

Original Article

Effects of different powers and the duration of Er:YAG laser therapy on the early osteogenic differentiation of human periodontal ligament cells

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Abstract: To compare the effects of erbium-doped yttrium aluminum garnet laser (Er:YAG) at a wavelength of 2,940 nm under different powers and durations on the early osteogenesis of human periodontal ligament cells (hPDLCs), the fourth passage of hPDLCs was exposed to the Er:YAG laser for the same time period using different irradiation powers (0 W, 0.45 W, 0.6 W, and 0.75 W for 10 s) or the same irradiation power but for different time periods (0 s, 10 s, 30 s, and 60 s at 0.6 W). The hPDLCs were then cultivated under common conditions and conditions for osteogenic differentiation. Western blot was used to determine the expression of alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx2) and osteocalcin (OCN) after 3, 5 and 7 days during early osteogenic differentiation. Comprehensively considering the test results under the two conditions, when the power of 0.45 W and 0.6 W were used with an irradiation time of 10 s each, the expression of the bone formation-related proteins ALP and Runx2 was higher than that in cells not irradiated by the laser ($P < 0.05$) on days 3, 5, and 7. The expression reached the maximum value on day 5. OCN expression was higher than that of cells not irradiated by the laser ($P < 0.05$) on days 3, 5, and 7 and reached a maximum value on day 7. It was observed in this study that The Er:YAG laser can promote the early osteogenic differentiation of hPDLCs at an appropriate irradiation level and duration.

Keywords: Durations, early osteogenesis, ER:YAG laser, hPDLCs, irradiation powers

Introduction

Periodontitis is a chronic infectious disease characterized by periodontal pocket formation and alveolar bone loss, which subsequently results in the destruction of periodontal support tissues. Periodontal therapy intends to control infection and reconstructs damaged soft and hard tissues [1, 2]. Currently, routine periodontal therapy is effective in controlling infection, but it is difficult to create ideal regenerating conditions for periodontal soft and hard tissues. Human periodontal ligament cells (hPDLCs) are a group of tissue cells with the characteristics of stem cells. They have the potential for multiple types of differentiation, including osteogenic differentiation, and play an important role in the regeneration of peri-

odontal supporting tissues, which comprise the periodontal ligament, alveolar bone and cementum [3]. Early osteogenic differentiation produces critical effects during osteogenesis when induced by periodontal ligament cells, and decides the final outcomes of differentiation and maturation of osteoblasts as well as new bone formation at late phase. It is believed that early expression of alkaline phosphatase (ALP) during osteogenic differentiation is the early marker for maturation of extracellular matrix [4, 5]. Osteocalcin (OCN) expression continues to increase, peaks in late phase of osteogenic differentiation and promotes hydroxyapatite sedimentation in extracellular matrix [6, 7]. As the specific transcription factor for osteogenesis, runt-related-transcription factor 2 (Runx-2) exerts effects at early phase of osteogenic differ-

entiation. Various osteogenesis related signals can directly or indirectly act on this critical point, thus regulating expression of the downstream target gene [8, 9]. Therefore, detection of changing trend of osteogenesis related parameters in early osteogenic differentiation is helpful for us to learn about osteogenic differentiation of periodontal ligament cells and the trend of osteogenic differentiation in late phase.

The 2940-nm erbium-doped yttrium, aluminum and garnet (Er:YAG) laser is one of the lower-powered lasers that has been extensively utilized in periodontal therapies, as it can be easily absorbed by water and causes less thermal injury to adjacent tissues [10, 11]. This laser has desirable hemostatic effects, presents a good aseptic technique, allows for clean tissue cutting and offers pain relief [11, 12], and has the potential to promote new bone formation [13]. However, very few studies have determined whether the Er:YAG laser can affect the osteogenic differentiation of periodontal ligament cells. This study intended to investigate the effects of the Er:YAG laser on the early osteogenic differentiation of periodontal ligament cells cultivated in vitro under different irradiation powers and duration. This study thus sought to provide a reference point for the future selection of an appropriate irradiation power and duration for periodontal laser therapy in clinical practice.

Materials and methods

Main reagents and materials

hPDLs (human periodontal ligament cells) were purchased from ScienCell Inc. (Carlsbad, CA, USA). Other materials were used as follows: α -Minimum Essential Medium (MEM) (Gibco, Grand Island, NY, USA), fetal bovine serum (HyClone, South Logan, UT, USA), 0.25% trypsin-EDTA (Gibco, Grand Island, NY, USA), Penicillin-Streptomycin Solution (containing 10000 U/mL and 10000 μ g/mL of penicillin-streptomycin) (HyClone, South Logan, UT, USA), dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), vitamin C (BioSharp, Hefei, China), β -Glycerophosphate disodium (BioSharp, Hefei, China), dimethyl sulfoxide (DMSO) (Ameresco, Solon, OH, USA), RIPA protein lysis solution (Beyotime, China), protein marker (Fermentas, Burlington, Ontario, Canada), SDS-PAGE gel kit

(KeyGEN, China), β -ACTIN antibody (Bioworld, St. Louis Park, MN, USA), RUNX2 antibody (Bioworld, St. Louis Park, MN, USA), OCN antibody (Bioworld, St. Louis Park, MN, USA), Fotona double wave laser (Fidelis, Slovenia), ImageQuant LAS 4000 gel imaging system (GE Healthcare, USA), and conventional instruments for experimental cell biology.

Cell culture and laser irradiation

The fourth passage of human periodontal ligament cells (hPDLs) was used in this study. hPDLs were inoculated into a 60-mm culture dish at a density of 3×10^5 /dish, and the culture solution was discarded after 60% of cells fused. Then, under different laser powers for the same time period (0 W, 0.45 W, 0.6 W, 0.75 W; 10 s) or for different time periods under the same laser power (0 s, 10 s, 30 s, 60 s; 0.6 W), the 2940-nm erbium-doped yttrium, aluminum and garnet (Er:YAG) laser were used to irradiate at a distance of 5 mm from the bottom of the dish.

Osteogenic differentiation

After irradiation, common culture medium (medium A, MA) with α -MEM, 10% fetal bovine serum and 1% Penicillin-Streptomycin Solution and culture medium for osteogenic differentiation (medium B, MB) with α -MEM, 10% fetal bovine serum, 1% Penicillin-Streptomycin Solution, 10^{-7} mol/L dexamethasone, 50 mg/L vitamin C and 10 mM β -Glycerophosphate disodium were used to culture cells for 3, 5 and 7 days, respectively. Culture medium was discarded at different times, then flushed with pre-cooled PBS two consecutive times. RIRP lysis solution was used for cell lysis to extract total proteins.

Western blot test

A polyacrylamide gel was prepared (4% concentrated gel, 10% separating gel); 30 μ g/channel were injected, and the gel was developed at a constant voltage of 60 V until bromophenol blue reached the bottom. The gel was then stripped under a constant current of 300 mA to transform the PVDF membrane. Further, milk was used for blocking for 2 h; first, the antibodies of alkaline phosphatase (ALP), osteocalcin (OCN), runt-related-transcription factor 2 (Runx2) and β -actin were blocked and maintained overnight at 4°C. The temperature returned to the origi-

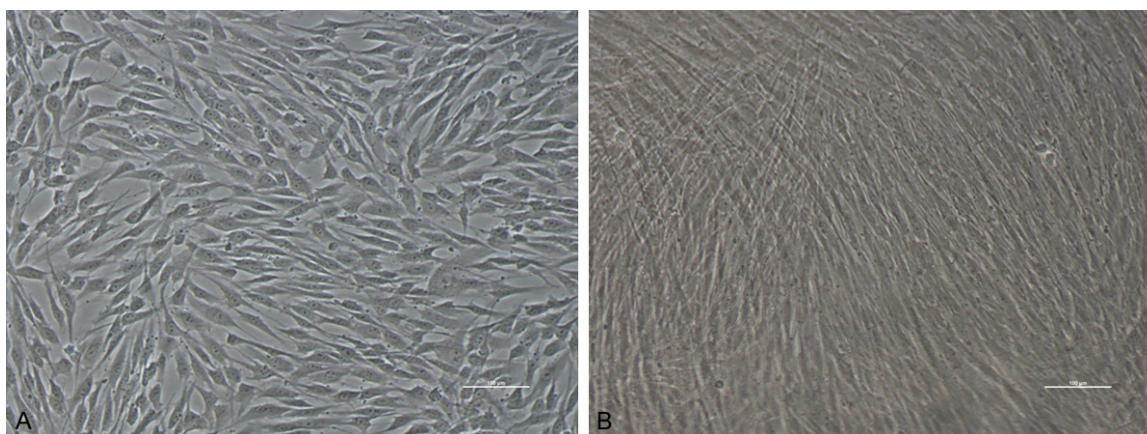


Figure 1. A: Morphologies of periodontal ligament cells of the fourth generation ($\times 100$); B: Morphologies of periodontal ligament cells on the 7th day of osteogenic induction ($\times 100$).

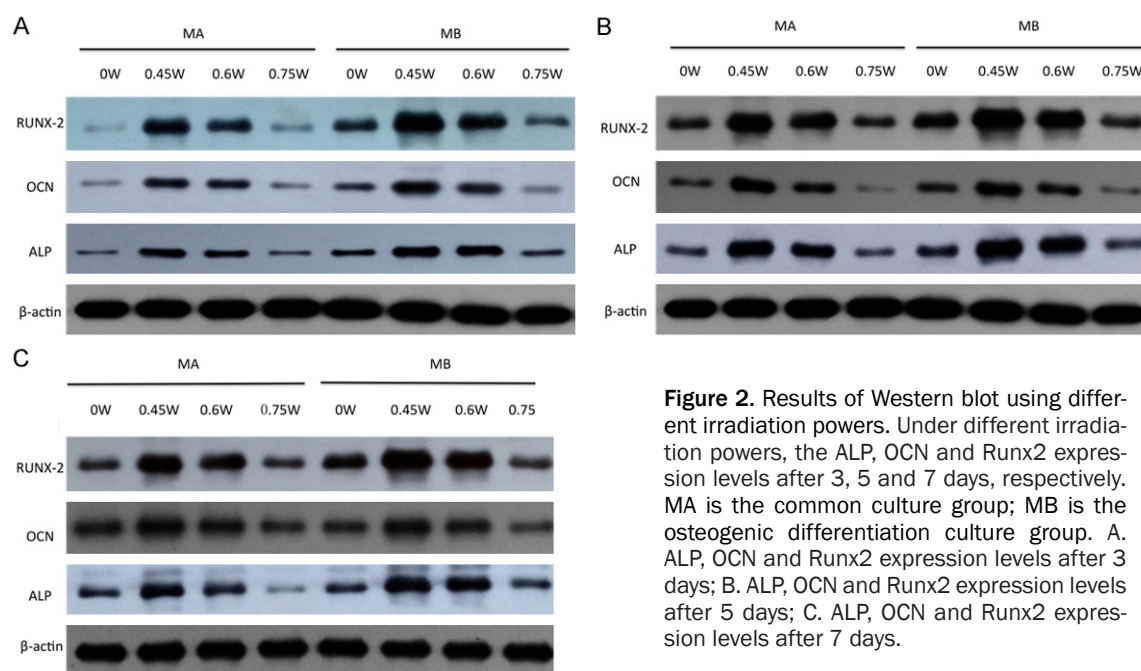


Figure 2. Results of Western blot using different irradiation powers. Under different irradiation powers, the ALP, OCN and Runx2 expression levels after 3, 5 and 7 days, respectively. MA is the common culture group; MB is the osteogenic differentiation culture group. A. ALP, OCN and Runx2 expression levels after 3 days; B. ALP, OCN and Runx2 expression levels after 5 days; C. ALP, OCN and Runx2 expression levels after 7 days.

nal level for 1 h the next day, and the membrane was washed at least 5 times with PBST. A secondary antibody was added and incubated for 1 h at room temperature. An enhanced chemiluminescent agent was added, and the IMAGE gel imaging system was used for exposure and imaging. A Gel-Pro Analyzer was used for grey value analysis. All experiments were repeated at thrice.

Data analysis

All Datas above are presented as means \pm SD. The SPSS18.0 statistical software was used for

statistical analysis. After the test of normality and homogeneity of variance (the homogeneity test level $\alpha=0.05$), one-way ANOVA analysis was used to evaluate statistical significance of differences between two groups. $P<0.05$ was considered statistically significant.

Results

Culture of periodontal ligament cells and early osteogenic differentiation

After periodontal ligament cells were recovered with common culture media and passaged to

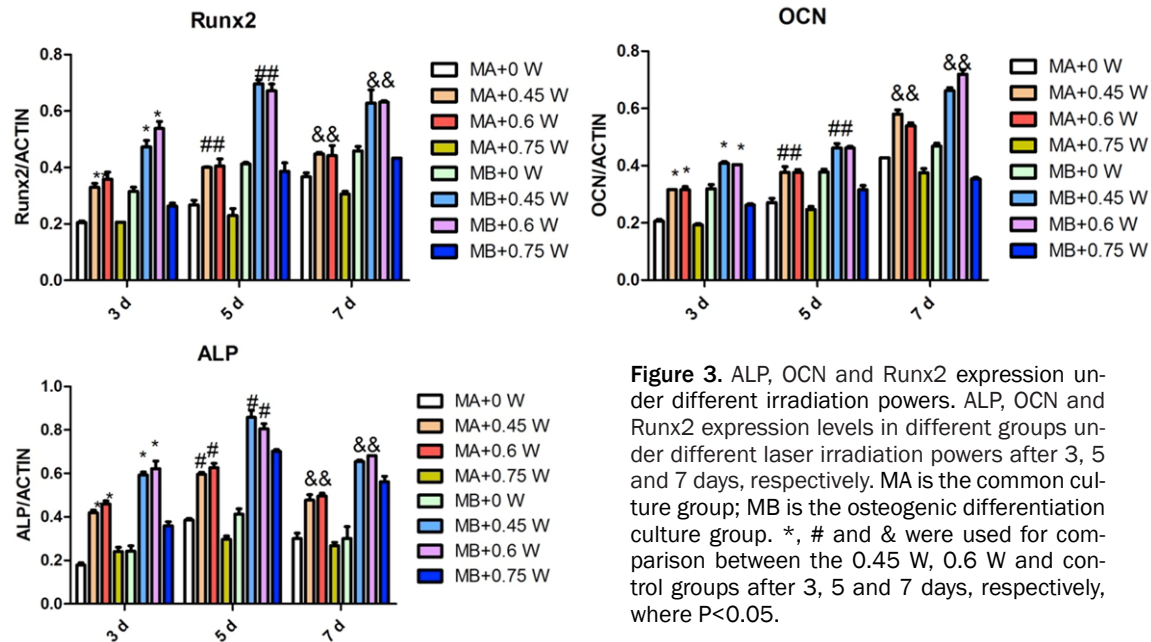


Figure 3. ALP, OCN and Runx2 expression under different irradiation powers. ALP, OCN and Runx2 expression levels in different groups under different laser irradiation powers after 3, 5 and 7 days, respectively. MA is the common culture group; MB is the osteogenic differentiation culture group. *, # and & were used for comparison between the 0.45 W, 0.6 W and control groups after 3, 5 and 7 days, respectively, where $P < 0.05$.

the fourth generation, microscopic findings showed good cell growth. Cells were elongated with typical fibroblast morphology and grew in a spiral shape with 70%-80% cells fused (**Figure 1A**). After osteogenic differentiation, medium was changed and cells were induced till the 7th day. Microscopic findings showed completely fused cells densely grown, elongated and mesh stacked, while no experiment group had obviously mineralised nodules (**Figure 1B**).

Effects of the 2940-nm erbium-doped yttrium, aluminum and garnet (Er:YAG) laser under different powers and the same time period on the early osteogenic differentiation of human periodontal ligament cells (hPDLCs)

With 0.45 W and 0.6 W irradiation power and 10 s duration, protein expressions associated with osteogenic regeneration, including those of alkaline phosphatase (ALP) and runt-related transcription factor 2 (Runx2), were higher than those in the group not receiving laser irradiation (control group). This was observed 3, 5 and 7 days later ($P < 0.05$), reaching a maximum value 5 days later. osteocalcin (OCN) expression was higher than that of the group that did not receive laser irradiation (control group) 3, 5 and 7 days later, and reached maximum value 7 days later. The expression of ALP, Runx2 and OCN in the osteogenic differentiation culture group was higher than that in the common cul-

ture group under the same conditions (**Figures 2, 3**).

Effects of the Er:YAG laser at different durations and the same power on the early osteogenic differentiation of hPDLCs

When the irradiation duration was 10 s and the power was 0.6 W, proteins associated with osteogenic expressions, including ALP and Runx2, were higher than those in the group not irradiated with the laser (control group) 3, 5 and 7 days later, where ($P < 0.05$), reaching a maximum value 5 days later. OCN expression was higher than that of the control group 3, 5 and 7 days later ($P < 0.05$) and attained a maximum value 7 days later. Under similar conditions, ALP, Runx2 and OCN expressions in the osteogenic differentiation culture group were higher than those in the common culture group (**Figures 4, 5**).

Discussion

Choi et al. were the first to study the effects of a low-dose laser on the osteogenic potential of fibroblasts obtained from human gingiva. They concluded that laser therapy has the capacity to promote fibroblast proliferation in human gingiva [14]. Zhao Y et al. demonstrated in clinical studies that the erbium-doped yttrium aluminum garnet (Er:YAG) laser can be combined

Effects of Er:YAG laser on the osteogenic differentiation of hPDLs

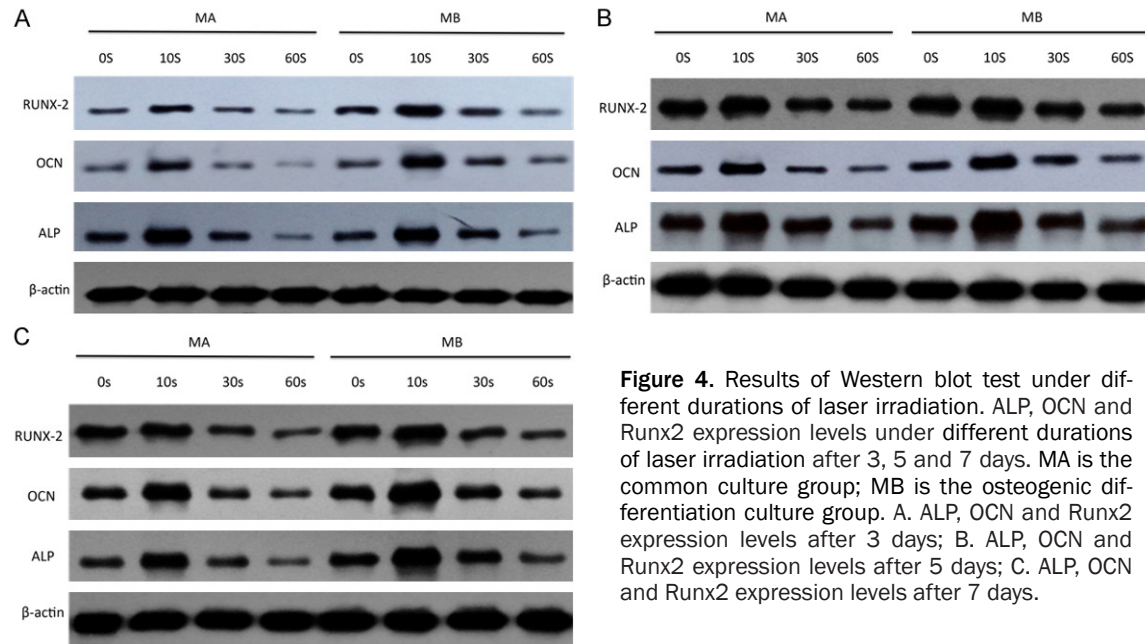


Figure 4. Results of Western blot test under different durations of laser irradiation. ALP, OCN and Runx2 expression levels under different durations of laser irradiation after 3, 5 and 7 days. MA is the common culture group; MB is the osteogenic differentiation culture group. A. ALP, OCN and Runx2 expression levels after 3 days; B. ALP, OCN and Runx2 expression levels after 5 days; C. ALP, OCN and Runx2 expression levels after 7 days.

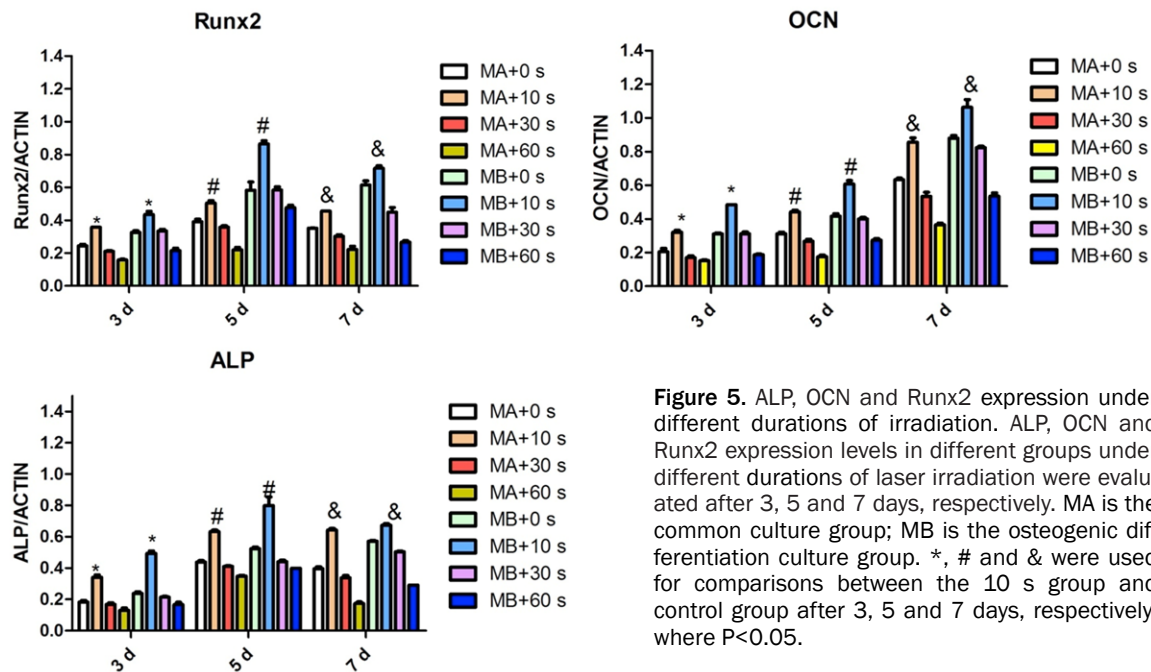


Figure 5. ALP, OCN and Runx2 expression under different durations of irradiation. ALP, OCN and Runx2 expression levels in different groups under different durations of laser irradiation were evaluated after 3, 5 and 7 days, respectively. MA is the common culture group; MB is the osteogenic differentiation culture group. *, # and & were used for comparisons between the 10 s group and control group after 3, 5 and 7 days, respectively, where $P < 0.05$.

with scaling and root planing to treat chronic periodontitis with certain therapeutic benefits [15]. Fawad Javed et al. have found in their study that Nd:YAG laser-assisted non-surgical periodontal therapy is more effective in reducing periodontal inflammatory than non-surgical periodontal therapy alone [16]. However, there are few studies on whether the Er:YAG laser can promote the osteogenic differentiation of

hPDLs during periodontal therapy. To demonstrate this, Er:YAG lasers at different powers and the same duration or different durations and the same irradiation power were used to treat hPDLs. alkaline phosphatase (ALP), osteocalcin (OCN), and runt-related-transcription factor 2 (Runx2), which are closely related with osteogenic differentiation, were selected for analysis, and their protein expression levels

were detected in the early osteogenic differentiation of hPDLs. ALP and Runx2 are expressed in early osteogenesis, while OCN is expressed later in osteogenesis [17]. With reference to available experiments and studies on Er:YAG lasers and by combining the results of preliminary experiments in the early phase, a power output of 0-1 W and irradiation duration of 0-60 s were selected as the reference range for parameters under formal experimental conditions.

Alkaline phosphatase (ALP) is closely related to bone calcification and can produce phosphoric acid via dephosphorylation and calcium phosphate with calcium. ALP has an important effect in the early phase of osteogenic differentiation, and ALP levels in periodontal ligament cells are considered markers for early osteogenic differentiation [4]. Studies indicate that hPDLs can express ALP in vitro [5], and the capacity of periodontal ligament cells' osteogenic differentiation can be reflected by determining ALP activity [4]. runt-related- transcription factor 2 (Runx2) is a specific osteoblast transcription factor that has major regulatory effects on the differentiation of osteoblasts. It is one of the early parameters for the osteogenic differentiation of cells and is critical for bone formation [5, 6]. Osteocalcin (OCN) is one of the γ -carboxyglutamic acid-containing proteins and is a non-collagenous protein in the extracellular matrix. It is composed of 49 amino acids, mainly excreted during the late phase of osteogenic differentiation [7, 8]. OCN is the marker for osteoblast maturity and has important effects on bone metabolism.

The results indicated that when different laser irradiation power and the same irradiation duration are used, ALP, OCN and Runx2 expression was higher in the 0.45 W and 0.6 W groups than in the group that did not receive laser irradiation (control group) ($P < 0.05$); the expression was slightly reduced in the 0.75 W group ($P > 0.05$). ALP and Runx2 attained their maximal expression levels 5 days after irradiation, while OCN reached the maximum expression 7 days later. In this study, the osteogenic expression of the associated protein was reduced in the group exposed to 0.75 W without any obvious statistical significance. However, this study had only 4 different power sets, which were not increased further. If the laser power is too high,

it may have inhibitory effects on osteogenic differentiation of hPDLs; this needs to be proven in further studies.

When different irradiation durations and the same power are used, a 10-s duration is ideal to promote ALP, OCN and Runx2 expression ($P < 0.05$). An irradiation duration of 60 s can inhibit ALP, OCN and Runx2 expression ($P < 0.05$), which indicated that appropriate laser irradiation can promote the osteogenic differentiation of hPDLs, but excessive irradiation exposure may inhibit osteogenic differentiation. This suggests that irradiation parameters, especially with respect to duration, must be established appropriately in clinical practice.

Chen YJ et al. have demonstrated that Nd:YAG laser with lower pulse energy and corresponding pulse rate should be selected to minimize the damage on adjacent soft tissue [18]. Jyun YW et al. found that irradiation by a low-power diode laser can promote the proliferation and osteogenic differentiation of hPDLs [19]. Previously, Saracino S et al. demonstrated that the low-power laser can promote osteogenesis-related marker expression in osteoblasts, such as ALP and OCN [20]. Huang TH et al. found that the low-power diode laser can promote the proliferation and osteogenic differentiation of hPDLs [21]. The study results mentioned above are related to this study's results to a certain extent.

In this study, the expression levels of the 3 proteins in the osteogenic differentiation culture group were higher than those in the common cell culture group under the same conditions, with statistically significant differences. The reason may be that osteogenic differentiation culture medium can increase the activity of alkaline phosphatase and induce the osteogenic differentiation of many cells, including periodontal ligament cells [22]. Therefore, the osteogenic differentiation culture group may provide better conditions for the osteogenic differentiation of hPDLs. These can be considered as routine culture conditions in future studies on osteogenic induction.

This study compared the effects of the Er:YAG laser on the early osteogenic differentiation of hPDLs with respect to irradiation power and duration. It was observed that by choosing different irradiation powers and durations, the

Er:YAG laser can affect the osteogenic differentiation of hPDLs. In this study, the output powers of 0.45 W and 0.6 W with an irradiation time of 10 s were the most favorable for the early osteogenic differentiation of hPDLs. This suggests that the irradiation output power and duration cannot be increased randomly during clinical procedures, such as the removal of dental plaque and tartar. However, it is necessary to remove dental plaque and tartar prior to periodontal therapy. Available results from clinical studies in recent years suggest that manual or ultrasonic instruments can be used to perform Er:YAG laser therapy in basic and supporting periodontal therapies [23, 24], to optimally promote the osteogenic differentiation of hPDLs in affected regions.

This study's results provide a reference point to select an appropriate irradiation output power and duration for periodontal therapy with lasers. All output powers and durations were not tested in this study. Additionally, further animal studies may be required to validate this study's results because the osteogenic differentiation of cells is different in vivo than in vitro. The osteogenic differentiation of cells is a complicated process, involving many signaling pathways and signaling molecules. Therefore, future studies may be required to further investigate the specific mechanism of the Er:YAG laser in promoting the osteogenic differentiation of cells.

Conclusion

The Er:YAG laser can promote the early osteogenic differentiation of hPDLs at an appropriate irradiation level and duration.

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Disclosure of conflict of interest

None.

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