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Osteogenic differentiation of dental pulp stem cells under the influence of three different materials

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Abstract

Background: Regeneration of periodontal tissues is a major goal of periodontal therapy. Dental pulp stem cells (DPSCs) show mesenchymal cell properties with the potential for dental tissue engineering. Enamel matrix derivative (EMD) and platelet-derived growth factor (PDGF) are examples of materials that act as signaling molecules to enhance periodontal regeneration. Mineral trioxide aggregate (MTA) has been proven to be biocompatible and appears to have some osteoconductive properties. The objective of this study was to evaluate the effects of EMD, MTA, and PDGF on DPSC osteogenic differentiation.

Methods: Human DPSCs were cultured in medium containing EMD, MTA, or PDGF. Control groups were also established. Evaluation of the achieved osteogenesis was carried out by computer analysis of alkaline phosphatase (ALP)-stained chambers, and spectrophotometric analysis of alizarin red S-stained mineralized nodules.

Results: EMD significantly increased the amounts of ALP expression and mineralization compared with all other groups ($P < 0.05$). Meanwhile, MTA gave variable results with slight increases in certain differentiation parameters, and PDGF showed no significant increase in the achieved differentiation.

Conclusions: EMD showed a very strong osteogenic ability compared with PDGF and MTA, and the present results provide support for its use in periodontal regeneration.

Keywords: Dental pulp stem cells, Osteogenesis, MTA, EMD, PDG

Background

The major goal of periodontal therapy is to regenerate tooth-supporting structures destroyed by periodontal disease [1]. Periodontal tissue engineering involves complex interactions between different cells and signaling molecules, as well as biological scaffolds [2].

In an attempt to mimic the original developmental events, the integrated use of precursor cell populations with specific biologic stimulants is under investigation [3, 4]. Stem cells represent primitive non-specialized cells with wide capabilities for differentiation and tissue regeneration. To date, mesenchymal stem cells have been successfully isolated from several body organs [5], including multiple tissues with dental origins [6–9].

Such dental tissue-derived stem cells were found to retain potent capacity for specific differentiation into dental tissue-forming cells [6, 10, 11]. Gronthos and colleagues successfully isolated human dental pulp stem cells (DPSCs), and proved both their multipotency and self-renewal capability [11, 12]. Further studies confirmed their findings [13, 14]. This multipotency, in addition to their relative accessibility, made DPSCs an appealing source of cells for application in regenerative medicine [15–18]. In fact, several papers have proved their superiority in different aspects, including osteogenic differentiation [19, 20], which supported their use for regeneration of craniofacial defects [21, 22], as well as alveolar bone defects [23, 24]. Additionally, the similar embryonic origins of dental pulp cells and periodontal cells [25] and their presence within protective layers of tooth structure have encouraged their use for periodontal tissue regeneration [26, 27].

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Studies on tissue engineering have used biological mediators to selectively enhance the recruitment of cellular populations into periodontal wounds [28]. Enamel matrix derivative (EMD) is a protein harvested from developing porcine teeth that has been reported to induce cementum formation and periodontal regeneration [29]. At the cellular level, EMD was proven to have regulatory effects on multiple periodontal cell types [28, 30].

Platelet-derived growth factor (PDGF) is a very powerful regulatory factor that initiates nearly all wound healing events. The main function of PDGF is to stimulate cell replication (mitogenesis) of healing-capable stem cells and partially differentiated osteoprogenitor cells, which are part of the connective tissue–bone healing cellular make-up [31]. Significant increases in bone and cementum formation have been reported histologically [32]. At the cellular level, PDGF increased the number of collagen-synthesizing cells [33] and stimulated bone sialoprotein transcription [34].

Another material with the ability to induce regeneration is mineral trioxide aggregate (MTA). MTA is a mixture of dicalcium silicate, tricalcium silicate, tricalcium aluminate, gypsum, and tetracalcium aluminoferrite [35]. Torabinejad et al. [36] reported a favorable biologic performance of MTA when in direct contact with bone, through the deposition and formation of hydroxyl apatite on its surface. The material was also found to enhance cellular production of type I collagen, osteocalcin, alkaline phosphatase (ALP), bone sialoprotein, and osteopontin [37]. A systematic review on the histological responses of the periodontium to the material concluded that MTA promoted healing toward regeneration [38].

The above findings suggest similar clinical performances for the three materials with no previous attempts for direct comparisons. Accordingly, the purpose of the present study was to examine and compare the effects of EMD, PDGF, and MTA on the osteogenic differentiation of DPSCs.

Results

Cell isolation and characterization

Dental pulp stem cells in the primary cultures started to appear in 5–14 days and became attached to the plate surfaces (Fig. 1a). Cells from the second passage successfully formed multiple colonies, with around 50 cells per colony (Fig. 1b). Flow cytometry analyses confirmed positive expressions of stromal cell-associated markers, with negative expressions of hematopoietic and endothelial markers (Fig. 1g). Cells that underwent osteogenic induction showed increased ALP staining compared with negative control cells (Fig. 1c, d), while cells cultured in the adipogenic medium exhibited several oil red O-positive lipid granules (Fig. 1e, f).

Material application

ALP staining

The samples showed different degrees of ALP staining (Fig. 2). One-way ANOVA revealed significant differences among the compared groups ($P < 0.0001$) (Table 1).

For all parameters examined, EMD was significantly higher than all other groups ($P < 0.05$). EMD revealed significantly higher percent total positive staining area, average optical density, and histological scores (95.6 ± 4.7 %, 0.35 ± 0.03, 221.99 ± 23.8) than MTA (64.19 %, 0.26 ± 0.02, 114.34 ± 20.90; $P < 0.05$) PDGF (48.8 % ± 12.62, 0.24 ± 0.02, 82.33 ± 28.3; $P < 0.05$) and reference control.

In contrast, MTA gave inconsistent findings, although it increased the ALP activity in a similar manner to the reference control when evaluated by the average optical density, the material resulted in reductions of the other parameters compared with the reference control, although those reductions were not always significant ($P > 0.05$).

With regard to PDGF, ALP expression generally revealed lower results compared with the reference control for the three parameters respectively, and these reductions were consistently significant ($P < 0.05$; Table 1).

Alizarin red S staining

There were obvious differences in the amounts of mineralization among the groups (Fig. 3). One-way ANOVA revealed these differences to be significant ($P < 0.0001$) (Table 2).

The EMD group had a significantly increased amount of mineralized nodule formation compared with all other groups, giving a mean absorbance of 1.2 ± 0.13 ($P < 0.05$).

The MTA group significantly increased amount of mineralization (absorbance: 0.16 ± 0.12), relative to the negative control group (0.08 ± 0.01), and PDGF group (0.09 ± 0.01).

Although the mean absorbance of the PDGF group (0.09 ± 0.01) appeared to be slightly different than the other groups, these differences were statistically non-significant ($P > 0.05$; Table 2).

Discussion

In this study, successful isolation of dental pulp cells was achieved through the application of enzymatic digestion with certain modifications to the protocol of Gronthos et al. [11]. The obtained cells underwent several investigations to evaluate their properties. According to the International Society for Cellular Therapy [39], the minimal criteria for defining multipotent mesenchymal stromal cells include: (1) adherence to plastic dishes; (2) multipotent differentiation potential; and (3) expressions of specific stromal surface markers (CD73, CD90, CD105) with lack of expressions of hematopoietic markers (CD45, CD34, CD14 and/or CD11b, CD19, CD79α) and the
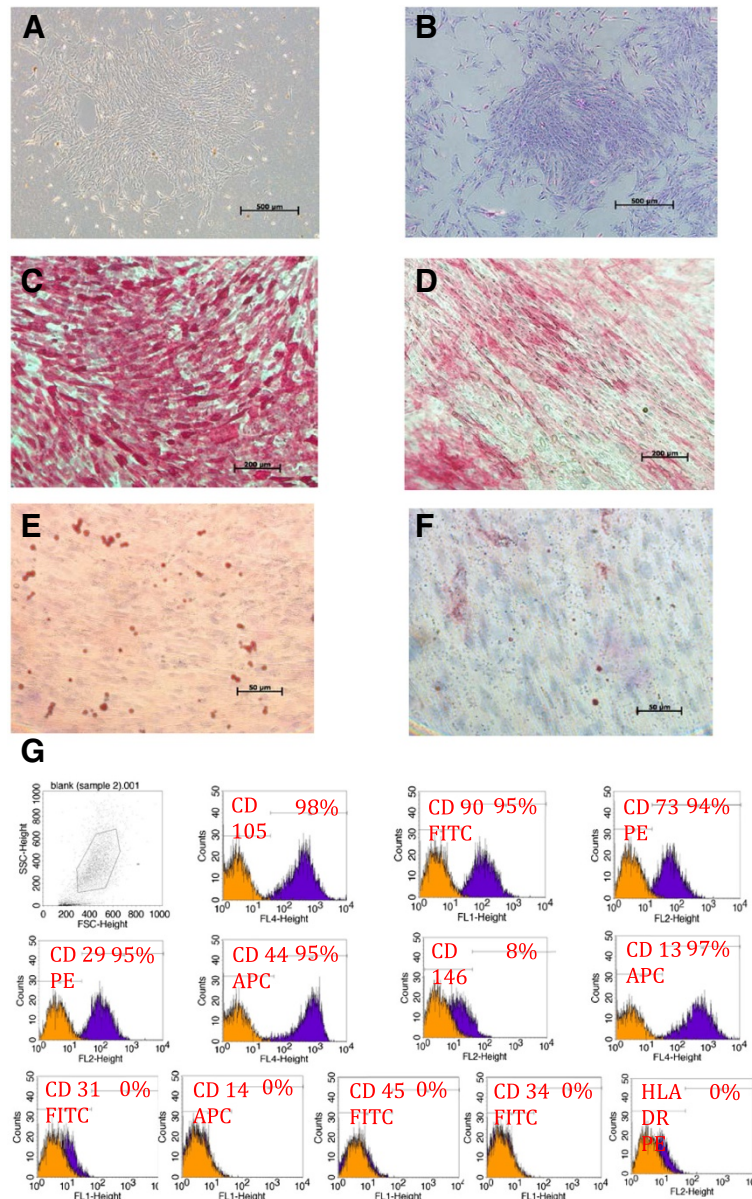
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Fig. 1 Inverted light microscopic images showing **a** dental pulp mesenchymal stem cells at primary culture, Magnification 5×. **b** Colony forming unit Fibroblast (CFU-F) magnification 5×, **c**, **d** Alkaline phosphatase staining for DPSCs 14 days after osteoinduction (**c**) versus negative control (**d**), magnification 10×, and Oil red O staining for DPSCs 14 days after adipogenic induction (**e**) versus negative control (**f**), magnification 40×. **g** FACS analysis results of a representative dental pulp cell line

HLA-DR marker. The isolated cells in this study presented all of the above features.

Different material concentrations were evaluated, and the concentrations with the best differentiation were selected. These concentrations were 200 μg/ml for EMD, 5 ng/ml for PDGF, and 0.05 mg/ml for MTA. The same concentrations were previously used in other studies [34, 40, 41]. In this study, computer

analysis for ALP activity and a semiquantitative evaluation technique for alizarin red S staining were selected, as these two techniques were reported to give results with relative sensitivity, and have been applied in previous studies [42, 43].

For EMD, the results revealed significant increases in ALP expression and abundant mineralization enhancement following its application. These findings are in
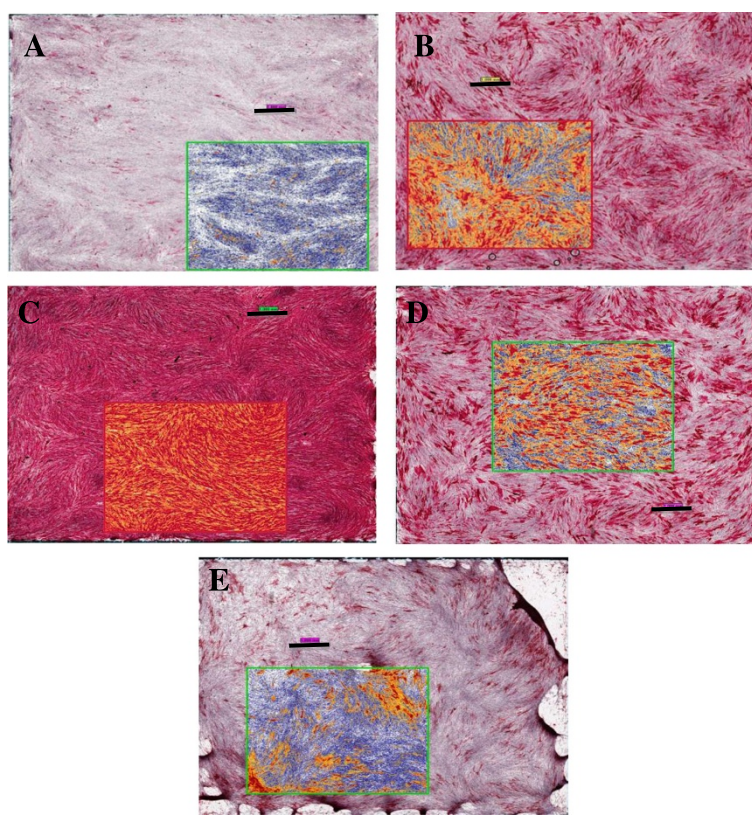
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Fig. 2 Scanoscope images for ALP stained different experimental groups of DPSCs. The internal image represents the evaluated field, which makes around 1/4th of the image. Original magnification 1.4×. Scale bar 1 mm. **a** Negative control, **b** Reference control (OT), **c** EMD, **d** MTA, **e** PDGF

accordance with several other studies evaluating the effects of this material on multiple cell lines [40, 44–48]. Duan et al. [44] found that EMD enhanced the osteogenic differentiation of induced pluripotent stem cell, as evidenced by increases in RUNX2 mRNA expression. Kémoun et al. [45, 46] evaluated the effects of EMD on follicular cells [45] and periodontal ligament stem cells [46]. In both studies, EMD was found to enhance ALP release and calcium deposition, in addition to the elevation of several mineralization markers. Another study by Guven et al. [47] found that Emdogain was the most effective material for enhancing both proliferation and odontogenic differentiation of human tooth germ stem cells through the evaluation of ALP activity, Von Kossa staining, and RT-PCR analyses for dentin sialophospho-protein (DSPP), and immunostaining for collagen type I and DSPP. A study by Wang et al. [48] found that Emdogain enhanced the mineralization of DPSCs as well as their osteogenic/odontogenic marker expression. However, studies with contradictory findings are also available [49, 50]. It was reported that EMD might not have appreciable effects on osteoblastic differentiation in periodontal ligament cells [49] or rat bone marrow cells [50].

Although the exact control mechanism remains unclear, these effects were explained by differences in the degrees of cellular immaturity, i.e. the material was thought to enhance cellular proliferation of more immature cells, but differentiation of cells at later stages of maturity [51].

In the present study, MTA gave inconsistent findings. The material revealed mineralization enhancement in comparison with the reference control, reductions in certain ALP parameters (percent total positive staining area and histological score), and maintenance of other parameters (average optical density). Although Yasuda et al. [52] and Lee et al. [53] reported that MTA increased ALP production and/or mineralized nodule formation compared with control cells, both Koh et al. [54] and Nakayama et al. [55] reported similar ALP expression between MTA-treated cells and negative control cells. These inconsistencies suggest that further evaluation of the different parameters guiding and affecting the performance of this material is warranted.

With regard to PDGF in the present study, it was observed that ALP expression generally revealed lower results in comparison with the negative control group as well as all of the other material groups, and the differences were

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Table 1 Represents the alkaline phosphatase analysis results for all groups

	Material/Group	Average	Standard Deviation (SD)	Post hoc Tukey's test for significance among groups
Percent Total Positive	Negative control	16.29	4.95	OT*
				EMD*
				MTA*
				PDGF*
	OT	72.92	9.24	Negative control*
				EMD*
				MTA*
				PDGF*
	EMD	95.59	4.69	Negative control*
				OT*
				MTA*
				PDGF*
	MTA	64.19	9.95	-ve control*
				OT*
				EMD*
				PDGF*
	PDGF	48.80	12.62	Negative control*
				OT*
				EMD*
				MTA*
Average Optical density	Negative control	0.18	0.01	OT*
				EMD*
				MTA*
				PDGF*
	OT	0.26	0.02	Negative control*
				EMD*
				MTA
				PDGF*
	EMD	0.35	0.03	Negative control*
				OT*
				MTA*
				PDGF*
	MTA	0.26	0.02	Negative control*
				OT
				EMD*
				PDGF
	PDGF	0.24	0.03	Negative control*
				OT*
				EMD*
				MTA
Histological Score	Negative control	20.633	7.034	OT*
				EMD*
				MTA*
				PDGF*
	OT	132.974	22.944	Negative control*
				EMD*
				MTA
				PDGF*
	EMD	221.992	23.818	Negative control*
				OT*
				MTA*
				PDGF*
	MTA	114.340	20.914	Negative control*
				OT
				EMD*
				PDGF*
	PDGF	82.330	28.254	Negative control*
				OT*
				EMD*
				MTA*

N.B.Intergroup comparison was statistically significant using ANOVA test, $P < 0.0001$
*Indicates statistical significance with $P < 0.05$

always significant. Regardless of the material's action in proliferative enhancement, PDGF-BB appeared to have no additional benefit for osteogenic differentiation, according to the parameters evaluated in this study. Several other authors observed similar results [33, 56]. In fact, PDGF enhanced bone collagen degradation [33], and disrupted or inhibited bone matrix formation [56]. Nakashima et al. [57] found that PDGF increased DNA synthesis, while causing 40–65 % inhibition of ALP activity. Tanaka and Liang [58] reported that the material exerted no effect on cellular ALP activity or collagen synthesis. Yokose et al. [59] reported that PDGF-BB significantly reduced the ALP activity of DPSCs.

Conclusions

Favorable cell-surface interactions with EMD were demonstrated, including ALP expression and abundant mineralization. EMD gave superior results compared with MTA and PDGF regarding osteogenic differentiation of DPSCs. The effects of MTA on osteogenesis of DPSCs were inconclusive and further studies are required. Moreover, our data on PDGF did not support its ability to induce osteogenic differentiation of DPSCs. However, PDGF did facilitate cell attachment and
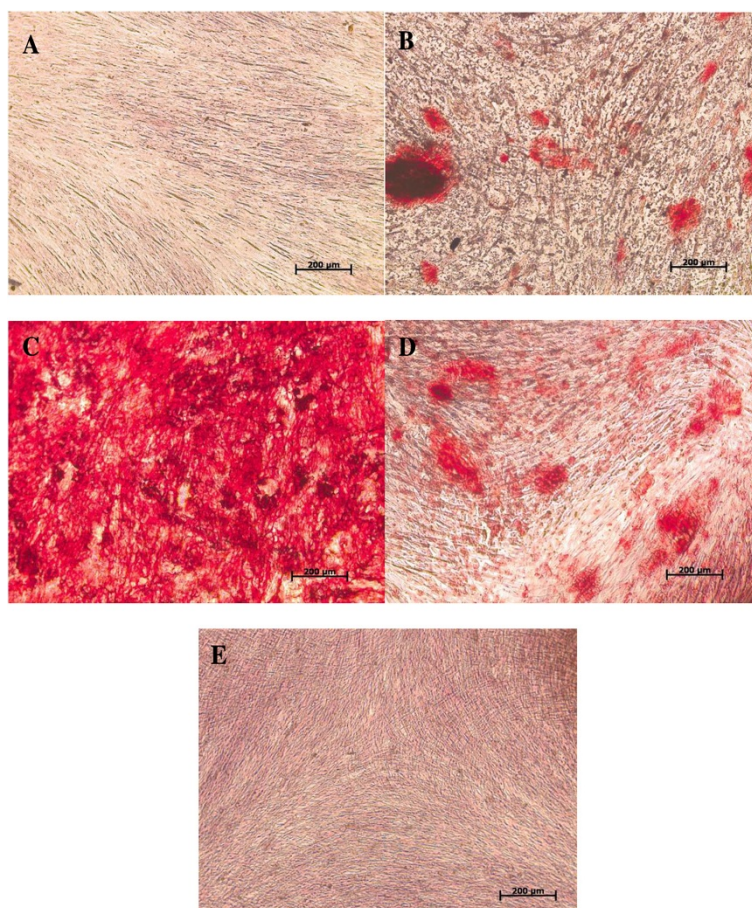
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Fig. 3 Inverted light microscopic image presenting Alizarin red S staining for different DPSCs experimental groups. Original magnification 10×. Scale bar 200 μm. **a** Negative control, **b** Reference control (OT), **c** EMD, **d** MTA, **e** PDGF

growth, suggesting a different mechanism of action that worth further investigation.

Methods

Isolation of stem cells

Human DPSCs were isolated and characterized by the authors in the Stem Cell Unit, King Saud University, Kingdom of Saudi Arabia (unpublished data). Teeth were collected from patients after they provided signed informed consent, according to a protocol approved by the institutional ethical committee (College of Dentistry Research Center-CDRC).

Briefly, the pulp contents of freshly extracted molar teeth were combined and subjected to 20–40 minutes of enzymatic digestion using collagenase type I (1 mg/ml) and dispase (5000 caseinolytic units). Subsequently, the cells were allowed to grow under regular cell culture conditions (37 °C, 5 % CO_2), using Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 % fetal bovine serum (FBS), 1 % penicillin-streptomycin (Pen-Strept), and

1 % non-essential amino acids (all purchased from Gibco-Invitrogen, USA).

Characterization of stem cells

Colony forming unit-fibroblasts (CFU-F)

CFU-F were evaluated by culturing 2.5×10^3 cells at the second passage in 6-cm culture dishes. At day 14, the cells were fixed with 1 % paraformaldehyde, stained with 0.5 % crystal violet, and subjected to microscopic evaluation using a phase-contrast inverted light microscope (Zeiss, Leica, Germany).

Flow cytometry

Fourth passage cells (1.5×10^6) were washed with FACS buffer (1× phosphate-buffered saline, 5 % FBS, 0.1 % sodium azide), and diluted in 1.5 ml of phosphate-buffered saline. Next, PE-conjugated mouse anti-human CD146, CD73, CD29, and HLA-DR, FITC- conjugated mouse anti-human CD34, CD90, CD45, CD13, and CD31, and APC-conjugated mouse anti-human CD105, CD14, and

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Table 2 Represents the average absorbance rate for Alizarin red S stained chambers of all groups

Material/Group	Average	Standard Deviation (SD)	Post hoc Tukey's test for significance among groups
Negative control	0.079	0.007	OT
			EMD*
			MTA*
			PDGF
OT	0.107	0.016	Negative control
			EMD*
			MTA
			PDGF
EMD	1.197	0.132	Negative control*
			OT*
			MTA*
			PDGF*
MTA	0.163	0.117	Negative control*
			OT
			EMD*
			PDGF*
PDGF	0.097	0.010	Negative control
			OT
			EMD*
			MTA*

OT reference control for osteoinduction, EMD Emdogain, MTA Mineral trioxide aggregate, PDGF Platelet derived growth factor-BB
N.B.Intergroup comparison was statistically significant using ANOVA test, $P < 0.0001$
*Indicates statistical significance with $P < 0.05$

CD44 antibodies were prepared in dark (all from BD Biosciences, USA, except for the monoclonal antibody against human CD105, which was purchased from R&D Systems, USA) and utilized. In each FACS tube, 100 μl of cells was mixed with 10 μl of the corresponding antibody, and incubated for 30 minutes in the dark at 4 °C. The expressions of cellular markers were assessed using a Becton Dickinson FACSCalibur Flow Cytometer (BD Biosciences, USA), and the resulting data were analyzed using Cell Quest Pro Software Version 3.3, BD bioscience, USA).

Osteogenic and adipogenic differentiation
Cells at the fourth passage were cultured on 6-well plates. At 60–70 % confluency, osteogenic differentiation was induced using osteoinduction medium prepared according to the protocol of Vishnubalaji et al. [60], and composed of DMEM supplemented with 10 % FBS, 1 % Pen-Strept, 50 μg/ml L-ascorbic acid (Wako Chemicals GmbH, Germany), 10 mM glycerol phosphate disodium salt (β-glycerophosphate), 10 nM dexamethasone, and 10 nM calcitriol (1α,25-dihydroxyvitamin D3)

(Sigma, UK). Cells maintained in the regular culture medium served as controls. The resultant osteogenesis was evaluated after 14 days through cytochemical staining for ALP.

Adipogenic differentiation was also induced using standard adipogenic medium [60], composed of DMEM supplemented with 10 % FBS, 10 % horse serum, 1 % Pen-Strept, 100 nM dexamethasone, 0.45 mM isobutyl methyl xanthine, 3 μg/ml insulin (all purchased from Sigma, UK), and 1 μM rosiglitazone (BRL49653; Novo Nordisk, Denmark). The resultant differentiation was assessed at 14 days through the use of oil red O staining.

Material application
Initially, a pilot study was carried out to evaluate three different concentrations for each material, and the concentrations yielding the highest amount of differentiation were selected for the comparisons (Fig. 4). Thereafter, cells at the fourth passage were cultured and divided into five groups as shown below.

1. **Negative Control:** Cells maintained in the regular cell culture medium for the entire experiment (DMEM with 20 % FBS, 1 % Pen-Strept, 1 % non-essential amino acids).
2. **Reference Control (OT):** Cells cultured in the osteoinduction medium, prepared according to the protocol of Vishnubalaji et al. [60].
3. **EMD Group:** Cells cultured in the osteoinduction medium supplemented with 200 μg/ml EMD (Straumann, USA).
4. **PDGF Group:** Cells cultured in the osteoinduction medium supplemented with 5 ng/ml PDGF-BB (Osteohealth, USA).
5. **MTA Group:** Cells cultured in the osteoinduction medium supplemented with 0.02 mg/ml MTA (Dentsply, USA).

The achieved differentiation was analyzed by evaluation of ALP expression through ALP staining and calcium ion deposition through alizarin red S staining.

ALP activity
Cells were plated on 8-chamber slides at the density of 0.02×10^6 cells/chamber and allowed to attach and grow to 50 % confluency. Thereafter, the slides were divided into the above-mentioned five different groups and regular or osteogenic medium was applied accordingly. On day 5, the cells were fixed and stained for ALP with Naphthol-AS-TR-phosphate solution (Sigma, UK). Next, the chambers were evaluated under a high-resolution digital microscope where the whole stained chambers were scanned with a ScanScope slide scanner (Aperio Technologies Inc., USA) at 40× objective magnification. The digital images of

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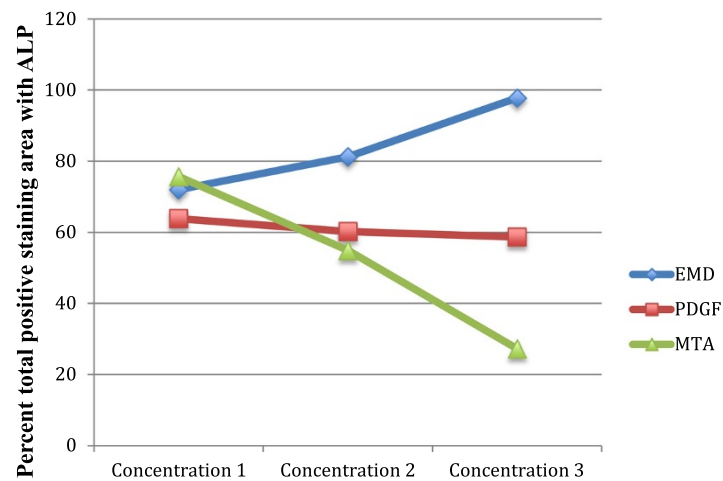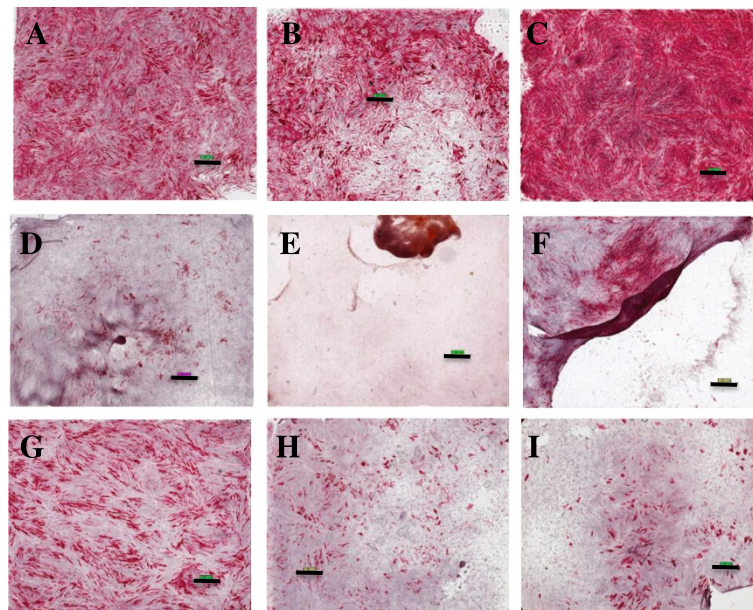
Fig. 4 Scanoscope images for ALP stained different experimental groups of DPSCs. Original magnification 1.4×. Scale bar 1 mm. **a**, **b**, **c** EMD at concentrations of 50, 100, 200 μg/ml respectively. **d**, **e**, **f** PDGF at concentrations of 5, 10, 20 ng/ml respectively. **g**, **h**, **i** MTA at concentrations of 0.02, 0.2, 2.0 mg/ml respectively. **j** Percent total positive staining area for different concentrations of each examined material

six different chambers from each trial were viewed and analyzed using the viewing and image analysis tools of Aperio Image Scope software (Version 10.2.2.2352; Aperio Technologies Inc.). The whole experiment was repeated three times independently, giving a total of 18 chambers/group for analysis. The analysis output results were exported to Excel sheets, focusing mainly on the percent total positive staining area, average optical density, and histological score as the parameters for statistical analysis and comparison.

Alizarin red S staining

In the same manner, cells were cultured on 24-well plates, and the five different groups were established. Media were replaced twice per week with freshly-prepared regular or osteogenic media. On day 12, the cells were stained with 40 mM AR-S Alizarin Red (Sigma, UK), and subjected to spectrophotometric evaluation according to the protocol of Gregory et al. [61] using a microplate reader (Gen5™, version 1.10; BioTek Instruments Inc., USA) to measure the absorbance at 405 nM. The same protocol was

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repeated three times independently, giving nine different readings for each trial.

Statistical analysis

Data was analyzed using SPSS statistical software (version 16.0; SPSS, USA). Descriptive statistics (mean and standard deviation) were used to describe the quantitative outcome variables. One-way analysis of variance (ANOVA) was used to compare the mean values of outcome variables across the categorical variables (groups), followed by a post-hoc Tukey test for pairwise comparisons. Values of $P < 0.05$ were considered to indicate statistical significance.

Abbreviations

ALP: Alkaline phosphatase; AR-S: Alizarin red S stain; BRL: Rosiglitazone; °C: Degree celsius or degree centigrade; CD: Cluster of differentiation; CFU-F: Colony forming unit-fibroblast; DMEM: Dulbecco's modified eagles medium (with high glucose, sodium pyrovate and L-glutamine); DNA: Deoxy-ribionucleic acid; DPSCs: Dental pulp stem cells; EMD: Enamel matrix derivatives; FACS: Fluorescence-activated cell sorting (Flow cytometric analysis); FBS: Fetal bovine serum; FITC: Fluorescence iso thioocyanide; Mg: Milligrams; ml: Milliliter; μl: Micro liters; mRNA: Messenger ribonucleic acid; MTA: Mineral trioxide aggregate; PBS: Phosphate buffered saline; Pen/Strept: Penicillin/Streptomycin; PDGF: Platelet derived growth factor; mRNA: Ribonucleic acid.

Competing interest

The authors declare that they have no competing interests.

Authors contributions

SA participated in different aspects of laboratory studies including cell characterization, and material application, in addition to preparing of the primary draft for this paper. NA helped in the development of the main research idea, prepared the basic study design, and provided critical review for whole paper writing. Additionally, she arranged for obtaining the dental test materials. AD provided general technical support especially in cell characterization and differentiation analysis, in addition to his role in getting all basic laboratory materials. MN have helped in cellular osteogenic and adipogenic differentiation studies, and supervised the writing of the technical part of the study (materials and methods). All authors read and approved the final manuscript.

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