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HEALTH SCIENCES

Effects of low-intensity pulsed ultrasound exposure on rats tibia periosteum

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Abstract: The periosteum is a rich source of osteoprogenitor cells and periosteal grafts can be used as an alternative method to replace bone grafts. The low-intensity pulsed ultrasound (LIPUS) has often been used as a noninvasive method to stimulate osteogenesis and reduce the fracture healing time. The aim of this study was to evaluate the effects of the ultrasound exposure on the rat tibia periosteum. Group I (7 animals) received LIPUS therapy on the left tibia for 7 days and group II (7 animals) on the left tibia for 14 days. After euthanasia, the tibias were processed. Number of periosteal cells and vessels and thickness of the periosteum were analyzed. The number of periosteal cells was higher in stimulated periosteum compared to controls at 7 and 14 days, but the number of vessels and the thickness only were higher in the group stimulated at 14 days. Furthermore, the ultrasound treatment for 14 days was more effective than 7 days. The ultrasound stimulation of the periosteum prior to grafting procedure can be advantageous, since it increases periosteal activity, and LIPUS may be an alternative method for stimulating the periosteum when the use of periosteal grafts in bone repair is needed.

Key words: tibia, blood vessels, periosteal cells, periosteum, low-intensity pulsed ultrasound.

INTRODUCTION

Tumor resection, mechanical trauma and congenital malformation can cause bone defects with tissue loss, which represent a challenge for reconstructive surgery (Wan et al. 2006, Zhang et al. 2014). The autogenous bone graft is the most used method for the treatment of bone defects and presents some advantages such as absence of immune response and the presence of cells with osteogenic, osteoinductive and osteocondutor potentials (Yoshikawa et al. 2004, Precheur 2007). However, the use this type of graft causes some degree of postoperative morbidity, additional surgical site to obtain the graft, bone resorption and possible risk of infection and hemorrhage (Oreffo & Triffitt 1999).

In some situations in which the mechanical resistance of the graft is not a determining factor, for example in the repair of craniofacial defects, periosteal grafts can be used as an alternative method to replace bone grafts (Ueno et al. 2003, 2007, Soldado et al. 2012, Esfahanian et al. 2014, Zhang et al. 2014, Chen et al. 2015). The advantage of this type of graft is the decreased morbidity of the donor site compared to the bone graft (Olivos-Meza et al. 2010). The periosteum is also a rich source of osteoprogenitor cells (Colnot et al. 2012, Bisseret et al. 2015, Roberts et al. 2015) and provides growth factors and matrix (Langer & Vacanti 1993). In addition, the periosteum-derived cells may be associated with biomaterials (Hattori et al. 2005, Ueno et al. 2007).

Some factors may compromise the osteogenic potential of periosteal cells, such as age, location of the donor area and technique used for removal of the periosteum (Chang & Knothe Tate 2012). Thus, methods for safe extraction were studied. Brownlow et al. (2000), for example, utilized a periosteal elevator to effect its detachment and ensuring the removal of the innermost layer of the periosteum which is strongly adhered to the bone surface. Other researchers investigated alternative methods to increase the osteogenic potential of periosteal tissue. Simon et al. (2003) surgically stimulated periosteum of the tibia of goats and observed abundant angiogenesis and increased cell proliferation in inner cambium layer with subperiosteal bone formation after 16 days. Kanou et al. (2005) surgically stimulated periosteum of the tibia of rats through its release, lifting and repositioning immediately on the bone, but maintaining its blood supply. After seven days, the stimulated periosteal was transplanted to the produced defect in the skull of the same animal and was verified an increase in osteogenic potential compared to unstimulated graft.

The low-intensity pulsed ultrasound (LIPUS) therapy has often been used as a noninvasive method to stimulate osteogenesis and reduce the fracture healing time in animal (Azuma et al. 2001, Hantes et al. 2004, Katano et al. 2011, Martinez de Albornoz et al. 2011) and human models (Martinez de Albornoz et al. 2011, Urita et al. 2013). Ultrasound can modulate cellular events in bone tissue by mechanical stimulation or heat transfer (Kruse et al. 2008). The ultrasound waves promote electrical polarization in the tissue and this polarization is determined by the piezoelectric effect and the bone microarchitecture (Pilla 2002, Tam et al. 2008). This polarization alters the membrane potential of bone cells such as osteoblasts and permits ion exchange and nutrient uptake (Pilla 2002).

Other studies investigated the effect of LIPUS during the fracture healing process in which there is impairment of periosteal tissue and observed recruitment of osteogenic progenitor cells to the site where these cells are deficient (Kumagai et al. 2008, 2012). Moreover, it has been shown that treatment with ultrasound enhances angiogenesis in the periosteum surrounding the bone callus (Katano et al. 2011) and increases the expression of cytokines and growth factors in cultured periosteal cells (Pilla 2002, Leung et al. 2004).

Considering the advantages of using periosteal graft in the repair of bone defects and assuming that the LIPUS exposure is a safe and non-invasive method that stimulates the activity of bone cells *in vivo* and periosteal cells *in vitro*, we hypothesized that treatment with ultrasound could stimulate the osteogenic potential of periosteal cells from a donor area prior to grafting procedure. Thus, the aim of this study was to evaluate through histological and morphometrical methods the effects of LIPUS exposure on the rat tibia periosteum.

MATERIALS AND METHODS

Animals

Fourteen albino Wistar rats (males), eight weeks of age, were obtained from the Center for Biological Investigation - CEMIB (State University of Campinas, Campinas, SP, Brazil). The rats were housed under standard conditions with 12 h L:12 h D cycle. Animals were provided with commercial rat feed and water ad libitum. The experiment was conducted in accordance with the ethical guidelines adopted by the Brazilian College of Animal Experimentation (COBEA) and the study was approved by the Ethics Committee

on Animal Experimentation (CEEA) of Unicamp (protocol 2072-1).

Division of groups

The animals were divided into two main groups (7 animals per group). The animals of group I received ultrasound exposure on the left tibia for 7 days and the animals of group II received ultrasound exposure on the left tibia for 14 days. The right tibia (non-stimulated) of each animal was used as control for its specific pair (left tibia). Considering these information, the samples (tibias) were divided into four groups in this study: group 7S (left tibia stimulated for 7 days); group 14S (left tibia stimulated for 14 days); group 7NS (right tibia not stimulated for 7 days); group 14NS (right tibia not stimulated for 14 days).

Low-intensity pulsed ultrasound stimulation

The animals were immobilized and the medial surface of proximal third of the left tibia of each animal received LIPUS exposure for five minutes per day. The animals of groups I and II were treated for a period of 7 and 14 consecutive days, respectively. The right tibia (control) received the same procedure performed with the left tibia, but the ultrasound device was turned off. In all cases, the stimulation was always done at the same time. It was used the device of the brand Bioset, Sonacel Dual (Continuous and Pulsound, 1 & 3 MHz) model (Bioset - Indústria de Tecnologia Eletrônica Ltda, Rio Claro, SP, Brazil), with frequency of 1MHz, intensity of 0.5 W/cm^2 (Spatial Average Temporal Average, SATA), duty cycle of 20% and pulse-modulated frequency of 100 Hz. The verification of the intensity emitted by the ultrasound device was held at the beginning of the experiment following the norms and standards of the manufacturer.

Indian ink-gelatin vascular injection

After completing the experimental period the animals were sacrificed by an overdose (0.30 mL/100 g) of ketamine hydrochloride and xylazine hydrochloride (1:1), and the aorta was exposed through a longitudinal laparotomy. The aorta was cannulated and perfused with a solution of heparin and saline. The inferior vena cava was cut to drain the blood and saline. Next 30 ml of solution of India ink and gelatin in buffer was injected through the abdominal aorta. After gelatin precipitation, the tibias were dissected, macroscopically analyzed and placed in fixative solution.

Light microscopy

The samples were fixed in 10% buffered formalin for 48 hr at room temperature and decalcified in an ethylenediaminetetraacetic acid (EDTA) solution. The bone segment that received ultrasound stimulation was dissected and used. Then, the samples were dehydrated in alcohol gradient, diaphanized in xylene and embedded in liquid paraffin at 60°C to produce paraffin blocks. Cross-sections (6µm thick) were obtained and stained with hematoxylin-eosin. The histological sections were examined under a Nikon 80i photomicroscope (Nikon Corporation, Shinagawa-ku, Tokyo, Japan) equipped with 40X objectives and the images were captured with a Nikon DS-Ri1 camera (Nikon Corporation, Shinagawa-ku, Tokyo, Japan).

Morphometric analysis

The morphometric measurements were made on the images of the histological sections using the NIS-Elements Advanced Research software (Version 3.0, Nikon Corporation, Shinagawa-ku, Tokyo, Japan). For each sample were randomly selected 5 fields in the region of each tibia that received or not received ultrasound exposure. In each field the following parameters were

evaluated: a) number of blood vessels in the periosteum; b) Number of cells in the periosteum; and c) the thickness of periosteum (μm).

Statistical analysis

Two-way analysis of variance for repeated measures, followed by the Tukey test if necessary, was used for statistical analysis. The results are reported as the mean ± standard deviation. A level of significance of 5% was adopted for all tests ($p < 0.05$).

RESULTS

Two-way ANOVA showed that the LIPUS and time of treatment (days) affected all morphometric parameters of the rat tibia periosteum (Table I). There was significant interaction between the effects of treatment and time for all the analyzed properties (Table I) and we could accomplish in our results the multiple comparisons of means by Tukey test (Figures 1-3).

Regarding the number of cells of the periosteum, the group 14S demonstrated a statistically significant difference compared to the other three groups. The group 7S presented a higher number of cells than its corresponding control group (group 7NS). However, groups 7S and 14NS were statistically equal. The control group of 14 days showed more cells than the control group of 7 days (Figure 1).

The number of blood vessels in the periosteal region of the stimulated left tibia for 14 days (group 14S) was approximately three times higher as compared to the other three groups. However, the groups 7S, 7NS 14NS were not statistically different from each other (Figure 2).

The results showed that the thickness of the periosteum followed the same pattern found in the statistical test for the number of newly formed vessels. The stimulated group for 14 days was significantly thicker than the other groups. The groups 7S, 7NS and 14NS presented equal mean values (Figure 3).

Table I. Statistical comparison by two-way ANOVA of effects of low-intensity pulsed ultrasound therapy and time of treatment (days) in the morphometric parameters of the rat tibia periosteum.

Measurement	Two-way ANOVA, p value		
	LIPUS	Time (days)	Interaction
Number of cells	< 0.0001	< 0.0001	0.0417
Number of blood vessels	0.0001	< 0.0001	0.0002
Thickness of the periosteum	0.0007	< 0.0001	0.0093

LIPUS, low-intensity pulsed ultrasound.

Analysis of histological sections clearly demonstrated that the group 14S had the highest concentration of cells and blood vessels in the periosteal layer when compared to the other groups (Figures 4a-d). Furthermore, it is also possible to observe a thicker and denser periosteum in group 14S (Figure 4d).

DISCUSSION

The LIPUS therapy has been used as a noninvasive alternative to stimulate osteogenesis to decrease the healing time of bone tissue (Azuma et al. 2001, Hantes et al. 2004, Katano et al. 2011, Martinez de Albornoz et al. 2011, Urita et al. 2013). The function of the periosteum in the bone repair process is fundamental since it is a source of osteogenic cells and has osteoinductive action (Colnot et al. 2012, Bisseret et al. 2015, Roberts et al. 2015). Due to these characteristics, some researchers have stimulated periosteal cells to promote and accelerate bone formation (Simon et al. 2003, Leung et al. 2004, Kanou et al. 2005, Tam et al. 2008, Zhang et al. 2014). Thus, the objective of this study was to analyze the effect

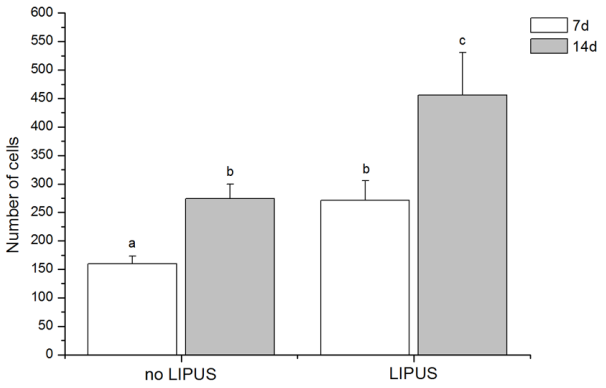

Figure 1. Number of periosteal cells of rat tibia periosteum in the four groups studied. Different letters indicate statistical differences among the non-stimulated and stimulated samples with LIPUS at seven or fourteen days of treatment.

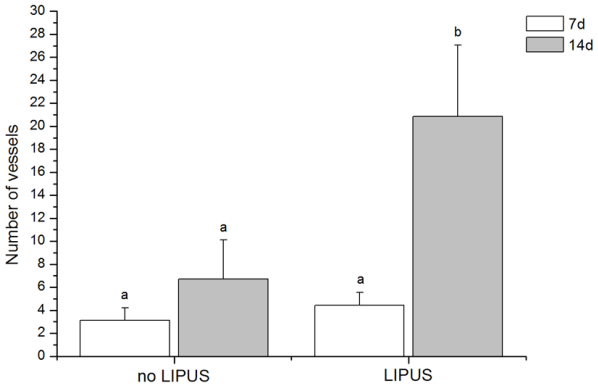

Figure 2. Number of blood vessels of rat tibia periosteum in the four groups studied. Different letters indicate statistical differences among the non-stimulated and stimulated samples with LIPUS at seven or fourteen days of treatment.

of stimulation of the periosteum of the rat tibia (non-fractured) with LIPUS.

It was found in this study that the number of periosteal cells on the fourteenth day of stimulation was higher compared to the seventh day, indicating that ultrasound is able to cause morphological changes in the periosteal tissue. These results suggest that LIPUS treatment stimulated cell biogenesis and metabolism in the periosteum similarly to other studies which have also verified the reaction of the periosteal tissue through different experimental protocols (Azuma et al. 2001, Kanou et al. 2005). According to Azuma et al. (2001), LIPUS emits pressure waves that cause micromechanical deformations on the living tissue, promoting biochemical changes at the cellular level, as it occurs in certain cellular responses involved in the regeneration process of a bone fracture. Kanou et al. (2005) observed increase in periosteal cells on the seventh day after surgical stimulation of the periosteum, corroborating this aspect with our results. Thus, our data showed that the time of ultrasound treatment (in days) induced significant proliferation of periosteal cells.

After 2-4 days of treatment, Leung et al. (2004) found that LIPUS increased the activity of human periosteal cells studied *in vitro*, such as cell proliferation, VEGF expression, alkaline phosphatase activity and mineralization. Also analyzing human periosteal cells *in vitro*, Tam et al. (2008) demonstrated that proliferation and total number of these cells and expression of alkaline phosphatase was higher in the sixth day post-treatment with LIPUS, but no difference was observed after eighteen days. Similar to studies of Leung et al. (2004) and Tam et al. (2008), the present study also showed that the time of treatment (days) is a determining factor on cellular activity in the periosteum. However, it is important to consider that *in vitro* studies may have different responses to ultrasound stimulation when compared to *in vivo* studies. Thus, cells grown in culture are exposed to a more intense exposure since there are no biological tissues to offer resistance to ultrasound waves.

An interesting fact was observed between the control groups (7NS and 14 NS). Similarly to the treated groups, the group 14NS presented more cells than the group 7NS, but it was

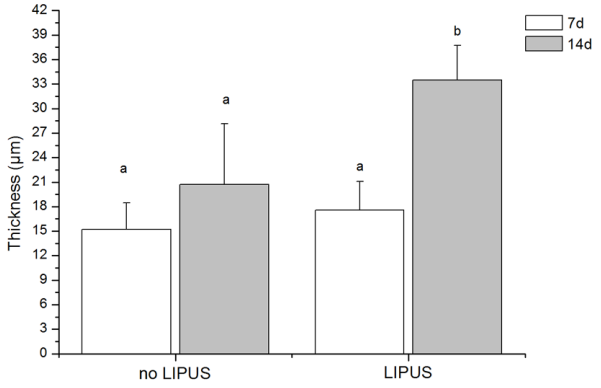

Figure 3. Thickness of the periosteum of rat tibia periosteum in the four groups studied. Different letters indicate statistical differences among the non-stimulated and stimulated samples with LIPUS at seven or fourteen days of treatment.

statistically equal to the group stimulated for seven days. This may have occurred in function of natural bone remodeling resulting from the osteogenic capacity of the periosteum (Colnot et al. 2012, Bisseret et al. 2015, Roberts et al. 2015) due to the bone tissue maturation process or loads from impact with the ground (Perry et al. 2009). In this regard, periosteal cells are naturally capable of synthesizing basic fibroblast growth factor (b-FGF or FGF-2) and vascular endotherial growth factor (VEGF) (Kanou et al. 2009) and provoke changes in the tissue.

Another interesting fact found was the increased number of blood vessels in the periosteum stimulated for 14 days but not on the seventh day. This fact showed that the number of days of ultrasound exposure (according to the protocol and parameters used) was a factor which favored the vasculogenesis (angiogenesis) in the periosteum. This probably occurred due to the increased synthesis of some growth factors, such as FGF-2 (Ying et al. 2012) and VEGF (Mayr-Wohlfart et al. 2002, Katano et al. 2011, Ying et al. 2012). In this regard, Katano et al. (2011) evaluated the healing of fractures of rat femur at 40 weeks of age and found increased expression of VEGF and abundant neovascularization in periosteal tissue surrounding only the bone callus in the treated group for 10 days with LIPUS, but not on the seventh day. In cases of bone repair, the increase in the number of blood vessels provided greater blood flow to the site to be repaired (Beamer et al. 2010, Kidd et al. 2010), favoring the tissue healing by diffusion of nutrients, oxygen and anabolic at the site of injury.

The significant increase in the thickness of the periosteum only in the group treated for fourteen days could be a reflex of the deposition and remodeling of the extracellular matrix of the periosteum under the effects of the LIPUS. This fact seems to occur due to the increased amount of periosteal cells and increased number of blood vessels, since the angiogenesis is influenced by growth factors, extracellular matrix components and cytokines (Reher et al. 1999).

The references cited in the present study specifically analyzed the fractures repair and observed positive effects of LIPUS on bone regeneration (Azuma et al. 2001, Hantes et al. 2004, Katano et al. 2011, Urita et al. 2013). Interestingly, there are other studies in the literature that found no effect of LIPUS on bone regeneration during osteogenic distraction (Medeiros et al. 2015, Simpson et al. 2017). In the study by Simpson et al. (2017), for example, 32 patients treated with LIPUS after a corticotomy in the proximal tibial metaphysis were analyzed followed by an application of an Ilizarov frame. No significant difference was observed between the parameters analyzed (distraction length, time to regenerate maturation and regenerate maturation index) comparing the treated to the untreated groups. Medeiros et al. (2015) compared the effect of LIPUS and therapeutic laser applications on osteogenic distraction in the rabbit mandible. The animals were divided into four distinct groups (control, treated with laser, treated with LIPUS, treated with laser +

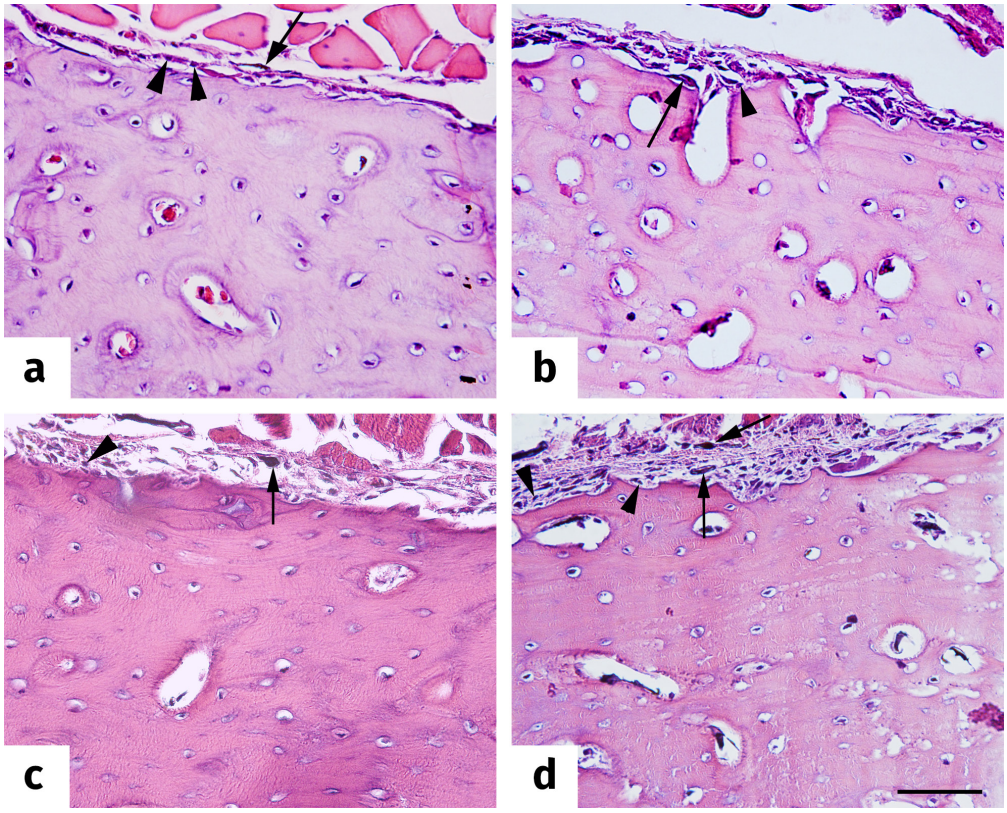

Figure 4. Light microscopy images of the rat tibia periosteum treated (b and d) and not treated (A and C) with LIPUS. a, right tibia not stimulated for 7 days (7NS); b, left tibia stimulated for 7 days (7S); c, right tibia not stimulated for 14 days (14NS); d, left tibia stimulated for 14 days (14S). Arrowheads indicate the periosteal cells and arrows indicate the blood vessels. The histological sections were stained with hematoxylin–eosin. Bar = 50µm.

LIPUS) and the researchers observed that the area of new bone formed was significantly larger only in the groups treated with laser or laser combined with ultrasound. In this context, it seems that the mechanical stimulus of LIPUS presents contradictory effects when used in fracture repair and in osteogenic distraction, since they are different bone regeneration processes (Ai-Aql et al. 2008). Although these processes show similar steps in bone healing, each one has specific molecular and cellular mechanisms during tissue repair, being guided by different levels and times of expression of some markers, such as molecular mediators of angiogenesis and inflammation (Ai-Aql et al. 2008).

Besides the differences between the specific molecular mechanisms of each regeneration process, a possible hypothesis to explain the previously mentioned situation would be based on the definition of both procedures. Fracture repair is a complex process that begins as a response to injury, while osteogenic distraction is a surgically controlled process that benefits itself from mechanical stress to assist in the tissue repair process. Thus, we suggest that the mechanical stress promoted by the osteogenic distraction could override the mechanical stimulus of LIPUS. In this context, our results showed a positive and stimulating effect of osteogenic cells on exposure to LIPUS, since the treated bones were not injured. In addition,

it is interesting to mention that Matsumoto et al. (2018) found evidences that LIPUS promotes osteoblastic differentiation through hedgehog signaling in fracture repair and cell culture experiments. At the fracture site, they observed that the amount of Gli2-positive cells, such as osteoblasts, was higher in the LIPUS-treated group when compared to the control. In the culture of MC3T3-E1 cells exposed to LIPUS, they noted up-regulation of hedgehog signaling molecules (SHH, Gli1, and Gli2) and an increase in the number and length of primary cilia. Therefore, Matsumoto et al. (2018) mentioned that primary cilia acts as a mechanosensor in bone development.

Considering the aspects presented, it is possible to conclude that the ultrasound stimulation of the periosteum prior to grafting procedure can be advantageous, since it increases periosteal activity causing an increase in the number of osteogenic cells and possibly in the production of growth factors. Thus, this study demonstrated that stimulation of the periosteum by LIPUS may be an alternative method for stimulating the periosteum when the use of periosteal grafts in bone repair is needed.

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Jaqueline Martins Batista and Evelise Aline Soares carried out the experiments (low-intensity pulsed ultrasound therapy and animal raising and care) and morphological analysis. Wilson Romero Nakagaki performed the statistical and morphological analysis, supervised the laboratory work and drafted the article and figures. José Angelo Camilli was responsible for the conception and design of the study. All authors revised and reviewed the manuscript.