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Supraspinatus and deltoid muscle fiber composition in rotator cuff tear conditions

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Background: Rotator cuff (RC) tears are associated with RC muscle atrophy and changes in composition that are crucial to the prognosis of RC repair. The aim of this study was to characterize muscle fiber composition in the supraspinatus (SS) muscle under tear conditions.

Methods: Muscle biopsies were obtained from 21 patients undergoing surgery for an RC tendon tear. Biopsies were obtained from the musculotendinous junction of the SS muscle, and control biopsies were harvested from the deltoid muscle (DT). Biopsies were immunohistochemically processed for detection of type 1 (slow type) and type 2 (fast type) fibers and analyzed using unbiased, stereological principles. We counted the total numbers of type 1 and 2 muscle fibers/mm², and fiber diameter was used to estimate muscle fiber atrophy and hypertrophy.

Results: We found significantly more type 2 cells/mm² in the SS compared with the DT (P < .01). In addition, we found a significantly higher fraction of type 1 fibers than type 2 fibers in the DT (P < .01), whereas both fiber types were equally present in the SS. The diameters of SS cells were generally smaller than those of DT cells. Atrophy of especially SS type 2 fibers was also demonstrated. Fiber atrophy was more pronounced in men than women.

Conclusion: The changes in the composition of SS muscle cell types suggest a shift from type 1 to type 2 muscle fibers and atrophy of both type 1 and 2 fibers. This composition indicates loss of endurance and rapid fatigue of the SS muscle under RC tear conditions.

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The 4 muscles in the rotator cuff (RC) coordinate shoulder movements and provide structural stability to the shoulder joint.1 The RC is vulnerable to considerable morbidity,22,30 and RC tears are common with a life prevalence of 10%-20%.38,39 The musculotendinous unit of the supraspinatus (SS) is especially prone to lesions,4 but only sparse information is available regarding the pathophysiological response of the SS muscle to traumatic tear conditions. The importance of the compositional and degenerative changes in the RC muscles as causes of surgical failure, however, has become increasingly apparent.13,29

Skeletal muscle consists of different fiber types characterized by their specific myosin heavy chain (MHC) isoforms. Skeletal muscles in humans are basically composed of a mixture of MHC type 1 (slow twitch) and MHC type 2 (fast twitch) fibers. The 2 types differ in their function. In humans, myosin ATPase hydrolysis rates for fast twitch fibers are 2-3 times greater than those for slow fibers26,36 and the growth capacity of type 2 fibers is approximately 50% greater than that of type 1 fibers.1 In addition, type 1 fibers mainly depend on aerobic/oxidative energy metabolism, whereas type 2 fibers mainly depend on anaerobic/glycolytic metabolism.32,33 This is significant for the movement of the joints, where type 1 fibers...
enable slow contractions and endurance, whereas type 2 fibers are responsible for fine motor control and power with the ability to perform rapid contractions.\textsuperscript{19}

Nonpathologic SS muscles in cadaveric 65-year-old (±12 years) men and women were shown to consist of approximately 54% type 1 fibers,\textsuperscript{16,17} but physical activity and tear of the SS tendon may alter the fiber-type composition.\textsuperscript{13,14} Fiber-type composition is also influenced by the training activity of the individual, and muscle disuse or denervation may be associated with a type 1 to type 2 shift.\textsuperscript{12,15} Also age is suggested to affect muscle fiber composition, as older men have smaller type 2 muscle fibers than younger men,\textsuperscript{21} though other studies show no age-dependent difference in fiber type.\textsuperscript{16,17} Gender appears to affect muscle fiber size, as in men type 2 muscle fibers tend to be slightly larger than type 1 fibers, whereas in women the diameters of the 2 fiber types are identical.\textsuperscript{15} This gender-dependent difference in thickness is believed to be caused by greater muscle strength in men,\textsuperscript{20} and muscle strength is correlated with the size of type 2 muscle fibers.\textsuperscript{24}

Several of these factors, such as fiber atrophy, loss of muscle fibers, and fatty infiltration, are also main predictors of RC function. Therefore, a comprehensive characterization of SS muscle composition and degree of atrophy may lead to a better understanding of the pathogenesis of muscle wasting/pathology in relation to RC tear. This is a prerequisite tool in the design of therapeutic interventions appropriate for healing of RC tears. The aim of the present study was therefore to analyze muscle fiber profiles of the SS muscle in tear conditions using design-unbiased stereology.\textsuperscript{15,17} Furthermore, fiber atrophy of the SS muscle was assessed by measuring fiber diameter, and gender-dependent differences in fiber-type composition were established.

Materials and methods

 Patients

Twenty-one consecutive patients (mean age, 60.3 ± 4.0 years; range, 45-73 years) with a lesion of 1 or more RC tendon tears involving the SS tendon in all cases were included in this study. Fourteen males and 7 females, with an SS tendon tear, had biopsies taken from the SS muscle. The musculotendinous junction of the SS muscle was gently debrided from fascia and bursal tissue using a blunt shaver. Twenty-one biopsies were taken approximately 1 cm medial to the tendon under direct visualization from the arthroscopic RC tendon repair. In addition and for comparison, 20 biopsies from assumed healthy, ipsilateral deltoid (DT) muscles were taken as well. Inclusion criteria were shoulder trauma, magnetic resonance imaging–confirmed RC lesion, and willingness to undergo surgery. All tears were confirmed at surgery. Exclusion criteria were severe retraction of tendons, fatty infiltration higher than grade 2,\textsuperscript{14} diabetes, autoimmune diseases, previous shoulder surgery, fractures, or a dislocated shoulder.

Informed written consent was obtained from all patients. The study was reported to the Danish Data Protection Agency.

 Immunohistochemistry, fiber typing, and histology

Biopsies were immersion fixed in 10% phosphate-buffered formaldehyde, embedded in paraffin, and cut into 4-μm-thick microtome sections. For immunohistochemical staining, sections were deparaffinized, demasked in cell conditioning 1 (Roche Tissue Diagnostics, Hvidovre, Denmark) buffer, and blocked in 1.5% H2O2 in Tris-buffered saline (pH 7.4), after which they were loaded onto a Bench-Mark ULTRA IHC/ISH staining module (Ventana Medical Systems, Tucson, AZ, USA). Sections were initially stained for myosin fast-type heavy chain (type 2 fibers) using a monoclonal mouse antiamyosin (skeletal, fast) antibody (1:8000, NovoCastra, clone MY32 [Leica Biosystems, Buffalo Grove, IL, USA]), followed by heat inactivation of the immune complex, and then stained for myosin slow-type heavy chain (type 1 fibers) using a monoclonal mouse antiamyosin (skeletal, slow) antibody (1:50, NovoCastra, clone WB-MHCs). The OptiView DAB IHC Detection Kit (Roche Tissue Diagnostics, Hvidovre, Denmark) was used for the development of myosin fast-type heavy chain fibers and the ultraView Universal Alkaline Phosphatase Red Detection Kit (Roche Tissue Diagnostics) for the development of myosin slow-type heavy chain fibers. Hematoxylin was used for identification of nuclei using standard protocols at the Department of Pathology, Odense University Hospital.

 Stereology

The 41 biopsies were analyzed blinded twice by 2 independent raters. All biopsies were analyzed using an Olympus BX50 microscope equipped with an Olympus DP70 digital camera attached to a PC with newCAST software (Visiopharm Integrator System, Hoersholm, Denmark).

Type 1 and type 2 cell profiles sampled by the 2D unbiased counting frame within the delineation along with corners hitting tissue were quantified using the counting tool.\textsuperscript{16} In practice, a masking tool was used to delineate biopsies at a 4× lens, followed by analysis of type 1 and type 2 cells using a 20× lens (Fig. 1). Each biopsy was analyzed using approximately 30 fields of views obtained by systematic uniformly random sampling throughout the biopsy cross-section. The measurement tool, set on the diameter setting, was used to measure the greatest diameter perpendicular to the longest axis of the cross-sectioned cell profiles inside the frame.

Estimation of the total number of type 1 and type 2 cell profiles per area

The number of type 1 and type 2 cell profiles per area, $Q_{\text{a}}$ (cells), was estimated as follows:

$$Q_{\text{a}} = \left( \frac{\sum Q(\text{cells})}{\text{a(frame)}} \right) \left( \frac{\sum P(\text{biopsy})}{4} \right)$$

where $Q(\text{cells})$ is the total number of either type 1 or type 2 cell profiles sampled by the counting frame, $a$(frame) is the size of the frame.

Figure 1 Stereological setup. A typical step in analyzing a biopsy, showing the counting frame with exclusion (red) and inclusion (green) guards, muscle cells, and the delineation (yellow) of the biopsy.
counting frame (55,000 μm²), and P(biopsy) is the number of count frame corners (4) hitting biopsy tissue.16

Fractions of the counted fiber types were calculated in both rating 1 and rating 2, and a mean fraction was found for each fiber type in the 2 muscles.

Estimation of fiber diameter

To determine the changes in fiber size, the greatest diameter perpendicular to the longest axis of the sampled cell profiles was measured.6 A mean diameter was found for both fiber types in each biopsy. One biopsy was eliminated due to technical artifacts.

Estimation of hypertrophy and atrophy factors in the SS and DT muscles

Feret’s diameter was used to quantify the degree of atrophy and hypertrophy.10 Atrophy and hypertrophy factors were calculated by the following equation:

\[
\text{The products of all the atrophied fibers multiplied} \quad \frac{\text{The total number of cell profiles}}{1000}
\]

The upper limits were compared with the upper limits found in biceps brachii (BB) muscle.10 The upper limits for atrophy were 150 in both type 1 and 2 cell profiles for men. The upper limits for women were 100 in type 1 cell profiles and 150 in type 2 cell profiles. Hypertrophy upper limits for men were 300 for type 1 and 500 for type 2, and for women 200 and 150, respectively.10 While analyzing these data, 1 biological outlier was identified and as a result a single biopsy was eliminated from this analysis.

ELISA for fatty acid binding protein 4

SS (n = 16) and DT (n = 16) muscle samples were homogenized at 4°C in Mesoscale Lysis buffer (150 mM NaCl, 20 mM Tris, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100; pH 7.5) containing Phosphatase Inhibitor Cocktail 2 and 3 (Sigma-Aldrich, Søborg, Denmark) and Complete Mini EDTA-free Protease Inhibitor (Roche). Lysates were rotated end-over-end for 1 hour at 4°C and cleared by centrifugation at 13,000 g at 4°C for 20 minutes. Protein content in the lysates was measured by the bicinechonic acid method using the Thermo Scientific Micro BCA Protein assay Kit (Pierce Chemical Co., Dallas, TX, USA). ELISA for fatty acid binding protein 4 (FABP4) was performed using a FABP4 ELISA kit (Nordic Biosite, Täby, Sweden) according to the manufacturer’s instructions.

Statistical analysis

Data are reported as mean (standard deviation [SD]) and \(P < .05\) was used to indicate significant differences. Comparisons were done using 2-way analysis of variance (ANOVA) followed by the relevant post hoc test and Student’s t-test. The Rout test was used to detect outliers. A simple correlation analysis was performed to assess the inter- and intrarater correlation. In addition, an intrarater correlation was examined using the coefficient of variance (CV) and coefficient of error (CE) and a ratio between these 2 coefficients. While analyzing the data, a single biopsy from rater 1’s first rating was identified as a technical outlier due to the tissue quality and therefore eliminated from this analysis. Removal of one outlier did not affect results.

CV, calculated from the 2 raters’ total number of type 1 and type 2 cells/mm², was used to make an intravariance correlation of both raters. The equation used was as follows:

\[
CV = \frac{SD}{mean}
\]

Thereafter the CVs from the first and second ratings were used to make a CV_mean for type 1 cells/mm² and a CV_mean for type 2 cells/mm² using the following equation:

\[
CV_{mean} = \left( \frac{CV_1^2 + CV_2^2}{2} \right)^{0.5}
\]

In normal muscle, the CV is less than 0.25. Any measure greater than this is considered abnormal variability. SD was calculated from the number of cells that had their diameter measured.23

CE was used to determine the precision of the estimates from the double estimates.18

Results

On the basis of unbiased stereological estimation of the total number of type 1 and type 2 fibers in the SS musculotendinous unit after the RC tendon tear, we found a total number of 54.6 (21.6) type 1 cells/mm² and a total number of 53.5 (19.1) type 2 cells/mm² (Fig. 2, A). In the DT muscle, we found a total number of 47.1 (18.1) type 1 cells/mm² and a total number of 33.7 (13.8) type 2 cells/mm² (Fig. 2, A). When comparing the SS and DT muscles, we found significantly more type 2 fibers/mm² in the SS muscle compared with the DT muscle (2-way ANOVA: muscle type \(**P < .01, F_{1.78} = 11.2\)), whereas the number of type 1 fibers/mm² was comparable (Fig. 2, A).
In the SS muscle, 49.6 (13.0)% of the cells were type 1 fibers and 50.4 (13.0)% were type 2 fibers (Fig. 2, B). In the DT muscle, 57.5 (13.7)% were type 1 fibers and 42.5 (13.7)% were type 2 fibers (Fig. 2, B). When comparing SS and DT muscle tissue, we found significantly different type 1 and 2 fractions in the DT muscle (2-way ANOVA: interaction **P < .01, F1.78 = 7.25, fiber type *P < .05, F1.78 = 5.8). In contrast, the fractions were comparable in the SS muscle (Fig. 2, B).

In total, fiber diameter was estimated on 1366 SS fibers and 907 DT fibers (Fig. 3). Means were calculated for each biopsy. Afterward, an overall mean and SD were found for each fiber type subdivided in muscle types and gender. In the SS muscle, the mean diameter of type 1 fibers in women was 42.2 (13.1) μm and in men 48.9 (16.6) μm. In the SS muscle, the mean diameter of type 2 fibers in women was 36.3 (13.3) μm and in men 48.3 (18.3) μm. In the DT muscle, the mean diameter of type 1 fibers in women was 53.0 (12.5) μm and in men 60.4 (17.7) μm. In the DT muscle, the mean diameter of type 2 fibers in women was 42.3 (12.3) μm and in men 55.9 (20.8) μm.

When comparing SS and DT muscle diameters, we found a significant difference in diameter between type 1 fibers in both men (P < .001, Student’s t-test) and women (P < .001) and between type 2 fibers in both men (P < .001) and women (P < .001) with larger fiber diameters in DT compared with SS. In general, fiber diameter appeared to be smaller in SS compared with DT (please compare the displacement of the SS histogram to the left compared with the histogram for DT in Fig. 3, A-D). As atrophy was apparent in most SS biopsies (Fig. 4), fiber diameters measured were used to find atrophy and hypertrophy factors (Table I). The results for men showed atrophy for both type 1 and type 2 muscle fibers in SS (Fig. 3, A and B, and Table I) and atrophy for type 2 fibers in women (Fig. 3, C and D, and Table I). Hypertrophy was not apparent in any fiber type in women and men (Fig. 3, A-D, and Table I). Fatty infiltration, assessed by FABP4, revealed comparable and low levels in the SS (753.6 ± 384.7 pg/mg) and DT (660.3 ± 412.3 pg/mg) muscles (P = .53) (Fig. 5).

**Inter-rater and intrarater reliability**

Results from the simple correlation analysis showed decent and comparable intrarater R2 values for type 1 and 2 fibers for both raters. For rater 1, the intrarater value for type 1 fibers was 0.56 and for type 2 fibers 0.48. For rater 2, the intrarater value
for type 1 fibers was 0.69 and for type 2 fibers 0.59. Inter-rater $R^2$ values were subsequently good for both type 1 (0.65) and type 2 (0.65).31

Mean CE2/CV2 intrarater analysis for rater 1 was 0.16 for type 1 fibers and 0.22 for type 2 fibers. For rater 2, the mean results were 0.16 for type 1 fibers and 0.20 for type 2 fibers.

**Discussion**

RC tears affect many patients’ daily lives, as untreated lesions in turn lead to atrophy and fatty infiltration of the RC muscles.4,13,27 Because tear-induced muscle changes can influence the outcome of tendon surgery, the aim of this study was to map the extent of muscle damage through histochemical evaluation of the micro-architectural properties of the SS musculature in tear conditions. This study demonstrated only minor changes in SS muscle fiber type composition, but atrophy of both type 1 and 2 fiber profiles prevailed in RC tear conditions.

| Table 1 | Atrophy and hypertrophy factors in type 1 and 2 cells for men and women |
|---------|---------------------------------------------------------------------|
|         | Female | Male     |
| Supraspinatus |       |         |
| Type 1   | Atrophy factor | 14.60 | 101.73 |
|          | Hypertrophy factor | 149.64 | 183.98 |
| Type 2   | Atrophy factor | 134.02 | 210.65 |
|          | Hypertrophy factor | 56.70  | 199.07 |
| Deltoid  | Atrophy factor | 4.42   | 62.71  |
|          | Hypertrophy factor | 336.28 | 481.85 |
|          | Type 2   | Atrophy factor | 15.75  | 146.02 |
|          |          | Hypertrophy factor | 133.86 | 469.03 |

In cadaveric RC muscles without long-term diseases or known myopathies, type 1 SS muscle fiber content has been shown to be 54%.26 This result is based on a small number of samples but represents the best normative data on the fraction of muscle fiber types within the SS muscle. In comparison, our results revealed a decreased fraction (50%) of type 1 cells in the SS muscle after the RC tendon tear, suggesting a minor change in muscle-type composition. This is in line with a previous study demonstrating reduced MHC1 content in SS muscle fibers obtained from patients with full-thickness RC tears compared with patients with partial-thickness RC tears.27 In contrast to previous studies showing a reduction in the fraction of type 1 fibers in the lesioned SS muscle compared with the DT muscle,13 we observed comparable fractions of type 1 and type 2 fibers in the RC tear conditions.
or type 2 fibers between the SS and DT muscles. We did, however, observe a significant difference in the fraction of type 1 and type 2 fibers in the presumably healthy DT, a difference that was not present in the SS muscle.

To our knowledge, this is the first study to take advantage of design-unbiased stereology in order to count the total number of type 1 and type 2 muscle fiber cell profiles per area in the SS muscle and in the ipsilateral DT muscle after the RC tendon tear. When comparing the number of type 1 cells/mm² between the SS and DT muscles, we did not find any significant differences. When comparing the number of type 2 fibers/mm², however, our results revealed a significantly increased number of type 2 cells/mm² in the SS compared with the DT muscle. This suggests that after the SS tendon tear there is either an increase in type 2 fibers or a loss in type 1 fibers. Our data thus support the suggestions by Gigliotti et al.,13 Lundgreen et al.,27 and Butt et al.7 that there is a decrease in type 1 fibers. Whether this is caused by a loss of muscle fiber or a conversion of type 1 to type 2 fibers remains a matter of debate.

Previous studies have demonstrated a decrease in fiber diameter and shift toward smaller-diameter fibers in the lesioned SS muscle compared with the DT muscle.12,13 In the present study, we demonstrated that in both women and men, there were significant differences in fiber diameter in both type 1 and type 2 fibers derived from the SS muscle compared with the DT muscle. Irlenbusch and Gansen18 found a greater reduction in type 2 muscle fiber diameter, compared with type 1 muscle fibers, when looking at biopsies taken at SS tendon surgery. They suggested that a disturbance of muscular coordination in patients with SS tendon rupture is responsible for the atrophy occurring. They also implied that type 2 fibers, responsible for fine motor control of the joint, often are impaired in these patients due to atrophy.

In line with our findings, Lundgreen et al.17 found statistically significant smaller average diameters of both type 1 and type 2 fibers in full-thickness tears, compared with partial-thickness tears. Despite findings of decreased fiber diameter in the SS muscle after RC tears, Butt et al.7 recently shown that the mean myofiber cross-sectional area had significantly increased in the SS muscle 1 year after SS tendon surgery. Repairing the SS tendon thus seems to induce regeneration of the muscle, thereby reversing the shift in muscle fiber type composition.7 These findings indicate that an SS tendon rupture with denervation and lack of activation of the muscle also induces a shift from type 1 muscle fiber isoforms to type 2 muscle fiber isoforms, as seen in our study, as well as suggested by Gigliotti et al.13 and Lundgreen et al.27

The measured diameters are influenced by shrinkage due to paraffin embedding. To discover hidden fiber atrophy or hypertrophy among the 2 types of fiber cells, we calculated atrophy and hypertrophy factors based on the measured diameters of the cell profile. Because the atrophy factor of the healthy SS muscle is unknown, the atrophy factors’ upper limits were compared with the anatomically similar BB muscle in order to determine whether hidden atrophy was present. The SS and BB muscles are somewhat similar in relation to fiber type 1 composition with BB having a proportion of 52% in elderly adults.9 In the present study, the calculated factors showed clear atrophy of both type 1 and type 2 SS muscle fibers in men, and for type 2 fibers in women, when compared with BB. Hypertrophy was not apparent in any fiber types.

With an average timespan of 11.2 months from lesion to surgery, our patients had experienced disuse of the RC for different lengths of time. Studies have shown that disuse of muscles can lead to a shift in expression from type 1 fibers toward faster type 2 MHC isoforms, making it difficult to identify atrophy in fibers that were originally slow.7 This might explain the lesser degree of type 1 atrophy found in this study. Furthermore, it raises questions as to whether the larger fractions of type 2 cells found in all SS estimates (50%) when compared with the fraction of type 2 cells found in normal muscle (46%)9 might be caused by the shift from type 1 to type 2 cells, or simply a loss of type 1 cells.13 This question cannot be answered with a biopsy but will require a full transect of the muscle.

Analysis of the degree of fatty infiltration, which is indicative of muscle weakness, was estimated for both the SS and DT muscle. In this study, fatty infiltration was determined based on the content of FABP4 protein levels in the SS and DT muscles, which we found were comparable between the 2 muscles. This in contrast to a recent study by Lee et al.,25 which used a different and semi-quantitative technique to assess FABP4 and demonstrated lower FABP4 levels, representative of the absence of fatty infiltration, in the DT muscle compared with the SS muscle.25 These findings are in favor of the use of DT as a reference muscle.

In this study, several limitations require consideration. The ipsilateral DT has been used as a standard of reference in a number of studies12,13 justifying the use of paired statistics and increasing the power of the analyses. The rationale for using the DT muscle for comparison could, however, be challenged, as it may be asymptotically affected.

Another limitation inherent in muscle biopsy studies is that changes in muscle composition may not be uniformly distributed throughout the muscle. Our priority was to harvest all biopsies from the musculotendinous junction of the SS muscle during an arthroscopic approach from the subacromial space, thereby ensuring uniformity of the biopsy procedure.

The strength of the present study was that the biopsies from SS and DT were analyzed using design-unbiased stereology giving very precise estimates.15,27 In addition, in this study, analysis of the samples was done blinded twice by 2 independent raters. In contrast, previous studies used less precise methods such as photographically enlarged paper prints of the samples and semiautomated image analyses.12,13,15,27 Our results showed a large degree of coherency between the 2 raters, as well as individually for each rater.

CE²/CV² ratios were used for both types of cells for both raters. To reach an acceptable level of precision when using stereology, CE² should be half or lower than CV².15 The results indicate this was the case.

Normal adult muscle fibers are between 40 and 80 μm in diameter in males and between 30 and 70 μm in diameter in females.10

Conclusions

In this study, we found evidence of atrophy of both type 1 and 2 muscle cells and changes in the composition of SS muscle cell types indicative of a shift from type 1 to type 2 muscle fibers. The fiber atrophy and compositional change also indicate loss of endurance and rapid fatigue in the SS muscle after the RC tendon tear. Furthermore, we found that an SS tendon tear affects the composition of adjacent muscles such as the DT.

Availability of data and materials

All data are hosted at OPEN (Odense Patient Explorative Network), which allows data sharing on request (https://www.sdu.dk/da/om_sdu/institutter_centre/klinisk_institut/forskning/forskningsenheder/open.aspx).

Disclaimer

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