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Low level laser therapy modulates viability, alkaline phosphatase and matrix metalloproteinase-2 activities of osteoblasts

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ABSTRACT

Low level laser therapy (LLLT) has been shown to stimulate bone cell metabolism but their impact on the matrix metalloproteinase (MMP) expression and activity is little explored. This study evaluated the influence of LLLT at two different wavelengths, red and infrared, on MC3T3-E1 preosteoblast viability, alkaline phosphatase (ALP) and MMP-2 and -9 activities. To accomplish this, MC3T3-E1 cells were irradiated with a punctual application of either red (660 nm; InGaAlP active medium) or infrared (780 nm; GaAlAs active medium) lasers both at a potency of 20 mW, energy dose of 0.08 or 0.16 J, and energy density of 1.9 J/cm^2 or 3.8 J/cm^2, respectively. The control group received no irradiation. Cellular viability, ALP and MMP-2 and -9 activities were assessed by MTT assay, enzymatic activity and zymography, respectively, at 24, 48 and 72 h. The treatment of cells with both red and infrared lasers significantly increased the cellular viability compared to the non-irradiated control group at 24 and 48 h. The ALP activity was also up modulated in infrared groups at 24 and 72 h, depending on the energy densities. In addition, the irradiation with red laser at the energy density of 1.9 J/cm^2 promoted an enhancement of MMP-2 activity at 48 and 72 h. However, no differences were observed for the MMP-9 activity. In conclusion, when used at these specific parameters, LLL modulates both preosteoblast viability and differentiation highlighted by the increased ALP and MMP-2 activities induced by irradiation.

1. Introduction

Low level laser therapy (LLLT) has been used to assist conventional treatments due to its anti-inflammatory and analgesic effects and ability to promote wound healing acceleration [1]. Besides these properties, recent studies show that phototherapy is an important strategy used to stimulate and/or increase bone formation [2–5].

The LLLT effect in cultured bone cells has been extensively studied over the past two decades. Irradiation with a pulsed Nd:YAG laser at 1.5 J/cm^2 stimulated the proliferation and differentiation of human osteoblasts [6]. The phototherapy in the murine preosteoblastic cell line MC3T3-E1 resulted in an increased mineralization by a mechanism dependent on laser-induced bone morphogenetic proteins (BMPs) [7]. Moreover, it was shown that a low intensity pulsed laser applied to the cultures of primary osteoblasts obtained from rat calvaria or a cell line induces greater mitochondrial activity [8], proliferation [9–12], DNA and RNA synthesis [12], cellular differentiation [13–14], and smaller RANKL/OPG relation. This information suggests that LLLT promotes enhancement of bone formation while decreasing osteoclastic differentiation and activity [9]. The effect of laser irradiation on human osteoblast stimulation was recently demonstrated by our group. LLLT and Light Emitting Diodes (LED), at 10 and 50 J/cm^2, differently modulated the metabolism of those cells, increasing proliferation by mechanism dependent or not of ERK signaling activation and osteogenic differentiation markers such as type I collagen and osteonectin [13]. Moreover, LLLT at the energy density of 0.43 J/cm^2 for two consecutive days induced an enhancement of cell proliferation, and expression of osteopontin and bone sialoprotein [14,15]. The osteogenic differentiation of human periodontal ligament cells was increased by low-intensity pulsed laser at a frequency of 1.5 MHz and intensity of 90 mW/cm^2,

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through BMP-Smad signaling pathway [16]. All these properties may positively associate laser irradiation and bone surgery, optimizing both the orthopedic and dental areas.

Although both *in vivo* and *in vitro* effects of LLLT on osteogenesis is largely studied, little is known about its impact on the expression and/or activity of some proteins, *i.e.* the family of matrix metalloproteinases (MMPs). MMPs are an important family of metal-dependent endopeptidases and represent the largest class of enzymes responsible for the degradation or resorption of all the components of the extracellular matrix [17]. These enzymes play an important role in tissue remodeling associated with various physiological and pathological conditions such as morphogenesis, angiogenesis, tissue repair, cirrhosis, arthritis and metastasis [18]. During the bone remodeling cycle, osteoblasts express and secrete abundant quantities of MMP-2 and -9, which are responsible for degrading small fragments of collagen. However, little is known about the effect of LLLT on their modulation.

In this study, we sought to evaluate the effect of two different LLLT, red and infrared lasers, in preosteoblast viability, alkaline phosphatase (ALP) and MMP-2 and -9 activities.

2. Material and Methods

2.1. Cell Culture

Mouse preosteoblast MC3T3-E1 (subclone 14) cells were obtained from ATCC and cultured in MEM culture medium supplemented with 10% fetal bovine serum (FBS) and nucleosides. Cells were maintained at 37 °C in an atmosphere containing 5% CO_2 and 95% air. When appropriated, the cells were cultured in osteogenic medium containing 50 μg/ml acid ascorbic and 10 mM β-glycerophosphate in MEM/10%FBS, as previously described [19].

2.2. Phototherapy

Irradiation was done with the Twin Flex Evolution low intensity laser diode (MM Optics-São Carlos, SP, Brazil). A red laser with a wavelength of 660 nm and InGaAlP active medium and an infrared with a wavelength of 780 nm and GaAlAs active medium were utilized. Both were once applied in the continuous irradiation mode and beam profile Gaussian at a potency of 20 mW, power density of 0.5 W/cm^2, energy density of 1.9 or 3.8 J/cm^2, total energy dose of 0.08 or 0.16 J, and time of irradiation exposure of 4 or 8 s. The laser equipment had its power measured with the Laser Check PowerMeter (Coherent Inc., Santa Clara, CA, USA). The area of the laser spot is 0.04 cm^2. A single application of the device was performed directly on the bottom side of the plate to assure that irradiation would penetrate into the monolayer cells. The plates were wrapped in a black cardboard mask with holes located in the position of the wells of the experimental groups so that only the uncovered well could be irradiated [20,21].

2.3. Cellular Viability

MC3T3-E1 viability was evaluated by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay, as described previously [13]. Cells were plated on 96-well plates at a density of 2×10^3 cells/well in MEM containing 10% FBS. After 6 h, the adherent cells were cultured in MEM medium/5% FBS to promote nutritional cellular stress, allowing that the phototherapy effects to present itself [22]. Cells were irradiated either with red (660 nm, 20 mW) or infrared laser (780 nm, 20 mW) at the energy density of 1.9 or 3.8 J/cm^2 and evaluated after 24, 48 and 72 h. Control cells did not receive irradiation.

2.4. ALP Activity

Cells were plated in 24-well plates at a density of 4×10^4 cells/well in MEM with 10% FBS. Adherent cells were cultured in osteogenic medium and irradiated as described before. After 24, 48 and 72 h, the total cellular protein extract was obtained by lysis buffer containing 10 mM Tris pH 7.5, 0.5 mM $MgCl_2$ and 0.1% Triton X-100. The ALP activity was measured by enzymatic assay using *p*-nitrophenyl phosphate (pNPP) as a substrate [22]. For the ALP activity assay, a solution containing 25 mM glycine buffer (pH 9.4), 2 mM $MgCl_2$ and 1 mM p-NPP were added to 96-well plates. After incubation for 30 min in a water bath, each sample was added in duplicate. The plate was kept at 37 °C for a time required for a reaction to occur, and then stopped with 1 M NaOH. The final product (*p*-nitrophenol) was quantified at 405 nm and the results were expressed as unit of enzyme activity/mg of protein where one unit was defined as the amount of enzyme that converted 1 μmol of substrate to product per minute. Protein concentrations were determined by the Bradford method.

2.5. MMP-2 and -9 Activities

The cells were plated in 24-well plates at a density of 5×10^4 cells/well in osteogenic medium 5% FBS followed by irradiation or not with either red or infrared laser. MMP-2 and -9 activities in cell supernatants were evaluated by gelatin zymography. Six hours before each supernatant collection, the complete medium containing FBS was replaced with medium without FBS. 20 μg of total protein of each group were applied in 11% acrylamide gels containing 10 mg/ml gelatin. For the loading control, recombinant MMP-2 and -9 (Calbiochem, San Diego, CA, USA) were utilized. After electrophoresis, the gels were stained with 0.5% Coomassie Blue G-250 and bleached by a solution containing 30% methanol and 10% acetic acid. The gels were performed in triplicate. Gelatinolytic activities were detected as transparent bands against the background of Coomassie blue-stained gelatin. To quantify the activities of the detected enzymes, zymograms were imaged using the Image Scanner®. The intensities of the separate bands were analyzed using the software Scion Image (NIH, USA). All values were deducted from the background. Bands from the active fraction (~62 kDa for MMP-2 and ~82 kDa for MMP-9) were taken for analysis. Conditioned medium from non-irradiated cells was used as a MMP-2 and -9 reference standard.

2.6. Statistical Analysis

Results are expressed as mean ± SD or mean ± SEM (as indicated) from one out of three independent experiments. Distribution of all variables was tested for normality by using the Kolmogorov and Smirnov (GraphPad Instat). Statistical analyses were performed using the ANOVA, followed by Tukey's *post hoc* test (GraphPad Prism 4). $P < 0.05$ was considered statistically significant.

3. Results

3.1. LLLT Enhances Preosteoblast Viability

At 24 and 48 h, phototherapy with red and infrared lasers, both at energy densities of 1.9 and 3.8 J/cm^2, increased cell viability compared to the non-irradiated cells ($P < 0.01$; $P < 0.001$) (Fig. 1). At 72 h, only the infrared laser with the energy density of 3.8 J/cm^2 enhanced it compared to control ($P < 0.05$). No statistical differences were observed among the lasers and their energy densities in the evaluated periods (Fig. 1).

3.2. Modulation of ALP Activity by Irradiation Therapy

At 24 h, the infrared laser at the energy density of 1.9 J/cm^2 increased the ALP activity compared to the control ($P < 0.001$). At the energy density of 3.8 J/cm^2, both red laser and infrared induced greater ALP activity when compared to the control at 72 h ($P < 0.05$ and $P < 0.001$,