Original

Effect of Low-intensity Pulsed Ultrasound (LIPUS) with Different Frequency on Bone Defect Healing

Kazuya Monden^{1,2}, Hodaka Sasaki^{1,2}, Masao Yoshinari² and Yasutomo Yajima^{1,2}

¹⁾ Department of Oral and Maxillofacial Implantology, Tokyo Dental College, Chiba, Japan

²⁾ Division of Oral Implants Research, Oral Health Science Center, Tokyo Dental College, Chiba, Japan

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Abstract: Low-intensity pulsed ultrasound (LIPUS) is known to promote bone defect healing. The frequency of the LIPUS is known to influence directivity and depth of penetration, but the differences on bone healing remains unknown. This study was to investigate the effect of LIPUS with different frequencies on bone defect healing. Bone defects of 1.6 mm in diameter were created in femurs of ten-week-old male Long-Evans rats (n=36). Experimental groups were exposed LIPUS (intensity: 30 mW/cm², burst width: 200 μ s, time: 15 min/day) and divided into a low frequency (LF, 1.5 MHz) group and a high frequency (HF, 3.0 MHz) group. Non-exposed LIPUS group were used as control. After 3, 5, 7, and 10 days, femurs were removed and radiological, histomorphological and molecular biological evaluations were conducted. Micro-CT images showed that the depression in cortical bone was reduced in LF and HF groups. 3D bone morphological analysis at 10 days revealed that LIPUS increased cortical bone volume / tissue volume (BV/TV) and decreased BV/TV in the lower layer of cancellous bone (P<0.05). Histomorphologically, clot retraction was seen in the both LIPUS groups but not in the control group at 3 days. These effects were observed at a deeper layer in the LF group than in the HF group. No significant difference in osteopontin (OPN) gene expression was observed. However, osteocalcin (OCN) gene expression was significantly elevated in the HF group relative to the control group at 10 days (P<0.05). Immunohistochemical staining revealed that newly formed bone exhibited a positive reaction to OPN and OCN in both LIPUS groups. Healing of the bone defect area was noted in both LIPUS groups, but there was no clear difference in histomorphology between the LF and HF groups. LIPUS frequencies of 1.5 MHz and 3.0 MHz promote increased cortical bone mass and remodeling of cancellous bone in rat femurs with bone defects.

Key words: Low-intensity pulsed ultrasound, Frequency, Bone healing, Dental implant

Introduction

In recent years, titanium dental implants that restore occlusal function by achieving osseointegration with the jaw bone have come to be widely used as a treatment for tooth missing¹⁾. With the dental implant treated commonly, it has also become necessary for patients with implant risk factors such as osteoporosis and diabetes to receive implants. Therefore, a large amount of research has been reported on improving aspects of implants such as surface topography and chemistry in order to securely achieve osseointegration more quickly and increase the success rate of implant in these patients²⁻⁴⁾. However, although many studies have been conducted on the effects of improving the implant body as graft side, very few studies have been conducted on methods of improving the jaw bone as host side. One method of improving the jaw bone condition is to inject drugs such as bone

morphogenetic protein-2⁵⁾, fibroblast growth factor 2⁶⁾ and simvastatin⁷⁾ into the extraction socket, which has been shown to promote bone healing. However, the effectiveness of these methods are based on the drug chemistry, and these drugs present various issues regarding the risk of side effects, the dose, and the limited areas in which they can be used. Therefore, it is necessary to establish a method of promoting extraction socket healing and improving jaw bone condition that is safe for the body.

In the field of orthopedic surgery, low-intensity pulsed ultrasound (LIPUS), a non-invasive technique that causes no drug-related side effects and utilizes physical stimulation, is used in clinical settings to promote healing of normal⁸⁾ and intractable^{9,10)} bone fractures. *In vitro* studies have shown that LIPUS stimulation increases expression of osteoblast differentiation markers and accelerates calcification¹¹⁻¹⁴⁾. Furthermore, *in vivo* studies have shown that LIPUS promotes healing and increases bone mineral density in fractured rat femurs^{15,16)}. LIPUS has also been shown to promote bone fracture healing in rat models of osteoporosis¹⁷⁾

Correspondence to: Dr. Hodaka Sasaki, Department of Oral and Maxillofacial Implantology, Tokyo Dental College, 2-9-18 Misaki-cho, Chiyoda-ku, Tokyo, 101-0061 Japan; Phone: +81-3-6380-9257; E-mail: hosasaki@tdc.ac.jp

Figure 1. Bone defect model in rat femur and experimental protocol
IHC: Immunohistochemical staining, qRT-PCR: quantitative RT-PCR

and diabetes¹⁸⁾ known as model of delayed bone healing. In addition, LIPUS is also known to promote bone defect healing in rats of the cranial and tibia bone^{19,20)}. Studies in the field of oral implantology have shown that LIPUS exposure improves the contact rate of newly formed bone in implants placed in rabbit femurs²¹⁾ and promotes the formation of new bone tissue in areas of bone augmentation in the maxillary sinus of rabbits²²⁾. The findings of these studies indicate that LIPUS is also useful in implant therapy because it promotes achieving osseointegration and extraction socket healing process.

The parameters for LIPUS include intensity, exposure time and frequency. Because LIPUS is a type of ultrasound and its wave is dispersed, scattered, and weakened by tissues²³⁾, LIPUS exposure effect is strongly influenced by these parameters. Differences in the intensity of LIPUS, which indicates the strength of the sound waves, are known to contribute to osteocyte differentiation, and optimal parameter is defined^{11,24)}. In addition, it has been shown that the healing period can shorten dose-dependent LIPUS exposure time²⁵⁾. The frequency of LIPUS is known to contribute to directivity and the depth of penetration. Although the directivity of ultrasonic energy improves at higher frequencies, the depth of penetration decreases. Therefore, in clinical practice, 3 MHz is used for superficial wounds whereas 1 MHz is used for deep wounds and when there is a large amount of subcutaneous fat²³⁾. Thus, if different frequencies of LIPUS could be used in implant therapy to selectively promote healing of cortical and cancellous bone in the jaw bone or to effectively promote bone healing in extraction sockets of different sizes and shapes, it would be a useful method for improving the host side. However, not only have the effects of the frequency of LIPUS on achieving osseointegration and extraction socket healing process, its effects on bone defect healing also remain unstudied.

The purpose of this study is to investigate the effect of low and high frequency LIPUS exposure in the rat femur bone defect healing process by radiological, histomorphological, and molecular biological evaluations.

Materials and Methods

Surgical bone defect creation and LIPUS stimulation

Ten-week-old male Long-Evans rats (Sankyo Labo Service Corporation, Inc, Tokyo, Japan; n=36) were used in this study. After peritoneal injections of pentobarbital sodium (Somnopenyl®, Kyoritsu Seiyaku Corporation, Tokyo, Japan; 0.9 µl/g) were administered as general anesthesia. For surgery, the hind legs of the rats were shaved considerably and the outside skin of the distal femur was incised longitudinally, and the femur was exposed by stripping the periosteum. The bone defects were created in both femurs at 3 mm from the articular surface of the knee joint using motor handpiece (G3, Urawa Corporation, Kuki city, Japan) with a round bur (1.6 mm diameter, Dentsply Maillefer, Ballaigues, Switzerland). The depth of the bone defect was created to reach the opposite side of the cortical bone perforation. After the bone defect was created, the periosteum was replaced and the surgical wound sutured. Starting from one day after bone defect creation, the bone defect area of the right femur was transcutaneously exposed to LIPUS (intensity: 30 mW/cm², burst width: 200 µs, time: 15 min/day, transducer size: M [3.2cm diameter], frequency: 1.5 MHz or 3.0 MHz) with gel as a conductive medium using ST-SONIC (Ito Co, Ltd, Tokyo, Japan). The frequency parameters for LIPUS were low frequency (LF, 1.5 MHz) and high frequency (HF, 3.0 MHz). The left femurs that composed the non-LIPUS group were used as the control group (Fig. 1). Six samples were taken for radiological and histological evaluation after 3, 5, 7, and 10 days (n=24), and three

Figure 2. Scheme of regions of interest in 3D bone morphological analysis

samples were taken for quantitative RT-PCR after 7 and 10 days ($n=12$). All experiments were performed according to the Guidelines for the Treatment of Animals at Tokyo Dental College (approval ID: 253002).

X-ray micro-CT

Rats were sacrificed with pentobarbital sodium after 3, 5, 7 and 10 days, and perfusion fixation was performed with 10% neutral buffered formalin (Wako Pure Chemical Industries, Osaka, Japan) ($n=6$ for each femur). Micro-CT images of bone defect area were taken with the *in vivo* micro X-ray CT system R_mCT2 (Rigaku Corporation, Tokyo, Japan). Scanning parameters were as follows: tube voltage, 90 kV; tube current, 140 μ A; magnification, $\times 10$; slice width, 20 μ m; and scanning time, 2 min.

Radiological analysis of newly formed bone

Micro-CT image data at 10 days that had no artifacts were selected (each $n=3$) and the 3D structure of newly formed bone was measured using TRI/3D-BON 3D Trabecular bone structure analysis software (Ratoc System Engineering Corporation, Tokyo, Japan). The Region of interest (ROI) was a cylindrical section in the center of the bone defect area that was 1.1 mm in diameter and contained bone from the top of the cortical bone area of the bone defect to the inner surface of the cortical bone area of the deep part of the bone defect. This ROI was divided into a cortical bone area and a cancellous bone area. The cancellous bone area was further divided into an upper layer and a lower layer (Fig. 2). Bone volume/tissue volume (BV/TV) was used for evaluation and measured 3 times for each samples. The Tukey test was used for statistical processing ($P<0.05$).

Histological evaluation

Samples that had been used for micro-CT image scanning were fixed in 10% neutral buffered formalin for 7 days and decalcified with 10 % EDTA (pH 7.0–7.5; Wako Pure Chemical Industries,

Osaka, Japan) over 6 days. Paraffin sections of 3 μ m in thickness were prepared and stained with hematoxylin-eosin (H-E) according to the standard procedure. Histological observations were made using a universal photo microscope (Axiophot2, Carl Zeiss, Oberkochen, Germany).

RNA extraction and quantitative RT-PCR (qRT-PCR)

The rats were sacrificed with pentobarbital sodium after 7 and 10 days which observed newly bone formation in Histological evaluation, and bone tissue samples for RNA extraction was collected using trephine bar (2.8mm internal diameter, Micro Tech Corp, Tokyo, Japan) from the center of the bone defect area (each $n=6$). Collected bone tissue was kept in RNeasy RNA stabilization reagent (Applied Biosystems, Foster City, CA, USA) and then homogenized (tungsten carbide beads; 5 mm diameter, 28 Hz, 2 min) using a Tissue Lyser (QIAGEN, Alameda, CA, USA). Total RNA was extracted from the lysate using an RNeasy® Mini kit (QIAGEN) according to the manufacturer's protocol and quantified with a NanoDrop® Spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). The mRNA expression levels of *osteopontin* (AssayID Rn01449971_g1) and *osteocalcin* (AssayID Rn00566386_g1) were confirmed by qRT-PCR using a TaqMan® MGB probe (Applied Biosystems) and normalized against β -actin (Applied Biosystems). Total RNA was reverse-transcribed using QuantiTect® Reverse Transcription (QIAGEN), and qRT-PCR was performed with TaqMan® Fast Universal PCR Master Mix (Applied Biosystems) and an ABI 7500 Fast Prism Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. This quantification for each sample was duplicated, and results were analyzed using the $\Delta\Delta$ Ct method. Values are expressed as the mean and standard error and were analyzed with the Tukey test.

Immunohistochemical staining

Paraffin sections of histological evaluation at 10 days were deparaffinized with xylene and rehydrated in ethanol. They were then washed in 10 nmol/l phosphate-buffered saline (PBS, pH: 7.4) and immersed in 0.3% hydrogen peroxide in ethanol for 30 min to block endogenous peroxidase activity. After the sections were washed in PBS, they were blocked with 3 % normal bovine serum (BSA; Roche Applied Science, Indianapolis, USA). After reacting the sections with the primary antibodies, rabbit anti-osteopontin (Millipore Corporation, Billerica, MA, USA; diluted 1:200) and rabbit anti-osteocalcin (Bioss Inc, MA, USA; diluted 1:200), for 1 hour at room temperature, they were reacted with the secondary antibody, biotinylated anti-rabbit IgG antibody (Histofine MAX-PO [MULTI]; Nichirei, Toyo, Japan), for 30 minutes at room temperature. After washing in PBS, the sections were stained with 3,3'-diaminobenzidine (DAB) (DAB substrate kit, Nichirei, Tokyo, Japan) at room temperature. After

Figure 3. Radiological evaluation of bone defect area by micro-CT scanning
Control group: A, D, G, J, Low frequency (LF) group: B, E, H, K, High frequency (HF) group: C, F, I, L, Bar:1mm

Figure 4. Measurement of newly formed bone at 10 days by 3D bone morphological analysis
Measurement item: Bone Volume / Tissue Volume (BV/TV) In cortical bone, BV/TV was significantly higher in both LIPUS groups (LF and HF) than in the control group ($P < 0.05$). And at the lower layer of cancellous bone was significantly lower in both LIPUS groups than in the control group ($P < 0.05$).

counterstaining with a hematoxylin solution, they were dehydrated and enclosed according to the established protocol, and then were observed with a universal photo microscope (Axiophot 2).

Results

Radiological observation of micro-CT

Micro-CT images of the bone defect area taken at 3, 5, 7 and 10 days after LIPUS exposure were evaluated (Fig. 3). At 3 days, only radiolucent region that indicated the bone defect were observed (Fig. 3A-C). At 5 days, some radiopaque findings that indicated new bone formation were observed (Fig. 3D-F), but there was no difference among three groups. At 7 days, although growth of newly formed bone and radiopacity were increasing in the bone

defect area, there was no difference among all groups (Fig. 3G-I). At 10 days, formation of flat new bone continuous with existing bone was observed in the cortical bone of the LF and HF LIPUS groups, and newly formed bone in cancellous bone defect area was assimilated to existing bone (Fig. 3K, L). In the control group, the cortical bone in the defect area was depressed (Fig. 3J).

3D bone structural measurement of newly formed bone at 10 days

BV/TV of newly formed bone was calculated (Fig. 4). In cortical bone, BV/TV was significantly higher in both LIPUS groups (LF and HF) than in the control group ($P < 0.05$). In the upper layer of cancellous bone, there was no significant difference.

Figure 5. Low magnification of histomorphological evaluation
Low magnification image of H-E staining section at 3, 5, 7 and 10 days; Control group: A, D, G, J, Low frequency (LF) group: B, E, H, K, High frequency (HF) group: C, F, I, L, Bar: 1mm

Figure 6. High Magnification of histomorphological evaluation
High magnification image of H-E staining section at 3, 5, 7 and 10 days; central cancellous bone defect area of Control group: A, D, G, M, Low frequency (LF) group: B, E, H, N, High frequency (HF) group: C, F, I, O, and cortical bone defect area of Control group: J, LF group: K, HF group: L, Bar: 0.2mm, Arrow: osteoclast

On the other hand, BV/TV at the lower layer of cancellous bone was significantly lower in both LIPUS groups than in the control group ($P < 0.05$).

Histomorphological evaluation

Fig. 5 showed low magnification images of H-E staining

sections and Figure 6 were high magnification of central cancellous bone defect area (A-I, M-O) and cortical bone defect area (J-L). At 3 days after LIPUS exposure, cortical bone defect and blood clots in bone marrow area were observed in the all groups (Fig. 5A-C). These blood clots were recognized in all layers in the control group (Fig. 6A), whereas blood clot was retracted

Figure 7. Gene expression quantification for osteopontin (OPN) and osteocalcin (OCN) by quantitative RT-PCR at 7 and 10 days
FC: Fold Change

Figure 8. Immunohistochemical staining for osteopontin (OPN) and osteocalcin (OCN) at 10 days
Control group: A, D, Low frequency (LF) group: B, E, High frequency (HF): group: C, F, Bar: 200 μ m, Arrow: positive reaction, EB: existing bone, NB: newly formed bone

in both LIPUS groups. In addition, the regression of blood clots in the LF group observed deeper layer area (Fig. 6B) than the HF group (Fig. 6C). At 5 days, there were still blood clots in the control group (Fig. 5D), whereas they were disappeared in both LIPUS groups (Fig. 5E, F). The granulation tissue containing red blood cells and newly bone formation at boundary zone of bone defect area was observed in control groups (Fig. 6D). On the other hands, newly bone formation with osteoblast lining from existing cancellous bone was observed in all layers of the bone defect area in both LIPUS groups (Fig. 6E, F). At 7 days, callus bone was

filled in bone defect area in all groups (Fig. 5G-I). The callus bone in central cancellous bone defect area became more mature than at 3days and capillary vessel formation in connective tissue was observed in all groups (Fig. 6 G-I). At 10 days, densification of newly-formed cortical bone and trabeculation of newly-formed cancellous bone was observed in all groups (Fig. 5 J-L). In cortical bone area, a depression was observed in the control group (Fig. 6J) but not in both LIPUS groups (Fig. 6K, L). In cancellous bone area, capillary formation between trabeculae and bone resorption by osteoclasts (arrowed) were observed in the LIPUS

groups (Fig. 6N,O) more than control group (Fig. 6M).

Quantitative RT-PCR (qRT-PCR)

The gene expression of *osteopontin* (OPN) and *osteocalcin* (OCN) was quantified using samples at 7 and 10 days (Fig. 7). No significant difference in OPN gene expression was observed in samples taken after 7 or 10 days. OCN gene expression had no significant difference among three groups at 7 days. However, that of HF group significantly increased than control group at 10 days ($P < 0.05$).

Immunohistochemical staining

Immunolocalization of OPN (Fig. 8A-C) and OCN (Fig. 8D-F) in newly-formed cortical bone at 10 days after LIPUS exposure was investigated by immunohistochemical staining. Immunoreaction of OPN in the control group was recognized newly formed bone adjacent to stump of existed bone (Fig. 8A). However, positive reaction of OPN in osteoblasts around the newly formed bone was seen not only stump of existed bone side, but also center side of bone defect area in both LIPUS groups (Fig. 8B, C). A positive immunoreaction to OCN was observed in newly formed bone at the boundary with existing bone in the control group (Fig. 7D). In both LIPUS groups (Fig. 7E, F), a positive reaction to OCN was observed in osteoblasts around newly formed bone from the stump of existing bone to the middle of the bone defect area.

Discussion

In this study, the effects of different frequencies of LIPUS exposure on bone defect healing process were compared in rat femur bone defect models by radiological, histomorphological, and molecular biological evaluations. LIPUS is a type of ultrasound energy that passes through living tissues²³⁾ and is known to promote healing of fractures and bone defects^{8,15,19)}. The parameters for LIPUS include intensity, exposure time and frequency. Some previous studies have examined the effects of differences in LIPUS intensity on bone tissue^{11,26)} and reported an intensity of 30 mW/cm² LIPUS promotes osteoblast differentiation *in vitro*¹⁴⁾ and promotes fracture healing in a rat femur model *in vivo*¹⁵⁾. It was also revealed LIPUS exposure at 30 mW/cm² promoted fracture healing and was safety in the clinical study^{8,27,28)}. From these results, the United States Food and Drug Administration currently recommends that an intensity of 30 mW/cm² be used when using LIPUS for human bone fracture. An intensity of 30 mW/cm² was used in this study as well.

At 10 days LIPUS exposure, the reduction of depression, increase of BV/TV and immunoreactions for OPN and OCN in newly formed cortical bone were observed in newly formed bone in both LIPUS groups (LF and HF), however not in the control group. LIPUS is known to promote cell proliferation and increased

bone differentiation marker expression in cultured rat bone marrow derived stromal cells¹²⁾ and human periosteal cells²⁹⁾, and to accelerate healing process of bone fracture and bone defect. Moreover, Yoshida A et al.³⁰⁾ found that LIPUS reduced the depression and accelerated densification of cortical bone in bone defect areas of mouse femurs by promoting periosteal cell differentiation. Naruse K et al.³¹⁾ also reported that LIPUS increased osteocalcin expression in periosteal cells and promotes periosteal cell-derived stem cells differentiation into osteoblasts in organ culture of rat femurs. These results suggested that LIPUS exposure matured newly formed bone in cortical bone area by increasing bone differentiation marker; OCN and OPN and reduced the depression by promoting periosteal cell differentiation.

On the other hands, BV/TV were decreased in the lower layer of cancellous bone in both LIPUS groups compared to control group. The process of bone healing divided into 4 stages; hematoma formation stage, fibrocartilaginous callus formation stage, bony callus formation stage, and bone remodeling stage. The samples taken at 10 days after bone defect creation in this study showed matured callus bone and partial bone resorption by osteoclast in both LIPUS group and it was indicated that the latter part of the bone healing process, which is transition between the bony callus formation stage and bone remodeling stage. In the some studies of LIPUS exposure with distraction osteogenesis has been shown to promote bone remodeling by callus maturation and bone resorption^{32,33)}. Therefore, it was suggested, LIPUS exposure brought about callus resorption in lower layer of cancellous bone by accelerating the transition into the bone remodeling stage in this study.

The frequency range for LIPUS is considered to be 0.75-3 MHz²³⁾. In this study, the frequencies were set to 1.5 MHz for the LF group and 3.0 MHz for the HF group. In the histomorphological evaluation at 3 days after LIPUS exposure, blood clot retraction and accompanying granulation tissue was seen in both LIPUS groups but not in the control group. Blood clot retraction tended to occur at a deeper level in the LF group than in the HF group. LIPUS has been reported to activate macrophages¹⁵⁾ and increase the expression of platelet-derived growth factor³⁴⁾, fibroblast growth factor³⁵⁾, and vascular endothelial growth factor³⁶⁾ which were known to involved in the transition between hematoma formation stage and fibrocartilaginous callus formation stage. It is also known that lower frequencies of LIPUS have a greater depth of penetration²³⁾. Based on these result, it is suggested the early stages of bone healing was faster in the LF group because the effects of LIPUS penetrated more deeply than in the HF group.

In samples taken after 10 days, gene expression of OCN was significantly higher in the HF group than in the control group. It is known that directivity improves as the frequency of LIPUS increases. In this study, gel was applied to the skin around rat femurs as a conductive medium, and the femurs were exposed to

LIPUS for 15 min/day using a transducer with a diameter of 3.2 mm. Therefore, the direction of propagation was not always constant, and it is suggested that differences in the directivity of LIPUS could have influenced the effects observed in samples taken after 10 days. However, there is not recognized histomorphological differences in bone healing were observed between the LF and HF groups at 10 days in this study. The depth of penetration of LIPUS is known as 1-2 cm for the 3 MHz frequency and 3-5 cm for the 1 MHz frequency²³. Thus, differences caused by the depth of penetration would not appeared in this study model using rat femurs even if width of soft tissue includes skin and muscle (approximately 2 cm), and the diameter of the femur (approximately 3 cm) were combined because the total depth is still less than 5 cm.

In this study, 1.5 MHz (LF) and 3.0 MHz (HF) frequencies of LIPUS both promoted healing in the bone defect area. This indicates that LIPUS could be useful in implant therapy to promote healing of the extraction socket before implant placement and achieving implant neck region of osseointegration. Additionally, LIPUS exposure in oral cavity is difficult to maintain a standard direction of propagation, it is better to use a high frequency to improve directivity in areas where the depth of penetration is 5 cm or less, including the cortical bone of the alveolar crest and bone surrounding the implant neck region. However, the effect of the frequency of LIPUS on the cancellous area of the human jaw bone, where the depth of penetration is greater than 5 cm, will need to be examined in future studies.

In conclusion, bone defects in rat femurs, LIPUS frequencies of 1.5 MHz and 3.0 MHz promote increased cortical bone mass and remodeling of cancellous bone.

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