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Comparison of Bacterial Leakage Resistance of Various Root Canal Filling Materials and Methods: Confocal Laser-Scanning Microscope Study

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Summary: This study evaluated the bacterial leakage resistance and root canal lining efficacy of various root canal filling materials and methods by using confocal laser-scanning microscope (CLSM). Sixty extracted human premolars with mature apex and single root canal were randomly divided into 2 control groups and 4 experimental groups. Group CW was filled with continuous wave technique using gutta-percha and AH Plus sealer. Group GC was coated with AH-Plus sealer and then obturated with soften GuttaCore. Group GF was obturated using GuttaFlow and gutta-percha. Group EM was filled with EndoSeal MTA and gutta-percha using ultrasonic vibration. The AH-Plus, GuttaFlow, and EndoSeal were labeled with Hoechst 33342 to facilitate fluorescence. The obturated root tip was incubated with Carboxyfluorescein diacetate succinimidyl ester (CFSE)-stained *E. faecalis* for 14 days. CLSM was performed to evaluate the sealer distribution and bacterial leakage for the apical 1-, 2-, 3-mm specimens. Statistically significant differences were determined by 1-way ANOVA with Tukey's post-hoc test and Pearson's correlation analysis. Group EM showed the better sealer distribution score than the other groups ($p < 0.05$). Group CW and group GC exhibited the less bacterial leakage than the group GF, while group EM showed the similar bacterial leakage score with the groups CW and GC. There was no significant correlation between the sealer distribution and bacterial leakage ($p > 0.05$). Under the conditions of this study, different root canal filling materials and methods showed different efficacy for canal distribution and bacterial leakage resistance. SCANNING 37:422–428, 2015. © 2015 Wiley Periodicals, Inc.

Key words: bacterial leakage, confocal laser scanning microscope, GuttaCore, GuttaFlow, root canal sealer, sealer distribution

Introduction

Root canal fillings aim to seal the root canal system to prevent reinfection of the periapex. Obturation should eliminate all routes of leakage from the oral cavity and the periradicular tissues into the root canal system by creating a fluid-tight seal. Therefore, ideally, root canal filling materials, and endodontic sealers should seal the canal laterally and apically and have good adaptation to the root canal dentin (Özcan *et al*., 2011; Chandra *et al*., 2012). The materials and methods used for root canal obturation are one of the critical determinants for the success or failure of endodontic treatment (Bodrumlu and Tunga, 2007).

Obturation of the root canal space has been performed using various techniques. The most commonly used technique is cold lateral condensation using gutta-percha (GP) cones which allows good length control and is predictable (Gutmann *et al*., '93). The "continuous wave of condensation" which was designed to simplify the vertical condensation method has been found superior or similar to that provided by the lateral condensation (Buchanan '98; DuLac *et al*., '99; Smith *et al*., 2000; Özcan *et al*., 2013). It is claimed that the heat source allows sufficient heat for the apical GP to be softened and adapted to the irregularities of the intracanal anatomy (Kytridou *et al*., '99; Gilbert *et al*., 2001). Thermoplasticized GP techniques have

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been advocated for root canal obturation because they may provide a more homogeneous obturation and better adaptation to the canal walls (Kytridou *et al*., '99) which might result in a lower leakage compared with lateral condensation. GuttaCore (DENTSPLY Tulsa Dental Specialties, Tulsa, OK) is one of the carrier-based GP obturation system. As an improvement over the previous Thermafil technique, GuttaCore obturator carriers are not made from plastic, but from a GP elastomer with intermolecular cross links (Li *et al*., 2014). This makes the procedures not only rapid and high-quality three-dimensional root canal obturaion, but also easy post space preparation and root filling removal in a case where retreatment is required. EndoSeal MTA (Maruchi, Wonju, Korea) was recently introduced in a premixed paste type MTA-based sealer with the characteristics of hardening even at the complex and moist canal environment. The company also claims that the ultrasonic method using the EndoSeal MTA may reduce lateral and vertical forces applied to the root dentin during filling procedure. GuttaFlow (Coltene Whaledent, Alstatten, Switzerland), a new flowable root canal filling paste, was introduced as a non-heated flowable obturation system that combines both the sealer and the gutta-percha in 1 injectable system (Li *et al*., 2014). The manufacturer claims that this material has good homogeneity and adaptation to the root canal walls owing to its better flow properties.

As above, new obturation biomaterials and methods have been introduced over the past decades to improve the seal of the root canal system. However, it is not clear whether they really produce a three-dimensional impervious seal that is important for reducing root canal reinfection. Therefore, the purpose of this study was to evaluate the bacterial leakage resistance and root canal lining of various root canal filling materials and methods by using confocal laser-scanning microscope (CLSM).

Materials and Methods

Preparation of Teeth and Canal Obturation

Sixty extracted human premolars with mature apex and single root canal were used in this study. After preparing a conventional access cavity, a size 10 K-file was inserted into the canal until it was just visible at the apical foramen. Working length was determined by subtracting 0.5 mm from this length. The root canal of each tooth was cleaned and shaped using sizes S1, S2, F1, and F2 ProTaper Universal nickel-titanium files (Dentsply Maillefer, Ballaigues, Switzerland) and finally with the size #35/0.04 taper nickel-titanium file (BLX; B&L Biotech, Ansan, Korea). The root canals were irrigated profoundly using 2.5% sodium hypochlorite between each instrument. Then canals were rinsed with 17% EDTA solution (MD-cleanser; Meta Biomed, Chungju, Korea) for 1 min to remove smear layer, followed by flushing again with 2 ml of sodium hypochlorite. The root canals were dried with paper point. After biomechanical preparation, all the teeth were sterilized in an autoclave for 20 min at 121˚C. The teeth were randomly divided into two control groups and four experiment groups using continuous wave of condensation technique, GuttaCore obturation system, GuttaFlow system, and EndoSeal MTA, respectively ($n = 10$). To facilitate fluorescence under confocal microscopy, AH Plus, GuttaFlow, and EndoSeal were labeled with Hoechst 33342 (Sigma, St. Louis, MO).

Group CW (Continuous wave of condensation technique with GP and AH Plus sealer): Each canal was filled with epoxy resin-based sealer (AH Plus; Dentsply Caulk, Milford, DE) and a size #35/0.04 taper GP master cone using Duo-Alpha and Duo-Beta system (B&L Biotech). The GP was down-packed with a Duo-Alpha heat source to the 4 mm from the working length and backfilled using a Duo-Beta.

Group GC (GuttaCore obturation system with AH Plus sealer): The canals were coated with a thin layer of AH Plus sealer using K-file. The size 30 GuttaCore obturator was softened in the dedicated GuttaCore oven and slowly inserted to the working length. The carrier was twisted off and the filled material was compacted at the orifice.

Group GF (GuttaFlow obturation system with GP): Teeth were obturated using the GuttaFlow and GP as the manufacturer's instructions. GuttaFlow provided in a special capsule was mixed in the triturator for 30 s. The capsule was then loaded on the dispenser with attached canal tip. GuttaFlow was layered slowly into the apical canal part. The master GP cone was coated with GuttaFlow and inserted in the canal. The master GP cone was cut at the orifice level.

Group EM (Ultrasonic condensation with EndoSeal MTA and GP): The EndoSeal MTA was prepared as the manufacturer's instruction and loaded in a metal tip (Centrix; Centirx, Shelton, CT). EndoSeal MTA was then injected into coronal and middle root canal. By using lentulo spiral, EndoSeal MTA was adjusted to middle and apical root canal. Then the master GP cone was inserted into the canal. By ultrasonic vibration to the pincette which hold the master cone, the GP cone could reach all the way to working length. The master GP cone was cut at the orifice level.

Group PC (Positive control; $n = 10$): The canals of this group were not filled.

Group NC (Negative control; $n = 10$): The canals of this group were filled with GP and AH plus sealer. The negative control group was sealed around entire root surface with colored nail varnish.

Except the Group NC, the roots of 50 teeth were sealed with colored nail varnish, except for the apical 1 mm around the apical foramen.

Bacterial Leakage Experiment

Enterococcus faecalis (*E. faecalis* strain ATCC 19433) were grown in Brain Heart Infusion (BHI) broth (DIFCO, BD science, San Jose, CA) under aerobic conditions at 37˚C. The bacteria were resuspended in phosphate buffer saline (PBS) at 5×10^7 CFU/ml and stained with cellTrace Carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular probes, Life technology, Carlsbad, CA) following the manufacture's instruction. Briefly, *E. faecalis* were stained with 10 μM of CFSE at 37˚C for 10 min. After incubation, cold PBS was added to stop the staining and *E. faecalis* were thoroughly washed. The prepared CFSE-stained *E. faecalis* were inoculated in BHI broth. After cutting the tapered end of 2 ml Eppendorf plastic tube (Eppendorf-Elkay, Shrewsbury, MA), the obturated root tip was inserted into the tube containing BHI broth inoculated with CFSE-stained *E. faecalis* until the root tip protruded through the end. The entire assembly was then incubated at 37˚C for 14 days.

Specimen Preparation and Confocal Laser-Scanning Microscope for Scoring

The samples were washed with PBS and specimens were sectioned at every 1 mm from 1 to 3 mm level of apex using a low speed diamond disk (Horico, Berlin, Germany). The procedure was done with minimum pressure under water cooling to minimize the smearing of the GP.

All samples were observed by CLSM (LSM 700; Carl Zeiss, Oberkochen, Germany) at each 1 mm specimen. Each 10 samples were evaluated for a blue fluorescent ring around the canal wall, indicating the sealer-dye distribution. The ratio of bacterial penetration was detected for a green spot around the canal wall, indicating the CFSE-stained *E. faecalis*. At each level, tooth surface was divided into eight parts of same arc and if there was fluorescence (Blue or Green) in a eighth, 0.125 point was given (Fig. 1). Because the score given in each level is not clinically available to evaluate the efficiency of the materials and methods, the final NET score for each specimen was given by summing up of the points from the levels of 1 mm to 3 mm of apical root.

Statistics

Quantitative data were tabulated and analyzed for 1-way analysis of variance (ANOVA) and Tukey's post-hoc tests using Graphpad Prism software (GraphPad Software, San Diego, CA). The correlation between sealer distribution and bacterial leakage was presented by Pearson's correlation analysis. The statistical significance was set at a confidence level of 95%.

Results

The representative CLSM results of sealer distribution and bacterial leakage of the tested groups are shown in Figure 2. The specimens of positive control group show a consistent green fluorescent ring around the canal wall, indicating the bacterial penetration into the canal (Fig. 2(A)). The blue fluorescent ring around the canal wall of negative control group represents the sealer distribution in canal wall (Fig. 2(B)).

The results of the quantitative evaluation of the penetration of AH Plus, GuttaFlow, and EndoSeal MTA stained with Hoechst 33342 are shown in Table I and Figure 3 (A and B). Group EM showed the better sealer distribution score than the other groups ($p < 0.05$). The results of bacterial leakage scores at 1, 2, and 3 mm levels from the apex and net score are presented in Table I and Figure 3 (C and D). Group CW and group GC exhibited the less bacterial leakage than the group GF, while group EM showed the similar bacterial leakage score with the groups CW and GC (Table I). As shown in Figure 3E, there was no significant correlation between the sealer penetration and bacterial leakage ($p > 0.05$).

Discussion

The elimination of bacteria from the root canals by cleaning and shaping procedures and the prevention of recontamination of the obturated root canal are fundamental for the successful treatment of apical periodontitis (Ray and Trope, '95). Healing of periapical disease involves a combination of bacterial eradication during treatment through chemomechanical means along with sealing of the root canal (Ray and Trope, '95). Recently, new materials for root canal filling and methods for these materials were introduced. This study evaluated the various root canal filling materials and methods by comparing the sealer distribution to canal wall and bacterial leakage resistance under the CLSM.

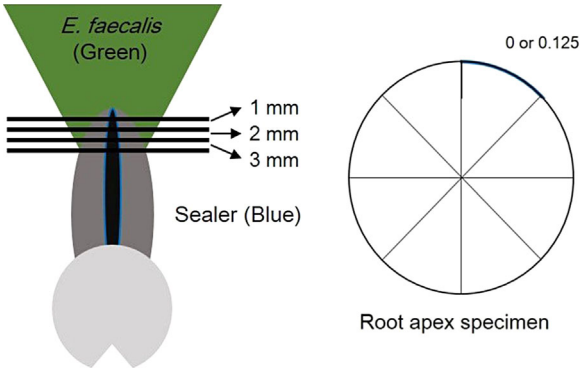

Fig. 1. The experimental designs for specimen preparation and scoring of fluoresences.

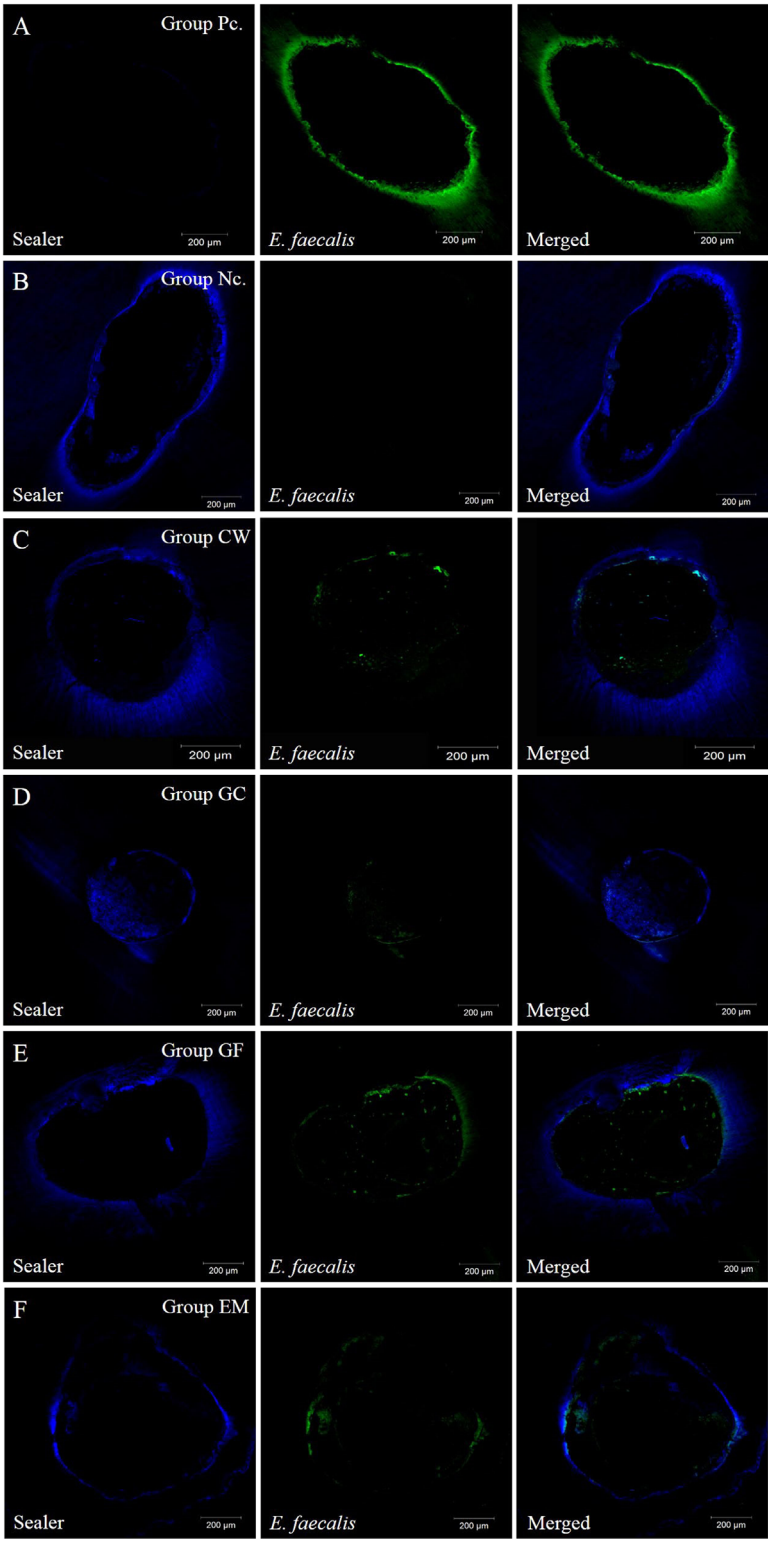

Fig. 2. The representative CLSM results of control groups and experimental groups: A. Group Pc (Positive control). B. Group Nc (Negative control). C. Group CW (continuous wave of condensation technique with GP and AH Plus sealer). D. Group GC (GuttaCore system with AH Plus sealer). E. Group GF (GuttaFlow obturation system with GP). F. Group EM (Ultrasonic condensation with EndoSeal MTA and GP).

TABLE I The sealer distribution score (%) and bacterial leakage score (mean ± SD)

Group	Level Score	1 mm	2 mm	3 mm	Net
Group CW	sealer distribution	85 ± 8	91 ± 8	91 ± 6	89 ± 4A
	bacterial leakage	0.35 ± 0.15	0.33 ± 0.11	0.29 ± 0.12	0.33 ± 0.07a
Group GC	sealer distribution	86 ± 9	86 ± 9	90 ± 5	88 ± 3A
	bacterial leakage	0.46 ± 0.15	0.27 ± 0.05	0.29 ± 0.10	0.32 ± 0.09a
Group GF	sealer distribution	85 ± 9	90 ± 5	88 ± 7	88 ± 4A
	bacterial leakage	0.54 ± 0.06	0.46 ± 0.05	0.36 ± 0.04	0.44 ± 0.07[b]
Group EM	sealer distribution	90 ± 8	93 ± 6	97 ± 5	93 ± 4[B]
	bacterial leakage	0.38 ± 0.12	0.39 ± 0.12	0.32 ± 0.06	0.37 ± 0.08a[b]

CLSM technique may be useful as a complement to the established microbiological, histologic, standard electron microscopy, and PCR-based techniques for the identification of viable bacteria (Scivetti *et al*., 2007). In the present study, CLSM offers advantages to evaluate clearly the distribution and interfacial adaptation of root canal sealers and the bacterial infiltration (Gharib *et al*., 2007). Meanwhile, because *E. faecalis* is also one of the most commonly isolated microbes from the root canal in secondary infections (Baumgartner and Falkler, '91; Timpawat *et al.*, 2001), this bacterial species was used in this study.

Ideally, a root canal sealer should be capable of producing a bond between the core material and the root dentin for preventing leakage effectively. A wide variety of root canal sealers are commercially available, however

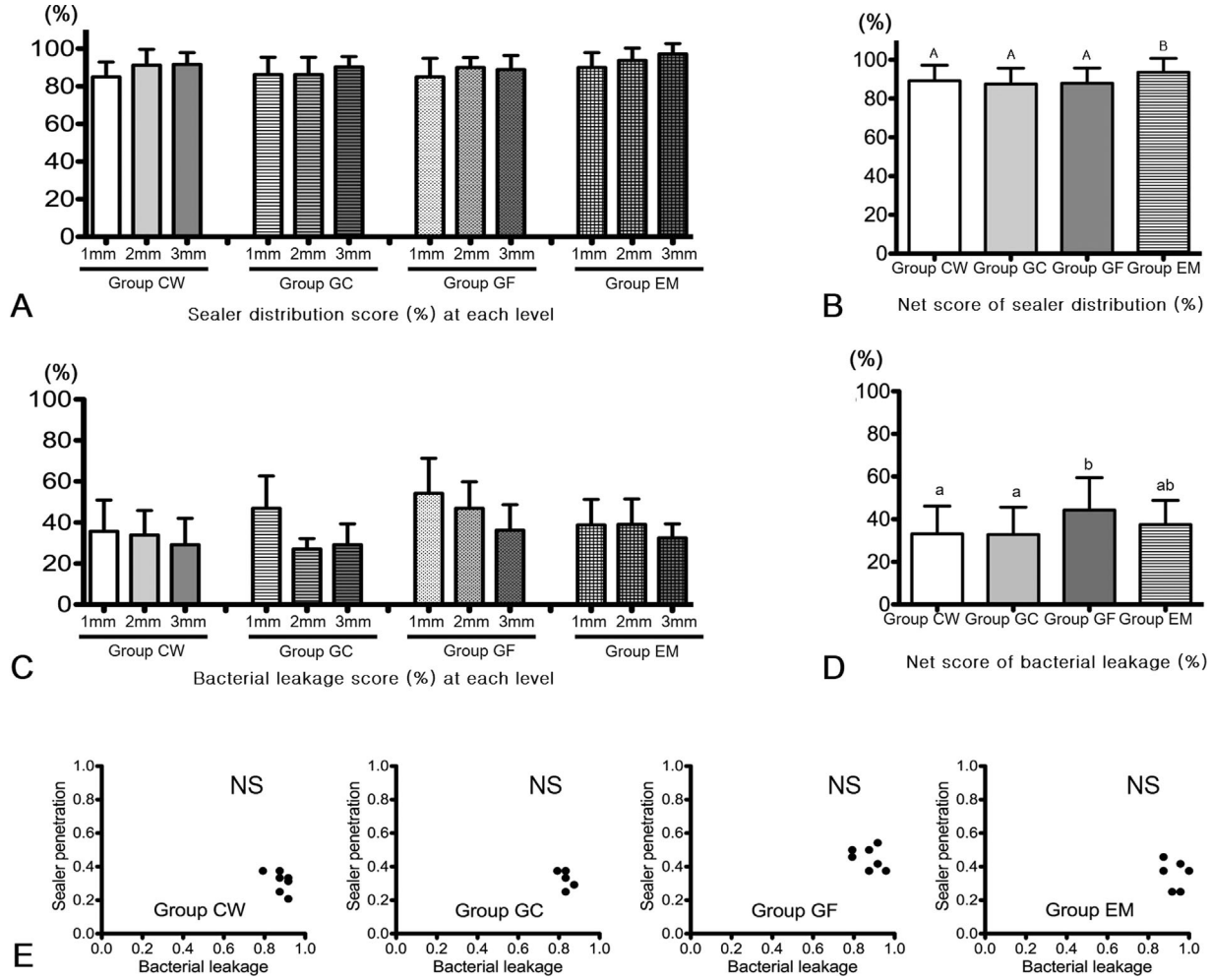

Fig. 3. A. The ratio of sealer distribution (%) in the 3 different levels. B. The net score of sealer distribution (%) of each group (groups with different superscripts have significant difference, $p < 0.05$). C. The score of bacterial leakage in the 3 different levels. D. The net score of bacterila leakage of each group (groups with different superscripts have significant difference, $p < 0.05$). E. The correlation between sealer penetration and bacterial leakage has no statistical significance (Pearson analysis, NS: not significant).

there is lack of consensus on which material seals the most effectively. AH Plus is an epoxy resin-based sealer. Some studies reported the apical seal of resin-based sealers are superior to the sealing ability of other sealers and shown to effectively seal the root canal (Oguntebi and Shen, '92; Cobankara *et al*., 2002; Wu *et al*., 2002; Gençoglu *et al*., 2003). These were reported as to be related to the slight expansion during their setting reactions (Ørstavik *et al*., 2001). In present study, it could be a same reason that the two groups of CW and GC had the less bacterial leakage than the groups which were AH Plus used. These results are coincidence with some previous reports regarding the efficacy of filling techniques (DuLac *et al*., '99; Silver *et al*., '99; Smith *et al*., 2000; Wu *et al*., 2001). The continuous wave of condensation technique has been found to be superior to some other canal filling techniques in terms of less apical leakage (DuLac *et al*., '99; Smith *et al*., 2000). However, some studies have found poor adaptation of GP to the canal wall by this technique (Silver *et al*., '99; Wu *et al*., 2001). Wu *et al*. (2001) reported that the apical GP ratio was low in some specimens and the sealer was too thick.

Meanwhile, the present results showed that group GF had significantly higher bacterial leakage than the continuous wave of condensation technique and GuttaCore system. However, on the contrary to the present results, Brackett *et al.* (2006) found no significant difference in sealing ability between Gutta-Flow and vertically compacted GP used with AH Plus sealer. In a study by Hammad *et al.* (2008) GuttaFlow also showed good penetration into the dentinal tubules, and thus to help in preventing leakage. The good sealing ability exhibited by the GuttaFlow root-canal filling material could be attributed to its ability to flow into lateral grooves and depressions (Zielinski *et al.*, 2008).

On the other hand, considering the sealer distribution score, group GF had the similar efficacy with the groups CW and GC to line the canal wall but lower than the group EM which had the highest distribution score. The use of "ultrasonic" condensation method seemed to make the higher distribution score in the group EM. Ultrasonic vibration may have improved the flowability to ease in filling procedure or adjusting for varying working lengths and complex anatomy.

The EndoSeal MTA is similar to MTA Fillapex (Angelus, Londrina, PR, Brazil) which is a new calcium silicate-based sealer containing MTA and is known to possess favorable biocompatibility, antimicrobial activity, and good sealing ability (Torabinejad and Parirokh, 2010). EndoSeal MTA has a special concept of ultrasonic condensation (12). The ultrasonic vibration for sealer condensation may have brought positive effects to seal the canal and make higher sealer distribution scores and lining efficacy in this study. MTA Fillapex which has the similar composition mainly with MTA was also found to have greater flow values than AH Plus (Zhou *et al*., 2013).

GuttaCore represents the latest generation of carrier-based root canal filling material that uses thermoplasticised GP as the core materials. In this present study, GuttaCore obturation system used with AH Plus sealer exhibited the less bacterial leakage than group GF and similar with the others. The reason for this finding might be similar with that found in group CW used with AH Plus sealer. The core-carrier may have enhanced the adaptation of GP to the canal wall and the flow of the molten GP material into irregular canal spaces. Previous studies reported that canals obturated with core-carrier techniques had the highest GP content within the filled canal space (Gençoglu, 2003; De-Deus *et al*., 2006).

The flow of a sealer determines how effectively it fills accessory canals, irregularities on the dentinal wall, and spaces between the core filling materials (Zhou *et al*., 2013). However, as found in Figure 3E in this study, the correlations of sealer distribution and bacterial leakage were not significant. It might be due to the technical sensitivity in filling procedures, although sufficient preliminary tests were done to minimize the procedural errors before the experimental tests. Probably the chemical and mechanical properties may have brought the different bacterial leakage resistances which were not correlated with the sealer distribution and penetration extents. This phenomenon should be studied further to see any potential correlations. Clinically, it is highly recommended to follow the manufacturers' instructions and do some sub-clinical practices using extracted teeth.

It is important to assess microbial leakage not only immediately after root canal filling but also with some time elapsed, because root canal sealing needs to be long lasting for clinical effectiveness. Thus, further studies are required to test the bacterial leakage with some time elapsed as well as thermos-mechanical loading considering clinical effects.

Conclusion

Within the limitations of present study, four different root canal filling materials and methods showed significant differences in their canal sealing efficiency and bacterial leakage resistance without correlations between the two variables.

Acknowledgment

Ji Hee Hwang and Jin Chung contributed equally to this work and have the first authorship shared.

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Dynamic Intratubular Biomineralization Following Root Canal Obturation With Pozzolan-Based Mineral Trioxide Aggregate Sealer Cement

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Summary: The application of mineral trioxide aggregates (MTA) cement during the root canal obturation is gaining concern due to its bioactive characteristic to form an apatite in dentinal tubules. In this regard, this study was to assess the biomineralization of dentinal tubules following root canal obturation by using pozzolan-based (Pz-) MTA sealer cement (EndoSeal MTA, Maruchi). Sixty curved roots (mesiobuccal, distobuccal) from human maxillary molars were instrumented and prepared for root canal obturation. The canals were obturated with gutta-percha (GP) and Pz-MTA sealer by using continuous wave of condensation technique. Canals obturated solely with ProRoot MTA (Dentsply Tulsa Dental) or Pz-MTA sealer were used for comparison. In order to evaluate the biomineralization ability under different conditions, the PBS pretreatment before the root canal obturation was performed in each additional samples. At dentin-material interfaces, the extension of intratubular biomineralization was analyzed using scanning electron microscopy (SEM) and energy dispersive spectroscopy. When the root canal was obturated with GP and Pz-MTA sealer, enhanced biomineralization of the dentinal tubules beyond the penetrated sealer tag was confirmed under the SEM observation ($p < 0.05$). Mineralized apatite structures (calcium/phosphorous ratio, 1.45–1.89) connecting its way through the dentinal tubules were detected at 350–400 μm from the tubule orifice, and the pre-crystallization seeds were also observed along the intra- and/or inter-tubular collagen fiber. Intratubular biomineralization depth was significantly enhanced in all PBS pretreated canals ($p < 0.05$). Pz-MTA cement can be used as a promising bioactive root canal sealer to enhance biomineralization of dentinal tubules under controlled environment. SCANNING 38:50–56, 2016. © 2015 The Authors. *Scanning* Published by Wiley Periodicals, Inc.

Key words: biomineralization, dentinal tubule, pozzolan-based MTA sealer, pre-crystallization seeds, scanning electron microscopy

Introduction

Endodontic treatment is an ongoing process to eliminate infection source and to create a fluid-tight seal of the root canal system (Siqueira, 2001). Theoretically a root canal filling material which can completely seal the root canal system would be of ideal in practice. However, several studies confirmed that currently available root canal obturation materials such as gutta-percha (GP) and/or polymer-based materials showed incomplete sealing even with the aid of sealers or dentin bonding systems (Zmener *et al*., 2008; Santos *et al*., 2010; Brosco *et al*.,2010; Punia *et al*., 2011).

Mineral trioxide aggregate (MTA) has been widely used in variety of applications including root-end filling, perforation repair, or apical/coronal sealing material during regenerative endodontic procedures (Parirokh and Torabinejad, 2010). The most favorable property, leaving its biologic properties aside, is the superior sealing ability

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which comes from the water-resistant final product after hydration. This sealing ability of MTA is largely attributable to its bioactive capacity to form an apatite layer when it is in contact with phosphate-containing physiological fluids (Tay *et al.*, 2007; Reyes-Carmona *et al.*, 2009; Gandolfi *et al.*, 2010; Han *et al.*, 2010). Such characteristic features of MTA appear to be important in biomineralization of dentinal tubules for enhanced sealing of the root canal system (Tay *et al.*, 2007; Reyes-Carmona *et al.*, 2010; Yoo *et al.*, 2014), thus makes it a good candidate for root canal filling material of choice.

However, MTA cannot be recommended as a routine orthograde root canal filling material because the sandy property and irretrievability of the substance (Bogen and Kuttler, 2009) have made it challenging to be used in a complicated root canal system. Inadequate water-to-powder ratio, insufficient packing also impedes adaptation of MTA to the canal wall (Fridland and Rosado, 2003; El-Ma'aita *et al.*, 2012; Saghiri *et al.*, 2012). In order to overcome such limitations of MTA as a root canal filling material, recent study has utilized MTA sealer cement during the root canal filling procedure (Camilleri *et al.*, 2011).

EndoSeal MTA (Maruchi, Wonju, Korea), a finely pulverized pozzolan-based MTA was recently introduced. The pozzolan cement, the main component of this sealer, gets cementitious properties after pozzolanic reaction which includes calcium hydroxide and water, and enables sufficient flow of the pre-mixed substrate though injection tip with adequate working consistency. The favorable mechanical characteristics such as fast setting time (around 4 min), higher washout resistance than other commercially available MTAs, and biologic effects including biocompatibility, mineralization potential, and odontogenic effect of the pozzolan cement had been previously reported (Choi *et al.*, 2013; Jang *et al.*, 2013a,b; Park *et al.*, 2014; Song *et al.*, 2014). However, no study has confirmed the intratubular biomineralization ability of this sealer material when it is applied in the root canal yet. Therefore, this study was aimed to investigate and compare the biomineralization ability of this pozzolan-based (Pz-) MTA sealer cement under various root canal obturation conditions.

Materials and Methods

Tooth Preparation

The protocol of this study was approved by the Institutional Review Board of the Seoul National University Dental Hospital, Seoul, Korea. A total sample size of 56 roots was calculated to be sufficient to detect significant differences (alpha at level 0.05, 90% power). Sixty curved roots (mesiobuccal and distobuccal roots) less than 30 degree (Schneider, '71) with fully formed apices from human maxillary molars were used in this study. Teeth with root cracks or defects confirmed under a microscopic evaluation (OPMI Pico; Carl Zeiss, Germany) were excluded from the study. All teeth were radiographically examined to evaluate the canal curvature, by measuring the angle between the long axis of the root and the line connecting the point that begins to move away from the long axis of the root to the apex (Schneider, 1971). The overall mean canal curvature was 15.28 ± 7.08 degree, and the roots were assigned to 6 sets (10 canals each) according to the canal curvature by block randomization (Fig. 1(A)).

The teeth were accessed with #4 round carbide burs and Endo-Z burs. The canal patency was gained using a size #10 stainless-steel (SS) K-file (Dentsply Maillefer, Ballaigues, Switzerland) until the tips were visible at the apical foramen. The working length was determined as 1 mm short from the measured length. After coronal flaring using Gates Glidden burs #2 to #4 (Komet, Rock Hill, SC), root canals were instrumented to an apical size of #35 and 0.06 taper with a crown-down technique using ProFile 0.04 and 0.06 Ni-Ti rotary instruments (Dentsply Maillefer). The root canals were irrigated with 2 ml of 5.25% sodium hypochlorite (NaOCl) solution between instrumentation, and immersed with 17% ethylenediaminetetraacetic acid (EDTA) solution (pH 7.2) for 1 min before final flush with 2.5 ml of 5.25% NaOCl solution. All irrigants were activated by using ultrasonic devices (P5 Newtron® XS; Satelec, Acteon group, Mérignac, France). Then, the canals were copiously rinsed with sterile distilled water and dried with sterile paper points.

For the root canal obturation procedure, 10 canals were obturated with MAF size- and taper- tailored GP cone and Pz-MTA sealer (EndoSeal MTA) by using continuous wave of condensation technique (CW). For the comparison control, another 10 canals were obturated solely with Pz-MTA per se. The pre-mixed Pz-MTA sealer cement was released via injection syringe and tip system, and the final coronal portion was tidied up with SS hand pluggers. The canals ($n = 10$) filled with ProRoot MTA (Dentsply Tulsa Dental, Tulsa, OK) according to the obturation technique suggested by Bogen and Kuttler (2009) were also used as a positive control. The ProRoot MTA was mixed with distilled water according to the manufacturer's instructions and placed incrementally with a carrier gun. An SS K-file, 1 or 2 sizes smaller than master apical file (MAF) was used to compact the apical 3–4 mm, then a progression of K-files sizing upward incrementally were used for further compaction. The final coronal portion was tamped by using SS hand pluggers to complete the root canal obturation.

For the phosphate buffered saline (PBS) pretreatment, additional 10 canals were assigned to each experimental group. They were immersed in sterile PBS solution for a minute and dried with sterile paper points before root canal filling.

Following the obturation, the teeth were stored at 37°C with 100% humidity for a day to allow complete

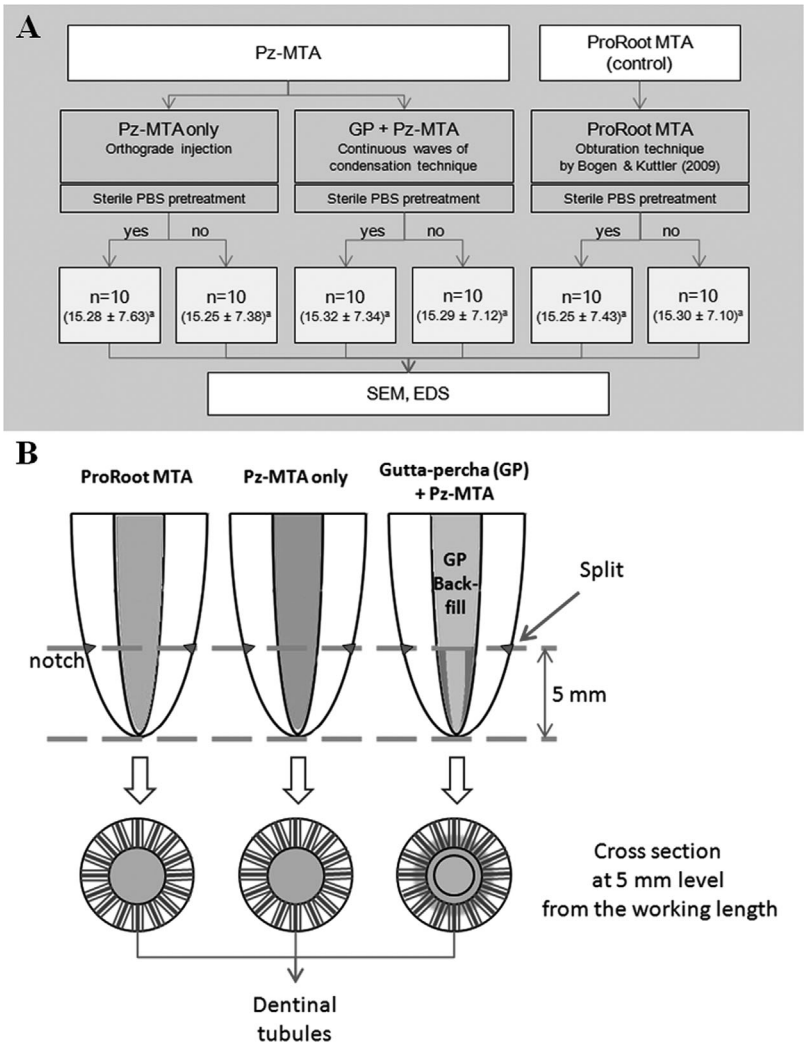

Fig 1. A: Schematic diagram of the experimental groups. The number in parenthesis shows the mean and standard deviation of the canal curvature of each set of roots, and their same lowercase superscripts show no significant differences among the mean values of the canal curvatures ($p > 0.05$). B: Schematic diagram of the root specimen preparation for SEM evaluation. MTA, mineral trioxide aggregate; Pz-MTA, pozzolan-based mineral trioxide aggregate; GP, gutta-percha; PBS, phosphate buffered saline; SEM, scanning electron microscopy; EDS, energy dispersive spectroscopy.

setting of the filling materials, and sealed the access cavities using intermediate restorative material (IRM; Dentsply Caulk, Milford, DE). The teeth were stored at 37˚C with 100% humidity until further analysis.

Assessment of Dentinal Tubule Biomineralization

After 12 weeks, the specimens were evaluated by scanning electron microscopy (SEM; S-4700, Hitachi, Tokyo, Japan) to characterize microstructural variations of the dentinal tubules. Each tooth was embedded in an acrylic block, and each mesiobuccal or distobuccal root was separated from the teeth with a slow-speed, water-cooled diamond saw (Isomet Low Speed Saw; Buehler, Lake Bluff, IL). The separated roots were split in horizontal direction for cross-section analysis at 5 mm level from the apex (Fig. 1(B)). Root segments were briefly washed in distilled water and sputter coated with platinum for SEM observation at an accelerating voltage of 15 kV. At the interface of main canal and obturation material, the depths of material penetration into dentinal tubules and intratubular mineralization were recorded. The elemental composition of intratubular mineralized precipitates were analyzed by using energy dispersive spectroscopy (EDS; 7200-H, Horiba, Northampton, England).

Statistical Analyses

The data were analyzed with one-way ANOVA and Tuckey *post hoc* test with SPSS software (SPSS Inc., Chicago, IL) to assess the differences among

experimental groups. For each group, the effect of PBS pretreatment on the intratubular biomineralization was investigated using a two-sample t-test. The significance level was set at $\alpha = 0.05$.

Results

The scanning analysis of GP with Pz-MTA sealer obturated samples showed the direct tubular penetration of Pz-MTA, and further formation of apatite crystals densely packing the dentinal tubules were detected (Fig. 2(A–C)). On the other hand, a close adaptation of the material to the main canals and intratubular biomineralization near the tubule orifices were observed in the ProRoot MTA obturation samples (Fig. 2(E–G)). The mineralized structure connecting the material-dentin interface was confirmed at the orifice level, although there was a lacking of direct penetration of ProRoot MTA into the tubules. The patterns of apatite crystallization appeared similar at the entrance of dentinal tubules in both GP with Pz-MTA sealer and ProRoot MTA obturated samples (Fig. 2(B and F)). However, they simultaneously changed along the tubule pathway; the agglomerated precipitates sparsely clogged the dentinal tubules in ProRoot MTA obturated samples (Fig. 2(G)), whereas continuous and successive crystallization along the tubule pathway was observed in GP with Pz-MTA sealer group (Fig. 2(C)). EDS evaluation indicated that the intratubular mineralized precipitates from all groups contained primarily calcium (Ca), phosphorous (P), oxygen, and trailed amount of silica with similar Ca/P ratio (1.45–1.89) (Fig. 2(D and H)).

In GP with Pz-MTA sealer group, there was a variety of precipitate nanostructures, mainly petals and flakes, in stratified or organized spherical form, or mixed (Fig. 3(A–D)). Interestingly, mineralized apatite structures connecting its way through the dentinal tubules were confirmed at 350–400 μm from the tubule orifice, and the pre-crystallization seeds were also observed along the intra- and/or inter-tubular collagen fiber (Fig. 3(E–H)).

The depth of intratubular mineralization is presented in Table I. The roots in GP with Pz-MTA sealer group demonstrated the direct tubular penetration of the sealer with significantly greater depth of dentinal tubule biomineralization than the other groups ($p < 0.05$). Pretreatment with PBS significantly promoted the biomineralization depth in all groups ($p < 0.05$). The roots solely obturated with either of ProRoot MTA or Pz-MTA sealer showed the minimum biomineralization depth without penetration of the materials into the tubules.

Discussion

Seeking for the better root canal filling material is the utmost concern of the clinicians. However the technological advancement of the obturation method had not been shown to have a statistically relevant impact on

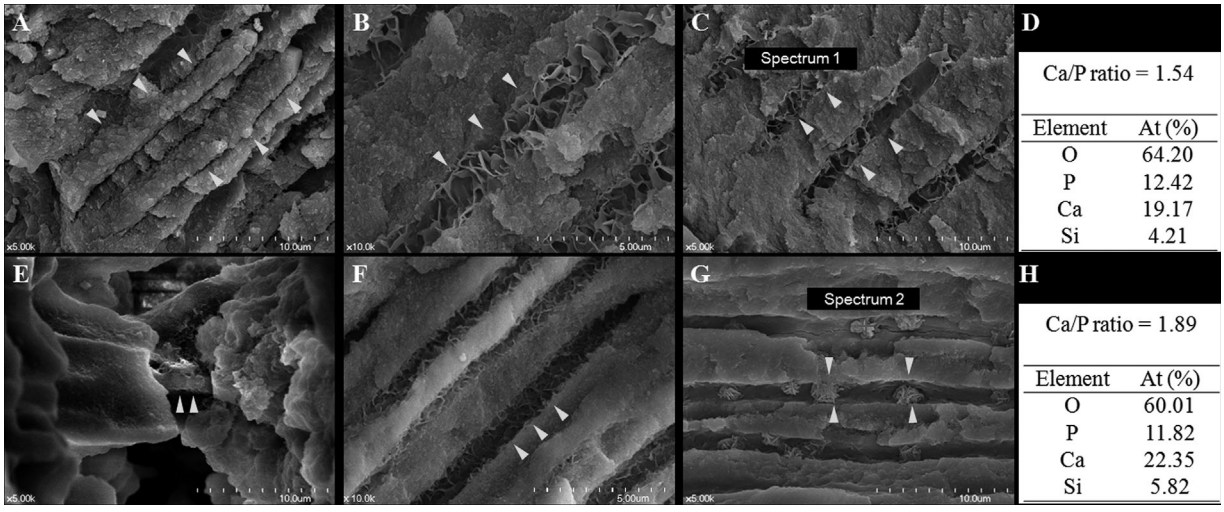

Fig 2. Representative scanning electron microscope images of gutta-percha with pozzolan-based (Pz-) MTA sealer cement- (A–D) or ProRoot MTA- (E–H) filled roots with phosphate buffered saline pretreatment. A: The Pz-MTA sealer cement penetrated into the dentinal tubules (arrowheads) at orifice level (×5,000). B: Further biomineralized dentinal tubules (arrowheads) beyond penetrated Pz-MTA cement at 50–100 μm distance showing densely packed dentinal tubules with organized apatite nanoprecipitates (×10,000). C: Successively biomineralized dentinal tubules (arrowheads) at 100–150 μm distance (×5,000). D: The semiquantitative chemical composition showing Ca/P ratio of the pointed area (red) of (C). E: Intermediate layer of ProRoot MTA connecting the material and dentinal tubule (arrowheads) at orifice level (×5,000). F: Biomineralized dentinal tubules at 50–100 μm distance (×10,000). G: Biomineralized dentinal tubules at 100–150 μm distance (×5,000). The agglomerated precipitates induced by ProRoot MTA (arrowheads) sparsely clogged the dentinal tubules. H: The semiquantitative chemical composition showing Ca/P ratio of the pointed area (red) of (G).

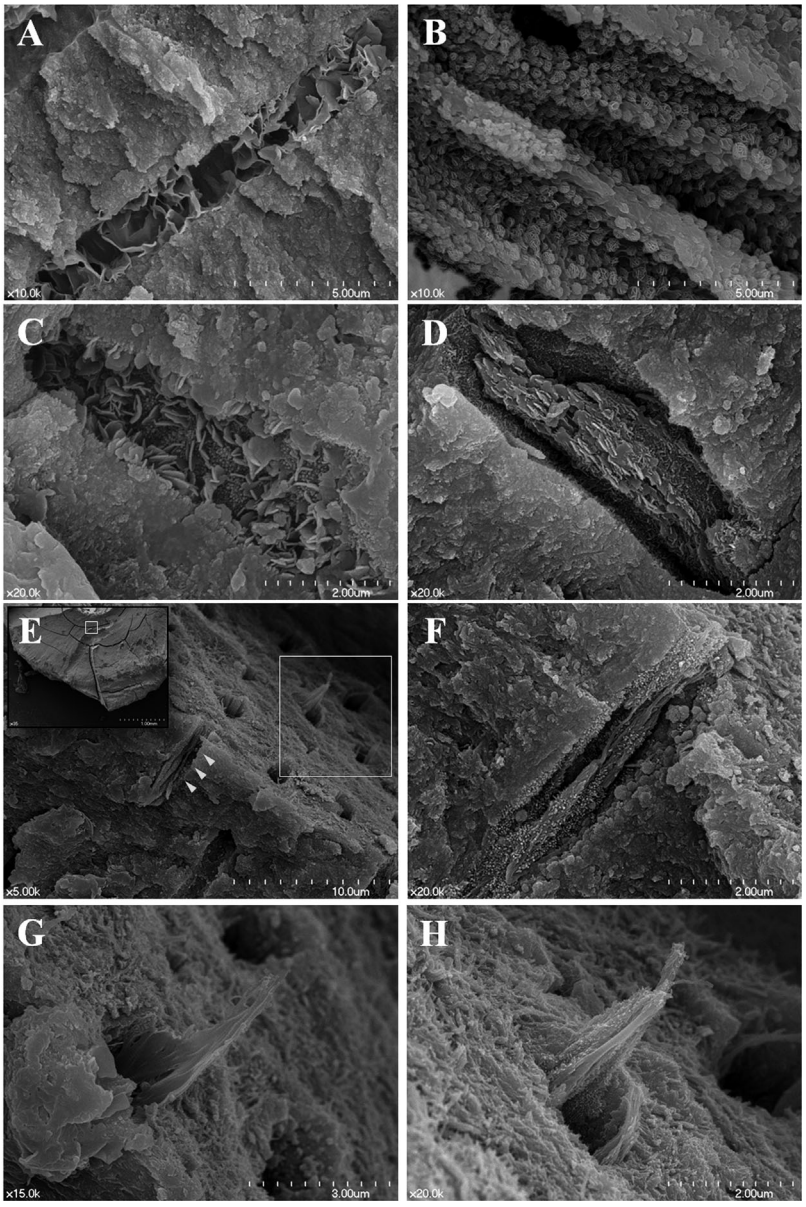

Fig 3. A–D: Diverse nanocrystallographs of pozzolan-based (Pz-) MTA sealer cement induced intratubular biomineralization. A: Organized nanoflakes (×10,000), (B) microsphere (×10,000), (C) mixed (×20,000), or (D) organized plates (×20000) are intergrown and exploited to seal the dentinal tubules. (E-F) Successive intratubular biomineralization of gutta-percha and Pz-MTA sealer filled canals. E: Boxed area (yellow) of left upper lower magnification image (×35) from the horizontal split specimen obturated with gutta-percha and Pz-MTA sealer cement showing the interface at 350–400 μm distance from dentinal tubule orifice (×5,000). (F) The pre-crystallization seeds observed along the collagen fiber (arrowheads of (E), ×20,000). (G, H) A higher magnification of upper right boxed area of (E) showing successive intratubular biomineralization in either (G) plate-like form or (H) agglomeration of precipitation seeds along the collagen fibrils (×20,000). Scanning electron microscope images.

treatment outcome (Peng *et al*., 2007). The clinical radiographs with densely filled root canals do not support the biologically sealed root canal system from surrounding periapical tissue. In fact, several methodologies such as micro-computed tomography proposed to assess the sealing ability of root canal filling materials did not fully provide sufficient amount of information about the sealing of the root canal system. In this regard, confirmation of the dentinal tubule mineralization may provide the secondhand evidence showing the possible ultimate sealing of root canal system, and for this purpose, the present study was conducted to extrapolate and compare the biomineralization capacity of currently available Pz-MTA cement with enhanced clinical conveniences.

In the present study, the Pz-MTA sealer cement showed further intratubular biomineralization up to significantly deeper level of the tubules. This provides compelling evidence of Pz-MTA as a bioactive root canal sealer when it was coupled with core material (GP)

TABLE I Depths of material penetration into the dentinal tubules and intratubular mineralization (mean ± standard deviation)

Root canal obturation material	GP with Pz-MTA sealer	Pz-MTA sealer only	ProRoot MTA only
Tubular penetration depth (μm)	23.77 ± 2.48	Not detected	Not detected
Intratubular mineralization depth (μm)			
PBS pretreatment			
No	350.25 ± 36.50^{Ab}	62.55 ± 9.56^{Bb}	68.20 ± 11.20^{Bb}
Yes	392.69 ± 39.43^{Aa}	98.12 ± 14.45^{Ba}	130.51 ± 20.21^{Ba}

GP, gutta percha; MTA, mineral trioxide aggregate; PBS, phosphate buffered saline; Pz-MTA, pozzolan-derived mineral trioxide aggregate. Same uppercase alphabet superscripts in row show no significant differences among the mean values of experimental groups ($p > 0.05$). Same lowercase alphabet superscripts in column show no significant effect of PBS pretreatment on the mean values of intratubular mineralization within each group ($p > 0.05$).

and vertical condensation pressure. In addition, PBS pretreatment before final obturation enhanced intratubular mineralization of both Pz-MTA sealer- (110–150%) and ProRoot MTA- (130–190%) obturated canals. Although the intratubular mineralization depth of MTA was rather limited when compared to previous researches (Reyes-Carmona *et al.*, 2009; Reyes-Carmona *et al.*, 2010) in which the specimens were immersed continuously in regularly refreshed PBS solution, the strategic importance to boost biomineralization ability is the incorporation of phosphate ion as an initial precipitation seed. Preconditioning with the phosphate ions derived from PBS soaking sequence might have enhanced the nucleation formation, which is known as polymer-induced liquid precursor (PILP) process (Gower, 2008). Phosphate anions in PBS are considered to make the intratubular environment even more labile in PILP process, and enhance the formation of the prenucleation cluster and its subsequent crystal growth. The collagen fibers exposed after smear layer removal with EDTA pretreatment also might have been directed the crystallization process once it is infiltrated with the amorphous precursors. However, there have not been any reports on the clinical use and tubular biomineralization inducing ability of PBS as a final soaking solution before root canal obturation. In this regard, the results of our SEM analysis (Fig. 3) is the first report of biomineralized apatite confirmed at 350-400 μm level of dentinal tubules with variety of precipitate nanostructures, such as petals and flakes, in stratified or organized spherical form.

It is noteworthy that although bulk obturated materials were closely adapted to the canal wall, occasionally clogging the tubule orifices as previously reported (Bird *et al.*, 2012), they could not penetrate into the tubules regardless of their different particle sizes. Rather, they showed the mineralized tag-like structures connecting the material-dentin interface at the orifice level. These structures are supposed to be the flocculated crystals formed on the material surface, which have been grown from the precursor–precipitation phase (Reyes-Carmona *et al.*, 2009; Reyes-Carmona *et al.*, 2010). In fact, precedent researches reported that such tag-like structures were the result of biomineralization potential of Portland cement and MTA, which could be enhanced by the interaction with phosphate-containing solution (Reyes-Carmona *et al.*, 2009; Reyes-Carmona *et al.*, 2010). However, the tags found in dentinal tubules of GP with Pz-MTA sealer group are the results of material penetration aided by the vertical condensation pressure transmitted via thermoplasticized gutta-percha, and are clearly distinct from such "tag-like structures" in the other groups.

Further, the small particle size would have contributed to induce more stable precursors for guiding an effective diffusion of the ions than the higher molecular weight particles of ProRoot MTA (Huang *et al.*, 2008). The mean particle size for white ProRoot MTA is 10 μm, with all particles being smaller than 50 μm (Komabayashi and Spangberg, 2008). The slurry made from such aggregates of particles become rheopectic when orthograde-filled in root canals. In contrast, finely pulverized Pz-MTA cement, with a mean particle size of 1.5 μm, becomes thixotropic when the material is released via needle tip and further compressed by vertical pressure. It then infiltrates or grouts toward dentinal tubules to form sealer tags and apatite precursors for further intratubular biomineralization. Such stable precursors may induce the propagation of crystallization along the dentinal tubules by secondary nucleation among individual nanoparticles of the disordered phase, providing successive biomineralization densified into deeper tubules. In fact, ProRoot MTA treated teeth showed sparsely clogging discrete agglomerates in limited depth.

The crystallographs of Pz-MTA cement-induced precipitates were also notable. They were in various shapes, relatively smaller in size than those induced by ProRoot MTA, within elemental composition of Ca/P ratio similar to hydroxyapatite. Among the various apatite structures, the particles with a grain size less than 100 nm in at least one direction have higher surface activity and ultrafine structure, resulting in enhanced bioactivity than coarser crystals (Vallet-Regi and Gonzalez-Calbet, 2004). We could confirm the seamless flow of mineralization precipitates in GP with Pz-MTA sealer group, extended beyond 300 μm-depth regardless

of the PBS pretreatment. In that, Pz-MTA cement as a sealer presented a favorable biomineralization pattern for the sealing of root canal system, while the canals solely filled with Pz-MTA sealer or ProRoot MTA lacked such fine structures. The in-depth investigation on the correlation among crystallography and elemental composition of Pz-MTA cement-induced biomineralization of dentinal tubules requires further researches.

Within the limitations of this study, the use of Pz-MTA cement as a sealer in conjunction with well-fit gutta-percha cone and vertical pressure resulted in consistent dentinal tubule biomineralization. Preconditioning with PBS before root canal obturation promoted PILP process, and led to enhanced biomineralization of the dentinal tubules beyond the penetrated Pz-MTA sealer tag in various crystallographs. The Pz-MTA cement as a root canal sealer thus figuratively renders a new possibility of bio-tight sealing of the root canal system.

Acknowledgements

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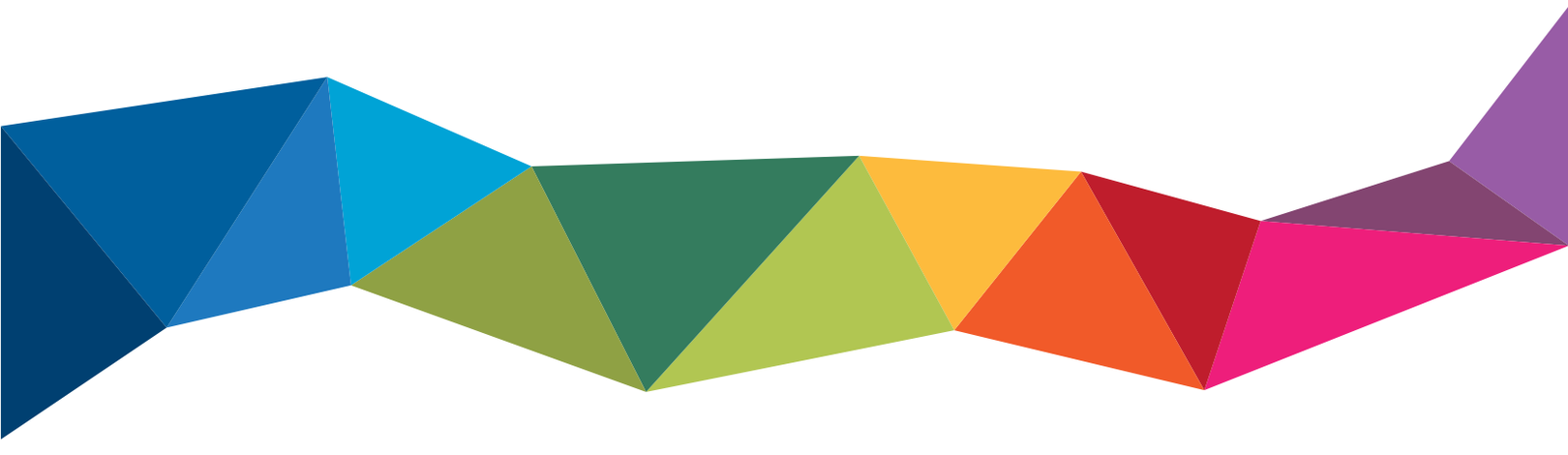

Biological Effects and Washout Resistance of a Newly Developed Fast-setting Pozzolan Cement

J Endod 2013, 39, 467-472

Washout resistance of fast-setting pozzolan cement under various root canal irrigants

Restor Dent Endod 2013, 38(4), 248-252

Tooth Discoloration after the Use of New Pozzolan Cement (Endocem) and Mineral Trioxide Aggregate and the Effects of Internal Bleaching

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Biological Effects and Washout Resistance of a Newly Developed Fast-setting Pozzolan Cement

Yoorina Choi, DDS, Su-Jung Park, DDS, MSD,* Seoung-Hoon Lee, PhD,†‡ Yun-Chan Hwang, DDS, PhD,§‖ Mi-Kyung Yu, DDS, PhD,¶ and Kyung-San Min, DDS, PhD¶*

Abstract

Introduction: Mineral trioxide aggregate has been widely used as a retrograde filling material. The aim of this study was to evaluate the biological effects of a newly developed fast-setting, mineral trioxide aggregate–derived pozzolan cement (Endocem). Furthermore, we explored whether this cement is resistant to washout in comparison with ProRoot. **Methods:** Biocompatibility was evaluated on the basis of cell morphology and a viability test. The expression of osteogenic genes was evaluated by performing real-time polymerase chain reaction, and calcified nodule formation was assessed by alizarin red S staining. The setting time was measured, and washout testing was performed by placing the material into fetal bovine serum. **Results:** The biocompatibility and osteogenicity of Endocem were similar to those of ProRoot. Moreover, Endocem showed a higher resistance to washout than ProRoot did. **Conclusions:** These results suggest that Endocem can be used as an available retrograde filling material because it sets faster and shows similar biological effects when compared with ProRoot. *(J Endod 2013;39:467–472)*

Key Words

Biocompatibility, fast-setting, mineral trioxide aggregate, osteogenic, pozzolan, washout

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Endodontic surgery is the preferred approach when conventional orthograde endodontic therapy fails or cannot be performed appropriately. The key to success in endodontic surgery is hermetic sealing of the root-end filling. Therefore, root-end filling materials should have adequate sealing ability (1) and should also be biocompatible with the host tissue, insoluble in tissue fluids, and dimensionally stable (2, 3). Although various materials have been advocated as root-end filling materials such as intermediate restorative material (IRM) and SuperEBA, mineral trioxide aggregate (MTA) is preferred because of its superior sealing ability and biocompatibility (4, 5).

Several studies have shown that MTA is a biocompatible material, because it shows less cytotoxicity than any other restorative materials in various conditions and cell lines (6–9). It has been reported that MTA induces an osteogenic phenotype, as reflected by the up-regulated expression of mineralization-related genes. Therefore, MTA is regarded as a bioactive material that is osteogenic, cementogenic, and odontogenic (10–12).

Some drawbacks of MTA also have been reported, including its long setting time (3). After endodontic surgery, the retrograde-filled MTA that is not set may encounter blood or tissue fluid. Kim et al (13) reported that washout of MTA could be mediated by continuous exudates or tissue fluid. Although MTA has excellent sealing ability, its washout can prevent the complete sealing of the retrograde preparation site of the tooth and thus eventually cause failure (13, 14).

There have been efforts to overcome the long setting time of MTA by using various additives (15–17). Although the setting time of MTA with additives is shorter than that of the original form of MTA, the reported setting time is still too long to reflect clinical significance. Moreover, various studies have shown that adding additives to MTA to accelerate the setting time may have an adverse effect on its physical and biological properties (18, 19).

Recently, an MTA-derived pozzolan cement (Endocem) was introduced. Endocem sets quickly without the addition of a chemical accelerator because it contains small-particle pozzolan cement. However, the biological effects, including biocompatibility and mineralization potential, of Endocem have not been evaluated. Therefore, the purpose of this study was to evaluate the biological effects of Endocem, fast-setting MTA-derived material. Furthermore, we explored whether this cement is resistant to washout when compared with a previously marketed MTA (ProRoot).

Materials and Methods

Cell Morphology Analysis by Scanning Electron Microscopy

Under aseptic conditions, ProRoot (Dentsply, Tulsa, OK), Endocem (Maruchi, Wonju, Korea), and IRM (Dentsply-Caulk, Milford, DE) were mixed according to the manufacturers' instructions. Then the materials were condensed into 1-mm × 5-mm round wax molds and allowed to set for 24 hours in a humidified incubator at 37°C. The disks were all placed at the bottom of 24-well tissue culture plates. Then MG63 cells were seeded at 1×10^5 cells per well on the prepared materials. After a 72-hour incubation period, the dishes were fixed with 2.5% glutaraldehyde (Sigma-Aldrich, St Louis, MO) for 2 hours. The samples were then dehydrated in increasing concentrations of ethanol (70%, 80%, 90%, 95%, and 100%) for 20 minutes at each concentration, immersed in n-butyl alcohol (Junsei Chemical Co, Tokyo, Japan)

for 20 minutes, and dried by using a CPD 030 critical point dryer (BalTec, Balzers, Liechtenstein). Scanning electron microscopy (SEM) was performed by using a JSM-6360 (JEOL, Tokyo, Japan) system operated at 10 kV.

Preparation of Extracts

ProRoot, Endocem, and IRM were mixed according to the manufacturers' instructions. The mixed cements were placed into a paraffin wax mold (1-mm thickness and 5-mm diameter), and the cements were stored in an incubator at 100% relative humidity and 37°C for 1 day of hydration. The cements were then sterilized in ultraviolet light for 1 hour. One tablet of each cement was stored in 10 mL of Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Invitrogen) along with 100 U/mL penicillin and 100 U/mL streptomycin (Invitrogen) for 3 days.

Cell Viability Test

As determined by hemocytometry, single-cell suspensions of MG63 cells were seeded in 24-well tissue culture plates at a density of 2×10^4 cells per well in DMEM containing 10% FBS along with 100 U/mL penicillin and 100 U/mL streptomycin and incubated in a humidified atmosphere of air and 5% CO_2 at 37°C for 24 hours. Then cells were treated with the prepared extract (experimental groups) or medium only (control group). After exposure to the extract of the materials for 12, 24, 48, and 72 hours, viable cells were detected by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, which forms blue formazan crystals on reduction by the mitochondrial dehydrogenase present in living cells. Briefly, 200 μL MTT solution (0.5 mg/mL in phosphate-buffered saline) was added to each well, and the wells were incubated for 2 hours. Subsequently, 200 μL dimethyl sulfoxide (Amresco, Solon, OH) was added to each well. The plates were then shaken until the crystals had dissolved, and the solution in each well was transferred to a 96-well tissue culture plate. The reduced MTT was then measured spectrophotometrically at 540 nm in a dual-beam microtiter plate reader. The statistical analysis of the data was performed by 1-way analysis of variance (ANOVA) followed by a multiple-comparison Tukey test, with the use of the SPSS program (SPSS 12.0; SPSS GmbH, Munich, Germany). Statistical significance was determined at $P < .05$.

Real-time Polymerase Chain Reaction Analysis

Cells (1×10^5) in DMEM containing 10% serum were seeded in 6-well tissue culture plates and incubated for 24 hours. The medium was then switched to the extract medium for the duration of the experiment. After exposure of the materials to the extract for 7 days, the cells were lysed to extract the total RNA by using Trizol reagent (Invitrogen), according to the manufacturer's instructions. In brief, the cells were lysed directly in the plates by using 1.0 mL Trizol reagent. After chloroform extraction, the total RNA was recovered from the aqueous phase and precipitated by using isopropanol and RNAse-free distilled water. Then reverse transcription of RNA was performed by using the Superscript First-Strand Synthesis kit (Invitrogen).

SYBR Green–based real-time polymerase chain reaction (PCR) was optimized and conducted by using the TOPreal qPCR premix Kit (Enzynomics, Cheongju, Korea). The final PCR mixture contained 2 μL each of the forward and reverse primers (final concentration of 0.4 μmol/L for each), 2 μL SYBR Green (2×), 1.6 μL $MgCl_2$ (final concentration, 3 mmol/L), and 5 μL of the template, and the volume was adjusted to 20 μL by using nuclease-free water. The sequences of the primers are listed in Table 1. All real-time PCR reactions (reactions, experimental samples, and controls) were performed in duplicate and conducted by using the StepOne Real-Time PCR System (Applied Biosystem, Singapore). The following protocol was used: 10 minutes at 95°C, followed by 30 cycles at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds. After the PCR cycles, a DNA melting curve was generated (0 second at 95°C and 15 seconds at 65°C, with a ramping time of 20°C/s, and 0 second at 95°C, with a ramping time of 0.1°C/s) to discriminate between specific and nonspecific amplification products. One-way ANOVA and Tukey tests were used for statistical analysis ($P = .05$).

TABLE 1. Real-time PCR Primers

Genes	Sequence
BSP	Forward: 5′-GCGAAGCAGAAGTGGATGAAA-3′ Reverse: 5′-GCTGCCGTTGCCGTTTT-3′
OC	Forward: 5′-CACTCCTCGCCCTATTGGC-3′ Reverse: 5′-CCCTCCTGCTTGGACACAAAG-3′
OP	Forward: 5′- GTGATGTCCTCGTCTGTAGCATCA-3′ Reverse: 5′- GTAGACACATATGATGGCCGAGG-3′
GAPDH	Forward: 5′-AAGGTGAAGGTCGGAGTCAAC-3′ Reverse: 5′-GGGGTCATTGATGGCAACAATA-3′

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Alizarin Red S Staining

After the MG63 cells were cultured in the presence of ProRoot, Endocem, or osteogenic medium supplemented with 50 μg/mL L-ascorbic acid (Sigma-Aldrich), 10 mmol/L β-glycerolphosphate (Sigma-Aldrich), and 100 nmol/L dexamethasone (Sigma-Aldrich) for 14 days, the cells were fixed in 70% ethanol for 30 minutes and rinsed with distilled water. The cells were stained with 40 mmol/L alizarin red S, at pH 4.2, for 10 minutes with gentle agitation. The cells were then washed with distilled water and allowed to dry. Images of the alizarin red S staining were obtained by using a scanner, and the stain intensity was analyzed by using an image analysis program (Image J; National Institutes of Health, Bethesda, MD). One-way ANOVA and Tukey tests were used for statistical analysis ($P = .05$).

Measurement of Setting Time

The test materials were mixed according to the manufacturers' instructions. The samples (n = 10) were tested just before their anticipated setting time and at 30-second intervals until they were fully set. A Gilmore apparatus was used with a stainless steel indenter and 1/4-pound indentation force for the initial setting time measurement; a 1-pound indentation force was used for the final setting time. The apparatus was applied at a right angle to the surface of the sample for 5 seconds. The setting time was defined as the time at which the indenter failed to leave a definite mark on the surface of the sample. One-way ANOVA and Tukey tests were used for statistical analysis ($P = .05$).

Washout Test

ProRoot, Endocem, and IRM were mixed according to the manufacturers' instructions. After mixing, each material was placed into a 5-mm × 2-mm Teflon mold (n = 10). Each mold was then placed into FBS (Invitrogen) immediately as shown in Figure 1*A*. After 24 hours, SEM was performed by using a JSM-6360 system operated at 10 kV. The washout score was evaluated by 3 dentists who had no knowledge about the source of the specimens, according to the criteria listed in Table 2. The Kruskal-Wallis test was used to evaluate the washout test score ($P = .05$).

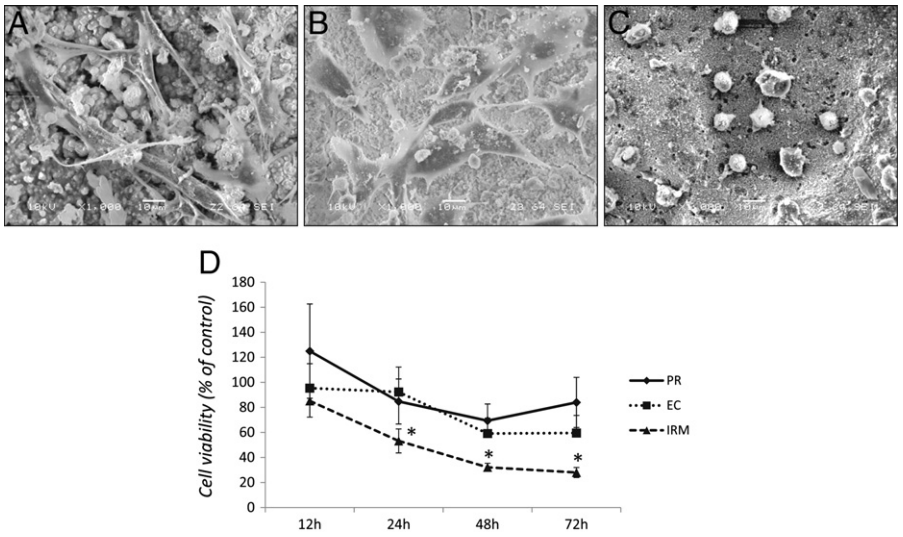

Figure 1. SEM observation of cells incubated for 72 hours on (*A*) ProRoot (original magnification, ×1000), (*B*) Endocem (original magnification, ×1000), and (*C*) IRM (original magnification, ×1000). (*D*) Effects of ProRoot (PR), Endocem (EC), and IRM on MG63 cells as measured by the MTT assay. Each point and bar represent the mean ± standard deviation. *Significant difference between each group; $P < .05$.

Results

Cell Morphologic Analysis

The cell growth and morphology of each material were evaluated by using SEM observation. As shown in Figure 2*A* and *B*, well-spread and flattened cells were observed in close contact with the surfaces of ProRoot and Endocem. In contrast, a few rounded cells were observed on the IRM surface, and no living cells were observed (Figure 2*C*). In the ProRoot and Endocem groups, many cellular extensions interacted with the surface of the cement and with the adjacent cells.

Cell Viability Test

To evaluate cell viability in the presence of the material extracts, an MTT assay was performed. As shown in Figure 2*D*, ProRoot and Endocem showed similar cell viability throughout the experimental period ($P > .05$). However, IRM showed lower cell viability after 24 hours when compared with the other groups ($P < .05$).

Real-time PCR Analysis

To evaluate the expression of mineralization-related markers, real-time PCR analysis was performed. Total RNA isolated from IRM-treated cells did not yield enough products for use with real-time PCR analysis. The expression of bone sialoprotein (BSP) and osteopontin (OP) mRNA increased in ProRoot-treated and Endocem-treated cells when compared with the medium only–treated cells of the control group ($P < .05$). The relative expression of BSP, osteocalcin (OC), and OP mRNA demonstrated no significant difference between the ProRoot-treated and Endocem-treated groups (Fig. 3*A–C*, $P > .05$).

TABLE 2. Scoring System for the Washout Test

Score	Description
0	No defect
1	Defect area ranges below 25% of total area
2	Defect area ranges from 25%–50% of total area
3	Defect area ranges from 50%–75% of total area
4	Defect area ranges above 75% of total area

Defect area is defined as hollow area on the surfaces of materials, not including marginal gaps around or porosities on the surfaces.

Alizarin Red S Staining

To investigate the effect of ProRoot and Endocem on mineralization, MG63 cells were stained with alizarin red S. After the addition of ProRoot and Endocem, the formation of mineralized nodules in MG63 cells was significantly higher than that in the medium only–treated cells of control group at day 14 (Fig. 3*D*, $P < .05$). Cells cultured in osteogenic medium served as the positive control.

Setting Time

The initial setting time of the ProRoot was 78 ± 5 minutes, and the final setting time was 261 ± 21 minutes. The initial setting time of the Endocem was 2 minutes ± 30 seconds, and the final setting time was 4 minutes ± 30 seconds. The initial setting time of the IRM was 6 minutes ± 30 seconds, and the final setting time was 10 minutes ± 30 seconds. The setting time of the Endocem was significantly lower than that of the ProRoot and IRM ($P < .05$) (Table 3).

Washout Test

As shown in Figure 1*B*, the washout score was higher in the ProRoot group than in the Endocem and IRM groups after 24 hours ($P < .05$). Representative images of ProRoot, Endocem, and IRM are shown in Figure 1*C–E*, respectively. Notably, ProRoot was washed out from the Teflon mold, whereas Endocem and IRM were maintained in the mold. However, a remarkable gap was observed between IRM and the mold.

Discussion

MTA fulfills many of the ideal properties of a root-end filling material, including biocompatibility and sealing ability (4). However, the long setting time, which is one of the major drawbacks of MTA, is still problematic for clinical applications of MTA. Notably, when the MTA is used as a retrograde filling material, MTA that was not set can interact with the tissue fluid until it sets completely. Tingey et al (20) and Nekoofar et al (21) reported that the physical properties of MTA set in tissue

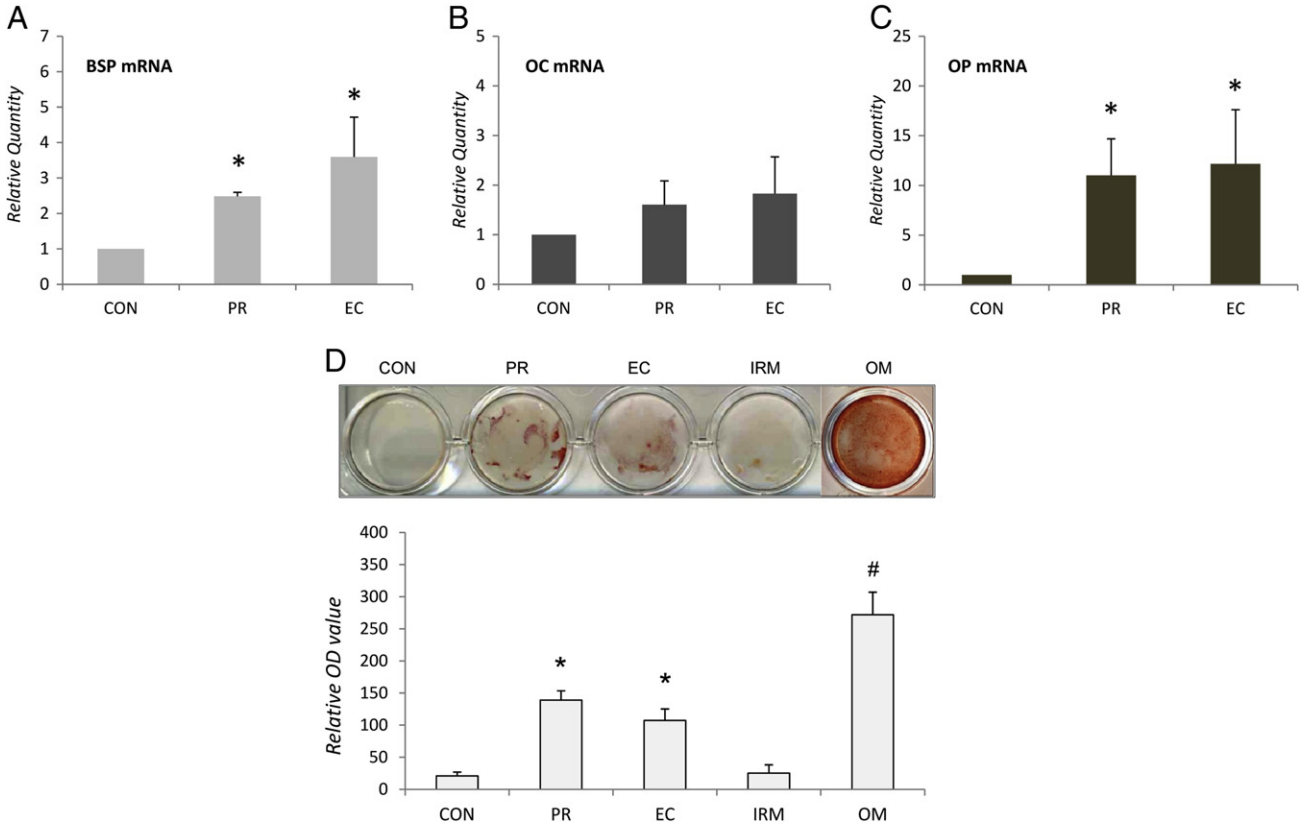

Figure 2. Effect of ProRoot (PR) and Endocem (EC) on expression of BSP (*A*), OC (*B*), and OP (*C*) mRNAs in MG63 cells cultured for 7 days. The relative mRNA levels were calculated by dividing the absolute levels of expression of BSP, OC, and OP mRNAs by the absolute level of expression of glyceraldehyde-3-phosphate dehydrogenase mRNA. *Significant difference among groups; $P < .05$. (*D*) Effects of PR and EC on the formation of calcified nodules in MG63 cells. Osteogenic medium (OM)-treated cells served as the positive control. The cells were cultured with MTA and OM for 14 days and stained with alizarin red S. A representative photograph of alizarin red staining is shown. Alizarin red S stain intensity was analyzed by performing densitometry. Groups identified by the same superscript symbols were not significantly different in the same gene group ($P > .05$). CON, control.

fluid, blood, or low pH conditions, which mimic clinical situations such as endodontic surgery, are different from those in moisture, which is the recommended setting condition. Furthermore, Kim et al (22) reported that MTA failed to set in the presence of FBS. There also have been reports that the washout of MTA can occur when MTA is used as a retrograde filling material (13, 14, 22). Initial looseness of MTA, ie, it is not

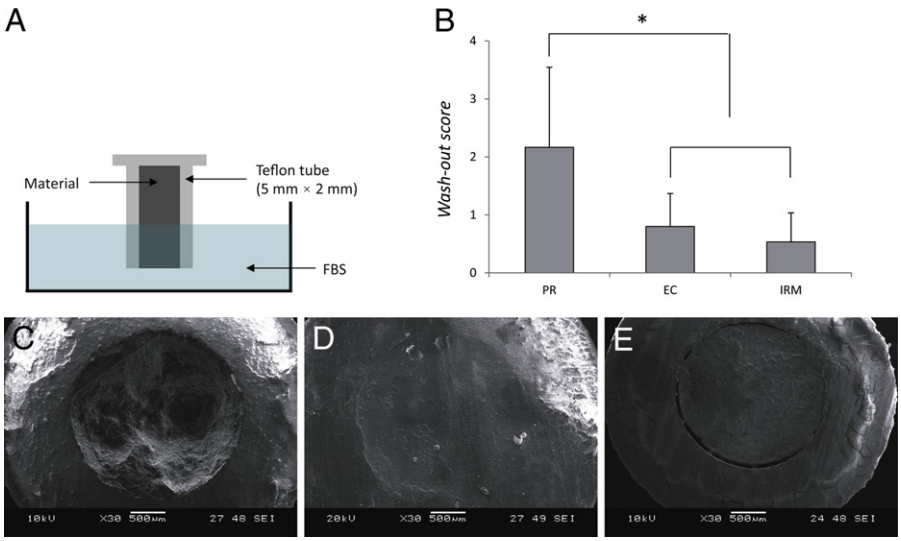

Figure 3. (*A*) Schematic illustration of the model used for the experiment. (*B*) Washout score of ProRoot (PR), Endocem (EC), and IRM. Each point and bar represent the mean ± standard deviation. *Significant difference among groups; $P < .05$. SEM observation indicating the washout tendency and marginal gap of PR (*C*), EC (*D*), and IRM (*E*).

TABLE 3. Means and Standard Deviations of Initial and Final Setting Times for Tested Materials

	Initial setting time (min)	Final setting time (min)
ProRoot	78 ± 5	261 ± 21
Endocem	2 ± 30 seconds*	4 ± 30 seconds*
IRM	6 ± 30 seconds#	10 ± 30 seconds#

Groups identified by the same superscript symbols were not significantly different in the same gene group ($P > .05$).

set or weakened physical condition, occurs in the presence of tissue fluid or blood flow and may threaten the hermetic sealing of communicating channels between the root canal system and the periradicular tissues, thereby resulting in the failure of endodontic surgery. This washout can be minimized if the root-end filling material sets quickly before it is exposed to blood or tissue fluid (17, 21, 23). In this respect, a short setting time in addition to biocompatibility and sealing ability is necessary for an ideal root-end filling material (17, 24).

The development of fast-setting MTA has been attempted by many researchers (14–17, 24). However, most of these approaches were based on the addition of chemical setting accelerators, some of which showed adverse physical and biological effects (1, 15, 16). Recently, Endocem, a fast-setting MTA-derived cement, was developed by using small particles of pozzolan cement without any chemical accelerators. A pozzolan is a siliceous or siliceous and aluminous material that possesses little or no cementitious value in itself but in finely divided form and in the presence of water will react chemically with calcium hydroxide at ordinary temperature to form compounds possessing cementitious properties (25). The chemical composition of Endocem is very similar to that of MTA, assembly of trioxide compound. Bismuth oxide (Bi_2O_3) is also added as a radiopacifier. The composition provided by the manufacturer, expressed as wt%, was 46.7 CaO, 5.43 Al_2O_3, 12.80 SiO_2, 3.03 MgO, 2.32 Fe_2O_3, 2.36 SO_3, 0.21 TiO_2, 14.5 H_2O/CO_2, and 11.0 Bi_2O_3. The pozzolanic reaction progresses like an acid-base reaction of lime and alkalies with oxides (SiO_2 + $A1_2O_3$ + Fe_2O_3) of the pozzolan (26). Two things happen. First, there is a gradual decrease in the amount of free calcium hydroxide with time. Calcium hydroxide has bad effects on mechanical and durability properties of material. Second, during this reaction there is an increase in formation of calcium silicate hydrate and calcium aluminate hydrate, stable crystals that are so effective on the strength of material (26). Furthermore, the use of small particles increases the surface contact of the particles with the mixing liquid and provides rapid setting and ease of handling (3, 27, 28). In the present study, Endocem demonstrated a much shorter setting time than ProRoot. The initial setting time of Endocem was 2 minutes ± 30 seconds, and the final setting time was 4 minutes ± 30 seconds, whereas the initial setting time of ProRoot was 78 ± 5 minutes, and the final setting time was 261 ± 21 minutes. The obtained value is similar to the value of 3 minutes and 15 seconds reported as the final setting time by the manufacturer's data sheet.

The cytotoxicity of root-end filling materials is of great concern because damage or irritation of the periapical tissue can delay wound healing (29, 30). In this study, SEM revealed that MG63 cells cultured on ProRoot and Endocem for 3 days appeared to be flat and exhibited well-defined cytoplasmic extensions that projected from the cells to the surrounding surface or adjacent cells, in contrast to cells cultured on IRM (Fig. 2*A–C*). Min et al (31) stated that cell attachment with multiple cytoplasmic extensions implies cell growth, proliferation, and differentiation. Furthermore, ProRoot and Endocem demonstrated significantly lower cytotoxicity than IRM in the MTT assay throughout the experimental period (Fig. 2*D*). These results indicate that Endocem permitted cell attachment and growth to a degree similar to that of ProRoot.

In addition to biocompatibility, osteogenic induction is regarded as an ideal characteristic for a retrograde filling material. Healing after endodontic surgery necessitates osseous repair of the medullary and cortical bone. It has been recognized that MTA stimulates the regeneration of osseous tissues surrounding the root end (4, 5, 7). In the current study, osteogenic differentiation markers including BSP, OC, and OP were examined, and alizarin red S staining was performed to evaluate the effects of the MTA-derived pozzolan cement on the formation of a mineralized matrix *in vitro*. Alizarin red S staining has been used for decades to evaluate calcium deposition by cells in culture (32). BSP is a major noncollagenous protein in mineralized connective tissue. BSP expression is highly specific for mineralizing tissues, including bone, mineralizing cartilage, dentin, and cementum (33). OC appears immediately before the start of mineralization and is a major noncollagenous protein synthesized by osteoblasts, odontoblasts, and cementoblasts. OP is believed to play a crucial role in modulating apatite crystal growth in bone (34). In this study, the relative quantity of BSP, OC, and OP mRNA was not significantly different between the ProRoot-treated and Endocem-treated groups (Fig. 3*A–C*). In alizarin red S staining, the extent of mineralization in cells treated with Endocem was similar to that in the cells treated with ProRoot and was significantly higher than that in the control and IRM groups (Fig. 3*D*). Collectively, these results indicate that the mineralization potential of Endocem is comparable to that of ProRoot.

To explore whether this fast-setting cement is resistant to washout when compared with the previously marketed MTA, a washout test was performed. FBS was used as the medium to replicate the clinical situation because of its biosafety and availability and because its biochemical profile is similar to that of human serum (20, 21). The degree of washout for ProRoot was significantly higher than that for Endocem (Fig. 1*B*). It is likely that the rapid setting of Endocem enhances its washout resistance (17). Most IRM samples showed a marginal gap between the materials and molds, whereas ProRoot and Endocem showed tight sealing between the material and the mold (Fig. 1*C–E*). This finding was in agreement with that of Torabinejad et al (34), who observed that the degree of marginal gaps was significantly higher for IRM than for MTA. On the basis of these results, Endocem can be concluded to have beneficial characteristics including "anti-washout" characteristic in tissue fluid–like medium and a lack of surrounding marginal gaps. The short setting time of Endocem may thus help to overcome the unfavorable clinical environment in endodontic surgery.

In this study, Endocem exhibited a biological effect similar to that of ProRoot. Thus, Endocem may be a substitute for ProRoot as an available retrograde filling material because of its short setting time and resistance to washout. However, long-term evaluation regarding the carcinogenicity or genotoxicity of Endocem should be performed.

Acknowledgments

The authors deny any conflicts of interest related to this study.

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Washout resistance of fast-setting pozzolan cement under various root canal irrigants

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Washout resistance of fast-setting pozzolan cement under various root canal irrigants

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Objectives: Fast-setting pozzolan cement (Endocem, Maruchi) was recently developed. The aim of this study was to investigate the effects of various root canal irrigants on the washout of Endocem in comparison to the previously marketed mineral trioxide aggregate (ProRoot; Dentsply) in a furcal perforation model. ***Materials and Methods:*** ProRoot and Endocem were placed into acrylic molds on moist Oasis. Each mold was then immediately exposed to either physiologic saline, 2.5% sodium hypochlorite (NaOCl), or 2% chlorhexidine (CHX) under gentle shaking for five minutes. Washout testing was performed by scoring scanning electron microscope (SEM) images. ***Results:*** Endocem exhibited higher washout resistance compared to ProRoot, especially in the NaOCl group. ***Conclusions:*** These results suggest that Endocem can be considered a useful repair material for furcal perforation, especially in a single-visit scenario. (*Restor Dent Endod* 2013;38(4):248-252)

Key words: Fast-setting, Furcal, Mineral trioxide aggregate, Perforation, Pozzolan, Washout

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Introduction

Furcal perforation may occur as the consequence of a procedural error or occasionally as the result of a pathologic process such as dental caries and root resorption.[1] Mineral trioxide aggregate (MTA) is a promising material that has been widely used in furcal perforation repair because of its excellent biocompatibility, superior sealing, and setting ability even in the presence of blood.[2,3] However, some shortcomings of MTA have also been reported, including its long setting time.[4] If a furcal perforation is repaired in this fashion, the clinician should apply wet cotton to the MTA and make another appointment for further treatment to provide the MTA the time to be set. Otherwise, "washout" of the unset MTA could occur during use of irrigation solution.

Recently, MTA-derived pozzolan cement (Endocem, Maruchi, Wonju, Korea) was introduced in the endodontic market. Endocem sets quickly without the addition of a chemical accelerator. Instead, it contains small particles of pozzolan cement to increases the surface contact with the mixing liquid and provides rapid setting. Recently, Choi *et al.* showed that Endocem set much quicker and was more resistant to "washout" mediated by fetal bovine serum than the previously marketed MTA (ProRoot, Dentsply, Tulsa, OK, USA) in a root-end filling model.[5] However, there is little information about the effects of various root canal irrigants including physiologic

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saline, sodium hypochlorite (NaOCl), and chlorhexidine (CHX). Therefore, the aim of this study was to investigate the effects of various root canal irrigants on "washout" of the MTA-derived pozzolan cement in a furcal perforation model.

Materials and Methods

ProRoot and Endocem were mixed according to the manufacturers' instructions. After mixing, each material was placed into a 1-mm × 2-mm acrylic mold on saline-moistened Oasis which simulated periodontal tissue in furcation area (n = 10) (Figure 1a). Each mold was immersed in either physiologic saline, 2.5% NaOCl, or 2% CHX immediately with gentle shaking (Compact Rocker-CR300, FINEPCR, Gunpo, Korea) for five minutes, and then was stored in an incubator at 95 ± 5% relative humidity and 37℃ for 24 hours. Scanning electron microscopy (SEM) was performed using a JSM-6360 system (JEOL, Tokyo, Japan) operated at 10 kV (Figures 2 and 3). The washout

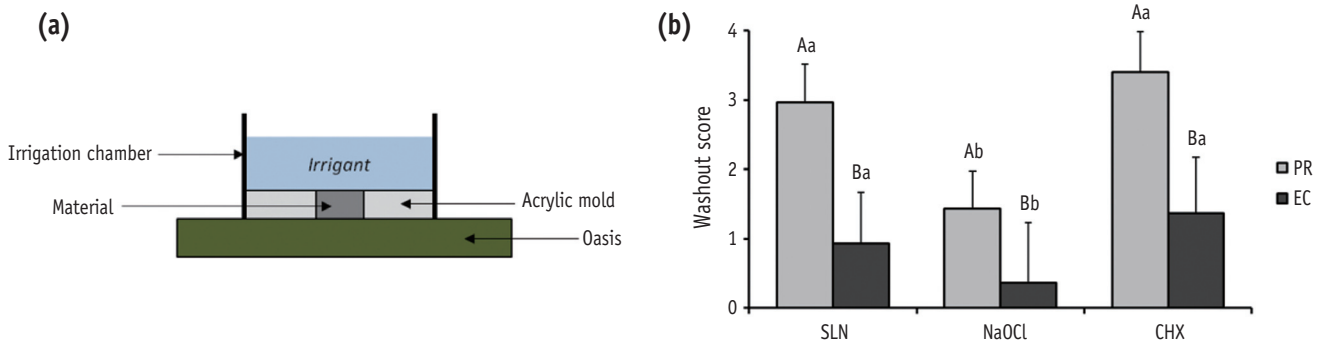

Figure 1. (a) Schematic of the model used for the experiment; (b) Washout scores of ProRoot (PR) and Endocem (EC). Each point and bar represents the mean ± SD. The capital letters represent significant differences between ProRoot and Endocem for each root canal irrigant, and the lowercase letters represent significant differences between root canal irrigants for each repair material. SLN, physiologic saline; NaOCl, sodium hypochlorite; CHX, chlorhexidine.

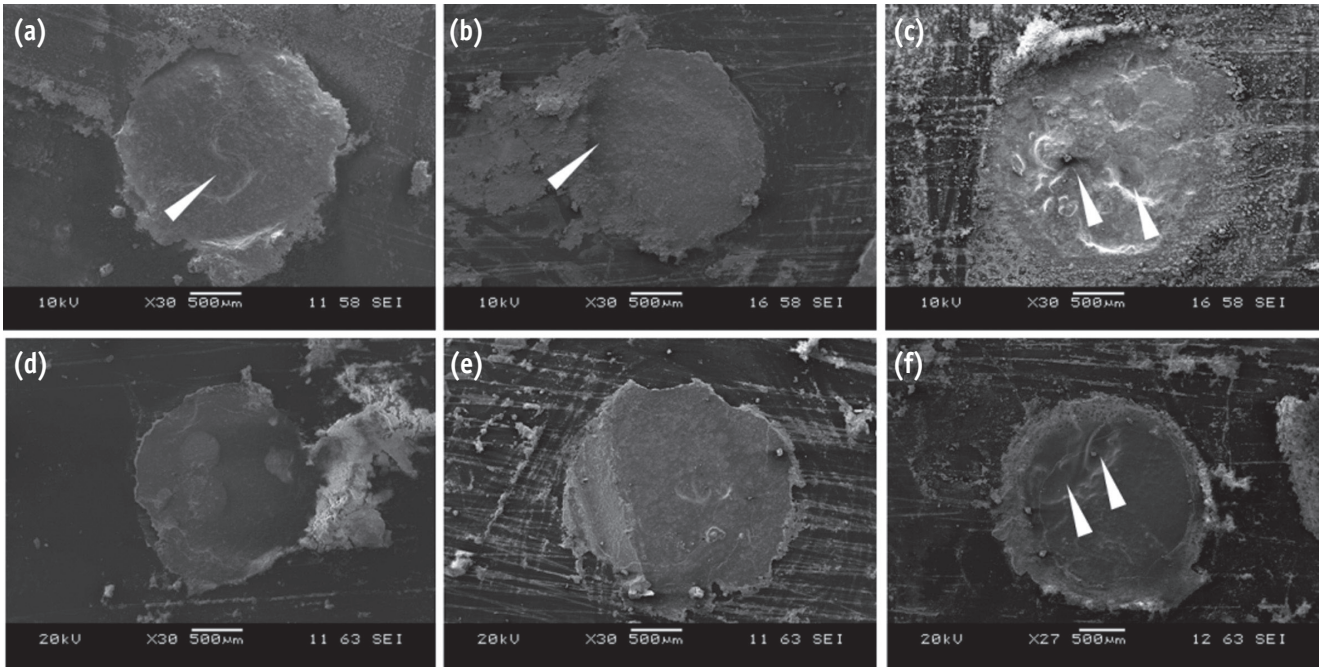

Figure 2. SEM observation indicating the washout tendency of ProRoot (a - c) and Endocem (d - f) in the presence of various root canal irrigants (×30). SLN, physiologic saline (a and d); NaOCl, sodium hypochlorite (b and e); CHX, chlorhexidine (c and f). White arrowheads indicate the areas where wash-out happened.

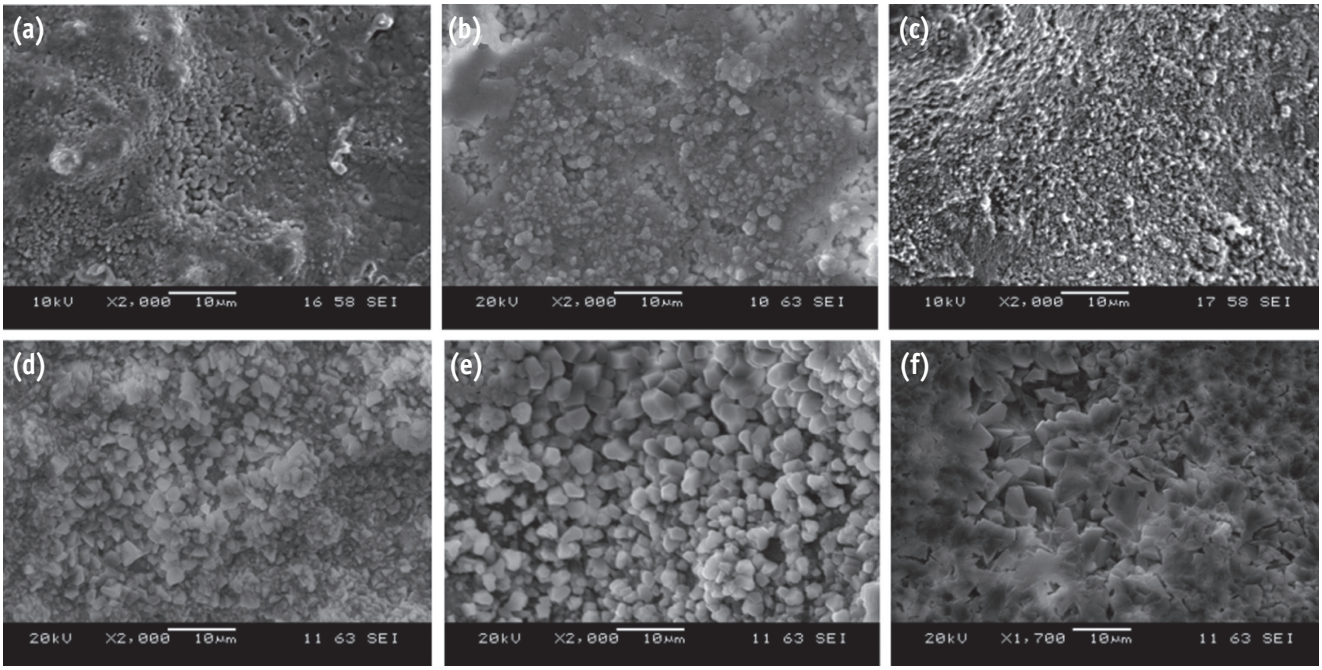

Figure 3. SEM observation indicating the microstructure of ProRoot (a - c) and Endocem (d - f) in the presence of various root canal irrigants (×2,000). SLN, physiologic saline (a and d); NaOCl, sodium hypochlorite (b and e); CHX, chlorhexidine (c and f).

score was calibrated and evaluated according to the criteria listed in Table 1 by three dentists who had no knowledge of the source of the specimens.

The data were then analyzed by an independent samples *t*-test to compare the two materials ($p = 0.05$). The Kruskal-Wallis test was used to evaluate the effects of the three irrigants on washout score ($p = 0.05$).

Table 1. Scoring system for the washout test

Score	Description
0	No defect
1	Defect area less than 25% of the total area
2	Defect area ranging from 25% to 50% of the total area
3	Defect area ranging from 50% to 75% of the total area
4	Defect area greater than 75% of the total area

The defect area was defined as the hollow area on the material surface, not including marginal gaps around or porosities on the surfaces.

Results

Washout scores were assigned to investigate whether the irrigation solutions affected the washout of ProRoot and Endocem. As shown in Figure 1b, ProRoot showed higher washout scores than Endocem under all irrigation solutions ($p < 0.05$). Furthermore, the washout scores of ProRoot and Endocem were significantly lower in the NaOCl-treated group compared to the saline- and CHX-treated groups ($p < 0.05$). Representative SEM images used for washout scoring are shown in Figure 2.

At the microstructure level, the crystal size of the NaOCl-treated group was greater than that of the saline- and CHX-treated groups in both ProRoot and Endocem samples. Notably, little crystalline formation and an amorphous layer were observed in the CHX-treated groups (Figure 3).

Discussion

For desirable results of furcal perforation repair, the material should have several ideal characteristics including biocompatibility, good sealing properties, insolubility in tissue fluids, and dimensional stability.[4,6] Among various materials, MTA has been recommended for the treatment of furcal perforations.[7-9] Despite its many favorable properties,

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MTA has some drawbacks such as a long setting time, which is still problematic for clinical applications. The slow setting results in handling difficulties and washout of the MTA newly placed in the repaired area during the subsequent irrigation procedure.[10,11]

Many researchers have attempted to develop fast-setting MTA or its derivatives.[11-16] However, most of these trials have focused on the addition of chemical setting accelerators, some of which proved to have detrimental physical and biologic effects.[15,16] Endocem, a fast-setting MTA-derived pozzolan type of cement, was recently developed. Choi *et al*. demonstrated that Endocem had a much shorter setting time and more washout resistance under the presence of fetal bovine serum compared to ProRoot.[5] They also suggested that the decreased setting time might be associated with the increase in the early strength of Endocem. Similarly, Endocem demonstrated a lower washout score than ProRoot under all irrigation solutions in the present study ($p < 0.05$).

We also observed that the washout scores of ProRoot and Endocem were significantly different depending on the irrigation solution used. The NaOCl-treated group showed significantly lower washout scores compared to the saline- and CHX-treated groups for both ProRoot and Endocem ($p < 0.05$) (Figure 1b). There were some studies that showed NaOCl decreased the setting time and microleakage of MTA. Kogan *et al*. reported that different additives produced a wide range of MTA setting times, and NaOCl gel decreased the setting time while saline and CHX gel resulted in an increase in setting time.[12] Furthermore, Uyanik *et al*. reported that the sealing of furcal perforation repaired with MTA was affected by the irrigation regimens, and the samples irrigated with NaOCl had the lowest leakage values.[17] Notably, NaOCl has an alkaline pH of 9.0 - 10.5 whereas CHX and saline have neutral pH.[18] The literature indicated that lower pH environments may affect various physical and chemical properties of MTA negatively.[19-21]

At the microstructure level, the CHX group demonstrated different crystal morphology than the other groups. Evaluation of the SEM images revealed a distinct lack of crystal structures in all groups exposed to CHX when compared to the saline and NaOCl groups (Figure 3). Recently, Hong *et al*. reported that CHX adversely affected the physical properties and hydration behaviors of MTA when it was in contact with MTA before initial setting.[22] The authors explained that crystal structures were not apparent on the surface of the CHX-treated samples, and they were not the typical calcium hydroxide crystals on energy dispersive x-ray spectroscopic analysis. These findings may explain why the washout resistance of the CHX-treated group was lower than that of the NaOCl-treated group in this study.

Conclusions

Our results indicate that Endocem exhibited superior washout resistance compared to ProRoot, especially in the NaOCl-treated group. Thus, within the limitation of this study, Endocem may be considered a substitute for ProRoot in a single-visit scenario of conventional root canal treatment with furcal perforation repair because it is less loosened during the setting reaction. Furthermore, NaOCl can be considered a more suitable irrigant than physiologic saline and CHX in a tooth with furcal perforation repaired using Endocem in terms of washout resistance. However, further studies regarding the biocompatibility of Endocem with periodontal tissue should be considered.

Conflict of Interest: No potential conflict of interest relevant to this article was reported.

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Tooth Discoloration after the Use of New Pozzolan Cement (Endocem) and Mineral Trioxide Aggregate and the Effects of Internal Bleaching

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Tooth Discoloration after the Use of New Pozzolan Cement (Endocem) and Mineral Trioxide Aggregate and the Effects of Internal Bleaching

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Abstract

Introduction: The aim of this study was to evaluate tooth discoloration after the use of mineral trioxide aggregate (MTA) and to examine the effect of internal bleaching on discoloration associated with MTA. **Methods:** Thirty-two teeth were endodontically treated. Three-millimeter plugs of MTA, ProRoot, Angelus, or Endocem were placed on the access cavities of 24 teeth. Eight teeth served as the control group. After 24 hours, the access cavities were restored, and the tooth color was recorded at baseline and at 1, 2, 4, 8, and 12 weeks. After 12 weeks, the MTA materials were removed under a microscope, and an internal bleaching treatment was performed. After removal of the MTA materials and after a 1-week bleaching treatment, the color changes were measured, and the MTA-dentin interfaces were observed under a microscope. **Results:** The ProRoot and Angelus groups displayed increasing discoloration during a period of 12 weeks. The discoloration associated with ProRoot and Angelus was observed at the MTA-dentin interface and on the interior surface of the dentin. However, the Endocem groups demonstrated no significant discoloration ($P < .05$). No marginal discoloration was observed around the material in the Endocem group. Removal of the discolored MTA was effective for resolving the discoloration in all of the experimental groups ($P < .05$). However, a subsequent internal bleaching treatment was not significantly effective compared with the removal of MTA. **Conclusions:** ProRoot and Angelus caused tooth discoloration. However, Endocem did not affect the contacting dentin surface. Removing the discolored MTA materials contributed more to resolving the tooth discoloration than post-treatment internal bleaching. *(J Endod 2013;39:1598–1602)*

Key Words

Internal bleaching, mineral trioxide aggregate, pozzolan cement, tooth discoloration

Mineral trioxide aggregate (MTA) is a biocompatible material with a high sealing ability and less cytotoxicity compared with conventional endodontic materials such as amalgam, Super EBA, and intermediate restorative material (1–4). MTA is a powder derived from Portland cement that consists of fine hydrophilic particles of tricalcium silicate, tricalcium aluminate, tricalcium oxide, and other mineral oxides. MTA is set in the presence of water, which results in the formation of a crystallized calcium silicate hydrate gel and calcium hydroxide (5). Setting also occurs in the presence of blood; however, no significant negative effect on the leakage of MTA was reported (6). On the basis of various experimental results, MTA is considered to be a reliable material for use in vital pulp therapy in dental traumatology and may replace calcium hydroxide (7–9).

The first developed MTA was gray (GMTA). GMTA has the potential to cause tooth discoloration. Discoloration occurred in 60% of treated cases when GMTA was used as a pulpotomy medicament in primary teeth (10, 11). Consequently, white MTA (WMTA) was developed, and WMTA displayed no significant difference in the pulp response compared with GMTA (12). The major difference in the chemical composition between WMTA and GMTA is the concentration of metal oxides such as Al_2O_3, MgO, and FeO (13), which were assumed to be the main causes of discoloration. The WMTAs used in this study were the ProRoot tooth-colored MTA formula (Dentsply, Tulsa, OK) and Angelus MTA (Angelus, Londrina, PR, Brazil). Still, unexpected tooth discoloration has been reported after using WMTA for vital pulp therapy (14–16).

Recently, a new type of MTA derived from pozzolan cement (Endocem MTA; Maruchi, Wonju, Korea) has been introduced. Endocem is advantageous because of its rapid setting and manipulation properties. Furthermore, the biocompatibility and osteogenicity of Endocem are similar to those of conventional MTA (17).

There have been several recommendations on how to overcome the discoloration caused by MTA. Belobrov and Parashos (16) reported on a case in which the tooth was bleached by using sodium perborate mixed with saline to resolve discoloration that occurred 17 months after MTA pulp capping. Akbari et al (18) observed that applying a dentin bonding agent before MTA could prevent tooth discoloration. Although the incidence of tooth discoloration induced by MTA is frequent, there have been few published reports addressing this issue.

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The aim of this study was to evaluate tooth discoloration after the use of MTA and to examine the effect of internal bleaching on MTA discoloration.

Materials and Methods

Sample Preparation

Thirty-two freshly extracted human single-rooted incisors were used in this study. The criteria for tooth selection included the tooth being free of caries, cracks, restorations, calcifications, and an absence of any signs of internal or external resorption. The external surfaces of the teeth were cleaned with curettes and stored in a physiological saline solution until use.

The access cavities were prepared, and the working lengths were determined by using stainless steel hand files (Dentsply Maillefer, Tulsa, OK) until the tips were observed at the apical foramen, which was calculated by subtracting 1 mm from the length. The canals were shaped and enlarged by using #2, #3, and #4 Gates Glidden drills and WaveOne Large reciprocating files (Dentsply Maillefer, Ballaigues, Switzerland). The canals were then irrigated by using a 2.5% sodium hypochlorite solution. The prepared root canals were filled with gutta-percha (GP) cones (Diadent, Seoul, Korea) and AH Plus sealer (Dentsply, Konstanz, Germany), and then the GP cones were cut off 3 mm below the cementoenamel junction.

The endodontically treated teeth were randomly divided into 4 groups that each contained 8 teeth. The experimental groups consisted of 3-mm-thick MTA plugs that were represented by (1) ProRoot, (2) Angelus, and (3) Endocem, each of which was placed directly over the GP cones. Periapical radiographs were taken to ensure that there were no voids in the MTA materials and to determine the thickness of each filling. The access cavities were cleansed, and wet cotton pellets were placed over the MTA plugs. The access cavities were sealed with temporary restoration material (Caviton; GC Corp, Tokyo, Japan) for 24 hours and immersed in artificial saliva. After that delay, the temporary restorative materials were removed, and the setting states of the MTA materials were confirmed. The dentin adhesives (AdheSE; Ivoclar Vivadent, Schaan, Liechtenstein) were applied over MTA materials according to the manufacturer's instructions, followed by composite restoration by using Filtek Z350 (3M ESPE, St Paul, MN). The teeth in the control group were restored with a composite resin placed directly over the GP cones. All samples were stored in artificial saliva (Taliva; Hanlim Pharm Co, Seoul, Korea) at room temperature and replenished every 2 weeks.

Measuring Tooth Discoloration

The tooth color was recorded at baseline and at 1, 2, 4, 8, and 12 weeks by using a spectrophotometer (VITA Easyshade Advance; Vita Zahnfabrik, Bad Sackingen, Germany). The measurements were performed under constant laboratory illumination by positioning the spectrophotometer at the incisal, middle, and cervical areas of the teeth. The color measurements were repeated twice for each sample, and the records were reported by using the CIE L*a*b* system. The value of L* is the lightness ranging from 0 (black) to 100 (white). The values of a* and b* are the chromaticity coordinates in the red-green axis and the yellow-blue axis, respectively. The comparison of the measured L*a*b* values obtained from the spectrophotometer is expressed as ΔE. In our study, ΔE describes the color difference between the baseline and each different measurement point, and it was calculated by using the following equation:

$$\Delta E = \left\{(L_2 - L_1)^2 + (a_2 - a_1)^2 + (b_2 - b_1)^2\right\}^{1/2} (19).$$

Effects of Internal Bleaching on Discoloration

After 12 weeks, the composite restorations and MTA materials were carefully removed with a carbide round bur #1 and ProUltra Endo tip #1 (Dentsply Tulsa, Tulsa, OK) by using an operating microscope (Zeiss OPMI pico; Carl Zeiss, Goettingen, Germany) at the range of ×7.5–15 magnification. A mixture of sodium perborate and 3% hydrogen peroxide solution was used as an internal bleaching agent. The bleaches were applied to the access cavities and sealed with intermediate restorative material for 1 week. The color changes were measured both after removing the MTA materials and after 1 week of application of the bleaching agent. The changes in the MTA-dentin interface were observed under a microscope at ×15 magnification to enhance visualization and to provide illumination.

Statistical Analysis

The time effects were analyzed at 3 different measuring levels and for 3 different materials. Repeated-measures analysis of variance was applied to examine the ΔE value as the dependent variable and time as a factor. The effects of the materials at each time point were analyzed by using a one-way analysis of variance. Scheffé and Bonferroni comparison tests were used to determine the statistical significance at a 95% confidence level. Statistical analysis was performed by using SAS 9.2 (SAS Inc, Cary, NC).

Results

Overall, the significant discoloration was observed only in the cervical area of the tooth samples, and the middle and incisal areas displayed no significant color changes. Table 1 displays the ΔE values from the tooth samples for the different groups, and it displays the time points for the measurements in the cervical area. The ProRoot and Angelus groups displayed an increasing discoloration pattern in the cervical area over time. Tooth samples from the Endocem group presented indistinct grayish color changes during the course of the 12 weeks. This group showed the largest ΔE value after the first week but the lowest ΔE change during the course of the remaining 11 weeks (Fig. 1).

After 12 weeks, the composite and MTA were removed, and the MTA-dentin interfaces were observed under a microscope. The ProRoot and Angelus groups revealed dark marginal discoloration that appeared to spread into the dentin in the interfacial layer. The Endocem group presented no marginal discoloration around the material (Fig. 2).

TABLE 1. Mean ΔE Values (standard deviation) of Tooth Samples for the Different Groups and Time Points Measured in Cervical Area

Groups	1 week	2 weeks	4 weeks	8 weeks	12 weeks
Control	3.95 (0.80)^{aA}	3.61 (1.42)^{aA}	3.98 (2.09)^{aA}	3.49 (1.87)^{aA}	3.59 (1.92)^{aA}
ProRoot	4.31 (1.57)^{aA}	4.69 (1.17)^{aA}	4.69 (0.69)^{aA}	7.60 (2.29)^{abA}	14.85 (6.36)^{bB}
Angelus	3.47 (1.13)^{aA}	4.69 (2.03)^{aA}	5.53 (2.55)^{aAB}	7.71 (3.74)^{abAB}	9.11 (4.07)^{abB}
Endocem	6.18 (3.18)^{bA}	6.87 (2.75)^{aA}	6.85 (3.06)^{aA}	8.30 (3.72)^{bA}	8.46 (3.47)^{aA}

Uppercase letters (in row) and lowercase letters (in column) indicate statistically homogenous subgroups (Scheffé and Bonferroni test, $\alpha = 0.05$ was used for every column and row). The same uppercase letters (within one MTA material, in a row) and lowercase letters (within one time point, in a column) indicate statistically similar groups (Scheffé and Bonferroni test, $\alpha = 0.05$).

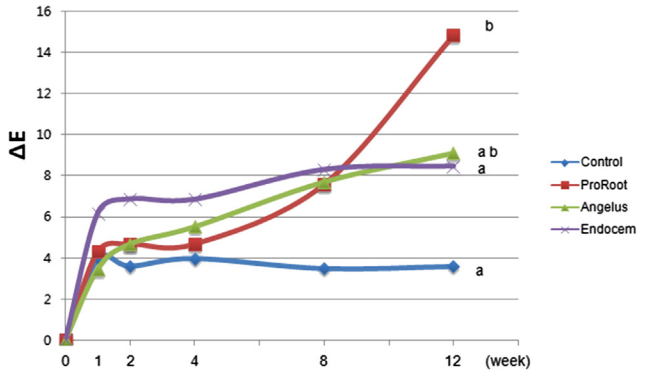

Figure 1. Change in the ΔE values during 12 weeks, measured at the cervical area. ΔE values indicate the differences in color, which were calculated by using the CIE L*a*b* values of 2 different measurements (L*, luminosity; a*, red-green parameter; b*, yellow-blue parameter). Different letters indicate statistically significant differences between the groups at 12 weeks ($P < .05$).

The ΔE values were decreased significantly in all of the experimental groups after removal of the discolored MTA ($P < .05$). However, a subsequent internal bleaching treatment did not significantly decrease the ΔE value compared with removal of MTA (Fig. 3).

Discussion

MTA satisfies many biological requisites for use as a vital pulp therapeutic material (6). However, tooth discoloration after MTA application is one of its main shortcomings, and this may be an esthetic concern when used on the anterior teeth. Several hypotheses have been proposed for the cause of MTA tooth discoloration (14, 15, 20); however, no report has clearly determined the mechanism.

In the present study, extracted teeth were used, and the teeth were endodontically treated so that we could apply the MTA materials over the GP cones. This pulpless tooth model is limited in its ability to appropriately reproduce the clinical and biological conditions of vital pulp therapy. However, this tooth model presented similar degrees and patterns of discoloration as did teeth treated with vital pulp therapy. To evaluate the tooth discoloration induced by endodontic materials, Lenherr et al (15) and Krastl et al (21) used extracted teeth and placed the endodontic materials within the dentin. Akbari et al (18) placed MTA materials over the GP cones in the dentinal root canals. These studies used extracted, nonvital teeth and reproduced significantly different tooth

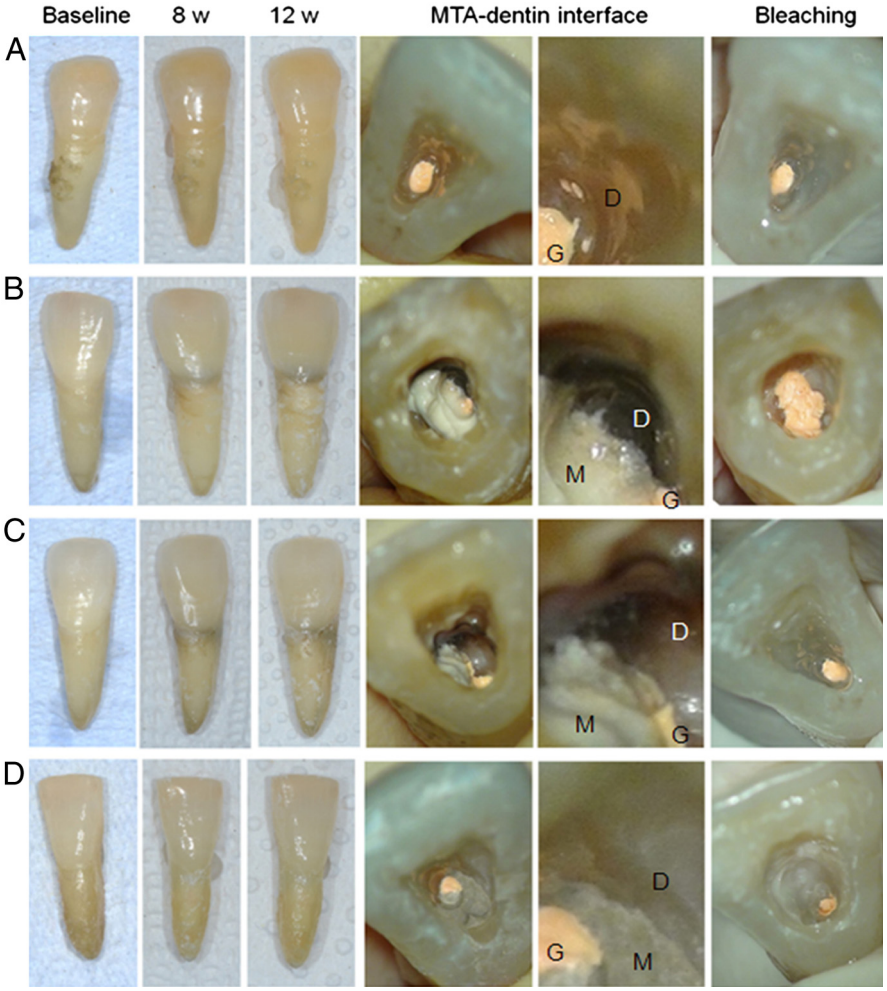

Figure 2. Photographs of discoloration at the time intervals after MTA application and microscopic images of the tooth sample after removal of MTA and bleaching treatment (original magnification, ×15). (*A*) Control, (*B*) ProRoot, (*C*) Angelus. In the ProRoot and Angelus groups, the cervical areas of the tooth samples showed dark discoloration, and the MTA-dentin interface revealed evident dark marginal pigmentation that appeared to be spreading into the dentin. (*D*) Endocem. In this group, the marginal discoloration pattern was absent. Note the gray color of the Endocem material itself, which is different from the ProRoot and Angelus group. D, dentin; G, gutta-percha; M, MTA material.

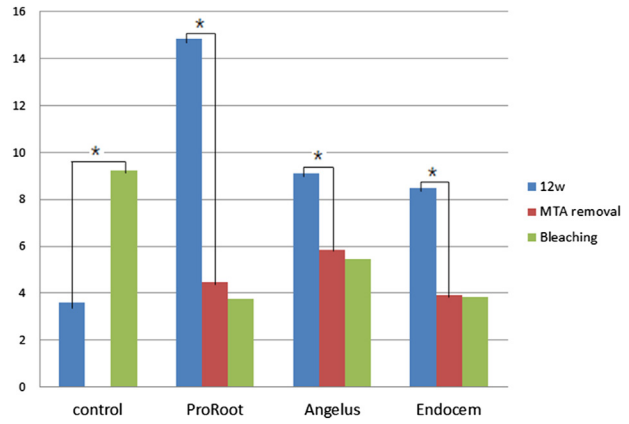

Figure 3. Change of the ΔE values after removal of MTA and bleaching. *Statistical significance ($P < .05$). ΔE values are coordinates of L*a*b* values between the baseline and each measuring point according to the following equation: $\Delta E = \{(L_2 - L_1)^2 + (a_2 - a_1)^2 + (b_2 - b_1)^2\}^{1/2}$. The control group presented an increase of the ΔE value after the bleaching treatment caused by the increase in the L* value. Other experimental groups presented significant decreases in ΔE value after removal of MTA because it contributed to recovering the tooth color as similar to its original L*a*b* values.

discolorations that were based on different endodontic materials, and the results from our pilot study corroborated these findings.

In our study, not only the gross observation of the teeth by the naked eye (Fig. 2) but also the ΔE values supported the discoloration caused by MTA materials. We used the CIE L*a*b* system to evaluate the color change. The mean color difference (ΔE) in the control group at 12 weeks was 3.59. The ΔE values for the experimental groups at 12 weeks ranged from 8.46–14.85 (Table 1). The CIE L*a*b* system is one of the most commonly used systems because it approximates uniform distances between the color coordinates, while entirely covering the visual color space (22, 23). In the previous studies it was shown that ΔE values over 2.0 and 3.7 were clinically detectable and could be obtained by calculating the L*a*b* values (23, 24). Our results for the color difference were coincident with those results, and we observed that all of the teeth in the experimental groups showed significant discoloration.

The ProRoot and Angelus (WMTA) groups demonstrated spontaneous increases in ΔE over time (Fig. 1), and the MTA-dentin interfaces revealed dark marginal discoloration that appeared to spread into the dentin (Fig. 2*B* and *C*). This observed tooth discoloration pattern was similar to the data provided in previous reports (14, 21). Recent *in vivo* studies demonstrated that the by-products of MTA hydration were deposited into the material surface or MTA-dentin interface and also into the intratubular dentin (25, 26). This has been reported to be the result of biomineralization, and some tag-like structures formed at the interfaces (26–28). Han and Okiji (29) noted that the width of the tag-like structure that formed along the MTA-dentin interface increased during a 90-day period. They suggested that the calcium ions released from the MTA reacted with phosphate ions that are available in the tissue fluid, and this reaction resulted in the precipitation of carbonated apatite. Although the methodology used in this study has limited ability to identify the mechanism of discoloration, we speculate that some component of the MTA may be bound to the phosphate ion or plasma protein in the dentinal fluid. After the chemical reaction between these components, the by-product might be oxidized, followed by transformation into a pigmented by-product. This hypothesis needs to be tested in future investigations.

In the results of the present study, the discoloration that spread into the interior of the dentin in the WMTA groups was absent in the Endocem group (Fig. 2*D*). The evident increase in the ΔE value during the first week in the Endocem group might be due to the material itself, which has a grayish color. The Endocem specimens used in the pilot study also presented little or no discoloration regardless of the storage medium. Endocem is a newly developed, fast-setting MTA-derived cement. The chemical composition of Endocem is similar to that of ProRoot. The composition provided by the manufacturer is CaO (46.7%), SiO_2 (12.8%), Al_2O_3 (5.4%), and other metallic oxides, and Bi_2O_3 (11%) is used as a radiopacifier. This pozzolan-based cement material chemically reacts in the presence of water, which is known as the pozzolanic reaction. The pozzolanic reaction occurs between calcium hydroxide, which is the product of cement hydration, and pozzolan. The reaction progresses in a manner similar to an acid-base reaction with oxides (SiO_2 + Al_2O_3 + Fe_2O_3) of the pozzolan (30). The small particle size of Endocem increases the surface contact of the particles while mixing with sterilized water, resulting in fast setting and ease of manipulation (17, 31, 32). A potential influence of the particle size might be the increase in surface area and hence the potential increase in the reactivity of the calcium silicate particles to form calcium hydroxide and calcium silicate hydrate phases. A recent *in vitro* study (17) demonstrated the rapid setting time of Endocem compared with ProRoot MTA. The final setting time of Endocem was 4 minutes ± 30 seconds, whereas that of ProRoot was 261 ± 21 minutes. This study also reported the biological effect of Endocem on the formation of the mineralization matrix by using osteogenic differentiation markers. The results indicated that the mineralization potential of Endocem was comparable with that of ProRoot. Further experimental studies are required to demonstrate the effect of rapid-setting Endocem on the time-consuming biomineralization behavior of conventional MTA.

In our study, the mean ΔE values in the experimental groups significantly decreased after removing the MTA (Fig. 3). Removing the MTA resulted in an increase in the L* value, and this caused the ΔE value to decrease significantly, which meant the tooth recovered its original color. After a subsequent bleaching treatment, the remnant pigments at the MTA-dentin interface were completely removed in all samples on microscopic observation. There were slight decreases in the ΔE values, but this did not statistically contribute to lightening the tooth. Our results were similar to those from the case report by Belobrov and Parashos (16).

Considering the indicated use of MTA, few clinical cases would allow for the removal of the entire MTA, followed by the application of a strongly acidic bleaching agent. Moreover, Tsujimoto et al (33) observed that the application of a bleaching agent over the MTA resulted in the structural deterioration of the MTA surface because of the bleaching agent's acidic pH. The use of a bleaching agent should be prescribed only in limited cases, and it may contribute a minor improvement in discoloration.

Keeping in mind the limitations of our study, we conclude that the MTA-derived Portland cement causes tooth discoloration during the course of 12 weeks. In contrast, MTA-derived pozzolan cement did not affect the contacting dentin surface. Tooth discoloration from MTA-derived Portland cement occurred at the MTA-dentin interface and in the interior of the dentin, whereas the discoloration of the MTA-derived pozzolan cement was caused by the color of the material itself. Removing the discolored MTA materials contributed more to improving tooth discoloration than post-treatment bleaching.

Acknowledgments

The authors deny any conflicts of interest related to this study.

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Abstract

Introduction: Mineral trioxide aggregate (MTA) is widely used as a pulp capping material. Recently, a MTA-derived fast-setting pozzolan cement (Endocem; Maruchi, Wonju, Korea) was introduced in the endodontic field. Our aim in this study was to investigate the odontogenic effects of this cement *in vitro* and *in vivo*. **Methods:** Human dental pulp cells (hDPCs) were cultured, and the effects of Endocem and a previously marketed MTA (ProRoot; Dentsply, Tulsa, OK) on biocompatibility were evaluated by assessing cell morphology and performing a cell viability test. Chemical composition of each material was analyzed by energy-dispersive X-ray spectroscopic analysis. Odontoblastic differentiation was analyzed by alkaline phosphatase activity and alizarin red S staining. The expression of odontogenic-related markers, namely dentin sialophosphoprotein, dentin matrix protein 1, and osteonectin, was evaluated by real-time polymerase chain reaction, Western blotting, and immunofluorescence analysis. Pinpoint pulp exposures were made on rat teeth and then capped with ProRoot or Endocem. After 4 weeks, reparative tertiary dentin formation and inflammatory responses were investigated histologically. **Results:** The biocompatibility of Endocem was similar to that of ProRoot. Energy-dispersive X-ray spectroscopic analysis showed that ProRoot and Endocem contained similar elemental constituents such as calcium, oxygen, and silicon. Alkaline phosphatase activity and mineralized nodule formation increased in ProRoot- and Endocem-treated cells compared with medium only–treated cells in the control group ($P < .05$). The expression of odontogenic-related markers was significantly higher in the ProRoot- and Endocem-treated groups than the control group ($P < .05$), but there was no significant difference in the expression of these markers between the 2 experimental groups ($P > .05$). Four weeks after the pulp capping procedure, continuous tertiary dentin had formed directly underneath the capping materials and the pulp exposure area in all samples in the 2 treated groups. Furthermore, most specimens either had no inflammation or minor pulpal inflammation. **Conclusions:** Our results indicate that ProRoot and Endocem have similar biocompatibility and odontogenic effects. Therefore, Endocem is as effective a pulp capping material as ProRoot. (*J Endod 2014;■:1–8*)

Key Words

Dental pulp, fast setting, mineral trioxide aggregate, odontogenic, pozzolan, tertiary dentin

Pulp capping, which involves sealing the pulp to stimulate the formation of tertiary dentin, is indicated to treat reversible pulpal injuries caused by physical or mechanical trauma. Mineral trioxide aggregate (MTA) has been successfully used for pulp capping in both experimental and clinical settings (1–5). However, it requires a long setting time; therefore, clinicians usually apply wet cotton to MTA and then make another appointment for further treatment to allow the MTA sufficient time to set. Numerous attempts have been made to shorten the setting time of MTA by adding chemical accelerators to MTA (6–9). However, there are 2 related problems with this approach. First, although these approaches did decrease the setting time, it was still too long to offer a significant clinical advantage. Second, additives might have an adverse effect on the physical and biological properties of MTA (9–11).

An MTA-derived pozzolan cement (Endocem; Maruchi, Wonju, Korea) that sets quickly has recently been marketed to endodontists. Choi et al (12) reported that Endocem has a much shorter setting time (around 4 minutes) and higher washout resistance than the previously marketed MTA brand (ProRoot; Dentsply, Tulsa, OK). They also showed that it had similar biocompatibility and mineralization potential as ProRoot in MG63 cells. However, they conducted their studies using a root-end surgery model, not a pulp capping model. No previous study has examined the odontogenic effects of Endocem versus ProRoot. Therefore, we investigated the odontogenic effects of these 2 cement types both *in vitro* and *in vivo* by examining their effects on odontoblastic differentiation of human dental pulp cells (hDPCs) and assessing tertiary dentin formation on capped rat teeth, respectively. Our 2 null hypotheses were as follows: (1) ProRoot and Endocem induce odontoblastic differentiation of hDPCs to a similar extent and (2) there is no difference between these 2 materials with respect to tertiary dentin formation or pulpal inflammation in experimentally exposed rat teeth.

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Materials and Methods

Primary Culture of hDPCs

Human dental pulp tissue was obtained from a freshly extracted human third molar. Pulp tissue was cut into fragments and cultured in minimal essential medium alpha (MEM-α; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS, Invitrogen) along with 100 U/mL penicillin and 100 U/mL streptomycin (Invitrogen). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. Cell cultures between the fourth and sixth passages were used. All experimental procedures were approved by the Institutional Review Board (IRB#: 2013-02-010-003) of Chonbuk National University Hospital, Jeonju, Korea.

Preparation of Material Extracts

ProRoot and Endocem were mixed according to the manufacturers' instructions. Mixed cement was placed into a paraffin wax mold (1-mm thickness and 5-mm diameter). Then, the cement was stored in an incubator at 95% ± 5% relative humidity at 37°C for 1 day. One tablet of each cement was stored in 10 mL MEM-α containing 10% FBS for 3 days to produce the extracts used for the treatment of hDPCs.

Cell Viability Test

Cells were seeded in 24-well culture plates at a density of 2×10^4 cells per well and preincubated in growth medium for 24 hours. Then, cells were treated with the prepared extracts (experimental groups) or medium only (control group). After exposure to the material extracts for 1, 2, 3, and 7 days, cell viability was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 200 μL MTT solution (0.5 mg/mL in phosphate-buffered saline [PBS], Invitrogen) was added to each well followed by a 2-hour incubation. Then, 200 μL dimethyl sulfoxide (Amresco, Solon, OH) was added to each well. The plates were then shaken until the crystals had dissolved, and the solution in each well was transferred to a 96-well tissue culture plate. Spectrophotometric absorbance was measured at 540 nm using an enzyme-linked immunosorbent assay plate reader (Synergy 2; Bio-Tek, Winooski, VT). Statistical analysis was performed by 1-way analysis of variance (ANOVA) followed by the multiple-comparison Tukey test ($P = .05$).

Measurement of pH

Specimens (1-mm thickness and 5-mm diameter) of ProRoot or Endocem were prepared and allowed to set for 1 day. After setting, 1 tablet was placed in 10 mL deionized water. The pH value of the water was then measured using a pH meter (Orion 3 Star; Thermo Scientific, Singapore). Before measurement, the pH apparatus was calibrated with solutions with pH levels of 7.0 and 4.0. Between each measurement, the electrode was washed with ultrapure water and blot dried. Data were then analyzed by an independent samples *t* test to compare the 2 materials ($P = .05$).

Scanning Electron Microscopic Analysis

Materials were condensed into 1 × 5-mm round wax molds under aseptic conditions. The materials were allowed to set for 24 hours in a humidified incubator at 37°C. Next, the disks were placed at the bottom of 24-well tissue culture plates. Cells were seeded at 1×10^5 cells per well on the prepared materials. After a 72-hour incubation period, the dishes were fixed with 2.5% glutaraldehyde (Sigma-Aldrich, St Louis, MO) for 2 hours. Samples were then dehydrated in increasing concentrations of ethanol (70%, 80%, 90%, 95%, and 100%) for 20 minutes at each concentration and immersed in n-butyl alcohol (Junsei Chemical Co, Tokyo, Japan) for 20 minutes. Scanning electron microscopy was performed using the SN-3000 system (Hitachi, Tokyo, Japan) operated at 10 kV.

Energy-dispersive X-ray Spectroscopic Analysis

Energy-dispersive X-ray spectroscopic (EDS) analysis was executed using the Apollo-X detector (EDAX, Mahwah, NJ), which was attached to a scanning electron microscope, for chemical element analysis of the surface of ProRoot and Endocem. The high magnification of ×10,000 was selected to discern the chemical compositions of specific crystal types within a sample. Via this process, a spectrum was obtained, and elements could be identified. Semiquantitative, standardless analyses of these spectra were performed to derive the atomic percent concentrations of constituent elements.

Alkaline Phosphatase Activity

Cells (5×10^4) were inoculated in 6-well culture plates and preincubated in medium for 24 hours. After hDPCs were incubated for 1, 2, and 3 days in the presence of material extract, cells were scraped into cold PBS and then sonicated with a cell disruptor (Heat System Ultrasonics, Plainview, NJ) in an ice-cold bath. Alkaline phosphatase (ALP) activity in the supernatant was determined using the method reported by Lowry et al (13) using p-nitrophenyl phosphate as a substrate. Absorbance was measured at 410 nm using an enzyme-linked immunosorbent assay reader (Beckman DU-650; Beckman Coulter, Fullerton, CA). One-way ANOVA and Tukey tests were used for statistical analyses ($P = .05$).

Alizarin Red S Staining to Detect Mineralized Nodule Formation

Cells were placed in a 24-well plate at a density of 2×10^4 cells per well and cultured for 24 hours for initial attachment. After exposure to the extract medium (experiment group) or medium only (control group) for 14 days, mineralization was assessed by staining with alizarin red S (Sigma-Aldrich). In brief, cells were fixed with 4% formalin for 1 hour at 4°C, washed 3 times with distilled water, and then stained with 40 mmol/L alizarin red (pH = 4.2) solution. After being washed with deionized water, the stained cell culture plate was moved to a scanner, and the stained image was acquired. For quantitative evaluation, the sample was reacted with 10% cetylpyridinium chloride (pH = 7.0) solution at room temperature for 15 minutes to dissolve the stain, and absorbance was measured at a wavelength of 540 nm with a standard solution. One-way ANOVA and Tukey tests were used for statistical analyses ($P = .05$).

Real-time Polymerase Chain Reaction Analysis

Cells (2×10^5) in MEM-α containing 10% FBS were seeded in 6-well tissue culture plates and incubated for 24 hours. The medium was then switched to the extract medium. After exposure to the extract medium for 3 days, cells were lysed, and total RNA was isolated using Trizol reagent (Invitrogen). After chloroform extraction, total RNA was recovered from the aqueous phase and precipitated using 75% isopropanol and RNase-free distilled water (USB, Cleveland, OH). Then, reverse transcription of RNA was performed using the Superscript First-Strand Synthesis Kit (Invitrogen).

SYBR green-based real-time polymerase chain reaction (PCR) was conducted and optimized using the TOPreal qPCR Premix Kit (Enzynomics, Cheongju, Korea). The final PCR mixture contained 2 μL each of forward and reverse primers (final concentration of 0.4 μmol/L for each), 2 μL SYBR green (2×), 1.6 μL $MgCl_2$ (final

concentration of 3 mmol/L), and 5 μL template; the volume was adjusted to 20 μL using nuclease-free water. Primer sequences are provided in Table 1. All real-time PCR reactions were performed in triplicate and conducted using the StepOne Real-Time PCR System (Applied Biosystems, Singapore). The following amplification protocol was used: 10 minutes at 95°C followed by 30 cycles at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds. One-way ANOVA and the Tukey test were used for statistical analyses (P = .05).

Western Blotting

Cells (3×10^5) in MEM-α containing 10% FBS were seeded in 100-mm culture plates and incubated for 24 hours. The medium was then switched to extract medium. After exposure to the extract medium for 3 days, cell lysates from hDPCs were prepared by solubilizing the cells with protein lysis buffer (Pro-prep; iNtRON Biotechnology, Seongnam, Korea) for 10 minutes on ice. Cell lysates were centrifuged at 13,000 rpm for 10 minutes, and protein concentrations were determined with Bradford reagent (Bio-Rad Laboratories, Hercules, CA). Samples containing equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose transfer membranes (Protran; Whatman, Dassel, Germany). Membranes were blocked with 5% skimmed milk in Tris-Buffered Saline and Tween 20 (TBST) at room temperature for 30 minutes and incubated overnight at 4°C with primary antibodies against dentin sialophosphoprotein (DSPP; Santa Cruz Biotechnology, Santa Cruz, CA), dentin matrix protein 1 (DMP1, Santa Cruz Biotechnology), osteocalcin (ON, Santa Cruz Biotechnology), or glyeraldehyde-3-phosphate dehydrogenase (GAPDH) (Thermo Scientific, Rockford, IL) followed by incubation with HRP-conjugated secondary antibodies. Antibody-bound proteins were detected using the ECL Western Blotting Luminol Reagent (Santa Cruz Biotechnology). The intensity of protein expression after normalization with GAPDH was quantified using an image analysis program (Image J; National Institutes of Health, Bethesda, MD). One-way ANOVA and Tukey test were used for statistical analyses (P = .05).

Immunofluorescence Analysis

Glass coverslips were sterilized by dipping them in 90% ethanol and then carefully drying them over a flame. Then, a coverslip was placed in each well of a sterile 6-well tissue culture plate. Cell suspensions containing 1×10^4 cells/mL were added to each coverslip. After cells were incubated for 24 hours, the medium was switched to extract medium. After exposure to the extract medium for 3 days, cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature. They were then incubated in 0.1% Triton X-100 (Amresco, Solon, OH) in PBS for 15 minutes. After blocking with 10% goat serum for 1 hour at room temperature, cells were incubated for 2 hours with monoclonal mouse anti-DSPP (Santa Cruz Biotechnology), anti-DMP1 (Santa Cruz Biotechnology), or anti-ON antibodies (Santa Cruz Biotechnology) (1:100) in 10% goat serum. Then, the cells were incubated with fluorophore-conjugated secondary antibodies (anti–mouse-fluorescein isothiocyanate [FITC]) for 2 hours at room temperature. Coverslips were mounted onto slides using mounting solution. Fluorescent images were obtained using a fluorescence microscope (Carl Zeiss, Jena, Germany).

TABLE 1. Real-time PCR Primers

Genes	Sequence	Size
DSPP	Forward: 5′-GGGATGTTGGCGATGCA-3′ Reverse: 5′-CCAGCTACTTGAGGTCCATCTTC-3′	70
DMP1	Forward: 5′-AGCATCCTGCTCATGTTCCTTT-3′ Reverse: 5′-GAGCCAAATGACCCTTCCATT-3′	106
ON	Forward: 5′-ACCAGCACCCCATTGACG-3′ Reverse: 5′-AGGTCACAGGTCTCGAAAAAGC-3′	109
GAPDH	Forward: 5′-AAGGTGAAGGTCGGAGTCAAC-3′ Reverse: 5′-GGGGTCATTGATGGCAACAATA-3′	102

Surgical Procedure

Twenty healthy upper first molars from 10 eight-week-old male Wistar rats were used for this study. Occlusal class I cavities were prepared, and then pinpoint pulpal exposure was made on the occlusal surface of the upper first molar using a #1/8 round carbide burr at high speed under water cooling. Then, teeth were randomly divided into 2 test groups: one in which ProRoot (n = 8) was used to cap the teeth and the other in which Endocem (n = 8) was used. Materials were mixed according to the manufacturers' recommendations and then applied to the exposure site. The cap was covered with a thin layer of light-cured glass ionomer cement (Fuji II LC; GC, Tokyo, Japan). Four teeth in the control group were capped only with glass ionomer cement. After 4 weeks, rats were sacrificed by transcardial perfusion with 4% paraformaldehyde in PBS. These experimental procedures were approved by the Institutional Animal Care and Use Committees (IACUC#: WKU12-34) of Wonkwang University (Iksan, Korea).

Histologic Examination

Maxillary segments were dissected carefully, immersed in 4% paraformaldehyde, and kept at 4°C for 24 hours. After decalcification using 18% EDTA (Yakuri Pure Chemical, Osaka, Japan) solution, specimens were embedded in paraffin, sectioned (5-μm thickness), and stained with hematoxylin-eosin. Tertiary dentin formation and pulp inflammation were scored according to the criteria used in a previously published study with slight modifications (Table 2) (14). Pulp inflammation was determined by taking 3 central sections beneath the dentin bridge. The Mann-Whitney U test was used to evaluate tertiary dentin formation. A P value <.05 was considered statistically significant.

Results

Cell Viability Test

To evaluate cell viability in the presence of the material extracts, we performed an MTT assay. As shown in Figure 1*A*, ProRoot- and

TABLE 2. Scores Used for Histologic Evaluation of Dentin Bridge Formation and Pulp Inflammation

	Score	Characterization
Dentin bridge formation	1	Complete
	2	Little communication of the capping material with dental pulp
	3	Only lateral deposition of hard tissue on the walls of the cavity
	4	Absence of hard tissue bridge
Pulpal inflammation	1	No inflammation
	2	Minimal inflammation (scattered chronic inflammatory cells beneath the calcified bridge or capping area)
	3	Moderate inflammation (obvious number of chronic inflammatory cells without sign of necrosis)
	4	Severe inflammation (abscess formation, necrosis and presence of polymorphonuclear cells)

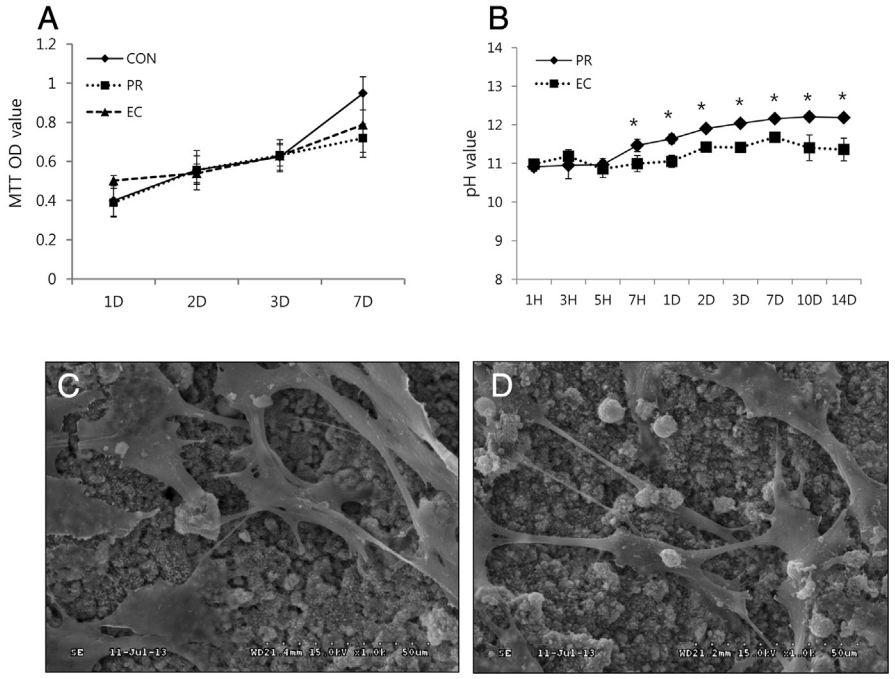

Figure 1. (*A*) Effects of ProRoot (PR) and Endocem (EC) on cell viability as measured by the MTT assay. *Significant differences between groups, $P < .05$. (*B*) pH values of PR and EC. *Significant difference between each group, $P < .05$. Scanning electron microscopic observations of cells incubated for 3 days on (*C*) ProRoot (×1000) and (*D*) Endocem (×1000).

Endocem-treated cells had similar cell viability as untreated control cells throughout the experimental period ($P > .05$).

Measurement of pH Value

ProRoot and Endocem were highly alkaline (pH between 10 and 12). There was no significant difference in pH values between the 2 cements until 5 hours ($P > .05$). However, the pH values of ProRoot were significantly higher than those of Endocem after 7 hours ($P < .05$) (Fig. 1*B*).

Cell Morphologic Analysis

To evaluate cell growth and morphology, cells were observed by scanning electron microscopy. Well-spread and flattened cells in close contact with the surfaces of ProRoot and Endocem were observed (Fig. 1*C* and *D*).

EDS Analysis

To investigate the chemical composition of the materials, EDS analysis was performed. The EDS spectra for elemental identification showed that both ProRoot and Endocem contained calcium, oxygen, and silicon as the major elemental constituents. Notably, the percentage of calcium was around 40% in both materials (42.45% in ProRoot and 39.87% in Endocem).

Effects of ProRoot and Endocem on ALP Activity

Compared with untreated control cells, cells treated with ProRoot and Endocem showed a significant increase in ALP activity after 2 and 3 days ($P < .05$) (Fig. 2*C*). However, there was no significant difference in ALP activity between the 2 experimental groups ($P > .05$).

Effects of ProRoot and Endocem on Mineralization

There was a significant increase in mineralization in the ProRoot- and Endocem-treated groups compared with the control group based on alizarin red S staining for calcium (Fig. 2*D* [$P < .05$]). However, there was no significant difference in mineralization between the 2 experimental groups ($P > .05$).

Real-time PCR Analysis

To investigate the expression of odontoblast-specific genes, real-time PCR analysis was performed. As shown in Figure 3*A–C*, the expression of DSPP, DMP1, and ON messenger RNA (mRNA) was higher in the ProRoot- and Endocem-treated cells than the medium only–treated cells in the control group ($P < .05$). However, there was no significant difference in the expression of these 2 markers between the 2 experimental groups ($P > .05$).

Western Blotting

To verify differentiation of hDPCs into odontoblast-like cells, the presence of specific markers was analyzed by Western blotting in whole cell lysates of hDPCs. Densitometric analysis was also performed on the bands for semiquantitative analysis of the amount of protein present. Similar to the real-time PCR results, the expression of DSPP, DMP1, and ON was higher in the 2 experimental groups than the control group ($P < .05$). However, there was no significant difference in the levels of these 3 proteins between the 2 experimental groups ($P > .05$) (Fig. 3*D* and *E*).

Immunofluorescence Analysis

Immunofluorescence labeling was performed to analyze the localization of DSPP, DMP1, and ON in hDPCs. DSPP, DMP1, and ON were localized in the cytoplasm, specifically in the perinuclear region of ProRoot- and Endocem-treated cells. Furthermore, the protein signals in

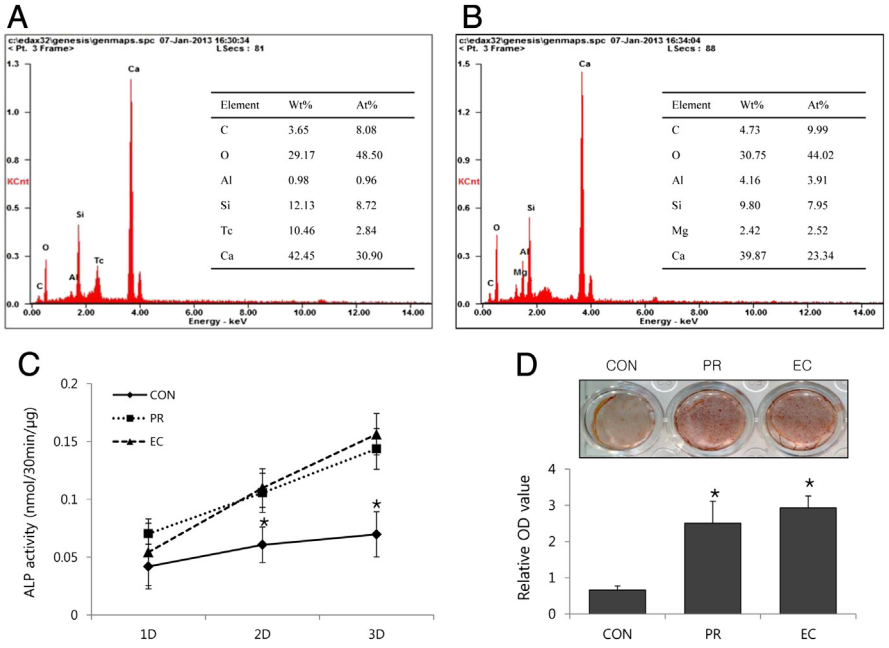

Figure 2. EDS analysis of the samples: (*A*) ProRoot and (*B*) Endocem. Effects of ProRoot (PR) and Endocem (EC) on (*C*) ALP activity and (*D*) the formation of calcification nodules in hDPCs. *A significant difference was determined at $P < .05$. CON, control.

the cells of the experimental groups were stronger than those in the cells of the control group (Fig. 4*A*–*I*).

Effects of ProRoot and Endocem on Tertiary Dentin Formation *In Vivo*

Four weeks after treatment, tertiary dentin with complete continuity had formed directly underneath the capping materials and the pulp exposure area in all samples in the 2 cement-treated groups (Table 3 and Fig. 5*A* and *B*). Most ProRoot and Endocem specimens did not have inflammation or showed only minor pulpal inflammation. Notably, odontoblastlike cells were polarized and appeared to be arranged in a palisade pattern (Fig. 5*D* and *E*). In contrast, there was no tertiary dentin formation in the pulp exposure area in the control group (Fig. 5*C*). Consequently, there was no significant difference between Endocem and ProRoot with respect to the continuity of tertiary dentin or

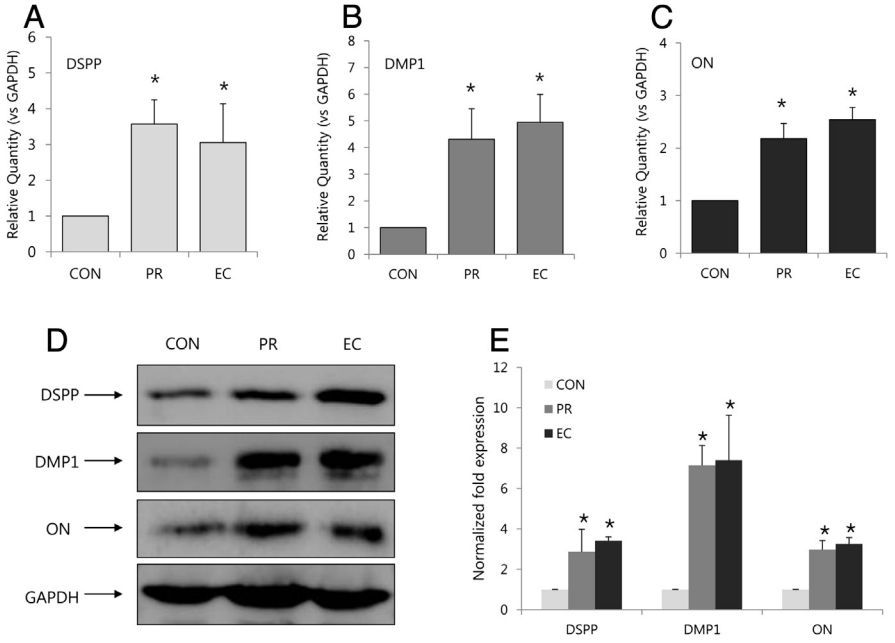

Figure 3. (*A*–*C*) Effects of ProRoot (PR) and Endocem (EC) on the expression of DSPP, DMP1, and ON mRNA in hDPCs. Relative mRNA levels were calculated by dividing the absolute levels of the expression of DSPP mRNA with the absolute level of expression of GAPDH mRNA. *Significant difference between groups, $P < .05$. (*D* and *E*) Effects of PR and EC on DSPP, DMP1, and ON protein expression in hDPCs. The graph shows quantification of protein expression by densitometry and data are presented as fold increases relative to control cells. *Significant difference between groups, $P < .05$.

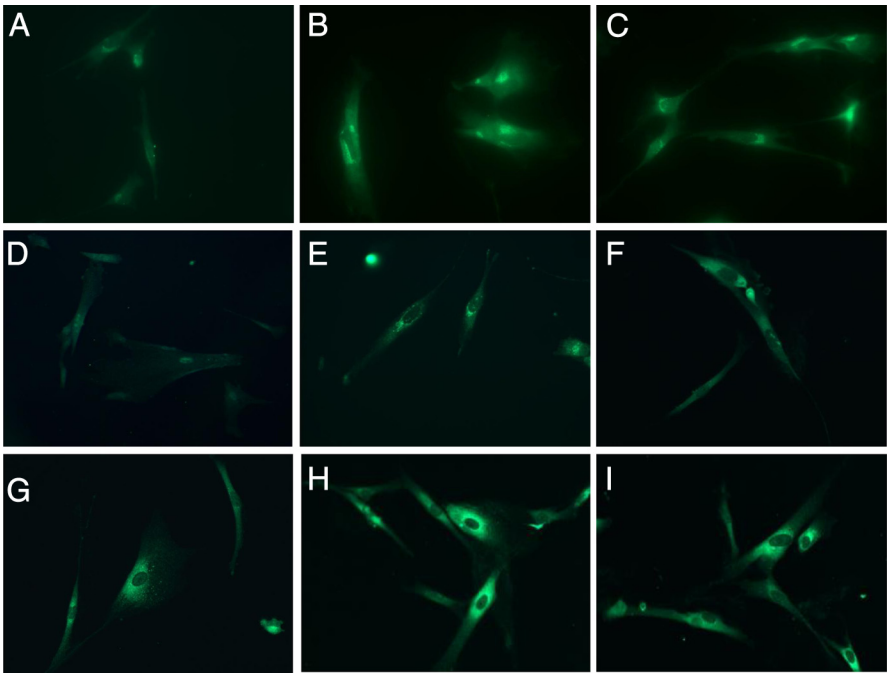

Figure 4. Immunofluorescence analysis of hDPCs treated with (*A*, *D*, and *G*) medium only, (*B*, *E*, and *H*) ProRoot, or (*C*, *F*, and *I*) Endocem. Fluorescence images showing (*A–C*) anti-DSPP, (*D–F*) anti-DMP1, and (*G–I*) anti-ON signals (*green*) of cells after 3 days of culture (×400).

the severity of pulp inflammatory responses to the pulp capping material ($P > .05$) (Table 3).

Discussion

Endocem, an MTA-derived cement, which is composed of small particles of pozzolan cement without any chemical accelerators, sets much quicker than ProRoot. We previously showed that Endocem sets at around 4 minutes and possesses similar biocompatibility to ProRoot in MG63 cells (12). However, to the best of our knowledge, no study has compared the effects of Endocem as a direct pulp capping material with that of ProRoot. Here, we investigated the odontogenic effects of both materials *in vitro* in hDPCs and *in vivo* in mechanically exposed rat dental pulp.

First, we compared the biocompatibility of the 2 materials by evaluating the effects of these materials on cell morphology and cell viability. It is generally considered favorable for a pulp capping material to be biocompatible because the material is then less likely to induce a response such as pulpal inflammation (15). In this study, ProRoot and Endocem had similar effects on cell viability in the MTT assay throughout the experimental period (Fig. 1*A*). Furthermore, scanning electron microscopic observations revealed that hDPCs cultured directly on ProRoot or Endocem for 3 days appeared to be flat and exhibited well-defined cytoplasmic extensions (Fig. 1*C* and *D*). These results indicate that Endocem allowed cell attachment and growth to a degree similar to ProRoot, which is consistent with our previous study in MG63 cells (12). In fact, the chemical composition of Endocem is very similar to that of ProRoot, which is a mineral trioxide aggregate. The short setting time of Endocem relative to ProRoot, even though the former does not contain any chemical accelerator, is because it consists of small-sized pozzolan particles that provide more surface contacts for the mixing liquid, resulting in rapid setting.

Next, we investigated whether ProRoot and Endocem facilitated odontoblastic differentiation of hDPCs *in vitro*. We showed that both ProRoot and Endocem promoted odontoblastic differentiation, as evidenced by the induction of ALP activity, the formation of mineralization nodules, and the increased expression of odontogenic-related markers (DSPP, DMP1, and ON). For the pulp capping procedure to promote tertiary dentin formation, undifferentiated dental pulp cells should differentiate into odontoblastlike cells that can secrete noncollagenous proteins. Among these proteins, DSPP, which is deposited primarily by odontoblasts, is considered a specific biochemical marker for functional odontoblasts (16). DMP1 is also considered an odontoblastic marker, even though it is less specific than DSPP (17). ON is a major noncollagenous protein of bone and dentin and is responsible for the mineralization properties of these tissues (18). As shown in Figure 3, the relative quantities of DSPP, DMP1, and ON mRNA and protein were significantly higher in ProRoot- and Endocem-treated cells than the medium only–treated cells of the control group ($P < .05$). However, there was no significant difference in the expression of these markers between the 2 experimental groups ($P > .05$). In

TABLE 3. The Number of Specimens Attributed for Each Group of Evaluation

		Tertiary dentin formation				Inflammatory response			
Group	No. of specimens	1	2	3	4	1	2	3	4
Control	4	0	0	0	4	0	0	0	4
ProRoot	8	8	0	0	0	8	0	0	0
Endocem	8	8	0	0	0	7	1	0	0

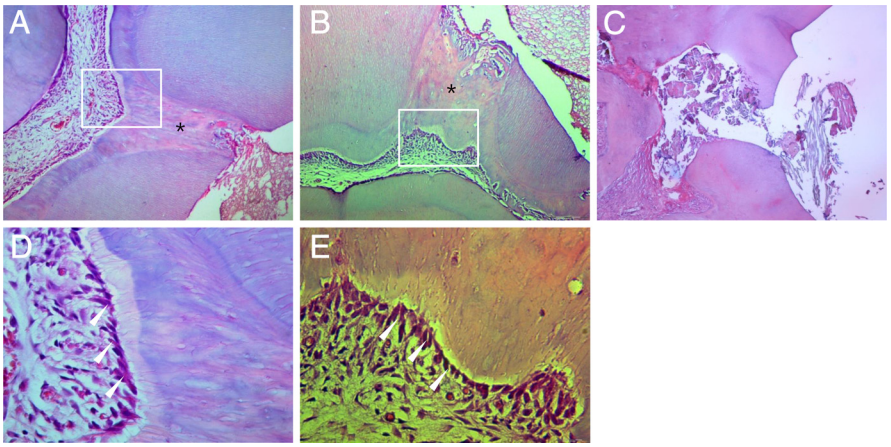

Figure 5. Capped pulps stained with hematoxylin-eosin 4 weeks after treatment with (*A*) ProRoot and (*B*) Endocem (×100). (*C*) A specimen in the control group that was capped without a pulp capping material. (*D* and *E*) High magnification of boxed areas in *A* and *B* (×400), respectively. Odontoblasts (*arrowheads*) are polarized and appear to be arranged in a palisade pattern. *Reparative tertiary dentin formed underneath the capping materials.

immunofluorescence analyses, we observed that the signals from DSPP, DMP1, and ON proteins were stronger in the ProRoot- and Endocem-treated cells than the control group cells (Fig. 4). Several recent studies also showed that MTA significantly up-regulates the expression of odontogenic markers in hDPCs (2, 19–21). It was also reported that even Portland cement, the base material of MTA, can increase DSPP mRNA expression (22). It is possible that calcium ions released from MTA-derived cements may affect odontoblastic/osteoblastic differentiation *in vitro*. Matsumoto et al (23) showed that an influx of calcium ions released from MTA into cells contributed to osteoblastic differentiation. Woo et al (21) also showed that a calcium channel blocker, nifedipine, attenuated MTA-induced odontoblastic differentiation in hDPCs and suggested that calcium ions released from MTA play an important role in the differentiation of hDPCs. EDS analysis revealed that Endocem contains around 40 wt% calcium, which is similar to ProRoot (Fig. 2*A* and *B*). Therefore, we suggest that calcium ions released from these materials affect the expression of DSPP in hDPCs *in vitro*.

Lastly, we investigated tertiary dentin formation induced by ProRoot or Endocem *in vivo*. Similar to our *in vitro* findings, there was no difference between ProRoot and Endocem regarding tertiary dentin formation. Tertiary dentin with complete continuity was formed directly underneath the capping materials and the pulp exposure area in all samples from the 2 cement-treated groups. It is generally believed that high pH values after mixing are caused by the mechanism of action of MTA on tertiary dentin formation *in vivo* (24). MTA dissolves into calcium hydroxide when it contacts pulp tissue, which results in a high pH (25, 26). The high pH of calcium hydroxide appears to result in mild stimulation of cell differentiation. In the present study, both ProRoot and Endocem had pH values that remained consistently high for 14 days. The high pH stimulated differentiation of dental pulp cells (Fig. 1*B*) and, consequently, might have influenced the formation of tertiary dentin *in vivo* according to the aforementioned mechanisms. Furthermore, the biocompatibility of capping materials plays a critical role in inflammatory cell responses (27). ProRoot and Endocem either did not cause inflammation or were associated with only minor pulpal inflammation (Table 3); most of the samples in the 2 groups were composed of normal tissue. This result, together with our *in vitro* experimental results, indicates that both ProRoot and Endocem have good *in vivo* biocompatibility.

It appears that Endocem induces tertiary dentin formation, both *in vitro* and *in vivo*, to a similar extent as ProRoot. Thus, we experimentally confirmed our 2 null hypotheses. In this respect, our results suggest that Endocem is potentially suitable for use as an effective pulp capping material. However, long-term clinical evaluation studies are required to confirm our findings.

Acknowledgments

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The authors deny any conflicts of interest related to this study.

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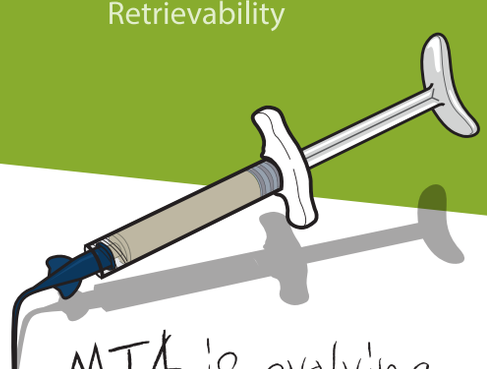

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