Normal aging alters *in vivo* passive biomechanical response of the rat gastrocnemius-Achilles muscle–tendon unit

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ABSTRACT

Predisposition to Achilles tendon (AT) ruptures in middle-aged individuals may be associated with age-related changes to inherent passive biomechanical properties of the gastrocnemius-Achilles (GC-AT) muscle–tendon unit, due to known muscle–tendon structural changes in normal aging. The goal of this study was to determine whether the passive biomechanical response of the GC-AT muscle–tendon unit was altered with age in 6 young (8 months) and 6 middle-aged (24 months) F344xBN hybrid rats from the National Institute on Aging colony. Fung's quasilinear viscoelastic (QLV) model was used to determine *in vivo* history and time-dependent load-relaxation response of the GC-AT. Effective stiffness and modulus were also estimated using linear regression analysis. Fung's QLV revealed a significantly decreased magnitude of the relaxation response (parameter C, $p=0.026$) in middle-aged animals compared to young animals (0.108 ± 0.007 *vs.* 0.144 ± 0.015), with similar time-dependent viscous GC-AT properties (τ_1, τ_2). The product of elastic parameters (A*B), which represents the initial slope of the elastic response, was significantly increased by 50% in middle-aged rats ($p=0.014$). Estimated GC-AT stiffness increased 28% at peak tensions in middle-aged rats (2.7 ± 0.2 N/mm) compared to young rats (1.9 ± 0.2 N/mm; $p=0.036$). While the limitations of this animal model must be considered, the changes we describe could be associated with the observation that GC-AT pathology and injury is more common in middle-aged individuals. Further studies are necessary to characterize the load-to-failure behavior of AT in middle-aged compared to young animals.

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1. Introduction

Achilles tendon (AT) pathology encompasses conditions ranging from AT tendinopathy to complete rupture. The annual incidence of AT ruptures is approximately 7/100,000, with an increased incidence in middle-aged individuals (Leppilahti et al., 1996; Suchak et al., 2005). The etiology of AT pathology is multifactorial involving both intrinsic and extrinsic factors (Kearney and Costa, 2012); however, over 80% of AT ruptures occur during recreational sports without any prodromal symptoms (Jarvinen et al., 2001; Leppilahti et al., 1996). The cause for the predisposition of healthy middle-aged men who occasionally participate in sports ("weekend warriors") to AT ruptures remains

unknown (Jarvinen et al., 2001; Leppilahti et al., 1996). A deeper understanding of the underlying mechanisms of AT pathology and associated changes in AT material response to imposed forces is imperative to guide prevention and treatment in middle-aged individuals at risk.

It has been suggested that overuse, in conjunction with an age-related decrease in muscle function and healing potential, and molecular and structural changes of skeletal muscle and tendons, may lead to AT pathology (Delbono, 2002; Larsson and Ramamurthy, 2000; Longo et al., 2009; Oh et al., 2010; Raj et al., 2010). The decrease in muscle mass and muscle fiber cross-sectional area (CSA), in combination with changes in collagen content and increased collagen disorganization in the tendon, can be expected to result in changes to the biomechanical response of the muscle and tendon tissue (Reeves et al., 2006). However, there have been contradictory findings assessing *in vivo* biomechanical changes of the AT during aging. Kubo et al. (2007) showed significantly decreased strain of the AT of middle-aged and older individuals

compared to young volunteers. Conversely, Onambele et al. (2006) found increased gastrocnemius tendon strain values in middle-aged and older individuals compared to young individuals. Furthermore, Karamanidis and Arampatzis (2006) did not find differences in AT stiffness. The impact of aging on the gastrocnemius (GC) muscle and AT as a comprehensive muscle–tendon unit has not been adequately assessed.

The GC-AT muscle–tendon unit has been found to act viscoelastically *in vitro* and *in vivo* (Kubo et al., 2001; Lin et al., 1999; Magnusson et al., 1995; Taylor et al., 1990), with contractile and elastic elements in series (Hill, 1938; Holmes, 2006). The evaluation of age-related changes in the *in vivo* assessment of muscle and tendon stress response may be achieved through the application of Fung's quasilinear viscoelastic (QLV) model (Fung, 1967, 1972). The QLV model has been used previously to characterize the *in vivo* time- and history-dependent changes in tendons, ligaments and muscle (Abramowitch et al., 2004, 2010; Lin et al., 1999; Magnusson et al., 1995; Peltz et al., 2010; Taylor et al., 1990). Abramowitch et al. (2004) used the QLV model to assess changes in the biomechanical stress-relaxation response of healing medial collateral ligaments in a goat model. They found a larger viscous response (parameter C) compared to control animals indicating a longer recovery time after removal of load. Mannava et al. (2011) used the QLV model to assess the passive biomechanical response of muscle–tendon units after acute and chronic supraspinatus tendon tear, and demonstrated that *in vivo* neural contribution to physiological muscle tone contributes significantly to the passive biomechanical response. Thus, *in vivo* measurements of passive response warrant consideration, but have rarely been included in previous studies.

We sought to assess the influence of aging on the *in vivo* passive biomechanical response of the GC-AT muscle–tendon unit in a rat model of aging using Fung's QLV analysis. We hypothesized that *in vivo* muscle–tendon unit QLV parameters describing passive viscoelastic behavior are altered during normal aging in this animal model.

2. Materials and methods

Twelve rats from the National Institute on Aging (NIA) Aged Rodent Colonies (Sprott, 1991) underwent *in vivo* biomechanical testing of the right GC-AT muscle–tendon unit. Six young (8 months old) and 6 middle-aged (24 months old) Fisher–Brown Norway F1-hybrid rats (F344xBN) were chosen to represent human age of approximately 18 and 52 years, based on the assumption that 13.7 rat days correlate to 1 human year (Quinn, 2005). The F344xBN is a validated animal model for studying aging of various organ systems and sarcopenia; animals show decreased muscle mass and function during aging similar to humans (Betik et al., 2008; Hagen et al., 2004; Lipman et al., 1996; Rice et al., 2005). All animals were sacrificed immediately following *in vivo* testing. Institutional Animal Care and Use Committee approval was obtained.

2.1. Surgical exposure and experimental apparatus

Animals were anesthetized with 3–5% isoflurane, the GC exposed, and the calcaneus transected distal to the AT insertion (Macintosh et al., 2011; Mannava et al., 2011). Animals were placed on a linear translating stage and the AT was attached to a 1000 g force transducer (FORT100; World Precision Instruments) (Fig. 1). The force transducer was connected to an amplifier (ps100W-2; EMKA Technologies) through an interface controller (EMKA Technologies) and integrated through a converter card (PCI-6023E; National Instruments) with a computer running Slow Wave Analyzer (EMKA Technologies). Animal body temperature was maintained with a heat lamp.

2.2. Passive load-relaxation testing

A starting preload of 0.05 N was set as 0 mm baseline displacement. Non-destructive load-relaxation consisted of intermittent, progressive translations of the stage, rapidly stretching (approximately 3 mm/sec) the muscle–tendon unit from 0 to 8 mm displacement in 2 mm increments. Between each testing interval,

the GC was allowed to rest for 180 s at 0 mm resting length. The particular displacement intervals were chosen to characterize the load-displacement relationship within the range of physiologic displacement encountered by the GC-AT in natural ankle motion while avoiding ultimate load-to-failure of the muscle–tendon unit (Stolov and Weilepp, 1966). Passive tension was recorded continuously for 175 s during displacement, including both peak and equilibrium load. Following passive biomechanical measurements, the animals were sacrificed, and the GC muscle was analyzed for weight, volume displacement, and cross-sectional area (CSA).

2.3. Data analysis

Fung's QLV model has been used to estimate the parameters of the instantaneous elastic response and viscous relaxation equations during load-relaxation experiments (Abramowitch et al., 2004; Mannava et al., 2011). Parameters for Fung's QLV analysis were calculated at 2, 4, 6, and 8 mm of displacement of the GC-AT muscle–tendon unit and the average assessed for each animal. The present study assumed the idealized case of an immediate and constant deformation applied to the tissue, such that $\varepsilon(t) = a$. Under this assumption, the stress–time relationship reduces to

$$\sigma(t) = \frac{A(e^{Ba}-1)[1+C(-E_1(t/\tau_1)+E_1(t/\tau_2))]}{1+C\ln(\tau_2/\tau_1)} \quad (1)$$

where E_1 denotes the Euler integral $E_1(y) = \int_y^\infty (e^{-z}/z)dz$ (Mannava et al., 2011). Parameters 'A' and 'B' characterize the linear and non-linear scaling, respectively, of the instantaneous elastic response. Parameter 'C' characterizes the relaxation scaling, and the parameters 'τ_1' and 'τ_2' the time course of fast and slow viscous relaxation (Mannava et al., 2011). The product 'AB' defines the initial slope (at $\varepsilon = 0$) of the instantaneous elastic response, $\sigma_e(\varepsilon) = A(e^{B\varepsilon}-1)$, where

$$\frac{\partial \sigma_e}{\partial \varepsilon} = ABe^{B\varepsilon}. \quad (2)$$

Parameters for the model were estimated using *nlinfit* non-linear least-squares regression function intrinsic to MATLAB (The MathWorks, Natick, MA) (Fig. 2).
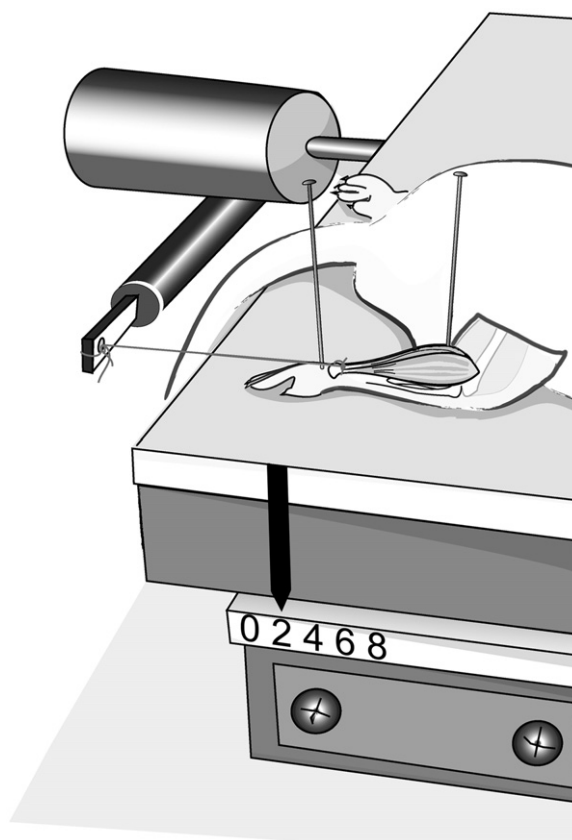

Fig. 1. Experimental setting for *in vivo* stress-relaxing testing. The GC-AT was exposed and the hindlimb stabilized with pins to isolate passive stretching of GC-AT and to limit artifact motion. The AT was then connected to a force transducer using stainless steel suture in line with the direction of force of the GC-AT. Passive muscle load was recorded continuously throughout the experiment.
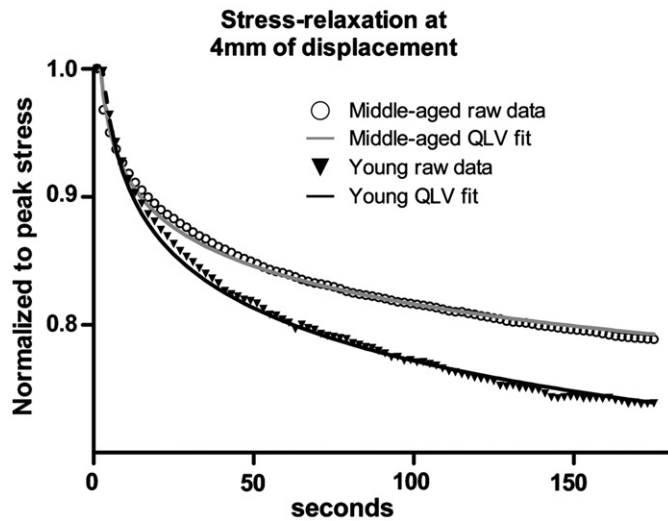
Stress-relaxation at 4mm of displacement

Fig. 2. Sample curve-fit of stress-relaxation data at 4 mm of displacement for a representative young and middle-aged animal.

Goodness-of-fit was quantified for elastic and viscous responses, respectively, via calculation of the coefficient of determination (R^2).

Estimates of the stiffness and modulus of the GC-AT for each animal were calculated from linear regression analysis of the peak and equilibrium load-displacement and stress–strain data points for each of the four imposed displacements. The slopes of the best-fit line for the load-displacement and stress–strain data were defined as the estimated stiffness and modulus, respectively. Stress was estimated by the quotient of load and muscle resting CSA. Strain was estimated by the quotient of displacement and muscle resting length. Mean stiffness and modulus for peak and equilibrium loading were calculated for the two age groups.

2.4. Statistical analysis

All values are expressed as mean±standard error of the mean (SEM) and statistical analysis performed using Prism 5 (GraphPad Software). For the estimated parameters obtained from the QLV analysis, a normal distribution was not assumed. Therefore, a non-parametric permutation test was employed to make comparisons between groups and detect statistical differences, which has been established as a standard method for comparing parameters that are not normally distributed (Kim et al., 1995). The initial calculation of a test statistic is followed by random permutation of data labels (resampling) with recalculation of the test statistic after each permutation. The maximum number of unique permutations determines the number of permutations performed: $N_{perm} = ((r+k)!/r!k!)$, where r and k are the number of samples taken from each group, respectively. The test statistic selected for this case was the difference of means between groups. The null hypothesis is rejected if $p = P(T \geq T_{obs}) < 0.05$, where $P(T \geq T_{obs})$ is the probability that the test statistic calculated for the resampled data, T, is greater than or equal to the original data, T_{obs}.

Statistical comparison between animal age groups of peak and equilibrium stiffness and modulus for each imposed displacement was performed using a Two-way Repeated Measures ANOVA with Bonferroni post-hoc tests with alpha=0.05. Animal weight, muscle weight, and muscle volume were compared using Student t-tests with alpha=0.05.

3. Results

Middle-aged animals (24 months) had greater total body weight compared to young animals (8 months, $p < 0.001$). While mean GC muscle weight ($p=0.239$) and mean GC muscle volume ($p=0.746$) were similar in both groups, the ratio of the gastrocnemius muscle to body weight was significantly decreased in middle-aged animals ($p < 0.001$, Table 1).

The Fung's QLV analysis was able to capture the response of the GC-AT muscle-tendon unit (Fig. 2). Fung's QLV analysis (Table 2) revealed similar linear scaling (parameter A) (Fig. 3A, $p=0.134$) and non-linear scaling (parameter B) (Fig. 3B, $p=0.180$) of the instantaneous elastic response between groups. The product of A and B describes the initial slope of the instantaneous

Table 1
Animal weight, gastrocnemius muscle wet-weight and volume (mean ± SEM).

Parameter	8 months	24 months	*p*-value
Animal weight (g)	387.7 ± 10.7	545.3 ± 6.8	< 0.001*
Wet-weight (g)	2.693 ± 0.129	2.880 ± 0.075	0.239
Volume (ml)	2.558 ± 0.108	2.517 ± 0.064	0.746
Ratio gastrocnemius/body weight	0.00694	0.00528	< 0.001*

* $p < 0.05$ was statistically significant.

elastic response, which represents the rate of increase in tensile stress with increasing displacement. The product AB was significantly increased by 50% in middle-aged animals compared to young animals (Fig. 3C, $p=0.014$). The magnitude of the relaxation response (parameter C) was significantly decreased in middle-aged animals (Fig. 4A, $p=0.026$). There was no significant difference between groups for the time constants of the relaxation response, τ_1 ($p=0.752$) or τ_2 ($p=0.360$) (Fig. 4B and C).

Estimated stiffness significantly differed between the two age groups (Table 3, Fig. 5A and B). Middle-aged animals demonstrated a 28% increase ($p=0.036$) in peak stiffness and a 30% increase ($p=0.004$) at equilibrium stiffness compared to young animals. Peak modulus was significantly increased by 35% ($p=0.005$) and equilibrium modulus was increased by 36% ($p=0.013$) in middle-aged animals compared to young animals (Table 3).

4. Discussion

This study revealed that age-related changes of the *in vivo* passive biomechanical response of the GC-AT muscle–tendon unit is associated with a significant increase of the product AB in Fung's QLV analysis in middle-aged animals compared to young animals. The product of A and B describes the initial slope of the instantaneous elastic response, which represents the rate of increase in tensile stress on the muscle tendon-unit with increasing displacement. On average, middle-aged animals experienced a 50% increase in peak tension proportional to initial displacement compared to young animals. We also found an increased estimated stiffness and an increased estimated modulus in middle-aged animals, consistent with the QLV analysis. These changes with age may be related to the predisposition of middle-aged individuals to experience AT ruptures. The primary injury mechanism of AT ruptures is a sudden stretch of an already pretensioned GC-AT muscle–tendon unit (Lin et al., 1999). The findings of this study suggest that circumstances in which high tensile stress is placed on the GC-AT could be more likely to result in failure in middle-aged GC-AT muscle–tendon units than young muscle–tendon units. However, this theoretical difference needs to be discerned in future load-to-failure analysis of young and middle-aged GC-AT muscle–tendon units.

There was a significantly decreased relaxation response to initial displacement (parameter C) in middle-aged animals (Table 2, Fig. 4A). During repetitive loading, the passive response of the muscle may result in a decreased muscle relaxation toward its normal resting stress. With continued repetitive loading, the muscle–tendon unit would not be allowed to return to its resting stress and may incrementally increase peak tensile stress with subsequent repetitive displacement. Repetitive loading with high tensions causes microtears in the muscle and/or tendon substance (Jarvinen et al., 2001; Maffulli et al., 2000). Sonographic evaluation of athletes revealed an increased number of microtears (Gibbon et al., 1999), particularly in the watershed area of the AT which receives limited blood supply (Kvist, 1994). The findings of

Table 2
Analysis of Fungs QLV (mean $\pm$ SEM).

Parameter	8 months	24 months	p-value
A (MPa/mm)	2.893 ± 1.181	5.735 ± 1.236	0.134
B	0.326 ± 0.045	0.255 ± 0.019	0.180
$A \times B$	0.714 ± 0.2	1.365 ± 0.171	0.014*
C	0.144 ± 0.015	0.108 ± 0.007	0.026*
τ_1 (seconds)	3.052 ± 0.287	2.923 ± 0.303	0.752
τ_2 (seconds)	2440593 ± 1764758	619685 ± 81098	0.360
R^2 elastic	0.990	0.980	
R^2 viscous	0.999	0.999	

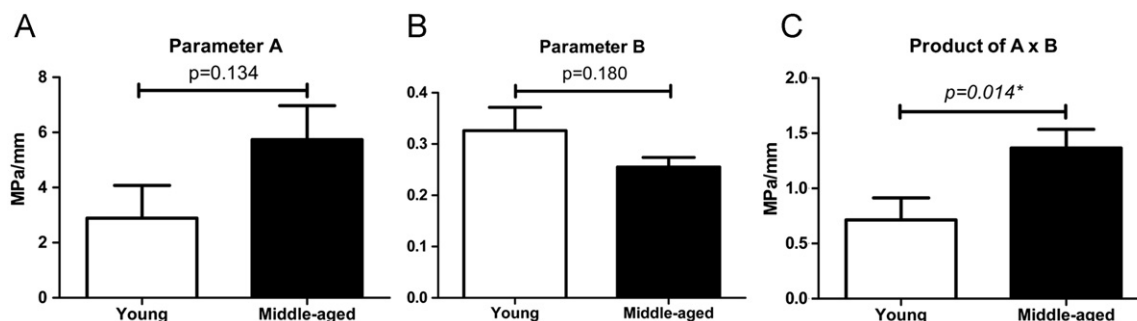* $p < 0.05$ was statistically significant; MPa, megapascal.

Fig. 3. Fung's QLV elastic response parameters. While parameter 'A' (A), and parameter 'B' (B) were found to be similar, the product of 'AB' was significantly increased in middle-aged animals (C). This finding revealed that peak tensile stress of middle-aged animals increased disproportionally with increasing displacement compared to young animals.
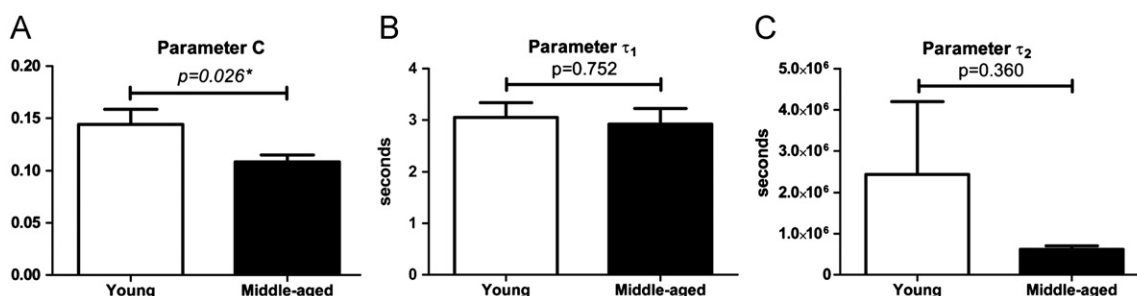

Fig. 4. Fung's QLV viscous response parameters. Parameter 'C' (A) was signficantly lower in middle-aged animals indicating a decrease in relaxation response. Parameters 'τ_1' (B) and 'τ_2' (C) were found to be similar during stress-relaxation between young and middle-aged animals.

this animal study imply that middle-aged individuals may be subjected to higher loading forces of the GC-AT with lower displacement, possibly increasing their susceptibility to overuse tendinopathy, specifically in sports involving repetitive loading at high tension (jumping, running, cutting actions such as in basketball or tennis). With further exercise and overuse, the number of microtears may increase leading to further tendon damage and mechanical impairment (Jarvinen et al., 2001).

There are inherent limitations of this study that must be considered when interpreting the results. This study uses an animal model to examine passive biomechanical response of the GC-AT muscle–tendon unit. While the anatomic structure of the GC-AT is similar between rats and humans, the animal model does not allow for an accurate scaling to the human condition. Because rats are quadrupedal, their GC-AT may experience different loading forces compared to bipedal humans. However, the rat animal model has been widely used to assess muscular and functional changes of the GC-AT following injury and during aging. A strength of this study is the preservation of in vivo blood supply and neural contributions of the GC-AT during biomechanical testing (Calvo et al., 2010; Macintosh et al., 2011), in contrast to ex vivo biomechanical studies. Similar in vivo testing

was used previously to assess the neural contributions to passive biomechanical response in the rat rotator cuff following Botulinum neurotoxin A injection into the muscle (Mannava et al., 2011). Using this chemical denervation of the supraspinatus muscle it was revealed that the nervous system influences the elastic component of the load-relaxation properties, and thus in vivo assessment of passive response is important to consider.

This study assessed the GC-AT composite tissue as a functional muscle–tendon unit, which does not allow for the analysis of the individual contributions of the muscle and tendon to changes in biomechanical response. Although most AT ruptures occur in the midsubstance of the tendon (Longo et al., 2009), the AT and GC function in concert, and the GC muscle fascicles contribute to the overall passive response during displacement. In a deformation applied to a muscle–tendon unit, approximately 75% of the strain is distributed to the muscle and 25% to the tendon (Kubo et al., 2001; Magnusson et al., 1995; Taylor et al., 1990). Given that the muscle does not have uniform CSA throughout its length and that we used resting CSA as opposed to instantaneous CSA, our method may underestimate the true stress applied to the muscle–tendon unit. This would result in underestimation of the modulus. One potential limitation of this study thus lies in

Table 3
Linear regression analysis for passive biomechanical properties of the GC-AT muscle–tendon unit in young and middle-aged animals (mean $\pm$ SEM).

Parameter	Slope	R^2	F	p-value
Peak load vs. displacement	Stiffness (N/mm)			
Young	1.996 ± 0.199	0.816	9.320	0.036*
Middle-aged	2.781 ± 0.178	0.918		
Equilibrium load vs. displacement	Stiffness (N/mm)			
Young	1.390 ± 0.138	0.822	8.778	0.004*
Middle-aged	1.984 ± 0.146	0.894		
Peak stress vs. strain	Modulus (MPa/(%dL/L))			
Young	157.3 ± 19.9	0.740	6.891	0.005*
Middle-aged	243.4 ± 26.28	0.796		
Equilibrium stress vs. strain	Modulus (MPa/(%dL/L_0))			
Young	111.4 ± 14.1	0.739	6.629	0.013*
Middle-aged	173.7 ± 19.87	0.777		

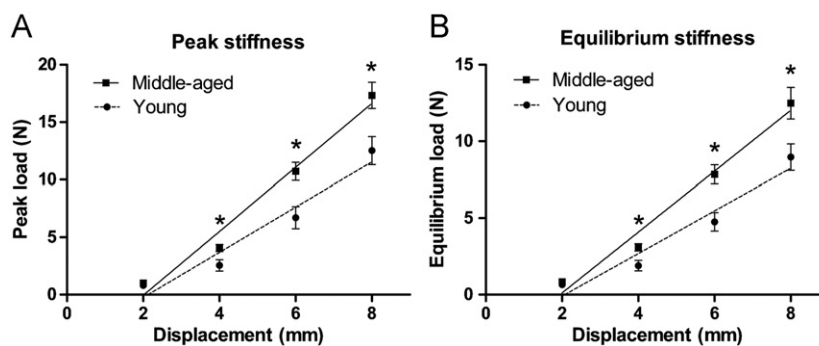* $p < 0.05$ was statistically significant; dL, change in length; L_0, resting length.

Fig. 5. Peak and equilibrium loads vs imposed displacement for young and middle-aged animals. (A) Peak load was significantly increased in middle-aged animals compared to young animals at 4 mm ($p=0.030$), 6 mm ($p=0.009$), and 8 mm ($p=0.016$) of displacement. (B) Equilibrium load was also significantly increased in middle-aged animals at 4 mm ($p=0.019$), 6 mm ($p=0.005$), and 8 mm ($p=0.026$) of displacement. Estimated effective stiffness of GC-AT for peak and equilibrium loading conditions was determined from the slope of the best fit from regression analysis of the load-displacement data for each loading condition.

whether there is a difference in the relative degree of under-estimation of the true stress and modulus of the young animals as compared to the aged animals, due to age-related muscle atrophy. Actual differences between groups could be lower than their estimated value as a result of such a discrepancy. However, it should be noted that we did not observe significant differences in muscle weight or volume between young and aged animals in this study. Therefore, this limitation is likely negligible with respect to the interpretation of our results. However, future studies will analyze the load-to-failure behavior of the GC-AT in young and middle-aged animals to circumvent these limitations in inter-pretation of the data by directly assessing the relative propensity for tendon rupture.

The classical approach to studies of the musculoskeletal stress response has been to report stiffness and modulus of both the peak and equilibrium responses as constant values (Abramowitch et al., 2010; Kay and Blazevich, 2009; Lin et al., 1999). However, early experiments by A.V. Hill (1938) demonstrated that the stress response of soft tissue, particularly muscle, is inherently non-linear (Holmes, 2006). Even though we have calculated an estimated stiffness and modulus in this study, the goodness-of-fit for the non-linear Fung's QLV analysis was greater. For the linear regression analysis to estimate stiffness and modulus in this study from individual load-displacement and stress–strain data points, the R^2 revealed a moderate fit of the observed data to a straight line (Fig. 5, Table 3). The R^2 of non-linear Fung's QLV analysis for the instantaneous elastic and viscous response was close to 1 resulting in an improved fit of the data. This finding

reveals that muscle load-relaxation is inherently non-linear and we believe the QLV analysis more accurately depicts the stress response of the muscle–tendon unit.

An important assumption in our approach to estimating the parameters of the QLV model is the use of an instantaneous, constant deformation on the muscle–tendon unit (Dortmans et al., 1984). However, strain rate was dependent on advancing the translating stage by the investigator, allowing for a small window of viscous accommodation in the strain before the theoretical peak strain was reached. We believe the contribution of this effect was minimal, but it may have contributed to the variability observed in the viscous response parameters (C, τ_1, and τ_2). The experimental setup did not allow for strain to be measured continuously. Therefore, a true dynamic analysis of the stress–strain relationship was not performed, but rather extrapolated from the stress-relaxation experiments using linear regression analysis. Furthermore, we applied a prescribed dis-placement to the muscle–tendon unit as the boundary condition. Elastic and viscoelastic properties may also be tested using a prescribed load to analyze creep or load-to-failure. However, in the case of the ankle joint, displacement may be a physiologically valid boundary condition, given that the kinematic motion of the ankle that imposes a stretch on the GC-AT muscle–tendon unit is relevant to GC-AT pathology.

In summary, this study revealed that normal aging signifi-cantly influences the passive biomechanical response of the GC-AT muscle–tendon unit. In particular, the muscle–tendon unit in middle-aged animals demonstrated an increased slope of the

instantaneous elastic response, a decreased relaxation response, and increased estimated stiffness and moduli at both peak and equilibrium loads. While the limitations of this animal model must be considered, the changes we describe could be associated with the observation that GC-AT pathology and injury is more common in middle-aged individuals. Further studies are necessary to characterize the load-to-failure behavior of AT in middle-aged compared to young animals.

Conflict of interest statement

No benefits in any form have been or will be received from a commercial party related directly or indirectly to the subject of this manuscript. There is no conflict of interest.

Acknowledgments

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