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Low-intensity pulsed ultrasound increases bone volume, osteoid thickness and mineral apposition rate in the area of fracture healing in patients with a delayed union of the osteotomized fibula ☆

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ABSTRACT

Introduction: Low-intensity pulsed ultrasound (LIPUS) accelerates impaired fracture healing, but the exact mechanism is unknown. The aim of this study was to investigate how LIPUS affects bone healing at the tissue level in patients with a delayed union of the osteotomized fibula, by using histology and histomorphometric analysis to determine bone formation and bone resorption parameters.
Materials and methods: Biopsies were obtained from 13 patients (9 female, 4 male; age 42–63) with a delayed union of the osteotomized fibula after a high tibial osteotomy, treated for 2–4 months with or without LIPUS in a randomized prospective double-blind placebo-controlled trial. In the histological sections of the delayed union biopsies, 3 areas of interest were distinguished, i.e. 1) area of new bone formation at the fracture ends, 2) area of cancellous bone, and 3) area of cortical bone. Histomorphometrical analysis was performed to determine bone formation and bone resorption parameters (as well as angiogenesis).
Results: In LIPUS-treated delayed unions, endosteal callus formation by direct bone formation without a cartilage intermediate as well as indirect bone formation was observed, while in untreated controls only indirect bone formation was observed. In the area of new bone formation, LIPUS significantly increased osteoid thickness by 47%, mineral apposition rate by 27%, and bone volume by 33%. No increase in the number of blood vessels was seen in the newly formed bony callus. In the area of cancellous bone, bone volume was significantly increased by 17% whereas no effect on osteoid thickness and mineral apposition rate was seen. LIPUS did not affect osteoid volume, osteoid maturation time, number of osteocytes, osteocyte lacunae, or osteoclast-like cells in any of the areas of interest.
Conclusions: Our results suggest that LIPUS accelerates clinical fracture healing of delayed unions of the fibula by increasing osteoid thickness, mineral apposition rate, and bone volume, indicating increased osteoblast activity, at the front of new bony callus formation. Improved stability and/or increased blood flow, but probably not increased angiogenesis, might explain the differences in ossification modes between LIPUS-treated delayed unions and untreated controls.

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Introduction

Fracture healing is a complex process requiring the recruitment of the appropriate cells and expression of the appropriate genes at the right time in the right place [1]. The majority of clinical fractures heal spontaneously, only 5–10% of the fractures show impaired healing [1]. Impairment of fracture healing leads to a delay in union or may even result in nonunion. The goal of therapy for fracture healing in general is to provide sufficient fracture stability by conservative or surgical means. Unstable fracture results in delayed consolidation, i.e. as occurs in the osteotomized non-fixated fibula [2]. If fracture healing is impaired, it can be initiated or enhanced by surgical and/or non-surgical means [1].

Fracture healing is modulated in response to external stimuli, such as growth factors, hormones, and mechanical forces [1,3]. Insight in the molecular biological mechanisms involved in fracture healing has resulted in the development of new treatment modalities for impaired fracture healing, such as bone morphogenic proteins, extracorporeal shock wave treatment, electro-stimulation, and low-intensity pulsed ultrasound (LIPUS) [4–8]. LIPUS and electro-stimulation have the advantage over surgery that they are non-invasive, do not cause any side affects, and can be used in an outpatient setting making them less expensive [9,10].

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LIPUS (30 mW/cm^2) is a form of mechanical energy transmitted transcutaneously by high-frequency acoustic pressure waves [11]. The intensity of LIPUS (30 mW/cm^2) is within the range of ultrasound intensities used for diagnostic purposes (1–50 mW/cm^2) and is regarded as non-thermal and non-destructive [9,12]. Bone cells are sensitive to strains caused by physical loading [13,14]. Mechanoreceptors convert biophysical stimuli into biochemical responses that alter gene expression and cellular adaptation [15]. The micro-mechanical stress produced by LIPUS may provide a surrogate for the forces normally applied on bone by physical loading according to Wolffs' law [16,17]. Although the strain induced by LIPUS at the tissue level is several orders of magnitude lower than the peak strains generated by functional load bearing [13], high-frequency low-magnitude strains can result in strong regulatory signals to bone tissue [18–20].

LIPUS increases prostaglandin E_2 production via the induction of cyclooxygenase-2 in MC3T3-E1 osteoblastic cells *in vitro* [21]. LIPUS treatment of mouse bone-marrow-derived ST2 cells upregulates osteocalcin and IGF-I mRNA levels in a biphasic manner, suggesting an anabolic effect of LIPUS on osteoblast activity [22]. *In vivo* animal studies on the effects of LIPUS on fracture healing have shown enhanced mechanical properties of the healing callus, [11,17,23] as well as more extensive bone bridging at the fracture site [11]. LIPUS may affect different processes involved in fracture healing as shown by increased vascularity around the fracture site in the osteotomized ulna fracture model in dogs [24]. Randomized clinical trials showed acceleration of clinical fracture healing by LIPUS in fresh fractures and osteotomies [2,25,26]. LIPUS also restores disrupted fracture repair in nonunion cases [5,6]. The exact mechanism by which LIPUS affects clinical bone healing is however still unknown.

The positive effect of LIPUS on fracture healing may be caused by a stimulation of the different cellular processes involved in fracture repair and bone formation, such as angiogenesis, chondrogenesis, and intramembranous and endochondral ossification [11]. Therefore, the aim of the present study was to investigate how LIPUS affects bone healing at the tissue level in patients with a delayed union of the osteotomized fibula, by determining bone formation and bone resorption parameters using histology and histomorphometric analysis.

Materials and methods

Patient selection

Biopsies of delayed unions of the human osteotomized fibula after a high tibial osteotomy were obtained from 9 female and 4 male patients (age 42–63 years) treated with or without LIPUS (7 LIPUS, 6 controls) in a randomized prospective double-blind placebo-controlled clinical trial (Table 1). The procedure of a closed high tibial osteotomy for medial located osteoarthritis of the knee included a diaphyseal non-fixated oblique osteotomy of the fibula. The osteotomized fibula has a tendency of impaired healing resulting in a delay in union [2]. Patients treated by high tibial osteotomy were evaluated 6 months post-surgery with regard to healing status of the fibula. The patients were invited to participate in this study if radiological healing of the fibula was not yet accomplished within 6 months. After informed consent was obtained, patients were submitted to either treatment by LIPUS or sham-treatment by a placebo device. Mean fracture age at inclusion was 192 days (range 180–214, median 185 days) for the sham-treated controls, and 222 days (range 180–331, median 190 days) for the LIPUS-treated patients. Trial approval was obtained from the Medical Ethical Review Board of the Vrije Universiteit Medical Center, registration number 2004-005.

Table 1
Patient and fracture characteristics at trial inclusion, and duration of LIPUS treatment of delayed unions of the osteotomized fibula at the time of the biopsy procedure

Treatment modality	Sex	Age	Smoking	Fracture age (days)	Delayed union type	Treatment time (days)
LIPUS	M	63	Yes	274	Hypertrophic	75
"	F	43	No	187	Hypertrophic	61
"	M	63	Yes	331	Hypertrophic	115
"	F	46	No	185	Atrophic	84
"	F	52	No	209	Hypertrophic	90
"	M	42	Yes	190	Hypertrophic	94
"	F	57	No	180	Atrophic	90
Control	M	61	No	187	Atrophic	87
"	F	48	No	183	Atrophic	72
"	F	54	No	180	Hypertrophic	83
"	F	53	No	183	Hypertrophic	88
"	F	57	No	202	Hypertrophic	81
"	F	44	No	214	Hypertrophic	89

LIPUS treatment

The patients enrolled in the clinical trial used the EXOGEN 2000+® low-intensity pulsed ultrasound device (Smith & Nephew Inc, Memphis, TN, USA) at home for a daily 20-minute treatment. The LIPUS device produced a 200 μs burst of 1.5 MHz acoustic sine waves, that repeated at a modulation frequency of 1 kHz, and provided a peak pressure of 30 mW/cm^2. The LIPUS device registered the duration of a single daily 20-minute treatment and the total number of treatments. Computerized randomization of active and placebo devices was performed, and devices were distributed among patients in the order of inclusion of the patients in the clinical trial. Unblinding of the trial was performed after completion of the histomorphometric and histologic analysis, and after all patients included in the trial completed their 5 month clinical treatment phase.

Biopsy procedure

Two to 4 months after the start of LIPUS treatment, a biopsy was taken from the delayed union of the fibula. Patients received LIPUS treatment for 87 days (range 61–115, median 90 days) or sham-treatment for 83 days (range 72–89, median 85 days). To determine the mineral apposition rate (MAR), patients received 200 mg tetracycline 4 times per day for two consecutive days, at 3 weeks and 1 week in advance of the biopsy procedure [27]. The standardized biopsy procedure (Fig. 1) was performed under general or spinal anaesthesia, with the use of a hollow trephine burr (ITI-Straumann, Basel, Switzerland) (Fig. 1). The hollow trephine burr (2.5 mm inner diameter, 3.5 mm outer diameter) was placed in a drilling device, and drilling was performed in a straight angle to the longitudinal axis of the fibula. The direction of drilling with the trephine burr was from the lateral towards the medial cortex of the fibula. The diameter of all harvested cylindrical biopsies was 2.5 mm, while the biopsy length ranged from 5 to 15 mm.

Histology

All biopsies of the delayed unions of the fibula were immediately fixed in 4% formaldehyde solution in 0.1 M phosphate buffer, pH 7.3, at 4 °C for 24 h. The biopsies were then rinsed 3 times with 0.1 M phosphate buffer and stored in 70% ethanol at 4 °C, until ready to be embedded. All biopsies were embedded in methylmethacrylate without decalcification [27]. Longitudinal sections of 5 μm thickness were made using a heavy duty Jung K microtome (R. Jung, Heidelberg, Germany). Three sets of 4 consecutive sections were collected, with a distance of 305 μm between the last section of one set and the first section of the following set.

One section of each set of consecutive sections was stained with Goldner's trichrome method in order to distinct mineralized (green) and non-mineralized (red) bone tissue. Another section was stained with toluidine blue (0.1% in H_2O) to determine the presence of chondrogenic tissue [28]. A third section was stained for tartrate resistant acid phosphate (TRAP) to detect any osteoclast-like cells. Finally, one section was used for fluorescence microscopy to visualize tetracycline labeling to determine the mineral apposition rate (MAR).

Histologic and histomorphometric analysis

All analyses were performed using a Leica DMRA microscope connected to a computer using an electronic stage table, and a Leica DC 200 digital camera [27]. All measurements were done using the Leica QWin© computer program (Leica Microsystems Image Solutions, Rijswijk, The Netherlands). For every biopsy three Goldner trichrome-stained sections were analyzed, with a 305 μm interval between each section. Within each section, 3 areas of interest were distinguished with regard to the bone architecture and histological appearance (Fig. 2). The fracture end was used as a distinct landmark and guideline to indicate the beginning of the area of new bone formation. The areas of interest were: 1) area of new bone formation, representing the newly formed bony callus within 0.6 mm distance from the fracture ends, 2) area of cancellous bone, and 3) area of cortical bone. Using these 3 areas of interest we were able to account for the differences in bone density and metabolic capacity between cortical, cancellous and newly formed bony callus at the fracture ends. In eight cases cortical bone tissue was lost during the biopsy procedure, and therefore the histological sections of these biopsies did not represent the area of cortical bone. In the area of cancellous and cortical bone, 2–4 measurement fields were selected for measurement of histomorphometric parameters. The number of fields was depended on the length of the biopsy. The measurement fields were evenly distributed along the longitudinal plane of the sections. In the area of new bone formation two measurement fields were selected, which were evenly distributed along the fracture plane. Nomenclature, symbols, and units used are as recommended by the Nomenclature Committee of the American Society for Bone and Mineral Research [29]. Bone volume (BV) was calculated

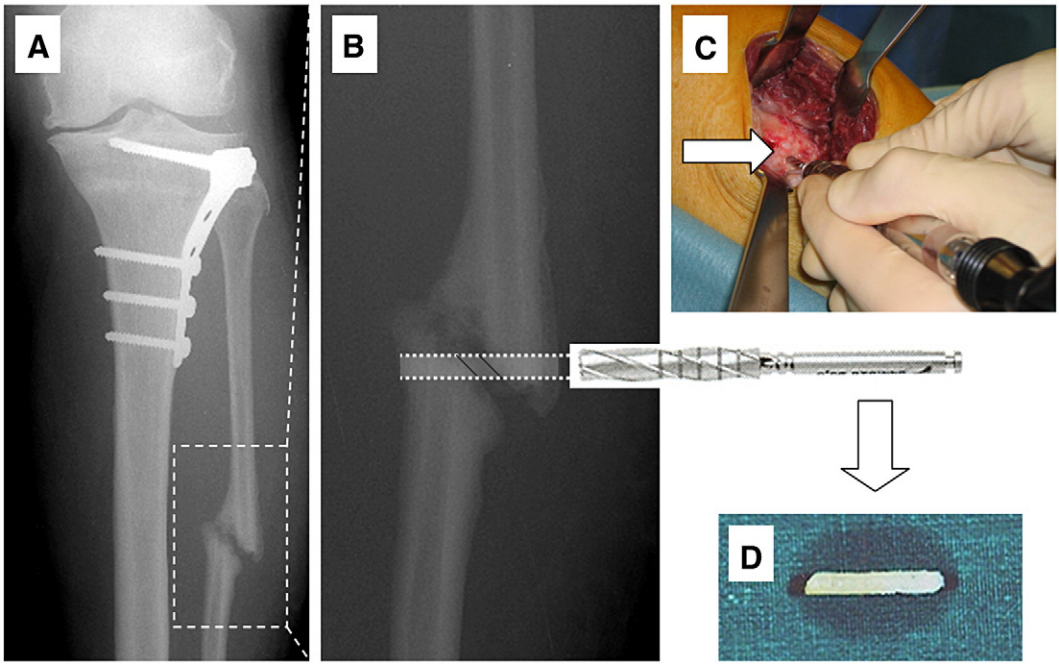

Fig. 1. Biopsy procedure in a delayed union of a human osteotomized fibula after a high tibial osteotomy procedure. (A) Anterior to posterior X-ray of the left tibia and fibula of a 52 year old female patient with a hypertrophic delayed union of the fibula. The delayed union is characterised by a discontinuity of the cortices, as shown by the radiolucent area (rectangle). (B) Magnification of the hypertrophic delayed union area of the fibula (rectangle in Fig. 1A). The direction of drilling with the hollow trephine burr (2.5 mm inner diameter, 3.5 mm outer diameter) from the lateral towards the medial cortex of the fibula is indicated by the dotted lines. (C) Three Homan retractors were placed around the anterior and posterior cortex to expose the fibula delayed union site. The arrow indicates the location of the fibula delayed union. The hollow trephine burr was placed in a drilling device, and drilling was performed in a straight angle to the longitudinal axis of the fibula. (D) Cylindrical biopsy of a delayed union of a human fibula, 2.5 mm core diameter after retrieval from the hollow trephine burr.

as the amount of mineralized bone tissue (Mineralized volume, Md.V) plus the amount of osteoid tissue (Osteoid volume, OV) as a percentage of the total tissue volume (BV/TV × 100%). The absolute osteoid volume was calculated as the amount of osteoid tissue as a percentage of the total tissue volume (OV/TV × 100%). The relative osteoid volume was calculated as percentage of the total bone volume (OV/BV × 100%). For every measurement field, osteoid thickness (O.Th) was calculated by averaging the osteoid thickness values (10 to 30 measurements obtained at fixed intervals along the osteoid surface). Within the area of new bone formation, osteoid thickness refers to mean thickness of the seam of osteoid located at or closest to the fracture end. The number of osteocytes (N.Ot) as well as the number of osteocyte lacunae (N.lac) were expressed per area of mineralized bone tissue (mm^2). The number of TRAP-positive cells was also expressed per total tissue area (mm^2).

The rate of bone formation was expressed as the mineral apposition rate (MAR). The MAR is the average distance between the corresponding edges of two consecutive fluorescent bone labels divided by the number of days between start of first administration period of tetracycline (3 weeks before taking the biopsy), and second administration period of tetracycline (1 week before taking the biopsy) [27]. The distance of consecutive fluorescent tetracycline labels was measured in unstained sections using an excitation wavelength of 354–425 nm and emission wavelength of 470 nm. In the area of new bone formation, distance measurements of double tetracycline labels were performed at or closest to the fracture ends. The MAR was determined in 12 patients enrolled in the study. In one LIPUS-treated patient the MAR could not be determined, since the patient did not receive tetracycline.

To assess the effect of LIPUS on angiogenesis, a quantitative analysis was performed to assess the number of blood vessels in the area of new bone formation. Immediately adjacent to the fracture end and within the fracture gap, we could not identify mature blood vessels. The determination of the number of blood vessels was therefore restricted to the soft connective tissue within the newly formed fracture callus. The number of blood vessels (N.Bv) was expressed per area of soft connective tissue (mm^2) [30].

Statistical analysis

Statistical analysis of the data was performed using a Student's independent *t*-test (two-tail). The values of the histomorphometric parameters are expressed as mean ± SEM. A p-value of < 0.05 is considered significant.

Results

Area of new bone formation

All biopsies showed vital fracture ends with low numbers of osteocyte lacunae, as well as bony callus formation by osteoblasts which deposit osteoid at the fracture ends (Fig. 3). The area of new

Fig. 2. Histological section of a biopsy of a delayed union of a human fibula showing the 3 areas of interest for histologic and histomorphometric analysis. The 3 areas of interest are: 1) Area of new bone formation, 2) Area of cancellous bone, and 3) Area of cortical bone. (1) The area of new bone formation represents the newly formed bony callus over a width of 600 μm to the fibrous and/or cartilaginous tissue adjacent to the fracture ends. New trabeculae (arrows) are formed by endochondral ossification replacing the cartilage matrix adjacent to the fracture end. (2) The area of cancellous bone consists of lamellar bone and more recently formed woven bone trabeculae divided by large areas of soft connective tissue. (3) Haversian canals surrounded by lamellar bone (osteons) form the structural elements of cortical bone, in the area of cortical bone. Goldner's trichrome-stained section; osteoid and soft tissue, red; mineralized bone, green; cell nuclei, black. Original magnification, ×50; Scale bar, 500 μm.

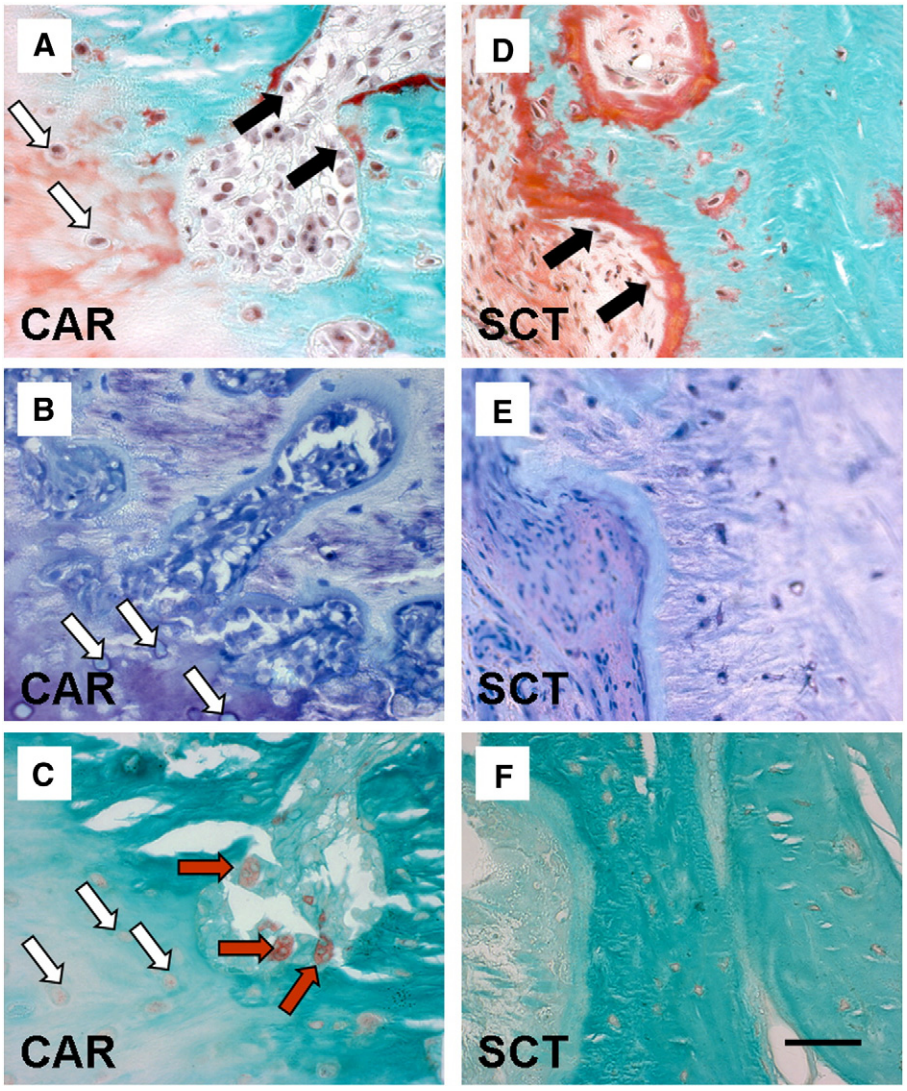

Fig. 3. Histological sections of delayed unions of the human fibula treated with or without LIPUS showing differences in type of bone formation in the area of new bone formation. (A, B, C) Consecutive sections of an untreated control biopsy showing endochondral ossification. (A) Newly formed woven bone replaces the cartilage matrix (CAR) at the fracture end. Osteoblasts deposit osteoid at the woven bone surface (black arrows). (B) The cartilage matrix adjacent the fracture end contains high numbers of hypertrophic chondrocytes (white arrows). (C) TRAP-positive osteoclast-like cells (red arrows) resorb the mineralized cartilage matrix. (D, E, F) Consecutive sections of a LIPUS-treated delayed union showing direct (intramembranous) bone formation. (D) Osteoblasts (black arrows) located in the soft connective tissue (SCT), deposit osteoid at the woven bone surface. (E) Cartilage tissue with hypertrophic chondrocytes is absent adjacent the fracture end. (F) TRAP-positive osteoclast-like cells are absent. A, D, Goldner's trichrome stained sections; osteoid and soft tissue, red; mineralized tissue, green. B, E, Toluidine blue-stained sections; cartilage, dark-purple; bone, blue. C, F, TRAP-stained sections; TRAP-positive cells, red; bone, soft tissue, green. Original magnification, ×400. Scale bar, 50 μm.

bone formation showed endosteal callus formation in all 13 biopsies (6 control, 7 LIPUS). In all controls, cartilage was present adjacent to the fracture ends (Figs. 3A–C). Four controls showed endochondral ossification, in which the (mineralized) cartilage matrix containing hypertrophic chondrocytes is resorbed by TRAP-positive cells (Fig. 3C), followed by the deposition of osteoid by osteoblasts at the surface of the eroded cartilage. Two controls showed complete mineralization of the cartilage matrix, which contained chondrocyte-like cells and smaller osteocyte-like cells. No TRAP-positive cells were present in the histological sections of these 2 controls (data not shown). Cartilage was present in 4 biopsies of LIPUS-treated delayed unions. These biopsies showed endochondral ossification as well as direct (intramembranous) bone formation (data not shown). The other 3 biopsies of LIPUS-treated delayed unions showed only direct bone formation (Figs. 3D–F). One delayed union case treated with LIPUS showed consolidation of the fracture ends on the radiographs taken 1 week in advance of the biopsy procedure. The histological sections of this clinically healed delayed union showed bridging trabeculae at the fracture ends, alternated by areas of new bone formation to obtain complete bridging (data not shown).

In the area of new bone formation, LIPUS treatment resulted in a 47% increase ($p < 0.05$) in osteoid thickness (Table 2). A discrepancy was seen in osteoid thickness of a clinically healed LIPUS-treated delayed union (10.1 μm), and clinically non-healed LIPUS-treated delayed unions (mean 18.1 μm; range 15.1–22.0 μm). In the newly formed bony callus, total bone volume and mineralized volume were increased by respectively 33% and 34% ($p<0.05$), whereas the absolute osteoid volume was slightly (26%) but not significantly increased as a result of LIPUS treatment. The relative osteoid volume remained unchanged in the area of new bone formation (Table 2). LIPUS did not affect the number of osteocytes nor the number of empty osteocyte lacunae per area of mineralized bone tissue (Table 2). Four of the 6 controls showed the presence of TRAP-positive cells. In 3 controls TRAP-positive cells were present in all 3 evaluated sections while in one control TRAP-positive cells were present in only one section. The 3 controls showing TRAP-positive cells in all evaluated sections showed

Table 2
Histomorphometric data of delayed unions of the osteotomized fibula treated with or without LIPUS

	Area of new bone formation			Area of cancellous bone			Area of cortical bone		
	Control	LIPUS		Control	LIPUS		Control	LIPUS	
	(n=6)	(n=7)	p-value	(n=6)	(n=7)	p-value	(n=2)	(n=3)	p-value
Age (mean±SD)	52±6	52±9		52±6	52±9		52±12	52±11	
Male/female	1M/5F	3M/4F		1M/5F	3M/4F		1M/1F	2M/1F	
BV/TV (%)	39.8±3.1	52.9±3.5	0.02*	58.3±1.5	70.0±2.1	<0.01*	88.1±2.6	78.0±4.3	0.16
Md.V/TV (%)	34.5±2.9	46.2±3.9	0.04*	54.3±2.0	64.8±2.3	<0.01*	88.6±2.6	77.6±4.5	0.17
OV/TV (%)	5.3±0.4	6.7±1.7	0.46	3.9±0.8	3.1±0.8	0.46	0.5±0.2	0.4±0.2	0.88
OV/BV (%)	13.7±1.2	13.3±3.2	0.91	6.9±1.5	4.6±1.1	0.24	0.6±0.3	0.6±0.3	0.91
O.Th (μm)	11.5±1.9	16.9±1.4	0.04*	13.5±1.9	13.1±1.5	0.88	7.3±0.9	8.3±0.8 (n=2)	0.48
MAR (μm/day)	1.8±0.1 (n=6)	2.3±0.2 (n=6)	0.04*	1.6±0.1 (n=6)	1.6±0.1 (n=6)	0.91	0.9±0.1 (n=2)	1.1±0.0 (n=1)	0.55
N.Oc/T.Ar (/mm^2)	9.6±4.8	0.5±0.3	0.12	2.0±1.2	0.3±0.3	0.15			
N.Ot/Md.Ar (/0.01 mm^2)	4.1±0.4	3.9±0.2	0.54	3.4±0.3	3.3±0.3	0.82	1.2±0.1	1.7±0.4	0.29
N.Lac/Md.Ar (/0.01 mm^2)	0.1±0.1	0.2±0.1	0.33	0.2±0.1	0.1±0.0	0.56	0.6±0.1	0.3±0.2	0.37

Histomorphometric data is presented for the 3 areas of interest: 1) area of new bone formation, representing the newly formed bony callus within 0.6 mm distance from the fracture ends, 2) area of cancellous bone, and 3) area of cortical bone. BV/TV, bone volume of total tissue volume (%); Md.V/TV, mineralized volume (%); OV/TV, absolute osteoid volume (%); OV/BV, relative osteoid volume (%); O.Th, osteoid thickness (μm); MAR, mineral apposition rate (μm/day); N.Oc/T.Ar, number of osteoclasts per tissue area (mm^2); N.Ot/Md.Ar, number of osteocytes per mineralized tissue area (0.01 mm^2); N.Lac/Md.Ar, number of empty osteocyte lacunae per mineralized tissue area (0.01 mm^2). Values are mean±SEM. Statistical analysis of the data was performed using two-tailed Student's independent t-test. *Significant effect of LIPUS, $p<0.05$.

high numbers of TRAP-positive cells (range 13–30/mm^2, mean 19/mm^2), which were associated with resorption of the cartilage matrix adjacent to the fracture ends. TRAP-positive cells were present in 3 of the 7 biopsies of LIPUS-treated delayed unions. In these 3 biopsies, only 1 of the 3 sections per biopsy showed TRAP-positive cells (range 2–5/mm^2, mean 4/mm^2). Statistical analysis did not reveal any differences between the number of osteoclast-like cells in the biopsies of the delayed unions treated by LIPUS when compared to the untreated controls (Table 2). Tetracycline labeling of the newly formed and mineralizing woven bone at the front of new bony callus formation was evident. The tetracycline labeling of the newly deposited woven bone was diffuse, and in some biopsies the adjacent calcified cartilage matrix also displayed tetracycline labeling. Double tetracycline labeling was visible in the newly formed bone enabling us to determine the rate of bone formation (MAR). The MAR was increased by 27% ($p<0.05$, Table 2) in the biopsies of LIPUS-treated delayed unions when compared to untreated controls. The osteoid maturation time (Omt, calculated as O.Th/MAR), which is the time interval between the onset of matrix deposition and the onset of mineralization, was not affected by LIPUS treatment (control, 6.3±0.7 days; LIPUS 7.1±0.6 days; mean±SEM; $p=0.42$). The number of blood vessels per area of soft connective tissue within the area of new bone formation showed a slight but not significant increase of 8% as a result of LIPUS treatment (control, 39.5±7.4 /mm^2; LIPUS 42.7±10.2 /mm^2; mean±SEM, $p=0.81$). Three of the 7 patients treated with LIPUS were smokers whereas the untreated control patients did not smoke. Smoking however did not affect the outcome of the histomorphometric parameters analyzed in biopsies of LIPUS-treated delayed unions (data not shown).

Area of cancellous bone

Within the area of cancellous bone, bone volume was increased by 17% and mineralized volume by 20% ($p<0.001$) whereas osteoid thickness and MAR remained unchanged as a result of LIPUS treatment (Table 2). LIPUS-treated delayed unions and untreated controls showed similar values for osteoid thickness and MAR in the area of cancellous bone, and these values were similar to those found for untreated controls in the area of new bone formation (Table 2). None of the other evaluated histomorphometric parameters in the area of cancellous bone showed a significant difference between the LIPUS-treated delayed unions and the untreated controls (Table 2). Bone remodelling was present in the area of cancellous bone as shown by the formation of osteoid and the presence TRAP-positive osteoclast-like cells (Table 2). Biopsies of 3 controls showed in all sections TRAP-positive osteoclast-like cells (range 2–8/mm^2, mean 4/mm^2), whereas in only one section of one LIPUS-treated delayed union TRAP-positive osteoclast-like cells (5/mm^2) were present.

Area of cortical bone

Few osteoid seams and low numbers of osteoblasts were present at the surface of the Haversian canals, indicating low bone-forming activity in the area of cortical bone. In this bone area no significant differences between LIPUS-treated delayed unions and controls were found regarding the histomorphometric parameters evaluated (Table 2). For LIPUS-treated delayed unions and untreated controls osteoid thickness and MAR were approximately 40% lower in the area of cortical bone than in the area of cancellous bone (Table 2). Osteoid maturation time remained unchanged in cortical bone tissue (control, 8.1±0.1 days; LIPUS 8.4± days; Mean±SEM) when compared to cancellous bone tissue (control, 8.5±0.9 days; LIPUS 7.9±0.6 days). No TRAP-positive osteoclast-like cells were present in histological sections of LIPUS-treated delayed unions or untreated controls.

Discussion

In this study histomorphometric and histologic analysis was performed to determine bone formation and resorption parameters in LIPUS-treated delayed unions of the osteotomized fibula and sham-treated controls in a double-blind clinical trial. This allows us to report for the first time on the influence of LIPUS treatment on clinical fracture healing at the tissue level. To account for the differences in bone density and bone surface area as well as amount of soft tissue between cortical, cancellous and newly formed bony callus at the fracture end, 3 areas of interest were distinguished within the histological sections of the retrieved biopsies. The areas of interest were: 1) area of new bone formation, representing the newly formed bony callus within 0.6 mm distance from the fracture ends, 2) area of cancellous bone, and 3) area of cortical bone. All retrieved biopsies showed a vital fracture and new bony callus formation, although fracture healing was impaired in the delayed unions of the osteomized fibula. The present study suggests that LIPUS accelerates clinical fracture healing by increasing bone formation through increased osteoblast activity. Direct (endosteal) bone formation was only observed in the LIPUS-treated delayed unions, which might be explained by increased stability and/or blood flow.

LIPUS treatment resulted in a significant increase in bone volume of the newly formed bony callus, and a significant increase in osteoid

thickness at the front of new bony callus formation. This increased osteoid thickness was not caused by impaired mineralization of the newly deposited osteoid at the fracture ends, since LIPUS treatment also significantly increased the mineral apposition rate, and did not change osteoid maturation time. The increase in osteoid thickness as a result of increased osteoid apposition suggests an anabolic effect of LIPUS on the activity of osteoblasts. Cultured mouse bone-marrow-derived ST2 cells have been shown to respond to LIPUS with elevated levels of IGF, osteocalcin, and bone sialoprotein mRNA, suggesting that LIPUS induces a direct anabolic reaction of osteogenic cells leading to bone matrix formation [22]. The nature of the LIPUS-induced anabolic response is of a nature similar to that in physically loaded bone [22]. The accelerated fracture repair and distraction osteogenesis by LIPUS depend on the stimulation of osteoblastic cells at relatively early stages of osteogenic lineage [22,31]. LIPUS treatment of fetal mouse metatarsal rudiments *in vitro* has been shown to stimulate endochondral ossification, which resulted from a direct effect of LIPUS on osteoblasts and ossifying cartilage by stimulation of cell activity and/or differentiation, but not proliferation [32,33]. This may explain our current findings that LIPUS increased osteoid thickness and mineral apposition rate at the front of new bony callus formation, whereas no increase in osteoid thickness and mineral apposition rate was found in the older or pre-existing bone. Our data shows that LIPUS affects clinical fracture healing at the bone tissue level, and underscores that the acceleration of clinical fracture healing by LIPUS, as least in part, depends on the stimulation of osteoblastic cells [22,31,33].

LIPUS did not affect osteoblast activity in the area of cancellous bone nor in the area of cortical bone. Bone volume was significantly increased in the area of cancellous bone formation as a result of LIPUS treatment, but this increase was less profound than in the area of new bone formation. LIPUS did not affect any of the other bone formation and bone resorption parameters. These findings could relate to LIPUS specifically affecting osteoblast differentiation as has been suggested previously [32,33], but it is also possible that a large part of the given energy remitted by LIPUS does not penetrate the intact bone [31,34]. Interestingly, the biopsy retrieved from one LIPUS-treated delayed union showing partial bridging of the fracture ends did not show increased osteoblast activity when compared to the other LIPUS-treated delayed unions. This suggest that LIPUS is effective in stimulating osteoid deposition at the front of bony callus formation during the consolidation phase, but that it has little or no effect on bone remodelling.

In LIPUS-treated delayed unions, direct bone formation without a cartilage intermediate as well as endochondral ossification was observed, while untreated controls only showed endosteal callus formation through endochondral ossification. In distraction osteogenesis, a shift from endochondral ossification to predominantly intramembranous bone formation occurs in time [35]. Bone healing in a delayed union of the osteotomized fibula may resemble bone healing in distraction osteogenesis. The initial displacement of the fracture ends after a non-fixated fibula osteotomy results in a gap between the fracture ends [2], and therefore bone healing must occur through replacement of fibrous or cartilagenous tissue by bone tissue within the fracture gap. The profound cartilage matrix mineralization and absence of TRAP-positive osteoclast-like cells in 2 biopsies from untreated non-unions suggests transchondroid bone formation. This type of bone formation, in which a tissue intermediate between cartilage and bone is formed, has been shown in the distraction gap during distraction osteogenesis of rat tibiae [35]. Intramembranous bone formation as seen in delayed unions after 2 to 4 months of LIPUS treatment could therefore reflect a more advanced stage of fracture healing in comparison to untreated controls.

Bone healing in delayed unions of the human fibula may resemble bone healing as seen in distraction osteogenesis, but there is one major difference. Distraction osteogenesis is a controlled procedure were certain fracture stability is maintained by surgical means, whereas the non-fixated fibula osteotomy provides an unstable fracture [2]. Excess motion at the fracture site of fibular bone is related to disruption of vasculature and blood supply, resulting in a hypoxic environment favourable for formation of cartilaginous tissue [36,37]. Alterations in the mechanical environment by advanced union and/or increased bone volume of the bone fracture ends could therefore enable blood vessel formation and invasion of fibrous and/or cartilaginous tissue within the fracture gap. The oxygenic environment may lead to intramembranous bone formation through differentiation of mesenchymal cells into osteoblasts, but not chondrocytes [36]. LIPUS treatment stimulated bone bridging at the fracture site in a rat closed femoral fracture model [11]. The stimulation of bridging by LIPUS was accompanied by a significant increase in maximum torque and stiffness [11]. The differences in ossification modes between LIPUS-treated delayed unions and untreated controls, as observed in the current study, might also be explained by improved mechanical stability.

Vascular invasion of the fibrous and/or cartilagenous tissue within the fracture gap may result in an oxygenic environment suitable for intramembranous bone formation [36]. LIPUS increased blood flow around the fracture site in a dog osteotomized ulna fracture model, suggesting that LIPUS stimulates vascularity during fracture healing [24]. Analysis of neovascularization within the newly formed fracture callus within the area of new bone formation, showed no significant increase in numbers of blood vessels per area of soft connective tissue. Increased blood flow around the fracture site could provide an explanation for the endosteal callus formation without a cartilage intermediate as observed in the LIPUS-treated delayed unions in this study. Our findings however suggest that a possible increase in blood flow to and around the fracture gap site is probably not related to an increase in new blood vessel formation within the newly formed fracture callus.

In conclusion, our results show that LIPUS accelerates clinical fracture healing of delayed unions of the fibula by increasing osteoid thickness, mineral apposition rate, and bone volume, indicating increased osteoblast activity, at the front of new bony callus formation. Improved stability and/or increased blood flow, but probably not increased angiogenesis, might explain the differences in ossification modes between LIPUS-treated delayed unions and untreated controls.

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