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Low intensity lasers differently induce primary human osteoblast proliferation and differentiation

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ABSTRACT

Among various compounds used in research and clinic for degenerative bone diseases, low level laser therapy (LLLT), comprising low level lasers (LLL) and light emitting diodes (LEDs), has been investigated regarding its effects on bone metabolism. They have specific wavelengths but in general act as a cellular biomodulator, and as a therapeutic agent, rebalancing and normalizing their activity. However, they are not standardized yet, since their parameters of use are relevant for the effects and mechanisms of action. Therefore, the aim of this study was to compare the influence of two spectrums of LLL and LED phototherapy, at the same energy densities (10 and 50 J/cm^2), on human osteoblasts proliferation and differentiation. The involvement of ERK signaling on proliferation was also investigated by evaluating its activation during proliferation under different phototherapies by western blotting and CFSE-based osteoblast proliferation was measured in a presence or absence of the ERK-specific inhibitor. Osteogenic differentiation was evaluated through in vitro mineralization and gene expression of type I collagen (COL1A1) and osteonectin (SPARC) by Real Time- PCR. Increases in viable cells and proliferation were obtained after irradiation, regardless of LLLT type. However, only red at 10 J/cm^2 and infrared at both doses, but not LED, induced ERK1/2 activation. In the presence of ERK inhibitor, the LLL-induced proliferation was prevented. In addition, while COL1A1 gene expression was upregulated by red laser, SPARC does so by infrared stimulation. However, LED, at both doses, increased both COL1A1 and SPARC expression. All LLLT increased mineralization, dependent on the dose and time. Thus, LLL and LED differently modulated the metabolism of human osteoblasts, increasing proliferation by mechanism dependent or not of ERK signaling activation and osteogenic differentiation markers.

1. Introduction

Low level laser therapy (LLLT) is a treatment based on red and infrared lasers or light-emitting diodes (LEDs) that promotes changes at cellular levels through photobiomodulation. The therapeutic use of low-level laser (LLL) has become widespread in biology, dentistry and medicine. Several benefits have been accompanied by its use such as pain relief, stimulation of mitochondrial activity [1], recovery from nerve injury [2,3], angiogenesis [4], modulation of inflammation [5], vasodilation [6], and tissue healing [7]. Mechanisms involved in LLL-based tissue healing are dependent on the increased cell turnover, recruitment, proliferation and differentiation. Target cells include mesenchymal cells [8], keratinocytes, fibroblasts [9], endothelial cells [10], and osteoblast [11]. Considering the variety of laser, time of exposure, cell types and application protocols, it is still difficult to standardize positive clinical LLL based-protocols. Moreover, the fact that the mechanisms by which they play an effective role in several biological processes have not been fully understood.

In this context, several cellular and molecular mechanisms have been proposed to the LLL-induced biological responses. Data from our laboratory has determined that two different LLL, red and infrared lasers, both at a potency of 20 mW and energy density of 1.9 J/cm^2 or 3.8 J/cm^2 increases mouse preosteoblast MC3T3-E1 proliferation, alkaline phosphatase (ALP) and metalloproteinase (MMP)-2 activity at early time point but not of MMP-9 (unpublished results). Also, it is extensively described that the stimulatory effect of LLL in bone cells is dependent on intracellular signaling protein activation [12]. A recent study demonstrated that LLLT at 70 mW power and energy density of 3 J/cm^2 stimulated primary human alveolar bone-derived osteoblast differentiation when cultured on titanium discs [13].

However, there still no data about the influence of light exposition on human primary osteoblast at late periods under osteogenic stimulus. Neither the impact of LLL-induced mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinases (ERK) activation on cellular proliferation and gene transcription codifying extracellular matrix formation markers and mineralization. The aim of this study was to

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evaluate the effects of two doses of red, infrared and LED on proliferation and differentiation of primary human osteoblast for driving bone regeneration.

2. Material and Methods

2.1. Human Primary Osteoblast Isolation and Culture

Cells were collected from bone explants during orthopedic surgeries. The procedure was approved by the local Ethics Committee, Fluminense Federal University (protocol # 232/08) and the Ethics Committee of the Bauru Dental School-University of Sao Paulo (protocol # 95/2011). Explants were treated with 0.25% collagenase for 2 h and then placed in 25 cm^2 flasks in Dulbecco's Modified Eagle Medium (DMEM) culture medium supplemented with 10% fetal bovine serum (FBS). Cells were maintained at 37 °C in an atmosphere containing 5% CO_2 and 95% air. After single cells were attached to the plastic flask, non-adherent cells were removed by changing the medium. When confluent, the cells were sub-cultured with 0.25% trypsin-EDTA. Cells of passage 9 were used for the experiments. When appropriated, cells were cultured or not in osteogenic medium containing 50 μg/mL acid ascorbic and 10 mM β-glicerophosphate in DMEM/ 10% FBS.

2.2. Phototherapy

Low intensity laser irradiations were done by using the Twin Flex Evolution diode laser (MM Optics-São Carlos, SP, Brazil). Red laser at a wavelength of 660 nm and InGaAlP active medium, infrared at a wavelength of 780 nm and GaAlAs active medium or light emitting diode (LED) at a wavelength of 637 ± 15 nm were applied to cells. They were set up as continuous mode, potency of 40 mW, power density of 1 W/cm^2, energy density of 10 and 50 J/cm^2, energy dose of 6 and 31 J, and exposure time of 10 and 50 s, respectively. All parameters are described in Table 1. Power measurements were checked by using the Laser Check PowerMeter (Coherent Inc., Santa Clara, CA, USA). The irradiation with different lasers was performed under the plate [14]. In 96 well plates, the stimulation was performed with static probe mode since the diameter of each well and the laser spot area is 6.4 mm and 4 mm^2, respectively. In 24-well plates (15.4 mm diameter), irradiation was done with a scanning mode [15]. Single or double application protocols were performed for cell viability and proliferation where the last was applied with an interval of 6 h. For differentiation and mineralization assays, the irradiation was performed every 6 days. The assays were performed at least in triplicate.

2.3. Cell Viability

The cell viability was assessed after 1, 2 and 3 days after the last irradiation by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) and Crystal violet assays. Cells were plated in 96-well plates at a density of 2×10^3 cells in DMEM/10% FBS. Upon adhesion, 5% FBS-containing medium was replaced, to highlight the effects of the phototherapies [15]. As follows, adherent cells were irradiated with single or double application of each phototherapy. Control cells were not treated but received the same experimental conditions.

For MTT assay, the cells were washed with phosphate-buffered saline (PBS) followed by the addition of solution containing 0.5 mg MTT per mL of medium. After incubation at 37 °C for 4 h protected from light, DMSO was added to the cells. The absorbance was read in the spectrophotometer (Fluostar OPTIMA, BMG Labtech, Offenburg, Germany) at 562 nm. For crystal violet assay, the cells were washed and then added 100% methanol for 10 min. After been removed, the solution containing 0.2% crystal violet in 2% ethanol was added to the cells for 3 min followed by additional wash and incubation with 0.05 mol/L in 50% ethanol solution for 10 min. The plates were read at 540 nm.

2.4. Western Blotting

Cells were plated in 24-well plates at a density of 4×10^4 cells. Adherent cells were irradiated with different phototherapies (n = 24 for each condition). After 10 min at 37 °C, the cells were lysed with buffer solution containing 50 mM Tris-HCl (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$, and 0.2% Nonidet P-40 supplemented with protease inhibitors (Roche Diagnostics) and phosphatase inhibitor (0.2 M sodium orthovanadate - Calbiochem). The lysates were pooled, sonicated and centrifuged at 1000 rpm for 10 min at 4 °C. Protein samples (40 μg) were applied to electrophoresis in Tris-HCl 10% polyacrylamide gel and subsequently transferred to PVDF membrane. It was immuno-labeled with rabbit polyclonal anti-phospho-ERK or anti-ERK primary antibodies (Cell Signaling) followed by secondary anti-rabbit IgG conjugated to HRP (Horseradish Peroxidase - Cell Signaling) and ECL reagent (enhanced chemiluminescence) detection (Amersham Biosciences). The relative densities of the bands were determined by densitometry analysis using Image J software (National Institutes of Health, NIH Image). The density values obtained were corrected by subtraction of the background values.

2.5. Proliferation Assay

Cell proliferation was performed by labeling cells with carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes) followed by flow cytometry analysis. Osteoblasts at a density of 3×10^5 (n = 3) were incubated with 10 μM CFSE for 10 min at 4 °C, according to the manufacturer's protocol. After washing with PBS, the labeled cells were plated in 24-well plates followed by double application phototherapies. When appropriate, the cells were pretreated with 20 μM ERK inhibitor (PD98059) for 30 min at 37 °C. After 3 days of the last irradiation, cells were trypsinized and acquired in FACsorting (BD Biosciences). Analyzes were performed using the CellQuest program (CellQuest software, BD).

2.6. Real Time RT-PCR

5×10^2 cells in 96-well plates in different conditions had the mRNA extracted and transcribed into cDNA using TaqMan® Gene Expression Cells-to-CT™ (Applied Biosystems) kit according to manufacturer's recommendations after 7, 14, 21 and 28 days of culture. The cDNA samples were incubated with Taqman® Gene Expression Master Mix and Taqman® Gene Expression assay (Applied Biosystems) for SPARC (secreted protein acidic and rich in cysteine-also named osteonectin) or COL1A1 (type I collagen) and were read in the Real-Time RT-PCR System ViiATM7 (Applied Biosystems). PCR amplification was performed

Table 1
Irradiation parameters.

Light source	Wavelength (nm)	Output power (mW)	Power density (W/cm^2)	Energy density (J/cm^2)	Energy dose (J)	Exposure time (s)	Application mode
Red laser (InGaAlP)	660	40	1	10	6	10	Continuous
	660	40	1	50	31	50	Continuous
Infrared (GaAlAs)	780	40	1	10	6	10	Continuous
	780	40	1	50	31	50	Continuous
LED	637 ± 15	40	1	10	6	10	Continuous
	637 ± 15	40	1	50	31	50	Continuous