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Effects of the enamel matrix derivative on the proliferation and odontogenic differentiation of human dental pulp cells

Yuanyuan Wang, Yuming Zhao [*], Lihong Ge* [1]

School and Hospital of Stomatology, Peking University, Beijing, China

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ABSTRACT

Objective: The enamel matrix derivative (EMD) has a positive effect on the proliferation of human periodontal ligament cells and the healing of periodontal tissues. The aim of this study was to evaluate the effects of EMD on the proliferation and differentiation of human dental pulp cells (hDPCs) in vitro.

Methods: hDPCs were isolated from human impacted third molars and cultured in vitro. After treatment with100 μg/mL EMD, the proliferation of hDPCs was determined by a cell counting kit 8 (CCK8) assay. After incubation in EMD osteogenic induction medium for 14 days, the osteogenic differentiation of hDPCs was evaluated by alkaline phosphatase (ALP) activity, alizarin staining and the expression of osteogenesis-related genes.

Results: The EMD osteogenic induction medium enhanced the proliferation of hDPCs. After osteogenic induction, EMD increased the osteogenic potential of hDPCs, as measured by alkaline phosphatase activity and calcium accumulation; the expression levels of osteogenesis-related genes, such as ALP, DSPP, BMP, and OPN were also upregulated. In addition, the expression levels of odontogenesis-related transcription factors Osterix and Runx2 were upregulated.

Conclusions: EMD could enhance the mineralization of hDPSCs upregulated the expression of markers for odontoblast/osteoblast-like cells. Further studies are required to determine if EMD can improve pulp tissue repair and regeneration.

1. Introduction

The health of teeth depends on the integrity of the hard tissue and the activity of the pulp and periodontal tissues, which are responsible for supplying nutrition to the teeth. Lack of nourishment provided by the pulp tissue can increase the risk of tooth fracture. In immature permanent teeth, impaired root development is another outcome associated with lack of nutritional support of the pulp. Maintaining the viability of the residual pulp tissue as far as possible is the main consideration in the procedure of pulp therapy. Direct pulp capping and pulpotomy are therapeutic approaches for exposed vital pulp, in which the formation of reparative dentine is facilitated by sealing the pulpal wound with a dental material.[1] After direct pulp capping and pulpotomy, the differentiation and proliferation of dental pulp cells (DPCs) are influenced by the activity of dental materials.[2,3]

Emdogain (Straumann AG, Basel, Switzerland) is a commercial enamel matrix derivative (EMD), derived from porcine developing enamel matrix. The main component of EMD is amelogenins. In addition, EMD contains low concentrations of

* *Corresponding author at*: Department of Pediatric Dentistry, Peking University School and Hospital of Stomatology, #22 Zhongguancun Nandajie, Haidian District, Beijing 100081, China. Tel.: +86 10 82195306.
E-mail addresses: yumingzhao70@gmail.com, cwyyd@126.com (Y. Zhao).
[1] Co-equal senior authors.
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matrix metalloproteinases and growth factors, including transforming growth factor β1 (TGF-β1), BMP-2 and BMP-4.[4] Several studies have shown that EMD influences the migration, attachment, proliferative capacity and biosynthetic activity of periodontal ligament cells.[5,6] Thus, it is considered effective in improving the healing process of replanted teeth and teeth with periodontal diseases.[7,8] Recently, it was suggested that EMD could also be used for pulp regeneration. Previous studies showed that amelogenin participates in the maturation and growth of dental pulp cells during tooth formation.[9] Animal experiments and clinical studies showed that EMD promotes reparative processes in the dental pulp.[10]

The addition of EMD can promote periodontal cell proliferation[11]; therefore, it was hypothesized that EMD exerts its therapeutic effect by providing an extracellular matrix that forms a more natural microenvironment for cells, stimulating cell attachment and differentiation.[12] EMD is also reported to induce a process mimicking normal odontogenesis and can thereby serve as a biologically active pulp dressing agent, which specifically induces pulpal wound healing and hard tissue formation without affecting healthy pulp.[13,14] It has been demonstrated that when mineral trioxide aggregate (MTA) and EMD were applied to human DPCs together, the cells differentiated into odontoblast-like cells, suggesting a synergistic effect of these two materials.[15] A recent study reported that Emdogain combined bismuth oxide containing Portland cement could improve cell growth and differentiation of human DPCs (hDPCs).[16] However, the direct effects of EMD on dental pulp, and the underlying mechanisms remain unclear. The aim of this study was to evaluate the effects of EMD on the proliferation and differentiation of hDPCs in vitro.

2. Materials and methods

EMD gel (30 mg/mL and 0.7 mL) (Emdogain; Biora AB, Malmö, Sweden) was diluted with Eagle's medium (α-MEM, GIBCO/BRL, Grand Island, NY, USA) to a final concentration of 100 μg/mL.

2.1. Cell culture

Human impacted third molars were collected from an adult (22 years, male) at the clinic of the Peking University School of Stomatology and used to culture hDPCs. The patient provided written informed consent, and the ethical committee of the Medical School of Peking University approved the protocol to obtain extracted teeth. After the teeth surfaces had been cleaned, the teeth were cut around the cementum-enamel junction with sterilized dental fissure burs to expose the pulp chamber. The pulp tissue was gently separated from the crown and root, and subsequently digested in a solution of 3 mg/mL collagenase type I (Sigma, St. Louis, MO, USA) and 4 mg/mL dispase (Sigma) for 1 h at 37 °C. Single-cell suspensions were obtained by passing the cells through a 70-mm strainer (Falcon, BD Biosciences, San Jose, CA, USA).

Single-cell suspensions (0.5–1.0 × 10^3/well) of hDPCs were seeded into 6-well plates (Costar, Corning Life Sciences, Tewksbury, MA, USA) containing α-MEM supplemented with 15% foetal bovine serum (FBS; Hyclone Thermo Scientific, Logan, UT, USA), 100 μg/mL penicillin, 100 mg/mL streptomycin (Sigma, St. Louis, USA), 100 μg/mL EMD and incubated at 37 °C in 5% CO_2. The control medium contained α-MEM, antibiotics and 15% FBS.

To induce differentiation, cells were cultured in a control medium with osteogenic induction media (OSTEO), comprising 50 m g/mL ascorbic acid (Sigma), 10 mmol/L β-glycerophosphate (Sigma), and 0.1 m mol/L dexamethasone (Sigma), as described previously.[6] EMD (100 μg/mL was added to the OSTEO as the experimental group (EMD).

2.2. Determination of EMD concentration

hDPCs (1 × 10^3/well) expended ex vivo were seeded into 96-well plates, cultured with EMD (0, 1, 10, 100 μg/mL) for 24 h in 37 °C. A cells counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan) assay was then carried out and repeated five times for each sample to evaluate the number of viable cells, according to the manufacturer's instructions. Untreated cells were used as the control group. 10 μL of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt was added to each well before the culture plate was incubated at 37 °C for 4 h. Absorbance was measured at 450 nm in a microplate reader. The mean values of the optical density were calculated and analyzed statistically for cell number at each dilution of the samples (version 13.0; SPSS, Chicago, IL, USA). The dilution of EMD at which hDPCs had the highest cell viability was chosen for subsequent studies.

2.3. Growth tendency study

According to the above experiment, 100 μg/mL EMD was chosen for the growth tendency study, with the untreated hDPCs as the control group. hDPCs (1 × 10^3/well; expanded ex vivo) were seeded into five 96-well plates separately. At 1, 3, 5 and 7 days after cell seeding, a CCK-8 assay was carried out with eight replications to evaluate the number of viable cells, following the same procedure as described in ''determination of EMD concentration''. The growth curves of the two groups were analyzed using SPSS software (version 13.0; SPSS, Chicago, IL, USA).

2.4. Alkaline phosphatase activity

After 7 and 14 days culture in the OSTEO, EMD and normal control medium, cells were rinsed three times in phosphate-buffered saline (PBS) and then lysed for 10 min in 100 mM Tris base with 1% Triton-X100. Alkaline phosphatase activity (AKP activity) was determined in the lysate by measuring the release of p-nitrophenol using SIGMAFAST™ p-nitrophenyl phosphate (Sigma) as a substrate after 45 min at 37 °C. Absorbance was measured at 420 nm using a Microplate Reader (ELx808IU, BioTek, Winooski, VT, USA).

2.5. Alizarin Red staining

After 7 and 14 days of culture in the EMD, OSTEO and normal control medium, the hDPCs were fixed in 4% paraformaldehyde for 30 min and washed in PBS, and the mineralization of the extracellular matrix was detected by staining with 1% Alizarin

Table 1 – Semi-quantitative real-time PCR primers.

Gens	Primer sequence (5′-3′)	GenBank number
ALP	F: 5′-ATGGGATGGGTGTCTCCACA-3′ R: 5′-CCACGAAGGGGAACTTGTC-3′	XM_005245818.1
DSPP	F: 5′-TTTGGGCAGTAGCATGGGC-3′ R: 5′-CCATCTTGGGTATTCTCTTGCCT-3′	XM_005555378.1
BMP-1	F: 5′-GCCACGTTTCCATCGTTCG-3′ R: 5′-AGAATGTGTTCCGAGCGTAATG-3′	XM_003823603.1
OPN	F: 5′-CTCCATTGACTCGAACGACTC-3′ R: 5′-CAGGTCTGCGAAACTTCTTAGAT-3′	XM_003265675.2
OSTERIX	F: 5′-GAGGCAACTGGCTAGGTGG-3′ R: 5′-CTGGATTAAGGGGAGCAAAGTC-3′	XM_005268643.1
RUNX2	F: 5′-TGGTTACTGTCATGGCGGGTA-3′ R: 5′-TCTCAGATCGTTGAACCTTGCTA-3′	XM_005696518.1
GAPDH	F: 5′-ATGGGGAAGGTGAAGGTCG-3′ R: 5′-GGGGTCATTGATGGCAACAATA-3′	XM_005569913.1

Red S for 10 min. Pictures of Alizarin Red S staining were scanned using a scanner and the density of staining in each group was analyzed by Scion image software (Scion Corporation, Maryland).

2.6. *Semi-quantitative real-time PCR*

After 14 days of culture in the EMD, OSTEO and normal control medium, the of hDPCs were rinsed three times in PBS and the total RNA of the cells was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommendations. Isolated RNA was then subjected to reverse transcription using an Oligo dT primer and superscript II reverse transcriptase (Invitrogen), according to manufacturer's instructions. Semi-quantitative real-time polymerase chain reaction (PCR) was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) with SYBR green (Roche, Shanghai, China). The reaction conditions comprised 70 °C for 5 min; 42 °C for 60 min and 95 °C for 10 min. Primer sequences are detailed in Table 1.

2.7. *Statistical analysis*

Statistical analysis was performed using the paired Student's t test in SPSS. The level of statistical significance was set at $P \leq 0.05$.

3. Results

3.1. *Determination of the most effective EMD concentration*

hDPCs viability increased in an EMD dosage-dependent manner. Among the EMD concentration used, hDPCs showed the highest cell viability compared to the untreated group ($P \leq 0.05$) at an EMD concentration of 100 μg/mL (Fig. 1A).

3.2. *Effects of EMD on the growth of hDPCs*

The CCK-8 results showed that cells incubated with EMD had a higher growth rate than those incubated with normal medium alone at 1, 3, 5 and 7 days of the cultivation ($P < 0.05$) (Fig. 1B).

3.3. *Effects of EMD on the odontoblastic differentiation of hDPCs*

To investigate the effect of EMD on the odontoblastic differentiation of hDPCs, we assessed the levels of ALP activity, mineral nodule deposition by Alizarin Red S staining, and messenger RNA expression of differentiation markers. The results showed that the ALP activity of the EMD group increased compared with that of the OSTEO group at 7 and 14 days of cultivation (both $P < 0.05$). The ALP activity of the OSTEO group was higher than the control group at 7 and 14 days of cultivation (both $P < 0.05$) (Fig. 1C). There were more calcium deposition in the EMD group than in the OSTEO group at 7 and 14 (both $P < 0.05$) days of induction (Fig. 2). EMD significantly increased the expression levels of the odontogenic marker genes ALP ($P < 0.05$), dentine sialophosphoprotein (DSPP) ($P < 0.05$), bone morphogenetic protein 1 (BMP1) ($P < 0.05$) and osteopontin (OPN) ($P < 0.05$) (Fig. 3A), and the transcription factors OSTERIX ($P < 0.05$), RUNX2 ($P < 0.05$) (Fig. 3B) compared with the OSTEO and the control group at 14 days of cultivation.

4. Discussion

Vital pulp therapy, including direct pulp capping and pulpotomy, minimizes pulpal injury by protecting the pulp tissue from the toxic effects of chemical, bacterial, mechanical or thermal insult.[17] Therefore, vital pulp therapy treats reversible pulpal injuries by sealing the pulp and stimulating the formation of tertiary dentine,[18] which is classified as either reactionary or reparative. Reactionary dentine, which is formed by surviving odontoblast cells in response to milder stimuli, is of higher quality than reparative dentine, which is formed by dental pulp cells in response to stronger stimuli.[18] Milder stimuli are recommended for pulp-capping materials to induce the more tubular and calcified tertiary dentine.[18]

The extent to which growth or differentiation agents applied directly to pulp tissue can induce reparative dentine has been a focus of many biomedical trials. As it is commercially available and economical compared with other bioactive agents, clinicians have recently become interested in using EMD as a pulp capping material. EMD is a gel-type agent

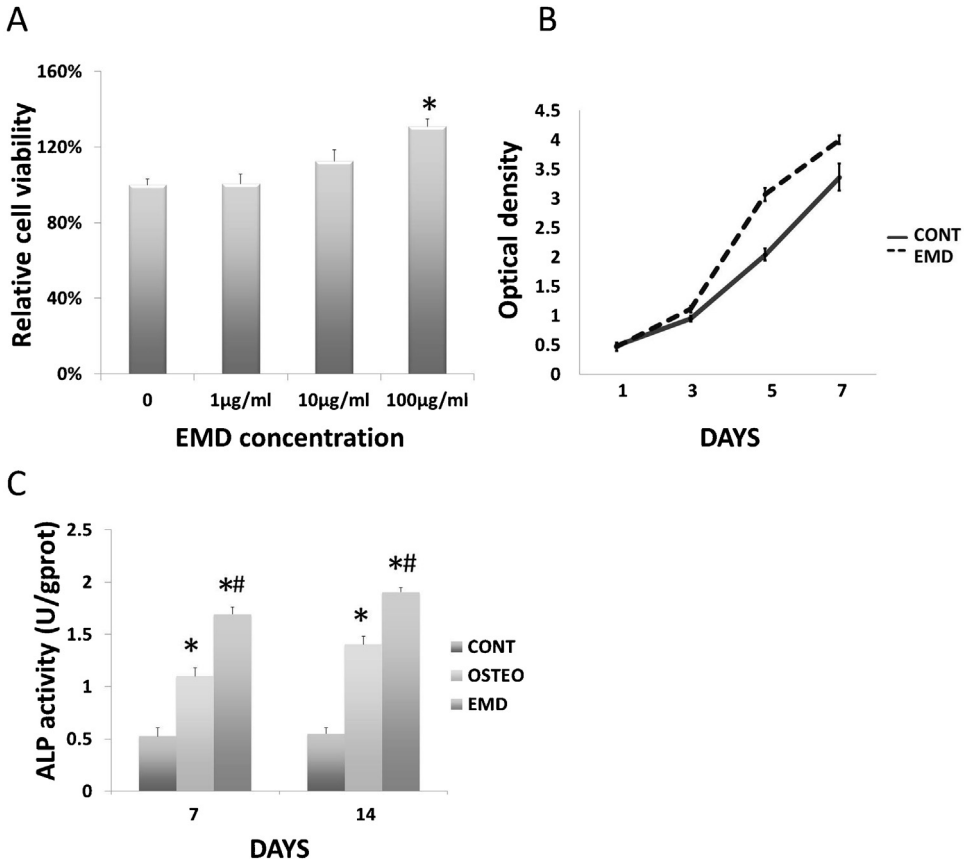

Fig. 1 – The effects of EMD on the proliferation and ALP activity of hDPCs. The determination of the effective EMD concentration by dosage-dependant experiment (A). The hDPCs growth curve for the EMD-treated group and control group (B). The effects of EMD and osteogenic induction on ALP activity of hDPCs (C). * comparison between control and OSTEO group; # comparison between OSTEO and EMD group.

that does not rapidly diffuse into the pulp tissue.[19,20] EMD has also been shown to induce reparative dentine and odontoblast differentiation in experimental pulp capping.[21,22]

The differentiation and mineralization of osteoblasts and odontoblasts involve an initial period of extracellular matrix biosynthesis and proliferation, which is followed by cell differentiation.[23] In the early stage of this process, the matrix matures, and specific proteins associated with the pulp cells phenotype, such as ALP, can be detected. hDPCs have been well characterized and can be induced to further differentiate into odontoblast/osteoblast-like cells.[24] In the current study, EMD-treated hDPCs exhibited more rapid proliferation and a

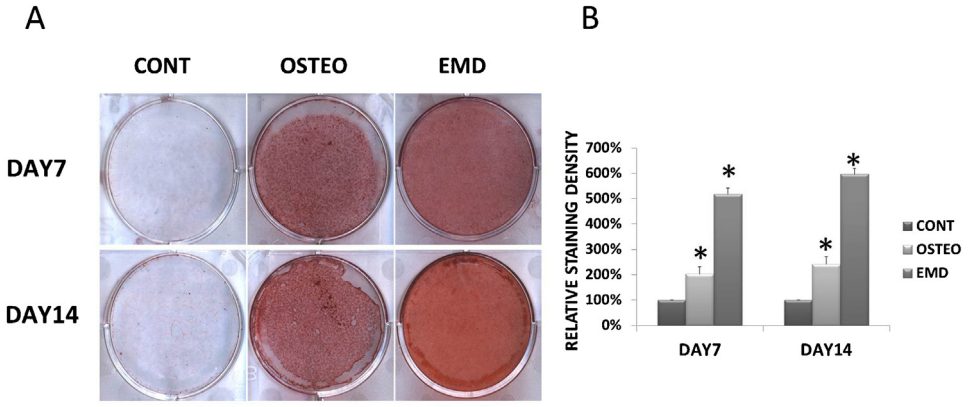

Fig. 2 – The effects of EMD on the formation of calcification nodules in hDPCs. hDPCs were cultured with osteogenic induction medium alone and EMD for 7 days and 14 days and then stained with Alizarin Red. A representative photograph of Alizarin Red staining was shown (A). The relative staining density was set as 100%, and the statistic results were shown (B). * comparison between the control and other groups.

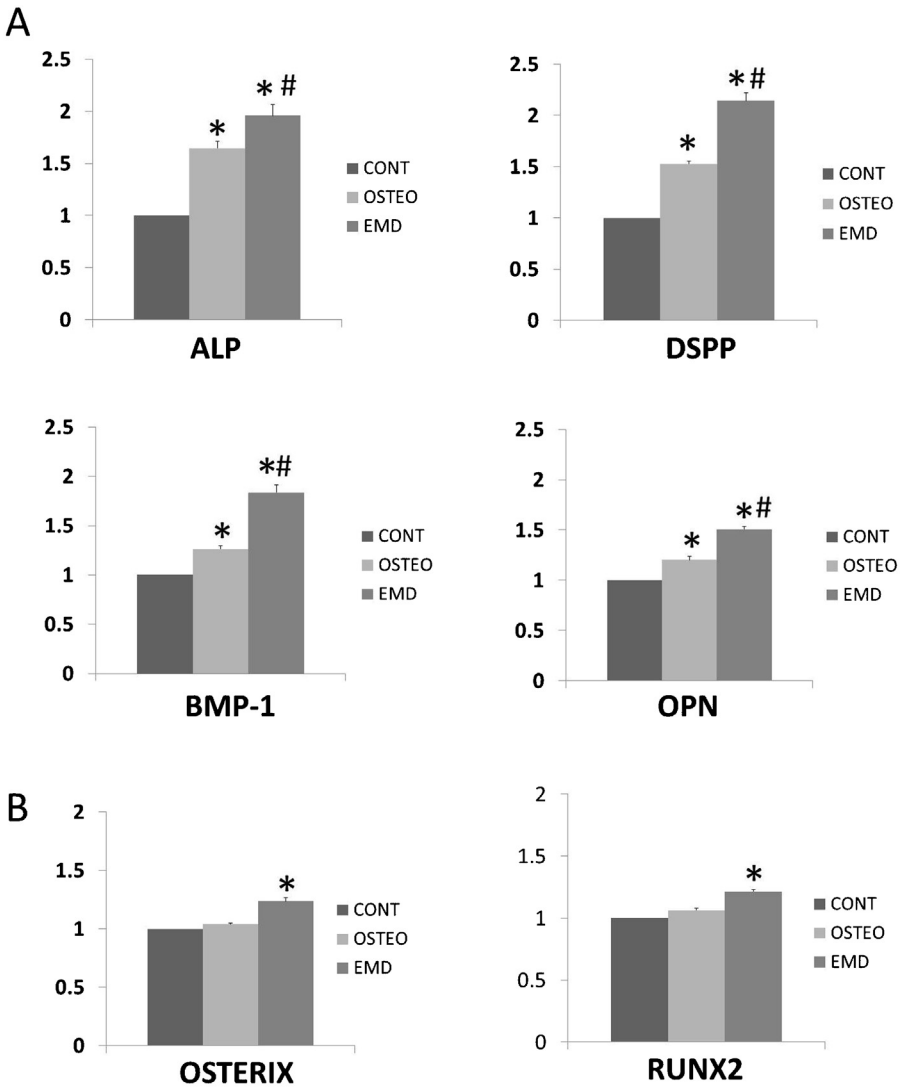

Fig. 3 – The effects of EMD on messenger RNA expression of (A) odontogenic (ALP, DSPP, BMP1, and OPN) differentiation markers and (B) transcription factors (Osterix and Runx2) in hDPCs. The relative gene expression level was normalized against GAPDH messenger RNA, and the control was set as 1.0. * comparison between control and OSTEO group; # comparison between OSTEO and EMD group.

higher level of ALP activity (Fig. 1), which suggested that EMD facilitates the regeneration of pulp and dentine.

Alizarin Red S staining has been used for decades to evaluate calcium-rich deposits by cells in culture.[25] Thus, Alizarin Red staining shows the presence of calcium deposits in the extracellular matrix. The EMD group exhibited a higher level of calcium deposits compared with the osteogenic induction group at day 7 and day 14 (Fig. 2), which is suggested that EMD enhanced the mineralization process.

The cell phenotype of differentiation-induced hDPCs presents several crucial characteristics of odontoblasts, as shown the increased ALP activity and the expression of the odontogenic genes, such as DSPP, and osteoblastic genes, such as ALP, BMP1 and OPN. DSPP is believed to play a regulatory role in the mineralization of reparative dentine, and serves as a specific marker for odontoblasts.[26] Like DSPP, BMP-1 is present in the extracellular matrix of dentine and bone as a processed fragment.[27] OPN is a secreted glycophosphoprotein that is found in both mineralized and non-mineralized tissues. Based on its strong inhibition of hydroxyapatite formation in vitro, OPN is believed to play a crucial role in modulating apatite crystal growth in bones.[28]

Osterix, is also an essential transcription factor for osteoblast differentiation and bone formation: osterix-deficient mice were deficient in bone formation because of maturation arrest of osteoblasts.[29] Runt-related gene 2 (Runx2) is another essential transcription factor for osteoblast differentiation and bone formation: Runx2-deficient mice were also deficient in bone formation because of maturation arrest of osteoblasts.[30,31] These two transcription factors govern the critical regulation of osteoblast differentiation and bone formation.

In the current study, ALP, DSPP, BMP1, OPN, osterix and Runx-2 were selected as differentiation markers for odontoblasts in hDPCs. We observed a significant increase in ALP

activity, mineral nodule deposition, and upregulation of markers for odontoblastic differentiation, such as ALP, DSPP, BMP1 and OPN in hDPCs treated with EMD. In addition, the results also showed that essential transcription factors, such as Runx2 and osterix, were upregulated in the EMD-treated group compared with the OSTEO group (Fig. 3). These results suggested that EMD plays a supplemental role in the mineralization process of hDPCs.

The mechanism by which EMD influences the function of pulp cells is not completely understood. In a previous report, the function of odontoblasts or pulp cells might have been stimulated directly by EMD to produce collagen matrix for calcification.[22] It was also suggested that transforming growth factor-β1 or amelogenin peptides present in EMD are involved in cell signalling to stimulate matrix formation and mineralization.[32,33] Recently, Kaida et al.[10] reported that BMP-expressing macrophages induced by EMD might play important roles in reparative dentine formation. Further research on the mechanisms of EMD's effect on pulp regeneration is required.

5. Conclusions

EMD could enhance the mineralization of hDPCs and increased the expression of markers for odontoblast/osteoblast-like cells. Further studies are required to determine whether EMD can improve pulp tissue repair and regeneration.

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