Published in final edited form as:

J Biomech. 2016 September 6; 49(13): 3056–3060. doi:10.1016/j.jbiomech.2016.06.034.

How does tissue preparation affect skeletal muscle transverse isotropy?

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Abstract

The passive tensile properties of skeletal muscle play a key role in its physiological function. Previous research has identified conflicting reports of muscle transverse isotropy, with some data suggesting the longitudinal direction is stiffest, while others show the transverse direction is stiffest. Accurate constitutive models of skeletal muscle must be employed to provide correct recommendations for and observations of clinical methods. The goal of this work was to identify transversely isotropic tensile muscle properties as a function of post mortem handling. Six pairs of tibialis anterior muscles were harvested from Giant Flemish rabbits and split into two groups: fresh testing (within four hours post mortem), and non-fresh testing (subject to delayed testing and a freeze/thaw cycle). Longitudinal and transverse samples were removed from each muscle and tested to identify tensile modulus and relaxation behavior. Longitudinal non-fresh samples exhibited a higher initial modulus value and faster relaxation than longitudinal fresh, transverse fresh, and transverse rigor samples ($p < 0.05$), while longitudinal fresh samples were less stiff at lower strain levels than longitudinal non-fresh, transverse fresh, and transverse non-fresh samples ($p < 0.05$), but exhibited more nonlinear behavior. While fresh skeletal muscle exhibits a higher transverse modulus than longitudinal modulus, discrepancies in previously published data may be the result of a number of differences in experimental protocol. Constitutive modeling of fresh muscle should reflect these data by identifying the material as truly transversely isotropic and not as an isotropic matrix reinforced with fibers.

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Conflicts of Interest Statement

The authors have no financial or other conflicts of interest to report.

Keywords

mechanical properties; rigor mortis; constitutive modeling; material testing

1. Introduction

The human body relies on skeletal muscle, supported by other orthopaedic tissues, for locomotion and posture. Passive properties of muscle are governed by two components of the tissue: the protein titin at the sarcomere level which gives muscle fibers passive stiffness (Magid and Law, 1985; Tskhovrebova and Trinick, 2002), and the collagen rich extracellular matrix which organizes muscle fibers in a hierarchical structure and dominates passive stiffness at the tissue level (Brown et al., 2012; Meyer and Lieber, 2011). In the case of skeletal muscle these passive properties have a multifaceted purpose: allowing for the transmission of internal force generated at muscle fibers to tendons (Gindre et al., 2013; Huijing, 1999), storing energy during locomotion (Cavanagh and Komi, 1979; Ettema, 1996), and maintaining proper resting length for maximum force generation (Fridén and Lieber, 1998). Muscle fiber alignment results in tissue transverse isotropy (Morrow et al., 2010; Pietsch et al., 2014; Takaza et al., 2012; Van Looke et al., 2006) as the material properties of the aligned fibers differ from those of the organized extracellular matrix (Meyer and Lieber, 2011).

Finite element analyses of biological soft tissues provide important insight into tissue behavior for clinical recommendations and observations. However, inaccurate constitutive models could present erroneous data, thus hampering clinical relevance. Some modeling studies of passive skeletal muscle assume the longitudinal direction is stiffer than the transverse direction (Calvo et al., 2010; Grasa et al., 2011; Hernández-Gascón et al., 2013; Lu et al., 2010). While this is supported by some experimental work (Morrow et al., 2010), there is also data which identifies a stiffer transverse response as compared to the longitudinal direction (Nie et al., 2011; Takaza et al., 2012). These differences may be the result of disparities in experimental protocol and anatomical or species variations, although they are more likely the result of rigor mortis, which results in a stiffening of the tissue (Van Ee et al., 2000; Van Looke et al., 2006). As rigor mortis is a complex phenomenon related to the actin-myosin complex (Huff Lonergan et al., 2010; Roth et al., 2006), it most likely influences the longitudinal mechanics to a greater extent than in the transverse direction. Thus, it is hypothesized that the large observed differences in passive transversely isotropic skeletal muscle behavior is a function of non-fresh testing conditions and/or experimental protocol. Data supporting this hypothesis would provide two important recommendations for future studies of skeletal muscle: 1) all mechanical testing should be conducted on fresh, never frozen tissue, and 2) computational models of passively stretched muscle should reflect the true transverse isotropy in that the longitudinal direction is less stiff than the transverse direction.

The goals of this work were thus to evaluate the effects of orientation and post mortem handling on the material properties of skeletal muscle. Specifically, we aim to answer the

question: “How does tissue preparation affect the transversely isotropic stiffness and time dependence of skeletal muscle?”

2. Methods

Six Giant Flemish Rabbits were obtained with Colorado State University Institutional Animal Care and Use Committee approval. Following euthanasia, whole tibialis anterior muscles were isolated from each hind limb and stored in a refrigerator for either fresh testing (left or right limb randomly) or to allow for the onset of rigor mortis (contralateral limb). As rigor mortis begins 6–8 hours post mortem (Van Ee et al., 2000; Van Loocke et al., 2006) fresh muscles were tested within four hours to reduce these effects, while the contralateral muscle was subject to non-fresh testing following a freeze-thaw cycle (Figure 1A). Each tibialis anterior yielded two samples, one longitudinal, and one transverse (Figure 1B). As the pennation angle of the New Zealand White Rabbit is very low (Lieber and Blevins, 1989), the longitudinal direction was assumed to be parallel with the axis of force transmission.

Tensile tests were conducted on a servo hydraulic material test system (MTS, Eden Prairie, MN). Cross sectional area and gauge length were measured optically with a 1.4 megapixel monochrome camera and ImageJ software (National Institutes of Health, Bethesda, MD). Graphite powder was used to track strain with digital image correlation (DIC) software (Matlab, Mathworks, Natick, MA) on a region of interest (ROI) (Figure 2A). All specimens underwent an initial ramp phase of 10% strain at 10% second⁻¹ followed by 300 seconds of relaxation and finally a constant ramp pull at 1% second⁻¹ until specimen failure (Figure 2B). Cauchy (true) stress and Euler strain were converted from force-displacement data and used to calculate tangent moduli. For specifics on the testing procedure and data analysis see Supplementary Materials. Tangent moduli were calculated at the initial peak, end of the relaxation phase, and at 20% strain, which is the higher end of the physiological range for the rabbit tibialis anterior (Davis et al., 2003; Winters et al., 2009). Relaxation ratio was calculated as the fraction of stress relaxation over three separate time periods following the initial ramp: 0–5 seconds, 5–50 seconds, and 50–300 seconds. The raw relaxation data were fitted to a power law equation (Equation 1, where σ is Cauchy stress, t is relaxation time, and a and b are constants which characterize the relative stress level and rate of relaxation, respectively) for identification of relaxation rate.

$$\sigma = at^b \quad (1)$$

Mean and standard deviations for all data were calculated, including raw stress-strain and stress-time data as well as moduli, relaxation ratios, and power law b terms (Equation 1). A oneway ANOVA was performed with a Tukey's post-hoc analysis ($p < 0.05$) to identify differences in moduli, relaxation ratios, and power law b parameter across all four groups.

3. Results

All data passed the Anderson-Darling normality test ($p > 0.05$), suggesting the data were normally distributed. This supported the use of an ANOVA to identify statistical differences between groups. Visual inspection of raw data and calculated moduli values show the peak modulus for longitudinal fresh samples is lower than all other groups ($p = 0.044$, $p < 0.0005$, and $p = 0.014$ for transverse fresh, longitudinal rigor, and transverse rigor, respectively), while longitudinal rigor is higher than both transverse groups ($p = 0.008$, $p = 0.027$ for transverse fresh and rigor, respectively) (Figure 3A and 3C). Equilibrium moduli for longitudinal fresh was lower than all other groups ($p = 0.006$, $p = 0.004$, and $p = 0.001$ for transverse fresh, longitudinal rigor, and transverse rigor, respectively), while values calculated at 20% strain during the constant rate phase again showed the longitudinal fresh group was lower than all other groups ($p = 0.001$, $p = 0.004$, $p = 0.015$ for transverse fresh, longitudinal rigor, and transverse rigor, respectively) (Figure 3). The longitudinal fresh samples showed a highly nonlinear shape compared to the other groups (Figure 3B).

Longitudinal rigor samples showed faster initial relaxation rate visually, as characterized by the power law b coefficient ($p = 0.017$, $p < 0.0005$, and $p < 0.0005$ for transverse fresh, longitudinal rigor, and transverse rigor, respectively), and when comparing the initial relaxation phase (0–5 seconds, $p = 0.029$, $p < 0.0005$, and $p < 0.0005$ for transverse fresh, longitudinal rigor, and transverse rigor, respectively) (Figure 4).

4. Discussion

The increased tangent moduli values and increase in relaxation rate of longitudinal non-fresh samples show that post mortem handling plays a key role in the alteration of muscle mechanical properties, which agrees well with previous experimental data on muscle properties as a function of rigor mortis (Van Ee et al., 2000). However, it remains unclear how the mechanical properties of skeletal muscle are affected separately by rigor mortis and a freeze/thaw cycle. To the authors knowledge this work represents the first investigation of both transverse isotropy and time dependence of skeletal muscle as a function of post mortem handling. As rigor mortis is a complex biochemical phenomenon (Huff Lonergan et al., 2010; Roth et al., 2006), it remains unclear exactly which mechanisms are contributing to these particular results. While this study employed a relatively small sample size ($n = 6$), the statistical significance and observable differences were quite clear despite the sample number.

While skeletal muscle tensile studies are common (Abraham et al., 2012; Calvo et al., 2010; Gras et al., 2012; Morrow et al., 2010; Takaza et al., 2012; Wheatley et al., 2016), separate investigations have determined the tissue to be stiffer in the longitudinal direction (Morrow et al., 2010) or the transverse direction (Takaza et al., 2012). Our data for fresh samples agree well with Takaza et al for both transverse isotropy (transverse stiffer than longitudinal) and in terms of general stress-strain shape (longitudinal is nonlinear, transverse appears linear). Takaza et al show higher stress values at similar strain levels when compared to our data, as they reported ~100 kPa versus ~50 kPa reported here at 30% strain for longitudinal fresh samples and ~110 kPa versus ~25 kPa for transverse samples at 15% strain. However,

this may be due to variations in species, anatomy, or experimental protocol, as stress values at the end of the relaxation phase from the data presented here were very low (<4 kPa) and Takaza et al did not incorporate a relaxation phase. Data from Morrow et al appears more linear in the longitudinal direction, which agrees fairly well with our longitudinal rigor samples, but is again stiffer than our samples. Unfortunately there is poor agreement between transverse samples, as Morrow et al found the transverse direction to be less stiff and more extensible (~ 60 kPa at 25% strain here vs ~ 25 kPa at 100% strain).

The data presented here can be utilized to improve constitutive modeling efforts of skeletal muscle, as some studies in the past have assumed muscle is stiffer in the longitudinal direction (Calvo et al., 2010; Grasa et al., 2011; Hernández-Gascón et al., 2013; Lu et al., 2010). Future studies should model muscle as stiffer in the transverse direction similar to other works (Blemker et al., 2005; Jenkyn et al., 2002; Odegard et al., 2008; Yucesoy et al., 2002).

5. Conclusions

The transversely isotropic mechanical properties of skeletal muscle were evaluated with a specific focus on post mortem handling. While it remains unclear exactly how a non-fresh conditions and a freeze/thaw cycle independently affect the tensile transverse isotropy of skeletal muscle, to prevent any alterations in mechanical properties muscle tissue should be tested prior to the onset of rigor mortis without a freeze/thaw cycle. These data should be utilized to improve future modeling efforts for skeletal muscle with a specific focus on the anisotropic constitutive formulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by the National Institutes of Health: National Institute of Child Health & Human Development for financial support [R01HD31476-12].

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Figure 1.

A) Specimen groupings and testing timeline, showing the fresh testing group and the group subject to non-fresh conditions and a freeze/thaw cycle. Each of these groups yielded longitudinal and transverse samples for a total of four groups. (B) Dissection orientations show that each muscle yielded two samples, one in the longitudinal direction and one in the transverse direction.

Figure 2.

Specimen testing procedures. A: Experimental setup showing speckled sample with gauge length (black arrow), three width measurement locations (white arrows), and digital image correlation region of interest (DIC ROI – light blue dotted box). B: Testing outline (not to scale) highlighting initial ramp phase, relaxation phase, and final ramp phase to failure (strain of 0.3 given as an example), with representative stress shown as solid black line and strain in dotted gray line.

Figure 3.

A) Raw data results showing stress relaxation step stress-time data with standard deviation. The loading phase and initial relaxation are highlighted for clarity on left with no standard deviation. (B) Quasi-static testing stress-strain data with standard deviation. (C) Moduli values with standard deviation for all four groups at the peak of the stress relaxation step, at equilibrium of the stress relaxation step, and at 20% strain of the quasi-static testing phase (* denotes statistically different from longitudinal fresh samples and # denotes different from longitudinal non-fresh samples, $p < 0.05$).

Figure 4.

A) Logarithmic plot of the mean relaxation behavior for the four experimental groups. (B) Mean and standard deviation power law b values for all four groups (# denotes significantly different than longitudinal non-fresh samples, $p < 0.05$). (C) Mean and standard deviation relaxation ratio for all four groups between 0–5 seconds, 5–50 seconds, and 50–300 seconds (# denotes significantly different than longitudinal non-fresh samples, $p < 0.05$).