Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.elsevier.com/locate/etap

Study on osteogenesis promoted by low sound pressure level infrasound in vivo and some underlying mechanisms

Hua Long[a,1], *Liheng Zheng*[b,1], *Fernando Cardoso Gomes*[c], *Jinhui Zhang*[b], *Xiang Mou*[d,**], *Hua Yuan*[d,*]

[a] *Department of Orthopaedics, Tangdu Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710038, PR China*
[b] *Department of Orthotraumatology, Centro Hospitalar Conde de Sao Januario, Macau*
[c] *Department of Rehabilitation, Centro Hospitalar Conde de Sao Januario, Macau*
[d] *Department of Physiotherapy and Rehabilitation, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710032, PR China*

ARTICLE INFO

Article history:
Received 7 October 2012
Received in revised form
15 April 2013
Accepted 24 April 2013
Available online 22 May 2013

Keywords:
Infrasound
Osteogenesis
Neuro-osteogenic network
Innervation
CGRP
NPY

ABSTRACT

To clarify the effects of low sound pressure level (LSPL) infrasound on local bone turnover and explore its underlying mechanisms, femoral defected rats were stabilized with a single-side external fixator. After exposure to LSPL infrasound for 30 min twice everyday for 6 weeks, the pertinent features of bone healing were assessed by radiography, peripheral quantitative computerized tomography (pQCT), histology and immunofluorescence assay. Infrasound group showed a more consecutive and smoother process of fracture healing and modeling in radiographs and histomorphology. It also showed significantly higher average bone mineral content (BMC) and bone mineral density (BMD). Immunofluorescence showed increased expression of calcitonin gene related peptide (CGRP) and decreased Neuropeptide Y (NPY) innervation in microenvironment. The results suggested the osteogenesis promotion effects of LSPL infrasound in vivo. Neuro-osteogenic network in local microenvironment was probably one target mediating infrasonic osteogenesis, which might provide new strategy to accelerate bone healing and remodeling.

1. Introduction

Bone defects resulting from trauma, infections, tumors and abnormal skeletal development represent a significant health problem. Approximately 2 million cases of delayed and nonunion fractures occur annually in the world, and most of which have underwent the regular treatments including reduction, fixation and functional exercise (Rodriguez-Merchan and Forriol, 2004). Clinicians are always looking for effective and non-invasive therapeutic agents to help recalcitrant fracture.

Infrasound is a mechanical wave with oscillation frequency below 20 Hz, which exists in natural and artificial environment extensively (Leventhall, 2007). As a main source of noise pollution, high level infrasound can cause functional and structural impairment. While infrasound with low sound pressure level was found to have potential for physical therapy. Over the last decades, clinical and experimental evidence has accumulated that LSPL infrasound can regulate local microenvironment and

* *Corresponding author*. Tel.: +86 29 84775437.
** *Corresponding author*. Tel.: +86 29 84775433.
E-mail addresses: longhuadr@gmail.com (H. Long), cheanglekhang@yahoo.com.hk (L. Zheng), drfcko@yahoo.com (F.C. Gomes), petercheungmc@yahoo.com.hk (J. Zhang), pro.mu@fmmu.edu.cn (X. Mou), yuanhua@fmmu.edu.cn (H. Yuan).
[1] The first two authors contributed equally to this paper.
1382-6689/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.etap.2013.04.015

tissue regeneration via different mechanisms, including vessel recanalization, deep massage effect, anti-oxygenization and so on (Obrubov and Tumasian, 2005). Osteotropic effect in bone turnover was also reported, which was boiled down to low frequency resonance produced by LSPL infrasound generally. But it should be pointed out that the majority of these results were based on cytological study but without enough in vivo evidences.

This study aims to investigate the effects of LSPL infrasound on bone metabolism in vivo, and also to clarify its mechanisms of osteogenesis promotion. By which it may provide new strategy to accelerate fracture healing and remodeling.

2. Materials and methods

2.1. Animals and operative techniques

Total 46 male Sprague-Dawley rats (10 weeks old, weighting 280 ± 20 g), obtained from the Center of Experimental Animal in the Fourth Military Medical University (FMMU, Xi'an, China) were maintained with food and water available ad libitum in an air-conditioned room (12/12 h light/dark cycle at a temperature of $23 \pm 2\,^{\circ}C$ and a relative humidity of $60 \pm 5\%$). All experiments were approved by the Committee of Animal Use for Research and Education in FMMU.

In the present fracture model, a 2.5 mm gap between the bone fragments was created and the femoral osteotomy site was stabilized with a single-side external fixator as described previously (Strube et al., 2008). A template was used to ensure the accurate and reproducible placement of the pins. Between the two middle pins, an osteotomy was performed with a reciprocating saw under irrigation. A 2.5 mm fracture gap was distracted through screwing the blot along the compressed shaft, and soft tissue was then reapproximated.

2.2. Treatment

The special electric-actuated infrasound generator (Infrasound 8^{TM}, Chi Corporation, USA) was used for local infrasound exposure, which could generate LSPL infrasonic wave. A real-time ultra-low frequency signal acquisition system was used to collect and analyze its characteristic parameters. Three-grade intensity in device could generate infrasound at frequency 12–20 Hz, and its sound pressure was less than 90 dB confirmed by analyzing system.

After osteotomy, all rats were allocated into experimental and control group randomly. The experimental group was exposed to LSPL infrasound for 30 min twice daily for 42 days, starting on the fifth postoperative day. To remove other infrasonic effects, the animals were held on a closed tube in prone position, with the surgery hind-limb exposed and fixed. The device surface was placed 3 cm from the posterior face of the thigh, and the center of the generator was positioned above the fracture zone. The control group underwent the same procedure but without infrasonic exposure.

2.3. Radiographic evaluation of bone healing

The progress of fracture healing and the alignment of the osteotomized bone fragments were monitored by anteroposterior radiographs of the study extremity under anesthesia every 2 weeks. A mobile C-arm X-ray system (Siemens, Germany) was used, and radiographic measurements were done manually on magnified printout X-ray pictures.

2.4. pQCT measurement

A quantitative determination of callus development was performed with pQCT (Research SA+, StraTEC Medizintechnik, Pforzheim, Germany) at 6 weeks. Fracture femur specimens were analyzed according to standard protocol. A scout view was performed prior to the actual scan to enable exact positioning of the bone specimens. The fracture segment was analyzed with five transverse scan sections, each with a thickness of 0.5 mm and a pixel size of 0.1 mm. The region of interest selected was fracture gap exactly, based on reference plane between the two middle pins. Mean BMD (mg/cm^3) and BMC (mg/mm) were measured for all five sections, which reflected the degree of calcifying in the entire segment of fracture. In addition, area of higher density callus (mm^2) within the fracture gap was also measured to assess progression of mineralization. The thresholds used for separating soft tissue from bone and higher density callus from soft callus were 280 mg/cm^3 and 550 mg/cm^3.

2.5. Morphology and semi-quantitative analysis of nerve-derived neuropeptides

Five rats in each group were killed on days 7, 14, 28 and 42. The rats were anesthetized with sodium pentobarbitone (60 mg/kg, i.p.). In vivo intra-aortic perfusion and demineralization of bone specimen were performed with conventional methods (Long et al., 2010). The samples were soaked in 20% sucrose for at least 2 days. Each decalcified femur was divided sagittally in two halves, and medial half samples were sectioned at thickness of 6 and 15 μm consecutively from middle to the medial aspect. Two interval sections, i.e., one close to middle part and another close to medial part of femur at each time point, were chosen for H&E staining and immunostaining according to the biotin–avidin system. Immunostaining was then performed with the use of antibody for CGRP and NPY following steps: 10 min hydration in 0.01 M PBS, 30 min incubation in 10% normal goat serum at room temperature. Sections were then incubated in a humid atmosphere with polyclonal antibody to CGRP (1:6000, Sigma) and NPY (1:5000, Sigma). After 2 min × 5 min rinse in PBS, sections were incubated with biotinylated goat anti-rabbit antibody (1:10,000, Invitrogen) for 40 min, and then in fluorochrome Cy3-conjugated avidin (1:5000, Amersham). Control staining was performed by omitting the primary antibody. Staining sections were examined using an epifluorescence microscope (H-7100, Hitachi, Japan). The analysis focused on the fracture segment as well as closed area. The density of positive nerve fibers in and adjacent to the fracture area was quantified according to tissue type by computerized image analysis program. The mean density