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Ultrasound therapy modulates osteocalcin expression during bone repair in rats

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ABSTRACT

The aim of this study was to measure the temporal pattern of the protein expression of RUNX2, RANKL, OPG, and osteocalcin after ultrasound therapy during the process of bone healing by immunohistochemistry. The animals were randomly distributed into two groups: control or ultrasound-treated group. A non-critical size bone defects were surgically created at the upper third of the tibia. The treatments started 24 h post-surgery, and they are performed for 3, 6, and 12 sessions, with an interval of 48 h. A low-intensity pulsed ultrasound (1.5 MHz, 1:4 duty cycle, intensity SATA 30 mW/cm^2, 20 min/session, stationary mode application) was used. On days 7, 13, and 25 post-injury, rats were killed individually by carbon dioxide asphyxia. The tibias were removed for analysis. The histopathological analysis pointed out no remarkable differences between groups for all periods evaluated. However, immunohistochemical data revealed that ultrasound therapy produced an up-regulation of osteocalcin at day 7th and 13th post-surgery. Taken together, our results indicate that ultrasound therapy modulates osteocalcin expression during bone repair in rats as depicted by differential immunopression at the initial and intermediate stages of recovery.

1. Introduction

Million of fractures occur every year worldwide, with 6.2 million of fractures reported per year in United States [1]. Among them, 5–10% show delayed healing, many persist for more than 9 months, and some termed in nonunions fractures. Many factors can impair fracture consolidation including bone loss caused by diseases, trauma, or tumor resection [2].

In this context, there is a critical need to develop technologies to increase and to promote bone healing [2]. One promising treatment is the use of low-intensity pulsed ultrasound (LIPUS). LIPUS began to be widely used clinically to treat fresh fractures in the early 1990s and nonunions or delayed unions in the late 1990s. It is a form of mechanical energy that is transmitted through and into living tissue as acoustic pressure waves. It has been theorized that the micromechanical strains produced by these pressure waves in biological tissues may result in biochemical events that accelerate tissue healing. This therapeutic modality is well established, approved by the FDA (US Food and Drug Administration) and in frequent use.

Many in vivo experimental studies suggest that LIPUS affects the earlier inflammatory or callus formation phases of healing. Yang et al. [3] showed that aggrecan gene expression in LIPUS-treated femora was significantly higher at day 7 and significantly lower at day 21 compared to untreated controls. Some *in vitro* works have showed that LIPUS is able to increasing prostaglandin E2 (PGE2) in a mouse osteoblastic cell line [4], to elevate gene expression for bone sialoprotein (BSP), insulin-like growth factor-1 (IGF-1), and osteocalcin (OC) to up-regulated the expression of early response genes (c-jun, c-myc, COX-2, Egr-1, TSC-22) as well as the bone differentiation marker genes, osteonectin and osteopontin [5]. Also, Rawool et al. [6] suggested that LIPUS may affect the angiogenesis phase of fracture healing.

Moreover, it seems that LIPUS treatment has been shown to accelerate restoration of mineral density and mechanical properties of bone callus in experimental studies [7,8]. Shakouri et al. [9] observed that LIPUS increased callus mineral density in rats. Esteki et al. also found an increase in the biomechanical properties after osteoperfuration treated with LIPUS in rabbits. In human randomized trials, it has been shown that LIPUS can reduce the time to normal fracture repair and in distraction osteogenesis with treatment periods lasting more than 70 days [10,11].

However, the mechanism by which LIPUS acts on osteoblast cells and bone healing is not fully understood and, for many, the use of LIPUS as a treatment modality is still controversial. Thus, there is a clear clinical need to understand the molecular details of the pathways that control bone formation after LIPUS treatment

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that might be possible to accelerate the healing of fractures and to treat the 5–10% of fractures that fail to heal satisfactorily [12].

It is well know that bone regeneration is a complex temporal and spatial interaction of the osteoclasts and osteoblasts cells, regulated by a series of cell-signaling molecules, cytokines, proteins, and growth factors, which induce or modulate osteoproducing cells to create a competent bone mass. RUNX2 is a multifunctional transcription factor that controls skeletal development by regulating the differentiation of chondrocytes and osteoblasts and the expression of many extracellular matrix protein genes during chondrocyte and osteoblast differentiation [13].

Osteoclast formation, differentiation, and activity are regulated by three recently described proteins, members of the tumor necrosis factor ligand, and receptor superfamilies [14]. These include receptor activator of nuclear factor kappa B ligand (RANKL), expressed as membrane-bound or soluble forms by different cell types, including bone-forming cells, fibroblasts, T cells, and others [15]. Ligation of RANKL to its cognate receptor, RANK, results in the fusion, differentiation, and activation of osteoclasts. Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL that inhibits the interaction between RANKL and RANK [16]. The up-regulation of RANKL and the down-regulation of OPG have been implicated during bone repair as well as in a range of diseases, including osteoporosis and rheumatoid arthritis [14]. Although few studies have demonstrated the importance of the RUNX2, osteocalcin and RANKL–OPG system during bone repair, the expression of these molecules in rats treated with LIPUS remains to be elucidated.

In a recent study conducted by our group, we were able to evaluate the temporal expression of osteogenic genes during the process of bone healing in low-intensity pulsed ultrasound (LIPUS)-treated bone defects by means of histopathologic and real-time polymerase chain reaction (PCR) analysis. The results showed that quantitative real-time polymerase chain reaction (RT-qPCR) showed an up-regulation of and Runx2 gene 7 days after the surgery. In the intermediary period, there was no increase in the expression. The expression of Runx2 was significantly increased at the last period [17]. Herein, it would be interesting to know if, and to what extent, mRNA expression of these genes as well as other closely related to bone resorption could determine protein expression.

As a result and because the lack of scientific evidence, the aim of the present study was to investigate the expression of RUNX-2, osteocalcin, RANKL, and OPG in rats submitted to LIPUS therapy, after days 7, 13, and 25 of bone post-injury.

2. Materials and methods

2.1. Animals

Male Wistar rats (weighing 300 ± 20 g, 12–13 weeks, $N = 60$) were divided randomly into two groups: control or LIPUS irradiated group. They were maintained under controlled temperature (22 ± 2 °C), light–dark periods of 12 h, and with free access to water and commercial diet. All animal handling and surgical procedures were strictly conducted according the Guiding Principles for the Use of Laboratory Animals. This study was approved by the Animal Care Committee guidelines of the Federal University of São Paulo. As described below, a non-critical size bone defects were performed on both tibias. Rats were killed 7, 13, and 25 days after surgery.

2.2. Surgery

A non-critical size bone defects were surgically created at the upper third of the tibia (10 mm distal of the knee joint). Surgery was performed under sterile conditions, and general anesthesia induced by intra-peritoneal injection of xilazin (Syntec®, 20 mg/kg, IP) and ketamin (Agener®, at 40 mg/kg, IP). The medial compartment of the tibia was exposed through a longitudinal incision on the shaved skin and muscle tissue. A standardized 2.5-mm-diameter bone defect was created by using a motorized drill (Kavo®, Joinville, Santa Catarina, Brazil) (12,356 rpm) under copious irrigation with saline solution. The cutaneous flap was replaced and sutured with resorbable polyglactin, and the skin was disinfected with povidone-iodine. The animals received analgesia (i.e., 0.05 mg/kg buprenorphine) and were returned to their cages. The health status of the rats was monitored daily.

2.3. Treatments

The treatments started 24 h post-surgery, and they are performed for 3, 6, and 12 sessions, with an interval of 48 h. Both treatments were performed through the contact technique on the skin, above the site of the bone injury. A low-intensity pulsed ultrasound (Exogen, Tennessee, USA) (1.5 MHz, 1:4 duty cycle, intensity SATA 30 mW/cm^2, 20 min/session, stationary mode application) was used. The dose was chosen because this is a therapeutic modality widely used to accelerate bone healing, and it is approved by the FDA (US Food and Drug Administration) and in frequent use [18].

On days 7, 13, and 25 post-injury, rats were killed individually by carbon dioxide asphyxia. Both tibias were removed for analysis.

2.4. Histopathological analysis

For the qualitative histopathological analysis, the right tibiae were used. They were removed, fixed in 10% buffer formalin (Merck, Darmstadt, Germany) for 48 h, decalcified in 4% EDTA (Merck), and embedded in paraffin blocks. Five-micrometer slices were obtained in a serially sectioned pattern and stained with hematoxylin and eosin (H.E stain, Merck). A descriptive qualitative histopathological evaluation of the total area of the bone defect was performed by a pathologist (blinded to the treatment), under a light microscope (Olympus, Optical Co. Ltd., Tokyo, Japan), at 25× magnification. Any changes in the bone defect, such as presence of woven bone, bone marrow, inflammatory process, granulation tissue, or even tissues undergoing hyperplastic, metaplastic, and/or dysplastic transformation were investigated per animal.

2.5. Immunohistochemistry

Sections at 3 μm were deparaffinized in three changes of xylene and rehydrated in a graded series of ethanol to distilled water. For antigen retrieval, slides were placed in 0.01 M citrate buffer pH 6.0 and heated in a steamer for 30 min. Endogenous peroxidases were quenched by incubating in 3% H_2O_2 for 20 min at room temperature. Sections were incubated overnight at 4 °C with primary antibody: RUNX-2 mouse polyclonal antibody (1:200), osteocalcin mouse polyclonal antibody (1:200), RANKL mouse polyclonal antibody (1:200), or OPG mouse polyclonal antibody (1:200), (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Subsequently, sections were incubated with biotinylated secondary antibody (LSAB, Dakocytomation) for 30 min, washed in PBS, and incubated with streptavidin–peroxidase conjugate (LSAB, Dakocytomation) for 30 min. Finally, the reaction was developed using 3,3′-Diaminobenzidine tetrahydrochloride (Sigma) for 5 min. Slides were briefly counterstained in hematoxylin and dehydrated, and cover slips added. Negative controls were made simultaneously. They were represented by eliminating the primary antibody.

Immunostaining was scored by two trained independent observers (ACMR and DAR) without prior knowledge of the