Structural and functional assessment of intense therapeutic ultrasound effects on partial Achilles tendon transection

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aDepartment of Biomedical Engineering, bAerospace and Mechanical Engineering, cDepartment of Orthopaedic Surgery, The University of Arizona, 1657 E. Helen St., Tucson, Arizona 85721, USA; dGuided Therapy Systems, 33 S Sycamore # 4, Mesa, Arizona 85202, USA

ABSTRACT

Tendinopathies and tendon tears heal slowly because tendons have a limited blood supply. Intense therapeutic ultrasound (ITU) is a treatment modality that creates very small, focal coagula in tissue, which can stimulate a healing response. This pilot study investigated the effects of ITU on rabbit and rat models of partial Achilles tendon rupture. The right Achilles tendons of 20 New Zealand White rabbits and 118 rats were partially transected. Twenty-four hours after surgery, ITU coagula were placed in the tendon and surrounding tissue, alternating right and left legs. At various time points, the following data were collected: ultrasound imaging, optical coherence tomography (OCT) imaging, mechanical testing, gene expression analysis, histology, and multiphoton microscopy (MPM) of sectioned tissue.

Ultrasound visualized cuts and treatment lesions. OCT showed the effect of the interventions on birefringence banding caused by collagen organization. MPM showed inflammatory infiltrate, collagen synthesis and organization. By day 14-28, all tendons had a smooth appearance and histology, MPM and OCT still could still visualize residual healing processes. Few significant results in gene expression were seen, but trends were that ITU treatment caused an initial decrease in growth and collagen gene expression followed by an increase. No difference in failure loads was found between control, cut, and ITU treatment groups, suggesting that sufficient healing had occurred by 14 days to restore all test tissue to control mechanical properties. These results suggest that ITU does not cause harm to tendon tissue. Upregulation of some genes suggests that ITU may increase healing response.

Keywords: Achilles tendon, Gene expression, Healing response, Intense therapeutic ultrasound, Ultrasound

1. INTRODUCTION

1.1 Background

Lower extremity tendinopathies and tears are extremely common, with a prevalence of greater than 11 per 1000 person-years1. These disorders have a significant impact on quality of life and lead to a substantial amount of time lost to work and sporting activities2. Initial treatment for tendinopathy consists of anti-inflammatory medications, activity modification, and physical therapy including eccentric exercise3. Even with these treatments, healing occurs in only about 80% of patients and often takes many months4,5. Reasons for delayed healing include poor vascularity of the tendon tissue and myxoid degeneration within the tendon5. Patients who fail to heal often go on to surgery that aims to stimulate a healing response within the degenerated tendon. Surgical treatment is successful in the majority of patients but is costly and exposes the patient to the risks that are inherent in surgery (infection, nerve damage, tendon rupture, etc)6. A nonsurgical alternative that could accelerate the healing process or stimulate tendon healing in those that have failed conventional therapy is greatly needed.

Energy therapies such as low intensity ultrasound, extracorporeal shockwave therapy (ESWT) and low level laser therapy (LLLT) have been employed to speed the healing of tendon ruptures and other tendinopathies. For low intensity ultrasound, there is evidence for a mechanism of action in a rat model of Achilles rupture (inflammation is increased, as well as induction of TGF 1 and collagen I and III)7. Animal studies suggest that ESWT can increase blood flow and induce an inflammatory-mediated healing process8. LLLT may promote more effective tissue healing by promoting type I collagen and decorin synthesis9. Meta-analyses have shown limited to moderate effectiveness of ESWT10 and LLLT11 over alternative non-operative treatment strategies. A challenge with each of these modalities is that the tissue of interest is not directly targeted, and the dosage at the Achilles tendon is variable and unknown.
1.2 Intense Therapeutic Ultrasound

Intense Therapeutic Ultrasound (ITU) is a versatile modality that uses a highly directive source geometry to focus the ultrasound energy into a tightly confined region (<1 mm3) to cause selective tissue thermal coagulation. The size and location of the lesions can be precisely controlled, ITU is similar to high intensity focused ultrasound (HIFU), but is specifically designed to create multiple, very small coagulative lesions, with the specific purpose of stimulating a reparative tissue response, rather than ablating macroscopic tissue regions leading to necrosis. ITU can operate in two functioning modes: imaging (which is used to locate the region of interest) and treatment (which delivers a series of high-energy ultrasound exposures at a given depth within the tissue.). The overlying tissue is spared, and an accurate, known dose can be placed precisely into the tissue of interest. Because it actually creates a lesion, ITU shares some commonality (confined intentional damage) with percutaneous tenotomy, which has been shown to be effective in treating tendinosis when conservative management fails. Unlike tenotomy, ITU does not have risks of hematoma or infection, nor does it cause damage to overlying tissues.

ITU has been used clinically for treating the facial skin, with the effect of “rejuvenation” noted, and it has been shown to be safe. Histologically, it has been proven that ITU induces greater dermal collagen with thickening of the dermis and straightening of elastic fibers in the reticular dermis. Based on promising results in dermal tissues, two studies were performed to assess the feasibility of using ITU in the treatment of partial Achilles rupture in a rabbit and a rat model. The study assessed expression of cytokine, growth factor, and collagen production genes, visualized the time course of healing by imaging ultrasound, assessed the final strength characteristics of treated and control tendons, and imaged in situ and explanted tendons with optical coherence tomography (OCT) and histology.

2. METHODS

2.1 Animal Models

Both rabbits and rats were used in two separate studies. All procedures were performed under a protocol approved by the University of Arizona Institutional Animal Care and Use Committee. For the first study, 20 New Zealand White rabbits weighing approximately 2.5 kg were obtained from Harlan Laboratories (Indianapolis, Indiana, USA) and acclimated for one week prior to use in the study. Rabbits were anesthetized with a mix consisting of 2cc (10mg/ml) Acepromazine Maleate + 5cc (100mg/ml) Ketamine HCl + 8cc (20mg/ml) Xylazine, 1 ml/kg intramuscular (IM). Prior to surgery, and at time points of 4, 16, 28, and 40 hours after surgery, the animals were given Buprenorphine, 0.05 mg/kg SC. The hindlimbs of the animals were shaved and depilated, and an incision made in the skin. The Achilles tendon complex was isolated, and an incision made in the paratenon. On the right hindlimb, the lateral 50% of the Achilles tendon was divided 1.5 cm above the calcaneus. The left hindlimb underwent the same procedures except that the Achilles tendon was not divided (operative control). The paratenon was repaired with running absorbable monofilament suture and the overlying skin sutured with running nylon suture. The surgical site was protected with gauze and self-adhering wrap. The rabbits were confined to their cages for the first 7 days, after which they were allowed every-other-day exercise. Skin stiches were removed at 7 days or at the time of explant if earlier.

For the second study, 118 Harlan Sprague Dawley outbred male rats weighing between 250-275g were utilized for the study. A procedure similar to that detailed in Akamatsu et al. was performed. They were anesthetized with either ketamine/xylazine (60/7.5mg/kg) or 1-5% isoflurane. On the right hindlimbs, a cut in the skin and partial laceration of the Achilles tendon was made. A small incision in the skin of the left leg over the Achilles tendon was also made, matching the length of the incision on the right leg, but including only the skin and not the underlying Achilles tendon (sham surgery). The incisions were sealed with surgical glue. On some animals, an 18G needle was inserted into the left dorsal tail tendon bundle at a 45° angle along the longitudinal axis, such as to puncture the bundle through its entire depth. Three punctures were made ~2 mm apart. Antibiotic ointment was applied to both hindlimb and tail wounds, long-acting Buprenorphine was provided for pain control, and animals were monitored until ambulatory.

2.2 ITU Treatment

A commercial system (Guided Therapy Systems, Inc. Gen IITM, Mesa, Arizona, USA) was utilized for treatment 24-48 hours after surgery. The animals were lightly anesthetized (0.5 ml/kg Rabbit mix, or 1-2% isoflurane for the rats) for the procedure. On the rabbits, the wrap and gauze was removed and the Achilles (and the cut, on right leg) was located in imaging mode. The transducer was positioned to avoid treatment directly over the skin and paratenon incision. The following source conditions were utilized: 7.5 MHz frequency, 1.5J energy, 100 msec exposure time, 25 mm length of
exposure line, 1.3 mm distance between coagula, and 66 msec time delay after each coagulation. Four lines of 20 coagula were made parallel to the longitudinal axis of the Achilles tendon, for a total of 80 coagula in the tendon. On the rats, a similar procedure was performed, although there was no wrap to remove on the leg. The following source conditions were used: 7.5 MHz frequency, 0.4J energy, 10 msec exposure time. Ten individual coagula were placed into the Achilles tendon and surrounding tissue. For both rats and rabbits, treatment was performed on only one leg, alternating right and left legs. Thus there were four categories of Achilles tendons: uncut and untreated (UC/UT; control), uncut and treated (UC/T), cut and untreated (C/UT) or cut and treated (C/T). On some rats, the left dorsal lateral tail tendon was treated with 10 individual coagula at the same parameter used for the Achilles tendon.

2.3 Ultrasound Imaging

For ultrasound imaging of rabbits, no anesthesia was necessary; the animals were carefully restrained with a towel wrap. A high frequency ultrasound system (Ardent Sound Spark™, Mesa, Arizona, USA) was used to visualize the entire rabbit Achilles tendon before surgery, before and after ITU therapy, and at time of explant. Some rabbits were imaged at intermediate time points as well. Rats were only imaged with ultrasound before and/or after ITU treatment and at the time of explant, when they were already anesthetized.

2.4 Explant

After humane euthanasia (sedation prior to placement in a CO2 chamber), the Achilles and/or tail tendon was exposed and photographed. For gene expression analysis by polymerase chain reaction (PCR), a segment of tissue with the incision at the midpoint was removed and immediately placed in the RNA stabilization and storage solution RNALater (Qiagen, Venlo, Netherlands). Rat tendons were immediately placed on dry ice then transferred to -80°C. or mechanical testing, the entire tendon from the calcaneus to the muscle was explanted, wrapped in saline-soaked gauze, and frozen at -20°C. For tendons undergoing histology, the tissue was placed in Histochoice (Amresco, Solon, Ohio, USA).

2.5 Optical Coherence Tomography and Histological Imaging

OCT imaging was performed on in situ but exposed tendons in the rats, and explanted tendons for the rabbits. OCT is a near-infrared imaging modality capable of obtaining cross sectional images of tissue at micro-scale resolution. It is rapid, non-contact, and does not affect subsequent procedures. OCT has been shown to visualize tendon crimp period and birefringence21, and alterations of these OCT image features have been seen in ruptured tendon22. In rabbits, some tendons explanted at day 21 after ITU treatment were imaged with OCT prior to freezing and mechanical testing. A commercial OCT system (Thorlabs SROCT, 1040 nm center wavelength, 10 x 13 µm resolution in air) was used and image cubes 8 mm longitudinal x 3 mm lateral x 2 mm deep centered over the incision location, were obtained. After mechanical testing, the same tendons were fixed, paraffin embedded, sectioned longitudinally and stained with hematoxylin and eosin (H&E). In rats, tendons were imaged with OCT at days 1, 3, 7, 14, and 21 after ITU treatment. Birefringence period was quantified when the tendon could be visualized. Then the tissue was fixed and histological sections prepared as for the rabbit tendons. Images of histological sections were obtained with a brightfield microscope at varying magnifications to examine the healing processes in the tendons.

2.6 Mechanical Testing

Mechanical testing was performed on rabbits at day 21 after ITU treatment. Mechanical testing was performed on a uniaxial tensile testing device (Test Resources 200R, Shakopee, MN). The tendons were clamped at the proximal end near the insertion point into the muscle, and the distal end at the calcaneus. Tendons were pulled at 1 mm/s to failure. Graphs of Cauchy stress vs. stretch were obtained. Based on a measurement of tendon diameter, and assuming a circular cross-section, ultimate tensile strength and maximum tangential modulus were calculated from the data. Values between categories were compared using a 2-sided students t-test with significance set at p < 0.05. For rats, a similar procedure was followed, although testing was performed at day 14 and day 28 after ITU treatment. The ends of explanted rat tendons were clamped with a pneumatic clamping system, and the clamps were attached to a MTS 810 test frame (MTS, Eden Prairie, MN). The axial applied load was a ramp function at a rate of approximately 4 mm/sec. The load was measured with an Interface load cell (Interface Inc., Scottsdale, AZ), and the data were collected on a desktop computer with a custom designed LabView (National Instruments, Austin, TX) VI recording the displacement and force over time.

2.7 PCR

Relative gene expression was measured with PCR, performed by the University of Arizona Genomics Core. Expression of the following genes was measured in rabbits: vascular endothelial growth factor (VEGFα), tumor necrosis factor...
(TNFα), interleukin-1 beta (IL1β), transforming growth factor beta (TGFβ1), collagen type I (COL1A1 and COL1A2), and collagen type 2 (COL2A1). These genes provided a view of inflammatory, growth, reparative, and remodeling processes. Results of COL1A1 and COL1A2 were nearly identical so averaged results are presented as COL1. Results were normalized to a housekeeping gene (18s) and to control tendons (uncut/untreated) to account for effects of surgery. Further, ratios of experimental groups were computed. Gene expression analysis was performed at day 4, 7, or 14 following ITU treatment.

In rats, expression of a slightly different set of genes was measured: VEGFα, TNFα, IL1β, TGFβ1, COL1A1, Collagen type 3 (COL3A1), insulin-like growth factor-1 (IGF-1), and heat shock protein 70 (Hsp70). Results were normalized to a housekeeping gene (GAPDH). Gene expression analysis was performed at day 1, 3, 7, 14, or 28 following ITU treatment. On day 28, only collagen gene expression was measured.

3. RESULTS

Surgery was successful on all animals. One unexpected rabbit death occurred due to complications from anesthesia during ITU treatment. The number of tendons included in histology/OCT, gene expression analysis, and mechanical testing is given in Table 1. In rabbits, histology was performed on all mechanical testing tendons, and OCT imaging was performed on one each of UC/UT, UC/T, C/UT, and C/T tendons. In rats, OCT and histology were performed on the same animals.

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3.1 Ultrasound Imaging

Ultrasound imaging was able to visualize the tendon cut and ITU coagula in both rabbits and rats. Figure 1 shows example images of a cut, and ITU coagula visualized in the Achilles tendon immediately after treatment in a rabbit. Subsequent time point imaging with ultrasound revealed that coagula were no longer visible 4 days after treatment (3 days in rats). Cuts were still visible at 4 and 7 days after treatment (3 and 7 days in rats, sometimes visible) but could not be visualized by ultrasound at day 14 or 21 (14 or 28 in rats). Figure 2 shows an example ultrasound image sequence for a cut only leg and a cut/treated leg, with images prior to surgery, after surgery and prior to treatment, and at days 7, 14,
and 21 post treatment, in a rabbit. The cut is visible after surgery, at day 7 the hematoma can be seen, and progressive normalization of the tendon seen at days 14 and 21.

Figure 1. Representative ultrasound images of rabbit Achilles tendon 1 day after surgery. Top: immediately before treatment; large arrow points to cut. Bottom: immediately after treatment; small arrows point to individual coagula in a treatment line.

Figure 2. Ultrasound image sequence for one cut/untreated tendon (left) and one cut/treated tendon (right). The cut is visible in both tendons at day 0 (after surgery and before treatment). At day 7, both tendons appear thickened due to residual hematoma. At days 14 and 21 both tendons are achieving a more normal appearance. Hypointense regions may indicate edema, which appears more severe in the cut/untreated tendon.

3.2 Gross Explant Imaging and Histology

At time of explant, the tendons were examined grossly. Uncut tendons at all time points appeared visually smooth with no effect of ITU treatment visible. In rabbits, cut (right) tendons at days 4 and 7 after treatment exhibited incomplete healing, with hematoma visible. By day 14, most cut tendons appeared smooth and white but some still showed resolving hematoma. There was no consistent visual difference between treated and untreated tendons at time points 4, 7, or 14. By day 21, all tendons appeared normal with no visible difference between any categories of cut or treated. In rats,
findings were similar, with perhaps a slightly faster healing response, as might be expected in the more metabolically active small rat, less hematoma but still visible fibrin clot at days 1, 3, and 7. By day 14, tendons appeared essentially normal from a gross perspective, and appeared completely normal at day 28. As with the rabbits, there was not consistent visual difference between treated and untreated tendons at any time point. Figure 3 shows a gross image, histology of the cut, and histology of an ITU lesion at days 1, 3, 7, 14, and 28 (no histology section was obtained of a lesion at day 7).

![Figure 3](https://www.spiedigitallibrary.org/conference-proceedings-of-spie)

Both the gross images and histology confirm the expected healing response. On day 1, histology of cut tendons revealed a large “V”-shaped cut (indicating tendency of tendon fibers to retract) with a fibrin clot. There is nuclear rowing present at the site of the cut, and neutrophils inside the cut along with fibrin, which extends out from the cut. Fibroblasts are also present in the collagen. By day 3, there is a pronounced web of fibrin connecting the two sides of the cut. Above it, there is a higher cellular density indicating inflammation. There are fibroblasts and fibrocytes present in the collagen and inside the cut. This process continues on day 7 with collagen synthesis, and by day 14 a “V” shaped cut is no longer visible, but a cap of fibroblasts and organizing collagen is still present in histology (but not visible grossly). By day 28, the cap is smaller but still visible in histology, with collagen continuing to organize. Histology of lesions shows a dense, darkly staining “melted” area of collagen on day 1 with little noticeable tissue reaction. However, by day 3, there exists a thick cap of inflammatory infiltrate on top of the tendon adjacent to the lesion. By day 14 the lesion is darkly staining and necrotic with less but still evident infiltrate and lots of fibroblasts. By day 28, the lesions present are small and infiltrate is absent but fibroblasts are still present in large number especially at the margins of the lesion. These findings suggest that both cutting and ITU treatment elicit a healing response with inflammation and collagen production.
3.3 OCT Imaging and Histology

OCT imaging and histology confirmed healing of rabbit tendon at day 21. Figure 4 shows OCT and histology images of cut and uncut, treated and untreated tendon. The crimp pattern, evidenced by vertical squiggly dark band, is clearly evident in this normal tendon tissue. OCT images all appear similar, with the exception that small hypointense voids were seen in the cut and untreated tendon, possibly indicating residual edema. Histology of all tendons showed well-aligned collagen fibers and fibroblasts. No histological evidence of damage or healing processes was seen at this 21 day time point.

Figure 4. Histology (left) and optical coherence tomography (OCT, right) images of rabbit tendons at 21 days post treatment. A: uncut/untreated, B: cut/untreated, C: uncut/treated, D: cut/treated tendon. Arrows in OCT image B point to small hypointense voids, possibly edema. Otherwise, histology and OCT images of all categories are similar and indicate substantially complete healing of tissue.

OCT images of rat tendon clearly showed the birefringence banding (horizontal dark banding) caused by rotation of light in healthy organized tendon collagen. However, crimp pattern was not normally seen, perhaps because the crimp period in rats is too small. Damage to collagen is often reflected in an increase in banding period. OCT was unable to regularly image directly over the cut in Achilles tendon until about day 7 due to the presence of the thick fibrin clot. However, analysis of banding period was obtained for measurements immediately adjacent to the cut when possible. Analysis of the Achilles tendon banding period show no statistically significant difference between treated and untreated left (uncut) tendons, between treated and untreated right (cut) tendons, or between treated or untreated tail (punctured) tendons. This suggests that ITU treatment does not appreciably affect the collagen integrity on a macro (mm) scale. Comparison of the banding period between left (uncut) and right (cut) tendons showed that the right leg banding period was significantly higher, reflecting damage due to the cut. OCT was not regularly able to visualize damaged tail tendon. The method of tail tendon damage (puncture) was less severe than the cutting, and did not appear to cause the same healing response. One clear case of visualizing tail tendon puncture was found at day 3. OCT was not able to regularly to see the ITU lesions, suggesting that either the scattering contrast between lesion and tendon is low, or the lesions were too deep. Two cases of lesions were seen, one at 14 days and one at 28 days. It may be that by day 14-28, the remaining material in the lesion area is necrotic and high contrast. A compilation of OCT images of rat tendon is given in Figure 5. In Achilles tendon, a clear progression of healing from the cut is seen in OCT images. At day 1, only the fibrin clot is seen as a diffuse hypointense layer (too thick to visualize the cut). At day 3, in this animal, the cut can be seen with the fibrin clot extending into the “V”-shaped cut. At day 7, a similar appearance is seen but with the cut smaller and the overlying clot thinner. By day 14, The material inside the cut is becoming more hyperintense, reflecting a more highly scattering material containing more collagen. At day 21, the cut is still seen but the filling material intensity is approaching that of normal tendon. Possible beginnings of weak birefringence banding are seen, reflecting that the newly-formed collagen is becoming organized.
3.4 Mechanical Testing

Ultimate load and maximum tangential modulus for each category of rabbit tendon are shown in Figure 5. All tendons ruptured at the calcaneus, indicating that the cut had healed to achieve near, at least, the pre-injury strength. On average, the uncut and treated tendons had a higher ultimate load, and the control (uncut and untreated) tendons had a higher maximum tangential modulus. However, due to large variability there was no statistically significant difference between any category. In the rats, a larger number of tendons were tested, however there was still no statistically significant difference between failure loads for any category or time point. At 14 days, means varied by nearly 50%, but standard deviations were high. At 28 days, all means were nearly equal. As the histology shows a cut no longer evident by day 14 (although collagen is still being synthesized and organized), apparently the healing process has progressed far enough that there is no significant difference in strength. Histology showed that ITU lesions could be still visible at 14 and 28 days, but they do not appear to noticeably affect the mechanical strength.
3.5 Rabbit Tendon PCR
Analysis of rabbit tendons showed changes in gene expression. Due to the very small number of tendons, no statistical comparisons are made—only means are compared. Cutting the rabbit Achilles tendon caused upregulation of cytokines IL1β and TNFα, as well as growth factors TGFβ1 and VEGFα, that peaked at day 7 but continued through day 14. Collagens were initially downregulated at day 4 but were upregulated by day 7 and 14. Treatment by itself caused unremarkable and variable changes, with no clear trend in upregulation or downregulation in any gene expression at any time. However, adding treatment to a cut tendon appeared to enhance gene expression, as shown in Figure 7, which shows the ratio of gene expression of cut/treated tendons to cut/untreated tendons, for all genes and time points. Cytokines and growth factors were upregulated at all time points, with a peak at day 7, where the effect of treatment was to cause a 2.5 - 4.5 fold increase in the expression of VEGFα, TNFα, IL1β, and TGFβ1. At day 14, the upregulation had subsided, but was still 2 - 2.5 fold increased. Collagen expression of both COL1 and COL2A1 was suppressed at day 4, but robustly increased (2.5 – 3 fold) at day 7. The upregulation of COL1 continued at day 14.

3.6 Rat Tendon PCR
For rat Achilles tendons, the means and standard error over time for genes can be seen in Figure 8. By day 14 and 28, there is no statistically significant difference in any gene. Some statistically significant differences or near-differences are seen at days 1, 3, and 7. It appears the cutting and ITU appear to cause similar changes of a reduction in several of the gene’s expression, especially at days 3 and 7, compared to sham. Examination of the means shows that fairly consistently, ITU treatment downregulates expression at time points 3 and 7 and upregulates at 14 and 28. Cutting has mixed effect at day 1, downregulation at days 3 and 7, mixed at day 14 and mostly down at day 28. Comparison of cut and treated vs. cut only shows that the additional effect of treatment is to upregulate nearly all genes at days 3, 14 and
21, and downregulate at day 7, although differences are relatively small. For tail tendon, no statistically significant difference is seen in any gene at any time point between ITU treated or not punctured tendons, although trends were in line with Achilles tendon, albeit smaller changes. Overall, the results are consistent with the healing process seen in histology, which shows a similar response of inflammatory infiltrate, then collagen synthesis, to either cutting or ITU treatment.

![Figure 8. Ratio of gene expression in rat tendons. Left, cut tendons compare to sham (uncut/untreated). Middle, treated tendons compared to sham. Right, cut and treated tendons compare to cut only.](image)

### 4. DISCUSSION

The animal protocol for both rabbits and rats was successful, with a reproducible partial transection obtained in all Achilles tendons. This protocol models a partial Achilles rupture and can also be applicable to tendinitis, or incomplete structural disruption of the tendon leading to an inflammatory response. These inflammatory conditions are distinct from tendinosis, a degenerative condition without significant inflammatory component, for which this model would not be an appropriate choice.

Treatment with ITU did not appear to have a noticeable effect on normal (uncut) tendon in the rabbit. Histology, OCT, mechanical testing, and PCR failed to show any evident or statistically significant differences between uncut/untreated and uncut/treated legs. The visual and histological appearance as well as the mechanical performance of all categories of tendons at 21 days was similar. In the rat, ITU lesions were seen in histology and OCT of Achilles tendon in one animal, even at 28 days, suggesting that the treatment could still be actively influencing physiology at later time points. However, there was no difference in mechanical properties between treated and untreated tendons. PCR showed that ITU treatment caused initial down regulation of inflammatory, growth factor, and collagen genes, followed by upregulation at later time points.

In the small number of rabbit Achilles tendons, a trend of increased cytokine and growth factor expression at all time points, and increased collagen expression at 7 and 14 days, was seen in the cut and treated tendons compared to the cut only tendons. A relative decrease in collagen expression at day 4 may be due to this enhanced inflammatory response, which was tamped down by day 7 but still relatively higher in cut and treated tendons than cut only tendons. In the larger number of rats, differences were not statistically significant, but the addition of treatment appears to cause a sustained increase in gene expression. Differences seen between rabbits and rats may reflect the difference in size of the tendons. The Achilles tendon in the rat is only 2-3 times larger diameter than the ITU lesion size.

Our findings are consistent with the three overlapping phases of healing, which have been described previously. First is an initial inflammatory phase with erythrocytes and inflammatory cells entering the wound, which is evidenced by the hematoma seen in Figure 3, time points 1 and 3 days. During the later portions of this phase, angiogenesis is initiated, and tenocytes are recruited. The second phase, remodeling, includes peak synthesis of collagen, seen in the upregulation of collagen genes at later time points in both rabbits and rats, and the progressively normalizing appearance of the explanted tendons at days 14 though 28. Others have also noted the promotion of collagen, for example upregulation of collagen gene expression seen at 14 days in a rat model of tendon rupture treated with low-intensity pulsed ultrasound, or collagen production by tenocytes 7 days after treatment with low-energy, low-impulses shock waves. Water content remains high, which may be the origin of the hypointense regions- likely edema- seen in the day 21 ultrasound and OCT images of rabbit tendon in Figures 2 and 4, respectively. The final stage, remodeling, is likely just commencing at day 21.
(rabbits) and 28 (rats). In this phase the healing tissue restructures, and growth factors and collagen synthesis decrease. In our model, while collagen remodeling is still continuing, the tendon has returned to functional strength at day 21 (rabbits) and day 14 (rats), as mechanical testing shows rupture at the insertion to the calcaneus, not the cut in all tendons in both species, and histology shows well ordered tendon with organized fibers in the rabbit. The rat, the cut is completely visually healed at day 28, but OCT shows that birefringence is not quite normalized, suggesting that the collagen is still remodeling into its final, highly organized form.

Overall, our results suggest that intense therapeutic ultrasound does not cause a decrease in mechanical strength of tendon, nor any other evidence of detrimental effect. It upregulates genes associated with inflammation and healing, with a trend of increased collagen gene expression at middle to later time points. The effects of ITU histologically and as seen with PCR are similar to cutting of the tendon, with an inflammatory reaction and recruitment of fibroblasts and fibrocytes. Therefore, our results, suggest that ITU may be particularly beneficial in promoting healing in degenerative diseases characterized by a lack of inflammation and healing response, such as tendinosis. Unlike other treatments for tendinosis that involve delivering controlled damage to elicit a healing response (such as surgery or tenotomy), ITU does not involve breaking the skin and appears to have no side effects. In this model of rupture or tendinitis, ITU may enhance the healing response already occurring.

REFERENCES