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Original Article

The biocompatibility and mineralization potential of mineral trioxide aggregate containing calcium fluoride—An in vitro study

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Abstract *Background/purpose:* MTA is used to induce hard tissue regeneration in various procedures. This study evaluated the biocompatibility and mineralization potential of mineral trioxide aggregate (MTA) containing calcium fluoride (CaF_2). To verify if the change of components affected physical properties, the setting time, solubility, and surface roughness were measured.
Materials and methods: Human dental pulp cells (HDPCs) were treated with powder and set MTA containing CaF_2 (0, 1, 5, and 10 wt %). The proliferation of HDPCs was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The mineralization potential of HDPCs was investigated with the relative gene expression of alkaline phosphatase (ALP), collagen type I (ColI), osteocalcin (OCN), and runt-related transcription factor 2 (Runx2) using real-time reverse transcription polymerase chain reaction (RT-PCR). For investigating the physical properties, setting time and solubility were tested. Surface profiles of material were analyzed by a non-contact surface profiler and a scanning electron microscope (SEM).
Results: MTA-5% CaF_2 mixtures increased the proliferation and the mineralization-related gene expression of HDPCs to a greater degree than pure MTA. The addition of CaF_2 to MTA delayed the setting, but the difference was only significant in the MTA-10% CaF_2. Solubility and surface roughness was not altered.

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Conclusion: The addition of more than 5% CaF_2 can be considered to increase the regeneration potential of pulp cells without adverse effects on physical property.

Introduction

Mineralized tissue formation is the ultimate goal of pulp treatment procedures. Dental pulp tissues exposed by caries or trauma result in odontoblastic destruction and fibroblast injury. The pulp tissues are primarily fibroblasts but can differentiate into odontoblasts.[1] Similar to osteoblastic cells, they show high ALP activity and form calcified nodules in long-term cultures.[2,3]

MTA is 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.[4,5] Most current studies regarding the modification of MTA composition have aimed to decrease setting time or improve handling properties.[6,7] Relatively little attention has been concentrated on the biocompatibility or regeneration potential of MTA supplemented with additives.[8,9]

Since the 1940s, fluoride has been used for dental caries prevention.[10] Fluoride also stimulates the proliferation of osteoblasts, ALP activity, collagen production, and OCN synthesis in bone cells.[11,12]

In this study, MTA was modified by fluoride addition. Previous studies regarding MTA with fluoride mainly used NaF and investigated the apatite formation on the cement surface or effects on the osteoblasts.[8,13]

The solubility of NaF may result in structural loss; thus, CaF_2 was used instead of NaF in this study. CaF_2 is a widely-used fluoride composite. The lower solubility of CaF_2 may prevent the rapid release of ions under clinical situations.[14,15] CaF_2 composite hydrogel dressings have shown good biocompatibility and antibacterial properties,[16] and a significant reduction in bacterial growth has been observed for composite resin modified with 1.5 wt% CaF_2.[17]

The objectives of this study were to evaluate the biocompatibility and mineralization potential of MTA after the addition of CaF_2. And to verify if there is a negative influence of CaF_2 on the physical property of MTA, setting time, solubility, and surface roughness were measured.

Materials and methods

MTA-CaF_2 sample preparation

For investigating the properties of the mixture of MTA and CaF_2, ProRoot MTA (Lot number: 212,470; Dentsply Sirona, York, PA, USA) and CaF_2 powder (99.99%, Sigma—Aldrich, San Jose, CA, USA) were used. MTA and CaF_2 were mixed in various concentrations (0,1, 5, and 10 wt% CaF_2).

For evaluating the direct effect of MTA-CaF_2, the mixture powder was used. MTA-CaF_2 powder samples were prepared under aseptic conditions as the previously reported method with some modifications.[18] Briefly, MTA-CaF_2 powder (2g) and sterile distilled water (DW; 5 ml) were mixed for 30 s using a vortex mixer (Scientific Industries Inc., Bohemia, NY, USA) and centrifuged at 3000×*g* for 5 min. The supernatant was used for the MTA-CaF_2 powder test.

For the set MTA-CaF_2 test, MTA-CaF_2 powder (1 g) was mixed with 300 μl of DW. The mixtures were transferred into flexible, circular, acrylic molds (5 mm diameter, 3 mm height) and solidified in an incubator at 37 °C with a relative humidity of 95% for 48 h.

HDPC culture

HDPCs (Axol Bioscience Ltd, Cambridge, UK) were cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin sulfate; Gibco Laboratories, Grand Island, NY, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. Passage 7 to 10 cells were used.

HDPC proliferation

The HDPCs were inoculated in a 12-well microplate (SPL Lifesciences, Pocheon, Korea) at 5×10^3 cells per well in the medium and incubated until the growth of 50% of the wells. The cells were treated with supernatant from MTA and MTA-CaF_2 after changing to fresh medium without FBS for the MTA powder test. For the set MTA test, after removing the medium, the Transwell cell culture insert (3.0 μm pore size; Corning Inc., Lowell, MA, USA), including the MTA disc, was placed in the well. They were then incubated for 24 and 48 h at 37 °C and 5% CO_2 in the medium.

HDPC proliferation was investigated using MTT assay. The HDPCs were treated with 0.4% MTT solution and incubated for 4 h at 37 °C under 5% CO_2 after removing the culture medium. The cells were washed with phosphate-buffered saline (pH 7.2) to remove the non-reacted MTT solution and treated with 95% ethanol for 30 min to dissolve the formazan. The optical densities of the solutions were measured at a wavelength of 570 nm using a spectrophotometer (Biotek, Winooski, VT, USA).

Mineralization-related gene expression

Gene expression was tested with a real-time PCR. The HDPCs were seeded on a 12-well microplate at 5×10^3 cells per well in the medium and incubated until the growth of 80% of the wells. After both MTA powder and set MTA groups were treated in the same way as the proliferation test, HDPCs were incubated at 37 °C under a 5% CO_2 atmosphere in the medium for 24 h.

Real-time RT-PCR

Using TRIzol reagent (Invitrogen Life Tech, Carsbad, CA, USA), total RNA was isolated from the HDPCs. The total RNA (1 μg) was mixed with a Maxime™ RT-premix kit (iNtRON, Seongnam, Korea) and incubated at 45 °C for 1 h to synthesize complementary DNA (cDNA). The cDNA was analyzed with semi-quantitative PCR on an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster, CA, USA). The primers used in this study were ALP, Coll, OCN, Runx2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1). The source of primer list was provided in the previous study.[19]

The mixture underwent a thermal cycle as follows: initial denaturation for 4 min at 95 °C and 40 thermal cycles of 15 s at 95 °C, 15 s at 60 °C, and 33 s at 72 °C. GAPDH, the house-keeping gene, was used to normalize the expression level of the target gene. For investigating the specific amplification of the target gene, the PCR products were analyzed by amplifying the dissociation curve.

Physical properties

Setting time

This test was performed according to the ISO 9917—1 protocol[20] with a Gilmore needle.

MTA-CaF_2 powder (total weight of 1 g) was mixed with 300 μl of DW and placed in an acrylic mold (10 mm diameter, 2 mm height). The samples were incubated at 37 °C and 95% relative humidity. The setting time was taken as the point at which Gilmore needle (113.4 g; 2.12 mm diameter) could no longer indent the surface of the disc. The tests were performed at 1 min intervals, 2 h after mixing the MTA or MTA-CaF_2. The compositions of each group were 0,1, 5, and 10 wt% CaF_2 ($n = 6$).

Solubility test

The solubility of the samples was evaluated according to the ISO 6876 protocol.[21] MTA-CaF_2 (total weight of 1g) were mixed with 300 μl of DW and placed in the acrylic mold (20 mm diameter and 1.5 mm height). The samples were stored for 48 h at 37 °C and 95% relative humidity.

The set samples and dish were weighed to establish a baseline value (W_0). The same material discs were placed in the dish with 50 ml of DW. The dish was covered with plastic wrap and incubated at 37 °C for 24 h. The discs were rinsed with 3 ml DW, and the discs and the dish were dried at 55 °C for 3 days in the dry oven. The discs and the dish were weighed (W_{24}). The solubility of the MTA-CaF_2 was calculated by the difference in weight after 24 h as a percentage of the original weight ($n = 6$).

Table 1 List of primers used for real-time RT-PCR.

Primer		Sequence
GAPDH	F	5′-GTG GTG GAC CTG ACC TGC-3′
	R	5′-TGA GCT TGA CAA AGT GGT CG-3′
Runx2	F	5′-CAC TGG CGC TGC AAC AAG A-3′
	R	5′-CAT TCC GGA GCT CAG CAG AAT AAT-3′
ALP	F	5′-GGA CCA TTC CCA CGT CTT CAC-3′
	R	5′-CCT TGT AGC CAG GCC CAT TG-3′
OCN	F	5′-CGG TGC AGA GTC CAG CAA AG-3′
	R	5′-TAC AGG TAG CGC CTG GGT CT-3′
Coll	F	5′-CTG CTG GAC GTC CTG GTG AA-3′
	R	5′-ACG CTG TCC AGC AAT ACC TTG A-3′

F; forward, R; reverse.
GAPDH; glyceraldehyde-3-phosphate dehydrogenase, ALP; alkaline phosphatase, Coll; collagen type I, OCN; osteocalcin, Runx2; runt-related transcription factor 2.

Surface roughness

Surface profiles of the discs of the material were analyzed by a non-contact surface profiler (Contour Elite K; Bruker, Billerica, MA, USA) and an SEM (EM-30AX plus; COXEM, Deajeon, Korea). The test was repeated at each group before and after soaking in water ($n = 8$).

Statistical analysis

The program used for statistical analysis was SPSS 23.0 (IBM Software, Armonk, NY, USA). 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 were described as median and interquartile range.

Results

HDPC proliferation

MTA and CaF_2 significantly affected HDPC proliferation. MTA groups showed higher proliferation than the control group ($p < 0.05$). However, the MTA-1% CaF_2 group showed no difference compared to the pure MTA group, except the group after 24 h in MTA powder. Comparing 1% and 5% CaF_2 groups, the 5% group showed higher proliferation ($p < 0.05$), but there was no difference between 5% and 10% CaF_2 groups. The differences among groups became less prominent in set MTA than MTA powder (Fig. 1).

Mineralization-related gene expressions of HDPCs

Gene expression profiles of the HDPCs revealed that MTA and CaF_2 increased the mineralization potential. MTA groups showed higher gene expressions ($p < 0.05$). Among the MTA groups, the pure MTA results did not differ from those of the MTA-1% CaF_2. Differences were identified between the 1% and 5% CaF_2 groups, except in Runx2 of the set MTA ($p < 0.05$). When the 5% and 10% CaF_2 groups were compared, the gene expressions showed differences, except in the Runx2 of the MTA powder. When MTA powder and set MTA groups were compared, the expression levels decreased in the set MTA (Fig. 2).

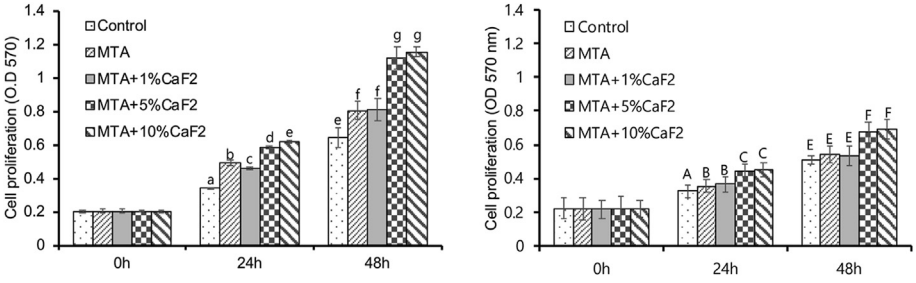

Figure 1 The proliferation of HDPCs treated with MTA powder (left) and set MTA (right). The proliferation of HDPCs after treatment with MTA and MTA-CaF_2 read with a spectrophotometer at 570 nm. Different letters indicate significantly different cell proliferation ($p < 0.05$). MTA and MTA-CaF_2 treatment increased the proliferation of HDPCs ($p < 0.05$). In MTA powder test, after 24 h, each MTA-CaF_2 group showed different effects ($p < 0.05$). After 48 h, there were no differences between pure MTA and 1% CaF_2 groups, or between 5% and 10% CaF_2 groups. In the set MTA test, after 24 h, MTA groups increased the proliferation of pulp cells compared to the control group ($p < 0.05$). There were no differences between pure MTA and 1% CaF_2 groups, or between 5% and 10% CaF_2 groups. After 48 h, the cell proliferation of the control group became similar to those of pure MTA and MTA-1% CaF_2 groups.

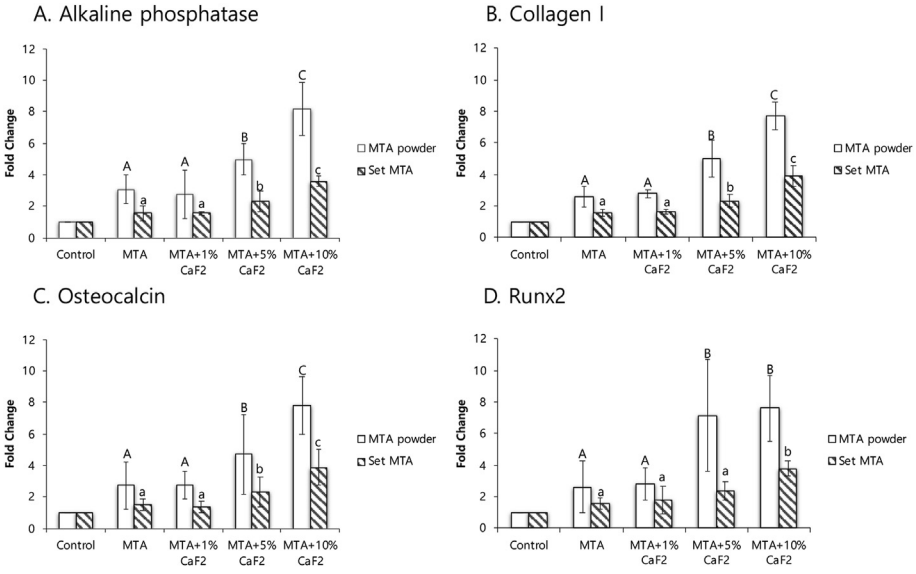

Figure 2 Mineralization-related gene expressions after treatment with powder and set MTA. Different superscripts indicate different expression levels ($p < 0.05$). The odontogenic potential of HDPCs treated with MTA-CaF_2 powder and set MTA-CaF_2 was investigated with relative gene expression using real-time PCR. MTA groups showed higher ALP, ColI, and OCN gene expressions than the control groups ($p < 0.05$). The expression levels increased with the concentration of CaF_2 ($p < 0.05$). Pure MTA and MTA-1% CaF_2 showed similar levels in both powder and set MTA groups ($p > 0.05$). Runx2 showed different odontogenic gene expression profiles to the other genes. MTA groups showed higher expression levels than the control groups ($p < 0.05$). In MTA powder groups, there were no differences between pure MTA and 1% CaF_2 groups, or between 5% and 10% CaF_2 groups. In the set MTA groups, only MTA-10% CaF_2 showed higher expression than the other MTA groups ($p < 0.05$).

Table 2 Setting time and solubility of MTA-CaF_2 mixture.

Groups	MTA	MTA+1%CaF_2	MTA+5% CaF_2	MTA+10% CaF_2
Setting Time (min)	221.0 (11)	224.0 (13)	226.0 (13)	238.0 (10)*
Solubility (%)	1.750 (0.336)	1.871 (0.354)	1.786 (0.218)	1.750 (0.415)

Pure MTA set in 221.0 (11) min, and differences among the groups are 3—17 min. But the statistical difference is detected only at MTA-10% CaF_2 group ($p < 0.05$). * means different setting time ($p < 0.05$).
Solubility of MTA and MTA-CaF_2 was calculated by the weight differences as a percentage of the original weight. The tested materials showed a 1.750—1.871% solubility. However, there were no differences among the groups ($p > 0.05$). All data are described as median and interquartile range.

Physical properties

Setting time

Setting time increased after the addition of CaF_2. Pure MTA set in 221.0 ± 11 min and differences among the groups are 3–17 min. But the statistical difference is detected only at MTA-10% CaF_2 ($p < 0.05$) (Table 2).

Solubility

The tested materials showed a 1.750–1.871% solubility. However, there were no differences among the groups ($p > 0.05$) (Table 2).

Surface roughness

Surface profiles showed the different surface heights. The addition of CaF_2 or soaking in the water resulted in a bit of difference (Table 3). MTA and MTA-CaF_2 showed diffuse deposits of different sizes and shapes from the SEM images. The depositions were more evident on MTA-CaF_2 discs than pure MTA discs, and washed after soaking (Fig. 3). However, there was no statistical difference among the groups.

Discussion

According to the previous studies, the addition of NaF to calcium silicate cement causes a delay in setting time and increases expansion and long-term apical sealing in the root canal.[22,23] When the concentration of NaF increased, the solubility of F-treated MTA also increased.[22–24] In the investigations of calcium silicate cement with CaF_2, CaF_2-treated tricalcium silicate showed better bioactivity, a lower heat generation, higher compressive strength,[25] and faster apatite formation[26] than pure tricalcium silicate.

Since endodontic materials are placed in close contact with vital pulp or periradicular tissues, they should be biocompatible. Clinically, MTA is inserted into the pulp chamber or root canals immediately after mixing. For investigating the direct effect of MTA-CaF_2, the eluates from MTA-CaF_2 powder were also used.

MTA improved the proliferation of HDPCs, and the addition of CaF_2 increased this effect. This result corresponds to previous studies that fluoride and MTA stimulate the proliferation of pulp cells and osteoblasts.[9,27] The current study suggests that 5% CaF_2 may be a good recommendation to increase the proliferation of HDPCs. MTA or MTA-CaF_2 improved HDPC proliferation after 24 h in both treatments of MTA powder and set MTA. Cell proliferation decreased in set MTA groups. It is speculated that the soluble fraction released from set MTA-CaF_2 was less than that of MTA-CaF_2 powder. Only the soluble fraction from MTA and CaF_2 can potentially affect the results.

Mineralization-related markers, such as ALP, Coll, OCN, and Runx2 were investigated. These markers showed higher expression in MTA groups than the control. These results are consistent with the previous studies those calcium silicate cement and fluoride upregulated odontogenic differentiation.[11,12,28,29] Based on the results, the addition of at least 5% of CaF_2 is recommended for improving the mineralization potential of MTA.

Former studies have shown improved osteogenic gene expression or more apatite formation with 1% NaF addition.[8,13] In this study, the addition of more than 5% CaF_2 increased mineralization-related gene expression. As the solubility of CaF_2 is lower than that of NaF, higher concentrations of CaF_2 may be required.[14,15]

On the other hand, 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.[25] Therefore, it could be presumed that the mineralization effect of MTA-CaF_2 is related to the fluoride more than the alkalinizing activity of MTA. The dissolved fluorine from CaF_2 could be the key to the proliferation and osteogenic stimulation of the HDPCs. However, the amount of fluorine and calcium ion released from CaF_2 and MTA, or the effect of CaF_2 on calcium release from MTA were not analyzed in this study. For a better understanding, chemical analysis is also needed.

Long setting time is one of the significant disadvantages of MTA.[4] The addition of NaF to calcium silicate delayed setting time,[22] so the effect of CaF_2 on MTA setting time was tested. From our experiment, the addition of CaF_2 delayed the setting of MTA; however, only the MTA-10% CaF_2 group showed a significantly longer setting time ($p < 0.05$).

Even though initial solubility is necessary for the hydration of MTA to form alkaline calcium silicate gel, high solubility after setting will affect the sealing effect and result in microleakage. Most studies reported low solubility for MTA,[30–33] but some long-term studies reported increased solubility with time.[34] In this study, the tested materials showed 1.750–1.871% solubility, which is lower than the 3% specified by the ISO 6876 protocol.[21] There was no difference among the groups.

Dental materials come in contact with tissues, and therefore, the surface morphology is important, at least for in vitro cell culture studies, in relation to cell attachment.[35] After soaking in DW, the roughness changed, but the difference was not significant. From this result, the addition of CaF_2 could be considered not to reduce HDPC adhesion. In

Table 3 Surface roughness of MTA-CaF_2 mixture before and after soaking in DW (Ra, μm).

Soaking	MTA	MTA+1% CaF_2	MTA+5% CaF_2	MTA+10% CaF_2
Before	2.80 (1.79)	2.88 (1.35)	2.63 (0.94)	2.80 (0.668)
After	2.78 (0.81)	3.07 (0.61)	2.53 (1.98)	3.35 (1.25)

A non-contact surface profiler analyzed the surface profiles of the materials. Ra (arithmetical mean roughness value) is the average of a set of individual measurements of surface peaks and valleys.
The addition of CaF_2 or soaking in the water resulted in a bit of difference. However, there was no statistical difference among the various MTA groups ($P > 0.05$). After soaking, roughness increased in 1% and 10% CaF_2 groups, but differences were not significant. The data are represented as median and interquartile range.

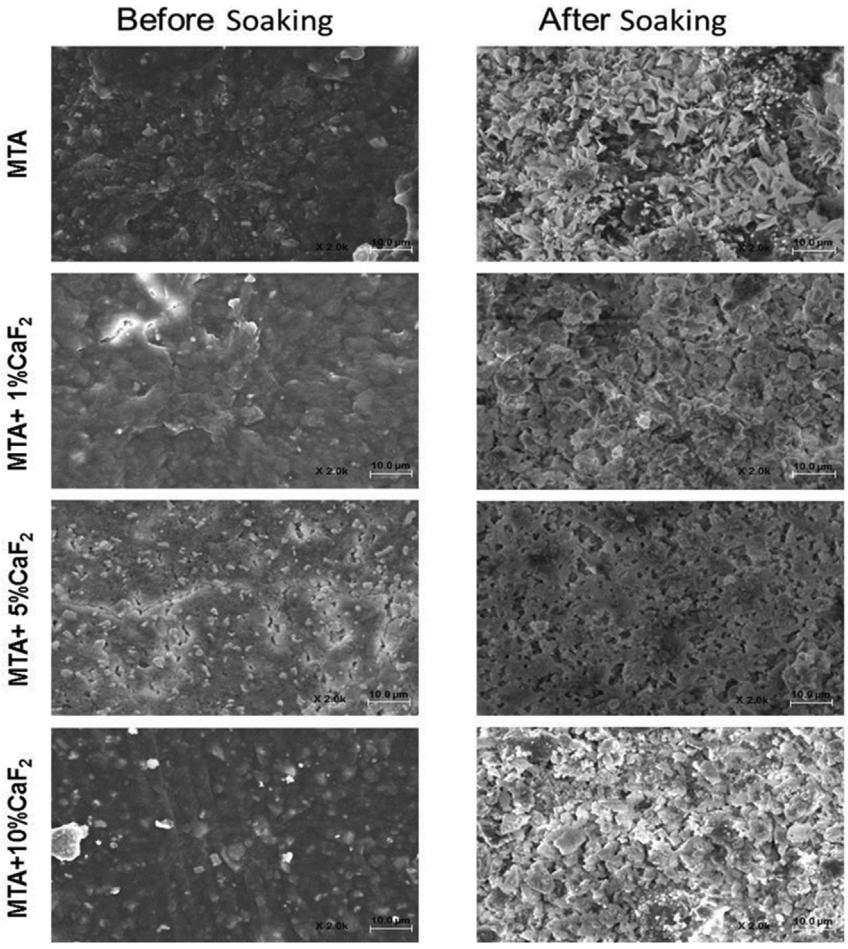

Figure 3 SEM images (x2000) of MTA and MTA-CaF_2 discs before and after soaking in DW. SEM images of soaked and non-soaked discs of the same material were taken with an SEM. MTA and MTA-CaF_2 showed diffuse deposits of different sizes and shapes. The depositions were more evident on MTA-CaF_2 than MTA, washed after soaking.

the previous study, fluoride-enriched MTA produced a more granular surface, and the osteoblasts attached readily to MTA regardless of NaF addition. Therefore, the function of fluoride should be focused on biochemical signals, not on the adhesion-related effect.[8]

MTA is more soluble in DW than in isotonic solutions, which do not simulate clinical conditions. The test also provided enough setting time for the cement to reach its final hardness. For clinical relevance, solubility should be tested immediately after application, and could involve slow submergence, preferably into a physiological solution or into the blood.[36]

In conclusion, the addition of more than 5% CaF_2 could be considered to increase the regeneration potential of pulp cells without adverse effects on physical property. However, clinical conditions, such as long-term irritation after pulp exposure and inflammation, may lead to another outcome. Therefore, further studies, including in vivo study and, chemical analysis, long-term evaluation, are necessary for clinical application.

Declaration of competing interest

The authors declare no conflicts of interest.

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