Low-level laser irradiation enhances the proliferation and osteogenic differentiation of PDLSCs via BMP signaling

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Abstract
The aim of this in vitro study was to evaluate the effects of low-level laser therapy (LLLT) at different energy intensities on proliferation and osteogenesis of periodontal ligament stem cells (PDLSCs). We designed one control group, without irradiation and four testing groups, treated with LLLT (Nd:YAG; 1064 nm) at 2, 4, 6, and 8 J/cm² for human PDLSCs. Cell proliferation was measured using colony-forming unit fibroblast assay and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Osteogenic capacity of cells was determined by alkaline phosphatase (ALP) staining, ALP activity assay, Alizarin Red S staining, and the gene levels of runt-related transcription factor 2 (Runx2), ALP, osteocalcin, and bone morphogenetic protein 2 (BMP2). The effects of LLLT on secretion of TNF-α and IL-1β in PDLSCs were measured by enzyme-linked immunosorbent assay. BMP/Smad pathway was measured through the expression of Smad1/5/8 phosphorylation (P-Smad1/5/8). LDN-193189, an inhibitor of the BMP/Smad pathway, was used to explore the underlying effects of BMP/Smad signaling on the process of LLLT regulating the proliferation and osteogenesis of PDLSCs. Our results demonstrated LLLT could promote the proliferation and osteogenesis of PDLSCs at 2–6 J/cm² and LLLT at 8 J/cm² significantly suppress osteogenic differentiation of PDLSCs. Moreover, LLLT stimulated the secretion of TNFα and IL-1β. Finally, we found the irradiation positively modulates the P-Smad1/5/8 level. When the cells were treated with LDN-193189, the proliferation and osteogenic effects of LLLT on PDLSCs were attenuated. In conclusion, LLLT may upregulate the proliferation and bone formation ability of PDLSCs via the BMP/Smad signaling.

Keywords Periodontal ligament stem cells (BMSCs) · Low-level laser therapy (LLLT) · Proliferation · Osteogenesis · BMP/Smad pathway

Introduction
Chronic periodontitis is a kind of chronic inflammatory disease characterized by periodontal tissue damage and eventually leads to teeth loss [1]. At present, how to repair damaged periodontal tissues becomes a focus in current research. Traditional treatments including periodontal scaling and root planning, guided tissue regeneration (GTR), and guided bone regeneration (GBR) could only obtain limited clinical outcomes [2]. To regenerate destroyed periodontal tissues with stem cells will be a new strategy for curing chronic periodontitis. Periodontal ligament stem cells (PDLSCs), which derived from periodontal ligament and have various abilities of differentiation, are the main candidate stem cells in periodontal regeneration. A wide array of studies confirms that PDLSCs could generate cementum and periodontal ligament-like tissues in vitro and in vivo [3–5]. At present, repairing the periodontal defect bone tissues through
PDLSCs has entered the clinical trial. A clinical study revealed that autologous PDL-derived cell sheets showed high stability and efficacy during mid-long-term follow-up in patients with periodontitis [6]. In periodontal regeneration, hard tissue repairing is the difficulty and the emphasis of current research. Therefore, improving the osteogenic differentiation is an important goal in the field of PDLSCs.

Low-level laser therapies (LLLT) including low light laser therapy, red light therapy, cold laser, and soft laser were first reported in 1960 [7]. LLLT induces a wide range of effects including wound healing, inflammation, and pain reduction [8–11]. Studies showed LLLT play a critical role in promoting bone formation of mesenchymal stem cells [12, 13]. Our research demonstrates LLLT could modulate the proliferation and the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) under healthy and inflammatory conditions [14]. In chronic periodontitis, different low-level lasers have been used in periodontal treatments with benefits of coagulation, antibacterial effects, root surface detoxification, and smear layer removal, and even bone recontouring [15, 16]. Nobuhiro and his team showed that high-power, red light-emitting diode irradiation significantly enhances proliferation, osteogenic differentiation, and mineralization of PDLSCs via ERK signaling pathway [17]. Among many types of lasers, neodymium-doped:yttrium–aluminum–garnet (Nd:YAG) laser appears to be the most suitable to use in periodontal treatments [18]. However, it has not been clarified the effects of Nd:YAG laser on the proliferation and osteogenic differentiation of PDLSCs. The aim of this study is to evaluate the effects and mechanism of Nd:YAG laser at different energy intensities on proliferation and osteogenic differentiation of PDLSCs.

Materials and methods

Cell culture

Primary human PDLSCs were obtained from 15 individuals, 5 male and 10 female, aged 15–35 years, undergoing routine premolar procedures for orthodontic reason extractions. None of the volunteers had periodontal disease, systemic diseases, smoking history, radiotherapy, and chemotherapy history. All samples were collected at the College of Stomatology, Xi’an Jiaotong University (Xi’an, China). Each volunteer provided written informed consent, and the study was approved by the Hospital’s Ethics Committee (license no. xjkqll[2020]NO.028). Tissues were scraped from the periodontal ligament of teeth and digested with type 1 collagenase (0.66 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for more than 20 min. Primary PDLSCs were cultured in α-minimum essential medium (α-MEM) (Gibco; Thermo Fisher Scientific, Inc., MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), glutamine, penicillin, and streptomycin. PDLSCs at passage 3 were isolated through immunomagnetic beads (M2450; Dynal Biotech, Wirral, UK) with STRO-1 antibodies (340,106; Biolegend, CA, USA), as previously described (14). In this study, cells at 3–5 passages were used.

LLLT treatment

Here, PDLSCs were divided into 5 groups, one control group (without irradiation) and four testing groups (irradiation at 2, 4, 6, and 8 J/cm²). When PDLSCs reached 80% confluence, cells were treated with LLLT as previously described (14). Briefly, a neodymium-doped yttrium aluminum garnet laser (Nd:YAG;1064 nm; Fidelis Plus III, Fotona; Ljubljana, Slovenia) was applied using a single-probe laser handpiece perpendicular to the surface, scanning the cells. The beam angle was 90°, and the energy intensity applied to cell was adjusted to approximately 0–8 J/cm² by applying 0.25 W output power for 20 s every other day.

Colony-forming unit fibroblast assay

Single-cell suspensions (200 cells) were seeded in Petri dishes. After 7 days, cells were fixed with 4% paraformaldehyde fixation and then stained with 0.1% toluidine blue. Colonies were defined as aggregates containing more than 50 cells. The colonies were analyzed by microscopy and colony-forming unit fibroblast (CFU-F) assay. Efficiency was determined by the number of colonies relative to the total number of seeded cells in each plate.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

PDLSCs were seeded in 96-well at a density of 5 × 10³ cells/well for 24 h. Subsequently, the proliferation of cells was measured using the MTT assay (Sigma, MO, USA) according to the manufacturer’s instructions for 7 days and the absorbance was determined at 490 nm.

Osteogenic differentiation

PDLSCs were seeded in 6-well at a density of 1 × 10⁵ cells/well until they reached 80%. Subsequently, cells were cultured in osteogenic medium (100 nM dexamethasone, 50 mg/ml ascorbic acid, and 5 mM b-glycerophosphate; Sigma, MO, USA) for 7 or 21 days.

Alkaline phosphatase activity

After induced in osteogenic medium for 7 days, alkaline phosphatase (ALP) activity was measured using an ALP kit under
the guidance of the manufacturer's instructions (Jiancheng, Nanjing, China). ALP activity was normalized to the total protein content and expressed in nanomoles of produced p-nitrophenol per minute per milligram of total protein (nmol/min/mg protein). Total protein content was determined in the same sample with the bicinchoninic acid (BCA) method using the Pierce protein assay kit (Thermo, MA, USA). ALP activity was normalized to the total protein content and expressed in nanomoles of produced p-nitrophenol per minute per milligram of total protein (nmol/min/mg protein).

**Alizarin red staining**

After induced in osteogenic medium for 21 days, cells were washed with 10% FBS in PBS twice and fixed in 60% isopropanol for 1 min. Subsequently, cells were washed twice and stained with Alizarin Red S (pH 4.2) (Kermel, Tianjin, China) for 30 min at room temperature. Alizarin red-stained nodules were measured using a calcium colorimetric assay kit (BioVision, San Francisco, CA, USA). The amount of calcium activity was normalized to the total protein content and expressed in nanomoles of produced p-nitrophenol per minute per milligram of total protein (nmol/min/mg protein). Total protein content was determined in the same sample with the bicinchoninic acid (BCA) method using the Pierce protein assay kit (Thermo, MA, USA).

**Quantitative real-time polymerase chain reaction**

Total RNA was isolated using TRIzol reagent (Life Technologies; Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions and converted into cDNA using a PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). Reverse transcription of mRNA was performed using the Prime Script RT reagent kit (Takara, Bio, Otsu, Japan). qPCR analysis was performed using the SYBR Premix Ex Taq II kit (Takara Biotechnology Co., Ltd.) and detected on the ABI Prism 7500 HT sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). β-actin was used for quantitation of mRNAs. The data were analyzed using the 2-ΔΔCq relative expression method. The primer sequences were as follows:

Runx2: 5′-CCCCTGCGCCTTCAAGGT-3′, 5′-GCATCCCGCCTAATGAGTA-3′;
ALP: 5′-GGACCA TTCCACGTCCTCC-3′, 5′-CCTGTAGCCAGGCCCATGG-3′;
OCN: 5′-CCACGCGCTACCTGTATCAA-3′, 5′-GGTCAGCCAACTCGTCAGTC-3′;
β-actin: 5′-TGGGACCCAGCAAATGGAA-3′, 5′-CTAGTCATAGTCGCCCTAGGAC-3′;
BMP2: 5′-TGGGACCTTTAGAGGAGAAC-3′, 5′-AGGGTATAGCCCCGAGG-3′

**Western blot analyses**

Total protein was extracted using lysis in RIPA buffer and using a protein assay kit to determine the protein concentration (Beyotime Institute of Biotechnology) according to the manufacturer’s instructions. Subsequently, 20 to 50 mg of the cell lysate samples were separated by 10% SDS-PAGE and then transferred to a polyvinylidene fluoride (PVDF) membrane (BioRad, Hercules, CA, USA). The membranes were blocked with 5% milk for 2 h and then incubated with antiphospho-Smad1 (Cell Signaling Technology, Beverly, MA, USA), anti-Smad1 (Cell Signaling Technology, Beverly, MA, USA), and anti-β-actin (Cell Signaling Technology, Beverly, MA, USA) primary antibodies. The secondary antibodies were horseradish peroxidase-conjugated antirabbit or anti-mouse IgG antibodies (Boshide, Beijing, China). Finally, the bands were imaged and analyzed by using the Western-Light Chemiluminescent Detection System (Peiqing, Shanghai, China).

**Cytokine expression assays**

The secretion of TNF-α and IL-1β in PDLSCs was determined using ELISA kits (R&D Systems, MN, USA) according to the manufacturer’s protocols. The results were normalized to cell number and expressed as pg/10⁶ cells.

**LDN-193189 treatment**

The cells were cultured until they reached 80%. Then, cells were cultured in medium with LDN-193189 (PeproTech, Rocky Hill, NJ, USA) (0.5 μmol/L) and the medium was changed in 2 days. Then, the cells were cultured in osteogenic-inducing media for 7 or 21 days to induce osteogenic differentiation.

**Statistical analysis**

All experiments were repeated at least three times in this study. Statistical significance was assessed using a χ² test and an independent sample t test and presented them by means ± SD. Multiple group tests were analyzed by the Bonferroni correction for post hoc analysis and statistical significance was defined as *P* < 0.05.

**Results**

The effects of LLLT on proliferation of PDLSCs

To find out optimal stimulation, we measured the proliferation ability of PDLSCs using CFU-F assay and MTT after treated with LLLT. In PDLSCs, irradiation could significantly increase the MTT activity ranging at 0–8 J/cm²;
the highest MTT activity was induced at 6 J/cm² at day 7 (Fig. 1a, b). The CFU-F assay showed similar results. Cells induced by a dose of 6 J/cm² LLLT formed the maximum quantity of adherent clonogenic cell clusters (Fig. 1c).

The effects of LLLT on osteogenic differentiation of PDLSCs

To measure the effects of LLLT on osteogenic differentiation of PDLSCs, we analyzed osteogenic genes level, ALP activity, and mineralized nodule formation after inducing cells in osteogenic media for 7 or 21 days. In this part, we found the BMP2 level was most sensitive to LLLT (Fig. 2b). Irradiation at 2–8 J/cm² all could promote the expression of BMP2 in PDLSCs and the highest expression level was induced by LLLT at 4 J/cm² (Fig. 2b). Other results including the gene levels of Runx2, ALP, and OCN and mineralized nodule formation showed that LLLT could enhance the bone formation of PDLSCs at 2–6 J/cm² (Fig. 2a, c–e). However, the osteogenic indicators (except BMP2) of PDLSCs were significantly inhibited by LLLT at 8 J/cm² (Fig. 2a, c–e).

Effects of LLLT on the secretion of TNF-α and IL-β1 in PDLSCs

In this part, we detected the effects of LLLT on secretion of TNF-α and IL-β1 in PDLSCs using ELISA assay. The results suggested irradiation could significantly promote the secretion of TNF-α and IL-β1 in PDLSCs (Fig. 3a). Moreover, the TNF-α and IL-β1 secretion increased more greatly measured after 7 days of culture at low seeding density. Data represent mean ± S.D. *P < 0.05; **P < 0.01; NS, not significant.
when irradiation reached stronger within the range of 2–8 J/cm² (Fig. 3a).

The effects of LLLT on BMP/Smad signaling pathways

To explore the underlying mechanisms of how LLLT treatments promote PDLSCs osteogenic differentiation, we detected BMP/Smad signaling pathways based on the expression of Smad1/5/8 phosphorylation (P-Smad1/5/8). The irradiation could significantly promote P-Smad1/5/8 level compared with the untreated control group. The highest expression level was induced by LLLT at 4 J/cm² (Fig. 3b, c).

Role of BMP/Smad signaling pathways in LLLT-mediated regulation of proliferation and osteogenesis of PDLSCs

In this part, to investigate the effects of LLLT on BMP/Smad pathways, irradiated PDLSCs were cultured with the BMP/Smad pathway inhibitor, LDN-193189. When the BMP/Smad pathway inhibited, the P-Smad1/5/8 expression of PDLSCs, irradiated with laser at 4 J/cm² or not, significantly decreased (Fig. 4a, b). After treated with LDN-193189, LLLT could not promote MTT activity and colony forming ability. Irradiation at 6–8 J/cm² even inhibited the proliferation of PDLSCs (Fig. 4c–e). Moreover, after treated with LDN-193189, only the expression of Runx2 was promoted by LLLT at 2 J/cm² (Fig. 5a). Other osteogenic gene levels, ALP activity, and mineralized nodule formation could not be enhanced by LLLT. Irradiation at 6–8 J/cm² significantly reduced ALP activity and mineralized nodule formation of PDLSCs (Fig. 5a, c–e). However, LDN-193189 could not influence the effects of LLLT on the BMP2 gene level (Fig. 5b).

Discussion

This study investigated the effects of LLLT on the proliferation and osteogenic potential of PDLSCs. We treated cells with Nd:YAG laser ranged from 0 to 8 J/cm² and showed for the first time that (1) LLLT could modulate the proliferation and osteogenesis of PDLSCs at 2–6 J/cm²; (2) LLLT at 8 J/cm² could significantly suppress osteogenic differentiation of PDLSCs; (3) LLLT could significantly promote the TNF-α and IL-β1 secretion; (4) LLLT enhances the osteogenic differentiation and mineralization through the activation of the BMP/Smad signaling pathway. These findings provide new insights into the application of laser irradiation, a non-invasive mechanical stimulation that played a substantial role in regulating the bone formation capacity of PDLSCs.

LLLT or rather called photobiomodulation (PBM) therapy has long been recommended to alleviate wound healing because it is a non-invasive therapy with biostimulatory and anti-inflammatory properties [19–21]. Periodontics is a dentistry field that has adopted LLLT in its surgical and nonsurgical treatments as an adjunctive treatment with many successful results. Qadri et al. found low level lasers could significantly reduce probing pocket depth, plaque, and gingival indices and significantly attenuate periodontal inflammation [22]. It has also been demonstrated that LLLT is an effective treatment for periodontal defects. It is reported that LLLT may improve the bone repairing effects of platelet-rich fibrin (PRF) and nanohydroxyapatite alloplast (NanoHA) in induced periodontal intrabony defects in rabbits [23]. Recently, studies showed the bone repair may enhance by LLLT through regulating osteogenesis of stem cells. Study by Santinoni et al. showed LLLT could accelerate bone healing by enhancing angiogenesis, cell proliferation, osteoblast differentiation, and mineralization [24]. Moreover, both proliferation and differentiation of PDLSCs were increased by high-power, red LED irradiation through the involvement of the ERK1/2 pathway [17]. In our study,

![Fig. 3](image_url)  
**Fig. 3** Effects of LLLT on the secretion of TNF-α and IL-β1 in PDLSCs and effects of LLLT on the BMP2/Smad pathway. a The concentration of secreted cytokines TNF-α and IL-β1 in the PDLSCs culture medium was determined by ELISA. P-Smad1/5/8 level was examined by Western blot analysis (b) and scanning densitometer (c). Actin was used as the internal control. Data represent mean ± S.D. *P < 0.05; **P < 0.01; NS, not significant.
we found LLLT (Nd:YAG; 1064 nm) significantly promotes PDLSCs proliferation at 2–8 J/cm² and 6 J/cm² was the most effective energy intensity (Fig. 1). However, the optimal dose in regulating osteogenic differentiation was 4 J/cm². Irradiation at 8 J/cm² even suppresses the bone formation of PDLSCs (Fig. 2). According to the Arndt–Schultz law, the desirable biological reactions must be triggered within a therapeutic window [25]. Doses below that range are not sufficient to make a difference, and doses over that range may have inhibitory effects. Our results indicated that, compared with proliferation, the osteogenesis of PDLSCs was more sensitive to irradiation therapy.

A large body of evidence has demonstrated that LLLT could suppress inflammatory reaction through decreasing the expression of inflammatory cytokines [26]. However, our findings indicate laser irradiation significantly increased the effects of LLLT on the proliferation of PDLSCs. e The colony-forming rate (%) of cells was measured after 7 days of culture at low seeding density. Data represent mean ± S.D. *P < 0.05; **P < 0.01; NS, not significant

Fig. 4 Role of BMP/Smad signaling pathways in LLLT-mediated regulation of proliferation. P-Smad1/5/8 level was examined by Western blot analysis (a) and scanning densitometer (b). Actin was used as the internal control. c, d The MTT was measured for 7 days to show

Fig. 5 Role of BMP/Smad signaling pathways in LLLT-mediated regulation of osteogenesis. a, b The expression levels of the osteogenic genes ALP, Runx2, OCN, and BMP2 were measured by real time PCR at day 7 after osteogenic differentiation induced. β-actin was used as endogenous normalization controls for quantitation of mRNAs. c ALP activity was measured by ALP activity assay at day 7 after osteogenic differentiation induced. d, e Osteogenic differentiation was determined by Alizarin Red S staining at day 21 after osteogenic differentiation induced. Calcium concentration stained by Alizarin Red S discussed above was examined by calcium level analysis. Data represent mean ± S.D. *P < 0.05; **P < 0.01; NS, not significant. The scale bar in the micrographs represents 100 μm
the expression of inflammatory factors TNF-α and IL-1β (Fig. 3). The differences between results suggested that different microenvironment may influence the effects of LLLT treatments. In previous studies, tissue or cells are in inflammatory condition and the expression of inflammatory cytokines is at high rate. In this research, PDLSCs were healthy and expressed TNF-α and IL-1β less. LLLT triggers low level inflammation of PDLSCs as a type of external stimulation. C-y Mao et al. found low level of IL-1β activates the BMP/Smad signaling pathway to promote the osteogenesis of PDLSCs, but higher doses of IL-1β inhibit BMP/Smad signaling through the activation of nuclear factor-κB (NF-κB) and mitogenactivated protein kinase (MAPK) signaling, inhibiting osteogenesis [27]. Considering these findings, we assume IL-1β and BMP/Smad pathway play an essential role in osteogenic regulation.

BMP2 is an important growth factor of osteogenic differentiation. BMP2 signals have been reported to work through activating BMP2/Smad signaling pathway. A growing body of literature has shown that BMP2/Smad signaling is closely implicated in regulation of proliferation and osteogenic differentiation in human PDLSCs [28, 29]. This signaling can be influenced by inflammatory factors, including TNF-α and LPS [30, 31]. Our findings show that LLLT significantly increase the expression of BMP2 in PDLSCs and promote the activation of the BMP/Smad signaling pathway (Fig. 3). When we inhibited Smad signaling by LDN-193189, the effects of irradiation on proliferation (Fig. 4) and osteogenic differentiation (including ALP activity, mineralized nodule formation, and gene level of ALP and OCN) were attenuated (Fig. 5). However, we found LLLT at 2 J/cm² could significantly increase the gene level of Runx2; this effect was not completely canceled by LDN-193189 (Fig. 5b). Some studies have indicated that BMP/Smad signaling can function independent of Runx2 [27, 32]. LLLT may regulate Runx2 through other mechanisms which need further investigation to be proven.

In conclusion, the present study established that LLLT at optimal energy intensity upregulates osteogenesis and the proliferation of PDLSCs. The underlying mechanism may involve BMP/Smad signaling. Further animal studies are required to verify the function and safety of LLLT in promoting the osteogenesis capacity of PDLSCs in vivo.

Conclusion

In conclusion, our study demonstrated for the first time that LLLT could significantly promoted PDLSCs osteogenic differentiation and proliferation at optimal energy intensity via BMP2/Smad signaling pathways in vitro. These findings may provide new therapeutic strategies for periodontitis and periodontal regeneration.

Author contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Liying Wang and Chen Liu. The first draft of the manuscript was written by Fan Wu, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Ethics approval This article does not contain any studies with human participants performed by any of the authors. Human cancellous bone fragments and blood were collected based on the treatment needs unrelated to this study. Each donor provided written informed consent. All procedures performed in studies were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Hospital’s Ethics Committee (license no. xjkqll[2020]NO.028).

Competing interests The authors declare no competing interests.

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