Accumulation of tendon fatigue damage detected in situ using automated histological quantification

INTRODUCTION: Fatigue loading of tendon has been shown to cause accumulation of microstructural damage in the extra-cellular matrix, as demonstrated by progressive fiber kinking and microrupture [1-2]. This degeneration has been observed in clinical overuse pathology and is believed to lead to tendon weakening and rupture [3]. We have published an automated technique for quantifying such damage in second harmonic generation (SHG) images of rat tendon generated from prepared slides [4]. In this study, our goal was to perform SHG imaging on mouse tendon in situ and validate our automatic damage quantification technique for use in mice.

METHODS: Adult female C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were used. All procedures were approved by the Institutional Animal Care and Use Committee. All mice were fatigued in accordance with our previously validated model [5]. Briefly, 18 mice were continuously administered isoflurane anesthesia (2% by volume, flow rate 0.3 L/minute). Once surgical anesthesia was attained, each animal was secured supine on a Plexiglas frame. The frame was attached to an adjustable vise to facilitate positioning of the animal and to maintain a physiologic knee flexion angle of ~30° during the loading tests. A 1 cm longitudinal incision was made to expose the tibia, patellar tendon and patella. The tibia and patella were gripped and the tendon was preconditioned for 10 cycles at 2 Hz between 0 N and 1 N. The tendon was then cyclically loaded in tension at 2 Hz, between 0.5 N and 4 N (~50% of monotonic failure load). 6 mice were fatigued for 1 hour (4 N, 1 hour group) and 6 mice were fatigued for 2 hours (4 N, 2 hour group). 6 additional mice were loaded between 0.5 N and 6 N (~75% of monotonic failure load) for one hour (6 N, 1 hour group). 12 mice underwent sham surgery (n=6 for 1 hour and n=6 for 2 hours). All mice were killed immediately after loading.

Superficial fascia was removed and SHG imaging was performed on the exposed, unfixed, unstained tendon in situ with a physiologic knee flexion angle of ~30°. Imaging was performed on a multiphoton microscope with a 60× water immersion objective lens. 8-bit grayscale images were acquired with 1024 × 1024 pixel resolution on a field of view of 205 mm through the entire thickness of the tendon.

One image per tendon (n=58, two tendons were excluded for poor image quality) was analyzed to quantitate damage area fraction (DAF), using our previously published technique [4]. Briefly, a grid of windows was digitally superimposed, and each window was then manually determined to be damaged or non-damaged using an established set of rules [2] by a blinded user. The number of damaged windows was then normalized to the total number of windows in the image to determine its DAF. A subset of images was analyzed three times to determine the user’s repeatability, which was assessed using a repeated measures ANOVA.

The 58 images were then analyzed automatically using a previously published technique [4]. Briefly, each image was divided into subsections that were analyzed using Fourier transform to calculate fiber orientation, which was then compared to the fiber orientation of the adjacent subsections. A 4° change in adjacent fiber orientation angles was defined as damage. Accuracy of the automatic analysis was measured by correlating the results of the manual and automatic analysis methods. P ≤ 0.05 represents statistical significance for all statistical tests. All two-group comparisons were made using a paired, two-tailed T-test.

RESULTS: SHG images of physiologically positioned, unstained, unfixed tendon in situ revealed collagen fibril orientation clearly (Fig. 1). There were no significant differences between the user’s repeated analyses (data not shown). The DAF was measured using the manual and automatic methods correlated well, yielding an R²=0.63 and best fit line of DAF_{Automatic} = 1.07 * DAF_{Manual} + 4.29, which was significant (Fig. 2).

DISCUSSION: Using our previously reported mouse model of tendon overuse, we induced damage using three distinct loading regimens. SHG imaging of the unstained, unfixed tendon in situ revealed that collagen fibril orientation was clearly visible (Fig. 1), suggesting that in overuse, we induced damage using three distinct loading regimens. The 6 N, 1 hour and 4 N, 2 hour groups exhibited significant DAF increase in the fatigue loaded limb relative to contralateral controls.

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Fig. 1. SHG image of mouse tendon in situ, revealing damage induced kinks in collagen orientation (center of image).