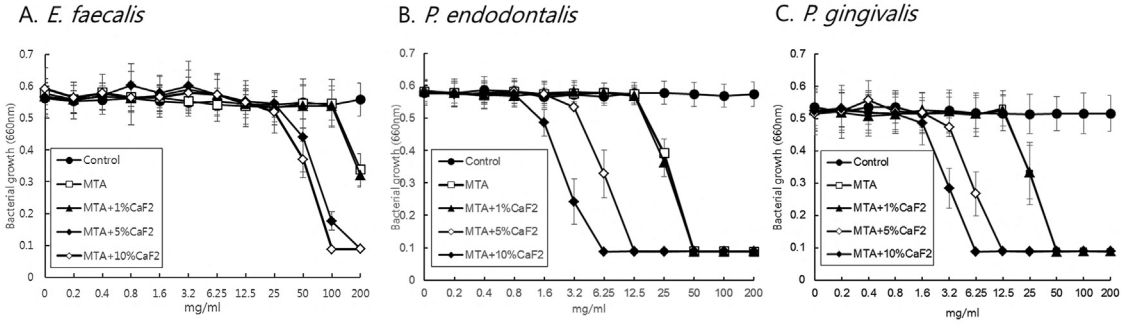

Figure 1 Antibacterial activity of MTA and MTA-CaF_2 eluates. *E. faeclais*, *P. endodontalis* and *P. gingivalis* were cultivated in the presence or absence of the eluate from MTA and MTA-CaF_2 mixture. Growth was measured via a spectrophotometer. The growth inhibition of *E. faecalis* began at 50 mg/ml of the MTA-5% CaF_2 and MTA-10% CaF_2, but pure MTA and MTA-1% CaF_2 groups showed inhibition effects only at 200 mg/ml ($p < 0.05$). The MTA-1% CaF_2 and the MTA-5% CaF_2 groups showed differences at 100 and 200 mg/ml. The MTA-5% CaF_2 and MTA-10% CaF_2 groups showed differences at 50 and 100 mg/ml. However, there was no difference between 5% and 10% CaF_2 groups at 200 mg/ml. The growth *P. endodontalis* and *P. gingivalis* were inhibited from 1.6 mg/ml of the MTA-10% CaF_2, while pure MTA showed an effect from 25 mg/ml ($p < 0.05$). Differences between MTA-5% CaF_2 and MTA-10% CaF_2 were detected from 1.6 to 6.25 mg/ml ($p < 0.05$). Differences among the CaF_2 addition groups disappeared after 50 mg/ml.

Investigations with direct contact with MTA power

Results of each bacterium are described below and shown in Table 2 and Fig. 2.

1) *E. faecalis*

MTA groups showed an antibacterial effect on *E. faecalis* ($p < 0.05$). Pure MTA and MTA-1% CaF_2 groups showed similar effects ($p > 0.05$). The addition of 5% and 10% CaF_2 significantly reduced the CFU/ml ($p < 0.05$) (Fig. 2).

2) *P. endodontalis*

MTA groups showed an antibacterial effect on *P. endodontalis*. The addition of CaF_2 further reduced the CFU/ml ($p < 0.05$) (Fig. 2).

3) *P. gingivalis*

MTA groups showed a significant antibacterial effect on *P. gingivalis*. The antibacterial effects increased with the concentration of CaF_2 ($p < 0.05$) (Fig. 2).

Discussion

Antibacterial activity is derived from MTA's main components, such as tricalcium silicate and dicalcium silicate. These components hydrate to form an alkaline calcium silicate gel when mixed with water. Calcium hydroxide from this calcium silicate matrix releases hydroxyl ions, resulting in high alkalinity, inhibiting microbial growth.[24,25] MTA was reported to maintain a high pH in the range of 11–12 for 78 days.[26]

For investigating the antibacterial effect of MTA-CaF_2 mixtures, three bacteria were used in this study: *P. gingivalis*, *P. endodontalis*, and *E. faecalis*. *P. gingivalis*, a bacterium related to periodontal disease was included because it is also frequently detected in subgingival plaque from endo-perio combined lesions, necrotic pulp,[27] and odontogenic abscesses in patients not suffering from periodontal disease.[28] *E. faecalis* is the most frequently isolated bacterium in recalcitrant infections after endodontic treatment.[29,30]

Previous studies have reported conflicting results regarding the antibacterial activity of MTA on *E. faecalis*. MTA was reported to fail to inhibit the growth of *E. faecalis*.[14,15] However, other studies have reported that MTA has antibacterial effects on *E. faecalis*,[31,32] *E. faecalis* is retarded at a pH of 10.5–11.0, and the bacteria cannot survive at a pH higher than 11.5.[33]

In this study, MTA and CaF_2 showed antibacterial effects on the bacteria tested. As CaF_2 is less soluble, there could be a difference between direct contact with MTA-CaF_2 powder and its use as an eluate. Therefore, the antibacterial activities of direct contact with MTA-CaF_2 powder were also investigated. Contact with powder is related to the direct influence of MTA, and effects are limited to the surface it contacts. The effect of MTA eluate is limited to the soluble fraction and could affect the distant area.

Table 2 CFU ($\times 10^3$)/ml with direct MTA-CaF_2 powder contact.

Groups	*E. faecalis*	*P. endodontalis*	*P. gingivalis*
Control	957.500 ± 80.00^{a}	1200 ± 195.00^{e}	1170 ± 150.00^{j}
MTA	155.000 ± 34.31^{b}	42.000 ± 16.437^{f}	40.875 ± 16.75^{k}
MTA + 1% CaF_2	160.250 ± 24.75^{b}	24.375 ± 18.25^{g}	17.500 ± 7.75^{l}
MTA + 5%CaF_2	9.850 ± 1.356^{c}	1.035 ± 20.75^{h}	1.240 ± 0.255^{m}
MTA + 10%CaF_2	5.650 ± 1.612^{d}	0.431 ± 0.075^{i}	0.515 ± 0.173^{n}

CFU; colony-forming unit.
Different superscripts indicate significantly different antibacterial activity for different bacteria ($p < 0.05$) (median ± interquartile).

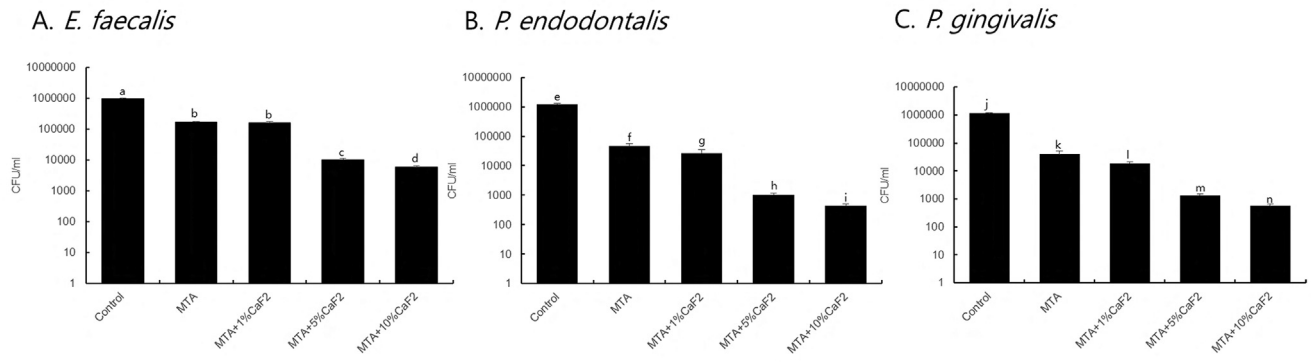

Figure 2 CFU/ml after direct MTA and MTA-CaF_2 powder contact. Dilution of *E. faecalis*, *P. endodontalis* and *P. gingivalis* mixed with MTA and MTA-CaF_2 powder was spread on a BHI agar plate. After incubation, bacterial colonies were counted. MTA groups reduced CFU/ml, and the antibacterial effects increased with the concentration of CaF_2 ($p < 0.05$). In *E. faecalis*, pure MTA and MTA-1% CaF_2 showed similar antibacterial effect. Different superscripts indicate different antibacterial activity ($p < 0.05$).

Both MTA as a powder and as an eluate showed antibacterial effects that increased with CaF_2. MTA inhibited the growth of three bacteria. The addition of CaF_2 improved this antibacterial effect. *E. faecalis* was more resistant to MTA powder and eluates than the other bacteria groups.

In eluate test, MTA-10% CaF_2 and MTA-5% CaF_2 inhibited the growth of *E. faecalis* from 50 mg/ml. Pure MTA and MTA-1% CaF_2 showed the antibacterial effect only at 200 mg/ml. In *P. gingivalis* and *P. endodontalis*, MTA-10% CaF_2 MTA inhibited the growth of three bacteria from 1.6 mg/ml and MTA-5% CaF_2 from 3.2 mg/ml. Pure MTA and MTA-1% CaF_2 showed the antibacterial effect from 25 mg/ml. However, from 50 mg/ml MTA-CaF_2, all MTA groups showed similar antibacterial activity. As a result, *E. faecalis* needs more concentrated extracts than the other two bacteria for growth inhibition.

In the powder test, the addition of CaF_2 improved the antibacterial activity of MTA. These antibacterial effects increased with the concentration of CaF_2. In *E. faecalis*, pure MTA and MTA-1% CaF_2 showed a similar antibacterial effect. From these results, in *E. faecalis*, more than 5% CaF_2 addition is recommended.

From the previous study, less soluble CaF_2 decreased calcium silicate cement hydration, resulting in a lower alkalinizing activity, and CaF_2-treated tricalcium silicate showed a lower pH than pure MTA.[34] Therefore, it could be presumed that the antibacterial activities of MTA-CaF_2 are more related to the inhibition of bacterial metabolism by fluoride than by the alkalinizing activity of MTA.

Torabinejad et al. reported that mixing MTA with chlorhexidine improved its antibacterial properties, but adversely affected biocompatibility.[35] However, from our previous study[23] and the current study, CaF_2 addition to MTA improved both antibacterial effect and biocompatibility. It is speculated that the fluorine level within the eluate was not high enough to be toxic to human dental pulp cells because only the soluble fraction of MTA or CaF_2 could affect the cells. The dissolved fluorine from CaF_2 could be the key to the proliferation and osteogenic stimulation of the pulp cells and the antibacterial effects. Importantly, CaF_2 is regarded as a relatively benign fluoride composite due to its low solubility and ionization.[36]

We conclude that the addition of more than 5% CaF_2 shows better antibacterial effects against *E. faecalis*, *P. endodontalis*, and *P. gingivalis* than pure MTA both in its powder form or in its eluate. The addition of CaF_2 can be considered to increase the antibacterial activity of MTA. However, further studies, including long-term evaluations, are necessary for clinical applications.

Declaration of competing interest

The authors declare no conflicts of interest.

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