

● Original Contribution

EFFECT OF LOW-INTENSITY PULSED ULTRASOUND ON JOINT INJURY AND POST-TRAUMATIC OSTEOARTHRITIS: AN ANIMAL STUDY

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Abstract—This study investigated the therapeutic potential of low-intensity pulsed ultrasound (LIPUS) in post-traumatic osteoarthritis (PTOA). Intra-articular fracture of the medial tibial plateau was surgically created in 30 rats. LIPUS was applied to the operated joints either for the first 2 wk (LIPUS₁₋₂ group) or in weeks 4 and 5 after intra-articular fracture (LIPUS₄₋₅ group). In controls, the operated knees were not treated with LIPUS (LIPUS₀ group). The rats were monitored with weekly gait analysis and euthanized at week 8. Among the altered gait parameters, the maximal and average paw print areas in the LIPUS₁₋₂ and LIPUS₄₋₅ groups, but not the LIPUS₀ group, had either reached baseline or significantly recovered (70%, $p < 0.05$) by week 8. PTOA pathology in both the LIPUS₁₋₂ and LIPUS₄₋₅ groups was less severe than that in the LIPUS₀ group (Mankin score: 5.4 and 4.5 vs. 8.8, $p < 0.05$). In conclusion, LIPUS treatment partially improved the gait of the affected limbs and reduced cartilage degeneration in PTOA. (E-mail: zijun.zhang@medstar.net) © 2018 World Federation for Ultrasound in Medicine & Biology. All rights reserved.

Key Words: Post-traumatic osteoarthritis, Ultrasound, Cartilage, Gait, Intra-articular fracture.

INTRODUCTION

Post-traumatic osteoarthritis (PTOA) accounts for about 12% of symptomatic osteoarthritis (OA) cases and is difficult to manage clinically (Brown et al. 2006; Buckwalter and Brown 2004; McKinley et al. 2010). Joint injury disrupts tissue matrix, causes cell death and bleeding, and triggers traumatic inflammation (Lotz and Kraus 2010). Inflammatory cytokines are detrimental to the integrity of articular cartilage as they activate matrix-degradation enzymes, inhibit matrix production and induce chondrocyte apoptosis (Lotz 2001). In severely injured joints, increased inflammatory cytokines, including tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β), in joint fluid could persist for as long as 6–12 mo and deliver secondary damage to the joint—the onset of PTOA (Marks and Donaldson 2005).

In the tissue, low-intensity pulsed ultrasound (LIPUS) produces no thermal effects, but generates mechanical stresses that stimulate a range of cellular responses to

promote cell proliferation and tissue formation (Zhang et al. 2003a). In cartilage, LIPUS stimulates chondrocyte proliferation and matrix production in various experimental settings (Choi et al. 2006; Ikeda et al. 2006; Korstjens et al. 2008; Naito et al. 2010; Zhang et al. 2002, 2003b). LIPUS does not induce chondrocytes in hyaline cartilage to terminal differentiation (Zhang et al. 2002). This is important because terminally differentiated chondrocytes eventually become apoptotic, a common pathology of OA (Hwang and Kim 2015).

LIPUS suppresses inflammation in several ways: reduction of leukocyte infiltration; augmentation of macrophage phagocytosis; and suppression of TNF- α and IL-1 β expression by synovial cells (Nakamura et al. 2011; Signori et al. 2011; Zhou et al. 2008). In addition, LIPUS treatment inhibited the activity of matrix metalloproteinase 13 (MMP-13) and improved OA pathology in animal models (Gurkan et al. 2010; Li et al. 2011; Zeng et al. 2012).

To examine the effect of LIPUS on PTOA, a valid animal model of joint injury is essential. Surgical meniscectomy and/or transaction of the anterior cruciate ligament to destabilize the knee and shift focal loading stresses on articular cartilage are the most common approaches to induction of PTOA in animals (Li et al. 2011;

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Zeng *et al.* 2012). Intra-articular fracture, where both bone and articular cartilage are fractured, is a type of severe joint injury. Concomitant intra-articular fracture increases the risk of PTOA of an injured joint as much as 20-fold (Schenker *et al.* 2014). Intra-articular fracture, including both closed (Furman *et al.* 2007) and surgical (Llinas *et al.* 1993; Trumble and Verheyden 2004), has been used in animal studies to induce PTOA. An open surgery ensures the accuracy and consistency of intra-articular fracture produced in the joints, and the simultaneously applied fracture fixation simulates clinical management of the intra-articular fracture (Zahoor *et al.* 2016). The outcome and pathology of a PTOA model are often evaluated with end-of-the-study histology, although joint pathology evolves from injury to degeneration. Gait reflects joint injury and cartilage degeneration in the lower extremity with alterations of a group of gait parameters (Naili *et al.* 2017). In follow-up of the rats with knee OA, gait analysis sensitively and accurately detected antalgic and shuffling gait in accordance with the stages of the OA development (Jacobs *et al.* 2017). Gait analysis is particularly valuable for animal models of PTOA, where clinical symptoms and physical signs are lacking.

Although LIPUS enhances fracture healing and improves cartilage repair (Bayat *et al.* 2017; Cook *et al.* 2001; Heckman *et al.* 1994), whether LIPUS treatment prevents or delays the development of PTOA secondary to intra-articular fracture is unknown. In this study, intra-articular fracture was surgically created in rat knees for PTOA induction. LIPUS was applied to the rat knees with intra-articular fracture at either the early stage of injury or the later osteoarthritic stage. The effects of LIPUS on PTOA development were longitudinally monitored with gait analysis and evaluated with histology at the end of the study.

METHODS

In this study, 30 male Sprague-Dawley rats at 12 wk of age were used (approved by MedStar Health Research Institute Institutional Animal Care and Usage Committee). The Sonic Accelerated Fracture Healing System (Bioventus, Durham, NC, USA) delivered ultrasound at an intensity of 30 mW/cm² (spatial-average, temporal-average intensity), with a sinusoidal waveform of frequency 1.5 MHz. The pulse burst frequency was 1 kHz, with a burst duration of 200 μ s. The devices were calibrated by the manufacturer before applications and validated at the completion of this study.

Intra-articular fracture model

The rats were anesthetized by inhalation of isoflurane. After skin preparation, a para-patellar incision was made

on the left knee joint of a rat to expose the medial tibial plateau as described previously (Zahoor *et al.* 2016). The middle point between the tibial anterior crest and the outermost edge of the medial plateau was used as a reference of osteotomy. A surgical blade (#11) was placed at the mid-point of the medial tibial plateau and in perpendicular to the front edge of the joint surface. The medial tibial plateau was osteotomized vertically, including the covering articular cartilage. The fractured medial plateau was reduced and fixed with two needles (23 gauge) transversely. After the wound was closed, rats were returned to their cages without immobilization. All animals survived the surgical procedure and exhibited no signs of infection.

Application of LIPUS

Under anesthesia, the rats were shaved around the operated left knees. Coupling gel was applied on the skin on the medial side of the knees, where an ultrasound transducer was secured in place with tape (Fig. 1). For the experimental groups, the LIPUS device was activated for 20 min/d, 5 d/wk, for 2 consecutive wk (Wang *et al.* 1994). For the control group, the device was affixed to the rat knees as scheduled for the experimental groups, but was not activated.

For LIPUS treatment, 30 rats with an intra-articular fracture of the medial tibial plateau were divided into three groups evenly. In the LIPUS₁₋₂ group, LIPUS was applied to the operated knees for the first 2 wk, starting on the second day post-surgery. In the LIPUS₄₋₅ group, LIPUS was applied for 2 wk, during weeks 4 and 5 after creation of the intra-articular fracture. In the LIPUS₀ group,

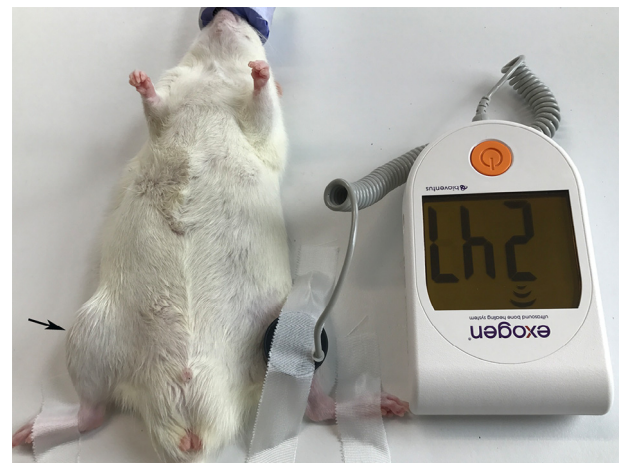


Fig. 1. Setup for application of LIPUS. Under anesthesia, the rat is in supine position and the hind limbs are secured to the table with hip in full abduction and knee in 90° bending. The transducer of the ultrasound device is attached to the medial side of the knee with a piece of tape. The transducer is positioned directly above the intra-articular fracture on the medial tibial plateau. The arrow indicates the level of the opposite knee.

Table 1. Modified Mankin score of osteoarthritis

Structure	Grade	Cells	Grade	Safranin-O staining	Grade
Normal	0	Normal	0	Normal	0
Surface irregularities	1	Diffuse hypercellularity	1	Slight reduction	1
Pannus and surface irregularities	2	Cloning	2	Moderate reduction	2
Clefts to transitional zone	3	Hypocellularity	3	Severe reduction	3
Clefts to radial zone	4			No dye noted	4
Clefts to calcified zone	5				
Complete disorganization	6				

the operated knees were not treated with LIPUS. All rats were euthanized at week 8 after surgery.

Gait analysis

On the day before surgery and every week after LIPUS treatment began, the gait of the rats was recorded using CatWalk system (Version 7.1, Noldus, Leesburg, VA, USA). The pixel intensity threshold was set at 1 (in a range of 0–255). A threshold of 55 pixels was used to register a paw print. Each rat walked through the platform three times voluntarily for gait recording, and from each walk, at least three consecutive paw prints were used for gait analysis. The output of the gait analysis included max area (the maximum area of a paw in contact with the walking platform), print area (the total floor area contacted by the paw during the stance phase), paw angle (the angle between the paw long axis and walking direction), stride length (the distance between two consecutive steps of the same paw), duty cycle (the percentage of stance duration over a full step), initial contact, maximum contact, intensity, paw print width, print length, stand, swing, swing speed and stand index. The procedure, setup of apparatus and definitions and units of the parameters of the CatWalk system are detailed in the manufacturer's reference manual and summarized in [Supplementary Table S1](#) (online only, available at <https://doi.org/10.1016/j.ultrasmedbio.2017.09.014>).

Histology

The harvested left rat knees were decalcified in 10% ethylenediaminetetraacetic acid (EDTA). The knees were bisected along the frontal plane to ensure tissue sections were collected from the midjoint, with full length of both tibial plateaus. Tissue blocks were embedded in OCT medium (Sakura Finetek USA, Torrance, CA, USA) and cut with a cryostat at a thickness of 10 μ m. Tissue sections were stained with Safranin O/fast green and graded for a modified Mankin score, according to cartilage tissue structure, cellular abnormality and matrix staining (Mankin et al. 1971). A higher score is indicative of more severe cartilage degeneration (Table 1). From each joint, three slides were stained and graded for PTOA pathology by two investigators who were blinded to the experimental

conditions. The consistency of the Mankin scores given by the two raters was assessed with the intra-class correlation coefficient, which was 0.95. The average Mankin score of each joint was used for analysis.

The thickness of articular cartilage on the medial tibial plateau was measured. On each tissue section, three measurements were performed, with the central point located at the narrowest joint space at the lesion site in the LIPUS₁₋₂, LIPUS₄₋₅ and LIPUS₀ groups. The cartilage thickness in the normal knees from the non-operated limbs was also measured in the same way, with the central point at the center of the narrowest joint space. The measurements of cartilage thickness in each joint were averaged for comparison among LIPUS₁₋₂, LIPUS₄₋₅, LIPUS₀ and normal groups.

Statistical analysis

Gait parameters were compared between a LIPUS group and the corresponding controls with the Wilcoxon–Mann–Whitney *U*-test. The Mankin scores, subcategory scores and average articular cartilage thickness of the LIPUS₁₋₂, LIPUS₄₋₅ and LIPUS₀ groups were analyzed with one-way analysis of variance (ANOVA), followed by *post hoc* Tukey's test. In the LIPUS₁₋₂ and LIPUS₄₋₅ groups, significantly altered gait parameters were also correlated with the corresponding Mankin scores in the same groups, using Spearman's rank correlation. Significance was set as $p < 0.05$. Statistical analyses were performed using Minitab 17 (Minitab, State College, PA, USA).

RESULTS

In the first week after intra-articular fracture, rats were able to stand and walk using the operated limbs. Gait recording was successful for all 10 rats in the LIPUS₁₋₂ group and 9 rats of the LIPUS₄₋₅ group (Fig. 2). Two rats in the LIPUS₀ group failed to register paw prints for this study, and one additional rat missed gait recording at week 8.

During the first 2 wk of intra-articular fracture, the max area of the paw in contact with the walking platform in both the LIPUS₀ and LIPUS₁₋₂ groups was reduced to less than 10% of the baseline (Fig. 3A). It slightly increased in the following weeks. By weeks 7 and 8, the max area in the LIPUS₀ group was less than half of its baseline

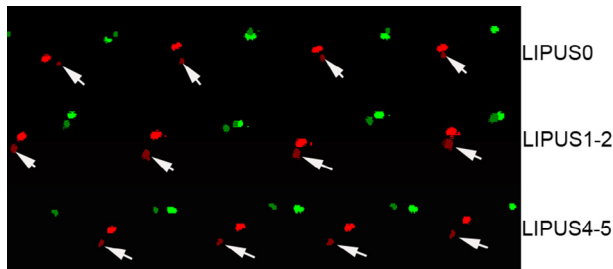


Fig. 2. Representative paw print images recorded at week 8. Images of paw prints were individually labeled for analysis. The paw prints of the right limbs are labeled *green* (prints of the hind limb in *dark green*). The paw prints of the left limbs are *red* (prints of the operated hind limb in *dark red*, indicated with *arrows*, were analyzed for comparison among LIPUS₀, LIPUS₁₋₂ and LIPUS₄₋₅ groups).

($p = 0.03$), but in the LIPUS₁₋₂ group, it did not significantly differ from its baseline ($p = 0.1$). Compared with the LIPUS₀ group, the max area of the LIPUS₄₋₅ group was increased in weeks 4 and 5 and remained so in week 8 (Fig. 3B). The max area of the LIPUS₄₋₅ group at week 8 was not significantly different from its baseline ($p = 0.8$).

Immediately after intra-articular fracture, paw print area during the stance phase was reduced to nearly 10% of the baseline (Fig. 3C). It gradually increased in the following weeks, with fluctuations, in both the LIPUS₀ and LIPUS₁₋₂ groups. By week 8, the paw print area of the LIPUS₁₋₂ group was about 60% of its baseline, but greater than that of the LIPUS₀ group ($p = 0.006$). The average paw print area of the LIPUS₄₋₅ group was greater than that

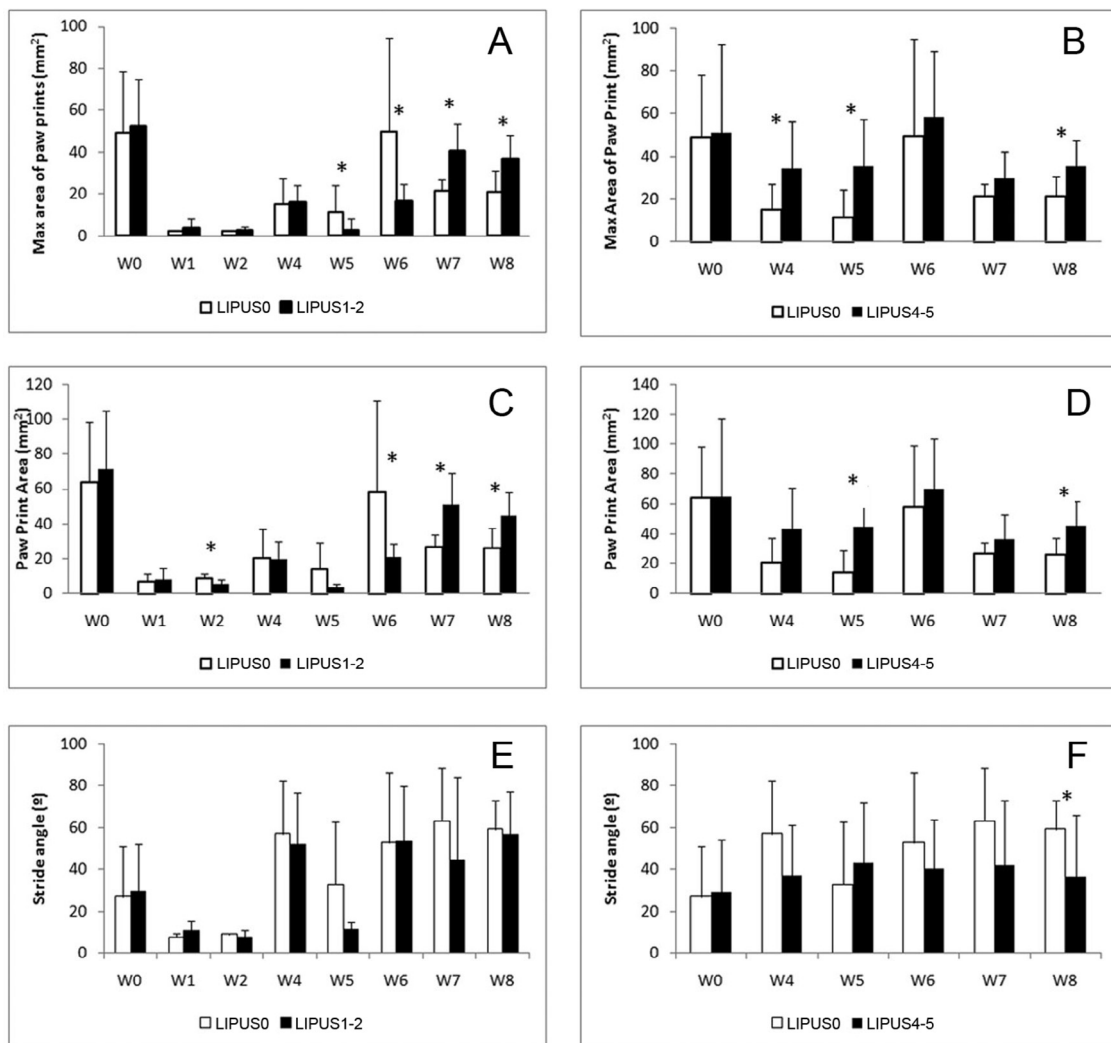


Fig. 3. Paw print features of LIPUS₁₋₂ and LIPUS₄₋₅ groups, in comparison with LIPUS₀ group. The maximum area of paw print in LIPUS₁₋₂ and LIPUS₄₋₅ groups changes at multiple time points and is consistently increased in weeks 7 and 8 (A, B). Paw print area is increased at weeks 7 and 8 in the LIPUS₁₋₂ group and week 8 in the LIPUS₄₋₅ group, compared with the LIPUS₀ group (C, D). Stride angle is not changed in LIPUS₁₋₂ group and is decreased in LIPUS₄₋₅ group at week 8 (E, F).

* $p < 0.05$, LIPUS treatment group compared with LIPUS₀ group. LIPUS = low-intensity pulsed ultrasound.

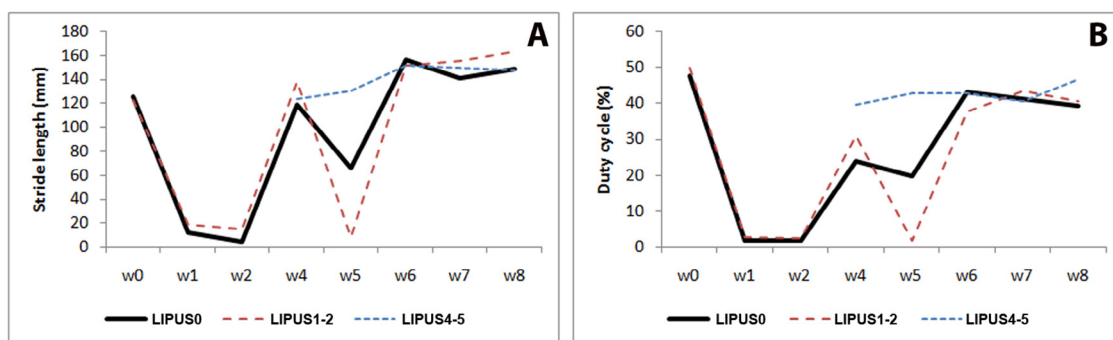


Fig. 4. Stride length and duty cycle of the rats with an intra-articular fracture in the knee. In the LIPUS₀ group, stride length is significantly decreased in weeks 1 and 2, and fluctuated and increased in the following week (A). Although stride length in the LIPUS₁₋₂ group follows the same trend as for the LIPUS₀ group, it is steady in the LIPUS₄₋₅ group. The duty cycle is significantly reduced in the first 2 wk of intra-articular fracture in the LIPUS₀ group (B). It gradually increases in the following weeks, but is still about 83% of the baseline at week 8. The duty cycle in the LIPUS₁₋₂ group generally follows the pattern of the LIPUS₀ group. In the LIPUS₄₋₅ group, however, duty cycle is relatively consistent and reaches its baseline level at week 8.

of the LIPUS₀ group and became statistically significant at week 8 ($p = 0.006$) (Fig. 3D). More importantly, the paw print area in the LIPUS₄₋₅ group at week 8 was comparable to its baseline ($p = 0.2$).

The paw angle with walking direction was reduced after intra-articular fracture, but became greater than the baseline angle in all three groups at week 4 (Fig. 3E, F). In the LIPUS₄₋₅ group, paw angle was reduced at week 8 compared with the LIPUS₀ group ($p = 0.01$). Although the angles in both the LIPUS₀ and LIPUS₁₋₂ groups at week 8 remained greater than their baselines ($p = 0.007$ and 0.004 , respectively), the paw angle in the LIPUS₄₋₅ group did not significantly differ between the baseline and week 8 ($p = 0.3$).

Stride length was drastically reduced in the LIPUS₀ group, from 120 mm at week 0 to 12 mm in week 1 ($p = 0.004$) and 5 mm in week 2 ($p = 0.004$) (Fig. 4A). The stride length in the LIPUS₀ group returned to baseline level in weeks 4 and 5 ($p = 0.9$ and 0.1). After week 6, the stride length in the LIPUS₀ group became longer than the baseline: It increased 25% at week 6 ($p = 0.006$), 13% at week 7 ($p = 0.04$) and 19% at week 8 ($p = 0.02$). At the same time point, differences in stride length among the LIPUS₀, LIPUS₁₋₂ and LIPUS₄₋₅ groups were not statistically significant.

Duty cycle was sharply reduced after intra-articular fracture in the LIPUS₀ group, from nearly 50% at week 0 to 4% at week 1 and 2% at week 2 ($p = 0.004$) (Fig. 4B). The duty cycle in the LIPUS₀ group increased in the following weeks, but was still less than its baseline in weeks 4 and 5 ($p = 0.01$). It became insignificantly different from the baseline in weeks 6 and 7 ($p = 0.09$ and 0.07), but was slightly reduced at week 8 ($p = 0.04$). Although the LIPUS₁₋₂ group had a duty cycle pattern similar to that of the LIPUS₀ group, the LIPUS₄₋₅ group had a steady duty cycle. Importantly, at week 8, the duty cycle of the LIPUS₄₋₅

group was not only significantly greater than that LIPUS₀ group ($p = 0.01$), but also nearly even with its baseline ($p = 0.45$).

Other gait parameters, including initial contact area, max contact area, print length and width, intensity, swing, swing speed and stand index, exhibited no meaningful difference among LIPUS₀, LIPUS₁₋₂ and LIPUS₄₋₅ groups.

On histology, fractures of medial tibial plateau healed in all three groups, as no recognizable fracture lines existed in the medial tibial plateau. Destruction of articular cartilage on the medial tibial plateau, however, was extensive (Fig. 5). Cartilage lesion included clefts and loss of articular cartilage, extending as deep as subchondral bone. Other prominent pathologies were depletion of proteoglycans from the cartilage matrix, ranging from partial reduction to complete loss, and the formation of chondrocyte clusters. Femoral articular cartilage opposite to the site of tibial osteotomy was fibrillated with depletion of proteoglycans. Articular cartilage in the lateral compartment of the knees remained intact. The average Mankin scores of the LIPUS₁₋₂ and LIPUS₄₋₅ groups were similar (5.4 ± 1.5 and 4.6 ± 1.7 , respectively, $p = 0.7$), and both were significantly less than the score for the LIPUS₀ group (8.8 ± 1.4 , $p = 0.009$ and 0.002 , respectively) (Fig. 6A). Analyses of the Mankin score subcategories revealed that, compared with the LIPUS₀ group, the cellular abnormality scores in both the LIPUS₁₋₂ and LIPUS₄₋₅ groups were reduced (both $p = 0.001$). In the subcategory of structural integrity, the score of the LIPUS₄₋₅ group was significantly reduced compared with that of the LIPUS₀ group ($p = 0.02$). The subcategory scores of matrix reduction, assessed by SafraninO staining, did not significantly differ among the LIPUS₀, LIPUS₁₋₂ and LIPUS₄₋₅ groups ($p = 0.4$). The average articular cartilage thickness around the PTOA lesion in the LIPUS₀,

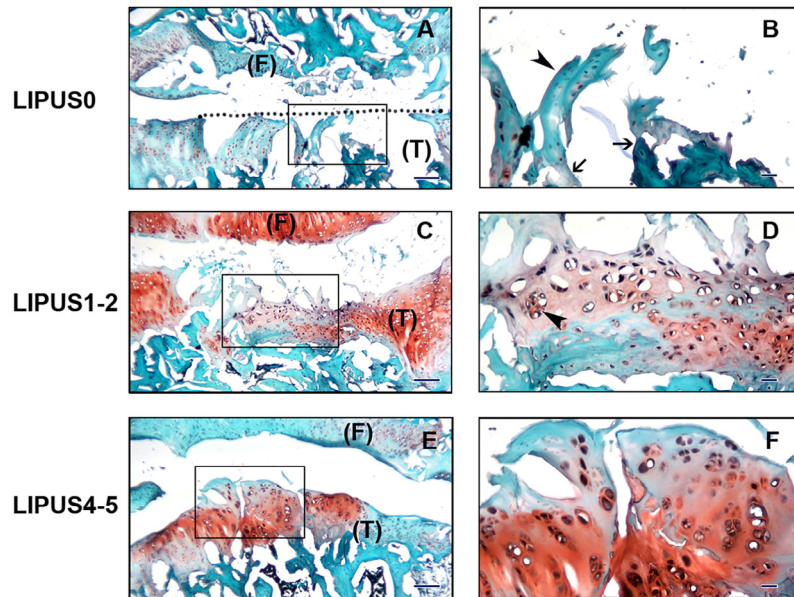


Fig. 5. Histology of the post-traumatic osteoarthritic knees in the LIPUS₀, LIPUS₁₋₂ and LIPUS₄₋₅ groups. Articular cartilage destruction involves both sides of the medial tibial plateau and the opposite femoral condyle. Around the lesion, articular cartilage defect extends into subchondral bone (*dotted line* marks the supposed cartilage surface) (A). The framed area at higher magnification exhibits severe depletion of proteoglycans from the residual cartilage, hypocellularity (*arrowhead*) and clefts extending into subchondral bone (*arrows*) (B). In this knee of the LIPUS₁₋₂ group, articular cartilage surface on the medial tibial plateau is irregular with large clefts, which extend into the deep layer of cartilage (C). The selected area at higher magnification reveals the formation of chondrocyte clusters (*arrowhead*) and regional depletion of proteoglycans (D). This knee joint from the LIPUS₄₋₅ group has areas of depletion of proteoglycans, in both tibial and femoral cartilage, with uneven cartilage surfaces (E). Higher magnification reveals clefts and proliferative chondrocytes in articular cartilage (F). Safranin O/fast green/hematoxylin staining. (F) = femoral condyle; (T) = medial tibial plateau. Bar = 100 μ m (A, C, E) and 50 μ m (B, D, F).

LIPUS₁₋₂ and LIPUS₄₋₅ groups was significantly reduced from that of the normal knees ($p = 0.003$) (Fig. 6B). Although cartilage thickness did not differ between the LIPUS₀ and LIPUS₁₋₂ groups, it was slightly increased in the LIPUS₄₋₅ group compared with LIPUS₀ group ($p = 0.03$).

In the LIPUS₁₋₂ group, the Mankin score and gait parameters, including max area and paw print area, at week 8 did not correlate. In the LIPUS₄₋₅ group, the max area and Mankin score were negatively correlated ($r = -0.76$, $p = 0.03$) (Fig. 6C). The Mankin score and paw print area in the LIPUS₄₋₅ group were also negatively correlated at week 8 ($r = -0.85$, $p = 0.007$) (Fig. 6D). The Mankin score and paw angle in the LIPUS₄₋₅ group were not correlated at week 8.

DISCUSSION

In the LIPUS₀ group, the operated joints exhibited severe cartilage destruction and had great Mankin scores, indicating that the intra-articular fracture on the medial tibial plateau induced PTOA. Joint injury, such as torn anterior cruciate ligament, increases inflammatory cytokines in

the joint fluid within 24 h (Irie *et al.* 2003). In most PTOA models, cartilage degradation becomes the dominant pathology in 3–4 wk (Furman *et al.* 2007). This study separately investigated the effect of LIPUS on two distinct pathologic events of PTOA: (i) joint injury and (ii) cartilage degeneration. In the LIPUS₁₋₂ group, LIPUS was applied when the operated joints were predominantly inflammatory. The LIPUS₄₋₅ group was used to examine the effect of LIPUS on the established pathology of cartilage degradation.

A pain gait, which features reduced stride length and duty cycle (Angeby-Möller *et al.* 2008; Boettger *et al.* 2009; Gabriel *et al.* 2007; Jacobs *et al.* 2017), was observed in the LIPUS₀ group, particularly in the first 2 wk after intra-articular fracture. LIPUS applied at the early stage of intra-articular fracture (LIPUS₁₋₂) had little effect on a typical pain gait resulting from the fracture, compared with the controls (LIPUS₀), but did immediately reduce print area, which may relate to pain and other limb disabilities (Gabriel *et al.* 2007; Liu *et al.* 2013). These immediate gait reactions to LIPUS treatment in the LIPUS₁₋₂ group could be signs of inflammation suppression in the operated joints, because LIPUS can inhibit the expression of inflammatory

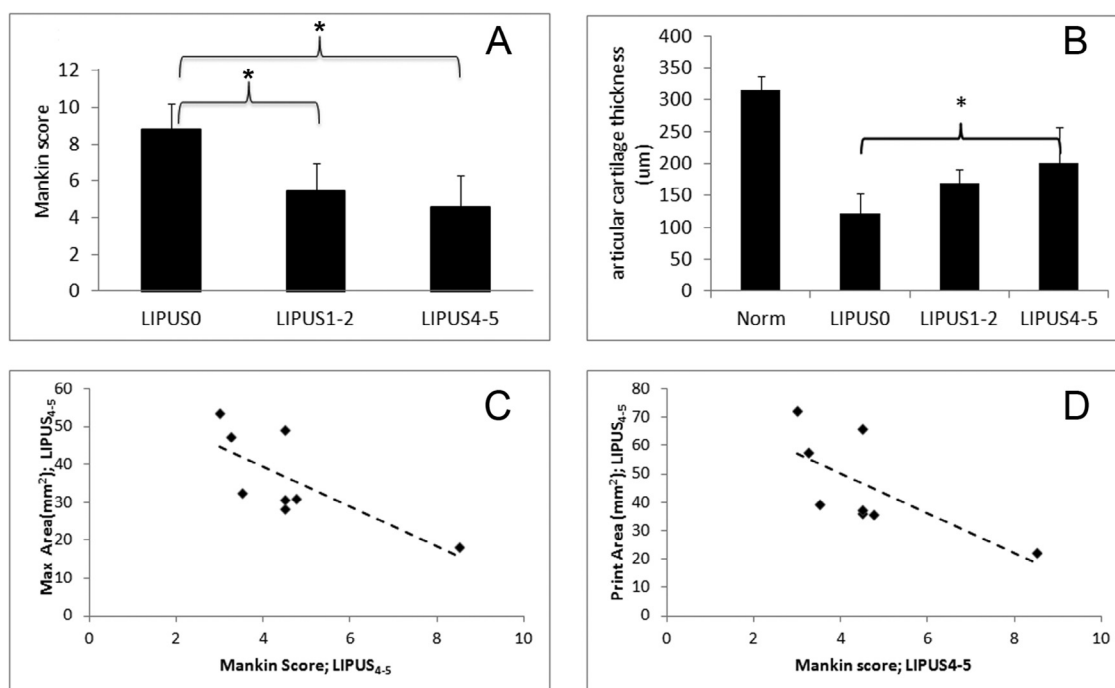


Fig. 6. Quantification of PTOA pathology in the rat knees from the LIPUS₀, LIPUS₁₋₂ and LIPUS₄₋₅ groups (A). The average Mankin score of the LIPUS₀ group is the highest among the three groups. The Mankin scores of the LIPUS₁₋₂ and LIPUS₄₋₅ groups are about the same and decreased compared with those of the LIPUS₀ group. The thickness of articular cartilage is significantly reduced in the LIPUS₁₋₂, LIPUS₄₋₅ and LIPUS₀ groups, compared with the normal knees. Articular cartilage thickness is greater in the LIPUS₄₋₅ group than in the LIPUS₀ group (B). The Mankin scores and paw print maximum areas in the LIPUS₄₋₅ group at week 8 are negatively correlated (C). The Mankin scores and paw print area are negatively correlated in the LIPUS₄₋₅ group at week 8 (D). * $p < 0.05$. LIPUS = low-intensity pulsed ultrasound; Norm = normal knees.

mediators and neutrophil infiltration in synovium (Chung et al. 2012). Interestingly, in the LIPUS₁₋₂ group, the max area, print area, duty cycle and stride length at weeks 7 and 8 were close to their baseline values. This suggests that LIPUS applied at the early stage of joint injury could have lasting effects on the recovery of the joints. LIPUS generally stimulates cell proliferation and matrix production (Zhang et al. 2003a), through which LIPUS therapy enhances tissue healing in bone and cartilage (Cook et al. 2001; Heckman et al. 1994). This study was not designed to investigate the effect of LIPUS on soft tissue and bone healing because rodents quickly heal bone and soft tissue injury in 2–3 wk (Katano et al. 2011). By week 8, the bony fracture in the medial tibial plateau had healed in all the animals, including the LIPUS₀ group. However, it was possible that LIPUS treatment enhanced tissue healing in the operated joints and stabilized the joints, which ultimately prevent or delay the development of PTOA.

In the LIPUS₄₋₅ group, compared with the LIPUS₀ group, the max area, print area, stride length and duty cycle were increased during the period of LIPUS treatment (weeks 4 and 5). These immediate gait responses were different from or opposed to what observed in the LIPUS₁₋₂ group. It is likely that LIPUS signals interfere with

stage-specific pathologic pathways in PTOA. The different effects of LIPUS on early injury and later joint degeneration leave the opportunity to further optimize LIPUS application, including timing, for PTOA treatment and prevention. In the LIPUS₄₋₅ group, nearly normal max area, print area, paw angle, stride length and duty cycle were detected in weeks 7 and 8. These results indicate that LIPUS treatment partially improved the function of the joints where PTOA had established.

In both the LIPUS₁₋₂ and LIPUS₄₋₅ groups, the knees with intra-articular fracture remained osteoarthritic at week 8, but the severity of PTOA was reduced, compared with the LIPUS₀ group. Analyses of the subcategories of Mankin scores revealed that LIPUS treatment particularly improved cellular morphology in the PTOA knees and structural integrity (LIPUS₄₋₅ group only). On average, the cellularity score in both the LIPUS₁₋₂ and LIPUS₄₋₅ groups was between normal and diffuse hypercellularity, whereas it was between hypercellularity and cloning in the LIPUS₀ group. This is consistent with previous studies that treated PTOA and osteoarthritic cartilage with LIPUS (Naito et al. 2010; Tan et al. 2015). In a rabbit study, LIPUS treatment increased the volume of reparative cartilage and reduced degenerative changes in osteochondral

defects (Cook *et al.* 2001). Like an osteochondral defect, the intra-articular fracture created in the present study broke the barriers between the bone marrow cavity and cartilage. LIPUS could stimulate mesenchymal stem cells in bone marrow to migrate into damaged cartilage and differentiate into the chondrogenic lineage for cartilage repair (Jang *et al.* 2014; Lee *et al.* 2006). In a 27-patient clinical trial, LIPUS treatment increased the thickness of articular cartilage in OA knees about 90 μm (Loyola-Sánchez *et al.* 2012).

In the LIPUS₄₋₅ group, LIPUS was applied to the injured joints, where the predominant pathology is cartilage matrix breakdown and chondrocyte apoptosis (Del Carlo and Loeser 2008). Studies have reported that LIPUS treatment reduces chondrocyte apoptosis and downregulates expression of the collagenolytic MMP-13 in OA joints (Jia *et al.* 2016a; Li *et al.* 2011). Although there are indications that LIPUS treatment increases the contents of type II collagen and aggrecan in cartilage (Naito *et al.* 2010; Zhang *et al.* 2003b), depletion of proteoglycans was not improved in either the LIPUS₁₋₂ or LIPUS₄₋₅ group. The subcategory scores of Safranin-O staining in the LIPUS₀, LIPUS₁₋₂ and LIPUS₄₋₅ groups were close and in the range between moderate and severe reduction.

In both the LIPUS₁₋₂ and LIPUS₄₋₅ groups, LIPUS treatment achieved similar improvement of PTOA pathology, according to Mankin scores. Other studies, however, have reported that the timing of LIPUS application during the development of PTOA is linked to the outcome (Gurkan *et al.* 2010). It is possible that, at the time of LIPUS application in the LIPUS₄₋₅ group, PTOA was not as advanced as in the other studies. In a pilot study, focused LIPUS was able to relieve pain and increase the range of motion of the OA knees (Jia *et al.* 2016b). The interaction between the physical signals of LIPUS and the molecular pathology of OA, however, may prove to be complex. Further research on this subject is required to determine the clinical indications for LIPUS treatment and maximize its value as an OA therapy (Rothenberg *et al.* 2017).

CONCLUSIONS

LIPUS treatment partially improved the gait and PTOA pathology of the animals with intra-articular fracture in the knee. LIPUS applied either at the early stage of intra-articular fracture or during PTOA development had lasting effects on the PTOA pathology in rat knees. This study demonstrated the therapeutic potential of LIPUS for the prevention and treatment of PTOA.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ultrasmedbio.2017.09.014>.

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