Rat Achilles Tendon *In vivo* Overload Injury Response is Loading Magnitude and Duration Dependent

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INTRODUCTION: Tendinopathy is a degenerative condition associated with pain, decreased quality of life, and diminished functional abilities, affecting 30 to 50% of the athletic, elderly, and workplace populations (1). Tendinopathy is a result of accumulated fatigue injuries and insufficient recovery, leading to a failed healing response and incomplete recovery of preinjury strength and function. However, there is limited understanding of its pathogenesis, as its later stages largely characterize it. Increased collagen disruption, upregulation in inflammation, and matrix turnover have been reported to precede symptoms of tendon injury and rupture, with the initial healing response involving an inflammatory phase from 24 hours to 1-week post-injury. This study aims to assess the rat Achilles tendon's mechanical and biological response due to repetitive cyclic fatigue loading indicative of exercise and overload injuries during the inflammatory healing phase.

METHODS: This study was performed under IACUC-approved methods. Forty-eight 13-week-old male and female Sprague Dawley rats were anesthetized, and their left Achilles tendons underwent cyclic loading according to a previously published method (2, 3). Briefly, the rat's left foot was attached to an ankle joint that performed passive ankle dorsiflexion up to 45˚relative to full plantarflexion. To isolate the Achilles tendon, the left hindlimb was fixed in full extension. Each loading bout consisted of the ankle being cyclically dorsiflexed at ~1 Hz to mimic normal gait for 500 cycles, with pre- and post-cyclic loading mechanical measurements performed to assess the loading regimen's effect on tendon *in vivo* mechanical properties. The rats were divided into 2 groups, where each group was subjected to loading bouts of 20% (exercise) or 40% (overload injury) of the Achilles tendon's ultimate failure load, obtained from pull-to-failure tests. Each group had 3 sub-groups, where animals received 1, 2, or 3 bouts of 500 loading cycles with two days of cage activity between each bout (n = 8/group, 4 females, 4 males). The rats were euthanized via CO_2 inhalation seven days post-injury (dpi). Six tendons per group were harvested for RT-qPCR gene expression analysis; the remaining tendons were dissected and frozen for other assays. The percentage change in mechanical properties (peak torque, hysteresis, and linear stiffness) of the torque vs. angle curves was calculated. The effect of each load, with differences between post-injury and the baseline properties, and the effect of consecutive loads, with differences between post and pre-injury, were calculated for the 20% and 40% load groups for 1, 2, and 3 days of loading. Statistical analysis was done using GraphPad with $p < 0.05$ considered statistically significant. A two-way ANOVA test was used to test the significance between the loading magnitude and duration.

RESULTS: The mechanical properties following each cyclic loading bout had consistent changes across loading days for the 20% and 40% groups. In comparison, there was a statistically larger decrease in mechanical properties for the 40% loading group (**Figure 1A**). Mechanical properties due to consecutive loads demonstrated decreased changes across all properties and loads for the 40% loading group, while the 20% loading group had lower decreases comparatively, with increases in hysteresis (**Figure 1B**). Gene expression of collagen genes 7 dpi showed no statistical changes, while MMP2 had a statistical upregulation between the 20%-2 load and 40%-3 load and 20% and 40% 3 load groups. TNFα and TGFβ showed a decreasing trend in expression following consecutive loading bouts, although not significant. VEGF demonstrated an elevated expression level for the 40%-2 load group compared to both 3 load groups. IL-1β had decreased expression with consecutive loading bouts of 7 dpi for the 20% loading group but no expression in the 40% loading groups, with statistical significance compared to the control group.

DISCUSSION: The changes in mechanical properties demonstrated that the 20% and 40% loading magnitudes damaged the tendon with each bout, with a higher amount of damage caused by the 40% group. With consecutive loads, the 40% group caused more cumulative damage than the 20% group, while the increase in hysteresis for the 20% group may indicate a beneficial response to the tendon. Analysis of stress-strain data will better inform of changes to tendon material properties. Despite being in the inflammatory healing phase, all loading regimens caused decreased IL-1β expression, with no expression for the 40% group. The decrease and lack of inflammation may implicate the negative effects on matrix turnover, as the steady expression of collagen 1 and 3 suggests the lack of matrix turnover. Analysis of apoptotic gene expression, histological evaluation, and composition will be carried out to understand further the implications of exercise and overload injuries on the tendon's healing response during the inflammatory phase.

SIGNIFICANCE/CLINICAL RELEVANCE: This study assessed the effects of different loading magnitudes with consecutive cyclic loading of a rat Achilles tendon in a noninvasive and *in vivo* setting. These results, with further analysis of remaining data, will help us understand the alterations to the inflammatory phase of healing following overload injury to meet the unmet clinical understanding of the initiating factors of tendinopathy.

REFERENCES: (1) J.F. Kaux (2011). *J Sports Sci Med*. (2) P. Williamson et al. (2023). *J. Biomechanics*. (3) P.H. Chainani et al. (Accepted, 2023). *JoVE*.

IMAGES AND TABLES:

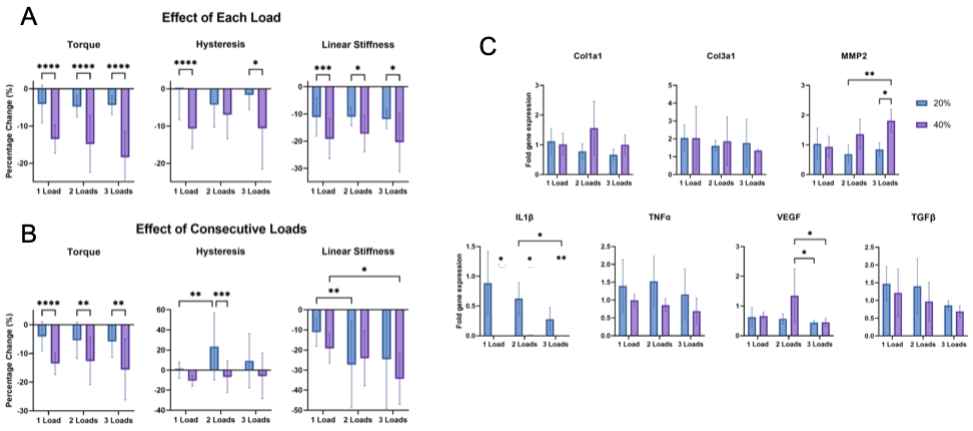

Figure 1. Sprague Dawley rats were subjected to cyclic fatigue loading to 20% or 40% of the failure load with 2 days of rest between loads and euthanized 7 days post final injury. Panels A and B shows changes in mechanical properties due to each injury (**A**) and due to consecutive injuries (**B**). Panel **C** shows RT-qPCR fold gene expression of collagen, matrix, and inflammatory cytokines for the 1, 2, and 3 loading groups 7 days post final injury with asterisks (*) denoting significance compared to the control group (not shown) and each injury group.