Tendon Degeneration is Not Due to Altered Local Tissue Strains in Fatigue-Loaded Mouse Achilles Tendons

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INTRODUCTION: Tendinopathy is characterized by altered structure and cellular degeneration in the form of increased tissue catabolism, inflammation, and the accumulation of abnormal tissue deposits[1]. It is hypothesized that cellular degeneration is a response to fatigue loading and the resultant changes in physical stimuli. However, the physical stimuli responsible for initiating tendinopathy remain unknown. Previous in vitro studies suggest that high strains are responsible for tendon cell degeneration[2]. However, in vitro studies fail to replicate important aspects of the native tendon environment (e.g., cell-cell communication, cell-matrix interaction, hierarchal tissue structure). Hence, it is important to study tendon degeneration in the native cell environment. Explant models enable the investigation of mechanobiology in the native cell environment while also ensuring precise control over loading, environmental conditions, and pharmaceutical agents to determine the mechanisms driving cell behavior. Therefore, the **objectives of this study** were to a) establish an explant model for fatigue-induced tendon degeneration, b) confirm that tendon degeneration is driven by cell mechanobiology (i.e., actin-mediated contractility), and c) identify the physical stimuli responsible for tendon degeneration. We hypothesized that hypoxia and actin contractility are necessary to induce degeneration in response to fatigue loading and that cellular degeneration would colocalize with regions of high tensile strains.

METHODS: Achilles tendons were harvested from male C57BL/6 mice under an approved IACUC study. Explant Model: Mouse Achilles tendons were gripped and statically loaded at ~0.2MPa in culture medium at 30°C for 24 h to acclimate them to culture conditions. The explants were then cyclically loaded to a physiological stress of 5 MPa[3] at 0.5 Hz for 12 h each day for 4 days at atmospheric (20%, n = 6), physiological (8%, n = 7)[4,5], or hypoxic (3%, n = 7) oxygen levels. This loading protocol represents approximately 80% of the fatigue lifetime (i.e., cycles to failure) of mouse Achilles tendons[3]. An identical number of samples were statically loaded to ~0.2MPa as controls to prevent tissue catabolism due to unloading[6]. An additional 7 samples were statically and fatigue loaded at 3% O_2 with 10 uM cytochalasin D (CytoD) to disrupt the actin cytoskeleton. After loading, samples were immediately frozen, cryogenically homogenized, and processed for qPCR. Local Strains and Mitochondrial Health: Five mouse Achilles tendons were fatigue loaded as previously mentioned and then stained with 10 nM Tetramethylrhodamine, methyl ester (TMRM), 20 uM fluorescein diacetate (FDA), and 5 uM DRAQ5. TMRM stains for cells with healthy mitochondria, FDA stains live cells, and DRAQ5 stains all cell nuclei. The explants were then transferred to a microtensile device and stretched to 2% strain increments until the tendon reached a stress of 5 MPa. Volumetric images were acquired at each strain increment. TMRM signal at 0% strain was binarized to distinguish between healthy and unhealthy mitochondria. A previously generated code[7] was used to calculate and generate spatial maps of the longitudinal, transverse, and shear strains at each strain increment. Statistics: Gene expression of fatigue loaded samples was compared to static samples within each treatment condition using Mann Whitney tests. The average and standard deviation of the local tissue strains were compared between fresh and fatigue-loaded tendons using an ANCOVA. A logistic regression was used to determine if any of the strains were predictors of mitochondrial dysfunction. Goodness of fit of the logistic regression was determined with McFadden's R^2. Statistical significance was set at $p < 0.05$.

RESULTS: Fatigue loading produced tendon degeneration (i.e., upregulation of catabolic, inflammatory, chondrogenic, adipogenic markers) only under hypoxic conditions, which was eliminated with CytoD treatment (Fig. 1). Surprisingly, there was no change in the average or standard deviation of the longitudinal, transverse, or shear strains between fatigue-loaded and freshly harvested tendons (only longitudinal strains shown in Fig. 2). Finally, the logistic regression model determined that none of the strains were predictive of healthy mitochondria (McFadden's $R^2 = 0.04$, Table 1).

DISCUSSION: We developed the first explant model of fatigue induced tendon degeneration that exhibits not only an increase in catabolic and inflammatory markers but also an upregulation of chondrogenic (Sox9) and adipogenic (LPL) genes that are associated with the deposition of abnormal tissue components. The fact that this was observed only under hypoxic conditions is consistent with clinical studies suggesting that hypoxia is a driver of tendon degeneration[8]. Additionally, the lack of a degenerative response to fatigue-loading with CytoD treatment suggests that mechanotransduction via an intact actin cytoskeleton is necessary to induce tendon degeneration. This is in agreement with a previous in vitro study that showed that non-tenogenic differentiation of tendon progenitor cells induced by cyclic loading can be prevented by inhibiting actin contractility[9]. Finally, we surprisingly found that there were no changes in local strain fields with fatigue loading. While counterintuitive, these findings are consistent with prior work[10] and help explain why we also found that the local tissue strains were not predictive of mitochondrial dysfunction. While previous in vitro studies of isolated cells show that high tensile strains (8%) can produce degenerative cell behavior[2], our findings indicate that the longitudinal strains in mouse Achilles tendons never exceed 2%, which suggests that the loading conditions in prior experiments are supraphysiologic. Rather than altered tissue strains, it is possible that local structural damage (e.g., collagen disorganization or denaturation) is responsible for initiating cellular degeneration in fatigue-loaded tendons. To investigate this further, future studies will measure the colocalization of gene expression indicative of tendon degeneration (e.g., Sox9, LPL, proteases, cytokines) with structural damage and altered tissue strains.

SIGNIFICANCE/CLINICAL RELEVANCE: The causes of tendon degeneration and tendinopathy in response to fatigue loading are unknown. Our findings suggest that tendon degeneration is dependent on actin-mediated mechanotransduction but that altered tissue strains are not the physical stimuli that induce a degenerative cellular response. This is important for understanding the origin of tendon degeneration and developing treatments to prevent pathology.

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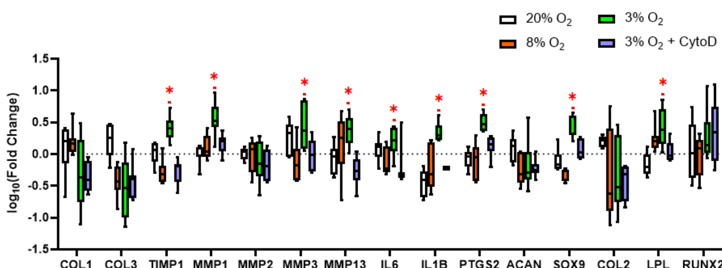

Fig.1. Gene expression of fatigue loaded tendons relative to statically loaded tendons in 20%, 3% or 3% O_2, without or without CytoD treatment. *$p<0.05$

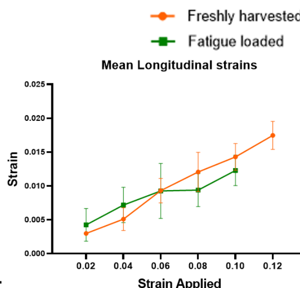

Fig.2. Longitudinal strains in fatigue loaded and freshly harvested tendons

Coefficients	Variable	Estimate	Odds ratio	p value	p value summary
β0	Intercept	-0.3560	0.7005	0.0095	**
β1	Area	0.02471	1.025	<0.0001	****
β2	x coordinate	-0.0001592	0.9998	0.2534	ns
β3	y coordinate	0.001465	1.001	0.0003	***
β4	Longitudinal strains	-0.04803	0.9531	0.9785	ns
β5	Transverse strains	-0.1991	0.8195	0.6544	ns
β6	Shear	-0.4524	0.6361	0.6392	ns
β7	Label (Sample 2)	-0.07408	0.9286	0.4712	ns
β8	Label (Sample 3)	-0.01516	0.9850	0.8718	ns
β9	Label (Sample 4)	-0.6131	0.5417	<0.0001	****
β10	Label (Sample 5)	0.2317	1.261	0.1719	ns

Table.1. Logistic regression data