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The impact of photobiomodulation on angiogenic differentiation of two different dental derived stem cells using two irradiation protocols: an in vitro investigation

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Abstract

The present study aimed to compare the effect of photobiomodulation with different energy densities on the angiogenic differentiation of human periodontal ligament stem cells (hPDLSCs) and stem cells from human exfoliated deciduous teeth (SHED). Photobiomodulation therapy with a 660 nm diode laser (2.4 J/cm^2 and 3.9 J/cm^2) on two consecutive days post-culture was applied to two types of stem cells (hPDLSCs and SHED). The Quantitative Real-time Polymerase Chain Reaction (RT-qPCR) test was undertaken to investigate Vascular Endothelial Growth Factor-A (VEGF-A) and Angiopoietin I (ANG-I) genes on days 1, 3, 5, 7, and 10 after the first session of laser application. The 4',6-diamidino-2-phenylindole (DAPI) staining and Methyl Thiazolyl Tetrazolium (MTT) test were conducted on days 1, 3, and 5 after the first session of laser application, to assess the cell viability. The Two-way ANOVA with Tukey post hoc test was used to analyze the outcomes of the MTT and RT-qPCR tests. The results of the MTT and DAPI convergently illustrated that the groups receiving photobiomodulation with 2.4 J/cm^2 had higher cell viability compared to 3.9 J/cm^2. All experimental groups showed an upregulation of VEGF-A and ANG-I gene expression from day 1 to 5, followed by a downregulation from day 5 to 10. The groups with cultured hPDLSCs and SHED receiving photobiomodulation using 2.4 J/cm^2 had the most amounts of VEGF-A and ANG-I gene expression on day 5, respectively. In conclusion, the 660 nm mediated photobiomodulation therapy of cultured SHED and hPDLSCs with 2.4 J/cm^2 energy density may be associated with higher angiogenic differentiation (the expression of VEGF-A and ANG-I) as well as higher cell viability compared to the photobiomodulation therapy with 3.9 J/cm^2.

Keywords Angiogenesis, In vitro techniques, Lasers, Low-level light therapy, Mesenchymal stem cells

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Introduction

Photobiomodulation (PBM) is a nonthermal light therapy that triggers the special photo acceptors within the cells and leads to the modification of cellular reactions. Photobiomodulation has the ability to enhance cell proliferation and differentiation, induce the production of cytokines, and boost granulation tissue formation [1–3]. Moreover, PBM has been used to enhance wound healing and tissue repair as well as reduce pain and inflammation [4, 5]. It has shown promising effects on tissue regeneration compared to high-intensity light [6]. Previous studies showed that the combination of the PBM and stem cells led to enhanced angiogenesis through stimulation of angiogenic gene expressions like vascular endothelial growth factor (VEGF) and angiopoietin (ANG) 2 [2]. This was found by an increased number of H-type arteries, which are characterized by high levels of CD31 and EMCN expression [3, 7]. Recent studies have demonstrated the efficacy of PBM in enhancing angiogenesis in various models. For instance, an investigation on the chorioallantoic membrane burn wound model illustrated that PBM promoted angiogenesis by increasing the number of emerging blood vessels, significantly [8]. Other studies showed PBM's effect on angiogenesis and osteogenesis, highlighting a substantial upregulation in blood vessel formation and new bone generation in PBM-treated groups [3, 9]. Furthermore, it has recently been shown that using the ERK/p38 MAKP signaling pathway, PBM interestingly promoted melanoma growth by the induction of angiogenesis [10]. These findings underscore the potential of PBM as a therapeutic intervention to enhance angiogenesis and improve clinical outcomes in various medical fields.

Angiogenesis, defined as the formation of new blood vessels from existing vasculature, plays a crucial role as a source of necessary oxygen and nutrients and is an unelectable component in regeneration [11]. Additionally, it guides stem cells to the specific area of regeneration through arteries [12]. Angiogenesis can be controlled through the expression of a variety of genes; it has been shown in studies that angiogenesis can be mediated by genes like VEGF (through mitogen-activated protein kinase (MAPK)) [13] or ANG I – IV (via Tie-2 and Tie-1) [14].

PBM's effect depends on the laser parameters like wavelength and energy density [15]. Mesenchymal stem cells from different origins can vary in their proliferative and differentiative ability. For instance, stem cells from human deciduous teeth (SHED) have a superior ability for proliferation compared to human dental pulp stem cells (hDPSCs) and human periodontal ligament stem cells (hPDLSCs) [16], or it has been demonstrated that SHED possesses a higher angiogenic ability compared to hDPSCs [17]. Furthermore, despite the positive effect of PBM on dental derived stem cells, it has been shown that the response to PBM may vary according to the nature of the cells and tissues [15].

Given the essential importance of angiogenesis in the process of tissue regeneration, it is imperative to augment this aspect in order to achieve a favorable outcome in tissue regeneration. The present investigation planned to assess the influence of 660 nm-PBM using different energy densities on angiogenic differentiation of the hPDLSCs and SHED.

Materials and methods

The present study design has received approval from the Ethics Committee at the Research Institute of Dental Sciences, Shahid Beheshti University of Medical Sciences (IR.SBMU.DRC.REC.1402.124).

Materials

Cells

The authors sourced hPDLSCs from the Iranian National Center of Genetic and Biologic Resources (IBRC C11326). The surface indicators of hPDLSCs were identified as CD90+ (99.6%), CD105+ (95.6%), CD34- (1.27%), and CD45- (1.08%). SHED with CD73+ (100%), CD90+ (100%%), CD105+ (99.8%), CD34- (2.08%), and CD45- (0.83%) surface characteristics were obtained from the Research Institute for Dental Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Both cell types were used from three passages.

Culture and test materials

From Boisera, France, the authors obtained trypsin/Ethylenediaminetetraacetic acid (trypsin/EDTA), Fetal bovine serum (FBS), High-glucose Dulbecco's modified Eagle medium (DMEM), and Penicillin/Streptomycin (pen/strep). The Phosphate-buffered Saline (PBS), paraformaldehyde, dimethyl sulfoxide (DMSO), 4′,6-diamidino-2-phenylindole (DAPI) solution, and Methyl Thiazolyl Tetrazolium (MTT) solution were acquired from Sigma Aldrich, United States. The authors bought Ethanol from Merck, United States. YTzol Pure RNA and cDNA synthesis kits were procured from Yekta Tajhiz Azma, Iran. Lastly, the 2x SYBR® Green master mix Kit™ was purchased from Ampliqon, Denmark.

Assessment tools and devices

Diode laser with 660 nm wavelength: Konftec, Taiwan.

Fluorescent Microscope: Cytation3, BioTek, United States.

LightCycler 96: Roche, Switzerland.

ELISA Reader Device: ELX800, BioTek, United States.

NanoDrop™ 2000/2000c Spectrophotometers: Thermo Fisher Scientific, United States.

Table 1 Description of the treatment details in study groups

	Groups	Description
1	PDL	Cultured hPDLSCs
2	SHED	Cultured SHED
3	PDL + PBM (2.4)	Cultured hPDLSCs + PBM using 660 nm diode with 2.4 J/cm^2 energy Density (Days 1 and 2 post-culture)
4	SHED + PBM (2.4)	Cultured SHED + PBM using 660 nm diode with 2.4 J/cm^2 energy Density (Days 1 and 2 post-culture)
5	PDL + PBM (3.9)	Cultured hPDLSCs + PBM using 660 nm diode with 3.9 J/cm^2 energy Density (Days 1 and 2 post-culture)
6	SHED + PBM (3.9)	Cultured SHED + PBM using 660 nm diode with 3.9 J/cm^2 energy Density (Days 1 and 2 post-culture)

PBM: Photobiomodulation; SHED: stem cells from human deciduous teeth; hPDLSC: Human Periodontal Ligament Stem Cell

Study design

Experimental groups

Table 1; Fig. 1 elicit the study timeline, different experimental groups, the details regarding PBM therapy for each group, and the dates of laboratory tests. The authors considered two control groups containing cultured hPDLSCs and SHED, without any laser intervention, for the study. PBMs were carried out using a 660 nm diode (8 mm tip; 0.5 cm^2 area) with a 1 mm distance from the cultured surface. In the groups with PBM (2.4), the laser parameters were (150 mW power; continuous mode; 8 s radiation; 2.4 J/cm^2 energy density; 0.3 W/cm^2 power density), and in the groups with PBM (3.9), the laser parameters were as (150 mW power; continuous mode; 13 s radiation; 3.9 J/cm^2 energy density; 0.3 W/cm^2 power density), in two consecutive days post-culture. The laser parameters and timing of application were adopted considering Etemadi et al. [18], Mylona et al. [19], Bergamo et al. [20], and Mahmoudian et al. [21] studies.

Cell culture

High-glucose DMEM supplemented with 15% FBS and 1% pen/strep was used as the standard medium culture in this study. Following the count with a hemocytometer, each well of a 48-well plate was populated with 4000 hPDLSCs and SHED and filled with 0.5 mL of the standard medium. The conditions were maintained at 98% humidity, 5% CO_2, and 37 °C. To avoid the PBM therapy on a well affecting its adjacent wells, the authors ensured there was an empty row and column between the wells with cultured cells. The standard medium was refreshed every other day. After reaching 80% confluency, all the experiments and tests were carried out three times for accuracy.

MTT assay

The MTT solution, at a concentration of 0.5 mg/mL, was introduced to each well on days 1, 3, and 5. The cultures were then incubated for 4 h in an environment with 5% CO_2, 37 °C temperature, and 98% humidity. Following this, 1 mL of dimethyl sulfoxide was added to the wells.

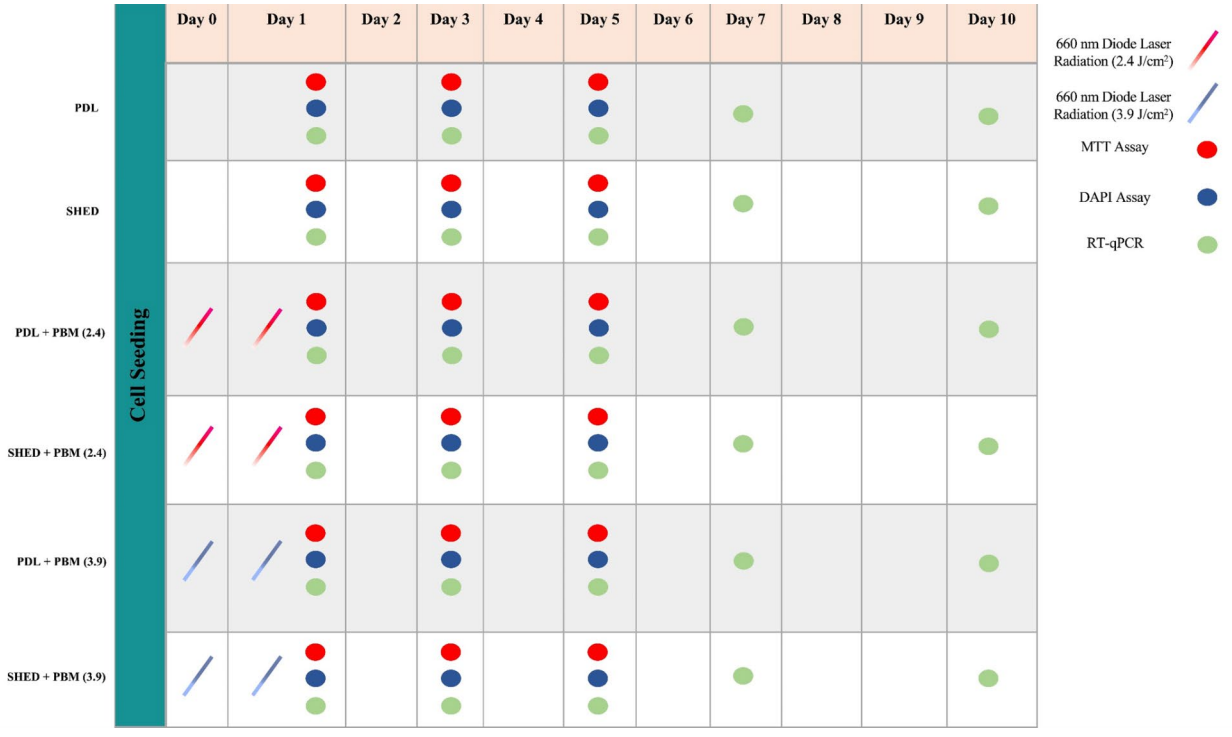

Fig. 1 Characterization of experimental groups and the timeline of study

The medium was subsequently moved to 96-well plates for analysis using the ELISA reader device at a wavelength of 570 nm.

DAPI staining

On days 1, 3, and 5, the medium was removed from the wells using PBS. A 4% concentration of paraformaldehyde solution was then applied for fixation. Subsequently, the wells were rinsed with PBS, and a DAPI solution with a concentration of 1 mg/mL was introduced to each well. The plates were then stored in a dark room at ambient temperature for 45 s. Finally, the wells were rinsed three times with PBS. The plates were then examined under a fluorescent microscope to visualize the blue-colored cell nuclei.

Total RNA extraction, cDNA synthesis, and real-time quantitative polymerase chain reaction (RT-qPCR)

The RT-qPCR analysis was performed to monitor the expressions of the VEGF-A and ANG-I genes on days 1, 3, 5, 7, and 10 after cell seeding. The expression of angiogenic genes was evaluated for up to 10 days because previous literature demonstrated that angiogenic gene overexpression would happen in the early days of the healing process (2 to 5 days) [22, 23]. The forward and reverse primer sequences for the targeted and housekeeping genes are displayed in Table 2. For RT-qPCR analysis, cells were gathered and preserved at -80 °C until the extraction of mRNA. The YTzol Pure RNA kit was used to isolate total cellular RNAs. The concentration and purity of the RNA extracted were assessed using NanoDrop™ 2000/2000c Spectrophotometers. The cDNA Synthesis Kit was used to synthesize Complementary DNA (cDNA) following the manufacturer's guidelines. The qPCR was conducted in triplicate on a Roche lightcycler-96 using the 2x SYBR® Green master mix Kit™. Each qPCR reaction included 1 µL (20 ng) of cDNA, 0.5 µL of each primer (10 pmol/L), 10 µL of 2x Ampliqon Master Mix, and 8 µL of double-distilled water. The 2−ΔΔCT method was utilized to analyze the expression of the target gene. The housekeeping GAPDH mRNA level was used for normalization.

Table 2 The sequences of target gene expression primers used for quantitative real-time polymerase chain reaction analysis

Name	Description	Primers Sequences (5′ to 3′)
VEGF-A	Vascular Endothelial Growth Factor A [Homo sapiens (human)]	F: GAACTTTCTGCTGTCTTGGG R: CTTCGTGATGATTCTGCCCT
ANG-I	Angiopoietin I [Homo sapiens (human)]	F: CAACAGTGTCCTTCAGAA GCAGC R: CCAGCTTGATATACATCTG CACAG
GAPDH	glyceraldehyde-3-phosphate dehydrogenase [Homo sapiens (human)]	F: CCACTCCTCCACCTTTGACG R: CCACCACCCTGTTGCTGTAG

Statistical analysis

The outcomes of the MTT assay and RT-qPCR analyses from various experimental groups were normalized against the control group from their own cell type. All statistical evaluations were conducted on three samples per group using GraphPad Prism v9 software. The Shapiro-Wilk test was employed to verify the normality assumption of the data. Any significant difference in each parameter among the groups was assessed using Two-way ANOVA. A *P*value of less than 0.05 was deemed significant.

Results

The Shapiro-Wilk analysis revealed that none of the MTT assay and RT-qPCR analysis data of the experimental groups deviated from the normal distribution.

MTT assay

Figure 2 presents the MTT assay results, comparing different days and groups. A Two-way ANOVA indicated a significant interaction between experimental groups and time ($P=0.006$).

Comparison between different days in each group

Within the PDL+PBM (2.4) group, cell proliferation was significantly higher on day 5 compared to days 1 and 3 ($P=0.01$). Moreover, cell proliferation was significantly higher on day 3 compared to day 1 ($P=0.03$). No significant differences were observed between days in other groups (Fig. 2A).

Comparison between different groups within each day

Comparing different groups on each day, the PDL+PBM (2.4) group showed significantly higher cell proliferation than the PDL+PBM (3.9) ($P=0.005$) and SHED+PBM (3.9) ($P=0.003$) groups on day 3. Similarly, on day 5, the PDL+PBM (2.4) group had higher cell proliferation compared to the PDL+PBM (3.9) ($P=0.02$) and SHED+PBM (3.9) ($P=0.003$) groups (Fig. 2B).

DAPI staining

Figure 3 illustrates the results of DAPI staining on days 1, 3, and 5. Qualitatively, the results are in convergence with the outcomes of the MTT assay, and it can be concluded that the groups PDL+PBM (2.4) and SHED+PBM (2.4) showed the most amounts of cell proliferation on days 3 and 5 among groups with cultured hPDLSCs and SHED, respectively.

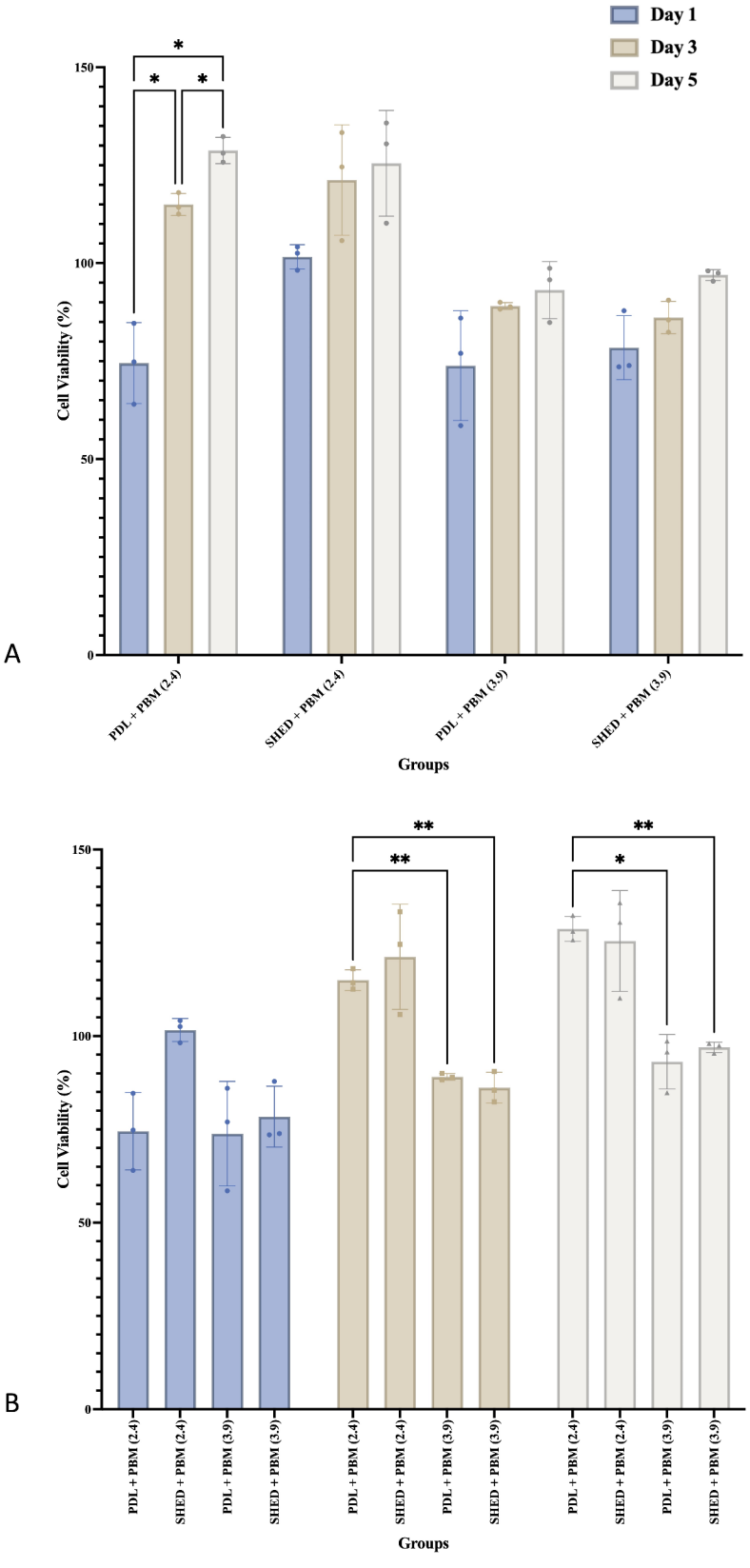

Fig. 2 Comparison of the effect of photobiomodulation with 2.4 and 3.9 J/cm^2 energy densities on the viability of hPDLSCs and SHED after 1, 3, and 5 days. (A) and (B) sections demonstrate the comparison between cell viability on different days within each group and the comparison between cell viability of different groups within each day, respectively. Each experiment was done in triplicate ($n=3$), and columns represent their data. ($P<0.05$ = *, $P<0.01$ = **)

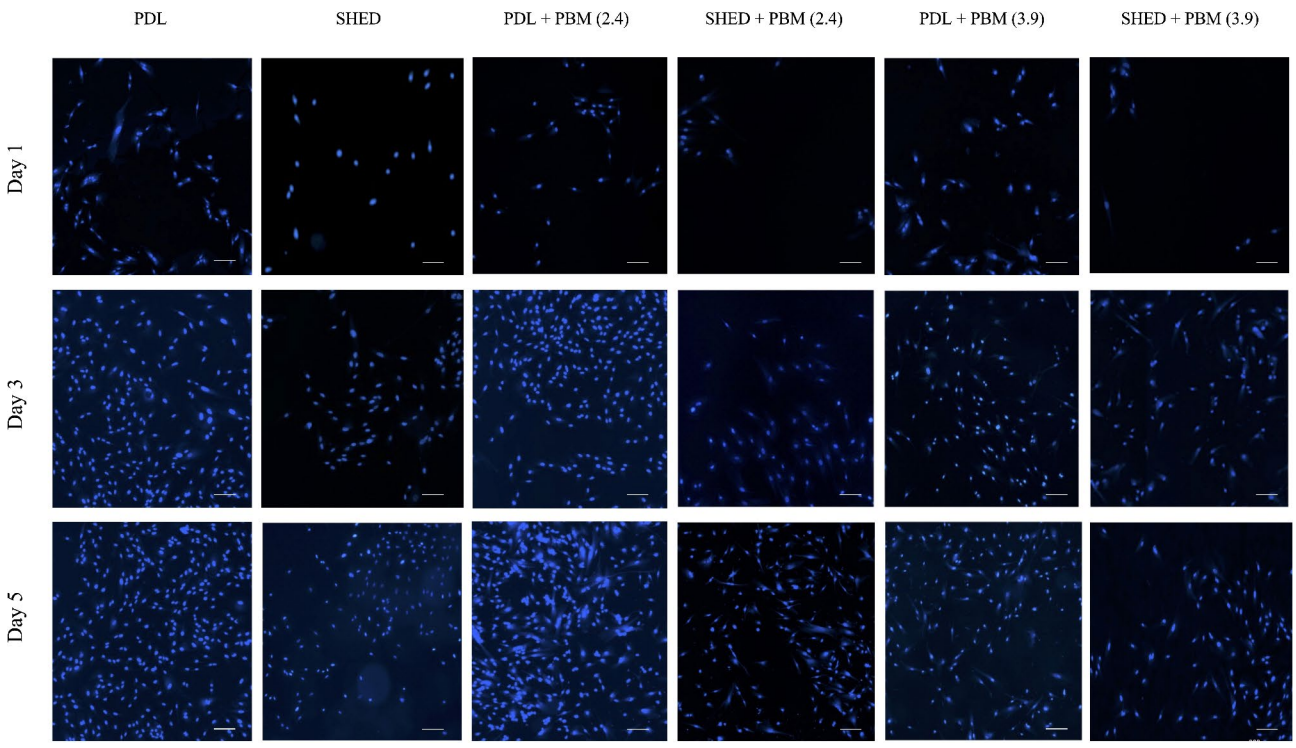

Fig. 3 Results of the 4′,6-diamidino-2-phenylindole (DAPI) staining for qualitative comparison of the effect of photobiomodulation with 2.4 and 3.9 J/cm^2 energy densities on the viability of hPDLSCs and SHED after 1, 3, and 5 days. Each experiment was done in triplicate ($n=3$). Scale bar: 100 µm

RT-qPCR analysis

VEGF-A

Figure 4 depicts the results of the RT-qPCR test for tracing the expression of VEGF-A based on the comparison between different days and groups. Two-way ANOVA showed a significant interaction between the experimental groups and time ($P<0.0001$).

Trend of the gene expression on different days within each group All experimental groups demonstrate the same pattern in the expression of the VEGF-A gene, with an overexpression of the gene from day 1 to day 5, followed by a downregulation in the expression from day 5 to day 10. Hence, the most amount of gene expression can be seen on day 5 (Fig. 4A).

Comparison between different groups on day 5 Among groups with cultured hPDLSCs, PDL+PBM (2.4) (3.08 ± 0.13 expression fold) group showed significantly higher VEGF-A expression than PDL (1.99 ± 0.13 expression fold; $P=0.003$) and PDL+PBM (3.9) (2.39 ± 0.11 expression fold; $P=0.01$) (Fig. 4B).

Among groups with cultured SHED, SHED+PBM (2.4) (2.69 ± 0.07 expression fold) demonstrated significantly higher VEGF-A expression than SHED ($P=0.0007$) and SHED+PBM (3.9) ($P=0.03$) (Fig. 4B).

Comparison between the effect of PBM on hPDLSC and SHED PDL+PBM (2.4) and SHED+PBM (2.4) groups illustrated the highest amounts of gene expression among groups with cultured hPDLSCs and SHED, respectively. Comparing PDL+PBM (2.4) and SHED+PBM (2.4), significantly higher VEGF-A gene expression was found on days 3 ($P=0.02$), 7 ($P=0.03$), and 10 ($P=0.02$); however, they have no significant difference on day 5 ($P=0.09$).

Table 3 represents complementary details of the results of the Two-way ANOVA regarding the comparison in the VEGF-A gene expression between each pair of the experimental groups.

ANG-I

Figure 5 depicts the results of the RT-qPCR test for tracing the expression of ANG-I based on the comparison between different days and groups. Two-way ANOVA showed a significant interaction between the experimental groups and time ($P<0.0001$).

Trend of the gene expression on different days within each group All experimental groups demonstrate the same pattern in the expression of the ANG-I gene, with an overexpression of the gene from day 1 to day 5, followed by a downregulation in the expression from day 5 to day 10. Hence, the most amount of gene expression can be seen on day 5 (Fig. 5A).

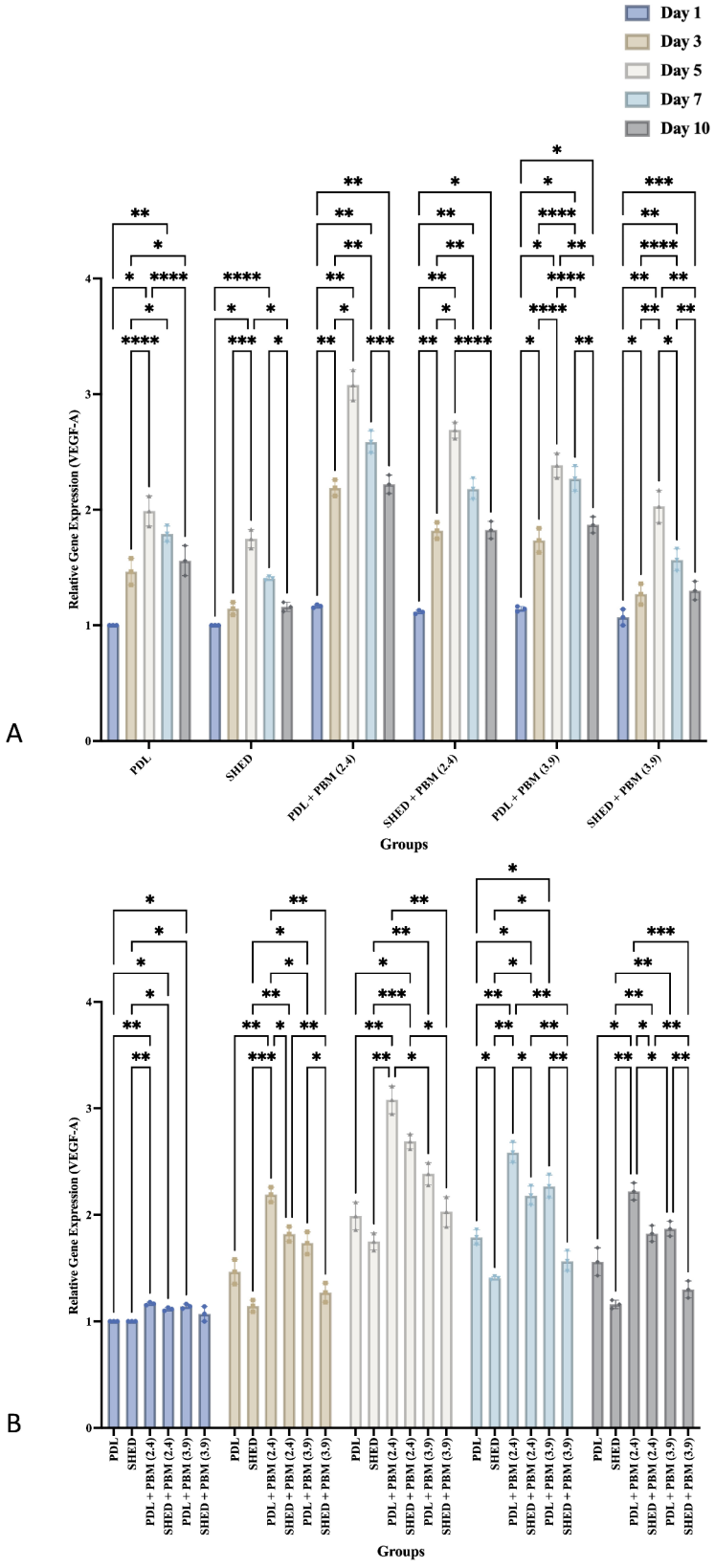

Fig. 4 Comparison of the effect of photobiomodulation with 2.4 and 3.9 J/cm^2 energy densities on the vascular endothelial growth factor A (VEGF-A) mRNA expression and angiogenic differentiation of hPDLSCs and SHED. (A) and (B) sections demonstrate the comparison between gene expression on different days within each group and the comparison between gene expression of different groups within each day, respectively. Each experiment was done in triplicate ($n=3$), and columns represent their data. ($P<0.05$ = *, $P<0.01$ = **, $P<0.001$ = ***, $P<0.0001$ = ****)

Table 3 League table containing *p*-values associated with comparing each pair of experimental groups' relative vascular endothelial growth factor (VEGF) gene expression resulting from two-way ANOVA and Tukey post hoc

1.00 ± 0.00	Day 1	**PDL**					
1.47 ± 0.12	Day 3						
1.99 ± 0.13	Day 5						
1.79 ± 0.07	Day 7						
1.56 ± 0.13	Day 10						
1.00 ± 0.00	Day 1	N/A	**SHED**				
1.15 ± 0.06	Day 3	0.11					
1.75 ± 0.08	Day 5	0.27					
1.41 ± 0.02	Day 7	0.03 *					
1.16 ± 0.04	Day 10	0.10					
1.17 ± 0.01	Day 1	0.01 *	0.01 *	**PDL + PBM (2.4)**			
2.19 ± 0.07	Day 3	0.01 *	0.0003 *				
3.08 ± 0.13	Day 5	0.003 *	0.002 *				
2.59 ± 0.10	Day 7	0.002 *	0.007 *				
2.22 ± 0.08	Day 10	0.02 *	0.001 *				
1.12 ± 0.02	Day 1	0.02 *	0.02 *	0.07	**SHED + PBM (2.4)**		
1.82 ± 0.07	Day 3	0.08	0.002 *	0.02 *			
2.69 ± 0.07	Day 5	0.02 *	0.0007 *	0.09			
2.18 ± 0.09	Day 7	0.03 *	0.01 *	0.03 *			
1.83 ± 0.08	Day 10	0.22	0.003 *	0.02 *			
1.14 ± 0.02	Day 1	0.03 *	0.03 *	0.62	0.57	**PDL + PBM (3.9)**	
1.74 ± 0.11	Day 3	0.20	0.02 *	0.03 *	0.83		
2.39 ± 0.11	Day 5	0.09	0.009 *	0.02 *	0.09		
2.27 ± 0.11	Day 7	0.03 *	0.02 *	0.10	0.87		
1.87 ± 0.07	Day 10	0.15	0.002 *	0.03 *	0.96		
1.07 ± 0.07	Day 1	0.62	0.62	0.43	0.86	0.62	**SHED + PBM (3.9)**
1.27 ± 0.09	Day 3	0.36	0.46	0.001 *	0.008 *	0.03 *	
2.03 ± 0.14	Day 5	1.00	0.23	0.004 *	0.03 *	0.14	
1.57 ± 0.10	Day 7	0.16	0.32	0.001 *	0.007 *	0.006 *	
1.30 ± 0.08	Day 10	0.23	0.29	0.0009 *	0.007 *	0.005 *	

The first column at the left demonstrates the amount of relative gene expression (mean ± standard deviation) related to each group on each day compared to the control group. Each experiment was done in triplicate ($n=3$). * sign indicates a significant difference between groups in each day. N/A = not applicable

Comparison between different groups on day 5 Among groups with cultured hPDLSCs, PDL + PBM (2.4) (2.02 ± 0.13 expression fold) possesses a higher amount of ANG-I expression compared to the other groups; however, no significant difference was observed between them (Fig. 5B).

Among groups with cultured SHED, SHED + PBM (2.4) (1.99 ± 0.11 expression fold) demonstrated significantly higher amounts of ANG-I expression ($P=0.03$) compared to the SHED group (1.38 ± 0.03 expression fold) (Fig. 5B).

Comparison between the effect of PBM on hPDLSC and SHED PDL + PBM (2.4) and SHED + PBM (2.4) groups illustrated the highest amounts of gene expression among groups with cultured hPDLSCs and SHED, respectively. Comparing these two groups, no significant differences were found in the expression of ANG-I on any of the experiment days.

Table 4 represents complementary details of the results of the Two-way ANOVA regarding the comparison in the ANG-I gene expression between each pair of the experimental groups.

Discussion

The aim of this investigation was to evaluate the effect of 660 nm-PBM with different energy densities on the angiogenic differentiation of hPDLSCs and SHED. The results showed that despite the PBM treatment with 3.9 J/cm^2 energy density, the application of PBM with 2.4 J/cm^2 energy density can positively affect the cell viability in both hPDLSCs and SHED. Moreover, treatment with PBM (2.4 J/cm^2 energy density) enhanced the angiogenic differentiation of both hPDLSCs and SHED more than treatment with PBM (3.9 J/cm^2 energy density). The application of PBM (2.4 J/cm^2 energy density) resulted in higher amounts of VEGF-A expression in the hPDLSCs compared to SHED; however, the expression of ANG-I was not different between hPDLSCs and SHED. Lastly, it was found that the expression of angiogenic genes was

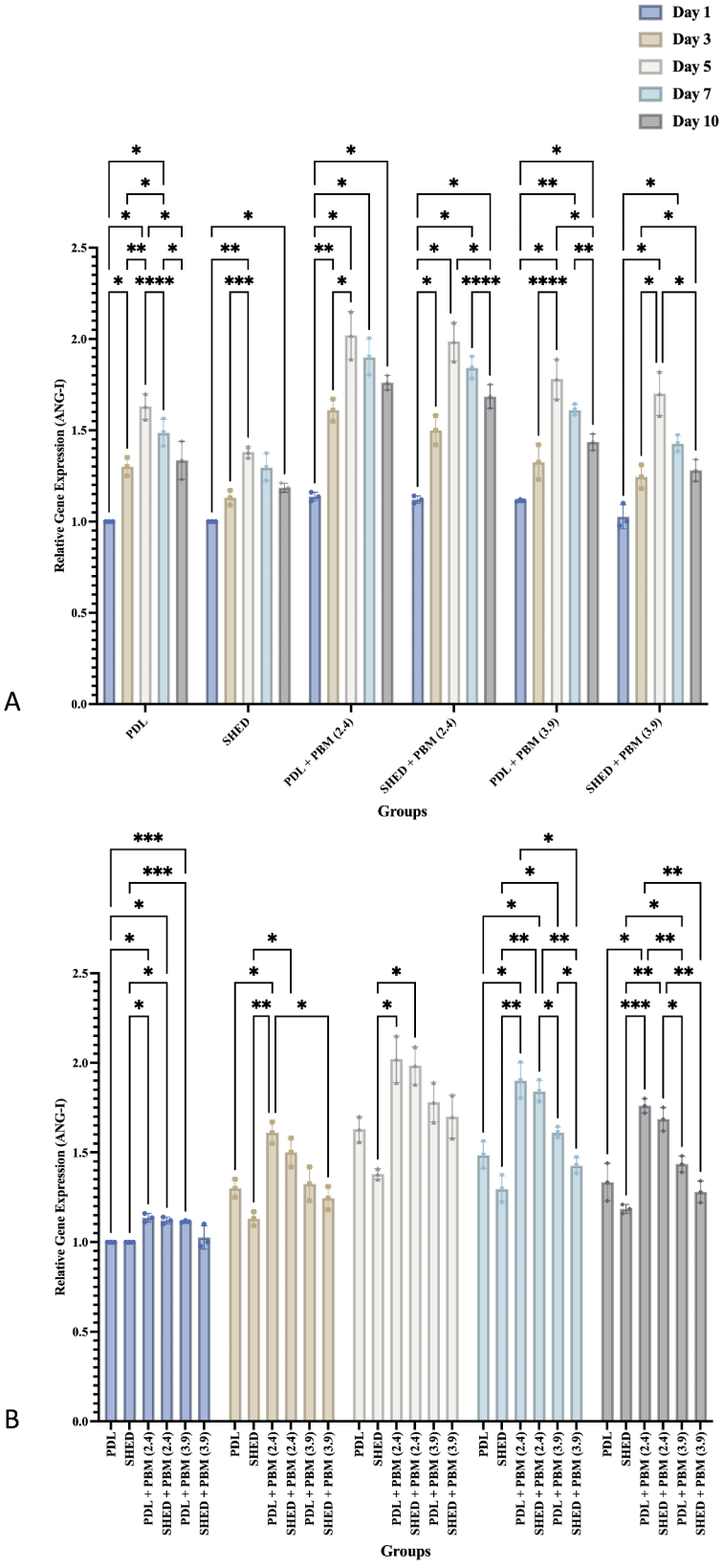

Fig. 5 Comparison of the effect of photobiomodulation with 2.4 and 3.9 J/cm^2 energy densities on the Angiopoietin I (ANG-I) mRNA expression and angiogenic differentiation of hPDLSCs and SHED. (**A**) and (**B**) sections demonstrate the comparison between gene expression on different days within each group and the comparison between gene expression of different groups within each day, respectively. Each experiment was done in triplicate ($n = 3$), and columns represent their data. ($P < 0.05$ = *, $P < 0.01$ = **, $P < 0.001$ = ***, $P < 0.0001$ = ****)

Table 4 League table containing *p*-values associated with comparing each pair of experimental groups' relative Angiopoietin I (ANG-I) gene expression resulting from Two-way ANOVA and Tukey post hoc

1.00 ± 0.00	Day 1	**PDL**					
1.30 ± 0.05	Day 3						
1.63 ± 0.07	Day 5						
1.49 ± 0.08	Day 7						
1.34 ± 0.11	Day 10						
1.00 ± 0.00	Day 1	N/A	**SHED**				
1.13 ± 0.04	Day 3	0.06					
1.38 ± 0.03	Day 5	0.06					
1.30 ± 0.08	Day 7	0.18					
1.19 ± 0.03	Day 10	0.40					
1.14 ± 0.02	Day 1	0.04 *	0.04 *	**PDL + PBM (2.4)**			
1.61 ± 0.06	Day 3	0.02 *	0.003 *				
2.02 ± 0.13	Day 5	0.09	0.04 *				
1.90 ± 0.10	Day 7	0.03 *	0.008 *				
1.76 ± 0.04	Day 10	0.04 *	0.0007 *				
1.12 ± 0.02	Day 1	0.03 *	0.03 *	0.93	**SHED + PBM (2.4)**		
1.50 ± 0.08	Day 3	0.14	0.03 *	0.51			
1.99 ± 0.11	Day 5	0.06	0.03 *	1.00			
1.84 ± 0.06	Day 7	0.02 *	0.004 *	0.93			
1.69 ± 0.07	Day 10	0.06	0.01 *	0.60			
1.11 ± 0.01	Day 1	0.008 *	0.008 *	0.69	0.99	**PDL + PBM (3.9)**	
1.33 ± 0.10	Day 3	1.00	0.21	0.08	0.32		
1.78 ± 0.11	Day 5	0.48	0.07 *	0.32	0.35		
1.61 ± 0.03	Day 7	0.32	0.04 *	0.11	0.04 *		
1.44 ± 0.05	Day 10	0.68	0.01 *	0.005 *	0.04 *		
1.03 ± 0.07	Day 1	0.97	0.97	0.31	0.40	0.44	**SHED + PBM (3.9)**
1.25 ± 0.07	Day 3	0.84	0.30	0.01 *	0.07	0.82	
1.70 ± 0.11	Day 5	0.93	0.14	0.18	0.18	0.94	
1.43 ± 0.05	Day 7	0.82	0.31	0.03 *	0.005 *	0.03 *	
1.28 ± 0.06	Day 10	0.95	0.35	0.004 *	0.008 *	0.13	

The first column at the left demonstrates the amount of relative gene expression (mean ± standard deviation) related to each group on each day compared to the control group. Each experiment was done in triplicate ($n=3$). * sign indicates a significant difference between groups in each day. N/A = Not applicable

upregulated from day 1 to day 5 (maximum), and afterward, it faced a downregulation to day 10.

Angiogenesis is crucial for wound healing and granulation tissue formation processes, especially in the early days of healing. This process provides cells (both progenitor and inflammatory) nutrition, oxygen, and growth factors [23]. The present study showed upregulation of VEGF-A and ANG-I in all treatment groups until day 5. Therefore, it can be suggested that two sessions of PBM treatments can promote angiogenic differentiation of hPDLSCs and SHED in the early days of healing. A previous investigation demonstrated that PBM enhances the migration, proliferation, and angiogenesis of human umbilical vascular endothelial cells (HUVECs) through activation of the PI3K/AKT pathway, which leads to an increase in the levels of angiogenesis-related genes (VEGF-A, eNOS, and Hypoxia-inducible factor (HIF)-1α) [24]. In coherence with these results, PBM, with an energy density between 4 and 8 J/cm^2, elicited a positive influence on the angiogenic phase of bone healing in the different mesenchymal stem cells (adipose-derived stem cells and bone marrow-derived stem cells) through increasing platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), VEGF, and activation of the ROS/HIF-1α pathway [25]. Regarding the impact of PBM on other cell types, Bergamo et al. [20] demonstrated that PBM can improve the angiogenic differentiation of human dental pulp fibroblasts. It has been found that the expression of the FGF-2 gene, which is attributed to the angiogenesis, was more upregulated in the groups with 2.5 J/cm^2 and 3.7 J/cm^2 compared to the groups with higher energy densities. Considering the different impacts of PBM on the different natures of the investigated cells [15], the different achieved results can be counted as justified. The present study's findings can complete the previous results and provide complementary insight into the application of PBM for the enhancement of angiogenesis.

Cell therapy is now an expanding treatment strategy in the field of medical sciences, which, alongside tissue

engineering, can be applied to many conditions as a treatment, like cancer, cardiovascular diseases, digestive system diseases, liver diseases, and arthritis [26, 27]. Dental derived stem cells like hPDLSCs, SHED, and dental pulp stem cells can be harvested from the extracted teeth and are easier and more accessible to be retrieved compared to other sources of stem cells; hence, they can be counted as serious options for future stem cell therapies. Therefore, underscoring the importance of angiogenesis, the results of the present study will be beneficial for further investigations in the field of stem cell therapy.

The novelty of the present study is that it compares the effect of PBM in the angiogenic differentiation of two different mesenchymal stem cell types with each other to bring complementary insight to the future perspective of regenerative therapies. Furthermore, exploring the target gene expressions over time will offer a more comprehensive vision of angiogenesis and help to understand its mechanisms better. Some limitations are also evident in this study. To gain better insight into the effect of PBM on angiogenesis, further complementary In vivo studies, with longer follow-up periods for the observation of long-term outcomes, followed by clinical studies on this subject, are strongly recommended. Moreover, the authors suggest future studies to investigate the effect of PBM with different laser parameters and energy densities on angiogenesis. Eventually, protein-level explorations will help the literature achieve comprehensive findings regarding the angiogenic differentiation of SHED and hPDLSCs. Other sources of mesenchymal stem cells and non-mesenchymal stem cells should be investigated in future studies, as biological variability can affect the reproducibility of the result. Furthermore, the investigation of other various angiogenic gene expressions, growth factors production, and underlying molecular mechanisms and signaling pathways are crucial for the comprehensive insight into the effect of PBM on angiogenesis.

Conclusion

Based on the present study's limitations, it can be concluded that two consecutive session applications of PBM with 660 nm diode laser at an energy density of 2.4 J/cm^2 have more positive effects on the angiogenic differentiation and cell proliferation of hPDLSCs and SHED compared to 3.9 J/cm^2 energy density. Furthermore, the expression of the angiogenic genes (VEGF-A and ANG-I) was found to be maximum on day 5.

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Author contributions
Fazele Atarbashi-Moghadam: Conceptualization, Methodology, Supervision, Resources, Writing – Original Draft, Writing – Review and EditingArian Samadi Rad: Conceptualization, Investigation, Writing – Original Draft, Writing – Review and EditingNeda Hakimiha: Methodology, Investigation, Writing – Original Draft, Writing – Review and EditingNiloofar Taghipour: Methodology, Investigation, Writing – Original Draft, Writing – Review and EditingAmirhosein Mahmoudian: Methodology, Investigation, Writing – Original Draft, Writing – Review and EditingAli Azadi: Conceptualization, Methodology, Formal Analysis, Project Administration, Investigation, Software, Visualization, Writing – Original Draft, Writing – Review and EditingHanieh Nokhbatolfoghahaei: Conceptualization, Investigation, Methodology, Project Administration, Validation, Writing – Original Draft, Writing – Review and Editing. All authors read and approved the final manuscript.

Data availability
The data that support the findings of this study are available from the corresponding author.

Declarations

Ethical approval
The study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.DCR.REC.1402.124).

Consent to participate
Not applicable.

Consent to Publish
Not applicable.

Competing interests
The authors declare no competing interests.

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