Passive Mechanical Properties of Rat Abdominal Wall Muscles Suggest an Important Role of the Extracellular Connective Tissue Matrix

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ABSTRACT: Abdominal wall muscles have a unique morphology suggesting a complex role in generating and transferring force to the spinal column. Studying passive mechanical properties of these muscles may provide insights into their ability to transfer force among structures. Biopsies from rectus abdominis (RA), external oblique (EO), internal oblique (IO), and transverse abdominis (TrA) were harvested from male Sprague–Dawley rats, and single muscle fibers and fiber bundles (4–8 fibers ensheathed in their connective tissue matrix) were isolated and mechanically stretched in a passive state. Slack sarcomere lengths were measured and elastic moduli were calculated from stress–strain data. Titin molecular mass was also measured from single muscle fibers. No significant differences were found among the four abdominal wall muscles in terms of slack sarcomere length or elastic modulus. Interestingly, across all four muscles, slack sarcomere lengths were quite long in individual muscle fibers (>2.4 μm), and demonstrated a significantly longer slack length in comparison to fiber bundles ($p < 0.0001$). Also, the extracellular connective tissue matrix provided a stiffening effect and enhanced the resistance to lengthening at long muscle lengths. Titin molecular mass was significantly less in TrA compared to each of the other three muscles ($p < 0.0009$), but this difference did not correspond to hypothesized differences in stiffness. © 2012 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 30:1321–1326, 2012

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Abdominal wall muscles play a vital role in generating controlled movement of the lumbar and thoracic spine. The unique anatomical arrangement of the four muscles [rectus abdominis (RA), external oblique (EO), internal oblique (IO), and transverse abdominis (TrA)] has inspired descriptions and related hypotheses regarding its function as a composite-laminate structure.[1–3] This composite laminate-like morphology has been hypothesized to enhance the ability of the abdominal wall to transfer force and stiffen the spine. Material properties of the components of this composite must be understood to define the structural basis for the properties of the abdominal wall. However, the passive mechanical properties of the abdominal wall muscle fibers and extracellular matrix remain unclear and thus motivated this study.

Muscle passive mechanical properties have been used to describe structural function,[4–6] differentiate between healthy and diseased muscle,[7,8] and characterize muscle adaptation to joint-related injury.[9] Studies of abdominal wall muscle passive properties were performed previously,[3,10,11] identifying varying differences in stiffness among muscles. However, these studies were limited by three main factors: (i) no study quantified and compared the four muscles; (ii) no sarcomere length measurements were made to normalize strain or extension data; and (iii) only large muscle strips were tested, thereby not elucidating differences between intracellular and extracellular sources of passive stiffness. Previous work demonstrated that differences that are not apparent on one scale (e.g., a single muscle fiber) may become apparent on a different scale (e.g., a bundle of muscle fibers and connective tissue matrix), highlighting the importance of studying muscle at both the single cell and muscle fiber bundle levels.[7,8]

Architectural analyses of human abdominal wall muscles predicted that regional fascicles of EO and IO may lengthen towards or beyond the end of force–length relationship during maximal contralateral bend.[12] Passive mechanical properties of the abdominal wall muscles and the interaction among the muscles dictate the passive stress and strain that muscle fibers and connective tissues will experience during such lengthening. Comparison of abdominal wall muscle architectural properties between human and rat demonstrated similar physiological cross-sectional areas, relative fascicle and sarcomere lengths, and fiber orientations, suggesting that they have similar functional roles in moving and supporting the spine.[2] Thus, the use of a rat model to study material properties may provide insight into the structure and function of the human abdominal wall muscles.

This study was designed to determine the passive mechanical properties (stiffness and slack sarcomere length) of rat abdominal wall muscles at the single fiber and fiber bundle levels. Molecular mass of titin, the protein most often suggested to dominate passive muscle behavior,[4] was also measured to provide insights into potential differences in mechanical properties among the muscles.

METHODS

All procedures were approved by the local IACUC. Six adult male Sprague–Dawley rats were euthanized, and biopsies of RA, EO, IO, and TrA were immediately obtained and placed

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in storage solution at −20°C to prevent hyperpolarization and destruction of the muscle tissue.[8]

All muscles were tested within 2 weeks of harvest. Testing was performed in a relaxing solution[8] to ensure muscle properties were assessed in a passive state. Single fibers and small fiber bundles (comprised of 4–8 fibers ensheathed in their connective tissue matrix) were isolated and tied with 10-0 silk thread to pins secured at one end to a force transducer (Model 405A, Aurora Scientific, Aurora, ON, Canada), and at the other end to a high-speed motor (Model 318B, Aurora Scientific) to manipulate specimen length. Typically, two single fibers and two fiber bundles were tested from each muscle. A 7 mW diode laser transilluminated the specimen and generated a diffraction pattern, enabling sarcomere length measurement during testing.[13]

Specimens were initially lengthened until they started to generate a measurable passive force above the noise level of the force transducer; further tests were initiated from this length (termed slack sarcomere length). The motor was controlled to provide rapid (100 fiber lengths/second) stretch to the specimens in increments of ~0.25 μm/sarcomere (~10% strain). After each stretch, the fiber or fiber bundle was held at constant length for 3 min to allow stress relaxation to occur. A minimum of seven stretches were performed on each specimen. Sarcomere length changes relative to initial slack length were used to compute specimen strain. Force at the end of each 3-min relaxation period was normalized to fiber or fiber bundle cross-sectional area (calculated at slack sarcomere length by measuring specimen diameter via cross-hairs under a microscope and assuming a circular area) to calculate stress.

Pilot data demonstrated that single muscle fibers behaved differently compared to fiber bundles; stress–strain profiles of single fibers displayed a small toe region followed by a linear elastic region, whereas fiber bundles displayed a nonlinear response throughout the range of stretch. Therefore, elastic moduli were calculated for single fibers as the slope of the linear region of the stress–strain curve (corresponding to at least four stretches) and for fiber bundles as the 1st derivative of 2nd-order polynomials fit to the stress–strain data. Fiber bundle moduli were then calculated and compared among muscles at a sarcomere length of 3.2 μm (~1/2 the length of the descending limb of the force–length relationship for rat muscle).[14] Also, as a second method for comparing fiber and fiber bundle properties, calculations were made of the sarcomere length at which fiber bundles reached a stiffness (from the 1st derivative of 2nd-order polynomials) that exceeded the mean stiffness of individual fibers from each muscle.

Titin molecular mass was quantified in single fibers that had been tested mechanically from each muscle group. Procedures for this vertical agarose gel electrophoresis (VAGE) method were published previously.[15] Briefly, single fibers were boiled in sample buffer solution, and SDS–VAGE analysis was used the quantify titin migration relative to the migration of titin standards (human soleus titin and rat cardiac titin, masses of 3,700 and 2,992 kD, respectively).

Slack sarcomere lengths and elastic moduli were compared statistically using two-way ANOVA with factors of: muscle (RA, EO, IO, TrA); and specimen size (individual fibers vs. fiber bundles). Titin mass was compared among muscles (for single fibers and fiber bundles) using one-way ANOVAs. Where appropriate, Tukey's HSD test was used for post hoc analysis. The significance level (α) was set at $p < 0.05$.

RESULTS

Elastic Modulus

No significant differences in elastic modulus were found among the four abdominal wall muscles for individual fibers ($p = 0.35$) or fiber bundles ($p = 0.84$), nor between individual fibers and fiber bundles at a sarcomere length of 3.2 μm ($p = 0.15$) (Fig. 1). Mean ± SEM r^2 values for curve fits were 0.99 ± 0.002 for linear fibers and 0.99 ± 0.003 for nonlinear bundles. Single fibers displayed a toe region followed by a linear elastic region, whereas fiber bundles displayed a nonlinear stress–strain relationship best characterized by a 2nd-order polynomial (Figs. 2 and 3). Thus, to further compare properties between fibers and bundles, we calculated the sarcomere length for each muscle at which fiber bundles became stiffer than single fibers.

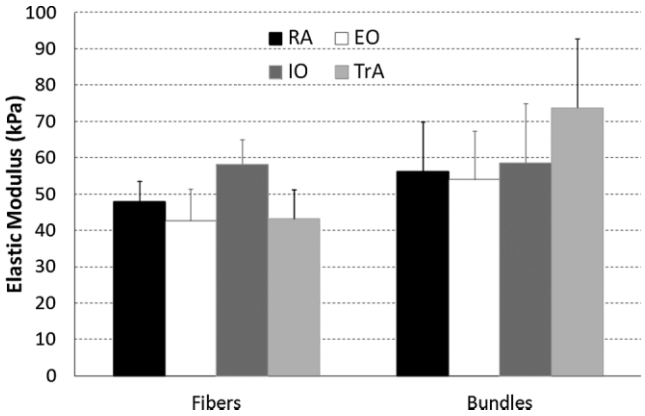

Figure 1. Mean elastic modulus of single fibers and fiber bundles for each abdominal wall muscle. Moduli of single fibers were calculated as the slope of the linear region of the stress–strain relationship. Moduli of fiber bundles were calculated by fitting 2nd-order polynomials to the stress–strain data of each sample, differentiating and solving for modulus at a sarcomere length of 3.2 μm. No significant differences were found among any of the muscles nor between fibers and bundles at this sarcomere length. Error bars represent standard error of the mean.

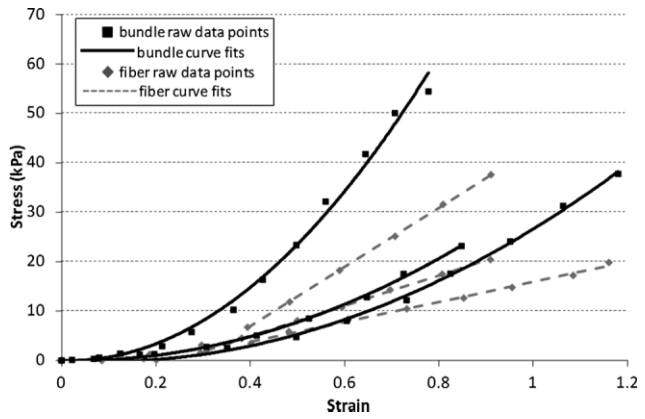

Figure 2. Representative stress–strain raw data points and curve fits for three individual RA muscle fibers and three RA fiber bundles. Individual fibers were linearly fit after exceeding an initial toe region; fiber bundles were fit with 2nd-order polynomials over the stress–strain relationship.

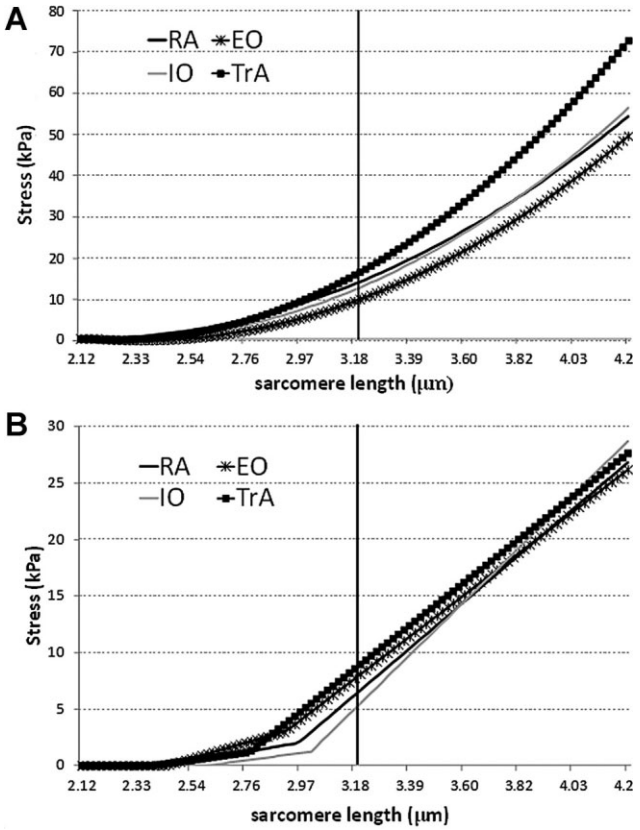

Figure 3. (A) Second-order polynomial fits of fiber bundle stress–sarcomere length relationship across all animals for each muscle. Data were fit to the stress–strain relationship for each tested fiber bundle, and coefficients were averaged across all samples for each muscle. Strain was then converted to sarcomere length based on average slack sarcomere lengths for fiber bundles for each muscle. Stress SEM, calculated from the polynomial fits at sarcomere length 4.24 μm, were: RA 14.1 kPa; EO 31.0 kPa; IO 23.6 kPa; and TrA 24.5 kPa. (B) Piece-wise linear fits of individual fiber stress–sarcomere length relationship across all animals for each muscle. Data were fit to the stress–strain relationship for each tested fiber, and coefficients were averaged across all samples for each muscle. The steeper linear region for each muscle represents the modulus values reported in Figure 1. In both A and B vertical lines represent sarcomere length 3.2 μm where comparisons were made in Figure 1.

These lengths were: RA = 3.05 μm; EO = 3.06 μm; IO = 3.20 μm; TrA = 2.80 μm.

Slack Sarcomere Length

Slack sarcomere lengths were not significantly different among the four abdominal wall muscles ($p = 0.38$, Fig. 4). Individual muscle fibers and fiber bundles did have significantly different slack sarcomere lengths ($p < 0.001$), with bundles being shorter (Fig. 4), as previously reported for human upper extremity muscles.[8]

Titin Isoform Size

Titin molecular mass of the TrA was significantly smaller ($p < 0.001$) compared to the other three abdominal wall muscles (Fig. 5).

DISCUSSION

Our results demonstrate that the four abdominal wall muscles have similar passive mechanical properties in the rat. The most interesting finding is that, for each muscle, individual muscle fibers behaved differently compared to bundles consisting of fibers ensheathed in their connective tissue matrix. Specifically, fiber bundles had a shorter slack sarcomere length and demonstrated a nonlinear stress–strain response, whereas individual fibers had long slack sarcomere lengths and

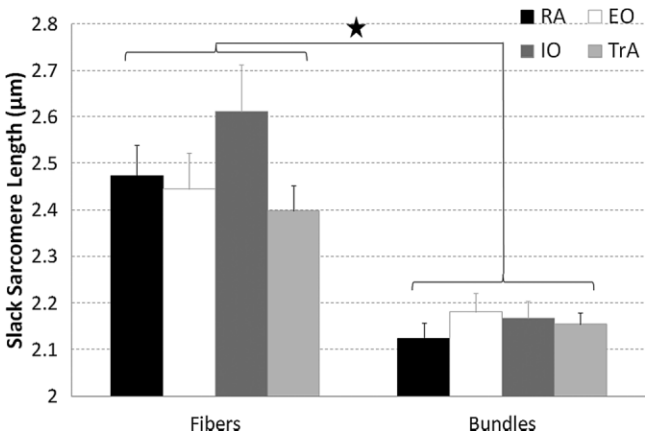

Figure 4. Mean slack sarcomere length of single fibers and fiber bundles for each muscle. A significant difference was observed between single fibers and fiber bundles ($p < 0.0001$). Error bars represent standard error of the mean.

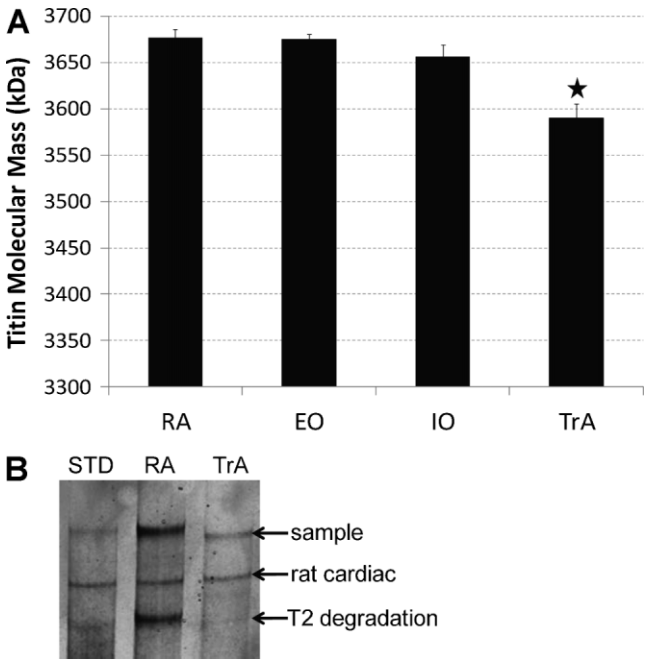

Figure 5. (A) Mean titin molecular mass for single fibers from each muscle. Star indicates TrA titin was significantly smaller compared to each of the other three muscles ($p < 0.0009$). Error bars represent standard error of the mean. (B) Example SDS–VAGE gel lanes. Molecular masses were calculated by the migration of titin relative to standards of known mass (human soleus and rat cardiac). STD represents the standard lane containing titin from human soleus (sample) and rat cardiac muscle. RA represents the lane containing titin from RA (sample) and rat cardiac muscle. TrA represents the lane containing titin from TrA (sample) and rat cardiac muscle. T2 degradation bands are degradation forms of the intact titin molecule.[4]

demonstrated a more linear response outside of an initial toe region. The nonlinear stress–strain response of fiber bundles makes them effectively much stiffer than their constituent fibers at longer lengths, suggesting an important stiffening effect of the connective tissue matrix. Recently, by comparing fiber bundles to "groups" of fibers that were tied together, we showed that this nonlinearity is due to the material properties of the extracellular matrix itself and not due to nonhomogeneities among the fibers.[16] The importance of this stiffening effect is addressed in context of the role that these muscles play in controlling and stabilizing the spine.

Mean slack sarcomere lengths of individual abdominal wall muscle fibers were equal to or longer than active optimal sarcomere length (~2.40 μm for rat). These slack lengths are much longer, relative to optimal length, than for previously reported human muscles.[6,17] Bundle slack sarcomere lengths, however, were much shorter than fiber slack sarcomere lengths. This may be explained by two phenomena. First, the connective tissue matrix of intact abdominal wall muscle may have begun to bear load and resist lengthening well before individual muscle fibers that make up the majority of the mass of the tissue. Alternatively, slack sarcomere lengths in fiber bundles may be determined simply by the "least slack" individual fiber, rather than by the extracellular matrix (ECM) itself. This second possibility may explain the highly nonlinear response of fiber bundles, characterized by an initial region of low stiffness, where individual fibers bear load, followed by a rapidly stiffening response at longer lengths, where ECM begins to bear load (Fig. 3). In this case, the substantial stiffening effect in fiber bundles at longer lengths may be necessitated by the range of lengths these muscles experience in vivo. Brown et al.,[12] predicted, based on architectural analyses of human abdominal wall muscles, that, as the lumbar spine moves through its full range of motion, abdominal wall muscles (in particular EO and IO) lengthen well beyond the plateau of the force–length relationship. If fibers in these muscles begin to resist load at low sarcomere lengths (low slack lengths), they would experience high strain and stress at such long lengths, which would be amplified during active lengthening. This could potentially damage muscle fibers[18,19] or influence strain and/or stress induced cell signaling.[20,21] By having greater intrinsic slack lengths, individual fibers experience lower strain and stress when lengthened. The stiffer connective tissue matrix observed in the fiber bundles provides the stiffening effect that may be necessary to transmit loads and stiffen the spine at moderate and long muscle lengths. Second-order polynomial curves fit to the bundle data suggest that intact muscle becomes stiffer, when compared to isolated fibers, on the early (TrA) to mid-range (RA, EO, IO) of the descending limb of the force–length relationship.

The important stiffening role of the matrix was suggested in previous work on abdominal wall muscles. Boriek et al.,[22] based on microdissection of canine IO and TrA muscles, found that these muscles were primarily comprised of short muscle fibers linked lengthwise to form long fascicles. They suggested that intramuscular extracellular connections are necessary to transmit force between fibers along the muscle, as was previously suggested for similar muscles.[23–25] Intrafascicular terminating fibers have since been identified in human abdominal wall muscles;[26] however, this has not been examined for rat abdominal muscles. On a more macroscopic scale, Brown and McGill[27] established that connective tissue networks play a role in transmitting force and stiffness between abdominal wall muscle layers, demonstrating that the matrix tissue may perform a load transmitting function at multiple levels.

While no significant differences were found for passive mechanical properties among the four muscles, modeled data suggests that TrA may behave in a somewhat stiffer fashion compared to the other three muscles. Specifically, TrA bundles become stiffer than their constituent isolated fibers at shorter lengths (2.80 μm) compared to the RA (3.05 μm), EO (3.06 μm), and IO (3.20 μm). Further, as TrA continues to lengthen, it stiffens to a greater extent than the other three muscles (Fig. 3), although not by a statistically significant amount (Fig. 1). Previous work also indicated that TrA displays greater passive stiffness than EO as the muscles are stretched beyond optimal active length.[10] Significance for this may lie in work that suggested TrA plays a specialized role in spine control, based on findings that it activates prior to other abdominal wall muscles in certain controlled settings[28] and is more tonically active compared to the other muscles during generation of opposing spine flexion/extension moments and movements.[29] As the deepest of the abdominal wall muscles, under certain conditions, early and/or tonic activation of the stiff TrA may serve a role of establishing a stiff foundation upon which the larger IO and EO muscles can generate more substantial forces and moments.

Titin isoforms in TrA were smaller than in each of the other muscles, consistent with its slightly stiffer fiber behavior. Titin is a giant protein that spans the half-sarcomere by its anchors on the Z-disc and M-line, and has been thought to be the primary determinant of muscle fiber passive stiffness[4] and slack sarcomere length.[30] Despite the TrA having smaller titin isoforms, which should make fibers stiffer with shorter slack lengths, no difference in fiber stiffness was found between TrA and the other muscles (Fig. 1); yet while not significant, TrA did have a shorter slack sarcomere length than the other three (Fig. 4). The lack of significant differences for stiffness and slack length for TrA may not be surprising, as the significant difference in titin isoform size is a relatively small fraction of the titin mass (~2%) reported for skeletal

muscle (3,300–3,700 kDa[4]). Further, other recent evidence indicates that titin size does not correlate as strongly with fiber stiffness as may have been previously thought,[6] suggesting that other structures within the muscle fiber may also contribute to passive stiffness.

Muscles of the low back and abdominal wall region play a vital orthopedic role in stabilizing and controlling motion of the lumbar spine.[31–33] Recent work focusing on mechanical and structural properties of these muscles demonstrated their unique muscular design for controlling the complex motion and loading patterns of the spinal column.[6,12,34] Our study further describes such unique properties in abdominal wall muscles. This information can be used: to guide protocols to train these muscles for low back injury prevention and rehabilitation; to establish baselines to examine how these muscles adapt to spinal disorders; and to refine surgical techniques to optimize maintenance of natural structure and function of these muscles.

The morphological, vascular, and innervation complexities of the abdominal wall muscles, combined with their proximity to internal organs, makes use of an animal model necessary to study much of their basic physiological and mechanical behavior. Previous work established morphological and architectural similarities between the human and rat abdominal wall muscles,[2] indicating that they have similar functional and relative force-generating capabilities. However, our results should be extrapolated to humans with caution. Specifically, the nature of the connective tissue within the muscles has not been compared between human and rat. Also, intact whole muscles (with complete fascial and connective tissue components) may behave differently than their component fibers and fiber bundles. For example, passive properties of larger segments of RA, EO, and TrA were compared previously with EO being more compliant than TrA (Arnold et al.,[10] in hamsters) and RA (Farkas and Rochester[11] in dogs). These results are not supported by our data in rats.

Our work demonstrates that in rat abdominal wall muscles the extracellular connective tissue matrix plays an important role in regulating passive mechanical behavior. Specifically, the connective tissue matrix provides a substantial stiffening effect at long sarcomere lengths (beyond the range of 2.80–3.20 μm). While no significant differences in passive mechanical behavior were found between the four abdominal wall muscles, TrA fiber bundles did display a slight trend of greater stiffness and lower slack sarcomere lengths.

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