Effects of Low-Intensity Pulsed Ultrasound on Tendon–Bone Healing in an Intra-articular Sheep Knee Model

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Purpose: This study reports the mechanical and histologic properties of intra-articular tendon–bone healing with the application of low-intensity pulsed ultrasound (LIPUS) in an ovine knee model. **Methods:** A single digital extensor tendon autograft from the right hoof was used as the graft in 89 adult sheep. Femoral fixation was achieved with an EndoButton (Smith & Nephew Endoscopy, Andover, MA) and tibial fixation by tying over a bony post. LIPUS treatment was performed daily for 20 minutes over the femoral and tibial tunnels until sacrifice in all groups, apart from the 26-week group, which was treated only for the first 12 weeks. Histology was performed at 3, 6, 12, and 26 weeks. Mechanical testing was performed at 6, 12, and 26 weeks. **Results:** The LIPUS-treated group showed increased cellular activity at the tendon–bone interface and general improvement in tendon–bone integration and vascularity. Stiffness and peak load were greater compared with the control group at 26 weeks after surgery ($P < .05$). **Conclusions:** The application of LIPUS appears to improve healing at the tendon–bone interface for soft tissue grafts fixed with a suspensory fixation technique. Histology supports a benefit based on increased integration between tendon and bone and a biologically more active interface, which would account for the improved mechanical properties. **Clinical Relevance:** The indications of LIPUS may be expanded to include tendon–bone healing, for example, in anterior cruciate ligament reconstruction. **Key Words:** Anterior cruciate ligament—Ultrasound—LIPUS—Tendon—Bone healing.

Anterior cruciate ligament (ACL) reconstruction is a common orthopaedic procedure; more than 100,000 cases are reported annually in the United States alone.[1] Graft failure or instability occurs in as many as 10% of cases.[2] The gold standard method of ACL reconstruction uses autologous tendon grafts, such as hamstrings or patellar tendon, which are inserted into bone tunnels in the femur and the tibia and are anchored at each end through a variety of methods.

It has been suggested that the weakest point in the reconstructed knee is the fixation of the tendon within these bone tunnels,[3] and that a significant amount of slippage between tendon and bone may occur when the graft is fixed.[4] Healing of the reconstruction is achieved at the interface between the bone tunnel and the tendon graft. Placing a tendon graft into a tunnel creates a new tendon–bone interface along the entire tunnel. Peterson and Laprell[5] and Robert and coworkers[6] reported a fibrous interface in femoral and tibial tunnels in human biopsy specimens obtained at revision surgery. The time needed to develop such an interface in humans was reported to be much longer than that reported in animal models.[7-10] An increased rate of tendon–bone healing in ACL reconstruction would provide a great advantage in reducing the time required to restore normal functional behavior in the knee.

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Ultrasound has been widely used in medicine as a diagnostic, therapeutic, and disruptive (surgical) tool. In surgery, ultrasound is delivered at intensities ranging between 5 and 300 W/cm^2 to fracture kidney stones or to assist in the removal of polymethylmethacrylate bone cement. Therapeutic ultrasound makes use of tissue-heating capabilities with intensities ranging from 1 to 3W/cm^2; diagnostic ultrasound lies in the lower energy range with 1 to 50 mW/cm^2 and is considered to be nonthermal and nondestructive.[11]

Low-intensity pulsed ultrasound (LIPUS) has a frequency of 1.5 MHz, which is administered in bursts of 200 microseconds with a duty cycle of 0.2. Energy is delivered at 30 mW/cm^2 and has been shown to significantly reduce time to bone union in fresh fractures and delayed unions/nonunions.[12-16] LIPUS has also been reported to increase the amount of callus, strength, and stiffness in fractures,[16-18] to accelerate bone healing in other defects,[19] and to improve healing of spinal fusion.[20] LIPUS has been reported to alter surface irregularities and the histologic appearance of cartilage defects[21] and possibly to enhance the early healing of medial collateral ligament injuries in a rodent model.[22]

Ultrasound provides mechanical stimulation to the tissues by means of high-frequency, small-amplitude pressure waves.[11,13,23] LIPUS has been shown to have positive biological effects at all stages of bone healing, including increased angiogenic, chondrogenic, and osteogenic activities.[11] An increase in prostaglandin E_2 (PGE_2) production,[16] osteoblast and fibroblast proliferation,[16,23] and increased collagen, interleukin, and vascular endothelial growth factor production[23] have been reported to occur with the application of LIPUS.

Given the positive effects of LIPUS administration on healing of bone and ligament, the purpose of this study was to determine the efficacy of daily LIPUS in improving the healing of the tendon–bone interface in an intra-articular model of ACL reconstruction in adult sheep. The null hypothesis tested was that LIPUS treatment would have no effect on tendon–bone healing in this sheep model.

METHODS

Experimental Design

Adult cross-bred wethers (18 months) were randomly allocated to 2 groups (control or LIPUS) with end points of 3, 6, 12, and 26 weeks (Table 1).

Table 1. *Experimental Design*

	3 weeks	6 weeks	12 weeks	26 weeks*
Histology				
Control	n = 2	n = 2	n = 2	
LIPUS	n = 5	n = 5	n = 5	
Mechanical				
Control	n = 8	n = 8	n = 8	n = 10
LIPUS	n = 8	n = 8	n = 8	n = 10

*Histology samples used were taken after mechanical testing was conducted at 26 weeks. LIPUS (low-intensity pulsed ultrasound) treatment was performed daily for 3, 6, and 12 weeks. The 26-week LIPUS group was treated for the first 12 weeks and was sacrificed at 26 weeks after surgery.

Surgery

After ethical approval was obtained, open intra-articular reconstruction was performed in 21 animals in the histology portion of the study and 68 animals in the mechanical testing study in accordance with an extensor tendon model. The native ACL was removed and 4.5-mm bone tunnels drilled in the femur and the tibia. A single digital extensor tendon autograft from the right hoof was used as the graft. The graft was harvested with the use of a single incision distally at the hoof, and the tendon removed with a tendon stripper. All digital extensors harvested were at least 12 cm long; after doubling over, this resulted in a graft that was 6 cm long with a diameter of 4.5 mm. A guidewire was drilled through the stump of the native ACL from the tibia to the femur. A 4.5-mm bone tunnel was drilled in the femur and tibia. The graft was passed through the tibia and into the femur and was fixed with an EndoButton (Smith & Nephew Endoscopy, Andover, MA) with No. 2 Ethibond (Ethicon, Somerville, NJ) on the femoral side and was secured to the tibia over a bony post with the use of No. 2 Ethibond. Animals were housed 2 per pen for the first 5 to 7 days after surgery and in paddocks thereafter.

Treatment

Ultrasound treatment (200-microsecond bursts of sine waves at 1.5 MHz repeated at 1 kHz, 30 mW/cm^2) was provided daily for 20 minutes on the lateral aspect of the femur and the anteromedial aspect of the tibia in the treatment group for 3, 6, and 12 weeks, and animals were sacrificed. The wool on the lateral aspect of the femur was removed at the time of surgery and approximately every 2 weeks during the treatment phase with clippers. The 26-week LIPUS group was treated for the first 12 weeks and was sacrificed at 26

weeks after surgery. The ultrasound transducer was coupled to the skin at the site of application with coupling gel and was held in place using Elastoplast (Smith & Nephew). The site of the transducers corresponds to the closest point of proximity to the sites of the bone tunnels on the lateral aspect of the femur and the medial aspect of the tibia. A treatment regimen of 20 minutes per day was chosen on the basis of clinical use of this system for fracture healing.[14] Ultrasound treatment was performed with the animals in single pens for the 20-minute period, after which they were returned to the paddock.

Mechanical Testing

Mechanical testing to failure of the operative side was performed at 3, 6, 12, and 26 weeks after surgery. Samples were tested at room temperature while kept moist with phosphate buffered saline spray. Mechanical testing was performed with the use of an MTS 858 Bionix testing machine (MTS Systems, Eden Prairie, MN). The testing technique for the ovine knee was based on a previously reported method.[24] The femur and tibia were placed into a drill template jig, with the knee capsule intact, in preparation for mounting. Two drill holes were placed in the diaphysis of the femur and tibia to match the mounting template to ensure reproducible placement of the samples. The mounting template oriented the samples in 45° of flexion to enable measurement of the load and displacement properties in an anterior draw loading profile.

The knee was dissected after the mounting holes were drilled. The capsule was carefully reflected through an anteromedial incision. The medial and lateral menisci and the PCL were removed with meticulous dissection so as not to damage the intra-articular graft, which was macroscopically examined. Samples were tested in anterior draw orientation (45°) with a preconditioning profile of 10 cycles. The EndoButton femoral fixation and tibial suture fixation were cut before testing was conducted to evaluate healing of the tendon–bone interface in the bone tunnels rather than the properties of mechanical fixation. Properties of tendon–bone healing were assumed to be equivalent to the shear properties measured by applying a tensile load to the graft and pulling it from the bone tunnels. Testing was conducted to failure at 50 mm/minute, and peak load, energy, linear stiffness, and failure mode were determined for all samples. Mechanical data were analyzed through a 2-way analysis of variance followed by a Tukey post hoc multiple comparisons test with the use of the Statistical Package for the Social Sciences (SPSS) for Windows (SPPS, Chicago, IL).

Histology

Histology was performed immediately after sacrifice on the 21 animals in the histology study and on all other animals after mechanical testing. Samples were fixed for 48 hours in phosphate buffered formalin before decalcification in 10% formic acid–phosphate buffered formalin. Tibial and femoral bone tunnels were grossly sectioned into 5-mm sections perpendicular to the bone tunnel for paraffin embedding. This resulted in serial blocks and sections of the tibial and femoral tendon–bone tunnel interface. Five-micron sections were cut with a Leica Microtome (Leica MicroSystems, Düsseldorf, Germany). Serial sections from the tibial and femoral tunnels were stained with hematoxylin and eosin and with Masson trichrome for examination under light microscopy by 2 trained observers. Qualitative histologic comparisons were made from slices taken approximately 10 mm from the articular surfaces. Histology was assessed for degree of bone–tendon incorporation, vascularity at the interfaces, and overall tendon quality within the tunnel. Vascularity was graded in a semiqualitative manner with the use of a scale from 0 to 5 (0 = no new blood vessels at the interface and tendon graft, and 5 = extensive new blood vessels). Data were analyzed with a Mann Whitney *U* test with the use of SPSS for Windows.

RESULTS

All animals recovered with no adverse events after surgery. No adverse events were encountered during daily ultrasound treatment. All animals were healthy and were ambulating normally before sacrifice. Macroscopic dissection revealed the presence of the intra-articular graft between the femur and the tibia with no differences noted between LIPUS-treated and control animals.

Mechanical Testing

Mechanical testing to failure of the operative side was performed at 3, 6, 12, and 26 weeks after surgery. Graft pullouts from the femoral tunnel and intra-articular graft failure were the failure modes observed in this study. No tibial pullout of the graft was observed in any sample (control or LIPUS treated) at any time point. All samples at 3 weeks in the control and LIPUS-treated groups failed because of pullout of the

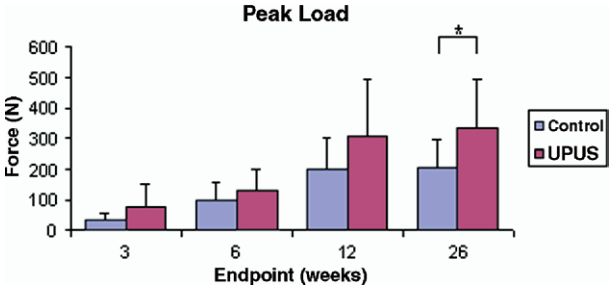

FIGURE 1. Peak load of low-intensity pulsed ultrasound (LIPUS) and control ACL reconstructions showed significant differences only at 26 weeks ($P < .05$, mean ± standard deviation).

graft from the femoral tunnels. At 6 weeks, all controls continued to fail because of femoral pullout, and a mixed mode of failure was observed in the LIPUS-treated group with some femoral pullout (4 of 8), as well as intra-articular failure of the graft itself (4 of 8). By 12 weeks, some femoral pullout continued to occur in the control group (2 of 8), and the entire LIPUS-treated group sustained graft failure. At 26 weeks, failure occurred by graft failure at midsubstance or at the femoral insertion site; however, no differences were noted between control and LIPUS-treated groups.

Peak load increased over time in LIPUS-treated and control groups. LIPUS treatment resulted in a greater peak load compared with that of controls at each end point (Fig 1). However, statistical significance was reached only at 26 weeks ($P < .05$). Similar to peak load, stiffness increased with time, and the LIPUS-treated constructs were stiffer than controls at each end point (Fig 2). This was found to be significant at 3 weeks ($P < .02$) and at 26 weeks ($P < .05$). Energy was greater for the LIPUS-treated group than for controls, but the difference was not statistically significant.

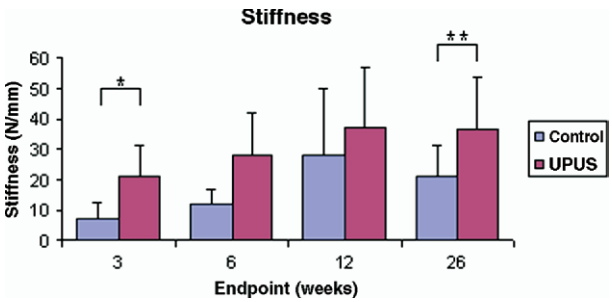

FIGURE 2. Stiffness of low-intensity pulsed ultrasound (LIPUS) and control ACL reconstructions showed significant differences at 3 and 26 weeks ($P < .05$ mean ± standard deviation).

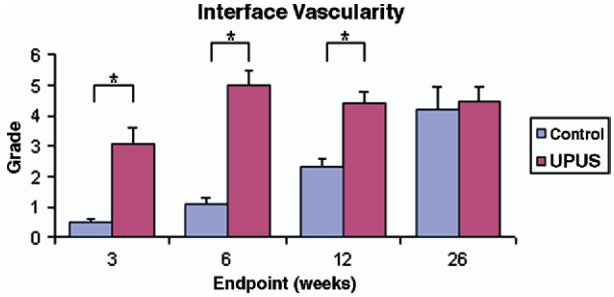

FIGURE 3. Vascularity of the tendon–bone interface based on semiquantitative grading showed improvement with low-intensity pulsed ultrasound (LIPUS) treatment at 3, 6, and 12 weeks ($P < .05$, mean ± standard deviation).

Histology

Histology at the tendon–bone interface showed generalized improvement in appearance over time in the control and LIPUS-treated groups. No evidence of a negative effect of LIPUS treatment was observed. In general, the LIPUS-treated group showed more mature organization at the tendon–bone interface and healthier cellular activity in the tendon graft and bone at all end points compared with controls. Vascularity at the tendon–bone interface was superior in the LIPUS-treated group compared with controls at 3, 6, and 12 weeks (Fig 3) ($P < .05$). The control group showed a steady increase in new blood vessels; in comparison, the LIPUS group showed a significantly accelerated rate of angiogenesis. This vascularity was found primarily within the new fibrous connective tissue at the interface between tendon graft and bone and was observed in the femoral and tibial sections.

Evidence of new bone at the margins of the bone tunnels was found in the LIPUS-treated animals; disorganized connective tissue was found to dominate the response in control animals at 3 weeks. The interface in the LIPUS group was characterized by the presence of Sharpey's fibers as early as 6 weeks (Fig 4A); however, this did not occur in the control group at this time point (Fig 4B). Histology at 12 weeks continued to show a maturing tendon–bone interface with Sharpey's fibers in the LIPUS-treated groups compared with controls. The interface in the LIPUS-treated group at 26 weeks again revealed significant differences compared with controls. In both groups, the tendon in the bone tunnel remained present and was not replaced with bone at 26 weeks. The interface in the LIPUS-treated group revealed Sharpey's fibers and a continuum between the tendon and the bone (Fig 5A). In contrast, the controls at 26 weeks had

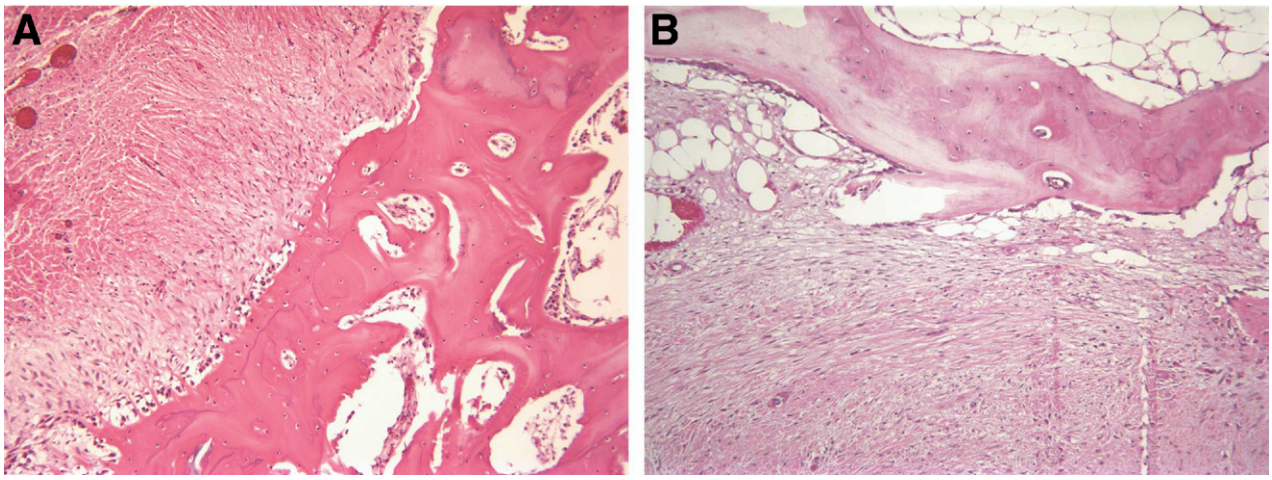

FIGURE 4. (A) Low-intensity pulsed ultrasound (LIPUS) treatment at 6 weeks (10× objective) showed an active interface with plump cells, blood vessels, and Sharpey's fibers. (B) Controls at 6 weeks (10× objective) showed a significant difference compared with LIPUS-treated animals at 26 weeks (Fig 5). No Sharpey's fibers were present.

some discrete areas of Sharpey's fibers and discontinuous contact around the perimeter of the tendon within the bone tunnel (Figure 5B).

DISCUSSION

The tendon–bone interface is highly specialized and vital to musculoskeletal function. Injuries that require some form of tendon–bone healing may be intra-articular (e.g., anterior cruciate ligament rupture) or extra-articular (e.g., rotator cuff injury). Methods of improving or accelerating the healing of tendon within a bone tunnel may be advantageous in reconstruction by reducing the time required before return to activity and reducing the likelihood of repeated injury. Healing of connective tissue, however, involves a complex cascade of coordinated molecular events that cannot be replicated by simple administration of associated factors. The current study examined the effects of LIPUS on tendon–bone healing. LIPUS, which appears to be a mechanism that may stimulate the normal biological response to injury, is a noninvasive, low-risk treatment alternative.

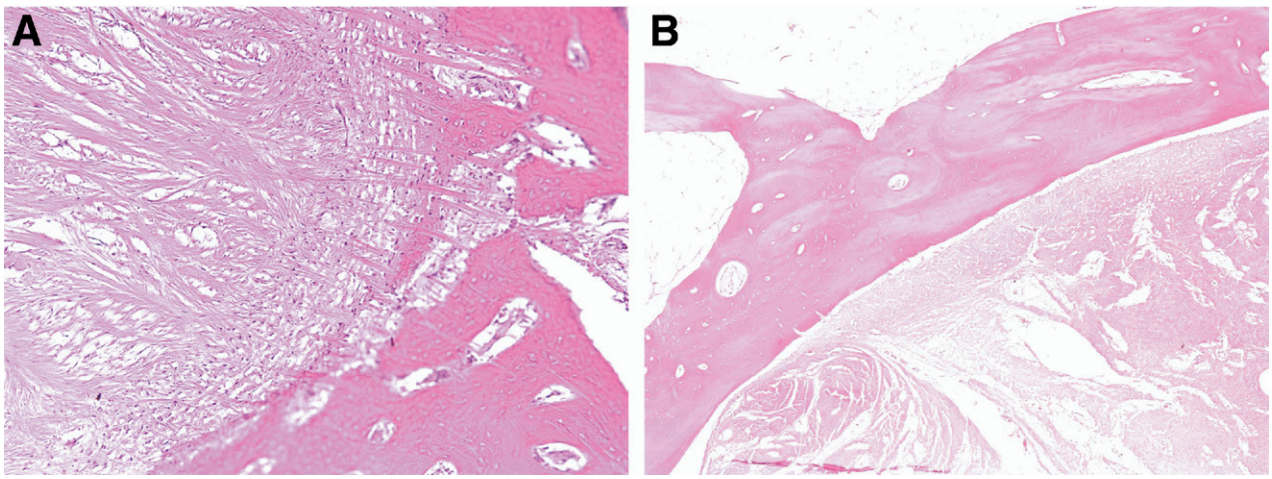

FIGURE 5. (A) Tendon–bone interface at 26 weeks in the low-intensity pulsed ultrasound (LIPUS)-treated group (original magnification ×4). The presence of Sharpey's fibers is noted at 26 weeks, even though LIPUS treatment was performed only for the first 12 weeks. Some tendon degeneration is noted at this time point. (B) The tendon–bone interface of the 26-week control group (original magnification ×4) showed some focal areas of attachment.

The US Food and Drug Administration approved LIPUS as treatment for the accelerated healing of fresh fractures in 1994 and for nonunions in 2000.[11] Smith & Nephew (Exogen) currently markets a LIPUS Sonic Accelerated Fracture Healing System (SAFHS) for the treatment of fresh fractures and nonunions. The SAFHS device is applied by the patient at home for 20 minutes a day, 7 days a week. LIPUS was chosen in the current study because it is already clinically available and is a proven safe technology.

Daily application of LIPUS for 20 minutes per day improves healing of bone under a wide range of circumstances such as fresh fractures, bone defects, and delayed unions or nonunions.[11,15,17,18,20] Furthermore, these improvements have been seen in cartilage defects[21] and ligament injuries.[22] It was hypothesized that this pattern of improved and accelerated healing with the application of LIPUS may be extended to the healing of a tendon within a bone tunnel, as is commonly required in reconstruction of the ACL. A treatment time of 20 minutes per day was chosen because this is what is clinically used for bone.

Biomechanical testing of the tendon–bone interface revealed that a weak link in the reconstruction during early time points is indeed tendon–bone healing in the femoral tunnel. We did not examine the material properties (stress and modulus) in the current study and chose to report only the structural properties (load and stiffness), considering that failure modes varied between time points and that the test considered tendon–bone interface healing. Actuator displacement as opposed to a noncontacting strain technique was used to monitor deformation. This was done because the testing was designed to evaluate tendon–bone interface properties and to monitor deformation of the tendon within the tunnels; the graft was achieved through actuator displacement. Macroscopically, no differences were detected between the size and the appearance of the intra-articular portion of the graft. This is not surprising given that extensor grafts were consistent between animals.

The mode of failure at 3 weeks was always femoral pullout, and this continued to be the case in all animals in the control group at 6 weeks. The tendon–bone interface in this sheep model remained relatively immature at 3 weeks with disorganized fibrous tissue present in controls; some new bone formation was noted in LIPUS-treated animals. Pullout of the tendon graft at the early time points is not surprising in that the EndoButton fixation was removed and healing between tendon and bone tunnel provided the only resistance to loading. By 26 weeks, reconstructions failed in the midsubstance of the graft in both groups; hence, it was difficult to establish the true properties of tendon–bone healing at this time point. Significant differences were found for peak load and stiffness (Figs 2 and 3) with LIPUS treatment at 26 weeks. This suggests that with LIPUS, improvements extended into the tendon graft midsubstance by 26 weeks, expanding beyond simple healing at the tendon–bone interface. This finding was consistent with those of Takakura,[22] who found improvement in healing of the medial collateral ligament with LIPUS treatment in a rodent model.

Histologic analysis of the tibial and femoral tunnels and the intra-articular tendon graft showed generally better improvement with LIPUS treatment compared with controls. Angiogenesis based on the presence of new blood vessels was stimulated earlier and with greater proficiency at the tendon–bone interface and within the tendon graft itself. Increased vascularity observed with ultrasound treatment did not appear to interfere with the healing process, as can be seen in the general improvement of the histologic appearance of the interface and in mechanical properties over time. Whether excessive vascularity has a potentially negative effect on the properties of the reconstruction is beyond the scope of this study.

Given their plump morphology, osteoblastic and fibroblastic cells appeared to be more active in the LIPUS group at all end points. Overall, tendon–bone healing was improved and accelerated in the LIPUS-treated group. Bone ingrowth was accelerated in the LIPUS group, which was evidenced by the presence of Sharpey's fibers at 6 weeks (Fig 4A) in contrast with controls (Fig 4B). The beneficial effects of LIPUS treatment were still evident at 26 weeks (Fig 5A), even though LIPUS treatment ceased at 12 weeks. This may be due to the biological head start that ultrasound provides to the healing site. Tissue remodeling continued to occur.

The exact mechanisms that produce these biological effects in response to ultrasound signals are unknown; however, at least 2 major systems are at work. First, because ultrasound is transmitted in longitudinal pressure waves, micromechanical loads on the order of 10 mg[20] are applied to the bone; this may trigger the remodeling phenomenon described by Wolff's law.[11,13] Second, the effect of these pressure waves is to distort the cellular membranes, which may directly alter the expression of particular genes and/or modify transmembrane channels, thereby influencing the transportation of ions across cellular boundaries.[13]

The benefits of LIPUS application at the cellular level have been extensively reported for bone[11,14,15,18,19,23,25,26]; however, to our knowledge, this is the first study undertaken to address tendon–bone healing. Although the present study confirms past work into bone healing with LIPUS treatment, these results may also represent a future indication for the use of LIPUS in the healing of tendon within a bone tunnel.

Ligament insertions have been classified as direct or indirect. A direct interface is composed of 4 layers—tendon, unmineralized fibrocartilage, mineralized fibrocartilage, and bone; an indirect interface is made up of 3 layers—tendon, Sharpey's fibers, and bone. These insertions have been described in humans[27,28] and animals[28-30] and in numerous animal models of healing between tendon and bone.[7-10,31-37] Tendon–bone histology from human patients after revision ACL surgery has also been reported.[5,6,38] These reports include information on range of fixation techniques, time to failure, and graft types, but to some degree, they also provide evidence of the formation of an indirect tendon–bone interface.

Direct comparisons between abundant animal data and limited human data are difficult. Differences between models in terms of species, age, surgical technique, graft choice, fixation hardware, time periods, and end points represent a major limitation in this area of research. We chose to use a single extensor tendon soft tissue autograft placed in the tibial and femoral tunnels to examine tendon-to-bone healing. This model, however, is limited in that we performed an open reconstruction with a single doubled-over tendon, and this may not replicate or represent the biological environment present in current arthroscopic ligament reconstruction. The model did, however, allow us to examine the in vivo response of the extensor tendon when it was placed into a bone tunnel in the femur and tibia, as well as the effects of LIPUS. In this sheep model, Sharpey's fibers had not developed at 6 weeks in control animals, but they were abundant in those given LIPUS treatment. This appears to be in contrast to the findings of Kanazawa[7] and Demirag,[8] who reported use of a rabbit semitendinosus graft model and the presence of Sharpey's-like fibers at 4 and 5 weeks after surgery. Grana and coworkers,[10] using an autograft in a rabbit model of ACL reconstruction, reported intra-articular graft failure and the appearance of indirect insertion at 3 weeks after surgery. This may reflect differences between animal models in terms of fixation, graft movement, and loading that may play an important role at the healing interface, as well as variations in technical aspects of mechanical testing. Mechanical testing in the present study was designed to evaluate the properties of the interface between tendon and bone. Thus, we chose to isolate the tendon–bone interface by removing the EndoButton and tibial sutures before testing. This may account for our graft pullout at 6 weeks and midsubstance failure only at later time points after substantial healing had occurred.

The sheep model in the current study used a single extensor tendon graft doubled over, as opposed to 4 stranded repairs, which are often used clinically. As a result, the biomechanical properties after reconstruction do not represent a replacement of the native ACL but rather healing of the tendon–bone interface. This study is also limited in that we did not explore any dose effects of LIPUS treatment. The 20 minutes per day protocol was chosen on the basis of previous reports of fracture healing. Whether a shorter or longer treatment period would be effective is beyond the scope of the current study. It is interesting to note that Cook and coworkers[21] reported that a 40-minute treatment protocol improved the histologic quality of cartilage defects with LIPUS.

Finally, we explored only a single method of fixation (suspensory fixation with an EndoButton) with a soft tissue graft in this model. The effects of ultrasound on tendon–bone healing when other fixation techniques (i.e., screws, transfix posts) are used remain unknown.

CONCLUSIONS

Findings in the current study show that the application of LIPUS appears to improve healing at the tendon–bone interface for soft tissue grafts fixed with the use of a suspensory fixation technique. Histologic testing supports a benefit based on increased integration between tendon and bone and a biologically more active interface, which would account for the improved mechanical properties observed.

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