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ORIGINAL ARTICLE

Combined effects of mineral trioxide aggregate and human placental extract on rat pulp tissue and growth, differentiation and angiogenesis in human dental pulp cells

Seok-Woo Chang[a]*, Ji-Youn Kim[b]*, Mi-Joo Kim[b], Ga-Hyun Kim[b], Jin-Kyu Yi[a], Deok-Won Lee[c], Kee-Yeon Kum[d] and Eun-Cheol Kim[b]

[a]Department of Conservative Dentistry; [b]Department of Oral and Maxillofacial Pathology and Research Center for Tooth and Periodontal Regeneration (MRC); [c]Department of Oral and Maxillofacial Surgery School of Dentistry, Kyung Hee University, Seoul, Republic of Korea; [d]Department of Conservative Dentistry, Seoul National University Dental Hospital, Seoul, Republic of Korea

ABSTRACT
Objective The aim of this study was to evaluate the combined effects of mineral trioxide aggregate (MTA) and human placental extract (HPE) on cell growth, differentiation and *in vitro* angiogenesis of human dental pulp cells (HDPCs) and to identify underlying signal transduction mechanisms. *In vivo* dental pulp responses in rats for a pulp-capping agent were examined. **Materials and methods** MTS assay. ALP activity test, alizarin red S staining and RT-PCR for marker genes were carried out to evaluate cell growth and differentiation. HUVEC migration, mRNA expression and capillary tube formation were measured to evaluate angiogenesis. Signal transduction was analysed using Western blotting and confocal microscopy. The pulps of rat maxillary first molars were exposed and capped with either MTA or MTA plus HPE. Histologic observation and scoring were performed. **Results** Compared to treatment of HDPCs with either HPE or MTA alone, the combination of HPE and MTA increased cell growth, ALP activity, mineralized nodules and expression of marker mRNAs. Combination HPE and MTA increased migration, capillary tube formation and angiogenic gene expression compared with MTA alone. Activation of Akt, mammalian target of rapamycin (mTOR), p38, JNK and ERK MAPK, Akt, and NF-κB were significantly increased by combining HPE and MTA compared with MTA alone. Pulp capping with MTA plus HPE in rats showed superior dentin bridge formation, odontoblastic layers and dentinal tubules and lower inflammatory cell response, compared to the MTA alone group. **Conclusions** This study demonstrates for the first time that the use of MTA with HPE promotes cell growth, differentiation and angiogenesis in HDPCs, which were associated with mTOR, MAPK and NF-κB pathways. Direct pulp capping with HPE plus MTA showed superior results when compared with MTA alone. Thus, the combination of MTA and HPE may be useful for regenerative endodontics.

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Introduction

Mineral trioxide aggregate (MTA) is a modified preparation of Portland cement that has several clinical applications in endodontic treatment including pulp capping,[1] repair of root perforation, retrograde filling and one visit apexification.[2] We previously reported that MTA yielded better dentin bridge formation and less pulpal inflammation than calcium hydroxide (CH) in human pulp capping.[3] However, MTA has several disadvantages, such as a long setting time, high cost, high pH, low compressive strength and potential for discolouration.[4,5] Moreover, subcutaneous implantation of white and grey MTA in rats was shown to provoke an initial moderate-to-severe inflammatory cell reaction that lasted for 7 days.[6] To improve the drawbacks associated with MTA, new calcium silicate-based materials such as Biodentine (Septodont, Saint Maur des Fosses, France) have been developed. We recently demonstrated that the biocompatibility, inflammatory response and odontoblastic differentiation of Biodentine are similar to those of Ortho-MTA (BioMTA, Seoul, Korea) and Angelus-MTA (Angelus, Londrina, Brazil) in human dental pulp cells (HDPCs).[7] In this respect, the development of less toxic and more bioactive agents is needed for pulp capping.

Growth factors and cytokines play key roles in the regulation of cell differentiation and tissue wound healing. The combination of fibroblast growth factor-2 (FGF-2) and MTA was recently shown to enhance proliferation and differentiation of HDPCs compared to MTA alone.[8] Moreover, we demonstrated that the combination of MTA and an enamel matrix derivative (EMD) promotes more rapid differentiation of HDPCs than MTA alone.[9] In addition, the combination of MTA and bone morphogenetic protein-2 (BMP-2) promotes more rapid

CONTACT Eun-Cheol Kim eckim@khu.ac.kr Department of Oral and Maxillofacial Pathology, Research Center for Tooth and Periodontal Regeneration (MRC), School of Dentistry, Kyung Hee University, 26, Kyungheedae-ro, Dongdaemun-gu, Seoul, 130-701, Republic of Korea
*These authors contributed equally to this study.

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differentiation in MC3T3-E1 cells than MTA/EMD treatment during early mineralization.[10]

Human placenta had been used on wound healing, such as burns, chronic ulcers and skin defects.[11–16] Aqueous human placental extract (HPE) is currently available and licensed for post-surgical dressings, burn injuries and chronic wounds in many countries and for liver disease in some countries.[11,17] HPE is a rich resource of various bioactive substances such as polydeoxyribonucleotides enriched in enzymes, nucleic acid, vitamins, amino acids, steroids, fatty acids and minerals.[18] HPE possesses immunotrophic, antioxidative and anti-inflammatory effects in experimental osteoarthritis,[19] hypoxic-ischaemic brain injury,[20] CCl4-injured liver [21] and rheumatoid arthritis.[22]

In addition, HPE promotes growth in human fibroblasts [23] and chondrocytes [19] and *in vitro* hepatogenic differentiation in placenta-derived stem cells.[24] Although mouse PE inhibits *in vitro* osteoclast differentiation of RAW 264.7 macrophages,[25] the effects of HPE on odontoblastic/osteoblastic differentiation have not yet been reported. Since HPE affects wound healing, we hypothesized that combined stimulation with MTA and HPE would augment odontoblastic differentiation and angiogenesis to a greater degree than treatment with MTA alone. Thus, the aim of this study was to assess the combined effects of MTA and HPE on growth, odontogenic differentiation and *in vitro* angiogenesis compared with intermediate restorative material (IRM) in HDPCs. *In vitro* signal transduction pathways and *in vivo* pulpal response of rats for pulp capping were also examined.

Materials and methods

Cell culture

Immortalized HDPCs, transfected with human telomerase catalytic component were kindly provided by Professor Takashi Takata (Hiroshima University, Japan). The cells were pre-cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. To induce differentiation, cells were cultured with osteogenic supplement (OS) including 50 mg/mL ascorbic acid and 10 mM β-glycerophosphate as described previously.[9] Human umbilical vein endothelial cells (HUVECs) from ATCC (Manassas, VA) were cultured in endothelial cell medium (ECM, ScienCell, Carlsbad, CA) at 37 °C in a 5% CO_2 atmosphere.

Test materials and groups

White ProRoot MTA (Dentsply Tulsa Dental, Tulsa, OK) and intermediate restorative material (IRM, Dentsply Tulsa Dental) were mixed according to the manufacturer's instructions. Each sample (diameter = 10 mm; thickness = 2 mm) was allowed to set for 24 h at 37 °C in 100% humidity. MTA was sterilized by gamma radiation with 37.2 Gray before being used to culture cells. Commercially available HPE was obtained from Melsmon (Tokyo, Japan). A preliminary dose-response study (0.1, 1, 10 and 20 μg/mL) identified 1 μg/mL HPE as the optimal concentration with the highest induction of ALP activity. HDPCs were seeded at 1×10^5 cells per well on the prepared MTA and cultured with HPE for 14 days. Experiments were performed in triplicate wells per condition and repeated in three independent experiments.

Cell viability

Cell viability was measured with a cell proliferation assay kit (Cell Titer 96 Aqueous One Solution; Promega, Madison, WI) using a microplate reader (Bio-Rad, Hercules, CA) at a wavelength of 490 nm.

Alkaline phosphatase (ALP) activity

ALP activity was measured using p-nitrophenyl phosphate (3 mM) as the substrate in 0.7 M 2-aminomethyl-1-propanol (pH 10.3) and 6.7 mM $MgCl_2$. Absorbance was measured at 410 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Rad).

ALP and alizarin red S staining

An ALP staining kit (86R-1KT, Sigma-Aldrich, St. Louis, MO) was used according to the manufacturer's instructions. Briefly, cultured cells were fixed with 4% paraformaldehyde for 30 min at room temperature. Alkaline-dye mixture was added at 200 μL/well and the plate was wrapped with aluminum foil and incubated for 20 min at 37 °C under dark conditions. For Alizarin red stain, fixed cells were stained with 40 mM Alizarin red S (pH 4.2) for 10 min with gentle agitation. Stained cells were photographed under a microscope with a digital imaging system (Olympus, Tokyo, Japan).

In vitro migration assay

The migration assay was performed with a QCM chemotaxis 24-well colourimetric cell migration kit (Chemicon, Temecula, CA) following the manufacturer's protocol. HUVECs were seeded in triplicate in the upper compartment of the chamber (2.5×10^4 cells/50 μl per well). The lower compartment was filled with 30 μl of conditioned media (CM) from MTA and HDPCs treated with HPE for 3 days. Cells that migrated through the polycarbonate membrane were incubated with Cell Stain Solution and then subsequently extracted and assessed with a microplate reader at 560 nm.

Endothelial tube formation assay

In vitro angiogenesis was assessed using an Endothelial Tube Formation Assay kit (CBA-200, Cell Biolabs Inc., San Diego, CA). Briefly, extracellular matrix (ECM) gel was thawed at 4 °C overnight and then bottom-coated in a 96-well plate (50 μl per well) at 37 °C for 1 h. Next, 150 μl of medium containing HUVECs ($1–2 \times 10^6$) was seeded on the ECM gel and cultured with CM at 37 °C in a 5% CO_2 atmosphere for 18 h. Capillary tube images were photographed under a microscope with a digital imaging system (Olympus).

RNA isolation and RT-PCR

Total RNA was extracted using Trizol reagent (Life Technologies, Gaithersburg, MD) and then 1 µg was reverse-transcribed and amplified using AccuPower RT PreMix (Bioneer, Daejeon, Korea). Primer sequences, annealing temperatures and cycle numbers for *ALP*, *OCN*, *BSP*, *DSPP*, *DMP-1*, *FGF-2*, *Ang-1*, *VEGF* and β-*actin* are detailed in Table 1. The PCR products were loaded on a 1.2% agarose gel stained with ethidium bromide.

Table 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) primers and conditions.

Genes	Primer sequences (5′-3′)	Annealing temp (°C)	Cycle number	Product size (bp)
ALP	F:5′-ACGTGGCTAAGAATGTCATC-3′ R:5′-CTGGTAGGCGATGTCCTTA-3′	55	30	476
OCN	F:5′-AGCCCTCACACTCCTCGCCCTAT-3′ R:5′-AGCCTGGTTCACCCCAGCTCA-3′	61	31	301
DSPP	F:5′-GCAAAAGTCCAGGACAGTGGGCC-3′ R:5′- GTCAAATTTCCACCTCAGTTGGCCA-3′	61	35	342
DMP-1	F:5′-AGAGAGAGATGGGAGAGCTGCGC-3′ R:5′- TGACCTTCCATCTGCCTCCTGTTC-3′	61	35	202
FGF-2	F:5′-CGCTCCTCTGGGTTGAACAT-3′ R:5′-GACTTCTTTGGCTCTGCCCT-3′	61	35	415
Ang-1	F:5′-GACGCCGCGCGGAAAAGATG-3′ R:5′-ACCACCCACAATTTGGCCCTGC-3′	61	35	159
VEGF	F:5′CAATGGACGTGTCCCCGCGT-3′ R:5′-GCTGCCCTCTCCAACCGGTG-3′	60	36	365
β–actin	F: 5′-CATGGATGATGATATCGCCGCG-3′ R: 5′-ACATGATCTGGGTCATCTTCTCG-3′	55	34	371

Western blotting

Samples were lysed in RIPA lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 1% SDS, 1% Nonidet P-40, 1:100 proteinase inhibitor cocktail, 50 mM β-GP, 50 mM sodium fluoride). Protein concentrations were measured using the Bradford protein assay (Bio-Rad). Thirty micrograms of protein was loaded per lane on a 10% SDS-PAGE gel for electrophoresis then transferred to 0.22 µm PVDF membranes (Bio-Rad). Western blot analysis was performed as described previously.[7] The proteins were visualized using an enhanced chemiluminescence system (Amersham, Piscataway, NJ).

In vivo experiments

Ten Sprague-Dawley male rats (Hanlim Inc, Seoul, Korea) weighing between 300–350 g were used in this study. Experimental protocols were approved by the Institutional Animal Care and Use Committee of Kyung Hee University (Seoul, Korea). The rats were anaesthetized intramuscularly with ketamine and then Class I cavities were prepared on the occlusal surface of the right maxillary first molar using a low-speed tapered round diamond bur (0.84 mm in diameter, Intensiv, Swizerland). The pulp was then exposed at the cavity floor using a dental explorer (0.35 mm in tip diameter, Maritn, Germany) and directly capped with MTA and HPE. The remainder of the cavity preparation was sealed with flowable resin (tetric N flow; Ivoclar Vivadent, Schaan,

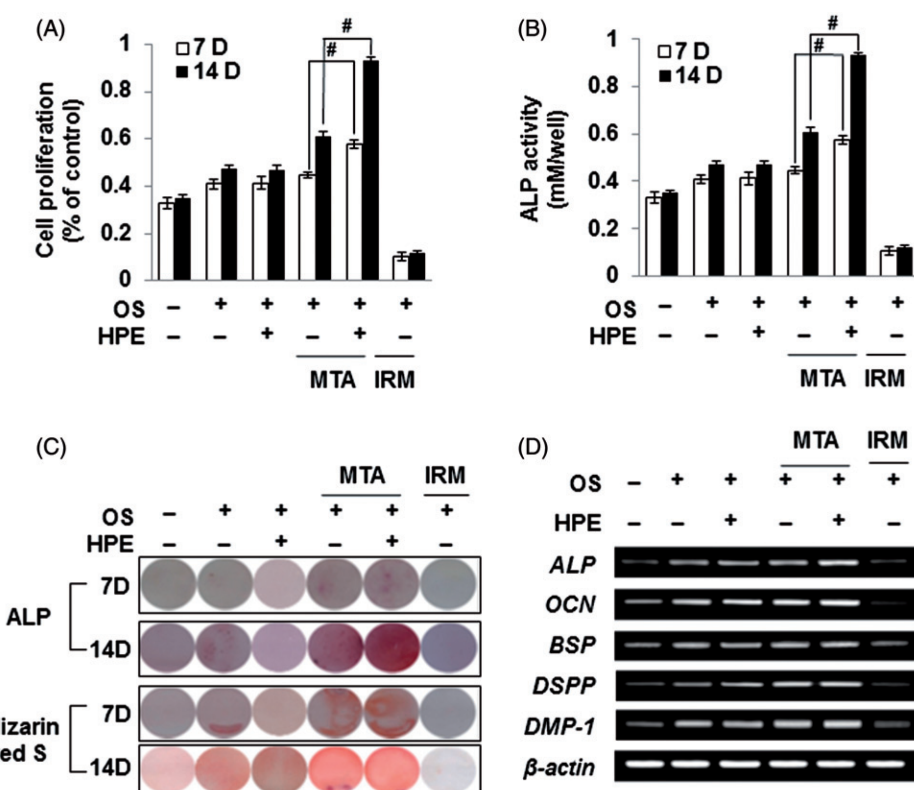

Figure 1. Combined effects of human placental extract (HPE) and mineral trioxide aggregate (MTA) on cell growth (A) and odontogenic differentiation (B–D) in human dental pulp cells (HDPCs). HDPCs were cultured with osteogenic supplement (OS) containing 10 mM β-glycerophosphate and 50 mg/mL ascorbic acid for 7 and 14 days. (A) Cell proliferation was examined by MTS assay. Differentiation was determined as (B) ALP activity, (C) formation of calcification nodules and (D) expression of odontogenic markers in HDPCs. Data are representative of three independent experiments. #$p < 0.05$.

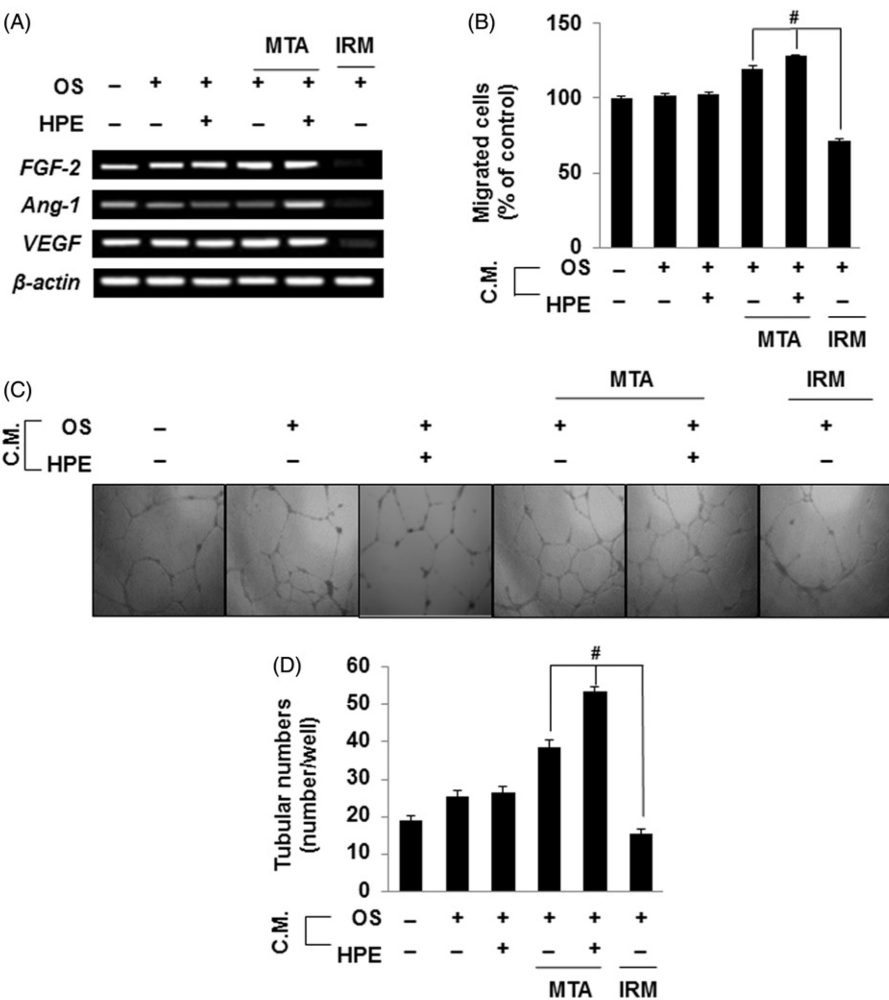

Figure 2. Combined effects of HPE and MTA on *in vitro* angiogenesis. Conditioned medium (CM) from HDPCs was obtained by 72-h incubation of HPE and MTA with a serum-free medium. (A) Cell migration of human umbilical vein epithelial cells (HUVECs) by CM was assessed and quantified. (B) The mRNA expression of angiogenic cytokines was evaluated after 3 days of HDPC culture. Angiogenic activity of CM was examined via tube formation assay in HUVECs (C, D). Data are representative of three independent experiments. #$p < 0.05$.

Liechtenstein). MTA was mixed with the HPE (Melsmon; 2 mL in 1 ampule with 200 mg of active ingredient) in a 3:1 ratio (v/v) using a metal spatula and the subsequent direct pulp capping process was the same. The animals were sacrificed 4 weeks after surgery.

Histological observation and histomorphologic analysis

Tissues were removed and fixed immediately in 10% formalin solution for 1 day, decalcified in 10% ethylenediaminetetraacetic acid (pH 7.4) for 4 weeks and then embedded in paraffin. Each sample was cut mesiodistally into 5 μm serial sections and stained with hematoxylin and eosin (H-E). The sections were analysed in a light microscope (Eclipse Ti -U, Nikon, Tokyo, Japan) and evaluated by an experienced examiner in a blind manner. The dentin bridge formation, odontoblastic layer and pulp inflammation were graded according to modified criteria that were based on a scoring system from a previous study.[26] The areas of newly formed hard tissue and exposure cavity were measured using imaging software (NIS Elements, Basic Research, version 4.40, Nikon). From the ratio of these areas, calcific barriers rate was calculated. Calcific barriers rate (%) $= B/A \times 100$, where A is the area of the entire exposure cavity of teeth and B is the area of the newly formed calcific barriers.

Statistical analysis

Values were calculated as the mean and standard deviation. Statistical significance was evaluated by one-way ANOVA using SPSS software (version 21.0; SPSS Inc., Chicago, IL) and defined as $p < 0.05$.

Results

Combined effects of MTA and HPE on cell proliferation and odontogenic differentiation in HDPCs

Proliferation of HDPCs cultured with MTA or HPE was not significantly different compared with OS at days 7 and 14. Cells cultured with MTA plus HPE proliferated significantly more than with MTA alone after 7 and 14 days ($p < 0.05$). Cell *growth* of IRM was markedly less compared to the other groups (Figure 1A).

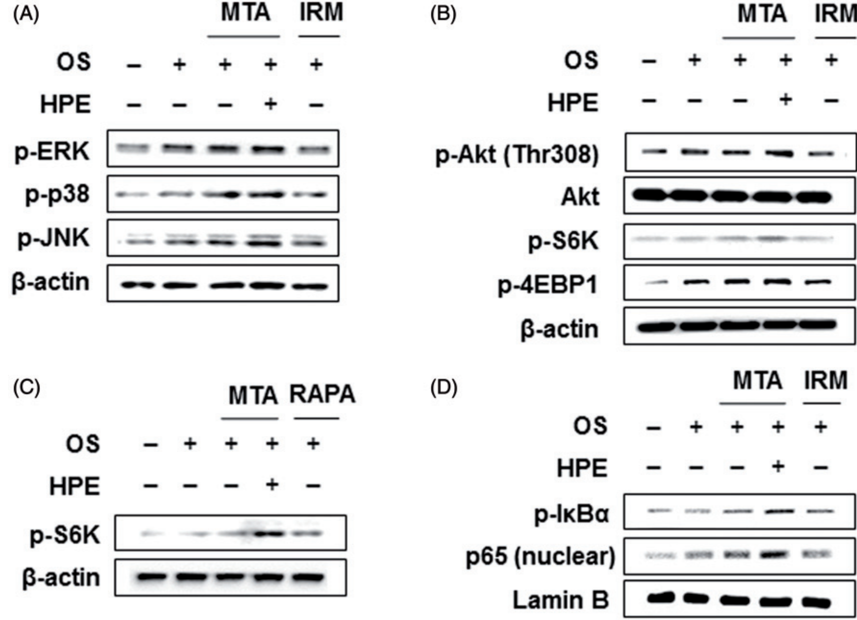

Figure 3. Combined effects of HPE and MTA on signalling pathways in HDPCs. HDPCs were incubated with MTA and HPE in OS media for 30 min (A–C) or 45 min (D, E). The signalling pathway was examined by Western blot (A–D) and confocal microscopy (E). Cells were pre-treated with rapamycin (100 ng/mL) for 30 min then incubated with MTA and HPE for 30 min. Arrows represent the translocation of p65 in cells. These data are representative of three independent experiments.

ALP activity and biomineralization as measured by ALP and Alizarin red S staining in the MTA and HPE-treated group was greater than that of the MTA-alone group after 7 and 14 days of cultivation (Figures 1B and C). HPE plus MTA increased mRNA expression levels of odontogenic marker genes, ALP, osteocalcin (OCN), bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP) and dentin matrix protein-1 (DMP-1) compared to MTA or HPE alone after 14 days of cultivation (Figure 1D).

Combined effects of MTA and HPE on in vitro angiogenesis

To evaluate the effects of MTA and HPE in *in vitro* angiogenesis, we assessed mRNA expression for angiogenic genes in HDPCs and phenotypic alterations of HUVECs using migration and capillary tube formation assays. Treatment with both MTA and HPE led to a significant increase in migration and capillary tube formation compared with MTA or HPE alone (Figures 2A, C and D). The mRNA expression of fibroblast growth factor (FGF-2), Ang-1 and vascular endothelial growth factor (VEGF) was upregulated after 18 h in the MTA and IRM group compared with the MTA alone group (Figure 2B).

Combined effects of MTA and HPE on signal pathways

To identify the molecular mechanisms involved in the response to MTA and HPE, activation of mitogen-activated protein kinase (MAPK), Akt/mammalian target of rapamycin (mTOR) and NF-κB pathways were examined (Figure 3). The phosphorylation of ERK, JNK and p38 was higher in HDPCs cultured on MTA and HPE than those cultured on MTA alone. In addition, protein levels of Akt, mTOR targets S6 kinase (S6K) and eukaryotic initiation factor 4E binding protein 1 (4EBP1) phosphorylation were slightly higher in MTA plus HPE-treated cells compared to those treated with MTA alone, but the level of total Akt was not changed (Figure 3B). Moreover, rapamycin, a selective mTOR inhibitor, attenuated combined MTA and HPE–induced phosphorylation of S6K (Figure 3C). Similarly, Western blot and confocal images showed a marked increase in NF-kB p65 in the nucleus of combined MTA and HPE-treated cells relative to those treated with MTA alone (Figures 3D and E).

In vivo response of the rats pulp to capping with MTA and HPE

To compare the *in vivo* response of HPE, rat dental pulps were capped with MTA and HPE. Inflammatory infiltrate and micro-organisms were observed in the sham control (pulp exposure) group (Figures 5A–C). The MTA group and MTA plus HPE group showed healthy dentin bridge formation, an intact odontoblastic layer and were free of inflammatory cells, when compared with the control group (Figures 5D–I). The scores for superior dentin bridge formation, odontoblastic layers, dentinal tubules and inflammatory cell response were superior in the MTA plus HPE group compared to in the MTA alone group (Table 2). Moreover, MTA plus HPE treated rat pulp tissue exhibited significantly increased newly formed calcific barrier rates, compared with MTA alone (Figure 6).

Discussion

Application of bioactive molecules or recombinant growth factors to the injured site to enhance the regeneration of dentin has been investigated for repair of dentin lost.[27]

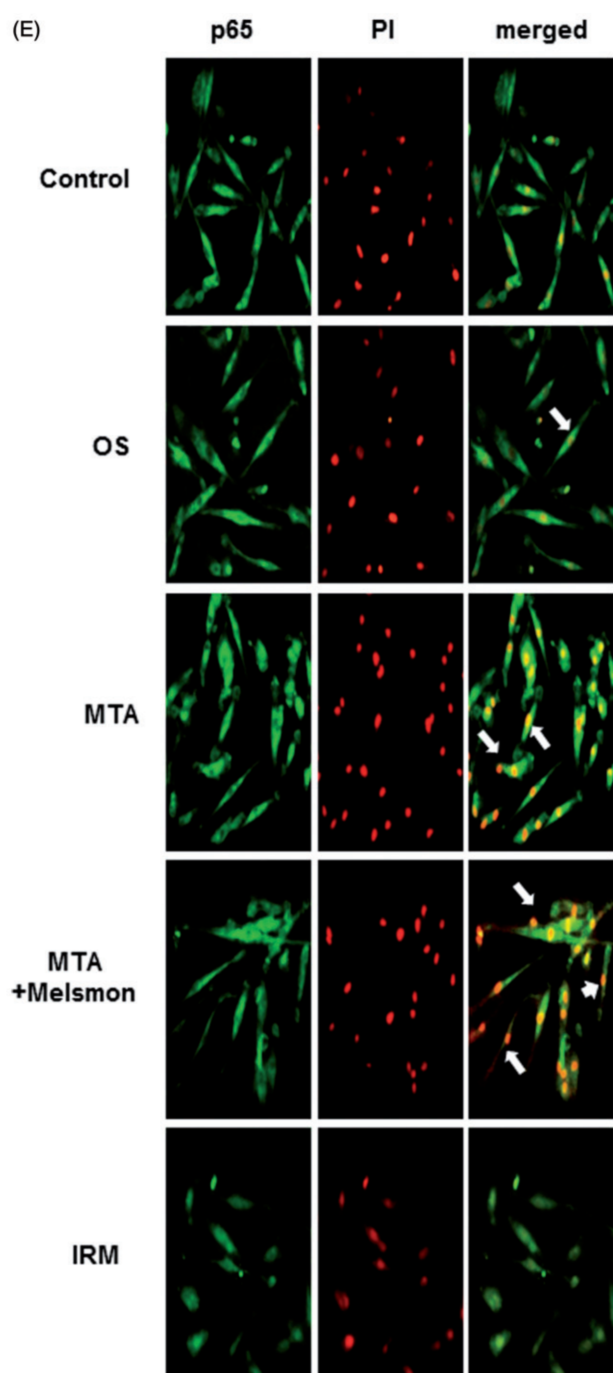

Figure 3. Continued.

In case of pulp revascularization, HPE would be used with the similar method of PRP (platelet rich plasma) application. After root canal disinfection, HPE soaked in collagen sponge is applied in root canal and the upper part of the canal could be sealed with MTA [28]. In case of pulp capping, HPE plus MTA can be applied in exposed pulp by several manners. After root canal disinfection, HPE soaked in collagen sponge would be applied in the root canal and the upper part of the canal could be sealed with MTA.[29] In the case of pulp capping, HPE along with MTA could be applied in exposed pulp in several manners. First, HPE can be directly applied on the exposed pulp with a syringe and the exposed pulp could be capped with MTA.[30] Otherwise, a collagen sponge soaked with HPE could be placed on the exposed pulp and the exposed pulp could be restored with MTA.[31] Based on these findings, application of HPE along with MTA using a syringe [32] or collagen sponge [33] could be considered in pulp capping or pulp regeneration.

Although HPE exhibits anti-inflammatory, anti-anaphylactic and anti-oxidative effects,[20–22] little biological information exists about the mechanism by which exogenous HPE acts as a supplement for pulp capping materials or as a reparative dentin inducer. To our knowledge, this report is the first to examine the effect of combined HPE and MTA treatment on angiogenesis and differentiation in an *in vitro* cell culture model using HDPCs and HUVECs.

MTA-treated cultures exhibited similar levels of cell viability to those treated with OS and superior cell viability over those treated with IRM. The observed biocompatibility of MTA is consistent with previous studies.[7,34,35] IRM is cytotoxic *in vitro* and is used as a positive control in cytotoxicity studies.[7,34,35] In addition, the combination of MTA and HPE increased proliferation of HDPCs compared with either factor alone.

HDPCs proliferate and differentiate into odontoblast-like cells and secrete type I collagen and other non-collagenous proteins, followed by a mineralized matrix.[7,35,36] In addition, increased angiogenesis is very important for pulp healing. Angiogenic factors such as VEGF, FGF-2, PECAM-1, and VE-cadherin have been implicated in the proangiogenic effects of HDPCs.[37] We found that treatment with HPE plus MTA led to a high angiogenic potential and differentiation-inducing capacity compared with MTA alone, as evidenced by increased ALP activity, deposition of mineralized nodules and up-regulation of marker genes, as well as increased levels of angiogenic factors, migration and capillary tube formation. The growth-, differentiation- and angiogenesis-promoting effects of HPE might be explained by a broad spectrum of migratory and angiogenic factors, as well as bio-active substances identified in the placenta such as FGF-2, VEGF and placental growth factor (PGF).[11,18]

MAPK is a proline-directed serine/threonine kinase consisting of three-enzyme modules; its targets, ERK, JNK and p38 kinases, are involved in MTA-induced odontoblastic differentiation.[38] MTA-induced NF-κB activation is also an important signal transduction pathway in rat dental pulp cells.[39] In the present study, the phosphorylation of ERK, p38 and JNK and activation of NF-κB were significantly greater in HDPCs cultured with combined HPE and MTA than in those cultured with MTA alone. These findings are consistent with previous evidence that DNA-binding activities of NF-κB are stimulated by HPE in THP-1 cells.[40] Our results indicate that MTA plus HPE might act through MAPK pathways to induce NF-κB activation in HDPCs.mTOR is one of the downstream serine/threonine kinases of the PI3K/Akt pathway that regulates cell growth and survival.[41] mTOR regulates translation rates and cell proliferation, in part by phosphorylating two major targets, 4E-BP1 and ribosomal protein S6 kinases (S6K1 and S6K2).[42] Recently, it was demonstrated that mTOR signalling plays an essential role in osteoblast differentiation *in vitro*.[43] It is notable that rapamycin, an inhibitor of mTOR, inhibits osteogenesis both *in vitro* and *in vivo*.[44] Furthermore, rapamycin treatment inhibits odontoblastic differentiation and mineralization of stem cells

HPE+MTA
Akt (Thr)
MAPK
mTOR
Cytosol
NFκB
Nucleus
Odontoblast differentiation ↑
Angiogenesis ↑

Figure 4. Schematic diagram illustrating the mTOR, MAPK and NF-κB signalling pathways triggered by the exposure to MTA and HPE, which ultimately stimulate the growth, odontogenic differentiation and angiogenesis of HDPCs.

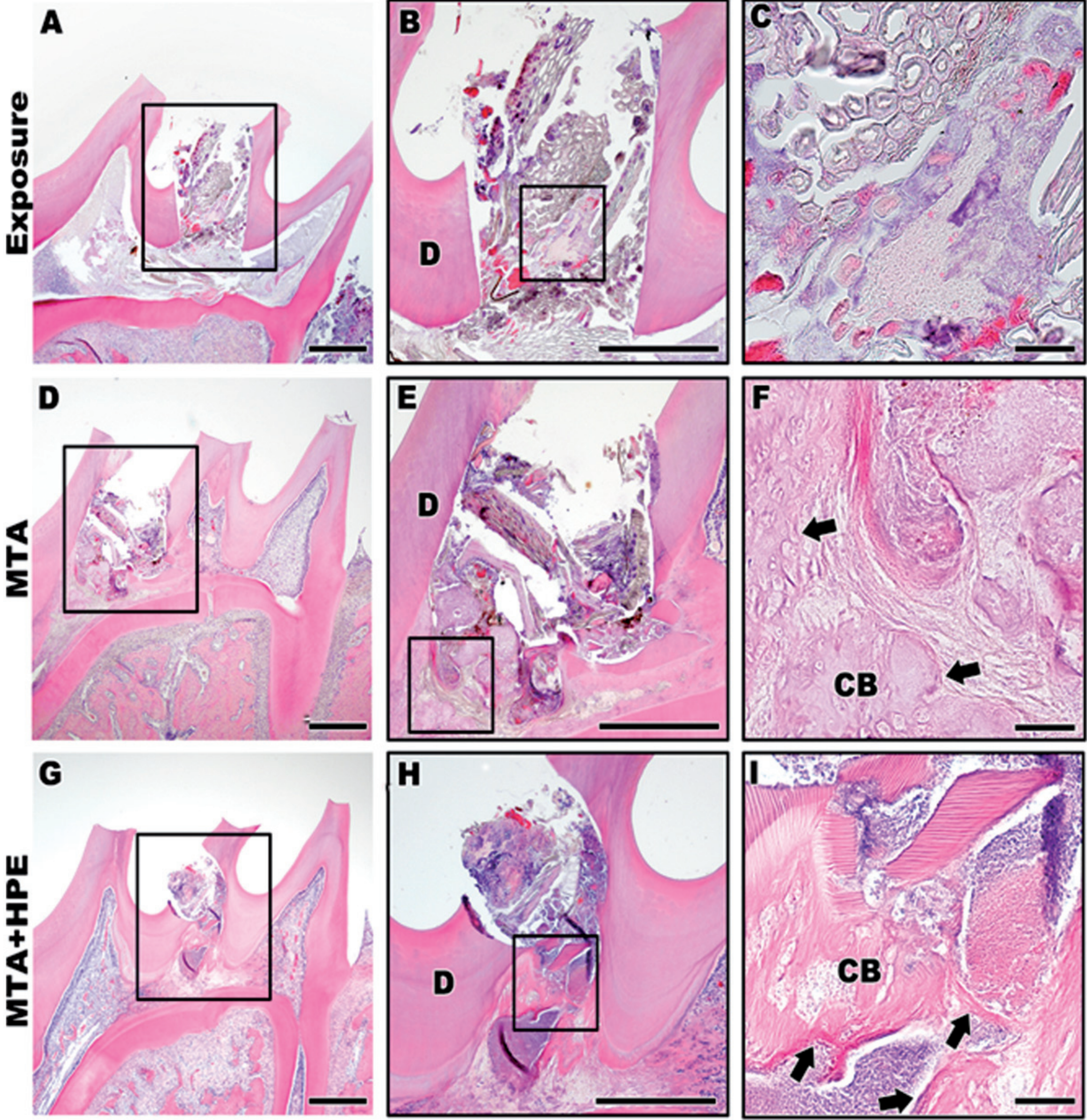

Figure 5. Representative histologic finding in rats pulp to capping with MTA and HPE . Experimental groups are divided into control (A–C), MTA (D–F) and MTA plus HPE (G–I). Scale bars = 500 μm (A, D, G). The newly formed calcific barrier can be seen in higher magnification views (scale bars = 50 μm). Black arrows indicate the newly formed calcific barrier. CB, calcific barrier; D, dentin.

Table 2. Score percentages for calcific barriers and inflammatory responses.

	Dentin bridge formation (%)				Odontoblastic layer (%)				Inflammatory cell response (%)				Dentinal tubules (%)	
Groups	0	1	2	3	0	1	2	3	0	1	2	3	0	1
exposure	50 (2/4)	50 (2/4)	—	—	100 (4/4)	—	—	—	—	25 (1/4)	50 (2/4)	25 (1/4)	100 (4/4)	—
MTA	12.5 (1/8)	50 (4/8)	37.5 (3/8)	—	75 (6/8)	25 (2/8)	—	—	—	37.5 (3/8)	50 (4/8)	12.5 (1/8)	87.5 (7/8)	12.5 (1/8)
MTA + M	—	—	75 (6/8)	25 (2/8)	—	62.5 (5/8)	25 (2/8)	12.5 (1/8)	12.5 (1/8)	75 (6/8)	12.5 (1/8)	—	25 (2/8)	75 (6/8)

*Numbers in parentheses are set as number of teeth receiving the score over total number of teeth evaluated.

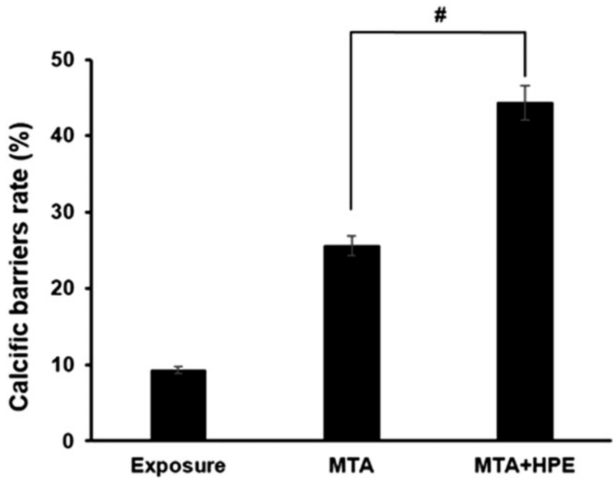

Figure 6. Combined effects of HPE and MTA on newly formed calcific barrier rate (%) *in vivo* pulp capping. #$p < 0.05$.

from human exfoliated deciduous teeth (SHED).[45] These data suggest a new role for mTOR in the regulation of dental stem cell differentiation and mineralization. In our study, MTA and HPE stimulated the phosphorylation of Akt, 4E-BP1 and S6K, suggesting that MTA and HPE may influence HDPC functions through the mTOR pathway. Moreover, mTOR-selective inhibitor rapamycin seems to suppress additive effects of HPE-induced phosphorylation of S6K rather than MTA alone. Because S6K1 is a positive regulator of protein synthesis downstream of mTOR,[41] the mTOR pathway seems to be a master mediator of MTA and HPE-induced odontoblastic differentiation. Collectively, mTOR, MAPK and NF-κB pathways, that are engaged in stimulating growth, differentiation and angiogenesis of HDPCs via MTA and HPE, are schematically drawn based on the current findings, as depicted in Figure 4.

In the present study, we examined histopathologic analysis comparing HPE with MTA in the pulpal response to direct pulp capping in a rat pulp injury model. The findings of our study indicate that the application of MTA showed an excellent histological response with the formation of a dentin bridge, odontoblastic layers and dentinal tubules in almost all cases with low inflammatory infiltrate. Moreover, newly formed calcific barrier rates were significantly higher in the HPE plus MTA group than in MTA alone. These results suggested that HPE plus MTA produced favourable *in vivo* pulpal responses

In summary, this is the first study to demonstrate an additive effect of combined MTA and HPE treatment on *in vivo* pulpal responses and odontoblastic differentiation and angiogenesis through Akt/mTOR, p38/JNK/ERK MAP kinase and NF-κB signalling pathways. The combination of HPE and MTA may offer a new therapeutic approach for pulp capping and regenerative endodontics.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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