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In Vitro Photobiomodulation Effects of Blue and Red Diode Lasers on Proliferation and Differentiation of Periodontal Ligament Mesenchymal Stem Cells

Ferena Sayar[1], Ahmad Garebigloo[2], Sogol Saberi[3*], Ardavan Etemadi[1*]

[1]Department of Periodontics, Faculty of Dentistry, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran
[2]Tehran University of Medical Sciences, Tehran, Iran
[3]Laser Research Center of Dentistry, Dentistry Research Institute, Tehran University of Medical Sciences, Tehran, Iran

***Correspondence to**
Sogol Saberi
Emails: S-saberi@razi.tums.ac.ir, Saberisogol@gmail.com;
Ardavan Etemadi,
Email: a-etemadi@farabi.tums.ac.ir

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Abstract

Introduction: This study aimed to assess the photobiomodulation effects of blue and red lasers on the proliferation and osteogenic differentiation of periodontal ligament mesenchymal stem cells (PDLMSCs).

Methods: PDLMSCs were cultured and tested in 4 groups. The first two groups were exposed to 445 nm diode laser irradiation (200 mW, 6 and 12 J/cm^2), and the third group was exposed to 660 nm diode laser irradiation (50 mW, 4 J/cm^2). The fourth group was also considered as the control group without irradiation. Cell viability/proliferation was assessed by MTT assay. RUNX2, alkaline phosphatase (ALP), collagen type 1 (col1), and osteocalcin (OCN) were evaluated by RT-PCR, and Alizarin red was used to evaluate the colonization. The data were analyzed by means of one-way analysis of variance.

Results: The results of our study showed that cell survival/proliferation in the second group was significantly lower than that in the control group on days 1 and 7 ($P<0.05$). RT-PCR showed a significant increase in osteogenic genes in all three laser groups compared to the control group ($P<0.05$). All groups showed a significant increase in calcium content compared to the control group ($P<0.05$). ALP activity also confirmed the osteoblastic differentiation of cells in laser groups.

Conclusion: 445 nm and 660 nm lasers with the studied parameters showed positive effects on the proliferation and osteoblastic differentiation of PDLMSCs.

Keywords: Low-level light therapy; Lasers; Cell differentiation; Mesenchymal stem cells.

Introduction

Alveolar bone resorption is one of the primary pathological approaches in periodontal disease and peri-implantitis.[1] Therefore, the prevention of bone loss and the induction of tissue regeneration are among the main treatments for this disease because the lack of treatment causes further complications of the disease.[1]

Currently, reconstructive surgery and bone grafting methods are used to replace the lost bone structure. However, these treatments have limitations such as the risk of tissue necrosis.[2] Therefore, efforts are ongoing to find alternative methods for tissue engineering to overcome such limitations.[3]

Low-level laser therapy (LLLT) is a new auxiliary technique in tissue engineering.[4] The effect of LLLT on the proliferation and differentiation of mesenchymal stem cells (MSCs) has been previously investigated. However, the results of the studies have been widely controversial.[5-8] Borzabadi-Farahani [9] and Mylona et al[10] confirmed the positive effects of LLLT on the proliferation of human MSCs in a systematic review. However, another systematic review conducted by Marques et al[11] showed no significant effect of LLLT on the proliferation and differentiation of MSCs. Another study on the effects of different laser wavelengths on MSCs showed that red and infrared laser wavelengths had a positive effect on the proliferation and bone differentiation of MSCs, while the blue laser did not show such an effect.[12] Yuan et al[13] showed that the blue laser had an inhibitory effect on the proliferation and differentiation of MSCs derived from bone marrow. However, Kushibiki and Awazu[14] showed the positive effects of blue laser irradiation on the differentiation of MSCs. Due to these different results, researchers have not been able to establish specific guidelines for LLLT, and further studies on this topic are necessary. Since such characteristics as wavelength, energy density, and beam spot size may affect the results, reviewing such studies can be effective. Regarding the energy density, the higher energy densities have been less investigated in studies. Concerning the wavelength, since not many studies have been done on the 445 nm wavelength and the available studies also differed, this study was conducted to investigate the effects of red and blue laser photobiomodulation on the osteogenic

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differentiation of periodontal ligament mesenchymal stem cells (PDLMSCs).

Materials and Methods

In this in vitro, experimental study, human PDLMSCs (Ibrc-c11326) were obtained from the cell bank of Iranian Stem Cell Organization.

According to a study by Zhu et al[1] (alpha = 0.05, beta = 0.2, mean standard deviation of 0.65, and effect size of 0.86), the sample size for reverse-transcription polymerase chain reaction (RT-PCR) was calculated to be 6 for each of the four groups, and for the methyl thiazolyl tetrazolium (MTT) assay, it was calculated to be 9 in each of the four groups (alpha = 0.05, beta = 0.2, mean standard deviation of 0.12, and effect size of 0.62) using one-way ANOVA power analysis of PASS 11.

Cell Isolation and Culture

Human PDLMSCs were cultured in DMEM (Dulbecco's modified Eagle's medium) with 10% FBS (fetal bovine serum), 100 µg/mL streptomycin, 100 U/mL penicillin, 2.5 µg/mL amphotericin B, and 2 mmol/L glutamine and incubated at 37 °C and 5% CO2. After reaching 80% confluence, the cells were detached from the bottom of the flask using trypsin/EDTA and passaged.

Laser Irradiation Protocol

The cell culture plates were randomly divided into three experimental groups for laser irradiation and one control group without laser irradiation. The first and second experimental groups were subjected to irradiation of 445 nm blue diode laser (Siroblue laser, Dentsply Sirona, Germany) with 200 mW power and 6 and 12 J/cm^2 energy density for 15 and 30 seconds; however, the third experimental group underwent 660 nm red diode laser irradiation (Siroblue laser, Dentsply Sirona, Germany) with power of 50 mW and energy density of 4 J/cm^2 for 40 seconds. The handpiece tip diameter was 8 mm.

Evaluation of Cell Viability and Proliferation by the MTT Assay

We performed the MTT assay on days 1, 4, and 7 after laser irradiation to investigate cell viability and proliferation. MTT solution (5 mg/mL) was added to the cells after changing their culture medium, and they were incubated for 3 hours. DMSO (dimethyl sulfoxide) was added to dissolve the formed formazan crystals, and the obtained solution was read at a 570 nm wavelength. The color intensity has a direct correlation with cell viability and proliferation. Accordingly, the color intensity was read by using a standard reference.

Assessment of Cell Differentiation

After reaching adequate confluence, PDLMSCs were passaged and removed from the culture medium, and the osteogenic medium containing dexamethasone, ascorbic acid, and beta-glycerophosphate was added to the cells. The cells were incubated at 37 °C and 5% CO2.

The standard real-time RT-PCR was used to assess the expression of osteogenic markers (RUNT-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), collagen type 1 (col1), and osteocalcin (OCN) by PDLMSCs) after 7 and 14 days. For this purpose, the total RNA was first extracted, and cDNA was synthesized. After ensuring the optimal quality of cDNA, specific primers were added, mixed with SYBR Green, and subjected to RT-PCR. The data were analyzed by REST software, and GraphPad Prism software was used for the final analysis.

DMEM supplemented with 10% fetal bovine serum, 10 nm dexamethasone, 35 µg/mL ascorbic acid, and 1 mM beta-glycerophosphate was used as an osteogenic medium in order for us to assess osteogenic differentiation. The cells were transferred to the wells, and DMEM supplemented with 10% fetal bovine serum was also added. After reaching adequate density, the osteogenic medium was added to the cells. The cells were exposed to the osteogenic medium for 14 days. The medium was refreshed every 3 days.

Alizarin Red Staining

The cells were rinsed with PBS and were then fixed with 2.5% glutaraldehyde in order for us to assess the mineralization and formulation of calcified nodules after 14 days of culture. They were rinsed again with PBS and stained with 2% alizarin red (pH of 4.2 to 4.4) for 5 minutes at 37°C. The stained areas were photographed under an inverted microscope.

Assessment of ALP Activity

ALP activity was assessed by the evaluation of the degradation of pyrophosphates in vitro. After the cells were rinsed, they were placed in the lysis buffer and centrifuged at 12 000 rpm for 10 minutes. ALP activity in lysed cells was measured as a substrate in the presence of nitrophenol phosphate using the ALP measurement kit. The optical density was measured at a 405 nm wavelength. The test was repeated in all groups after 7 and 14 days.

Measuring the Amount of Calcium

The cells were rinsed with phosphate-buffered saline and lysed with 6 N HCl. Next, they were centrifuged at 12 000 rpm for 10 minutes. A calcium measurement kit was used for this purpose. Calcium forms a purple complex in the presence of cresolphthalein. The intensity of the final color depends on the amount of calcium.

Statistical Analysis

The data were analyzed by one-way ANOVA using SPSS version 22 (IBM Corp., Armonk, NY, USA). All experiments were repeated at least 3 times and the level of

statistical significance was defined as P value < 0.05.

Results

Results of MTT Assay

The percentage of cell viability and proliferation in four groups on days 1, 4, and 7 is shown in Figure 1. On day 1, the viability/proliferation of PDLMSCs subjected to 445 nm blue laser/12 J/cm² energy density was significantly lower than the control group (P < 0.05). On day 4, no significant difference was noted in the cell viability of the experimental and control groups (P > 0.05). On day 7, the percentage of cell viability in the 445 nm blue laser/12 J/cm² group was significantly lower than in the control group (P = 0.002).

Results of RT-PCR

The expression of four genes related to osteogenic differentiation was evaluated.

OCN (Figure 2): On day 7, the expression of OCN in all laser groups was significantly higher than that in the control group (P = 0.000). A comparison of laser groups with each other showed that blue laser/6 J/cm² had significantly lower expression of OCN than blue laser/12 J/cm² (P = 0.002) and red laser (P = 0.000) groups. No significant difference existed between blue laser/12 J/cm² and red laser (P = 0.907). However, the latter two groups showed higher expression of OCN than the control group (P < 0.05). On day 14, the expression of OCN in all laser groups was significantly higher than that in the control group (P = 0.000). A comparison of laser groups with each other showed maximum expression of OCN in red and blue laser/12 J/cm² groups, with no significant difference with each other (P = 0.760). The latter two groups indicated significantly higher expression of OCN than blue laser/6 J/cm² and control groups (P = 0.000).

ALP (Figure 3): On day 7, the expression of ALP in red and blue laser/12 J/cm² groups was significantly higher than that in the control group (P = 0.000). The difference in ALP expression was not significant between the blue laser/6 J/cm² group and the control (P = 0.760) and red laser (P = 0.220) groups. However, ALP expression in the blue laser/12 J/cm² group was significantly higher than that in the red laser group (P = 0.000). On day 14, ALP expression in all laser groups was significantly higher than that in the control group (P < 0.05). The difference between the red laser group and the blue laser/12 J/cm² group was not significant in ALP expression (P = 0.089), but they showed higher ALP expression than other groups.

RUNX2 (Figure 4): On day 7, the expression of RUNX2 in the red and blue/12 J/cm² groups was significantly higher than that in the control group (P = 0.000). However, its expression in blue laser/6 J/cm² was not significantly different from that of the control group (P = 0.940). No

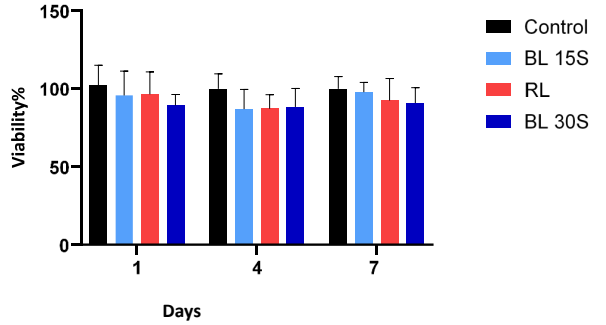

Figure 1. Evaluation of PDLMSCs Proliferation After Laser Irradiation on Days 1,4 and 7. (BL: Blue Laser, RL: Red Laser)

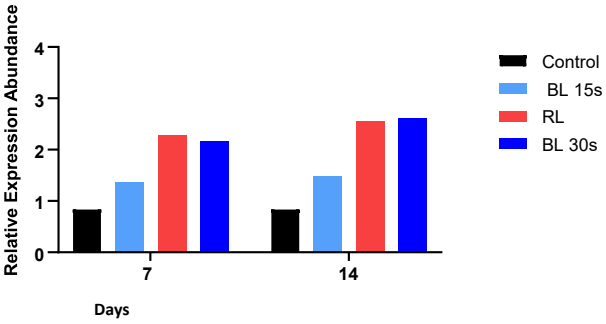

Figure 2. Osteocalcin Gene Expression After Different Laser Irradiations During Osseous Differentiation on DAYS 7 and 14 (BL: Blue Laser, RL: Red Laser). BL and R laser groups had significant differences compared to control groups (P value < 0.05)

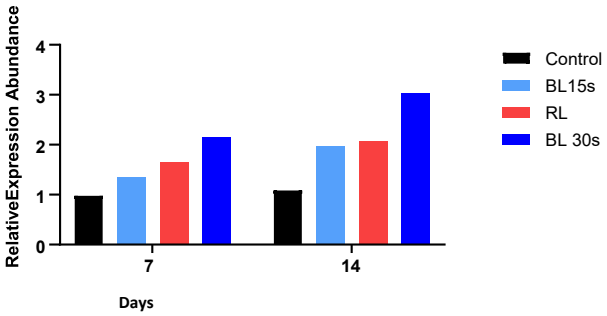

Figure 3. Alkaline Phosphatase Gene Expression After Different Laser Irradiations During Osseous Differentiation on Days 7 and 14 (BL: Blue Laser, RL: Red Laser). On day 14, ALP expression in all laser groups was significantly higher than that in the control group (P < 0.05)

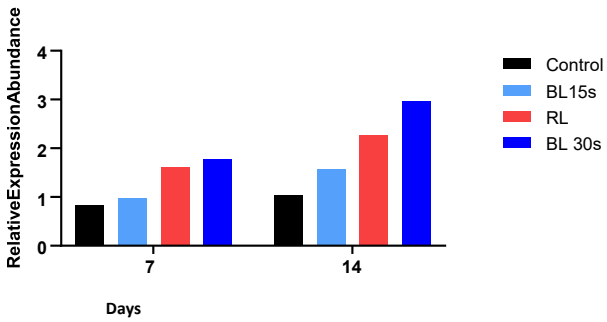

Figure 4. Runt-Related Transcription Factor 2 Gene Expression After Different Laser Irradiations During Osseous Differentiation on Days 7 and 14. (BL: Blue Laser, RL: Red Laser). On day 14, the expression of RUNX2 in all laser groups was significantly higher than that of the control group (P < 0.05)

significant difference was noted between the red laser group and the blue laser/12 J/cm² group ($P=0.220$), and they both showed significantly higher expression of RUNX2 than other groups ($P<0.05$). On day 14, the expression of RUNX2 in all laser groups was significantly higher than that of the control group; the expression of RUNX2 in the red laser and blue laser/12 J/cm² groups was higher than that in other groups. All laser groups had significant differences with each other in this parameter ($P=0.000$).

COL1 (Figure 5): On day 7, the expression of COL1 in all laser groups was significantly higher than that in the control group ($P=0.000$). Significant differences were also noted between all laser groups ($P=0.000$). On day 14, the expression of COL1 in the blue laser/12 J/cm² group was significantly higher than that in the control group ($P=0.000$). No other significant differences were noted ($P>0.05$).

Assessment of the Amount of Calcium

The amount of calcium was evaluated in all four groups on days 7 and 14 (Table 1). On day 7, the amount of calcium in the blue laser/12 J/cm² group was significantly higher than that in the control group and the other two laser groups ($P=0.000$). No other significant differences were noted ($P>0.05$). On day 14, all laser groups showed a significantly higher amount of calcium than the control group ($P<0.05$). The difference between the red laser group and the blue laser/6 J/cm² group was not significant in this regard ($P=0.530$). The blue laser/12 J/cm² group also showed a significantly higher calcium level than the blue laser/6 J/cm² ($P=0.003$) and red laser ($P=0.000$) groups.

ALP Activity

Table 2 presents ALP activity in the groups on days 7 and 14. On day 7, ALP activity in the blue laser/12 J/cm² group was significantly higher than that of the control group ($P=0.000$). ALP activity in the red laser group was significantly lower than that in the blue laser/12 J/cm² group ($P=0.002$). The difference between the blue laser/12 J/cm² and blue laser/6 J/cm² ($P=0.420$) and blue laser/6 J/cm² and red laser groups ($P=0.530$) was not significant. On day 14, significant differences were noted between all groups except between the red laser and blue laser/12 J/cm² groups ($P=0.753$).

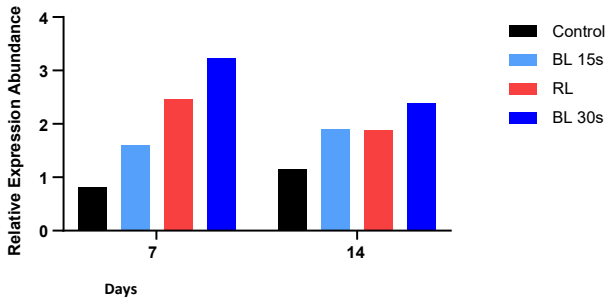

Figure 5. Collagen Type 1 Gene Expression After Different Laser Irradiations During Osseous Differentiation on Days 7 and 14 (BL: Blue Laser, RL: Red Laser). On day 7, the expression of COL1 in all laser groups was significantly higher than that in the control group ($P<0.05$)

Alizarin Red Staining

To assess mineralization, we inspected all groups under a microscope on days 7 and 14. Increased calcification was noted in all groups (Figure 6).

Discussion

This study showed the effects of red and blue lasers on the proliferation and differentiation of PDLMSCs. Periodontal ligament stem cells are important cells in the regeneration process of damaged periodontium. The results showed significant effects of laser irradiation on the differentiation of PDLMSCs; the blue laser with a 445-nm wavelength and 12 J/cm² energy density had the greatest effect in all groups. The results of our study showed that cell proliferation in the 445nm and 12 J/cm² group was significantly lower than the control group on days 1 and 7 ($P<0.05$). RT-PCR showed a significant increase in osteogenic genes in all three laser groups compared to the

Table 1. Amount of Calcium (µg/dL) in the Groups on Days 7 and 14 (n=9 in Each Group)

Time	Group	Mean
7 days	Control	0123.7
	Red	3331.7
	Blue 6 J/cm²	6039.7
	Blue 12 J/cm²	9960.8
	Total	7364.7
14 days	Control	9335.7
	Red	6553.9
	Blue 6 J/cm²	1373.9
	Blue 12 J/cm²	4984.12
	Total	8061.9

Table 2. ALP Activity in the Groups on Days 7 and 14 (n=9)

Time	Group	Mean
7 days	Control	0.1528
	Red	0.1742
	Blue 6 J/cm²	0.1970
	Blue 12 J/cm²	0.2207
	Total	0.1862
14 days	Control	0.2369
	Red	0.3148
	Blue 6 J/cm²	0.3109
	Blue 12 J/cm²	0.4459
	Total	0.3271

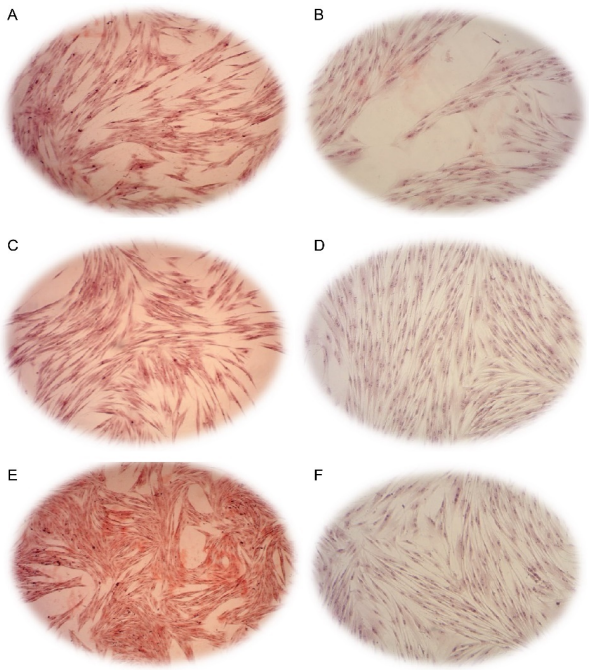

Figure 6. Mineralization (A) 660 nm, 7 Days; (B) 660 nm, 14 Days; (C) 445 nm/15 s, 7 Days; (D) 445 nm/15 s, 14 Days; (E) 445 nm/30 s, 7 Days; (F) 445 nm/30 s, 14 Days

control group ($P < 0.05$). All groups showed a significant increase in calcium content compared to the control group ($P < 0.05$). ALP activity also confirmed the osteoblastic differentiation of cells in laser groups.

Zhu et al[1] assessed the effect of the blue laser on undifferentiated gingival MSCs. They irradiated the laser with 1, 2, 4, and 6 J/cm^2 energy density and noticed the inhibitory effect of the blue laser on the proliferation of MSCs. They also evaluated the expression of RUNX2, COL1, and OCN, and their results showed that laser irradiation of cells with 2 and 4 J/cm^2 energy density significantly increased the expression of RUNX2, COL1, and OCN genes by MSCs and led to increased osteogenesis, which was similar with our results. Yuan et al[13] evaluated the effect of the irradiation of the blue LED (light emitting diode) on the proliferation, cell death, and differentiation of bone marrow MSCs. They irradiated the cells for 1, 5, 10, 30 and 60 minutes and assessed cell proliferation after 6 hours. They found no significant difference in cell proliferation between the control group and the 1-minute irradiation group, while other groups experienced a significant reduction in the cell count. Our study showed that cell proliferation in the 445nm and 12 J/cm^2 group was significantly lower than that in the control group, but on day 4, no significant difference was noted in the cell viability of experimental and control groups. Gholami et al studied the effect of laser photobiomodulation on periodontal ligament stem cells, and they showed that no statistically significant difference was seen between MTT levels of the laser groups and controls.[15] Kushibiki and Awazu[14] evaluated the effect of the blue laser on the differentiation of MSCs and extracellular matrix calcification. They evaluated murine bone marrow MSCs and assessed the expression of the CRY1 gene on days 7 and 14 to assess cell differentiation and calcification. Their results indicated an increase in the amount of extracellular calcium, which was in line with the present findings. However, they only evaluated one laser wavelength (405 nm) with 4 different powers, while in the present study, cells were subjected to red and blue lasers with two different energy densities. Fekrazad et al[16] evaluated the effect of photobiomodulation with single and combined wavelengths of laser on MSCs derived from the iliac bone marrow of rabbits. They evaluated 8 laser groups including 660 nm red, 880 nm infrared, 532 nm green, 485 nm blue, and a combination of infrared-red, infrared-blue, red-green, and blue-green laser wavelengths. Daily irradiations were performed for 21 days. They evaluated the proliferation and differentiation of cells to bone and cartilage and also assessed the expression of ALP, COL1, and OCN for the bone. They found an increase in cell proliferation in the red laser, but there was no difference between the control and the blue laser. However, in the present study, only the blue laser/12 J/cm^2 group on days 1 and 7 significantly decreased cell proliferation, and there was no difference between other groups. The energy densities used in our study were higher than those in Fekrazad and colleagues' study, and this could be one of the reasons for this difference. The ALP increased in the blue and red laser groups, which was similar to our study. There was no significant difference between the control group and the blue and red laser groups in the expression of OCN. This was different from our study in which we assessed that the expression of OCN in all laser groups was significantly higher than that in the control group, and the blue laser/12 J/cm^2 group and the red laser group had no difference. This can also be due to the difference in the studied energy density and different assessment time points for gene expression. They indicated the stimulating effects of red and infrared lasers on osteogenesis, which was similar to our study.[16] Fekrazad et al showed an increase in COL1 expression in blue and red laser groups, which was similar to our study. It appears that the effect of irradiation of single or combined wavelengths of laser on osteogenic differentiation has yet to be clearly understood.

Borzabadi-Farahani[9] evaluated the effect of LLLT with 660, 810, and 980 nm laser wavelengths on the proliferation of MSCs derived from the adipose tissue, dental pulp, and PDL. They evaluated bone marrow MSCs at 20, 24, 48, and 168 hours after irradiation, which were different from the assessment time points in the present study. They indicated that the irradiation of the 660 nm laser had a greater effect on the proliferation of MSCs. The present study also showed that blue and red lasers can induce the differentiation of PDLMSCs to osteoblasts. Thus, in addition to the red laser, the blue laser can also be

used to induce osteogenic differentiation.

This study was conducted on two different laser wavelengths and two different intensities of the blue laser. Further studies on different parameters of lasers and different durations of irradiation are required to find the most suitable protocol for the induction of osteogenic differentiation of PDLMSCs. Moreover, similar studies on other types of stem cells are required to finally achieve a guideline for this purpose. Animal studies are also required to verify the present findings in the clinical setting.

Conclusion

The present results showed positive biomodulation effects of the 445 nm laser and the 660 nm laser on the proliferation and osteogenic differentiation of PDLMSCs.

Authors' Contribution

Conceptualization: Ferena Sayar, Ardavan Etemadi.
Data curation: Sogol Saberi, Ahmad Garebigloo.
Formal analysis: Sogol Saberi, Ahmad Garebigloo.
Funding acquisition: Ardavan Etemadi, Ferena Sayar.
Investigation: Sogol Saberi, Ahmad Garebigloo.
Methodology: Sogol Saberi, Ferena Sayar, Ardavan Etemadi.
Project administration: Sogol Saberi, Ferena Sayar, Ardavan Etemadi.
Resources: Sogol Saberi, Ferena Sayar, Ardavan Etemadi.
Software: Sogol Saberi, Ferena Sayar, Ardavan Etemadi.
Supervision: Sogol Saberi, Ferena Sayar, Ardavan Etemadi.
Validation: Sogol Saberi, Ferena Sayar, Ardavan Etemadi.
Visualization: Sogol Saberi, Ferena Sayar, Ardavan Etemadi.
Writing–original draft: Sogol Saberi, Ferena Sayar, Ardavan Etemadi, Ahmad Garebigloo.
Writing–review & editing: Ahmad Garebigloo, Sogol Saberi, Ferena Sayar, Ardavan Etemadi.

Competing Interests

None.

Ethical Approval

Not applicable.

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