ISCHEMIA INDUCES CHANGES IN SOD ACTIVITY AND NEUROMUSCULAR REGENERATION

Research Option Thesis

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Abstract

Critical limb ischemia (CLI) is characterized as a neuromuscular disorder of aging that develops due to an occlusion of blood flow to the affected lower limbs. Following ischemic injury, disruption of vasculature interrupts delivery of oxygen and nutrients to motor neurons and subsequently results in degeneration of the neuromuscular junctions (NMJs). This induces further myofiber atrophy due to the lack of innervation from the presynaptic motor neurons to the myofibers. Recent regenerative medicine approaches have investigated the role of redox homeostasis in the regeneration of ischemic skeletal muscle. Superoxide dismutases (SOD1 and SOD2) are key enzymes that regulate reactive oxygen species and help to maintain redox homeostasis and the regenerative capacity of myofibers. Research investigating the redox signaling in the crosstalk between ischemic myofibers and motor neurons has been limited. Our research question is: how does redox signaling impact neuromuscular regeneration following injury? We hypothesize that dysregulated SOD enzymes correlates to altered morphology of the neuromuscular junctions following ischemia. To test our hypothesis, we induced ischemia in a mouse model and performed microscopic imaging and biochemical assays in muscle and nerve cells in various time points following ischemic injury. Our results indicate that ischemic injury results in denervation of the motor neuron and fragmentation of postsynaptic acetylcholine receptors (AChR). We also concluded that there is a disruption in the SOD enzymes in the muscle microenvironment upon injury that corresponds with the morphological changes in the NMJs that were witnessed. By understanding this correlation between the innervation and fragmentation of NMJs and the SOD specific activity, the essential role of redox homeostasis in maintaining and regenerating a functional neuromuscular system can be better understood.

Introduction

Critical limb ischemia (CLI) is the most severe form of peripheral artery disease (PAD), which affects 8-12 million individuals in the United States and is classified as an atherosclerotic syndrome that causes blockage of the peripheral arteries that terminate in the legs (Hirsch et al., 2001). Risk factors for PAD include smoking and diabetes (Hirsch et al., 2001). Approximately 1% of the total population of PAD patients are affected by CLI; however, CLI patients have the greatest mortality rate of 70% within 10 years of diagnosis (Varu et al., 2010).

Although there is no cure for CLI, current goals of treatment are to relieve pain, improve mobility, treat ulcers, and reduce cardiovascular risk factors (Slovut and Sullivan, 2008). Varying treatments have demonstrated differing levels of success in preventing amputation and improving quality of life. Surgical revascularization, a procedure that restores blood flow to the ischemic limbs, is one option for eligible patients but has similar survival outcomes as endovascular therapy, a non-surgical option (Iida et al., 2017). CLI patients who are not able to undergo surgical revascularization may receive therapeutic angiogenic treatments to help promote new blood vessel formation. An example of this type of treatment is with basic fibroblast growth factor (bFGF), a strong angiogenic factor that was injected intramuscularly within hydrogel microspheres (Marui et al., 2007). This has shown improvements in distance walked, transcutaneous oxygen pressure, and ankle brachial index (Marui et al., 2007). Another example of angiogenic treatments is intramuscular injection of vascular endothelial growth factor (VEGF) to the symptomatic leg that increases collateral vascularization to tissues (Cooke and Losordo, 2015). However, these treatments have had limited success, possibly due to the ischemia-induced degeneration of the muscle that hosts newly formed blood vessels.

Alternatively, the regenerative capacity of skeletal muscle may offer a more effective potential target for regenerative therapies. CLI has demonstrated significant disruptions in all niche components of skeletal muscle tissue. This encompasses the vascular system, motor neurons, muscle fibers, and muscle stem cells. Disrupted vasculature, as it occurs in ischemia, interrupts proper innervation of the motor neuron due to the lack of oxygen and nutrients that would be supplied by the blood. Motor neurons require a constant supply of oxygen and nutrients in order to meet the high energy demands required to maintain the membrane potential. The close proximity of blood vessels to the motor neuron axons demonstrates that there is some interdependence present between the two tissues in order to promote a functional neurovascular system (Martin and Lewis, 1989). Additionally, the muscle atrophy induced by ischemia is further exacerbated by degeneration of the motor axon (Vignaud et al., 2010) (Rowan et al., 2012). Muscle satellite cells (MuSCs) are stem cells that proliferate and differentiate to fuse into myotubes, and eventually myofibers, and are critical to the regeneration of skeletal muscle. Depletion of MuSCs has been shown to disrupt neuromuscular junction (NMJ) morphology while regenerating NMJs correlate with an increase in neighboring MuSC activity (Liu et al., 2015). Furthermore, severe disruption of NMJs diminishes crosstalk between MuSC and motor neurons (Choi et al., 2020). These findings highlight the essential role of communication between MuSCs and nerve cells in maintaining a functional neuromuscular environment.

Superoxide dismutases (SOD1 and SOD2) are reactive oxygen species (ROS) regulatory enzymes that maintain redox homeostasis and work to promote the regenerative capacity of

skeletal muscle, or myofibers, by converting the highly reactive superoxide into the more stable hydrogen peroxide (H_2O_2) signaling molecule (Fukai et al., 2011). Knockout of SOD1 in mice has shown denervation and motor deficits, developing into a chronic peripheral axonopathy (Flood et al., 1999). Additionally, SOD1 knockout has been shown to increase the denervation associated with age progression (Kostrominova 2010). This finding is important to our research in CLI because it demonstrates the importance of redox homeostasis, as it is regulated by SOD1/2, in maintaining a healthy neuromuscular system.

Research investigating the crosstalk, or communication, between ischemic myofibers, motor neurons, and vasculature and the corresponding redox signaling has been limited. Additionally, there has been limited work comparing neuromuscular remodeling and redox signaling in the aged compared to young models. Considering that CLI is primarily prevalent in the aged human population, this relationship is an important aspect to investigate, despite the majority of therapeutic research for ischemic injury taking place in young animals. Taking advantage of this crosstalk, we plan to correlate changes in redox signaling and neuromuscular remodeling, via SOD1 and SOD2 specific activity and content in the motor neurons and myofibers following ischemia. Our specific methodology includes enzymatic activity assays, western blots, and fluorescent and confocal imaging on samples of ischemic motor neurons, muscles, and spinal cord extractions. The research question we aim to answer is: how does ischemia affect SOD levels and NMJ morphology separately and how might this differ between aged and young models? We hypothesize that dysregulated SOD1 and SOD2 enzymes will correlate with altered NMJ morphology. This damage to the muscle microenvironment is hypothesized to be exacerbated in the aged model. By understanding this correlation, the essential role of redox homeostasis in maintaining and regenerating a functional neuromuscular system can be assessed. This provides potential therapeutic targets in promoting stronger nerve-muscle interactions in CLI patients. By fully understanding this interaction, we aim to promote increased mobility and improved quality of life in CLI models.

Literature Review

Critical limb ischemia (CLI) (Varu et al., 2010) is the most severe form of peripheral artery disease (PAD) (Hirsch 2001) that is characterized by extreme loss of blood flow to the affected extremities. Damage to the vasculature following ischemic injury has shown to correspond with degeneration of the neuromuscular junction (NMJ) (Martin et al., 1989) and further muscle atrophy due to the lack of nutrient and oxygen delivery that is normally carried out by the vasculature (Vignaud et al., 2010). Current treatments for relieving PAD symptoms include exercise and angiogenic therapies that increase vascular endothelial growth factor (VEGF) expression in order to increase vascularization to tissues (Cooke et al., 2015). New evidence may suggest that muscle satellite cells (MuSCs) promote not only muscle regeneration, but additionally a remodeling of the neurovascular niche (Liu et al., 2015).

Crosstalk evident between the motor neuron, vasculature, and myofibers regulates the functional neurovascular and neuromuscular system. Disruption of blood flow caused by ischemia also disrupts the delivery of nutrients and oxygen to the motor neurons, thereby disrupting proper innervation. This disrupted innervation also exacerbates myofiber atrophy (Rowan et al., 2012).

Reactive oxygen species (ROS) generation has been associated with cellular damage and key regulatory enzymes, such as SOD1 and SOD2, play a role in maintaining redox homeostasis. These enzymes convert the superoxide species induced by ischemia into the less reactive hydrogen peroxide in order to attenuate oxidative damage to myofibers and neurons (Fukai et al., 2011).

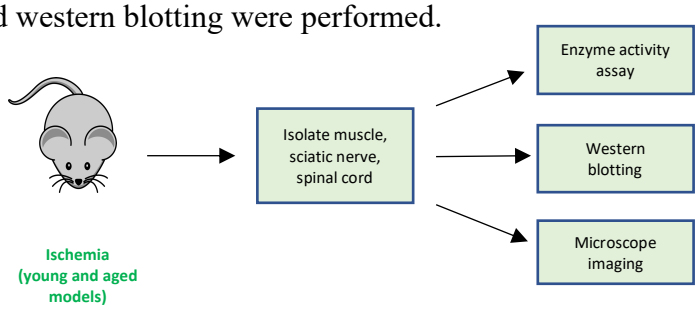
A subset of familial amyotrophic lateral sclerosis (ALS) cases has been shown to be linked with a mutation in SOD1 (Wong et al., 1995). Since both ALS and CLI are neuromuscular disorders, it makes sense to focus on how ROS production disrupts the nerve-vessel interactions present in CLI.

Scalabrin et al. (2019) investigated the changes in reactive oxygen species (ROS) generation following denervation of skeletal muscles in the hind limb. The overarching question this study addressed is to what extent does denervation of the muscle contribute to ROS release and how does the muscle respond to this change in the microenvironment. This relates to our fundamental research question of how redox signaling and oxidative stress impact regeneration of the neuromuscular system. It was hypothesized that short term increase in ROS may actually induce a protective mechanism for myofibers, but long-term ROS generation may induce atrophy. They found that hydrogen peroxide was generated from 7 to 21 days post denervation injury. It was also hypothesized that additional sources of peroxides could come from monoamine oxidases, NADPH oxidase, and phospholipases.

The findings of this study are relevant because it provides a basis for understanding the temporal scale of how ROS production changes in a denervation model. Since denervation is a characteristic of the dysfunctional NMJ, it would be interesting to see if a similar time scale of ROS production would be evident in a CLI model. This gap in the field could be useful to assess how oxidative stress changes and impacts the remodeling of the nerve-muscle interaction.

Methods and Materials

Ischemic surgery was performed on mice, both young and aged, and subsequently, hindlimb muscle, spinal cord, and sciatic nerves were dissected at various timepoints. Samples were then imaged under fluorescence or confocal microscope and biochemical assays, such as enzyme activity assays and western blotting were performed.



Animals

Both male and female mice aged 3 to 6 months were used in this study and are considered young adults. Aged mice were those 19 months and older. Thy1-YFP transgenic reporter mice were used to assess presynaptic motor neurons. Pax7-TdTomato mice were used to obtain analyses of muscle satellite cells (MuSCs). C57B16/J mice were used to assess SOD1 and SOD2 activity. Mice were euthanized following 0, 7, 14, 28, and 56 days post ischemic surgery.

Ischemia surgery

A murine hindlimb ischemia surgical model was applied to observe the effects of CLI. To perform this, the femoral artery was ligated on one leg. A sham surgery, whereby a similar methodology was used but no ligation, was applied on the contralateral leg as a control. Mice were maintained for 3-56 days following the CLI surgery.

Sciatic nerve and spinal cord extraction

Contralateral and ipsilateral sciatic nerves from mice euthanized after 28 days were isolated. Spinal cord extracts were flushed with 1X PBS from each animal using an 18-gauge needle and 1mL syringe.

Western blotting

Nerve and muscle samples were homogenized in homogenization tubes and RIPA lysis buffer supplemented with Roche Complete Mini Protease Inhibitor and PhosSTOP Phosphatase Inhibitor. The ratio used for muscles were 1mL lysis buffer for every 100mg of tissue. Nerve samples used a total of 250 uL lysis buffer per nerve sample. Following three freeze-thaw cycles in liquid nitrogen, homogenates were centrifuged at 14,000 rpm at 4 degrees C for 10 minutes, after which the supernatants were collected. A bicinchoninic acid assay (BCA assay) kit was used to determine overall protein concentration of each sample. Samples were normalized to the lowest protein concentration. A 45 ug protein solution mixed with beta-mercaptoethanol and

laemlli buffer were loaded into a 4-20% Criterion TGX gel at a constant 150V for 1 hour and 12 minutes. The gel was transferred to a PVDF membrane, using the Trans-Blot Turbo System for 7 minutes at 2.5A. The membrane was blocked for 30 minutes with blocking buffer and stained with primary antibodies overnight. Following washed with TBST, the membrane was stained with secondary antibodies. Antibodies used in this study include SOD1 and SOD2. Membranes were then imaged on the Li-Cor Odyssey CLx-1050 Infrared Imaging System and bands were quantified on Li-Cor Image Studio. Ponceau solution was then used to stain the membrane to visualize all proteins and was imaged on the Amersham under epi-illumination.

SOD activity gel

Native running buffer was made using Trizma, Glycine, and 0.5M EDTA. Using homogenized samples, 25ug of protein in 30uL volume was loaded with 5X SOD loading dye in a 14% Tris-Glycine gel. The gel ran at 4 degrees Celsius and a constant 50mA for some time until the bromophenol blue reached the end of the gel, approximately 4 hours. After running, the gel was stained in the dark for 1 hour with SOD activity staining solution composed of 1M K2HPO4, 1M KH2PO4, NBT, and Riboflavin. TEMED was added on top of the staining solution. After the stain was completed, the solution was removed, and the gel rinsed with ddH2O. Bands began to appear following exposure to light overnight. The gel was imaged on the Amersham using trans-illumination. Bands were quantified with mean gray value on Fiji/ImageJ software.

Immunostaining

Following euthanization of animals, the tibialis anterior (TA) and extensor digitorum longus (EDL) muscles were isolated and fixed in 4% paraformaldehyde. Samples were stained with bungarotoxin and DAPI for visualizing the post-synaptic acetylcholine receptor (AChR) and myonuclei respectively.

Microscope imaging

Samples were imaged on the Zeiss 700 Laser Scanning Confocal microscope at 63x magnification with oil immersion. Z-stack images were taken and analyzed on Fiji/ImageJ.

Quantification of NMJs

Following microscopic imaging, NMJs were quantified on Fiji/ImageJ using the methodology described by Minty et al. (2020). Images were cleaned up of any noise and measurements for nerve terminal and postsynaptic AChR perimeter and area were taken. Complexity, compactness, overlap, and fragmentation of NMJs were determined.

3D reconstruction of images

Using CZI files obtained from z-stack images taken on confocal microscopy, 3D reconstruction of both NMJs and MuSC was accomplished using Volocity software.

Statistical Analyses

Statistical analyses in this study were performed in GraphPad Prism 7 software and data is represented as mean +- standard deviation. One way ANOVA with Dunnet's multiple comparison test was used between the mean of control and each time point. A p-value of less than 0.05 was considered statistically significant.

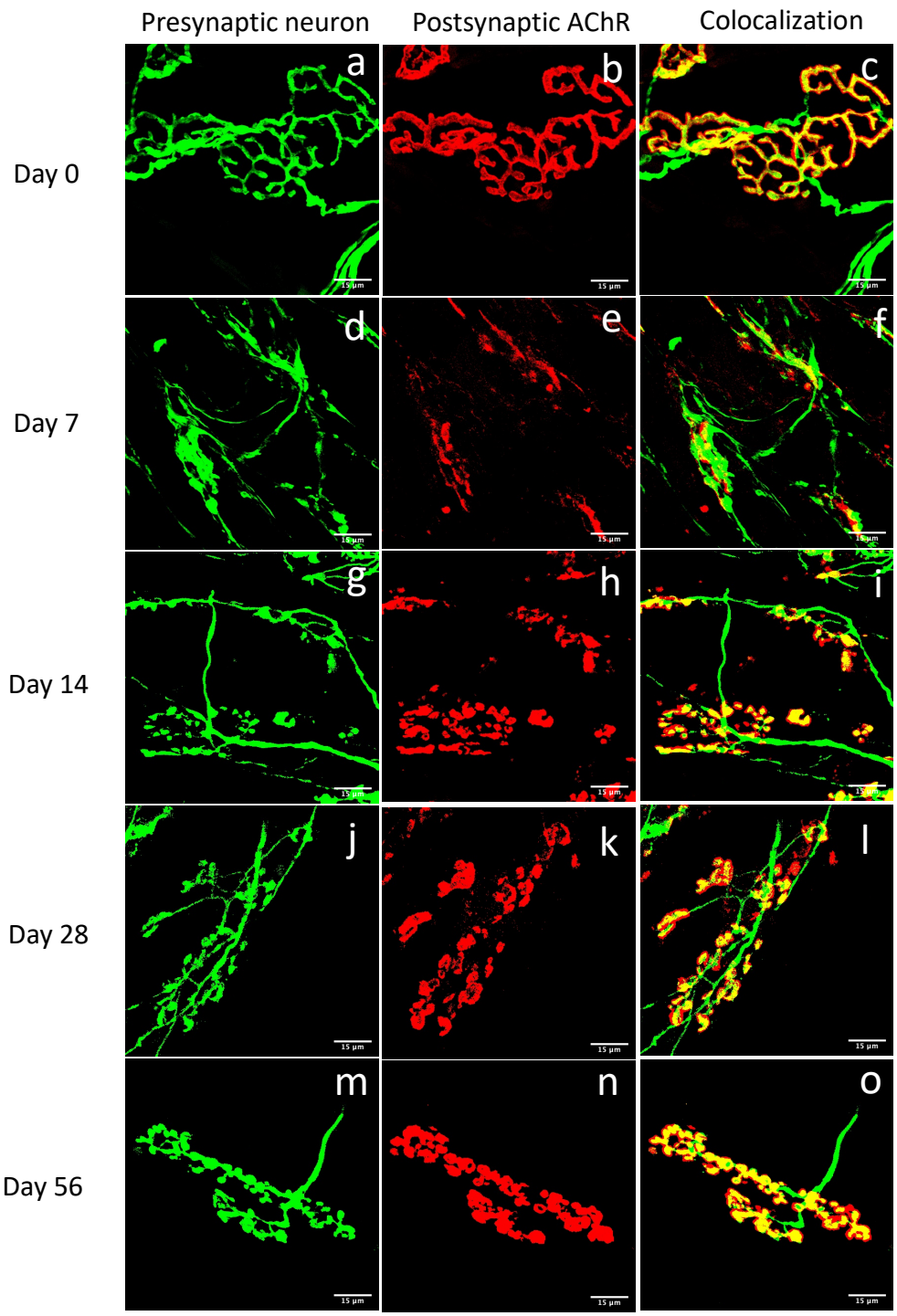
Results

1. Remodeling of neuromuscular junctions following ischemic injury

Initially, we assessed the morphological changes to the NMJs and characterized how the muscle and motor neuron regenerate following ischemia. Ischemic injury occludes blood flow to the extremities, which subsequently prevents the proper delivery of nutrient and oxygen to the skeletal muscle. This disrupts the neuromuscular system by resulting in Wallerian degeneration of the presynaptic motor neuron and fragmentation of the postsynaptic acetylcholine receptor (Fig. 1). The greatest fragmentation of the AChR and most denervation occurs at day 7 post ischemic injury (Fig.1d,1e,1f, 1q), followed by a remodeling of the NMJ up to 56 days following injury (Fig.1m,1n,1o).

2. Area of neuromuscular junction correlates with number of subsynaptic nuclei

Following a disruptive cycle, denervation induces further myofiber atrophy. Furthermore, due to their close proximity to one another in the muscle microenvironment, MuSCs can help maintain the NMJ by fusing into the underlying myofiber as subsynaptic nuclei (Fig. 2a). At day 14, where there is evidence of synaptic remodeling (Fig. 1g, 1h, 1i), there is also an increase in the number of subsynaptic nuclei in single muscle fibers. These subsynaptic nuclei synthesize the proteins that are essential for the development of a proper NMJ. Analysis of subsynaptic myonuclei reveals a correlation with the overall area of the NMJ (Fig. 2b). The drastic accumulation of subsynaptic nuclei beginning at day 14 corresponds with the time point where the NMJ begins remodeling and increasing in area. This accumulation persists up to day 56 (Fig 2b). MuSCs fuse into the myofiber as subsynaptic nuclei, so we assessed the morphological changes in MuSCs following a sciatic nerve transection, another form of denervation injury (Fig. 2c). 3D reconstruction of the MuSCs demonstrate long projections near the NMJ following a denervation injury (Fig. 2c). Depletion of MuSCs has been shown to disrupt the formation of functional NMJs and the regeneration of NMJs has been correlated with an increase in the number of MuSCs (Liu et al., 2015). Our observations indicate the presence of crosstalk evident between the motor neurons and myofibers.


Presynaptic neuron
Postsynaptic AChR
Colocalization
Day 0
Day 7
Day 14
Day 28
Day 56
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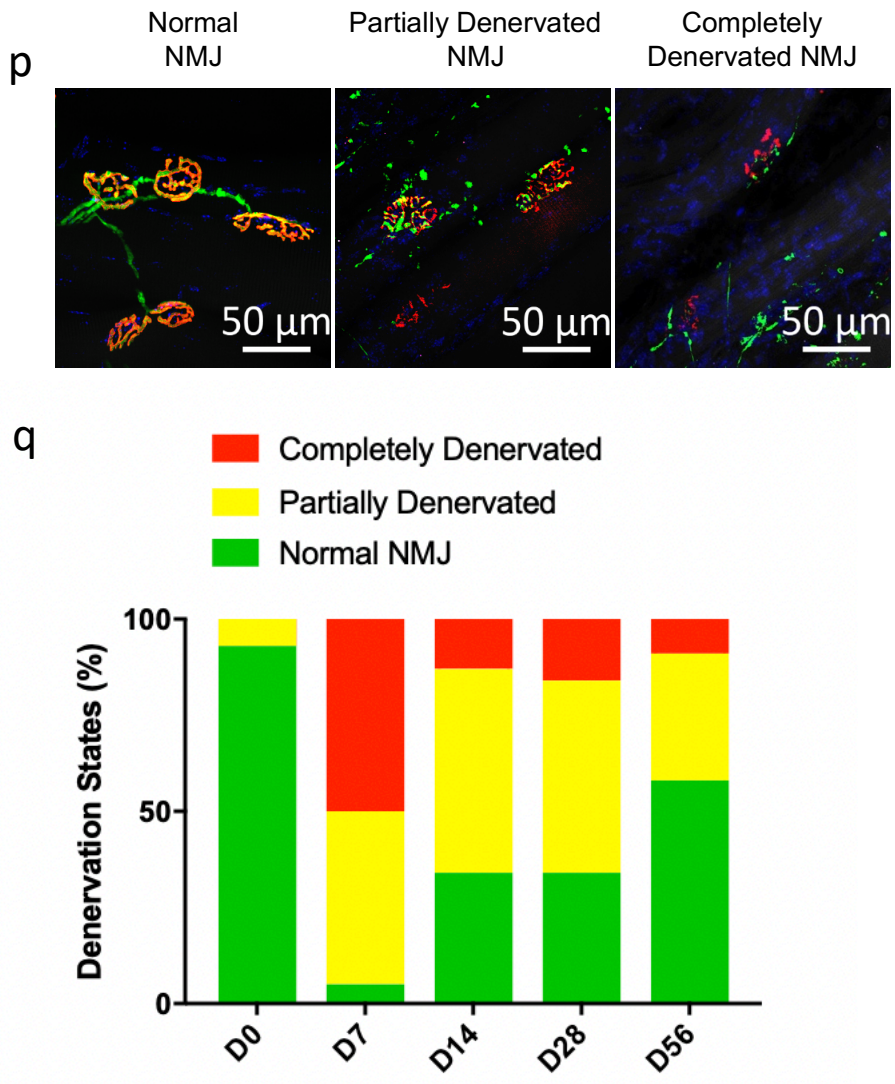
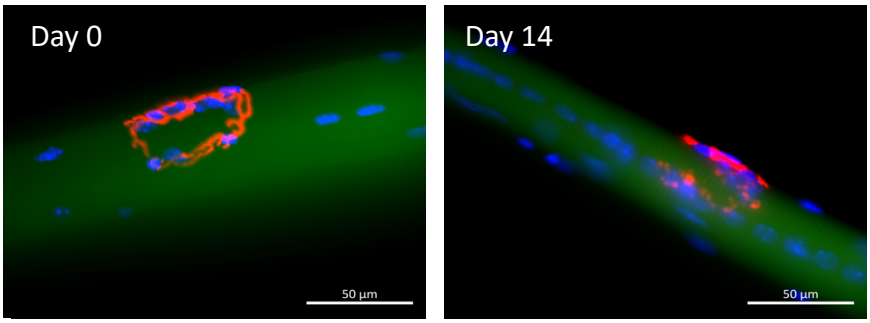


Figure 1: *NMJ remodeling throughout timepoints up to day 56 following ischemia.* (a-o) The greatest fragmentation of the AChR and most denervation occurs at day 7 post ischemic injury, followed by a remodeling of the NMJ up to 56 days following injury. (p-q) Quantification of denervation shows greatest complete denervation at 7 days post injury. Images were taken using 63X magnification on confocal microscope. Presynaptic neuron was stained with Thy1-YFP and postsynaptic neuron was stained with Bungarotoxin 555.

a



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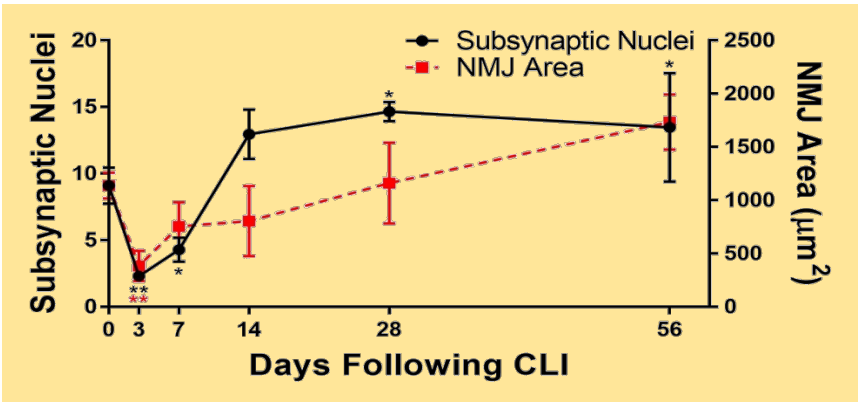

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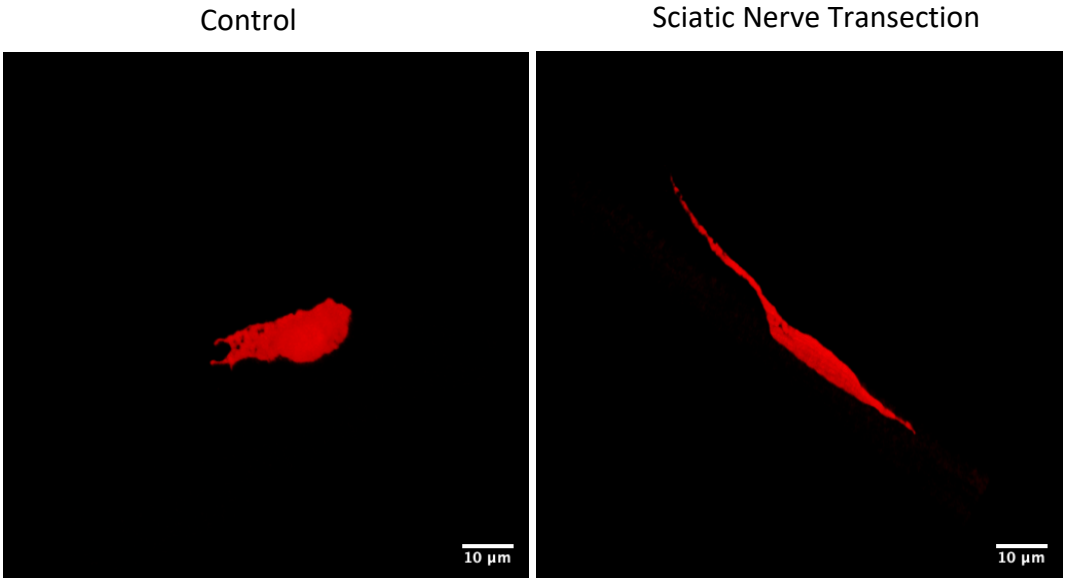


Figure 2: *Subsynaptic nuclei, NMJ area, and MuSCs* (a) Single muscle fiber images were taken on confocal microscope. AChR has been pseudo-colored red, the muscle fiber shown in green, and the subsynaptic nuclei in blue. (b) Graph showing trends in number of subsynaptic nuclei and NMJ area up to 56 days following CLI. (c) 3D reconstruction of MuSC following sciatic nerve transection. Statistical significance shown with p-value less than 0.05

3. Denervation in aged samples is exacerbated following ischemia

The effect of age on ischemia induced NMJ regeneration has not been thoroughly studied but is an important factor to consider due to the higher likelihood of CLI in the aged population. To investigate the impacts of age on the restoration of the neuromuscular system, we observed differences in NMJ morphology between the young and aged ischemic samples. Results demonstrate that the aged control mouse samples show greater fragmentation and less complete innervation of the NMJ when compared to young control samples (Fig. 3a, 3c). This is likely due to cycles of denervation and innervation that occur naturally in aged muscle. Aged samples 28 days post ischemic injury also display greater fragmentation of the postsynaptic AchRs (Fig. 5) and less complete overlap of the presynaptic motor neuron and post-synaptic receptor when compared to young (Fig. 4). Suggesting that aged motor axons are not able to reinnervate the NMJ as effectively as young (Fig. 3).

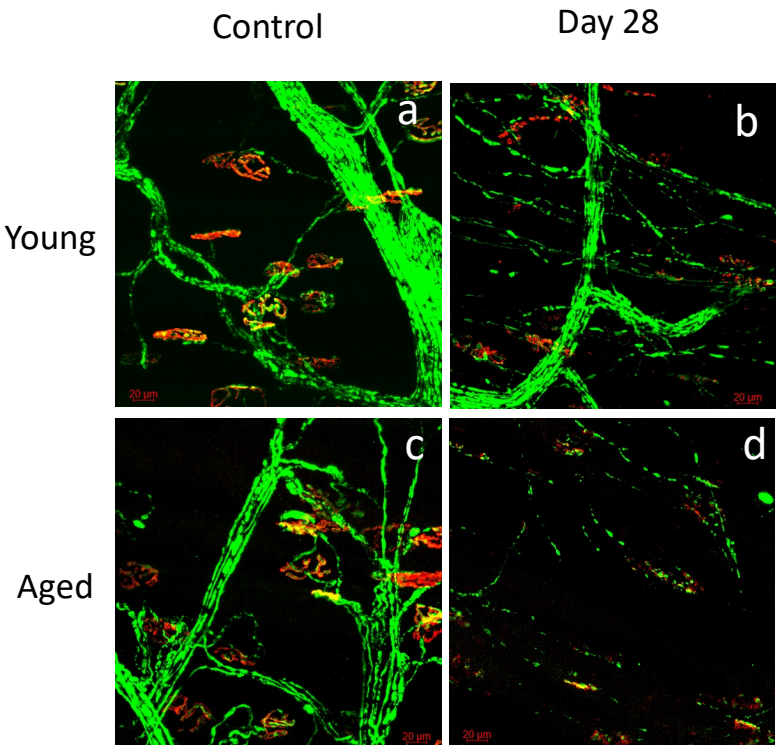


Figure 3: *Comparison of young and aged control and 28 days post ischemia.* Aged control samples show greater denervation compared to young. Images taken on confocal microscope with 20X magnification. Green represents the presynaptic motor neuron and red represents the postsynaptic AChR

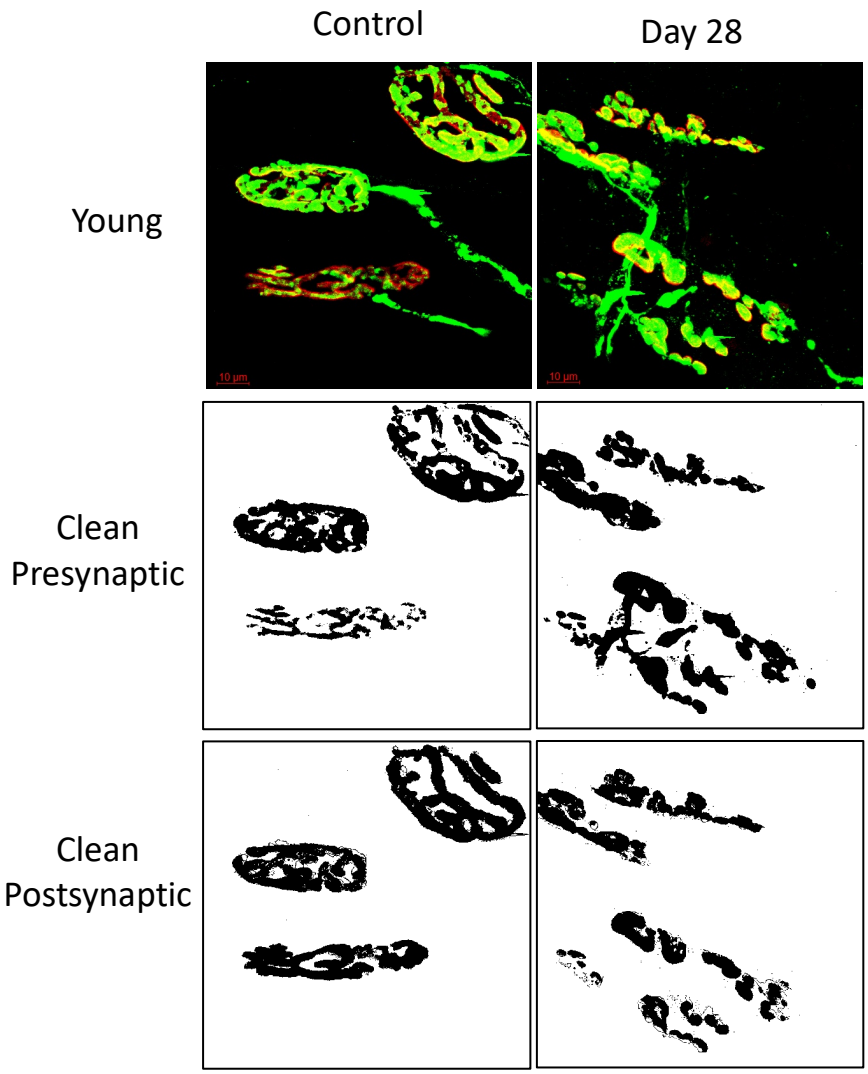


Figure 4: *Cleaned NMJ of young control and 28 days post ischemia.* Images were analyzed in ImageJ and the presynaptic and post-synaptic sides were isolated to obtain a clearer synapse profile.

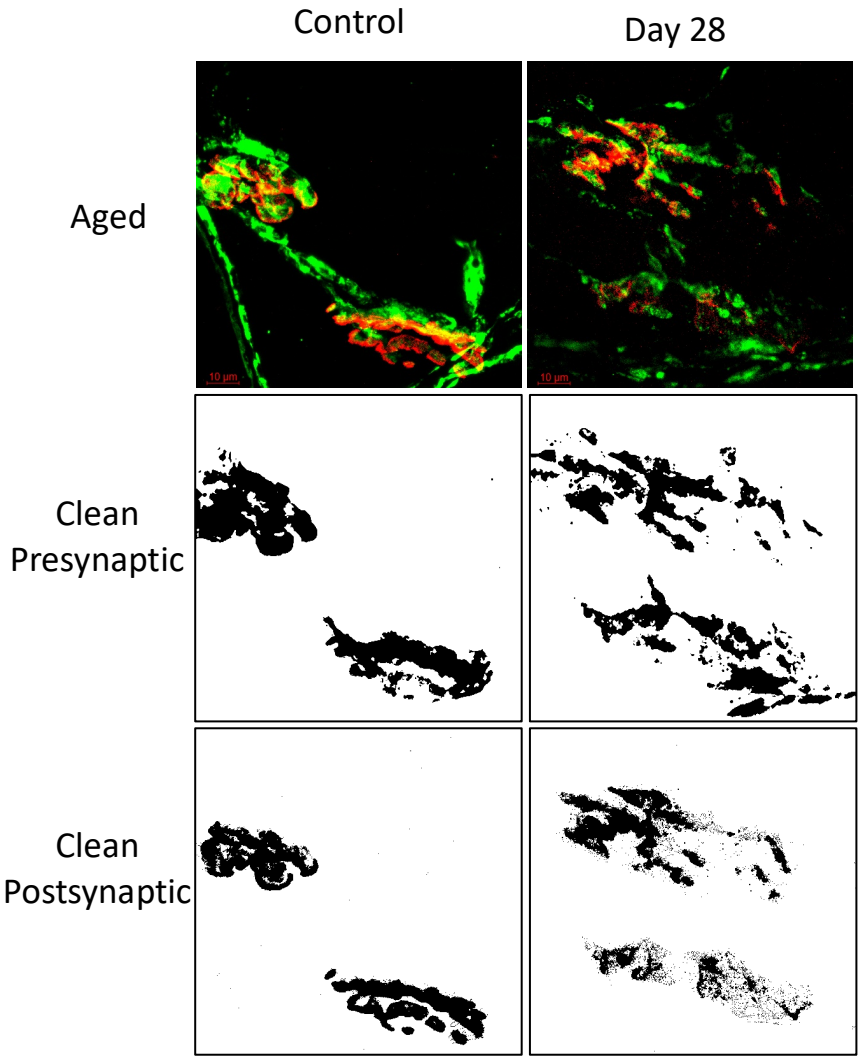


Figure 5: *Cleaned NMJ of aged control and 28 days post ischemia.* Aged control samples show greater fragmentation and less complete NMJ innervation. This difference is exacerbated following ischemic injury. Images were analyzed in ImageJ and the presynaptic and post-synaptic sides were isolated to obtain a clearer synapse profile.

4. Disruption in SOD regulatory enzymes following ischemic injury

For our analysis of ROS regulatory enzymes, we initially assessed redox changes in young muscle samples in both control and ischemic conditions. Upon performing an SOD activity gel in muscle samples, we observe a significant decline in SOD2 activity at 7 days post ischemic injury (Fig. 6a) which corresponds with the timepoint of the greatest denervation and fragmentation (Fig. 1d, 1e, 1f). A western blot was used to determine if there was a correlation with the content of SOD2 in the muscle. Experimental results indicate an increase in SOD2 content from day 7 to day 28 post injury, indicating an increase in SOD2 as the muscle attempts to repair itself (Fig. 6b). This corresponds with the regeneration present in the NMJ from day 7 to day 28 as the presynaptic and postsynaptic sides of the NMJ attempt to reinnervate (Fig. 1d, 1e, 1f). Upon looking at the specific activity of SOD2, a measure of the activity of enzyme per the total quantity, we observe a significant decline in specific activity at day 7 (Fig. 6c). Therefore, although there is an increase in SOD2 protein content, the activity of the enzyme appears to decrease, indicating that the enzyme is losing its catalytic activity, possibly from other factors present in the muscle microenvironment and is likely trying to compensate by increasing its total content.

Our next step was to investigate the content and activity of SOD enzymes in ischemic nerve samples in young mice. Upon performing an SOD activity assay on control spinal cord and muscle samples, we observe higher SOD2 and SOD1 enzyme activity in the spinal cord compared to the sciatic nerves, located in the lower limbs, and muscles (Fig 7a., Fig. 7c) Western blot analysis of SOD1 indicates significantly greater SOD1 content in the spinal cord when compared to muscle samples (Fig. 7b, Fig. 7c).

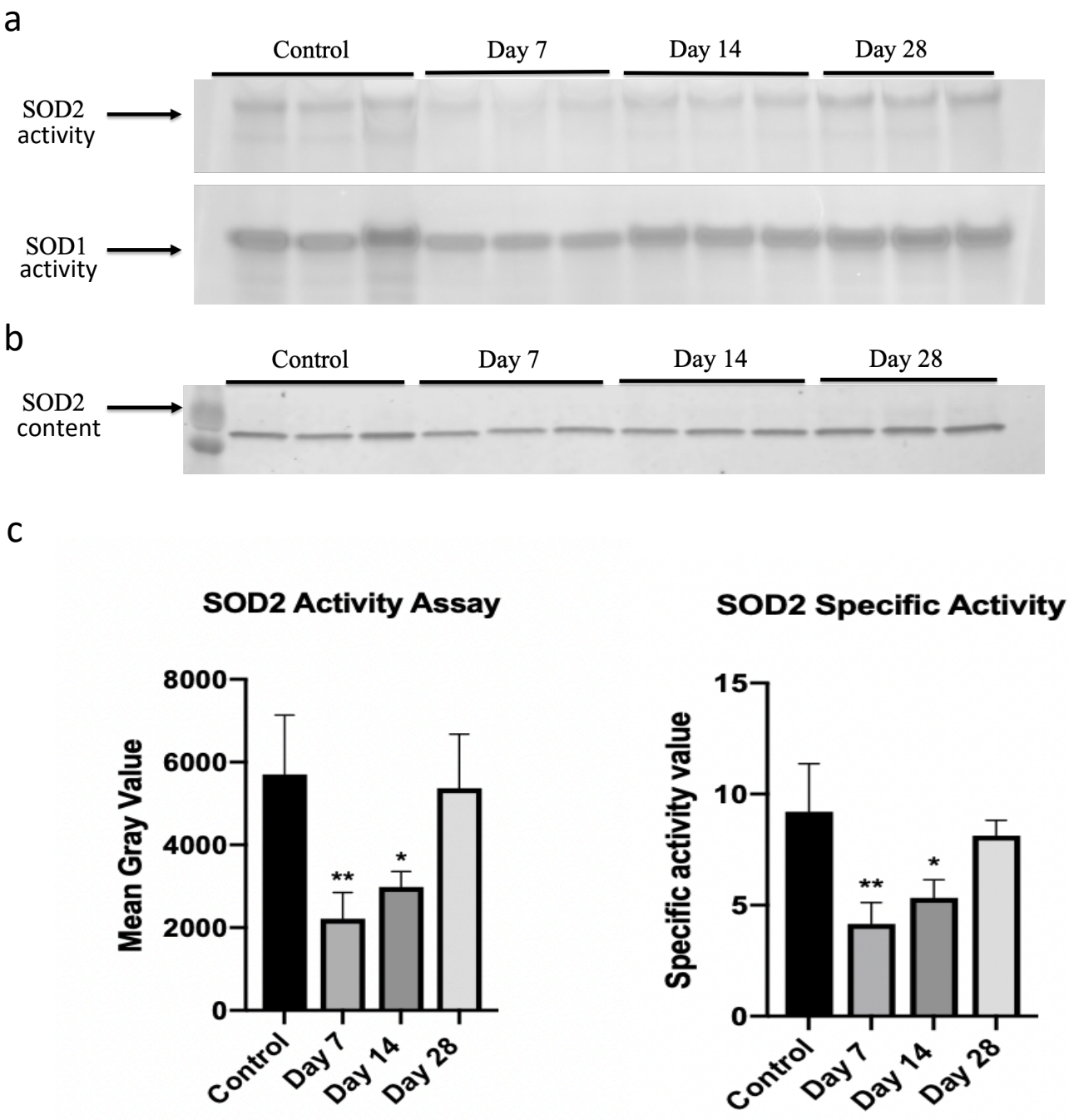
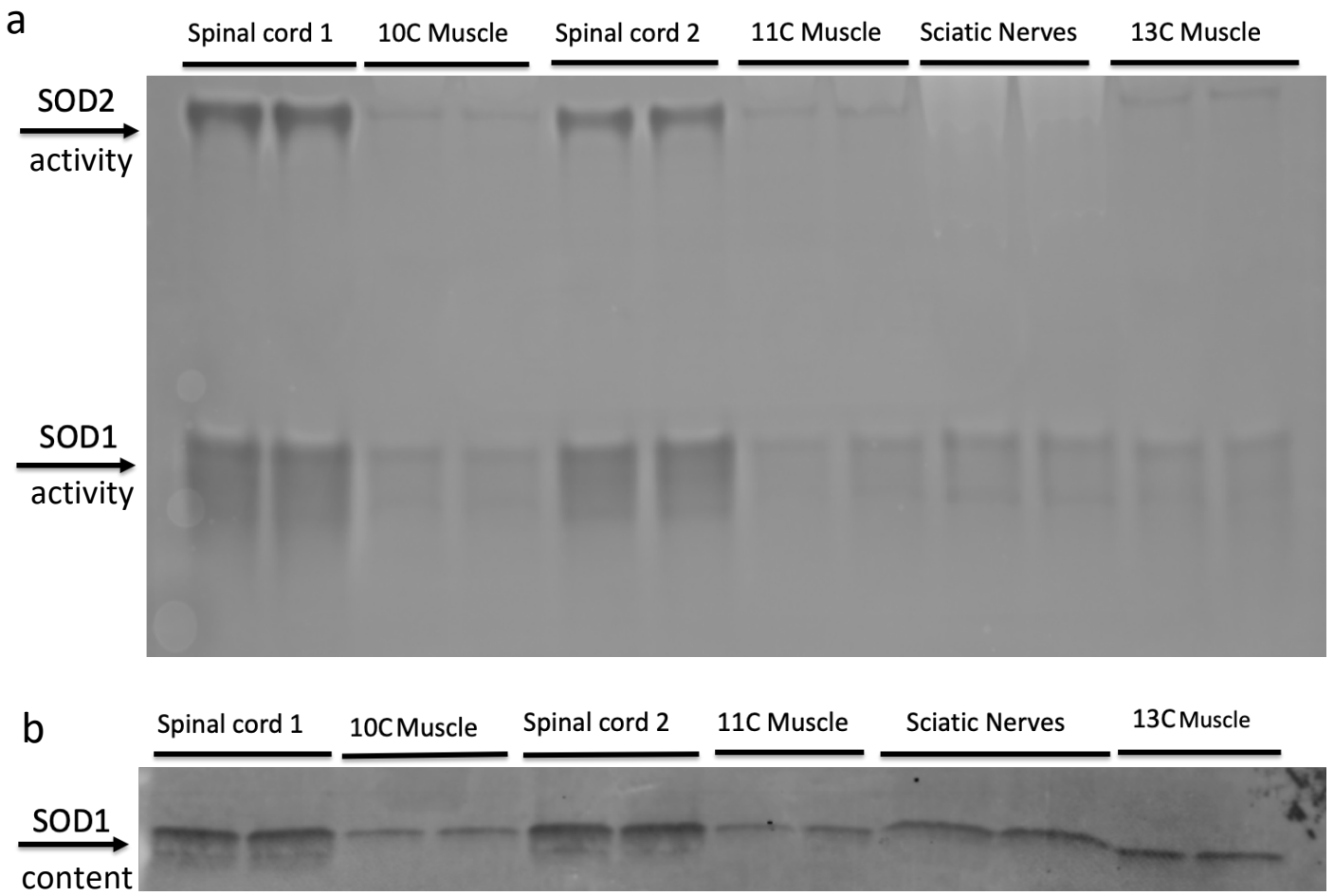
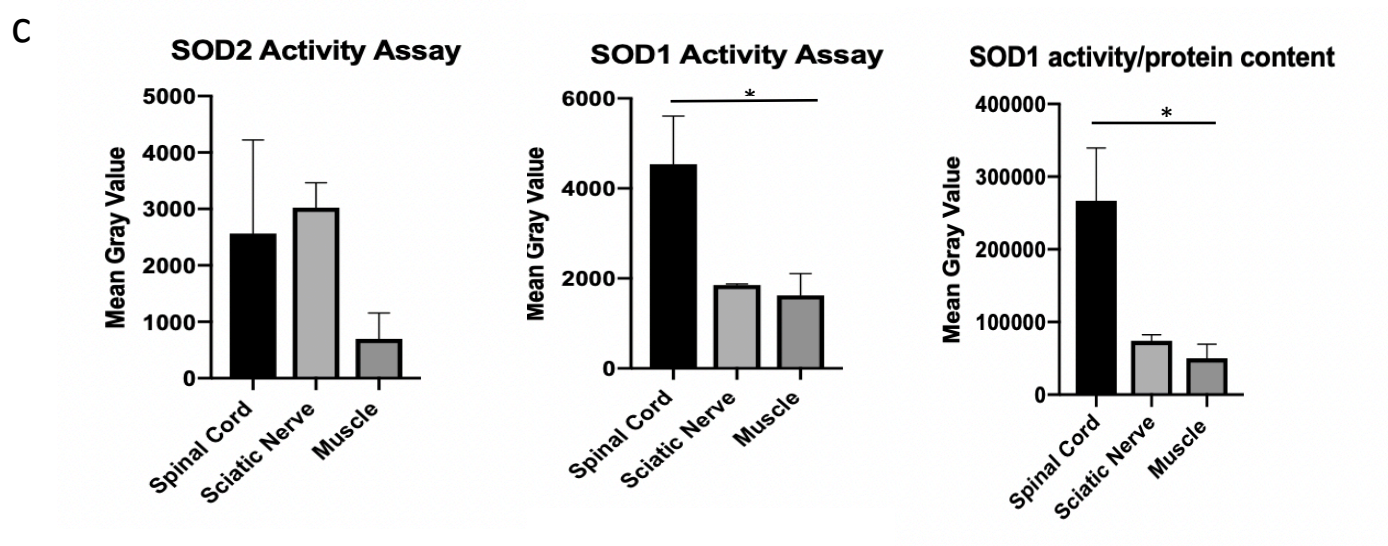


Figure 6: *SOD1/SOD2 enzyme activity and western blot analysis in muscle following ischemia* (a) SOD2 and SOD1 activity assay on muscle samples following ischemic injury show significant decline in SOD2 activity at 7 days post ischemic injury (b) SOD2 western blot on muscle samples following ischemic injury shows an increase in SOD2 content from day 7 to day 28 (c) quantification of activity and specific activity of SOD2 demonstrates that at 7 days post ischemic injury, there is a significant decline in specific activity of SOD2 in the muscle. Ordinary one way ANOVA with multiple comparisons test was used.


a
Spinal cord 1
10C Muscle
Spinal cord 2
11C Muscle
Sciatic Nerves
13C Muscle
SOD2
activity
SOD1
activity
b
Spinal cord 1
10C Muscle
Spinal cord 2
11C Muscle
Sciatic Nerves
13C Muscle
SOD1
content


c
SOD2 Activity Assay
Mean Gray Value
5000
4000
3000
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0
Spinal Cord
Sciatic Nerve
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SOD1 Activity Assay
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Spinal Cord
Sciatic Nerve
Muscle
SOD1 activity/protein content
Mean Gray Value
400000
300000
200000
100000
0
*
Spinal Cord
Sciatic Nerve
Muscle

Figure 7: *SOD1/SOD2 enzyme activity assay in spinal cord, muscle and sciatic nerve controls.* (a) Spinal cord demonstrates the greatest fluorescence for both SOD1 and SOD2 in comparison to the other samples. (b) SOD1 western blot demonstrates the greatest protein content in spinal cord samples. (c) SOD1 and SOD2 activity is significantly less in sciatic nerve and muscle samples compared to spinal cord. Statistical significance indicated by p-value less than 0.05

5. *Aconitase content decreases in aged samples following ischemic injury*

Aconitase is a citric acid cycle enzyme, localized in the mitochondrial matrix, that has iron-sulfur clusters that are prone to oxidative damage from superoxide, a reactive oxygen species. Following oxidative damage, as induced by hind limb ischemia (HLI), aconitase is degraded by ion protease, thus resulting in decreased content in the aged model (Fig. 8).

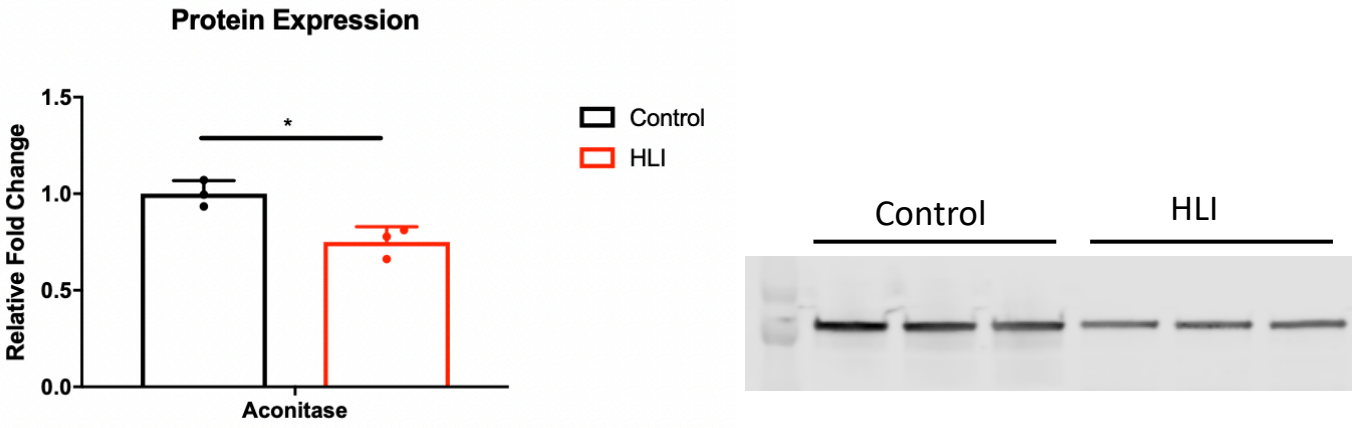

Figure 8: *Aconitase content in aged ischemic samples* Western blot analysis of wild type aged muscle samples demonstrates decrased aconitase content following ischemic injury. Two-way ANOVA with multiple comparisons test was used to test statistical significance with p-value less than 0.05.

6. Hydrogen peroxide (H2O2) production in aged ischemic samples

H2O2 levels are representative of superoxide production, thus elevated levels of H2O2 in ischemic myofibers suggests that there is more superoxide production. Both basal and complex-I linked activity (with the addition of glutamate) show increased levels of H2O2 production following ischemia (Fig. 9). However, with the addition of succinate, demonstrating complex-II linked activity, the control samples produce more superoxide which can efficiently get converted into H2O2 via SOD enzymes (Fig. 9). This isn't present in the ischemic samples, indicating that the SOD system is dysregulated and cannot convert the increased superoxide into H2O2.

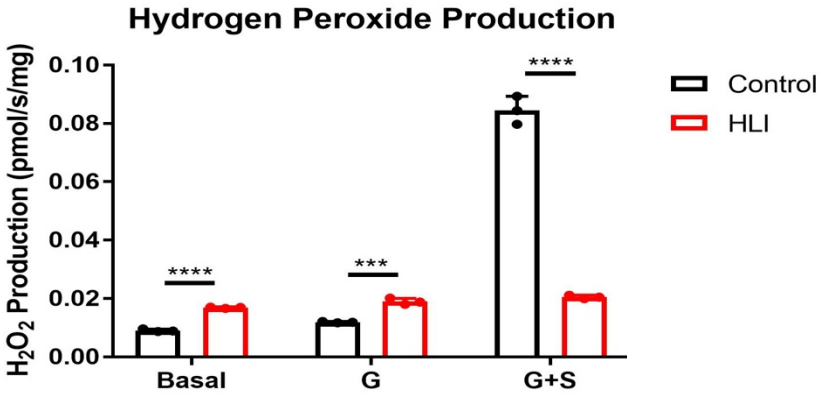

Figure 9: *H2O2 production in mitochondrial electron transport chain following ischemia in aged* Basal represents H2O2 production using only endogenous substrates, glutamate (G) represents complex-I linked activity, and glutamate + succinate (G+S) represents complex-I and complex-II linked activity. Statistical significance is determined by p-value less than 0.05.

Discussion

In this report, we demonstrate changes that occur in SOD activity and neuromuscular regeneration following ischemic injury. We additionally briefly investigated the differences present in neuromuscular regeneration in aged samples compared to young.

Our results indicate that there are significant changes to the morphology of NMJs following an ischemic injury. There is denervation of the presynaptic motor neuron from the muscle fiber and fragmentation of the AChR. These characteristics are exacerbated in the aged model, indicative of changes in the aged muscle microenvironment that prevent proper neuromuscular regeneration. This remodeling of the NMJs persists for up to 56 days but demonstrate the greatest ischemia-induced denervation at day 7. Additional studies have demonstrated partially segmented endplates and increased nerve terminal branching in the aged model when imaged under scanning electron microscope (Fahim et al., 1983). Additionally, the increase in subsynaptic nuclei during time points of greatest denervation following ischemia in young mice supports the idea that muscle satellite cells (MuSCs), muscle stem cells that fuse into the myofiber as new nuclei, have a role in the regeneration and repair of the NMJs (Schultz, 1978).

The disrupted vasculature resulting from ischemia induces myofiber atrophy in skeletal muscle and subsequently damages motor neurons (Mohiuddin et al., 2019). It has also been shown that stimulating NMJs by inducting contraction of the skeletal muscle ultimately increases the capillary-to-fiber ratio (Shiragaki-Ogitani et al., 2019), providing further evidence for cellular crosstalk amongst various components of the muscular niche, primarily the myofiber, motor neurons, vasculature, and mitochondria. Due to damage to various components of this system, remodeling of the neuromuscular domains is hindered. Furthermore, the aged model demonstrates greater denervation in control conditions, which is only exacerbated following ischemia. It is likely due to an underlying disruption in the crosstalk amongst the niche components in aged conditions that hinders proper neuromuscular structure formation. Considering the remodeling of NMJs occurs before any muscle atrophy present in aging (Deschenes et al., 2010), it is not surprising to see unhealthy NMJs in the uninjured aged controls.

The communication between muscle and nerve cells is influenced by the presence of ROS such as superoxide and hydrogen peroxide, which is regulated by the mitochondria. However, the role of ROS regulatory enzymes, such as superoxide dismutase (SOD), in maintaining the regenerative capacity of muscles and nerves has not been thoroughly investigated. How the role of these enzymes may differ in the aged model when compared to young ischemia has also not been fully researched. SOD1 and SOD2 are key enzymes that convert the highly reactive superoxide, induced by ischemia, into the less reactive hydrogen peroxide. By assessing the content and enzymatic activity of SOD1 and SOD2 in muscles and nerves following ischemic injury, we can better understand the redox signaling associated with neuromuscular regeneration. In future studies, we suspect the aged model to demonstrate lower SOD levels, due to mitochondrial dysfunction being present before any myofiber atrophy (Spendiff et al., 2016).

In conjunction with our data concerning the morphological changes to NMJs following ischemia, we are able to gather a more comprehensive biochemical understanding of the changes occurring

in the muscle microenvironment during neuromuscular regeneration by assessing changes in SOD1 and SOD2. Our results indicate that there is a disruption of SOD enzymes in muscle cells upon injury, which correlates with the timepoint of the most severe neuromuscular degeneration. By assessing H2O2 production in the mitochondrial electron transport chain, we can confirm that the SOD enzyme system is dysregulated and unable to convert increased superoxide levels into H2O2. We are also able to conclude the presence of oxidative stress within the mitochondrial matrix, due to the decrease in aconitase enzyme content following ischemic injury.

Evidence of crosstalk amongst cellular components of the muscle microenvironment indicates the likely presence of paracrine signaling. Thus, it is possible that any altered redox state of one cell type would consequently alter the redox state of a neighboring cell. Although we were unable to test SOD activity in ischemic nerve cells in comparison to muscle cells, it has been demonstrated that denervation in mouse muscle results in decreased mitochondrial respiratory capacity (Spendiff et al., 2016). Due to the mitochondria playing the major role in regulating ROS, it can be hypothesized that ischemia induced denervation will affect SOD activity in nerve cells as well. Shiragaki-Ogitani et al. (2019) demonstrated that stimulation of NMJs also increases the number of oxidative muscle fibers, suggesting improved usage of oxygen in the injured limbs. The high concentration of mitochondria present on the presynaptic and postsynaptic sides of NMJs (Rygiel et al., 2016) also provides implications for mitochondria playing a role in the remodeling of NMJs.

Prior studies assessing SOD1 mutations in ALS have shown enlarged sciatic nerves and increased diffusivity of sciatic nerves with more advanced stages of damage, when imaged with MRI (Riva et al., 2014). Electron microscopy imaging also reveals a remodeling of the blood-nerve barrier, characterized by expanded tight junction gaps and increased mitochondrial number (Riva et al., 2014). This not only provides evidence for communication amongst vasculature and nerve cells, but additionally indicates the essential role SOD1 plays in maintaining the blood-nerve barrier.

In summary, we demonstrate the occurrence of remodeling of NMJs following ischemia that is further exacerbated in the aged model. Cellular crosstalk amongst various components of the muscle microenvironment supports the notion that biochemical changes in one cell type likely influences corresponding changes in another. In our study, this is most evident with redox signaling induced by the production of ROS following ischemic injury. By understanding how SOD activity changes in individual cell types, we can better visualize how they impact one another and the overall structural changes in the neuromuscular system.

Future direction

Due to time constraints, we were unable to assess the aged phenotype on a more biochemical level. For future studies, we hope to fully investigate the specific activity of SOD1 and SOD2 enzymes in aged mice. By assessing both the biochemical and the morphological changes that occur following ischemia induced oxidative stress in the aged model, we can gain a better understanding of how neuromuscular regeneration is impacted by age. We also plan to assess the specific activity of SOD1 and SOD2 in more sciatic nerve and spinal cord samples to gain a more thorough understanding of the overall changes in the NMJs.

Conclusion

The aim of this research study was to examine the relationship between oxidative stress and neuromuscular regeneration following ischemic injury and understand how this regeneration may be altered in the aged model. These experiments have shown that ischemic injury results in denervation of the presynaptic motor neuron and fragmentation of the postsynaptic AChR. This degeneration of the NMJ is exacerbated in the aged model and demonstrates prolonged fragmentation and denervation. Additionally, our results indicate a disruption in regulatory enzymes SOD1/SOD2 upon injury. These findings suggest that SOD1 and SOD2 play an essential role in the remodeling of the NMJs and consequently in promoting a healthy neuromuscular system. In the field of neuromuscular diseases, there has been limited work focusing on redox signaling and neuromuscular remodeling in both young and aged models. The results from this present study contributes to our understanding of the crosstalk evident between muscle cells and motor neurons and how this communication amongst different cell types plays an essential role in maintaining redox homeostasis. Continued efforts are needed to further assess the aged disease model and gain a more thorough understanding of redox changes and their impacts on maintaining a healthy neuromuscular system.

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