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

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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

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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

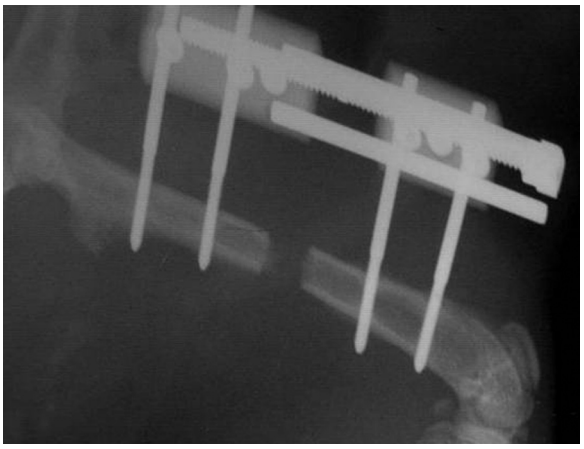

Fig. 1 – Postoperative X-ray film of rat femoral fracture model with monolateral external fixator.

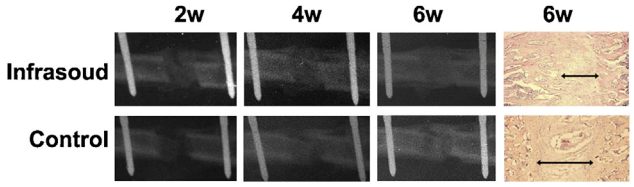

Fig. 2 – Representative radiographs of fracture area specimens from infrasound and control group in different time points showed LSPL infrasound exposure significantly accelerate fracture healing process. Satisfied union occurred at 6 w postoperation in infrasound group, whereas radiolucent area was clear and the lower density callus around the same area was still increasing in control group. The results were consistent with histological observation.

(neuronal immunofluorescence/mm^2) was determined based on 12 microscopic fields.

2.6. *Statistical analyses*

All data were expressed as mean ± S.E. Statistical analyses were performed using SPSS Statistics 13.0 Software (SPSS Company, USA). Differences between groups were determined by two-tailed independent sample *t*-test. $p < 0.05$ was considered significant.

3. Results

3.1. *Surgery quality assurance*

The rats regained weight bearing within a few hours after operation and gait returned to normal around 5 days post fracture. There were no infections, either at the surgical site or around the pin tracks. However, two rats from the infrasound group and one from control were excluded from the study, because of pin broken or complication related to the anesthesia. The equal width well-aligned fracture gaps were observed in radiographs after surgery. And the placement of osteotomies was just right between the two middle pins in the mid-diaphyseal region (Fig. 1).

3.2. *Effects of infrasound on femoral fracture healing*

A consecutive of radiographs revealed that regenerate formation could be observed in both infrasound and control group at 2 weeks postoperation. From X-ray films at 2 and 4 weeks after surgery, the callus filled with the radiolucent gap in infrasound group was much more than that in control clearly, and this tendency continued until the dissection day. Satisfied union occurred at 6 weeks after surgery in infrasound group. The mature callus in fracture segment was consolidated and part of callus in non-force line was absorbed, whereas radiolucent area was clear and the lower density callus around the same area was still increasing in control group (Fig. 2).

Besides the difference in the volume and the rate of regenerated callus formation from radiographs, pQCT was used to assess the progression of mineralization accurately at the consolidation stage. In infrasound group, mean BMD (593.5 ± 27.6 vs 437.1 ± 47.5, $p < 0.01$) and BMC (1.36 ± 0.26 vs 1.01 ± 0.18, $p < 0.05$) at fracture gap were significantly higher than that in control. The total area of highly mineralized callus determined by pQCT was increased remarkably than that in control (5.85 ± 1.06 vs 3.84 ± 0.37, $p < 0.01$).

Histological sections, made 6 weeks after infrasound exposure, showed much more extensive new bone formation in fracture area and the trabecular bone adjacent to bone defect. The fracture gap was narrow and unclear, in which filled with osteoblast-like cells and chondrocyte. In control group, the segment was wide and clear, where the main cell types were fibroblasts, inflammatory cells and less chondrocytes. Less new bone could be observed in and closed to fracture area, though there were more immature callus and proliferated connective tissue (Fig. 2). These findings were consistent with the radiographic data.

3.3. *Morphology and distribution of CGRP- and NPY-positive nerve fibers in fracture microenvironment*

As previously described (Strube et al., 2008), nerve fibers immunoreactive to CGRP were distributed predominantly in the periosteum, bone marrow and connective tissue adjacent to fracture gap, while NPY-positive fibers were mainly observed around the blood vessels in both groups. In control group, ingrowth of nerve fibers containing CGRP and NPY was already observed at 1 week postfracture in hematoma as free nerve terminals. Between days 14 and 28, many CGRP-positive fibers were seen forming networks in or around the walls of new blood vessels in the hypertrophic periosteum and in bone marrow. Especially in the fracture segment, some CGRP fibers were distributed in non-vascular cartilaginous callus and new woven bone. A similar phenomenon occurred in NPY immunofluorescence at 2 weeks postoperation, some NPY-positive fibers were observed sprouting into callus from the deep layers of the periosteum or close to the chondroid cells. CGRP fibers maintained high intensive distribution until 6 weeks, while NPY-positive fibers were seen to retract from

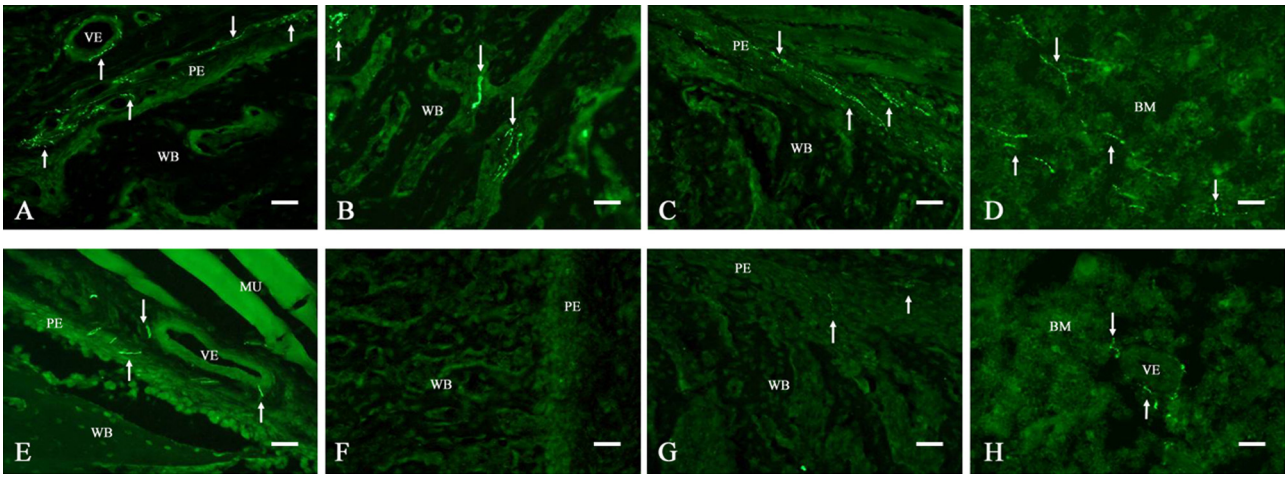

Fig. 3 – Immunofluorescence photomicrographs of fracture area in control (A–D) and infrasound group (E–H) showed nerve fibers (arrow) stained for NPY at 4 w postoperation. In new woven bone, many positive fibers were observed around the blood vessel (A), or distributed in non-vascular cartilagious callus as free nerve terminals (B) in control, whereas positive fibers almost disappeared in the same area (F) after infrasound exposure except less fibers along blood vessels (E). Some NPY fibers could be seen in periosteum (C and G) and bone marrow (D and H) in both groups, but the number of positive fibers in control was much more than that in infrasound group. 20× objective. Bar represents 50 μm. NB: new woven bone; VE: vessel; PE: periosteum; BM: bone marrow.

the fracture site and be present as vascular fibers in the periosteum from 2 weeks.

Until 2 weeks after operation, CGRP and NPY innervations in local microenvironment displayed similar expression pattern in two groups. At 4 weeks after LSPL infrasound treated, more CGRP fibers were found penetrating into the new woven bone or distributed as varicose nerve terminals in fibrocartilage callus. Contrarily, infrasound treatment reduced the number of NPY-positive fibers significantly, NPY fibers even almost disappeared in new woven bone. While many NPY-positive fibers still appeared around the fracture area in control group (Fig. 3).

The total neuronal immunoreactivity (the nerve fiber immunofluorescent area around the fracture gap) within 12 microscopic fields including periosteum, bone marrow, connective tissue and woven bone was measured and compared. The semi-quantitative data showed that at 6 weeks after infrasound treatment, the total neuronal CGRP immunofluorescent area exhibited an upward tendency around fracture area (109.6%, $p = 0.03$), and CGRP immunoreactivity in new woven bone increased significantly (140%, $p < 0.01$). At the same time, the total NPY immunofluorescent area decreased notably (83.3%, $p < 0.01$). NPY immunoreactivity in new woven bone and periosteum represented a decrease of 56.7% and 17.3% respectively as compared to that in control (Fig. 4).

4. Discussion

In previous in vitro data, infrasound at certain range of intensity and frequency could enhance the proliferation, differentiation and secretion of osteoblast-like cells, therefore promote osteogenesis and prevent osteoporosis (Wang et al., 2006). Because inconsistent intervention protocols and technical variations in different models resulted in different, even controversial outcomes, little is known accurately about its effects on the fracture healing in vivo.

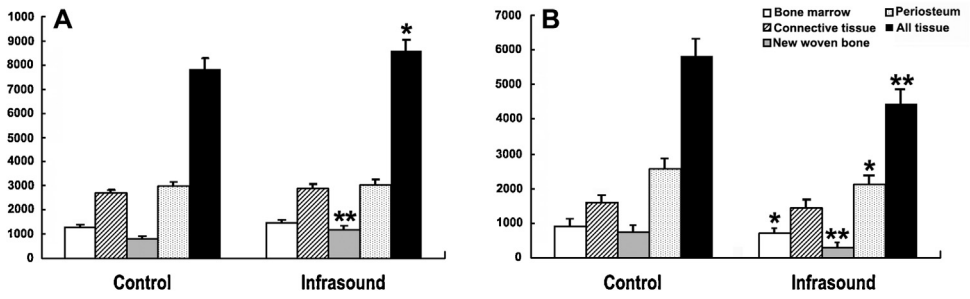

Fig. 4 – CGRP (A) and NPY (B) mean content at 6 weeks in different tissues around the fracture area in infrasound and control group ($n = 5$). At 6 weeks after infrasound exposure, CGRP immunofluorescence increased especially in new woven bone. While NPY immunofluorescence decreased significantly. Data were shown as mean ± S.E.; $^{*}p < 0.05$, $^{**}p < 0.01$ vs control group.

In the present research, the special electric-actuated infrasound generator and standardized animal model were used. Infrasound 8™, cleared for clinical use by FDA, was suitable for local exposure in small animal, and avoided other system effects of infrasound effectively. Real-time ultra-low frequency signal acquisition system confirmed the sound pressure level less than 90 dB. Femoral fracture model, stabilized with a monolateral external fixator experimentally mimiced the actual clinical condition. These ensured the experimental results to be reliable and reproducible.

The radiographic measurements did not show any significant differences at beginning between two groups. So the potential differences found between the two groups in the fracture healing process could be assumed to be the effects of LSPL infrasound treatment.

Consistent with cytological results, this study demonstrated that LSPL infrasound could significantly accelerate bone healing process in vivo. Both the infrasound group and control group showed the typical fracture healing process: callus formation, bridge, mature, consolidation and modeling. But the LSPL infrasound group was shown to have significantly accelerated healing process. At the early stage, the X-ray results showed the faster callus formation and faster bridge (about 2 weeks after surgery) in the LSPL infrasound group. At later stage, the pQCT results showed the accelerated mineralization by the LSPL infrasound as the higher value of BMC, BMD and the total area of highly mineralized callus at 6 weeks after LSPL infrasound treatment. It suggested that the LSPL infrasound accelerated the augmenting mineral deposition, leading to a narrowing fracture gap and increased new bone shown by the histology assessment (Fig. 2). It was also supported by the histological finding showed that the less new bone and more immature callus in the control group.

It is known that bone formation must depend on suitable mechanical stimulation. As LSPL infrasound is a kind of mechanically compressed wave which could produce low frequency stress by resonance, it is naturally to attribute the effects to the response of bone to stress. Chao et al. reported that vibration with a low frequency of 0.5–3 Hz, which was within the range of infrasound could promote new bone generation (Chao et al., 1998). Presumably, low frequency stress produced by infrasound exerted marked effects on the process of long bone fracture healing through activation of osteoblast-like cells. It seemed to be involved in promotion of callus and new woven bone formation at intermediate stage from our radiographic observation largely.

But not all the findings in vivo could be completely explained by resonance theory. Object would appeared maximum vibration amplitude when its natural frequency was close to the frequency of driving force. The natural frequency of femur was in the range of 59–620 Hz based on finite element analysis in different degree of freedom (Jiang and Ge, 2007), which far exceeded the frequency spectrum of infrasound. Some researchers even thought that infrasound could not stimulate resonance of mature bone tissue effectively. Secondly, some previous experiments showed that simple stress wave with low frequency and wide amplitude only promoted the sensitivity of bone to mechanical load, and multiple low frequency oscillation like physical exercise had valid osteogenesis effects (Ye et al., 2007). In our present study, the local bone turnover in the stage of consolidation displayed complicate and biphasic characteristics in infrasound group.

Besides mechanical loading, the regulation of bone remolding has been conventionally linked to hormones and autocrine/paracrine signals. It is also under the influence of central and peripheral neural control which was recognized as neuro-osteogenic network. In this network, CGRP, SP and NPY were most studied.

In order to reveal the mechanisms by which infrasound promoted the bone healing, the current study focused on the related components in fracture microenvironment which could affect the bone development. We compared the local vascularization, innervation and collagen anabolism of two groups. The results suggested the neuro-osteogenic network to be a candidate factor mediating infrasonic effects. As a monitor and executor, the peripheral nervous system (PNS) especially sensory and automatic nerve played an incontestable role in bone development. Some classical neuropeptides like CGRP, NPY and their corresponding receptors were regarded as the most important osteo-neuromediators, which could be released and exert paracrine biological effects on bone cells close to the nerve endings (Franquinho et al., 2010).

In the present study, the accelerated fracture healing by LSPL infrasound was always accompanied with spatial–temporal change of CGRP and NPY innervation. In the process of bone healing, CGRP-positive fibers were found to distribute in some metabolically active area of skeleton, like periosteum and marrow. Compared with control group, more neoformative CGRP fibers were penetrating into the new woven bone or distributed as varicose nerve terminals in fibrocartilage callus after infrasound exposure. CGRP belongs to the 'calcitonin family', a group of peptide hormones act as physiological regulators of bone metabolism. CGRP has pleiotropic effects on bone cells, and both osteoclasts and osteoblasts have functional CGRP receptors. CGRP is anabolic to osteoblasts by stimulation of several classical signalings and by inhibition of osteoblast apoptosis. Furthermore, CGRP inhibits maturation of osteoclasts and bone resorption (Wang et al., 2010). So, the relative increased density of CGRP-positive fibers in bone could be an important determinant of bone mass increase after LSPL treatment.

While the effects of infrasound on NPY exhibited a more complex model. LPSL infrasound treatment increased the NPY positive fibers at earlier stage which might be a response to injury with the possible stimulatory effects on cell proliferation and angiogenesis leading to enhanced osteogenesis. The early inflammatory phase of fracture healing lays the foundation for bone healing as inflammatory cells secrete a number of proinflammatory cytokines which may initiate the regenerative healing cascade such as matrix synthesis, cell proliferation, angiogenesis and neurogenesis.

In the late consolidation phase, NPY positive fibers decreased obviously and almost disappeared in new woven bone. NPY system acts as an antiosteogenic factor in the regulation of bone homeostasis through central and peripheral mechanisms.

At the central nervous system level, NPY exerts its inhibition function in bone homeostasis through hypothalamic Y2 receptor. On the other hand, locally in the bone, via Y1

receptor, NPY directly inhibits the differentiation of mesenchymal progenitor cells as well as the activity of mature osteoblasts (Lundberg et al., 2007). The presence Y1 receptor in osteoblasts and peripheral tissues also suggests that system factors may also interact with Y1. As its inhibiting actions, the decreased NPY-positive fibers after infrasound treatment might enhance the bone formation and contribute to the promoted bone formation.

We had reason to believe that LSPL infrasound also could effect on the PNS innervation in local microenvironment besides force mechanism, which further contributes to osteotropic effects.

In summary, our results indicated that LSPL infrasound could promote osteogenesis and fracture healing in vivo. Even though there was no conclusive evidence to implicate spatial–temporal regular change of sensory and automatic nerve innervation in infrasound effects on bone turnover, the present results combined with the previous reports highlighted the possibility of neuro-osteogenic network in local microenvironment being one target besides stress contribution. Therapeutic application of the LSPL infrasound would emerge as a promising method in clinical orthopedics after its optimal working parameters were recognized.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Corrigendum

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