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BRIEF REPORT

Effects of laser therapy on the proliferation of human periodontal ligament stem cells

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Abstract Low-level laser irradiation (LLLI) stimulates the proliferation of a variety of cell types. However, very little is known about the effect of laser therapy on dental stem cells. The aim of the present study was to evaluate the effect of LLLI (660 nm, 30 mW) on the proliferation rate of human periodontal ligament stem cells (hPDLSC), obtained from two healthy permanent third molars extracted due to surgical indication. Culture cells were either irradiated or not (control) with an InGaAlP diode laser at 0 and 48 h, using two different energy densities (0.5 J/cm², 16 s and 1.0 J/cm², 33 s). Cell proliferation was evaluated by the Trypan blue exclusion method and by measuring mitochondrial activity using the MTT-based cytotoxicity assay at intervals of 0, 24, 48, and 72 h after the first laser application. An energy density of 1.0 J/cm² improved the cell proliferation in comparison to the other groups (control and laser 0.5 J/cm²) at 48 and 72 h. The group irradiated with 1.0 J/cm² presented significantly higher MTT activity at 48 and 72 h when compared to the energy density of 0.5 J/cm². It can be concluded that LLLI using infrared light and an energy density of 1.0 J/cm² has a positive stimulatory effect on the proliferation of hPDLSC.

Keywords Periodontal ligament · Stem cells · Lasers · Cell proliferation

Introduction

Dental stem cells were first identified in the pulp of permanent teeth [1] and later in dental and periodontal sources, including the periodontal ligament [2]. These cells are able to differentiate into osteoblasts, fibroblasts, and cementoblasts, a property that is essential for the repair of periodontal tissues [2, 3].

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The ability of low-level laser irradiation (LLLI) to stimulate the proliferation of a variety of cell types has been indicated as its most important physiological effect [4]. Studies have shown that LLLI promotes an increase in the proliferation rate of cells such as fibroblast [5, 6], endothelial cells [6], osteoblasts [7], epithelial cells [8], and lymphocytes [9].

With respect to the proliferation of mesenchymal stem cells, a positive effect of LLLI on bone marrow [4, 10] and adipose tissue stem cells has been reported in the literature [11-13]. However, very little is known about the effect of laser therapy on adult stem cells derived from other sites. Therefore, the objective of the present study was to evaluate the effect of LLLI on the proliferation rate of human periodontal ligament stem cells (hPDLSC).

Materials and methods

The study was approved by the Ethics Committee of the Federal University of Rio Grande do Norte, Brazil. Human PDLSC were obtained from two healthy permanent third molars extracted due to surgical indication and isolated according to the protocol of Vasconcelos et al. [14]. After careful root scraping and enzymatic digestion of the periodontal ligament extract, the cells were cultured on plates containing α-MEM medium supplemented with 15 % fetal bovine serum and the culture medium was changed at intervals of 3 days until the cells reached 80–95 % confluence.

Flow cytometry revealed positive staining of the periodontal ligament cells for CD29/integrin beta1 (BD Biosciences, USA), a surface marker expressed on stem cells. Next, the cells were cultured in osteogenic and adipogenic differentiation media (StemPro® Differentiation kits, Invitrogen Corp., USA) for 21 days to confirm their multipotent nature. Examination by light microscopy showed the characteristic morphology of osteoblastic and adipose cells.

P3 cells (third subculture) were irradiated with an InGaAlP diode laser (Kondortech, Brazil) using the parameters shown in Table 1. The cells were irradiated at 0 and 48 h (Fig. 1), with the laser probe fixed perpendicular to each plate at a distance of 0.5 cm from the cells. Cell viability and proliferation were analyzed at 0, 24, 48, and 72 h after the first laser application in the control (not irradiated) and irradiated groups using two methods, respectively: evaluation of mitochondrial activity by reduction of MTT and Trypan blue exclusion method. The cells were plated in such a way that there was always one (for 24-well plates) or three (for 96-well plates) empty wells between seeded wells in order to minimize the unintentional dispersion of light between wells during laser application.

For the MTT assay, the cells were cultured in 96-well plates at a density of 5×10^3 cells/well. One 96-well plate was used for each time point, with four wells per group (control, 0.5 and 1.0 J/cm^2) and absorbance of the samples was monitored in an ELISA reader at 570 nm. For Trypan blue staining, the cells were cultured in 24-well plates at a density of 3×10^4 cells/well. The number of Trypan blue-stained cells in each well was counted in a Neubauer chamber at each time point. The cell counts were performed by two blinded and previously calibrated examiners. Differences between groups at each time point studied were analyzed by the Kruskal–Wallis and Mann–Whitney tests, adopting a level of significance of 5 % ($p < 0.05$).

Results

Table 2 demonstrates the number of hPDLSC analyzed by the Trypan blue exclusion method in the different groups. An increase of cell proliferation over time was observed in all groups. Comparison of the mean number of cells between the irradiated groups and the control group only revealed significant differences for the group irradiated with an energy density of 1.0 J/cm^2 at 48 and 72 h. The number of cells was similar in the group irradiated with an energy density of 0.5 J/cm^2 and the control group at all time points studied. When the groups submitted to laser irradiation were compared, a significant difference was observed at 48 and 72 h, with a higher growth rate in the group irradiated with the energy density of 1.0 J/cm^2. Cell viability analyzed by the Trypan blue exclusion method was similar in the control and irradiated groups at all time points studied.

Table 1 InGaAlP laser parameters used

Parameter	Value
Power	30 mW
Wavelength	660 nm
Mode of action	Continuous
Output area	0.03 cm^2
Tip diameter	0.01 cm^2
Output fiber diameter	2 mm
Energy density	0.5 and 1.0 J/cm^2
Irradiation time	16 s (0.5 J/cm^2) and 33 s (1.0 J/cm^2)

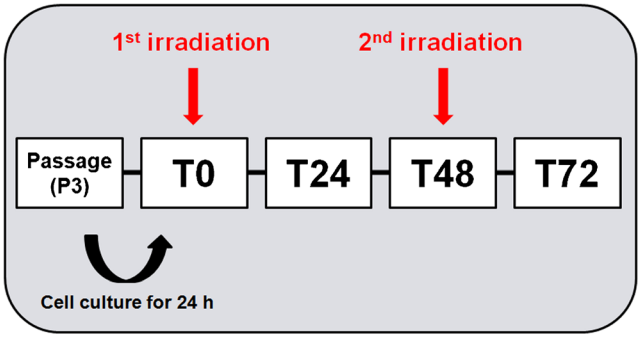

Fig. 1 Experimental design of laser irradiation

The pattern of mitochondrial activity of hPDLSC analyzed by the MTT assay is illustrated in Fig. 2. Mitochondrial activity in the irradiated groups followed the pattern observed by the Trypan blue exclusion method. Irradiation with an energy density of 1.0 J/cm^2 promoted significantly higher number of cells when compared to the group irradiated with 0.5 J/cm^2 at the last two time points studied (48 and 72 h).

Discussion

Studies investigating the biomodulatory effect of LLLI on dental stem cells are scarce in the literature and there are no reports on stem cells derived from the periodontal ligament. Submitting cells to LLLI, some authors observed higher proliferation rates of gingival fibroblasts [15], dental pulp stem cells from permanent teeth [16], and stems cells derived from bone marrow and adipose tissue [10-12], in agreement with the present study.

The controversial aspects of laser therapy in the literature include the ideal light spectrum, power level, and energy density and wavelength to obtain the desired results or to maximize these results. In vitro studies have shown that both the visible and invisible light spectrum can stimulate cell proliferation. However, most of the studies that reported satisfactory results have used the visible light spectrum [12, 16, 17]. This spectrum was also used in the present study and only the irradiation dose was modified, with the observation of more favorable results when a dose of 1.0 J/cm^2 was used.

The laser therapy wavelength seems to influence its effect on cell cultures. Previous studies have shown that wavelengths

Table 2 Number and percent viability of human periodontal ligament stem cells at different times after laser therapy in the groups studied

	Control	Laser 0.5	Laser 1.0	*p* value[a]		
				Control vs. 0.5	Control vs. 1.0	0.5 vs. 1.0
T0	2.5±0.6	2.3±0.9	1.8±0.3	0.6423	0.0885	0.6423
	100 %	100 %	90 %			
T24	5.8±0.3	5.0±1.2	5.5±0.6	0.6423	0.6423	0.6423
	88.5 %	94.4 %	96.2 %			
T48	6.8±0.3	7.8±0.9	9.8±0.9	0.0885	0.0284*	0.0284*
	100 %	97.2 %	100 %			
T72	14.8±3.2	16.3±3.2	25.3±6.1	0.3065	0.0284*	0.0284*
	96.6 %	100 %	100 %			

Values are reported as the mean ± standard deviation ($\times 10^4$ cells/ml) and percent viability

*$p < 0.05$, significant difference

[a] Mann–Whitney test

in the red light spectrum, ranging from 600 to 700 nm, promote an increase of cell proliferation and differentiation [18, 19]. In contrast, light in the infrared spectrum at a wavelength ranging from 810 to 830 nm has been associated with the inhibition of proliferation of some cell types [5, 6]. On the basis of these findings, we chose a wavelength of 660 nm which promoted positive stimulatory effects similar to those reported by Eduardo et al. [16] and Horvat-Karajz et al. [17] for other cell types.

The energy density is another parameter that needs to be considered when the objective is cellular biostimulation. According to Tuner and Hoder [20], biostimulation can be achieved with doses as low as 0.001 J/cm^2 and even higher doses, with 10 J/cm^2 being the highest dose used for this purpose. Eduardo et al. [16] reported successful proliferation of pulp stem cells derived from permanent teeth using a wavelength of 660 nm and energy density of 3.0 J/cm^2. According to Karu [21], an energy density increase can damage the photoreceptors, with a consequent reduction in the biomodulatory effect of the laser. Using lower energy densities (0.5 and 1.0 J/cm^2), laser application promoted biostimulation in the present study.

In addition to energy density, power and wavelength, the ideal number of laser applications for different illnesses is also

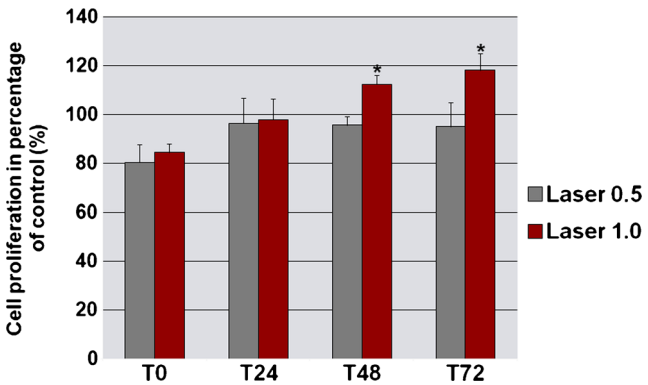

Fig. 2 Proliferation of human periodontal ligament stem cells incubated for different periods of time. Values are the mean percentage of control ± standard deviation (*$p < 0.05$).

controversial. The present results showed that a power of 30 mW in combination with a dose of 1.0 J/cm^2 has positive effects and a lower dose seems to have little influence on the proliferation rate of hPDLSC. With respect to the interval between laser applications, an interval of 48 h between applications seems to exert a positive effect on cell proliferation. Similar results have been reported by Li et al. [22] who compared the proliferation of bone marrow stem cells irradiated in a single session and at an interval of 48 h over a period of 13 days.

According to Huang et al. [23], laser therapy exerts a dose-dependent effect on biological responses and seems to have a cumulative effect at each new dose applied. This fact was confirmed in the present study by the response of hPDLSC to laser therapy, with the observation of an ascending growth curve at the doses tested and a cumulative effect over time. The response was more pronounced when the cells were irradiated with the dose of 1.0 J/cm^2 compared to 0.5 J/cm^2, as indicated by the higher proliferation rates at the last two time points (48 and 72 h), after the second irradiation.

Different approaches, including LLLI and stem cell therapies, have been used in an attempt to restore lost periodontal tissue. In this respect, several studies demonstrated improvement in the regeneration potential of lost alveolar bone when laser therapy was applied as an alternative in periodontal regeneration [24]. Furthermore, therapy with stem cells derived from different sources has shown satisfactory results in terms of the regeneration of supporting periodontal tissues [2, 25].

In summary, the present results showed that LLLI using infrared light and an energy density of 1.0 J/cm^2 has a positive influence on the in vitro proliferation of hPDLSC. These findings and literature data suggest that the combination of laser therapy and tissue engineering using stem cells may further improve the outcomes of periodontal regeneration reported in previous studies. However, further studies using other LLLI protocols and investigating the effects of this therapy on cell functions are needed to establish parameters of laser therapy that permit satisfactory clinical results.

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