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● *Original Contribution*

LOW- INTENSITY PULSED ULTRASOUND PRODUCED AN INCREASE OF OSTEOGENIC GENES EXPRESSION DURING THE PROCESS OF BONE HEALING IN RATS

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Abstract—The aim of this study was to measure 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. Animals were randomly distributed into two groups (*n* = 30): control group (bone defect without treatment) and LIPUS treated (bone defect treated with LIPUS). On days 7, 13 and 25 postinjury, 10 rats per group were sacrificed. Rats were treated with a 30 mW/cm^2 LIPUS. The results pointed out intense new bone formation surrounded by highly vascularized connective tissue presenting a slight osteogenic activity, with primary bone deposition was observed in the group exposed to LIPUS in the intermediary (13 days) and late stages of repair (25 days) in the treated animals. In addition, quantitative real-time polymerase chain reaction (RT-qPCR) showed an upregulation of bone morphogenetic protein 4 (BMP4), osteocalcin and Runx2 genes 7 days after the surgery. In the intermediary period, there was no increase in the expression. The expression of alkaline phosphatase, BMP4 and Runx2 was significantly increased at the last period. Our results indicate that LIPUS therapy improves bone repair in rats and upregulated osteogenic genes, mainly at the late stages of recovery. (E-mail: a.renno@unifesp.br) © 2010 World Federation for Ultrasound in Medicine & Biology.

Key Words: **Bone repair, Rats, Low-intensity pulsed ultrasound, Osteogenesis, Genes, Quantitative PCR.**

INTRODUCTION

Over 6,200,000 fractures of the skeleton occur in the United States each year, with almost 10% complicated by disrupted patterns of bone healing (Hadjiargyrou et al. 2002). Even with a majority of fracture healing appropriately, over 30,000,000 days each year are lost because of disability or confinement of patients, leading to a tremendous loss of productivity and income. Given the great potential of both tissue and genetic engineering, it is anticipated that exogenous acceleration of fracture healing could increase the overall number of fractures that heal successfully, as well as reduce the number of lost patient days due to incapacity (Yang et al. 2005).

It is clear the importance of the development of innovative clinical approaches to accelerate bone metabolism and to repair damage to bone tissue. One promising treatment method is the use of low-intensity pulsed ultrasound (LIPUS). LIPUS 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 (Claes and Willie 2007). This therapeutic modality is well established, approved by the FDA (U.S. Food and Drug Administration) and in frequent use (Claes and Willie 2007).

Many *in vitro* works have showed that LIPUS increases prostaglandin E2 (PGE2) production *via* the

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induction of cyclooxygenase-2 (COX-2) in a mouse osteoblastic cell line (Kokubu et al. 1999), induces the transient expression of the immediate-early response gene c-fos and elevates gene expression for bone sialoprotein (BSP), insulin-like growth factor-1 (IGF-1), osteocalcin (OC) and Runx2 (Naruse et al. 2003). LIPUS also was shown to upregulate 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 (Sena et al. 2005). Moreover, it seems that LIPUS can accelerate bone formation, callus maturation and increase bone stiffness in tibial osteotemies in rats and sheep (Chang et al. 2002; Hantes et al. 2004). Some authors suggested that LIPUS might affect the angiogenesis phase of fracture healing, resulting in an increase of the vasculature and decreasing the time of consolidation (Rawool et al. 2003). In human randomized trials, it has been shown that LIPUS can reduce the time to normal fracture repair (Mayr et al. 2001; Tsumaki et al. 2004).

However, the mechanism by which LIPUS acts on osteoblast cultures and bone healing is not fully understood and, for many, the use of this therapy as a treatment modality is still controversial (Mayr et al. 2001). Thus, there is a clear clinical need to understand the molecular details of the pathways that control bone formation after LIPUS application, which might be possible to accelerate the healing of fractures and to treat the 5% to 10% of fractures that fail to heal satisfactorily (Khosla et al. 2008).

In this context, it is well known that bone regeneration is a complex temporal and spatial interaction of cells, regulated by a series of cell-signaling molecules such as cytokines, and growth factors, which induce or modulate osteoproducing cells to create a competent bone mass (Brick et al. 2009). Osteoblasts are effector cells for bone formation with the widely known ability to form bone tissue by secretion of alkaline phosphatase, type I collagen, proteoglycan, bone sialoprotein and osteopontin. In addition, bone morphogenetic proteins (BMPs) are involved in osteoblast differentiation and bone regeneration. Evidences suggested that BMP derived from mesenchymal cells and osteoblasts could exhibit chemotatic properties to stimulate differentiation of mesenchymal cells into osteogenic/chondrogenic lineage and increase expression of alkaline phosphatase and osteocalcin (Proff and Romer 2009). In addition, BMP affects bone remodeling through the regulation of osteoclast bone-resorbing activity (Kochanowska et al. 2007). BMP have been reported as having a role in the mechanical stimulation of fracture healing and chondrocyte differentiation (Wang et al. 2003). Four members of the BMP family, BMP-2, BMP-3, BMP-4, and BMP-7, have shown positive effects on facilitating fracture healing and bone formation (Wang et al. 2003). Another transcriptional factor involved in skeletal development is the Runx2. It regulates the differentiation of chondrocytes and osteoblasts and the expression of many extracellular matrix protein genes during chondrocyte and osteoblast differentiation (Komori 2009).

Although many authors have shown the positive effects of LIPUS on tissue repair, the mechanism by which this therapy acts on bone is not fully understood (Stein et al. 2008). Since this modality is widely used to accelerate the process of tissue healing, the aim of this study was to extend prior histologic descriptions of LIPUS on bone healing by characterizing the temporal–spatial pattern of the expression of bone formation genes. We used quantitative real-time polymerase chain reaction (qPCR), along with histology, to assess gene expression following LIPUS treatment on created bone defects in tibias of rats.

METHODS

Animals

Male Wistar rats (weighing 300 ± 20 g, 12–13 weeks, n = 60) were assigned randomly to one of two groups, control or ultrasound 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 noncritical size bone defects were performed on both tibias. On days 7, 13 and 25 after surgery, 10 rats per group were killed.

Surgery

Noncritical 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 intraperitoneal injection of xilazin (Syntec®, 20 mg/kg, IP, Syntec, São Paulo, São Paulo, Brazil) and ketamin (Agener®, at 40 mg/kg, IP; Agener, Fortaleza, Ceara, Brazil). 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 under copious irrigation with saline solution (12500 rpm; Biomed Drill, São Paulo, São Paulo, Brazil). The cutaneous flap was replaced and sutured with resorbable polyglactin and the skin was disinfected with povidone iodine. The animals received analgesia (IM, 0.05 mg/kg buprenorphine) and were returned to their cages. The health status of the rats was monitored daily.

Treatments

Treatments started 24 h post-surgery and it was performed for 3, 6 and 12 sessions with an interval of 48 h by using the contact technique on the skin, above the site of the bone injury. A low-intensity pulsed ultrasound at 1.5 MHz, 1:4 duty cycle, intensity SATA 30 mW/cm^2, 20 min/session, stationary mode application were used (Exogen; Smith and Nephew, San Francisco, CA, USA). On days 7, 13 and 25 postinjury, rats were sacrificed individually by carbon dioxide asphyxia. The tibias were removed for analysis.

Histopathologic analysis

For the histopathologic analysis, the right tibiae were used. They were fixed in 10% buffer formalin (Merck, Darmstadt, Germany) for 48 hours, 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 histopathologic 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.

Quantitative RT-PCR (RT-qPCR)

Immediately postmortem, right tibias were dissected (periostium intact) and rapidly frozen in liquid nitrogen. The ends of each tibia and the callus region were removed and stored (−80°C) until analysis by quantitative real-time polymerase chain reaction (qPCR). Total RNA was isolated using standard protocols. Trizol reagent (1 mL; Invitrogen, São Paulo, São Paulo, Brazil) was added to the sample and allowed to thaw. The mixture was transferred to a polypropylene tube and incubated (room temperature, 5 min). Chloroform (0.2 mL, Sigma, São Paulo, São Paulo, Brazil) was added, mixed vigorously, and the mixture was transferred to a 2 mL tube (Eppendorf) and centrifuged (2°C, 15 min). The nucleic acid phase was decanted and an equal volume of RNase-free 70% ethanol was added. Potential DNA contamination was removed by RNase-free DNase I (Invitrogen). RNA integrity was verified by RNA gel electrophoresis and spectrophotometry. Four genes of interest (Table 1) were selected representing processes associated with osteogenesis. For each gene, rat specific primers were designed for real-time PCR around exon junctions when possible. All real-time primers were initially tested against standards and a standard curve was generated. First strand cDNA was synthesized (M-MLV RT; Invitrogen, Carlsbad, CA, USA) from total RNA (1 μg). qRT-PCR reactions were carried out at 50 μL total volume. Following an initial denaturing step for each primer described in Table 1, genes of interest were amplified through 40 cycles (Rotor-Gene, R 3000; Robert Research, Mortlake, New South Wales, Australia). Gene amplification was measured by SYBR green (Applied Biosystems, Carlsbad, CA, USA) fluorescence during the annealing/elongation phase. All samples were run in duplicate and the average was used for further analysis. Measures of real-time PCR threshold cycles were normalized to the expression of ribosomal protein S18 (RPS18) for each tibia. For comparison between experimental groups, RPS18-normalized expression from each tibia was divided by the normalized gene expression from the control group to obtain a fold increase in gene expression of LIPUS group and control group in 7, 14 and 25 days (Table 1).

Statistical analysis

The normality of all variables' distribution was verified using Shapiro–Wilk's W test. Two-way analysis of variance (ANOVA) was used to assess the effect of displacement level and time. Post hoc, multiple comparisons were made using Fisher's protected least significant difference tests with statistical significance defined as $p < 0.05$.

RESULTS

Histological analysis

Seven days postsurgery, the defects in the control group were filled by high cell density granulation tissue. Eventually, new bone formation was observed on the

Table 1. Primers and the expected PCR product size at indicated annealing temperatures for each gene analyzed

Gene	Forward primer	Reverse primer	PCR product size (bp)	Annealing temperature (°C)
OC	CTGCATTCTGCCTCTCTGACCT	GCCGGAGTCTATTCACCACCTT	108 bp	60°C
ALP	CGAGCAGGAACAGAAGTTTGC	TGGCCAAAAGGCAGTGAATAG	105 bp	60°C
BMP4	GAGCGCCATTTCCATGT	CTCCACCACCATCTCCTGATAATT	76 bp	56°C
Runx2	ATGGCCGGGAATGATGAGAA	TCTGTCTGTGCCTTCTTGGT	151 bp	56°C
RPS18	CTAGTGATCCCCGAGAAGTTTC	TGTCTGCTTTCCTCAACACC	142 bp	60°C

OC = osteocalcin; ALP = alkaline phosphatase; BMP4 = bone morphogenetic protein 4; Runx2 = transcriptional factor.

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surface of some bone particles (Fig. 1A). Specifically, this was noticed in five animals. Woven bone formation was observed in six animals. Moreover, the animals treated with LIPUS also showed high cell density granulation tissue composed by collagen fibers and mesenchymal cells (Fig. 1B). Seven animals presented woven bone formation.

On day 14 after the surgery, the control group demonstrated new bone formation and remodeling bone trabecula surrounded by granulation tissue (Fig. 2A) marked by basophilic reversal lines. Bone marrow presented high vascularization as well. At the same period, animals treated with LIPUS, presented new bone formation on the walls of the bone defect, surrounded by granulation tissue and with deposition of primary bone (Fig. 2B). In addition, it seems that the amount of neoformed bone was higher in the LIPUS treated group.

On day 25, mature bone trabeculas were noted filling the bone defect in the control group (Fig. 3A). In addition, a good deal of remodeling trabecula was visualized in the group treated with LIPUS. Bone marrow showed intense vascularization as well (Fig. 3B).

Alkaline phosphatase

Figures 4, 5, 6 and 7 represent the osteogenic gene expression in the control and LIPUS treated group.

Day 7. Of the four genes reflecting osteogenic coupling potential in this model, BMP4, osteocalcin and Runx2 were consistently and significantly upregulated at this time point in the treated animals. At day 7, BMP4 expression was significantly elevated by an average of 4.7-fold in bone defect treated with LIPUS compared with nontreated control (Fig. 5). Osteocalcin (Fig. 6) and Runx2 (Fig. 7) expression were significantly elevated, up by an average of 1.9-fold and 2.2-fold in LIPUS-treated bone defect. Interestingly, the expression of alkaline phosphatase was significantly downregulated in the LIPUS group compared with the control (Fig. 4).

Day 13. At this intermediary point during the process of bone healing, no significant expression of any of the four genes analyzed was observed after the treatment with LIPUS compared with the nontreated control.

Day 25. On day 25, a significant increase in the ALP expression was observed in the treated animals compared with the control (up by an average of 2.5-fold) (Fig. 4). Similarly, the expression of BMP4 (Fig. 5) and Runx2 (Fig. 7) were also increased in the LIPUS treated group compared with the nontreated group. At day 25, BMP4 expression was significantly elevated, up by an average of 5.3-fold in bone defect treated with LIPUS compared with the nontreated control. In addition, the Runx2 increased 4.5-fold.

DISCUSSION

The present study aimed to investigate the effects of LIPUS on histologic modifications (descriptive analysis) and on the expression of osteogenic genes (alkaline phosphatase, osteocalcin, Runx 2 and BM4) during the process of bone healing in tibias of rats. The histologic analysis showed that the treated animals presented more mature trabeculas, higher amount of bone deposition and highly vascularized connective tissue, especially at the intermediate and late stages of repair (14 and 25 days after surgery) compared with the control. In addition, RT-qPCR analysis showed that, at day 7 postsurgery, LIPUS induced a significant upregulation of the osteogenic genes. Interestingly, this treatment

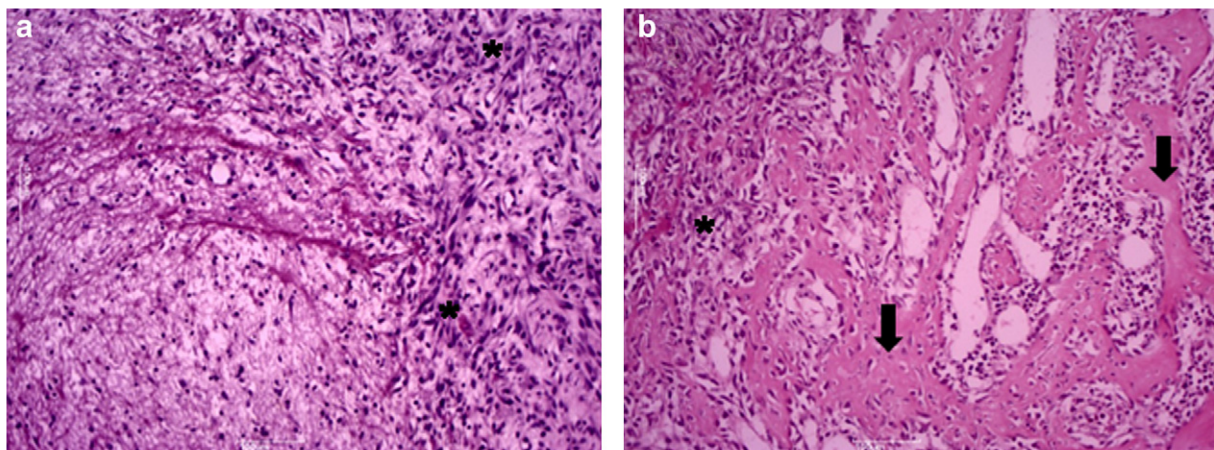

Fig. 1. Bone defects after 7 days of surgery from control group (a) displaying granulation tissue (asterisks); and (b) group treated with ultrasound showing granulation tissue (asterisk) and early woven bone formation (arrow) (H.E. stain ×10).

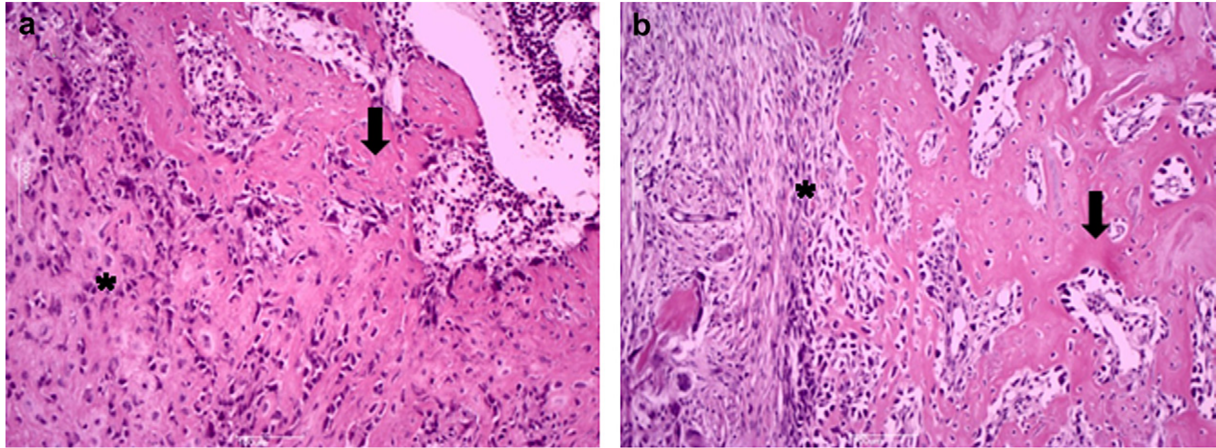

Fig. 2. Bone defects after 13 days of surgery from control group (a) displaying granulation tissue (asterisk), new bone formation as well as bone trabeculas (arrow) inside the defect; and (b) group treated with LIPUS showing granulation tissue (asterisk) and woven bone formation in the walls of the bone defect (arrow) (H.E. stain ×10).

modality did not have any effect on gene expression at the intermediary time post-surgery (day 14). Moreover, in the last period evaluated, LIPUS stimulated the increase of ALP, BMP4 and Runx2 expression. To the best of our knowledge, these results have not yet been demonstrated.

LIPUS has been emerged as an efficient noninvasive treatment to stimulate osteogenesis, to accelerate bone healing and to stimulate protein and osteogenic gene expression (Diniz et al. 2009). Such findings are in line with other studies investigating osteoblastic cell proliferation and bone repair in rats (Nissan et al. 2006; Pretel et al. 2007). Gebauer et al. (2005) found that LIPUS enhanced the healing of nonunion fractures in humans.

Our study showed that LIPUS was able to increase the expression of osteoblast differentiation markers (BMP4 and OC) and Runx2 at early stages compared with the control animals. This is in agreement with Suzuki et al. (2009) who observed an increase of Runx2 expression after LIPUS treatment in osteoblastic cells. The authors state that Runx2 appears to be a crucial transcriptional factor for osteoblast differentiation (Suzuki et al. 2009). BMP-4, which is a member of the transforming growth factor (TGF) superfamily, is known to have an important role in osteoblast differentiation from mesenchymal precursor stem cells and it is upregulated when the osteoblastic recruitment are more active (Ai-Aql et al. 2008). In addition, in this period, the osteocalcin was upregulated.

Interestingly, there was no increase in the ALP expression in the first period evaluated in the present study. Although ALP is an important marker of early osteoblast differentiation, its function is less clear (Proff and Romer 2009). It seems that ALP expression are stimulated later rather than early time points after LIPUS

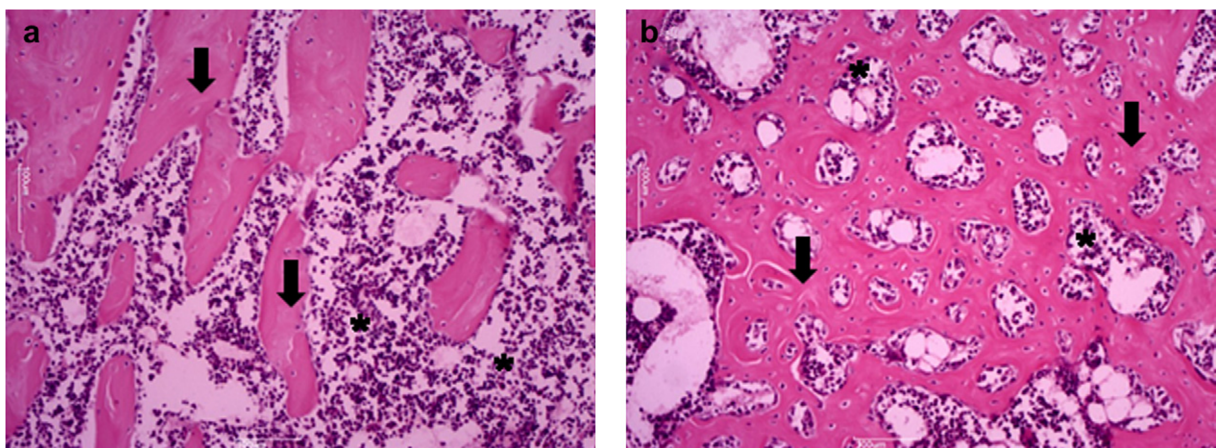

Fig 3. Bone defects after 25 days of surgery from control group (a) displaying granulation tissue (asterisks) and bone tissue (arrows); and (b) group treated with LIPUS showing granulation tissue (asterisks) and intense remodeling trabeculas (arrows) throughout the bone defect (H.E. stain ×10).

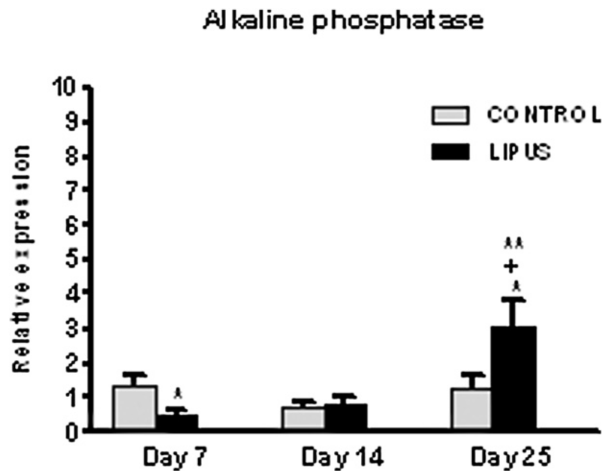

Fig 4. Means and SD of the changes in the expression of the ALP gene in the control and LIPUS treated animals measured by the qPCR. * $p < 0.05$ vs. control; + $p < 0.05$ vs.7 days; ** $p < 0.05$ vs. 13 days.

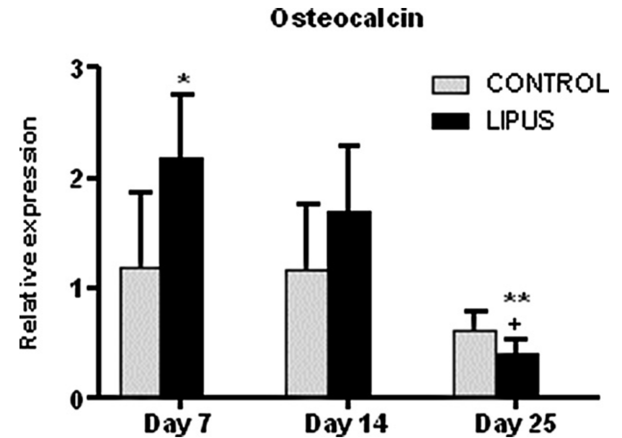

Fig. 6. Means and SD of the changes in the expression of the osteocalcin gene in the control and LIPUS treated animals measured by the RT-PCR. * $p < 0.05$ vs. control; + $p < 0.05$ vs.7 days; ** $p < 0.05$ vs. 13 days.

treatments (Sena et al. 2005) and it is more involved in the bone mineralization process (Proff and Romer 2009). These facts could explain the lack of statistical difference in the expression of ALP.

Sena et al. (2005) demonstrated that LIPUS, at the same parameters used in this study, resulted in elevated transient expression of early response of the genes c-jun, c-myc, COX-2 and Egr-1 as well as the bone differentiation marker genes, osteonectin and osteopontin, at 3 h after irradiation. This induction of early response genes as well as extracellular matrix genes associated with cell proliferation and differentiation may represent the effect of LIPUS to cells of osteoblastic lineage. In addition, Naruse et al. (2003) and Rawool et al. (2003) found that LIPUS elevated mRNA levels for insulin-like growth factor-I, osteocalcin and bone sialoprotein (BSP), which is consistent with a bone-forming response. LIPUS has also been shown to stimulate mRNA expression of osteocalcin and another bone matrix protein, alkaline phosphatase (ALP), in UMR-106 cells (Tsumaki et al. 2004). Suzuki et al. (2009) observed that in osteoblast cell cultures, LIPUS stimulated the expression of Runx2 and Msx2 mRNA.

Conversely, we found no significant expression of any of the osteogenic bone markers in the treated group compared with the nontreated group at 14 days postsurgery. It is very difficult to explain these findings at this moment. In the histologic analysis, the presence of higher amount of granulation tissue with a few deposition of primary bone was detected. It can be hypothesized that LIPUS stimulated initial tissue response by accelerating the acute inflammation period immediately after the injury and an earlier osteoblast cell recruitment, which could be indicated by the upregulation of Runx2, BMP4 and osteocalcin at earlier stages. Moreover, the intermediary period evaluated could be a phase of osteoclast recruitment, which may be participating in the process

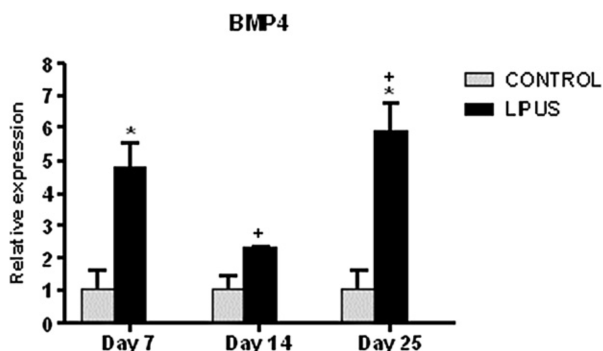

Fig. 5. Means and SD of the changes in the expression of the BMP4 gene in the control and LIPUS treated animals measured by the RT-qPCR. * $p < 0.05$ vs. control; + $p < 0.05$ vs. 7 days.

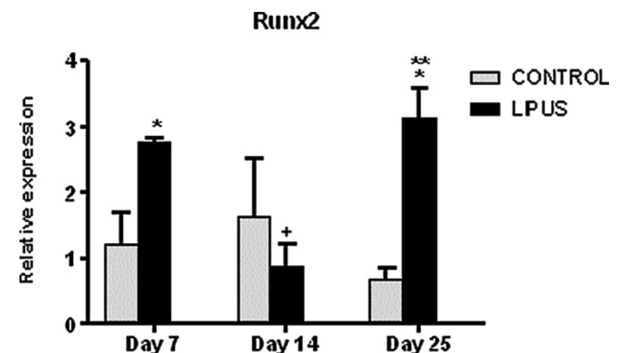

Fig. 7. Means and SD of the changes in the expression of the ALP gene in the control and LIPUS treated animals measured by the RT-PCR. * $p < 0.05$ vs. control; + $p < 0.05$ vs.7 days, ** $p < 0.05$ vs. 13 days.

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of bone remodeling. However, we did not evaluate the activity of these cells and further works are warranted to clarify these points.

In addition, another interesting finding is the significant increase in ALP, BMP4 and Runx2 expression at the last period studied in the treated animals. The upregulated expression of the genes supporting the bone formation at this period corresponds to the higher number of bone cells presented at this period and the higher amount of organized neoformed bone tissue at the bone defect area detected in the histologic descriptive analysis. Probably, the increased expression of ALP, BMP4 and Runx2 in LIPUS treated animals was able to stimulate osteoblast differentiation and increase bone matrix deposition at this stage.

We considered the methodology of our study highly adequate to investigate the effects of LIPUS on bone healing. The model of bone defects have been used by many authors (Kreisler and Al Haj 2003; Lirani-Galvão et al. 2006). In addition, it was found that the LIPUS parameters had a stimulatory effect on cell recruitment and stimulating bone formation genes. This therapeutic modality is approved by the U.S. Food and Drug Administration (FDA) and in frequent use (Lirani-Galvão et al. 2006).

The results of this work highlight the stimulatory effects of LIPUS on bone formation genes. However, the reasons for the stimulatory effects of this therapy and the suite of parameters to be used in clinical therapies do warrant further investigation.

CONCLUSION

The present study has demonstrated the positive effects of LIPUS on bone healing as depicted by histopathologic analysis. In addition, we have showed that LIPUS was able to upregulate genes related to the osteogenic differentiation during bone healing, mainly at early and late stages. Such findings would allow us to obtain preliminary data on the mechanism of action of this therapy and its efficacy as an effective treatment for nonunion fractures or pseudoarthrosis.

Despite these results, further studies are required to study other possible response mechanisms that may explain the positive effects of the LIPUS on bone tissue. Such future studies will undoubtedly contribute to a better understanding of the safety of this therapy and to design future research strategies using human experiments.

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