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Original Article

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

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Abstract

Objective

Low-intensity pulsed ultrasound (LIPUS), a non-invasive technique that utilizes physical stimulation, is known to promote bone fracture healing. The LIPUS of frequency is known to influences aspects such as directivity and the depth of penetration, but the effect of its differences on bone healing remains unknown. This study was to investigate the effect of LIPUS with different frequency on bone defect healing.

Methods

Bone defects of 1.5 mm in diameter were created in both femurs of ten-week-old male Long-Evans rats (n=36). Starting from the following day, right femurs were exposed to LIPUS (intensity: 30 mW/cm², burst width: 200 µs, time: 15 min/day). The LIPUS group was divided into a low frequency (LF, 1.5 MHz) group and a high frequency (HF, 3.0 MHz) group. The left femurs that composed the non-LIPUS group were used as the control group. After 3, 5, 7, and 10 days, both femurs were removed and radiological, histomorphological, and molecular biological evaluations were conducted.
Results

Micro-CT images of samples taken after 10 days showed that the depression in cortical bone was reduced in both LIPUS groups (LF and HF) but not in the control group. 3D bone morphological analysis at 10 days revealed that LIPUS increased cortical 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. In samples at 10 days, a depression in cortical bone was seen in the control group but not in either both LIPUS groups. 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.

Conclusion
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.
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 missing\(^1\). 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 patients\(^2,3,4\). 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-2\(^5\), fibroblast growth factor 2\(^6\) and simvastatin\(^7\) 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 normal\(^8\) and intractable\(^9,10\) bone fractures. *In vitro* studies have shown that LIPUS stimulation increases expression of osteoblast differentiation markers and accelerates calcification\(^11,12,13,14\). 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 osteoporosis\(^17\) and diabetes\(^18\) 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 femurs\(^21\) and promotes the formation of new bone tissue in areas of bone augmentation in the maxillary sinus of rabbits\(^22\). 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 tissues\textsuperscript{23)}, 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\textsuperscript{11,24). In addition, it has been shown that the healing period can shorten dose-dependent LIPUS exposure time\textsuperscript{25}). 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 fat\textsuperscript{23}). 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

1. 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 (Somnopentyl® 0.9 µl/g, Kyoritsu Seiyaku Corporation, Tokyo, Japan) 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 a round bur (1.5 mm diameter). 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 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).

2. 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 µA; magnification, ×10; slice width, 20µm; and scanning time, 2 min.

3. 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).

4. 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 EDTA (pH 7.0–7.5; Wako Pure Chemical Industries, Osaka, Japan) over 6 days. Paraffin sections of 3 µm 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).

5. 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 RNAlater RNA stabilization reagent (Applied Biosystems) and then homogenized (tungsten carbide beads; 5 mm diameter, 28 Hz, 2 min) using a TissueLyser (QIAGEN). 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 ΔΔCt method. Values are expressed as the mean and standard error and were analyzed with the Tukey test.

6. Immunohistochemical staining

Paraffin sections of histological evaluation at 10 days were deparaffinized with xylene and rehydrated in ethanol. They were then washed in 10nmol/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 (Histofine MAX-POMULTI; Nichirei, Tokyo, Japan). 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 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

1. 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, B, C). At 5 days, some radiopaque findings that indicated new bone formation were observed (Fig. 3D, E, F), but there was no difference among three groups. At 7 days, although growth of newly formed bone and radiopacities were increasing in the bone defect area, there was no difference among all groups (Fig. 3G, H, 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).

2. 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. 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).

3. Histomorphological evaluation

At 3 days after LIPUS exposure, blood clots were observed in the bone defect
area. These blood clots were recognized in all layers in the control group (Fig. 5A),
whereas blood clot was retracted in both LIPUS groups. In addition, the regression of
blood clots in the LF group observed deeper layer area (Fig. 5B) than the HF group (Fig.
5C). At 5 days, there were still blood clots in the control group (Fig. 5D), whereas
newly bone formation from existing cancellous bone was observed in all layers of the
bone defect area in both LIPUS groups (Fig. 5E, F). At 7 days, callus bone maturation
was observed in all layers of the bone defect area in all groups (Fig. 5G, H, I). At 10
days, densification of newly-formed cortical bone and trabeculation of newly-formed
cancellous bone was observed in all groups. In cortical bone area, a depression was
observed in the control group (Fig. 5J) but not in both LIPUS groups (Fig. 5K, L). In
cancellous bone area, capillary formation between trabeculae and bone resorption by remodeling of callus was observed in the LIPUS groups but not in the control group.

4. quantitative RT-PCR (qRT-PCR)

The gene expression of osteopontin (OPN) and osteocalcin (OCN) was quantified using samples at 7 and 10 days (Fig. 6). 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).

5. Immunohistochemical staining

Immunolocalization of OPN (Fig. 7A-C) and OCN (Fig. 7D-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. 7A). 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 LPUS
groups (Fig. 7B, 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 tissues and is known to promote healing of fractures and bone defects. 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 and reported an intensity of 30 mW/cm² LIPUS promotes osteoblast differentiation in vitro and promotes fracture healing in a rat femur model in vivo. It was also revealed LIPUS exposure at 30 mW/cm² promoted fracture healing and was safety in the clinical study. 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 depression in cortical bone was reduced, BV/TV was increased, and immunoreactions for OPN and OCN were observed in
newly formed bone in both LIPUS groups (LF and HF), however not in the control group. Moreover, BV/TV were decreased in the lower layer of cancellous bone in both LIPUS groups compared to control group. LIPUS is known to promote cell proliferation and increased bone differentiation marker expression in cultured human periosteal cells\textsuperscript{29}. Naruse et al.\textsuperscript{30} 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. Moreover, Yoshida A et al.\textsuperscript{31} found that LIPUS accelerated densification of cortical bone in bone defect areas of mouse femurs. They also found that LIPUS significantly increased the volume of newly formed bone that was continuous with the periosteum in a mouse model of senile osteoporosis. 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 were in the latter part of the bone healing process, which is transition between the bony callus formation stage and bone remodeling stage. LIPUS exposure with distraction osteogenesis has been shown to promote increased callus formation and bone
These results indicate LIPUS promoted bone healing relative to the control group by activating periosteal cells and accelerating callus formation and maturation in cortical bone, and bringing about callus resorption in 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 MHz\textsuperscript{23}). 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 macrophages\textsuperscript{15)} and increase the expression of platelet-derived growth factor\textsuperscript{34)}, fibroblast growth factor\textsuperscript{35)}, and vascular endothelial growth factor\textsuperscript{36)} 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 penetration\textsuperscript{23}). 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 frequency\textsuperscript{23)}. 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.

Conclusion

In 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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Figure legends

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

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

Fig 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

Fig 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).

Fig 5. Histomorphological evaluation of bone defect area
Control group: A, D, G, J, Low frequency (LF) group: B, E, H, K, High frequency (HF) group: C, F, I, L, Bar: 1mm
At 3 days, the blood clots were recognized in all layers in the control group (A), the regression of blood clots in the LF group observed deeper layer area (B) than the HF group (C). At 5 days, there were still blood clots in the control group (D), whereas newly bone formation was observed in the bone defect area in both LIPUS groups (E, F). At 7 days, callus bone maturation was observed in the bone defect area in all groups (G, H, I). At 10 days, in cortical bone area, a depression was observed in the control group (J) but not in both LIPUS groups (K, L).

Fig 6. Gene expression quantification for osteopontin (OPN) and osteocalcin (OCN) by quantitative RT-PCR at 7 and 10 days
FC : Fold Change

Fig 7. Immunohistochemical staining for osteopontin (OPN) and osteocalcin (OCN) at
10 days
Fig. 1

Process:
1. Bone defect creation
2. Femoral distal epiphysis
3. LIPUS exposure (right side)
   - 15 min/day
4. Non-exposure (left side)
5. Sacrificed
6. Micro CT scanning
7. H-E staining
8. IHC staining
9. qRT-PCR
10. 1.5 MHz (LF)
11. 3.0 MHz (HF)
12. Control
Fig. 2

Cortical bone area

Upper layer of Cancellous bone

Lower layer of Cancellous bone

1.5mm

1.1mm
Fig. 3
Fig. 4

* $P < 0.05$  

- Cortical bone Area
- Upper layer of Cancellous bone
- Lower layer of Cancellous bone

* $P < 0.05$  

control  
LF  
HF
Fig. 5

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Fig. 6
Fig. 7