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From Cell Biology to Tissue Engineering

Low-intensity pulsed ultrasound increased blood vessel size during fracture healing in patients with a delayed-union of the osteotomized fibula

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Summary. Disturbed vascularity leads to impaired fracture healing. Since low-intensity pulsed ultrasound (LIPUS) increases new bone formation in delayed-unions, we investigated whether LIPUS increases blood supply in delayed-unions of the osteotomized fibula, and if LIPUS-increased bone formation is correlated to increased blood supply. Blood vessel parameters were analysed using histology, immunohistochemistry, and histomorphometric analysis as well as their correlation with bone formation and resorption parameters. Fibular biopsies of thirteen patients with a delayed-union of the osteotomized fibula treated for 2-4 months with or without LIPUS originating from a randomized prospective double-blind placebo-controlled clinical trial were studied. In histological sections of the fibular biopsies parameters of blood vessel formation were measured and were related to histomorphometric bone characteristics of newly formed bone of the same samples analysed in our previously published study on the effects of LIPUS on bone healing at the tissue level in delayed-unions. LIPUS-treated delayed-unions and sham-treated delayed-unions as well as healed delayed-unions and failed-to-heal delayed-unions were compared. The volume density of blood vessels was increased in LIPUS-treated delayed-unions compared to sham-treated controls. LIPUS did not change blood vessel number, but significantly increased blood vessel size. Healed delayed-unions as well as LIPUS-treated and sham-treated delayed-unions showed significant correlations between blood vessel size and osteoid volume. LIPUS increases blood vessel size, essential for fracture healing, in bone from patients with a delayed-union of the osteotomized fibula. The increased osteoid volume in delayed-unions can largely be explained by increased blood supply and perfusion.

Key words: Low-intensity pulsed ultrasound, Vascularization, Fracture healing, Delayed-union, Histomorphometry

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Introduction

Fracture healing requires the recruitment of appropriate cells and expression of appropriate genes at the right time in the right place (Einhorn, 1995). Most clinical fractures heal spontaneously (Einhorn, 1995). Impairment of fracture healing, appearing in 5-10% of fractures, leads to a delay in union or may even result in a non-union. The goal of therapy for fracture healing in general is to provide sufficient fracture stability and healthy bone tissue by conservative or surgical means. Unstable fracture results in delayed consolidation.

Low-intensity pulsed ultrasound (LIPUS) is a non-invasive treatment modality used to accelerate fracture healing (Heckman et al., 1994; Kristiansen et al., 1997; Nolte et al., 2001a; Rutten et al., 2007, 2016) and to stimulate osteogenesis (Nolte et al., 2001b; Favaro-Pipi et al., 2010). Recently, postoperative use of LIPUS after intramedullary nailing for a tibial shaft fracture did not

improve functional recovery or accelerate radiographic healing in a clinical trial (Busse et al., 2016). On the other hand, LIPUS does not accelerate fracture union in patients with operatively treated fresh fractures, but enhances fracture healing by increased bone formation in patients with delayed and/or impaired fracture healing (as reviewed by Rutten et al., 2016). Therefore research focusing on fractures with prolonged natural healing tendency as in the current study is needed.

The intensity of LIPUS therapy is below the upper limit of diagnostic intensities (<100 mW/cm^2) (Padilla et al., 2014). LIPUS (30 mW/cm^2) is considered as non-thermal and non-destructive. This therapy transmits high frequency acoustic pressure waves resulting in acoustic streaming and microstreaming (Harrison et al., 2016). It is proposed that the LIPUS signal can produce nanomotion at the fracture site (Harrison et al., 2016). The biomechanical wave is then converted into a biochemical signal within the cell that has a biological effect (Harrison et al., 2016). LIPUS triggers intracellular signal transduction and subsequent gene transcription leading to changes in cell proliferation and differentiation (Padilla et al., 2014). It is not yet clear if the stimulatory effect of LIPUS on endochondral ossification is likely due to stimulation of bone cell differentiation alone (Korstjens et al., 2004; Rutten et al., 2009), or to a more complex combination of accelerated proliferation, differentiation and maturation. It has been demonstrated in human delayed-unions of the osteotomized fibula that LIPUS does not increase the number of osteogenic cells, but likely affects osteogenic cell differentiation (Rutten et al., 2008). Ding et al. (2009) found an accelerated bone formation and increased bone mineral density on the experimental LIPUS-treated side compared to the untreated side in dogs during mandibular distraction osteogenesis. The new bone volume remained unchanged. For a review see Padilla et al. (2014).

Vascularization plays an important role in fracture healing by angiogenesis and blood flow to the fracture site (Saran et al., 2014). Angiogenesis is essential in the early stages of fracture healing in humans (Hausman et al., 2001). It occurs predominantly before the onset of osteogenesis (Saran et al., 2014). Inhibition of angiogenesis completely prevents fracture healing (Hausman et al., 2001). Stability and loading are important for the angiogenic component of fracture repair (Hankenson et al., 2011). A relationship between mechanical loading and angiogenesis has been suggested (Yao et al., 2004; Cheung et al., 2011). Accelerated osteoporotic fracture healing by enhanced angiogenesis, callus formation, and remodeling in LIPUS-treated rats has been observed (Cheung et al., 2011). Angiogenesis is indispensable for exercise-induced bone gain, which has been demonstrated in treadmill running rats (Yao et al., 2004). Vascularization is required for both intramembranous and endochondral bone formation and for bone repair (Hankenson et al., 2011). LIPUS-treatment results in highly vascularized connective tissue and improved angiogenesis and tissue perfusion in rats (Barzelai et al., 2006; Favaro-Pipi et al., 2010). It also stimulates human osteoblasts to respond with increased nitric oxide (NO) production and angiogenic gene expression (Wang et al., 2004). Whether LIPUS also stimulates vascularization in patients with delayed- or non-unions is still largely unknown, but sufficient blood supply is a prerequisite for fracture healing.

LIPUS is known to stimulate fracture healing and osteogenesis (Bashardoust Tajali et al., 2012; Rutten et al., 2016). Previously we showed that LIPUS increases bone volume, osteoid thickness, and mineral apposition rate (Rutten et al., 2008). Since blood flow to the fracture site in the fracture healing process is highly relevant, we focused in this study on the influence of LIPUS on blood supply around the fracture gap using bone biopsies from patients included in a randomized clinical trial (Rutten et al., 2008). The aim of the present study was to investigate whether LIPUS increases blood supply in delayed-unions of the osteotomized fibula, and if LIPUS-increased bone formation is correlated to increased blood supply. Blood vessel parameters were analysed using histology, immunohistochemistry, and histomorphometric analysis as well as their correlation with bone formation and resorption parameters.

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) treated with or without LIPUS (7 LIPUS, 6 controls) originating from a randomized prospective double-blind placebo-controlled clinical trial (Rutten et al., 2008). 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. When radiological healing of the fibula was not accomplished within 6 months, patients could participate in this study after informed consent was obtained. The only inclusion criteria were a delayed union of the fibula after a high tibial osteotomy and an age between 18 and 75 years. Exclusion criteria were pregnancy and lactation, pathological bone disease, receiving long term calcium channel blockers or bisphosphonates, apparent alcoholism and/or nutritional deficiency. None of the patients was suffering from diabetes. Two LIPUS-treated patients and 3 sham-treated patients demonstrated hypertension. Medication usage did not show any difference between the sham-treated and LIPUS-treated groups, except for the fact that 3 smokers were among the LIPUS-treated delayed unions. Alcohol consumption was comparable for both groups. 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.

The trial was registered and approved on 3 May

2004 (retrospectively registered) at the Medical Ethical Review Board of the VU University Medical Center, Amsterdam, registration number 2004-005. The patients were recruited at the Spaarne Hospital (Hoofddorp) and Tergooi Hospitals (Hilversum), The Netherlands.

LIPUS treatment

The Exogen 2000+® LIPUS device (Smith & Nephew Inc., Memphis, TN, USA) was used at home during 20 min per day for 5 months (Rutten et al., 2008). 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. Computerized randomization of active and placebo devices was performed by the Exogen® device manufacturer (Smith & Nephew Inc.) for blocks of 8 devices (1:1). Devices were shipped as blocks of 8 devices (4 active 4 control) to each investigational center and dispatched in order of inclusion. The allocation sequence remained concealed to the investigators and patients participating in the trial upon termination of the study treatment phase of the patients enrolled. The active and control devices were identical in appearance. The trial was unblinded after all patients completed 5 months LIPUS treatment, and after completion of the histomorphometric and histologic analysis. Radiographs were taken to diagnose if the delayed-unions were healed or failed-to-heal.

Biopsy procedure

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 (Zerbo et al., 2003). A biopsy was taken under general or spinal anaesthesia with the use of a hollow trephine burr (ITI-Straumann, Basel, Switzerland) as described earlier (Rutten et al., 2008). One delayed-union case treated with LIPUS showed radiographic consolidation when the biopsy was taken.

Histology

The biopsies were fixated in 4% formaldehyde in 0.1 M phosphate buffer at pH 7.3 and 4°C for 24 h. For histomorphometric analysis, the biopsies were embedded in methylmetacrylate without decalcification (Zerbo et al., 2003). Longitudinal 5 μm thick sections were cut, and 3 sections at 305 μm distance from each other were stained with Goldner's trichrome method (mineralized tissue: green; osteoid: red). For immunohistochemistry, the biopsies were decalcified with 5% EDTA, 1% formaldehyde at pH 7.3 at 4°C for 8 weeks. Biopsies were dehydrated by ethanol, cleared in xylene, and embedded in paraffin at 56°C. Sections of 6-7 μm thickness were cut, mounted on polylysine-coated glass slides, and dried overnight in a 37°C stove.

Histomorphometric analysis

Three Goldner trichrome-stained sections per patient were quantitatively measured at 200x magnification by a Leica DMRA microscope connected to Leica Qwin computer software (Leica Microsystems Imaging Solutions, Cambridge, UK). Blood vessel formation was measured in the zone of newly formed bone, which represents the far most active bone area within 2.5 mm from the fracture end (Fig. 1). To account for differences in differentiation, the zone of newly formed bone was divided into 3 domains with regard to the bone architecture and histological appearance. Domain-1 was located within 0.6 mm distance from the fracture end, domain-2 was located from 0.6-1.3 mm distance from the fracture end, and domain-3 was located from 1.8-2.5 mm distance from the fracture end, just before the end of the zone of newly formed bone (Fig. 1). Since reliable quantification of very small blood vessels in domain-1 was not possible, we did not further analyze this area. For our measurements we used therefore domain-2 and domain-3 which allowed to distinguish relatively "immature" versus "mature" bone.

The definition of blood vessels and blood vessel parameters was according to Amir et al. (2006). Number of blood vessels and soft connective tissue parameters were quantified (Fig. 2). Volume density of blood vessels was calculated as total blood vessel area/total soft connective tissue area x 100% (Blv.V/SCT.V x 100%). Blood vessel size was calculated as the total blood vessel area/total number of blood vessels expressed as μm^2 (Blv.Ar/N.Blv). The number of blood vessels per unit soft connective tissue was determined per mm^2 (N.Blv/SCT.Ar (/mm^2).

The data obtained from the 3 sections of each patient were averaged and were related to data from our previously published study on the effects of LIPUS on bone healing at the tissue level in delayed-unions (Rutten et al., 2008). Nomenclature, symbols, and units used are as recommended by the Nomenclature Committee of the American Society for Bone and Mineral Research (Parfitt et al., 1987). The following parameters, as described earlier (Rutten et al; 2008), were measured: Bone volume (BV): amount of mineralized tissue (Mineralized volume, Md.V) plus the amount of osteoid tissue (Osteoid volume OV) as a percentage of the total tissue volume (BV/TV x 100%). Absolute osteoid volume: amount of osteoid tissue as a percentage of the total tissue volume (OV/TV x 100%). Relative osteoid volume: the amount of osteoid tissue as a percentage of the total bone volume (OV/BV x 100%). Osteoid thickness (O.Th): average of the osteoid thickness. Mineral apposition rate (MAR): average distance between corresponding edges of two consecutive fluorescent bone labels divided by the number of days between start of the first administration

period of tetracycline and the start of the second administration period of tetracycline (Zerbo et al., 2003; Rutten et al., 2008). TRAP-positive osteoclasts (N.Oc): number expressed per total tissue area (mm^2) (N.Oc/T.Ar).

Immunohistochemistry

Tissue sections were deparaffinized, rehydrated, and subjected to antigen retrieval in 10 mM citrate buffer (pH 6.0) of 60°C for 48 h. Sections were incubated in 50 mM NH_4CL for 10 min to inactivate formalin, blocked in 0.5% BSA for 30 min, and incubated with the primary antibody to laminin, a basement membrane component of vessels (Abcam, Cambridge, UK; catalog number ab 11575; laminin was purified from the basement membrane of Englebreth Holm-Swarm (EHS) sarcoma (mouse)), at 1:25 dilution, for 2 h at room temperature. The second antibody goat anti-rabbit IgG-HRP (horse radish peroxidase) (Envision kit, Dako, Carpinteria, CA) was applied according to the manufacturer's instructions. After development with DAB substrate, sections were counterstained with hematoxylin. Endothelials cells of vessels stained positive for laminin.

Statistical analysis

Data are expressed as mean±SEM. Univariate analysis of variance (UNI-ANOVA) was performed for sham-treated and LIPUS-treated patients, and for failed-to-heal delayed-unions and healed-delayed-unions, since it is known that in the long run some delayed-unions heal spontaneously without LIPUS treatment while not all delayed-unions heal after LIPUS treatment. Pearson correlation test was used to examine correlation between blood vessel and bone formation parameters. $p<0.05$ was considered statistically significant.

Results

As reported previously, after 5 months of LIPUS- or sham-treatment, the healing time was slightly but not significantly decreased by 29% as a result of LIPUS-treatment (Rutten et al., 2012). However 1 year after treatment started, LIPUS-treatment significantly decreased the healing time by 57% (Rutten et al., 2012).

Histology

Blood vessels were found throughout the zone of newly formed bone in biopsies from both sham-treated and LIPUS-treated patients. Active bone formation was observed by osteoblasts depositing osteoid at the bone surface (domain-1; Fig. 3). Sham-treated controls showed only endochondral ossification at the fracture ends, while direct/intramembranous bone formation was also seen in biopsies of the LIPUS-treated delayed-unions (Rutten et al., 2008). Blood vessels were present in close vicinity to active ostoblasts depositing osteoid. In domain-1 numerous small developing blood vessels were seen in sham-treated delayed-unions, and even more in LIPUS-treated delayed-unions (Fig. 4a,b). Therefore domain-1 seems an active area in which initiation of blood vessel formation occurred. In domain-2 large blood vessels were

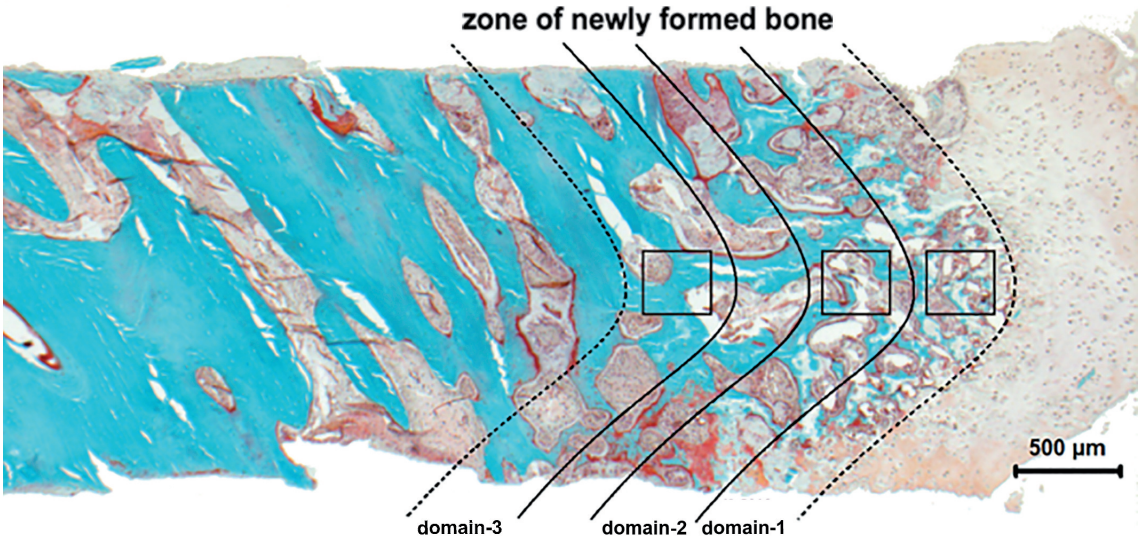

Fig. 1. Histological section of a biopsy of a delayed-union of a human fibula showing the zone of newly formed bone, which represents the far most active bone area within 2.5 mm from fracture end. The zone of newly formed bone was divided into 3 domains with regard to the bone architecture and histological appearance: 1) domain-1 is located within 0.6 mm distance from the fracture end, 2) domain-2 is located from 0.6-1.3 mm distance from fracture end, 3) domain-3 is located from 1.8-2.5 mm distance from fracture end. Goldner's trichrome stained section; osteoid and soft tissue, red; mineralized bone, green; cell nuclei, black. x 50.

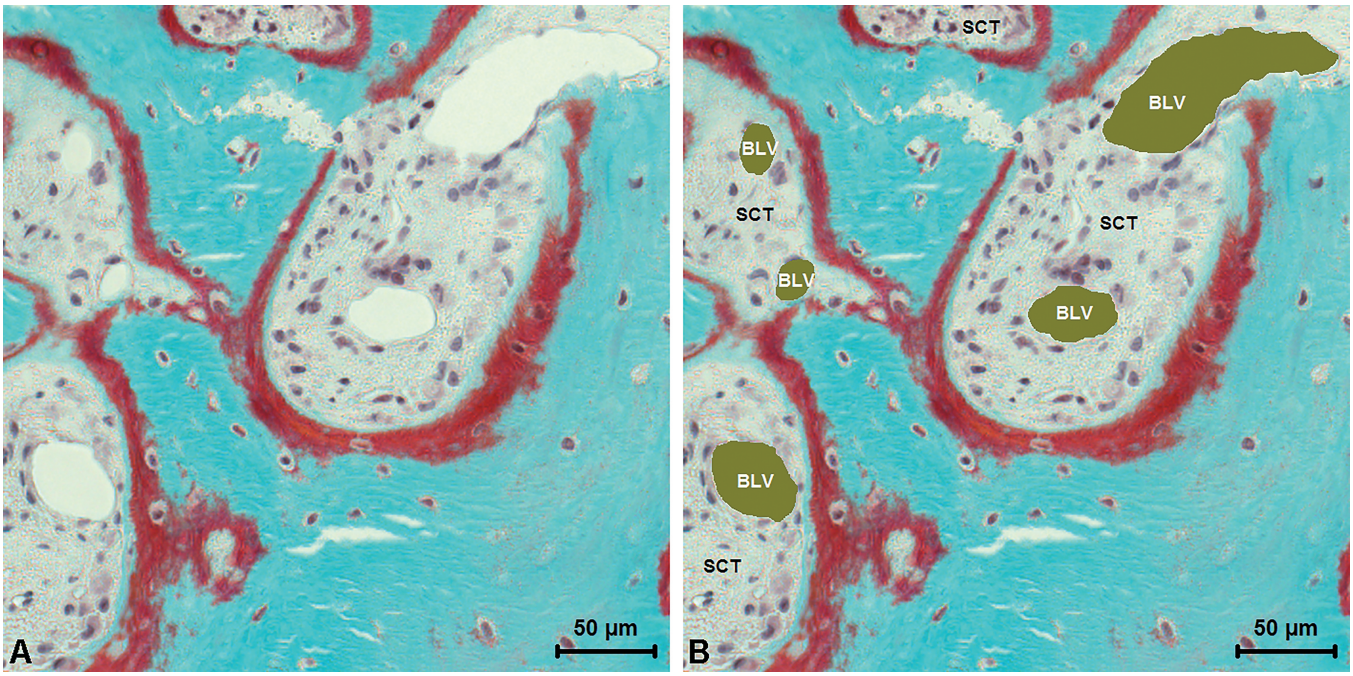

Fig. 2. Drawing of the area of blood vessels for vascular histomorphometry. **A.** Histological section. **B.** Histological section with blood vessels indicated. Goldner's trichrome stained section; blood vessel, BLV; soft connective tissue, SCT; osteoid, red; mineralized bone, green; cell nuclei, black. x 200.

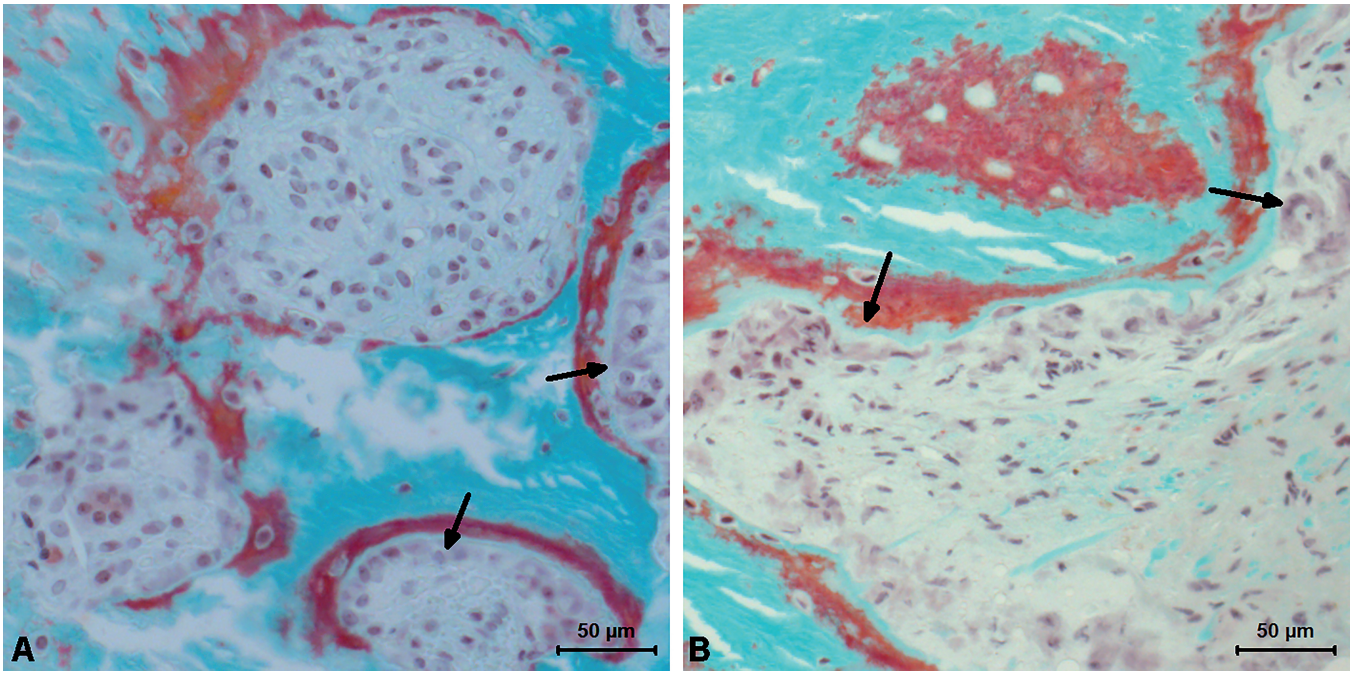

Fig. 3. Histological section of a biopsy of a delayed-union of a human fibula. High cell density and some clusters of osteoblasts surrounded by osteoid can be seen (domain-1). **A.** Sham-treated delayed-unions. **B.** LIPUS-treated delayed-unions. Arrows indicate osteoblasts. Osteoid, red; mineralized bone, green; cell nuclei, black. x 200.

clearly visible in sham-treated and LIPUS-treated nonunions (Fig. 4c,d). Larger blood vessels were seen in the LIPUS-treated delayed-unions compared to sham-treated delayed-unions (domain-2; Fig. 4c,d). Domain-3 showed similar results as domain-2.

Histomorphometry

Using UNI-ANOVA no significant interaction effects between LIPUS-treated and sham-treated delayed-unions, and between failed-to-heal and healed delayed-unions were found (Table 1). A significant influence of LIPUS on vascularity and blood flow was only found in domain-2. LIPUS increased blood vessel size by 70% (p=0.021) compared to sham-treatment (Table 1). In domain-3 LIPUS resulted in a comparable trend of increased blood vessel size of 64% (ns, p= 0.163) compared to sham-treatment (Table 1). We also found a trend of increased volume density of blood vessels by 65% in domain-2 (ns, p=0.259), and by 60% in domain-3 (ns, p=0.239) compared to sham-treatment (Table 1). The number of blood vessels in domain-2 and domain-3 of LIPUS-treated and sham-treated delayed-unions was similar (Table 1). No significant differences were found between healed delayed-unions and failed-to heal delayed-unions.

Significant Pearson correlations between blood vessel parameters and bone formation parameters were found in domain-3 for sham-treated and LIPUS-treated patients (Table 2). LIPUS-treated patients showed a significant positive correlation between blood vessel size and relative osteoid volume (ϱ=0.774, p=0.041). Sham-treated patients showed a significant positive correlation between blood vessel size and absolute osteoid volume (ϱ=0.827, p=0.042) and relative osteoid volume (ϱ=0.871, p=0.024).

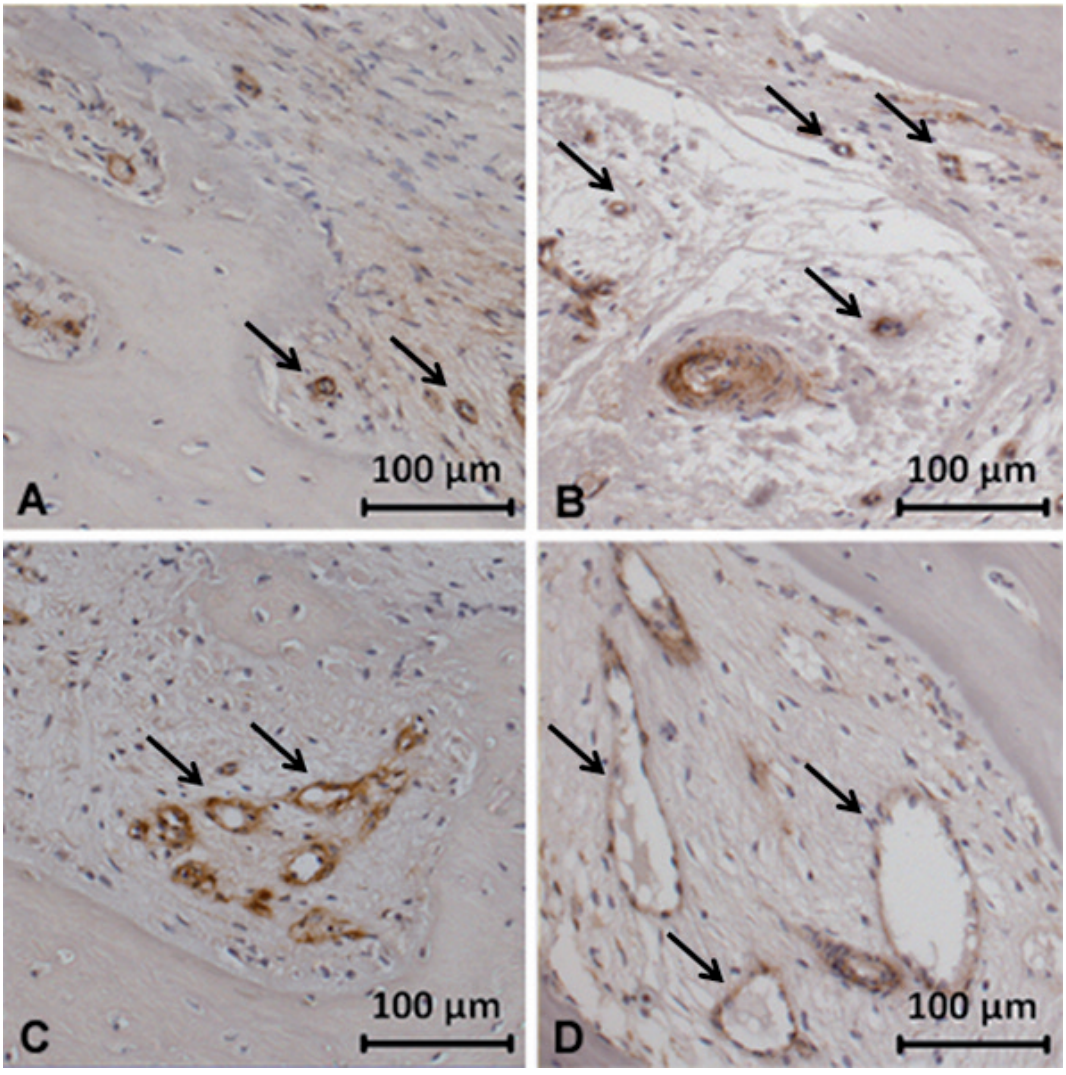

Fig. 4. Immunostained histological section of a biopsy of a delayed-union of a human fibula. **A.** Domain-1, sham-treated. **B.** Domain-1, LIPUS-treated. Small developing blood vessels (brown) can be seen. **C.** Domain-2, sham-treated. **D.** Domain-2, LIPUS-treated. The LIPUS-treated delayed-unions showed increased blood vessel size. Arrows indicate blood vessels. x 200.

Table 1. Histomorphometric data of blood vessels of delayed-unions of the osteotomized fibula analysed by treatment (with or without LIPUS) and by healing status (failed or healed).

		Sham-treated N=6	LIPUS-treated N=7	Uni-anova
	Male / Female	1M / 5F	3M / 4F	
	Age (Mean ± Sd)	52±8	52±9	
	Blood vessel parameter	Mean ± SEM	Mean ± SEM	p-value
Domain 2	Blv.V/SCT.V (%)	7.15±1.54	11.78±2.96	0.259
	Blv.Ar/N.Blv (μm^2)	1811±320	3023±333	0.021*
	N.Blv/SCT.Ar (/mm^2)	48.47±9.11	44.80±12.81	0.640
Domain 3	Blv.V/SCT.V (%)	5.86±1.75	9.37±1.49	0.239
	Blv.Ar/N.Blv (μm^2)	1524±229	2492±534	0.163
	N.Blv/SCT.Ar (/mm^2)	35.04±6.89	46.19±10.25	0.446
		Failed N=5	Healed N=8	Uni-anova
	Male/ Female	0M / 5F	4M / 4F	
	Age (Mean ± Sd)	51±7	53±9	
	Sham-treated/LIPUS-treated	2 / 3	4 / 4	
	Blood vessel parameter	Mean ± SEM	Mean ± SEM	p-value
Domain 2	Blv.V/SCT.V (%)	6.97±1.92	11.31±2.59	0.239
	Blv.Ar/N.Blv (μm^2)	2778±551	2267±317	0.524
	N.Blv/SCT.Ar (/mm^2)	33.70±11.52	54.48±9.86	0.282
Domain 3	Blv.V/SCT.V (%)	7.78±2.51	7.73±1.33	0.949
	Blv.Ar/N.Blv (μm^2)	2391±646	1829±360	0.552
	N.Blv/SCT.Ar (/mm^2)	29.36±7.34	48.35±8.51	0.163

Histomorphometric data is presented for domain-2 and domain-3 in the zone of newly formed bone. Blv.V/SCT.V (%), volume density of blood vessels (%); Blv.Ar/N.Blv (μm^2), blood vessel size (μm^2); N.Blv/SCT.Ar (/mm^2), number of blood vessels per mm^2 soft connective tissue. * Significant effect of LIPUS, $p<0.05$.

Table 2. Pearson correlations between blood vessel parameters and bone formation parameters in domain-2 and domain-3 for sham-treated delayed unions and LIPUS-treated delayed-unions in biopsy of the human fibula.

		Sham-treated				LIPUS-treated			
		Domain-2		Domain-3		Domain-2		Domain-3	
		ρ	p-value	ρ	p-value	ρ	p-value	ρ	p-value
Blv.V/SCT.V	BV/TV	-0.173	0.743	-0.015	0.977	0.409	0.362	-0.546	0.205
	Md.V/TV	-0.206	0.696	-0.151	0.775	0.483	0.272	-0.451	0.310
	OV/TV	0.306	0.556	0.747	0.088	-0.282	0.540	-0.082	0.861
	OV/BV	0.203	0.700	0.643	0.168	-0.345	0.448	0.072	0.879
	O.Th	-0.595	0.213	-0.032	0.953	-0.027	0.954	-0.316	0.490
	MAR	-0.644	0.167	-0.034	0.949	0.531	0.278	0.225	0.668
	N.Oc/T.Ar	0.410	0.419	0.637	0.174	0.192	0.679	0.500	0.253
Blv.Ar/N.Blv	BV/TV	-0.442	0.380	-0.034	0.949	-0.079	0.867	-0.415	0.354
	Md.V/TV	-0.397	0.436	-0.200	0.704	-0.081	0.862	-0.647	0.116
	OV/TV	-0.458	0.361	0.827	**0.042***	0.028	0.953	0.656	0.110
	OV/BV	0.006	0.990	0.871	**0.024***	0.135	0.773	0.774	**0.041***
	O.Th	-0.677	0.139	0.293	0.573	0.061	0.897	0.417	0.352
	MAR	-0.319	0.538	0.376	0.462	0.637	0.174	0.283	0.587
	N.Oc/T.Ar	-0.128	0.809	0.411	0.419	0.747	0.054	0.527	0.224
N.Blv/SCT.Ar	BV/TV	0.264	0.613	-0.154	0.771	0.332	0.467	0.039	0.935
	Md.V/TV	0.241	0.645	-0.248	0.636	0.435	0.329	0.281	0.542
	OV/TV	0.429	0.396	0.547	0.262	-0.331	0.468	-0.582	0.170
	OV/BV	-0.076	0.886	0.500	0.313	-0.419	0.349	-0.605	0.150
	O.Th	-0.054	0.919	0.025	0.963	-0.116	0.804	-0.551	0.200
	MAR	-0.415	0.413	-0.008	0.988	0.130	0.806	0.044	0.933
	N.Oc/T.Ar	0.416	0.412	0.319	0.538	-0.099	0.832	-0.105	0.823

Pearson correlation coefficients (ρ) between blood vessel parameter and bone formation parameter are presented for domain-2 and domain-3 in the zone of the newly formed bone. Blv.V/SCT.V, volume density of blood vessels (%); Blv.Ar/N.Blv, blood vessel size (μm^2); N.Blv/SCT.Ar, number of blood vessels per soft connective tissue area (/mm^2); 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 TRAP-positive cells per tissue area (/mm^2). * Significant correlation, $p<0.05$.

Table 3. Pearson correlations between blood vessel parameters and bone formation parameters in domain-2 and domain-3 for delayed-unions that failed to heal and healed delayed-unions in biopsy of the human fibula.

		Failed-to-heal				Healed			
		Domain-2		Domain-3		Domain-2		Domain-3	
		ρ	p-value	ρ	p-value	ρ	p-value	ρ	p-value
Blv.V/SCT.V	BV/TV	-0.755	0.140	-0.645	0.240	0.800	**0.017***	0.369	0.369
	Md.V/TV	-0.786	0.115	-0.663	0.222	0.904	**0.002***	0.342	0.407
	OV/TV	0.777	0.122	0.622	0.263	-0.232	0.580	0.056	0.895
	OV/BV	0.809	0.097	0.681	0.205	-0.486	0.222	-0.012	0.978
	O.Th	-0.156	0.802	-0.175	0.778	0.213	0.612	0.274	0.512
	MAR	-0.031	0.960	0.334	0.582	0.688	0.088	0.369	0.415
	N.Oc/T.Ar	0.250	0.685	0.092	0.882	0.031	0.942	0.329	0.425
Blv.Ar/N.Blv	BV/TV	0.179	0.773	-0.329	0.589	0.268	0.521	0.120	0.776
	Md.V/TV	0.100	0.872	-0.391	0.515	0.236	0.574	-0.201	0.634
	OV/TV	0.311	0.611	0.621	0.264	0.094	0.825	0.904	**0.002***
	OV/BV	0.300	0.624	0.643	0.242	0.021	0.961	0.901	**0.002***
	O.Th	0.631	0.254	0.375	0.534	-0.144	0.733	0.578	0.133
	MAR	0.968	**0.007***	0.816	0.092	0.271	0.557	0.056	0.906
	N.Oc/T.Ar	-0.814	0.094	-0.438	0.461	0.027	0.949	0.243	0.562
N.Blv/SCT.Ar	BV/TV	-0.727	0.164	-0.613	0.272	0.626	0.097	0.494	0.213
	Md.V/TV	-0.663	0.223	-0.578	0.308	0.780	**0.022***	0.671	0.069
	OV/TV	0.209	0.735	0.288	0.639	-0.365	0.374	-0.495	0.212
	OV/BV	0.274	0.656	0.368	0.542	-0.608	0.110	-0.638	0.088
	O.Th	-0.827	0.084	-0.654	0.231	0.251	0.549	0.112	0.792
	MAR	-0.719	0.171	-0.161	0.796	0.406	0.367	0.568	0.184
	N.Oc/T.Ar	0.912	**0.031***	0.502	0.389	-0.092	0.829	-0.005	0.991

Pearson correlation coefficients (ρ) between blood vessel parameter and bone formation parameter are presented for domain-2 and domain-3 in the zone of the newly formed bone. Blv.V/SCT.V, volume density of blood vessels (%); Blv.Ar/N.Blv, blood vessel size (μm^2); N.Blv/SCT.Ar, number of blood vessels per soft connective tissue area (/mm^2); 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 TRAP-positive cells per tissue area (/mm^2). * Significant correlation, $p<0.05$.

Significant Pearson correlations between blood vessel parameters and bone formation parameters were found in domain-2 and domain-3 for healed delayed-unions, and in domain-2 for failed-to-heal delayed-unions (Table 3). Healed delayed-unions showed positive correlations in domain-2 between volume density of blood vessels and bone volume (ϱ=0.800, p=0.017), between volume density of blood vessels and mineralized volume (ϱ=0.904, p=0.002), and between number of blood vessels and mineralized bone volume (ϱ=0.780, p=0.022). In domain-3, healed delayed-unions showed positive correlations between blood vessel size and absolute osteoid volume (ϱ=0.901, p=0.002), and between blood vessel size and relative osteoid volume (ϱ=0.901, p=0.002). Failed-to-heal delayed-unions showed positive correlations in domain-2 between blood vessel size and mineral apposition rate (ϱ=0.968, p=0.007), and between number of blood vessels and number of TRAP-positive cells (ϱ= 0.912, p=0.031).

Discussion

Histomorphometric and histologic analysis of bone biopsies of LIPUS-treated and sham-treated delayed-unions of the osteotomized fibula was performed to evaluate the effect of LIPUS-treatment on vascularity in a double-blind randomized clinical trial. It was found that LIPUS increased blood vessel size, which enables improved blood flow resulting in enhanced oxygen delivery and increased bone formation in and/or around the fracture gap. The increased osteoid volume in healed-delayed-unions, LIPUS-stimulated delayed-unions, and sham-treated delayed-unions can therefore largely be explained by increased blood flow and perfusion.

Histology showed a cell-rich area with active osteoid-depositing osteoblasts and only very small blood vessels next to the fracture gap. VEGF, which is upregulated by mechanical loading and LIPUS, plays an important role in angiogenesis (Padilla et al., 2014). It regulates mitogenesis and the recruitment of endothelial cells, and is involved in osteoblast differentiation and osteoclast activation (Padilla et al., 2014). LIPUS treatment results in higher VEGF mRNA expression and improved blood flow and angiogenesis in rats with hind-limb ischemia (Barzelai et al., 2006). Therefore our findings might suggest improved blood flow in LIPUS treated delayed-unions since we found increased blood vessel size. Formation of blood vessels precedes bone growth in the fracture gap (Saran et al., 2014). Visual inspection revealed that the number of very small newly formed blood vessels next to the fracture gap was higher

after LIPUS treatment.

Early bone formation necessitates high energy consumption, requiring adequate blood supply (Amir et al., 2006; Saran et al., 2014). We found that LIPUS increased blood vessel size. This suggests that oxygen diffuses to the hypoxic fracture gap resulting in an oxygen-rich environment, which allows, in combination with a stable mechanical environment, direct bone formation without a cartilage intermediate (Le et al., 2001; Thompson et al., 2002; Barzelai et al., 2006; Hankenson et al., 2011). The rate of the blood flow is directly proportional to the fourth power of the vessel radius (Poiseuille's Law), which indicates that the blood vessel diameter is a determining factor in the rate of blood flow through a vessel. Earlier we speculated that endosteal callus formation without a cartilage intermediate is the result of increased blood flow around the fracture site, which is not necessarily related to increased new blood vessel formation (Rutten et al., 2008). Although biopsies were available from only 13 patients, this study indeed demonstrated a significant increase in blood vessel size, which likely increases blood supply in the LIPUS-treated delayed-unions. In addition, not only a significant increase in blood vessel size but also a trend towards increased blood vessel volume density was seen.

Re-establishment of the circulation is essential to the early stages of fracture healing (Hausman et al., 2001; Hankenson et al., 2011; Saran et al., 2014). The key elements blood supply and stability ensure the timely and accurate reconstitution of bone tissue. Angiogenesis is required for osteogenesis (Hankenson et al., 2011; Saran et al., 2014). Our findings show an increase in osteoid volume for sham-treated and LIPUS-treated delayed-unions, which might be explained by increased blood vessel size resulting in increased blood flow and perfusion. Remarkably, some sham-treated delayed-unions healed and some LIPUS-treated delayed-unions failed to heal. To better understand the process of healing of delayed-unions, we questioned whether a particular condition exists resulting in healing. Therefore we divided the delayed-unions into two groups i.e. 'failed-to-heal delayed-unions' and 'healed delayed-unions'. Interestingly, healed delayed-unions showed comparable significant correlations between osteoid volume and blood vessel size as sham-treated and LIPUS-treated delayed-unions. The failed-to-heal delayed-unions did not show significant correlations between osteoid volume and blood vessel size. Therefore, we suggest that a positive correlation between osteoid volume and blood vessel size might stimulate fracture healing. In addition, since we found that LIPUS significantly increases the blood vessel size, this will likely result in a further increase in osteoid volume necessary for fracture healing.

Conclusions

Our data show that LIPUS increases blood vessel size in bone from patients with a delayed-union of the osteotomized fibula. Larger vessels improve blood supply, which is essential for accelerated fracture healing. The increased osteoid volume in delayed-unions can largely be explained by increased blood supply and perfusion.

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