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Photobiomodulation Effect of Different Diode Wavelengths on the Proliferation of Human Buccal Fat Pad Mesenchymal Cells

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Abstract: This study aimed to determine the most effective wavelength for the proliferation of Human Buccal Fat Pad Mesenchymal Stem Cells (BFPMSCs) in cell culture. These cells can be used for different purposes such as regenerative periodontal procedures. Materials and Methods: The wells containing BFPMSCs were subjected to laser irradiation at 635, 660, 808, and 980 nm wavelengths with 1, 1.5, 2.5, and 4 J/cm^2 energy densities. Cell proliferation and viability were evaluated after 1, 3, and 5 days with the methyl thiazolyl tetrazolium (MTT) assay. Result: The proliferation rate of human Buccal Fat Pad Mesenchymal Cells (BFPMSCs) was increased on the first and third days at a wavelength of 808 nm and day five at a wavelength of 980 nm in comparison to the control group. Our findings distinguished that PBMT with 635, 660, 808, and 980 nm wavelengths increased the proliferation of BFPMSCs. Conclusion: The best laser radiation setting, which led to the highest proliferation rate of the cells, included a wavelength of 808 nm with 2.5 J/cm^2 energy density.

Keywords: mesenchymal stem cells; MTT; photobiomodulation therapy; cell proliferation; buccal fat pad

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1. Introduction

Periodontal problems are one of the most critical dental diseases in developing countries. This condition is harmful to the health of tissues that support teeth. Some microorganisms cause this disease by harming the alveolar bone and the periodontal ligament, appearing as a periodontal pocket, bone loss, or both [1]. Mesenchymal Stem Cells are multipotent and undifferentiated cells that can transform into different tissues according to the signals they receive. These cells are obtained from other sources such as bone marrow, orofacial tissue, skin, and adipose tissue [2,3]. Adipose tissue has been introduced as a promising source of MSCs, which can be obtained with minimal discomfort for patients, as subcutaneous adipose tissue is usually discarded after cosmetic surgery procedures. In addition, several studies have shown that Mesenchymal Cells derived from fat tissue possess a yield that is 100–500 times higher than cells derived and aspirated from bone marrow [4–6]. Recently, Farre-Guasch et al. isolated Adipose-Derived Stem Cells (ADSCs) from a mass of fatty tissue in the oral cavity called the Buccal Fat Pad. Under appropriate conditions, it has been shown that ADSCs derived from the Buccal Fat Pad (BFP) are differentiated into chondrocytes, osteoblasts, or fat cells in laboratory conditions [7]. Khojaste and Sadeghi have recently used (BFPSCs) in connection with iliac bone block grafting and increased the rate of new bone formation and decreased secondary bone resorption. This was performed in extensively atrophic jaws [8].

Lasers are one of the most widely used tools in dental activities, creating many developments in various fields in the last 35 years of the 20th century [9]. For the first time in phototherapy treatments, Mester et al. reported the wound healing rate by using a low-level laser [10]. In 1993, the first research on the use of low-level lasers in dentistry was performed by Fernando. He wanted to investigate the impact of the low-level laser in decreasing pain and swelling after the bilateral extraction of the mandibular-impacted third molar. This research observed no difference between the laser and the placebo [11]. In other studies, the low-level laser was used for increasing cell proliferation [12], osteoblastic differentiation of Mesenchymal Stem Cells [13], increasing functional attachment of titanium implants to bones, and improving bone healing and mineralization [14]. In periodontal treatments, the low-level laser has been shown as an additional treatment after non-surgical therapies [15–17], gingivectomy [18–21], and regenerative treatments [22]. In addition, low-level laser therapy is now considered as photobiomodulation (PBM) therapy [23]. Photobiomodulation therapy means that tissues are exposed to lasers or LEDs (Light Emitting Diodes). According to the light parameters, this treatment stimulates and inhibits the responses [24]. An increase in the speed of healing is probably related to the fact that the laser increases cell proliferation. The laser energy is absorbed by intercellular chromophores and converts them into metabolic energy, which is used in the mitochondrial respiratory chain to produce ATP, increase DNA activity, and synthesize RNA and proteins [25]. The impact of PBM is related to photons taken by the chromophore cells and tissues and is changed by different factors such as wavelength, energy density, and power density. Wavelength is the most crucial factor between them [26]. Thus, parameter standardization helps improve the impact of this technology [27]. We performed this study because of heterogeneities in studies that compare different energy densities with various wavelengths of lasers in PBM [27–29]. As a result, this research compared the impacts of different wavelengths and energy densities of diode lasers on the proliferation of human Buccal Fat Pad Mesenchymal Stem Cells.

2. Materials and Methods

2.1. Cell Culture

Human Buccal Fat Pad-Derived Mesenchymal Stem Cells (98-BA-2-7) were purchased from the Shahid Beheshti dental research institute. It should be noted that these cells have been evaluated in terms of the expression of mesenchymal markers and differentiated into all three types: bone, fat, and cartilage. These cells were the third passage. Mesenchymal stem cell characterization was performed by the evaluation of cell surface markers such as CD105 (99.9%), CD90 (99.9%), CD73 (100%), and CD45 (1%) using flow cytometry (Tecan Company, Grödig, Austria). Cells were cultured in a DMED medium (Biowest, Nuaillé, France) containing 10% FBS and penicillin–streptomycin under 5% CO_2 to reach proper confluency. Cells were cultured in 96-well plates 24 h before laser treatment. A total of 6 wells were considered for each group.

2.2. Groups and Irradiation Protocol

The prepared 96-well plates were randomly divided into 4 study groups and a control group (no laser radiation). Before irradiation, each well was placed in a separate stand to protect other wells from receiving laser radiation. Group 1 was diode laser radiation at a wavelength of 635 nm, 220 mW power, 0.33 w/cm^2 power density, irradiation time of 3, 5, 7, and 12 s, and energy densities of 1, 1.5, 2.5, and 4 J/cm^2. Group 2 was diode laser radiation at a wavelength of 660 nm, 150 mW power, 0.25 w/cm^2 power density, irradiation time of 4, 6, 10, and 16 s, and energy densities of 1, 1.5, 2.5, and 4 J/cm^2. Group 3 was diode laser radiation at a wavelength of 808 nm, 250 mW power, 0.4 w/cm^2 power density, irradiation time of 2, 4, 6, and 10 s, and energy densities of 1, 1.5, 2.5, and 4 J/cm^2. Group 4 was diode laser radiation with a wavelength of 980 nm, power of 100 mW, a power density of 0.25 w/cm^2, irradiation time of 4, 6, 10, and 16 s, and energy densities of 1, 1.5, 2.5, and 4 J/cm^2. All irradiation was performed at the edge of the well (the tip area of

0.5 cm^2) (Table 1). All irradiation was in continuous mode 1 mm above the wells in a fixed position. Then, cell viability and proliferation were investigated 24 h, 72 h, and five days after laser irradiation. A total of 24 wells were considered for each wavelength (6 wells for each energy density).

Table 1. Laser characteristics of the study groups.

Groups	Wavelength (nm)	Power (mW)	Power Density (mW/cm^2)	Energy Density (J/cm^2)
1	635	220	0.33	1, 1.5, 2.5 and 4
2	660	150	0.25	
3	808	250	0.4	
4	980	100	0.25	

2.3. MTT Assay

The viability and proliferation of cells in 4 groups were evaluated 24 h, 72 h, and five days after laser irradiation using the colorimetric test of 5-Diphenyltetrazolium Bromide and 3-[4,5-Dimethylthiazol-2-yl]-2 MTT. The basis of this test is the conversion of MTT to formazan crystals by living cells, which will determine the level of mitochondrial activity. In most cell populations, the total mitochondrial activity is related to the number of living cells. MTT is soluble in water and has a yellow color, which is absorbed by living cells and is reduced by the activity of mitochondrial dehydrogenase to create formazan, which is insoluble in water, has a purple color, and must be dissolved for colorimetric measurement. To achieve this objective, 100 µL of MTT solution (5 mg/mL MTT (Sigma-Aldrich, Taufkirchen, Germany) (in phosphate-buffered saline)) was added to the cell culture wells and incubated at 37 °C and 5% CO_2 for 3–4 h. Tetrazolium salt, which exists in MTT, was absorbed by biologically active cells and caused the purple color of the formazan granules. This medium was carefully removed, and the intercellular formazan was dissolved by adding 60µL DMSO into each well and placing the plates in a shaker for 15 min. Then, 50 µL of the supernatant of each well was transferred to a clean 96-well plate, and the absorbance was determined at 570 nm using a microplate reader (BioTek, Santa Clara, CA, USA). Then, the optical density was evaluated at 24 h, 72 h, and five days [30,31].

2.4. Statistical Analysis

Three repetitions were considered for the MTT assay. In this study, after collection, the data were analyzed with SPSS25 software. One-way ANOVA and Dunnet's supplementary test were used to compare the study groups with the control, and three-way ANOVA and the Tuckey HSD test were used to investigate the effect of wavelength, energy density, and time on MTT. The statistical significance was adjusted at 5% (p-value of less than 0.05).

3. Results

On the first and third days, cell proliferation in all of the groups was insignificant compared to the control group, so the energy density in all of the groups showed no significant difference; however, the wavelengths were significant compared to the control group ($p < 0.05$). The 808 nm wavelength was significantly higher than the other three wavelengths with regard to cell proliferation (Tables 2 and 3) (Figures 1 and 2).

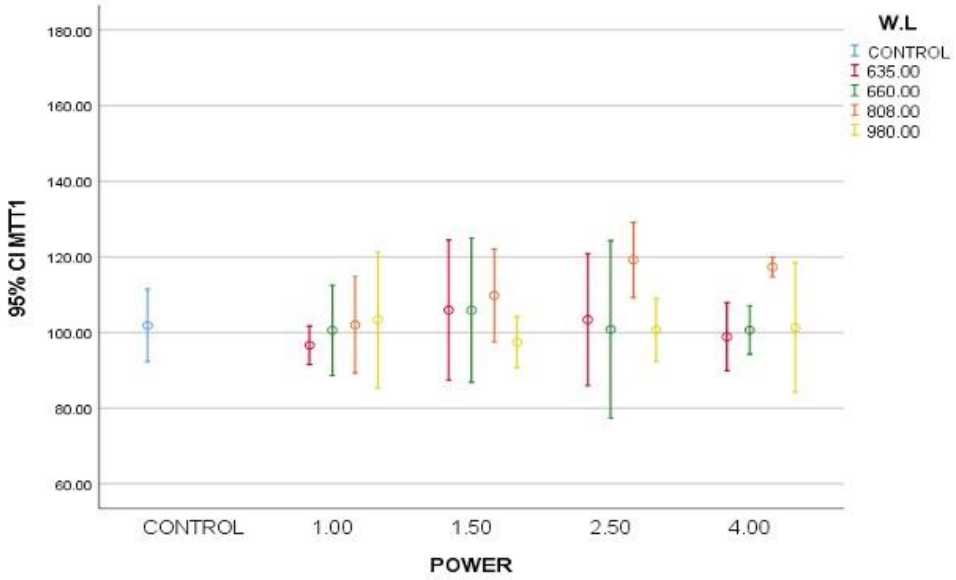

Figure 1. Comparing the effect of wavelengths and energy densities on the proliferation of BFP stem cells on the first day.

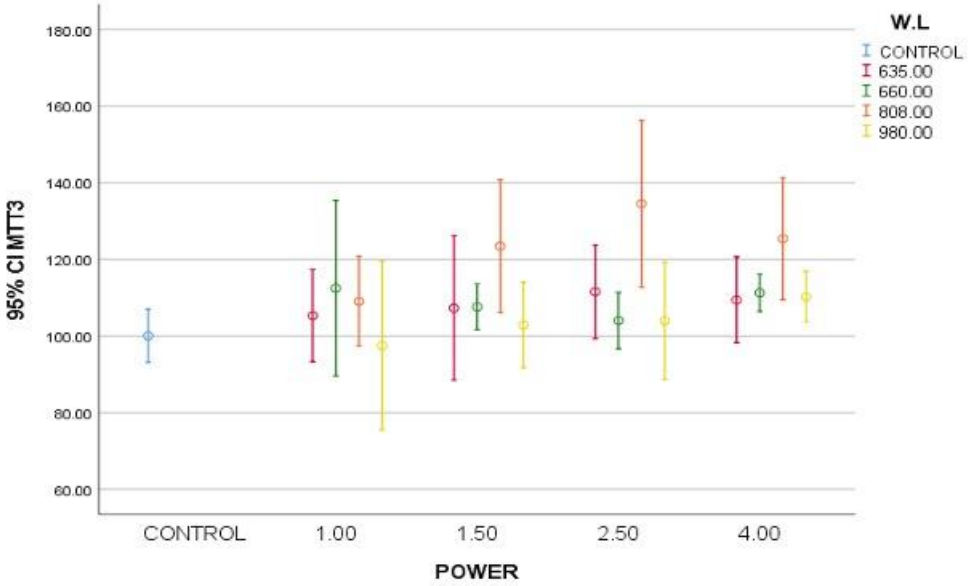

Figure 2. Comparing the effect of wavelengths and energy densities on the proliferation of BFP Stem Cells on the third day.

Table 2. The viability of cells (%) on the first day (items marked with * are $p < 0.05$ in comparison to the control group). [One-way ANOVA and Dunnet's supplementary tests were used to compare the study groups with the control].

Energy Density	1 J/cm^2	1.5 J/cm^2	2.5 J/cm^2	4 J/cm^2
Wavelength				
control	100	100	100	100
635 nm	81.6018	105.898	95.8477	98.8391
660 nm	100.561	105.862	100.796	100.602
808 nm *	102.007	109.765027	119.181	117.269
980 nm	103.347	97.3933	93.1032	101.313

Table 3. The viability of cells (%) on the third day (items marked with * are $p < 0.05$ in comparison to the control group). [One-way ANOVA and Dunnet's supplementary tests were used to compare the study groups with the control.

Energy Density	1 J/cm^2	1.5 J/cm^2	2.5 J/cm^2	4 J/cm^2
Wavelength				
control	100	100	100	100
635 nm	105.28	107.26	111.52	109.38
660 nm	112.45	107.59	104.01	111.24
808 nm *	109	123.42553	134.52	125.39
980 nm	97.459	102.86	103.94	110.23

However, on the fifth day, the interaction became significant compared to the control group (p value = 0.007), and it was necessary to investigate each of the subgroups.

At wavelengths of 635 nm, 660 nm, and 808 nm with different energy densities, there was no significant difference in cell proliferation compared to the control group; however, at 980 nm, a significant difference was seen compared to the control group, so the energy density of 4 J/cm^2 was different from energy densities of 1 and 1.5 J/cm^2. In addition, the most cell proliferation was related to an energy density of 4 J/cm^2 and the least cell proliferation was related to an energy density of 1.5 J/cm^2 at this wavelength.

At the energy density of 1 J/cm^2, there was a significant difference compared to the control group, so the wavelength of 808 nm is greater than the other three wavelengths regarding cell proliferation. At the energy density of 1.5 J/cm^2, there was a significant difference compared to the control group (p value: 0.031), so the wavelength of 808 nm is better than the wavelengths of 635 nm and 660 nm, and the wavelengths of 635 nm and 660 nm are better than the wavelength of 980 nm (Table 4) (Figure 3).

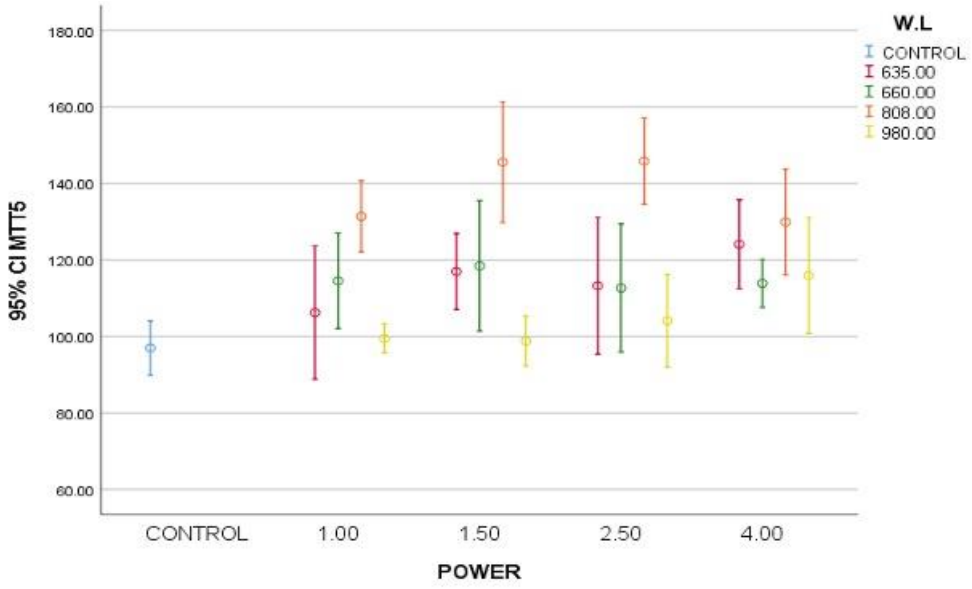

Figure 3. Comparing the effect of wavelengths and energy densities on the proliferation of PDL stem cell on the fifth day.

Table 4. The viability of cells (%) on the fifth day (items marked with * are $p < 0.05$ in comparison with the control group). [One-way ANOVA and Dunnet's supplementary tests were used to compare the study groups with the control].

Energy Density	1 J/cm^2	1.5 J/cm^2	2.5 J/cm^2	4 J/cm^2
wavelength				
control	101.11	101.11	101.11	101.11
635 nm	98.706	116.94	105.71	124.08
660 nm	114.48	118.43	112.64	113.84
808 nm	131.37 *	145.55961 *	145.84 *	129.9
980 nm *	99.442	98.755	104.1	115.86

4. Discussion

The purpose of regenerative periodontal treatments is to restore physiological dental function by reconstructing periodontal tissues such as the alveolar bone, gingiva, periodontal ligament, and cementum. With tissue regeneration, destructive periodontal tissues can be rebuilt by using stem cells, growth factors, or extracellular matrix scaffolds. Until now, various types of stem cells have been used to regenerate periodontal tissues; today these include Mesenchymal Stem Cells (MSCs) [32]. In 2010, Farre-Guasch et al. isolated Adipose-Derived Stem Cells (ADSCs) from a mass of fatty tissue in the oral cavity called the Buccal Fat Pad. Under appropriate conditions, it has been shown that ADSCs Derived From the Buccal Fat Pad (BFPADSCs) differentiate into chondrocytes, osteoblasts, or fat cells [7]. This study used Human Buccal Fat Pad mesenchymal cells to regenerate periodontal tissues.

Laser therapy is widely used to increase the speed of tissue regeneration. Studies have shown an increase in the proliferation rate of cells such as fibroblasts and osteoblasts. In mesenchymal cells, studies have shown that PBMT has a positive effect on mesenchymal stem cells obtained from bone marrow and adipose tissue [33,34]. One of the crucial issues that should be considered in choosing the wavelength is the optical window, in which the effective permeability of light into the tissue is the maximum. The optical window is approximately in the range of 650 nm to 1200 nm. The amount of absorption and diffusion of light in the blue region of the spectrum is higher than in the red region because the main chromophores in tissue (hemoglobin and melanin) have the highest absorption and scattering of light at shorter wavelengths. In addition, water absorbs infrared light at wavelengths greater than 1100 nm. Therefore, the use of PBMT in patients and animals is in the range of red and near-infrared light (600–1100 nm). [35]. In our study, our wavelengths were 635 nm, 660 nm, 808 nm, and 980 nm, which are in the abovementioned range.

Another parameter that should be considered in cell stimulation is energy density. According to the studies of Tuner and Hoder, cell stimulation can be achieved at low doses, such as 0.001 J/cm^2, and even at higher doses, with 10 J/cm^2 being the maximum amount [36]. In this study, energy densities were 1, 1.5, 2.5, and 4 J/cm^2, which are in the abovementioned range.

According to published studies, wavelengths in the range of 810 nm are one of the most used wavelengths for PBM treatment. The mechanism of their function is that the photons of these wavelengths are absorbed by the chromophores in cytochrome C oxidase and stimulate mitochondrial metabolism by increasing MMP and O2 absorption; therefore, they will increase the production of ATP [37].

In this study, the 808 nm wavelength was significantly higher on the first and third days than the other three wavelengths. In addition, the 808 nm wavelength was greater on the fifth day than other wavelengths at 1, 1.5, and 2.5 J/cm^2.

In the study by Wang et al., the mechanism of the effect of 810 and 980 nm wavelengths on fat stem cells was investigated. They wanted to test the hypothesis that the 980 nm chromophore is intercellular water that can create a microscopic temperature gradient during laser irradiation. To achieve this, 4 °C cold medium was added during laser

irradiation, and cells were incubated at 42 °C; both works eliminated the effect of the 980 nm wavelength but had no impact on the 810 nm wavelength. They concluded that the 980 nm wavelength affected temperature-dependent calcium channels, but the 810 nm wavelength had the greatest impact on mitochondrial cytochrome c oxidase [38].

In research performed by Gholami et al. in 2020, they investigated near-infrared (940 nm) photobiomodulation on stem cells derived from a Buccal Fat Pad. In this research, after separating the cells from the sample of the human BFP, the third passage of the cells was irradiated twice a day for three consecutive days. Irradiation was performed with six different laser settings. These settings consisted of two continuous pulse conditions (50% cycle), energy densities of 3 and 6 J/cm^2, and two different output powers (0.1 W and 0.3 W). This experiment was repeated on three different days and each time, the cells were evaluated and repeated using a MTT assay at intervals of 24, 48, and 72 h. The highest proliferation was observed in 3 J/cm^2, 0.3 W, pulsed radiation at 24 h and 48 h; however, after 72 h, the greatest increased proliferation was related to 6 J/cm^2, 0.1 W, pulsed. This setting was used for the osteoporotic differentiation method. Microscopic analysis of Alizarin red staining showed that cells under 3 J/cm^2, 0.3 W pulse irradiation also increased the mineralization of BFPSCs cultured in an osteogenic induction medium compared to the negative control [39]. In our research, we concluded that the wavelength of 980 nm (0.1 W) with an energy density of 4 J/cm^2 compared on the fifth day was significant compared to other energy densities. As both wavelengths are in the near-infrared region, they can be used for the proliferation of BFP stem cells.

In the study by Gholami et al., the effect of PBM on proliferation, viability, and osteogenic differentiation in stem cells isolated from human PDL was investigated, and the 940 nm diode laser with an energy density of 4 J/cm^2 and a power of 100 mW was used for three sessions every 48 h. Cell viability was evaluated 24, 48, and 72 h later. In this research, the cells were divided into three groups: C−: without osteogenic medium, C+: with osteogenic medium and without laser, and L+: with osteogenic medium and laser. In this research, the results of MTT did not show a difference between the control and study groups. However, after 14 and 21 days, both the C+ and L+ control groups showed an increase in mineral tissue formation compared to the C− group [40]. In the current study, the same energy density and power were used, but the wavelength we used was 980 nm, the radiation was done in two sessions, and the stem cell used was human BFP. In the current study, the wavelength of 980 nm on the fifth day was significant compared to the control group.

In the study of Fekrazad et al., the effect of PBMT and mesenchyme stem cells on bone regeneration was investigated. In this research, 48 lesions with a diameter of 6 mm were created in rabbit calvarial bone. These lesions were divided into four groups: no treatment, treatment with PBMT, treatment with mesenchyme stem cells, and a combination of stem cells and laser. The wavelength of the laser was 810 nm, the power density was 0.2 W/cm^2, and the energy density was 4 J/cm^2. In this research, it was concluded that the group that was only under laser radiation produced significant results compared to the other two groups and the control group. In addition, the amount of inflammation decreased significantly in the group treated with laser compared to the other two treatment groups [41]. Our study used a different cell source, and the wavelength was 808 nm. IN addition, the power density was 0.4 W/cm^2, although the energy density was similar. In the current study, the wavelength of 808 nm was significantly different from the control group on days 1 and 3. Furthermore, on the fifth day, the energy densities of 1, 1.5, and 2.5 J/cm^2 of the wavelength of 808 nm were significant compared to the control group. Therefore, lower energy densities can be effective in the long-term.

Research conducted by Zaccara et al. in 2015 aimed to investigate the effect of PBMT on the proliferation and viability of dental pulp stem cells. In this research, cells were isolated from two third molars. The laser used in this research was InGaAlP with a wavelength of 660 nm and a power of 30 mW, and densities of 0.5 J/cm^2 and 1 J/cm^2. Proliferation, viability, and mitochondrial activity were evaluated 24, 48, 72, and 96 h later. In addition,

apoptosis and events related to the cell cycle were investigated by flow cytometry. In this research, they concluded that the group that was irradiated with an energy density of 1 J/cm^2 had increased proliferation and had a significant difference at 72 h and 96 h compared to the control group [42]. In our study, although a different cell source was used, the laser had a similar wavelength (660 nm) and energy densities (1, 1.5, 2.5, 4 J/cm^2). However, the power we used was 150 mW, and MTT was checked 1, 3, and 5 days after laser irradiation. In the current study, it was observed that the results obtained from the wavelength of 660 nm with an energy density of 1 J/cm^2 on days 1, 3, and 5 were insignificant compared to the control group, which is in line with the previous study. In addition, at the wavelength of 660 nm at any time, the difference in energy densities was not significant. These results can be attributed to the lower power of the laser device and the lower penetration of this wavelength.

In the study by Fekrazad et al., the effect of different wavelengths on the proliferation and differentiation of bone marrow stem cells was investigated. This research extracted mesenchymal stem cells from rabbit iliac bone marrow. A control group and eight laser groups were considered: Infrared (810 nm), Red (660 nm), Green (532 nm), Blue (485 nm), IR-R, IR-B, R-G, and B-G. The energy density was 4 J/cm^2. Laser therapy was repeated every day for 21 days, and proliferation, bone, and cartilage differentiation were checked. Cell proliferation was evaluated after 10 days. The combination of 532 nm and 485 nm had the most cell proliferation, and 532 nm without any combination decreased proliferation. There was no significant difference between the control and other groups except for the 485 nm and 532 nm groups. Wavelengths at 660 nm and 810 nm increased proliferation, and 660 nm had a better effect. Wavelengths at 660 nm and 810 nm had a stimulating effect on osteogenesis, but the impact of the wavelength at 532 nm was inhibitory [33]. In the current study, BFP stem cells with wavelengths of 635, 660, 808, and 980 nm were used during two sessions of laser therapy with a similar energy density, and we concluded that the wavelength of 808 nm was significant on the first and third days compared to the control group which was in line with the mentioned study. On the fifth day, the wavelength of 808 nm with energy densities of 1, 1.5, and 2.5 J/cm^2 was significant compared to the control group, and the wavelength of 980 nm was significant compared to the other wavelengths, showing that higher wavelengths in the near-infrared region are more effective. This can be related to the different mechanisms of signaling pathways and needs further study in the future.

The study of Pereira et al. investigated the effect of PBMT on the proliferation and differentiation of HDPSC isolated from Healthy Dental Pulp and inflamed pulp. Healthy and inflamed dental pulp stem cells were prepared from different patients and were exposed to laser radiation at a wavelength of 660 nm with four densities of 0.05, 0.30, 7, and 42 J/cm^2. In this research, the experimental and control groups' differences were insignificant in terms of proliferation and differentiation [43]. In the current study, the cell source and energy densities were different, but the wavelength was the same as the study of Pereira. The wavelength of 660 nm, which was used in both studies, produced no significant differences compared to the control group in terms of energy densities and days. In addition, the difference between this wavelength's energy densities was insignificant.

In a systematic review published by Ginani et al., the effect of PBMT on the proliferation of mesenchymal stem cells was investigated. This study used the PubMed/Medline database and articles published in the last 12 years. In total, nineteen articles were selected. Sixteen articles used wavelengths in the range of red visible light, and three articles used wavelengths in the range of infrared light. The lowest energy density was 0.05 J/cm^2, the most used was 0.5 J/cm^2, and the highest energy density was 42 J/cm^2. Twelve articles investigated the effect of bone marrow stem cell proliferation, four articles investigated the effect on adipose tissue, two articles investigated the effect on dental pulp, and one article investigated the effect on the periodontal ligament. Most of the articles showed an increase in proliferation, and it was concluded that laser therapy has a positive effect on stem cell proliferation, but more research is needed [33]. In the current study, wavelengths

and energy densities were in the mentioned range. Limitations of this study include the possibility of cell contamination and the lack of availability of materials needed for cell culture. It is suggested to use a combination of wavelengths or a pulsed mode of irradiation for the proliferation of HBFP cells. Future research is required to more thoroughly understand how PBMT affects the proliferation and differentiation of Buccal Fat Pad Stem Cells, which are crucial to periodontal regeneration.

5. Conclusions

The results of this study proved that PBMT at a wavelength of 808 nm increased the proliferation of Human Buccal Fat Pad (HBFP) stem cells. In more detail, for achieving the highest proliferation rate of the cells, the 808 nm wavelength with 2.5 J/cm^2 energy density on the fifth day is the most desirable laser radiation setting. In addition, the 660 and 635 nm diode laser showed favorable proliferation of HBFP stem cells but the rate was lower compared to the 808 nm diode laser. The 980 nm diode laser does not possess a suitable wavelength for the proliferation of HBFP stem cells.

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