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Skeletal Muscle Fibrosis and Stiffness Increase After Rotator Cuff Tendon Injury and Neuromuscular Compromise in a Rat Model

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ABSTRACT: Rotator cuff tears can cause irreversible changes (e.g., fibrosis) to the structure and function of the injured muscle(s). Fibrosis leads to increased muscle stiffness resulting in increased tension at the rotator cuff repair site. This tension influences repairability and healing potential in the clinical setting. However, the micro- and meso-scale structural and molecular sources of these whole-muscle mechanical changes are poorly understood. Here, single muscle fiber and fiber bundle passive mechanical testing was performed on rat supraspinatus and infraspinatus muscles with experimentally induced massive rotator cuff tears (Tenotomy) as well as massive tears with chemical denervation (Tenotomy + BTX) at 8 and 16 weeks post-injury. Titin molecular weight, collagen content, and myosin heavy chain profiles were measured and correlated with mechanical variables. Single fiber stiffness was not different between controls and experimental groups. However, fiber bundle stiffness was significantly increased at 8 weeks in the Tenotomy + BTX group compared to Tenotomy or control groups. Many of the changes were resolved by 16 weeks. Only fiber bundle passive mechanics was weakly correlated with collagen content. These data suggest that tendon injury with concomitant neuromuscular compromise results in extra-cellular matrix production and increases in stiffness of the muscle, potentially complicating subsequent attempts for surgical repair. © 2014 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 32:1111–1116, 2014.

Keywords: shoulder; rotator cuff; injury; muscle passive mechanics; muscle fibrosis

Rotator cuff tears are a common degenerative condition found in approximately 30% of patients over 60 years of age.[1] Symptoms have been reported in approximately 7% of all elderly patients, resulting in pain and loss of functional range of motion in the shoulder.[2] While surgical treatment and repair of the tendon is possible, structural failure rates have been reported as high as 20–94%, with increasing rates of failure coinciding with the size of the tear and the age of the patient.[3,4]

Increased passive stiffness of the rotator cuff muscle–tendon unit has been measured in both human and animals models and has been implicated as one reason for the difficultly in repairing massive rotator cuff tears[5,6] and for poor tendon-to-bone healing.[7] Previous work in sheep and canine models has suggested an association between increased passive stiffness and the proliferation of collagen in muscle following chronic rotator cuff tears.[8,9] However, it is unclear if the observed increases in whole muscle stiffness were due to changes at the single fiber and/or the fiber-bundle hierarchical levels of the muscles. Fiber- and fiber bundle-level changes in passive stiffness likely contribute to degenerative changes in rotator cuff muscle and are driven by accumulation and/or altered structure of a number of proteins such as titin[10] and collagen.[11] Likewise, traction to the suprascapular nerve and subsequent denervation of the rotator cuff muscles has been associated with massive rotator cuff tears, and may accelerate degenerative changes in the muscle and contribute to poor clinical outcomes.[12,13] Determining the scale and protein-level sources of passive tension following rotator cuff injury may yield insight into therapeutic targets for mitigating tear-related fibrosis in the rotator cuff and improve healing potential after surgical repair.

To address the aforementioned questions, a controlled investigation using a rodent model of massive rotator cuff injury was performed.[14] The objectives of our study were to identify muscle passive mechanical outcomes and protein-level adaptations at the single fiber and fiber bundle scales in a rodent model of massive rotator cuff tear with or without nerve injury. We hypothesized that fiber and fiber bundle passive stiffness would both increase with time and injury severity, and that these mechanical changes would be associated with smaller titin isoforms at the fiber level and increased collagen content at the bundle level.

METHODS

Animal Model and Surgical Methods

All animal procedures were approved by the institutional Animal Studies Committee. Twenty-four male Sprague–Dawley rats (506.6 ± 69.1 g at the time of sacrifice) were used in this study. In order to investigate the effect of a large rotator cuff tendon injury with and without denervation, animals were subjected to bilateral dual Tenotomy of the supraspinatus (SS) and infraspinatus (IS), with chemical denervation via botulinum toxin A (BTX) injection on the left shoulder and without BTX on the right shoulder. All surgeries were performed under sterile conditions. Briefly, animals

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were anesthetized using isofluorane carried by 1% oxygen. The injury site was sterilized with iodine preparation. Dual-tendon injury for both Tenotomy and Tenotomy + BTX shoulders was performed as follows: a 2-cm vertical incision was made over the scapulohumeral joint, and the deltoid was detached from the cranial and lateral aspects of the acromion using electrocautery. The acromion was elevated using a 3–0 Vicril suture passed around the spine of the scapula. The SS and IS tendons were exposed by supination of the forearm and transected via No. 11 blade at the insertion on the humeral head. For the left shoulders (Tenotomy + BTX group), the above tendon transections were performed following BTX injections, whereby BTX was diluted in sterile saline and injected into both the SS and IS muscle bellies (2.5 U/125 μl per muscle). The right SS and IS muscles of each animal were injected with a volume-matched injection of saline (Tenotomy group). Following Tenotomy and injections, the deltoid and trapezius muscles were then reattached using 3–0 Vicryl suture and the skin was closed using staples. Post-operative animal care was administered by an animal care technician, staples were removed within 10 days post-injury, and animals were allowed free cage activity until sacrifice time points of either 8 weeks (animal age ~4 months) or 16 weeks (animal age ~6 months) post-surgery. Normal, uninjured animals were time-zero age-matched to serve as comparative controls ($N = 6$). Control animals did not undergo sham surgery.

At the time of sacrifice, SS and IS muscles were individually dissected and divided in half along their intra-muscular tendons. The superior half of each muscle was placed in storage solution for passive mechanical testing and the inferior half was snap frozen in liquid nitrogen-chilled isopentane and stored at −80°C for subsequent biochemical analysis.

Passive Mechanics of Single Fibers and Fiber Bundles

Single fiber and fiber bundle testing was carried out as previously described.[15] Detailed methods and representative stress–strain curves (Fig. S1) are available in the Supplemental Materials.

Tangent modulus was defined as the tangent to the quadratic fit of the stress–strain curves between 2.0 and 4.0 μm. Tangent modulus was taken at the strain corresponding to a sarcomere length of 3.2 μm, as this is sarcomere length of human SS and IS muscles in the anatomical position.[16] This is within the physiological range of sarcomere lengths for rat skeletal muscle.[17] Three fibers and three fiber bundles were tested from each muscle sample and moduli were averaged. Samples were discarded if they did not produce a clear diffraction pattern, if any irregularities appeared along their length, or if they were severed or slipped at suture attachment points during testing.

Titin Molecular Weight Determination

Titin molecular weight was quantified using a previously developed method utilizing SDS–VAGE.[18] Detailed methods and a representative gel image (Fig. S2) are available in the Supplemental Materials.

Collagen Content

The hydroxyproline content of the muscles was used to determine collagen content (μg collagen/mg wet weight tissue) using a modification of a previously published protocol.[19] Detailed methods are available in the Supplemental Materials.

Myosin Heavy Chain

Muscle fiber type was estimated using myosin heavy chain (MHC) composition as previously described.[20] Detailed methods and a representative gel image (Fig. S3) are available in the Supplemental Materials.

Statistical Analysis

After screening the data for normality and homogeneity of variances, two-way ANOVA with post-hoc Sidak tests were used to determine significant differences between treatments, time-points, and between individual groups within each time-point. Linear regression was used to determine correlations between mechanical and protein-level variables. Statistical analyses were performed using SPSS 20.0 (IBM, Armonk, NY) and Prism 6.0b (GraphPad, Inc., La Jolla, CA). Significance was set at $p < 0.05$ and all data are presented as mean ± SD.

RESULTS

Single Fiber Passive Mechanics

At the single fiber level, the SS and IS muscles became less stiff between 8 and 16 weeks ($p = 0.018$ and $p = 0.001$, respectively), regardless of injury (Fig. 1A and B). In the SS muscle, there was also a significant interaction between time and injury ($p = 0.001$); muscle fibers from the Tenotomy + BTX injured muscles tended to be more stiff at 8 weeks and less stiff at 16 weeks. Although there were no significant differences between injury types at 8 weeks, by 16 weeks the Tenotomy (12.68 ± 3.86 kPa/μm) and Tenotomy + BTX (6.59 ± 2.35 kPa/μm) groups were significantly less stiff ($p < 0.05$) than controls (22.48 ± 10.87 kPa/μm; Fig. 1A). When comparing individual groups across time, the Tenotomy + BTX group was significantly less stiff ($p < 0.001$) at 16 weeks compared to 8 weeks (23.26 ± 6.77 kPa/μm). In the IS muscle, there were no injury type main effects or time main effects, but fiber stiffness significantly decreased from 8 to 16 weeks in both Tenotomy ($p = 0.041$) and Tenotomy + BTX groups ($p = 0.022$), Fig. 1B).

Titin Isoform

In the SS muscle, at 16 weeks, Tenotomy (3,521 ± 35 kDa) and Tenotomy + BTX (3,540 ± 36 kDa) resulted in significantly smaller titin isoforms compared to controls (3,633 ± 110 kDa) (Fig. 1C). There was an observed time effect in the IS muscle, as titin molecular weight significantly increased from 8 to 16 weeks ($p = 0.002$; Fig. 1D). Specifically, controls increased in titin molecular weight from 8 to 16 weeks ($p = 0.002$), with a similar trend observed in Tenotomy + BTX groups ($p = 0.055$). At 16 weeks, Tenotomy alone (3,591 ± 67 kDa) showed a trend ($p = 0.070$) toward decreased titin molecular weight, while Tenotomy + BTX (3,586 ± 38 kDa) resulted in significantly decreased titin molecular weight compared to control (3,668 ± 83 kDa; $p = 0.049$).

Muscle Fiber Bundle Passive Mechanics

In SS and IS muscles at both 8 and 16 weeks, Tenotomy + BTX fiber bundles tended to have increased

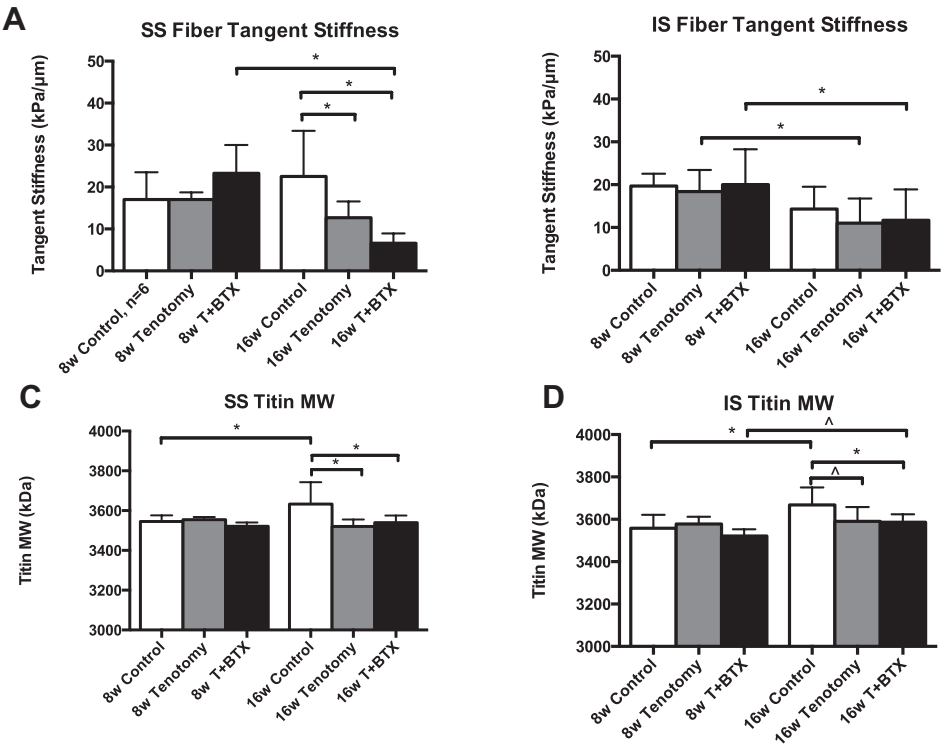

Figure 1. The SS (A) and IS (B) single muscle fibers became less stiff between 8 and 16 weeks, regardless of injury. Whole muscle titin molecular weight was lower in the injured SS (C) and IS (D) muscles compared to controls at 16 weeks. In both muscles, this effect appears to be related to larger titin isoforms in the control group at 16 weeks compared to 8 weeks. $N = 6$ for each group. * Indicates $p < 0.05$ for individual comparisons, and ^ indicates $p < 0.1$.

stiffness compared to controls or Tenotomy alone (Fig. 2A and B). At 8 weeks, Tenotomy + BTX (SS = 186.60 ± 44.54 kPa/μm; IS = 122.2 ± 37.64 kPa/μm) had significantly stiffer fiber bundles than controls (SS = 60.96 ± 16.57 kPa/μm, $p < 0.001$; IS = 58.67 ± 8.58 kPa/μm, $p = 0.002$) or Tenotomy alone (SS = 64.90 ± 19.01 kPa/μm, $p < 0.001$; IS = 54.61 ± 21.71 kPa/μm, $p = 0.001$). In the SS muscle at 16 weeks, Tenotomy + BTX fiber bundles (90.18 ± 56.72 kPa/μm) were significantly stiffer than controls (33.89 ± 7.73 kPa/μm) but not different than Tenotomy only. The SS muscles of all three groups were less stiff at 16 weeks than at 8 weeks ($p < 0.001$). In the IS muscle at 16 weeks, there was a trend for Tenotomy + BTX (64.00 ± 50.39 kPa/μm) fiber bundles to be stiffer than Tenotomy alone (22.90 ± 11.18 kPa/μm, $p = 0.066$), but were similar to control fiber bundles. Fiber bundle passive mechanics of the IS muscle in all three groups were less stiff at 16 weeks than at 8 weeks ($p < 0.001$).

Collagen Content

In SS and IS muscles, control (SS $p = 0.003$; IS $p = 0.008$) and Tenotomy (SS $p = 0.002$; IS $p = 0.001$) muscle collagen content significantly increased from 8 to 16 weeks, respectively (Fig. 2C and D). In SS muscle at 8 weeks, the Tenotomy + BTX (22.80 ± 7.93 μg/mg) fiber bundles had significantly increased collagen content compared to control (12.10 ± 4.08 μg/mg) and Tenotomy alone (11.79 ± 4.52 μg/mg) (Fig. 2C). In the IS muscle, there were no significant differences in collagen content between groups at 8 or 16 weeks (Fig. 2D).

Myosin Heavy Chain Composition

Results, figure (Fig. S4) and discussion related to myosin heavy chain composition are summarized in the Supplementary Materials.

Passive Mechanics and Biochemical Correlations

There was no observed correlation between single fiber titin molecular weight and single fiber tangent modulus across both muscles and time points (Fig. 3A). Similarly, there was no correlation between fiber bundle tangent modulus and collagen content across all muscles and time points. However, there was a significant, but weak, correlation between fiber bundle tangent modulus and collagen content within the 8-week samples ($R^2 = 0.20$, $p = 0.0046$; Fig. 3B).

DISCUSSION

The purpose of this study was to characterize the adaptations in rotator cuff muscle passive mechanics after injury at the micro- and meso-scale and to correlate these changes with the underlying protein-level adaptations in the muscles. Contrary to our hypothesis, results suggest that increases in muscle stiffness after injury do not originate at the single fiber level and are not supported by changes in titin

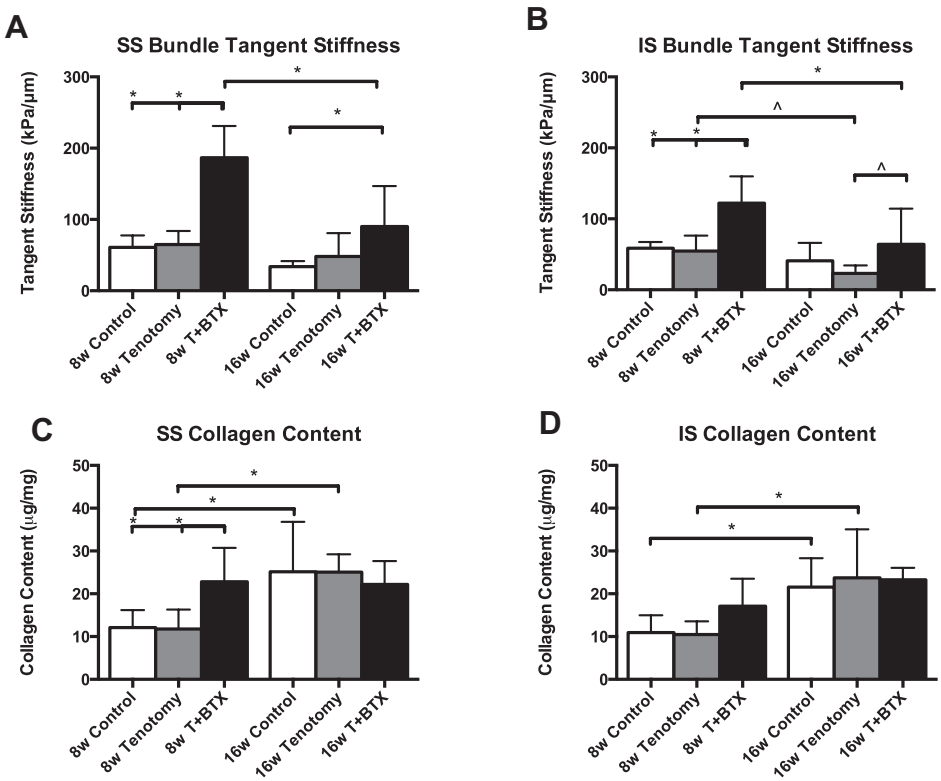

Figure 2. The SS (A) and IS (B) muscle fiber bundles were more stiff in the Tenotomy + BTX group at both 8 and 16 weeks, although this effect was attenuated by 16 weeks. At 8 weeks, there was also an increase in collagen content in the SS muscle (C). This trend was observed in the IS muscle (D), but it does not achieve significance. By 16 weeks, there were no differences between groups as control and Tenotomy groups had great collagen content at this later time point. $N = 6$ for each group. $^{*}p < 0.05$ for individual comparisons, and $^{\wedge}p < 0.1$.

molecular weight. We did observe a decrease in titin molecular weights at 16 weeks in both Tenotomy and Tenotomy + BTX groups compared to controls. We expected this to be associated with stiffer muscle fibers[10] compared to controls, but our micromechanical data at the fiber level did not support this relationship. However, results demonstrated that muscle fiber bundles had increased stiffness after injury when (BTX) was used in conjunction with Tenotomy. Consistent with this change, collagen content was elevated in this group, and positively correlated with fiber bundle stiffness at 8 weeks. At 16 weeks, collagen content was elevated in all groups, including controls, but the magnitude of the increased bundle stiffness was diminished. The observation that collagen content was elevated following massive tendon injury with BTX, indicating muscle fibrosis, supports previous whole-muscle experiments in rotator cuff muscles.[8,9] This is consistent with our recent work showing that collagen content is weakly correlated to fiber bundle

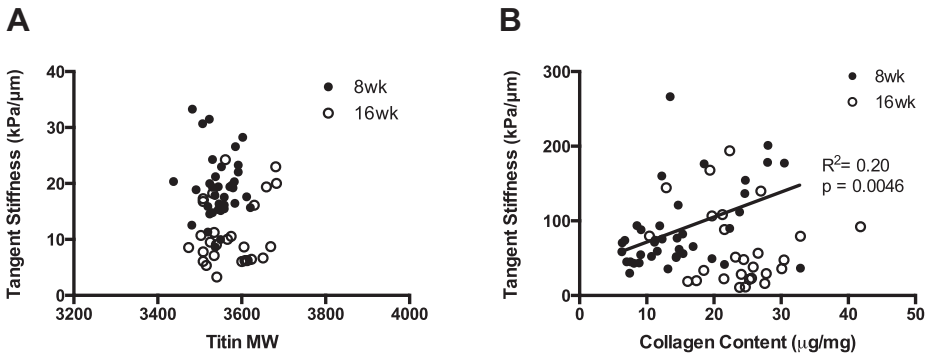

Figure 3. Single fiber stiffness was not related to titin molecular weight at 8 or 16 weeks (A). However, was a positive linear relationship ($R^2 = 0.20$, $p = 0.0046$) between collagen content and muscle fiber bundle tangent modulus at 8 weeks, but not at 16 weeks (B).

stiffness.[21,22] However, the weak correlations suggest that the structural adaptations, which lead to increased stiffness in rotator cuff tears may not simply be due to an increased collagen fraction in the muscle. Alternative sources and mechanisms of stiffness should therefore be explored. Sources of increased muscle stiffness may include changes to collagen cross-linking, increased collagen fibril diameter, or alternations in collagen isoform expression and are appropriate future experimental directions. Furthermore, the cellular sources and targets for suppression of fibrosis remain unknown.

The passive tangent stiffness data in this experiment were analyzed at a sarcomere length of 3.2 μm because this represents the length of the SS and IS muscles measured in humans in the anatomical position,[16] and is a meaningful length in terms of passive tension generation in muscle. Recent data from our laboratory have measured the sarcomere lengths of rat SS and IS muscles in the anatomical position to be closer to 2.3–2.4 μm.[23] However, when we analyzed the muscle tangent stiffness at 2.4 and 2.8 μm, we found no change in the pattern of differences between groups. Therefore, we believe that our conclusions, based on our measurements at 3.2 μm, are independent of our analytical methods.

Contrary to the original hypothesis, the passive stiffness of Tenotomy + BTX muscles decreased from 8 to 16 weeks. This surprising outcome may be due to a combination of factors, including aging and recovery of the muscle from a singular BTX injury over time. Recent data from our lab show that muscle architecture features of uninjured rotator cuff muscles in rats change with age,[23] so it is possible that biochemistry and passive mechanical properties also change as a result of aging. Prior animals studies using BTX injections to paralyze skeletal muscle have shown that the effects of BTX last beyond 8 weeks,[24–26] with some functional recovery observed after 100 days post-injection.[27] Further experiments are necessary to uncouple the effects of aging and recovery from BTX from changes in passive mechanical properties.

Clinically, repair of chronic, large rotator cuff tears becomes increasingly difficult over time. Chronic changes in the muscles include atrophy, fat accumulation, and general loss of plasticity. Beyond a certain level of retraction, the cuff becomes very difficult to mobilize and restore its normal length. As a result, tears potentially become irreparable or repairs are under such high tension that mechanical failure of repairs results. These changes likely have implications to muscle function as well, making it difficult to restore strength even after successful repair. This study demonstrates increased stiffness at the bundle level associated with a massive tear, especially when accelerated by further chemical impairment of neuromuscular function. While the exact mechanism remains unknown, we have established a model that can be used for further study. Furthermore, we also found a decrease in fiber stiffness with injury, both with and without BTX. This may reflect pathologic changes secondary to mechanical unloading and/or neuromuscular disruption that may have implications with regards to muscle function and deterioration with time.

The current study has several limitations. First, our study did not test whole muscle–tendon unit passive mechanics, which would ultimately determine the amount of passive stress in the muscle during repair. Whole muscle passive mechanical measurements in addition to fiber and bundle mechanics may be useful in corroborating the clinical manifestations of these observed changes. However, previous reports have already described increased tension in rotator cuff tendon–muscle units after Tenotomy, including in the rat.[5,6] Second, our assay for fibrosis, the hydroxyproline assay, only measures the changes in fractional amounts of collagen within the tissue. Future studies will be required to parse out the potential contributing factors leading to increased passive stiffness, including collagen type, collagen organization, and other non-collagenous components, which compose the muscle extra-cellular matrix. Third, although BTX is frequently used to produce chronic and severe rotator cuff degeneration in rat models, the causes of degeneration in patients with rotator cuff disease are not fully understood. This is an experimental issue that should be explored prior to searching for a source of increased passive mechanical stiffness in BTX-induced denervation models. Last, we studied a relatively short time period. Chronic muscle changes take years to develop in humans, and we studied relatively short-term responses. A longer time frame may have more effectively replicated the clinical scenario.

CONCLUSIONS

In summary, massive rotator cuff tears, combined with chemical denervation, result in increased passive stiffness at the muscle fiber bundle level in SS and IS muscles. Our findings suggest that adaptations leading to increased stiffness of the rotator cuff muscles occur not within the muscle fibers themselves, but at the fiber bundle level via contributions from newly deposited extra-cellular matrix. However, the molecular and protein-level mechanisms that lead to this increase in stiffness were only partially elucidated by the current study. Further study of these mechanisms of fibrosis will be required to determine therapeutic targets to mitigate muscle fibrosis and ultimately improve the success rate of rotator cuff repairs.

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SUPPORTING INFORMATION

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