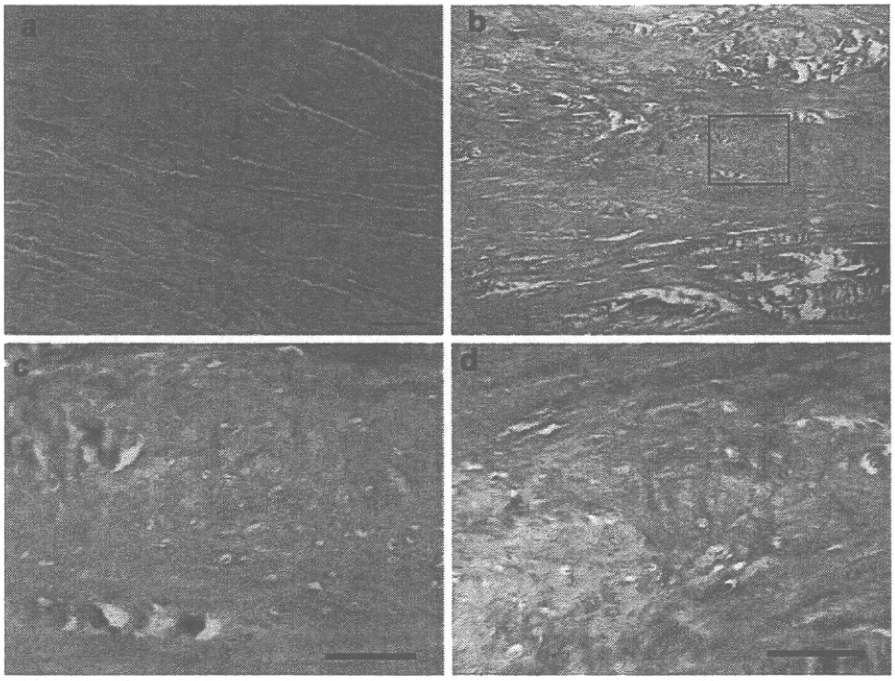

Fig. 4 Histology of migrated chondrocytes within the dense tissue. **a** H.E. stained section of solvent preserved meniscus with recombinant human BMP-2 (rhBMP-2). Migrated chondrocytes and newly-formed extracellular matrix were observed in H.E stained sections. High (×400) magnification is shown below right. **b** H.E. stained section of solvent preserved meniscus without rhBMP-2 as a control. **c** Safranin O stained section of solvent preserved meniscus with rhBMP-2. Migrated chondrocytes and newly-formed extracellular matrix were observed in Safranin O stained sections. High (×400) magnification is shown below right. **d** Safranin O stained section of solvent preserved meniscus without rhBMP-2 as a control

as scaffolds onto which patients' cells could be seeded, or as carriers for transplantation of genetically engineered cells. However, it is difficult to seed cells into high density regular connective tissue which has few interstitial spaces.

We turned our attention to cancer cells to solve the difficulty of cell induction into these connective tissues. Cancer cell migration is critical in the invasive growth of cancer into the connective tissues (Albini et al. 1987). Various growth factors and cytokines are involved in cellular infiltration into the narrow interstitial spaces in a relatively short time (Zigmond and Hirsch 1972). The purpose of this study was to investigate the potential of chondrocytes to infiltrate into dense meniscus tissue in relation to the chemotactic/chemokinetic effect of BMP-2.

BMP-2 has been reported to induce migration of bone marrow derived undifferentiated mesenchymal stem cells, osteoblasts, fibroblasts and cells derived from menisci. Although chondrocyte differentiation by BMP-2 has been well studied (Erickson et al. 1997), the migration response of chondrocytes to BMP-2 has not been investigated. Our results indicate that rhBMP-2 has a chemokinetic effect on chondrocytes. The optimal concentration, 10 ng/ml, for chondrocyte migration is one fifth of that required for induction of cellular differentiation (Erickson et al. 1997). Within the tissue, the local concentration of BMP-2 is predicted to gradually decrease in relation to the distance from the site where the cytokine is synthesized. Thus cell migration may be induced at sites distant from BMP-2 synthesizing cells, while cell differentiation may be induced at sites close to these cells.

We have shown that chondrocytes migrate deeply into the preserved meniscal tissues if induced by BMP-2. Characteristics of the scaffold such as pore size, shape, interconnectivity, and material stiffness or surface chemistry and degradability influence tissue regeneration. Of note, the scaffold pore size is highly related to the proliferation and differentiation of chondrocytes in culture (Bhardwaj et al. 2001; Malda

et al. 2005; Nehrer et al. 1997; Yamane et al. 2007). In our study, we show that BMP-2 induces chondrocyte migration and proteoglycan production throughout the meniscus tissue in vitro. By combining appropriate scaffolds with application of a low concentration of BMP-2, effective application of chemokinesis to cartilage tissue engineering is expected even with seeding of a small number of recipient cells.

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Quality of life, knee function, and physical activity in Japanese elderly women with early-stage knee osteoarthritis

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ABSTRACT

Purpose. To compare quality of life, knee function, and physical activity in 33 elderly women with or without early-stage knee osteoarthritis (OA).

Methods. 33 Japanese elderly women (mean age, 66 years) with (n=18) or without (n=15) early-stage knee OA symptoms (knee pain and decreased range of motion [ROM]) were recruited. The height, weight, and body mass index, disease severity, quality of life (according to the Japanese Knee Osteoarthritis Measure [JKOM]), knee function (knee extension strength, ROM, 10-m gait time), and the amount of physical activity (net energy expenditure and step count) of the 2 groups were compared.

Results. The 2 patient groups did not differ significantly with respect to mean patient age, height, and body mass index, except for weight. Regarding knee function, mean knee extension strength, ROM (extension but not flexion), and 10-m gait speed (comfortable and maximum) were significantly inferior in patients with knee OA than in controls. Regarding the mean amount of physical activity undertaken, patients with knee OA did not differ significantly from controls with respect to net energy expenditure (179 vs. 212 Kcal/day) and step count (8016 vs. 9729 steps/day). Net energy expenditure (r= –0.65, p=0.04) and step count (r= –0.62, p=0.02) correlated negatively with JKOM scores in patients with knee OA but not in the controls.

Conclusion. In Japanese elderly women with knee OA, quality of life (JKOM scores) correlated negatively with physical activity (net energy expenditure and step count). The 2 groups undertook similar amounts of physical activity, although those with knee OA exhibited less knee extension strength. Decreased knee extension strength coupled with high levels of physical activity may exacerbate the development of knee OA.

Key words: *activities of daily living; motor activity; muscle strength; osteoarthritis, knee; quality of life; range of motion, articular*

INTRODUCTION

In Japan, about 7 million people have knee

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osteoarthritis (OA), and the proportion continues to increase in the ageing population.[1] The prevalence of knee OA increases rapidly in people aged ≥40 years.[2] In elderly women with knee OA, their activities of daily living (ADL) are markedly hindered.[3] Mild exercise is recommended for prevention of lifestyle-related disease. The main symptoms of knee OA are knee pain and diminished knee function (range of motion and muscle strength), which may affect the ability to undertake physical activity.

The amount of physical activity in ADL can be measured quantitatively in terms of net energy expenditure (using an accelerometer), heart rate or step count.[4] All of which have been used in patients with diabetes mellitus and cardiac disease.[5,6] We therefore compared knee function, physical activity, and quality of life in 33 Japanese elderly women with and without early-stage knee OA.

MATERIALS AND METHODS

Between April 2006 and March 2007, 33 Japanese elderly women (mean age, 66 years) with (n=18) or without (n=15) early-stage knee OA symptoms (knee pain and decreased range of motion [ROM]) were recruited. The ethics committee of our university approved the study, and written informed consent of each subject was obtained. The height, weight, and body mass index of individuals in the 2 groups were compared using the Wilcoxon rank-sum test. In those with knee OA, disease severity was evaluated using the Yokohama City University Classification system (Table 1).[7]

Quality of life was measured using the Japanese Knee Osteoarthritis Measure (JKOM),[8] as the ADL of Japanese people often involves deep flexion of the knee, which is related to the symptoms of knee OA. Higher scores indicate better quality of life. The JKOM has been modified based on the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC),[9] which evaluates disease-specific quality of life in patients with hip or knee osteoarthritis. The JKOM is a self-administered questionnaire and consists of 25 items (each scores one to 5 points) including (1) the pain level during walking, standing or climbing stairs, (2) physical functions related to ADL, and (3) social functions including social participation. It has been validated for evaluating patients with knee OA using the Short Form-36 and WOMAC.

Knee function was measured in terms of the knee extension strength, ROM, 10-m gait speed (maximum and comfortable), and the degree of knee pain (using a visual analogue scale). Knee extension strength was measured using a handheld dynamometer, with the participant in a sitting position with 90° flexion of the knee and hip joints under isometric contraction. The 10-m gait speed and step count were measured using a 13-m walkway (composed of a 3-m approach way and a 10-m straight line).

The amount of physical activity (net energy expenditure and step count) was measured using an accelerometer[10,11] attached to the waist for 9 days (except during sleeping). Participants were instructed not to perform any unusual activities. The mean amount of physical activity over 5 days (excluding 2 days during weekends and the 2 days on which the accelerometer was attached and removed) was recalculated as net energy expenditure and a step count was derived.

The side with more severe knee OA (9 left and 9 right) was compared with the right knees of control subjects. The amount of physical activity between groups was compared using the analysis of covariance (ANCOVA) adjusted for body weight. Correlations between physical activity and the JKOM score were evaluated using a 2-sided Pearson's correlation coefficient. A p value of <0.05 was considered statistically significant.

Table 1
Grading of osteoarthritic knees according to the Yokohama City University Classification[7]

Grade	Feature on anteroposterior standing radiographs
0	Normal
1	Bone sclerosis or osteophyte formation
2	Narrowing of joint space (≤3 mm)
3	Obliteration of joint space or subluxation*
4	Defect of tibial plateau (<5 mm)
5	Defect of tibial plateau (≥5 mm)

* Subluxation indicates that the medial edge of the medial tibial plateau shows a lateral shift by >5 mm against the medial edge of the articular surface of the medial femoral condyle without including osteophyte

RESULTS

The 2 patient groups did not differ significantly with respect to mean patient age, height, and body mass index, except for weight (Table 2). In patients with early-stage knee OA, mean disease severity was grade 2. Regarding knee function, mean knee extension strength ($p=0.027$), extension ROM ($p<0.01$), and 10-m gait speed (comfortable [$p=0.004$] and maximum [$p<0.01$]) were significantly inferior in patients with knee OA than in controls (ANCOVA, Table 2). Regarding the mean amount of physical

Table 2
Comparison between patients with knee osteoarthritis and controls*

Characteristics	Patients with knee osteoarthritis (n=18)	Controls (n=15)	p Value
Age (years)	67±8	66±4	0.73
Height (m)	1.52±0.061	1.48±0.044	0.072
Weight (kg)	54±8	49±7	0.045
Body mass index (kg/m²)	23±3	22±3	0.29
Knee pain (visual analogue scale) [mm]	29±24	-	-
Knee function			
Knee extension strength (kg/weight x100)	32%±16%	48%±14%	0.027
Extension range of motion	-5°±3°	3°±2°	<0.01
Flexion range of motion	148°±10°	154°±4°	0.064
Comfortable gait speed (m/sec)	1.4±0.3	1.6±0.2	0.004
Maximum gait speed (m/sec)	1.7±0.3	2.1±0.3	<0.01
Physical activity			
Net energy expenditure (Kcal/day)	179±89	212±65	0.26
Step count (step/day)	8016±3283	9729±3586	0.20

* Data are presented as mean±standard deviation

activity undertaken, patients with knee OA did not differ significantly from the controls with respect to net energy expenditure (179 vs. 212 Kcal/day, p=0.26, ANCOVA) and step count (8016 vs. 9729 steps/day, p=0.20, ANCOVA). Net energy expenditure (r= –0.65, p=0.04) and step count (r= –0.62, p=0.02) correlated negatively with JKOM scores in patients with knee OA but not in the controls (r= –0.31, p=0.26 and r= –0.39, p=0.15, respectively) [Fig.].

DISCUSSION

In patients with early-stage knee OA, most have slight contracture of extension ROM but not flexion ROM. Flexion contracture is thus considered a feature of knee OA. Decrease in the knee extension strength precedes deterioration of other parameters of physical function, even in patients with no knee pain or muscle atrophy.[12] In Japan, the gait speed required to safely cross a road is 1.0 m/s or faster[13]; our patients with knee OA walked faster than that.

In a health and nutrition survey conducted in Japan in 2002,[14] elderly women (aged 60 to 70 years) walk approximately 7313 steps per day. Our patients with knee OA and controls walked more than that, probably because the accelerometer was more sensitive than a pedometer. Excessive physical activity in patients with early-stage knee OA may exacerbate the development of knee OA. The knee extension strength for maintaining gait ability among elderly people was 35% (kgf/kg [body weight] x100) or more.[13] Our patients with knee OA had mean knee extension strength of 32%, but their physical activity was similar to that of controls.

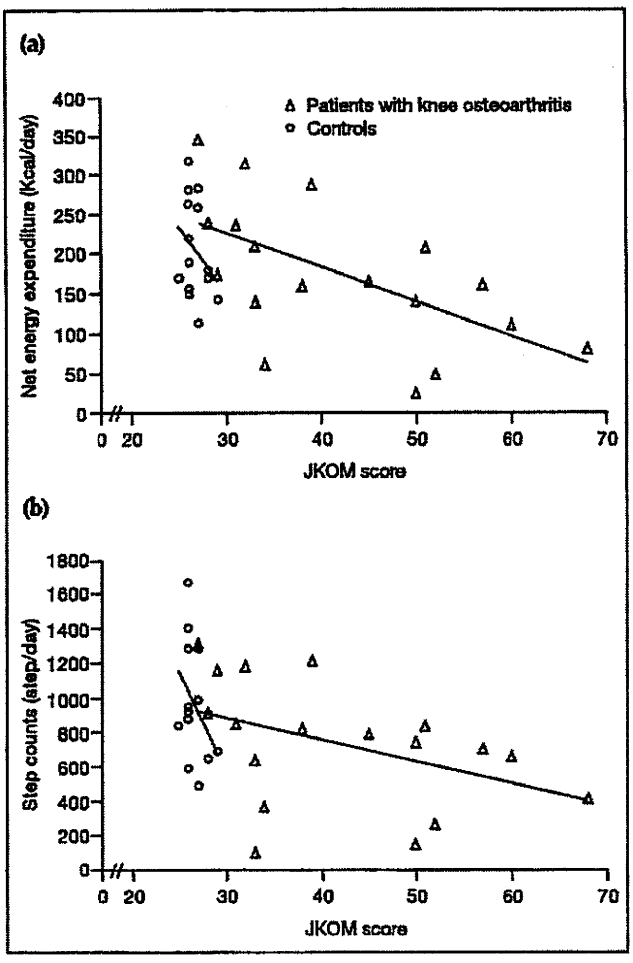

Figure (a) Net energy expenditure (r= –0.65, p=0.04) and (b) step counts (r= –0.62, p=0.02) are negatively correlated to Japanese Knee Osteoarthritis Measure (JKOM) scores in patients with knee osteoarthritis, but not in controls (r= –0.31, p=0.26 and r= –0.39, p=0.15, respectively).

If physical activity is limited by disease, physical function and ADL may also be limited. Physical activity (net energy expenditure and step count) correlated negatively with quality of life (JKOM scores) in patients with knee OA, but not in controls. As JKOM is a disease-specific quality-of-life index, the correlation coefficient for participants without knee OA was low. Therefore, when evaluating quality of life between a healthy and disease group, the non–disease-specific health-related quality-of-life index should be used.

Advancement of knee OA from early to late stage decreases physical function, ADL, the mental condition, and therefore quality of life. Understanding physical activity that elderly people with knee OA undertake may clarify the pathology of knee function including quality of life.

CONCLUSION

In Japanese elderly women with knee OA, quality of life (JKOM scores) correlated negatively with physical activity (net energy expenditure and step count). They maintained a similar amount of physical activity despite having inferior knee extension strength. Decreased knee extension strength coupled with high levels of physical activity may exacerbate the development of knee OA.

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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 in to 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 stimulation[7,8] 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.

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In the present study, we conducted radiological examination and mechanical testing using the 36-week-old Wistar rat closed femoral fracture model[12,13] 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 parapateller 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.[12,13]

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 cm^2 in diameter (Teijin Pharma Ltd., Tokyo) placed on the skin according to Azuma's method.[12]

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.[14]

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

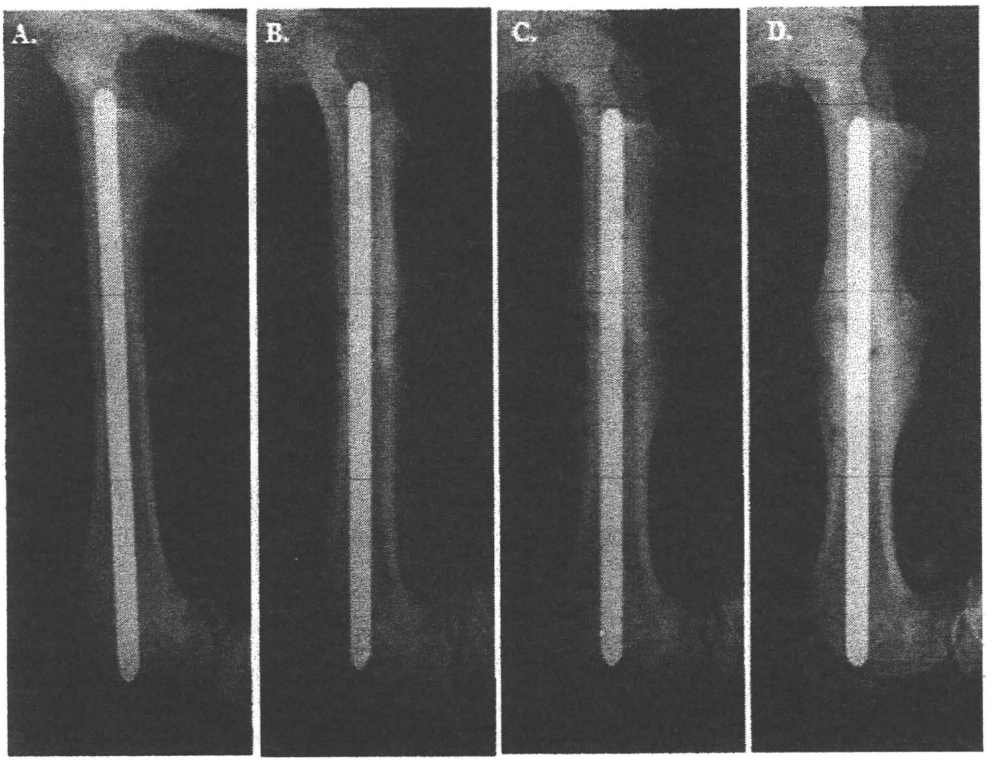

Figure 1. Changes in x-ray images of fractured femora of 10-week-old rats over time

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.

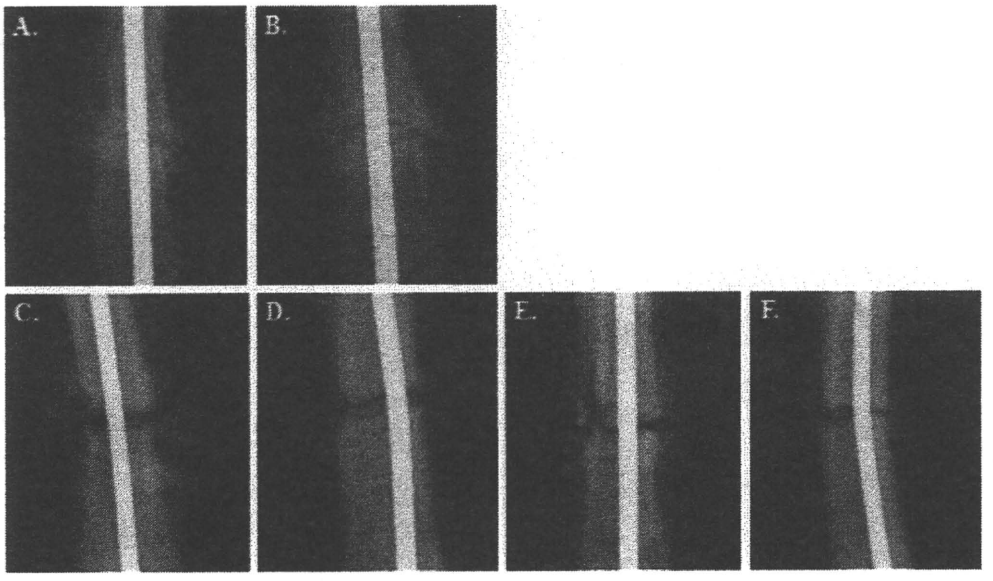

Figure 2. X-ray images of fractured femora of 10- and 36-week-old rats 3 weeks after fracture

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.[15] They demonstrated that LIPUS exposure stimulated the production of Cyclooxygenase-2 (COX-2) in osteoblasts, thereby inducing the production of PGE_2 (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.[16] 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.[17]

Table 1. The rate of hard-callus bridging and total callus area of 10- and 36-week-old rats 3 weeks after fracture

	10-week-old rats		36-week-old rats			
	Fracture		Fracture			
	LIPUS (-)	LIPUS (+)	LIPUS (-)	LIPUS (+) Daily	LIPUS (+) Alternate days	LIPUS (+) Once every 3 days
RHCB	100.0	100.0	0	28.5	37.5	25.0
TCA	31.6 ± 6.3[a]	28.3 ± 8.0	60.9 ± 13.2[b]	26.5 ± 9.6	39.4 ± 12.7[c]	40.0 ± 14.8[c]

RHCB, rate of hard-callus bridging; TCA, total callus area. TCA data represent the mean values ± SDs.

[a]$P < 0.05$, TCA in the 10-week-old-non-LIPUS group vs. TCA in the 36-week-old non-LIPUS group; [b]$P < 0.05$, 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; [c]$P < 0.05$, 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.

Table 2. Ultimate strength of femora of 10- and 36-week-old rats 3 weeks after fracture in torsion strength test

	10-week-old rats			36-week-old rats				
	Fracture		Non-fracture	Fracture				Non-fracture
	LIPUS (-)	LIPUS (+)		LIPUS (-)	LIPUS (+) Daily	LIPUS (+) Alternate days	LIPUS (+) Once every 3 days	
	323.1	344.21	306.72	-	182.9	165.1	111.8	532.8
	286.6	272.16	315.21	-	90.3	182.4	158.5	587.5
Maximam	207.0	454.11	340.32	-	-	144.9	-	696.4
torque	378.9	279.89	308.35	-	-	-	-	808.3
(N/mm)	340.5	307.92	296.16	-	-	-	-	745.9
	260.3	189.51	459.96	-	-	-	-	667.6
	220.2	191.28	390.72		-	-	-	868.3
						-	-	
Ave ± SD	288.1 ± 63.4	291.3 ± 91.6	345.0 ± 59.5					701.0 ± 118.2

In contrast, several studies have reported that LIPUS does not stimulate cellular proliferation.[15,17-21] Azuma et al. reported the effect of LIPUS in promoting fracture healing in detail, using the rat closed femoral fracture model.[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.[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.[24,25] Instability at the fracture site in the early stage of fracture is known to increase the size of callus.[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

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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