Accelerated fracture healing using low-intensity pulsed ultrasound in an aged rat closed femoral fracture model

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Objective: To examine the effect of low-intensity pulsed ultrasound (LIPUS), over a range of exposure frequencies, on the aging-related delay in fracture healing in rats.

Materials and Methods: Closed fracture was created in the right femur in Wistar rats aged 10 or 36 weeks. Rats with fracture were divided into non-LIPUS and LIPUS groups. The LIPUS group of 36-week-old rats was further subdivided into daily-, alternate-day-, and once-every-3-days-LIPUS groups. The rates of hard-callus bridging and total callus area were determined via radiography. Mechanical testing of right femora was performed 3 weeks after the fracture.

Results: Total callus area was significantly smaller in the LIPUS group than in the non-LIPUS group, in the 36-week-old rats but not in the 10-week-old rats. Among the 36-week-old rats, the callus was significantly smaller in the daily-LIPUS group than in the groups with different exposure frequencies. Mechanical tests revealed no differences related to exposure frequency. However, the positive rate of the maximum peak force to failure was 30% in the LIPUS group, versus 0% in the non-LIPUS group.

Conclusions: LIPUS accelerated delayed fracture healing due to aging. Our results suggest that daily exposure is required to obtain a significant effect.

Key words: low-intensity pulsed ultrasound, fracture healing process, aged rat

Introduction

There is an increasing number of fractures among the elderly, reflecting the aging of the Japanese society. These fractures are often characterized by underlying conditions of reduced bone mass, caused by low activity levels as well as estrogen-deficient osteoporosis. For this reason, fixation between osseous tissue and internal fixation materials tends to be unstable in surgical osteosynthesis. Therefore, internal fixation materials are currently being developed that can be efficiently fixed to osseous tissue with low bone mass.1-4 To date, however, no material has achieved sufficient stability. Moreover, in the elderly, fracture healing is delayed due to a lowered level of biological activity in some cases.5,6 Prolonged treatment periods not only contribute to higher healthcare costs, but also expose patients to higher risks of complications including pneumonia, muscular weakness, and joint contracture. Therefore, it is important to devise ways of accelerating healing and shortening the treatment period for the treatment of fractures in the elderly, and thereby avoiding lethal complications by prolonged periods of the patient being bedridden.

Clinically, methods to accelerate fracture healing by mechanical stimulation such as pulsed electromagnetic field stimulation7,8 and low-intensity pulsed ultrasound stimulation (LIPUS),9-11 have been used. Clinical evidence is gaining ground in the published reports about LIPUS.9-11

LIPUS has been reported to shorten fracture healing periods by as much as 40%,10 and accelerate the healing of nonunion and delayed-union fractures.9 We hypothesized that LIPUS accelerates fracture healing by reducing aging-related delays in healing. LIPUS is typically used in the form of home therapy, in which patients self-apply LIPUS once a day for 20 minutes. It can be difficult for elderly patients to remember the daily exposure. No report has yet been made about the direct relationship between exposure frequency and the acceleration of fracture healing.
In the present study, we conducted radiological examination and mechanical testing using the 36-week-old Wistar rat closed femoral fracture model to examine whether or not LIPUS exposure counteracts the prolongation of the fracture healing period due to advanced age and effectively accelerates healing. We also examined the relationship between the exposure frequency and the accelerating effect on fracture healing by comparing a control group without LIPUS and 3 groups receiving LIPUS every day, every other day, and once every 3 days.

Materials and Methods

Study design
The following experimental protocols were approved by the Kitasato University School of Medicine Animal Care Committee. Male Wistar rats (10 weeks old, n = 21; 36 weeks old, n = 36) (Japan Charles River Co., Ltd., Atsugi, Japan) were used in this study. Twenty-one rats aged 10 weeks were divided into 3 groups of 7 rats each: 1. non-LIPUS with fracture, 2. LIPUS with fracture, and 3. non-fracture. Thirty-six rats aged 36 weeks were divided into 5 groups: 1. non-LIPUS with fracture (6 rats), 2. daily LIPUS with fracture (7 rats), 3. alternate-day LIPUS with fracture (8 rats), 4. once-every-3-days LIPUS with fracture (8 rats), and 5. non-fracture (7 rats). All fractures were created in the right femur under anesthetic.

Fracture model
For anesthesia, we injected intramuscularly 0.05 ml/100 g of a 3:1:1 anesthetic of domitor (Nippon Zenyaku Co., Fukushima), mitazolam (Sand Co., Yamagata), and vetorphale (Meiji Seika Co., Tokyo). Anesthetized rats were prepared for surgery exposing the knee via a medial parapatellar incision. The intramedullary canal of the femur was reamed with an 18-gauge needle, and a Kirschner wire (1.2 mm diameter, 32 mm long) was inserted into the intramedullary canal. After closing the knee joint, the middiaphysis of the femur was fractured by applying a bending force. The biomechanical stages of fracture union were determined based on patterns of failure, according to White et al.

Method of LIPUS exposure
Beginning postoperative day 1, rats were treated for 20 minutes per day by LIPUS. During the treatment, the rats were sedated by intramuscular injection of the anesthetic (0.05 ml/100 g). The rats were kept in a prone position with a transducer 3.88 cm² in diameter (Teijin Pharma Ltd., Tokyo) placed on the skin according to Azuma's method.

Radiological assay
All the rats were analyzed once a week using an x-ray system (SOFTEX-CMB4; SOFTEX Corporation, Kanagawa) with 10 sec exposure time at 25 kV and 10 mA, using X-Ray IX industrial film (Fuji Photo Film Co., Ltd., Tokyo). Radiographic signals of each femur and the standard scale (aluminum) were captured as digitized images with a scanner (Epson ES-10000G) and stored on a Windows computer using Adobe PhotoShop CS4 software. The area of the total callus was measured by tracing the callus extent with NIH Image software, version 1.6 (NIH, Bethesda, MD, USA). Any hard-callus in which continuity was observed in 1 or 2 places in the unidirectional x-ray images was classified as hard-callus bridging. The rate of hard-callus bridging was expressed as the percentage of femora with hard-callus bridging among the total number of femora in each group.

Mechanical testing
The femora were collected on day 21 after the fracture. After the soft tissues had been dissected from the femora, the intramedullary pins were removed. Both bone ends were embedded in polyacrylic resins (GC-OSTORON; GC Dental Products Co., Ltd., Aichi). A custom-made jig ensured consistent alignment of the bone axis with the axis of the testing machine. All specimens were tested to failure in torsion at room temperature on an electromechanical testing machine (RV-E2; Mitsubishi Electric, Inc., Tokyo) at a rate of 1.5 °/second with 111.5 g of weight as axial load. Maximal torque until failure and torsional stiffness (the tangent at the point of maximal slope) were calculated from the load-deformation curve. The biomechanical stages of fracture union were determined based on patterns of failure, according to White et al.

Statistical analysis
Paired t-tests were used for the analyses of total callus area and mechanical measurement data. The significance of interexperimental group comparisons of mechanical measurement data was determined by a one-way analysis of variance (ANOVA) test. A confidence level of 95% (P < 0.05) indicated statistical significance.

Results
On x-ray images of femoral fractures in 10-week-old rats taken immediately after the fracture, a line of uncomplicated transverse fracture was found in the center of the diaphysis (Figure 1A). On images taken 1 week after the fracture, membranous ossification was observed
Figure 1. Changes in x-ray images of fractured femora of 10-week-old rats over time
A. Immediately after fracture, B. 1 week after fracture, C. 2 weeks after fracture, D. 3 weeks after fracture. Callus started to appear 1 week after the fracture, and its size increased over time. Hard-callus bridging was observed 3 weeks after the fracture.

Figure 2. X-ray images of fractured femora of 10- and 36-week-old rats 3 weeks after fracture
A, B. X-ray images of fractured femora of 10-week-old rats 3 weeks after fracture; A. Non-LIPUS group; B. LIPUS group; C, D, E, F. X-ray images of fractured femora of 36-week-old rats 3 weeks after fracture; C. non-LIPUS group; D. Daily-LIPUS group; E. Alternate-day-LIPUS group; F. Once-every-3-days-LIPUS group. Hard-callus bridging was observed in both A and B. The callus in C is the largest, and no continuity is found in it. The calluses in D, E, and F are smaller than that in C.
in the area surrounding the fracture site, and hard callus was confirmed near the fracture site (Figure 1B). Two weeks after the fracture, the images showed an increase in the size of hard callus (Figure 1C). Three weeks after the fracture, continuity in hard callus started to appear, indicating that hard-callus bridging had taken place (Figure 1D). The area of hard callus enlarged over time.

On x-ray images, hard-callus bridging was found in femoral fractures of 10-week-old rats 3 weeks after the fracture, whether or not LIPUS was applied (Figures 2A, B, and Table 1). In contrast, no hard-callus bridging was found in any of the 8 fractures in the 36-week-old rats that did not receive LIPUS exposure (Figure 2C, Table 1). Among 36-week-old rats with fracture, the rate of hard-callus bridging was 28.5%, 37.5%, and 25.0% in the daily-LIPUS, alternate-day-LIPUS, and once-every-3-days-LIPUS groups, respectively (Figures 2D, E, F, and Table 1). Among 10-week-old rats, there was no significant difference in the total callus area between the daily-LIPUS group and the non-LIPUS group (Table 1). We also compared the total callus area of the non-LIPUS groups of 10-week-old rats and 36-week-old rats. We found that the area was significantly larger in the non-LIPUS group, the alternate-day-LIPUS group, and the once-every-3-days-LIPUS groups of 36-week-old rats than in the non-LIPUS group of 10-week-old rats. There was no significant difference in total callus area between the non-LIPUS group of 10-week-old rats and the daily-LIPUS group of 36-week-old rats. The total callus area in the non-LIPUS group of 36-week-old rats was significantly larger than those in all the other groups of 36-week-old rats. The total callus area was significantly smaller in the daily LIPUS group of 36-week-old rats than in the other groups.

There was no significant difference in the ultimate strength of 10-week-old rats, as determined by mechanical strength tests conducted on the LIPUS and non-LIPUS groups. In the non-LIPUS group of 36-week-old rats, no peak (indicating the maximum breaking strength) was observed in the mechanical strength test. In the daily-LIPUS group, the alternate-day-LIPUS group, and the once-every-3-days-LIPUS group of 36-week-old rats, the peak for the ultimate strength was found in 2 of 7, 3 of 8, and 2 of 8 rats, respectively (Table 2). The proportion of rats with a peak was consistent with the rate of hard-callus bridging as determined based on the x-ray images.

**Discussion**

Kokubu et al. first reported the effect of LIPUS in cultured osteoblasts. They demonstrated that LIPUS exposure stimulated the production of Cyclooxygenase-2 (COX-2) in osteoblasts, thereby inducing the production of PGE2 (prostaglandin E2). We previously reported that LIPUS exposure increased the production of COX-2 in ST2 cells, the undifferentiated bone marrow stromal cells that can differentiate into osteoblasts. Increased COX-2 level leads to increased production of osteocalcin, a bone matrix protein, as well as bone sialoprotein. LIPUS is also demonstrated to promote osteoblast differentiation and production of many types of bone matrix proteins.

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<th>Table 1. The rate of hard-callus bridging and total callus area of 10- and 36-week-old rats 3 weeks after fracture</th>
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RHCB, rate of hard-callus bridging; TCA, total callus area. TCA data represent the mean values ± SDs. 

*P < 0.05, TCA in the 10-week-old-non-LIPUS group vs. TCA in the 36-week-old non-LIPUS group; 

* *P < 0.05, TCA in the 36-week-old-non-LIPUS group vs. TCA in the 36-week-old-daily-LIPUS group, the alternate-day-LIPUS group, and the once-every-3-days-LIPUS group; 

* P < 0.05, TCA in the 36-week-old-daily-LIPUS group vs. TCA in the alternate-day-LIPUS group, and the once-every-3-days-LIPUS group.
In contrast, several studies have reported that LIPUS does not stimulate cellular proliferation.\textsuperscript{15,17-21} Azuma et al. reported the effect of LIPUS in promoting fracture healing in detail, using the rat closed femoral fracture model.\textsuperscript{12} In the present study, comparison of the callus area at the fracture sites in the LIPUS and non-LIPUS groups of 10-week-old rats revealed that LIPUS exposure did not influence the increase in area, resulting in no significant difference between these groups. Based on the results of the past studies in cultured cells and animals, acceleration of fracture healing by LIPUS has been attributed not to cellular proliferation but to promotion of differentiation.\textsuperscript{12,15,22,23} Our results showing no significant difference in callus area between the LIPUS and non-LIPUS groups in the 10-week-old-rat fracture model were, therefore, consistent with existing reports.

The rate of hard-callus bridging 3 weeks after the fracture was 100\% in the 10-week-old rats with or without LIPUS irradiation. In contrast, the rate was 0\% in the non-LIPUS group of 36-week-old rats, i.e., none of the 8 rats exhibited hard-callus bridging. This suggested that the 36-week-old-rat-closed-femoral-fracture model could be considered as a model of delayed healing.

Many investigators have reported data pertaining to the relationship between the size of fracture callus and stability of the fracture site.\textsuperscript{24,25} Instability at the fracture site in the early stage of fracture is known to increase the size of callus.\textsuperscript{26} Current study also demonstrated that growth of callus continued until the appearance of hard-callus bridging (Figure 1). We speculate that when hard-callus bridging is completed, the fracture site becomes stable, and the callus stops growing in size. In the non-LIPUS group of 36-week-old rats, the callus was significantly larger than in the non-LIPUS group of 10-week-old rats; therefore, we speculate that this is due to the instability of the fracture sites in the older animals. The size of callus in the daily-LIPUS group of 36-week-old rats was not significantly different from that of the daily-LIPUS group of 10-week-old rats. Furthermore, the callus in the daily-LIPUS group of 36-week-old rats was significantly smaller than in the non-LIPUS group of similarly aged rats. This result suggests that the callus in the former group did not grow, basically because the fracture site became stable in the early stage of the healing process. Thus, LIPUS was shown to accelerate the fracture healing that was delayed due to old age.

Among 36-week-old rats, the callus area of the daily-LIPUS group was significantly smaller than in the alternate-day and once-every-3-days-LIPUS groups. We speculate that this is because the greatest acceleration of fracture healing occurred in the daily-LIPUS group. This finding indicates that, although LIPUS accelerates fracture healing to some extent when applied every other day or once every 3 days, daily exposure is most effective. It also suggests that daily exposure is appropriate when LIPUS is used for the treatment of delayed union or nonunion.

In the mechanical strength test of the femora of 36-week-old rats, the peaks indicating maximum force to failure were detected in 0 of 6 rats in the non-LIPUS group, and in 7 of 23 rats in the LIPUS group. Thus, the positive rate of the appearance of the peak force to failure

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<th>Table 2. Ultimate strength of femora of 10- and 36-week-old rats 3 weeks after fracture in torsion strength test</th>
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was as low as 30% among the 23 rats that received LIPUS exposure. However, the positive rate was largely consistent with the rate of hard-callus bridging, determined based on the x-ray images, which indicates that completion of hard-callus bridging resulted in a positive peak force to failure. The callus area on x-ray images clearly reflected the effect of LIPUS exposure. However, in the mechanical testing, it was not clear whether or not LIPUS resulted in acceleration of fracture healing. This is probably because fracture healing was delayed to a greater extent than expected in older rats, and the mechanical strength of the fracture site was not sufficiently recovered 3 weeks after the fracture.

The present study demonstrated that LIPUS accelerated fracture healing that was delayed due to aging. It also suggested that daily exposure is necessary to obtain a significant effect of LIPUS in acceleration of fracture healing. Because patients apply LIPUS at home by themselves, the frequency of exposure depends on their compliance. Therefore, the necessity of daily irradiation should be thoroughly explained to patients.

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