

Ex Vivo and In Vivo Effects of Focused Ultrasound (FUS) treatments on murine Achilles tendons

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INTRODUCTION: Tendon injuries are prevalent, debilitating and challenging to treat. It is widely accepted that mechanical loading-based treatments offer long-term symptomatic resolution and improved functionality [1]. However, as these protocols rely heavily on patient compliance and involve long rehabilitation periods, there is a critical need for the development of novel, non-invasive strategies to mechanically stimulate healing in injured tendons. Focused Ultrasound (FUS) is emerging as an attractive treatment option for soft tissue injuries as it can induce significant bio-effects in targeted tissues via thermal and mechanical mechanisms [2] and can be customized to target injured tendons with high spatial and temporal resolution. However, the safety profile and bio-effects of FUS in tendons have not been well characterized. Previously, we developed a custom experimental methodology to precisely treat murine Achilles tendons and designed FUS pulsing schemes that emphasize thermal (heating) effects in tendons [3]. The objective of the current set of studies was to design and examine the effects of thermal-dominant and mechanical-dominant (mechanical loading) FUS treatments on material and extracellular matrix (ECM) properties of murine Achilles tendons *ex vivo* and *in vivo*.

METHODS: A custom 1.1MHz FUS transducer (H-101, Sonic Concepts) was mounted onto a stereotactic positioning system and driven using a signal generator (TEK AFG31102, Tektronix, Inc.) and a radiofrequency amplifier (Electronics & Innovation, 2100L) combination [Figure 1]. For the *ex vivo* study, uninjured Achilles tendons of 12-week-old C57B1/6 male mice were treated *in situ* immediately following euthanasia. Hind limbs were shaved using Nair and ultrasound coupling gel was applied. FUS treatments were applied for five minutes each at two locations on the tendon proper (tendon mid-body and distal aspect) according to the assigned protocol for each limb, and the order of treatment application was randomized (n=10 per group; n=8 for mechanical testing and n=2 for histology) [Table 1: Set 1, Set 2]. For the *in vivo* study, under IACUC approval, uninjured Achilles tendons of 12-week-old male C57B1/6 mice were prepared and treated as described previously (n=10 per group). Under isoflurane anesthesia, each mouse received 4 treatment sessions spaced 24 hours apart during a 1-week time span and euthanized 24 hours after the last session. In a separate study (*in vivo*, injured tendon), Achilles tendons of 12-week-old C57B1/6 male mice (n=10; n=8 for mechanical testing and n=2 for histology per experimental group) were injected with TGF- β 1 to induce tendinopathy [4]. 24 hours following injury induction, tendons were treated as described above, for 1 week according to the assigned protocol [Table 1: Sets 1, 2, 3]. For all *in vivo* studies, mice were euthanized 24 hours after the final treatment session and prepared for mechanical testing and histological assessments (Safranin-O and Toluidine blue staining).

RESULTS: *Ex Vivo treatment of uninjured tendon:* No significant differences were found when comparing material properties (maximum stress, yield stress, elastic modulus) of naïve (untreated) tendons in comparison to those treated with thermal- or mechanical-dominant FUS. Histologic analysis revealed collagen fiber disorganization and disruption along with increased cellularity in tendons treated with thermal-dominant pulsing, while tendons treated with mechanical-dominant pulsing displayed moderately increased cellularity near the calcaneal insertion site, myotendinous junction and in the tendon body. ECM disorganization was not as pronounced compared to the thermal treatment, but presented as mild fiber separation and fraying. *In vivo treatment of uninjured tendon:* Mice showed no signs of distress during and after treatments (4 sessions/ 10 min per session/ one week). Maximum stress and elastic modulus were similar between the treated (thermal and mechanical) tendons but were significantly ($p=0.004$ and $p=0.001$ respectively) reduced compared to naïve tendons. Yield stress was similar among the three groups. Histology showed more prominent (compared to *ex vivo* treatments) evidence of matrix disorganization and hypercellularity in the tendons treated with thermal-dominant pulsing while the mechanical-treatment group showed mild disorganization and fiber separation and moderately increased cellularity compared to single session *ex vivo* treatments. *In vivo treatment of injured tendon:* Cross-sectional area (CSA) was significantly elevated in the injured, untreated tendons, and all three treatments resulted in significantly reduced CSA (relative to injured/untreated). Maximum stress of the injured/untreated, thermal, moderate mechanical and high mechanical treatment groups was significantly lower than that of native group. Elastic modulus of the injured/untreated and moderate mechanical treatment group were significantly reduced relative to the naïve group. However, no significant differences were observed between the elastic modulus of the naïve, thermal and high mechanical treatment groups. Yield stress for injured/untreated tendons, thermal and moderate mechanical groups were significantly lower than that of the naïve group. Histologic analyses of injured tendons showed hypercellularity throughout the fat pad, peritenon and tendon body including rounded, chondrocyte-like cells in all treatment groups. Collagen disorganization close to these cells was evident as mild fiber separation. While signs of injury persisted after 1-week of treatments, in the treatment regions (irrespective of the treatment type), there appeared to be fewer rounded cells and sulfated glycosaminoglycan deposits [Figure 2].

DISCUSSION: Characterizing the effects of FUS *ex vivo* and in uninjured tendons *in vivo* is crucial to establish safety and inform methodologies for treating injured tendons. Results from our *ex vivo* study revealed that distinct FUS pulsing (ranging from moderate to high pressures) did not significantly alter tendon biomechanical integrity. Further, our *in vivo* feasibility study revealed that both treatments were well tolerated and results indicated that bioeffects were confined to the tendon. Biomechanical properties of uninjured, FUS-treated tendons were altered and correlated with changes in tendon microstructure, likely indicative of heat- and loading-induced matrix disorganization. However, our results demonstrate the encouraging therapeutic potential of FUS as both thermal and mechanical stimulation elicited an increase in material properties of injured tendons. Future studies will quantify matrix strains (using high-frequency ultrasound) and molecular responses in order to elucidate mechanisms through which FUS promotes tendon healing.

SIGNIFICANCE/CLINICAL RELEVANCE: Herein, we present pre-clinical FUS approaches to precisely target murine Achilles tendons and examine distinct bioeffects of thermal-dominant and mechanical-dominant treatments. We investigated the safety and feasibility of applying FUS pulsing to murine Achilles tendons *ex vivo* and *in vivo* and demonstrated that FUS can be applied without any deleterious effects in surrounding tissues. When applied to injured tendons, mechanical dominant schemes appeared to drive larger improvements in material properties compared to thermal-dominant pulsing. Such strategies may mimic clinical rehabilitation protocols that improve biomechanical integrity and function of injured tendons.

REFERENCES: [1] Yu et al. Am J Phys Med Rehabil 2013; [2] Best et al. Intern Med Rev 2016 [3] Meduri et al. ORS Tendon Section 2022 [4] Rezvani et al. J Appl Physiol 2021



Figure 1: Experimental FUS setup; A: murine hind limb; B: transducer and coupling cone

Set #	Treatment	Pressure (MPa)	Pulse Repetition Freq (Hz)	Duty Cycle (%)
1	Thermal	0.5	Continuous FUS	100
2	High Mechanical	5	10	1
3	Moderate Mechanical	2	10	1

Table 1: Experimental Parameters for *ex vivo* (Sets 1&2) and *in vivo* (Sets 1-3) treatments

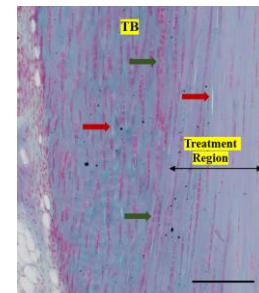


Figure 2: Changes in tendon body (TB) after FUS (mechanical) treatment; scale bar: 100 microns; red arrows: matrix disorganization; green arrows: matrix disorganization