In vivo bone regenerative effect of low-intensity pulsed ultrasound in rat calvarial defects

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Objectives. Low-intensity pulsed ultrasound (LIPUS) is a biophysical intervention in a bone repair process. However, neither the healing events of the flat bones of the skull using LIPUS nor the optimal stimulation settings are fully understood. The aim of this study was to evaluate the bone regenerative effect of LIPUS in rat calvarial flat bone defects by using in vivo microfocus computerized tomography (micro-CT).

Study design. The calvarium was exposed, and noncritical-sized 2.7-mm defects were prepared. LIPUS (1.6 MHz, repeating 1.0-kHz pulsation, and 30-mW/cm² intensity) was applied for 20 minutes daily. Bone regeneration was evaluated by image analysis using micro-CT and histologic examination.

Results. Within 2 weeks, LIPUS-treated rats demonstrated 7.0% reossification of the original surgical defect, whereas control rats demonstrated 3.6%. At 3 and 4 weeks, a significant difference in the reossification ratio was observed (12.0% vs. 5.8% and 18.1% vs. 9.8%, respectively; \( P < .05 \)).


Sizable adult populations around the world have bone loss within the oral cavity owing to oral traumatic injuries, periodontal diseases, and other conditions. In many of these cases, a bone augmentation procedure is required, and oral surgeons or periodontists must choose among several techniques to foster the bone regenerative process. Recently, approaches other than scaffolds and growth factors have focused on enhancing bone healing using different forms of biophysical stimulation, such as ultrasound. 

The pressure waves produced by ultrasound are transmitted into the body as high-frequency acoustic pressure waves in tissues and can cause biochemical events at the cellular level; they may also promote bone formation. Low-intensity pulsed ultrasound (LIPUS) represents a very mild mechanical stimulation of bone, and its effect has been studied in vitro studies, \(^2,5\) animal models, \(^6,7\) and clinical settings \(^8,9\) involving fractures and other healing responses. Although reports have discussed the influence of LIPUS on fracture healing in long bones, \(^10\) neither the healing events of the flat bones of the skull (such as the parietal, parts of the temporal, and parts of the maxilla) nor the optimal stimulation settings promoting the development of new bone are fully understood.

Microfocus computerized tomography (micro-CT) enables the observation of microscopic bone structures. The micro-CT technique seems to be promising for the qualification of bone formation inside bony defects. Lavandier et al. \(^11\) estimated LIPUS reossification ability from micro-CT images in the rat calvarium. However, it is difficult to observe the dynamics of bone augmentation in the same animal continuously, making it necessary to kill many animals. The in vivo micro-CT (R_mCT; Rigaku, Tokyo, Japan) system is characterized by high resolution, quick operation, and low effective dose. The R_mCT system produces clear images of the bones in small living experimental animals within a short time. Kochi et al. \(^12\) observed the dynamics of bone augmentation with R_mCT in the rat calvarium.

In the present study, we examined the bone regenerative effect of LIPUS using R_mCT in rat calvarial flat bone defects.

MATERIAL AND METHODS

Animals

Eighteen 12-week-old male Fischer rats weighing 250-300 g each were used. The animals were housed in an experimental animal room (22°C, 55% humidity, and 12-h light/dark cycle) and fed a standard laboratory diet.
and water. The Animal Experimentation Committee of the Nihon University School of Dentistry approved this study.

**Surgical procedure**

After general anesthesia was established with sodium pentobarbital (30 mg/kg intraperitoneally [IP]; Somnopentyl; Schering-Plough, Munich, Germany), the surgical area was shaved, and the skin was washed with a 70% ethanol. Local anesthesia with an intraperiosteal injection of 0.5 mL 2% lidocaine (Xylocaine; AstraZeneca, Osaka, Japan) was performed to control bleeding and to provide additional local anesthesia.

A horseshoe-shaped skin incision over the head was made, the parietal area was exposed under aseptic conditions, and the periosteum was elevated to expose the bone. A 2.7-mm defect was created on the midsagittal suture as described by Intini et al.13 (Fig. 1). Defects were created using a dental surgical drilling unit with a trephine constantly cooled with sterile saline; subsequently, the calvarial disk was carefully removed to avoid tearing the dura. After thoroughly rinsing the area with physiologic saline solution to wash out any bone fragments, skin closure was accomplished using 4-0 silk suture (Ethicon, Somerville, NJ). The day of surgery was designated as day 0.

The 18 rats were randomly divided into 2 groups: the LIPUS treatment group (LIPUS rats) and the control group (control rats). For bone labeling, all rats were injected subcutaneously with calcein (Dojin, Kumamoto, Japan) at a dose of 8 mg/kg every 4 days.

**LIPUS exposure**

LIPUS rats were exposed to an application of LIPUS for 20 minutes daily. The application of LIPUS was started on day 1 and continued to day 28. An original LIPUS exposure system (Asahi, Irika Co, Saitama, Japan), which was a modification of a commercially available clinical device (Exogen 2000+, Smith & Nephew, Memphis, TN, USA), was used. The LIPUS exposure apparatus was set to work with a 1.6-MHz 200-μs burst-width sine wave, repeating pulsation at 1.0 kHz, and an intensity of 30 mW/cm² spatial and temporal average (SATA). The intensity of the ultrasound produced was 30 mW/cm², which is similar to diagnostic ultrasound levels used in sonogram procedures and 1%-5% of the intensity level used in conventional therapeutic ultrasound. LIPUS rats were anesthetized slightly with dilute sodium pentobarbital (10 mg/kg IP; Somnopentyl) before LIPUS treatment. LIPUS applications were performed after placing the animal in a box that restricted excessive movements (Fig. 2). Ultrasound gel was applied directly on the LIPUS rats’ heads before LIPUS treatment.

**Imaging system**

The R_mCT system uses a microfocus X-ray tube with a focal point of 7 μm (L9181S; Hamamatsu Photonics, Hamamatsu, Japan), and the X-ray sensor has a 4-inch image intensifier. The X-ray source and image intensifier are connected by a basal plate, and the I-arm rotates in a vertical plane, driven by a direct-drive motor. LIPUS and control rats were anesthetized with sodium pentobarbital and placed on the stage, and images of the areas of interest were captured. Repeated R_mCT imaging was performed from 1 to 4 weeks after surgery.

**Microfocus computerized tomography analysis**

The exposure parameters were 90 kV and 88 μA. The images were reconstructed on a personal computer.
using specially designed I-View software. On day 0, we made a screen cylinder, which had a height of 0.27 mm and diameter of 2.70 mm, to overlap the initial bone defect (Fig. 3). We measured the bone volume (BV) within the cylinder from voxel images using BV-measuring software (Kitasenjyu Radiest Dental Clinic, I-View Image Center, Tokyo, Japan). Using the BV-measuring software, the gray values and number of voxels with the corresponding gray value were calculated in regions of interest (ROIs). A histogram of the X-ray absorption rate as the x-axis and CT voxel number as the y-axis was calculated for the field of view of the CT imaging area. The histograms of the X-ray absorption rates showed the peaks for hard and soft tissues. The threshold was set at the value for the trough between these peaks (Fig. 4). The number of voxels for the X-ray absorption rates exceeding the threshold was counted. Finally, the bone volume was calculated, and the number of voxels was multiplied by the voxel volume. The bone volume in the ROIs was measured on day 0 and again each week under the same conditions. Then the increase in bone volume was calculated by subtracting the bone volume on day 0 from each of the subsequent values. The increase in bone was considered to be defect reossification. Thus, we calculated the defect reossification ratio every week.

Statistical analysis

Means and standard deviations were calculated for the reossification ratio every week. Comparisons of the 2 groups’ mean reossification ratios were tested using the Mann-Whitney U test. The significance level for statistical analysis was set at $P < .05$. Statistical analysis was performed with SPSS 16.0 J for Windows (SPSS, Chicago, IL, USA). We carried out a sample size calculation, based on the results of Lavandier et al. The difference of the mean reossification ratio among 2 groups was about 5.7 (SD 4.0). From these data, a sample size of 9 per group was required for detection of a significant difference in reossification ratio (80% power, 2-sided 5% significance level).

Histologic analysis

Four weeks later, the animals were killed by deep anesthesia with sodium pentobarbital (100 mg/kg IP; Somnopentyl). The skin was dissected, and the defect sites were removed along with surrounding bone and soft tissues. The first sections were stained with Villanueva bone stain for calcein fluorochrome and polarized light analyses. The bone specimens were fixed in ethyl alcohol overnight and prepared for histologic analysis. They were immersed in Villanueva bone-stain solution (5 mg/mL in 70% methanol; Maruto Instrument Co., Tokyo, Japan) for 72 hours and then embedded in methyl methacrylate resin using routine methods. Each plastic-embedded bone specimen was cut with a low-speed saw (Maruto Instrument Co., Tokyo, Japan) as coronal sections. The specimens, taken from the 100-mm-thick section centered on the bone defect, were prepared and then ground to a thickness of 30 μm. The ground sections were photographed with a fluorescent microscope (Model BHF-142; Olympus, Tokyo, Japan) under ultraviolet light.

The second sections were stained with hematoxylin and eosin. Defect sites were removed along with surrounding bone and soft tissues and were fixed in 10% formalin. The specimens were then decalcified with formic acid–sodium citrate decalcification solution for
1 week and embedded in paraffin. Five-μm-thick coronal sections through the center of the circular defect were prepared and processed for hematoxylin and eosin staining. Histologic examination was performed under a light microscope equipped with a morphometric system connected to a personal computer.

RESULTS

One LIPUS rat and 2 control rats died during the observation period. Healing progressed uneventfully with no complications in all of the other animals. Therefore, we analyzed 15 animals with micro-CT images.

Micro-CT images revealed defect reossification over time in 15 animals. Images of 1 representative subject of each group are presented in Figs. 5 and 6. New bone tissues were observed at the defects of both groups as early as 2 weeks after surgery. The reossification developed by extensions of growth from the bony rims on the lateral sides of the bone defects. The reossification around the midsagittal suture was hardly seen in all 15 animals from beginning to end.

Data from the microfocus computerized analysis are presented in Fig. 7. In microfocus computerized analysis using the reossification ratio, the ratio varied widely among the subjects in each group (Fig. 7). Within 2 weeks, LIPUS rats showed 7.0% reossification of the original surgical defect, whereas control rats showed 3.6%. At 3 and 4 weeks, a significant difference in the reossification ratio was observed (12.0% vs 5.8% and 18.1% vs 9.8%, respectively; \( P < .05 \)) (Fig. 7). In vivo micro-CT images were similar to optical microscopic images (Figs. 8 and 9).

The ground sections, stained by the Villanueva bone-staining method, revealed clear bone labeling in the edges of bone defects. In fluorescence microscopy images, calcein was observed as a green color. We regarded the labeled section as new bone. Comparing LIPUS rats and control rats, we confirmed vigorous new bone formation in LIPUS rats (Figs 8 and 9). There was an apparent difference between LIPUS rats and control rats, especially on the periosteal side.

In histologic analysis, with hematoxylin and eosin staining, more osteoblast-like cells were observed around the bony rim in LIPUS rats than in control rats (Figs. 10 and 11).

DISCUSSION

This study showed that the application of LIPUS enhanced the bone regenerative effect of noncritical bone defects in rats as confirmed by in vivo micro-CT.

To our knowledge, only ~6 studies have carried out an in vivo association study between LIPUS and bone defects, not fractures or bone distraction. Duarte\(^{14}\) made small holes with diameters of 1.5 mm in the cortex of the femur in rabbits and showed that daily 15-minute ultrasound treatments for 2 weeks stimulated the callus formation inside the holes compared with the contralateral nontreated holes. Yang and Park\(^{15}\) made small and large ulnar defects in dogs and demonstrated
a LIPUS bone regenerative effect. Both of those studies focused on long bones and therefore do not link directly to our oral surgical or periodontal daily practice. Azuma et al.\textsuperscript{16} reported actions of cartilage tissue, chondrocytes, and chondroclasts on the LIPUS bone regenerative effect in long bones using histology. The maxilla and mandible form primarily by intramembranous ossification, in contrast to long bones, which form by endochondral ossification.

Schortinghuis et al.\textsuperscript{17} created a circular mandibular defect (5.0 mm outer diameter). Three groups were studied: an ultrasound treatment group, a placebo treatment group, and a control group. Ultrasound and placebo treatments involved a daily treatment for 20 minutes at the site of the defect. LIPUS settings were 1.5 MHz, 200 μs burst width, and 30 mW/cm\textsuperscript{2} intensity. They concluded that LIPUS does not stimulate bone defect healing in the case of a large mandibular defect.
in the rat. They also performed the same investigations using expanded polytetrafluoroethylene membranes or collagen membranes to avoid growth of soft tissues. The settings of LIPUS used in those 3 studies almost matched the settings used in the present study. Those 3 studies focused on mandibular and critical bone defects. In accordance with these results, we designed a LIPUS study of noncritical bone defects of the calvaria.

Lavandier et al. investigated LIPUS reossification ability from micro-CT images in 3-mm noncritical bone defects of the rat calvarium. Two intensities of ultrasound (1 MHz, 100-Hz pulse repetition frequency, and 20% duty cycle) were investigated: 100 and 300 mW/cm² SATA. They obtained a significant LIPUS bone regenerative effect with 300 mW/cm² but not with 100 mW/cm². The mean 60-day bone reconstruction ratio of the 300 mW/cm² group was about 20% of volume. In the present study, a 1.6-MHz LIPUS with 200 μs burst width and 30 mW/cm² intensity was investigated. At 3 and 4 weeks, a significant difference in the reossification ratio was observed. We were able to demonstrate effectiveness of LIPUS with a relatively lower power. This is an important difference between the present study and the report from Lavandier et al.

Few studies have examined the optimization of LIPUS intensity. Pilla et al. compared different LIPUS intensities (1.5, 7.5, 15, 30, and 45 mW/cm²) in a rabbit fracture model. They revealed that the LIPUS effect is maximal and constant when the intensity reaches 30 mW/cm². Yang et al. compared different LIPUS intensities (50 and 100 mW/cm²) in restoring the mechanical properties of rat femora after fracture. They measured an increased maximum torque and a slightly decreased torsional stiffness using 50 mW/cm² compared with 100 mW/cm². They also showed that the 100-mW/cm²-treated femora had lower maximum torque and torsion stiffness compared with the 50-mW/cm²-treated femora. Mizuta et al. observed the effect of tissue thickness on LIPUS intensity in a tissue-mimicking phantom and pig limb. They revealed that the farther from the surface the defect is, the more significant the LIPUS attenuate becomes. In the present study, the transducer was set just above the bone defect. Therefore, we think that the attenuation must be very small in the present study, and we needed minimal LIPUS intensity to give bone cells sufficient strain by mechanical vibration. Instead, we must emphasize the transducer setting, treatment timing, and observation methods.

In the present study, high-resolution in vivo micro-CT was used to measure the bone volume within the defect. Kochi et al. used the same micro-CT system to examine bone augmentation beyond the skeletal envelope in the rat calvarium and concluded that the micro-CT system enabled the continuous observation and measured bone volume of guided bone augmentation in the rat calvarium. Using R_mCT, we can obtain bone images of living experimental animals without killing them. Thus, R_mCT enables us to follow the dynamics of bone change in the same animal over time. The observation of 3 dimensions might be useful for measuring bone volume precisely.

Though various in vivo and in vitro studies have been performed, the underlying mechanism has remained unclear. Gleizal et al. verified a LIPUS effect in vitro with osteoblasts obtained from mouse calvaria. They suggested faster proliferation with ultrasound treatment, as determined by cell counting over 4 days. Suzuki et al. studied osteoblasts originating from rat osteosarcoma (ROS) cells and showed that LIPUS stimulation did not affect proliferation. They showed that daily LIPUS stimulation increased bone morphogenetic protein 2 gene expression and the formation of mineralized nodules in ROS cells. This result indicated that LIPUS stimulation promotes the formation of bone by osteoblasts. In addition, bone sialoprotein was expressed in the last stage of osteoblast differentiation. Therefore, they concluded that LIPUS might stimulate not only the differentiation but also the maturation of osteoblasts. The nature of the LIPUS effect (proliferation, differentiation, or maturation) on osteoblasts remains unclear. Gleizal et al. used osteoblasts obtained from calvaria, and Suzuki et al. used osteoblasts originating from rat osteosarcomas, and there may be some essential differences. Our present in vivo study of rat calvaria bone defects showed more osteoblast-like cells...
around the bony rim in LIPUS rats than in control rats. These results are not inconsistent with the findings obtained from Gleizal et al.\textsuperscript{23}

In conclusion, our results show the bone regenerative effect of LIPUS treatment on rat noncritical calvaria defects, as confirmed with in vivo micro-CT. Although
further studies, such as examination of the effects of different settings, are still required for clinical use consideration, the present study may provide insight for future clinical repair of bone defects.

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REFERENCES


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