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Fiber type composition of the architecturally distinct regions of human supraspinatus muscle: A cadaveric study

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Summary. The human supraspinatus muscle is clinically important as it is frequently injured in older adults and the elderly. We have previously shown that the supraspinatus has a complex architecture with two distinct regions each consisting of three parts. Further we have found dynamic changes in architectural parameters such as fiber bundle length markedly vary between these regions. Fiber types of the supraspinatus have not been thoroughly investigated throughout its volume and are of interest to clinicians treating supraspinatus pathologies. In this study we investigated the distribution of fiber types within the distinct regions and parts of supraspinatus. Samples of supraspinatus were excised from six distinct parts of each muscle from five formalin embalmed specimens (one male, four female; mean age 77±11.1 years) free of tendon pathology. Samples were frozen in liquid nitrogen and then cryosectioned. Serial sections were labeled using immunohistochemical techniques and antibodies against fast or slow myosin heavy chain isoforms. The mean percentage of Type I (slow) fibers ranged from 56.73% to 63.97%. Results demonstrated significant variations in fiber type distribution. The middle part of the anterior region has a significantly greater percentage of Type I fibers compared to that of the posterior. The superficial part of the anterior region has a greater percentage of Type II (fast) fibers compared to the middle and deep parts. Findings aid in highlighting the distinct functions of the anterior and posterior regions, and prompt the need to re-evaluate assessment and treatment techniques established on a limited understanding of the fiber type distribution.

Key words: Rotator cuff, Shoulder, Muscle fibers, Immunohistochemistry, Myosin heavy chain

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Introduction

The human supraspinatus muscle is a one of the four rotator cuff muscles of the shoulder girdle which work together to provide dynamic stability. It is a clinically important muscle as it is most commonly involved in rotator cuff tendon tears. Rotator cuff tendon tears affect approximately 30% of patients over the age of 60 (Lehman et al., 1995) and can cause shoulder pain that interferes with self-care ability and functional independence in older adults (Harryman et al., 1991; Lin et al., 2008).

The gross morphology and function of the supraspinatus are complex. Cadaveric and imaging studies have demonstrated that the supraspinatus muscle consists of anterior and posterior regions, each of which attach to different parts of the supraspinatus tendon (Vahlensieck et al., 1994; Roh et al., 2000). In a three dimensional computer modeling study using cadaveric specimens, we demonstrated that in addition to the anterior and posterior regions, each region was further subdivided into superficial, middle and deep parts based on fiber bundle arrangement (Kim et al., 2007). A distinct functional difference between anterior and posterior regions of the muscle with respect to humeral

rotation has been demonstrated using biomechanical testing (Gates et al., 2010). *In vivo* investigation of geometric properties of the normal supraspinatus using real time ultrasonography supports these findings as we found that dynamic changes of the musculotendinous architecture vary between anterior and posterior regions with muscle contraction and various shoulder positions (Kim et al., 2010).

Muscle function is directly correlated with histochemical and biochemical features of the constituent muscle fibers. Human skeletal muscles can be categorized into two main types based on their metabolic and electrophysiological characteristics: Type I and Type II. Type I fibers are more fatigue resistant, more dependent on aerobic metabolism and slower contracting than Type II fibers. Immunohistochemical methods on both formalin fixed (Behan et al., 2002; Lovering and Russ, 2008) and unfixed tissues (Lee et al., 2006; Srinivasan et al., 2007) are commonly used to determine fiber types in human skeletal muscles.

The fiber type composition within a muscle can vary between architecturally distinct regions (Gonyea and Ericson, 1977; Chanaud et al., 1991; English et al., 1993). To date, the fiber type distribution throughout the supraspinatus muscle volume has not been well described. Previous fiber type studies (see Table 1) of the supraspinatus muscle have harvested samples from the superficial part of the muscle (Johnson et al., 1973; Srinivasan et al., 2007) and have not been correlated to either the anterior or posterior region (Lovering and Russ, 2008). An in-depth understanding of the fiber type composition within the architecturally distinct regions and parts of supraspinatus would provide important insight into the contraction velocity, endurance properties and propensity for injury for various components of the muscle, and support the development of new evidence based rehabilitation protocols. It could also establish a fiber type database to which future studies of pathologic muscles could be compared. Therefore, the purpose of this study was to investigate the fiber type composition within the architecturally distinct regions and parts of the supraspinatus using immunohistochemical methods. We hypothesized that fiber type composition will vary between architecturally distinct regions and parts of the human supraspinatus.

Materials and methods

Cadaveric specimens

Five formalin (3.3% solution) embalmed human cadaveric shoulder specimens (one male, four female) for tissue excision were obtained from the Department of Anatomy and Cell Biology, University of Saskatchewan. Mean age of specimens was 77±11.1 (range 66-94) yrs. According to available medical information, none had any history of neuromuscular disease. Specimens were dissected to expose the supraspinatus muscle and the lateral end of the acromion was cut away to allow full viewing of the rotator cuff. Each specimen was then carefully assessed to ensure the rotator cuff was free from any tendon pathology and/or gross bony changes. Ethics approval was obtained from the Biomedical Research Ethics Board, University of Saskatchewan (Bio#08-197).

Tissue sampling

Samples of muscle tissue, each approximately 20-30 mm in length and 5-10 mm in diameter, were excised from either the left or right supraspinatus muscle of each specimen. Samples of each specimen's supraspinatus were obtained from each of the six architecturally distinct parts, as defined by Kim et al. (2007). These components consisted of anterior and posterior regions, which were each further subdivided into superficial, middle and deep parts. All samples were taken *in situ* from the center of each part along the midpoint of the fiber bundles except for the superficial part of the anterior region. For this part of the muscle, samples were taken from the anterior portion (Fig. 1). Following excision each sample was coated with Tissue Tek OCT Compound (Sakura Finetek, Torrance, CA, USA), immediately frozen in 2-methylbutane cooled by liquid nitrogen (Dubowitz and Sewry, 2007) and then stored at -20°C.

Cryosectioning and microscope slide preparation

Serial cross-sections of each muscle sample were cut at a thickness of 12 microns at a temperature of -23°C, in

Table 1. Summary of fiber type studies of the human supraspinatus.

Author	Sample	Site of Tissue Sample	Mean Percentage of Type I
Lovering and Russ (2008)	N=9 (6 formalin fixed; 3 unfixed) 3 male, 3 female, mean age 65±12 years (fixed specimens) 2 male, 1 female, mean age 73±13.5 years (unfixed specimens)	Proximal and distal aspect of muscle No specification of layer	54%±6% No significant findings between fixed and unfixed specimens
Srinivasan et al. (2007)	N=3 (unfixed) 3 male, mean age unknown	Not specified	50%±15%
Johnson et al. (1973)	N=6 (unfixed) 6 male, mean age 21.8±5.7 years	Not specified	59.3% Range 41.4%-85.0%

the chamber of a Minitome PLUS Cyrostat (Triangle Biomedical Sciences, Durham, NC, USA). Each pair of successive serial sections from each sample were picked up on a chilled ProbeOn Plus charged microscope slide (Fisher Scientific, Nepean, ON, Canada). Sections were quickly thawed onto the slides, which were then air dried for approximately 30 minutes at room temperature. Consecutive slides, containing pairs of successive cross-sections, were numbered and stored at -20°C.

Immunohistochemistry

Immunohistochemical techniques follow our earlier protocols (Rosser et al., 2000), and were applied to several consecutive slides of each of the six parts of the supraspinatus acquired from each specimen. Briefly, slides were removed from the freezer and air dried for 15 minutes. Blocking solution consisting of 1% bovine serum albumin and 5 mM ethylenediaminetetraacetic acid in phosphate buffered saline (0.02 M sodium phosphate buffer, 0.15 M sodium chloride, pH 7.2) was applied to each slide for 30 minutes. Subsequently, two primary mouse monoclonal antibodies, either A4.951 or A4.74 (supernatant; Developmental Studies Hybridoma Bank, University of Iowa, IA, USA) were used to label myosin heavy chains of Type I fibers and Type II fibers respectively. A4.951 labels all Type I (slow-twitch) fibers in human skeletal muscle, and A4.74 all Type II (fast-twitch) fibers. Each primary antibody solution consisted of 1:20 dilution of antibody in blocking solution, and was applied overnight at 4°C.

The secondary antibody process utilized the Avidin Biotin Complex (ABC) method, and two commercially available kits; the ABC kit (Vector laboratories Burlingame, CA, USA; PK 6200) and the DAB kit (Vector laboratories Burlingame, CA, USA; SK 4100). The ABC kit included a universal secondary biotinylated anti-mouse IgG antibody that was used to label A4.951 or A4.74. A 3% hydrogen peroxide solution in methanol was then used to prevent background staining. A preformed avidin and biotinylated horseradish peroxidase macromolecular complex reagent, prepared from the ABC Kit, was then applied to the slides and subsequently left for 60 minutes in the dark at room temperature. Finally, using the DAB Kit, a peroxidase substrate containing 3,3' diaminobenzidine was added to effect a specific color reaction visible with light microscropy. Slides were then mounted with Aquamount (Lerner Laboratories, Pittsburg, PA, USA) and stored at 4°C.

Image analysis

Images of prepared tissue sections were captured with a Sony Cybershot DSC V3 digital still camera (Sony, Tokyo, Japan) attached to Zeiss Axioskop 20 microscope (Carl Zeiss, Oberkochen, Germany). Images were then uploaded into an iMac Computer (Apple Computer, Cuperino, CA, USA) and saved in a high resolution jpeg format. A total of three to seven images were taken of each muscle part of each specimen, such that a minimum of one thousand fibers were photographed from both serial A4.951 and A4.74 treated slides for each muscle part.

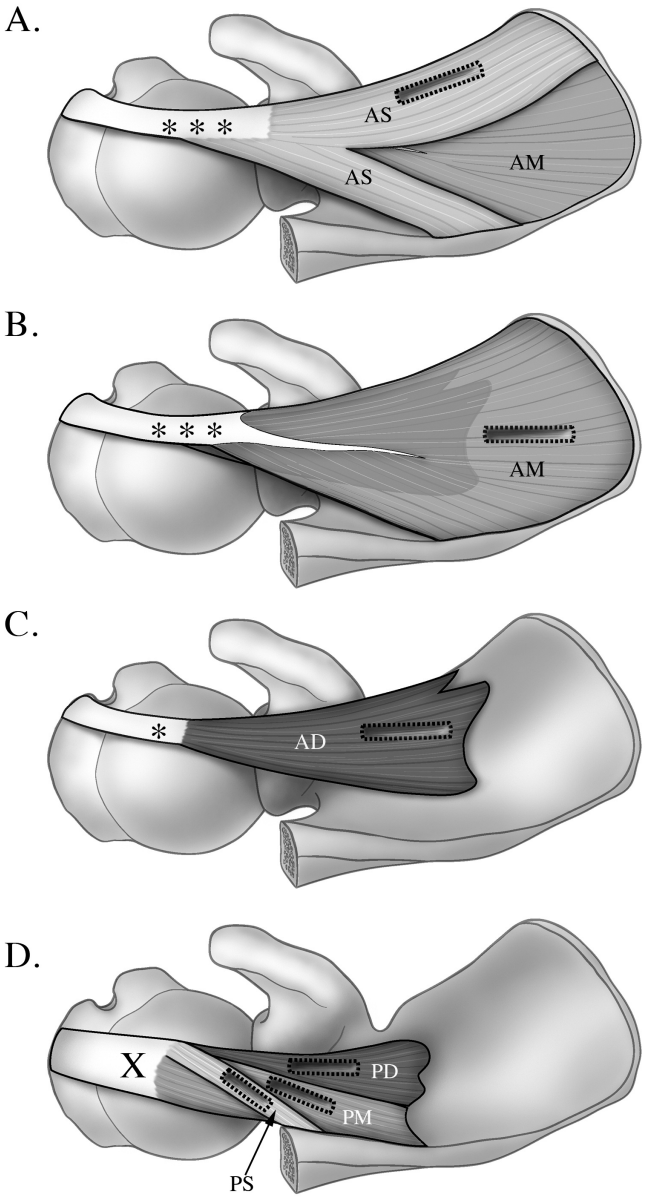

Fig.1. Supraspinatus muscle with architecturally distinct regions. Superior view with acromion removed. **A.** Superficial part of anterior region (AS). **B.** Middle part of anterior region (AM) with superficial part removed. **C**. Deep part of anterior region (AD). **D**. Posterior region with superficial (PS), middle (PM) and deep (PD) parts. Anterior region is not shown. Anterior part of supraspinatus tendon at different depths of the muscle (represented by *** in **A** and **B** and * in **C**). Posterior part of supraspinatus tendon (X). Rectangular box indicates the location of muscle sample.

Fiber type calculations

Stored images (Fig. 2) were printed using a commercial laser printer. Two investigators (Dyck and Lunn) used the antibody labeling evident in serial A4.951 and A4.74 images to independently classify fibers as Type I (labeled by A4.951 but not A4.74), Type II (labeled A4.74 but not A4.951) or hybrid (labeled by both A4.951 and A4.74). Data were entered into a Microsoft Excel (Microsoft, Redmond, WA, USA) spreadsheet. The mean percentages of Type I, Type II and hybrid fibers, in the fiber population of each muscle part of each specimen were determined.

Statistical analyses

The statistical package SPSS Version 18.0 (SPSS Inc., Chicago, IL, USA) was utilized for all statistical analyses. Descriptive statistics were used to derive mean, ± standard deviation and range for all variables. Independent t-tests and one way analysis of variance (ANOVA) followed by Tukey's post hoc test were carried out to compare means between regions and their respective parts. Correlation analyses were performed to establish inter-rater reliability of measurements. In all statistical evaluations, the level of significance was set at $P<0.05$.

Results

Investigator reliability

Correlation coefficients for inter-rater reliability of determining fiber types by two investigators demonstrated excellent reliability. Values for Type I, II and hybrid fibers were r=0.96 (95% confidence interval=0.94-0.97), r=0.97 (95% confidence interval= 0.95-0.97) and r=0.92 (95% confidence interval= 0.89-0.94) respectively. Given the high consistency, measurements of one investigator (Lunn) were used for analyses.

Percentage of fiber types

Mean percentage of fiber types within the distinct regions and parts of the supraspinatus muscle are presented in Figs. 3 and 4. Within all regions and parts of the supraspinatus muscle a mixed fiber type distribution was found with a significantly greater percentage of Type I fibers compared to Type II fibers ($P<0.05$). Mean percentage of Type I fibers ranged from 56.73% to 63.97%. For all regions and parts, mean percentage of hybrid fibers was less than 2.12%.

Comparing the anterior and posterior regions, no significant difference in mean percentage of fiber types was found within the superficial or deep parts (Fig. 3).

However, the middle part of the muscle showed a significantly greater mean percentage of Type I fibers in the anterior region compared to the posterior ($P=0.042$).

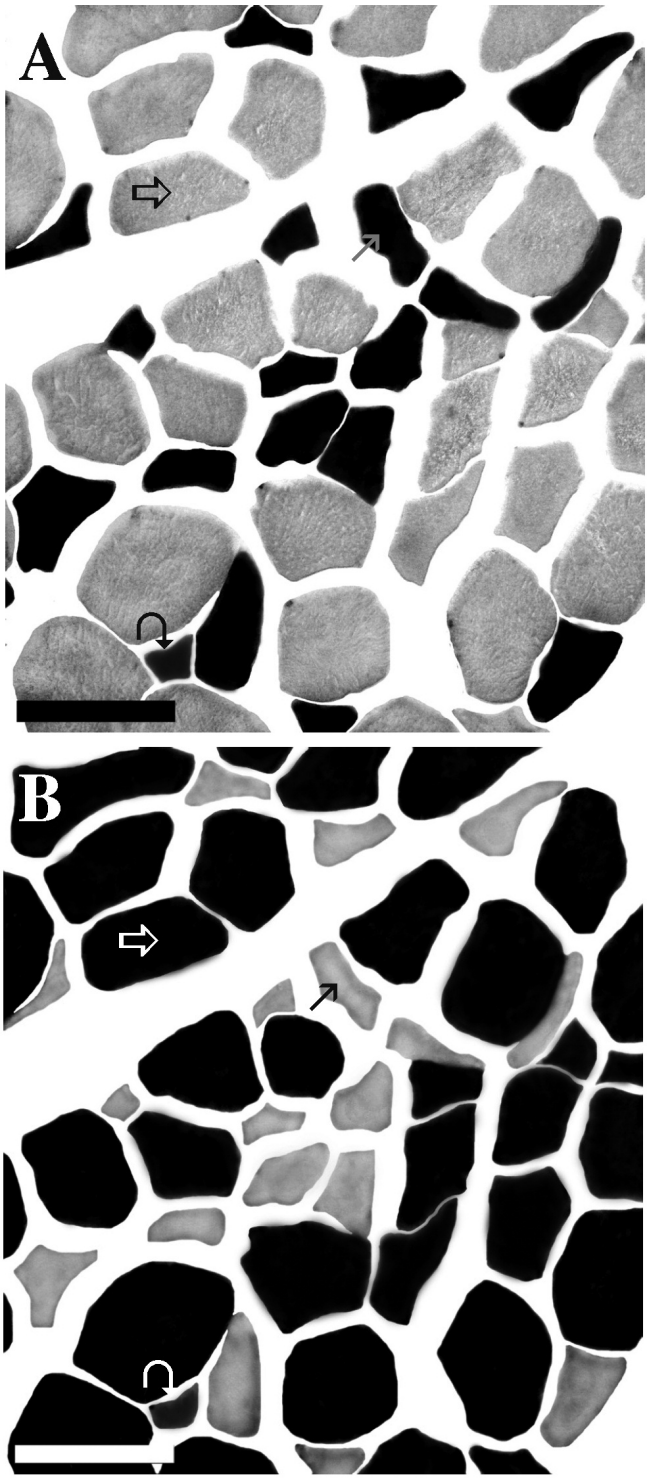

Fig. 2. Immunohistochemical labeling of serial cross-sections of supraspinatus. **A and B** indicate representative serial sections, respectively, labeled by antibody A4.74 against Type II (fast) and antibody A4.951 against Type 1 (slow) myosin heavy chains. Thick straight arrow indicates a Type I fiber, not labeled in A but labeled in B. Thin straight arrow indicates a Type II fiber, labeled in **A** but not labeled in **B**. Curved arrow indicates a hybrid fiber, labeled in both **A and B**. Scale bar: 50 microns.

Correspondingly, mean percentage of Type II fibers was significantly greater in the posterior region compared to the anterior (*P=0.031*).

Within the anterior region, mean percentage of fiber types varied between the different parts (Fig. 4). A greater mean percentage of Type II fibers was found in the superficial part compared to the middle (*P=0.020*) and deep parts (*P=0.012*). No difference was found in the mean percentage of Type II fibers between the middle and deep parts. Accordingly, a smaller mean percentage of Type I fibers was found in the superficial part compared to the middle (*P=0.024*) and deep parts (*P=0.023*). No difference was found in the percentage of Type I fibers between the middle and deep parts. Within the posterior region, no difference was found in the percentage of fiber types between the different parts (Fig. 4).

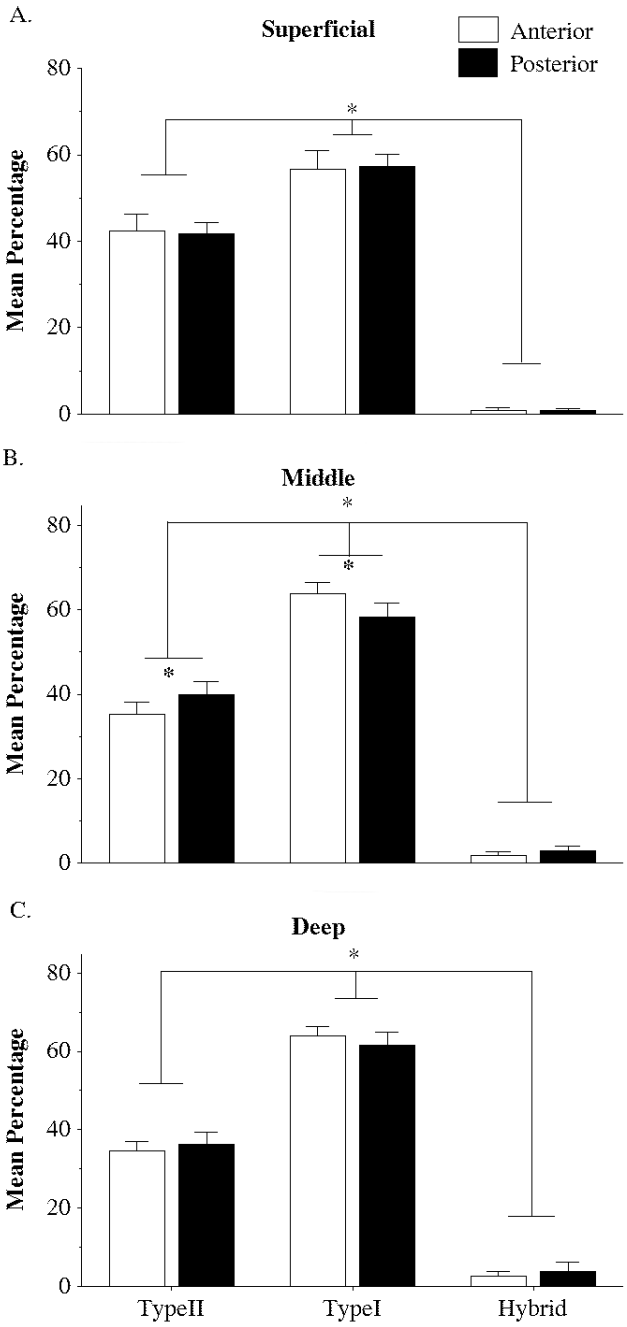

Fig. 3. Comparison of mean percentage of each fiber type between anterior and posterior regions of the three parts of supraspinatus: superficial **(A)**, middle **(B)** and deep **(C)**. Values are means ± 95% confidence interval. Statistical significance is indicated with * *P<0.05*. Results show mean percentage of both Type I and Type II fibers were significantly different between the anterior and posterior regions within the middle part of the muscle.

Morphology of muscle fibers

In general, Type I fibers presented as polygonal shapes whereas Type II fibers tended to have an irregular shape with unsmooth edges (Fig. 2). In all samples that

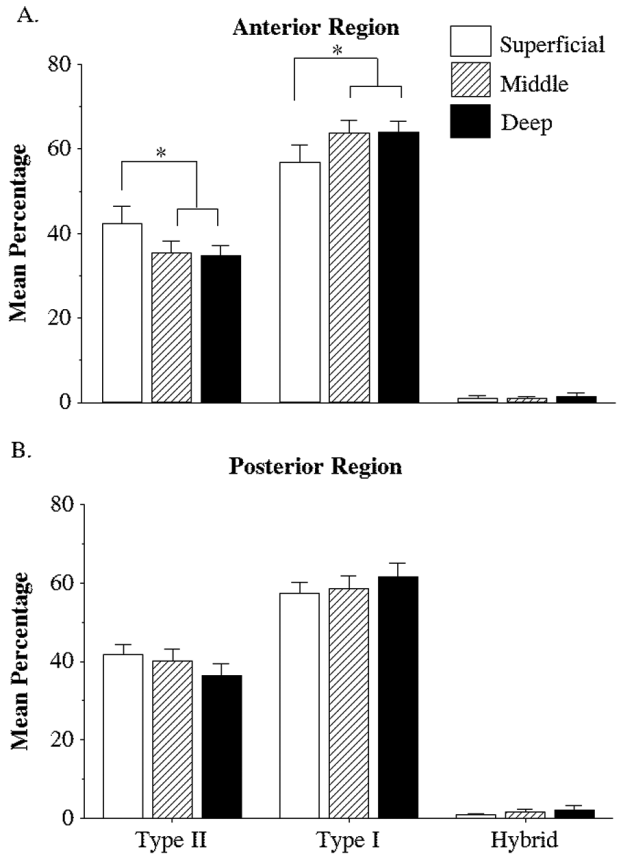

Fig. 4. Comparison of mean percentage of each fiber type within the superficial, middle and deep parts of anterior **(A)** and posterior **(B)** regions of supraspinatus. Values are means ± 95% confidence interval. Statistical significance is indicated with * *P<0.05*. Results show mean percentage of Type II fibers within the superficial part of the anterior region was significantly greater than that of the middle and deep. Mean percentage of Type I fibers within the superficial part of the anterior region was significantly less than that of the middle and deep.

we studied, Type II fibers were qualitatively smaller than Type I fibers. We did not quantify fiber size, however, as this parameter varied greatly between samples due to dehydration related shrinkage.

Discussion

The human supraspinatus muscle is prone to injury in older adults, and frequently implicated in rotator cuff injuries. The resultant limitations in self-care and functional independence are costly, both socially and economically. Despite its clinical importance, evidence regarding complex architectural aspects of the muscle is lacking. To our knowledge this is the first study to investigate the fiber type distribution throughout the muscle volume of human supraspinatus and to correlate findings with architecturally and functionally distinct regions. The principal findings of this study are (1) the anterior and posterior regions of supraspinatus have a mixed fiber type composition that significantly differs from each other and (2) within the anterior region the percentage of fiber types varies as a function of depth. Our findings support our hypothesis and both contrast with and confirm existing literature, thereby providing valuable insight and directions for further study and clinical practice.

The distribution of the two main fiber types, Type I and II, were mixed with a predominance of Type I fibers. This is consistent with other studies of the human supraspinatus (Johnson et al., 1973; Srinivasan et al., 2007; Lovering and Russ, 2008) and confirms our basic understanding of the supraspinatus; a muscle with postural function involving tonic activity and one which participates in movements involving phasic activity.

The larger percentage of Type I fibers within the middle part of the anterior region compared to the posterior indicates that the anterior region, overall, has a slower maximum shortening velocity and is more resistant to fatigue. Previous anatomical studies indicate the anterior region is able to produce greater forces than the posterior; indeed, the volume of the anterior region has been reported to be 3-6 times that of the posterior region (Roh et al., 2000; Kim et al., 2007). Moreover, the tensile strength of the anterior tendon is greater than that of the posterior (Itoi et al., 1995). Based on fiber bundle length changes observed *in vivo* it has also been suggested that the anterior region is more likely responsible for stabilization and the posterior region more for adjusting tension on the tendon (Kim et al., 2010). The significantly different fiber type compositions found between the anterior and posterior regions in this study add credence to previously made statements postulating the functions of these architecturally distinct regions. In addition, it highlights the need to reassess exercise protocols for the supraspinatus which are not yet based on the complex architecture or muscle fiber complement.

Within the anterior region, a significant difference in the mean percentage of fiber types was found between the different parts. A greater percentage of Type II fibers was found in the superficial part compared to the middle and deep. This difference in fiber type distribution between superficial and deeper sites is not surprising as it has been demonstrated previously in studies of other human muscles. Dahmane et al. (2005) found a predominance of Type II fibers at the surface and Type I fibers in the deep regions of upper and lower limb muscles. Similarly, compared to more superficial regions, greater numbers of Type I fibers are found within deeper regions of the musculature of a variety of mammalian species (Matsumoto et al., 2007; Schilling, 2009). Despite the difference in fiber bundle orientation between the middle and deep parts of the anterior region of the supraspinatus muscle (Kim et al., 2007), a difference in the fiber type distribution was not found. A more uniform distribution of fiber types was found within the posterior region. The difference in fiber types between the layers of the anterior region in addition to architectural differences may be of clinical relevance to the location and point of initiation of degenerative rotator cuff tears. Rotator cuff tears have been found to typically initiate in the anterior portion of the supraspinatus tendon (Codman, 1990; Lehman et al., 1995) and can be classified as a partial-thickness tear or a complete tear. Partial-thickness tears can be located on the bursal or articular side of the tendon. It has been theorized that architectural differences within the anterior region observed *in vivo* may be related to shear stress along the anterior tendon and contribute to the pathogenesis of articular sided rotator cuff tears (Kim et al., 2010). Future research is needed to further test the influence of muscle architecture on tendon stresses. The new insights about fiber type of the supraspinatus muscle gained from this study will inform subsequent study of tear processes.

This study provides a more accurate representation of fiber type distributions throughout muscle volume in relation to architecturally distinct regions of the supraspinatus. Previous studies of the supraspinatus have not provided a true representation of the fiber type distribution for the majority of the muscle volume. Fiber type distributions reported in previous studies are reflective of the superficial part of the muscle, where the muscle samples have been taken or at least alluded to be (Johnson et al., 1973; Srinivasan et al., 2007; Lovering and Russ, 2008). Mean percentages of Type I fibers previously reported range from 50-59.3%. This agrees quite closely with values for the superficial part of the muscle in this study. It is important to note the superficial part of the supraspinatus as defined by Kim et al. (2007), both in the anterior and posterior regions, is a thin layer of muscle and accounts for only a small portion of the entire muscle volume. The middle part of the anterior region, distinct in its fiber bundle orientation and lateral attachment sites onto the tendon from the superficial and deep parts, accounts for most of the muscle volume. Thus, investigation of samples from this region is more representative of the muscle volume.

Hybrid muscle fibers express more than one myosin heavy chain isoform, unlike more typical mature fiber types each of which normally contains just one isoform (Stevens et al., 2004). Hybrid fibers are of common occurrence in human muscles (Neunhäuserer et al., 2011) as is the case for various other vertebrate muscles and species (Stephenson, 2001). The presence of hybrid fibers may be related to fibers undergoing transformation, or just typical fiber phenotype, and may be correlated with changes of the contractile properties of those fibers affected (Stevens et al., 2004; Stephenson, 2001). Notably, unremarkable frequencies of hybrid fibers were observed in the present study.

The two main fiber types in the muscles of humans, and other mammals, are Type I and Type II (Schiaffino and Reggiani, 2011). Many studies of human muscle have centered on just these two fiber types (Dubowitz and Sewry, 2007). However, in most mature human muscles, Type II fibers have two subtypes; IIA and IIX (Ahmetov et al., 2012). Type I fibers have the greatest aerobic capacity, and are adapted for fatigue resistance endurance performance. Type IIA fibers are utilized for medium term anaerobic exercise, and Type IIX fibers for short bursts of rapid powerful contraction (Ahmetov et al., 2012). Mature human muscles contain mainly Type I and IIA fibers, IIX fibers being a minor component (Schiaffino, 2010). Each fiber type has its own myosin heavy chain isoform, which is under the regulation of a different gene (Schiaffino and Reggiani, 2011). Hybrid fibers may be the result of differential gene expression by myonuclei along the lengths of the multinucleated skeletal muscle fibers (Neunhäuserer et al., 2011). Human muscle fibers that transform type do so in a prescribed sequence, thus creating hybrid fibers; Type I to I-IIA to IIA to IIA-IIX to IIX. (Schiaffino, 2010). As the hybrid fibers in our current study contained the Type I myosin heavy chain, they would have also contained the Type IIA myosin heavy chain. This could have been either more I than IIA, or more IIA than I (Neunhäuserer et al., 2011). In addition to myosin, there are many other genes and proteins that modify the phenotypes of muscle fibers (Ahmetov et al., 2012).

The difficulties inherent to obtaining young cadaveric specimens are well known. To date, it is still not clear whether fiber type proportions change with aging (Lexell et al., 1988; Brunner et al., 2007; Narici and Maffulli, 2010), nor whether such changes influence risk of injury. The mean fiber type percentages in this study, at least for the superficial part, correlate well with those presented by Johnson et al. (1973) where specimens were much younger (Table 1). Type II fibers were qualitatively smaller and more angular in shape than Type I fibers, in each muscle sample we studied. While Type II fiber atrophy can result from alcohol or steroid usage (Dubowitz and Sewry, 2007), it is also a well-known characteristic of aged skeletal muscle fibers (Brunner et al., 2007; Narici and Maffulli, 2010).

Although the number of specimens we investigated was small, this number is comparable to previous fiber typing studies of the supraspinatus (Srinivasan et al., 2007; Lovering and Russ, 2008). In addition, with the limited number of specimens available for research at our institution and subsequent to employing our inclusion and exclusion criteria, an even number of male and female specimens could not be obtained. Finally, it is difficult to comment on the influence of handedness or the amount of physical activity of the specimen donors as this type of information was limited.

In conclusion, the results of this study establish an accurate spectrum of fiber type distribution of the architecturally complex supraspinatus muscle in normal older adults. The findings provide further insight into functional differences which have been suggested in previous cadaveric (Roh et al., 2000; Kim et al., 2007) and *in vivo* (Kim et al., 2010, 2013) studies. It confirms the anterior region is mainly responsible for force production and better suited for tonic activity whereas the posterior region is more suited for phasic activity and perhaps adjustment of tension on the tendon and rotator cuff. As fiber type variations in human myopathies are frequently reported, this study will serve as an important database for future comparisons and aid in elucidating the influence of neuromuscular changes on rotator cuff tendon function. In patients with impingement syndrome and rotator cuff tendon tears, for example, significant alterations in fiber type proportions have been reported (Irlenbusch and Gansen, 2003). Future studies investigating fiber type proportions within the different functional regions in patients with different pathologies or myopathies and comparing results to those without pathology will help ascertain important functional implications for the shoulder.

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