WHOLE BODY HEAT SHOCK ATTENUATES IMPACT-INDUCED SKELETAL MUSCLE DAMAGE IN MICE

A Thesis
by
JOSHUA SCOTT GODWIN

Submitted to the Graduate School
at Appalachian State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

May 2019
Department of Health and Exercise Science

WHOLE BODY HEAT SHOCK ATTENUATES IMPACT-INDUCED SKELETAL MUSCLE DAMAGE IN MICE

A Thesis
by
JOSHUA SCOTT GODWIN
May 2019

APPROVED BY:

R Andrew Shanely, PhD.
Chairperson, Thesis Committee

Kevin A. Zwetsloot, PhD.
Member, Thesis Committee

Alan R. Needle, PhD.
Member, Thesis Committee

Kelly J. Cole, PhD.
Chairperson, Department of Health and Exercise Science

Michael McKenzie, PhD.
Dean, Cratis D. Williams School of Graduate Studies

Abstract

WHOLE BODY HEAT SHOCK ATTENUATES IMPACT-INDUCED SKELETAL MUSCLE DAMAGE IN MICE

Joshua Scott Godwin
B.S., Lees McRae College
M.S., Appalachian State University

Chairperson: R. Andrew Shanely

Impact-induced muscle damage of IIMD is a common sports injury, often resulting in acute skeletal muscle contractile dysfunction. Whole-body heat shock is reported to attenuate skeletal muscle atrophy in animal models. **PURPOSE**: The purpose of this study was to test the hypothesis that whole-body heat shock attenuates IIMD related contractile dysfunction and accelerates the recovery of contractile function following IIMD. **METHODS**: Male mice (13.7 ± 0.6mo) were randomized to either the whole body heat shock group (WBHS n = 24) or the normal body temperature group (NBT n = 24). Under anesthesia, the *in vivo* torque-frequency relationship (1Hz-300Hz) of the anterior crural muscle group was measured in all mice. Rectal temperature was then raised to 41°C (WBHS) or maintained at 37°C (NBT) for 30 min and then all mice were allowed to recover consciousness. Twenty-four hours later all mice were anesthetized again and a single impact (IIMD) was delivered via the instrumented mass-drop technique (14.1 g steel ball was dropped through a tube from 115 cm onto an impactor directly striking the tibialis anterior),

all mice were then allowed to recover. Following 2-hours, 2-days, or 5 days of normal cage activity (2-hours, 2-days, or 5-days recovery), *in vivo* torque-frequency relationships were measured in all mice. After post-recovery *in vivo* torque-frequency testing, the tibialis anterior is removed and mounted for later histological analysis. Data were analyzed using a factorial ANOVA with an *a priori* level of significance of 0.05. Fisher's LSD pair-wise comparisons were made *post hoc*. **RESULTS**: There was a significant group-time-frequency interaction ($F = 3.41$, $p < 0.0001$). Within group pairwise comparison pre-to 5-days recovery revealed, WBHS fully recovered ($p = 0.901$), and NBT did not recover ($p < 0.0001$) muscle contractile function. Comparison pre-to 2-days recovery revealed, WBHS recovered ($p = 0.090$), and NBT did not recover ($p < 0.0001$) muscle contractile function. Comparison pre to 2-hours recovery revealed both WBHS and NBT did not recover muscle contractile function ($p = 0.001$, $p < 0.0001$ respectively), however at 250Hz, WBHS had greater torque compared to NBT ($p = 0.048$). Histological analysis revealed that mice in the WBHS groups had visually less edema compared to NBT groups at 2-days and 5-days. The number of centrally located myonuclei was higher in NBT compared to WBHS at 5-days ($p = 0.001$). The percentage of injured fibers did no differ at 2-hours and 2-days ($p = 0.501$, and $p = 0.342$, respectively); however, at 5-days NBT had significantly higher percentage of injured fibers compared to WBHS, ($p = 0.048$). **CONCLUSION**: Whole body heat shock treatment prior to a single impact induced muscle injury, accelerates the rate of recovery of *in-vivo* skeletal muscle contractile function within the 2-days and 5-days recovery period, as well as confers a degree of protection to the skeletal muscle at 2-hours.

Acknowledgments

I would like to extend my gratitude to all the members of my thesis committee. Dr. Alan Needle for giving a clinical perspective and aiding with statistics. Dr. Kevin Zwetsloot for aiding in all aspects of this project, particularly with sectioning and staining of tissue. Dr. R. Andrew Shanely for aiding in all aspects of this project. I would also like to extend my gratitude to Office of Student Research, Graduate Student Government Association and to the GRAM research assistant mentoring program for helping fund this project.

Dedication

To my thesis committee chairperson, Dr. R. Andrew Shanely, thank you for your guidance and assistance not only with this project, but throughout my entire time at Appalachian State University. You have been an amazing mentor and have helped me establish many skills that will help me excel in the future.

To Dr. Kevin A. Zwetsloot, thank you for your guidance and mentorship throughout my time at Appalachian State University. The research techniques you have taught me will be invaluable in future research projects.

To my wonderful fiancé Noel and the rest of my family, thank you for supporting me in everything I do as I continue to pursue future education.

Table of Contents

List of Figures

Foreword

The research detailed in this thesis plans on being submitted as a research article to *The Journal of Applied Physiology*, a peer-reviewed journal published by the American Physiological Society; it has been formatted according to the style guide for that journal.

Chapter 1. Introduction and Literature review

Introduction

In the sports world, impact-induced skeletal muscle damage (IIMD), contusions, are one of the most common injuries incurred, however little is known about proper treatment of IIMD due to the variability of the injury. In sports, IIMD are typically caused by blunt trauma directly impacting the skeletal muscle, causing a hematoma (9,25,39). IIMD are difficult to characterize in human clinical populations; therefore, Crisco et al. (6) developed a "mass-drop" method to induce IIMD in an animal model. Since the development of this technique, other groups (3,10,15,26,27,48) have studied the effect IIMD have on skeletal muscle function and recovery. These groups report that IIMD causes whole muscle damage, ranging from contractile dysfunction to overt structural damage. They also report that recovery time for IIMD, as verified by measuring contractile function and histological evidence, takes several days to weeks, which can lead to performance deficits in sport. Due to the lengthy recovery time associated with IIMD, many studies have been aimed at treatment methods (3,18,20,27); however, little is known about the recovery of IIMD in humans other than the rest, ice, compression, and elevation (R.I.C.E) technique (1).

Heat shock proteins (HSPs) are a family of proteins that are upregulated when the body is exposed to "stress" stimuli such as heat. Heat shock proteins have protective characteristics across a variety of different tissues (4,11). The protective characteristic of HSPs have been historically studied in cardiac muscle and have been shown to protect cardiac muscle in animal models of ischemia-reperfusion injury (4). Multiple studies (10,21,24,28,33,35) report that HSPs can play a role in attenuating oxidative stress and

atrophy in skeletal muscle during disuse atrophy models. Although HSPs play a protective role in both ischemia-reperfusion (cardiac) and atrophy (skeletal muscle), the degree to which HSPs attenuate IIMD is unknown.

Literature Review

Impact-Induced Skeletal Muscle Damage. Impact-induced muscle damage (IIMD), as stated by Naughton et al. (25), can occur from contact events resulting in blunt trauma impacts and is one of the most common sports related injuries that are often overlooked as injuries that frequently require treatment (25). IIMD is usually identified by hematoma from capillary rupture and swelling due to inflammation; the common term for this is "bruising" (5,6). IIMD is normally caused by a non-penetrating blunt trauma directly to the muscle and the crushing type force that is applied, results in muscle damage (39), characterized by structural damage, contractile dysfunction, and leukocyte infiltration (1,6,10).

Impact-Induced Skeletal Muscle Damage Models. The effects of IIMD on skeletal muscle are difficult to study using human subjects, given the large variability in IIMD that occur in every-day activities (5). Consequently, most research into the effects of IIMD on skeletal muscle function and recovery, has been done in animal models, primarily in rats. In order to obtain reliable and reproducible damage, a "mass-drop" technique was developed to induce IIMD (6). The IIMD mass-drop technique involves dropping a known mass from a set height onto the desired muscle group to induce a non-penetrating blunt trauma. By adjusting the mass of the dropped object or the height from which the object is dropped, the impact force can be altered to produce more or less serve IIMD. Following the development of the mass-drop model other groups have modified the design to allow for different levels of injury and other animal models, such as mice (48).

Type of Muscle Damage and Regeneration. Contractile function is often measured by the amount of force the muscle is able to produce at a given stimulation frequency. Following IIMD, skeletal muscle is damaged at the cellular level, including contractile dysfunction, structural damage, and leukocyte invasion (5,6). Contractile dysfunction is defined as a decrease in contractile force per unit area (or mass) of skeletal muscle. The cause of contractile dysfunction is due to altered structure or alignment of the muscle fibers (6,10,20). Multiple studies have demonstrated that contractile function is significantly impaired following IIMD (3,5,10,33). Each of these studies have demonstrated that contractile function of the damaged muscle group is impaired from low/submaximal stimulation frequencies through higher maximal stimulation frequencies. Crisco et al. (6) reported that from 7 to 24 days post-IIMD, contractile function remains depressed by 10 to 20%, compared to pre-injury/non-injured values. Another important factor of contractile function recovery is the rate of fatigue that occurs. Elmer et al. (10) report that even after several days of recovery, the onset of fatigue in IIMD is 15% faster, when compared to non-injured muscle. These results demonstrate the high degree of contractile dysfunction that is caused by IIMD and how this damage can negatively affect muscle performance days to weeks after injury.

Structural damage of the skeletal muscle fibers occurs due to the physical force and mechanical strain that is imparted onto the muscle during blunt force trauma (1,6,29,39). Structural damage is characterized by the disruption of individual muscle fiber alignment and between fibers and extracellular components (6,18,37,40-42). Structural damage in skeletal muscle may be studied using histological staining techniques, such as hematoxylin and eosin stain, in order to visualize infiltration of immune cells and muscle fiber disruption. Many labs

use these techniques to map the structural regeneration of the damaged muscle tissue (6,15,16,19,21,40-42, 46). By tracking the structural regeneration of the damaged skeletal muscle (as indicated by centralized myonuclei), the degree of recovery and persisting damage can be accessed. These results show measurable myofiber disruption hours after IIMD. At two days post-IIMD, severe inflammation and misaligned fiber disruption are observed (6,21). At five days post-IIMD vimentin and fibronectin are present, both of which are components of muscle fiber regeneration (6,15,18,21). At eight days post-IIMD, significant fiber regeneration and realignment are noted (6,18,21). At ten days post-IIMD close to normal/control muscle fiber arrangement is reported (6,16,21).

Blunt trauma IIMD is noted for the large amount of edema that is visible in the hours and days following the injury; this is largely due to the invasion of leukocytes that infiltrate into the damaged area (3). Leukocyte invasion is started by neutrophils which respond to chemicals secreted by the damaged skeletal muscle and is followed closely by the invasion of macrophages (22,26,29,48). With the infiltration of neutrophils and macrophages into the damaged tissue, further structural misalignment occurs (29,40-42). Even though the initial invasion of leukocytes is damaging, the phagocytic role of these cells is vital in the removal of damaged tissues and cellular structure to allow for necessary functional recovery (22,36,40-42).

Treatment of IIMD. The treatment of IIMD has not been well studied due to the inability to control the severity of IIMD in human trails. When IIMD occurs in a human, it is uncommon for the person to be hospitalized; therefore, the various treatments for IIMD are not well researched. Currently the most common type of treatment for IIMD is to apply the R.I.C.E. method which stands for rest, ice, compression, and elevation (1). The R.I.C.E. method is

widely used due to the ease of application and does not require pharmaceutical treatment. The R.I.C.E method was developed to decrease the hematoma and connective tissue scarring that occurs as a result of immune cell activation following IIMD. By aiming to decrease the swelling and connective tissue damage, the R.I.C.E. method is employed as a first treatment therapy and the best results of recovery occur when the method is applied immediately after the injury (1).

Another method used to treat IIMD is non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs are pharmaceutical drugs that decrease swelling and inflammation as the result of skeletal muscle injury (29,32). NSAIDs are often overused and can be detrimental to the healing process as inflammation is needed for normal recovery (29). Inflammation is the result of leukocyte invasion which is critical for the remove damaged tissue and is necessary for the recovery process (36). Further evidence against the over-use of NSAIDs comes from Rahusen et al. (32), who report that the use NSAIDs had no significant effect in the treatment of acute IIMD.

Regardless of the treatment that is selected for healing of IIMD, timing is vital in the effectiveness of regeneration (1,26). Baoge et al. (1) explains that the R.I.C.E. method is best when implemented directly after injury and the use of NSAIDs should not be used prior to 48 hours post-IIMD (1). The use of NSAIDs during the initial 48-hour post-injury period can interfere with the inflammatory response that is necessary for normal muscle repair (1). Regardless of the treatment type employed, mitigating the symptoms of IIMD injury is often the primary goal, which is why the R.I.C.E method is most commonly used.

Despite the ineffectiveness of some treatments to IIMD, it is worth mentioning that a study by Kim et al. (17), reports that heat therapy following eccentric exercise induced

muscle damage, accelerates the recovery of fatigue resistance compared to non-heat-treated human subjects (17). Given that eccentric exercise induced-injury is a common sport related injury, heat treatment could be a viable therapy option for treating IIMD.

***Heat Shock Protein Families*.** Heat shock proteins are a family/group of proteins whose name is derived from their primary function of chaperoning nascent proteins and to protect against denaturing of proteins during stressful conditions, mainly heat (13,45). The HSP family of proteins are divided into subfamilies based on the size of the molecules in kilodaltons (kDa), ranging from HSP10 to HSP110 (13). The HSP70 and HSP90 families of proteins have been reported to play an important role during physical exercise. HSP72 is a well-known, inducible protein that chaperones other HSPs as well as aiding in the folding of newly synthesized proteins (13). HSP90 is a major constitutively expressed protein that has been reported to be largely active in chaperoning the folding of newly synthesized proteins, while also aiding in myosin thick filament and sarcomere formation (12,13,45). The HSP110 family of heat shock proteins has been reported to play roles in supporting the immune system as well as aiding protein folding (44,51). HSP110 has also been reported to amplify inflammatory signals; however, little is known on the expression of HSP110 in response to exercise (13,44,51).

***Heat Shock Proteins in Cardiac Muscle*.** Cardiac and other cardiovascular related diseases are among the leading health problems (4,11,16,38), therefore any treatment or protective method that can be applied would have tremendous clinical impact. HSPs have been heavily researched for their role in protection of cardiac muscle, especially following a cardiac event (4,7). Ischemic events are characterized by insufficient blood flow to a tissue, often resulting in, stunning, necrosis or death of the tissue (7,16). HSP activation prior to an ischemic event

in the heart has been demonstrated to provide effective cardio-protection during these conditions (4,7,8,11). It has been demonstrated that activation of HSPs prior to the ischemic event enhances recovery of the cardiac muscle (7,8,38). Further studies conducted by Karmazyn et al. (16) have associated HSP activation with recovery of force in cardiac muscle following ischemic conditions. The same study also related the protective mechanisms of HSPs to attenuating ventricular damage, thus enhancing recovery. From these studies involving the protective role of HSPs in cardiac muscle, many researchers also believe heat shock proteins play a similar role in skeletal muscle (24,28,34). However further studies are needed to investigate if the mechanisms of protection provided by HSPs are similar in both cardiac and skeletal muscle.

Heat Shock Proteins in Hindlimb Suspension and Immobilization. Hindlimb suspension models were developed to simulate microgravity and results in disuse atrophy of the affected limbs. In these experimental animal models, mainly mice and rats, the hindlimbs are suspended so that the forelimbs bare the majority of the body mass and thus, the animal can no long use their hindlimbs for normal ambulation. The purpose of this technique is to study the effect of disuse or low contractile activity on skeletal muscle and increases in oxidative stress and atrophy of the muscle (24, 28). During the reloading phase of the suspension experiments, which occurs once the animal is allowed use of its hind limbs again, a large amount of eccentric damage is observed, which is characterized by increased inflammation and muscle fiber disruption (24). This heavy inflammation and muscle fiber disruption then leads to impaired contractile function, resulting in further loss of specific force production (37). It has been demonstrated that during the end of suspension and the beginning of the reloading phase, skeletal muscle HSP expression is low (28); however, following

inflammation and disruption of muscle fibers from reloading, HSP expression increases significantly (24,28). Once recovery of injured muscle is complete, HSP expression returns to pre-suspension values. These data indicate that HSP expression responds to damage and plays a role in the recovery of skeletal muscle health and function.

In practical applications, prevention or attenuation of damage is preferred, meaning activation of HSPs before hindlimb suspension or other insults attenuates muscle damage. Naito et al. (24) demonstrated that activation of HSPs prior to hindlimb suspension results in less skeletal muscle atrophy, suggesting that induction of HSPs also plays a role in protection of skeletal muscle from atrophy.

Immobilization is often used in clinical settings as an initial treatment for various injuries, however it can lead to skeletal muscle weakness caused by low contractile activity in the muscle (45). Similar to hindlimb suspension, low contractile activity in the skeletal muscle of the immobilized limb causes increased oxidative stress and muscle atrophy (35). The damage caused by immobilization results in loss of fiber size, decreasing overall force production (35). Selsby et al. (34) demonstrated that activation of HSPs prior to immobilization decreases the amount of atrophy and oxidative stress. In this study, HSPs were activated prior to immobilization of one hindlimb. After eight days of immobilization, HSP72 levels, antioxidant enzyme activity, and total muscle weight were measured. After eight days immobilization it was found that levels of antioxidant enzyme activity, and muscle mass were significantly higher in heat-stressed rats, compared to immobilized non-heat shocked control rats. These results demonstrate that the activation of HSPs prior to immobilization played a role in protecting against skeletal muscle oxidative stress and atrophy. This protective role displayed by HSPs is important as it demonstrates that HSP

activation may be used as a viable modality to prevent or attenuate skeletal muscle oxidative stress and atrophy during periods of disuse.

***Heat Shock Proteins in Mechanical Ventilation*.** Mechanical ventilation is a critical procedure in rehabilitating patients who cannot maintain adequate alveolar ventilation. In controlled mechanical ventilation, air is forced into the airways and controlled by an external source (15). However, controlled mechanical ventilation can cause significant diaphragmatic problems including oxidative stress, muscle fiber atrophy, and increased contractile dsyfunction (14,30,36,40,49,50). As shown by Ichinoseki et al. (14), HSP activation prior to controlled mechanical ventilation protects the diaphragm from oxidative stress and atrophy. This study demonstrates that activation of HSPs decreased the amount of oxidative stress caused by controlled mechanical ventilation. Oxidative stress is damaging to the muscle in that the build-up of harmful metabolites can impair function and decreased force production (39,49,50). Decreased oxidative stress was assessed by measuring the levels of oxidized proteins in HSP activated diaphragm when compared to a control of non-heat shock protein activated diaphragm (14). Ichinoseki et al. (14) also reported that when HSPs are activated, the overall weight of the mechanically-ventilated diaphragm and the fiber cross-sectional area did not decrease compared to normal non-mechanically-ventilated diaphragm. Controlled mechanical ventilation results in contractile dysfunction (31), however HSP activation prior to mechanical ventilation can protect against contractile dysfunction (49). Yoshihara et al. (49) demonstrated that with the activation of HSPs prior to controlled mechanical ventilation, the prevention of diaphragm contractile dysfunction at lower stimulation frequencies is observed. This is an important concept regarding the effects of HSPs, due to submaximal stimulation being more frequent in day-to-day activities.

Heat Shock Proteins Attenuate Eccentric Contraction-Induced Muscle Damage. It has been previously reported by McArdle et al. (23) that life-long overexpression of HSP70 in mice attenuates some of the force deficit following eccentric contraction-induced muscle injury. The study also showed an improved recovery time in both adult and old mice that overexpress HSP70 when compared to mice that do not overexpress HSP70. This study by McArdle et al. (23) shows that overexpression of HSPs aid in the recovery of a sports related injury.

Muscle Injury and HSP. Hindlimb suspension, immobilization, mechanical ventilation, eccentric muscle damage in skeletal muscle, and ischemic conditions in cardiac muscle cause oxidative stress and atrophy, both of which lead to a decrease in specific force production of muscle. Previous studies report that HSP activation has a protective effect in cardiac muscle and skeletal muscle, decreasing oxidative stress and protecting against atrophy, thus playing a role in recovery. The collective findings from these studies demonstrate that activation of HSPs can attenuate of a many types of muscle damage. However, it is unknown if prior activation of HSPs has a protective role in decreasing the amount of IIMD caused by a single impact.

Purpose of Study

IIMD is among the most common sports related injury and HSP activation provides a protective mechanism against muscle damage in a variety of muscle injuries. Therefore, the purpose of this study was to determine the degree to which whole body heat shock attenuates impact-induced skeletal muscle damage on the anterior crural skeletal muscle group in adult mice and to assess recovery following impact-induced skeletal muscle damage by measuring contractile function and histological analyses.

Hypothesis

I hypothesized that whole body heat shock prior to IIMD would decrease the amount of damage to the skeletal muscle, decreasing both contractile muscle function loss and structural damage; as well as, decrease the time to recover contractile function. Further, I hypothesize that whole body heat shock prior to IIMD will result in less overt structural damage compared to normal body temperature.

Chapter 2. Methods

Methods

Experimental Design. Adult C57BL/6 mice were randomly assigned to one of six groups and the body weight of each mouse was measured. Following this, mice were then anesthetized with 4% isoflurane and maintained with 2% isoflurane prior to undergoing *in vivo* contractile function testing (described below). After the initial contractile function testing, mice were either subjected to whole body heat shock (WBHS) or maintained at normal body temperature (NBT). 24-hours later mice were anesthetized and received one impact using the mass-drop method, all mice were then allowed to recover consciousness and allowed normal cage activity. Following the assigned recovery time of 2-hours, 2-days, or 5-days, mice were re-anesthetized and *in vivo* contractile function was retested. After the follow-up contractile function, the tibialis anterior muscle was harvested and mounted for histological analysis.

Animals. Male C57BL/6 mice aged 12-to-14 months, procured from the in-house colony at Appalachian State University, were randomly assigned into one of six groups (n=8 per group); whole body heat shock 2-hour, whole body heat shock 2-day, whole body heat shock 5-day, normal body temperature 2-hour, normal body temperature 2-day and, normal body temperature 5-day. All groups were tested for contractile function via *in-vivo* methods prior to heat treatment or normal body temperature conditions and again after the prescribed amount of time following IIMD. This study was approved by the Appalachian State University IACUC (16-18).

Injury Model. The IIMD model that was employed was developed by the Crisco lab (5,6) and later modified by Xiao et al. (48) for mice. The model used (48) is a mass-drop technique

where a 14.1g steel ball was dropped from a height of 115cm onto a metal impactor that directly impacts the belly of the tibialis anterior (TA), Figure 1. The force that is applied to the TA has been reported to induce whole muscle damage (5,6,33,47).

In Vivo Contractile Function. To test contractile function, a torque frequency curve was determined for each mouse prior to injury. Once the animal is anesthetized with 4% isoflurane and maintained with 2% isoflurane, isometric twitch contractions were used to obtain optimal electrode placement of the peroneal nerve to stimulate the anterior crural muscles, optimal placement was confirmed by 5-10 300Hz isometric contractions. Following electrode placement, a torque frequency curve tested the isometric torque produced by the anterior crural muscle group at 11 ascending 120ms train stimulations ranging from 1Hz to 300Hz with two minutes rest between each contraction. This torque frequency curve is designed to assess torque from submaximal, unfused tetanic contractions up to maximal fused tetanic contractions. This specific torque frequency curve was published by Baumann et al. (2) and has been conducted by our lab in previous investigations (see Figure 2).

Data Acquisition and Analysis. The muscle lever system (Aurora Scientific 1300A, Aurora, ON, Canada), stimulator and force transducer was connected to a signal interface (Aurora Scientific, Model 610A) that sends the analog signal to an analog to digital converter card (Model PCI-6221, National Instruments, Austin, TX, USA) on a computer with Dynamic Muscle Control software (Aurora Scientific, 310A). The force output data was analyzed utilizing the Dynamic Muscle Analysis software (Aurora Scientific, 610A).

Activation of Heat Shock Proteins. Whole body heat shock was used to activate heat shock proteins. The mice were anesthetized (as described above) and placed on an electric heating pad in the prone position. The rectal temperature of the animals was raised to 41° C and

maintained at this temperature for 30 minutes. The temperature of the animals was monitored by a rectal temperature probe (34-1402, Harvard Apparatus, Holliston, MA) throughout the entirety of the heating protocol. Animals in the normal body temperature groups, were maintained at 37° C for 30 minutes. All mice were then allowed to fully recover consciousness and resume normal cage activity.

Preliminary Data. Preliminary data from our lab shows that this method of whole body heat shock increased the expression of HSP72 ~5-fold (Figure 3). This was confirmed by Western blot analysis of homogenized skeletal muscle 24 hours post whole-body heat shock. Preliminary data also showed that whole body heat shock had no effect on the amount of torque produced. This was tested by comparing torque frequency curves pre- and 24hr post-whole body heat shock treatment, data not shown.

Histology. Tissue was harvested and mounted for histological analysis at 2-hours, 2-days, and 5-days post injury. Harvested tissue mounted on cork using optimal cutting temperature medium (Fisher healthcare, Houston, TX., USA), frozen in isopentane cooled in liquid nitrogen. Slide-mounted serial cross-sections, 10μm thick, were obtained from the proximal, medial, and distal portions of the tibialis anterior muscle. Mounted tissue sections were then stained using common histological techniques for cytosolic and nucleic components using Mayer's hematoxylin and eosin (H&E) solution. H&E stained sections were then analyzed using an EVOS imaging microscope (AMFC4300, Thermofisher) at magnification 20X. Several images were taken of each section, ~300 fibers/section. When quantifying the amount of muscle damage, the total number of damaged/regenerating fibers was taken as a percentage of the total number of fibers. The guidelines used for quantifying muscle damage

included counting the presence of: pale cytoplasm, extracellularly located phagocytes, intracellularly located phagocytes, and centrally located myonuclei (19,43).

Statistics. A three-way factorial ANOVA was used to evaluate muscle torque-generating capacity. Treatment group, recovery/harvest time post IIMD, and stimulation frequency were the three factors. Histological data were analyzed using a one-way ANOVA for each marker of damage. All statistical analysis used a *priori* level of significance set at $p < 0.05$ and Fisher's LSD pair-wise comparison were made *post hoc*. Data were analyzed using SPSS (IBM Inc., Chicago, IL, USA).

Chapter 3: Results

Contractile Function. At 5-days post-IIMD there was a significant interaction effect of time-by-frequency-by-group ($F = 3.602$; $p = 0.038$). Mice that received the WBHS treatment fully recovered torque values over the entire torque frequency relationship, ($F = 0.016$; $p = 0.901$, Figures 4 and 8), whereas mice that were maintained at NBT did not, ($F = 26.368$; $p = 0.001$, Figures 4 and 7). Further analysis of torque values at a submaximal frequency, 40Hz, and a maximal tetanic frequency, 250Hz, revealed that WBHS animals recovered torque at both frequencies, ($F = 0.078$; $p = 0.786$ and $F = 0.293$; $p = 0.602$, respectively), whereas animals in the NBT group did not, ($F = 23.943$; $p = 0.001$ and $F = 12.752$; $p = 0.027$, respectively, Figures 9 and 10).

At 2-days post-IIMD, there was a significant interaction effect of time-by-frequency-by-group ($F = 3.689$; $p = 0.024$). WBHS mice fully recovered torque values over the entire torque frequency relationship ($F = 0.090$; $p = 0.090$, Figures 5 and 8), whereas NBT mice did not, ($F = 23.071$; $p = 0.0001$, Figures 5 and 7). Further analysis of torque values at a submaximal frequency, 40Hz, and a maximal tetanic frequency250Hz, revealed that WBHS animals recovered torque at both frequencies, ($F = 0.888$; $p = 0.516$ and $F = 1.675$; $p = 0.217$, respectively), whereas NBT mice did not, ($F = 9.244$; $p = 0.009$ and $F = 21.583$; $p = 0.0001$, respectively, Figures 9 and 10.)

At 2-hours post-IIMD, there was a significant interaction effect of time-by-frequency-by-group ($F = 6.001$; $p = 0.0001$). The torque frequency relationship was significantly depressed in both groups, ($F = 4.075$; $p = 0.001$ for both groups, Figure 6) with WBHS IIMD function significantly greater than NBT ($F = 4.677$; $p < 0.05$, Figure 6). Further analysis of torque at 40Hz and 250Hz was significantly depressed compared to pre-injury for both

groups, (F = 104.462; p = 0.0001 and F = 79.628; p = 0.001, respectively, Figures 9 and 10). However, the WBHS group generated significantly more torque than the NBT group at 40Hz, at 250Hz no significant difference was found between NBT and WBHS, (F = 1.249; p = 0.195; F = 4.677; p = 0.048 respectively, Figure 9 and 10).

Skeletal Muscle Morphology. Hematoxylin and Eosin staining was used assess the changes in skeletal muscle morphology following IIMD; see Figure 11 for normal skeletal muscle morphology. Morphology was quantified using the following criteria; presence of extracellular phagocytes (Figure 12), pale cytoplasm (Figure 13), intracellular phagocytes (Figure 14), and centrally located myonuclei (Figure 15). At 2-hours post-IIMD, visually substantial edema and fiber disruption was present, while what appears to be the start of phagocyte infiltration is observed, (Figure 16). At 2-days post-IIMD, many extracellular phagocytes are present and a small quantity of centrally located myonuclei are present, (Figure 17). For intracellular located phagocytes, there was a significant main effect of group (F = 6.044; p = 0.001). Fisher LSD comparisons revealed, NBT group at 2-days post-IIMD had significantly greater intracellular phagocyte infiltration compared to un-injured control tissue (p = 0.009, Figure 19). The presence of centrally located myonuclei displayed a significant main effect of group (F = 7.675; p = 0.001). Fisher LSD comparisons revealed, both groups at 2-hours and 2-days post-IIMD did not differ (p = 0.354 and p = 0.355, respectively). However, the NBT had significantly more centrally located myonuclei at 5-days post-IIMD than the WBHS group, (p = 0.001, Figure 21). The percentage of injured fibers, as described by Tsivitse et al. (43), displayed a significant main effect of group (F = 7.385; p = 0.0001). Fisher LSD comparisons revealed both groups at 2-hours, or 2-days post-IIMD did not differ (p = 0.501, and p = 0.342, respectively). However, the NBT group at 5-

days post-IIMD had significantly more injured fibers than the WBHS group 5-days post-IIMD, ($p = 0.048$, Figure 22).

Chapter 3. Discussion

Discussion

The purpose of this study was to investigate the effects of WBHS, prior to IIMD, has on the recovery of contractile function in adult mice and its effects on attenuating the amount of skeletal muscle damage incurred. The novel findings of this study are that WBHS accelerated the rate of contractile function recovery at both 2-days and 5-days and afforded a degree of protection against contractile function loss 2-hours post-IIMD. Histological findings demonstrate that WBHS treatment reduced the number of intracellularly located phagocytes at 2-days and WBHS treatment resulted in a decrease in the number of centrally located myonuclei at day 5.

Accelerated Recovery of In-Vivo Contractile Function. The response of contractile function in NBT mice following IIMD was as hypothesized. Mice in the NBT groups had contractile function deficits of ~50% at 2-hours and ~40% at 2- and 5-days post-IIMD at a maximal tetanic contraction, 250Hz. These results align with previous *in vitro* contractile function data from Crisco et al. (6), who report depressed contractile function up 24 days following IIMD.

The rate of contractile function recovery from IIMD was accelerated in the WBHS treatment group. Mice that received WBHS recovered contractile function at both 2-days and 5-days (89% and 96% of pre-injury, respectively). At 2-hours post-impact injury, contractile function was significantly depressed in both treatment groups; however, at 250Hz, WBHS resulted in less functional decrements (30%), compared to NBT mice (50%; $p<0.05$), where at 40Hz there was no significant difference between WBHS and NBT, ($p=0.195$). These data align with the functional deficits reported by Crisco et. al (6) and Russ et. al (33), in that functional deficits of ~30-40% persist 7 days post-IIMD.

These results differ from previous studies by Yoshihara et al. (49) and Ichinoseki et al. (14), where these groups found WBHS prior to controlled mechanical ventilation did not attenuate the loss of *in vitro* diaphragmatic contractile function. The results of the current study are in line with results from McArdle et al. (23), where transgenic mice that had life long overexpression of HSP70 had increased contractile function recovery times and attenuated force deficits after eccentric contraction-induced skeletal muscle injury. Therefore, the contractile function results of the current study are in agreement with the hypothesis that activation of HSPs via whole body heat shock prior to skeletal muscle injury confers a degree of protection.

Histological Analysis. Impact-induced skeletal muscle damage has been characterized in previous studies by Crisco et al. (6) and Xaio et al. (48) as displaying large amounts of muscle fiber disruption and a large infiltration of phagocytic cells, namely neutrophils and macrophages. In the current study, several key histological markers for repairing/regenerating muscle fibers were used, as previously described by Koh et al. (19), and Tsivitise et al. (43). These markers include presence of pale cytoplasm, extracellular phagocytes, intracellular phagocytes, and centrally located myonuclei.

When examining H&E staining of both WBHS and NBT groups at all time points it is apparent that both groups have an increased presence of extracellularly located phagocytes. These findings are in-line with increased presence of phagocytes reported by Xaio et al. (48), i.e., the infiltration of phagocytes peaks 1-3 days post-injury. The progression of different phagocyte infiltration into the damaged muscle issue is explained in depth by Tidball (42). The infiltration of these phagocytes begins with the invasion of neutrophils, starting hours after injury, and is followed closely by the infiltration of M1-like (pro-inflammatory)

macrophages and later M2-like (anti-inflammatory) macrophages (42). This progression of phagocyte infiltration into damaged skeletal muscle was confirmed by Xaio et al. (48) in an IIMD model in mice. The time course for extracellular phagocyte infiltration matches the histological evidence seen from the current study, where at 2-hours post-IIMD some phagocytes can be seen (Figure 16), at 2-days post IIMD (Figure 17) increased levels of phagocyte invasion are observed, and at 5-days the levels of phagocyte infiltration is again minimal (Figure 18). Even though the amount of extracellularly located phagocytes was visual higher in all groups, the amount of intracellularly located phagocytes was lower than 5-days in both WBHS and NBT groups. This finding differs from Xaio et al. (48), where they reported mRNA data for CD163 M2-like macrophages was still elevated at 5-days post IIMD, suggesting intracellularly located phagocytes were expected to be elevated in the current study.

The number of centrally located myonuclei is higher in NBT mice at 5-day compared to WBHS mice at the same time point, while at 2-days both groups have approximately the same number of centrally located myonuclei. Given this decrease in centrally located myonuclei at 5-days in WBHS mice, a possible explanation for accelerated contractile function recovery is that WBHS could be affecting the number of centrally located myonuclei between 2-days and 5-days post-injury. The presence of centrally located myonuclei represent the shift from damaged fibers to regeneration/repairing fibers (40,42). Evidence of fewer centralized myonuclei at 5-days in WBHS mice, could mean that mice with WBHS treatment are reaching the fiber regeneration stage sooner compared to NBT mice. This decrease in central myonuclei at 5-days in WBHS mice could also be due to less initial damage in WBHS mice, evident by the contractile function data at 2-hour post-IIMD

(figure 6). Less initial damage would result in a both a decreased phagocyte response and fewer regenerating fibers meaning fewer centralized myonuclei. Further studies with time points between 2-days and 5-days post-injury are needed to determine the peak of centrally located myonuclei for WBHS mice.

While quantifying the markers of muscle damage, it is visually clear that animals in the WBHS treatment groups had less edema compared to animals in the NBT groups, (Figures 16-18). Less edema could be another possible mechanism for the accelerated recovery of contractile function in mice subjected to WBHS treatment. Less edema, as seen in WBHS groups, can result in less contractile dysfunction as shown by less force deficit at 2-hours and full recovery of contractile function at 2-days and 5-days post-IIMD. It has been proposed that edema increases the disruption of actin and myosin filaments, reducing the ability of cross bridge formation and ultimately resulting in a decrease in contractile function (6,10,25). Further investigation into quantifying the amount of edema is needed to determine if this is actually a mechanism involved in the protection afforded by whole body heat shock treatment.

Future Studies. Future studies should aim to discern the mechanism at which WBHS is affecting the skeletal muscle following IIMD. Future studies should include understanding the monocyte progression in the injured tissue by using monocyte markers. This would determine the effect WBHS has on the progression of macrophage phenotypes from M1-like pro-inflammatory to M2-like anti-inflammatory and aid in determining the stage of recovery at that time point. Another future study would be to use a sarcolemmal stain of calcium dye to understand how the release and reuptake of calcium is affected. Russ et al. (33), report that calcium uptake and release are impaired following IIMD with no intervention; therefore,

determining calcium handling after WBHS and IIMD could prove to be another potential mechanism of WBHS providing protection and accelerating recovery. Also, future studies should look into the difference between local single limb heating/HSP expression verse global whole body heating/broad HSP expression. Studies determining which HSPs are predominantly playing a role in skeletal muscle is needed. To do this, shRNA can be used to directly inhibit HSPs in the skeletal muscle, this could be used to determine which HSPs are primarily affecting recovery of skeletal muscle.

Limitations of the Study. While this study is the first to test if WBHS treatment prior to impact-induced muscle injury affects contractile function and histological markers of muscle damage, the investigation was not without limitations. Major limitations of this study include the following: muscle wet weight was not measured, this would have allowed for an estimate of the amount of edema resulting from IIMD and if WBHS attenuates the resulting edema, only three time points were tested, earlier post-injury time points (e.g., immediately post-IIMD, 30min and 60min post-IIMD) may help if WHBS attenuates edema.

CONCLUSION

This is the first study to report that WBHS prior to IIMD attenuates skeletal muscle damage and accelerates the rate of contractile function recovery. Furthermore, the 2-hour post-injury data suggest that WBHS treatment provides a protective mechanism to the skeletal muscle. Further analysis is needed to uncover the differing response in the amount of edema that is visually seen between WBHS and NBT groups. The findings from this study also warrant the need for further investigation into the mechanism with which HSP activation provides this protection.

References

1. **Baoge L, Van Den Steen E, Rimbaut S, Philips N, Witvrouw E, Almqvist KF, Vanden Bossche LC**. Treatment of skeletal muscle injury: a review. *ISRN Orthop* 2012. doi:10.5402/2012/689012
2. **Baumann CW, Rogers RG, Gahlot N, Ingalls CP**. Eccentric contractions disrupt FKBP12 content in mouse skeletal muscle. *Physiological Reports.* 2: 2014. doi:10.14814/phy2.12081
3. **Beiner JM, Jokl P, Cholewicki J, Panjabi MM**. The effect of anabolic steroids and corticosteroids on healing of muscle contusion injury. *Am J Sports Med.* 27: 2-9, 1999. doi:10.1177/03635465990270011101
4. **Benjamin IJ, McMillan DR**. Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. *Circ Res*. 83: 117-132, 1998.
5. **Crisco JJ, Hentel KD, Jackson WO, Goehner K, Jokl P**. Maximal contraction lessens impact response in a muscle contusion model. *J Biomech*. 29: 1291-1296, 1996.
6. **Crisco JJ, Jokl P, Heinen GT, Connell MD, Panjabi MM**. A muscle contusion injury model. Biomechanics, physiology, and histology. *Am J Sports Med.* 22: 702-710, 1994. doi:10.1177/036354659402200521
7. **Currie RW, Karmazyn M, Kloc M, Mailer K**. Heat-shock response is associated with enhanced postischemic ventricular recovery. *Circulation Research.* 63: 543-549, 1988. doi:10.1161/01.res.63.3.543
8. **Demirel HA, Powers SK, Zergeroglu MA, Shanely RA, Hamilton K, Coombes J, Naito H**. Short-term exercise improves myocardial tolerance to in vivo ischemia-

reperfusion in the rat. *Journal of Applied Physiology*. 91: 2205-2212, 2001. doi:10.1152/jappl.2001.91.5.2205

9. **Diaz JA, Fischer DA, Rettig AC, Davis TJ, Shelbourne KD**. Severe Quadriceps Muscle Contusions in Athletes: A Report of Three Cases. *The American Journal of Sports Medicine.* 31: 289-293, 2003. doi:10.1177/03635465030310022201
10. **Elmer S, McDaniel J, Mattson J, Martin J**. Effect of a contusion injury on muscular force, power, work, and fatigue. *Scand J Med Sci Sports.* 22: 488-494, 2012. doi:10.1111/j.1600-0838.2010.01276
11. **Gray CC, Amrani M, Yacoub MH**. Heat stress proteins and myocardial protection: experimental model or potential clinical tool? *Int J Biochem Cell Biol.* 31: 559-573, 1999.
12. **Hawkins TA, Haramis AP, Etard C, Prodromou C, Vaughan CK, Ashworth R, Wilson SW**. The ATPase-dependent chaperoning activity of Hsp90a regulates thick filament formation and integration during skeletal muscle myofibrillogenesis. *Developmen.* 135: 1147-1156, 2008. doi:10.1242/dev.018150
13. **Henstridge DC, Febbraio MA, Hargreaves M**. Heat shock proteins and exercise adaptations. Our knowledge thus far and the road still ahead. *J Appl Physiol.* 120: 683-691, 2016. doi:10.1152/japplphysiol.00811.2015
14. **Ichinoseki-Sekine N, Yoshihara T, Kakigi R, Sugiura T, Powers SK, Naito H**. Heat stress protects against mechanical ventilation-induced diaphragmatic atrophy. *J Appl Physiol.* 117: 518-524, 2014. doi:10.1152/japplphysiol.00170.2014

15. **Kami K, Masuhara M, Kashiba H, Kawai Y, Noguchi K, Senba E**. Changes of vinculin and extracellular matrix components following blunt trauma to rat skeletal muscle. *Med Sci Sports Exerc.* 25: 832-840, 1993.
16. **Karmazyn M, Mailer K, Currie RW**. Acquisition and decay of heat-shock-enhanced postischemic ventricular recovery. *Am J Physiol.* 259: 424-431, 1990. doi:10.1152/ajpheart.1990.259.2.424
17. **Kim K, Kuang S, Song Q, Gavin TP, Roseguini BT**. Impact of heat therapy on recovery following eccentric exercise in humans. *Journal of Applied Physiology*. 910: 2019. doi:10.1152/japplphysiol.00910.2018
18. **Kobayashi M, Ota S, Terada S, Kawakami Y, Otsuka T, Fu FH, Huard J**. The Combined Use of Losartan and Muscle-Derived Stem Cells Significantly Improves the Functional Recovery of Muscle in a Young Mouse Model of Contusion Injuries. *The American Journal of Sports Medicine.* 44: 3252-3261, 2016. doi:10.1177/0363546516656823
19. **Koh TJ, Brooks SV**. Lengthening contractions are not required to induce protection from contraction-induced muscle injury. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology.* 208: 2001. doi:10.1152/ajpregu.2001.281.1.r155
20. **Kruger MJ, Smith C**. Postcontusion polyphenol treatment alters inflammation and muscle regeneration. *Med Sci Sports Exerc.* 44: 872-880, 2012. doi:10.1249/MSS.0b013e31823dbff3

21. **Lawler JM, Song W, Kwak HB**. Differential response of heat shock proteins to hindlimb unloading and reloading in the soleus. *Muscle Nerve.* 33: 200-207, 2006. doi:10.1002/mus.20454

22. **Liu X, Zeng Z, Zhao L, Xiao W, Chen P**. Changes in inflammatory and oxidative stress factors and the protein synthesis pathway in injured skeletal muscle after contusion. *Experimental and Therapeutic Medicine*. 2017. doi:10.3892/etm.2017.5625

23. **Mcardle A, Dillmann WH, Mestril R, Faulkner JA, Jackson, MJ**. Overexpression of HSP70 in mouse skeletal muscle protects against muscle damage and age-related muscle dysfunction. *The FASEB Journal.* 18: 355-357, 2004. doi:10.1096/fj.03-0395fje

24. **Naito H, Powers SK, Demirel HA, Sugiura T, Dodd SL, Aoki J**. Heat stress attenuates skeletal muscle atrophy in hindlimb-unweighted rats. *J Appl Physiol.* 88: 359-363, 2000. doi:10.1152/jappl.2000.88.1.359

25. **Naughton M, Miller J, Slater GJ**. Impact-Induced Muscle Damage and Contact Sports: Etiology, Effects on Neuromuscular Function and Recovery, and the Modulating Effects of Adaptation and Recovery Strategies. *International Journal of Sports Physiology and Performance.* 13: 962-969, 2018. doi:10.1123/ijspp.2017-0268

26. **Nicholas J, Voss JG, Tsuji J, Fulkerson ND, Soulakova J, Schneider BS**. Time course of chemokine expression and leukocyte infiltration after acute skeletal muscle injury in mice. *Innate Immunity.* 21: 266-274, 2014. doi:10.1177/1753425914527326

27. **Nozaki M, Li Y, Zhu J, Ambrosio F, Uehara K, Fu FH, Huard J**. Improved Muscle Healing after Contusion Injury by the Inhibitory Effect of Suramin on

Myostatin, a Negative Regulator of Muscle Growth. *The American Journal of Sports Medicine.* 36: 2354-2362, 2008. doi:10.1177/0363546508322886

28. **Oishi Y, Taniguchi K, Matsumoto H, Kawano F, Ishihara A, Ohira Y**. Upregulation of HSP72 in reloading rat soleus muscle after prolonged hindlimb unloading. *Jpn J Physiol.* 53: 281-286, 2003.
29. **Peake JM, Markworth JF, Nosaka K, Raastad T, Wadley GD, Coffey VG**. Modulating exercise-induced hormesis: Does less equal more? *J Appl Physiol.* 119: 172-189, 2015. doi:10.1152/japplphysiol.01055.2014
30. **Powers SK, Jackson MJ**. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiol Rev* 88: 1243–1276, 2010.
31. **Powers SK, Shanely RA, Coombes JS, Koesterer TJ, McKenzie M, Van Gammeren D, Dodd SL**. Mechanical ventilation results in progressive contractile dysfunction in the diaphragm. *J Appl Physiol.* 92: 1851-1858, 2002. doi:10.1152/japplphysiol.00881.2001
32. **Rahusen FT, Almekinders LC**. Non-Steroidal Anti-Inflamatory Drugs (Nsaids) And Acetaminophen In The Treatment Of An Acute Muscle Injury. *Medicine & Science in Sports & Exercise.* 35: 2002. doi:10.1097/00005768-200205001-00575
33. **Russ DW, Garvey SM, Densmore C, Hawks T, Herman S, Pardi K**. Effect of acute muscle contusion injury, with and without dietary fish oil, on adult and aged male rats: Contractile and biochemical responses. *Experimental Gerontology.* 111: 241-252, 2018. doi:10.1016/j.exger.2018.08.001

34. **Selsby JT, Dodd SL**. Heat treatment reduces oxidative stress and protects muscle mass during immobilization. *Am J Physiol Regul Integr Comp Physiol.* 289: 134-139, 2005. doi:10.1152/ajpregu.00497.2004

35. **Selsby JT, Rother S, Tsuda S, Pracash O, Quindry J, Dodd SL**. Intermittent hyperthermia enhances skeletal muscle regrowth and attenuates oxidative damage following reloading. *J Appl Physiol.* 102: 1702-1707, 2007. doi:10.1152/japplphysiol.00722.2006

36. **Shanely RA, Zergeroglu MA, Lennon SL, Sugiura T, Yimlamai T, Enns D, Powers SK**. Mechanical ventilation-induced diaphragmatic atrophy is associated with oxidative injury and increased proteolytic activity. *Am J Respir Crit Care Med.* 166: 1369-1374, 2002. doi:10.1164/rccm.200202-088OC

37. **Smith C, Kruger MJ, Smith RM, Myburgh KH**. The inflammatory response to skeletal muscle injury: illuminating complexities. *Sports Med.* 38: 947-969, 2008. doi:10.2165/00007256-200838110-00005

38. **Snoeckx LH, Cornelussen RN, Van Nieuwenhoven FA, Reneman RS, Van Der Vusse GJ**. Heat shock proteins and cardiovascular pathophysiology. *Physiol Rev.* 81: 1461-1497, 2001. doi:10.1152/physrev.2001.81.4.1461

39. **Souza J, Gottfried C**. Muscle injury: review of experimental models. *J Electromyogr Kinesiol.* 23: 1253-1260, 2013. doi:10.1016/j.jelekin.2013.07.009

40. **Tidball JG, Dorshkind K, Wehling-Henricks M**. Shared signaling systems in myeloid cell-mediated muscle regeneration. *Development.* 141: 1184-1196, 2014. doi:10.1242/dev.098285

41. **Tidball JG, Villalta SA**. Regulatory interactions between muscle and the immune system during muscle regeneration. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology.* 298: 2010. doi:10.1152/ajpregu.00735.2009

42. **Tidball JG**. Regulation of muscle growth and regeneration by the immune system. *Nature Reviews Immunology.* 17: 165-178, 2017. doi:10.1038/nri.2016.150

43. **Tsivitse SK, Mcloughlin TJ, Peterson JM, Mylona E, Mcgregor SJ, Pizza FX**. Downhill running in rats: Influence on neutrophils, macrophages, and MyoD cells in skeletal muscle. *European Journal of Applied Physiology*. 90: 633-638, 2003. doi:10.1007/s00421-003-0909-0

44. **Tuttle JA, Castle PC, Metcalfe AJ, Midgley AW, Taylor L, Lewis MP**. Downhill running and exercise in hot environments increase leukocyte Hsp72 (HSPA1A) and Hsp90alpha (HSPC1) gene transcripts. *J Appl Physiol.* 38: 996-1005, 2015. doi:10.1152/japplphysiol.00387.2014

45. **Venojarvi M, Kvist M, Jozsa L, Kalimo H, Hanninen O, Atalay M**. Skeletal muscle HSP expression in response to immobilization and remobilization. *Int J Sports Med.* 28: 281-286, 2007. doi:10.1055/s-2006-924350

46. **Welle S, Thornton C, Jozefowicz R, Statt M**. Myofibrillar protein synthesis in young and old men. *Am J Physiol* 264: 693-698, 1993. doi:10.1152/ajpendo.1993.264.5.E693

47. **Wright-Carpenter T, Opolon P, Appell HJ, Meijer H, Wehling P, Mir LM**. Treatment of Muscle Injuries by Local Administration of Autologous Conditioned Serum: Animal Experiments Using a Muscle Contusion Model. *International Journal of Sports Medicine*. 25: 582-587, 2004. doi:10.1055/s-2004-821303

48. **Xiao W, Liu Y, Luo B, Zhao L, Liu X, Zeng Z, Chen P**. Time-dependent gene expression analysis after mouse skeletal muscle contusion. *Journal of Sport and Health Science.* 5: 101-108, 2016. doi:10.1016/jshs.2016.01.017

49. **Yoshihara T, Ichinoseki-Sekine N, Kakigi R, Tsuzuki T, Sugiura T, Powers SK, Naito H**. Repeated exposure to heat stress results in a diaphragm phenotype that resists ventilator-induced diaphragm dysfunction. *J Appl Physiol.* 119: 1023-1031, 2015. doi:10.1152/japplphysiol.00438.2015

50. **Zergeroglu MA, McKenzie MJ, Shanely RA, Van Gammeren D, DeRuisseau KC, Powers SK**. Mechanical ventilation-induced oxidative stress in the diaphragm. *J Appl Physiol.* 95: 1116-1124, 2003. doi:10.1152/japplphysiol.00824.2002

51. **Zuo D, Subjeck J, Wang XY**. Unfolding the Role of Large Heat Shock Proteins: New Insights and Therapeutic Implications. *Front Immunol.* 7: 75, 2016. doi:10.3389/fimmu.2016.00075

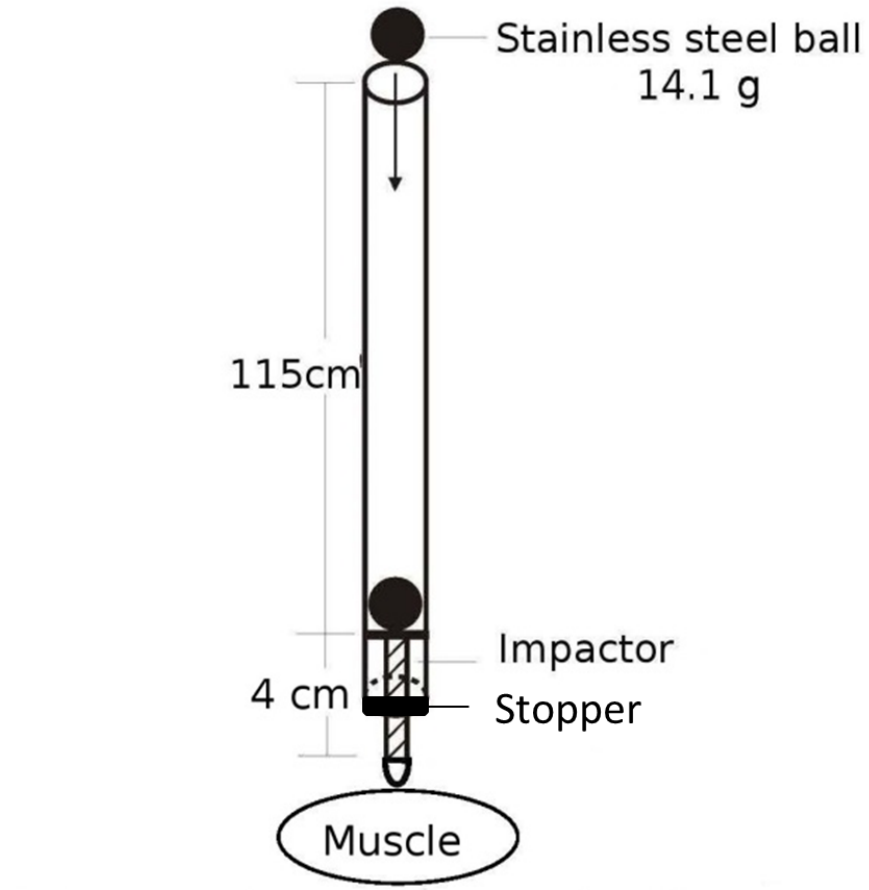

Figure 1: Schematic of the mass drop device used to cause impact-induced skeletal muscle damage.

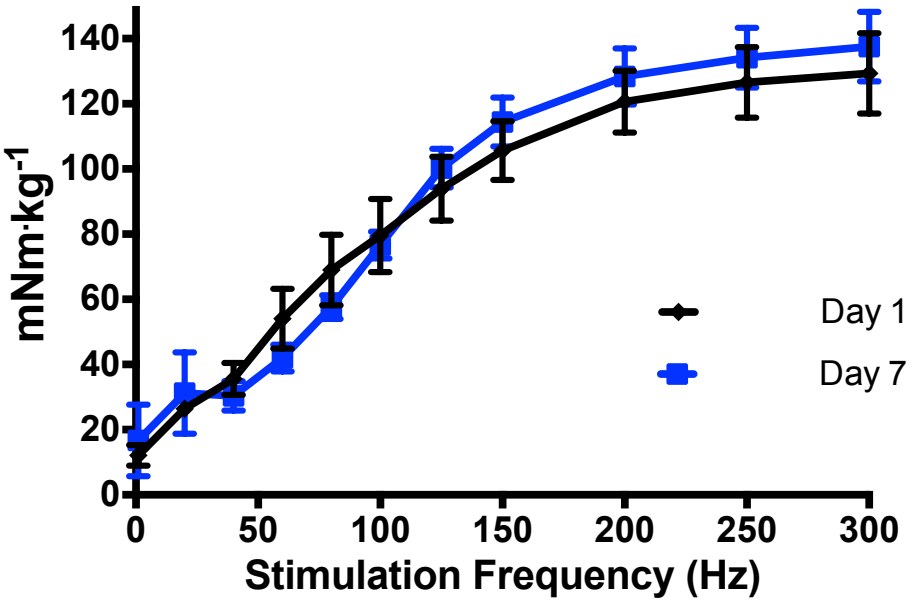

Figure 2: Preliminary data demonstrating the anterior crural *in-vivo* torque frequency relationship in young (3-6 mo old) mice (n = 20 for each group), P = 0.564. Mice were initially tested on Day 1 and the same *in-vivo* torque frequency relationship was measured 7-days later. These data show the repeatability of this technique.

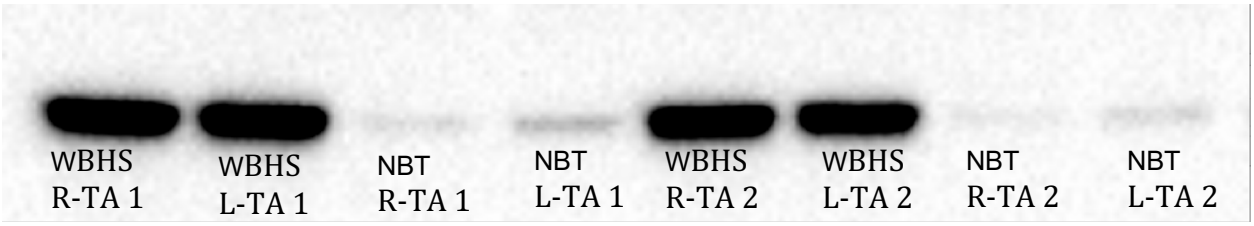

Figure 3: Preliminary study demonstrates that whole body heat shock increases HSP72 expression. Tibialis anterior (right and left) from whole body heat shock mice (n = 2) lanes 1, 2, 5, and 6. Tibialis anterior (right and left) from normal body temperature mice (n = 2) lanes 3,4,7, and 8.

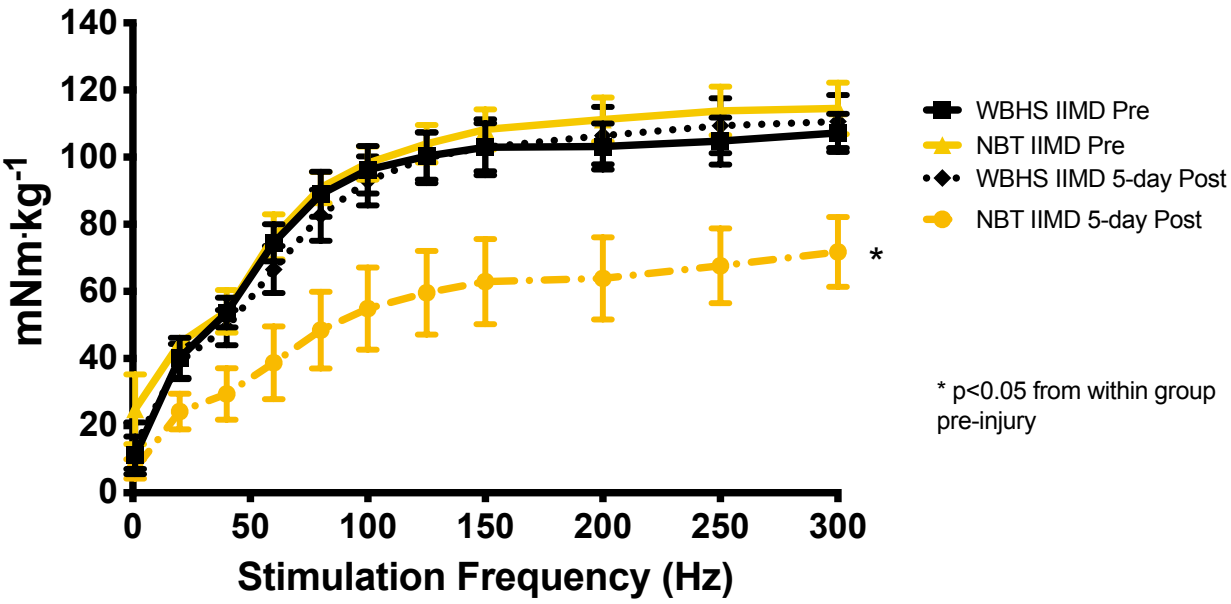

Figure 4: Contractile function pre-injury and 5-days post-impact induced muscle damage. Data from *in-vivo* torque frequency relationship, mean ± SEM.

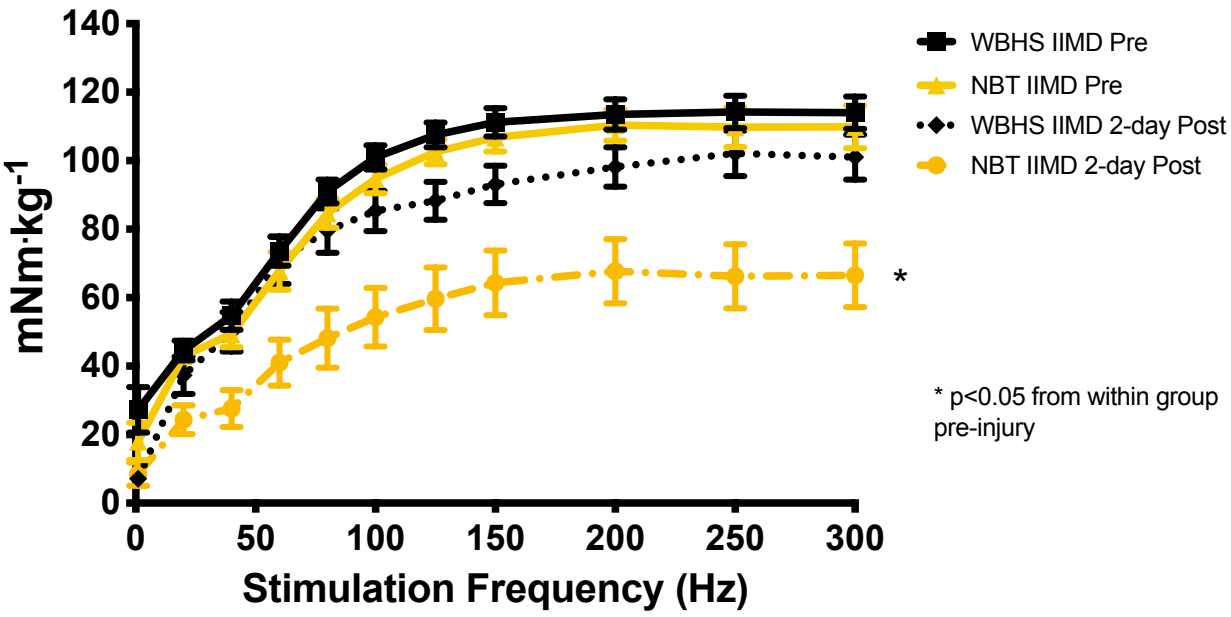

Figure 5: Contractile function pre-injury and 2-days post-impact induced muscle damage. Data from *in-vivo* torque frequency relationship, mean ± SEM.

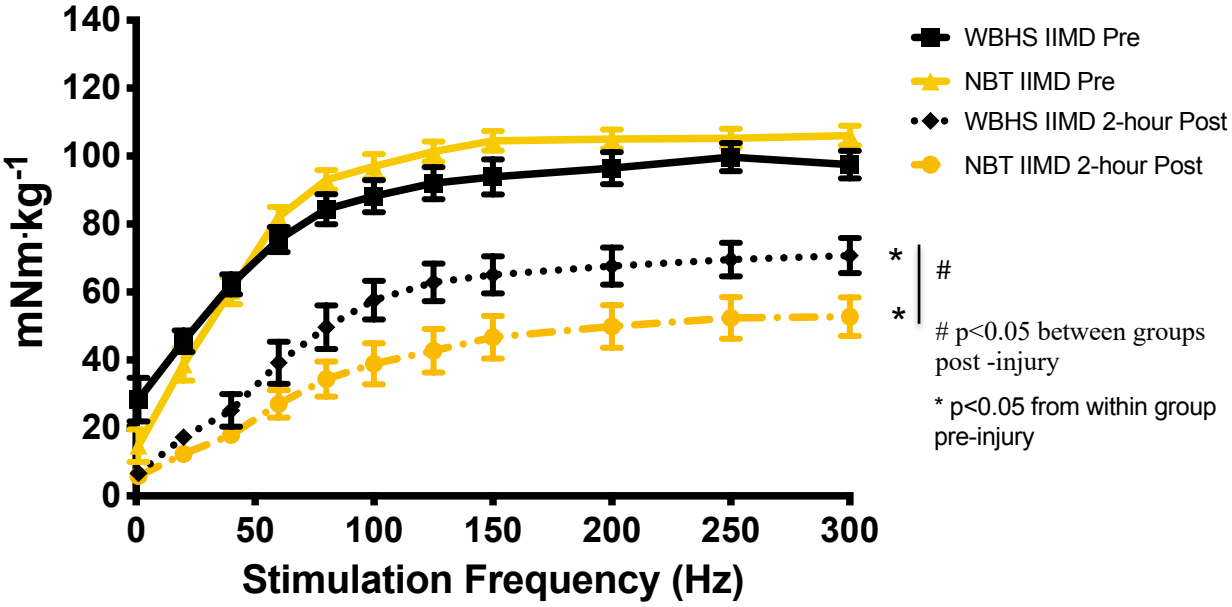

Figure 6: Contractile function pre-injury and 2-Hours post-impact induced muscle damage. Data from *in-vivo* torque frequency relationship, mean ± SEM.

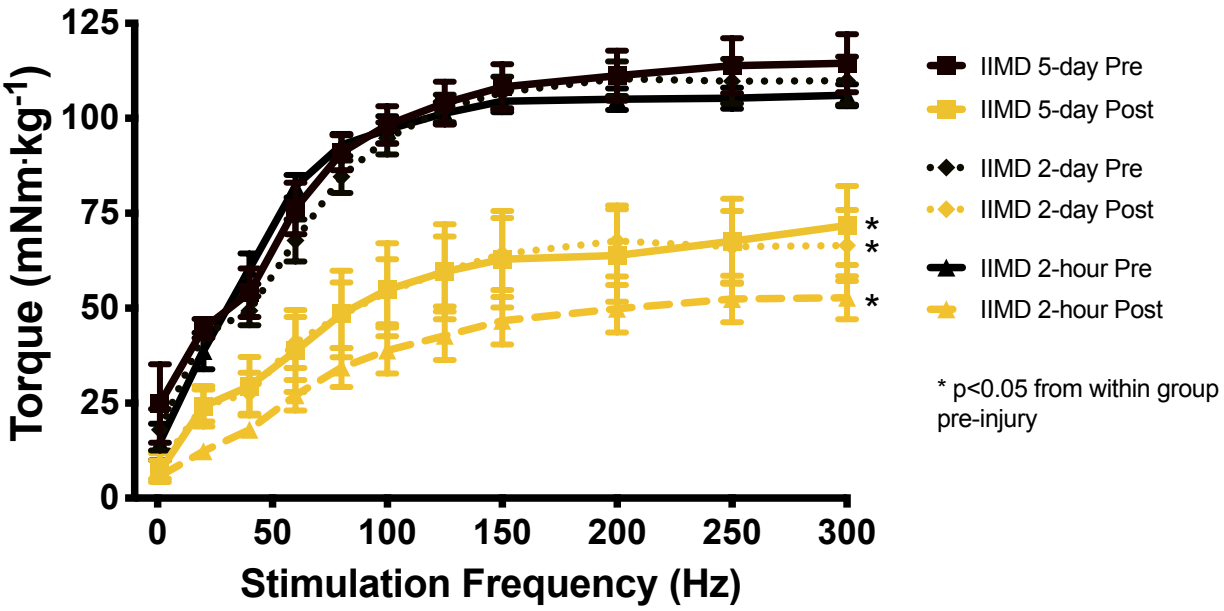

Figure 7: Contractile function data for normal body temperature groups pre-injury, 2-hour, 2-day, and 5-day post-impact induced muscle damage. Data from *in-vivo* torque frequency relationship, mean ± SEM.

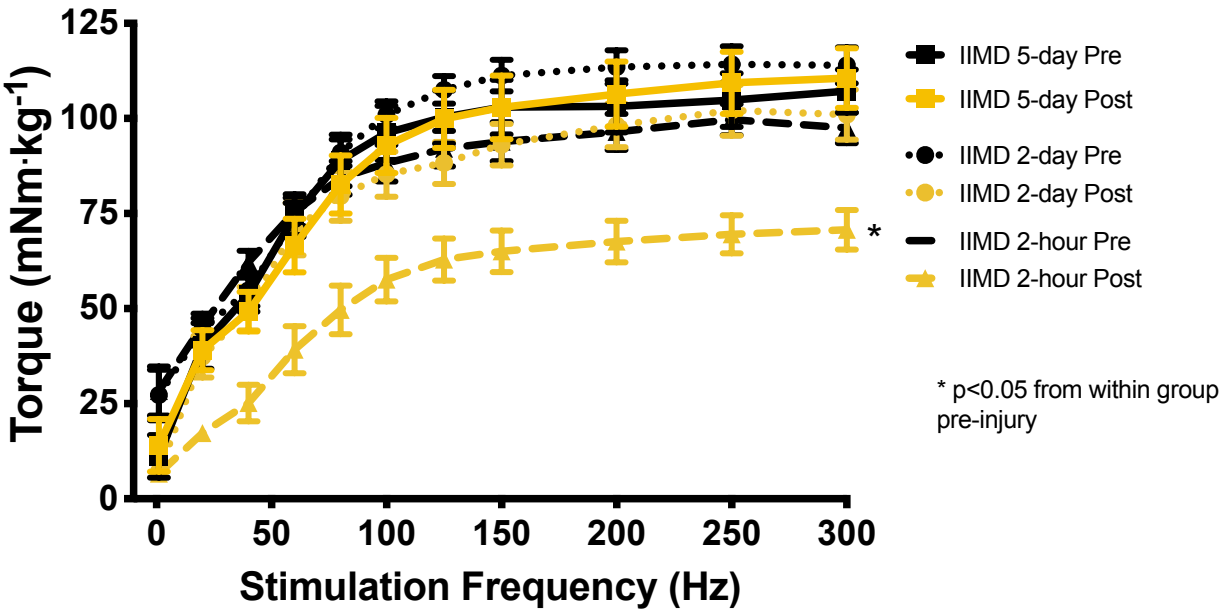

Figure 8: Contractile function data for whole body heat shock groups pre-injury, 2-hour, 2-day, and 5-day post-impact induced muscle damage. Data from *in-vivo* torque frequency relationship, mean ± SEM.

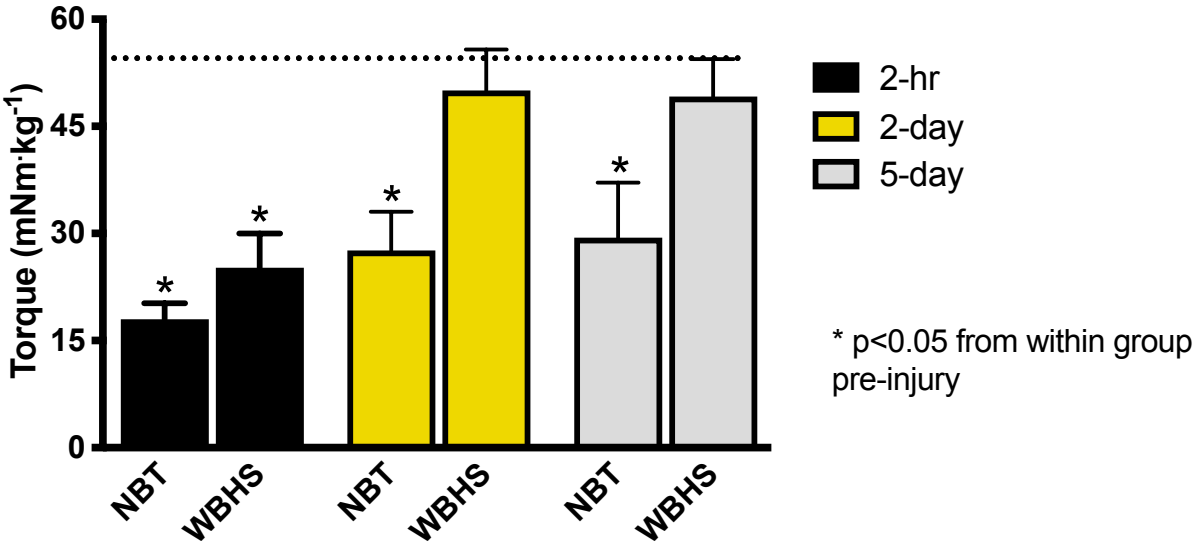

Figure 9: Submaximal 40Hz frequency for all groups at all time points. The dotted line represents the average pre-injury torque for all groups; NBT (normal body temperature), WBHS (whole body heat shock), mean ± SEM.

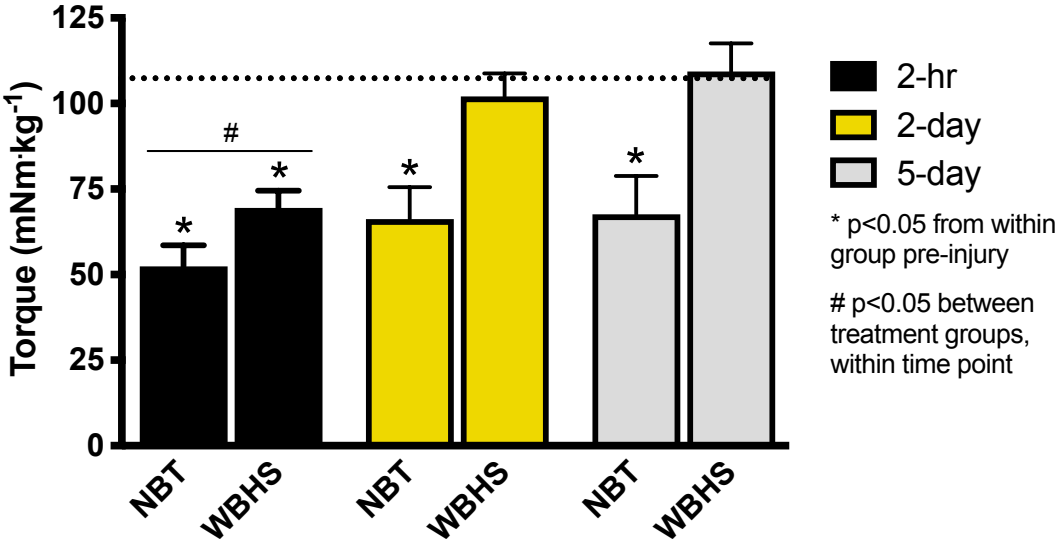

Figure 10: Maximal 250Hz frequency for all groups at all time points. The dotted line is average pre-injury value for all groups; NBT (normal body temperature), WBHS (whole body heat shock), mean ± SEM.

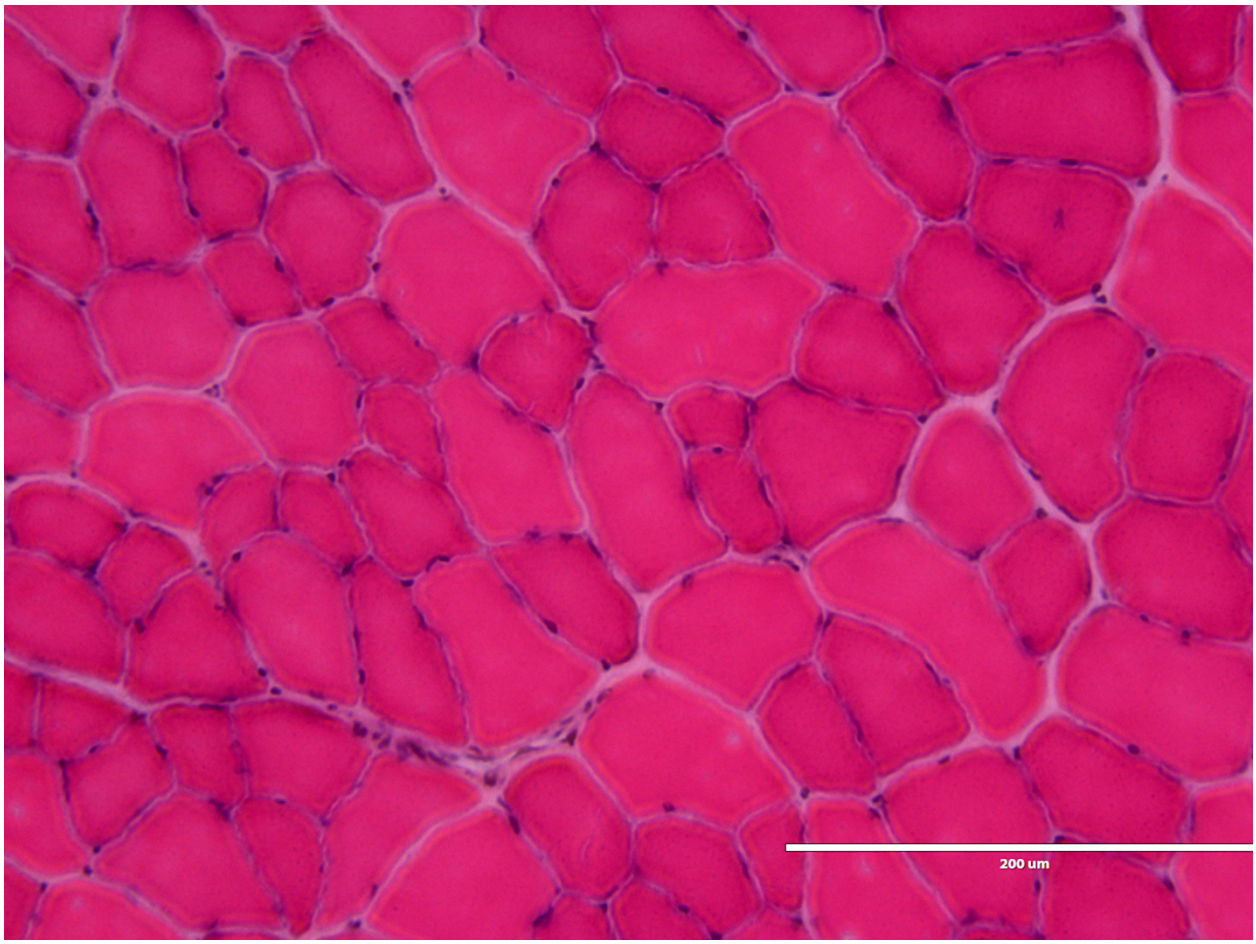

Figure 11: Hematoxylin and Eosin stain of uninjured tibialis anterior, 20X magnification.

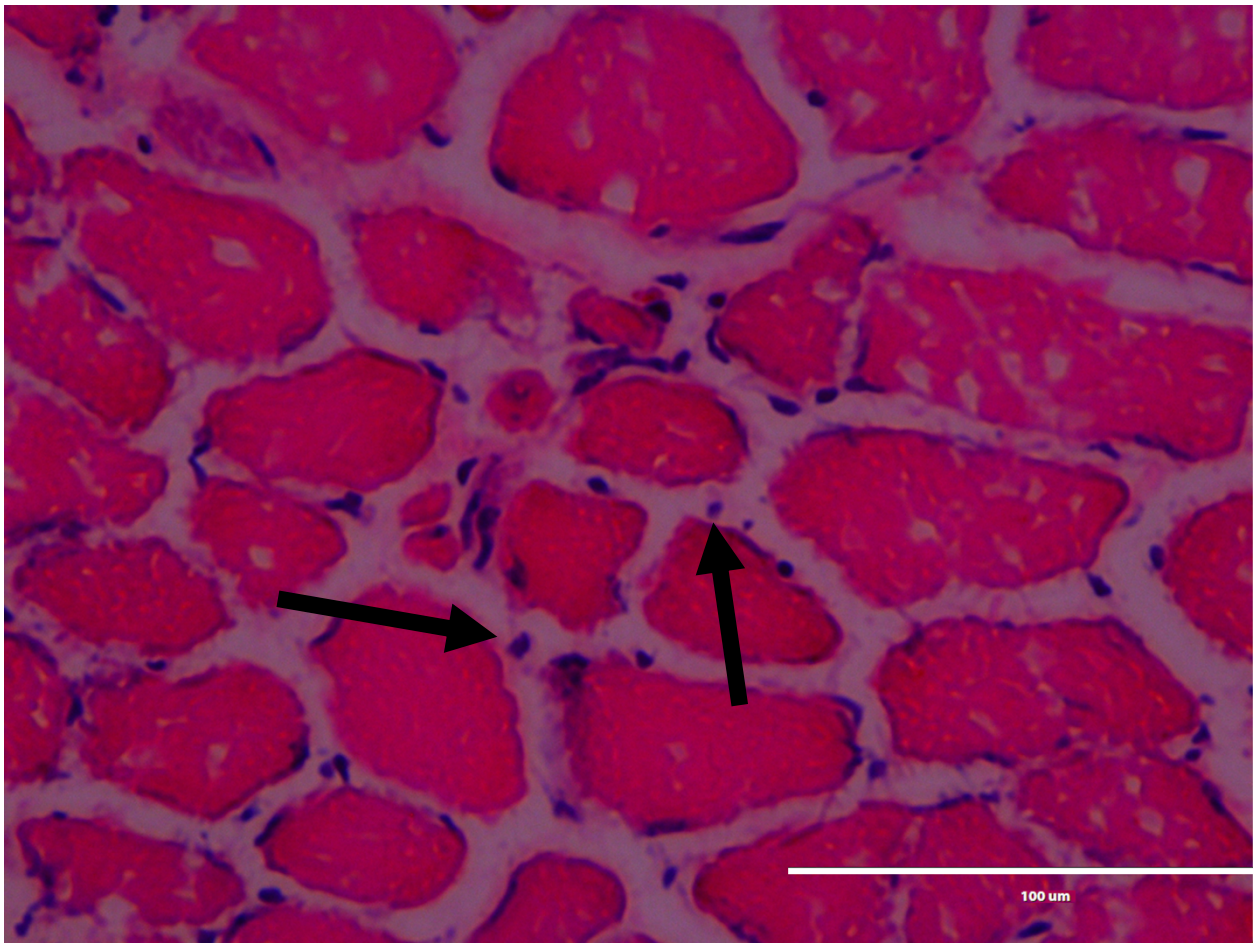

Figure 12: Hematoxylin and eosin stain showing extracellular phagocytes. Black arrows pointing to extracellularly located phagocytes, 40X magnification.

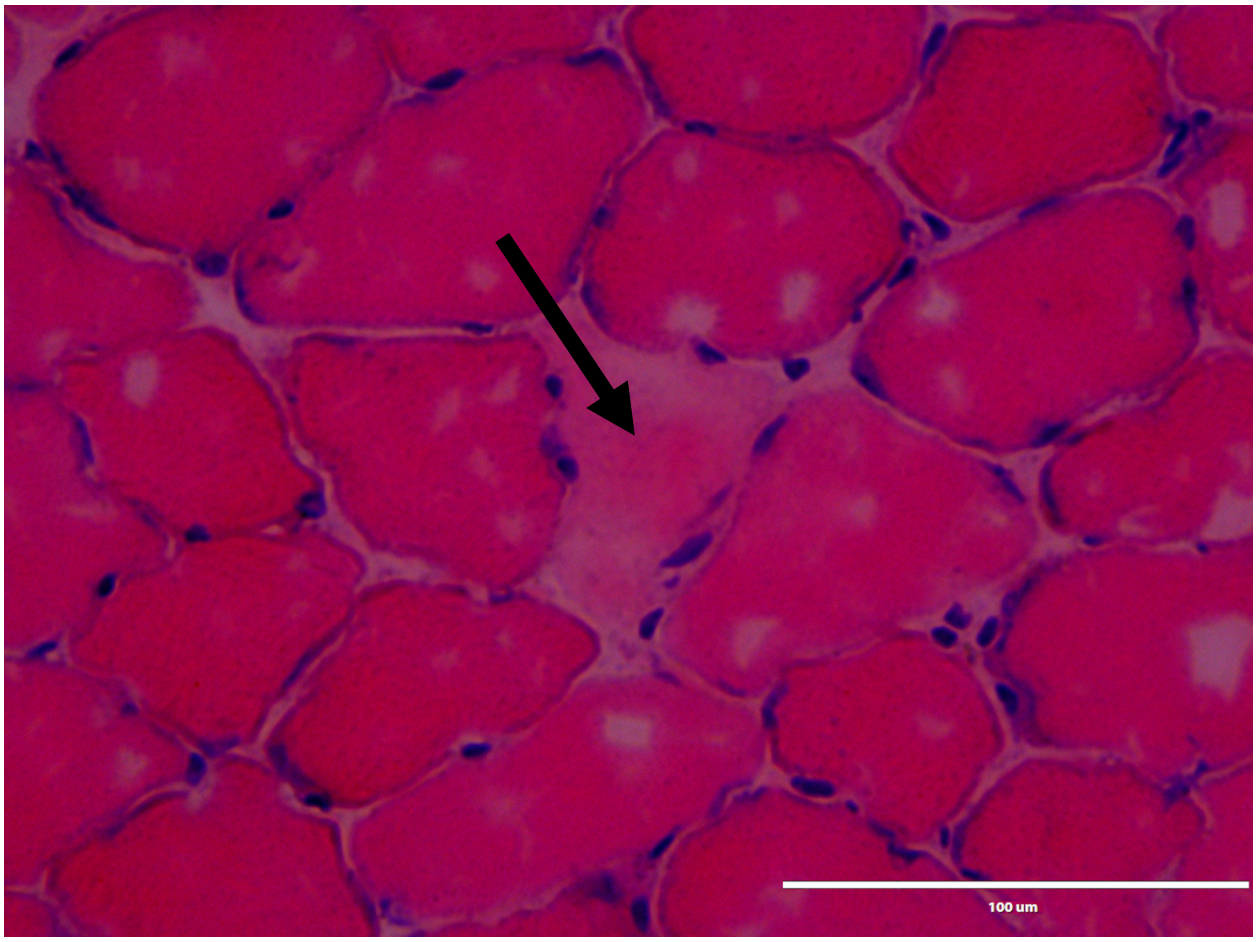

Figure 13: Hematoxylin and eosin stain showing pale cytoplasm. Black arrow pointing to pale cytoplasm, 40X magnification.

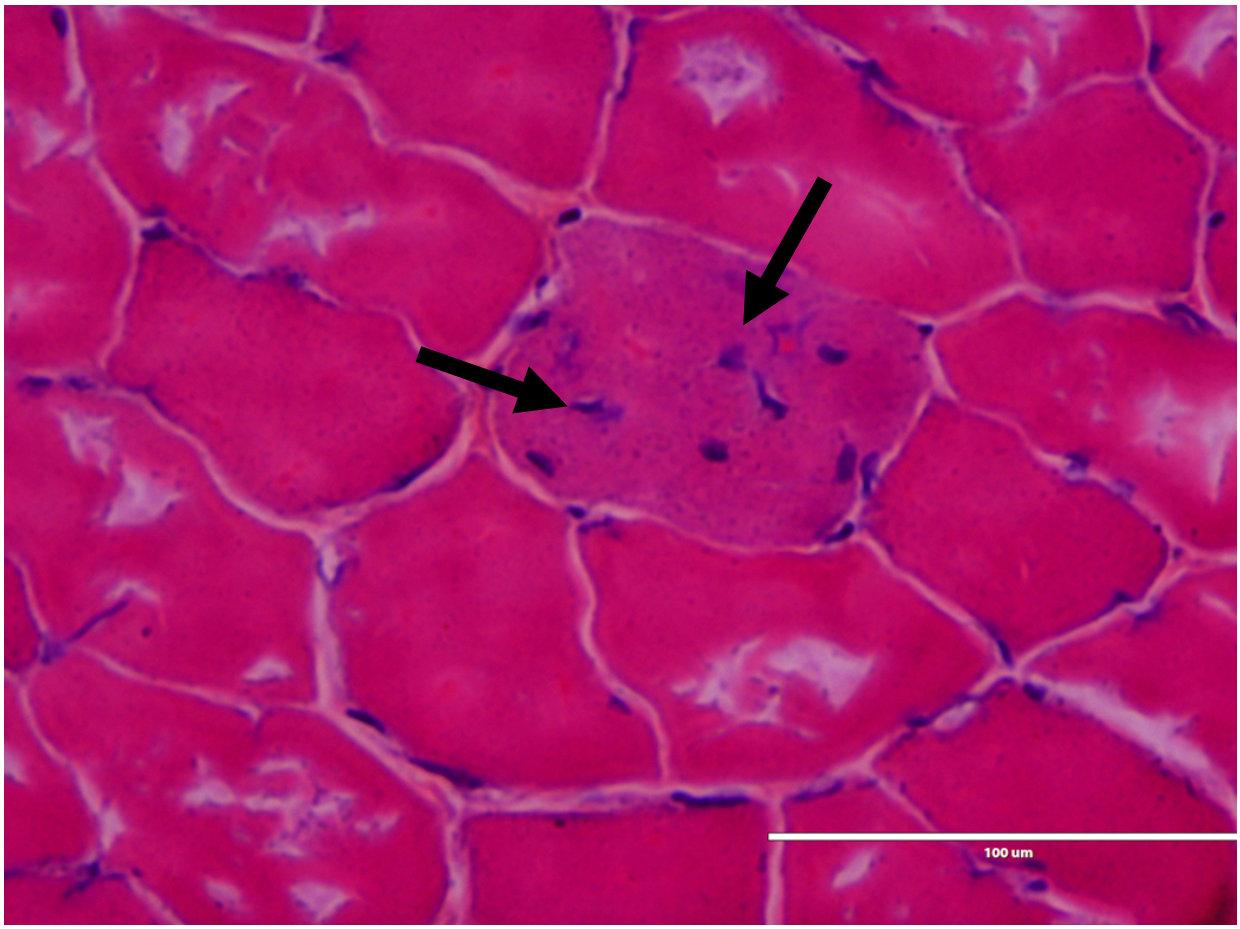

Figure 14: Hematoxylin and eosin stain showing intracellular phagocytes. Black arrow pointing to intracellularly located phagocytes, 40X magnification.

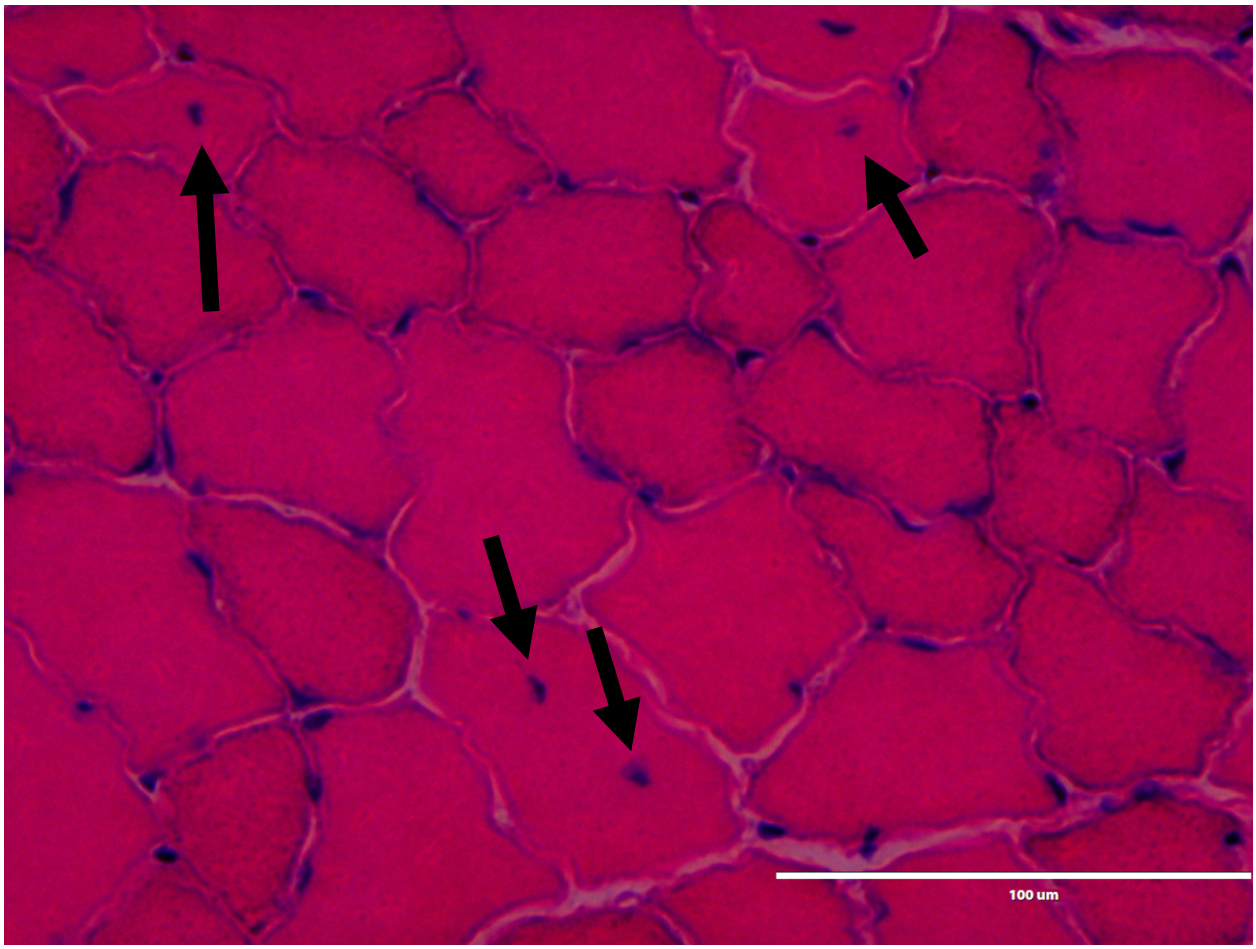

Figure 15: Hematoxylin and eosin stain showing central myonuclei. Black arrows pointing to centrally located myonuclei, 40X magnification.

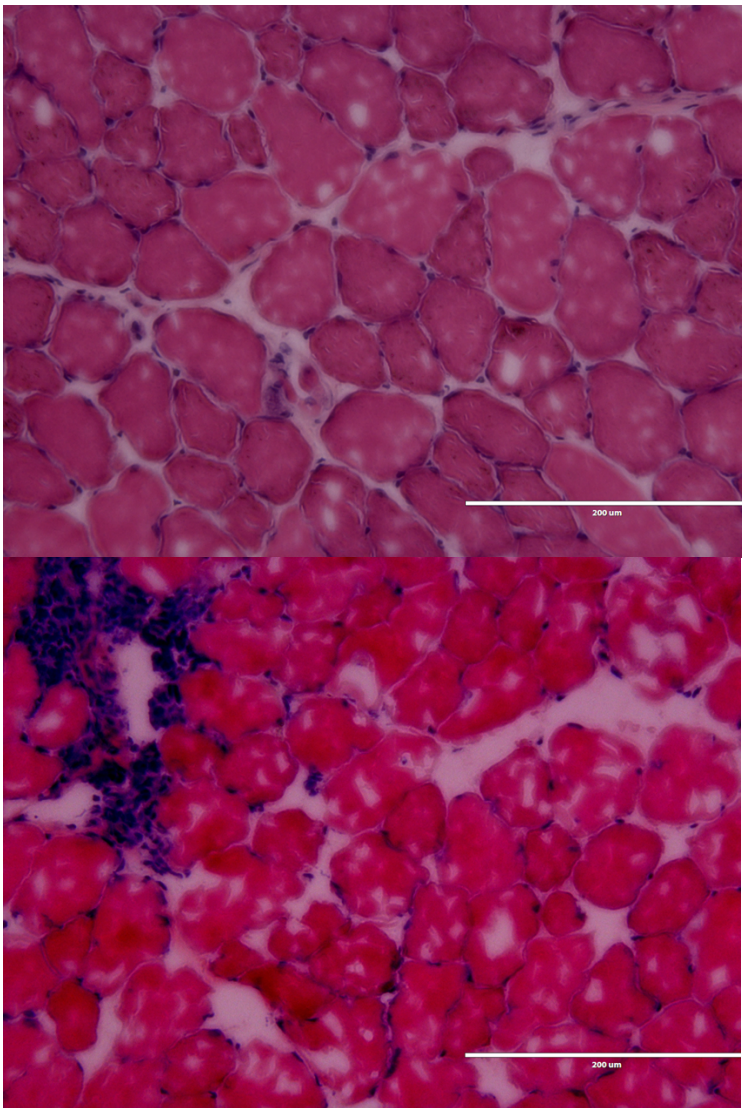

Figure 16: Hematoxylin and Eosin stain at 2-hour post-impact induced muscle damage. Top: image of WBHS 2-hour. Bottom: NBT 2-hour. Showing edema and extracellularly located phagocytes.

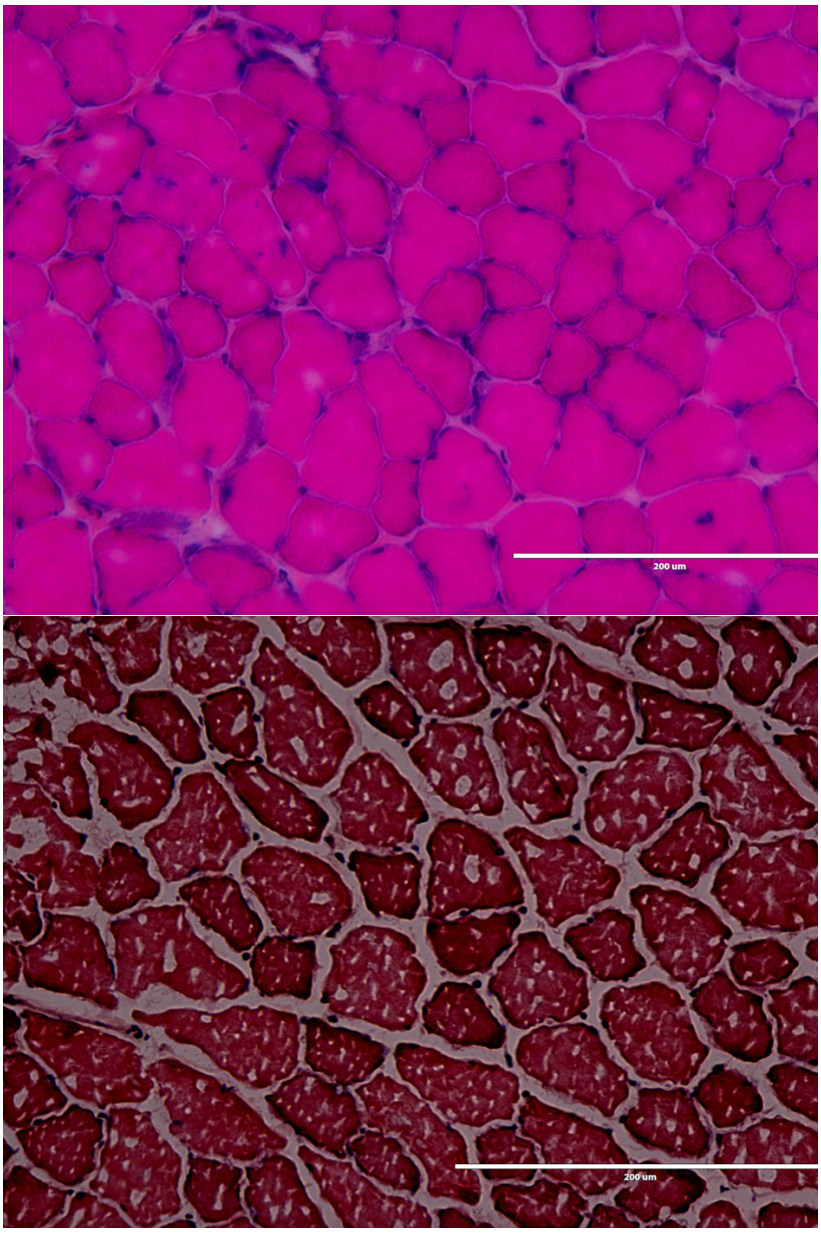

Figure 17: Hematoxylin and Eosin stain at 2-day post-impact induced muscle damage. Top: image of WBHS 2-day. Bottom: NBT 2-day. Showing edema and extracellularly located phagocytes.

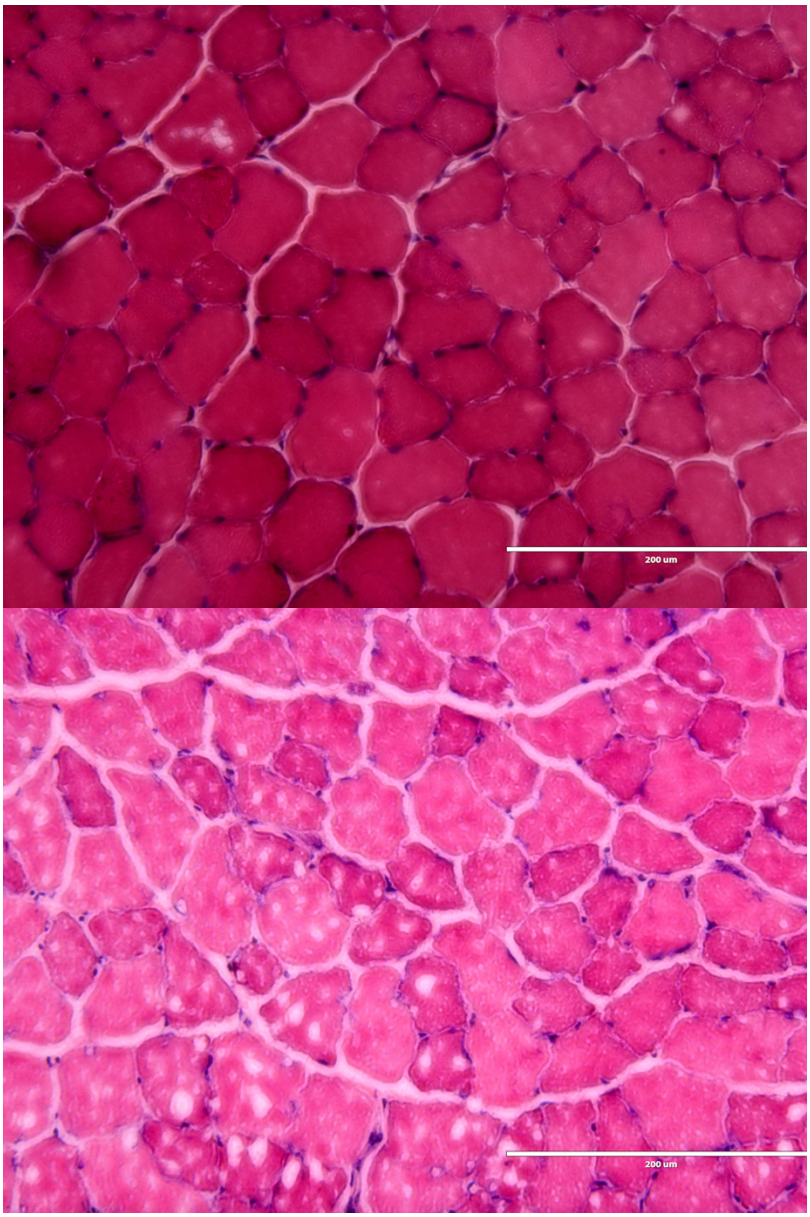

Figure 18: Hematoxylin and Eosin stain at 5-day post-impact induced muscle damage. Top: image of WBHS 5-day. Bottom: NBT 5-day. Showing edema and extracellularly located phagocytes.

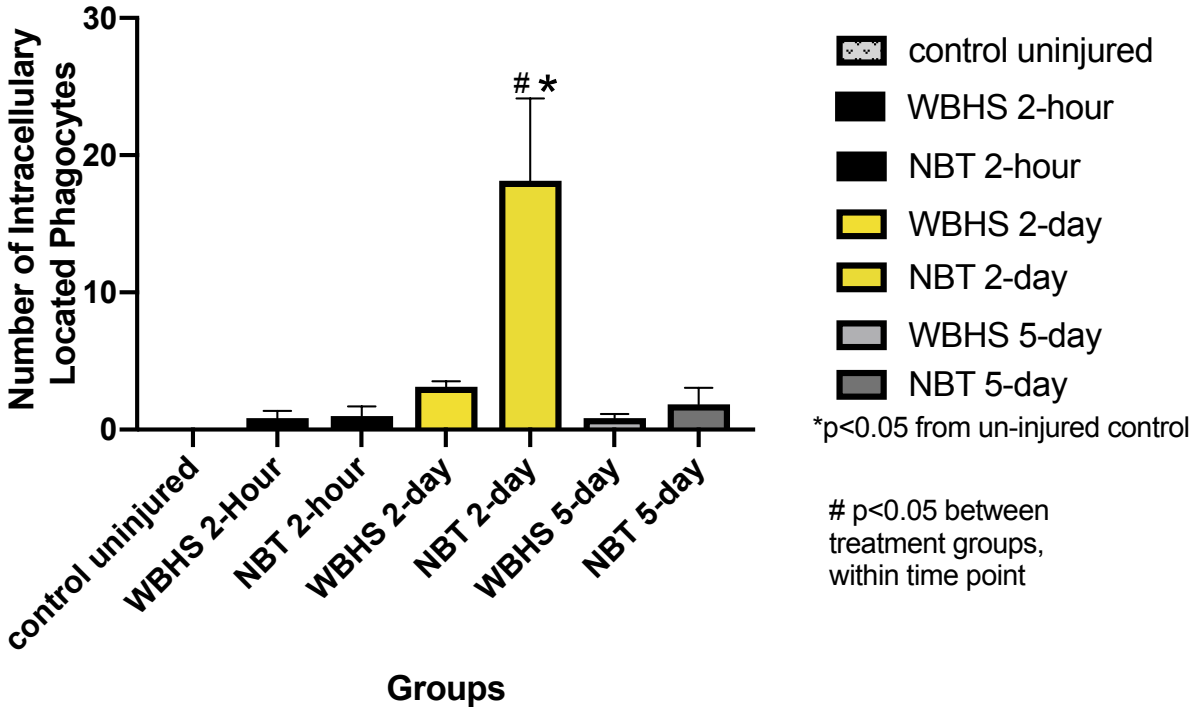

Figure 19: Quantification of intracellularly located phagocytes. Total number of myofibers with intracellularly located phagocytes are counted for each section, mean ± SEM

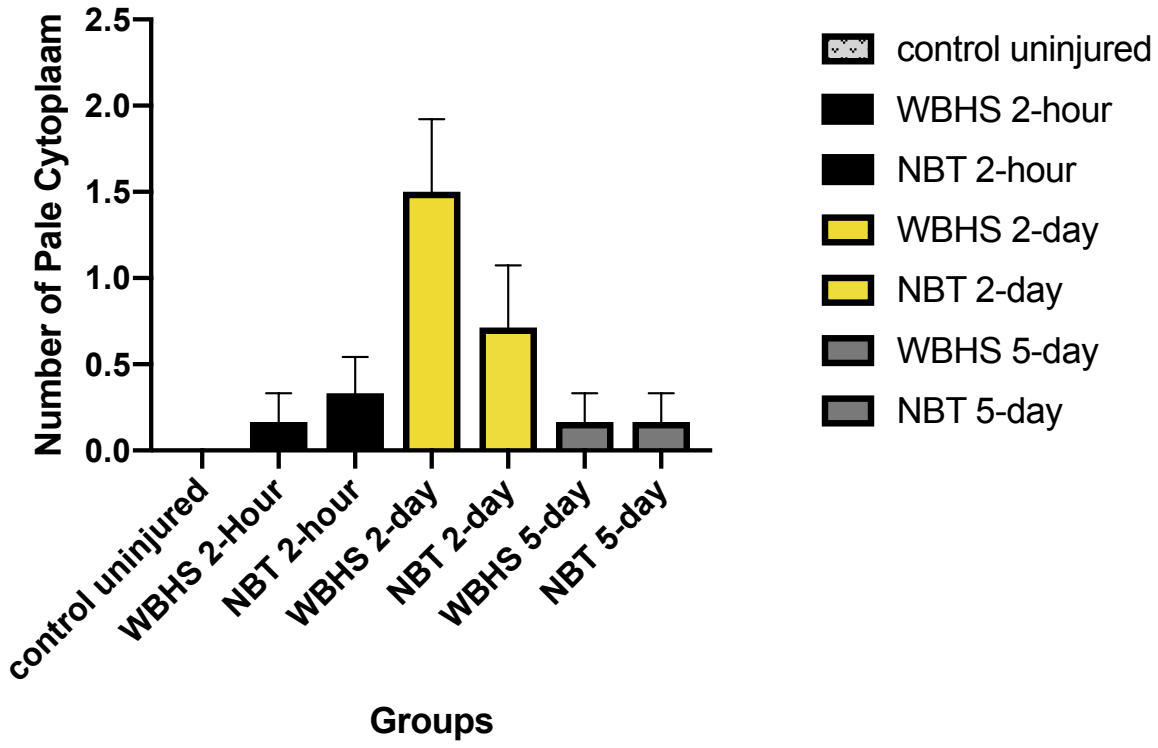

Figure 20: Quantification of pale cytoplasm. Total number of myofibers containing pale cytoplasm for each section, mean ± SEM

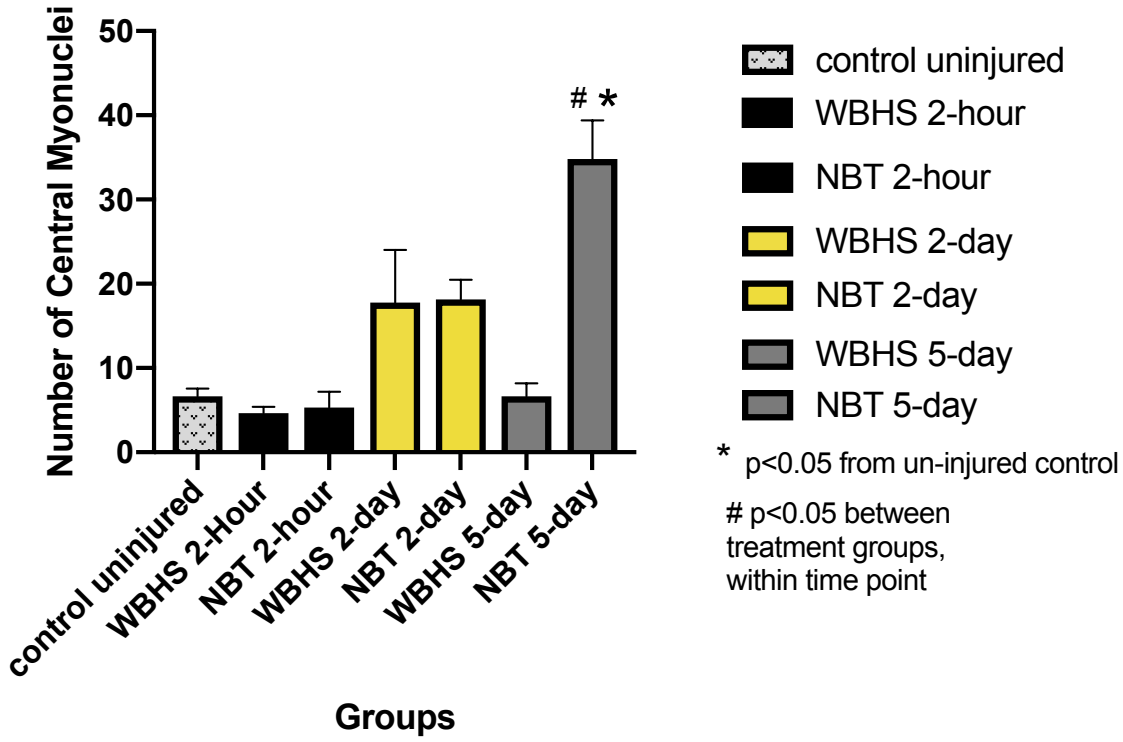

Figure 21: Quantification of centrally located myonuclei. Total number of myofibers with centrally located myonuclei for each section, mean ± SEM

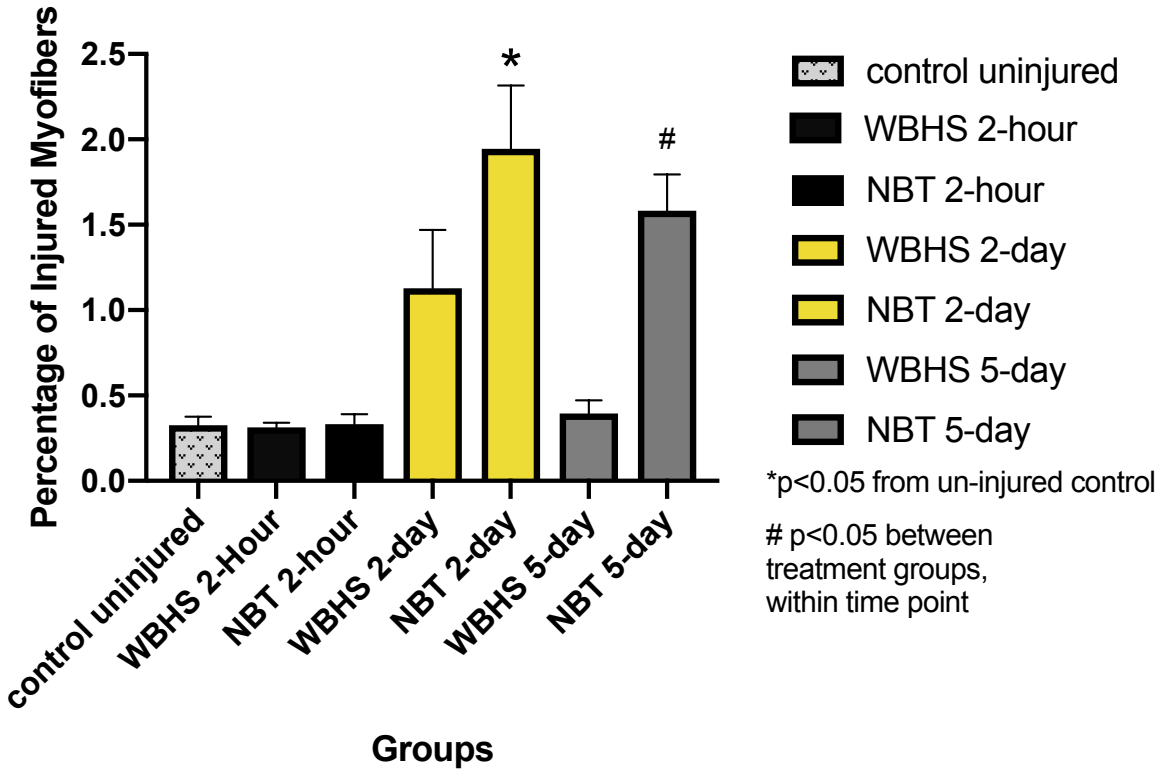

Figure 22: Percentage of injured fibers for each section. Percentage of injured fibers is determined by adding the amount of pale cytoplasm, intracellular phagocytes, and central myonuclei then dividing number of injured fibers by the total number of fibers, mean ± SEM

Vita

Joshua Scott Godwin was born in Greensboro, North Carolina, to Jeff and Michelle Godwin. He graduated from Providence Grove High School in June 2013. The following fall, he entered Lees McRae College to study Biology, and in June 2017 was awarded a Bachelor of Science degree. In the fall of 2017, he accepted a research assistantship in exercise science at Appalachian State University and began study toward a Master of science degree. The M.S. was awarded in May 2019.