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Atrophy, inducible satellite cell activation, and possible denervation of supraspinatus muscle in injured human rotator-cuff muscle

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Gigliotti D, Leiter JR, Macek B, Davidson MJ, MacDonald PB, Anderson JE. Atrophy, inducible satellite cell activation, and possible denervation of supraspinatus muscle in injured human rotator-cuff muscle. Am J Physiol Cell Physiol 309: C383–C391, 2015. First published July 1, 2015; doi:10.1152/ajpcell.00143.2015.—The high frequency of poor outcome and chronic pain after surgical repair of shoulder rotator-cuff injury (RCI) prompted this study to explore the potential to amplify muscle regeneration using nitric oxide (NO)-based treatment. After preoperative magnetic resonance imaging (MRI), biopsies of supraspinatus and ipsilateral deltoid (as a control) were collected during reparative surgery for RCI. Muscle fiber diameter, the pattern of neuromuscular junctions observed with alpha-bungarotoxin staining, and the γ:ε subunit ratio of acetylcholine receptors in Western blots were examined in tandem with experiments to determine the in vitro responsiveness of muscle satellite cells to activation (indicated by uptake of bromodeoxyuridine, BrdU) by the NO-donor drug, isosorbide dinitrate (ISDN). Consistent with MRI findings of supraspinatus atrophy (reduced occupation ratio and tangent sign), fiber diameter was lower in supraspinatus than in deltoid. ISDN induced a significant increase over baseline (up to 1.8-fold), in the proportion of BrdU+ (activated) Pax7+ satellite cells in supraspinatus, but not in deltoid, after 40 h in culture. The novel application of denervation indices revealed a trend for supraspinatus muscle to have a higher γ:ε subunit ratio than deltoid (P = 0.13); this ratio inversely with both occupancy ratio (P < 0.05) and the proportion of clusters at neuromuscular junctions (P = 0.05). Results implicate possible supraspinatus denervation in RCI and suggest NO-donor treatment has potential to promote growth in atrophic supraspinatus muscle after RCI and improve functional outcome.

Pax7; nitric oxide; acetylcholine receptor; supraspinatus; deltoid

“ROTATOR CUFF” MUSCLES (supraspinatus, infraspinatus, teres minor, and subscapularis) function to rotate and abduct the arm and stabilize the shoulder joint. Rotator-cuff injury (RCI) from damage to cuff muscles or their tendons may be either chronic and age-related (4, 5) or an acute result of sports injury (23, 26). RCI most commonly affects the supraspinatus, and secondary patholgy, including muscle atrophy and fibro-fatty infiltration, results in pain, weakness, loss of function, and biomechanical shoulder-joint instability (3, 12, 15). Most tears require reparative surgery; however, recurrence of tears following surgery is common, with failure rates ranging from 30 to 94% (12).

Regulation of muscle satellite cells (SCs), the stem cells for muscle regeneration and growth, is implicated in the response to and rehabilitation from RCI, since there are reportedly fewer SCs in RCI muscle (12). The SC population and its responsiveness to an activating stimulus needed for regeneration could potentially be targeted for new treatments to improve recovery, since surgical repair of a torn tendon may not restore joint stability and function.

SCs are mitotically quiescent and identifiable by their satellite position between the plasma membrane and myofiber basal lamina (27), and by the proteins they express, including Pax7 (4, 6, 27). Transit of a SC from the quiescent G0 state into the G1 (activated) stage of the cell cycle is regulated by hepatocyte growth factor (5, 35) and nitric oxide (NO) (2, 3). Declining SC function can lead to myopathy and accompanies conditions such as muscular dystrophy and age-related atrophy. However, while strategies such as treatment with the NO-donor drug, isosorbide dinitrate (ISDN), have been explored as possible treatment for age-related atrophy and muscular dystrophy (19, 26, 28), the potential of NO-based treatment for RCI has not been explored (3, 26). This study aimed to investigate whether NO treatment in culture would increase SC activation in pathological supraspinatus compared with uninjured deltoid muscle (as an internal control).

Acetylcholine receptor (AChR) distribution on the muscle fiber membrane and subunit composition reveal the innervation status of muscle, histologically and in protein assays, respectively. During development, before fibers are innervated, AChRs are arranged in a linear pattern on fibers. With innervation, AChRs cluster (45) at neuromuscular junctions (NMJs). Five subunits form each receptor, and the identity of one subunit changes during development: specifically, the gamma (γ) subunit, which shows fetal characteristics, transitions to a more efficient adult epsilon (ε) subunit after innervation. After muscle is denervated, both histological and subunit character change: AChR distribution becomes more linear on the sarcolemma, and the AChR subunit pattern reverts to the fetal, γ isoform (45). These functional transitions make AChR distribution and composition useful indicators of innervation status.

This study aimed to investigate myogenic and neurogenic aspects of RCI, by examining differences in fiber morphometry...
and innervation (AChR distribution and subunit composition) and SC responsiveness to an activating stimulus in biopsy samples of supraspinatus muscle in RCI. These features of atrophy in supraspinatus were compared with those in uninjured, ipsilateral deltoid muscle of the same participant, to identify the mechanisms underlying RCI and ultimately find potential target areas for preventing RCI or improving the outcome of RCI repair. The hypothesis was that supraspinatus muscle would demonstrate atrophy, indicators of denervation, a reduced baseline level of SC activation, and increased responsiveness of SCs to activation by ISDN compared with the deltoid.

**MATERIALS AND METHODS**

Participants were recruited to this study according to a protocol approved by the Human Ethics Review Board at the University of Manitoba (protocol no. B2010-074) and the Winnipeg Regional Health Authority Research Review Access Committee (WRHA 2010-019) and signed an informed consent form prior to starting the study. Participants had clinical evidence (history, physical exam, and MRI findings, below) suggesting a rotator-cuff tear and had failed at least 6 mo of conservative management of RCI (which usually includes physiotherapy) at the time they consented for surgery.

**MRI.** MRI scans took place at the Pan Am Clinic (Winnipeg, MB) and were performed using a 1.5-T Siemens MAGNETOM scanner (Siemens, Erlangen, Germany). The occupation ratio, defined as the cross-sectional area of the supraspinatus muscle belly in relation to the cross-sectional area of the supraspinatus fossa, was used as a quantitative measure of atrophy, and calculated from the scapular Y-view (37, 43). An occupation ratio of <50% indicates supraspinatus muscle atrophy (37). The tangent sign (43) is a qualitative indicator of atrophy. In the Y-view a line is drawn connecting the coracoid process to the scapular spine; a positive tangent sign is observed when the supraspinatus muscle belly lies completely below that line (37). To assess fatty infiltration, the classification system of Goutallier (10) for intramuscular fat, as follows. A grade of 0 indicates no intramuscular fat; 1 indicates small streaks of fat without significant accumulation; 2 corresponds to significant fat but less than the amount of muscle; 3 describes equal amounts of fat and muscle; and 4 indicates there is more fat than muscle.

**Tissue collection.** Biopsies of RCI-affected supraspinatus and healthy, ipsilateral deltoid muscle were obtained during arthroscopic surgical repair. The deltoid muscle was used as a control to the affected supraspinatus as it is not affected in patients reporting RCI symptoms, and the contralateral supraspinatus is often affected even though it may be asymptomatic (42). Another study demonstrating a reduction in fiber diameter with increasing pathology of the rotator cuff, from partial- to full-thickness tears (11), also used the deltoid muscle as a control and reported that in the presence of supraspinatus pathology on one side, changes were more evident in the contralateral supraspinatus muscle than the deltoid muscle ipsilateral to the RCI. To minimize bias, the identity of muscle samples was coded until after data analysis. Parts of each biopsy were prepared for histology, a culture study of the capacity for NO-induced SC activation, and for protein study by Western blotting.

**Histology.** Muscle was fixed in 4% paraformaldehyde in phosphate-buffered saline and prepared for sectioning (7 μm thick); sections were collected onto slides for morphometry after staining with hematoxylin and eosin (H & E) (35). Slides were visualized and photographed using a digital Sentea camera and imaging software (StCamSoftware v1.0.0.9).

A second set of slides was collected for immunostaining to analyze the pattern of AChR distribution at NMJs identified by direct fluorescence (14). In brief, slides were washed, fixed with acetone, incubated with fluorescein-conjugated α-bungarotoxin (Invitrogen, F1176, Carlsbad, CA), and mounted with Vectashield media (Vector Labs H1000, Burlingame, CA). Coded sections were scanned systematically, and up to 20 nonoverlapping fields (depending on biopsy size) were photographed using a 40X oil immersion lens on a Zeiss Atpomone (Jena, Germany) for analysis.

**SC activation in culture.** A portion of each biopsy was divided in two and cultured in 60-ml petri dishes at 37°C with 5% CO2 for 40 h in basal growth medium (41) with 0.65 μM BrdU, with or without 1 mM ISDN, based on our previous report (18). Each piece was initially a maximum of 2–3 mm in any dimension, and due to the nature of fibers collected and cut in the biopsy, small bundles of fibers fanned out, once immersed in the culture medium. The 40-h time point was selected to restrict the time for BrdU incorporation into DNA to only one S-phase of the cell cycle. The need to accommodate the anticipated time lag in activation (following biopsy manipulation) in participants aged 49–65 yr-of-age was based on previous reports in

### Table 1. Summary of demographic data and MRI findings

<table>
<thead>
<tr>
<th>Value</th>
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<tr>
<td>Age, yr</td>
<td>57.6 ± 1.5 (range 45–69)</td>
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<tr>
<td>Sex, Male, n = 8; Female, n = 5</td>
<td></td>
</tr>
<tr>
<td>Shoulder affected</td>
<td>Right, n = 9; Left, n = 4</td>
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<tr>
<td>Height, m</td>
<td>1.74 ± 0.03</td>
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<tr>
<td>Weight, kg</td>
<td>92.4 ± 5.6</td>
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<td>BMI, kg/m²</td>
<td>30.4 ± 1.4</td>
</tr>
<tr>
<td>Smoking history</td>
<td>Yes, n = 4; No, n = 9</td>
</tr>
<tr>
<td>Injury mechanism</td>
<td>Traumatic, n = 9; unknown, n = 4</td>
</tr>
<tr>
<td>MRI findings</td>
<td>SS, supraspinatus; FT, full tear; PT, partial tear; IS, infraspinatus; TM, teres major.</td>
</tr>
<tr>
<td>SS tear</td>
<td>1 PT, 12 FT</td>
</tr>
<tr>
<td>SS tear thickness, cm</td>
<td>2.24 ± 0.30 cm</td>
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<tr>
<td>Muscles affected by tear</td>
<td>SS, n = 9; SS+IS, n = 1; SS+TM, n = 2; md = 1</td>
</tr>
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<td>Tangent sign</td>
<td>Negative, n = 10; positive, n = 1, md = 2</td>
</tr>
<tr>
<td>Occupancy ratio</td>
<td>0.75 ± 0.06, md = 2</td>
</tr>
<tr>
<td>Goutallier classification</td>
<td>1.18 ± 0.23 (range 0 to 3), md = 2</td>
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Values are means ± SE. Demographic data are from 13 participants. For MRI findings, 2 participants had tear described and measured in MRIs, but other data were not available; these are recorded as missing data (md). BMI, body mass index; SS, supraspinatus; FT, full tear; PT, partial tear; IS, infraspinatus; TM, teres major.

Fig. 1. Representative preoperative MRI Y-shaped views of participants with a rotator-cuff tear, in which a line is drawn from the coracoid process (C) to the apex of the scapular spine (S). A: in this MRI, the supraspinatus muscle belly lies above the line, so the tangent sign is considered negative. B: in this MRI, the supraspinatus muscle belly lies below the line connecting the coracoid process and the apex of the scapular spine, indicative of a positive tangent sign. Orientation is indicated as superior (sup), inferior (inf), anterior (ant), and posterior (post), and supraspinatus is indicated by an asterisk (*)
old-roodent muscle (1, 17, 18). Following incubation, tissue was fixed and sectioned, and slides were stored at −20°C until use.

Sections were processed for immune detection of BrdU and Pax7 using double immunofluorescence to identify mitotically active SCs, as reported (13, 28). Overnight incubation at 4°C with a primary antibody solution containing mouse anti-BrdU (1:200, 11–170376001, Roche, Basel, Switzerland) and rabbit anti-Pax7 (1:150, ab34360, AbCam, Cambridge, MA) was followed by quenching of endogenous peroxidase with 3% hydrogen peroxide (10 min). Sections were washed and incubated (1 h) with secondary antibodies: Dylight 488-conjugated goat anti-rabbit (1:200, 111–487–003) and Dylight 649-conjugated goat anti mouse (1:200, 115–497–003, Jackson ImmunoResearch), washed, and mounted (Vectashield, Vector Labs).

Coded sections were scanned systematically and 20 nonoverlapping fields (depending on biopsy size) were photographed using a 40X oil immersion lens on a Zeiss ApoTome. Fluorescent SCs were counted and tabulated in Excel as activated (BrdU+/Pax7+) or not (BrdU−/Pax7−) in each section, and the proportion of activated SCs was calculated (the number of BrdU+/Pax7+ SCs divided by the total number of Pax7+ SCs). The change in activation induced by ISDN was calculated as the proportion of activated SCs after ISDN divided by the baseline level of activation in the paired untreated biopsy from the same participant.

Immunoblotting for AChR subunits. The levels of γ and ε AChR subunit proteins were quantified using Western blotting according to a standard protocol (19) with modifications to detect multiple epitopes from the same membrane (39). In brief, samples were placed in RNA-later stabilizing solution (Life Technologies, Burlington, Canada) and frozen until protein extraction. Total protein was assayed on a standard curve using a Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Rockford, IL). Samples (20 µg protein) were loaded on 9% polyacrylamide gels and run for 1 h before transfer (100 V for 1 h) to polyvinylidene fluoride membranes (Fisher Scientific, Ottawa, Canada). Membranes were first probed using goat anti-ε AChR (diluted 1:150, sc-1454, Santa Cruz, Dallas TX) and incubated with bovine anti-goat-HRP secondary antibody (1:300, sc-2350, Santa Cruz). Bands were visualized using chemiluminescence and imaged using a VersaDoc chamber linked to the QuantityOne software program (BioRad, Hercules, CA). Membranes were quenched in 27% hydrogen peroxide, and serially reprobed, visualized, and quantified for additional epitopes (39), including γ AChR subunit using rabbit anti-γ AChR (1:250, sc-13998, Santa Cruz) detected using donkey anti-rabbit-HRP secondary antibody (1:5,000, NA-9340V, GE Healthcare Life Sciences, Piscataway, NJ) and β-actin using a cocktail of mouse anti-β-actin primary antibody (1:1,000, sc-81178, Santa Cruz) and goat anti-mouse-HRP secondary antibody (1:5,000, A2304, Sigma Aldrich, St. Louis, MO). Membranes were stained in Ponceau Red (Sigma, St. Louis, MO) to visualize and quantify total protein loaded in each sample.

Statistics. Data were compiled in Microsoft Excel spreadsheets for analysis and graphing (mean, SE). Parametric tests of score data, nonparametric Chi-squared tests (to study distributions), and correlations and linear regressions were used to analyze the findings collected from assays in different parts of the biopsy from each participant, as appropriate, using Excel or JMP-SAS statistical software. A probability of P < 0.05 was used to indicate statistical significance. Power analyses were conducted post hoc, using data for the distribu-
tion of the AChR staining pattern at NMJs and the γ:ε ratio of AChR subunits.

RESULTS

Participants and surgery. Fifteen participants completed an informed consent form and were enrolled in the study. Two were excluded intraoperatively; one had no rotator-cuff tear at arthroscopy, and there were technical difficulties at the time of biopsy retrieval in the other. MRI data were missing in two participants; therefore the study included 13 participants overall, with 11 for MRI analysis. Demographic data are reported in Table 1. Average time from symptom onset to surgery was 142 wk, although one participant had surgery 10 yr after onset; without this participant, the average symptomatic period was 103 wk from injury to surgery. Arthroscopy confirmed a full-thickness rotator-cuff tear in 12 participants and a partial-thickness tear in one. Other diagnoses made at surgery included one labral tear, one partial subscapularis tear, three Type-2 superior labral tear from anterior to posterior (SLAP) lesions, one SLAP I lesion, an AC arthrosis in two participants, and one teres minor with mild to moderate muscle atrophy. Rotator-cuff repair was performed in nine participants (all full-thickness tears); biceps tenotomy \( (n = 9) \), acromioplasty \( (n = 12) \), bursectomy \( (n = 5) \), and labral debridement \( (n = 2) \) were also performed during surgery in these participants.

MRI. Representative MRIs are shown in Fig. 1. MRI findings are summarized in Table 1 \( (n = 11) \), including the size of the rotator cuff tear of supraspinatus, muscles affected by the tear, tangent sign, occupancy ratio, and Goutallier classification. Two participants had tear extension to the infraspinatus tendon. The occupancy ratio was 0.75 ± 0.06 (mean ± SE) with a median Goutallier classification score of 1 (range: 0–3).

AChR distribution at NMJs. NMJ staining appeared as tightly defined spots on fiber membranes in both muscles. NMJs were classified along a range of complexity as one of a singlet, a doublet, a cluster (3 or more dots less than 2.5 μm apart), or a line (three or more dots in a straight, linear arrangement), as illustrated in Fig. 4. There were 42–209 NMJs classified for each biopsy. In both muscles, the majority of AChRs appeared as single dots of α-bungarotoxin fluorescence with a diameter of 1.5–2.5 μm on the sarcolemma.

The pattern of NMJs was analyzed by the proportions of AChRs distributed as clusters (representing innervated fibers) or lines (representing denervated fibers) in each of the supraspinatus and deltoid muscles, as visualized with α-bungarotoxin fluorescence. Singlet and doublet patterns were not included as they could not be distinguished from lines or clusters sectioned in a different plane. The distribution of staining pattern (proportion of clusters vs. lines) did not differ

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Fig. 4. Representative micrographs of neuromuscular junctions (NMJs) observed by direct fluorescence using α-bungarotoxin staining. Singlet, doublet, and cluster describe one, two, or three or more dots in close proximity, respectively; a line is defined as three or more dots in a row. Pairs of images in fluorescence (top row) and DIC (bottom row) show a characteristic singlet (A), doublet (B), line (C), and cluster (D) from supraspinatus (A and C) and deltoid muscles (B and D). Scale bar, 10 μm.
between supraspinatus and deltoid ($P = 0.3$). The proportion of clusters was 0.49 in supraspinatus (923 NMJs photographed) and 0.51 in deltoid (675 NMJs photographed). The proportion of linearly arranged AChRs in the deltoid increased with age ($R^2 = 0.42, P = 0.02$); however, this correlation was not noted for the RCI supraspinatus.

**AChR subunits.** Two AChR subunits, $\gamma$ and $\varepsilon$, were quantified by Western blot, as an indicator of innervation status in the muscles, $\gamma$ representing AChRs on denervated fibers and $\varepsilon$ representing AChRs on normal, innervated fibers (Fig. 5, A and B). The amount of the $\gamma$ subunit protein was highly variable; relative to total protein, neither subunit differed between supraspinatus and deltoid. Supraspinatus showed a tendency toward a higher $\gamma:\varepsilon$ AChR ratio than the deltoid ($P = 0.13$, Fig. 5C). The ratio of $\gamma:\varepsilon$ AChR subunits in supraspinatus increased with age ($R^2 = 0.44, P = 0.02$), but this was not the case for the ratio from deltoid muscle. There was no statistical relationship between the $\gamma:\varepsilon$ AChR subunit ratio and the proportion of clustered AChRs at NMJs, analyzed for separate muscles or pooling data for the two muscles.

**SC activation.** Pax7+ cells were most often located in a peripheral position around muscle fibers, as expected for SCs (Fig. 6). The pattern of sarcomeres was retained in fiber segments throughout the biopsy, indicating that conditions including oxygenation were able to maintain tissues in culture. In all sections, the number of nonproliferating Pax7+/BrdU−SCs (Fig. 7) was larger than the number of activated and proliferating Pax7+/BrdU+ SCs. In cultured supraspinatus biopsies, ISDN increased the proportion of BrdU+/Pax7+ SCs compared with the baseline level in untreated supraspinatus ($P < 0.03$); by contrast, deltoid muscle SCs showed no change in activation in response to ISDN compared with baseline.

Scatterplots were used to consider possible statistical relationships between activation (baseline and change with ISDN treatment), NMJ staining (proportion of clusters), and age in supraspinatus and deltoid together (Fig. 8A) and between NMJ staining, fiber diameter, $\gamma:\varepsilon$ AChR subunit ratio, and occupancy ratio (MRI) in supraspinatus (Fig. 8B). Correlations were made on data compiled for each participant and included variables from assessment of any of the 3 parts of each biopsy.

There was no statistical relationship between the proportion of clusters at NMJs and the change in SC activation induced by ISDN (Fig. 8A1). The proportion of clusters at NMJs decreased with increasing age ($R^2 = 0.24, P < 0.05$, Fig. 8A2). Baseline SC activation only tended to be related to the proportion of clusters at NMJs ($R^2 = 0.05, P = 0.15$, Fig. 8A3). Interestingly, there was a negative relationship between baseline SC activation and the change in SC activation with ISDN, in that those biopsies with the lowest baseline had the highest increase in activation after ISDN ($R^2 = 0.28, P = 0.01$, Fig. 8A4). Considering only the RCI supraspinatus biopsies, the propor-
tion of clusters at NMJs was not significantly related to mean fiber diameter ($R^2 = 0.14$, Fig. 8B1). The occupancy ratio of supraspinatus by MRI was negatively related to the proportion of clusters at NMJs ($R^2 = 0.39$, $P = 0.05$, Fig. 8B2), negatively related to the $\gamma:\varepsilon$ AChR subunit ratio ($R^2 = 0.18$, $P = 0.05$, Fig. 8B3), and positively related to the mean fiber diameter in supraspinatus ($R^2 = 0.15$, $P = 0.05$, Fig. 8B4).

**DISCUSSION**

The most novel finding of this study was that SCs in the supraspinatus muscle with RCI responded to activation by ISDN whereas those in the control deltoid muscle did not. In the same participants, the supraspinatus was atrophic both by MRI assessment and histology, the latter compared with a control muscle, the contralateral deltoid. The finding of supraspinatus atrophy is consistent with previous literature on mouse models of RCI or denervation, and other studies on human muscle atrophy and neuromuscular disorders (9, 11, 15, 21, 24, 32, 38, 45). To examine the mechanism of atrophy (disuse vs. denervation), the innervation status of RCI supraspinatus muscle was analyzed for the first time using two indices in the same biopsy: the pattern of AChR staining in NMJs and the $\gamma:\varepsilon$ ratio of AChR subunits. Although neither index of innervation status gave significant results, at least partly due to a small sample size, the ratio of $\gamma:\varepsilon$ AChR protein subunits tended to be higher in the RCI supraspinatus than in control deltoid. Together with the findings of SC responsiveness and atrophy, the trend in innervation status is interpreted as leaving open the possibility that denervation plays a role in RCI pathophysiology. For instance, partial denervation of the supraspinatus muscle (not captured in this small sample as changes in NMJ clusters or the $\gamma:\varepsilon$ ratio of AChR subunits) could theoretically account for the responsiveness of SCs in that muscle to activation by the ISDN stimulus in culture due to disuse in RCI. It would be interesting to investigate whether there is fiber-type grouping and/or larger motor units in supraspinatus (due to collateral axon sprouting in compensation...
for partial denervation) in those with RCI who progress to reparative surgery. Notably in this study, the ipsilateral deltoid served as a control, since the contralateral supraspinatus is often affected asymptomatically in unilateral RCI. As well, the deltoid is reportedly not affected in patients with symptoms of RCI (42). Although the potential for some disuse atrophy in the ipsilateral deltoid (in accommodating the symptomatic supraspinatus) cannot be ruled out, the observed statistical differences between the two muscles would be even more important if the deltoid were also atrophied compared with deltoid in a healthy age-matched individual. Therefore, this study was designed to biopsy ipsilateral deltoid muscle as a comparison to the supraspinatus muscle of RCI participants, and control for influences of age, life history, and activity, on the two muscles. While all participants had failed at least 6 mo of conservative treatment when they consented for surgery, the effects of physiotherapy treatment on the supraspinatus and deltoid muscle of the affected shoulder were beyond the scope of this study and would be of future interest in relation to present findings on muscle fiber diameter, SC responsiveness to an activating stimulus, and innervation status.

SCs in supraspinatus were activated by a NO-donor drug, despite atrophy and possible contribution by denervation, whereas SCs in the control deltoid muscle were not. SCs from RCI supraspinatus were more activated after culture with ISDN relative to baseline and did not differ from the baseline of a healthy muscle, which could increase SC sensitivity to the NO-donor drug, and raise SC activation up to the baseline level seen in control muscle. This reasoning would fit with the proposed U-shaped model of SC activation as a function of NO concentration, previously reported (40), since the SC-activation response to ISDN observed in biopsies from individual participants ranged widely. For instance, two supraspinatus biopsies with the highest level of ISDN-induced SC activation (Fig. 8A1), a relative 1.8-fold increase from baseline, were from a 65 year-old male and a 56 year-old female. Both were smokers with relatively high BMI (35 and 36 kg/m²), and had surgery at 73 and 69 wk post-onset; their supraspinatus mean fiber diameter was 16 and 23.7 μm, respectively (Fig. 8B1). Conversely, injured supraspinatus may have a relatively low level of NO due to disuse (and/or possible partial denervation) compared with a healthy muscle, which could increase SC sensitivity to the NO-donor drug, and raise SC activation up to the baseline level seen in control muscle. This reasoning would fit with the proposed U-shaped model of SC activation as a function of NO concentration, previously reported (40), since the SC-activation response to ISDN observed in biopsies from individual participants ranged widely. For instance, two supraspinatus biopsies with the highest level of ISDN-induced SC activation (Fig. 8A1), a relative 1.8-fold increase from baseline, were from a 65 year-old male and a 56 year-old female. Both were smokers with relatively high BMI (35 and 36 kg/m²), and had surgery at 73 and 69 wk post-onset; their supraspinatus mean fiber diameter was 16 and 23.7 μm, respectively (Fig. 8B1). Two other supraspinatus biopsies showed a decrease in SC activation after ISDN to 0.7-fold of their respective baseline levels. These participants, both male (53 and 65 yr old), had lower BMI (27 and 25 kg/m²) and
supraspinatus mean fiber diameter (8 and 20 μm, respectively) at surgery (61 and 100 wk post-onset, respectively). Based on recent reports that semaphorin3A, a putative axon-guidance molecule, is secreted by SCs after activation and early differentiation (7, 7, 30, 34, 36), possible changes in SC-derived semaphorin3A with age, injury, or inflammation in humans may be related to innervation status and the potential for successful surgical repair of RCI.

Muscle atrophy was assessed using both histology and MRI occupancy ratio. Fiber diameter in supraspinatus was statistically related to occupancy ratio despite the variable interval between MRI and surgery in study participants (36.3 ± 4.5 wk). RCI and injury to the supraspinatus nerve (25) leads to muscle atrophy and fibro-fatty infiltration (15) after both partial- and full-thickness tears (24), and similar findings were reported in a mouse model of massive RCI (22). Fiber atrophy accompanied by fibro-fatty infiltration (Goutallier sign), low occupancy ratio, and a trend to a higher \( \gamma: \varepsilon \) ratio of AChR subunits would be consistent with denervation, as demonstrated in rat experiments (9, 21, 45), clinical reports on neuromuscular and neurogenic disorders (9, 45), and findings that supraspinatus nerve damage limits active movement by supraspinatus muscle (25). It was interesting that \( \gamma: \varepsilon \) AChR-subunit ratio and the proportion of AChR clusters at NMJs observed by α-bungarotoxin staining were not statistically related, possibly as the molecular-level change in subunit ratios would be consistent with denervation, as demonstrated in rat experiments (9, 21, 45), clinical reports on neuromuscular and neurogenic disorders (9, 45), and findings that supraspinatus nerve damage limits active movement by supraspinatus muscle (25). It was interesting that \( \gamma: \varepsilon \) AChR-subunit ratio and the proportion of AChR clusters at NMJs observed by α-bungarotoxin staining were not statistically related, possibly as the molecular-level change in subunit composition in each receptor complex may occur before or after redistribution of receptor clusters and NMJ remodeling at the cellular level (9, 45). As well, the distribution of AChRs in the staining pattern of NMJs could be influenced by the diameter range in each biopsy (smaller fibers would theoretically increase the number of NMJs sampled in a section through the motor end-plate region). Nonetheless, the novel application of two indices of denervation in arthrosopic biopsies of muscle in RCI suggests further study of the possible implication of denervation in RCI would be useful, now informed by power analyses showing for human RCI that the prevalence of injuries such as RCI with age (20). Although findings implicate denervation in RCI, they do not establish denervation in the etiology of RCI. However, the present findings suggest the value of exploring the innervation status of RCI supraspinatus in the clinic, as well as other factors such as vascularization, to assist decision making about the approach to treatment, since even anatomically effective tendon repair will not restore function in the absence of innervation. These experiments are the first, to our knowledge, to study the physiology of human SCs concurrent with cellular and molecular indicators of innervation status in RCI supraspinatus muscles. The clinical potential for a treatment based on NO delivery to muscle in RCI patients may be useful in promoting supraspinatus growth as a means of helping restore muscle strength to achieve maximal functional outcome after tendon repair.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


