

# Effect of Bioceramic Materials on Proliferation and Odontoblast Differentiation of Human Stem Cells from the Apical Papilla

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## Abstract

**Introduction:** In regenerative endodontic treatment (RET), practitioners favor the placement of bioceramics as sealing materials over blood clots. It is important to understand the interaction between sealing material and cells in the root canal. The purpose of this study was to compare the effectiveness of various bioceramic materials (ProRoot MTA [Dentsply, Tulsa, OK], Biodentine [Septodont, Saint-Maur-des-Fossés, France], and RetroMTA [BioMTA, Seoul, Korea]) as sealing materials in RET for the proliferation and differentiation of stem cells from the apical papilla (SCAPs). **Methods:** SCAPs were seeded at 20,000 cells/well and cultured with soluble agents of testing materials through a transwell culture plate. The proliferation of SCAPs was investigated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on days 1, 3, 7, and 14 of testing. Alizarin red staining and quantitative real-time polymerase chain reaction were used for SCAP differentiation at different time points (1, 7, 14, and 21 days). The odontoblast genes expressed are dentin matrix acidic phosphoprotein 1, dentin sialophosphoprotein, osteocalcin, and matrix extracellular phosphoglycoprotein, which were used in this study. The SCAPs were cultured in odonto/osteogenic induction medium and also contacted soluble agents from the testing materials. **Results:** All 3 tested biomaterials induced SCAP proliferation. The Biodentine, ProRootMTA, and RetroMTA groups showed significant SCAP proliferation on days 7 and 14 compared with the control. In regard to odontoblastic differentiation, only Biodentine showed positive alizarin red staining. The highest expressions of dentin matrix acidic phosphoprotein 1, dentin sialophosphoprotein, and matrix extracellular phosphoglycoprotein were found on day 21 in the Biodentine group. The expression of osteocalcin was found to be significant on day 7. **Conclusions:** Biodentine, ProRootMTA, and RetroMTA can induce

SCAP proliferation. Biodentine induced significant SCAP differentiation among the 3 materials. (*J Endod* 2018; ■:1–6)

## Key Words

Bioceramics, differentiation, proliferation, regenerative endodontic treatment, stem cells from the apical papilla

The advantages of regenerative endodontic treatment (RET) include the possibility of further root development, reinforcement of the dentinal walls by deposition of hard tissue, and posttreatment vitality response (1).

One of the most important aims in RET is to create a healing environment for regeneration so that sealing material can be placed on top of the blood clot. Thus, biocompatible sealing materials are essential. The sealing material should prevent bacterial or toxin leakage, show biocompatibility, and enhance cell proliferation and differentiation. Mineral trioxide aggregates (MTAs), bioceramics, and tricalcium silicate-based materials, such as Biodentine (Septodont, Saint-Maur-des-Fossés, France) and RetroMTA (BioMTA, Seoul, Korea), have been recommended for placement on top of blood clots as a sealing material (2). Bioceramics have several favorable properties, including the ability to induce hard tissue formation and biocompatibility; they are also nontoxic, nonresorbable, and unaffected by blood contamination (3). Moghaddame-Jafari et al (4) showed that white MTA can induce the proliferation of murine odontoblast-like and undifferentiated pulp cells. D'Antò et al (5) evaluated the ability of MTA to support human mesenchymal stem cell (hMSC) adhesion, proliferation, and migration. The results showed that MTA was able to enhance hMSC adhesion, growth, and migration. Biodentine has been reported to be biocompatible and bioactive and to be able to induce the osteogenic differentiation and mineralization of dental pulp and mesenchymal stem cells (6–11). An *in vitro* study showed that RetroMTA has a biocompatibility on human pulp-derived cells comparable with ProRoot MTA (Dentsply, Tulsa, OK) (12). This material is quite new, and only a few studies have investigated its properties.

## Significance

Guidelines on RET are underinvestigated. This study provides information for the selection of sealing materials for RET that should stimulate stem cell proliferation and differentiation, leading to pulp-dentin complex repair. SCAPs were used because they are more clinically relevant.

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## Basic Research—Technology

Bioceramics play an important role in RET; several reports have studied their effect on various stem cells, especially dental pulp stem cells. However, until now, little information has been available regarding their effects on proliferation and odontogenic differentiation of stem cells of the apical papilla (SCAPs) with those materials. The purpose of this study was to compare the effectiveness of various bio-ceramic materials (ie, ProRoot MTA, Biodentine, and RetroMTA) as sealing materials in RET for the proliferation and differentiation of SCAPs.

### Materials and Methods

#### Cell Culture

SCAPs were transferred from the University of Texas Health Science Center at San Antonio, San Antonio, TX (13). They were grown in alpha-minimum essential medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (Gemini, West Sacramento, CA), 2 mmol/L L-glutamine (Sigma-Aldrich), and 100 U/mL penicillin and streptomycin (Sigma-Aldrich). SCAPs were cultivated in a T75 flask at 37°C and 5%CO<sub>2</sub>. The culture medium was changed every other day.

#### Material Preparation

ProRoot MTA, Biodentine, and RetroMTA were mixed under aseptic conditions following the manufacturer's instructions and placed in a 5-mm-diameter, 3-mm-high silicone mold. The concentrations of ProRoot MTA, Biodentine, and RetroMTA were 0.13 mg/mL, 0.14 mg/mL, and 0.1 mg/mL, respectively. The mixed materials were allowed to set completely in an incubator set to 37°C and 95% relative humidity for 24 hours. After setting, the material tablet was demolded and transferred to the transwell culture insert.

#### Testing Procedure

The subcultured SCAP suspension was diluted to a concentration of 10<sup>5</sup> cells/mL. Two hundred microliters of cell suspension was seeded into the bottom chamber of a 24-transwell culture plate (Corning Inc, Corning, NY), and the material tablet was placed in the upper chamber ( $n = 3/\text{group}$ ). The proliferation control group contained only SCAPs incubated in cell culture medium. The differentiation assay–positive control group consisted of cells incubated in odonto/osteogenic induction medium. SCAPs and materials were incubated for 1, 3, 7, and 14 days for the proliferation experiment and 1, 7, 14, and 21 days for the differentiation experiment (14).

#### SCAP Proliferation Assay

To determine the proliferation of treated SCAPs, a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide metabolic activity assay (MTT, Sigma-Aldrich) was used. MTT solution (1 mg/mL) was added to each well for 2 hours in the culture incubator. Then, the MTT solution was discarded, and 1 mL isopropanol was added to each well. The plate was shaken for 30 minutes at room temperature to solubilize the formazan crystals. Two hundred microliters of isopropanol was transferred to a 96-well tissue culture plate. A spectrophotometer was used to measure the absorbance values of wavelengths of 570 nm (RI Technologies, Bangkok, Thailand).

#### Alizarin Red Staining

At the end of each time interval, the treated SCAPs were fixed for 10 minutes with cold 100% methanol (Sigma-Aldrich) and washed with sterile water twice and then stained with 2% alizarin red (Sigma-Aldrich) for 10 minutes at room temperature. The alizarin red was removed, and specimens were washed with sterile water to remove

excess color. The specimens were visualized under a light microscope for the presence of calcified nodules to determine positive or negative staining.

#### Quantitative Real-time Polymerase Chain Reaction

After 1, 7, 14, and 21 days, the cells were lysed and extracted to RNA. The total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions (14, 15). The iScript select cDNA Synthesis kit (BIO-RAD, Hercules, CA) was used to convert 1  $\mu\text{g}$  messenger RNA to complementary DNA. The conditions used for quantitative real-time polymerase chain reaction (PCR) analysis in this study were as follows. Two micrograms of complementary DNA, 0.4  $\mu\text{L}$  forward and reverse primer each (Table 1), PCR-grade water, and SYBR green of the KAPA SYBR Fast qPCR Master Mix Kit (KAPABIOSYSTEMS, Wilmington, MA) were mixed, resulting in a total of 20  $\mu\text{L}$  for 1 reaction. Forty PCR cycles were performed at the annealing temperatures of 60°C. After the PCR cycles ended, melting curve analysis was performed by heating to 95°C for 10 seconds followed by cooling to 65°C for 5 seconds and gradual heating to 95°C at 0.2°C/s in the C1000 thermal cycle of CFX96 real-time system (BIO-RAD). The data collected were presented as fold-relative messenger RNA levels to the control (undifferentiated SCAPs). All quantitative real-time PCR analyses were performed in triplicates.

#### Statistical Analysis

Statistical analysis was performed using SPSS Statistics Version 18 (SPSS Inc, Chicago, IL). The data from the proliferation assay and gene expression were analyzed using the Kruskal-Wallis test and the Mann-Whitney *U* post test. For alizarin red staining, the data were interpreted as present/absent, and the chi-square test was used for analysis.  $P < .05$  was considered significantly different.

## Results

#### SCAP Proliferation

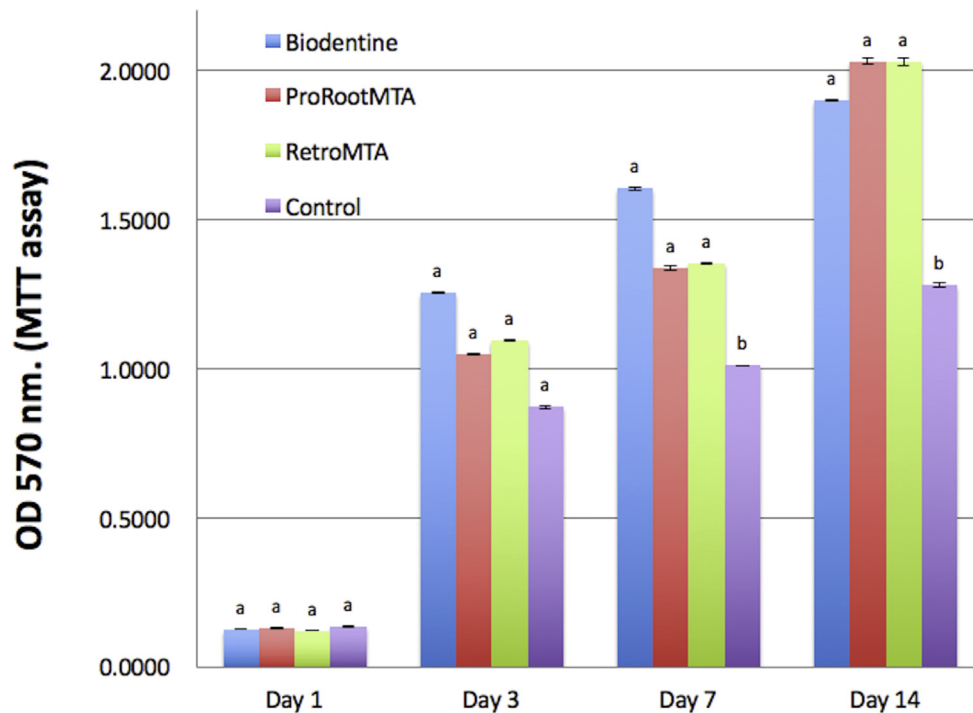
SCAPs of all experimental groups were well-spread spindle-shaped cells, and cell proliferation increased from day 1 to day 14 (Fig. 1). Significant differences in SCAP proliferation in Biodentine, ProRootMTA, and RetroMTA were found on days 7 and 14 when compared with the control group ( $P < .05$ ).

#### Odonto/Osteogenic Differentiation

Alizarin red staining was used to detect calcified nodule formations. The Biodentine (Fig. 2C) and positive control groups (Fig. 2B) were positive to mineralization staining on days 7, 14, and 21. This was significantly different from ProRoot MTA and RetroMTA

**TABLE 1.** Primer Sequences Used in Quantitative Real-time Polymerase Chain Reaction

Gene	Forward primer (F) Reverse primer (R)	Size (bp)
OCN	F: 5'-ATGAGAGCCCTCACACTCCTC-3' R: 5'-CGTAGAAGCGCCGATAGGC-3'	291
DSPP	The primer was purchased from PrimerPCR SYBR Assay (BioRad, Hercules, CA; lot no. 10025636)	—
MEPE	F: 5'-CCCTGGAAGAGAAGGAAACAGA-3' R: 3'TGAAACTCAACCTTCCCTTGGT-3'	393
DMP-1	F: 5'-CCCTTGGAGAGCAGTGAGTC-3' R: 5'-CTCCTTTCTGTGCTCCTG-3'	164
GAPDH	F: 5'-GTCAGTGGTGGACCTGACCT-3' R: 5'-AGGGGAGATTCACTGTGG-3'	395



**Figure 1.** The effects of bioceramic materials on SCAP proliferation. The control group was incubated with only the cell culture medium. The trend of SCAP proliferation was observed to increase in all groups. Significant differences of SCAP proliferation in the 3 testing materials were found on days 7 and 14 when compared with the control. The results suggested that all 3 materials induced SCAP proliferation. Different letters represent statistically significant differences between each testing period ( $P < .05$ ).

( $P < .05$ ). The undifferentiated SCAP (control group), ProRootMTA and RetroMTA groups were negative to mineralization staining through out the period of testing (Fig. 2A, 2D, and 2E, respectively)

Dentin matrix acidic phosphoprotein 1 (DMP-1), dentin sialophosphoprotein (DSPP), osteocalcin (OCN), and matrix extracellular phosphoglycoprotein (MEPE) are constituted markers of odontogenic/osteogenic differentiation. Concerning the positive control, the results showed that DMP-1, DSPP and OCN expressed on day 14 and 21 (Fig. 3A). DMP-1 expression on days 1, 7, 14, and 21 in the RetroMTA group did not differ from the control. The day 14 Biodentine group produced significantly greater DMP-1 ( $P < .05$ ). The highest expression of DMP-1 was found on day 21 in the Biodentine group (Fig. 3B).

The expression of DSPP on days 1 and 7 in all groups did not differ from the control. On day 14, the ProRoot MTA group produced significantly lower DSPP expression compared with the RetroMTA and Biodentine groups. On day 21, DSPP expression of the RetroMTA group was significantly less than that of the Biodentine, ProRootMTA, and RetroMTA groups ( $P < .05$ ) (Fig. 3C).

OCN expression in the ProRoot MTA and RetroMTA groups continuously increased from day 1 to day 21. On the other hand, the Biodentine group did not demonstrate OCN expression until day 14 and showed a great increase on day 21. There was a significant difference in OCN expression between Biodentine and ProRoot MTA on day 7 ( $P < .05$ ) (Fig. 3D).

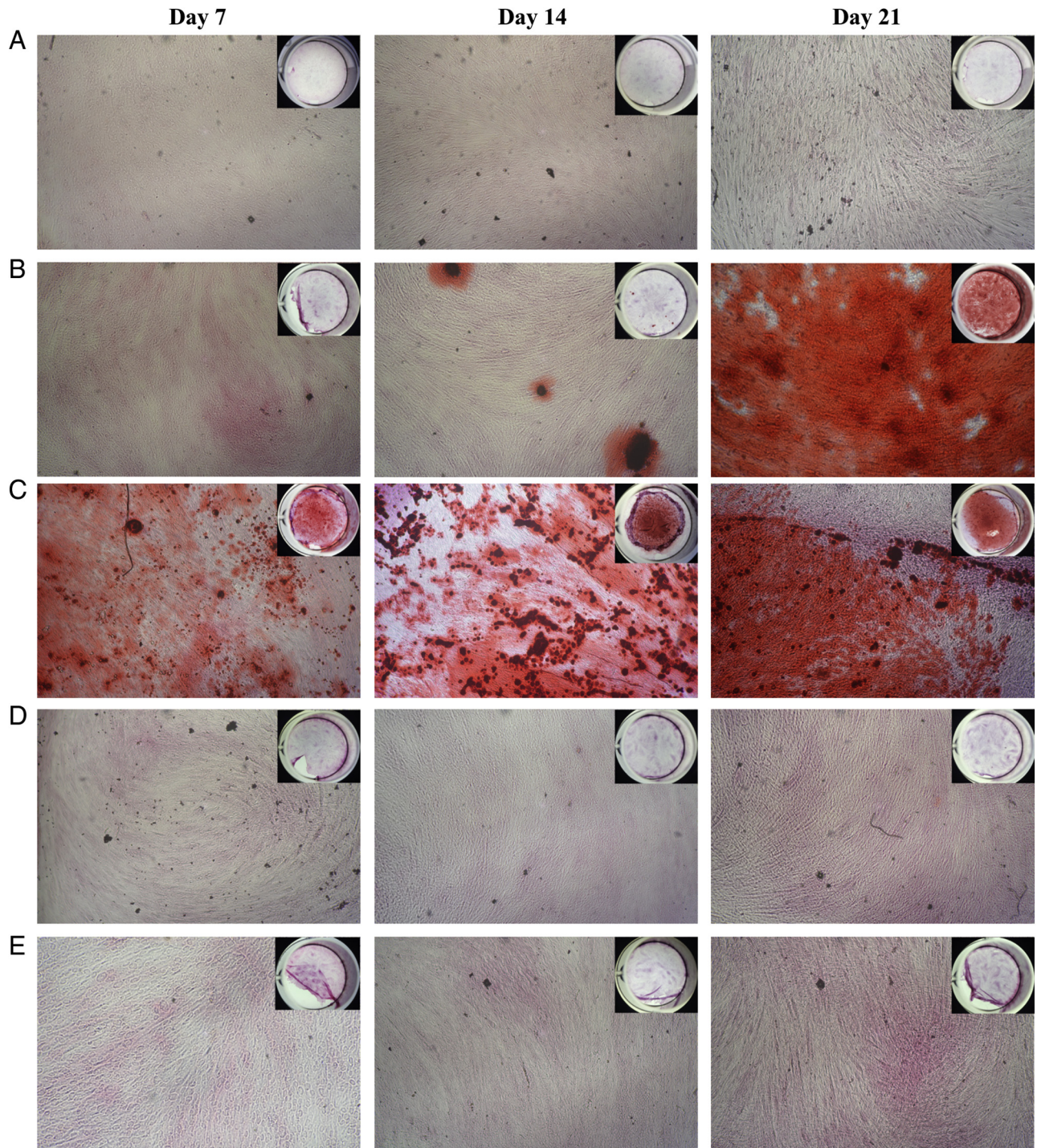
MEPE expression on days 1 and 7 in all groups did not differ from the control. The Biodentine group had an initial expression on day 14, which was significantly greater than that of the ProRoot MTA group, and had the highest expression on day 21. The RetroMTA group showed significantly lower gene expression than the Biodentine and RetroMTA groups on day 21 ( $P < .05$ ) (Fig. 3E).

## Discussion

In RET, once the blood and stem cells are delivered through the apical foramen to the root canal space, sealing material is required to protect the blood clot and stem cells. MTAs have been recommended for placement on top of blood clots as a sealing material, but they have several disadvantages (eg, difficulty in handling, long setting time, need for hydration while setting, and discoloration). A new generation of bioceramics has been developed. Biodentine, RetroMTA, and ProRootMTA were the focus in our study. To our knowledge, there is little information on SCAP cell proliferation and differentiation using those biomaterials.

SCAPs are an important population of hMSCs in RET. SCAPs have the potential to proliferate, differentiate, and express CD73, CD90, and CD105 through all cultures up to 20 passages (13, 16, 17). SCAPs have shown a greater proliferation rate than dental pulp stem cells (17). According to this study, the time interval of evaluation was from day 1 to day 21. Mesenchymal stem cell differentiation was divided into 3 stages (18). The first stage occurred from day 1 to day 4, in which the highest cell numbers were seen. Day 5 to 14 represents the second stage. Early cell differentiation was found, and alkaline phosphatase was detected in this period (19). The last stage, days 14 to 28, showed a high expression of bone-related proteins, including OCN, osteonectin, and calcium phosphate mineralization (18).

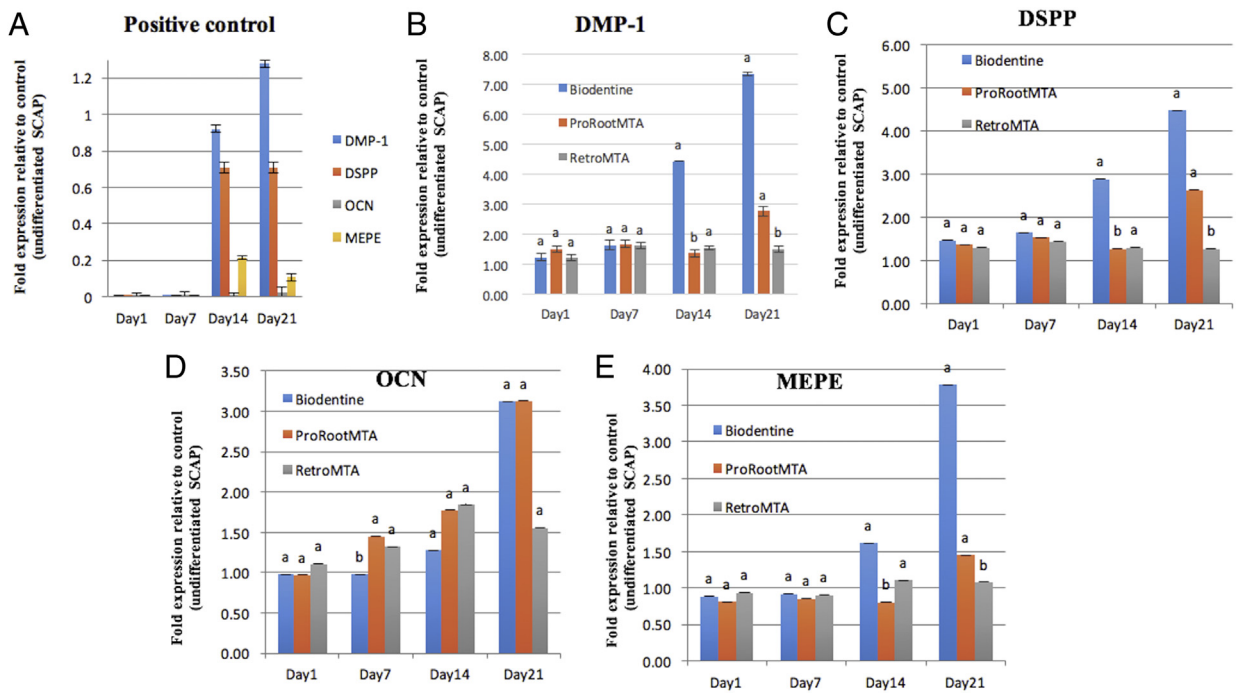
The purpose of RET is to replace damaged structures, including dentin and root structures, as well as the pulp-dentin complex. To achieve this, odontoblast-like cells are needed. The results of this study suggested that all 3 materials induced SCAP proliferation. SCAP proliferation was found to be significantly high on days 7 and 14 when compared with the control group. This finding is in agreement with other studies reporting that MTA and Biodentine have the ability to



**Figure 2.** Alizarin red staining of treated SCAPs during calcified nodule formation (original magnification: 100 $\times$ ). (A) The control group is undifferentiated SCAPs, which were negative to alizarin red staining. (B) The positive control is SCAPs cultured in odonto/osteogenic induction medium and (C–E) cocultured with odonto/osteogenic induction medium and different testing materials. (B) The positive control and (C) Biodentine group were positive to mineralization staining on days 7, 14, and 21 when compared with the control group. (D) The ProRootMTA and (E) RetroMTA groups were negative to mineralization staining, like the control group. This indicated that SCAPs had the capacity to differentiate to odontogenic/osteogenic cells when treated with Biodentine.

induce mesenchymal stem cells to odontoblast-like cells (5, 7). D'Antò et al (5) reported that MTA showed significantly higher hMSC adhesion, proliferation, and migration than Portland cement. Biodentine's effect on murine pulp cells in terms of its effect on proliferation was investigated. The results showed Biodentine to be a bioactive material because it increased murine pulp cell proliferation and mineralization (7). To

date, no information is available regarding RetroMTA and its effect on cell proliferation. To the best of our knowledge, our study is the first to study this material in this aspect. Our results showed that RetroMTA increased SCAP proliferation throughout the entirety of the experiment. The results were observed in a time-dependent manner for all 3 groups of testing material.



**Figure 3.** The expression profile of SCAPs treated with Biodentine, ProRootMTA, and RetroMTA. (A) The expression of the positive control group compared with control (undifferentiated SCAPs). (B–E) DMP-1, DSPP, and MEPE expression was found on days 14 and 21. OCN expression was found on days 7, 14, and 21. Different letters represent statistically significant differences between each testing period ( $P < .05$ ).

Odontoblast-like cells were investigated by alizarin red staining of calcified nodule formation and measurements of DMP-1, DSPP, OCN, and MEPE gene expression. Odontoblast-like cell differentiation shares a similar process to osteogenic cell differentiation in the bone marrow, which forms a calcified nodule (20). Thus, the expression of 1 of these genes may play a role in dentinogenesis (21, 22). The positive staining results of Biodentine were similar to another study by Zanini et al (7). Murine pulp cells differentiated into odontoblasts, and calcified nodule formation was found (7).

Regarding cell differentiation, calcified mineralization only appeared in the Biodentine group on days 7, 14, and 21. These results were confirmed with the gene expression experiment, and the Biodentine group was found to have a significantly greater expression of DMP-1, DSPP, OCN, and MEPE in comparison with the other materials.

DMP-1, DSPP, and MEPE were grouped as a family of small integrin-binding ligand N-linked glycoproteins. They are extracellular matrix proteins, which are expressed during odontoblast differentiation (23, 24). The small integrin-binding ligand N-linked glycoprotein family as well as OCN, osteonectin, and basic phosphatase have been used as particular mineralization markers for odontoblast/osteoblast-like cells (25). OCN was used in this study even though it comprised osteoblast gene expression because *in vivo* studies have reported the formation of bonelike tissue after RET (26). Wei et al (27) discovered OCN, DSPP, and MEPE expression in dental pulp stem cells incubated in osteoinductive medium.

The composition of each material affects their physical, chemical-biological properties, and differentiation (7, 8, 15). Both ProRoot MTA and Biodentine are composed of calcium silicate, which gives results in a constant release of calcium ions. Calcium ions are released when ProRootMTA and Biodentine are mixed with water, which is converted to calcium hydroxide. Calcium hydroxide then separates into calcium and hydroxide ions, resulting in an increased pH and calcium ion release. The amount of calcium ion released has been

proven to be important for osteoblast survival, differentiation, and mineralization of pulp cells (28–30). Calcium ions have been identified as an important factor in signaling molecules and controlling most cellular activities (28).

The major components of RetroMTA are inorganic compounds. The inorganic ions released from silicon-based materials influence cell proliferation and osteogenesis marker expression (31). In related studies, RetroMTA has shown the lowest osteogenic potential when compared with ProRootMTA, Micromega MTA (MicroMega, Besancon, France), and experimental calcium silicate cement (15). Another study also reported a slightly lower pulpal response and a smaller area of calcified barrier in RetroMTA compared with ProRootMTA (32). In our study, the RetroMTA group showed the lowest expression of odontoblastic markers, similar to the findings of the other studies.

From related studies, human dental pulp cells were treated with medium containing MTA and medium containing exogenous calcium ions. The results showed that human dental pulp cells in calcium-containing medium had increased dose-dependent proliferation after 12 days (28). Our study used SCAPs, but the results correlated well with those studies.

## Conclusion

Biodentine, ProRootMTA, and RetroMTA induced SCAP proliferation from day 1 to day 14. SCAPs had the greatest differentiation and expression of DMP-1, DSPP, OCN, and MEPE in the Biodentine group.

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

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