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The effect of low-intensity pulsed ultrasound on bone healing in SR-PLLA rod fixed experimental distal femur osteotomy in rat

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Abstract The effects of low-intensity pulsed ultrasound (30 mW/cm^2) were investigated in experimental cancellous bone fracture healing in bioabsorbable self-reinforced poly-L-lactide (SR-PLLA) rod fixed distal femur osteotomy in rats. A transverse transcondylar osteotomy was fixed with one SR-PLLA rod in 32 male Wistar rats of the age of 20 weeks. Half of the rats had a daily 20-min ultrasound exposure for three weeks. The follow-up times were three, six, and 12 weeks. Radiographical, histological, microradiographical, oxytetracycline labeling, and histomorphometrical analyses were performed. No foreign-body reactions were noted. The biocompatibility of SR-PLLA and ultrasound was found to be good. In the radiological and histological assessments there was a slight tendency for enhanced healing in the ultrasound group at three weeks, but at six and 12 weeks no differences were observed. The histomorphometrical and oxytetracycline labeling analyses showed that ultrasound exposure had no significant effects on bone healing.

The present study shows that there were no obvious findings to support the hypothesis that low-intensity pulsed ultrasound enhances bone healing in self-reinforced poly-L-lactide (SR-PLLA) rod fixed experimental metaphyseal distal femur osteotomy in rats. The observed good biocompatibility provides a safe starting-point for clinical trials on bioabsorbable fixation combined with low-intensity ultrasound.

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Introduction

Bioabsorbable implants, such as self-reinforced poly-L-lactide (SR-PLLA) rods used in the present study, are made of synthetic polymers and their biodegradation occurs by hydrolysis and non-specific enzymatic activity. The rate of degradation is strongly influenced by the molecular weight [1] as well as by the physical and chemical structure of the implant [2]. Bioabsorbable implants have been used in surgery since 1984 [3]. Thereafter many clinical studies have proved bioabsorbable fixation devices suitable for fixation of different kinds of fractures, osteotomies, and arthodeses [4, 5]. The benefit of using a bioabsorbable implant in fracture fixation is that it initially maintains the stability of fracture fixation during the time of healing and after that resorbs abolishing the need for implant removal.

Ultrasound is a form of mechanical energy that can be transmitted into the body as high-frequency acoustical pressure waves. Ultrasound is used in medicine both operatively and therapeutically (intensities ranging from 0.2 to 100 W/cm^2) as well as for diagnostic imaging (0.05 to 0.5 W/cm^2). Lower intensities, such as 30 mW/cm^2 used in the present study, do not increase the temperature on the exposed tissue. There are

reports on both experimental [6–8] and clinical [9–11] studies indicating that low-intensity (30 mW/cm^2) pulsed ultrasound may have beneficial effects on healing bone after a fracture.

It has been reported that ultrasound with an intensity of 1.5–2 W/cm^2 enhances the overall degradation kinetics of 50–50% copolymer of polylactic and polyglycolic acids in vitro [12]; the effect was seen both on molecular weight loss and on mass loss. Also, ultrasound with an intensity of 5 W/cm^2 has been reported to have up to a five-fold increase in the degradation rate of polylactic and polyglycolic acids [13]. However, low-intensity (30 mW/cm^2) pulsed ultrasound exposure of even 12 weeks has not been seen to have any effect on the mechanical or molecular properties and degradation behavior of SR-PLLA screws in vitro [14]. This indicates that low-intensity pulsed ultrasound exposure can be performed without a risk of an accelerated loss of mechanical strength of self-reinforced poly-L-lactide fixation devices.

In the present study we investigated the effects of low-intensity pulsed ultrasound (30 mW/cm^2) on cancellous bone fracture healing in self-reinforced poly-L-lactide (SR-PLLA) rod fixed distal femur osteotomy in rats. The hypothesis was that low-intensity ultrasound enhances bone healing.

Materials and methods

Animals, implants, and operative methods

A total of 32 male Wistar rats with a mean weight of 407 g (range 326–497 g) were operated on at the age of 20 weeks. The rats were randomly divided into two groups prior to the operation, 16 rats to the ultrasound (US) group and 16 to the control group. The US- and control groups were further divided into three follow-up subgroups, five rats for the three- and six-week subgroups, and six rats for the 12-week subgroup. The rats were anesthetized with subcutaneous injections of ketamine (Ketalar, Parke-Davis, Spain) 75 mg/kg and medetomidine (Domitor, Orion-Pharmos, Finland) 0.5 mg/kg. A single subcutaneous dose of 100.000 IU procaine penicillin (Procapen, Orion, Finland) was administered preoperatively for infection prophylaxis. The right hind leg was shaved and scrubbed with an antiseptic fluid (Neo-Amisept, Orion-Pharmos, Finland), and a medial parapatellar incision with a lateral dislocation of the patella was performed. The femoral medullary canal was opened through the intercondylar region and drilled up to the depth of 20 mm with a 2.0 mm pneumatic drill, and a transverse transcondylar osteotomy was created with an oscillating saw. The osteotomy was reduced exactly and fixed with one 15 mm (diameter 2 mm) SR-PLLA rod (Bionx Ltd, Tampere, Finland). The incision was closed in layers with 3-0 polyglycolide sutures (Dexon, Davis and Geck, United Kingdom). Postoperative radiographs were taken in antero-posterior and lateral views. After the recovery from anesthesia, the rats were returned to their gages, two rats in each. They were fed ad libitum with a regular laboratory animal diet and water. No external support of the operated limb was used.

Ultrasound exposure and three week daily anesthesia

The ultrasound exposure was performed with a Sonic Accelerated Fracture Healing System (SAFH) 2 device (Exogen Inc., USA). The treatment head module of the SAFH 2 device delivered an ultrasound pressure wave signal that was composed of a burst width of 200 μs containing 1.5 MHz sine waves, with a repetition rate of 1 kHz. The intensity of the pressure wave applied was 30 mW/cm^2. The daily 20-min ultrasound exposure was started on the second postoperative day for a period of 21 days (US-group). For the exposure, the rats were anesthetized with subcutaneous injections of medetomidine (Domitor, Orion-Pharmos, Finland) 0.15 mg/kg and immobilized in a ventral position with the right hind leg abducted and externally rotated. The treatment head module applied with coupling gel was fastened over the medial aspect of the distal femur and knee. The control group rats were anesthetized and immobilized similarly to the ultrasound group rats for 20 min from the second postoperative day on for a period of 21 days without an ultrasound exposure. This was done to avoid any bias caused by the effect of immobilization or nutritional intake on bone healing during the daily anesthesia and recovery period.

Preparing of specimens and radiographical analysis

The rats were killed with an overdose of sodium pentobarbital (Mebunat, Orion, Finland) 60 mg/kg at three weeks (five ultrasound and five control rats), six weeks (five ultrasound and five control rats), and 12 weeks (six ultrasound and five control rats) postoperatively. A dose of 50 mg/kg of hydrochloride oxytetracycline (Terramycin, Pfizer, Belgium) was injected intramuscularly three days before killing for oxytetracycline labeling studies. Both femora were dissected free, and radiographs were taken. The osteotomy was determined as united if 3/4 or 4/4 cortices were united

in the antero-posterior and lateral radiographic views. Any signs of infection or other complication were visually observed. The specimens were fixed in a series of ethanol immersions with increasing concentrations (70–99%) and embedded in methylmethacrylate.

Histological, microradiographical, and oxytetracycline labeling analysis

For histological and histomorphometrical evaluation, 5 μm thick longitudinal sections were cut with a Polycut S microtome (Reichert-Jung, Germany). The sections were stained using the Masson-Goldner trichrome method [15]. For microradiographic and oxytetracycline labeling studies, 80 μm thick longitudinal sections were cut with a Leitz Saw Microtome 1600 (Leitz, Germany). The microradiographs were made using the Faxitron Xray system Model 43855A (Hewlett Packard, USA) and high resolution ultraflat plates type 1 A (Imtec Products, USA). The histologic, oxytetracycline fluorescence, and microradiographic specimens were studied with a Diaplan microscope (Leitz, Germany). The fluorescence microscopic analysis was performed using an HBO 220 ultraviolet lamp (Osram, Germany) and a BG 812/6 primary filter (Leitz, Germany).

Histomorphometrical analysis

For the semiautomatic quantitative histomorphometrical analysis, a Leitz microscope was linked via a video camera (Color View II, Soft Imaging System GmbH, Münster, Germany) to a computer (Dell Precision 340, Ireland). Magnifications of 13.0× and 78.8× were used. The image analyzing software was AnalySIS docu 3.2 (Soft-Imaging Software GmbH, Münster, Germany). Both femora were analyzed in each rat, the left femur acting as a control. Four standardized sample fields were determined in each femur around the lateral implant channel 6.0 mm from the distal joint level of the lateral condyle and 1.5 mm apart in the horizontal direction (Fig. 1). The AnalySIS-program was used in the determination of the corresponding sample field. Within the 1.46 mm × 1.08 mm (1.57 mm^2) sample fields, the histomorphometrical variables were analyzed. The variables were as follows: total tissue area, total area of trabecular bone, total length of the trabecular bone circumference, total length of the osteoid, and total length of the osteoblast lines. All assessments of the sections were done by one investigator to avoid any bias caused by interobserver variation. The hypothesis was that the measured variables in the US-group were higher than those in the control group. The differences of means between the treatment groups, at given times, were compared using the two-tailed independent samples *t*-test.

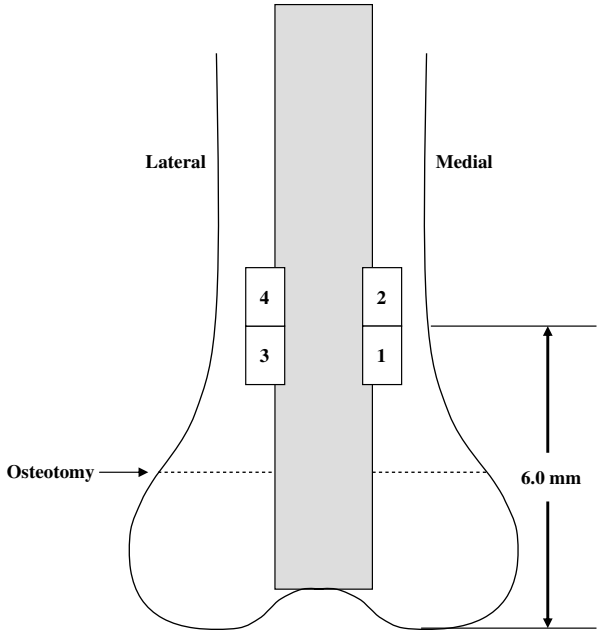

Fig. 1 Schematic anterior view of the distal rat femur showing positioning of the osteotomy, the implant, and the four standardized sample fields (1, 2, 3, 4) used in the histomorphometric analysis

Results

Clinical and macroscopical results

One 12-week control group rat had to be killed on the second postoperative day due to a primary operation-related iatrogenic femoral diaphyseal fracture. The rest of the rats tolerated the three-week daily anesthesia, resulting in 31 rats with a complete follow-up. However, the three-week daily anesthesia was observed to be a major exertion and stress for the rats. It was seen in the alteration of the body weights during the follow-up; during the first 21 days the rats lost 14% of their initial weight (mean weight loss 54 g, range 29–75 g) but after that started to gain weight again. Compared to the initial body weights (mean 407 g, range 326–497 g), at six weeks the mean body weight increased by 5% (mean 449 g, range 361–517 g) and at 12 weeks by 13% (mean 458, range 393–523 g). The alteration in the body weights was symmetrical in the ultrasound and control groups.

One three-week control group rat and one 12-week ultrasound group rat were observed to have a displaced

and non-united osteotomy due a technical failure at the primary operation resulting in an intercondylar fracture. No signs of wound healing problems, such as superficial infections or fistulas, were observed. Mild synovitis with osteoarthrotic lesions of the femur condyle was seen in four rats (two in the six-week ultrasound group, and one in the six-week and one in the 12-week control group).

Radiological, histological, microradiographical, and oxytetracycline fluorescence results

At three weeks, radiologically all the five osteotomies seemed united in the ultrasound group compared to two out of four osteotomies in the control group. No difference was observed in the visualization of the osteotomy line between the groups. Histologically at three weeks intense bony callus formation and solid bony union through the osteotomy line were seen in two out of five osteotomies in the US-group compared to none out of four and one out of four osteotomies in the control group, respectively. Microradiographically there was periosteal callus formation in all cases, but solid bony unions through the osteotomy line were observed in the same cases as seen in the histological analyses. There was strong oxytetracycline uptake periosteally and endosteally in both groups. The beginning of the bony rim around the implant was also seen in all cases.

After six weeks, radiologically all the osteotomies seemed united and the fracture lines were almost faded out both in the ultrasound and control group (Fig. 2). An external radiological callus formation was noted in three cases in both groups. Histologically at six weeks a solid bony union through the osteotomy line was seen in four out of five osteotomies in both groups (Fig. 3).

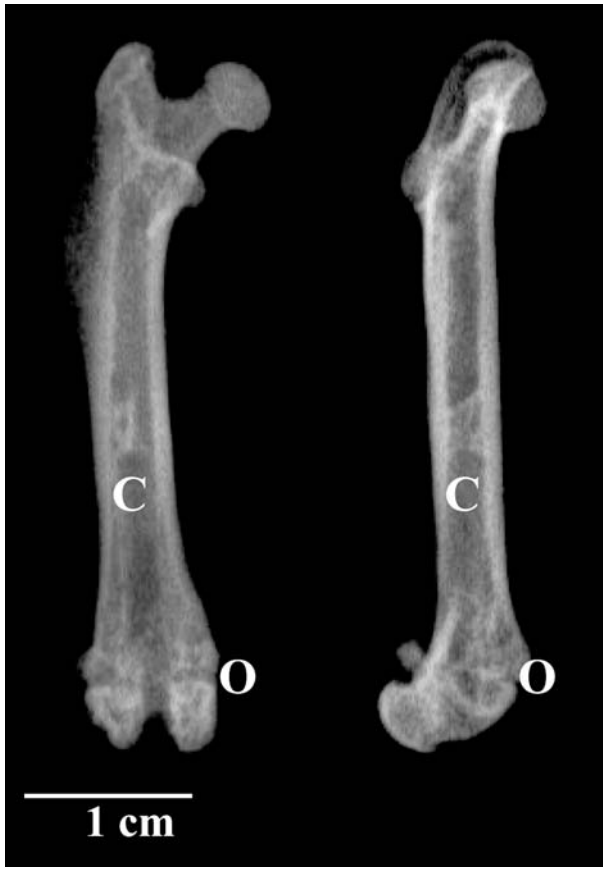

Fig. 2 Antero-posterior and lateral radiographs of the rat femur without 21-day ultrasound exposure (control group) at six weeks. Implant channel (white letter C) is seen clearly. The osteotomy line (white letter O) has faded and is hardly seen. Radiologically all the osteotomies seemed united both in the ultrasound and control group at six weeks

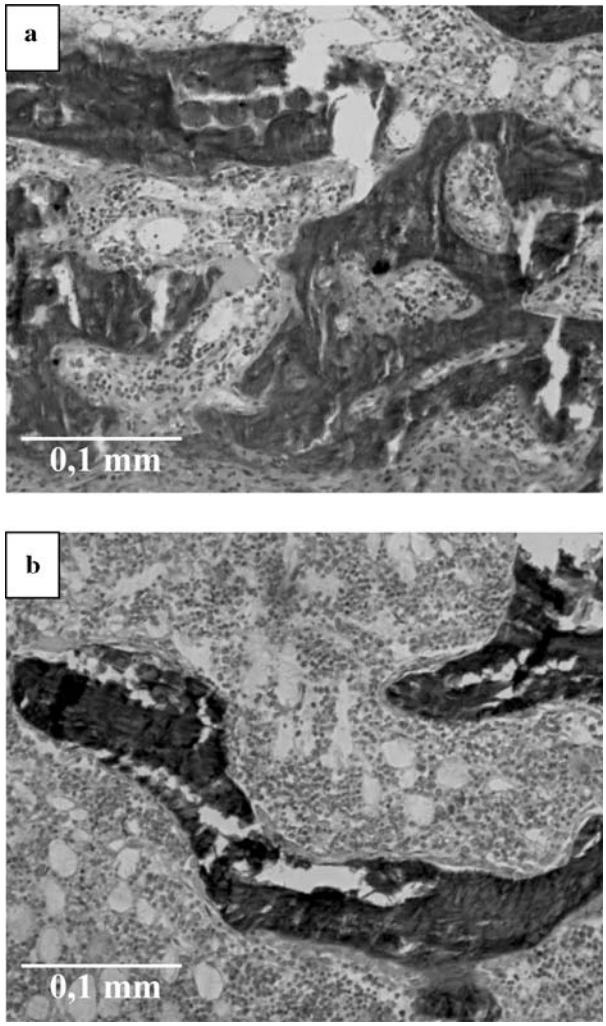

Fig. 3 (**a**) Photomicrograph of the osteotomized site after 21-day ultrasound exposure at six weeks. The osteotomy is consolidated. The bone structure is similar to the control group (no ultrasound exposure) (**b**). (Original magnification× 125)

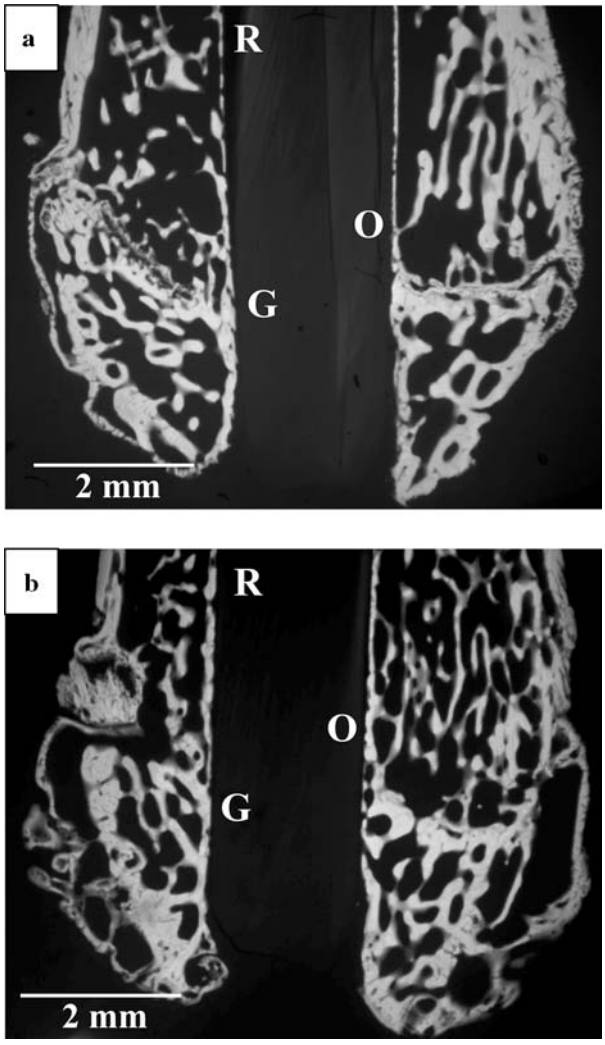

Fig. 4 (**a**) Microradiograph of the osteotomized site after 21-day ultrasound exposure at six weeks. The osteotomy (white letter O) is consolidated. Remnant of the growth cartilage (white letter G) is seen. The bony rim around the implant channel is seen clearly (white letter R). The bone structure and consolidation is similar to the control group (no ultrasound exposure) (**b**). (Original magnification× 20.6)

Intense bony callus formation was observed in four cases and in one case of the five osteotomies in the US- and control groups, respectively. Microradiographically there was periosteal callus formation in all cases, and one osteotomy in both groups was not completely united (Fig. 4). The periosteal oxytetracycline uptake was quite similar to that of the three-week uptake and it was even among the US- and control group. A clearly visible bony rim around the implant was observed in all specimens.

At 12 weeks, all the osteotomies seemed radiologically united both in the ultrasound and control groups, excluding one osteotomy with the above-mentioned iatrogenic and, later on, dislocated intercondylar femoral fracture in the US-group. An external radiological callus formation was seen in four US-group specimens, but, surprisingly, in none of the control group. Histologically a solid bony union through the osteotomy line was seen in three and four out of five osteotomies in the US- and control groups, respectively. New external bony callus formation was evenly smaller, but the bony rim around the implant was clearly seen in both groups. Microradiographically the new bone was mineralized as normal bone. Two osteotomies in the US-group and one in the control group were observed only partially united. The oxytetracycline uptake around the osteotomy line was evenly smaller in both groups compared to six weeks.

Histomorphometrical results

The histomorphometrical results are presented in Table I. In the US-group the mean total trabecular bone area fraction over the total tissue area was slightly decreased during the follow-up period. However, there was no statistical difference between the US-group and the control group at three, six or 12 weeks. The mean trabecular bone circumference

Table I Results of the histomorphometric analysis of the tissue-implant interface in the SR-PLLA rod fixed distal femoral osteotomy in rats after 21-day ultrasound exposure (US-group) or without it (Control group) at three, six, and 12 weeks (mean and SD)

Follow-up (weeks)	Total trabecular bone area fraction over the total tissue area (%)		Trabecular bone circumference fraction over the total tissue area (%)		Total osteoid length over the total tissue area (%)		Total osteoblast line length over the total tissue area (%)	
	US-group	Control group	US-group	Control group	US-group	Control group	US-group	Control group
3	0.27 (0.19)	0.24 (0.10)	4.18 (1.90)	4.98 (2.13)	0.52 (0.52)*	0.98 (0.58)	0.04 (0.08)*	0.07 (0.10)
6	0.20 (0.14)	0.23 (0.07)	4.23 (2.22)	5.07 (1.20)	0.67 (0.90)	0.58 (0.41)	0.07 (0.17)	0.02 (0.04)
12	0.21 (0.14)	0.23 (0.13)	3.87 (1.46)	3.54 (0.98)	0.50 (0.75)	0.80 (0.70)	0.03 (0.06)	0.00 (0.00)

t-test with two-tailed interpretation

* ($P < 0.05$)

fraction over the total tissue area rose slightly up to six weeks and then started to diminish towards 12 weeks. The alteration was symmetrical in both study groups, and no statistical differences were observed. The total osteoid length fraction over the total tissue area was statistically higher in the control group at three weeks (difference of means 0.463, 95% confidence interval 0.870–0.056). At six weeks it was higher in the US-group and at 12 weeks in the control group, but the differences were not statistically significant. Also the total osteoblast line length over the total tissue area was statistically higher in the control group at three weeks (difference of means 0.662, 95% confidence interval 0.884–0.439). At six and 12 weeks it was higher in the US-group, but the difference was of no statistical significance.

Discussion

There are no previous experimental studies on ultrasound exposure combined with bioabsorbable fixation material in vivo. The purpose of the present study was to investigate the effects of low-intensity pulsed ultrasound (30 mW/cm^2) on bone healing in self-reinforced poly-L-lactide (SR-PLLA) rod fixed distal femur osteotomy in rats. The rats had to be anesthetized for the ultrasound exposure, as otherwise it would have been impossible to hold them still for 20 min. Generally it took about from two to three hours for the rats to recover from the anesthesia after the daily ultrasound exposure. During the recovery period the rats did not eat much and moved only a little. Therefore also the control-group rats were anaesthetized every day during the three postoperative weeks, since daily immobilization period and cessation in the nutritional intake caused by the anesthesia during the recovery phase would probably have had an effect on the normal bone healing process. The observed changes in the mean body weights were symmetrical in the ultrasound and control groups; therefore it is assumed that the groups were comparable regarding mobilization and nutritional intake.

Previous experimental animal studies have shown that the biocompatibility of the bioabsorbable self-reinforced poly-L-lactide (SR-PLLA) is acceptable for internal fixation [16–18]. In the present study there were three operational failures related to an inadequate operational technique. The biocompatibility of SR-PLLA and ultrasound was found to be good. No foreign-body reactions were noted. Four rats had mild synovitis with osteoarthrotic lesions of the femur condyle, which were more probably related to the arthrotomy and operation itself than to poor biocompatibility.

In the radiological and histological assessments there was a slight tendency for enhanced healing in the ultrasound group at three weeks. After six weeks no differences were observed, since the distal femoral osteotomy is generally united by that time. Mobilization and weight bearing of the operated limb cause torque and micro-movement between the osteotomy lines until consolidation occurs, which may increase callus formation. However, ultrasound had no clear effect on the callus formation in the consolidation phase during the first three weeks. After six weeks, there was a tendency for increased callus formation in the ultrasound group, which is controversial to the assumption that ultrasound would cause the osteotomy to unite faster and, thus, cease the micro-movement in a shorter period. However, we did not find a correlation between the amount of callus formation and the rate of osteotomy consolidation.

Oxytetracycline labeling results in deposition of a fluorochrome label on bone surfaces undergoing active mineralization at the time of the labeling. In the present study, the oxytetracycline fluorescence was observed to be strong at three and six weeks, indicating that there was an intense ongoing mineralization. At 12 weeks, the rate of active mineralization was lower. However, the amount of the oxytetracycline uptake was observed to be even in the ultrasound and control groups during the follow-up, which suggests that ultrasound exposure had no obvious effect on the rate of mineralization around the healing osteotomy line or the adjacent periosteum. The histomorphometrical results showed that at 12 weeks the active osteoid formation was slow both in the US- and control group, indicating that the bone healing had occurred within normal time. However, the active osteoid formation was found to be faster in the control group at three weeks. Later on no difference was seen between the groups, indicating that ultrasound had no effect on the rate of new bone formation.

The results of the present study show that there were no obvious findings to support the hypothesis that low-intensity pulsed ultrasound enhances bone healing in self-reinforced poly-L-lactide (SR-PLLA) rod fixed metaphyseal distal femur osteotomy in rats. On the other hand, the observed good biocompatibility provides a safe basis for clinical trials on biodegradable fixation combined with low-intensity ultrasound.

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