INTRODUCTION

Many clinical situations such as pulp capping, apexification, root-end fillings, and perforation repairs need endodontic root repair materials. An ideal endodontic root repair material should possess biocompatibility, radiopacity, antibacterial activity, easy handling, and good sealing ability. Mineral trioxide aggregate (MTA) is a material of choice in these situations. It consists of fine hydrophilic particles of tricalcium silicate, tricalcium aluminate, tricalcium oxide, and silicate oxide. It sets in the presence of moisture. MTA has significant advantages over other materials due to its biocompatibility and osteoconduction ability. However, MTA has a long setting time. In addition, it is not easy to handle MTA. According to Nekoofar et al., such disadvantages of MTA are due to mixing inconsistency. That is, the amount of water released from a plastic container is inconsistent. This inconsistency in the amount of water inside ProRoot MTA packages may explain uncontrolled and undesirable characteristics of the material under various clinical and laboratory situations.

Based on outstanding biological properties of MTA, a new calcium silicate-based cement called EndoSequence Root Repair Material (ERRM; Brasseler USA, Savannah, GA, USA) has been developed. According to the manufacturer, unlike MTA, this material is in a ready-to-use and premixed putty form or syringe form with a working time of more than 30 min. Similar to MTA, setting reaction of ERRM is initiated by water. Its setting time is also dependent on the presence of moisture. Many studies have investigated the cytotoxicity and antibacterial effect of ERRM. Although Rifaey et al. compared the osteogenic potential of MTA and ERRM, there is little information about its osteoblastic differentiation ability. Interaction between retrograde filling material and periradicular tissue is a key factor in tissue repair or healing. Thus, the aim of this study was to evaluate the chemical property, surface texture and the effect of ERRM, a newly-developed retrograde filling material, on osteoblastic differentiation compared to MTA.

MATERIALS AND METHODS

Preparation of test materials
MTA (ProRoot MTA, Dentsply Tulsa Dental, Tulsa, OK, USA) was mixed with sterile water in a 3:1 powder to liquid ratio for 1 min following the manufacturer’s recommendations. For ERRM, a premixed putty was used. All specimens used in this study were fabricated using cylindrical polyethylene molds.

Scanning electron microscope (SEM)
Six samples (8.0 mm in diameter and 3.0 mm in height) of each test material were used. Surfaces of each test group were observed by SEM (S4700, Hitachi, Tokyo, Japan). These samples were mounted onto aluminum holders with adhesive carbon tape and sputter coated with 15 nm gold palladium using a vacuum evaporator.
at 0.5 mmHg (vacuum pressure) and 12 mA (discharge current) for 100 s. Scanning electron microscopic observation (magnification: ×500) was conducted using a secondary electron imaging technique operating at 20 kV and a high vacuum type.

\[ \text{pH} \]

The pH was measured by using a pH meter (ORION STAR A211, Thermo Scientific, Waltham, MA, USA) with an electrode for solid specimens (InLab Surface, Mettler Toledo, Schwerzenbach, Switzerland). Before the test, the apparatus was calibrated with standard solutions of pH 4.0, 7.0, and 11.0. Six samples (10.0 mm in diameter and 5.0 mm in height) of each material were used. Readings were taken at various time intervals after setting (after 3 h, 24 h, 7 days, and 14 days). Between each measurement, the electrode was washed with distilled water and blot dried.

\[ \text{Cell culture} \]

MC3T3-E1 cells were used for this study. They were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin, Invitrogen) at 37ºC in a humidified atmosphere containing 5% CO₂.

\[ \text{Material extracts} \]

Samples (5 mm in diameter and 3 mm in height) of each test material were kept at 37ºC with relative humidity of 95% for 24 h for complete setting. After setting, both surfaces of samples were exposed to ultraviolet light (1 kV and a high vacuum type). Between each measurement, the electrode was washed with distilled water and blot dried.

\[ \text{Cell viability assay} \]

MC3T3-E1 cells were seeded into 96-well culture plates at a density of 1×10⁴ cells per well and incubated in a growth medium (DMEM containing 10% FBS and 1% antibiotics) for 24 h to allow adhesion. The medium was replaced with 100 μL of each material extract and incubated for 24 h. MC3T3-E1 cells with growth medium were used as controls. To compare dose-dependent relationship, each material extract was gradually diluted with growth medium to obtain five dilutions (1, 1/2, 1/4, 1/10, 1/50). Cell viability was examined using 4-[3-(4-Idophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1 assay, EZ-Cytox, Daeil Lab Service, Seoul, Korea) according to the manufacturer's recommendations. Briefly, 10 μL of EZ-Cytox (tetrazolium salts) was added to culture medium and incubated with cells at 37ºC for 3 h. Absorbance was measured at wavelength of 420 nm with a background subtraction of 650 nm using a VERSAmax multiplate reader spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

\[ \text{RNA isolation and quantitative real-time polymerase chain reaction (PCR)} \]

MC3T3-E1 cells were seeded into 6-well cell culture plates at a density of 2×10⁴ cells per well and incubated in a growth medium for 24 h. The growth medium was replaced with a medium containing 1/4 dilution of each material extract. Untreated cells (medium only) were used as controls. To compare dose-dependent relationship, each material extract was gradually diluted with growth medium to obtain five dilutions (1, 1/2, 1/4, 1/10, 1/50). Cell viability was examined using 4-[3-(4-Idophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1 assay, EZ-Cytox, Daeil Lab Service, Seoul, Korea) according to the manufacturer's recommendations. Briefly, 10 μL of EZ-Cytox (tetrazolium salts) was added to culture medium and incubated with cells at 37ºC for 3 h. Absorbance was measured at wavelength of 420 nm with a background subtraction of 650 nm using a VERSAmax multiplate reader spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

\[ \text{ALP staining} \]

MC3T3-E1 cells were seeded into 24-well cell culture plates at a density of 2×10⁴ cells per well with growth medium. After 24 h, growth medium was changed to an induction medium containing 1/4 dilution of each material extract and cultured for five days. These cultured cells were washed with phosphate buffered saline (PBS) and fixed with 70% ethanol for 20 min at 4ºC. After fixation, cells were washed with deionized water 3 times. ALP staining solution (1-Step NBT/BCIP Solution, Thermo Fisher Scientific, Rockford, IL, USA) was then added to each well (300 μL per well) and incubated at room temperature under dark condition for 15 min. The results of the ALP staining were quantified using Image J (Version 1.47, National Institutes of Health, Bethesda, MD, USA). The density of the experimental group was normalized according to the density of control.

\[ \text{Statistical analysis} \]

Each experiment containing triplicate independent
samples was repeated at least twice and qualitatively identical results were obtained. One-way analysis of variance followed by Tukey’s post hoc test was used to determine any statistically significant difference according to test materials. All statistical analyses were performed using SPSS 18.0 software program (SPSS, Chicago, IL, USA). Differences were considered statistically significant at $p<0.05$.

**RESULTS**

*Surface texture*

Scanning electron microscopic images were obtained to reveal the texture of material surface. The surface of MTA (Fig. 1A) and ERRM (Fig. 1B) showed crystallographic features. Spindle-shaped crystals appeared in MTA while clusters of round-shaped nanosize crystals appeared in ERRM.

*pH*

The mean pH of all samples is presented in Fig. 2. ERRM presented lower pH in comparison to MTA at all time periods except for 7 days after setting. There was no statistically significant ($p>0.05$) difference in pH between MTA and ERRM. MTA and ERRM showed similar graph patterns according to time periods. The pH level was increased up to 7 days after mixing. It showed the highest value on 7 days after setting. After 14 days, pH values of the experimental group were decreased, although they remained relatively high.

![Fig. 1](image1.png)  
**Fig. 1** Scanning electron microscopic (SEM) images of material surfaces. Surfaces of MTA and ERRM showed crystallographic features. Spindle-shaped crystals appeared in MTA (A) while clusters of round-shaped nano-sized crystals appeared in ERRM (B).

![Fig. 2](image2.png)  
**Fig. 2** pH values of test materials. ERRM presented lower pH in comparison with MTA at all time periods except for 7 days after setting. There was no statistically significant ($p>0.05$) difference in pH between MTA and ERRM.

![Fig. 3](image3.png)  
**Fig. 3** Cell viabilities of MC3T3-E1 cells exposed to extracts of test materials. Cell viabilities in the presence of ERRM in serial dilutions were significantly ($p<0.05$) lower than those in the presence of MTA. Different capitals represent statistically significant ($p<0.05$) differences between test materials. Different small letters represent statistically significant ($p<0.05$) differences between serial dilutions of the test material.
Osteoblastic gene expression

To investigate the effect of test materials on osteoblastic differentiation of MC3T3-E1 cells, expression levels of ALP and OCN genes were evaluated. Expression levels of ALP and OCN are shown in Fig. 4. ALP expression level with MTA or ERRM was significantly ($p<0.05$) increased compared to that with the control (Fig. 4A) on day 3. For OCN expression, its mRNA level was increased significantly on day 3 in experimental groups with MTA or ERRM (Fig. 4B).

ALP activity analysis

ALP activity of MTA and ERRM was investigated with ALP staining. According to results of ALP staining (Figs. 5A and B), both materials at 5 days showed significant increase in ALP activity compared to the control ($p<0.05$). There were significant differences in ALP activity between the MTA and ERRM samples ($p<0.05$).

Cell viability

Cell viabilities with both material extracts are shown in Fig. 3. Although cell viabilities with ERRM in serial dilutions were significantly ($p<0.05$) lower than those with MTA, both materials showed good cell viabilities compared to the control.

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DISCUSSION

When nonsurgical root canal treatment fails or cannot be performed, surgical root canal treatment can be conducted. This procedure includes placement of a retrograde filling material which is in close contact with periradicular tissue. Therefore, physical and chemical properties as well as biocompatibility of the retrograde filling material are very important for the success of apical surgery\(^{11}\).

As a newly developed retrograde filling material, ERRM uses bioceramic technology to overcome some inconsistencies associated with the conventional MTA. Bioceramic refers to the combination of calcium silicate and calcium phosphate applicable for medical or dental use\(^{12,13}\). However, there are little studies about the osteoblastic effect of ERRM compared to MTA, a gold standard of retrograde filling material at present time. In this study, surface texture and chemical (pH) properties, cell viability, and osteoblastic effect (gene expression and...
enzyme activity) of MTA and ERRM were evaluated. Material’s physical structure and surface characteristics are known to influence tissue response to materials. In SEM analysis of this study, both MTA and ERRM showed crystallographic features. It has been shown that particle size can affect the early strength of a material and the ease of handling as well. Based on SEM analysis, the particle size of ProRoot MTA has been found to be less than 1 to approximately 30 μm. In comparison, the new bioceramic material ERRM has the largest particle size of 0.35 μm, with approximately 50% of its particles being nano size which allows the material to enter into dentinal tubules and interact with moisture present in these tubules. This creates a mechanical bond between the material and dentin.

ERRM showed high pH at 7 days after setting, similar to calcium hydroxide cement on 7 days. High pH level not only neutralizes lactic acid, an important factor in dissolution of mineral component of dentin, but also activates alkaline phosphatases that play an important role in hard tissue formation. Biomineralization is promoted by propagation of hydroxyapatite into the extracellular matrix and its deposition among collagen fibrils. Extracellular pyrophosphate inhibits hydroxyapatite formation. Alkaline phosphatase is an enzyme that can increase local concentration of inorganic phosphate, a mineralization promoter. It can also decrease the concentration of extracellular pyrophosphate, an inhibitor of hard tissue formation. These findings suggest that it is possible to induce hard tissue formation when ERRM is used as a retrograde filling material. To prove the mineralization potential of ERRM, osteoblastic gene expression analysis and ALP staining were conducted. In this study, mRNA expression levels of ALP and OCN in ERRM group were shown that particle size can affect the early strength of a material and the ease of handling as well. Based on SEM analysis, the particle size of ProRoot MTA has been found to be less than 1 to approximately 30 μm. In comparison, the new bioceramic material ERRM has the largest particle size of 0.35 μm, with approximately 50% of its particles being nano size which allows the material to enter into dentinal tubules and interact with moisture present in these tubules. This creates a mechanical bond between the material and dentin.

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High pH of ERRM also suggests that it has antibacterial effects because antibacterial effects of materials are known to be attributed to their high pH, hydrophilicity, and active calcium hydroxide diffusion. In a recent study, intracanal placement of ERRM and white ProRoot MTA has shown diffusion of hydroxyl ions across the dentin. Although antimicrobial assays were not measured in that study, the pH of the material might have contributed to its antibacterial activity.

Many studies have investigated the cytotoxicity of ERRM and found it to be as biocompatible as that of MTA. However, Modareszadeh et al. have shown that all ERRM elutes can significantly decrease both cell viability and ALP activity. This finding was consistent with that of Bonson et al., but different from findings of Rajan et al. Modareszadeh et al. have explained that differences in these results are due to the use of various cell lines or greater dilutions of elutes. Although cell viabilities with ERRM in serial dilutions were significantly lower than those with MTA in our study, cell viabilities with both materials were significantly higher than those with the control. Therefore, both materials are thought to be biocompatible.

CONCLUSION

ERRM and MTA showed similar cytotoxicities and alkaline pH during setting reaction. They both showed significant effects on osteoblastic differentiation. Therefore, ERRM can be used as a desirable alternative material to MTA for root-end filling. However, further studies are needed to evaluate the clinical feasibility of ERRM.

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REFERENCES