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Original Article

Effect of polydatin on the viability and odontogenic differentiation of human dental pulp stem cells: An *in-vitro* study

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KEYWORDS
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Abstract *Background/purpose:* Various materials have been used to promote human dental pulp stem cells (hDPSCs) differentiation to produce dentin bridge formation with less-than-optimal results. Polydatin (PD), a naturally present material with osteogenic properties can be a promising material in the pulp regeneration/repair process. The aim of this study was to evaluate the effect of (PD) on the viability and differentiation of human dental pulp stem cells.

Materials and methods: PD effect on hDPSCs in terms of cellular viability, alkaline phosphatase (ALP) production, and messenger RNAs (mRNA) of odontogenic markers production using quantitative reverse transcription polymerase chain reaction (RT-qPCR) were evaluated. In addition, mineral deposits were detected with Alizarin red stain.

Results: The viable hDPSCs in the presence of 0.01 μM and 0.1 μM PD were significantly higher than the control on days 3 and 7, respectively. In addition, ALP activity of hDPSCs was significantly increased with 0.01, 0.1, and 1 μM of PD. In addition, increased expression mRNAs of ALP, osteocalcin (OC), osteonectin (ON), osteopontin (OP), Runt-related transcription factor-2 (RUNX-2), dentin sialophosphoprotein (DSPP), and dentin matrix protein-1 (DMP-1) was observed after PD treatment, however, the difference was not statistically significant. Furthermore, increased size of mineral deposits was observed with PD.

Conclusion: PD promoted the expression of markers associated with odontogenic differentiation and mineralized tissue deposition in hDPSCs.

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Introduction

Human dental pulp has a number of functions including dentin production, neural functions, and defense mechanisms.[1] In response to various insults, pulp reacts by producing an inflammatory reaction. In particular, injuries resulting from bacterial infection of the pulp, if left untreated, will progress into an irreversible pulpal inflammation with eventual pulp necrosis.[2,3]

Traditionally, management of pulpal disease consisted of removal of the pulp or remnants thereof followed by cleaning the root canal and filling it with a biocompatible material. This process produced a predictable high success rate.[4,5] However, certain situations, such as a necrotic pulp in an immature permanent tooth, require different management to stimulate continued root development, root wall thickening, and apical narrowing prior to root canal filling.[6]

In addition, irreversibly inflamed pulps have been traditionally treated with root canal therapy. Although direct pulp capping has been considered for a long time, earlier outcomes when calcium hydroxide ($Ca(OH)_2$) was used were not encouraging.[7] However, more recent studies utilizing calcium silicates have shown promising results when used on asymptomatic teeth.[8]

The biological principles involved in "regenerative endodontics" and "direct pulp capping" are essentially the same. Both procedures rely on recruiting undifferentiated mesenchymal stem cells to undergo differentiation into cells involved in the regeneration of lost dentin pulp complex tissues.[9–11]

Since formed dentin bridge is a primary indicator for successful pulp therapy; *in vitro* studies with stem cells are carried out to evaluate the ability of potential materials or therapeutic agents to induce stem cell differentiation into odontoblasts or odontoblast-like cells.[12–14] Bone and dentine formation processes are related at the molecular level with similar non-collagenous proteins that direct the formation of calcified tissue. Bone markers such as osteocalcin (OC), osteopontin (OP), and alkaline phosphatase (ALP) can also indicate an odontogenic process as well. On the other hand, specific odontogenic markers that can be used to detect the differentiation of stem cells to odontoblasts or odontoblast-like cells include dentin sialophosphoprotein (DSPP) and dentin matrix protein-1 (DMP-1).[15,16]

Despite significant success of regenerative endodontics procedures, certain important shortcomings are still evident, for example, the tissues produced in "regenerative endodontics" are not true pulp tissues, but rather a periodontal ligament-like, bone-like, or cementum-like tissues.[17,18] In addition, formed dentin bridge at the site of vital pulp therapy procedures often contains atubular dentin, tunnel defects, and soft tissue inclusions.[19–21] Furthermore, inflammation is often present, at least in the earlier stages, of vital pulp therapy using pulp capping materials.[22,23]

A material capable of promoting odontoblastic differentiation while reducing inflammatory process might prove advantageous in dental pulp regeneration/repair procedures. Polydatin (PD) a precursor of Resveratrol (RSV) is a traditional Chineses medicine with known anti-inflammatory and osteogenic properties.[24,25] It is a naturally occurring product that was first extracted from the Japanese knotweed (*Fallopia japonica*) but is also present in other plants such as grapes, cocoa, and peanuts.[24,25] PD (Fig. 1) has a molecular weight of 390.4 g/mol.[24,26–28] Although PD and RSV have similar chemical structures and share many biological effects, PD has been reported to be more potent than RSV.[27]

PD has been reported to promote osteogenic differentiation of dental bud stem cells (DBSCs), while RSV has been shown to upregulate odontogenic and osteogenic messenger RNAs such as ALP, OC, DMP-1, and DSPP as well as mineralized nodule formation by hDPSCs.[27,29]

On the other hand, RSV had a protective effect against oxidative stress, while PD modulated the production of inflammatory cytokines associated with pulp capping materials application on bone marrow mesenchymal cells (BMSCs).[30,31]

To date, no study has evaluated the effect of PD on human dental pulp stem cells, therefore the aim of this study was to evaluate the effect of PD on the viability and differentiation of human dental pulp stem cells.

Materials and methods

Ethical approval

The research was approved by the Institutional Review Board (IRB) at King Saud University Medical City (KSUMC) with (IRB Project No. E–21-5997).

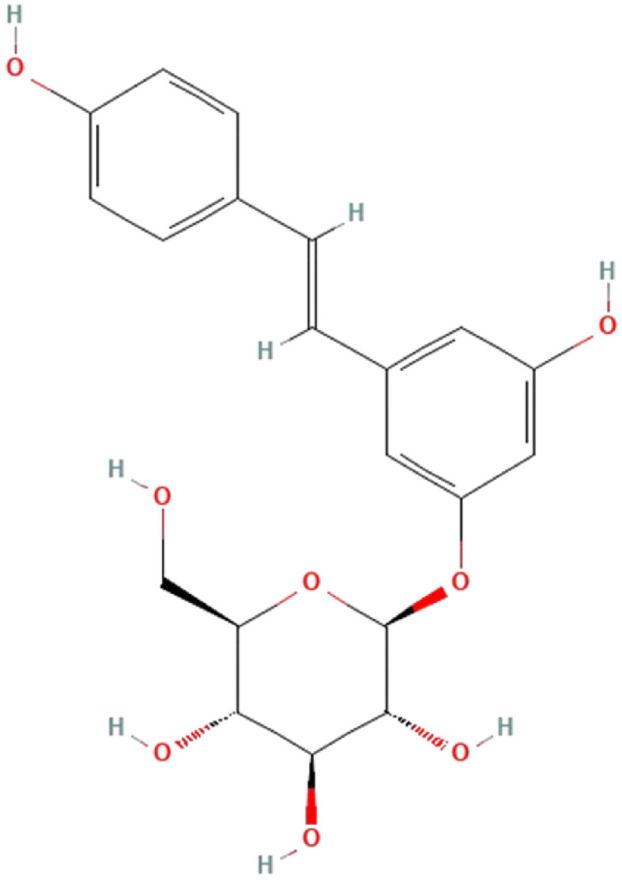

Figure 1 The chemical structure of Polydatin.[28]

Cells and cell culture condition

Cell expansion

Human dental pulp stem cells (hDPSCs) were purchased from Axol Bioscience LTD, (Catalog number ax3901, Axol, Cambridge, UK). Cells were expanded and cultured in alpha-modified minimum essential medium (α-MEM) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), 1% penicillin/streptomycin, and 1% MEM Non-Essential Amino Acids Solution (NEAA), hereafter referred to as supplemented α-MEM. All cells were cultured at 37 °C at 5% CO_2 and 95% humidity. After reaching 90% confluency cells were transferred to 96, 24, and 12 well plates with the supplemented α-MEM in preparation for the consequent experiments. Passages 3–5 of hDPSCs were used in the experiments.

Material preparation

Solution preparation

Polydatin. Polydatin (PD) (purchased from Shandong Zhi Shang Chemical LTD, Jinan, China, CAS 27208-80-6), was dissolved in 0.1% Dimethyl sulfoxide (DMSO) according to the manufacturer's instructions. PD effect on viability of hDPSCs was performed using 0.01 μM, 0.1 μM, 1 μM, 10 μM, and 100 μM compared to the control group with DMSO.[27] PD was re-added with each medium change.

Osteogenic differentiation medium. Osteogenic differentiation medium (OM) consists of (α-MEM) supplemented with 10% FBS, 1% Pen-strep, 50 μg/mL L-ascorbic acid (Wako Chemicals, Neuss, Germany), 10 nmol/L calcitriol (1α,25-dihydroxy vitamin D3 (Sigma–Aldrich, Burlington, MA, USA), 10 mmol/L β-glycerophosphate (Sigma–Aldrich), and 10 nmol/L dexamethasone (Sigma–Aldrich).

Cellular viability of human dental pulp stem cells exposed to polydatin

Alamarblue™ assay

The effect of PD on the viability of hDPSCs was evaluated at 5 concentrations 0.01 μM, 0.1 μM, 1 μM, 10 μM, and 100 μM on days 1, 3, and 7 using the AB assay (Thermo Fisher Scientific) as prescribed previously.[32,33] Cells were seeded in 96-well plates at a concentration of 5×10^3 cells/well. At the end of each time point, 10% AlamarBlue™ reagent (Bio-Rad Inc., Hercules, CA, USA) was added to each well. After 1 h of incubation, fluorescence was measured at an excitation and emission wavelength of 530 nm and 590 nm, respectively, using a fluorescence reader SpectraMax® M5/M5e Multimode Plate Reader (Molecular Devices, San Jose, CA, USA). Data was collected using the SoftMax® Pro 6 Microplate Data Acquisition and Analysis Software (Molecular Devices). The experiment was quadrupled in triplicates.[34]

Evaluating odontogenesis

Alkaline phosphatase activity and stain

Dental pulp stem cells were seeded in a 24 well-plate in α-MEM with a density of 1×10^5 cells/well for alkaline phosphatase (ALP) activity and 12 well plate with a density of 2×10^5 cells/well For ALP staining. After cells reached confluency of 70–80%, PD was added to each group with α-MEM for 24 h. The next day, cells were cultured with OM supplemented with 0.01 μM, 0.1 μM, or 1 μM PD. The expression of the biochemical marker for ALP activity and stain was assessed on days 7 and 14.[12,13] Briefly Alkaline Phosphatase was stained using (Sigma–Aldrich) following manufacturers' protocol. Samples were viewed by light microscopy for the detection of ALP stain and images were evaluated by two blinded examiners. On the other hand, Alkaline Phosphatase Diethanolamine Activity Kit (Thermo Fisher Scientific) was used to quantify ALP activity by absorbance level measured at 405 nm by SpectraMax M5/M5e Multimode Plate Reader (Molecular Devices). The ALP assay was duplicated in triplicates. ALP activity was expressed as units/mg protein normalized to the number of cells.

Table 1 List of primers sequences for ALP, RUNX-2, OC, OP, ON, DSPP, and DMP-1.

Gene	Sequence (5′-3′)
GAPDH	Sense (Forward primer)
	5′-CTGGTAAAGTGGATATTGTTGCCAT-3′
	Antisense (Reverse primer)
	5′-TGGAATCATATTGGAACATGTAAACC-3′
ALP	Sense (Forward primer)
	5′-GACGGACCCTCGCCAGTGCT-3′
	Antisense (Reverse primer)
	5′-AATCGACGTGGGTGGGAGGGG-3′
RUNX-2	Sense (Forward primer)
	5′-ACGIGGCTAAGAATGTCATC-3′
	Antisense (Reverse primer)
	5′-CTGGTAGGCGATGTCCTTA-3′
OC	Sense (Forward primer)
	5′-GGCAGCGAGGTAGTGAAGAG-3′
	Antisense (Reverse primer)
	5′-CTCACACACCTCCCTCCTG-3′
OP	Sense (Forward primer)
	5′-CAGTTCAGAAGAGGAGG-3′
	Antisense (Reverse primer)
	5′-TCAGCCTCAGAGTCTTCATC- 3′
ON	Sense (Forward primer)
	5′-GAGGAAACCGAAGAGGAGG-3′
	Antisense (Reverse primer)
	5′-GGGGTGTTGTTCTCATCCAG-3′
DSPP	Sense (Forward primer)
	5′-GCCATTCCAGTTCCTCAACTT-3′
	Antisense (Reverse primer)
	5′-CATGCACCAGGACACCACTT-3′
DMP-1	Sense (Forward primer)
	5′-CGGTTCCTGGAATACTGACC-3′
	Antisense (Reverse primer)
	5′-TCTTTGGCTGTGTTCTGGTG-3′

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX-2), osteocalcin (OC), osteopontin (OP), osteonectin (ON), dentin sialophosphoprotein (DSPP), dentin matrix protein-1 (DMP-1).

Quantitative reverse transcription polymerase chain reaction

Dental pulp stem cells were seeded in 12 well plate in OM with PD with a density of 2×10^5 cells/well. On day 7 cells were collected and RNA lysis buffer was added to the cell pellet. RNA was isolated using RNeasy mini kit (RNeasy; Qiagen, Hilden, Germany) then quantified using Nanodrop spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific). The extracted RNA was reverse transcribed by High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific) and cDNA was synthesized by Multigene thermocycler (Labnet International, Inc., Edison, NJ, USA). Thereafter, mRNA expression was analyzed using Fast SYBR™ Green PCR Master Mix (Thermo Fisher Scientific). The primer sequences for ALP, Runt-related transcription factor-2 (RUNX-2), OC, OP, osteonectin (ON), DSPP, and DMP-1 (Oligo™, Seoul, South Korea) are listed in Table 1. Groups were run in duplicates, cycle threshold (Ct) values were obtained after 45 amplification cycles. The Ct values for tested genes were normalized to the endogenous control Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) values. The comparative delta–delta Ct method ($2^{-\Delta\Delta}$ CT) was used to normalize the data based on the endogenous reference (GAPDH) to be expressed as the relative fold change in percentage compared to the values obtained from the control group.[35]

Evaluation of calcified deposits

Alizarin red staining

Alizarin red staining was performed on days 14 and 21 according to previously published studies.[12,13] Briefly, hDPSCs were exposed to PD in a 12-well plate in OM with a density of 2×10^5 cells/well. Wells were rinsed then fixed with 4% paraformaldehyde followed by incubation with 2% Alizarin red stain (Cat. No. 0223, ScienCell Research Laboratories, Carlsbad, CA, USA) for 30 min at room temperature and washed with dH_2O 4 times for 5 min each. Light microscope Pictures were registered and evaluated by two blinded examiners for each group. Experiments were duplicated in triplicates.

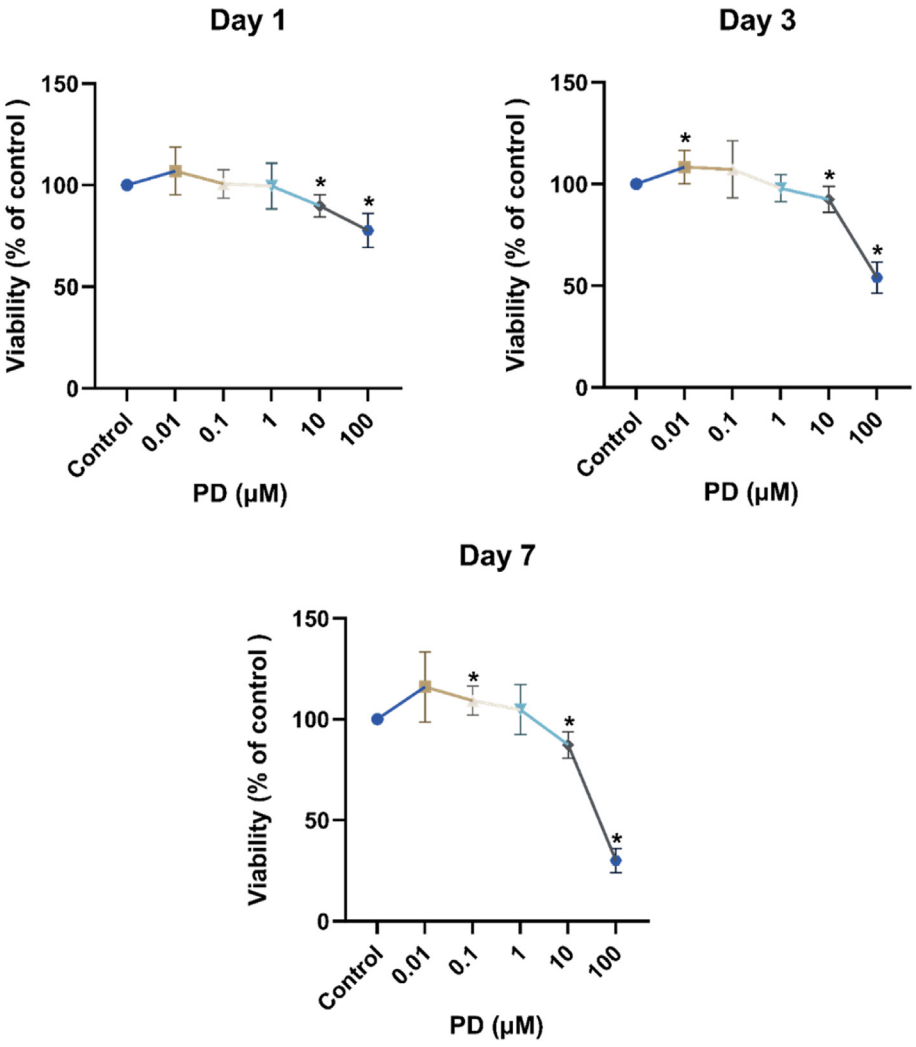

Figure 2 Viability of hDPSCs on Days 1,3, and 7. Data presented as mean ± SD. * Indicates a statistically significant difference $P < 0.05$ compared to the control group.

Statistical analysis

Data was expressed as the mean ± standard deviation (SD) of the number of observations. Treatment groups were normalized to the control group. Distribution was examined for normality using the Shapiro–Wilk test. When data is normally distributed, a parametric test (one-way ANOVA) was performed. Equality of variances verified using Levene test, if assumption is violated, Welch ANOVA with Games-Howell post-hoc tests was used. Statistically significant if *P*-values <0.05. Analysis was performed using IBM SPSS Statistics software (version 29). Graphs were designed using GraphPad Prism 10 software (GraphPad Software, San Diego, CA, USA).

Results

Viability of cells

The viable hDPSCs exposed 0.01 μM PD concentration was slightly higher than the control on day 1 and 7 and significantly higher on day 3 ($P = 0.043$). Similarly, 0.1 μM PD concentration had significantly increased viable cells on day 7 ($P = 0.010$). Whilst 1 μM PD concentration was similar to the control group in all days. Conversely, 10 and 100 μM PD had significantly decreased viable cells on Days 1, 3, and 7 compared to the control group (Fig. 2).

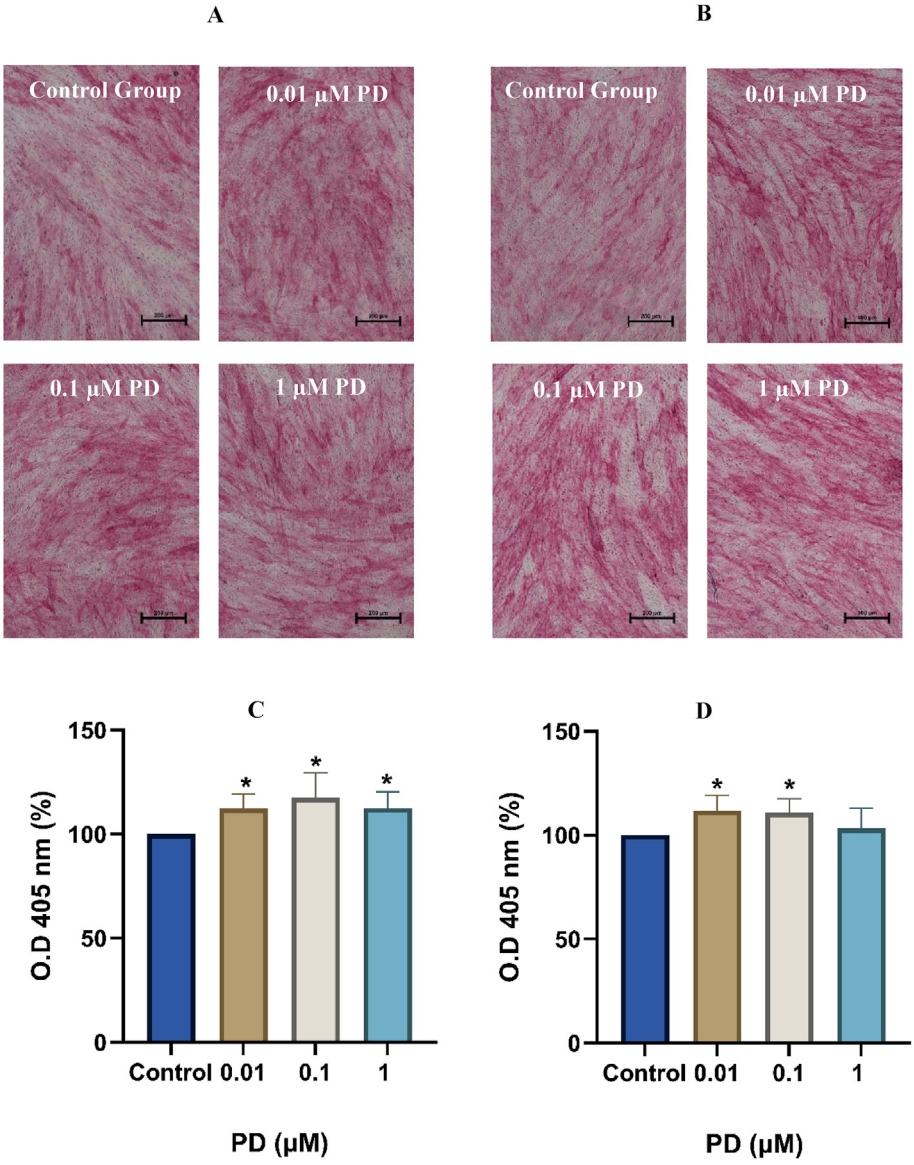

Figure 3 (A, C) ALP staining photomicrograph and ALP assay optical density (OD) in percentage change compared to the control group on day 7; (B, D) ALP staining photomicrograph and ALP assay in percentage change compared to control group on day 14. Data are presented as mean ± SD. Scale bar 200 μm; magnification 10x. * Indicates a statistically significant difference at $P < 0.05$ compared to the control group.

Alkaline phosphatase assay and stain

Alkaline phosphatase activity of hDPSCs on day 7 was significantly increased with all the PD concentrations compared to the control 0.01 μM ($P = 0.003$), 0.1 μM ($P = 0.010$), and 1 μM ($P = 0.005$). The highest ALP activity among the three concentrations was with 0.1 μM followed by 1 μM and 0.01 μM. On day 14, 0.01 μM had the highest activity followed by 0.1 μM, both were significantly higher than the control ($P = 0.007$, $P = 0.005$), respectively. ALP-stained wells showed denser stain of treated wells compared to the control group with slight differences between treated groups. Light microscope pictures of stained wells and ALP assay graph are shown in (Fig. 3).

Quantitative reverse transcription polymerase chain reaction

PD concentration 0.1 μM demonstrated the highest mean value compared to the control group for the following messenger RNA (mRNA): ALP, RUNX-2, OC, OP, ON. DSPP expression was highest with 0.01 μM, while DMP-1 expression was highest with 1 μM PD, however, the differences were not statistically significant (Fig. 4).

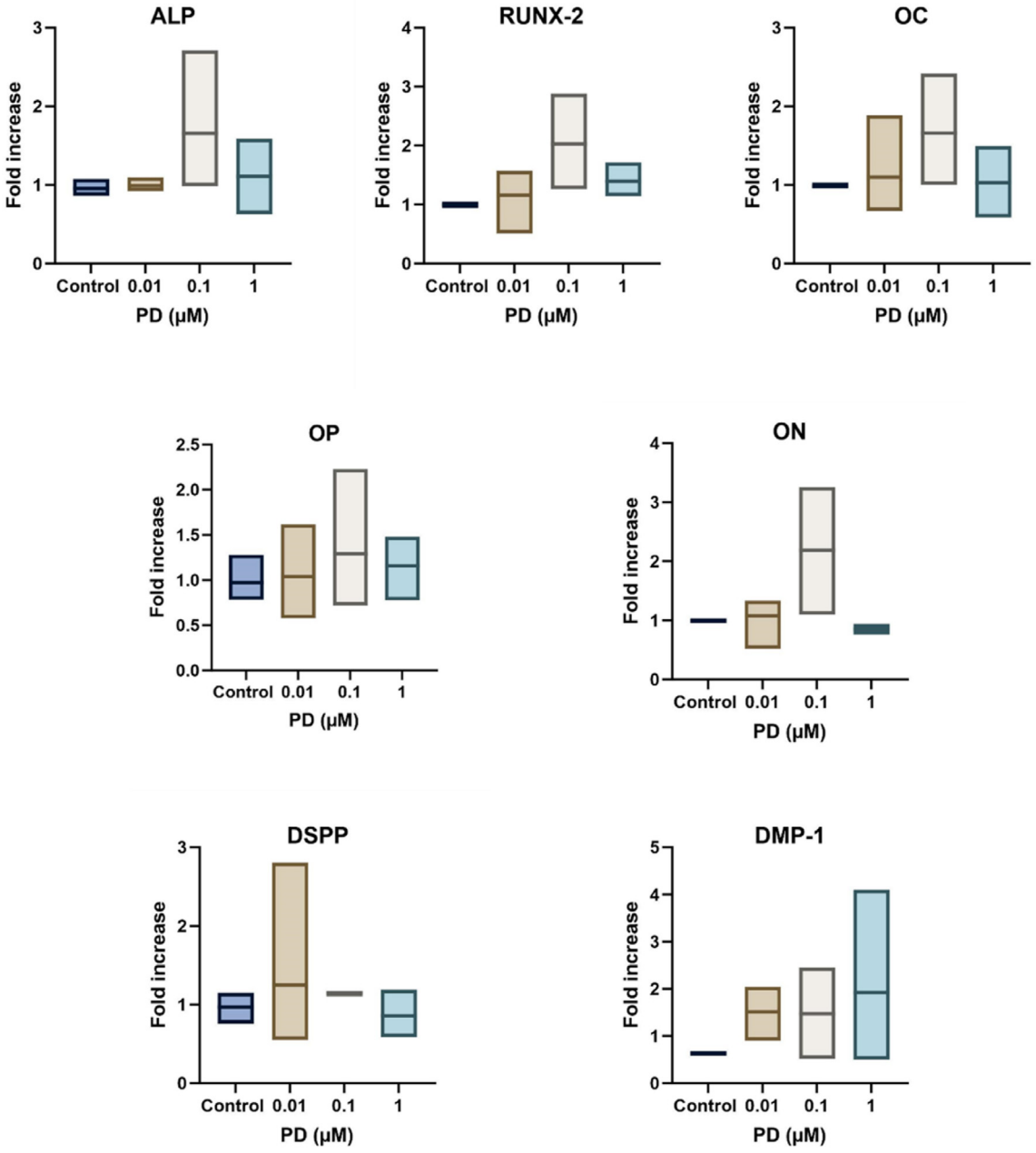

Figure 4 RT-qPCR of 0.01,0.1.1 μM PD on day 7 of alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX-2), osteocalcin (OC), osteopontin (OP), osteonectin (ON), dentin sialophosphoprotein (DSPP), and dentin matrix protein-1 (DMP-1) mRNA. Data are presented as mean, minimum, and maximum fold change increase of mRNA compared to the control group.

Alizarine red stain

On day 14, hDPSCs stained with alizarin red, showed slight increase in the size of calcified nodules in the PD treated groups compared to the control, however, similar staining intensity was observed for all PD treated groups. On day 21, PD groups showed an evident increase in the calcified nodules number, color, and size compared to the control group. Calcified nodules seen in 0.1 μM were more prevalent on day 21 than other groups (Fig. 5).

Discussion

The biological principle involved in "regenerative endodontics" and "direct pulp capping" rely on recruiting undifferentiated mesenchymal stem cells to undergo differentiation into cells that are involved in the regeneration of lost dentin pulp complex tissues.[10,11,36] This is the first study to investigate the effect of PD on hDPSCs in terms of viability and odontogenic differentiation. The findings suggest that PD promotes the viability of hDPSCs and induces odontogenic differentiation and mineralized nodule formation. Cell viability and mineralized tissue deposits are critical for dental applications aiming to replace missing pulp—dentin complex tissues.

The literature is limited on work studying odontogenic/osteogenic differentiation of human stem cells in response to PD and its derivative resveratrol.[27,29,37] However, the findings of the published papers are in line with our findings suggesting that PD promoted the production of odontogenic/osteogenic markers. In addition, viability of hDPSCs in the presence of PD was found to be dose-dependent where lower concentrations promoted viability, whilst higher concentrations (10 and 100 μM) reduced cell viability, this finding is in agreement with Di Benedetto et al. (2018) and Zhan et al. (2020) using DBSCs and BMSCs.[27,38]

Although mRNA levels provide evidence of odontogenic differentiation, the ultimate indicator for odontogenic differentiation is the formation of mineralized nodules and the production of molecules involved in mineralization such as ALP. In this study, ALP levels were significantly elevated with observed increased mineralized deposits in agreement with previous work on DBSCs.[27]

The findings of the current *in-vitro* study were shown on one type of stem cells with controlled variables, therefore, the effect on other types of stem cells as well as *in vivo* conditions need to be investigated.

Pulpal inflammation is a feature of pulp disease as well as a response to agents applied to the pulp to promote hard tissue repair.[22,23] PD has been shown to affect the expression of inflammatory cytokines in a manner favorable to reducing the inflammatory response.[26] The beneficial effect of PD on hDPSCs in terms of viability and mineralized tissue deposition might be augmented by the inflammation reducing properties making it a potential therapeutic agent for dental pulp procedures requiring hard tissue barrier formation, however, further work is required to determine the anti-inflammatory properties of PD on hDPSCs.

In conclusion, PD promoted cell viability and increased expression of markers associated with odontogenic differentiation, as well as mineralized tissue deposition in hDPSCs. The findings suggest that PD merits further investigation for its use as a therapeutic agent in endodontic applications that require dentin bridge formation.

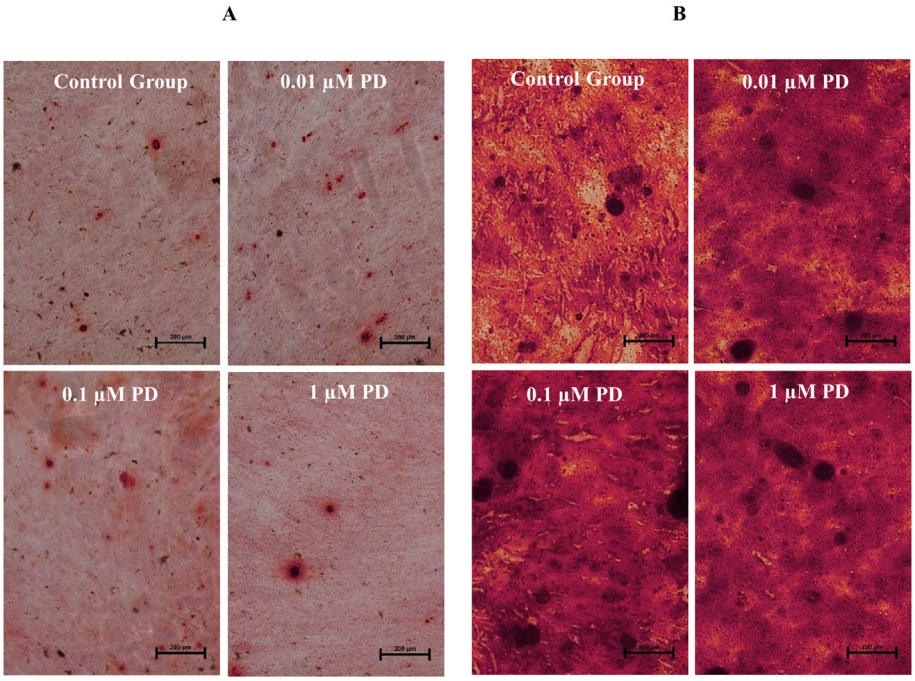

Figure 5 (A) Alizarin red stain photomicrograph on day 14; (B) Alizarin red stain photomicrograph on day 21. Scale bar 200 μm; magnification 10x.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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