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Effect of Biodentine and Bioaggregate on odontoblastic differentiation via mitogen-activated protein kinase pathway in human dental pulp cells

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Abstract

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Aim To compare the mineralization inductive capacity of Biodentine and Bioaggregate with Mineral trioxide aggregate (MTA) and to investigate possible signaling pathways of mineralization in human dental pulp cells (HDPCs).

Methodology Viability of HDPCs in response to Biodentine, Bioaggregate, and MTA was measured using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide. To investigate their potential to induce odontoblast differentiation, expression of dentine sialophosphoprotein (DSPP) and dentine matrix protein1 (DMP1) mRNA level was evaluated by RT-PCR. For the mineralized nodule assay, Alizarin red staining was performed. To determine the role of MAPK signaling in the odontoblastic differentiation of HDPCs, activated MAPKs were investigated by Western blot and the effect of MAPK inhibitor was examined by Alizarin red S staining. The results were statistically analysed using one-way ANOVA and the Bonferroni test.

Results The effects of MTA, Biodentine, and Bioaggregate on cell viability were similar. Biodentine and Bioaggregate enhanced DSPP and DMP1 mRNA expression compared to the control group, but to the same extent as MTA ($P < 0.05$). MTA, Biodentine, and Bioaggregate increased the area of calcified nodules compared to the control ($P < 0.01$). MTA, Biodentine, and Bioaggregate increased phosphorylation of extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK). MAPK inhibitors attenuated mineralized nodule formation, which was increased by MTA, Biodentine, and Bioaggregate, respectively ($P < 0.01$).

Conclusion Biodentine and Bioaggregate stimulated odontoblastic differentiation and mineralization nodule formation by activating the MAPK pathway as did MTA. This suggests that the new materials could be useful for regenerative endodontic procedures.

Keywords: bioaggregate, biodentine, MAPK, mineralization, odontoblast.

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Introduction

Mineral trioxide aggregate (MTA) is used as a pulp capping agent on exposed pulp surfaces because it can induce formation of dentine bridges and tertiary dentine. During reparative dentinogenesis, odontoblast-like cells differentiate from dental pulp cells to pro-

vide the replacement cells. MTA affects mineralization and differentiation of dental pulp cells (Yasuda *et al.* 2008, Masuda-Murakami *et al.* 2010) and the combination of MTA and enamel matrix derivative promotes odontoblastic differentiation of human dental pulp cells (HDPCs) (Min *et al.* 2009). Tricalcium silicate cement can also induce odontogenic differentiation of HDPCs and might be a candidate pulp capping agent (Peng *et al.* 2011).

Biodentine™ (Septodont, Saint-Maur-des-Fosses, France) has recently been developed and is marketed as a bioactive material. Biodentine induces differentiation of odontoblast-like cells and stimulates mineralization of murine pulp cells. Biodentine is considered a suitable pulp capping material (Zanini *et al.* 2012). Biodentine also increases expression of transforming growth factor-beta 1 (TGF-β1) expression in human pulp cells and induces mineralization foci in a human tooth culture model (Laurent *et al.* 2012).

Bioaggregate (Innovative Bioceramix, Vancouver, BC, Canada) has also been recently developed as a bioceramic material. Bioaggregate has cytotoxicity similar to MTA with human mesenchymal cells (De-Deus *et al.* 2009) and HDPCs (Chung *et al.* 2010). Bioaggregate induces mineralization associated gene expression in mouse osteoblast cell lines (Yuan *et al.* 2010). Bioaggregate is also similar to MTA in promoting odontoblastic differentiation of HDPCs (Chang *et al.* 2014).

Mitogen-activated protein kinases (MAPKs) are an essential component in many physiologic processes, such as cell growth, proliferation, differentiation, and apoptosis (Neary 1997). MAPKs are a family of serine-threonine kinases and include three familes, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 JNK (Johnson & Lapadat 2002). p38 MAPK is activated during odontoblast stimulation in tertiary dentinogenesis (Simon *et al.* 2010).

Previous studies have demonstrated that the mineralization and odontoblastic differentiation effects of MTA can be explained by MAPK pathways and calcium ion release from MTA. MTA induces phosphorylation of MAPK, and both MAPK inhibitor and calcium channel blocker attenuated the MTA-induced odontoblastic differentiation (Zhao *et al.* 2012, Woo *et al.* 2013). However, the mechanism of mineralization effect of Biodentine and Bioaggregate is unclear. The purpose of this study was to compare the mineralization effect of Biodentine and Bioaggregate with MTA and reveal the possible pathway of mineralization in HDPCs.

Material and methods

Preparation of MTA, Biodentine, and Bioaggregate, and material extract

MTA, Biodentine, and Bioaggregate were mixed according to manufacturer's instructions under sterile conditions and then were added to moulds (diameter 5.0 mm and height 3.0 mm). The materials were left to set for 4 h at 37 °C in a humidified 5% CO_2 incubator. Each disc was removed using sterile forceps. Discs of each material were placed into 50 mL of fresh alpha-minimal essential medium (α-MEM; Gibco Invitrogen, Grand Island, NY, USA) and incubated for 7 days at 37 °C in a humidified 5% CO_2 atmosphere. The medium was drawn off and sterilized by passage through a 0.2 μm pore size filter before use. The concentrations of MTA, Biodentine, and Bioaggregate extracts were 0.1, 1, 10, and 20 mg/mL for cell viability and 10 mg mL^{-1} for the other experiments.

Cell culture

HDPCs were isolated from freshly extracted maxillary third molars from three male patients. All procedures were conducted with informed consent. The institutional review board of the Chonnam National University Dental Hospital approved this study protocol (CNUDH-2013-002). Immediately after extraction, the teeth were split open and the pulp tissues aseptically removed and minced with a surgical knife. The minced tissues were placed in wells of 6-well cell culture plates containing α-MEM supplemented with 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. After culturing for 14–20 days, outgrowth of the cells from pulp tissue was observed. When the cells reached confluence, subculture was performed. Cells passaged 3–4 times were used for this study.

Cell viability assay

Cell viability was measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide MTT assay. Cells were seeded at a concentration of 2×10^4 cells/well in 96-well plates in α-MEM with 10% FBS. The concentrations of MTA, Biodentine, and Bioaggregate extract were 0.1, 1, 10, and 20 mg mL^{-1}, respectively, based on a previous report (Hakki *et al.* 2009). After incubation for 2 days, MTT

was added to each well for the last 4 hs of the experiment, and the reaction was stopped by the addition of dimethylsulfoxide. The optical density was determined at 570 nm on a multiwell plate reader. The percentage of cell viability in the control group (media only group) represented 100%.

Reverse transcription-polymerase chain reaction

HDPCs were incubated with 10 mg mL^{-1} of MTA, Biodentine, and Bioaggregate extract for 1 and 2 days. Total RNA of HDPCs was extracted using Trizol reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. RNA (2 μg) was reverse transcribed for first-strand complementary DNA (cDNA) synthesis (Gibco BRL, Rockville, MD, USA). The cDNA was amplified in a final volume of 20 μL containing 2.5 mmol/L magnesium dichloride, 1.25 U Ex Taq polymerase (Bioneer, Daejeon, Korea), and 1 mmol/L specific primers. Amplification was performed for 30 cycles in a DNA thermal cycler. Primer sequences for differentiation markers are detailed in Table 1. The PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide. Gels were scanned and bands were quantified by optical densitometry.

Alizarin red S staining

For mineralized nodule assay, HDPCs were seeded in 24 well plates at a density of 5 x 10^4 cells/well and treated using 10 mg mL^{-1} of MTA, Biodentine, and Bioaggregate extract with or without pretreatment of MAPK inhibitors (U0126, 10 μmol/L; SB203580, 10 μmol/L; and SP600125, 10 μmol/L) for 1 h. The culture medium was replaced with fresh culture medium every 2 days. After 14 days, the calcium deposition of HDPCs was studied using 0.1% Alizarin red S staining solution (Sigma-Aldrich, St. Louis, MO, USA). The samples were washed with phosphate buffered saline (PBS) and fixed with 70% ice-cold ethanol for 1 h at room temperature, rinsed twice with distilled water, and stained with 40 mmol/L Alizarin red S (pH 4.2) solution for 15 min with gentle agitation at room temperature. Alizarin red S staining was photographed using an Officejet pro L7580 scanner (HP, Palo Alto, CA, USA). Bound dye was solubilized in 10% cetylpyridinium chloride and quantitated spectrophotometrically at 562 nm.

Table 1 RT-PCR primer sequence

Gene	Sequence (5′-3′)	Size (bp)
DSPP	Forward: CAGTGATGAATCTAATGG	488
	Reverse: CTGATTTGCTGCTGTCTGAC	
DMP1	Forward: CAGGAGCACAGGAAAAGGAG	213
	Reverse: CTGGTGGTATCTTGGGCACT	
GAPDH	Forward: GAGTCAACGGATTTGGTCGT	185
	Reverse: GACAAGCTTCCCGTTCTCAG	

DSPP, dentine sialophosphoprotein; DMP1, dentine matrix protein1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Western blot analysis

HDPCs were incubated with 10 mg mL^{-1} of MTA, Biodentine, and Bioaggregate extract for 15, 30, 60, and 120 min. Cell lysates (50 μg) were placed in NP-40 lysis buffer (30 m mol L^{-1} Tris-Cl, pH 7.5, 1 mmol L^{-1} EDTA, 150 mmol L^{-1} NaCl, 1% NP-40, 1 mmol L^{-1} phenylmethylsufonyl fluoride, and protease inhibitor mixture containing 1 μg mL^{-1} aprotinin and leupeptin) and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The resolved proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, UK) according to standard procedures. The membrane was blocked in 5% non-fat dry milk for 1 h and incubated with anti-phospho-ERK, anti-ERK, anti-phospho-p38, anti-p38, anti-phospho-JNK, or anti-JNK antibody (Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. After incubation with the specific peroxidase-coupled secondary antibody (Sigma-Aldrich) for 1 h, the blotted bands were detected using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

Statistical analysis

All experiments were performed in triplicate. The data are expressed as the mean ± standard deviation and were analysed using a one-way analysis of variance followed by the Bonferroni *post hoc* test with the use of the SPSS 17.0 software program (SPSS, Chicago, IL, USA).

Results

Effect of Biodentine and Bioaggregate on cell viability in HDPCs

To determine the optimal concentration of MTA, Biodentine, and Bioaggregate, viability of HDPCs was

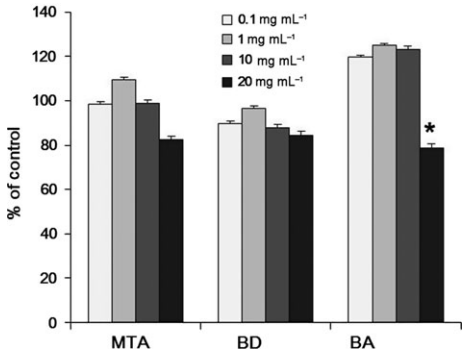

Figure 1 Effects of MTA, Biodentine, and Bioaggregate on HDPC viability. The cell viability in the control group (media only group) represented 100% as the reference value. The asterisk (*) indicates a significant difference when compared to other concentrations ($P < 0.05$). Each value represents the mean of three replicates.

evaluated. Overall, MTA, Biodentine, and Bioaggregate similarly affected HDPC viability. However, 20 mg mL^{-1} of Bioaggregate produced lower cell viability than other concentrations (Fig. 1). Therefore, 10 mg mL^{-1} was used in subsequent experiments.

Effect of Biodentine and Bioaggregate on the expression of DSPP and DMP1 mRNA in HDPCs

To investigate the effect of Biodentine and Bioaggregate on odontoblast differentiation in HDPCs, expressions of dentine sialophosphoprotein (DSPP) and dentine matrix protein1 (DMP1) mRNA were evaluated by RT-PCR and quantified with densitometry. Biodentine and Bioaggregate increased DSPP and DMP1 mRNA expression compared to the untreated control at day 1. MTA, Biodentine, and Bioaggregate increased DMP1 mRNA expression and Biodentine increased DSPP mRNA expression compared to the untreated control at day 2 (Fig. 2) ($P < 0.05$).

Biodentine and Bioaggregate induce mineralization nodules in HDPCs

To determine the mineralization effect of Biodentine and Bioaggregate in HDPCs, mineralized nodule formation in HDPCs was examined using Alizarin Red S staining. After 14 days of culture, MTA, Biodentine, and Bioaggregate enhanced the area of calcified nodules compared to the untreated control (Fig. 3) ($P < 0.01$).

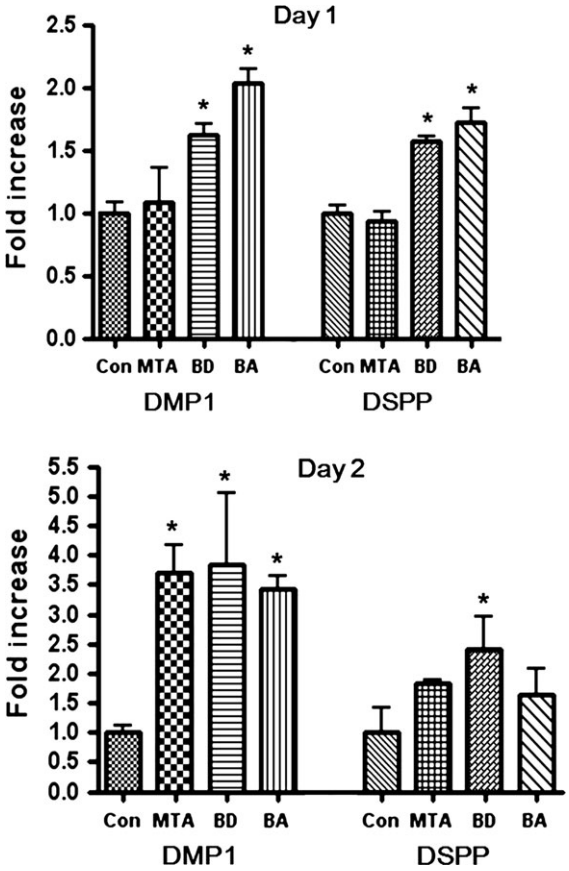

Figure 2 Effects of MTA, Biodentine, and Bioaggregate on HDPCs differentiation. he mRNA levels of DSPP and DMP1 were determined by RT-PCR and quantified by densitometry. Values are expressed as mean ± standard deviation of three separate experiments. *Statistically significant difference when compared to control ($P < 0.05$).

Biodentine and Bioaggregate induce MAPK activation in HDPCs

To investigate the activation of MAPK signaling in MTA-, Biodentine -, and Bioaggregate -induced odontoblastic differentiation in HDPCs, phosphorylated MAPK was investigated by Western blot. MTA, Biodentine, and Bioaggregate induced phosphorylation of ERK, p38, and JNK within 30 min of treatment (Fig. 4). To determinate the role of MAPK signaling on odontoblastic differentiation in HDPCs, cells were pretreated with 10 μmol L^{-1} of MAPK inhibitors (U0126, SB203580, and SP600125) for 1 h, cultured with 10 mg mL^{-1} MTA, Biodentine, and Bioaggregate extract for 14 days, and stained using Alizarin red. Inhibited activation of ERK, p38, and JNK in

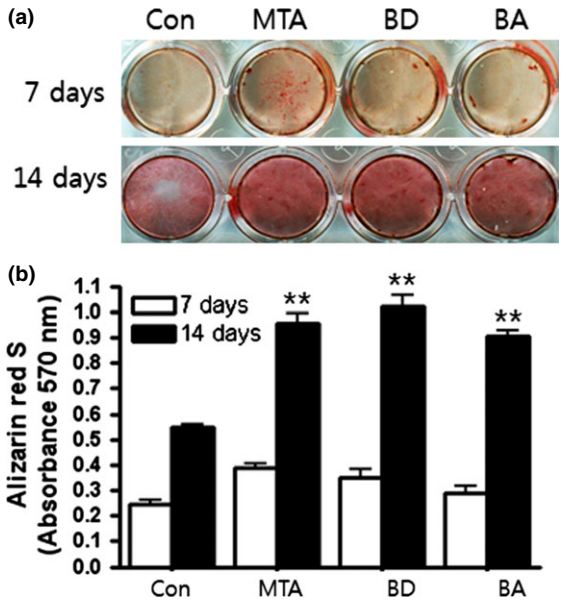

Figure 3 Effects of MTA, Biodentine, and Bioaggregate on mineralization of HDPCs. (a) Representative photograph of calcified nodule. (b) The quantification of Alizarin red S staining. The results are representative of three independent experiments. **Statistically significant difference when compared to control ($P < 0.01$).

MTA, Biodentine, and Bioaggregate extract-treated HDPCs with U0126, SB203580, and SP600125, respectively, reduced mineralization nodule formation, which was increased by MTA, Biodentine, and Bioaggregate extract (Fig. 5) ($P < 0.01$).

Discussion

MTA stimulates the odontogenic differentiation of HDPCs and the effects are mediated by the ERK pathway (Simon *et al.* 2010, Zhao *et al.* 2012, Woo *et al.* 2013). Biodentine or Bioaggregate also stimulate odontoblastic differentiation of HDPCs (Laurent *et al.* 2012, Chang *et al.* 2014). The present study focused on whether the effects of Biodentine and Bioaggregate are comparable to MTA in odontoblastic differentiation and mineralization, and whether MAPK pathways are involved in this process.

The evaluation of cytotoxicity is crucial when treating with Biodentine and Bioaggregate because they directly contact the pulpal tissues. Cell viability assay was performed to determine the difference in cell damage. In this study, Biodentine and Bioaggregate showed similar viability in HDPCs at 10 mg mL^{-1} compared with MTA-treated group.

The mineralization effect of the pulp capping material is very important in clinical situations. MTA has mineralization effects that include odontogenic or cementogenic activities (Tani-Ishii *et al.* 2007, Yasuda *et al.* 2008, Hakki *et al.* 2009). DSPP has a regulatory role in the mineralization of reparative dentine and is regarded as a specific marker of odontoblast differentiation (Liu *et al.* 2013). DMP1 is present in the extracellular matrix of dentine and bone, and serves as an essential factor for mineralization of hard tissue (Suzuki *et al.* 2012). In addition, calcium nodule production is a marker of mineralization (Chang *et al.* 2014). In this study, to determine the odontoblastic differentiation and mineralization effect of the Biodentine and Bioaggregate, odontoblastic differentiation marker genes, such as DMP1 and DSPP, were evaluated by RT-PCR and mineralized nodule formation was examined using Alizarin red S staining during HDPC culture with Biodentine and Bioaggregate extract. Biodentine and Bioaggregate up-regulated DSPP and DMP-1 mRNA. Interestingly, Biodentine and Bioaggregate showed higher expression than the MTA-treated group at

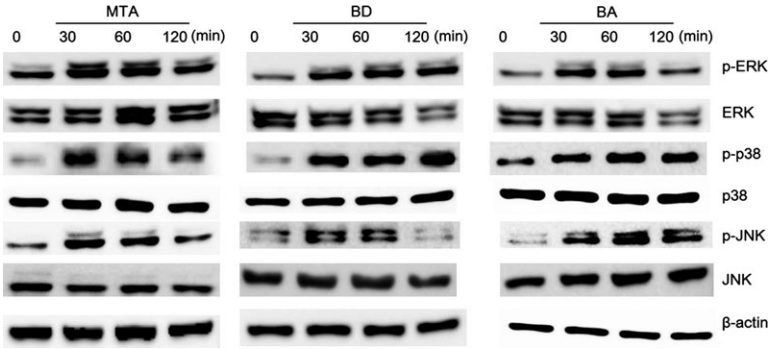

Figure 4 Effects of MTA, Biodentine, and Bioaggregate on MAPK phosphorylation in HDPCs. Phosphorylation of MAPK was detected by Western blot. The expression of β-actin was used as the control.

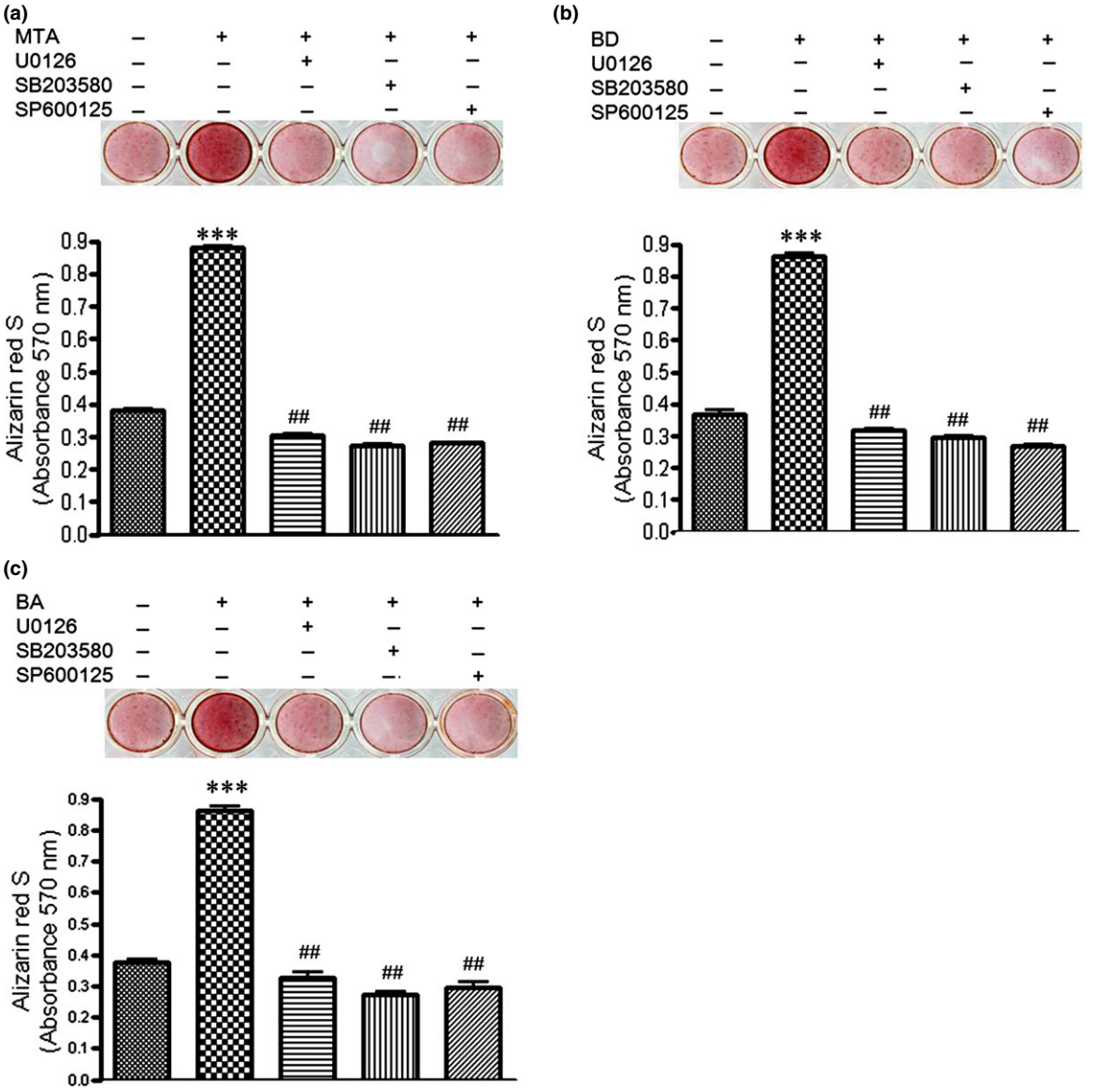

Figure 5 Involvement of MAPK activation in MTA-, Biodentine-, and Bioaggregate -induced mineralization in HDPCs. U0126, SB203580, and SP600125 reduced mineralizaed nodule formation which was increased by (a) MTA, (b) Biodentine, and (c) Bioaggregate. The results are representative of three independent experiments. ***Statistically significant difference when compared to control ($P < 0.001$). ##Statistically significant difference when compared to MTA-, Biodentine-, and Bioaggregate–treated group ($P < 0.01$).

1 day. In addition, Alizarin red S staining revealed that Biodentine and Bioaggregate induced similar levels of mineralization nodule formation compared with MTA-treated group after 14 days of HDPC culture. These results are consistent with the previous findings that Bioaggregate increases DSPP and DMP1 mRNA expression and mineralization nodule formation (Chang *et al.* 2014), and that Biodentine increases calcific nodule formation in immortalized murine pulp cells (Zanini *et al.* 2012).

Considering the present results, it seems that the clinical application of Biodentine and Bioaggregate as pulp capping agents promotes mineralization of dentine beneath the capping material, and then stimulates reparative odontogenesis from injured dental pulp tissue. Additional studies will be necessary to

compare cytokine production or inflammatory response with these cements and MTA in HDPCs for clinical application.

The mechanism by which MTA induces odontoblastic differentiation in HDPCs is not completely understood. MTA induces mineralization and differentiation by MAPK pathway and calcium ion release in HDPCs (Simon *et al.* 2010, Zhao *et al.* 2012, Woo *et al.* 2013). MTA also activates ERK, p38, and JNK MAPKs during odontoblast differentiation and MAPK pathways, particularly ERK, are involved in the MTA-induced odontoblast differentiation of HDPCs (Zhao *et al.* 2012). On the other hand, it is reported that p38 MAPK is activated during odontoblast stimulation in tertiary dentinogenesis (Simon *et al.* 2010). In addition, influx of calcium ion released from MTA stimulates odontoblastic differentiation and mineralization via MAPK pathway activation (Woo *et al.* 2013).

In this study, MAPK pathways were associated with Biodentine - and Bioaggregate -induced mineralization in HDPCs was investigated. Biodentine and Bioaggregate induced phosphorylation of ERK, p38, and JNK MAPKs. These results are consistent with previous results for MTA (Zhao *et al.* 2012). MAPK inhibitors (U0126, SB203580, and SP600125) abolished the mineralization nodule formation, which was increased by Biodentine and Bioaggregate extracts. These results indicated that ERK, p38, and JNK MAPK pathways play a crucial role in Biodentine and Bioaggregate -induced mineralization in HDPCs.

Conclusion

Collectively, these data demonstrate that Biodentine and Bioaggregate induce mineralization of HDPCs by activating the MAPK pathway. These results are comparable to those observed with HDPCs exposed to the gold standard, MTA, suggesting the value of Bioaggregate and Biodentine for regenerative endodontic procedures.

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