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Differential ubiquitin-proteasome and autophagy signaling following rotator cuff tears and suprascapular nerve injury

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Abstract

Previous studies have evaluated role of Akt/mTOR signaling in rotator cuff muscle atrophy and determined that there was differential in signaling following tendon transection (TT) and suprascapular nerve (SSN) denervation (DN), suggesting that atrophy following TT and DN was modulated by different protein degradation pathways. In this study, two muscle proteolytic systems that have been shown to be potent regulators of muscle atrophy in other injury models, the ubiquitin-proteasome pathway and autophagy, were evaluated following TT and DN. In addition to examining protein degradation, this study assessed protein synthesis rate following these two surgical models to understand how the balance between protein degradation and synthesis results in atrophy following rotator cuff injury. In contrast to the traditional theory that protein synthesis is decreased during muscle atrophy, this study suggests that protein synthesis is up-regulated in rotator cuff muscle atrophy following both surgical models. While the ubiquitin-proteasome pathway was a major contributor to the atrophy seen following DN, autophagy was a major contributor following TT. The findings of this study suggest that protein degradation is the primary factor contributing to atrophy following rotator cuff injury. However, different proteolytic pathways are activated if SSN injury is involved.

Keywords

rotator cuff tear; denervation; muscle atrophy; ubiquitin-proteasome; autophagy; protein synthesis

Introduction

Rotator cuff tears (RCTs) are one of the most common injuries seen by orthopaedic surgeons that result in chronic pain and limited shoulder function. One of the major pathological outcomes seen following a chronic tear is muscle atrophy[1, 2]. While previous studies have suggested that atrophy can be decreased and improved via surgical cuff repair, up to 70% of patients with a large tear suffer a re-tear or poor outcomes after repair due in part to progressive muscular atrophy and degeneration that impairs shoulder function[3–5]. Suprascapular nerve (SSN) entrapment injury at the scapular notch due to muscle retraction after large or massive cuff tears has been suggested to increase the degree of muscle atrophy and degeneration[6–8]. Our previous study has shown that rotator cuff muscle atrophy after SSN injury (Denervation, DN) is more severe compared to that after tendon transection (TT)[9]. This data suggested that there may be an intrinsic difference in rotator cuff muscle atrophy following TT and DN.

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Normal muscle mass is maintained by the balance between protein synthesis and protein degradation. Previous studies in other injury models have suggested that muscle atrophy results from the imbalance between these two critical components[10–14]. However, to our knowledge, the role of protein synthesis and degradation in rotator cuff muscle atrophy following RCTs has never been assessed.

The ubiquitin-proteasome pathway and autophagy are two key protein degradation mechanisms that have been reported in skeletal muscle atrophy[15–21]. In our previous study, we saw increased expression of two E3 ubiquitin ligases, MuRF1 and MAFbx, which are key members of the ubiquitin-proteasome pathway in supraspinatus muscles following DN but not TT[9]. This data suggested that there may be a dichotomy in protein degradation mechanisms following TT and DN in rotator cuff muscles. Other studies have reported that autophagy, a cellular process utilizing lysosomal machinery to remove excessive or damaged cellular components, plays a pivotal role in muscle atrophy [19–21]. Apart from a recent key study from Gumucio *et al.* that showed up-regulation of several autophagic markers[22], the role of autophagy in rotator cuff muscles remains largely unknown.

Despite increasing knowledge regarding the importance of muscle changes after RCTs, relatively few studies have investigated the molecular pathways responsible for the development of atrophy following this injury. The goal of this study is to determine how protein synthesis and two key protein degradation mechanisms, the ubiquitin-proteasome pathway and autophagy are regulated in the setting of progressive muscle atrophy after TT and DN. Based on our previous study that evaluated the mammalian target of rapamycin (mTOR) pathway in rotator cuff muscles following TT and DN[9], we hypothesize that muscle atrophy following TT is primarily driven by decreased protein synthesis and increased autophagy while muscle atrophy after DN is stimulated by increased proteasome-mediated protein degradation.

Materials & Methods

Animal Surgery

Thirty-six adult male Sprague Dawley rats (Charles River Laboratories Inc., Wilmington, MA) that initially weighed 250 g were divided into two groups. In order to simulate a RCT, the first group (N=18) underwent a complete supraspinatus and infraspinatus tendon transection (TT) on the right shoulder. As previously described, five mm long fragments at each tendon were removed in order to prevent tendon reattachment [9]. Suprascapular nerve denervation (DN) was performed on the second group (N=18) where a five mm long segment of the suprascapular nerve was removed while leaving the rotator cuff intact. Sham surgery was performed on the contralateral side to serve as an internal control. The animals in both TT and DN groups were then randomly divided into three subgroups to evaluate autophagy, the ubiquitin-proteasome pathway, and protein synthesis (N=6 per subgroup). All procedures were approved by San Francisco Veterans Affairs Medical Center (SFVAMC) Institutional Animal Care and Use Committee (IACUC).

Muscle harvest

All animals were sacrificed at two weeks after surgery as a majority of muscle atrophy was seen at the two week time-point. Supraspinatus muscles from both surgical and sham sides were harvested and the remaining tendon and scar tissue were removed at the muscle/tendon junction. The wet weight of supraspinatus muscles from both surgery groups was measured immediately for analysis of muscle atrophy.

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Evaluation of autophagy

(a) Western-blot analysis—Supraspinatus muscles were homogenized in 500 μL of T-PER solution (Pierce Biotechnology Inc., Rockford, IL.) with a protease inhibitor cocktail (Sigma-Aldrich Inc., St. Louis, MO) for total protein extraction[9]. The homogenates were centrifuged at 2,500 × *g* at 4 °C for 10 min. After determining the protein concentration, 50 μg of protein from cytosolic fractions was loaded on 10% NUPAGE Bis-Tris gels and transferred to PVDF membranes (Invitrogen Inc., Carlsbad, CA) that were blocked and incubated in primary and secondary antibodies as previously described[9]. The following rabbit-anti-rat primary antibodies were used at a dilution of 1:200 to 1:1000: anti-LC3B and anti-β actin. A HRP conjugated goat-anti-rabbit secondary antibody (Cell Signaling Technology Inc., Danvers, MA) was used at a dilution of 1:10,000. Blots were developed using enhanced chemiluminescence (ECL) plus reagent (GE Healthcare Inc., Piscataway, NJ), imaged, and quantified using ImageJ Software (NIH).

(b) Quantitative Polymerase Chain Reaction (qPCR)—To isolate total RNA, supraspinatus muscles were homogenized in 500 μL of Trizol® reagent according to the manufacturer's instructions. Isolated RNA was quantified and normalized to synthesize cDNA as previously described [9]. RT-PCR was performed to quantify the expression of autophagy markers in muscle samples using a SYBR Green I Master kit (Roche Applied Bioscience, Indianapolis, IN) with the following primers: LC3B: (forward) 5′-AGTGGAAGATGTCCGGCTCAT-3′ and (reverse) 5′-GCTGCTTCTCACCCTTGTATCG-3′; GABARAPL1: (forward) 5′-CATCGTGGAGAAGGCTCCTA-3′ and (reverse) 5′-ATACAGCTGTCCCATGGTAG-3′; ATG12: (forward) 5′-GGCCTCGGAGCAGTTGTTTA-3′ and (reverse) 5′-CAGCATCAAAACTTCTCTGA-3′; and ATG4B: (forward) 5′-CAGTGTCCTCAACGCTTTCCT-3′ and (reverse) 5′-TAGACTTGCCTTCGCCAACTC-3′. Gene expression was normalized to the house keeping gene, GAPDH[9]. Fold change in mRNA expression was calculated by using ΔΔCT.

(c) Cathepsin L Assay—Cathepsin L is a lysosomal protease that degrades proteins and other cellular components that are sent to the lysosome. It performs a major role in the autophagic process[23, 24]. Supraspinatus muscles from both surgical groups were lysed to measure Cathepsin L activity based on a method validated by Kim *et al.*[25]. Cathepsin L activity was measured using a fluorescence assay kit (BioVision Inc., Milpitas, CA). The plate was read using the Synergy 2 microplate reader (BioTek Instruments, Winooski, VT) at the appropriate wavelength for 1 hour. Cathepsin L activity was recorded every 5 minutes. The relative fluorescence unit reading of each sample was normalized to their protein concentration. The assay was performed in triplicates.

Evaluation of the ubiquitin-proteasome pathway

20S Proteasome Activity Assay—We chose to evaluate 20S proteasome activity as the 20S subunit is the catalytic core of the proteasome complex. To isolate proteasome from supraspinatus muscles, we used a previously established method[26] where muscle samples from each surgery group were homogenized in 1.5 mL of buffer (20 mM Tris, 20 mM KCl, 10 mM magnesium acetate, 2 mM DTT, 10% glycerol, pH 7.6). The homogenate was then centrifuged at 30,000 × *g* at 4 °C for 30 minutes. The resulting supernatant was then centrifuged at 100,000 × *g* at 25 °C for 6 hrs. The final pellet was washed and re-suspended in 1 mL of the aforementioned buffer. 50 μg of protein sample was used to measure 20S proteasome activity using the manufacturer's protocol (Millipore, Billerica, MA)[27, 28]. The assay was performed in triplicates and was read using a Synergy 2 microplate reader (BioTek Instruments) at the appropriate wavelength.

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Evaluation of protein synthesis

In Vivo SUnSET—To measure the change of *in vivo* protein synthesis, we used a recently developed nonradioactive technique called surface sensing of translation (SUnSET) [11, 29]. Rats from each surgery group were anesthetized and given an intraperitoneal injection of 0.04 μmol/g puromycin dissolved in 1 mL of PBS[29]. Rats were under general anesthesia with 2% isoflurane in oxygen after injection. After exactly thirty minutes, supraspinatus muscles were harvested and prepared for western blotting as described above. Gel loading was verified via coomassie blue staining.

Statistical Analysis

A paired t-test was used for data analysis between surgical and sham sides where appropriate. A paired t-test was also used to analyze 20S proteasome results. An ANOVA with a Tukey post hoc comparison was used for data analysis to compare differences among groups (TT, TT sham, DN, and DN sham) where appropriate. Significance was defined as a $p < 0.05$. Data are presented as the mean ± SD. For RT-PCR, data is presented as fold change of the surgery vs. sham groups (mean ± standard error).

Results

Muscle atrophy after two weeks

Two weeks after TT and DN surgery, the supraspinatus muscles (N=6 per surgical group) exhibited significant muscle weight loss. The wet weight of supraspinatus muscle reduced 25.5 ± 9.1 % after TT and 56.2 ±7.9% after DN compared to the contralateral control shoulder (p<0.007 vs. control). Consistent with our previous finding, DN caused significantly increased muscle weight loss compared to TT alone (p=0.002)[9]. (Figure 1)

LC3B II activity was up-regulated following tendon transection

The gold standard of autophagy, LC3BII, was significantly up-regulated following two weeks of TT compared to DN and control (N=6 per surgical group). The ratio of cytosolic LC3BII to LC3BI (also known as autophagic flux) was significantly higher following TT than DN (p<0.001). This difference was independent of sham surgery as no significant difference was observed between TT and DN sham (p=0.36). There was also a significant difference in flux between TT and TT sham (p<0.001). However, there was no difference between DN and DN sham (p=0.21). (Figure 2)

Up-regulation of autophagy markers following tendon transection

Real-time PCR results demonstrated that expression of LC3B and ATG12 at the mRNA level was significantly increased (8.4 ± 4.2 fold for LC3B and 13.3 ± 8.2 fold for ATG12) in supraspinatus muscles following TT (p<0.05) but not following DN (0.94 ± 0.1 fold for LC3B and 0.95 ± 1.2 fold for ATG12) (p=0.22) (N=6 per surgical group). While the expression level of GABARAPL1 was higher in supraspinatus muscles following TT, it did not reach significance (TT: p=0.06, DN: p>0.05) (2.2 ± 1.9 fold following TT and 1.4 ± 0.2 following DN). (Figure 3)

Increase in Cathepsin L activity following tendon transection

Lysates of supraspinatus muscles following TT showed a significant increase (p<0.01) in cathepsin L enzyme activity per μg of protein compared to lysates following DN (TT: 20.06 ± 1.84; DN: 14.87 ± 3.77) (N=6 per surgical group). A significant difference in Cathepsin L activity was also seen between TT and TT sham surgery (p<0.001) and between DN and DN sham surgery (p=0.007). However, no difference was observed between TT and DN sham

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surgeries (p=0.999). This again confirmed that the difference we observed in Cathepsin L activity between TT and DN is independent of sham surgery. (Figure 4)

Increase in 20S proteasome activity following denervation

The amount of 20S proteasome activity in relation to sham surgery was significantly greater in supraspinatus muscles after 2 weeks of DN (2.36 ± 1.09) than TT (0.84 ± 0.32) ($p<0.05$) (N=6 per surgical group). (Figure 5)

Increase in rate of protein synthesis following tendon transection and denervation

There was a significant difference seen in the rate of protein synthesis between TT and sham group ($p<0.0001$) (N=6). There was also a significant difference seen in the rate of protein synthesis between DN and sham group ($p<0.0001$) (N=6). Protein synthesis was higher in both surgical groups compared to sham surgeries as evident by quantification. (Figure 6)

Discussion

The goal of this study was to evaluate the molecular mechanisms of atrophy following tendon transection and SSN injury. As we had hypothesized, our results suggest that muscle atrophy following SSN denervation is mainly regulated by the ubiquitin-proteasome pathway and atrophy following tendon transection is mediated by autophagy. In contrast to our hypothesis, the protein synthesis rate was increased following both tendon transection and SSN denervation. This data suggests that activation of muscle protein degradation pathways is the primary factor responsible for muscle atrophy after RCTs.

Our current study, in addition to our previous finding[9], suggests that muscle atrophy following SSN denervation is primarily mediated by the ubiquitin-proteasome pathway. Interestingly, this pathway had a minimal contribution to the muscle atrophy observed following tendon transection. This is consistent with a recent study conducted by Gumucio *et al.* in rat RCT model[22]. Many previous studies have confirmed the role of ubiquitin-proteasome pathway in muscle atrophy following denervation. Using a sciatic nerve denervation model, Sacheck *et al.* demonstrated that MuRF-1 and MAFbx were significantly up-regulated after three days[17]. We saw a similar trend (18 fold increase in MuRF-1 and 11 fold increase in MAFbx) following two weeks of SSN denervation[9]. Other studies have shown that inhibition of these two genes results in a decrease in muscle atrophy following denervation, thereby providing further evidence of how critical the ubiquitin-proteasome degradation pathway is in atrophy following denervation of muscles[15, 16]. Though the detailed mechanisms remain unknown, our data in addition to other studies suggests that this pathway serves as a central regulator of muscle protein degradation following nerve injury.

While the ubiquitin-proteasome system was increased in denervated supraspinatus muscles, our results suggest that autophagy facilitates the atrophic process following tendon transection. Autophagy is characterized by the commitment of double membrane vesicles to autophagosomes, which ultimately fuse with the lysosome[18, 24, 30–32]. This commitment step is mediated by ubiquitin-like molecules such as LC3B and ATG12, which were significantly increased following tendon transection in our study. Though there are multiple methods to measure the autophagic process, there is general consensus that the ratio of LC3BII/LC3BI provides a reliable indication of the autophagic flux[33]. Our analysis of autophagic flux further supports that autophagy is activated in rotator cuff muscles following tendon transection. Though a slight increase of cathepsin L activity is also observed in rotator cuff muscles after denervation, no change of autophagy markers was observed. Thus, we think the slight increase of lysosome cathepsin L activity in denervated muscles is independent of autophagy.

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Previous studies have demonstrated that mTOR signaling plays a critical role in regulating autophagy by suppressing the autophagy initiation complex, ULK1/Atg13/FIP200[32, 34, 35]. In our pervious study, we have shown that phosphorylation of mTOR was decreased in rotator cuff muscles following tendon transection, but increased following denervation. This may explain the different pattern of autophagy activity we observed in rotator cuff atrophy following both surgical models. Increased mTOR signaling following denervation may suppress autophagy in rotator cuff muscles by inhibiting the formation of the ULK1/Atg13/FIP200 complex. Our finding is consistent with a previous study conducted by Quy *et al.,* in which they demonstrated that autophagy in hindlimb muscle is suppressed following sciatic nerve denervation[11].

To date, many animal studies support that muscle atrophy occurs due to the traditional theory, a decrease in protein synthesis and unparalleled increase in protein degradation[15, 16, 36, 37]. However, this was not the case in our surgical models. The rate of protein synthesis increased in rotator cuff muscles following both tendon transection and SSN denervation. Since the Akt/mTOR/S6K1 signaling pathway has been reported to play an important role in modulating protein synthesis, increased protein synthesis in rotator cuff muscles after denervation is consistent with our previous finding of increased phosphorylation of Akt/mTOR/S6K1 in denervated rotator cuff muscles. In line with our finding, Quy *et al.* demonstrated that sciatic nerve denervation does not solely prompt muscle atrophy but also adaptive muscle remodeling via an increase in mTOR signaling[11]. However, increased protein synthesis rate after tendon transection contradicts our previous observation of decreased Akt/mTOR/S6K1 phosphorylation in rotator cuff muscles after tendon transection. This data suggests that there are other signaling cascades rather than mTOR signaling that mediate the rate of protein synthesis following tendon transection. A previous *in vitro* study conducted by Schmidt *et al.* also demonstrated that in spite of a decrease in S6K1 activity, protein synthesis rate increased[38]. Since we evaluated the rate of protein synthesis two weeks post-surgery rather than immediately, it is likely that a remodeling process had started, possibly as a result of a local feedback pathway to offset atrophy progression. Future work is needed to elucidate the mechanisms of increased protein synthesis in rotator cuff muscles following tendon transection.

There are some limitations to the current study. We chose to examine the ubiquitin-proteasome and autophagy pathways in supraspinatus muscles only after two weeks. While longer time-points will be considered in future studies, we chose two weeks as rotator cuff muscle atrophy is an early event clinically and our previous studies have shown that the tendon begins to heal at later time-points[39, 40]. We did not investigate protein degradation or synthesis in the setting of a combined tendon transection and SSN denervation injury as we sought to understand the specific contribution of autophagy, ubiquitin-proteasome pathway, and protein synthesis to the muscle atrophy observed following both surgical models independently. Since our protein synthesis results differ from previous studies which evaluated protein synthesis at shorter time-points[16, 36, 37], we will consider shorter time-points in a future study.

Like other animal studies, the anatomy and mechanics of a rat shoulder are different from that of a human shoulder and, therefore the results from this study cannot be directly translated into clinical practice. Though our tendon transection model provides a proxy to what is seen following RCTs in humans as evident by our previous data[9, 41] and that of others who utilized our model[22, 42], it does not duplicate the typical tendinopathy that is seen clinically. Therefore, the autophagy results we found in rats following tendon transection may differ in the setting of tendinosis. However, our findings support a recent clinical study conducted by Wu B, *et al.*[43] which concluded that autophagy was a major protein degradation process that was up-regulated following rotator cuff injury. Lastly, while RCTs

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are not associated with transection of the SSN, many clinical studies have shown that the degree of atrophy and fatty infiltration are aggravated due to tension placed on the SSN[6–8]. It should be noted that our SSN denervation procedure differs from what is seen during neurapraxia (nerve compression). While significant muscle atrophy and fatty infiltration may be seen during neurapraxia, our SSN transection model generates significant muscle atrophy and fatty infiltration as seen following chronic massive RCTs in a shorter amount of time.

In summary, our study suggests that protein degradation is the primary factor responsible for rotator cuff muscle atrophy, at least at the early stage following RCTs. Autophagy and the ubiquitin-proteasome pathway are predominant protein degradation pathways in rotator cuff muscle atrophy following tendon transection and SSN injury respectively (Figure 7). Taken together, our results may guide the development of specific therapeutics for patients with rotator cuff muscle atrophy accompanied with or without SSN injury in the future.

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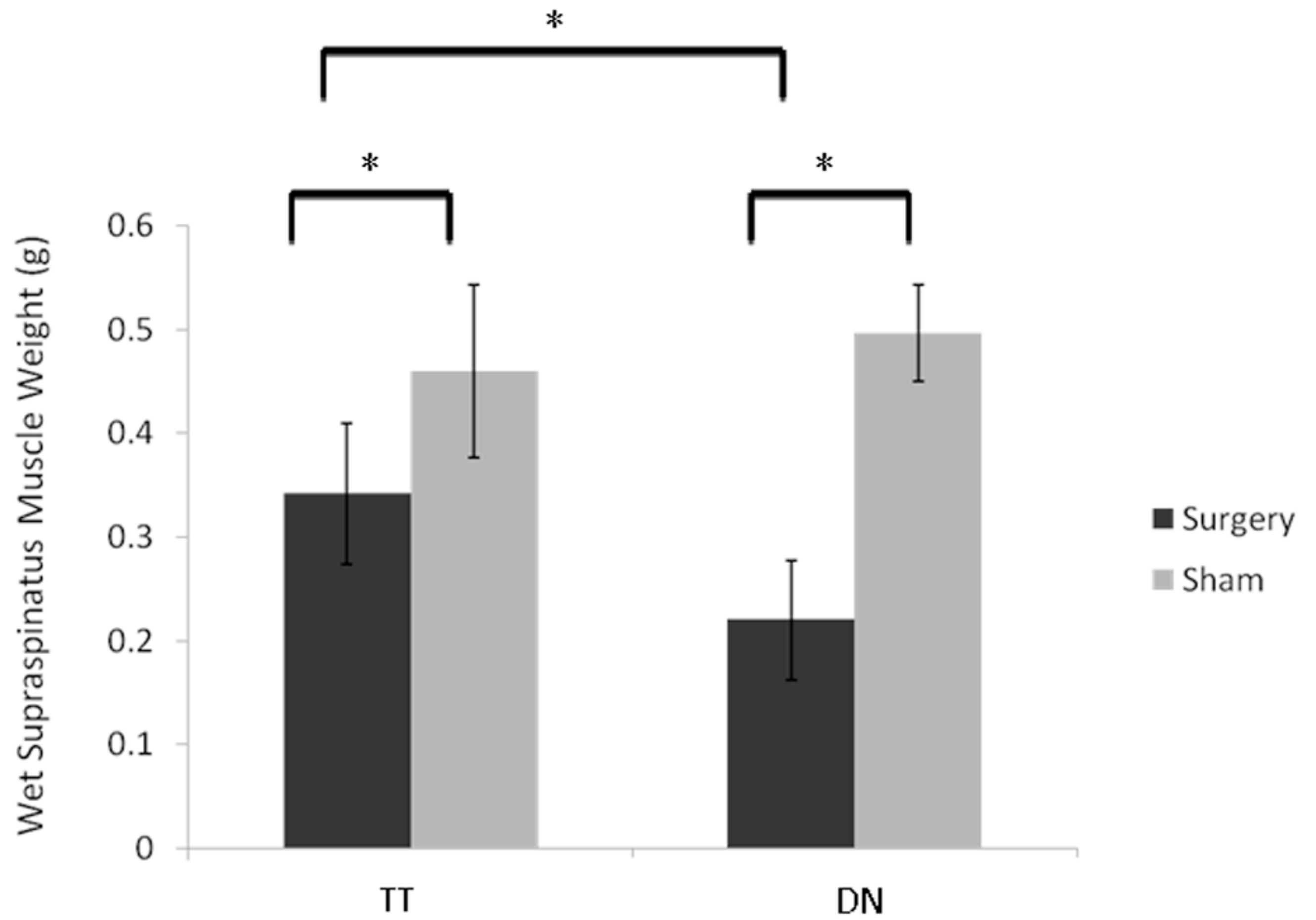

Figure 1.
Significant muscle weight loss was seen in supraspinatus muscles two weeks after tendon transection (TT) and suprascapular nerve denervation (DN) (* indicated P < 0.007).

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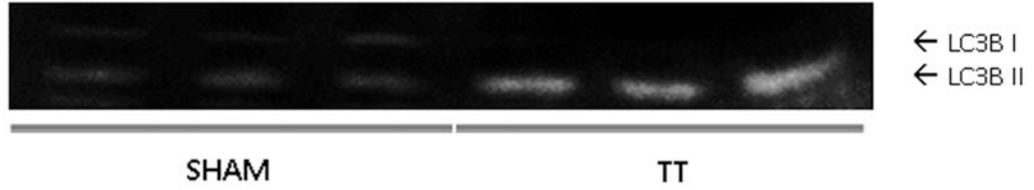

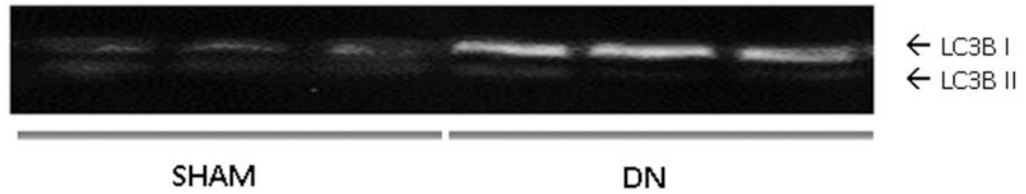

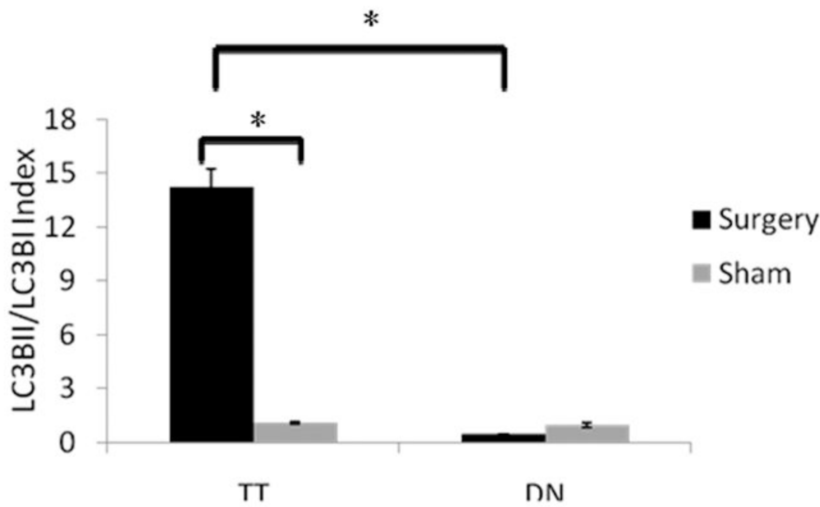

Figure 2.

Two weeks after TT surgery, activity of LC3BII was up-regulated in supraspinatus muscles of the surgery side compared to sham control as evident by western blot analysis **(A)**. The same trend was not seen following two weeks of DN **(B)**. ImageJ quantification of autophagic flux demonstrated that significant autophagy was seen in supraspinatus muscles following TT compared to sham surgery **(C)**. However, there was no significant difference in flux between supraspinatus muscles following DN compared to sham surgery. There was a significant difference in flux between TT and DN groups (* indicates $P<0.001$).

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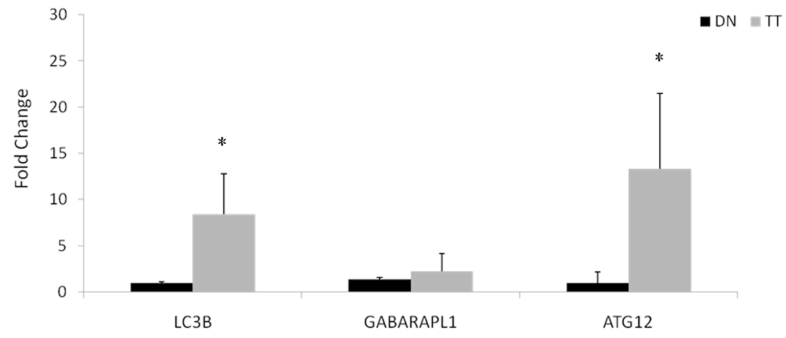

Figure 3.
Fold change of LC3B, GABARAPL1, and ATG12 in supraspinatus muscles two weeks after TT and DN surgery (* indicates P<0.05).

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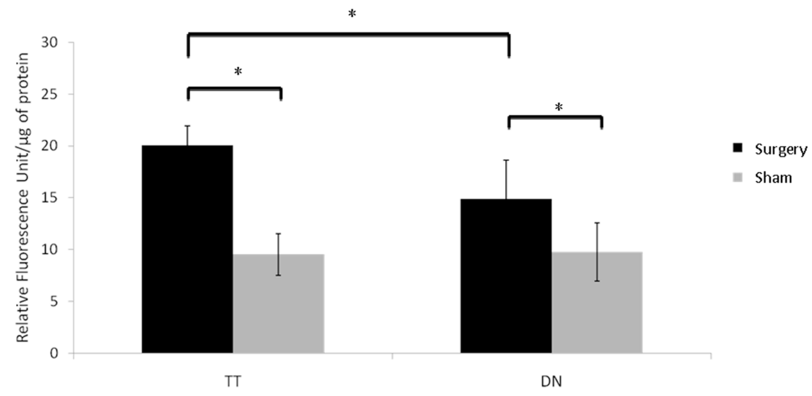

Figure 4.

Significant difference in Cathepsin L activity was seen between TT vs. TT sham, DN vs. DN sham, and TT vs. DN (* indicates P<0.05).

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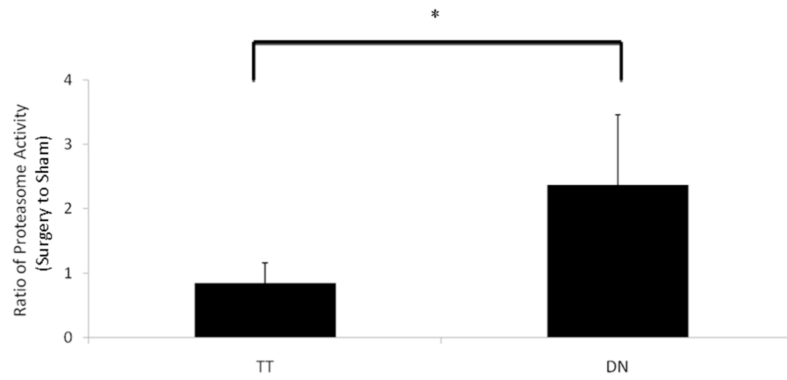

Figure 5.

Significantly greater 20S proteasome activity was seen following DN compared to TT (* indicates P<0.05). Data is graphed as a ratio of proteasome activity between surgery and sham groups.

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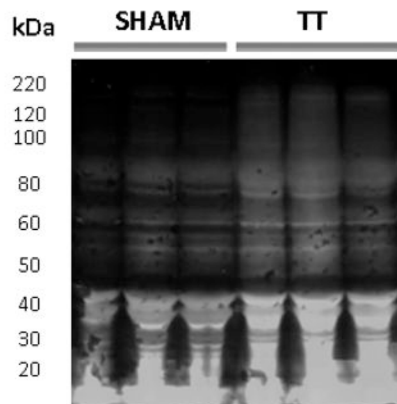
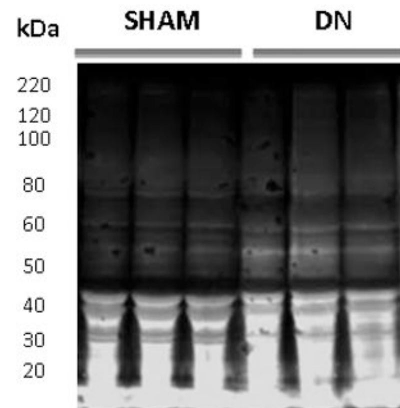
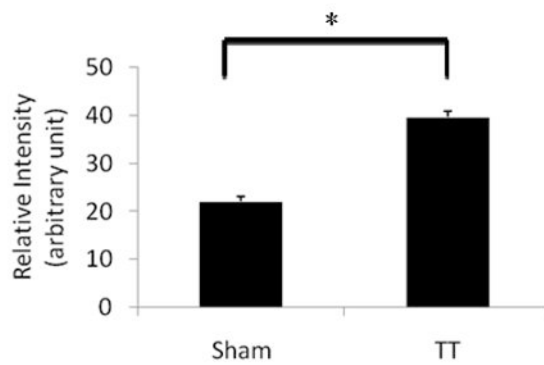
A. Tendon Transection (TT) SUnSET blot:



B. Denervation (DN) SUnSET blot:



C. TT Quantification:



D. DN Quantification:

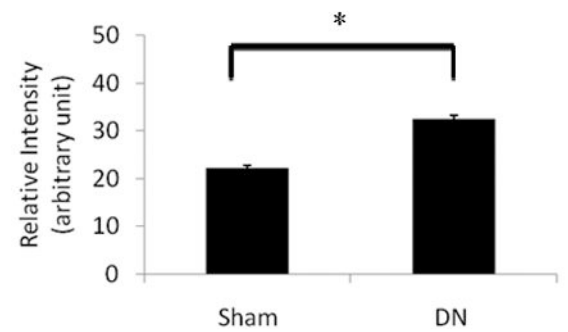


Figure 6.

The rate of protein synthesis was significantly increased following both TT and DN compared to sham surgery as evident by the western blot **(A, C)** and ImageJ quantification **(B, D)** (* indicates $P<0.05$).

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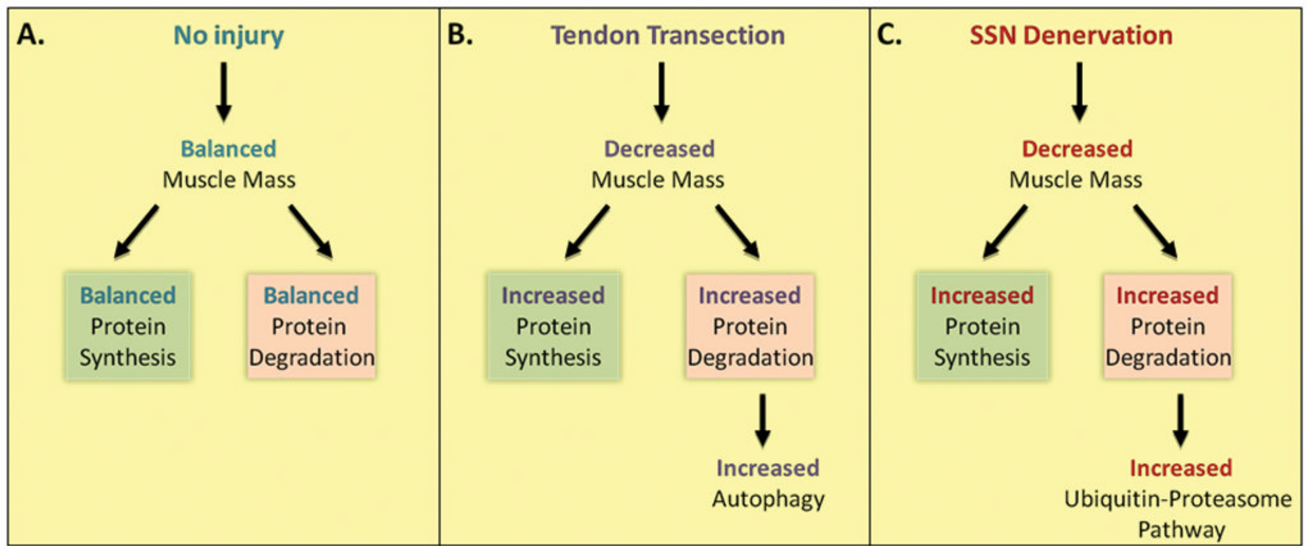

Figure 7.
Summary of mechanisms of muscle atrophy following rotator cuff injuries: No injury **(A)**, TT **(B)**, SSN Denervation **(C)**.