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The Effect of Low-Level Laser Therapy on the Viability of Human Dental Pulp Stem Cells

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Abstract

Introduction: This study assessed the effect of low-level laser (LLL) irradiation on the viability of dental pulp stem cells (DPSCs).

Methods: In this in vitro experimental study, human DPSCs were purchased from the cell bank of Iranian Genetic Resources and cultured in flasks containing Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum (FBS) at 37°C, 5% CO2, and 95% humidity. The cells were stored in semi-confluent form, and the culture medium was refreshed every two days. The cells in the control group were not laser-irradiated, but the cells in the experimental groups were irradiated with 660-nm and 808-nm diode lasers with 4.1 J/cm^2 energy density. Cell viability was assessed at baseline and after 24, 48, and 72 hours using the methyl thiazolyl tetrazolium (MTT) assay. The effects of laser irradiation, laser wavelength, and time on the percentage of cell viability were analyzed by two-way ANOVA and Tukey's test.

Results: The effects of laser irradiation and its wavelength ($P=0.04$), time of assessment ($P<0.001$), and the interaction effect of group and time ($P=0.02$) on cell viability were significant. Cell viability in 660-nm and 808-nm laser groups at 48 and 72 hours was higher than that of the control group; however, only the difference in cell viability between the 660-nm laser group and the control group at 72 hours was statistically significant ($P=0.03$).

Conclusion: Considering the optimal effect of diode laser irradiation (particularly 660 nm) on the viability of DPSCs, we conclude that it may be suitable for relevant clinical applications.

Keywords: Cell survival; Low-level light therapy; Dental pulp; Stem cells.

Introduction

Low-level laser (LLL) is a relatively novel technology that increases the metabolic activity of the cells and has shown promising results in bone regeneration[1] and wound healing.[2] LLLs have an energy level lower than 500 mW and are often irradiated in the visible electromagnetic or infrared spectrum.[3] Bio-stimulation, phototherapy, and photo-biomodulation are alternative terms for LLL therapy.[4] Different laser types such as diode and InGaAlP lasers are used for photo-biomodulation.

Intracellularly, laser light is absorbed by the final enzyme in the mitochondrial respiratory chain, which is cytochrome C oxidase, present in all cells. Mitochondria is the main site of biological interactions of LLLs. Evidence shows that LLL can inhibit the expression and release of pro-inflammatory mediators such as prostaglandin E2, tumor necrosis factor-alpha, cyclooxygenase 2, and interleukin 1B. Laser therapy decreases the infiltration of neutrophils and the volume of edema after soft tissue injury and controls inflammation.

The LLL bio-stimulation effects have yet to be fully understood. However, Karu suggested five mechanisms to be involved in this process, of which two mechanisms are explained by the reduction phenomena, two by oxidation pathways, and the last one by an intracellular temperature rise. These mechanisms include (I) the alteration of the reductive properties and electron exchange, (II) the relative inhibition of nitride oxide, which is an essential factor in the inhibition of the mitochondrial activity of the cells, (III) the production of superoxide, (IV) the generation of singlet oxygen (photodynamic therapy), and (V) changed biochemical activity by the induction of transient heat in intracellular chromophores. As mentioned earlier, laser therapy mainly affects mitochondria. Increased ATP synthesis in mitochondria improves cellular metabolism significantly when the cells are injured or in the stationary phase. Thus, laser therapy is more effective for injured or destructed

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tissues than sound tissues.[5]

Laser irradiation stimulates intracellular and tissue elements and enhances cell proliferation and regeneration. Induction and activation of DNA and RNA, enhanced cell oxygen uptake, stimulation of enzymes, mitochondrial activity, increased level of cytochrome oxidase, and increased ATP synthesis are among the other effects of LLLs.[6]

Laser therapy was first used to control inflammation in dentistry in 1985. It currently has several applications to reduce pain and discomfort following anesthetic injection or minor oral surgical procedures and alleviate temporomandibular joint pain or neuralgia. The biomodulation effects of LLLs accelerate cellular and tissue healing, and LLLs are now considered an alternative or adjunct to therapeutic modalities.[7-9]

Dental pulp stem cells (DPSCs) are the most suitable cells for tissue engineering because they can be easily isolated and replaced. DPSCs can repair dentin, and their potential for the regeneration of non-dental tissues has also been confirmed.[10] DPSCs have properties similar to mesenchymal stem cells and can differentiate into multiple cell types such as odontoblasts, osteoblasts, chondrocytes, and hepatocytes. Rat[11,12] and human[13,14] dental pulp is commonly used to isolate different odontoblastic cell lines. However, despite the possession of clonogenicity and tripotency, these cells may lose their potential for clinical implantation after isolation, which is a significant shortcoming in tissue engineering. Thus, LLL irradiation has been suggested to improve the biocompatibility of stem cells in different environments and enhance tissue regeneration.[15,16]

Laser light at a specific wavelength can stimulate and induce cell proliferation. DPSCs are no exception to this rule. However, since the dental pulp is a susceptible tissue and thermal damage caused by a laser can lead to pulp necrosis, researchers are trying to assess different wavelengths and powers of lasers to find the safest protocol with a maximum effect on the differentiation of DPSCs.

Tissue engineering has reported promising results in pulp regeneration. In regenerative endodontic therapy, a higher level of disinfection is necessary, and disinfecting agents must not harm the stem cell's ability to survive and proliferate. One of the essential elements of successful regeneration endodontic therapy is stem cells. It is worth investigating the effects of two diode laser wavelengths, 660 and 808 nm, used in the process of root canal disinfection on the viability of stem cells.[17]

Accordingly, this study aimed to assess the effects of 660-nm and 808-nm diode laser irradiation on the viability of human DPSCs.

Materials and Methods

In this in vitro experimental study, DPSCs were purchased from the Iranian Genetic Resources cell bank. The cells were cultured in Dulbecco's modified Eagle medium supplemented with L-glutamine and 20% fetal bovine serum (FBS). The cell culture flasks were incubated at 37°C with 5% CO_2 and 95% humidity. All procedures were performed under a laminar hood. The cells were stored in semi-confluent form to prevent their differentiation. Also, the culture medium was refreshed once every two days.

The control wells did not receive any laser irradiation. Six wells were assigned to each laser wavelength for assessment at each time point (24 wells for each wavelength).

The cells in laser groups were subjected to the near-infrared spectrum at a continuous wavelength of 808-nm LLL with 250-mW power and 660-nm LLL (red laser) with 150-mW power and 4.1 J/cm^2 energy density at a 1-mm distance (DX6182, Konftec, Taiwan). Considering the 0.5-cm^2 spot size, we irradiated the entire surface of each well of the 96-well plate with a laser. The laser device automatically adjusted the irradiation time to 9 seconds for the wavelengths. Laser irradiation was performed in a dimly lit room to prevent interference with the environmental light. Several empty wells were considered between the experimental and control wells to prevent unwanted irradiation of wells by the scattered radiation.

The viability of DPSCs was evaluated immediately after irradiation (time 0) and at 24, 48, and 72 hours using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) (evaluation of mitochondrial activity by the reduction of MTT) assay. Trypsin 0.25% in phosphate buffered saline (PBS) was used to remove the cells from culture flasks once they had reached about 80% confluence. Three PBS washes were performed on the cells. Following centrifugation, they were suspended in a growth medium. Tetrazolium bromide solution (Sigma, USA) at a concentration of 5 mg/mL was added to wells, and then they were incubated at 37°C for 4 hours. The medium was discarded without washing, and 100 µL of DMSO was used as a formazan crystal solvent. After 15 minutes of incubation, the viability of the cells in the well was measured by reading the optical density of the wells spectrophotometrically (Biotech, USA) by an ELISA-Reader.

The data were analyzed using GraphPad Prism V8. The mean and standard deviation of optical density were reported for the two wavelengths at different time points. The effects of laser irradiation (group), laser wavelength, and assessment time on the percentage of viability of DPSCs were analyzed by two-way ANOVA at a 0.05 level of significance.

Results

Table 1 presents the mean viability percentage of human DPSCs in the laser and control groups at different time points. Two-way ANOVA showed significant effects of group (660 and 808-nm lasers/control) ($P=0.04$) and

time ($P<0.001$) and their interaction effect ($P=0.02$) on the percentage of cell viability. In other words, the viability of pulp stem cells at 0, 24, 48, and 72 hours differed significantly. The interaction effects of time and group demonstrated that the viability of DPSCs at zero, 24, 48, and 72 hours and between the control and diode laser groups had significant differences in the two wavelengths.

Considering the significant effect of group (laser/control) on cell viability, we performed pairwise comparisons by Tukey's test (Table 2), which showed significantly higher cell viability in the 660-nm laser group than in the control group ($P=0.03$). Table 3 shows the results of Tukey's multiple comparisons test comparing the viability of pulp stem cells between 660 and 808 nm laser groups at different times. There were also no significant differences in the viability of DPSCs between the control and 808-nm diode laser groups ($P=0.5$). Also, no significant differences were seen in terms of the viability of DPSCs between the 808-nm diode laser and 660-nm diode laser groups ($P=0.33$)

Multiple comparisons of the groups and times are presented in Table 4. The differences were not significant in most comparisons. However, some differences were noted. For instance, at 72 hours, a significant difference was noted between the control and 660-nm laser groups in cell viability ($P=0.004$).

Discussion

This study used the MMT assay to evaluate cell viability and proliferation. A standard method for determining the metabolic activity of living cells is the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium test. The test is based on the enzymatic reduction of the light-colored tetrazolium salt to its spectrophotometrically quantifiable formazan of vivid purple-blue color. The resulting absorbance value is precisely related to the number of live and proliferative cells under optimal conditions. There are several tests available to determine cell viability and proliferation. The MTT test was used in this study, which has some limitations. One disadvantage of the MTT test is that it overestimates viability and its final solubilization step. Although other viability tests may have disadvantages, relying solely on the MMT test may be one of the limitations of the study.

It has been demonstrated that irradiation of LLL can enhance wound healing and accelerate the migration of cells. It can induce the proliferation of fibroblasts, collagen synthesis, phagocytosis by macrophages, and acceleration of the inflammatory phase of wound healing.[18-20] All these mechanisms can lead to cell proliferation and enhancement of wound healing.[21-23]

Different laser types have been used for photobiomodulation. However, diode lasers are most commonly used[24] since they are more cost-effective and easy to handle. Evidence shows that irradiation of lasers in infrared wavelengths (700 nm to 1 mm) and specific densities has more significant antibacterial effects than

Table 1. Mean percentage of viability of human DPSCs in the laser and control groups at different time points

Variable	Time	Mean	Standard Deviation
Control	0	100.0%	12.60%
	24 h	100.0%	10.19%
	48 h	100.0%	15.63%
	72 h	100.0%	18.24%
808 nm diode	0	90.48%	15.53%
	24 h	96.23%	18.32%
	48 h	111.98%	15.23%
	72 h	120.32%	18.94%
660 nm diode	0	93.05%	14.12%
	24 h	105.72%	9.61%
	48 h	105.83%	14.35%
	72 h	135.7%	7.28%

Table 2. Pairwise Comparisons of the Groups Regarding the Percentage of Cell Viability Using the Tukey's Test

Group 1	Group 2	Mean Difference	95% CI	*P* Value
Control	808 nm	4.754	5.376-14.88-	0.5
Control	660 nm	10.83	0.6965-20.96-	0.03
808 nm	660 nm	6.072	4.058-16.20-	0.33

Table 3. Pairwise Comparisons of the Time Points Regarding the Percentage of Cell Viability Using the Tukey's Test

Time	Laser Wavelength	Standard Deviation	Mean	Mean Difference	95% CI	*P* Value
Time 0	660 nm	14.12	93.05	2.566-	26.1-31.23-	>0.99
	808 nm	15.53	90.48			
24 h	660 nm	9.61	105.72	9.496-	19.17-38.16-	0.99
	808 nm	18.32	96.23			
48 h	660 nm	14.35	105.83	3.153	31.82-25.51-	>0.99
	808 nm	15.23	111.98			
72 h	660 nm	7.28	135.7	15.38-	13.28-44.04-	0.79
	808 nm	18.94	120.32			

Table 4. Multiple Comparisons of the Groups and Times

Time	Comparison of Control and Laser Groups	Mean Difference	95% CI	*P* Value
Time 0	660	6.948	-21.72-35.61	0.99
	808	9.515	-19.15-38.18	0.99
24 h	660	-5.722	-34.39-22.94	>0.99
	808	3.774	-24.89-32.44	>0.99
48 h	660	-8.831	-37.50-19.83	0.99
	808	-11.98	-40.65-16.68	0.95
72 h	660	-35.7	-64.37-7.037	0.004
	808	-20.32	-48.99-8.342	0.42

in higher doses; however, cell death is possible in higher wavelengths due to photothermal mechanisms of laser irradiation. Also, the risk of damage to the adjacent sound tissue exists.[24]

The present study used an 808-nm diode laser in the infrared wavelength range. A similar study evaluated the effects of a low-level diode laser with a 660-nm wavelength (in the visible range of 400-700 nm) on the proliferation of stem cells isolated from the exfoliated human deciduous teeth. They assessed the viability of cells by the MTT assay.[25] It has been reported that LLL irradiation with a 600-nm wavelength increases the number of stem cells, suggesting the possible role of LLL irradiation in tissue regeneration.[19,26]

Nutritional conditions can also affect the proliferation of DPSCs. Evidence shows that LLL irradiation can enhance the proliferation of different cell lines in nutritional deprivation.[27-29] In the present study, nutritional deprivation was performed to simulate clinical stress conditions. Evidence shows that the FBS percentage is correlated with the cell line and its passage. De Paula Eduardo et al[30] and Moura-Netto et al[31] reported nutritional deprivation in the presence of 10% FBS, which agreed with the present study.

The present study assessed the effects of 660-nm and 808-nm diode laser irradiation on the viability of human DPSCs. The results showed that cell viability in 660-nm and 808-nm laser groups at 48 and 72 hours was higher than that in the control group; however, only the difference in cell viability between the 660 nm laser and control groups at 72 hours was statistically significant ($P=0.03$).

Considering the changes in cell viability following the irradiation of a 660-nm diode laser and a significant difference between the 660 nm laser and control groups, we can conclude that the irradiation of a 660-nm laser is safe. It should be noted that the biocompatibility of this laser type increased with time, which calls for further investigations. Modalities used in endodontic treatment should have optimal antimicrobial activity and preserve their potential to induce the proliferation/differentiation of stem cells over time.[32,33]

However, cell reactions to LLL irradiation also depend on the frequency of irradiations. Moreover, a combination of specific laser parameters is required to ensure the optimal efficacy of LLL therapy. The available literature regarding the effects of a 660-nm diode laser on DPSCs agrees with the present findings. Borzabadi-Farahani[34] evaluated the effects of LLL irradiation on the proliferation of mesenchymal stem cells in a review study and showed increased proliferation of DPSCs isolated from primary and permanent teeth following their irradiation with 810-nm and 980-nm lasers. Also, a previous study showed that the irradiation of LLL with a density close to that used in the present study (4.1 J/cm^2) increased the proliferation of stem cells isolated from the exfoliated primary cells under nutritional deprivation.[31] A previous study used an InGaAlP diode laser with different densities and reported increased proliferation of human DPSCs at times longer than 48 hours compared with the control group.[35]

In the present study, cell viability in 660-nm and 808-nm laser groups at 48 and 72 hours was higher than that in the control group; however, only the difference in cell viability between the 660 nm laser and control groups at 72 hours was statistically significant. Considering all the above, it appears that LLL in specific wavelengths and energies can affect the viability of DPSCs and may be used for tissue engineering, vital pulp therapy, and regenerative treatments. In general, an increase in the proliferation of stem cells has been reported following LLL irradiation.[36]

The present results regarding increased cell viability at 72 hours agreed with those of a study by Koutná et al.[37] The difference in cell viability at the same time point between the two laser wavelengths of 660 and 808 nm may be due to the different affected sites or effects of lasers on the respiratory chain or cell cycle.

Despite the value of in vitro studies, it should be noted that their results cannot be directly generalized to the clinical setting since the confounding factors can be easily controlled in vitro while the situation is different intraorally. Future studies are required on the effects of LLL therapy with different wavelengths and energy densities on the viability of DPSCs and other stem cells such as the periodontal ligament stem cells and stem cells from the apical papilla over more extended periods.

Conclusion

Considering the optimal effect of diode laser irradiation (particularly 660 nm) on the viability of DPSCs, we conclude that it may be suitable for relevant clinical applications.

Conflict of Interests

The authors declare that they have no conflict of interest.

Ethical Considerations

The ethics committee at Shahid Beheshti University of Medical Sciences approved this study (IR.SBMU.DRC.REC.1398.129).

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