ABSTRACT

Title of Dissertation: BIOLOGICAL AND FUNCTIONAL CHANGES IN SUPRASPINATUS MUSCLE AFTER ROTATOR CUFF TEAR

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Rotator cuff (RTC) tears impair upper limb mobility and affect 20% of the adult population. Unfortunately, surgical repair of major RTC tears often fails to restore shoulder function and has a high risk of re-tear. RTC tears induce irreversible, degenerative changes to the muscle that may hinder the recovery of shoulder function. Currently, very few studies have comprehensively assessed RTC muscle function, thus, little is known about which markers may be able predict changes in function after RTC tear. In this dissertation, I present three studies designed to systemically determine the impact of a RTC tear on contractile function of the supraspinatus (SS), the muscle most commonly affected in the RTC.

In study #1 I developed a novel method to test *in vivo* SS contractile function using animal species common to RTC research. In study #2, I found that the SS exhibited a 30% loss in force prior to onset of muscle atrophy after acute RTC tear using the rat model. The initial loss of force was associated with a decrease in the size

and continuity of the neuromuscular junction (NMJ). The SS muscle was also more susceptible to injury, which was associated with a reduction in collagen packing density. Therefore, SS size is not the strongest predictor of force output with acute RTC tears. In addition, the increased susceptibility to injury could compound the dysfunction already apparent in the SS muscle after RTC tear.

In study #3, I found that the rabbit model experienced a 40% loss of force after 6 weeks of RTC tear that persisted at 12 weeks. Using a number of different *in vivo* and *ex vivo* imaging approaches I found the degree of fatty infiltration (FI) to be the strongest predictor of muscle force production after RTC tear. Surprisingly, the data suggested that muscle atrophy only explained the loss in force in torn muscles when little to no FI was present. Therefore, FI is a prognostic marker for muscle weakness after RTC tear, and can help clinicians predict the force generating capacity of the SS for surgery and rehabilitation decision-making.

Results from both studies found that SS contractile function was significantly impaired after RTC tear, and identified measureable markers beyond muscle atrophy that were associated with the loss in muscle force that may act as potential therapeutic targets to improve functional outcomes after RTC tear.

BIOLOGICAL AND FUNCTIONAL CHANGES IN SUPRASPINATUS MUSCLE AFTER ROTATOR CUFF TEAR

by

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List of Abbreviations

12W: 12 weeks after tenotomy

15D: 15 days after tenotomy

2D: 2 days after tenotomy

6W: 6 weeks after tenotomy

ADL: Activities of daily living

CSA: Cross sectional area

FI: Fatty infiltration

GH: Glenohumeral

IS: Infraspinatus

PCSA: Physiological cross sectional area

Plin1: Perilipin 1

RTC: Rotator cuff

SS: Supraspinatus

Chapter 1: Introduction and Specific Aims

The rotator cuff (RTC) consists of four muscles that provide motion and dynamic stability to the complex and inherently unstable shoulder (glenohumeral) joint. RTC tears are amongst the most common orthopedic injury, affecting 20% to 30 % of the adult population. The injury almost always involves the tearing of the supraspinatus tendon. [129] In humans, the shoulder joint has the greatest range of motion compared to other joints, so RTC tears drastically impair upper limb mobility and therefore quality of life. [90,118,136] RTC tears, particularly larger tears (e.g. full-thickness and massive), lead to degeneration of the RTC muscles and poor shoulder function that are often irreversible even after surgical repair. [19,43,47] Moreover, out of the 300,000 repairs done annually in the United States, and the re-tear rates have been documented to be as high as 90% in patients with larger tears. [37,138]

Although RTC tears involve the rupture of tendinous tissue, RTC muscle health plays a crucial role in the progression and recovery after RTC tears. One of the most notable topics in RTC research is the role of fatty infiltration that occurs in the RTC muscle after a tear. Fatty infiltration refers to the presence of fat within the muscle, which is detectable using histology and non-invasive imaging such as magnetic resonance imaging (MRI) and computed tomography (CT) scanning. Studies suggest that the degree of fatty infiltration in RTC muscles is the strongest prognostic factor for functional and surgical repair outcomes, [19,43,88] and is closely associated with the severity and duration of the RTC tear. [89] A systematic study reporting on 925 shoulders, found that RTC muscles with moderate to significant amount of fatty infiltration have a higher re-tear rate (59%) compared to muscles with

minimal fatty infiltration (25%). [63] Patients with a greater degree of fatty infiltration also had inferior shoulder function after repair (e.g range of motion and strength, ability to work at shoulder level) compared to patients with a lower degree of fatty infiltration. [13]

However, due to the nature of the studies using non-invasive imaging in people already experiencing a RTC tear, it is difficult to determine whether fatty infiltration is the root cause for the functional and surgical complications seen in patients with RTC tear. In fact, Gladstone et al reported that moderate to severe muscle atrophy is also strongly associated with higher re-tear rates, similar to those seen with fatty infiltration. Interestingly, they also reported that supraspinatus atrophy, but not fatty infiltration, correlated with post-operative shoulder function. [43] Supraspinatus muscle atrophy was also negatively associated with functional scores in a 67-month follow-up after RTC repair. [110]

Besides muscle atrophy, the cellular and intracellular composition of the supraspinatus muscle is altered in biopsies from elderly patients with RTC tear, including an increase in connective tissue, decrease in myofibril volume, and increase in intramyocellular lipid. [114] Animal models of RTC tear also show a variety changes in the muscle after RTC tear, including atrophy, [7,65] fibrosis, [7,78] altered architecture, [127] stiffness, and increased expression of adipogenic and atrophic-related genes [35,50,78]. Furthermore, it has been reported that it takes about 3 to 4 years for fatty infiltration to develop in patients with RTC tear after the onset of symptoms [89]. Therefore, changes in the musculature of the RTC that occur at earlier time points after a tear

and prior to fatty infiltration could be major contributors to the degeneration of muscle seen over time.

Since RTC muscles are deep muscles underneath the trapezius and deltoid, and the tendons sit under the strong bony arch of the acromion, it is challenging to obtain a muscle biopsy to study the biological aspects of RTC muscle in patients who are not already undergoing a surgical procedure. Therefore, the use of animals in RTC research has allowed the field to better understand the progression of muscular changes after a RTC tear. Rats and rabbits have a shoulder anatomy that strongly resembles that of the human. [30] In order to model massive RTC tears in animals, at least two RTC tendons are surgically transected resulting in tendon detachment and supraspinatus muscle atrophy. [7,65] Using this approach, the muscle's architecture is altered, including pennation angle, sarcomere length, sarcomere number, and muscle length. [127] RTC muscles also become fibrotic over time, with changes in collagen composition and in muscle stiffness, that may alter the function of the muscle and therefore contribute to poor shoulder function seen after RTC tears. [78]

Although the use of animal models has helped investigators understand the alterations induced by RTC tears, challenges remain as a result of differences in the recovery process across different models. For example, cutting the tendons alone in the rat model does not result in fatty infiltration to the same degree that is evident in humans with a RTC tear, [35,50,78] but in contrast, cutting the tendons alone in the rabbit model of RTC tear results in significant fatty infiltration within several weeks. [105] In order to induce fatty infiltration in the rat model, the suprascapular nerve is transected or the RTC muscles are treated with exogenous neurotoxins, which results in reduced

healing capacity after RTC injury.[64] However, nerve transection or induction of a neuropathy does not represent the majority of clinical cases since the incidence of neuropathy in RTC tears is only about 2-5%.[21] Thus, in order to assess the validity of the animal models it is necessary to comprehensively assess function of the muscles that contribute to the recovery process of the RTC after a tear.

One of the current issues with the animal models is that numerous investigations have used indirect biomarkers to assess muscle injury rather than direct assessment of muscle force production. Unfortunately, the biomarkers do not always correlate with the loss of contractile force,[24-1] like serum marker creatine kinase that is commonly used to infer muscle damage.[6] It is well accepted that direct measurement of muscle function (contractile force) is the most comprehensive measure of overall muscle health.[6] Only a few studies have assessed muscle function (whole-muscle contractility) of the RTC muscles,[28,84,85,100] but the results show largely incongruent values in force generation after RTC tear[10,28,33,34,84]. This gap in the literature is likely due to the unique challenges for testing RTC muscles, such as the depth of the tendons and the presence of an overlying acromion. In order to isolate the function of any muscle, any compensating movement by other joints should be avoided, therefore the scapula must be completely immobilized to obtain accurate measures of RTC function.[57] However, stabilization of the scapula is unique and challenging compared to hind-limb muscles that are commonly tested, because the scapula can move in multiple axes[97] (e.g. upward, downward, internal, and external rotation, and it can also tilt posteriorly and anteriorly). In addition, the external tendon of RTC muscles are particularly short compared to other muscles commonly tested

(e.g. soleus or tibialis anterior), which makes it challenging to attach the muscle to a load cell for measurement collection. Thus, development of a method that can overcome these challenges is necessary to reliably determine how RTC muscle function changes after a tear.

Despite the growing literature on RTC pathology no treatments have proved to be advantageous in the clinical setting. Most the literature on RTC tears is focused on the occurrence of fatty infiltration, while other aspects of muscle health have been generally neglected due to the limitations of the developed methodology. The use of animal models has provided more details on the progression of muscular changes after a tear, but there is very limited information as to how these changes affect muscle function. Currently a number of therapies have been explored in animals, such as platelet rich plasma [25,54] and stem cell treatment, [49] but none have had a significant clinical success. [59,102,123,128] Therefore, by assessing muscle contractility we can more comprehensively understand the pathophysiology of RTC tears, and also elucidate new mechanisms that are affected by RTC tears that could serve as promising therapeutic targets to improve functional outcomes and recovery.

This dissertation work includes a series of animal experiments to determine the impact of RTC tear on supraspinatus muscle function. Study #1 established an *in vivo* method to test supraspinatus contractile function in mice, rats, and rabbits. In the remaining two studies, I identified changes in contractile function of the supraspinatus muscle in the two most common animal models of RTC tear. Lastly, I determined the association between changes in contractile function and changes in histological markers, including atrophy, fatty infiltration, and fibrosis.

Specific Aim #1: Develop a method to test rodent and rabbit supraspinatus contractile function.

The purpose of this completed study (study #1) was to develop a method to test contractile function of the supraspinatus muscle *in vivo* in three species commonly used in RTC research, mice, rats, and rabbits (see Chapter 3). In summary, I described an *in vivo* method to assess supraspinatus muscle contractility (e.g. twitch, tetany, force-frequency, and rates of muscle fatigue) in the mouse, rat, and rabbit. I also provided morphological measurements of muscle and tendon lengths in all three species. I found the rabbit to have the highest specific force (force divided by physiological cross-sectional area), which could be due to differences in muscle architecture (greater fiber length-to-moment arm ratio and fiber length-to-muscle length ratio compared to rodents). I also found a lower tetany:twitch ratio in the rabbit, which could be due to differences fiber type composition compared to the rodent species. In addition to providing a detailed method to test supraspinatus contractility in species commonly used in RTC research, I provided normal values of supraspinatus mass and force in mice, rats, and rabbits that can be used to compare to injured muscles.

Specific Aim #2: To identify the progression of *in vivo* changes in contractile function of the supraspinatus muscle in animal models of RTC tear.

RTC tears that are large enough to involve more than one tendon (e.g. supraspinatus and infraspinatus) are referred to as massive tears, and they severely impair shoulder function and often lead to long-term disability. [20] Without

intervention, smaller tears (defined as partial-thickness tears) can become larger over time. [83,136] Therefore, I focused on two-tendon RTC tears for the remainder of this dissertation work.

A variety of muscles other than RTC are involved in shoulder function, [15,68,70] but RTC muscles provide critical stability and mobility to the shoulder. [70,121] Since RTC muscle contractility cannot be readily assessed in humans, and the evidence from animal studies is scarce and inconsistent, assumptions can only be made on muscle function based on the biomarkers previously measured by other groups. However, it is not empirically known how two-tendon tears affect RTC muscle contractile function.

Using the method described in specific aim #1, I measured supraspinatus contractile function at various time points after RTC tear. **The purpose of specific aim #2 was thus 1) To identify the extent to which supraspinatus contractile function is impaired after a tear, 2) To test whether supraspinatus muscle becomes more susceptible to contraction-induced injury after RTC tear, and 3) To determine if changes in contractile force are influenced by the duration of a RTC tear**. I employed the rat model of RTC tear in Study #2 and rabbit model of RTC tear in Study #3, not only because they are the most common animal models in the literature, but also because the muscle's response to RTC tear is not identical between species (e.g. fatty infiltration is greater in the rabbit than the rat). Although the method to test contractile function is suitable for testing mice, only rats and rabbits were used in subsequent studies. The rationale behind this decision is because smaller differences in force across time are more detectable using larger animals that

exert a greater amount of force. The animal model of RTC tear consists of surgically releasing the infraspinatus and supraspinatus tendons. Since no studies have comprehensively assessed contractile function, I included force frequency curves, rates of fatigue, twitch force, and maximal isometric force. Moreover, eccentric muscle contraction of RTC muscles has not been previously tested, even though eccentric movement of RTC muscles is commonly used in physical therapy and during activities of daily living. [68] Since muscle can be damaged by continuous eccentric contractions, I also determine whether there is a greater susceptibility to injury in muscle after RTC tear.

RTC tears can last several years in humans, and a gradual loss in shoulder function and pain occur after the onset of tendon detachment. [83] Atrophy and fatty infiltration are evident between 6 weeks to 4 years after RTC tear and progress significantly over time. [46,89] However, there is insufficient evidence to conclude that contractile function of RTC muscle will behave in a similar fashion and gradually decrease over time. Therefore, the purpose of Aim #2 was also to determine how contractile function of the supraspinatus muscle is affected over the duration of a tear. I assessed contractile function at time points that are associated with changes in atrophy and fatty infiltration. In the rabbit, fatty infiltration begins at 6 weeks following tendon detachment and progresses over the following weeks; therefore, I measured supraspinatus contractile function after 6 weeks and 12 weeks after RTC tear. Although the rat model does not develop the degree of fatty infiltration seen in the human or rabbit, [35] it still experiences atrophy and fibrosis, which tend to occur within 2 weeks after RTC tear. [33,51] Therefore, I tested supraspinatus contractile

function at 2 days and 2 weeks after RTC tear. Using both the rabbit and rat model of RTC tear I addressed the following hypotheses:

I) **Supraspinatus contractile force will be lower in animals with a RTC tear compared to control animals.**

II) **Supraspinatus muscle will be more susceptible to contraction-induced injury in animals with a RTC tear compared to control animals.**

III) **The attenuation in contractile force and the increase in susceptibility to injury will be more pronounced in supraspinatus muscle from animals with long-term RTC tears (2 weeks for rats, and 12 weeks for rabbits) compared to animals with short-term RTC tears (2 days for rats, and 6 weeks for rabbits).**

<u>Specific Aim #3</u>: To determine if an association exists between functional changes and histological markers in RTC muscles after a tear and in the period leading up to repair.

Skeletal muscle pathology is often assessed through histology, but histological markers are not always predictive of muscle function. There is insufficient evidence to conclude that the changes in muscle, such as atrophy, fibrosis, and fatty infiltration in RTC muscles mimic changes in contractile function. Therefore, the **purpose of this aim is to determine which histological markers are more closely associated to the changes in contractile function of the supraspinatus muscle after RTC tear**. Using the muscle samples collected and stored from Aim 2, I histologically

analyzed fiber size, fatty infiltration, and fibrosis and addressed the following hypotheses:

I) **The decrease in contractile force of supraspinatus will be proportional to a decrease in muscle fiber size after RTC tear.**

II) **The decrease in contractile force of supraspinatus will be associated with the presence of fatty infiltration in the supraspinatus muscle after RTC tear.**

III) **The increase in susceptibility to contraction-induced injury will be associated with an increase in fibrosis in supraspinatus muscle from RTC tear.**

Chapter 2: Review of Literature

Introduction

The rotator cuff (RTC) is made up of four muscles that move and stabilize the major joint of the shoulder (glenohumeral joint). A tear in the RTC is a common orthopedic problem that impairs shoulder function and results in disability [29,76,125]. Simple tasks such as combing hair, reaching for an object, or pulling a door open become challenging for people with RTC tears [55,125], and depression and anxiety are often consequences of shoulder disability [17]. Surgical RTC repair is commonly performed to improve the quality of life after a RTC tear, but often fails to restore muscle function and results in an unacceptably high rate of tendon re-tear over time [27,39,61]. While there have been efforts to improve tendon-to-bone healing, understanding the effects of RTC tendon tear on RTC muscle function may provide further insight into the pathophysiology of this condition, and further lead to more effective therapies to improve functional outcomes. Therefore, this review will focus on the muscular aspects of RTC tendon tear, particularly on how RTC tears impact shoulder function and the capacity of the RTC muscles to generate force.

Shoulder function is essential in activities of daily living

The shoulder allows the upper extremity to move through a large range of motion, but can be simplified into motions involving three spatial degrees of freedom. Anterior and posterior movements are flexion/extension respectively, lateral and medial movements are abduction/adduction respectively, and movements in the axis of the humerus are external/internal rotation (Fig. 1). Activities of daily living (ADL),

such as combing hair, reaching for an object, and getting dressed, require a complex combination of shoulder movements [82,95]. For instance, flexion and external rotation are required to place an object on a shelf, and extension and internal rotations are required to tuck in a shirt behind the back [95]. Studies that track the shoulder movement of patients through video-movement analysis software or electromagnetic tracking systems have shown that most ADLs do not require the full range of motion of the shoulder, but instead require approximately 120° of forward elevation, 45°of extension, 130° of abduction, 115° of cross body adduction, 60° degrees of external rotation, and 100° of internal rotation [95]. However, other activities such as throwing, swimming, and playing tennis require movement well beyond these ranges [31].

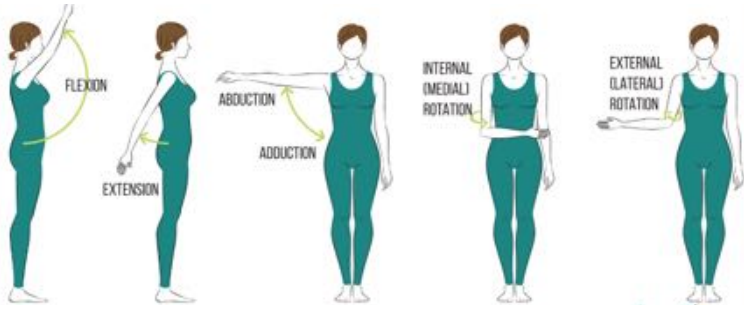

Figure 2.1: Basic movements of the shoulder. *Retrieved from sequencewiz.org*

Prevalence and severity of RTC tears

It is estimated that at least 20% of the population over 60 years old has a RTC tear [66,136]. Prevalence of RTC tears also increases with age, rising to 62% by age of 80 [119], and the likelihood of having a RTC tear is 3- fold higher every 10 years after the age of 65 [36]. However, the prevalence of RTC tears may be underestimated, as a portion of RTC tears are initially asymptomatic and gradually result in impaired

shoulder function and pain [66,83]. In fact, 16% of people without shoulder complaints may have an asymptomatic RTC tear [135] and 51% of patients with an asymptomatic tear develop symptoms over the course of 2.8 years [134]. Regardless of the patient report, both symptomatic and asymptomatic RTC tears are more common in advanced age [66,119,134].

RTC tears become more severe over time [118,134]. In fact, there is a 35-50% likelihood that a small RTC tear will become larger and more severe in only 2 years [61,86], with almost no probability for the tear to heal on its own [86]. The severity of the tear depends on the size of the tear and the number of tendons affected. Full-thickness tears can disrupt tendinous tissue more than 6 mm deep, which is more than a half of the thickness of the substance of the cuff [52]. The width of a full-thickness tear can also range from a small puncture wound to a tear that is larger in diameter. When the diameter of a full-thickness tear is larger than 5 cm, it is known as a *massive RTC tear*, and it spreads across the cuff affecting more than one of the four RTC tendons. Massive rotator cuff tears lead to the most disability, pain, and are also the most difficult ones to repair [27,39,53].

People with RTC tears have impaired shoulder function

The ability to perform ADLs is dependent on range of motion, comfort, and strength [82], which are all compromised in the shoulder of people with RTC tears [55,125]. In older adults with a full-thickness supraspinatus tear, kinematics for forward reach, functional pull, upward reach, perineal care, and hair combing are strikingly different compared to healthy subjects [125]. For instance, subjects with a full-thickness RTC tear

rely more on internal rotation during most movements, which may be an adaptive strategy to avoid pain [55]. The complexity of the shoulder can also account for the biomechanical differences in shoulder movement in subjects with RTC tears, as other muscles like the pectoralis major, biceps, and deltoid compensate for movements when RTC muscle function is poor [55,70,125].

However, the majority of the evidence of impaired shoulder function in patients with RTC tears is derived from validated assessments such as the Constant-Murley score (CMS) and the American Shoulder and Elbow Surgeons (ASES) standardized assessment form, which quantitatively evaluate shoulder function [20,36,76,90]. The CMS score includes a physical examination of the range of motion and strength of a single shoulder movement (abduction), and a self-reported assessment of pain, sleep, and ability to engage in recreation and work. The ASES evaluation scores self-reported pain and function through a visual analog scale without a physical exam. Despite the differences between these two commonly used tests, their scores are correlated and have been established as a valid assessment of shoulder function of people with RTC tears [133]. However, they do not provide a comprehensive assessment of shoulder function and do not always correlate with quantifiable strength deficits during shoulder movement [106].

Shoulder function is often not fully restored after surgical repair of RTC

In the past few years there has been a dramatic increase in the number of RTC repairs. In 1996 there were approximately 41 RTC repairs per 100,000 people, which more than doubled in 2006 with 98 repairs per 100,000 people [22]. The rise in surgical

intervention is partly attributed to new techniques such as arthroscopy versus open surgery, the rise in the population at risk for RTC tears (over 65 years of age), and growing popularity of outpatient surgery [22]. RTC surgical repair involves reattachment of torn tendons to the humeral head, with the goal of restoring shoulder function and reducing pain. Although the surgery initially improves patient reported outcomes, more than 30% of repairs do not improve shoulder function, and functional deficits can persist indefinitely [9,18,110].

Some studies compare functional outcomes after RTC repair in patients who experienced a recurrent tear to patients who did not (referred to as *intact repair*) [47,53]. Not surprisingly, shoulder strength, range of flexion, and ability to perform ADLs are inferior in shoulders with recurrent tears, but despite these impairments, patients' self-reported outcomes are not drastically different to those from patients with an intact repair [47,106]. In fact, self-reported outcomes tend to improve after RTC repair in patients with a recurrent tear, which could be attributed to a variety of factors such as reductions in pain, post-operative care, and physical therapy [9,47,53,106]. Thus, it would appear that shoulder function does not correlate with patient-reported outcome [106].

Despite improvements in pain and range of motion following a RTC repair, shoulder strength deficits and alteration in glenohumeral mechanics persist, even in the absence of a recurrent tear [9,106]. Therefore, surgical repair techniques, although necessary, are not optimal in restoring long-term shoulder function [9,37,106]. Interestingly, patients who manage their RTC tears non-operatively also have diminished pain and improved function [69]. One study examined 452 patients with full-thickness RTC tears who underwent a physical therapy program, which improved

shoulder function and reduced pain, and only 25% of the patients went on to have RTC surgery [69]. However, less is known about the long-term effectiveness of non-operative treatment [60].

Glenohumeral mobility is fundamental for shoulder function

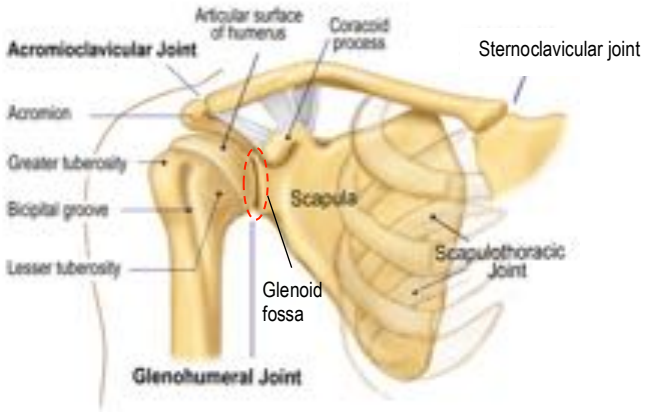

Figure 2.2. Bony anatomy of the shoulder (anterior view). The glenohumeral joint (dotted red circle) is a ball-and-socket joint made up by the articular surfaces of the humeral head and glenoid fossa. The other joints include the acromioclavicular, sternoclavicular, and scapulothoracic joints. *Image retrieved from greylynnosteopathy.co.nz*

The shoulder consists of four different joints (Fig. 2) (sternoclavicular, acromioclavicular, scapulothoracic, and GH joint) that differ in type and motion they provide. The sternoclavicular and acromioclavicular joints are classified as plane joints, which consist of flat articular surfaces that allow gliding movement, but permit a limited amount of motion. The scapulothoracic (ST) joint consists of the anterior surface of the scapula gliding on the posterior thoracic rib cage; however, it is technically not an anatomic joint because it lacks fibrous, cartilaginous, or synovial tissue. Although the ST joint contributes to shoulder movement, its function is beyond the scope of this review. The joints with the greatest range of motion are

ball-and-socket joints, also described as multiaxial for their ability to move in multiple axes. The GH joint is a ball-and-socket joint and consists of the round surface of the humeral head that can roll, slide, and spin on the surface of the glenoid fossa of the scapula [99,130]. The multiaxial design of the GH joint permits movements of flexion/extension, abduction/adduction, circumduction, and medial-lateral rotation [99], which can be combined to create a complex variety of movements previously described in this review.

Bony structure of GH joint is unstable

There is always a trade-off between mobility and stability of joints, thus, the extensive mobility provided by the bony structure of the GH joint occurs at the expense of stability [130]. Matsen et al. define GH instability as the unwanted displacement of the humeral head on the glenoid articular surface [87]. The movement of the center of the humerus on the glenoid fossa is defined as *humeral head translation* (aka "gliding"), and maintaining optimal humeral head translation without losing contact between the humerus and glenoid during movement is challenging due to the differences in sizes of the articular surfaces of the joint. Because the articular surface area of the humeral head is three to four times the size of the articular surface area of the glenoid, only 25-30% of the humeral head articular surface comes in contact with the glenoid surface at any one time [113,130]. The amount of articular contact is also dependent on the angle of the GH joint, with greatest contact occurring at 60° and 120° of abduction [113]. In addition to the asymmetry in size, the asymmetry in shape between the surfaces of the humerus and glenoid further contribute to GH

instability, as the glenoid surface is relatively flat while the humeral surface convex. The RTC provides compression of the humeral head onto the glenoid fossa, helping to compensate of the inherent structural instability [113,130].

RTC muscles stabilize the GH joint

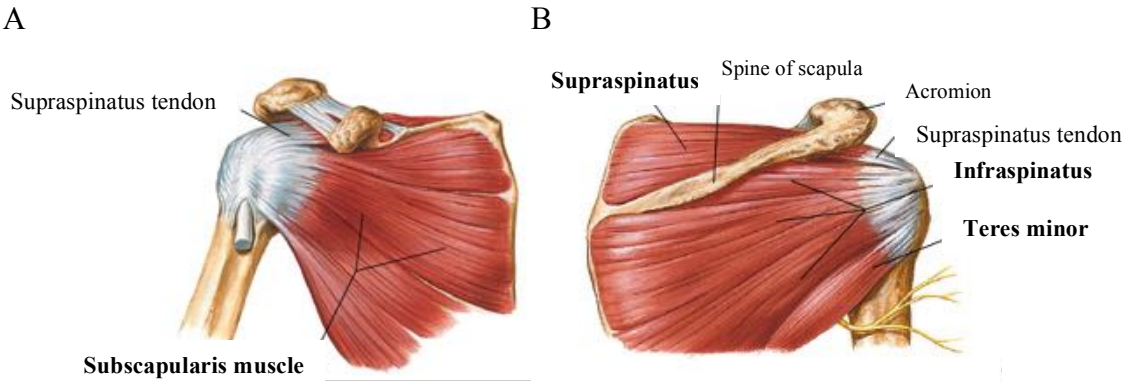

Figure 2.3. Anterior (A) and posterior (B) view of the RTC. Muscles (in bold) include the subscapularis, supraspinatus, infraspinatus, and teres minor. *Retrieved from Netter Images software.*

The merging of the supraspinatus, infraspinatus, teres minor, and subscapularis tendons on the humerus form the actual "cuff"(Fig 3A,B). The muscles compress the humeral head to the glenoid fossa [80]. All four muscles are active throughout the range of motion of the GH joint, and each muscles varies in force-generating capabilities [68]. The subscapularis muscle is the strongest, followed by the infraspinatus, supraspinatus, and lastly the teres minor [62]. Despite the differences in their ability to produce force, all four muscles contribute to joint stability [11], but GH kinematics are disrupted if balanced transverse moments are affected [121]. The *transverse force* couple is a term described by Burkhart to describe the compressive forces of the internal rotator (subscapularis) and external rotators (infraspinatus, and

teres minor) to hold the humerus onto the glenoid fossa, and is necessary for stable rotation during GH abduction [12]. An imbalance in the forces involved in the force couple leads to unwanted displacement of the humeral head on the glenoid articular surface. The proportion of muscle volume (subscapularis to infraspinatus and teres minor) tends to be around 1.0 irrespective of gender or age in a population without shoulder or muscular pathology [32]. Not only is proportional force in the transverse force couple important for joint stability, but the total absolute force of the muscles is also essential for GH stability, as a 50% decrease in RTC forces leads to a 50% increase in anterior displacement of the humeral head during external load [132].

RTC tears lead to GH instability and alterations in GH mobility

RTC tears disrupt GH stability provided by the RTC muscles, as evidenced by increased GH translation and altered contact sites between the humeral head on the glenoid. Detachment of the infraspinatus or subscapularis tendons leads to posteriosuperior translation of the humeral head, while detachment of infraspinatus and supraspinatus tendons leads to increased anteriosuperior translation [11]. The size and location of the tear also impacts the degree of GH instability. Tears involving the subscapularis have a greater impact on GH biomechanics than tears of the infraspinatus [115], and larger tears increase humeral displacement on the glenoid and cause a humeral shift in the direction of the tendon tear [56].

RTC tears also decrease the mobility of the GH joint according to the muscle affected. Massive tears involving the supraspinatus, infraspinatus, and part of the subscapularis, disrupt the contact between the humeral head on the glenoid during

abduction; whereas a single supraspinatus tear destabilizes the joint to a lesser degree [56]. Subjects with infraspinatus tears have weak external rotation and are often unable to raise their arms [12]. In cases where both the supraspinatus and subscapularis are affected, active elevation is significantly reduced, and external rotation is impaired when the supraspinatus and infraspinatus are affected [20]. Strength during GH movement is also reduced in patients with RTC tears, as gradual detachment of the infraspinatus tendon sequentially decreases abduction torque [94], and full-thickness supraspinatus tears are associated with reductions in strength during external rotation and abduction [125].

GH instability and shoulder dysfunction persist after RTC repair, likely due to RTC muscle weakness

RTC tears destabilize the GH joint by disrupting the transmission of force generated by RTC muscles on the joint. Interestingly, surgical reattachment of the tendon (repair) fails to restore GH stability and function. For instance, RTC repair fails to restore the point of contact between the humeral head and the glenoid in patients with repaired full-thickness supraspinatus tears [9], and also fails to restore dynamic joint excursion[6]. Therefore, factors other than tendon ruptures impair GH stability and function after RTC tear.

Weakness of RTC muscles can also lead to GH instability and dysfunction. In fact, paralysis of the supraspinatus muscle, used to model muscle weakness, has a greater effect on GH instability than a small RTC tear [56]. RTC weakness also results in poor shoulder function, as patients with infraspinatus paralysis due to a ganglion

compressing the inferior branch of the suprascapular nerve, have infraspinatus atrophy, shoulder pain, and weakness of external rotation and abduction [116]. Also, in an animal model of massive RTC tear, impaired compressive forces destabilize the GH joint and leads to unwanted contact between the humerus and acromial arch, which increases the likelihood of damage of the soft tissues between the two bony structures [15].

The forces provided by RTC muscles are necessary for GH function, and in the case of RTC repair, tendon reattachment may not restore shoulder function if the muscle is too weak. However, it is not empirically known if RTC tears result in RTC muscle weakness, or the extent to which contractile force may be affected by RTC tears. The next section will thus focus on determinants of muscle contractile force that are affected after RTC tear.

Tendon tears may impair contractile function of RTC muscles

Forces generated by RTC muscle contraction stabilize and mobilize the GH joint for proper shoulder function, and people with RTC tears have poor shoulder function that is often not fully restored after surgical repair. However, few studies have assessed how RTC tendon tears affect the muscle's ability to generate force. As described previously, a variety of muscles contribute to shoulder movement and it is impossible to isolate the force produced by the individual RTC muscles in a clinical setting. This differs from muscles like the quadriceps femoris, which can be tested through assessment of a subjects' ability to perform knee extensions [126].

In order to isolate function of each individual muscle, it would be necessary to release each muscle from its origin to attach the muscle to a load cell. To the best of our knowledge, only one study has performed this method in humans [40]. Supraspinatus muscle force was measured in thirteen patients with full-thickness supraspinatus tears while undergoing surgical repair. Because there was no healthy control group, it could not be determined whether supraspinatus force was reduced in these patients. However, authors concluded that supraspinatus force was associated with pre-operative cross sectional area of the muscle measured by MRI, but no significant correlations were made to support their statement. They found that muscle cross sectional area increased slightly after 1 year of RTC repair, but sharply decreased in supraspinatus muscles that experienced a re-tear. These findings could imply that RTC repairs improve supraspinatus force as long as there is no re-tear, but the study did not show that their method to measure muscle cross sectional area was an accurate and reliable predictor of supraspinatus contractile function. Shoulder function was also assessed through CMS scores, which improved after 1 year after repair in patients with intact RTC and those with a recurrent tear. Although no correlations were made between functional scores and supraspinatus strength, functional scores may not be accurate predictors of RTC strength since patients who experienced recurrent tears had improved scores despite decreased muscle cross sectional area. The study had various limitations, as it had a small sample size and did not control for sex, age, tear severity or symptoms. Therefore, a more controlled study would be needed to define how RTC function is altered after a tear and how it contributes to repair outcomes.

Only a few studies have assessed RTC muscle function in animal models of RTC tear. However, they vary significantly in the animal species used, methodology, and force values recorded [10,28,33,84,91]. In terms of force output, supraspinatus muscle of rabbits with a RTC tear generated less force at when stimulated at a low frequency compared to the contralateral control side, but maximal tetanic force was not measured in this study [34]. Maximum tetanic force was tested in a rat model of RTC, and it was 30% lower 12 weeks after RTC tear compared to contralateral control [28]; however, force values were only a fraction of forces documented by mammalian muscles of similar weights [79,124, 84]. Based on the evidence, further studies are needed to clarify how RTC tears affect RTC muscle function, specifically, the extent to which RTC tears affect RTC muscle maximal and submaximal force output, how contractile force is affected according to the severity and duration of RTC tears, and whether RTC repair restores or fails to restore RTC muscle function.

Structural determinants of muscle function are affected by RTC tears

Generation of force is the primary function of skeletal muscle, but contractile function has not been thoroughly assessed in RTC muscles. Since the structural organization of muscle strongly influences its function [74], the changes in muscle architecture after RTC may indicate how function of the RTC is affected after RTC tears. The strongest determinant of muscle force is physiological cross sectional area (PCSA) of the muscle. This value takes into account factors such as myofiber length, pennation angle, muscle density, and muscle mass, all of which influence PCSA as seen in the following equation:

$$PCSA\ (cm^2) = \frac{Muscle\ mass\ (g) \times \cos\ (pennation\ angle)}{Fiber\ length\ (cm) \times muscle\ density}$$

Myofibers are individual multinucleated muscles cells that are cylindrical in shape, and contain organelles necessary for energy production and calcium handling like the mitochondrial and sarcoplasmic reticulum. Most of the myofiber volume is made up of myofibrils, which contain filaments of actin and myosin that form long strings of sarcomeres that result in muscle contraction when actively shortened. The diameter of a myofiber determines its force output, and myofiber diameter decreases after a RTC tear in the rat by 32%, which was associated with a 43% in myofiber force [48]. Myofibers isolated from biopsies of RTC muscles from patients with chronic supraspinatus tears also exhibit a 20% to 30% reduction in force [90] and myofiber diameter tends to be reduced in patients with full-thickness tears [81].

Myofibers can also vary in length, which influences the velocity of the contraction and is correlated with increased force generation [14]. The optimal myofiber length (L_o) for maximum force generation is based on the length of the sarcomeres within myofiber. The sarcomere is the smallest contractile unit of muscle, as it contains a parallel arrangement of action and myosin filaments that form cross bridges that result in muscle contraction. Optimal sarcomere length ranges between 2.2 and 2.6 μm, which results in optimal overlap of the myosin and actin filaments [3]. When sarcomere length is too short, there is excessive overlap between the two opposing actin filaments in each sarcomere, which interferes with cross bridge formation and results in reduced force generation. On the other hand, when sarcomere length is increased beyond optimal length, there is a reduction in the overlap between

the actin and myosin filaments, thus reducing force generation. In animal models of RTC tear, sarcomere length initially decreases with muscle retraction, but returns to normal after weeks of RTC tear, indicative of sarcomere remodeling as a response to muscle fiber shortening [127]. In cadaveric shoulders with full-thickness RTC tears sarcomere length is strikingly similar to healthy RTC muscles, but myofiber length is shorter in the torn groups, suggesting there are fewer sarcomeres in series [67,122]. Although a decrease in serial sarcomeres does not change the maximum force of the myofiber, it decreases the active range of the fiber, which is directly related to the joint's range of motion [73,137]. Therefore, a decrease in the number of serial sarcomeres may affect the contribution of RTC muscles to joint movement. The decrease myofiber length in full-thickness RTC tears is associated with the severity of the tear, and sarcomere length is decreased in massive RTC tears, but not full-thickness tears [41].

The arrangement of muscle myofibers (i.e pennation angle) also influences the overall mechanical function of the muscle. When myofibers are parallel to the axis of the tendon, their forces are transmitted along the tendon axis resulting in greater forces produced compared to muscles whose myofibers are not parallel to that axis. In technical terms a greater pennation angle results in a muscle that produces less force compared to another muscle with all parallel myofibers (cosine of 0° equals 1) of the same mass and myofiber length. However, in healthy muscles a greater pennation angle allows for more myofibers to pack in a given area ("myofiber packing"), and therefore increase the number of contractile units. Pennation angle of RTC muscles increases in humans after RTC tear [139] and in some animal models of RTC tear [92,127].

However, the number of myofibers does not increase after RTC tear, and some argue that the increase in pennation angle without an increase in myofiber packing allows for fibroblasts and adipocytes to fill in the available space [92], which will be discussed further in this review. Changes in pennation angle may thus decrease the muscle's capacity to generate force.

Changes in muscle mass after RTC tear may be the most influential predictor of changes in force generating capacity of RTC muscles as RTC muscle atrophy is a common feature in patients [8,40,43,93,109]. Depending on the duration and severity of the RTC tear, supraspinatus mass decreases 10-50% [48,93,127]. There is also a significant decrease in the relative number of myofibers in supraspinatus muscles of patients with full-thickness tears [114]. Supraspinatus atrophy is correlated with impaired poor shoulder function and is also associated with poor functional scores in a 67-month follow-up after RTC repair [110]. Furthermore, normal muscle mass of RTC muscles does not recover even after successful RTC repair, similar to the pattern seen with functional outcomes after repair [26,109]. Therefore, RTC tears may lead to RTC muscle weakness by decreasing in muscle mass.

Muscle quality is affected by RTC tear

Muscle quality describes the ability of a muscle to produce force per unit mass [44]. Although changes in muscle mass influences PCSA and the force generating capacity of the muscle, there is evidence for additional factors to affect function of RTC muscles after RTC tear. For instance, isolated myofibers from rats and patients after RTC tear generate 18% to 29% lower force, respectively, compared to myofibers from untorn muscles despite changes in diameter [48,90]. Single fiber studies

suggest that factors other than structural aspects of the muscle may be involved in impaired muscle function after RTC tear. Our group has also found that contractile force of the supraspinatus muscle in a rat model of RTC tear is 30% lower 2 days after a tear (unpublished), even when muscle mass and PCSA are no different to contralateral control [107]. Therefore, factors other than PCSA may contribute to functional changes in the muscle.

Fatty infiltration of RTC muscles is associated with poor muscle quality

Fatty infiltration (FI) of RTC muscles is commonplace after a RTC tear, and is associated with the duration of the tear and its severity. The term FI is often not defined in the RTC literature, but according to Addison et al., FI in the muscle refers to the storage of lipids in adipocytes found underneath the fascia of the muscle [2]. Adipocytes may be found within the muscle between myofibers (intramuscular) and between different muscle groups (intermuscular) [38]. Non-invasive imaging of patients with RTC tears show increased FI in muscle, particularly in full-thickness and massive tears [8,43,89,110], and FI can make up more than 50% the volume of the muscle, particularly in massive RTC tears [8,89]. Low preoperative FI is associated with lower rates of re-tear following surgery, and higher degree of FI has been associated with reduced strength and limited range of external rotation [16,43,46], and one study found that absolute supraspinatus force is negatively associated to the degree of FI in the muscle in a sheep model of RTC tear [91].

Lipid can also be stored the form of small lipid droplets inside the myofiber (intramyocellular lipid,IMCL), which is associated with insulin resistance and

diminished anabolic signaling [103]. Although FI can be detected through magnetic resonance imaging (MRI), IMCL concentrations are not detected through this method [45], but histological analysis of supraspinatus biopsies from patients with massive RTC tears show adipocytes between muscle fibers as well as increased IMCL [114]. Despite the associations between FI and post-surgical outcomes, it is unclear whether FI is the cause for the high re-tear rates and poor functional outcomes after repair [63].

The degree of FI in skeletal muscle is associated with muscle weakness in muscular dystrophy, neurological patients, and in the elderly [44,77,96,104], and it may also play a role in muscle function of RTC muscles. Studies assessing the direct relationship between adipocytes and myofibers through co-culture experiments show that adipocytes impair contractile force and disrupt regeneration of myofibers [71- 117]. On the other hand, FI may also be a consequence of muscle dysfunction, as impairments in muscle function precede FI in muscular dystrophy, and fatty infiltration is associated with the duration of the disease [72].

It is also unclear as to why FI occurs in RTC muscles after a severe tear. It is possible that the increase in pennation angle after a RTC tear separates myofiber bundles and allows adipocytes to infiltrate the muscle [92]. However, no significant fatty infiltration is found in muscles after Achilles [101] or biceps tendon ruptures [120]. Since tenotomy of hindlimb muscles tends to increase pennation angle of the muscle [58], the fact that FI is not evident in other muscles after a tear may either disprove the spatial theory of FI, or may highlight the uniqueness of RTC tears.

ECM organization may decrease RTC muscle quality

The extracellular matrix (ECM) contributes to skeletal muscle structure, mechanical properties, transduction of mechanical force, and remodeling [98]. ECM is the major factor influencing passive loading, and it contributes to muscle elasticity [42,75], but excessive deposition of ECM proteins in the muscle is a hallmark of muscle pathology [75,111]. In a cross sectional area of healthy muscle, the ECM makes up about 5-10% the volume, but it can increase up to 10-fold in injured muscle [75]. The role of fibrosis on RTC tears is still unclear. Fibrosis is evident in some studies [48,78], but not in others [108,114]. Furthermore, collagen content, which is the main protein in ECM, was not affected in a rat model of RTC tear, despite changes in passive stiffness of the muscle [108].

The material properties of tissue are not only affected by the amount of ECM, but also by the organization of ECM components, particularly collagen [5]. The crosslinking of collagen fibers is associated with collagen turnover, and crosslinking could contribute to muscle stiffness and increase the mechanical strength of the muscle [5]. The passive stiffness of skeletal muscle in advanced age is likely driven by collagen crosslinking that increases collagen packing [131]. In some instances, stiffness in the muscle is not associated with collagen content, but rather with collagen organization [111,112]. However, it is still unclear how collagen organization contributes to stiffness in skeletal muscle, particularly in the RTC. Collagen packing density is a parameter of collagen organization, and densely packed collagen has been correlated with passive stiffness[102,106], while others suggest that collagen organized in parallel to the muscle fibers increases muscle stiffness [4,111,131]. To the best of our knowledge,

collagen organization has not been assessed after RTC tear. However, genes involved in collagen turnover (i.e. matrix metalloproteinase and tissue inhibitor metalloproteinase), which have an impact on collagen organization, are upregulated in a rat model of RTC tear [23], suggesting that organization may play a role RTC muscles. This would impact the mechanical properties of muscle that could affect muscle function.

Conclusion

RTC muscles contribute significantly to upper limb motion by moving and stabilizing the GH joint. Patients with RTC tears have poor shoulder function that is not fully restored after surgical repair and often results in re-tear. RTC muscle weakness results in GH instability and impaired mobility, but the extent to which RTC muscle contractile function is affected after RTC tear is unknown because of the lack of methodology to test RTC muscles in isolation. Tendon tears in the RTC induce changes in the architecture and quality of RTC muscles that may alter the muscles' force-producing capabilities. Muscle atrophy after RTC tear may be the most influential predictor of changes in force generating capacity, and muscle atrophy is often not reversible after repair, similar to impairments in shoulder function. Muscle FI of can be easily detected through non-invasive imaging, and is also associated with functional and surgical outcomes; however, it is unclear whether FI is a cause or consequence of decreased muscle function. Muscle contractile function is a comprehensive measure of muscle health, and changes in muscle function after RTC tear may explain the poor functional outcomes seen in patients with RTC tear. Further studies are necessary to understand how RTC muscle contractile function is affected

after a RTC tear; the extent to which contractile function is affected by the duration and severity of a tear; whether poor functional outcomes after RTC repair are a direct consequence of impaired RTC muscle function; whether RTC muscle contractile function influences RTC tendon healing.

Chapter 3: A method to test contractility of the supraspinatus muscle in mouse, rat, and rabbit

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A method to test contractility of the supraspinatus muscle in mouse, rat, and rabbit

(Journal of Applied Physiology article type: Innovative Methodology)

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ABSTRACT

The rotator cuff (RTC) muscles not only generate movement, but also provide important shoulder joint stability. RTC tears, particularly in the supraspinatus muscle, are a common clinical problem. Despite some biologic healing after RTC repair, persistent problems include poor functional outcomes with high re-tear rates after surgical repair. Animal models allow further exploration of the sequela of RTC injury such as fibrosis, inflammation, and fatty infiltration, but there are few options regarding contractility for mouse, rat, and rabbit. Histological findings can provide a "direct measure" of damage, but the most comprehensive measure of the overall health of the muscle is contractile force. However, information regarding normal supraspinatus size and contractile function is scarce. Animal models provide the means to compare muscle histology, imaging, and contractility within individual muscles in various models of injury and disease, but to date, most testing of animal contractile force has been limited primarily to hindlimb muscles. Here, we describe an *in vivo* method to assess contractility of the supraspinatus muscle and describe differences in methods and representative outcomes for mouse, rat, and rabbit.

INTRODUCTION

The shoulder joint (gleno-humeral joint) consists of a large humeral head articulating with a relatively small, shallow socket. Thus, the surrounding rotator cuff (RTC) muscles not only generate movement, but also provide important joint stability. RTC tears, particularly in the supraspinatus muscle, are a common problem encountered in orthopedics (12; 81). Large RTC tears can lead to irreversible muscle atrophy and fatty infiltration, especially in older patients (26; 38). Despite some biologic healing after RTC repair, problems include poor functional outcomes and re-tear rates after surgical repair, reportedly as high as 90% (24).

Muscle damage, which occurs after a RTC tear, has been defined and measured in many ways (e.g. inflammation, fatty infiltration, atrophy, changes in cell structure, etc.). Structural damage is evident in histological findings (9; 29; 46; 47; 63), but one problem with many of the biological markers used to assess muscle injury, including those used in animal studies, is that they may not correlate with the loss of force. There is a plethora of biological markers to assess muscle damage such as indicators of membrane damage (e.g., Evans blue dye) (29; 59; 77); disruption of the muscle fiber cytoskeleton (e.g., loss of desmin or titin) (42; 43; 71); changes in excitation-contraction coupling (33; 78; 84); alterations in force generating structures or force-transmitting structures (32; 64; 80); increases in serum markers (21); alterations in composition of the extracellular matrix (7; 71; 76); changes in neuromuscular junction structure and function (17; 35; 67); altered muscle fiber morphology (13; 30); and altered signals with non-invasive imaging, such as MRI

(20; 57; 73; 75). Muscle damage is often defined within the context of the assay used to examine it, however no single biological marker can account for the changes in contractility. Since full contractile function can persist despite the presence of biological markers indicating damage, muscle function may be the most valid and comprehensive measure of muscle health (4).

Several animal models are available to measure muscle contractility, but these have been limited to testing small, thin muscles *in vitro* (33), or testing the ankle (48; 49; 51) or knee (65; 66) muscles *in vivo*. Recently published works have provided information regarding the size and/or whole muscle contractility of the supraspinatus, but the number of papers is limited and it is difficult to obtain an overview from the piecemeal information (Table 1). Fatty infiltration after a RTC tear is a common clinical problem that also occurs in rabbits (25; 60; 70; 82), but not reliably in rats (18). Despite this advantage of the rabbit as an animal model for fatty atrophy, we could only identify two publications that examine contractility in the rabbit (15; 16). There are only 2-3 studies providing data on whole muscle contractility for the rat, and none for the mouse (Table 1). Of the handful of studies in all three species, the lack of data on both muscle mass and contractility make it difficult to normalize force to muscle size.

The overall goal of this work is to share detailed methods for testing contractile function of the supraspinatus muscle in commonly used research models such as mouse, rat, and rabbit. We also report normative values of muscle mass and contractile function for a given age and body weight. To the best of our knowledge, this is the first report of whole muscle contractile measurements for the supraspinatus

muscles in mice, and one of only a handful of studies for rats and rabbits. We also provide new information on tendon morphometry and contractile characteristics such as maximal twitch and tetanic tension, force-frequency comparisons, and rates of fatigue. This protocol, together with the contractile data provided for mouse, rat, and rabbit muscles, should be valuable to researchers currently studying the biology of RTC pathology and wishing to assess the functional outcomes or therapeutic interventions.

MATERIALS AND METHODS

Contractile function: All protocols were approved by the University of Maryland Institutional Animal Care & Use Committee. We used male mice (C57BL/10ScSn, body weight 23.6 ± 0.6 g, Jackson Laboratory, Bar Harbor, ME, N = 8), rats (Sprague-Dawley, body weight 242 ± 11 g, Charles River Laboratories, Germantown, MD, N = 8), and rabbits (New Zealand white, body weight 2.3 ± 0.6 Kg, Charles River Laboratories, Germantown, MD, N = 8), all approximately 3 months of age. Before each experiment, the animal was anesthetized (~ 4-5% isoflurane in an induction chamber, then ~ 2% isoflurane via a nosecone for maintenance) using a precision vaporizer (cat # 91103, Vet Equip, Inc, Pleasanton, CA). During the procedure, the animal was kept warm by use of a heat lamp.

In the anesthetized animal, the suprascapular nerve was stimulated via subcutaneous needle electrodes (J05 Needle Electrode Needles, 36BTP, Jari Electrode Supply, Gilroy, CA) placed at the suprascapular notch. Proper electrode position was determined by a series of isometric twitches. Impulses 1 ms in duration

were generated by an S48 square pulse stimulator (Grass Instruments, West Warwick, RI) and passed through a PSIU6 stimulator isolation unit (Grass Instruments, West Warwick, RI). In preliminary experiments (data not shown), the suprascapular nerve was dissected free through a small incision and clamped with a subminiature electrode (Harvard Apparatus, Holliston, MA), which was used to stimulate the supraspinatus. There were no differences between placement of needle electrodes and the subminiature clamp electrode.

Contractile function of the isolated supraspinatus muscle was measured before tissue harvesting, similar to methods described previously (50; 52). Although contractile testing is performed *in vivo*, the tendon was released and this was a terminal procedure. After proper anesthetic depth was confirmed by lack of a deep tendon reflex (no foot withdrawal in response to pinching the foot or ear), we used a custom-built rig to stabilize the scapula. This entails incising through the middle trapezius and rhomboid muscles in order to access the vertebral border of the scapula to place a clamp along the vertebral border near the infraspinatus fossa for complete immobilization of the scapula (Figure 3.2). A second rig was specifically developed for the rabbit, as the devices used for rodents were insufficient to preclude scapula movement with the large forces generated by the rabbit supraspinatus. Once the scapula was securely immobilized, the tendon of the supraspinatus muscle was released and attached to a load cell. Single twitches (rectangular pulse, 1 ms) were applied at different muscle lengths to determine the optimal length (resting length, L_0). TIP: The rat and rabbit tendons were attached to the load cell via suture (sizes 4.0 and 0 Ethicon silk suture, respectively), but the mouse tendons can be difficult to

suture; thus we left the tendon attached to the humeral head and sutured through the head for attachment to the load cell. At L_0, maximally fused tetanic contraction was obtained at ~100 Hz (300-ms train duration of 1-ms pulses at a constant current of 5 mA). We used 150% of the maximal stimulation intensity to induce maximal activation of contraction, P_0. We also generated a force-frequency plot, obtained by progressively increasing the frequency of pulses during a 200 ms pulse train. To provide an index of fatigue, maximal tetanic contractions were performed repeatedly (every 2 s) with the final tension expressed as percentage of P_0. The entire procedure, from anesthesia to the completion of contractile testing takes approximately 30 minutes for each animal, regardless of species.

Supraspinatus muscle-tendon length: Muscle length (origin of the muscle belly at superior angle of the scapula to the muscle-tendon junction) and tendon length (muscle-tendon junction to insertion on the humerus) were measured *in situ* using digital calipers to the nearest hundredth of a millimeter. We have used this method before to examine even smaller structures (22) and, although reliability was not formally tested, and all measurements were performed by the same investigator, and confirmed by a second investigator. After the supraspinatus muscle was harvested, we followed the tendon into the muscle belly via micro-dissection to examine the length of the tendon, which we refer to as internal tendon length.

Statistical analysis. Statistical analysis was not performed on obvious differences between species, such as body weight, muscle mass, and muscle force. To evaluate potential differences between groups for such variables as fatigue, normalized force, muscle length:tendon length ratios, external tendon:internal tendon

ratios, and tetany:twitch ratios, a 1-Way ANOVA was used (SigmaStat, San Rafael, CA). Significance was set at $p < 0.05$.

RESULTS

Anatomy: Figure 3.1 shows the general anatomy of the area. The supraspinatus muscle is completely covered by the upper trapezius muscle and its tendon of insertion is obscured by the deltoid (Figure 3.1A). For instructional purposes, Figure 1B shows a detached scapula with overlying muscles removed, allowing a view of the supraspinatus and its distal tendon disappearing under the acromion bone. For experiments, the supraspinatus tendon is identified and then its tendon released for contractile testing (separation of the RTC tendons is straightforward in animals; the tendons still form a "cuff", but they have a more distinct insertion than in humans (11; 72)). Figure 3.2A shows the overall apparatus setup and Figure 3.2B provides further orientation and detail regarding scapula immobilization.

Muscle mass and tendon length: As expected, there was a marked difference in muscle mass between the mouse, rat, and rabbit (32.5 ± 0.9 mg, 407 ± 9 mg, and 8035 ± 150 mg, respectively, Figure 3.3). The muscle length (length from proximal muscle belly at superior angle of scapula to the muscle-tendon junction) and total tendon length (external portion seen *in situ* and internal portion of the tendon observed after harvesting the muscle) were measured at the time of sacrifice. The overall muscle length:tendon length ratios were almost identical for all three species

(1.57 ± 0.08), but interestingly, the mouse had the greatest ratio of external:internal tendon length ($P < 0.05$, Figure 3.3B).

Contractile function: Figure 3.4A shows representative trace recordings from a mouse (green), rat (red), and rabbit (blue) supraspinatus muscle. The high forces generated by the rabbit supraspinatus muscle necessitate the rabbit scapula having its own separate stabilization device (see Methods and Figure 3.2B). Maximal isometric tension in the mouse, rat, and rabbit (0.26 ± 0.03 N, 2.98 ± 0.60 N, and 15.3 ± 0.86 N, respectively, Figure 3.4B) mirror the respective differences in mass and the previously reported cross-sectional area in these species (58). The tetany:twitch ratio was lower in the rabbits than in rodents ($P < 0.05$).

Representative traces of increasing muscle force with increasing stimulation frequencies are shown in Figure 3.5A. The mean force-frequency relationship for all animals is plotted on the graph in Figure 3.5A and indicates a shift to the right (arrows) for the rabbits compared to rodents. We also compared the degree of supraspinatus muscle fatigue after repeated tetanic contractions (representative recording is shown in Figure 3.5B). No significant differences were found in the rates of fatigue (bar graph in Figure 3.5B), but our test of repeated contractions was limited to two minutes, so differences at longer time points might still exist.

DISCUSSION

Methods for muscle function testing for some hindlimb muscles are well described, with a profuse amount of data generated by contractility studies (1; 3; 5; 10; 37; 39; 40; 42; 54; 61; 67; 68). The shoulder presents some unique challenges for

testing RTC muscles, such as the depth of the supraspinatus tendon and the overlying acromion (Figure 3.1). The shape of the scapula provides a challenge for bony fixation compared to the long lever arms in the lower extremity, but the apparatus (Figure 3.2) and methods to test the supraspinatus muscle described here are relatively straightforward. In the present study, we describe an *in vivo* (non-survival) model to assess supraspinatus muscle contractility (e.g., twitch, tetany, force-frequency, and fatigue) in the mouse, rat, and rabbit. We have also provided contractile data and morphological measurements of the muscle and tendon lengths in all three animal models.

There are advantages and disadvantages when studying various species. Large animals such as the rabbit can be easier to work with, including ease of testing contractility, feasibility of surgical interventions, and providing large structures for *in vivo* imaging. Furthermore, the high forces generated by the rabbit supraspinatus also make it an appealing model to work with, as differences after injury, tenotomy, repair, or treatment will likely be easier to discern. The small structures and correspondingly small forces generated by the rat, and especially mouse, can make them more difficult to work with. However, there are advantages to working with smaller animals including ready availability, comparability to previous studies in the literature, and having a known genome. Because of access to many genetically altered models, mouse studies can be better suited to study mechanisms underlying changes in muscle function.

As expected, the overall tendon length (internal and external combined, Figure 3.3B) scales with animal size, but the ratio of external:internal tendon length was

proportional in all three species, it was greatest for the mouse. While normative data is available for supraspinatus muscle mass and architecture (11; 15; 16; 26; 58; 74), information specific to the supraspinatus tendon length has not been reported. The supposition that the tendon length scales with overall body size and muscle mass among the three species assumes that supraspinatus function is identical in all three. However, differences between species in upper extremity use patterns during gait (8; 31) could preclude identical muscle and tendon architecture in all quadrupeds.

In addition to providing methods for testing contractility, this study provides normal values of muscle weight and contractile data for investigators wishing to study whole muscle contractility of the supraspinatus in various animal models. The absolute quantity of muscle mass is generally well correlated to muscle strength (23), and specific tension (contractile force normalized to physiological cross-sectional area, or PCSA) is similar in most mammals (44). Thus, the stepwise increase in muscle mass and contractile force from mouse to rat to rabbit was not surprising, but there were some unexpected findings. For instance, the tetany:twitch ratio was lower in the rabbits than in rodents. This could be due to muscle fiber type composition, as muscles with a higher number of slow muscle fibers can have a lower tetany:twitch ratio (6). Information regarding fiber type composition of the supraspinatus in animals is scarce; there are, to the best of our knowledge, only a few studies that report on the supraspinatus of the rat (2; 27; 28) and even fewer for the rabbit (14; 70) with little to no data regarding fiber type composition in the mouse supraspinatus. Despite the paucity of information, the consensus appears to be that, like humans, the fiber type in the supraspinatus of rodent and rabbit is mixed. However, specific or

quantitative comparisons are difficult based on the small number of studies, which have used a variety of methods (2; 14; 70).

The relationship between a muscle's activation frequency and isometric force is evident in the sigmoidal force-frequency curve, which shows a steep rise in force with increasing stimulation frequencies (Figure 3.5A). This curve can be shifted to the left or right (reaching peak force at lower or higher frequencies, respectively) for several reasons, such as changes in passive tension during tests or differences in fiber type composition within a muscle (19; 55). We observed a shift to the right of the force-frequency relationship in the rabbit compared to the rodents. This shift results in a reduced summation of force at lower stimulation frequencies in rabbit muscle, which is consistent with a lower tetany:twitch ratio, especially if there is a higher percentage of slow fibers within the rabbit supraspinatus. There are several alternative explanations for the lowered tetany:twitch ratio in rabbits compared to rodents. For example, length-dependent calcium sensitivity seems to be a major factor determining the magnitude of the shift of optimal muscle length (69), and differences have been documented in the geometry and function of the sarcoplasmic reticulum rodents and rabbits (34; 62), which could affect intracellular Ca^{2+} kinetics. It is possible that the response of force to different frequencies is also affected by potentiation of the contractile system, distribution of sarcomere length, and interactions between force exerted and aponeurosis length (69).

Fiber type composition affects the speed of a muscle contraction, but less so the specific tension (force per unit area). Specific tension of skeletal muscle is considered relatively constant (53; 56). Force depends not only on the size and

number of the fibers in the supraspinatus, but also on muscle architecture, and such variables have been well described for humans and animals (58; 83). Muscle mass can vary significantly based on species, age, and health of the animal. Since the maximal force per unit of cross sectional area (specific tension) of skeletal muscle is considered relatively constant, contractile force of a skeletal muscle can be estimated based on its physiological cross-sectional area (PCSA) (41), represented by the equation: PCSA (mm^2) = M(g) * cos θ / ρ(g/mm^3) * L_f(mm), where M is muscle mass, θ represents the angle of the fibers (pennation), ρ is muscle density (1.056 g/cm^3 in mammalian muscle) and L_f represents fiber length (estimated from length of the measured fiber bundle). We gleaned muscle fiber pennation angle and relative fiber length from published studies that have measured these architectural variables in mouse, rat, and rabbit supraspinatus muscles (58). Dividing P_0 by PCSA, the calculated specific tension was, as expected, similar between the mouse, rat, and rabbit supraspinatus muscles (4.48 ± 0.97 kg/cm^2). Although not significantly different, the rabbit generated the highest specific tension (5.42 kg/cm^2). This could be due to differences in architecture between rodents and rabbits. Mathewson et al. (58) did a careful study that compared RTC architecture between mouse, rat, and rabbits (among other species). They found that the architectural difference index (ADI), a combined measure of fiber length-to-moment arm ratio, fiber length-to-muscle length ratio and the fraction of the total RTC physiological cross-sectional area contributed by each RTC muscle, was higher (less like human architecture) for the rabbit than mouse or rat. Such differences between rabbit and rodent could help explain slight changes in normalized force.

There are large differences between humans and non-primates when comparing the percentage of the supraspinatus mass relative to total RTC mass. As expected, the supraspinatus in quadruped animals are comparatively larger than those of bipedal animals, such as primates and humans (58). Thus, even though the ADI of the rodent more closely resembles the human, the size of the supraspinatus in the rabbit is closest to the overall size of the human supraspinatus. There are also differences in the bony anatomy and, compared to rabbits, the rodent bony anatomy is more similar to the human (11; 74). Analogous to the human shoulder, the rodent acromion projects anteriorly over the humeral head to the clavicle, creating an enclosed arch over the supraspinatus tendon. The bony anatomy of rabbit diverges from human, in that the acromion, clavicle, and the coracoid process are generally minimal or nonexistent and do not cover the RTC (11).

The majority of full thickness RTC tears present in patients over 50 years of age (12; 81). Chronic tears can lead to fatty degeneration of the muscle, which is a poor prognostic factor for healing (45; 79). Despite many advantages of using a rodent model, there is no consensus regarding which animal model best mimics the pathophysiology of human RTC tears. The rabbit shoulder is well established as an animal model for chronic cuff tear and an induced tear (surgical tenotomy) in the rabbit RTC muscles results in fatty infiltration (60; 70; 82). Fatty infiltration after a RTC tear is a common clinical problem (26; 38) and desirable in an animal model. Substantial fatty infiltration does not occur in the rodent after a supraspinatus tear (18) unless both the muscle and its nerve (suprascapular nerve) are injured (36; 46; 47), whereas the rabbit mimics the human condition, with fatty infiltrate occurring

after tenotomy alone (60; 70; 82). Despite this advantage of the rabbit as a model for fatty atrophy and ease of surgical interventions, there is a paucity of information regarding average strength of the rabbit supraspinatus muscle (Table 1).

In summary, this work describes methods to immobilize the scapula bone and assess supraspinatus muscle contractility in a variety of animal models. Even though contractile force of a healthy skeletal muscle can be roughly estimated based on its mass and architecture, this assumption only stands for healthy muscle, and makes it difficult to compare between species. The methods detailed here provide the investigator with normal values for the supraspinatus mass and contractility, as well as detailed techniques that can be used to compare uninjured, healthy supraspinatus masses and forces to those obtained after various interventions or to compare healthy, injured, and dystrophic muscles.

FIGURES:

Table 1. *Published studies that include supraspinatus muscle weight and/or whole muscle contractile force*

Authors (Ref No.)	PMID	Species	Sex	Age	Body Mass	Muscle Mass	Contractile Force
Mathewson et al. (58)	24072803	Rabbit	—	—	3.16 kg	7.9 g	—
Rowshan et al. (70)	20926720	Rabbit	Male	—	3–4 kg	6.09 g	—
Uhthoff et al. (82a)	24743593	Rabbit	Female	—	3.4–4.3 kg	8 g*	—
Fabis et al. (16)	9930099	Rabbit	Male	—	3.2–4.5 kg	—	8.5–11.1 N
Fabis et al. (15)	10888165	Rabbit	Male	—	3.7–4.6 kg	—	§
Ditsios et al. (11a)	24981552	Rat	Male	13 mo	500 g	—	1.93 N†
Mannava et al. (56b)	21445691	Rat	Male	—	400–450 g	—	5.1 N
Mannava et al. (56a)	21938374	Rat	—	—	400–450 g	—	§
Plate et al. (64a)	23791493	Rat	Male	12 mo	—	—	5.4 N
Mathewson et al. (58)	24072803	Rat	—	—	570 g	770 mg	—
Sato et al. (71)	25834081	Rat	Male	—	—	500 mg*	—
Liu et al. (47)	20949443	Rat	Female	—	250 g	310–400 mg*	—
Davies et al. (9)	25974842	Rat	Female	—	—	418.3 mg	—
Gumucio et al. (27)	22696414	Rat	Male	6 mo	—	740 mg	—
Liu et al. (46)	22488625	Mouse	Female	12 wk	—	34.6 mg	—
Mathewson et al. (58)	24072803	Mouse	—	—	30 g	30 mg	—

PMID, PubMed ID. *Estimated specific muscle mass from bar graph, as specific values were not provided. †Published in units of grams, but converted to newtons for ease of comparison. §Contractile testing was performed, but data were expressed as a percentage of maximal contractile force, rather than absolute force.

Table 1: Published studies that include supraspinatus muscle weight and/or whole muscle contractile force.

* = Estimated specific muscle mass from bar graph, as specific values were not provided.

ŧ = published in units of grams, but converted to Newtons for ease of comparison.

§ = contractile testing was performed, but data were expressed as a percentage of maximal contractile force, rather than absolute force.

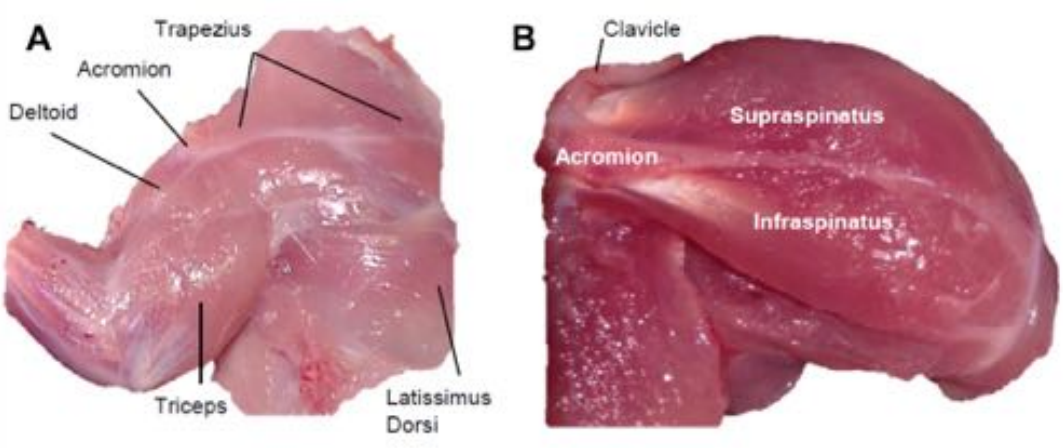


Figure 3.1: General anatomy of the shoulder. **A:** A euthanized animal is shown with the overlying skin removed. The major superficial muscles such as the trapezius, deltoid, and triceps are indicated, but the rotator cuff muscles are deeper and not visible. **B:** A detached scapula from an animal post-mortem is shown with the superficial muscles removed. The supraspinatus can be visualized, but its tendon of insertion is difficult to see due to the overlying acromion and clavicle bones (AC joint). As discussed in the text, the individual RTC tendons in lower species are more distinct than in humans.

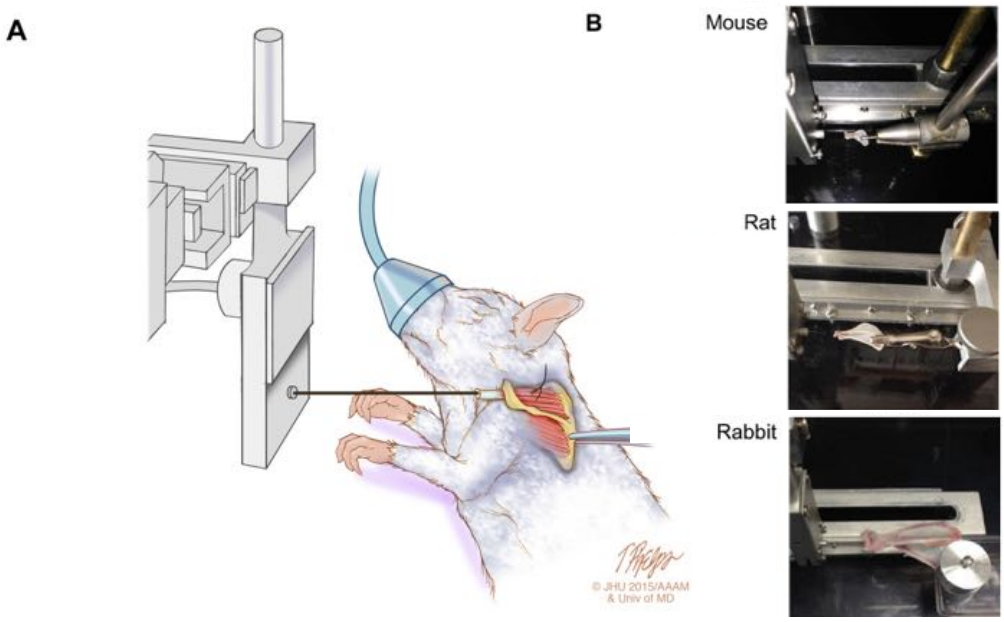


Figure 3.2: Apparatus used to assess *in vivo* supraspinatus contractile function. **A:** Drawing illustrates the general setup used to assess supraspinatus force. With care to avoid the supraspinatus muscle, the scapula is stabilized and the distal tendon of the supraspinatus is attached to the load cell. The load cell is mounted to a micromanipulator so that the muscle can be adjusted to resting length and aligned properly (i.e., adjusting the position between the origin and insertion of the muscle and the load cell so that there is a straight line of pull) in the X, Y, and Z directions. Subcutaneous electrodes are inserted at the suprascapular notch to stimulate the suprascapular nerve. Single twitches (1 ms duration) are induced at different muscle lengths (thus a length-tension, or L-T, curve) in order to determine optimal length (L_o). A maximal tetanic contraction is obtained to determine maximal contractile tension (P_o). **B:** The apparatus is shown without the animal. The custom-made devices we use to immobilize the scapula are adjustable in all three anatomical planes. A commercial clamp (provided it does not clasp the supraspinatus) would likely work too, but the forces generated by the rabbit are quite large, requiring a different rig (i.e., much more stable) than the one used for rodents.

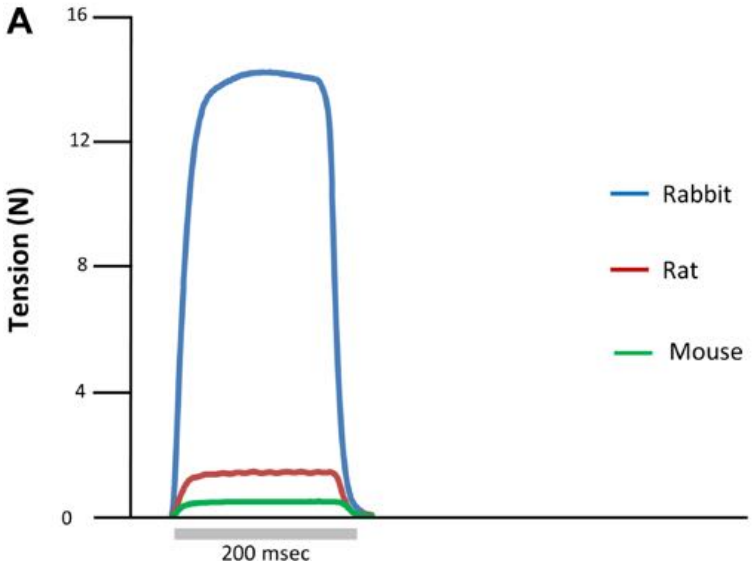
A

B

	MOUSE	RAT	RABBIT
Mass (mg)	32.5 ± 0.9	407 ± 9	8035 ± 150
Muscle length (mm)	10.6 ± 0.2	25.4 ± 0.7	72.2 ± 0.7
Tendon length (mm)	6.8 ± 0.7	16.2 ± 0.4	46.7 ± 1.0
Tendon L_{ext}/L_{int}	0.42 ± 0.09 *	0.29 ± 0.04	0.20 ± 0.04

Figure 3.3: Comparison of supraspinatus muscles from mouse, rat, and rabbit. **A:** Supraspinatus muscles from a mouse, rat, and rabbit are shown for size comparison. The figure shows the underside of the muscles, where the full length of the tendon diving into the muscle belly ("internal tendon") was identified by microdissection and measured. **B:** The table shows weights of supraspinatus muscles from all mice, rats, and rabbits (2-3 months old, N = 8 each species). In addition to muscle mass, muscle length and tendon length (combined length of both the visible external tendon and dissected internal tendon) is provided, as well as the ratios between the length that was external (L_{ext}, measured *in situ* from the superficial aspect) to the length that was internal (L_{int}, determined through microdissection). * = $P < 0.05$

A



B

	Twitch (N)	Tetany (N)	Tetany/Twitch
Rabbit	2.57 ± 0.06	15.3 ± 0.86	6.1 ± 0.5 *
Rat	0.42 ± 0.21	2.98 ± 0.60	7.1 ± 0.2
Mouse	0.04 ± 0.01	0.26 ± 0.03	6.8 ± 0.6

Figure 3.4: Comparison of maximal isometric contractile force from mouse, rat, and rabbit supraspinatus muscles. **A:** Representative *in vivo* trace recordings from a rabbit (blue), rat (red), and mouse (green) are shown for the supraspinatus muscle. In this example, muscles were stimulated for 200 ms at optimal muscle length (L_0) to induce a maximal tetanic contraction (P_0). **B:** Contractile data from mouse, rat, and rabbit supraspinatus muscles. Such absolute values shown can be normalized to muscle physiological cross-sectional area. For experiments, care should be taken to evaluate animals that are age-matched, gender-matched, and of the same strain and species. We did not compare obvious differences in absolute force between species, but statistical analysis for tetany:twitch ratio showed a lower ratio for rabbit compared to rodent. * = $P < 0.05$

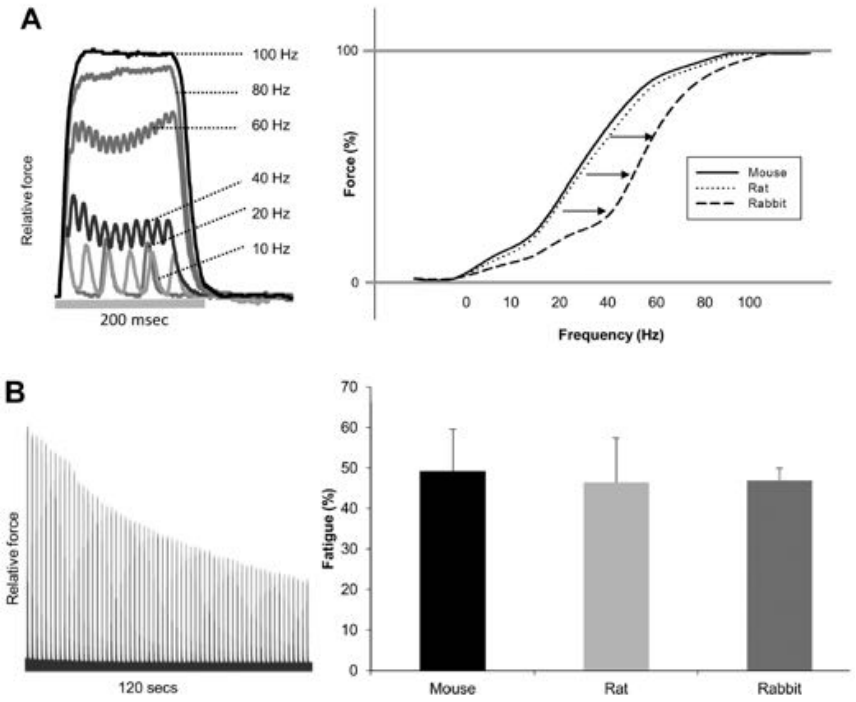


Figure 3.5: A: Comparison of force-frequency data from mouse, rat, and rabbit supraspinatus muscles. LEFT: Curves shown were produced from maximal contractions (200 ms duration) at incrementing higher frequencies (Hz) in the supraspinatus muscle in a rat. Note that maximal force is generated at ~ 80-100 Hz, which diminishes with higher frequency (not shown). This optimal frequency can vary depending on the muscle group tested and the conditions (e.g., direct nerve stimulation as here versus field stimulation when muscles are removed for in vitro experiments, not shown here). RIGHT: Rabbit force frequency curves showed a shift to the right (arrows) when compared to mouse and rabbit. **B:** LEFT: Maximal tetanic tension can be performed repeatedly over time with the final tension expressed as percentage of the starting optimal tension (P_o), providing an index of fatigue at a desired point in time. In this example, the supraspinatus was isolated, adjusted to optimal length (Lo), and then stimulated with a 200 ms tetanic contraction every other second for two minutes. RIGHT: Comparison of fatigue from mouse, rat, and rabbit supraspinatus muscles. There were no significant differences in muscle fatigue.

Chapter 4: Supraspinatus tenotomy results in muscle weakness and susceptibility to injury

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Supraspinatus muscle is more susceptible to injury after rotator cuff tear

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Running title: Supraspinatus contractile function after tenotomy

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ABSTRACT

Background: Rotator cuff (RTC) tears are a common clinical problem resulting in adverse changes to the muscle, but there is limited information comparing histopathology to contractile function. This study assessed supraspinatus force and susceptibility to injury in the rat model of RTC tear, and compared these functional changes to histopathology of the muscle.

Methods: Unilateral RTC tears were induced in male rats via tenotomy of the supraspinatus and infraspinatus. Maximal tetanic force and susceptibility to injury of the supraspinatus muscle were measured *in vivo* at day 2 and day 15 after tenotomy. Supraspinatus muscles were weighed and harvested for histologic analysis of the neuromuscular junction (NMJ), intramuscular lipid, and collagen.

Results: Tenotomy resulted in eventual atrophy and weakness. Despite no loss in muscle mass at day 2 there was a 30% reduction in contractile force, and a decrease in NMJ continuity and size. Reduced force persisted at day 15, a time point when muscle atrophy was evident but NMJ morphology was restored. At day 15, torn muscles had decreased collagen-packing density and were also more susceptible to contraction-induced injury.

Conclusion: Muscle size and histopathology are not direct indicators of overall RTC contractile health. Changes in NMJ morphology and collagen organization were associated with changes in contractile function and thus may play a role in response to injury. The most salient finding is that RTC tenotomy results in increased susceptibility to injury of the supraspinatus; therefore, active lengthening of the supraspinatus should be avoided early in post-repair rehabilitation.

INTRODUCTION

Rotator cuff (RTC) tears, particularly in the supraspinatus muscle, are a common orthopedic problem resulting in shoulder dysfunction and can result in disability.[17, 39, 70, 72] Despite substantial biologic tendon healing after a RTC repair, persistent problems include high re-tear rates and long-term functional deficits of the muscle-tendon unit that may persist even in the absence of a recurrent tendon tear.[6]

In RTC tears, loss of tendon continuity is clearly the initial, paramount problem, but associated changes in the muscle are a major obstacle to full recovery. Large RTC tears can lead to irreversible muscle atrophy and fatty infiltration, especially in older patients.[22, 37] Muscle weakness can result in gleno-humeral instability and poor shoulder function,[27, 67, 69, 74] but it is unclear how RTC tendon tears specifically impact strength of the RTC muscles. Much of the available data has been ascertained from studies on animals, which provide control over many variables (i.e. age, gender, history, etc.) and other advantages, such as a means to use identical injuries to study underlying mechanisms.

Previous work has suggested that the RTC muscles respond differently to injury from muscles in the hind limb,[13] and that damaged RTC muscles have fewer satellite cells[29] with decreased proliferative capacity,[44] all of which may help explain the poor outcomes observed after RTC tears compared to other muscle-tendon tears.[13] To the best of our knowledge, susceptibility to eccentric contraction-induced injury of torn RTC muscles has never been assessed, even though eccentric movement of the RTC is necessary for activities of daily living,[49] and is recommended for shoulder rehabilitation.[12, 30, 31, 76] The overall aim of this work was to assess contractile function

in the rat supraspinatus after a two-tendon RTC tear, and to compare such changes to biological markers such as atrophy, NMJ morphology, lipid content, and fibrosis. A second aim was to assess supraspinatus muscle susceptibility to eccentric injury after RTC tear. Such information on contractility and susceptibility to injury could help with decision making in the period leading up to repair and post-repair rehabilitation.

MATERIALS AND METHODS

All protocols were approved by the University of Maryland Institutional Animal Care & Use Committee. We used male rats (Sprague-Dawley, body weight 242 ± 11 g, Charles River Laboratories, Germantown, MD) at approximately 3 months of age. Rats were randomly assigned to three groups (N = 10 per group). Twenty rats underwent tenotomy 15 (15D) or 2 days (2D) prior to muscle testing, and the remaining ten rats were used as weight-matched controls and were tested on the same day as the tenotomized groups. Before each experiment, the animal was anesthetized (~ 4-5% isoflurane in an induction chamber, then ~ 2% isoflurane via a nosecone for maintenance) using a precision vaporizer (cat # 91103, Vet Equip, Inc, Pleasanton, CA). During the procedure, the animal was kept warm by use of a heat lamp.

Tenotomy: Since the histopathology of the rat supraspinatus better mimics the human condition of RTC tear when the supraspinatus and infraspinatus tendons are cut,[24, 34, 41, 59] both of these tendons were surgically released. Unilateral dual tenotomy of the supraspinatus and infraspinatus tendons were performed after induction of anesthesia. After shaving and cleaning the skin, a small longitudinal incision was made over the acromion and deltoid. The deltoid muscle was split to

expose the superior aspect of the RTC. The supraspinatus and infraspinatus tendons were transected as distally as possible, both to mimic the typical location of a tear and to provide sufficient tendon for attachment to the load cell for testing at later time points. Incisions were closed using sterile Vicryl 4.0 silk suture (Johnson & Johnson, New Brunswick, NJ). All animals were monitored until recovery from the inhalation anesthesia, and buprenorpophine was administered (0.05 mg/kg) subcutaneously as needed.

In vivo contractile function and susceptibility to injury: Contractile function of the supraspinatus muscle was measured *in vivo* as described previously[71]. Briefly, in the anesthetized animal, the scapula was immobilized in a custom designed rig as described previously[71] and the tendon of the supraspinatus muscle was released and attached to a load cell (FT03, Grass Instruments, Warwick, RI & QWLC-8M, Honeywell, Morris Plains, NJ). The suprascapular nerve was stimulated via subcutaneous needle electrodes (36BTP, Jari Electrode Supply, Gilroy, CA) placed at the suprascapular notch. Single twitches (1 ms, S48 square pulse stimulator, Grass Instruments, West Warwick, RI) were applied at different muscle lengths to determine the optimal length (resting length, L_o). At L_o, a force-frequency plot was obtained by progressively increasing the frequency of pulses during a 200 ms pulse train.

For muscle injury, a custom program on commercial software (Labview version 8.5, National Instruments, Austin, TX) was used to synchronize contractile activation and the onset of forced lengthening. A stepper motor (model T8904, NMB Technologies, Chatsworth, CA) was used to induce muscle lengthening. Injury

resulted from 30 forced lengthening contractions superimposed onto maximal isometric contractions spaced 0.5 minutes apart. The moment arm of the supraspinatus relative to the axis of rotation was ~3.7 mm, a 30° angular displacement represents a strain approximating 15% L_0 of the supraspinatus muscle, which is within the physiological range of supraspinatus lengthening. Maximal isometric force was obtained after 2 minute rest and it was compared to the maximal isometric force recorded before injury protocol.

Assessment of NMJ morphology: Supraspinatus muscles were dissected and stored in 4% paraformaldehyde until stained with α-bungarotoxin (α-BTX) conjugated to Alexa-488 (Molecular Probes B13423, Eugene, OR). A total of 80 NMJs were imaged (30 CTRL, 25 2D, 25 15D) and analyzed as described previously.[52-54] Digital images of NMJs from whole mount tissue preparations were obtained with a Zeiss 510 confocal laser-scanning microscope with pinhole set at 1.0 Airy unit. A maximum intensity flat plane projection was made from Z-stacked images in ImageJ software (NIH) to account for the depth of the NMJ. Only NMJs in a complete *en face* view were selected for analysis. After background was subtracted and noise despeckled, a Gaussian Blur filter with $\sigma = 2.00$ was applied. Binary images were then generated from which total area and total perimeter were quantified using tracing tools for the total NMJ endplate. Dispersion index (DI) was calculated as total stained area / total area * 100, describing NMJ density. To quantify continuity and branching of the NMJ, binary images were skeletonized and histograms describing the connectivity for each pixel were generated as previously described.[54] Histogram bins correspond to the number of neighboring pixels for each pixel. One

neighbor implies a terminal pixel, two neighbors imply a pixel along a single branch, and 3 or more neighbors indicate that a pixel exists at a branch node. Thus, discontinuities (terminal pixel) or branching (3+ neighbors) may be quantified within the motor endplate.[36]

Lipid Droplet staining: Muscles were sectioned at a thickness of 10 μm and were stained with BODIPY-493/503 (Invitrogen, Carlsbad, CA) at 1:200 dilution for one hour to identify neutral lipid in muscle (N = 6 per group). Sections were mounted in Vectashield. Sections were visualized using a confocal microscope (Zeiss 510), and fluorescence of ~600 muscle fibers per group was quantified using ImageJ software (NIH, Bethesda, MD) as previously described.[46] Briefly, the integrated density, mean gray value, and area were measured for individual muscle fibers (~100 myofibers per animal), along with several background readings. The fluorescence for each muscle fiber was calculated by the following equation: Integrated density – (area of muscle fiber × mean fluorescence of background readings) × 100.

Western Blotting: Supraspinatus muscles (50 mg) were homogenized in tissue-TEK lysis buffer (Invitrogen), and protein concentration was measured using BCA protein assay (Thermo Fisher Scientific). In a 4-15% gradient gel, 20 μg of protein were loaded, and separated proteins were transferred to a nitrocellulose membrane. Membranes were stained with Ponceau red (Sigma) to confirm successful transfer of protein and equal loading of lanes. Membranes were then blocked in 5% milk and incubated overnight in primary Anti-Ubiquitin antibody (Sigma, cat number U0508) at a 1:500 dilution. Membranes were visualized after incubation with HRP-

conjugated goat antibodies and ECL substrate (Thermo Fisher Scientific). Bands were quantified (N = 4 per group) using ImageJ software and normalized to total protein.

Sirius Red staining: Sections were stained for 1 hour with Sirius red (0.1% Direct Red saturated in aqueous picric acid, Sigma), and rinsed with acidified water (5% acetic acid) (N = 6 per group). Samples were mounted and imaged under brightfield microscopy followed by polarized light microscopy (Nikon). Pictures were taken under the same conditions and exposure time. Birefringent collagen was then analyzed as previously described.[62] Briefly, we determined the number of pixels with 8-bit hue thresholds for red, orange, yellow, and green using ImageJ. The proportion of each hue was calculated by dividing the pixels for each hue to the sum of total colored pixels.

MRI imaging: Small animal *in vivo* magnetic resonance imaging (MRI) was performed as described.[47, 55, 68] High-resolution dual-echo proton density and T2-weighted rapid acquisition relaxation-enhanced (RARE) MR images (TR/TEeff/NA, 1500.00ms/12.94ms/4) were on a 7 Tesla Bruker Biospec 7T/30 MR system (Biospec 7T/30; Bruker Biospin, Billerica, Massachusetts) with a four-channel phased array surface coil. T2-weighted images with and without fat-suppression were acquired for one animal at day 2 and day 15 after tenotomy to evaluate the fat content in supraspinatus muscle. Contralateral shoulder was used as a control. For *ex vivo* imaging, harvested supraspinatus muscles from each group were fixed in 4% paraformaldehyde, patted dry, and placed in a conical tube with Fluorinert FC-40 solution (Sigma). High-resolution T2-weighted RARE MR images (TR/TEeff/NA, 2500.00ms/30ms/1) with and without fat-suppression were acquired (2.5 hour scan).

Statistical analysis: Normality and homogeneity of variance were verified for all data before analysis (SigmaStat, San Rafael, CA). To evaluate potential differences between the three groups a One-Way ANOVA was used. Post-hoc Holm-Sidak test was performed to identify differences compared to the control group. Significance was set at $p < 0.05$ and data are represented as mean ± standard deviation.

RESULTS

Tendon transection resulted in supraspinatus muscle retraction of approximately 5 mm (Figure 4.1A) by day 2, or almost 20% of resting muscle length in the rat supraspinatus.[71] There was no further change in muscle shortening over time, but the tendon scarred down by day 15, in such a way that the space between the tendon and insertion site was filled by a fibrous-connective tissue, forming an ill-defined "pseudo-tendon" that reattaches the muscle to the humeral head (Figure 4.1A, inset).[4, 73] As expected and shown by others,[23, 41, 73] there was a loss of muscle mass 15 days after supraspinatus tenotomy ($P = 0.04$ Figure 4.1B). The progressive decrease in muscle mass after tenotomy was preceded by increased conjugation of ubiquitin to muscle proteins in total cell lysate from muscles at day 2 ($P = 0.01$); and remained elevated at day 15 ($P = 0.01$; Figure 4.1C), suggesting higher protein degradation via the ubiquitin-proteasome pathway.[8]

By day 15, there was a 10% decrease in muscle mass and a 20% reduction in muscle force compared to control ($P = 0.007$; Figure 4.2, blue bar). However, at day 2 there was a 30% decline in isometric force ($P = 0.0002$; Figure 4.2, green bar)

despite no significant loss in muscle mass ($P = 0.31$; Figure 4.1C). Our findings suggest that mechanisms beyond simple atrophy influence contractile force 2 days after tenotomy. Since there is a strong structure-function relationship for the NMJ, and its disruption likely results in altered EC coupling,[52] i.e. muscle activation, we assessed NMJ morphology. At the 2-day time point, NMJs exhibited significant reductions in area, perimeter, and continuity compared to the control ($P = 0.007$) and 15-day group ($P = 0.004$; Figure 4.3).

We have used small animal magnetic resonance imaging (MRI) previously to assess the overall structure of hindlimb muscles.[42, 43, 47, 51, 55, 75] Here, we applied this modality *in vivo* and *ex vivo* to detect fat in the supraspinatus muscle. We compared T2-weighted images with fat-suppression to T2-weighted images without fat-suppression. Although the technique was effective to visualize subcutaneous fat (Figure 4.4A, red arrows), *intramuscular* fat was not detected at any time point after RTC tear (Figure 4.4A-B, not all time points shown). This was consistent with absence of any increases in intramyocellular lipid content with histological staining ($P = 0.602$; Figure 4.4C).

There are conflicting results regarding fibrosis in the rat supraspinatus after RTC tear, with some investigators reporting fibrosis[23, 41] while others do not.[60, 65]. We did not find an increase in the percent area of interstitial collagen staining ($P = 0.492$; Figure 4.5A). However, based on the birefringent properties of collagen stained with Picosirius red under polarized light[1, 48, 62] (see Methods), there was a change in collagen organization at day 15. Collagen birefringence in control muscles had a greater proportion of pixels closer to the red spectrum ($P = 0.007$; Figure 4.5B) when

analyzed as a proportion of total colored pixels. However, supraspinatus muscles at 15D had a greater proportion of yellow and green pixels compared to control (P = 0.017, P = 0.007) indicating altered collagen organization (i.e. reduced collagen packing density, thin collagen).[62] No differences were evident at 2D compared to control.

The screenshot in Figure 4.6A shows an example of a lengthening contraction of the supraspinatus (closed arrow) superimposed onto a maximal isometric contraction (open arrow). We used a protocol of thirty eccentric contractions to the supraspinatus to induce injury and examined the loss in maximal isometric force. Figure 4.6B illustrates the loss in isometric force after each eccentric contraction for a representative animal from each group. Supraspinatus muscles at 15D were more susceptible to injury evidenced by a greater drop in isometric force followed by a short recovery period after injury (P = 0.009; Figure 4.6C).

DISCUSSION

RTC tears result in measurable histological changes to the RTC muscles[41, 59] but none of these indirect biological markers can account for the changes in contractile function.[25, 63, 64] Muscle contractile function is therefore considered the most valid and comprehensive measure of muscle health[10]. Our findings suggest that muscles become weaker and susceptible to injury after a simple tenotomy, even without direct trauma to the muscle fibers.

Given the high rate of poor outcomes after shoulder surgery, understanding the mechanisms leading to insufficient function is critical to develop effective

treatments. Similar to other studies, we found a significant loss in muscle mass of RTC muscles two weeks following RTC tear.[32, 40, 73] While the ubiquitin-proteasome pathway is the main protein degradation pathway of skeletal muscle, previous studies show no changes in expression of key ubiquitin ligases, e.g. muscle RING-finger protein-1 (MuRF)1 and muscle atrophy F-box (MAFbx), after RTC tear.[23, 40] However, ubiquitin ligase expression is not reliably upregulated in muscle atrophy, and their expression can be transient as atrophy progresses.[16] Furthermore, additional ubiquitin ligases have been identified to regulate muscle mass[7] that have not been assessed in torn RTC muscles. Given our finding of increased protein ubiquitination (a downstream process of ubiquitin ligase expression) preceding significant loss in supraspinatus mass after tenotomy, the ubiquitin-proteasome system may play a more significant role in muscle atrophy induced by RTC tear than previously suggested.[23,32]

Although muscle atrophy could be a contributing factor to the decrease in contractile function, the initial drop in contractile force was evident before a significant decrease in muscle mass, paralleling the transient change of NMJ morphology (Figure 3). The NMJ has been implicated as a possible contributing factor to loss of contractile force[19, 20, 45, 57], and the notion of NMJ morphology changing after muscle mechanical strain is not new, but this is one of the very few studies to examine the NMJ after RTC tear. The gross morphology of NMJs has been examined qualitatively in a small sample of biopsies in the human supraspinatus,[20] which classified acetylcholine receptor (AChR) staining along a range of morphologies (singlet dot, a doublet, a cluster, or a line) and concluded "the trend in innervation status is interpreted as leaving open the possibility that denervation plays

a role in RTC injury pathophysiology".[20] Here, we rigorously examined the NMJ using established methods[52-54] to provide precise, quantifiable measures of morphology. Others have reported no changes in the NMJ using an animal model of RTC tear,[19] but that study was conducted in different species (rabbit) and only examined at one time point late after injury (3 months). It is possible that assessing the NMJ at such a late time point accounts for those negative findings, as turnover of AChRs has been reported on a timeline of days.[3, 66] We examined NMJ morphology after tenotomy at a time point when changes at the NMJ occur after injury[53, 61] and found changes during the period when muscle atrophy could not explain the loss in muscle force.

Fatty infiltration of muscles in patients with RTC tear, involves the presence of adipocytes within the muscle (also known as intramuscular adipose tissue). Our findings agree with several studies showing that fatty infiltration is not substantial in the rat[41] compared to the levels seen in humans[5] or rabbits[58] after RTC tear. In addition to the formation of adipocytes in muscles, skeletal muscle fibers have the ability to store lipid in the form of small lipid droplets, and amount of lipid changes according to lipid oxidation, synthesis, and uptake.[9] Increased intramyocellular lipid is associated with insulin resistance and diminished anabolic signaling.[56] While myofibers from patients with RTC tear also have an increase intramyocellular lipid[65], intramyocellular lipid did not increase after RTC tear in our rat model.

The extracellular matrix (ECM) contributes to muscle structure, transduction of mechanical force, remodeling, passive loading, and elasticity,[38, 50] but excess accumulation of ECM in muscle (fibrosis) is common in pathological conditions.[21, 38]

Fibrosis is evident after RTC tear in some studies,[23, 41] but not others.[60, 65]. Mechanical properties of tissue are not only affected by the amount of ECM, but also by the organization of ECM components, particularly collagen.[2] Collagen *content* has been measured after a RTC model in animals, but collagen *organization* has not. Genes involved in collagen turnover (i.e. matrix metalloproteinase and tissue inhibitor metalloproteinase) have an impact on collagen organization, and are upregulated in a rat model of RTC tear.[14] Using the birefringent properties of collagen stained with Picosirious red, we determined that collagen organization was altered 15 days after RTC tear.[26, 62] Decreased crosslinking of collagen is associated with increased collagen turnover, and increased crosslinking could contribute to muscle stiffness, both affecting the mechanical function of the muscle.[2] Although we do not show causality, we found altered collagen organization in the supraspinatus when it was also most susceptible to injury by eccentric contractions.

Eccentric injury commonly induces muscle inflammation and fibrosis, so the repair of a muscle that is apparently healthy, but susceptible to injury, could compound the dysfunction already induced by the RTC tear alone. For instance, one group found that muscle fibers become injured at the time of surgical tendon repair in a rat model of chronic RTC tear.[15] Although, it is currently unknown if susceptibility to injury is preventable, such information could help with decision making for optimal timing for repair, repair tension, and post-operative rehabilitation.

A limitation in the rat in this study that prevented us from assessing contractile force at later time points is the spontaneous reattachment to the humerus via a pseudo-tendon. While some investigators make no mention of adhesions or

reattachment of the cut rotator cuff tendons in a rat model,[24] others report that re-attachment of the supraspinatus tendon occurs spontaneously after tendon transection in rats at time points exceeding 2 weeks.[4, 11, 73] Spontaneous reattachment of the supraspinatus tendon is associated with recovery of muscle mass and collagen content.[4, 73] Efforts to avoid reattachment include removing the distal fragment of tendon[28, 41] or using a membrane[18, 24] to prevent spontaneous reattachment, while other studies make no mention of these deterrents.[23, 24, 33-35] We initially tried using membrane and even a polymer gel (not shown) after tenotomy to prevent spontaneous tendon reattachment. Such methods not only failed to prevent the tendon from scarring down, but also resulted in massive inflammation and an increase of variability in the data.

CONCLUSIONS

This study describes histological and functional changes in the supraspinatus muscle in a rat model of RTC tear. The most salient findings of this work are the apparent dissociations between atrophy and muscle force soon after a RTC tear, as well as the finding that the supraspinatus becomes more susceptible to contraction-induced injury. Knowing when a torn RTC muscle is most susceptible to injury could be useful in surgical and rehabilitation planning, but additional work is needed to elucidate the specific timing and significance of this increased susceptibility to injury in patients.

FIGURES

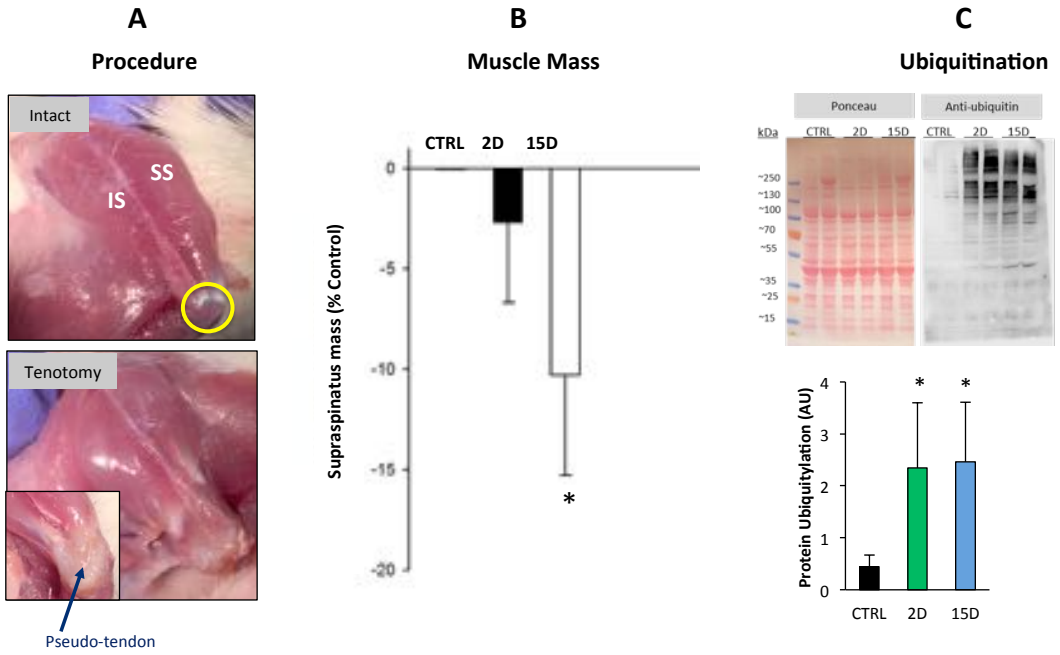


Figure 4.1: Supraspinatus tendon in a model of a RTC tear. **A:** *Top panel***.** Normal anatomy of the rat RTC (used as control) is shown, with the supraspinatus muscle (SS) and infraspinatus muscle (IS), including attachment of their tendons to the greater tubercle of the humerus (yellow circle). RTC tear was surgically induced by tenotomizing the supraspinatus and infraspinatus tendons. *Bottom panel***.** After two days (2D), the RTC tear results in retraction of the tendons. Fifteen days after RTC tear (15D), the space between the muscle tendon and insertion site is filled by a fibrous-connective tissue (arrow) that reattaches the supraspinatus to the humeral head (inset). **B:** As expected, supraspinatus muscle mass was slightly altered after tenotomy and significantly reduced by day 15 (N = 10). **C:** Western blot analysis was used to detect ubiquitinated proteins in total protein extracts of supraspinatus muscles (N = 4). Equal amounts of protein were loaded and confirmed with Ponceau and probed with anti-ubiquitin antibody. Total protein ubiquitination was upregulated at 2 and 15 days after tenotomy in supraspinatus muscle compared to control. All data are presented as mean ± SD, $p < 0.05$. ***,** indicates statistical significance compared to control.

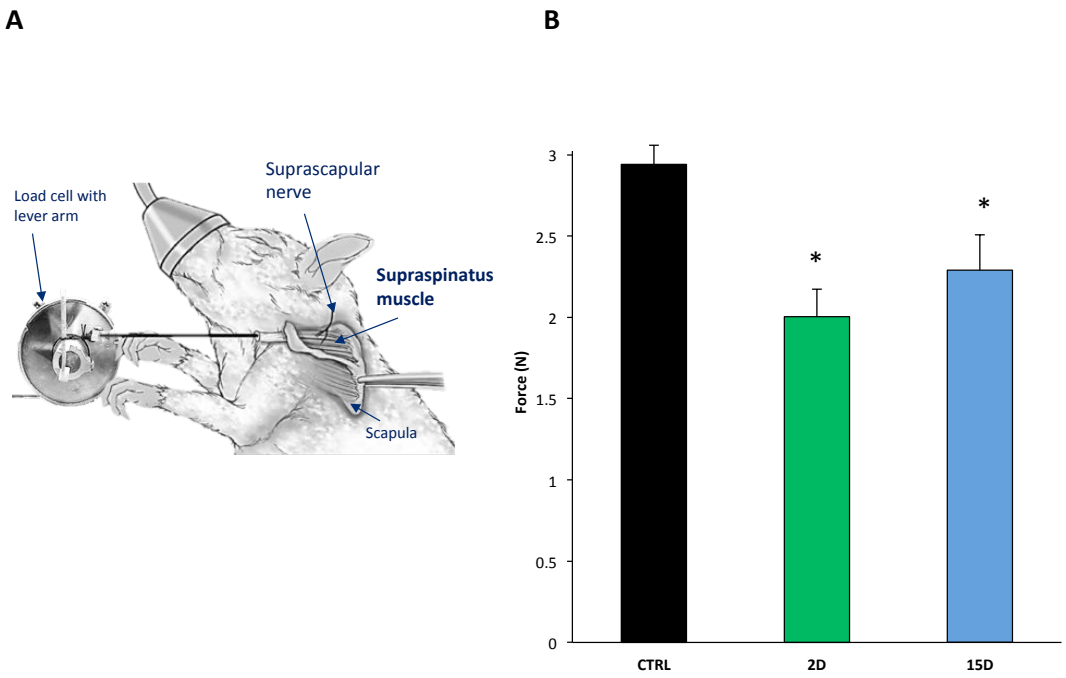
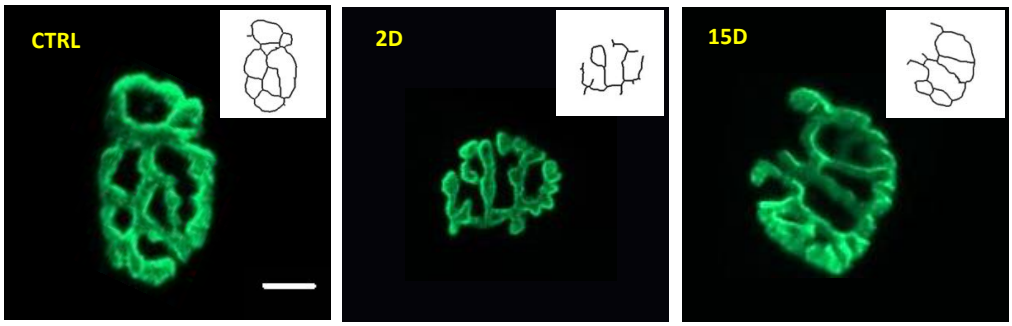


Figure 4.2: Maximal isometric force is lower in tenotomized supraspinatus at 2D and 15D.
A: Apparatus to measure muscle *in vivo* contractility and susceptibility to injury in the supraspinatus muscle. The insertion of the supraspinatus was released and the tendon tied to a load cell. The suprascapular nerve was stimulated via subcutaneous needle electrodes to activate the supraspinatus maximally. A series of maximal twitches was used to determine optimal length (L_0) and the force-frequency relationship was determined to obtain maximal isometric force. **B:** When compared to control, the mean of maximal isometric force per group was 30% lower at 2D and 20% lower at 15D. Maximal force was not different between the tenotomized groups. All data are presented as mean ± SD, $p < 0.05$. *****, indicates statistical significance compared to control.



NMJ morphology	CTRL	2D	15D
Area (μm²)	481.7 ± 42.5	316.4 ± 53.7*	507.5 ± 90.5 ŧ
Perimeter (μm)	98.5 ± 4.7	72.7 ± 8.6 *	91.85 ± 6.8 ŧ
Continuity (AU)	287.9 ± 24.0	197.8 ± 33.9*	318.1 ± 21.9 ŧ
Branching (AU)	27.3 ± 7.4	16.5 ± 5.2	27.4± 5.9
Dispersion index (%)	64.3 ± 7.1	65.2 ± 6.4	63.1 ± 9.6

Figure 4.3: NMJ morphology is altered in tenotomized supraspinatus at 2D, but recovers at 15D. Neuromuscular junctions (NMJs) of at least three supraspinatus muscles per group were fluorescently stained with an acetylcholine receptor binding neurotoxin (α-Bungarotoxin, BTX, green) and imaged using confocal microscopy. Z-stacked images were analyzed and quantified using ImageJ software. Skeletonized images are shown in the white panel for each NMJ to further illustrate continuity and branching of NMJs. At 2D, NMJs were smaller and morphology was altered, as evidenced by decreased continuity of NMJ branches. No significant differences were seen in NMJ morphology compared to control at 15D. Scale bar represents 10 μm. All data are presented as mean ± SD, $p < 0.05$. *, indicates statistical significance compared to control, and ŧ indicates statistical significance compared to 2D.

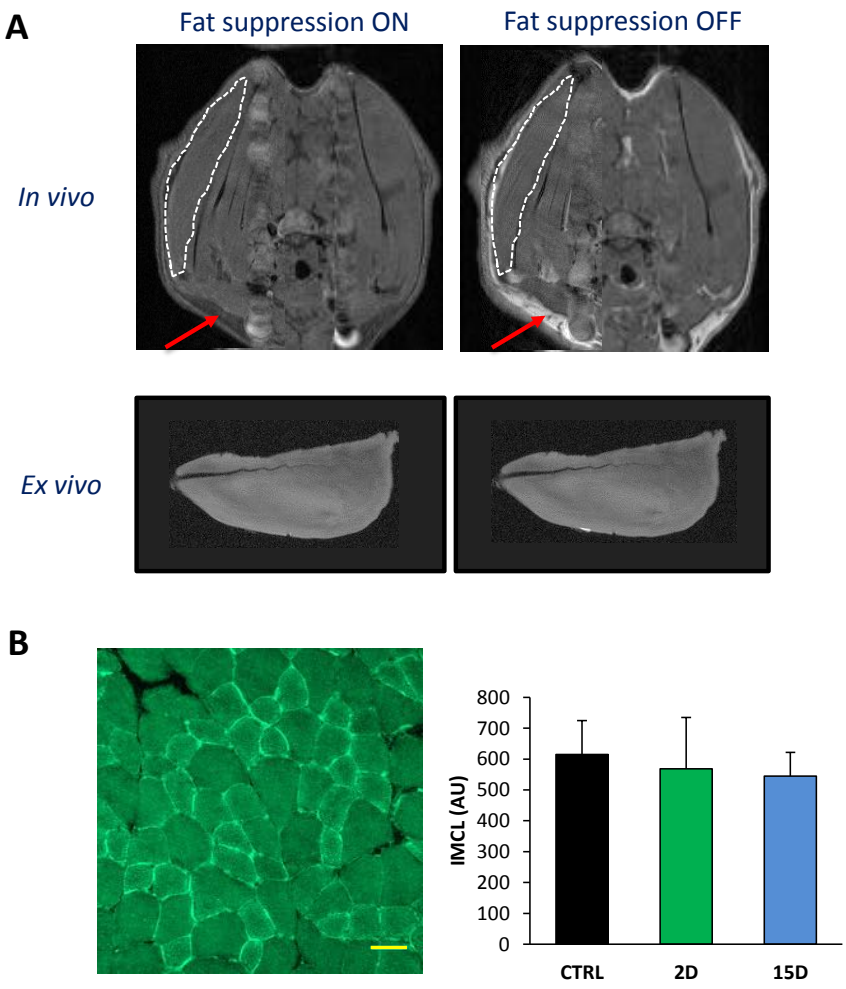


Figure 4.4: Lipid content is not altered in tenotomized supraspinatus at 2D or 15D. A. Axial sections of *in vivo* magnetic resonance imaging (MRI) of a rat 15 days after unilateral RTC tear. When fat suppression is turned off, the white signal represents fat (note the obvious white signal from subcutaneous fat, red arrow). Fat was not detected in the torn supraspinatus (outlined by white dotted line). A longer (> 2 h), more detailed MRI scan of supraspinatus muscles *ex vivo* corroborated this finding. **B**. Neutral lipids were stained in cross-sections of supraspinatus using Bodipy-493/503 and quantified using ImageJ. No differences in intramyocellular lipid at 2D and 15D were found (N = 6). Scale bar represents 50 µm. All data are presented as mean ± SD, $p < 0.05$.

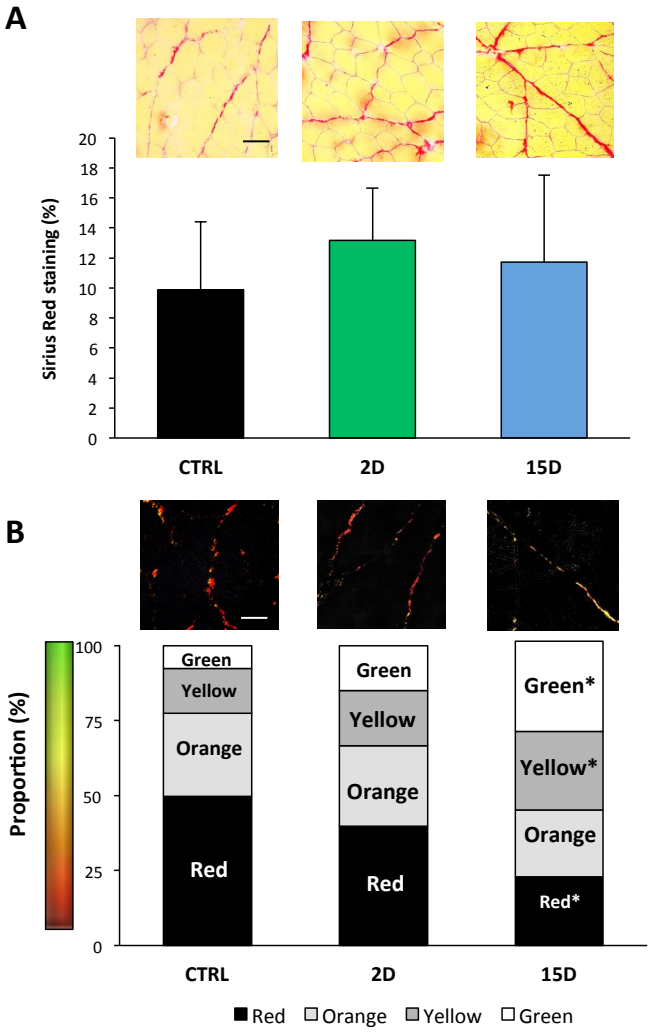


Figure 4.5: Collagen organization is altered in tenotomized supraspinatus at 15D.

A. Representative images of muscle cross-sections stained with Picosirius red viewed under brightfield microscopy. Collagen content was visualized using Picosirius red staining, and quantified using a threshold on ImageJ to calculate the percentage of pixels stained per area. No differences in total collagen content are evident between groups. **B**. Collagen organization was assessed using Picosirius red-stained sections viewed under polarized light. Red represents more densely packed collagen that is perpendicular to muscle fibers, and green represents loosely packed collagen that is parallel to the fibers. When analyzed as a proportion to total colored pixels, supraspinatus muscle at 15D has a lower proportion of red pixels and greater proportion of yellow and green pixels compared to control, indicating altered collagen organization (decreased collagen density, crosslinking, and thickness) at 15D after tear. No differences were evident at 2D compared to control. Scale bar represents 50 μm. All data are presented as mean ± SD, $p < 0.05$. *, indicates statistical significance compared to control.

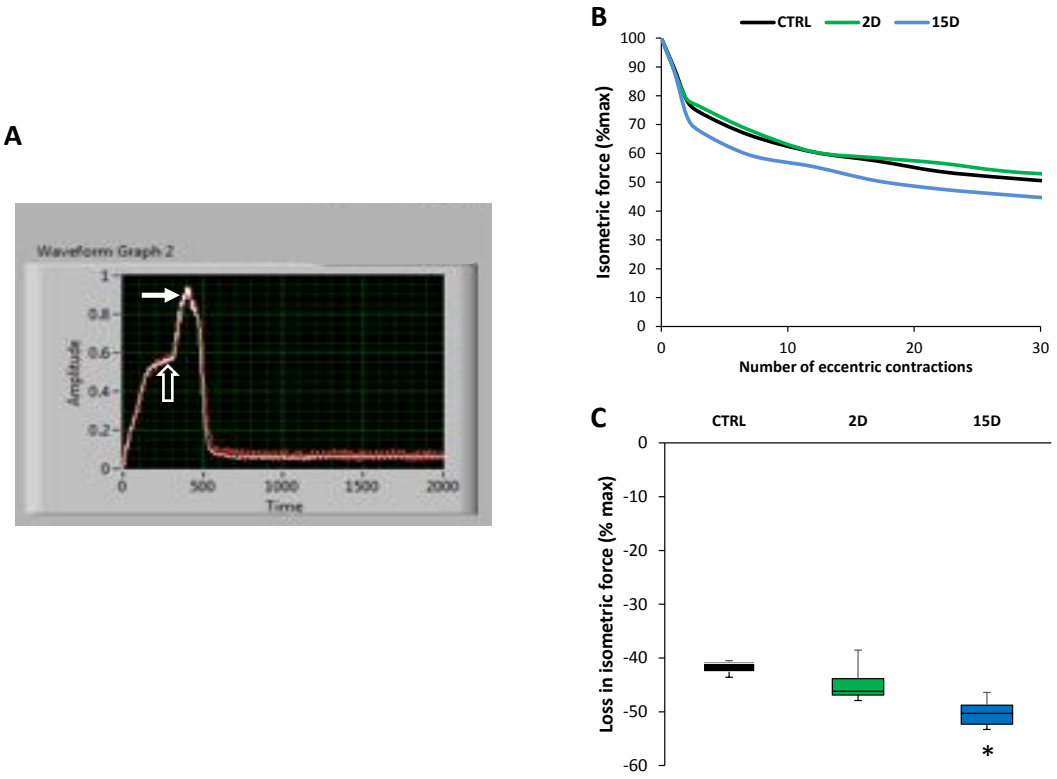


Figure 4.6: Tenotomized supraspinatus becomes more susceptible to injury at 15D but not at 2D.
The same apparatus used to collect isometric force (Figure 2) was also used to induce injury. The suprascapular nerve is used to stimulate the supraspinatus maximally while movement of the lever arm resulted in forced linear lengthening of the muscle (15% L_o). **A.** Representative screen shot showing force from a single eccentric contraction (closed arrow) superimposed onto a maximal isometric contraction (open arrow; y-axis in volts). **B.** Representative rep-by-rep isometric force loss for one animal in each group throughout eccentric contraction protocol. **C.** The mean loss of force for each group after the injury protocol. Despite an identical injury protocol, there is a greater drop in isometric force after injury at 15D (51.8 ± 2.5 %) compared to control. All data are presented as mean ± SD, $p < 0.05$. *, indicates statistical significance compared to control.

Chapter 5: Fatty infiltration as a prognostic marker of muscle function after rotator cuff tear

The following is a manuscript in preparation based on my final dissertation work.

FATTY INFILTRATION AS A PROGNOSTIC MARKER OF MUSCLE FUNCTION AFTER ROTATOR CUFF TEAR

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Running head: Supraspinatus fatty infiltration and contractile function

ABSTRACT

Background: Massive rotator cuff (RTC) tears begin as primary tendon injuries, and cause a myriad of changes in the muscle that influence rehabilitation. However, it is unclear which changes are most closely associated to muscle function. This study used a rabbit model of chronic RTC tear to compare supraspinatus maximal isometric force to histological and radiographic changes.

Methods: Unilateral RTC tears were induced in female rabbits via tenotomy of the supraspinatus and infraspinatus. Maximal isometric force and rate of fatigue were measured in the supraspinatus *in vivo* at 6 and 12 weeks after tenotomy. CT scanning was performed followed by histologic analysis of myofiber size, fatty infiltration (FI), and fibrosis.

Results: Tenotomy resulted in supraspinatus weakness, reduced myofiber size, FI, and fibrosis, but no differences were evident between 6 and 12 weeks after tenotomy except for increased collagen content at 12 weeks. Collagen content was not strongly correlated to maximal isometric force, even when normalized to muscle size. FI was a predictor of supraspinatus weakness, and was also strongly correlated to force after accounting for muscle size. While muscle atrophy accounted for the loss in force in tenotomized muscles with minimal FI, it did not account for the greater loss in force in tenotomized muscles with the most FI.

Conclusion: FI and atrophy have long been associated with poor outcomes after RTC tear. Following RTC tear, the loss in contractile force of supraspinatus results from atrophy, but exacerbated weakness is marked by increased FI. Therefore, the level of FI can predict contractile function of torn RTC muscles.

INTRODUCTION

Tears in the rotator cuff (RTC) affect a third of the population, [23,43,83] and massive RTC tears are the most debilitating and most challenging to treat. [16,25,35] While surgical repair of the tendon(s) is effective to treat less severe forms of RTC tear, [42] surgical repair is often discouraged for patients with massive RTC tears, [18] as the rates of re-tear are high (35-90%),[24,55,86] and shoulder dysfunction persists even in the absence of post-surgical re-tear. [4,11,66] The RTC muscles undergo a variety of changes after RTC tear, including atrophy, fibrosis and fatty infiltration (FI), which are often irreversible and may thus prevent the full recovery of muscle and shoulder function. [15,64] In addition, these muscle changes predict RTC re-tear after repair.[31] Current therapies to improve functional outcomes after massive RTC tears focus on tendon to bone healing, but show limited success. [41,60,80,82] New strategies are focused on decreasing or reversing muscle atrophy, fibrosis, or FI[26,33,67] but it is unclear if these changes contribute equally or disproportionately to changes in RTC muscle function.

The inability to test force of each RTC muscle in isolation in patients[44] and the inaccessibility of the RTC due to its deep position under the deltoid and acromion, present challenges to assessing RTC muscle function even in animals. We previously described a method to test contractile function of the supraspinatus in several animal species commonly used in RTC research. [81] Few studies have associated RTC muscle weakness to a marker of atrophy, specifically the decrease in cross-sectional area of the obtained from CT imaging; [27,52] however, the muscle also undergoes cellular changes beyond a decrease in size. [28,70] Although few studies have shown that torn

RTC muscles have poor function, [17,21,52] no studies have correlated muscle force to histological changes in the muscle, such as fibrosis, myofiber size, intramuscular adipocytes.

In the current study, dual tenotomy was used in New Zealand rabbits to mimic a massive RTC tear. The objective of the study was to determine which, if any, histological markers related to changes in muscle function. Since the rabbit supraspinatus better mimics the human condition following 6 weeks after tenotomy, [61] and changes in the muscle tend to progress over the course of the tear, [62] we assessed the muscle after six and twelve weeks following tenotomy. We tested the hypothesis that no one histological marker would correlate to function over another.

METHODS

Animals: All protocols were approved by the University of Maryland Institutional Animal Care & Use Committee. We used rabbits (New Zealand white, body weight 2.3 ± 0.6 Kg, Charles River Laboratories, Germantown, MD), all approximately 3 months of age. Animals were divided into two groups to serve as controls (intact RTC), or undergo unilateral tenotomy. Supraspinatus contractile testing and histological analysis was performed in control and tenotomized rabbits following 6 and 12 weeks of tenotomy. Contractile testing was performed in eighteen rabbits, from which twelve were also used for histological and correlation analyses. An additional five animals were included in the histological analysis in order to increase the power to detect differences in histological markers between the two time points after tenotomy.

Tenotomy: Surgical procedure was performed under inhalation anesthesia (~3% isoflurane) using a precision vaporizer (Vet Equip,Inc, Pleasanton, CA). The rabbit was shaved, cleaned (alternating scrubs of betadine and 70% alcohol to prevent seeding skin bacteria into the soft tissue) and a small (~2cm) incision is introduced just distal to the acromion process. The deltoid was reflected posteriorly and the supraspinatus and infraspinatus tendons were identified. Tendons were individually transected and the stumps are then sutured using a 5-0 prolene (Ethicon, Somerville, NJ) to a Penrose drain (Grafco, Memphis, TN) in order to identify the tendon in the following weeks for contractile testing.

Contractile function: Contractile function of the isolated supraspinatus muscle was measured as described previously.[81] Before each experiment, the animal was anesthetized (~ 4-5% isoflurane in an induction chamber, then ~ 3% isoflurane via a nosecone for maintenance) using a precision vaporizer (cat # 91103, Vet Equip, Inc, Pleasanton, CA). During the procedure, the animal was kept warm by use of a heat lamp. After proper anesthetic depth was confirmed by lack of a deep tendon reflex (no foot withdrawal in response to pinching the foot or ear), we used a custom-built rig to stabilize the scapula. This entails incising through the middle trapezius and rhomboid muscles in order to access the vertebral border of the scapula to place a clamp along the vertebral border near the infraspinatus fossa for complete immobilization of the scapula. The tendon of the supraspinatus muscle was released and attached to a load cell. The suprascapular nerve was stimulated via subcutaneous needle electrodes (J05 Needle Electrode Needles, 36BTP, Jari Electrode Supply, Gilroy, CA) placed at the suprascapular notch. Proper electrode position was

determined by a series of isometric twitches. Impulses 1 ms in duration were generated by an S48 square pulse stimulator (Grass Instruments, West Warwick, RI) and passed through a PSIU6 stimulator isolation unit (Grass Instruments, West Warwick, RI).

Single twitches (rectangular pulse, 1 ms) were applied at different muscle lengths to determine the optimal length (resting length, L_0). At L_0, maximally fused tetanic contraction was obtained at ~100 Hz (300-ms train duration of 1-ms pulses at a constant current of 5 mA). We used 150% of the maximal stimulation intensity to induce maximal activation of contraction, P_0. We also generated a force-frequency plot, obtained by progressively increasing the frequency of pulses during a 200 ms pulse train. To provide an index of fatigue, maximal tetanic contractions were performed repeatedly (every 2 s) with the final tension expressed as percentage of P_0. Although contractile testing is performed *in vivo*, the animal was euthanized through cardiac pucture after the procedure, and the shoulder girdles were harvested.

Micro CT imaging: After euthanasia, the shoulder girdle of twelve animals were harvested to undergo micro-computed tomography (μCT) scanning (Bruker Skyscan 1172, Madison, WI), which provides a higher resolution than a larger clinical scanner. The Y-shape view obtained from the CT sagittal oblique imaging plane was analyzed using the Medical Image Processing, Analysis and Visualization Program (MIPAV, National Institutes of Health) software to quantify the cross sectional area (CSA, cm^2) and density (Hounsfield units, HU) of the supraspinatus muscle. Measurements were performed in a blinded fashion by a licensed, board certified radiologist.

Fatty infiltration: Fixed supraspinatus muscles (4% paraformaldehyde) were used to make paraffin embedded cross-sections along the muscle belly. Sections were stained with hematoxylin and eosin (H&E) and viewed under a brightfield microscope (Nikon eclipse 50i). A sequence of ~300 adjacent images was captured using high-resolution color camera (Nikon DS-Fi2) using a 20x objective. The images were electronically stitched together to create a high magnification mosaic of the entire cross-section (NIS Elements Viewer). Stitched images were then analyzed on ImageJ to assess FI. The image was converted to an 8-bit grayscale, and the total stained area ($mean_{TSA}$) was selected using thresholds to obtained the mean (0-255). The area covered with adipocytes (unstained), was manually identified to calculate the mean for the total stained area plus adipocytes ($mean_{TSA+A}$). Percent area covered by adipocytes was calculated by dividing the $mean_{TSA+A}$ / $mean_{TSA}$ multiplied by 100.

Immunolabeling: Paraffin embedded sections were deparaffinized through 1-hour heating period (65ºC) followed by a 10-minute incubation in boiling sodium citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) and xylene washes. The sections were rehydrated in decreasing ethanol solutions and washed in PBS buffer. Sections were incubated overnight at 4º C in a 1:50 dilution of perilipin 1 (Plin1) antibody (kindly provided by Dr. Carole Sztalryd, University of Maryland, Baltimore). Sections were then incubated in a 1:100 dilution with secondary antibodies conjugated with Alexa-488 (Invitrogen, CA), and visualized using a fluorescent microscope (Nikon eclipse 50i).

Feret diameter: Stitched images taken at 20x magnification of H&E stained sections were also used to calculate myofiber size. Myofibers (100 per sample) were

selected at random across the whole section for each muscle, and manually outlined to calculate minimal Feret diameter using ImageJ. The minimal Feret diameter is the minimum distance between parallel tangents at opposing borders of the myofiber, and this measurement is "insensitive to deviations from the optimal cross-sectioning profile". [7] The mean Feret diameter per group was calculated, as well as the mean distribution of fibers according to their diameter.

Collagen content: Paraffin embedded sections were deparaffinized in xylene, rehydrated, and stained for 1 hour with Sirius red (0.1% Direct Red saturated in aqueous picric acid, Sigma), and rinsed with acidified water (5% acetic acid). Samples were mounted and imaged under brightfield microscopy followed by polarized light microscopy (Nikon). A sequence of adjacent images of stained sections was captured in a similar fashion as done for H&E images. Pictures were taken under the same conditions and exposure time. Stitched images were then analyzed on ImageJ. To assess the area of collagen of the entire section, the background was subtracted and thresholds were used to determine the total area of the section ($mean_{TSA}$). Another threshold was used to only include the area stained red (collagen) ($mean_{col}$). To calculate percent area stained with Sirius red, the $mean_{col}$ was divided by $mean_{TSA}$ and multiplied by 100.

Collagen organization: Collagen birefringence was analyzed as previously described.[57] Briefly, using images taken at magnification of 20x, we determined the number of pixels with 8-bit hue thresholds for red, orange, yellow, and green using ImageJ. The proportion of each hue was calculated by dividing the pixels for each hue to the sum of total colored pixels.

Statistical analysis: Normality and homogeneity of variance were verified for all data before analysis (SigmaStat, San Rafael, CA). To evaluate potential differences between the three groups a One-way ANOVA was used followed by a post-hoc Holm-Sidak test when ANOVA was significant. Significance was set at $p < 0.05$ and data are represented as mean ± standard deviation. Normality was not met for the histological assessment of %FI, as values for control muscles were close to the zero limit, skewing the data to the left. A Kruskal-Wallis test was used to compare %FI between the three groups, followed by a post-hoc Dunn's test when the Kruskal-Wallis was significant to determine differences in %FI between groups. Pearson correlation coefficients were calculated in the subset of muscles that underwent histological analysis and contractile testing to determine the association between maximal isometric force and histological markers. Histological markers were also entered as independent variables in a step-wise multiple linear regression analysis with maximal isometric force set as the dependent variable.

RESULTS

There was a 40-45% reduction in maximal contractile force in the supraspinatus muscle after 6 weeks ($P = 0.004$) and 12 weeks ($P = 0.003$) of tenotomy compared to control (16.1 N ± 1.7) (Figure 5.1A). Although lower than controls, maximal force was not different between 6 and 12 weeks (9.6 N ± 3.2 vs 8.8 N ± 5.7, $P = 0.713$). The amount of muscle fatigue, evidenced by a loss in force after fatigue protocol, was not different between any of the groups (-45.4% ± 4.7; $P = 0.867$; Figure 5.1B).

Histological analysis of the supraspinatus at 6 and 12 weeks after tenotomy showed FI, evidenced by the presence of intramuscular adipocytes (Figure 5.2A). We confirmed the identification of adipocytes by immunolabeling for perilipin 1, an adipocyte-specific lipid droplet-coating protein (Figure 5.2A, green). Adipocytes covered 7% ± 6 and 12% ± 7 of the tissue sections stained with H&E of supraspinatus muscle after 6 and 12 weeks of tenotomy respectively, and negligible in the control (0.3% ± 0.2) (Figure 5.2B, bar graph). No differences in adipocyte content were evident between 6 and 12 weeks. We also found the greatest concentration of adipocytes (79% ± 15) in perifascicular spaces of the muscle, specifically closer to blood vessels, and a smaller proportion of adipocytes (18% ± 14) in the intrafascicular space in tenotomized muscles (Figure 2B, bottom).

The distribution of myofiber size showed a shift to the left (i.e., toward smaller muscle fiber sizes) at both time points after tenotomy (Figure 5.3A). The mean myofiber diameter was 22% lower after 6 weeks and 24% lower after 12 weeks compared to control (116 ± 13 μm; $P = 0.0005$; Figure 5.3B), with no differences evident between 6 and 12 weeks ($P = 0.671$).

Fibrosis was evident only at 12 weeks after dual tenotomy by an increased proportion of area covered by collagen using Sirius red staining ($P = 0.004$; Figure 5.4A). Collagen covered 18% ± 7 of total tissue area at 12 weeks after tenotomy, which was significantly higher than control (6% ± 4). Collagen packing density evidenced by collagen birefringence was similar in all three groups ($P = 0.85$; Figure 5.4B).

The histological markers for FI, fibrosis, and myofiber size were all correlated to maximal tetanic force (Figure 5.5A). However, the marker for FI (% area covered by adipocytes) had the strongest correlation to muscle force (R = 0.819, $P = 0.001$), and was also the only predicting factor of muscle force when the three histological markers where incorporated to a stepwise multiple linear regression model ($P = 0.016$; Figure 5.5B). To better illustrate the characteristics of supraspinatus muscles based force generated, muscles were divided into three cohorts. Muscles with force values in the top third (17 N ± 1.2) were grouped into the *strongest* cohort, muscles with force values in the middle third (12.3 N ± 1.8) were grouped into the *weak* cohort, and muscles with force values in the bottom third (5.9 N ± 1.4) were grouped in to the *weakest* cohort (Figure 5.5C). The strongest cohort consisted of only intact control muscles, while the weak and weakest groups included tenotomized muscles of both 6 and 12 week time points. Compared to the strongest cohort, weaker muscles had FI (3.3 % ± 2.3), but the percent area covered by myofibers (muscle) was not significantly different (85.8 % ± 3.6, $P = 0.115$). The weakest muscles had the most FI (13.5 % ± $P = 0.033$) and a decreased percent area covered by myofibers (71.8 % ± 9.5, $P = 0.001$ vs strongest and $P = 0.010$ vs weak).

Cross sectional area (CSA) of the supraspinatus muscle belly was measured using μCT (Figure 5.6A, top). CSA area was lower than control (1.16 cm^2 ± 0.31) at 6 weeks (0.567 cm^2 ± 0.13, $P = 0.004$) and 12 weeks (0.757 cm^2 ± 0.19, $P = 0.025$) after tenotomy, but not different between the two time points after tenotomy ($P = 0.2$, Figure 5.6A, bottom). The mean CSA for each group was used to estimate the total area of collagen, fat, and muscle in the supraspinatus muscle, by multiplying it to the

proportions of collagen fat, and muscle previously calculated through histology (see Supplemental). There was a decrease in total muscle area at both time points after tenotomy. When we correlated maximal isometric force to the total amount of collagen, fat, and muscle (Figure 5.6B), we found a strong positive correlation between force and the total area of muscle (R = 0.791, P = 0.002), a strong negative correlation between force and total area of fat (R = 0.772, P = 0.003), but no significant correlation with the total area of collagen (R = 0.256, P = 0.422).

Since the duration of the tear did not have a significant effect on force, size, or composition of the muscle, we wanted to further examine the characteristic of the muscle according to their maximal force generated by dividing them into the three cohorts based on force generated (Figure 5.6C). Compared to the strongest cohort, the weak cohort had a smaller total area of muscle (0.53 cm^2 ± 0.07, P = 0.0003) that accounted for the difference in muscle force, but the muscle area in the weakest cohort was not significantly different from the weak cohort (0.44 cm^2 ± 0.03, P = 0.284) despite a significant reduction in force (P = 0.0002). The degree of FI was not different between the strongest and weak cohorts (P = 0.492). In contrast, the weakest group had a significant level of FI (0.09 cm^2 ± 0.05, P = 0.0216 vs. weak and P = 0.007 vs. strongest).

Based on our histological analysis, FI is strongly correlated to force, so we assessed FI of tenotomized muscles through CT by measuring muscle density in Hounsfield units. Figure 5.7A shows representative images of CT scans of the supraspinatus, and darker areas (arrow) are hypodensities, indicative of FI. Corresponding histological sections (inset) show the degree of FI in each

corresponding muscle. Muscle density was lower in tenotomized supraspinatus muscles compared to control (Figure 5.7B, $P = 0.002$ vs. 6W and $P < .001$ vs. 12W) with no differences between the two time points after tenotomy. Because we found the degree of FI in the muscle to be strongly correlated to muscle force, we verified whether measurements from CT could also be used to predict force of supraspinatus muscles in the muscles (control and tenotomized) that underwent both CT and contractile testing. We found for FI to be strongly correlated to force after accounting for muscle size (productof of CSA and HU) (R = 0.817, $P = 0.025$, Figure 5.7C).

DISCUSSION

The supraspinatus muscle undergoes a variety of changes such as atrophy, FI, and fibrosis after RTC tear[19,25,28,31,70,75] that may impair functional recovery; however, it is unclear if these changes contribute equally or disproportionately to changes in RTC muscle function. Our findings show that supraspinatus atrophy and FI are strongly correlated to its loss in contractile force after RTC tear, but the weaker tenotomized muscles were not characterized by exacerbated atrophy, but rather by marked FI.

The influence of RTC muscle weakness on shoulder dysfunction has been documented.[37,72] The loss of force of RTC muscles destabilizes the glenohumeral joint, 37 and results in unwanted contact between the humerus and acromion, increasing the likelihood of damage to soft tissues.[71] Therefore, improvement in shoulder function after RTC tear not only depends on the reattachment of the tendon, but also the ability of the repaired muscle to generate force.[53] In addition, the forces

generated by RTC muscles may also provide mechanical load necessary for optimal tendon healing after repair.[2,38] Functional recovery after RTC tear therefore depends on the restitution of normal muscle structure and function[53].

Clinicians make decisions for treatment based on the patient's size of the tendon tear, level of pain, age, as well as the degree of atrophy and FI in the muscle, but there is a "lack of clinical agreement about RTC surgery."[18] Some studies suggest that muscle atrophy can improve after repair,[20,79] but FI does not.[31-34] While the amount of atrophy is generally used to estimate the contractile capacity of the muscle,[53] atrophied muscles have varying degrees of FI. Therefore, based on findings from Study 3, an atrophied muscle with a high degree of FI is likely to be weak, and therefore less likely to improve glenohumeral function after repair. Conversely, a muscle with atrophy but little FI is more likely to be stronger than a muscle with significant FI, and therefore more likely to improve glenohumeral function after repair, especially if muscle mass improves after repair and rehabilitation. These concepts can help guide clinicians in the surgical and rehabilitation planning after RTC tear. Furthermore, defining the molecular pathways that lead to FI would provide an attractive target for future therapeutic interventions.

Our findings support various studies that have shown increased FI, fibrosis, and decrease in myofiber size in tenotomized RTC muscles,[40,62,63,76,78] but this is the first study to associate these factors to muscle force, and it is the first study to provide a histological marker for contractile function. We determined the influence of fibrosis, FI, and muscle atrophy on contractile force by accounting for changes in muscle size after RTC tear. While muscle atrophy accounted for the loss in force after

tenotomy, the weakest tenotomized muscles were also characterized by significant FI but not a significant degree of atrophy compared to the stronger torn muscles. Only a handful of studies have assessed contractile force of the supraspinatus,[6,17,21,22,50] and two of those also measured muscle density,[27,52] indicative of FI. These reports are consistent with our findings, as a greater degree of FI was evident in the weakest muscles after RTC tear.

We also found that fibrosis of the supraspinatus was not a strong influence on muscle force. This was unanticipated, as ECM contributes to skeletal muscle structure, mechanical properties,[29,47] transduction of mechanical force, and muscle remodeling[57]. Gradual deposition of ECM proteins in the muscle is common in muscle pathology,[47,68] and can increase up to 10-fold in injured muscle[47]. The role of fibrosis on RTC tears is still unclear, as fibrosis is evident in some studies using animal models of RTC tear,[32,48] but not in others,[63,70] but ECM may alter muscle stiffness which increases the repair tension necessary to reattach the muscle back to the humeral head[30] which may contribute to myofiber injury seen after repair.[13]

In healthy muscle, muscle mass is the strongest predictor of muscle force, but atrophy did not account for the significant weakness seen in muscles with the most FI. Myofibers from torn muscles have lower force generating capacity[32,51] that may be a cause of consequence of the development of FI after RTC tear. Myofiber degeneration can promote the differentiation of muscle resident stem cells into adipocytes[36,46] by reducing myogenesis by blocking Rho-signaling pathway.[8,36] The loss of muscle also reduces the secretion of myokines that have shown to have anti-inflammatory properties,[69] and enhance lipid breakdown and oxidation in

adipocytes.[65] On the other hand, the close proximity of adipocytes and myofibers in muscles with FI can also impair muscle function, as adipocytes can reduce the expression of contractile and structural proteins,[58] promote myofiber atrophy, and disrupt muscle regeneration.[58,73,10] Adipocytes secrete fatty acids,[46] adipokines,[74] cytokines and chemokines like IL-1β58 that promote inflammation and[58] induce oxidative stress,[9] which can further reduce force generating capacity of myofibers[45,1] by impairing NMJ activation,[39] excitation-contraction (EC) coupling,[3] and actin-myosin interactions.[12,56] In addition, intramuscular adipocytes can decrease the force generating capacity of muscle by altering its biomechanical properties.[59]

There are currently no effective treatments used to prevent or reduce FI after RTC tear in patients. In order to reduce FI, we need to better understand the source of adipocytes within the muscle. We found for a greater concentration of adipocytes in the perifascicular space, specifically near vascular walls, which has also been documented in RTC muscles from patients with RTC tears.[28] The significance of finding adipocytes close to vascular walls is that a number of muscle progenitor cells (i.e. fibro-adipogenic cells, pericytes, myogenic endothelial cells) reside in the same location, and contribute to muscle development and regeneration,[14,54,85] but in some cases, can also differentiate into intramuscular adipocytes.[5,84] While some have suggested for the source of FI to be muscle resident progenitor cells[49], there are a variety of progenitor cells that have not been explored in RTC injury.[14,54,84,85]

One of the limitations of our study was the time points chosen to detect a progression in FI, atrophy, and contractile force after tenotomy, were not spaced apart enough. FI tends to progress over time in the rabbit model of RTC tear,[62,79] but

differences are not always be detected in a time frame of 6 weeks or less.[75,77] Given our findings, neither FI nor contractile force worsened between 6 and 12 weeks, but since FI can progress over a longer period of time, muscle force may diminish within that time course as well. Another limitation was the small number of animals who underwent both CT scanning and contractile testing in this study. Although we found strong evidence for FI to be a predictor of force using histology, more studies are necessary to confirm that FI measured by CT is also a reliable predictor of force.

The main finding of this study was that the percent area covered by adipocytes in a histological section of the supraspinatus muscle is a strong indicator of muscle weakness. FI has long been associated with poor outcomes after RTC tear, and atrophied muscles with significant FI are significantly weaker than those with atrophy alone. Therefore, a patient with RTC muscle atrophy who also exhibits FI after RTC tear is likely to have worse outcomes after repair due to poor muscle function, compared to a patient with muscle atrophy with minimal FI. While FI is currently considered irreversible, therapies that can inhibit or even stop FI have the potential to significantly improve muscle function after RTC tear.

FIGURES

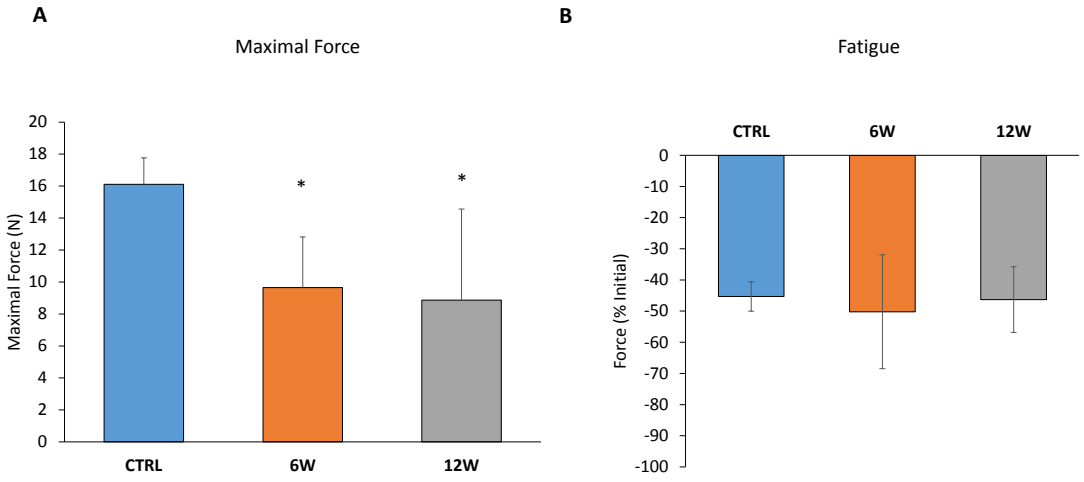
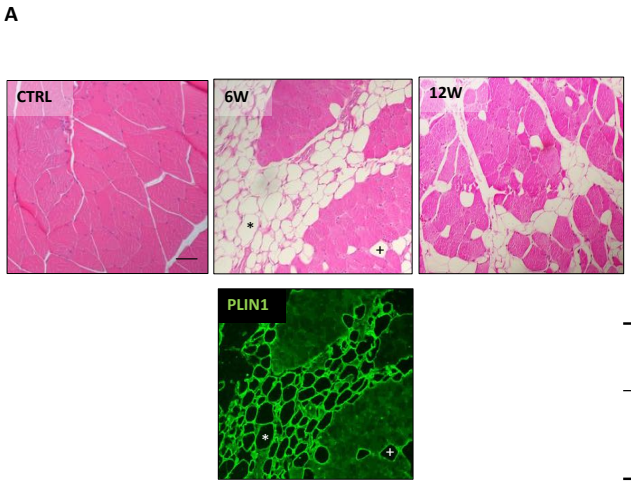


Figure 5.1: Maximal isometric force is lower at 6 and 12 weeks after RTC tear. **A:** Maximal tetanic isometric force (200-ms duration) of the supraspinatus was measured in vivo after setting the muscle at optimal length and stimulating the suprascapular nerve at incrementing frequencies (Hz). When compared to control, the mean maximal isometric force of the supraspinatus muscle was 40% lower at 6 and 12 weeks after tenotomy, with no differences between the two time points. **B:** The muscle was then stimulated with a 200-ms tetanic contraction every other second for 2 min to induce fatigue. The percent loss of force was not different between the three groups. All values are expressed as mean ± SD, * $P < 0.05$ compared to control. N = 5-7 per group.



Location of adipocytes (% adipose area)	6W	12W
PERIFASCICULAR (*)	75.5 ± 17.4	83.1 ± 11.4
INTRAFASCICULAR (+)	19.0 ± 17.0	16.9 ± 11.4

Figure 5.2: Histological assessment of FI after RTC tear.
A: Paraffin embedded section of the supraspinatus muscles were stained with H&E to quantify the percent area covered by adipocytes in the whole section (~300 stitched fields of view). Representative images show specific fields of view to note the FI in the muscle (viewed under 20x magnification). The presence of adipocytes in the perifascicular (*) and intrafascicular space (+) (6W image) was confirmed by labeling adipocyte-specific protein Perilipin 1 (Plin1 image) in serial sections. **B:** *Top:* Adipocytes covered an average of 9% of the total area of the cross-section (% FI), and %FI was not different between the two time points after tenotomy. *Bottom:* About 75% of the adipocytes were in the perifascicular space that surrounds muscle bundles and the remaining adipocytes were found in the intrafascicular space, within muscle bundles. Scale bar represents 100 µm. All values are expressed as mean ± SD, * = $P < 0.05$ compared to control, N = 5-7 per group.

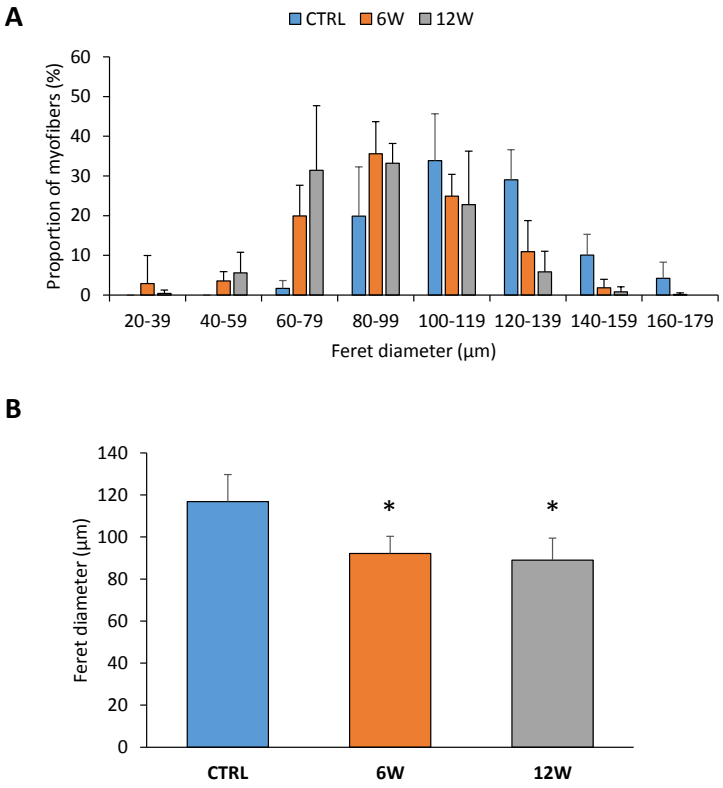


Figure 5.3: Histological assessment of myofiber size after RTC tear.
A: Minimal Feret diameter of 100 fibers per muscle was measured using ImageJ. Myofibers were binned according to their diameter to determine heterogeneity and shift in myofiber size. There was a larger proportion of smaller myofibers at both time points after tenotomy. **B:** The mean diameter was 22% lower in tenotomized muscles compared to control, and no differences were detected between the two time points. All values are expressed as mean ± SD, * = $P < 0.05$ compared to control, N = 5-7 per group.

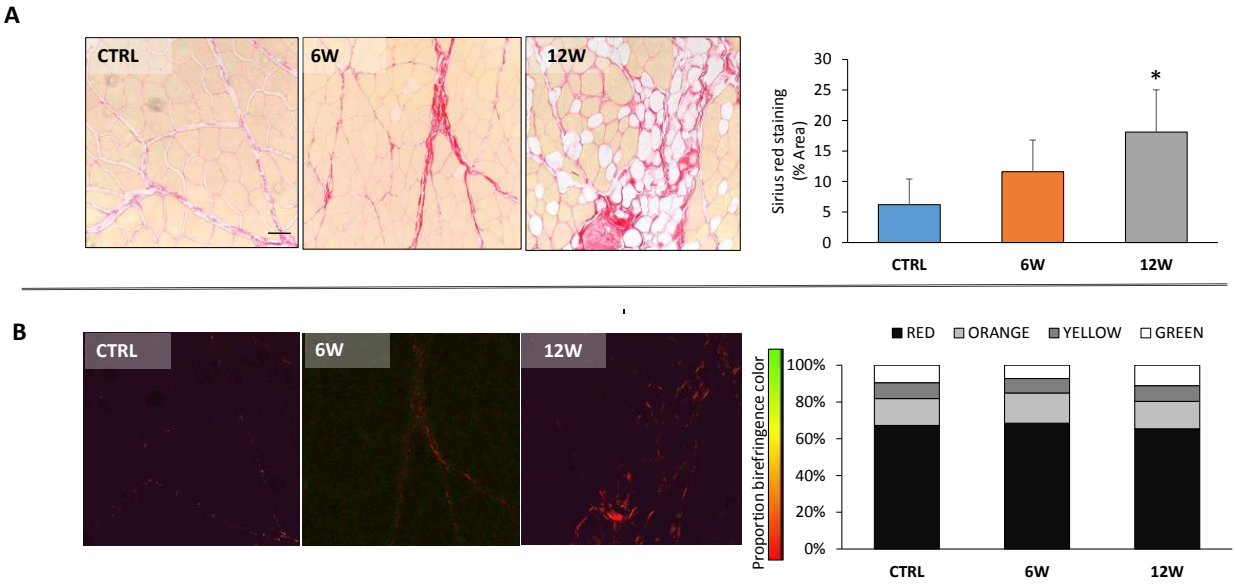
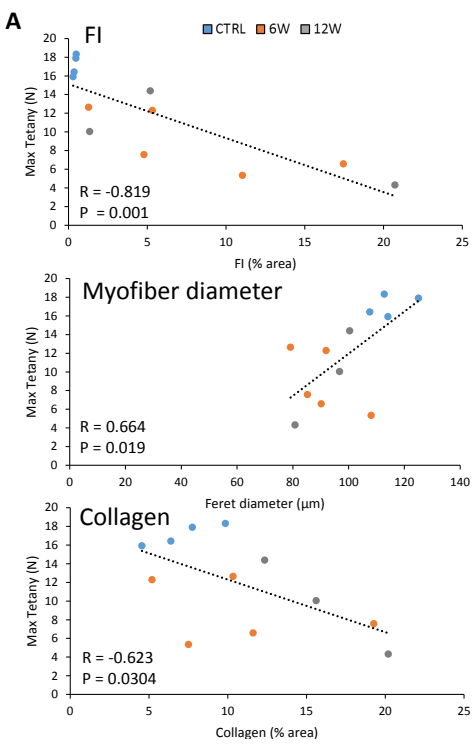


Figure 5.4: Histological assessment of fibrosis after RTC tear.
A. Collagen content was visualized using Picosirius red staining, and quantified using a threshold on ImageJ to calculate the percentage of pixels stained in the whole section (~300 fields of view). Representative images show specific fields of view (20x) showing collagen staining. Collagen covered 6% of the tissue area in control muscles, and 18% in muscles after 12 weeks of tenotomy. No significant differences were found at 6W.
B. Collagen organization was assessed using Picosirius red images viewed under polarized light. Red represents more densely packed collagen that is perpendicular to muscle fibers, and green represents loosely packed collagen that is parallel to the fibers. There were no differences in the proportion or red, orange, yellow, or green pixels between groups. Scale bar represents 100 μm. All data are presented as mean ± SD. * = $P < 0.05$ compared to control, N = 5-7 per group.



B

Predictor	β Coefficient	SE	P - value
FI (% area)	-0.607	13.98	**0.015**
Collagen (% area)	-0.177	19.91	**0.438**
Myofiber diameter (µm)	0.258	0.075	**0.272**

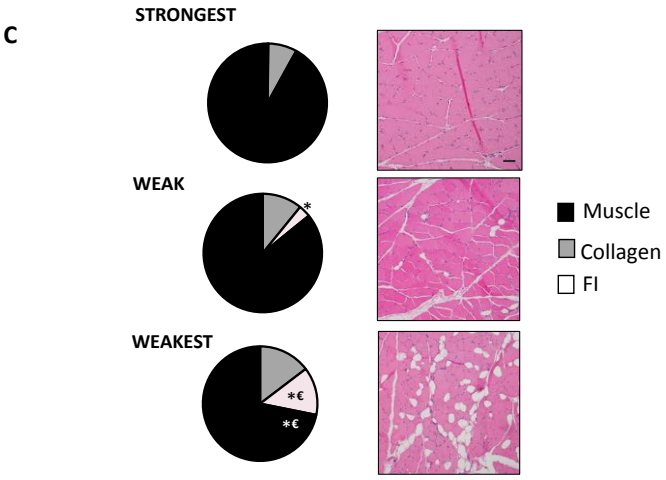


Figure 5.5: Correlation of histological markers for FI, myofiber size, and fibrosis to contractile function of supraspinatus muscle.
A: Pearson correlation analysis to determine the association between maximal isometric force and histological markers shows a significant positive correlation with degree of intramuscular fat, a moderate negative correlation with collagen, and moderate positive correlation with myofiber size (N = 12). **B:** Step-wise multiple linear regression analysis was performed using maximal force as dependent variable, and each histological marker as independent variables. The histological marker for FI was the strongest predictor of muscle function. **C:** To better illustrate the histological characteristics of muscle according to maximal force generated, the muscles in the top third (strongest) were compared, to those on the middle third (weak), and to the lowest third (weakest). *Left:* A pie chart for each group illustrates the mean proportions of FI, collagen, and muscle in a histological section (N = 4 per group). Compared to the strongest cohort, weaker muscles had FI. The weakest muscles had the most FI and a smaller proportion of muscle. *Right*: Representative H&E images (10x) for each group. Scale bar represents 100 µm. *R* represent the correlation coefficient. * = $P < 0.05$ compared to control. € = $P < 0.05$ compared to weak.

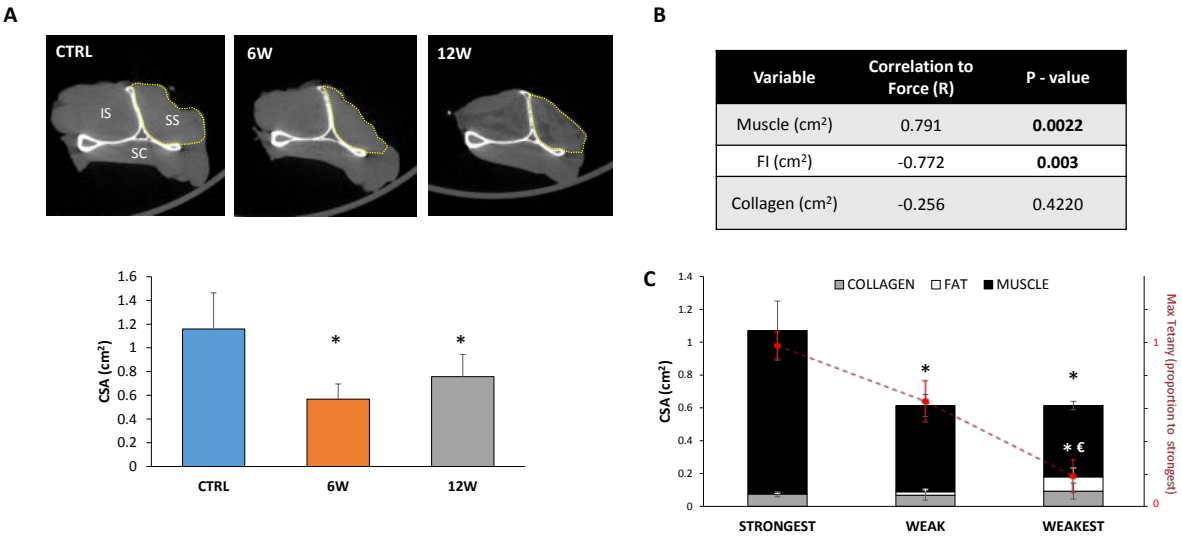
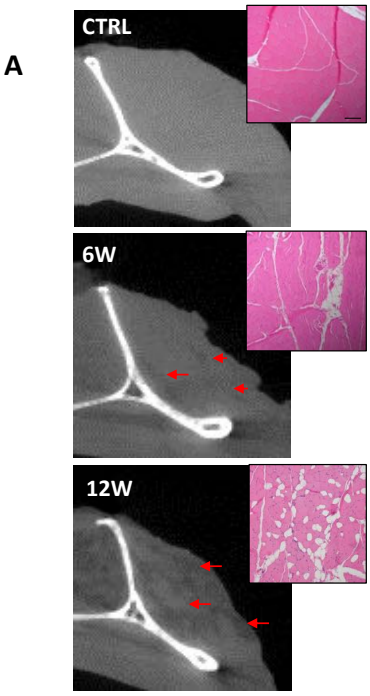
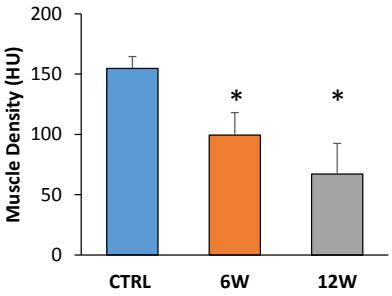


Figure 5.6: Cross-sectional area of the supraspinatus is reduced after RTC tear, and the amount of FI in the muscle is greatest in the weakest muscles.
A: Representative sagittal CT scans of rabbit RTC of control and 6 weeks after tenotomy. The supraspinatus cross-sectional area (CSA) (outlined) was quantified for each group and it was lower in animals with RTC tenotomy lasting 6 and 12 weeks (graph) (N= 3-5 per group). **B:** The mean CSA for each group was used to estimate the total area of collagen, fat, and muscle by multiplying it to the proportions previously calculated through histology. Pearson correlation analysis shows a significant positive correlation with total area of muscle and muscle force, a significant negative correlation with total area of FI and muscle force, and no significant correlation with total area of collagen and muscle force. **C:** The mean total area of collagen (gray), FI (white), and muscle (black) was calculated for the strongest, weak, and weakest muscles. The red line indicates the mean force in each cohort as the proportion to the strongest cohort. Compared to the strongest cohort, weak muscles had a smaller total area of muscle that accounted for the decrease in force; however, the weakest cohort did not have a significant decrease in muscle area to account for its loss in force, and was characterized by significant FI (N = 4 per group). All values are expressed as mean ± SD *R* represent the correlation coefficient. * = $P < 0.05$ compared to control (A) or strongest (C). € = $P < 0.05$ compared to weak.

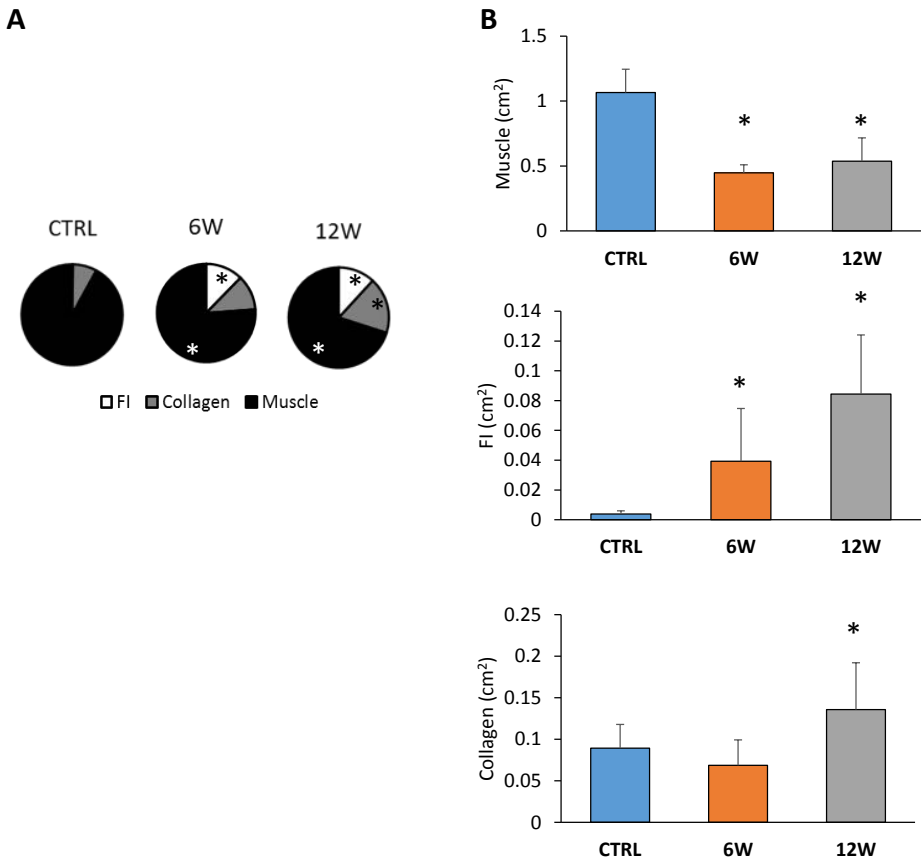




C

Variable	Correlation to Force (R)	P - value
CSA*HU	0.817	**0.025**
HU	0.715	0.0707
CSA	0.585	0.167

Figure 5.7: **FI measured through CT is strongly correlated maximal force after accounting for changes in CSA.**
A: Representative images of CT scans for control and tenotomized groups with their corresponding H&E image (10x). Arrows point to hypodensities, which are indicative of FI. **B.** The mean muscle density (HU) was lower in tenotomized supraspinatus muscles (N = 3-5 per group). CT scans were also performed in seven muscles that underwent contractile testing. **B.** Pearson correlation of muscle density and CSA to supraspinatus force indicates a strong positive correlation between the product of CSA and muscle density. Scale bar represents 200 µm. All data are presented as mean ± SD. R represents the correlation coefficient.* = $P < 0.05$ compared to control.



Supplemental figure: Estimation of total area of muscle, FI, and collagen in the supraspinatus of the control and tenotomized groups.
A. Pie charts representing the percentage of muscle, FI, and collagen measured through histology in each group. **B.** The total area for each tissue (cm^2) was estimated by multiplying the mean CSA of each group (CTRL, 6W, 12W) measured through CT, to the proportions of muscle, FI, and collagen measured through histology for every supraspinatus sample in each group (N = 5-7). All data are presented as mean ± SD.* = $P < 0.05$ compared to control.

Chapter 6: Summary, Limitations, and Future Directions

SUMMARY

Rotator cuff (RTC) tears, particularly massive tears, are debilitating and pose a great challenge for health professionals to treat.[15,16,21] Since the initial problem is the tearing of the tendon, most of the efforts have focused on tendon-to-bone healing;[37,50,59,62] however, no treatments are currently effective in restoring shoulder function following massive RTC tears.[6,10,22] Forces generated by the RTC muscles are necessary to stabilize and rotate the glenohumeral joint, but RTC tears induce irreversible, degenerative changes to the muscle (i.e. atrophy, fatty infiltration, and fibrosis) that may prevent the ability of the muscle to generate its expected force and hinder the recovery of shoulder function.[6,20,44] Therefore, targeting the muscle itself is therapeutically necessary to improve outcomes after RTC repair, but only a handful of studies have been able to assess contractile function of RTC muscles, and little is known about which markers may be able predict changes in function after RTC tear.

The studies presented in this dissertation provide new insight into functional changes of the supraspinatus muscle using *in vivo* models of massive RTC tear. RTC tears typically originate in the supraspinatus tendon and become larger with time, affecting additional RTC tendons resulting in what is termed a massive RTC tear.[41,63] Testing contractile function of RTC muscles presents unique challenges due to the depth of the supraspinatus tendon and an overlying acromion. Therefore, the purpose of **specific aim 1** was to develop a method to test supraspinatus contractile function,

and provide normative values of supraspinatus muscle force and mass in the most commonly used research models: the mouse, rat, and rabbit. Specific aim 1 was addressed in study #1 and was the first report of whole muscle contractile measurements for supraspinatus in mice, and contributed data to the field by complementing the few already existing studies for rats and rabbits (see chapter 3).

The methods developed in study # 1 were then used to address **specific aim 2**, to identify the progression of *in vivo* changes in contractile function of supraspinatus muscle in the rat (study #2) and rabbit (study #3) models of RTC tear. Supraspinatus contractile force was lower in animals with a RTC tear compared to control, but was not influenced by the duration of the tear, contrary to what I hypothesized. However, supraspinatus force was associated to histological markers in the muscle that were identified as part of **specific aim 3** in both the rat and rabbit models of RTC tear.

Study #2 employed the rat model of RTC tear. The most salient findings of this study were the apparent dissociations between muscle mass and muscle force soon after a RTC tear, as well increased susceptibility to contraction-induced injury in the torn supraspinatus muscle. The initial drop in force after RTC tear was associated with a decrease in neuromuscular junction (NMJ) size and continuity, while the increased susceptibility to injury was associated with altered collagen organization in the muscle. Knowing when a torn RTC muscle is weak and also most susceptible to injury could be useful in surgical and rehabilitation planning, but additional work would be needed to elucidate the specific timing and significance of these findings in patients with RTC tear.

The rabbit is often considered the most suitable model to assess chronic changes in the muscle after a RTC tear, as it accurately mimics the clinical condition by evidence of atrophy, fibrosis, and FI in the supraspinatus muscle after tenotomy.[52,57] The rabbit model of RTC tear was employed in study #3. The main finding of this study was that the percent area covered by FI (specifically, intramuscular adipocytes) in a histological section of the supraspinatus was a strong indicator of muscle weakness (i.e. maximal isometric force), and a better indicator than myofiber size or fibrosis. While the decrease in force generation after tenotomy was partially due to atrophy (total decrease in muscle tissue), supraspinatus muscles with the same degree of atrophy had variations in force that were associated with the degree of FI. The descriptive findings of this study warrant further investigations to determine whether FI exacerbates muscle weakness in atrophied muscles after RTC tear. Moreover, this finding could markedly impact diagnosis, treatment and prognosis for patients after RTC tears, as FI can be measured non-invasively using imaging modalities such as Magnetic Resonance Imaging (MRI) and Computed Tomography (CT).

Clinicians make decisions for treatment based on a variety of factors (i.e. size of the tendon tear, level of pain, patient's age, level of atrophy and FI in the muscle), but there is a lack of clinical agreement about which factors to consider to refer a patient to RTC surgery.[16] Improvements in shoulder function after RTC tear not only depend on the reattachment of the tendon, but also the ability of the repaired muscle to generate its expected force.[6,20,44] While muscle atrophy can improve after repair,[18,58] FI does not decrease after repair.[22,30] The amount of atrophy, measured using MRI or CT, is generally used to estimate the contractile capacity of the muscle after

RTC tear,[44] but atrophied muscles have varying degrees of FI. Based on findings from study #2, a torn supraspinatus muscle can be weak in the absence of atrophy and FI, and may also be susceptible to contraction-induced injury, which may hinder the recovery of shoulder function, if not taken into consideration in rehabilitation planning. Additionally, based on findings from study #3, an atrophied muscle with a high degree of FI is likely to be exceedingly weak, and therefore less likely to improve glenohumeral function after repair compared to an atrophied muscle with minimal FI. These concepts can help guide clinicians in the surgical and rehabilitation planning after RTC tear. Furthermore, defining the molecular pathways that lead to susceptibility to injury and FI would provide an attractive target for future therapeutic interventions.

Collectively, studies #2 and #3 demonstrate that supraspinatus contractile function is significantly impaired after RTC tear, evidenced by substantial muscle weakness and susceptibility to injury. Muscle mass is a well-established determinant of muscle force, and it is thus expected for supraspinatus force to be diminished as a result from atrophy after RTC tear; however, both studies identified factors beyond muscle atrophy (morphology of the NMJ and the degree of FI in the muscle) that are associated with the loss of supraspinatus force after tenotomy. The compilation of these results not only provide accessible markers that are indicative of changes in the muscle's capacity to generate force, but also provide new potential of therapeutic targets to improve functional outcomes after RTC tear.

LIMITATIONS

Assessing contractile function of RTC muscles requires an invasive procedure that is not practical in the clinical setting. Therefore, this dissertation work used laboratory animals models to determine how supraspinatus contractile function was affected following RTC tear. While the method to test supraspinatus function was developed in three different animal species, the clinical relevance for each animal model varies greatly, as tendon scaring and muscular changes (i.e. FI, fibrosis) did not always mimic what is commonly seen in patients with RTC tear. The following paragraphs will discuss limitations found in each animal model.

The rat is generally employed due to similarities in biomechanics, bony anatomy of shoulder, and accessibility. However, the rat model of RTC tear did not develop FI or fibrosis, and the tendon re-attached spontaneously, unlike patients with massive RTC tears.[41,65] While others have reported similar findings using the rat model,[2,19,61] the rat has also been used to study FI after RTC tear.[12,35] In order for the RTC muscles to resemble the degree of FI seen in humans, communication with the motor neuron must be blocked through denervation (i.e. suprascapular nerve transection)[36] or pharmacological inhibiting acetylcholine release (botulinum toxin, botox).[12] However, the clinical relevance of denervation/botox in rodents is debatable, as the prevalence of nerve damage is low in patients with RTC tear[25] and may therefore not be the major contributor of FI.[1] Furthermore, denervation/botox precludes the stimulation of muscle contraction through the suprascapular nerve. As a result, I did not use denervation/botox in studies #2 and #3, as measuring muscle contractile activity was an essential aspect of my dissertation. Another limitation that

could have prevented the development of FI was the spontaneous reattachment of the muscle to the humeral head, making the rat a suboptimal model to study RTC tears of longer duration. While some investigators make no mention of adhesions or reattachment of the cut RTC tendons in a rat model,[13,27] others report that reattachment of the supraspinatus tendon occurs spontaneously after tenotomy at time points exceeding 2 weeks.[2,61] Despite the use of various methods to prevent reattachment of the muscle, including suture of a penrose drain on the tendon stump, cauterization of the humeral head, insertion of a custom-made resin mold on the humeral head, each method was not only ineffective in preventing tendon re-attachment, but also led to increased inflammation and damage of the supraspinatus and surrounding tissue.

On the other hand, the supraspinatus of rabbits did not reattach to the humeral head and developed substantial FI, similar to what occurs in patients with RTC tears. It is still unclear why substantial FI occurs in the rabbit model but not in the rodent model with only tenotomy. One possibility could be that the supraspinatus remains unattached for a prolonged period of time (unlike the rat) allowing for FI to develop. However, it is unknown why spontaneous tendon re-attachment occurs rodents, but not in humans or rabbits (also larger animal species). The striking differences in size between the two species may account for such phenomenon after RTC tenotomy, as minimal scar tissue would be necessary to reattach the supraspinatus muscle to the humeral head in a smaller animal like the rat. Although tendon reattachment does not occur in the rabbit, the tendon still has a capacity to spontaneously heal from smaller tears.[32] Tendon tears in humans, however, do not heal and tend to get larger with

time, which could be due to an underlying tendinopathy that results in a tendon tear, that cannot be mimicked by transecting healthy tendons by *in vivo* animal studies.[31] The use of animal models is therefore a limitation in the tendon literature as well, as it questions the applicability of healthy animal tendons to study tendon pathology in humans.[31,66]

RTC tears are most prevalent amongst people between 50-80 years of age, but the animals used in my studies were not of an age comparable to the people affected. Age may exacerbate the loss of force after RTC tear, as aging is also associated with decreased muscle mass, force,[48] quality[3] and decreased regenerative capacity.[26] Few studies have assessed the effects of age on tenotomized RTC muscles, and some suggest that age exacerbates the effects of tenotomy[29] while others report no significant influence of age.[19]

Another limitation in our study was the cross-sectional design to determine the progression of changes in contractile function after RTC tear. While a longitudinal design would have been preferable, the animals could not be tested repeatedly because supraspinatus testing was a terminal procedure. To overcome this limitation, three different groups of weight-matched animals were used to represent a different point in time; moreover, to prevent confounding factors like growth or weight gain, animals of each group were tested on the same day.

Considering the limitations of each animal model, the rabbit is more clinically relevant with regard to the muscular changes in the RTC that occur after tenotomy. Currently, the few therapies that are focused on treating the muscle after RTC tear have mainly been tested in the rodent model,[13,17,28,53,54] and to the best of my

knowledge, none have been tested in patients with RTC tear. While therapeutic interventions can begin to be tested in the rat, they should be tested in clinical-relevant models like the rabbit to gauge their effectiveness for clinical trials.

FUTURE DIRECTIONS

The compilation of this dissertation work shows that RTC tears result in significant supraspinatus weakness, which is associated with changes in NMJ morphology, muscle atrophy, and FI. While the NMJ morphology recovered in the rat model, further studies are currently being conducted in our lab to elucidate whether NMJ morphology is also affected in the rabbit model. Neuromuscular transmission can also be disrupted by changes in the integrity of the NMJ. [64]Although RTC FI can occur as a result of tenotomy, it can also occur in intact RTC muscles by blocking neuromuscular transmission from the motor neuron (denervation, or blocking neurotransmitter release). However, the low incidence of nerve damage in patients with RTC tear [1] does not account for the high prevalence in FI. [4] Currently no studies have determined the influence of NMJ morphology on the development of FI. Therefore, detecting changes in NMJ morphology after RTC tear could provide a connection between the loss of muscle force and increase in FI, and also provide a novel therapeutic target to improve muscle function after RTC tear.

One of the most intriguing findings was that tenotomized muscles with a similar degree of atrophy could significantly vary in strength according to the variations in FI. The close proximity of myofibers with adipocytes may promote cell-to-cell cross-talk that reduces myofiber contractile function, [38,47] impairs muscle

regeneration [47,55] and promotes adipocyte proliferation. [33,39] Adipocytes can reduce muscle quality through secretion of fatty acids [39] and adipokines,[47,56] that promote a pro-inflammatory environment [47] that can induce oxidative stress,[8 3] which impairs NMJ activation, [34] excitation-contraction (EC) coupling, [5] and actin-myosin interactions in myofibers. [11,46] Conversely, myofiber dysfunction can also promote the differentiation of muscle resident stem cells into adipocytes [33,39] by blocking signaling pathways necessary for myogenesis of muscle resident stem cells. [7,33] In addition to cell-to-cell cross-talk, intramuscular adipocytes can decrease the force generating capacity of muscle by altering its biomechanical properties. [49] While some have suggested for the source of FI to be muscle resident progenitor cells, [40] there are a variety of progenitor cells that have not been explored in RTC injury. [14,45,67,68] Further studies may thus seek to determine how a RTC tear promotes the commitment of muscle resident stem cells into an adipogenic lineage, and which muscle resident cells contribute to FI after RTC tear. Also, understanding how intramuscular adipocytes directly affect force-generating capacity of RTC muscles can help develop treatments that can improve contractile function of muscles with FI.

Currently, our laboratory is putting in place a study to use pharmacological agents to prevent FI in the rabbit muscle through inhibition of peroxisome proliferator-activated receptor gamma (PPAR-γ), a transcription factor involved in adipogenesis. [9] If this treatment can prevent FI and attenuate the loss in contractile force without having an impact on muscle atrophy, then it would support that FI is an additional, independent contributor to muscle force production. Conversely, if inhibition of PPAR-γ can also prevent muscle atrophy, then the study would provide a

target for therapeutic intervention for patients with RTC tear to improve functional outcomes by addressing both atrophy and FI. Another potential study in our laboratory is to treat RTC muscles with low-frequency electric stimulation (LFES) to maintain muscle mass after RTC tear. LFES can improve neural drive in skeletal muscle [24] and attenuate loss of muscle mass and strength after hindlimb suspension, [60] but LFES has not been tested in RTC muscles. If LFES could prevent the loss of muscle strength after RTC tear by attenuating atrophy, it would be interesting to test whether LFES could also influence the degree of FI in the muscle. Studies suggest that healthy muscle may be less likely to promote the differentiation of intramuscular stem cells into adipocytes, [33,39] but further evidence is necessary to determine whether FI is a cause of consequence of poor muscle function.

Understanding the role of FI in muscle contractile dysfunction can not only contribute to the development of treatments to improve shoulder function after RTC tear, but also improve other conditions where contractile tissue experiences FI, such as cardiac muscle after myocardial infarction, [23] injured skeletal muscle in diabetes, [42] and muscles of patients with spinal chord injury, [43] and chronic obstructive pulmonary disease. [51]

Appendix

Animal Care and Use Documentation

UNIVERSITY OF MARYLAND
SCHOOL OF MEDICINE
Office of Animal Welfare Assurance

655 W. Baltimore Street
BRB, Mezzanine Ste. M023
Baltimore, MD 21201-1559

email: iacuc@som.umaryland.edu
voice: (410) 706-7859/8470
Assurance Number: A3200-01

DATE: November 04, 2014

TO: Richard Lovering, Ph.D., PT
Department of Orthopaedics
AHB, Rm. 540

FROM: Institutional Animal Care and Use Committee

RE: IACUC PROTOCOL #1014007
"Mechanisms underlying muscle injury and muscle disease"

This is to certify that the Institutional Animal Care and Use Committee received your response to their queries and that your response was considered sufficient to grant FULL APPROVAL to your protocol.

An annual report must be submitted to the IACUC one month before each anniversary of the protocol. Please note that your protocol will expire on October 17, 2017. If you need to extend the protocol beyond this date, you must submit a new animal use protocol at least 3 months prior to the expiration.

If you have any questions, please do not hesitate to contact the Office of Animal Welfare Assurance by email (iacuc@som.umaryland.edu) or by phone (706-7859 / 8470).

John B. Sacci, Jr., Ph.D.
IACUC Chair

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email: iacuc@som.umaryland.edu
voice: (410) 706-7859/8470
Assurance Number: A3200-01

DATE: April 08, 2015

TO: Mohit Gilotra, MD
Department of Orthopaedics
22 S. Greene St. S11B

FROM: Institutional Animal Care and Use Committee

RE: IACUC PROTOCOL #1214004
"Rotator Cuff Fatty Infiltration in a Rabbit Injury Model"

This is to certify that the Institutional Animal Care and Use Committee received your response to their queries and that your response was considered sufficient to grant FULL APPROVAL to your protocol.

An annual report must be submitted to the IACUC one month before each anniversary of the protocol. Please note that your protocol will expire on December 17, 2017. If you need to extend the protocol beyond this date, you must submit a new animal use protocol at least 3 months prior to the expiration.

If you have any questions, please do not hesitate to contact the Office of Animal Welfare Assurance by email (iacuc@som.umaryland.edu) or by phone (706-7859 / 8470).

John B. Sacci, Jr., Ph.D.
IACUC Chair

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Chapter 1 & 2

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Chapter 3

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Chapter 5

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Chapter 6

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