Low-intensity pulsed ultrasound promotes the osteogenic effect induced by bone allograft

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Objectives: The aim of this study was to investigate the combined effects of bone allograft and low-intensity pulsed ultrasound (LIPUS) on osteogenesis using a bone allograft model.

Methods: Femora and tibiae were harvested from 8 Wistar rats. These long bones were used for bone allografts. Subsequently, two groups were studied. In the bone allograft group, freeze-dried bone was fixed unilaterally to the anterior surface of the femur in 42 Wistar rats. In the sham-operation group, the same procedure was performed but without a bone allograft in 8 rats. The right femora were exposed to LIPUS for 20 minutes per day, beginning the day after surgery and continued until harvest in both groups. X-ray, three-dimensional micro-computed tomography (3D micro-CT) and histological analysis were performed.

Results: In the bone allograft group, x-ray, 3D micro-CT, and histology showed that LIPUS promoted intramembranous ossification induced by the bone allograft, and suggested that LIPUS accelerated the differentiation of mesenchymal stem cells in the cambium layer.

Conclusions: LIPUS facilitates the osteogenic effect induced by a bone allograft. LIPUS has been successfully used to accelerate fracture healing. Therefore, we anticipate that earlier clinical application of LIPUS treatment to bone allograft may represent a promising approach for accelerating bone healing.

Key words: low-intensity pulsed ultrasound, bone allograft, osteogenesis, intramembranous ossification, neovascularization

Introduction

Autologous or allogenic bone grafting is effective in accelerating and initiating fracture repair in cases of delayed union or pseudarthrosis. Some growth factors, such as bone morphogenetic proteins (BMPs), transforming growth factor-β1 (TGF-β1), fibroblast growth factors (FGF) and insulin-like growth factors (IGFs), have been shown to be sustainably released from grafted bone;1-3; these factors modulate cellular phenomena that inhibit fracture healing. Although these bone morphogenetic factors for fracture healing have beneficial effects, it is not yet completely clear how best to promote the osteogenic effect induced by bone grafting.

The repair of long bone after fracture is a unique multistep process including many cellular events, such as inflammation, angiogenesis, intramembranous ossification, chondrogenesis, endochondral ossification, and bone remodeling.4 These repair processes are regulated by systemic and hormonal factors, as well as by local factors such as growth factors.5 A number of reports have shown that administration of several growth factors by local injection accelerates the fracture healing process.6-11 In the clinic, several methods have been used to accelerate fracture healing by mechanical stimulation; these include pulsed electromagnetic field stimulation12-17 and low-intensity pulsed ultrasound stimulation (LIPUS). In particular, there is a high level of clinical evidence for the effectiveness of LIPUS.18-24 The LIPUS mechanism of action of has been revealed through extensive basic research on physical interactions,25,26 biological responses,27-32 transduction processes,33-36 and integrin
LIPUS enhanced osteogenesis resulting by bone allograft

Several growth factors are closely related to the acceleration of fracture repair by LIPUS. LIPUS also promotes the synthesis of growth factors such as IGF-I and IGF-II and TGF-β1 in osteoprogenitor cells and osteoblasts. These factors play important roles in osteogenesis and bone matrix formation through the production of proteins such as osteocalcin and bone sialoprotein. The findings that LIPUS promotes osteogenesis during fracture repair via regulation of several growth factors led us to suggest the hypothesis that LIPUS would accelerate the osteogenic effect induced by bone grafting.

The aim of this study was to investigate the combined effects of allogenic bone grafting and LIPUS on osteogenesis using a bone graft model in the rat femur. We harvested the long bones from rats, produced bone allografts by defatting and freeze-drying the bones, and implanted these bones on the anterior surface of rat femora. The site of the bone graft was exposed to LIPUS for 20 minutes daily. The difference in new bone formation in the rat femur with or without LIPUS exposure was evaluated by radiographs, three-dimensional micro-computed tomography (3D micro-CT) and histology.

Materials and Methods

Production of freeze-dried bone grafts

The following experimental protocols were approved by the Kitasato University School of Medicine Animal Care Committee. The long bones, 16 femora and 16 tibiae, were harvested from 8 male Wistar rats at 8 weeks of age (Japan Charles River Co., Ltd., Atsugi). Proximal and distal epiphyses were cut from the metaphysis, and the bone marrow cavity was washed with 1× phosphate buffer solution (PBS) to remove bone marrow tissue. The sample was defatted in 1 L of a 50:50 mixture of methanol/chloroform for 24 hours, and the sample was then freeze-dried using an F26 freeze-drying system (Labconco, Kansas City, MO, USA). Using a rotating saw, bone grafts 5 mm wide and 10 mm long were generated from the freeze-dried bones for further processing.

Freeze-dried bone grafting model and experimental design

The bone graft surgery group: The freeze-dried bone graft experiment was conducted in a total of 42 Wistar rats (40 weeks old). Onlay grafts were performed on the bilateral femora. For grafting of the freeze-dried bone, rats were sedated by subcutaneous injection of ketamine (80 mg/kg) and xylazine (16 mg/kg). The hair was shaved, the skin was prepared, and a lateral skin incision was made at the center of the thigh. The femur was exposed and the bone graft was fixed to the periosteum of the anterior surface of the femur by tying it with 3-0 nylon thread. After grafting, the fascia and skin were sutured. Starting 1 day after the operation, grafts on the right femora were treated every day with LIPUS for 20 minutes for the duration of the 42-day experiment. In 6 of the 42 rats, morphological changes that occurred over time were evaluated by radiographs once a week. The remaining 36 rats were killed using deep anesthesia in groups of 6 at days 7, 14, 21, 28, 35, and 42 after surgery. Bilateral femora were harvested subsequent to killing. The harvested femora were fixed in ice-cold 4% paraformaldehyde for 48 hours followed by 1× PBS(-), then used for 3D micro-CT evaluation and histochemical assays.

The sham-operation group: Under the same anesthesia protocol, the same operation was performed without a bone graft in 8 Wistar rats (40 weeks old). The femur was tied with 3-0 nylon thread but without a bone graft. Beginning 1 day after this sham operation, LIPUS was applied to the right femur daily for 7 days. Morphological changes were postoperatively evaluated by radiography on days 0, 3, and 7.

Method of LIPUS exposure

Starting 1 day after the operation, only the right femora of all rats in both groups were treated for 20 minutes per day with LIPUS. During this treatment, rats were sedated by subcutaneous injection of ketamine (80 mg/kg) and xylazine (16 mg/kg). They were kept in a prone position, with a transducer 3.88 cm² in diameter (Teijin Pharma Ltd., Tokyo) placed on the skin at the site of bone grafting, according to Azuma’s method.43

Radiological assay and 3D micro-CT evaluation

The samples were analyzed using an x-ray system (Softex-CMB4; Softex Corp., Kanagawa) with a 10-second exposure at 25 kV and 10 μA and exposed on Industrial X-ray Film (Fuji Photo Film Co., Ltd., Tokyo). A 3D micro-CT equipped with a microfocus x-ray tube (focus size 8×8 mm; MCT-100MF; Hitachi Medical Corp., Chiba) produced a 3D image from 201 image slices. Tube voltage, tube current, and voxel size were 70 kV, 100 μA, and 85.0×85.0×85.0 μm, respectively. A 3D imaging software program (TRI/3D BON; Ratoc System Engineering Co., Ltd., Tokyo) was used to construct 3D images.
Figure 1. X-ray findings in rat in the sham operation group (without bone graft) on days 0, 3, and 7 after surgery. Radiographic changes over time in the same animal are shown. a-c show the low-intensity pulsed ultrasound (LIPUS)-treated femur, and d-f show the femur without LIPUS treatment. a and d, day 0 after surgery; b and e, day 3 after surgery; c and f, day 7 after surgery. Significant new bone formation was not seen up to 7 days after surgery in either the LIPUS-treated or the untreated femurs.

Figure 2. X-ray findings on days 0, 7, 14, and 21 after surgery in the bone graft surgery group. Radiographic changes over time in the same animal are shown. a-d show the LIPUS-treated femur; e-h show the untreated femur. a and e, day 0 after surgery; b and f, day 7 after surgery; c and g, day 14 after surgery; d and h, day 21 after surgery. Black arrows indicate new bone formation. In the LIPUS-treated femur, the size of newly formed bone increased and peaked at 14 days after surgery.
Figure 3. X-ray findings at 28, 35, and 42 days after surgery in the bone graft surgery group. Radiographic changes over time in the same animal are shown. a-c show the LIPUS-treated femur; d-f show the untreated femur. a and d, day 28 after surgery; b and e, day 35 after surgery; c and f, day 42 after surgery. In the LIPUS-treated femur, the size of new bone formation did not change after postoperative day 28. In the untreated femur, the size of newly formed bone gradually increased until postoperative day 42.

Figure 4. Micro-3D CT findings at 28, 35, and 42 days after surgery in the bone allograft group. a-f are micro-3D-CT findings from different rats. a-c are the LIPUS-treated femora, and d-f are the untreated femora. a and d are at day 28 after surgery. b and e are at day 35 after surgery. c and f are at day 42 after surgery. The newly formed bone contains trabecular bone and intertrabecular bone marrow cavities (black arrows).
Figure 5. Histological findings at 3 days after surgery with bone allograft (proliferating cell nuclear antigen (PCNA) immunohistochemical staining) and at 5 days after surgery with bone allograft (Hematoxylin & Eosin [H&E] staining). a and b are the LIPUS-treated femora; c and d are the untreated femora. Each scale bar represents 50 μm. Subperiosteal cell proliferation (detected by PCNA staining) at day 3, and intramembranous ossification (detected by H&E staining) at day 5, are observed in both the LIPUS-treated and untreated femurs.

Figure 6. Histological findings at 21 days after surgery in the bone graft surgery group (H&E staining). a and b are the LIPUS-treated femora; c and d are the untreated femora. b and g are high-magnification fields of a and c. GB, grafted bone; WB, woven bone. Each scale bar represents 100 μm. Black arrows indicate neovascularization. The trabecular structure in the newly formed bone progressed further after LIPUS treatment. Marked neovascularization is seen in the soft tissue around the newly formed bone in B.
Histochemical assay
For the histochemical studies, the fixed bilateral femora were further decalcified in 3M EDTA (ethylenediaminetetraacetic acid) for 3 weeks. Sections (3-μm thick) were cut from paraffin-embedded blocks, and stained with hematoxylin and eosin (H&E) or tartrate-resistant acid phosphatase (TRAP Staining Kit; Primary Cell Co., Ltd., Hokkaido) and hematoxylin staining. Slides were also immunohistochemically stained using a rabbit polyclonal primary antibody against proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the streptavidin-biotin-peroxidase method (Histofine SABPO Kit; Nichirei, Tokyo).

Results
In the absence of a bone graft, no changes were seen in the radiological findings, regardless of whether or not LIPUS was administered (Figure 1). When a freeze-dried bone was grafted to the anterior surface of the femur, radiographs showed a low level of new bone formation in the periostenum under the grafted bone beginning 14 days after the operation (Figure 2c, g). The size of newly formed bone gradually increased over the 42-day period (Figure 3d-f). When LIPUS was applied to the grafted bone, new bone formation was observed from 7 days after the operation (Figure 2b). The size of newly formed bone increased and peaked 14 days postoperatively and remained unchanged at 21 days or later (Figures 2c, d, 3a-c). When LIPUS was applied to the grafted bone, 3D micro-CT at 28 days after surgery revealed that newly formed bone had both trabecular bone and an intertrabecular bone marrow cavity (Figure 4, black arrows). In contrast, in the group without LIPUS exposure, neither trabecular bone nor a bone marrow cavity was observed in the newly formed bone until 42 days after grafting.

Histological examination by H&E staining showed intramembranous ossification at day 5, and PCNA staining confirmed proliferation of subperiosteal cells at day 3 both with and without LIPUS treatment (Figure 5).

Figure 7. Histological findings at 21 days in the bone graft surgery group (tartrate-resistant acid phosphatase [TRAP] staining with toluidine blue stain). a-c show the LIPUS-treated femur; d and e show the untreated femur. b and e show high-magnification images of the left and right rectangles in a. e is a high-magnification image of the rectangle in d. GB, grafted bone; WB, woven bone. Each scale bar represents 100 μm. The cells stained red indicate TRAP-positive osteoclasts. In a, remodeling is already in progress in the newly formed bone, and almost no TRAP-positive cells are seen around the trabecular bone. In b, a large number of TRAP-positive cells are seen around the grafted bone, and resorption of grafted bone is proceeding. (a magnification ×100, b magnification ×400, c magnification ×400, d magnification ×100, e magnification ×400)
The area of newly formed bone was clearly larger in the LIPUS-treated femur than in the untreated femur (Figure 6). In addition, the LIPUS-treated femur had an increased number of capillary blood vessels in the soft tissue under the grafted bone (Figure 6). TRAP staining showed that remodeling of trabecular bone in the newly formed bone was more extensive in the LIPUS-treated femur than in the untreated femur. Marked TRAP-positive cells were seen on the surface of the grafted bone, and progression of resorption of grafted bone was greater in the LIPUS-treated femur than in the untreated femur (Figure 7).

Discussion
Azuma et al.43 showed that LIPUS increased the rate of fracture healing at each stage of the process, with the biggest impact occurring when the treatment was used throughout all stages. They concluded that LIPUS acts on cellular phenomena involved in each phase of the healing process, including inflammation, angiogenesis, chondrogenesis, intramembranous ossification, endochondral ossification, and bone remodeling. There is clinical evidence indicating an acceleration of healing time of 38% in conservatively-treated closed, grade-I open cortical tibial fractures, and cancellous radial fractures.20,21 Without LIPUS treatment, new bone formation was seen from 14 or 21 days after surgery, while in the LIPUS-treated femora, new bone was observed by radiography starting at 7 days. Because no new bone formation after LIPUS treatment was evident in femora without a bone graft, the promotion of bone formation by LIPUS treatment was thought to represent an enhancement of the bone formation that results from bone graft alone (Figure 2).

Localization of newly formed bone under the grafted bone observed in the present study suggested that bone morphogenetic factors released from the bone graft were closely linked to intramembranous ossification. Previously, we reported that LIPUS promotes cell differentiation from mesenchymal cells to immature osteoblasts, and from immature to mature osteoblasts.27,38 We have also demonstrated that LIPUS accelerates periosteal bone formation in organ cultures derived from 2-day-old rat femur.44 These studies suggested that LIPUS promotes the differentiation of mesenchymal stem cells into mature osteoblasts in the cambium layer of the periosteum, resulting in acceleration of the intramembranous ossification induced by bone graft.

Histological evaluation revealed some neovascularization in the soft tissue around the grafted bone in the absence of LIPUS treatment. LIPUS treatment accelerated neovascularization and new bone formation (Figure 6). LIPUS treatment increases the blood flow in the fracture area,10 and accelerates the production of angiogenic factors, such as VEGF and HIF-1 alpha, by osteoblasts.41,42 Also, neovascularization around the matured callus has been observed after LIPUS treatment following distraction osteogenesis.45 The neovascularization observed in the present study supports these earlier reports, and it may be caused by a similar biological mechanism of action.

TRAP staining revealed marked TRAP-positive cells around the grafted bone and demonstrated the progression of resorption of the grafted bone following LIPUS treatment. In newly formed bone exposed to LIPUS, remodeling was already in progress and cancellous marrow cavity formation was seen at 14 days. Azuma et al. reported that in the presence of LIPUS treatment, cellular reactions were accelerated during the remodeling stage of fracture healing.33 Freeman et al. showed that LIPUS affected bone remodeling.34 These papers support our findings. The increased supply of osteoclast precursors due to marked neovascularization may also be related to the acceleration of bone remodeling and bone allograft resorption.

To our knowledge, there are only a few studies that have reported that the osteogenic effect induced by a bone graft is accelerated by LIPUS treatment.46-49 The present study provides important science-based information linked to clinical treatment. LIPUS treatment is well established as an effective conservative treatment in the field of orthopedics. Therefore, it would be immediately possibly to apply LIPUS to the treatment of bone grafts in the clinic. We expect that LIPUS treatment will be widely accepted as a component of fracture healing via bone grafting, in the hope of promoting an osteogenic effect on the bone graft and achieving better final outcomes.

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