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Effects of low level laser therapy on inflammatory and angiogenic gene expression during the process of bone healing: A *microarray* analysis

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ABSTRACT

The process of bone healing as well as the expression of inflammatory and angiogenic genes after low level laser therapy (LLLT) were investigated in an experimental model of bone defects. Sixty Wistar rats were distributed into control group and laser group (830 nm, 30 mW, 2,8 J, 94 seg). Histopathological analysis showed that LLLT was able to modulate the inflammatory process in the area of the bone defect and also to produce an earlier deposition of granulation tissue and newly formed bone tissue. *Microarray* analysis demonstrated that LLLT produced an up-regulation of the genes related to the inflammatory process (MMD, PTGIR, PTGS2, Ptger2, IL1, 1IL6, IL8, IL18) and the angiogenic genes (FGF14, FGF2, ANGPT2, ANGPT4 and PDGFD) at 36 h and 3 days, followed by the decrease of the gene expression on day 7. Immunohistochemical analysis revealed that the subjects that were treated presented a higher expression of COX-2 at 36 h after surgery and an increased VEGF expression on days 3 and 7 after surgery. Our findings indicate that LLLT was efficient on accelerating the development of newly formed bone probably by modulating the inflammatory and angiogenic gene expression as well as COX2 and VEGF immunoexpression during the initial phase of bone healing.

1. Introduction

Fracture healing is a multistage repair process that involves complex and well-orchestrated steps which are initiated in response to the injury and with the purpose of recovering bone mechanical functions [1,2]. In general, bone tissue has the ability of healing by itself [3]. However, under critical conditions, such as in larger bone defects and fractures with inadequate or interrupted vascularization, a delay in the healing process or even a nonunion may happen [4]. Therefore, innovative clinical approaches such as low-level laser therapy (LLLT) [5,6] have been developed in order to stimulate the bone healing process.

LLLT irradiation stimulates mitochondrial metabolism, which results in an increased expression of adenosine triphosphate (ATP), molecular oxygen production [6] and transcription factors [7]. These effects may increase the synthesis of DNA, RNA and cell-cycle regulatory proteins, therefore stimulating cell proliferation [8,9]. Moreover, LLLT could modulate the expression of some inflammatory mediators such as interleukin 1β, (IL1β), interleukin 6 (IL6), interleukin 10 (IL10) and tumor necrosis factor α (TNFα) [6,10–12]. Furthermore, it has been demonstrated that LLLT stimulates angiogenesis, which is an essential part of the healing process [13,14]. Based on the effects of LLLT aforementioned, this therapeutic modality has been used to promote wound healing, to accelerate tissue repair and to modulate the inflammatory processes after injuries [15,16]. Furthermore, the effects of LLLT on the process of bone consolidation have been demonstrated and showed an increased osteoblastic activity [17], neoangiogenesis as well as higher newly formed bone deposition at the site of the fracture [15]. In a recent *in vivo* study, Bossini *et al.* [13] observed that LLLT (830 nm) could increase newly formed bone and angiogenesis at the site of the fracture in an experimental model of bone defect in the tibias of rats.

Despite the encouraging data concerning the osteogenic potential of LLLT, the molecular and cellular mechanisms by which this therapy acts on tissues remain unclear [18,19]. Moreover, works describing the effects of LLLT in expression of inflammatory and angiogenic genes in early fracture healing are still scarce. Thus, with the advent of more sophisticated techniques in gene expression analysis (*i.e. microarrays*), it

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has become possible to examine the global gene expression and to investigate entire pathways that command biological processes. Therefore, *microarray* is very helpful in identifying possible genes regulated by LLLT during the process of bone healing [20].

In view of the aforementioned information, it was hypothesized that LLLT can regulate the expression of genes involved in the inflammatory process and angiogenesis, which in turn may accelerate the process of bone repair. Thus, the present study aimed to investigate the histological modifications in the bone callus and to study the expression of genes related to the inflammatory process and the formation of new blood vessels after LLLT irradiation in the initial stages of healing.

2. Materials and Methods

2.1. Experimental Design

Sixty male Wistar rats (12 weeks old; weighing ~300 g) were used in this study, which was conducted in accordance with the Guide for Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of the Federal University of São Carlos (010/2011).

Anesthesia was induced by an intra-peritoneal injection of Ketamine (Agener®, 40 mg/kg, IP) and Xylazine (Syntec®, 20 mg/kg, IP). Bilateral bone defects (3.0 mm diameter) were surgically created at the tibia (10 mm distal of the knee joint). In order to do this, the tibia was exposed through a longitudinal incision on the shaved skin, and after that, bone defect was created by using a motorized round drill (BELTEC®, Araraquara-SP, Brazil), under constant physiologic saline solution irrigation. Once the defect had been done, it was packed with sterile cotton gauze to stop bleeding. Thereafter, the cutaneous flap was joined and sutured with resorbable Vicryl® 5–0 (Johnson & Johnson, St. Stevens-Woluwe, Belgium). The animals were divided into 2 groups ($n = 30$ each group): bone defect control group (CG) (bone defects without any treatment) and bone defect laser irradiated group (LG). In order to minimize post-operative discomfort, the animals received analgesia (i.m., 0,02 mg/kg buprenorfiine — Temgesic; Reckitt Benckiest Healt Care Ltd. Schering-Plow, Hoddesdon, UK) directly after the operation and subcutaneously for 2 days after surgery. Rats were individually euthanized by carbon dioxide asphyxia in different set points (36 h, 3 days and 7 days after surgery).

2.2. Low Level Laser Therapy

A laser (Thera laser, DMC®, São Carlos, Brazil), CW, 830 nm, 0.6 mm beam diameter, 30 mW, 94 s, 2.8 J was used in this study. LLLT sessions were applied immediately after the surgery and repeated every 24 h at two, three and seven days. Treatments were performed by the contact technique, at one point, above the site of the injury.

2.3. Retrieval of Specimens

The right tibias, used for gene expression evaluation ($n = 10$ per group), were dissected, rapidly frozen in liquid nitrogen and stored in a freezer at −80 °C until *microarray* analysis was carried out. For the histopathological analysis, the left tibias ($n = 10$ per group), were removed and immediately fixed in 10% formaldehyde (Merck, Darmstadt, Germany) for 24 h, then were decalcified in 4% diamine tetra-acetic acid (EDTA) (Merck, Darmstadt, Germany) and embedded in paraffin blocks. Therefore, thin sections (5 μm) were prepared in the longitudinal plane, using a micrometer (Leica RM-2145, Germany). Afterwards, the laminas were stained with hematoxylin and eosin (H.E stain, Merck, Darmstadt, Germany).

2.4. Histopathological Analysis

A descriptive qualitative histopathological evaluation of the total area of the bone defect was performed by two experienced observers (PB and CT) in a blinded manner, under a light microscope (Olympus, Optical Co. Ltd., Tokyo, Japan) [13,15]. Any changes in the bone defect, such as the presence of blood clots, fibrin, inflammatory processes, granulation tissue, woven bone or even tissues undergoing hyperplastic, metaplastic and/or dysplastic transformation were investigated in each animal.

2.5. RNA Sample Preparation

Total RNA was isolated using the TRIzol® reagent (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. After the RNA isolation, the samples were purified using the illustra RNAspin Mini RNA Kit (GE Healthcare Life Sciences, USA) according to the manufacturer's instructions. The RNA concentrations were determined using a NanoVue spectrophotometer (GE Healthcare Life Sciences, USA). The quality and integrity of the total RNA were evaluated with an Agilent 2100 Bioanalyzer (GE Healthcare Life Sciences, USA) and samples presenting RNA integrity numbered ≥8 were used for cRNA synthesis.

2.6. Microarray Hybridizations

Microarray hybridizations were performed with Agilent Whole Rat Genome *Microarray* 4 × 44 K. The labeling and *microarray* hybridizations were performed by Agilent using Two-Color *Microarray*-Based Gene Expression Analysis (Agilent Technologies, USA). Briefly, for cDNA synthesis and labeling 200 ng of total RNA were used. Afterwards, cDNA was transcribed into cRNA and was labeled using Agilent Low RNA input Fluorescent Linear Amplification Kit (Agilent Technologies, Santa Clara, CA, USA). Then, the labeled cRNA was purified, mixed with hybridization buffer and hybridized to an Agilent Whole Rat Genome *Microarray* 4 × 44 K for 17 h at 65 °C, according to the manufacturer's instructions. After hybridization, *microarray*s were sequentially washed: 1 min at room temperature in GE Wash Buffer 1 (Agilent Technologies, USA) then 1 min at 37 °C in GE Wash Buffer 2 (Agilent Technologies, USA), followed by 10 s in Acetonitrile Wash (Agilent Technologies, USA) and finally 30 s in Stabilization and Drying Solution wash (Agilent Technologies, USA). Afterwards, *microarray* slides were scanned using GenePix® 4000B *microarray* scanner (Molecular Devices, USA) with simultaneously scanning the Cy3 and Cy5 channels at a resolution of 5 μm. Laser was set at 100% and PMT gain was automatically adjusted for each slide using the program GenePix 4000B according to the intensity of the signal in each array.

2.7. Microarray Data Analysis

Microarray data analysis was performed as described by Castro *et al.*, [21]. Data files were generated using Agilent's Feature Extraction Software (version 11.5, Agilent) and the default parameters, which include Lowess based signal normalization. The dye-normalized values generated in the Feature Extraction data files were used to upload the software Express Converter (version 2.1, TM4 available at http://www.tm4.org/utilities.html) which conveniently converts the Agilent file format to MeV (MultiExperiment View) file format compatible to the TM4 softwares for *microarray* analysis (available at http://www.tm4.org/). The MeV files were then uploaded in the MIDAS software where the resulting data were averaged from replicated genes on each array, from three biological replicates, taking a total of 3 intensity data points for each gene. The MeV files generated were then loaded in MeV software where differentially expressed genes were identified using one-class t-test ($p > 0.01$). Significantly different genes were those whose mean log2 expression ratio over all included samples was statistically different from 0 which indicates the absence of gene modulation.