




# Combination of mineral trioxide aggregate and propolis promotes odontoblastic differentiation of human dental pulp stem cells through ERK signaling pathway

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**Abstract** The aim of this study is to investigate combined effects of mineral trioxide aggregate (MTA) and propolis on odontoblastic differentiation of human dental pulp stem cells (DPSCs) and to find a signaling pathway involved. Combination of MTA and propolis significantly up-regulated the expression of DSPP and DMP1, and facilitated a mineral nodule formation ( $p < 0.05$ ). Treatments with MTA, propolis or combined increased the phosphorylation of extracellular signal-regulated kinases (ERK), one of mitogen-activated protein kinases signaling cascades during odontogenic differentiation of DPSCs ( $p < 0.05$ ), and U0126, an inhibitor of ERK, decreased calcium deposits ( $p < 0.05$ ). Combination of MTA and propolis promotes odontogenic differentiation and mineralization of DPSCs through ERK pathway.

**Keywords** ERK signaling · Human dental pulp cells · Mineral trioxide aggregate · Odontoblastic differentiation · Propolis

## Introduction

Dental pulp tissue contains dental pulp stem cells (DPSCs) have a capacity to proliferate and differentiate into odontoblasts (Huang et al., 2009). After dental pulp injuries, DPSCs undergo reparative regeneration producing new odontoblasts, which form a dentin matrix and secrete both collagenous and non-collagenous proteins, such as type I collagen (COL1), alkaline phosphatase (ALP), osteocalcin (OCN), osteonectin (ON), osteopontin (OPN), dentin

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sialophosphoprotein (DSPP), and dentin matrix protein1 (DMP1) (Gronthos et al., 2002; Moreau and Xu, 2009).

Although DPSCs contain precursor cells for a formation of new barrier, the selection of pulp treatment materials has been an important factor for facilitating regeneration and hard tissue formation and for preventing bacterial infection and any leakage. Various materials have been used in vital pulp procedures, especially direct pulp capping. Calcium hydroxide has been extensively and regularly used for pulp treatment in clinical dentistry. However, the reparative dentin, which is formed by calcium hydroxide is porous and does not function as a complete barrier (Carrotte, 2004).

Mineral trioxide aggregate (MTA), a bioceramic material is mainly used for direct pulp capping, apexogenesis, apexification, and perforation repair (Utneja et al., 2015). It has excellent biocompatibility, sealing ability, and has a pH of 12.5, similar to calcium hydroxide. MTA can be a good choice as a cementing material, which triggers DPSCs differentiation and periodontal ligament regeneration although a precise mechanism of action has not yet been elucidated (Naik et al., 2014). Meanwhile, MTA has been demonstrated to increase bone sialoprotein (BSP) and alkaline phosphatase (ALP) expressions (Hakki et al., 2009).

Recently, propolis has been used as an alternative pulp treatment material (Ahangari et al., 2012; Qureshi et al., 2014). Propolis is a natural product produced by honey bees from tree resin. It has been used traditionally in the form of food and beverages for centuries for skin healing, infection, and inflammation control. In general, propolis is composed of 50% resin, 30% wax, 10% essential and aromatic oils, 5% pollen, and 5% various other substances (Hwu and Lin, 2014; Nieva Moreno et al., 1999). Other various substances consist of mostly organic compounds (phenolic compounds, flavonoids, terpenes, etc.), vitamins (B1, B2, B6, C and E), and minerals (Mg, Ca, K, Na, etc.). Therefore, propolis can be a good source of nutritional food and generates a variety of health benefits. It is being currently used as an ingredient or bioactive compounds of candies, foods and drinks (Hwu and Lin, 2014; Osés et al., 2016). Propolis prevents inflammation, a main cause for periodontitis-related diseases and alveolar bone loss, and reduces proliferation and maturation of osteoclast cells (Ahangari et al., 2012; Pileggi et al., 2009; Toker et al., 2008). Propolis imparts its significant effects to regeneration and recovery of DPSCs and exhibits excellent biocompatibility with periodontal ligament (Scheller et al., 1978). But, overdose could result in an allergic reaction or mucous irritation so minimal use is recommended.

It is known that propolis induces dentin regeneration in guinea pigs (Ahangari et al., 2012). MTA also promotes odontoblastic differentiation of DPSCs under culture

conditions in MTA cement-treated cells (Zhao et al., 2012). However, no research of the effects of the combination of propolis and MTA on odontoblastic differentiation in human DPSCs in vitro has been carried out. Our study aims to know if the combination of MTA and propolis would facilitate odontoblast differentiation of DPSCs, and to elucidate involved signaling mechanisms.

## Materials and methods

### Human DPSCs isolation and culture

To isolate and culture Human DPSCs, extracted teeth from the clinical practice of orthodontic therapy were used. All procedures were followed after obtaining informed consent and the approval of the IRB of Chonnam National University Dental Hospital (CNUDH-2013-002). The isolation and culture of human DPSCs were carried out with no use of collagenase as previously reported method (Hilkens et al., 2013; Raof et al., 2014). That is, pulp tissue was removed aseptically from sectioned teeth, rinsed with Hank's buffered saline solution and placed in a 60 mm petri-dish. It was, then, minced by using a blade into small fragments and cultured in an alpha minimum essential medium ( $\alpha$ -MEM, Gibco Invitrogen, Grand Island, NY, USA) including 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin. Human DPSCs culture was maintained at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. When grown enough to cover nearly all over the cell plate, trypsin was added and a new batch of subculture was kept. For our study, from 3 to 8 batches of sub-cultured cells were sampled.

### Preparation of propolis and MTA extract

Propolis (10 g), provided by Damyang Agriculture Technology Center (Damyang, Korea), was dissolved in 80% ethanol (50 mL) with gentle swirling for overnight. After filtration with Whatman No. 2, it was kept overnight in the freezer to remove hardened wax, then, dried with a rotary evaporator at 40 °C. ProRoot MTA (Dentsply Tulsa Dental, Tulsa, OK) was stirred in sterile water with a spatula, and poured into cylindrical polyethylene tubes (diameter = 5.0 mm, height = 3 mm) for molding. It was dehydrated for 24 h at 37 °C in a humidified 5% CO<sub>2</sub> incubator and sterilized by ultraviolet radiation.  $\alpha$ -MEM (50 mL) including 1% penicillin and streptomycin was added to MTA mold for infusion. After 7 days, media were filtered with a 0.2  $\mu$ m syringe filter, and diluted to a concentration of 1 mg/mL (1:20 dilution).

### Cell viability assay

To measure cell survival rate, a commercially available proliferation and cytotoxicity kit was used, based on tetrazolium salts (EZ-CYTOX, Daeil Lab, Daejeon, Korea). A suspension of DPSCs at a concentration of  $2 \times 10^4$  cells per well was seeded into 96-well plates with  $\alpha$ -MEM with 10% FBS. A different range of propolis (0, 50, 100, and 250 ng/mL) and/or MTA (1 mg/mL) was applied onto DPSCs. After 24 or 48 h of incubation, 10  $\mu$ L EZ-CYTOX was added to each well during the final 4 h of each experiment. The cell turbidity was determined at 450 nm by using a multi-well plate reader (Molecular Devices, Toronto, Canada).

### Real-time polymerase chain reaction (PCR)

TRIzol (Molecular Research Center, Inc, OH, USA) was used for total RNA extraction from DPSCs, following manufacturer's instructions. To synthesize single strand cDNA, total RNA (2  $\mu$ g) was denatured at 95 °C for 5 min, then, reverse transcription was conducted using M-MLV Reverse Transcriptase (Promega, Madison, USA) at 37 °C for 60 min. An aliquot of the synthesized cDNA from 0.3  $\mu$ g RNA was used as a template for PCR. Real-time PCR data were analyzed, following instructions from Rotor-Gene Q Thermal Cycler (Qiagen, Hidden, Germany). Real-time PCR was triplicated for data accuracy. Total volume (25  $\mu$ L) of each reaction mix contained  $2 \times$  RG SYBR PCR Master mix (Qiagen, Hidden, Germany), cDNA, primers (10 pmol of each), and sterile water. Briefly, 40 thermocycles of 95 °C, 15 s; 58 °C 30 s; and 72 °C, 30 s were run after denaturation of DNA at 95 °C for 10 min. Melting curve was recorded in 0.2 s interval from 65 to 95 °C. PCR was run with following specific primers: DSPP, (F) 5'-CAACCATAGAGAAAGCAAACGCG-3', (R) 5'-TTTCTGTTGCCACTGCTGGGAC-3'; DMP1, (F) 5'-ATGCCTATCACAACAAACC-3', (R) 5'-CTCCTTTATGTGACAACCTGC-3', or GAPDH, (F) 5'-CATCACCATCTTCCAGGAG-3', (R) 5'-AGGCTGTTGTCATACTTCT C-3'.

### ALP staining

Combined MTA (1 mg/mL) and propolis (10 or 50 ng/mL) were applied to human DPSCs ( $2 \times 10^4$  cells) in 24-well plates for 7 days of incubation. To start ALP staining, cells were fixed with 70% ethanol for 1 h after removing culture media. After 3 times of rinse with deionized water, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) solution (Sigma-Aldrich, St. Louis, MO) was added to each well for staining reaction, which was stopped by adding water. Staining was captured with

an Epson scanner (Epson perfection V700 PHOTO, Tokyo, Japan). For quantitative analysis, stain was extracted with 10% (w/v) cetylpyridinium chloride (Sigma-Aldrich) for 15 min and quantified by measuring the absorbance at 570 nm with an Absorbance Microplate Reader (Molecular Devices, Toronto, Canada).

### Alizarin red S staining

Human DPSCs ( $2 \times 10^4$  cells) in 24-well plates were cultured in presence of MTA (1 mg/mL) and propolis (10 or 50 ng/mL) for 14 days once an odontogenesis induction medium (OIM) including 50 mg/mL ascorbic acid and 10 mM  $\beta$ -glycerophosphate was added. Cells were fixed for 1 h at room temperature with 70% ice-cold ethanol and stained with 40 mmol/L alizarin red S (pH 4.2) for 30 min after washing with phosphate-buffered saline (PBS) buffer. Cells were, again, washed with PBS with  $Mg^{2+}$  or  $Ca^{2+}$ , and after residual staining solution was gently aspirated, they were washed 4 times with distilled water. Staining was photographed with an Epson scanner (Epson perfection V700 PHOTO). To quantify staining, stain was extracted with 10% (w/v) cetylpyridinium chloride (Sigma, USA) for 15 min. Stain intensity was measured by absorbance at 570 nm with an Absorbance Microplate Reader (Molecular Devices, Toronto, Canada).

### Western blot analysis

After cells were washed 2 or 3 times with cold PBS buffer, 1 mL of PBS-TDS (PBS, 1% Triton X-100, 0.05% sodium deoxycholate, 0.01% SDS, 0.5  $\mu$ g/mL leupeptin, 1 mM EDTA, 1  $\mu$ g/mL pepstatin and 0.2 mM PMSF) was added and left on ice for 15 min. Cell membranes were removed by centrifugation at  $15,000 \times g$  for 10 min, and total protein was quantified with BCA assay kit (Sigma, St. Louis, USA). Bovine serum albumin (BSA) was used as a reference protein. Isolated proteins (20  $\mu$ g) were separated on 12% polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) following standard SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) procedure, and transferred to a nitrocellulose membrane (Bio-rad, Hercules, USA) for 2 h at 100 V. The transferred membrane was blocked with 5% non-fat dry milk in PBS-T (PBS, 0.1% Tween 20) for 1 h at room temperature, and incubated with antibodies including anti-ERK (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-phospho-ERK (Cell signaling, Denver, MA) for 1 h after the antibodies were diluted to 1:1000 in PBS. As a second antibody, horse radish peroxidase (HRP)-bound anti-mouse IgG or anti-rabbit IgG (Sigma, USA) was used with a 1:5000 dilution for 1 h at room temperature. After 3 times of washing, chemiluminescent HRP Substrate was used for

luminescence reaction for 30–60 s and its detection was performed with Ez-capture chemiluminescence imaging system (Atto, Tokyo, Japan).

### Statistical analysis

All experiments were performed in triplicates. Each value was shown as a mean  $\pm$  standard deviation. Analysis of variance (ANOVA) with Dunnett's test was used for multiple comparisons. Differences with  $p < 0.05$  were considered statistically significant.

## Results and discussion

### Effects of propolis on the cell viability of human DPSCs

Effects of propolis on the cell viability of DPSCs was evaluated. As shown in Fig. 1A, the cell viability of DPSCs was unaffected in presence of various concentrations of propolis (0, 50, 100, and 250 ng/mL). Also, cells treated with a combination of MTA (1 mg/mL) and propolis (0, 50, 100, and 250 ng/mL) for 24 and 48 h showed no inhibition of cell growth (Fig. 1B).

### Effects of MTA and propolis on the expression of odontoblastic differentiation markers

To investigate the differentiation of DPSCs into odontoblasts after MTA (1 mg/mL) only, or combined MTA and propolis (10 or 50 ng/mL) treatments, expression levels of DSPP and DMP1, odontogenic differentiation markers, were quantified in DPSCs by using real-time PCR. The combination of MTA and propolis significantly up-regulated DSPP and DMP1 levels (Fig. 2). It also enhanced ALP activity (Fig. 3A). In addition, to determine effects of the combination of MTA and propolis on mineralization in DPSCs, a mineralized nodule formation in DPSCs was assessed by using alizarin red S staining. The combination of MTA (1 mg/mL) and propolis (10 or 50 ng/mL) significantly increased mineralization (Fig. 3B).

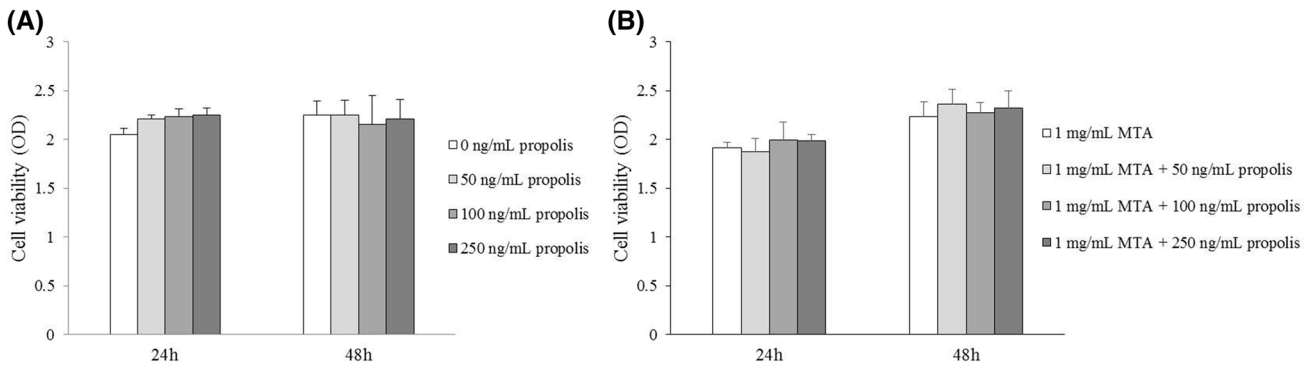
### Combination of MTA and propolis stimulates odontoblastic differentiation by activating an ERK signaling pathway

To investigate whether combined MTA and propolis effects is triggered through the activation of a ERK signaling pathway in DPSCs, ERK phosphorylation was evaluated by using a Western blot analysis with DPSCs in treatment of propolis (50 ng/ml) at different time intervals (0, 5, 10, 30, 60, 120 min). ERK phosphorylation was

induced in 5 min, reaching a maximum at 10 min, and disappeared after 30 min. However, propolis-induced ERK phosphorylation was suppressed at 10 min after pretreatment of ERK inhibitor U0126 (0.1 or 1  $\mu$ mol/L) 1 h (Fig. 4A). The combination of MTA (1 mg/mL) and propolis (10 or 50 ng/mL) showed more phosphorylation of ERK than MTA or propolis alone (Fig. 4B). To confirm the involvement of an ERK signaling pathway in combined MTA and propolis-induced odontoblastic differentiation and mineralization of DPSCs, cells were pretreated with U0126 (0.1 or 1  $\mu$ mol/L), an ERK inhibitor, for 1 h followed by treatment with combination of MTA (1 mg/mL) and propolis (50 ng/mL) for 2 days. Inhibition of an ERK pathway by an U0126 inhibitor reduced the phosphorylation of ERK (Fig. 4C). In addition, pretreatment with U0126 decreased a MTA and propolis-enhanced calcified nodule formation by staining with alizarin red S staining (Fig. 4D). These results demonstrated that an ERK signaling pathway was taken through in the effects of combination of MTA and propolis on the odontoblastic differentiation and mineralization of DPSCs.

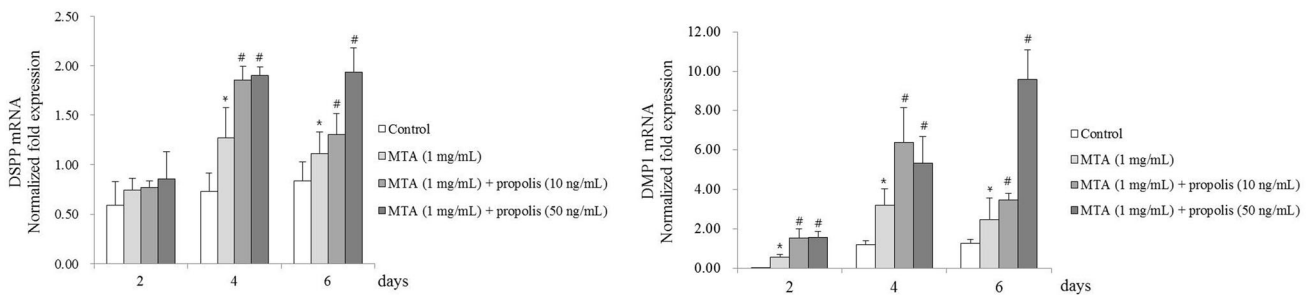
Potential therapeutic regulation of reparative dentin formation is a desirable goal in clinical dentistry. During dental pulp repair, the differentiation of dental pulp cells into odontoblast-like cells is important for reparative dentinogenesis. To initiate reparative dentinogenesis after dental pulp injuries, DPSCs must migrate, proliferate, and differentiate to set a new barrier. A new layer of barrier formation is set to protect pulp cells and prevent inflammation (Yamamura, 1985).

Propolis has been widely used as a traditional anti-inflammatory and anti-bacterial medicine and food for many centuries. It might include more than 300 active substances and its color and composition are diverse, depending on its place of collection and time (Hwu and Lin, 2014). For propolis to be consumed in the form of food, its concentration shall not be higher than 0.5% because it causes bitterness taste. But, within 0.1–0.5% it can be added to form a likeable product without compensating for deterrent taste. By including propolis in a regular diet, anti-inflammatory effects can be maximized for oral health (Osés et al., 2016). In recent dental practices, propolis has been applied for therapeutic purposes (Ahangari et al., 2018; Marcucci, 1995). Our study investigated combined effects of MTA and propolis on the odontoblastic differentiation and mineralization of DPSCs in vitro. At this stage, it is difficult to conclude that which specific chemical compound nature of propolis plays a key role in odontogenic differentiation and mineralization of DPSCs. One of active component of propolis, caffeic acid phenethyl ester (CAPE), which is a phenolic acid, attenuates osteoclastogenesis and bone resorption, thereby promoting osteoblast formation (Ang et al., 2009). Another active group of



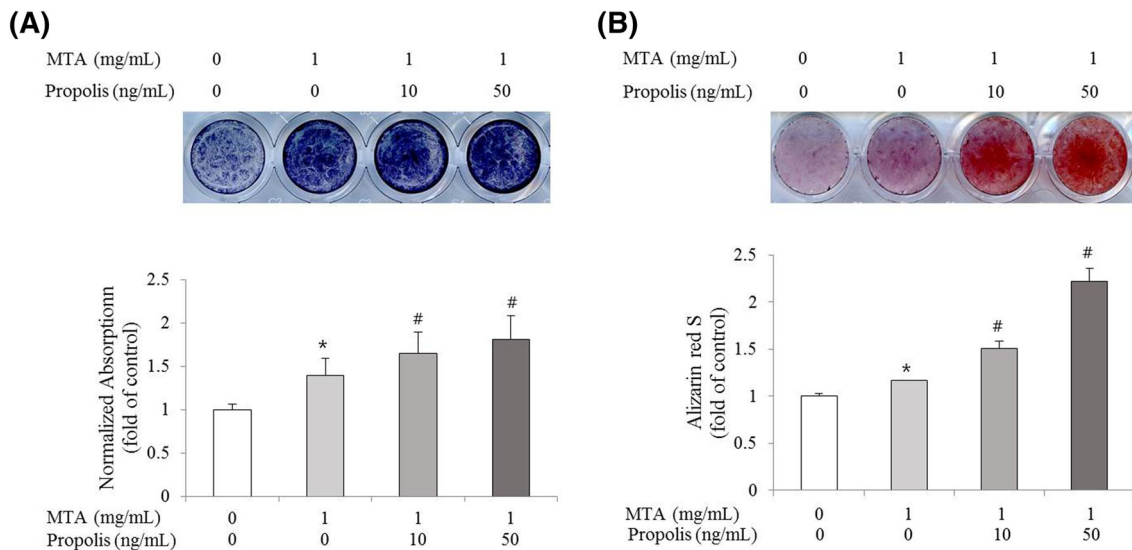
**Fig. 1** Effects of propolis and MTA on cell viability in DPSCs. (A) The viability of DPSCs under different concentrations (0, 50, 100, and 250 ng/mL) of propolis for 24 and 48 h was investigated by WST assay. (B) Cells cultured with a combination of MTA (1 mg/mL) and

propolis (0, 50, 100, and 250 ng/mL) for 24 and 48 h were investigated using WST assay. The results are the mean ± standard deviation of triplicate measures from 3 independent experiments



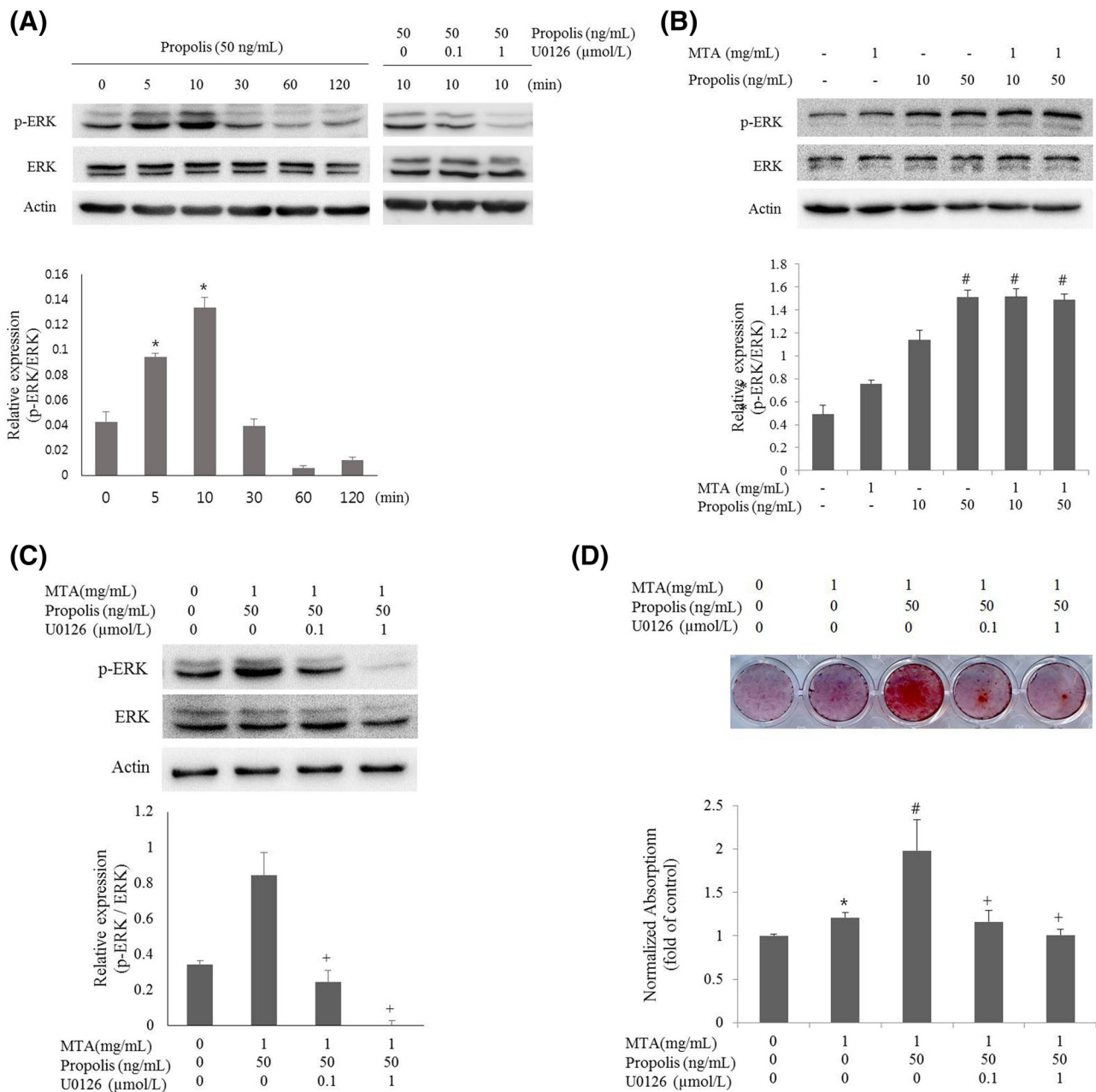
**Fig. 2** Effects of MTA with propolis on the expression of odontoblastic differentiation markers in DPSCs. Cells were cultured with MTA (1 mg/mL) or a combination of MTA (1 mg/mL) and propolis (10 and 50 ng/mL) for 2, 4, and 6 days. Expression levels of DSPP and DMP1 were measured by real-time PCR, and the relative level of

gene expression was normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Values are expressed as mean ± standard deviation of triplicate measures from 3 independent experiments. \**p* < 0.05 versus control group. #*p* < 0.05 versus MTA treated group



**Fig. 3** Effects of MTA with propolis on ALP activity and mineralization in DPSCs. (A) After cells were cultured with or without the treatment of MTA or a combination of MTA (1 mg/mL) and propolis (10 and 50 ng/mL) for 7 days, ALP activity was detected. (B) Cells were cultured with or without the treatment of MTA or a combination

of MTA (1 mg/mL) and propolis (10 and 50 ng/mL) for 14 days and stained with alizarin red S. Results were normalized to those of the control from 3 independent experiments. Values are expressed as mean ± standard deviation of triplicate. \**p* < 0.05 versus control group. #*p* < 0.05 versus MTA treated group



**Fig. 4** Combination of MTA and propolis stimulated odontoblast differentiation via ERK signaling in DPSCs. **(A)** The levels of ERK and the phosphorylation of ERK in propolis (50 ng/mL) were determined by Western blot analysis during various time intervals (0, 5, 10, 30, 60, and 120 min). **(B)** Cells were cultured with MTA (1 mg/mL) or propolis (10 and 50 ng/mL) or combination of MTA and propolis for 2 days. The levels of ERK and the phosphorylation of ERK were determined by Western blot analysis. **(C)** Cells were pretreated with or without U0126 (0.1 and 1 μmol/L), an ERK signaling inhibitor, for 1 h and then cultured with a combination of

MTA (1 mg/mL) and propolis (50 ng/mL) for 2 days. The levels of ERK and the phosphorylation of ERK were determined by Western blot analysis. **(D)** Calcified nodule stained with alizarin red S staining were detected in MTA, propolis or a combination of MTA and propolis treated DPSCs for 14 days with or without pretreatment of U0126 for 1 h. Values are as mean ± standard deviation of triplicate measures from 3 independent experiments. \* $p < 0.05$  versus control group; # $p < 0.05$  versus MTA treated group; + $p < 0.05$  versus MTA and propolis treated group

propolis, flavonoids, has potent inhibitory activity against protein kinases (protein kinase C, protein tyrosine kinases, phospholipase A2 and others), in particular, in activated cells of immune system, resulting in health promotion

(Middleton, 1998). Therefore, although propolis was used in our study, total phenolics including flavonoids and phenolic acids are most likely responsible for ERK-mediated DPSCs differentiation (Ahangari et al., 2018).

The differentiation from dental pulp cells into odontoblasts was evaluated with our results of expressions of genes associated with odontoblastic differentiation, such as DSPP, DMP1, ALP activity, and formation of mineralization. Previous studies have shown that DSPP, DMP1, and ALP genes are expressed in differentiated odontoblast cells from human DPSCs, so they could be used as molecular markers to evaluate the extent of DPSCs differentiation in a more rapid and efficient manner than any other conventional means, especially at early stages (Feng et al., 2003; George et al., 1995; Hwang et al., 2008). DMP1 is present in the bone tissues and highly expressed in osteoblasts and odontoblasts (Jacob et al., 2014; Narayanan et al., 2003; Narayanan et al., 2006). ALP can also be harnessed as an early stage differentiation marker (Schwab and Gargett, 2007). Our results showed that combined MTA and propolis further up-regulated expression of DSPP and DMP1. Moreover, the combination of 1 mg/mL MTA and 10 or 50 ng/mL propolis treatments increased an ALP activity and mineralized matrix formation in human DPSCs. These results suggest that combined MTA and propolis accelerates more odontoblastic differentiation in human DPSCs than MTA or propolis alone. Previous studies have shown that MTA promoted odontoblastic differentiation by up-regulating DSPP, OCN, and ALP in DPSCs and reported regeneration of the pulp cells following application of propolis on injured dental pulp (Scheller et al., 1978; Zhao et al., 2012). Several studies have identified the formation of reparative dentin in rat dental pulp cells in presence of propolis, and reported a partial dental bridge formation in rat dental pulp tissue capped with propolis after 4 weeks (Bretz et al., 1998; Sabir et al., 2005). In addition, another study has shown that a dentinal bridge formation and tubular dentin were more evident in presence of propolis or MTA (Parolia et al., 2010). Our study demonstrated further synergistic effects of combined MTA and propolis on odontoblastic differentiation in DPSCs.

Mitogen-activated protein kinases (MAPKs) are second messengers that play a critical role in cellular responses including growth, proliferation, differentiation, and apoptosis in mammalian cells (Yang et al., 2013). MAPKs are composed of three well-characterized families, including ERKs, JNKs, and p38 MAPK (Johnson and Lapadat, 2002). ERK signaling is known to be involved in the differentiation of mesenchymal stem cells and skeletal development (Ge et al., 2007; Greenblatt et al., 2010; Wu et al., 2012). It was also reported that MAPKs, particularly ERKs, are associated with MTA-induced odontoblastic differentiation of human DPSCs (Zhao et al., 2012). In our study, ERK was rapidly phosphorylated in presence of combined MTA and propolis, reaching a maximum in 10 min and its phosphorylation status decreased thereafter, while JNK1 and p38 were not noticeably phosphorylated at

the given time points (data not shown), suggesting that MTA and propolis exert their effects through an ERK signaling pathway. ERK phosphorylation was specifically decreased with U0126 treatment, an inhibitor of ERK, reversing the effects of ERK signaling by combined MTA and propolis on odontogenic differentiation of DPSCs. Increased calcium deposits by combined MTA and propolis was attenuated by U0126. In accordance with another capping material, MTA, which induced DPSCs differentiation through ERK, propolis strongly increased odontogenic differentiation and this was highly likely to be mediated through ERK signaling. The activated ERK MAPK signaling contributed to odontogenic differentiation in human dental pulp cells, regulates different upstream signaling mediators in differentiation marker gene regulation (Liu et al., 2014; Lv et al., 2016; Woo et al. 2015a, b). The present study suggests that ERK MAPK signaling activated by propolis and MTA combination might take part in the expression of odontogenic markers and mineralization in DPSCs. The exact mechanisms by which exact molecular interactions are intertwined or the nature of molecules playing crucial roles in the signaling pathway remain to be solved.

Our study revealed that the combined application of MTA and propolis promoted the differentiation of DPSCs into odontoblast-like cells through activation of an ERK signaling pathway and combined effects were synergistic. We hope these findings could help to provide novel insights on practical application of combined MTA and propolis to induce odontogenesis and facilitating dentin regeneration.

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#### Compliance with ethical standards

**Conflict of interest** The authors report no conflict of interest related to this study.

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