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Aging leads to inferior Achilles tendon mechanics and altered ankle function in rodents

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ABSTRACT

Spontaneous rupture of the Achilles tendon is increasingly common in the middle aged population. However, the cause for the particularly high incidence of injury in this age group is not well understood. Therefore, the objective of this study was to identify age-specific differences in the Achilles tendon-muscle complex using an animal model. Functional measures were performed *in vivo* and tissues were harvested following euthanasia for mechanical, structural, and histological analysis from young, middle aged, and old rats. Numerous alterations in tendon properties were detected across age groups, including inferior material properties (maximum stress, modulus) with increasing age. Differences in function were also observed, as older animals exhibited increased ankle joint passive stiffness and decreased propulsion force during locomotion. Macroscale differences in tendon organization were not observed, although cell density and nuclear shape did vary between age groups. Muscle fiber size and type distribution were not notably affected by age, indicating that other factors may be more responsible for age-specific Achilles tendon rupture rates. This study improves our understanding of the role of aging in Achilles tendon biomechanics and ankle function, and helps provide a potential explanation for the disparate incidence of Achilles tendon ruptures in varying age groups.

1. Introduction

Achilles tendon injuries are most common in middle-aged men, especially those involved in recreational sports. Various studies have each reported a consistent increased incidence rate since the 1950s that is greater than the rate of population increase alone (Lantto et al., 2015; Leppilahti et al., 1996; Suchak et al., 2005). Furthermore, there is evidence that the median age and overall incidence in older individuals specifically of Achilles tendon ruptures have both increased over the past decade (Huttunen et al., 2014). The mechanisms underlying this age-specific disparity are not well understood, but have been suggested to include increased participation in demanding sports by older individuals combined with tissue degeneration associated with aging (Ng et al., 2011; Peffers et al., 2015).

Specifically, age-dependent changes in Achilles tendon physiology and mechanics, such as decreased blood flow and increased tissue stiffness, have been implicated as potential causes for the high incidence of ruptures in this age group. However, although peritendinous blood flow is decreased while at rest in older individuals, blood flow in Achilles tendons during exercise is similar throughout ages, suggesting that vascular factors are not likely solely responsible for the increased incidence with age of Achilles tendon injuries (Langberg et al., 2001). Importantly, *in vivo* human studies evaluating biomechanical changes in aging Achilles tendon have yielded contradictory results as to whether Achilles tendon stiffness and strain in older individuals is decreased, increased, or similar to younger individuals (Karamanidis and Arampatzis, 2006; Kubo et al., 2007; Onambele et al., 2006). Additionally, there has been suggestion that regular physical activity as athletes age can help to avoid degeneration by increasing tendon size, strength, and nutrient delivery (Smith et al., 2002). Conversely, a recent study found no differences in Achilles tendon cross-sectional area, collagen content, or mechanical properties between adult long distance runners who were physically active or inactive in young age, suggesting that physical activity during youth did not improve Achilles tendon properties (Lenskjold et al., 2015). In summary, previous clinical studies have differing results which do not establish a clear relationship between age and Achilles tendon ruptures, likely due to an incomplete fundamental understanding of how aging affects Achilles tendon physiology and biomechanics. Given

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the practical limitations of human studies, there exists a clear need for a rigorous animal study that leverages the benefits of using a non-human model to better and more comprehensively define the role of aging in Achilles tendon structure and function.

Animal models offer a highly controlled system to study Achilles tendon biomechanics, and have demonstrated a potential explanation for the contrasting incidence in Achilles tendon rupture across sex (Pardes et al., 2016). However, it is unknown if aging also alters Achilles tendon mechanical behavior that could help explain the particularly high frequency of Achilles tendon ruptures in middle-aged men. There is preliminary support for this hypothesis, as changes in the viscoelastic response of the gastrocnemius-Achilles (GC-AT) muscle-tendon unit have been identified with increasing age (Plate et al., 2013). However, more comprehensive investigation is needed in order to better define the impact of aging on Achilles tendon-muscle properties and ankle function *in vivo* and *ex vivo*, ultimately helping to elucidate the mechanism underlying the age-specific incidence of ruptures observed clinically.

Therefore, the objective of this study was to identify functional, mechanical, and structural differences among Achilles tendon-muscle units from young, middle aged, and old male rats. We hypothesized that middle aged and old rats would exhibit increased joint stiffness and decreased Achilles tendon material quality compared to young rats, as well as decreased matrix organization.

2. Methods

2.1. Design

All procedures were approved by the University of Pennsylvania's Animal Care and Use Committee. Young (8 mo), Middle Aged (19 mo), and Old (28 mo) male F344XBN rats, approximating respective human ages of 18, 43, and 63 years (Quinn, 2005), were acquired from the National Institute of Aging ($n = 16$/group) and euthanized following *in vivo* functional testing. This animal model shows musculoskeletal changes similar to those observed in human aging and has been previously used specifically for the study of the rotator cuff and Achilles tendon-muscle units aging response (Mannava et al., 2011; Plate et al., 2013).

2.2. Gait analysis

Animals ($n = 12–16$/group) were acclimated to an instrumented walkway, and spatial, temporal, and kinetic parameters were quantified during autonomous locomotion as previously described (Fryhofer et al., 2016). Briefly, maximum ground reaction forces (medial/lateral, braking, propulsion, vertical) were calculated following isolation of the hindlimb on either one of two six degree-of-freedom force plates and reported as percent of animal body weight (%BW). Spatiotemporal parameters (stride length, stride width, speed, and stance time) were determined by semi-automated analysis of images captured by digital camera during locomotion using MATLAB (Mathworks; Natick, MA).

2.3. Passive joint function

Passive ankle range of motion (ROM) and stiffness were measured using a custom device while animals ($n = 16$/group) were anesthetized as previously described (Fryhofer et al., 2016). Bilinear fits were applied to torque-angle data (within a consistent torque range across all animals) in order to calculate toe and linear stiffness for both dorsiflexion and plantarflexion. Range of motion was measured relative to the geometric zero position (ankle and tibia oriented perpendicularly).

2.4. Tendon sample preparation

Animals were euthanized (mass [mean ± SD]: Young 393 ± 25 g, Middle Aged 521 ± 27 g, Old 504 ± 44 g) and Achilles tendon-foot units were harvested and either processed for histological assays or frozen until preparation for structural and mechanical analysis. Prior to all *ex vivo* testing and analyses, specimens were randomized and blinded to the study investigators. High frequency ultrasound/mechanical testing specimens (same specimens were used for both assays) were fine dissected to remove non-tendinous soft tissue and measured for tendon mid-substance cross sectional area (CSA) using a custom laser-based device (Freedman et al., 2016). Stain dots were applied for optical strain tracking, the calcaneus-foot complex was embedded in PMMA, and the proximal end of the tendon was attached between sandpaper using cyanoacrylate to leave a gauge length of 12 mm (Freedman et al., 2016).

2.5. High frequency ultrasound (HFUS)

Prior to mechanical testing, sagittal B-mode images of tendons loaded at 1 N in a 1× PBS bath ($n = 11–12$/group) were captured at 0.25 mm increments using a 40 MHz scanner (MS550D; VisualSonics, CA) as previously described (Riggin et al., 2014). A custom MATLAB program was used to analyze the 3–4 central-most images and determine tendon matrix alignment (reported as circular standard deviation) and density (reported as echogenicity).

2.6. Mechanical testing

Samples ($n = 11–12$/group/protocol) were tested to evaluate quasi-static properties (ramp to failure with optical strain tracking) or viscoelastic, dynamic, and fatigue properties (stress relaxation, low-strain frequency sweep, load-controlled fatigue testing) using one of two previously described protocols (Freedman et al., 2016; Pardes et al., 2016). Briefly, the Achilles tendon was maintained perpendicular to the foot to mimic *in vivo* loading and submerged in a 1× PBS bath at 37 °C. The Electropuls E3000 testing frame (Instron; Norwood, MA) was used with a 250 N load cell, while images were captured by a digital camera (Basler; Exton, PA) and 200 mm lens (Nikon; Melville, NY). Fatigue samples were tested until failure or completion of 20,000 cycles. Mechanical properties were evaluated at 50 cycles (early response) and 1500 cycles (latest possible cycle number to include data from >90% of all specimens). Load-displacement data for all tests were acquired by WaveMatrix (Instron; Norwood, MA) and imported into MATLAB for calculation of mechanical properties (Freedman et al., 2016).

2.7. Tendon histology

Achilles tendons ($n = 4–8$/group) were fixed, decalcified, and embedded in paraffin using standard techniques and sectioned sagitally at 7 μm (Fryhofer et al., 2016). Sections were then stained with Hematoxylin-Eosin (H&E) or Safranin-O/Fast Green and imaged at 100X. Images were evaluated for cell density and nuclear shape factor (0 = circle; 1 = line) using a commercial software (Bioquant Osteo II; Nashville, TN) (Rooney et al., 2016). Safranin-O positive staining was determined using a previously described thresholding method in ImageJ (Fryhofer et al., 2016).

2.8. Muscle histology

Gastrocnemius-soleus muscle mid-belly ($n = 7$/group) was excised at the time of sacrifice, flash frozen, and stored at −80 °C until axial cryosectioning at 10 μm, as described previously (Pardes et al., 2016). Immunofluorescence imaging was performed on sections stained for laminin and myosin heavy chain (MyHC) types 1, 2a, and 2b. The anti-MyHC antibodies developed by Stefano Schiaffino were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. MyHC2x expression was presumed from unstained fibers. After staining, three images were taken from both the superficial and deep regions of each muscle. The SMASH application in MATLAB was used to calculate muscle fiber size (minimum Feret diameter) and type distribution for each region (Smith and Barton, 2014).

2.9. Statistics

One-way ANOVAs were used to compare groups for functional, structural, and mechanical Achilles tendon-muscle properties, and significant relationships ($p < 0.05$) were further evaluated using post hoc Student's t-tests with Bonferroni corrections ($\alpha = 0.05/3$), except for cycles completed and tendon histological properties. For cycles completed, ranks were assigned (1, 0–5000 cycles; 2, 5000–10,000 cycles, 3, 10,000–15,000 cycles; 4, 15,000–20,000 cycles) and the non-parametric Kruskal-Wallis test with Dunn's post hoc tests was used. Non-parametric Kruskal–Wallis tests were also used to evaluate differences in tendon histological properties between groups ($\alpha = 0.05$). All data are presented as mean ± standard deviation except for fatigue cycles completed and tendon histology, which are reported as median (interquartile range).

3. Results

Gait analysis revealed that lateral force increased and propulsion force decreased with increasing age (Fig. 1A and B), while no significant differences in braking or vertical forces were detected (Fig. 1C and D). Animals also took slower, shorter, and wider steps as they aged (Fig. 1E–G).

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