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Temporal Healing in Rat Achilles Tendon: Ultrasound Correlations

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Abstract

The purpose of this study was to explore whether a new ultrasound-based technique correlates with mechanical and biological metrics that describe the tendon healing. Achilles tendons in 32 rats were unilaterally transected and allowed to heal without repair. At 7, 9, 14, or 29 days post-injury, tendons were collected and examined for healing via ultrasound image analysis, mechanical testing, and immunohistochemistry. Consistent with previous studies, we observe that the healing tendons are mechanically inferior (ultimate stress, ultimate load, and normalized stiffness) and biologically altered (cellular and ECM factors) compared to contralateral controls with an incomplete recovery over healing time. Unique to this study, we report: 1) Echo intensity (defined by gray-scale brightness in the ultrasound image) in the healing tissue is related to stress and normalized stiffness. 2) Elongation to failure is relatively constant so that tissue normalized stiffness is linearly correlated with ultimate stress. Together, 1 and 2 suggest a method to quantify mechanical compromise in healing tendons. 3) The amount and type of collagen in healing tendons associates with their strength and normalized stiffness as well as their ultrasound echo intensity. 4) A significant increase of periostin in the healing tissues suggests an important but unexplored role for this ECM protein in tendon healing.

Keywords

defect healing; ultrasound echo intensity; biomechanics; immunohistochemistry; periostin

Introduction

Complete tears or lacerations of tendons are common with the incidence of Achilles ruptures reported to range from 5.5–9.9 ruptures per 100,000 people in North America²². Healing results in a complex, coordinated series of events that form a neo-tendon which is more scar-like in character than the native tissue. The repair process may extend from months to years and the injured ligament never fully recovers its original mechanical properties.^{12, 13} An improved understanding of tendon healing, both mechanical and biological, are essential to improve treatment. Temporal data that describe the biological processes affecting the extracellular matrix and the functional compromise are particularly valuable. Mechanical

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data are typically obtained through *in vitro* experimentation.^{1, 5, 9, 18} However, a non-invasive method to acquire these data would allow direct, subject-specific, longitudinal evaluations of compromise in healing tendons. Ultrasound imaging has been used to examine tissue strain and other mechanical properties.^{6, 19–21} One ultrasound-based method, acoustoelasticity, is based on the principle that acoustic properties of a material are stiffness- and load-dependent and altered as a non-linear material is deformed and loaded.⁸ Changes in acoustic properties resulting from elastic deformation can be measured as changes in wave propagation velocity and reflected wave amplitude.^{10, 11} The acoustoelastic relationship between reflected wave amplitude and mechanical behavior (strain-dependent stiffness and stress) has been derived experimentally and corroborated via analysis of both A-mode 1-D and B-mode 2-D ultrasound waves.^{6, 10, 11}

The present study observes an acoustoelastic-like behavior in healing Achilles tendons and examines the relationship between ultrasonic echo intensity (gray scale brightness from standard clinical B-mode images) and the mechanical behavior during controlled *in vitro* loading. Confirming the existence of correlations between echo intensity and measured mechanical parameters under standardized test conditions is the critical first step towards assessing mechanical function *in vivo*. Additionally, this study characterizes some cellular and ECM properties of the healing tendons to further elucidate factors associated with tissue compromise.

Materials and Methods

Animal and tissue preparation

All experimental protocols were approved by the University of Wisconsin Institutional Animal Use and Care Committee. Thirty-two skeletally mature Wistar rats (275–299 g) were used for this healing study, eight per temporal group (day 7, day 9, day 14, and day 29), as well as eight intact control rats. Each rat in the healing study underwent unilateral surgical Achilles transection to create a gap-filling healing model, using sterile techniques. The gap-filling model created via surgical transection was created in order to reliably measure and provide a defect large enough to demonstrate a proof of concept correlation between ultrasound echo intensity and mechanical test parameters. A skin incision was made and both the plantaris and Achilles tendons were unilaterally transected at the midpoint axially. The contralateral side remained intact and served as a control. All animals were allowed unrestricted cage movement immediately after surgery. At 7, 9, 14, or 29 days post-injury, rats were humanely euthanized. Time of sacrifice was based on our previous ligament healing studies, indicating dynamic changes in granulation tissue formation up to day 14 post-injury.^{3, 4} Our reported biological changes in healing were less significant after day 14 and thus only day 29 was included in the current study.

Tendons from rats used for immunohistochemistry (3 rats/post-surgical day) were carefully dissected (all bone, muscle, and extraneous tissue removed) immediately after sacrifice, measured, weighed, and immediately placed in OCT (optimal cutting temperature) for flash freezing. Sagittal cryosections were then cut at a 5 μ m thickness, mounted on Superfrost plus microscope slides and maintained at -70°C . Rats used for mechanical testing (5 rats/post-surgical day) were stored *in toto*, at -70°C until time of testing. Prior to testing, rats were thawed at room temperature and Achilles tendons were dissected and surrounding tissue (including muscle) excised with care to keep the calcaneal insertion intact.

Mechanical Testing

Tendons remained hydrated using phosphate-buffered saline (PBS). Tendon length, width, and thickness were measured using a 0–150mm (0.01mm resolution) digital caliper

(measurements performed three times and averaged) and the cross-sectional area (assumed to be an ellipse) was calculated. Tendons were tested in a custom-designed load frame which held and loaded tendons along the longitudinal axis of the tissue (Figure 1). The calcaneus was trimmed and press-fit into a custom bone grip. The soft tissue end was fixed to strips of Tyvek® (E. I. du Pont de Nemours and Co.) with a cyanoacrylate adhesive, which were held between two plates on the soft-tissue grip. Tendons were carefully aligned along the loading direction to prevent twisting in the grips or out-of-plane movement during loading. Tendons were immersed in PBS to prevent dehydration and facilitate ultrasound wave propagation from transducer to tissue.

Mechanical testing was performed at room temperature. A low preload of 0.1N was applied in order to obtain a uniform zero point ¹⁷ prior to preconditioning (20 cycles at 0.5Hz) to 0.5%. Following a 10 minute rest period (to allow for viscoelastic recovery), pull-to-failure testing at a rate of 3.33mm/sec was performed on tendons. Force and grip-to-grip displacement information from the test system were recorded by Labtech Notebook data acquisition software (Laboratory Technologies Corp., Wilmington, MA) at 10Hz during testing.

Grip-to-grip displacement (inclusive of deformation occurring in the insertion site, the residual healthy tissue, and the healing tissue) was normalized by initial length to calculate overall tendon elongation (expressed as a percent of the initial tendon length). Force information was divided by average initial area to calculate stress. The slope of the linear portion of the line relating stress to elongation was used to calculate a normalized stiffness parameter.

Ultrasound

Cine ultrasound was recorded during testing using a GE LOGIQe ultrasound machine with GE 12L-RS Linear Array Transducer (General Electric, Fairfield, CT). The ultrasound transducer (operating at 12MHz) was held in a fixed position above the bath using a custom stand holder, oriented lengthwise along the midline of the specimen (Figure 1), in order to record cine B-mode ultrasound (20 frames per second) of the tendon during the mechanical testing outlined in the previous section. All ultrasound settings (i.e. frequency, gain, time gain compensation) were held constant across all testing to reduce variability and standardize image intensity across samples. The transducer length of 4cm (more than double the length of the average specimen at maximum elongation) accommodated viewing the entire tendon length during stretch. Careful specimen and grip alignment and uniaxial test protocol prevented motion in the transverse direction, such that the same plane was imaged throughout the test.

The overall echo intensity (average gray scale brightness in the B-mode image) of the tendon, averaged over the entire region between the grips, was calculated for each frame in order to record the echo intensity changes over time using post-processing software (EchoSoft™; Echometrix, Madison, WI). Briefly, this software tracks tendon movement and deformation during loading using digital image correlation (DIC). Then, echo intensity values for each frame are recorded via a Lagrangian (i.e. material) frame of reference.

Staining and Immunohistochemistry

To detect general morphology of the healing tendon, sections were stained with hematoxylin and eosin (H&E). Immunostaining was performed on frozen sections using mouse monoclonal or rabbit polyclonal antibodies. Cryosections were fixed with acetone, exposed to 3% hydrogen peroxide to eliminate endogenous peroxidase activity, blocked using Background Buster (Innovex Biosciences, Richmond, CA) and incubated with rabbit or

mouse primary antibodies. Sections were then incubated with biotin, and streptavidin-conjugated to horseradish peroxidase, respectively, using the Stat Q staining kit (Innovex Biosciences, Richmond, CA). The bound antibody complex was then visualized using DAB. Stained sections were dehydrated, cleared, cover-slipped and viewed using light microscopy. Negative controls omitting the primary antibody were included with each experiment. Positive controls of gut or spleen were also included.

Mouse monoclonal antibodies to markers, CD68, CD163, CD3, CD31, (all from Abcam-Serotec, Raleigh, NC at a dilution of 1:100), muscle actin (1:10 dilution, Abcam-Serotec, Raleigh, NC), and ki67 (1:25 dilution; Dako, Carpinteria, CA) were utilized, to identify the classically activated macrophages (M1), alternatively activated macrophages (M2), T-lymphocytes, endothelial cells and blood vessel lumen, myofibroblasts, and proliferating cells, respectively. Extracellular matrix factors were identified using antibodies to type I procollagen (straight; mouse SP1.D8; Developmental Hybridoma, Iowa City, Iowa), mouse type III collagen, (1:8000, Sigma-Aldrich, St. Louis, MO), and rabbit periostin (1:1000; Biovendor, Chandler, NC).

Quantification

After immunohistochemistry, micrographs were collected using a camera assisted microscope (Nikon Eclipse microscope, model E6000 with an Olympus camera, model DP79, Mellville, NY). Six random pictures were obtained from each stained cryosection including one image of the paratenon. Two to three sections were counted per animal. Endothelial cells, myofibroblasts, type I procollagen, type III collagen, tenomodulin, and periostin were then quantified with ImageJ (National Institutes of Health, Bethesda, MD). T-lymphocytes, blood vessel lumen, proliferating cells, and M1 and M2 macrophages were quantified manually.

Statistics

A one-way analysis of variance (ANOVA) was used to examine differences across time for all ultrasound, mechanical, and immunohistochemistry results. If the overall p-value for the F-test in ANOVA was significant, post-hoc comparisons were performed using the Scheffe method. Experimental data are presented as the means $\pm$ S.D. of replicates. All p-values reported are two sided. $p < 0.05$ was used as the criterion for statistical significance. Computations and figures were performed using Kaleidagraph, version 4.03 (Synergy Software, Inc.).

Results

Morphology

Tendon cross-sectional area significantly increased at all healing time points ($p < 0.0001$) compared to the intact tendon; area increased (from $1.62 \pm 0.42\text{mm}^2$, mean $\pm$ standard deviation) with time (to $9.41 \pm 1.83\text{mm}^2$, $12.03 \pm 2.20\text{mm}^2$, and $12.71 \pm 0.81\text{mm}^2$ at day 7, 9, and 14) until day 29, then decreased respective to other healing points (to $7.69 \pm 0.50\text{mm}^2$) but remained significantly greater than intact tendon ($p < 0.001$).

Mechanical Testing

All healing tendons failed in the transected region; two of the intact tendons failed near the proximal grip and the remaining three failed near the calcaneal insertion. Ultimate load at failure was lower at all time points following injury compared to the intact tendon (Table 1). Mean ultimate load appeared to increase over time (Table 1), but increases were not significant (Table 2). Ultimate stress also demonstrated a decrease at all time points following injury (Figure 2a, Table 1). Mean values remained unchanged from days 7, 9, and

14 (Tables 1, 2), and while ultimate stress appeared increased at day 29, it was not significantly higher than days 7, 9, and 14, and was still significantly lower than intact tendon (Table 2).

Normalized stiffness, measured from the linear portion of the stress-elongation curve, followed a similar pattern as ultimate stress (Figure 2b, Table 1). Normalized stiffness values were significantly lower than intact tendon at all time points (Table 2), and while mean normalized stiffness values at day 29 appeared higher than days 7, 9, and 14, values were not significantly different (Table 2). A linear regression model relating normalized stiffness to ultimate stress (Figure 2c) demonstrated a strong correlation (Table 3) with an R^2 value of 0.984. Ultimate elongation was relatively constant across all groups (Tables 1, 2).

Ultrasound Testing

The maximum ultrasound echo intensity reached during pull to failure (Figure 3a) generally follows the same pattern as ultimate stress and normalized stiffness (Figure 3b, c); linear regression models (Table 3) relating echo intensity to ultimate stress (Figure 3b) or normalized stiffness (Figure 3c) demonstrate a strong correlation, with R^2 values of 0.845 and 0.794, respectively (Table 3). Echo intensity values of the injured Achilles were significantly lower than intact tendon at all time points (Tables 1, 2), and maximum intensity at day 29 was significantly higher than days 7, 9, and 14 (Table 2) but remained well below intact values. Echo intensity increased steadily with elongation during the pull to failure, similar to stress (Figure 3d), until the ultimate elongation was reached.

Immunohistochemistry

Injury to the tendon resulted in a significant change to the typically hypocellular, collagen aligned structure (Figure 4a–c). Immunohistochemistry results indicate that proliferating cell number (Figures 4d–f and 5a; Table 4) was significantly changed ($p < 0.001$) across time, peaking at days 7 and 9 and decreasing thereafter. By days 14 and 29, cell numbers were similar to the intact tendon. Specific cells identified within the healing tendon, included the M1 and M2 macrophages, T-lymphocytes, myofibroblasts, and endothelial cells. Granulation tissue-localized M1 macrophages tended to increase ($p = .062$) at day 7 compared to the intact tendon (Figures 4g–i and 5b; Table 4). The number of macrophages in the healing tendon was not significantly different than macrophages in the intact tendon by day 9. M2 macrophages, T-lymphocytes and myofibroblasts were present within the healing tendon but were not significantly different across time ($p = 0.212$, $p = 0.084$, and $p = 0.079$, respectively; Table 4). Endothelial cells (Figures 4j–l and 5c; Table 4) were significantly higher than the intact tendon from days 7 through 14 ($p = 0.002$). By day 29, the number of cells was similar to the intact tendon. Similarly, the number of blood vessel lumen increased (Figures 4j–l and 5d, $p = 0.002$; Table 4) after injury and reduced to intact levels by day 29. The ECM factors, type I procollagen, type III collagen, and periostin were all significantly modified after injury (Figure 4m–o). Type I procollagen significantly decreased from the intact tendon at all time points (Figures 4m–o and 6a; Table 4). Although an increase in the mean value for type I procollagen was noted at day 29, compared to days 7, 9 and 14, results were not significant. Type III collagen (Figures 4p–r and 6b; Table 4), a typical marker for scar formation, was low in the intact tendon but was significantly higher across all time points after injury ($p < 0.001$). Similar to type III collagen, periostin (Figures 4s–u and 6c; Table 4) significantly increased at all time points compared to the intact tendon ($p = 0.001$).

The biological response of the ECM factors, type I procollagen, type III collagen and periostin can be related to both the mechanical and ultrasound parameters (Figure 7a–b).

Type I procollagen levels correlated linearly to normalized stiffness (Figure 7c) and nonlinearly to echo intensity (Figure 7d).

Discussion

In this study, mechanical, ultrasound, and biological experiments were performed on rat Achilles tendons as a model for healing with gap-filling. We observe that healing results in a compositionally and mechanically inferior tendon, indicated by significant changes from intact tendon in biological (cellular and ECM factors), mechanical (ultimate stress, ultimate load, and normalized stiffness), and ultrasonic (echo intensity) factors after 29 days post-injury. We demonstrate that the relationship between ultrasound echo intensity and mechanical properties previously described in healthy, intact tendons (Duenwald et al, 2011) also exist during the temporal healing of a completely transected Achilles tendon, with maximum ultrasound echo intensity exhibiting decreases similar to ultimate stress and normalized stiffness in the transected tissue, indicating the potential value of ultrasound image analysis. These correlated mechanical and ultrasound parameters can also be related to the biological response of the ECM factors including type I procollagen, type III collagen and periostin.

Mechanical parameters measured for the intact tendons in this study are in agreement with the normalized stiffness, ultimate force, and ultimate stress reported by Eliasson and colleagues (179 ± 36 MPa, 63 ± 5 N, and 45 ± 10 MPa, respectively; mean $\pm$ standard deviation), as is the average cross-sectional area measurement (1.39 ± 0.19 mm²).⁷ Ultimate stress in the healing rat Achilles study (measured at 1, 2, and 4 weeks) by Zhang and colleagues noted a similar behavior of low ultimate stress until the 2 week timepoint, followed by increased strength by day 29.²⁴

The relationship between echo intensity, ultimate stress and normalized stiffness is exhibited by the pull to failure data. Kobayashi and Vanderby^{10, 11} previously developed the acoustoelastic relationship between wave amplitude and mechanical behavior in a nearly incompressible material using A-mode 1-D ultrasound. More recently, Duenwald, *et al.*⁶ demonstrated that an acoustoelastic effect, using B mode ultrasound, was related to stress and strain in a tendon at physiologic strains. Using a rat model to analyze the temporal behavior of healing, this study further supports the use of ultrasound-based analysis of acoustoelastic-like behaviors to interpret mechanical properties of tissues. Successful estimation of stress and strain in a tissue via ultrasound provides an alternative to standard mechanical testing. *In vivo* applications similar to these must overcome many challenges such as interposing tissue, hand held transducers, etc. They must also investigate whether the correlations between echo intensity and mechanical properties are consistent across tendon types (including different locations and species), as collagen fibril type, orientation, and packing density may all impact echo intensity, as would the degree of organization in the tissue, tissue hydration, and initial loading conditions. Now that a relationship between echo intensity and tissue biomechanics has been identified, inverse dynamics, utilizing force plate and anatomical marker analysis to estimate loads and modulus values on the tissues *in vivo*¹⁵ in order to generate empirical relationships that account for skin, fat, and other tissue contributions, can be exploited. Strain can be measured by tracking anatomical markers or image texture in the ultrasound images to relate echo intensity to strain.¹⁴ If successfully translated into *in vivo* studies, this method could obtain ultrasound data on an individual basis and compute subject-specific mechanical data. Additionally, this noninvasive method may be able to track pathologies and healing over time in both animal models and human subjects.

Elongation measurements were based on grip-to-grip displacement measurements recorded by the machine (placement of the ultrasound transducer prevented the use of a camera for optical strain measurement). A recent study demonstrated the ability of digital image correlation (DIC) tracking techniques to measure tendon strain using ultrasound images to be as good as standard optical strain measurements.¹⁶ Thus, future experiments in this style could use the ultrasound images to calculate strain in place of grip-to-grip elongation methods. Such tracking methods would also allow for regional strain analysis, which could analyze strain in the inhomogeneous healing region which may be underestimated by overall strain measurements.

In this study, we observe a typical cascade of events during tendon healing (Figures 4–6). Cellular activity and changes in vascularity are essential to the formation of granular tissue in the tendon gap and the subsequent development of neo-tendinous tissue to replace the damaged native tissue. As this process occurs, we observe some notable associations between the mechanical, ultrasound, and biological data. Normal, intact tendon is comprised primarily of type I collagen while type III collagen increases after injury. We have previously demonstrated a temporal decrease in type I procollagen concomitant with an increase in type III collagen in a healing medial collateral ligament.^{2, 3} An increase in type III collagen is also associated with an upregulation of periostin during MCL healing.² Another report has indicated an association between echogenicity and collagen fibers during Achilles healing.²³ The current study further specifies that the decrease in type I procollagen (and increase in type III collagen and periostin) correlates to the reduced normalized stiffness, strength, and echo intensity (Figure 7a,b). We also show that tissue normalized stiffness is linearly correlated to procollagen I (Figure 7c), whereas echo intensity appears nonlinearly correlated to type I procollagen. Additionally, the increase in M1 macrophages, proliferating cells, and blood vessels within the two weeks of injury are associated with granulation tissue formation. Echo intensity, ultimate stress, and normalized stiffness are all lowest at these times. Taken together, these results suggest the reduction in stress and normalized stiffness measured via mechanical and ultrasonic methods, correlate well with the biological aspect of tendon healing.

During Achilles tendon healing, mechanical behaviors, biological changes in the ECMs, and ultrasound-based analyses have interrelated outcomes. Echo intensity correlates well with many mechanical and biological metrics that describe tendon healing. This relationship may allow standard clinical ultrasound systems to noninvasively and quantitatively probe mechanical and biological processes during wound healing.

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Figure 1. Mechanical and ultrasound testing setup. The ultrasound transducer is positioned above the midline of the specimen and aligned along the length to capture the entire length of the tissue.

Figure 2.

Mechanical testing indicating a) ultimate stress, b) normalized stiffness, and c) ultimate stress vs. normalized stiffness for the intact, day 7, 9, 14, or 29 post-injured rat Achilles tendons. Beneath graph, p value indicates group ANOVA results. Within a graph, bars without a common superscript letter differ (results of Scheffe's post-hoc pairwise analysis, $p < .05$). Error bars represent one standard deviation.

Figure 3.

Ultrasound results indicating a) maximum echo intensity for the intact, day 7, 9, 14, or 29 post-injured rat Achilles tendons, b) the relationship between echo intensity and ultimate stress in the healing rat Achilles tendon, c) the relationship between echo intensity and normalized stiffness in the healing rat Achilles tendon, and d) pull-to-failure (stress and echo intensity) results for intact and day 7 post-injured rat Achilles tendons. Beneath graph, p value indicates group ANOVA results. Within a graph, bars without a common superscript letter differ (results of Scheffe's post-hoc pairwise analysis, $p < .05$). Error bars represent one standard deviation.

Figure 4.

Representative Achilles tendon immunohistochemistry micrographs of the intact (first column: a, d, g, j, m, p, s), day 7 injured (second column: b, e, h, k, n, q, t) and day 29 injured (third column: c, f, i, l, o, r, u). H&E (a–c) staining indicates the increase in cellularity and disorganization associated with injury. Proliferating cells (d–f), M1 macrophages (g–i), and endothelial cells/blood vessel lumen (j–l) increase at 7 days post-injury compared to the intact tendon. By day 29, levels have started to decrease. Type I procollagen (m–o) is highest in the intact (m) tendon and decreases after injury (n–o). Type III collagen (p–r) and periostin (s–u) levels are low in the intact tendon (p, s) and increase after injury (q–r, t–u). All images were captured at 200X and include scale bars.

Figure 5. Immunohistochemistry of a) proliferating cells b) M1 macrophages c) endothelial cells and d) blood vessel lumen of the intact, day 7, 9, 14, or 29 post-injured rat Achilles tendon. Beneath graph, p value indicates group ANOVA results. Within a graph, bars without a common superscript letter differ (results of Scheffe post-hoc pairwise analysis, $p < .05$). Values are expressed as mean positive staining $\pm$ S.D.

Figure 6.

Immunohistochemistry of a) type I procollagen, b) type III collagen, and c) periostin of the intact, day 7, 9, 14, or 29 post-injured rat Achilles tendon. Beneath graph, p value indicates group ANOVA results. Within a graph, bars without a common superscript letter differ (results of Scheffe's post-hoc pairwise analysis, $p < .05$). Values are expressed as mean positive staining $\pm$ S.D.

Figure 7.

Relationship between proteins and mechano-ultrasound data. a) Time-course behavior of collagens I and III, periostin, ultimate stress, normalized stiffness, and echo intensity; b) time-course behavior of structural protein, procollagen I, and mechanical parameters, ultimate stress and normalized stiffness; c) positive correlation between normalized stiffness and procollagen I levels during healing and d) positive correlation between echo intensity and procollagen I levels during healing.

Table 1

Mechanical and Ultrasound Parameter Values (mean $\pm$ standard deviation)

	Intact	Day 7	Day 9	Day 14	Day 29
Ult. Load [N]	36.78 $\pm$ 12.23	11.44 $\pm$ 3.98	12.86 $\pm$ 5.57	16.49 $\pm$ 7.25	20.39 $\pm$ 5.82
Ult. Elongation [%]	15.22 $\pm$ 1.78	17.78 $\pm$ 3.36	16.30 $\pm$ 6.37	15.37 $\pm$ 4.80	15.35 $\pm$ 3.31
Normalized Stiffness [MPa]	106.87 $\pm$ 41.10	6.58 $\pm$ 2.53	5.11 $\pm$ 2.05	8.02 $\pm$ 3.26	16.87 $\pm$ 2.93
Ult. Stress [MPa]	15.94 $\pm$ 5.64	1.24 $\pm$ 0.49	0.90 $\pm$ 0.49	1.27 $\pm$ 0.53	2.67 $\pm$ 0.81
Max Echo Intensity	55.02 $\pm$ 6.96	27.70 $\pm$ 5.80	25.98 $\pm$ 4.87	24.71 $\pm$ 2.09	42.79 $\pm$ 6.09

Table 2

Statistical Significance for Mechanical and Ultrasound Results (p values)

	ANOVA	cx vs. 7	cx vs. 9	cx vs. 14	cx vs. 29	7 vs. 9	7 vs. 14	7 vs. 29	9 vs. 14	9 vs. 29	14 vs. 29
Ult. Load	0.001 *	<0.001 *	<0.001 *	0.001 *	0.028 *	0.999	0.935	0.163	0.919	0.275	0.528
Ult. Elongation	0.614	-	-	-	-	-	-	-	-	-	-
Normalized Stiffness	<0.001 *	<0.001 *	<0.001 *	<0.001 *	0.001 *	0.999	0.999	0.807	0.998	0.731	0.876
Ult. Stress	<0.001 *	<0.001 *	<0.001 *	<0.001 *	<0.001 *	0.999	0.999	0.083	0.998	0.068	0.083
Echo Intensity	<0.001 *	<0.001 *	<0.001 *	<0.001 *	0.006 *	0.871	0.661	0.002 *	0.995	<0.001 *	<0.001 *

* indicates statistical significance (p 0.05)

cx indicates intact tendon, numbers (7, 9, 14, 29) indicate days of healing

Table 3

Linear Regression Model Results

	Ult. Stress vs. Stiffness	Ult. Stress vs. Echo Intensity	Stiffness vs. Echo Intensity
Correlation	$\sigma_{ult} = 0.145(stiffness) + 0.277$	$\sigma_{ult} = \frac{1}{8.8}(echo - 17.046)$	$stiffness = \frac{1}{1.2}(echo - 18.470)$
R ² value	0.984	0.845	0.794

σ_{ult} = ultimate stress

echo = ultrasound echo intensity

Stiffness = normalized stiffness parameter (slope of linear portion of stress-elongation curve)

Table 4

Summary of Immunohistochemistry Results (mean $\pm$ standard deviation)

IHC Factors	Intact	Day 7	Day 9	Day 14	Day 29
Proliferating Cells (cells/mm ²)	0.13 $\pm$ 0.23	28.42 $\pm$ 1.67	19.29 $\pm$ 9.37	9.31 $\pm$ 2.19	2.58 $\pm$ 2.58
M1 Macrophages (cells/mm ²)	0.44 $\pm$ 0.77	50.00 $\pm$ 31.83	25.22 $\pm$ 11.74	34.72 $\pm$ 8.36	17.33 $\pm$ 14.73
M2 Macrophages (cells/mm ²)	4.06 $\pm$ 1.59	13.58 $\pm$ 5.19	27.12 $\pm$ 23.14	15.84 $\pm$ 5.58	11.11 $\pm$ 1.73
T-lymphocytes (cells/mm ²)	0.38 $\pm$ 0.12	9.22 $\pm$ 7.57	14.58 $\pm$ 10.51	4.45 $\pm$ 5.19	0.66 $\pm$ 0.29
Myofibroblasts (density/mm ²)	8.93E-05 $\pm$ 1.33E-04	3.00E-04 $\pm$ 2.22E-04	1.11E-03 $\pm$ 7.32E-04	4.68E-04 $\pm$ 3.48E-04	4.53E-04 $\pm$ 1.90E-04
Endothelial Cells (density/mm ²)	3.50E-04 $\pm$ 1.27E-04	2.44E-03 $\pm$ 3.18E-04	3.37E-03 $\pm$ 8.11E-04	2.22E-03 $\pm$ 5.26E-04	1.18E-03 $\pm$ 3.31E-04
Blood Vessel Lumen (lumen/mm ²)	0.59 $\pm$ 0.08	5.25 $\pm$ 1.55	5.88 $\pm$ 1.72	4.89 $\pm$ 1.32	2.97 $\pm$ 0.72
Type I Procollagen (density/mm ²)	2.44E-03 $\pm$ 6.11E-04	1.18E-04 $\pm$ 4.28E-05	7.28E-05 $\pm$ 1.09E-05	1.04E-04 $\pm$ 2.83E-05	4.51E-04 $\pm$ 3.45E-04
Type III Collagen (density/mm ²)	3.38E-03 $\pm$ 1.83E-03	4.12E-02 $\pm$ 1.06E-02	4.83E-02 $\pm$ 1.44E-03	4.27E-02 $\pm$ 3.43E-03	5.92E-02 $\pm$ 3.92E-02
Periostin (density/mm ²)	2.60E-03 $\pm$ 1.19E-03	3.51E-02 $\pm$ 6.17E-03	3.41E-02 $\pm$ 1.58E-02	3.77E-02 $\pm$ 4.48E-03	4.42E-02 $\pm$ 8.09E-03