MUSCLE OXIDATIVE CAPACITY AND PLASTICITY AFTER VOLUMETRIC MUSCLE LOSS: IMPLICATIONS FOR REGENERATIVE REHABILITATION

by

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(Under the Direction of Jarrod Call)

ABSTRACT

Volumetric muscle loss (VML) is characterized by a large volume of muscle tissue being removed from the body due to surgery or severe trauma. The remaining muscle after VML has poor function and unknown effects on oxidative capacity and adaptive potential during physical rehabilitation. We sought to investigate the oxidative capacity and plasticity of the remaining skeletal muscle after VML injury. We found that VML mitochondrial respiratory function at 3 and 7 days post injury was severely impaired (38% and 53% of control, $P<0.001$). In an attempt to correct these mitochondrial deficits, we subjected VML injured mice to voluntary wheel running (WR). After 4 weeks of WR, mitochondrial function was ~23% greater in the uninjured limb of VML+WR mice compared to VML without WR, but mitochondrial function in the injured limb of VML+WR mice was not different from VML alone, suggesting VML injury prevents oxidative adaptations to exercise ($P<0.001$). To determine if a faulty oxidative signaling cascade (i.e., PGC-1α) was responsible for impaired metabolic adaptation in the VML-injured muscle, direct muscle activation via sciatic nerve electrical stimulation was used to initiate oxidative gene transcription in control (CON) and VML mice. The stimulated muscle in CON mice had

~4-fold greater PGC-1α gene expression than the unstimulated muscle; however, there was no effect of stimulation on PGC-1α expression in VML mice (Interaction: $P<0.001$) suggesting VML injury attenuates oxidative gene regulation. PGC-1α (CMV promoter) transfection was performed to bypass endogenous PGC-1α activation pathways. Forced expression of PGC-1α resulted in ~33% and ~31% greater mitochondrial function in CON+ PGC-1α and VML+ PGC-1α mice, respectively, compared to VML alone ($P<0.001$), and VML+ PGC-1α mice had 47% greater muscle strength than VML alone ($P<0.001$). In conclusion, PGC-1α activation is the limiting factor impairing oxidative plasticity in VML-injured muscle; and improving oxidative capacity of the remaining muscle after VML injury improves recovery of strength.

INDEX WORDS: Volumetric muscle loss injury; muscle injury; inflammation; mitochondria; oxidative capacity; PGC-1α

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DEDICATION

This work is dedicated to my wife Lauren. Without her unwavering support, I would not have been able to complete this journey. She is truly the real MVP.

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CHAPTER 1

INTRODUCTION

In military populations, extremity injuries comprise the majority of combat wounds and cost the Department of Defense $42.4 billion in initial costs, which contributes to the $108.8 billion in lifetime disability benefits issued to injured service members (1). Over half of these extremity injuries are soft-tissue wounds involving extensive damage to the muscle (2). This type of injury is known as volumetric muscle loss (VML) and is characterized by a large volume of muscle tissue being removed from the body due to surgery or severe trauma. Currently, the pathophysiology of VML is poorly defined (3). As a result, clinical rehabilitation approaches are poorly defined and have limited efficacy. In fact, if a clinical intervention is employed, it often starts months after the injury occurs and offers little in the way of functional muscle recovery (1, 4-6). This leaves individuals who have suffered VML injuries with chronic muscle dysfunction and a reduced quality of life (4, 7).

A critical barrier to restoring muscle function in patients with VML is that the pathophysiology of VML has not been fully elucidated. The limited number of clinical trials and case studies that do describe VML pathophysiology primarily focus on the most notable feature of VML which is the loss of muscle strength. This strength loss is permanent and also out of proportion to the muscle volume removed (3), as the remaining tissue has 60% worse function than would be predicted for a given amount of tissue

removed. In addition to strength loss, VML is also characterized by extensive nerve damage (8), fibrotic infiltration into the injury site (4, 9, 10), as well as loss of force transmission through the muscle (9, 11). While these are all important aspects of VML pathophysiology, they may not entirely account for the suboptimal muscle function observed in the remaining tissue. Additional studies are needed to explore the VML pathophysiology at the cellular level within the remaining tissue. This area of research remains largely unexplored but could be critical to developing effective treatments strategies for VML patients.

One reason that the pathophysiology of VML remains poorly defined is that few studies have actually addressed this issue. Instead, a majority of VML research has focused on muscle fiber regeneration strategies. Muscle fiber regeneration is not possible following VML because of the complete removal of muscle tissue with the injury. While the muscle has a remarkable ability to repair itself after injury, VML injuries require de novo muscle fiber regeneration beyond the natural capacity of the muscle (4, 12, 13). Previous studies have tried to explore ways to reconstruct functional muscle within the void created by the VML. Some of the techniques have included implanting muscle tissue grafts (8) and stem cell-infused scaffoldings or hydrogels into the void in an attempt to provide an extracellular matrix-like structure for new muscle cell growth (6, 8, 9, 14-16). However, these approaches have achieved minimal success primarily due to poor vascularization of implanted tissue or materials. One study recently demonstrated that progressive delivery of muscle cells into the VML void might overcome previous vascularization limitations and be a promising option for functional muscle regeneration after VML (16). The technique involves delivery of C2C12 cells in layers shallow enough to allow the

vasculature to perfuse the new muscle creating a healthy and functional layer of tissue. However, despite the promising results of this method, it is still far from being ready to implement into human patients, as its success has so far only been established in mice. One reason for the limited success of these studies attempting to develop muscle regeneration strategies is that VML pathophysiology is not fully understood. Designing effective treatments for VML is difficult without a proper understanding of how VML affects the muscle. Moving forward, the focus of VML research should shift away from regeneration research and instead explore the pathophysiology of VML so that better, more effective therapies can be created.

With this project, I am seeking to advance the understanding of VML pathophysiology by investigating the oxidative plasticity of the remaining muscle after VML. Successful completion of this work will change concepts regarding VML because it will characterize the muscle oxidative capacity and plasticity of the remaining muscle after injury and use this information to create guidelines suggesting optimal rehabilitation therapies. Overall, this proposal will help to overcome the VML pathophysiology knowledge gap by contributing important scientific knowledge to the field of VML research.

Specific Aims and Hypotheses

Specific Aim 1: To determine the oxidative capacity and plasticity of the remaining muscle after a VML injury.

Hypothesis 1: Oxidative capacity and plasticity of the remaining muscle will be negatively affected by VML.

Specific Aim 2: To improve oxidative capacity of the remaining muscle after a VML injury.

Hypothesis 2: Improving oxidative capacity of the remaining muscle will have a positive effect on the function of the remaining muscle.

Significance of Study

A better understanding of VML pathophysiology is necessary to improve muscle function and quality of life in patients with VML. Therefore, the goal of this proposal is to determine the oxidative plasticity of the remaining muscle after VML. To accomplish this goal, a primary objective of this project is to characterize the oxidative plasticity of the remaining muscle. Understanding the oxidative plasticity of the remaining muscle will provide information about VML pathophysiology in the remaining muscle that is necessary for building a foundation upon which evidenced-based treatment strategies can be designed and validated. The second objective of this project is to improve the oxidative capacity of the remaining muscle. Understanding how improving oxidative capacity affects the function of the remaining muscle will shape VML treatment strategies. Greater muscle function after increasing oxidative capacity of the remaining muscle would indicate that clinicians should promote rehabilitation strategies that specifically target mitochondrial function. I hypothesize that the remaining muscle will have reduced oxidative plasticity and that improving the oxidative capacity of the remaining muscle will positively affect function of the remaining muscle. Overall, successful completion of this project will transform treatment strategies for patients with VML.

CHAPTER 2

REVIEW OF LITERATURE: A ROLE FOR MITOCHONDRIA IN SENSING AND RESPONDING TO SKELETAL MUSCLE INJURY[1]

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Abstract

Mitochondria are extensively involved in muscle cell physiology playing roles in energy homeostasis, ion balance, redox homeostasis, programmed cell death, and much more. Recently, mitochondria have emerged as powerful signaling centers that are capable of reacting to various forms of cellular stress. However, the extent to which mitochondria are involved in skeletal muscle stress remains unclear. In this review, we sought to 1) determine if mitochondria are capable of sensing skeletal muscle injury, and 2) determine if mitochondria can contribute to the post injury skeletal muscle response, namely inflammation. We demonstrate that mitochondria are indeed sensitive to skeletal muscle injury, in that they undergo alterations in network size and function in response to an injury. We also propose a role for mitochondria in which they help to coordinate the inflammatory response after muscle injury via stress-induced activation of the NLRP3 inflammasome. Overall, we conclude that mitochondria are cellular detectors of skeletal muscle stress and potentially important regulators of the inflammatory response after injury.

Introduction

The repair process after muscle injury is very complex and requires coordination across multiple systems including the mitochondrial network. Mitochondria are critical to skeletal muscle health as they regulate energy balance, cell death, ion homeostasis, redox homeostasis, and much more. Recent evidence suggests that mitochondria are needed for cell membrane repair and normal muscle regeneration (1, 2), and a growing body of literature suggests that mitochondria are signaling centers when cells are under stress (3-6). In theory, muscle injury could trigger a mitochondrial stress response where mitochondrial-derived signals could contribute to the characteristic skeletal muscle response, i.e. inflammation, observed after an injury.

Changes in mitochondrial function in response to muscle injury as well as the mitochondrial involvement in the post-injury repair process have not been well characterized. The purpose of this review is to address the question of whether mitochondria are sensitive to skeletal muscle injury, and if so, whether they might serve as the nexus for initiation of skeletal muscle responses to the stress. We postulate that mitochondria are sensitive to skeletal muscle injuries, and that the mitochondria respond in a distinctive way to the stress which may serve as the signaling axis to initiate skeletal muscle responses to the stress, e.g. inflammation, membrane repair, satellite cell mediated muscle regeneration. In the first section of this review, we will discuss whether mitochondria are sensitive to muscle injuries. To address this topic, we will present the known literature surrounding the impact of various muscle injuries on mitochondria. In the second section of this review, we will explore the interaction between inflammation and

mitochondrial signaling, as inflammation is a common element among the various muscle injuries presented. Overall, this review should demonstrate to readers that mitochondria are indeed sensitive to muscle injury and upon sensing muscle stress they may help coordinate the ensuing inflammatory response.

Overview of Mitochondrial Sensitivity to Skeletal Muscle Injury

Skeletal muscles will undergo a number of distinct stresses throughout their lifetime, each impacting the muscle in a unique way. For this review, we evaluated muscle stresses that specifically impact muscle contractility, which is the most reliable and physiologically relevant measure of muscle function. The scope of this review is therefore narrowed to only include forms of muscle injury that result in measurable strength loss, and includes burn, freeze injury, myotoxic injuries, contraction induced injury, volumetric muscle loss, and ischemia reperfusion, as different forms of non-disease related muscle stress. We consolidated these injuries into three distinct categories separated by their mechanism of action on the muscle: 1) skeletal muscle trauma, 2) contraction induced injury, and 3) mechanical and myotoxic muscle injury. Upon establishing these categories of muscle stress, we explored the extent to which mitochondria are sensitive to the various muscle stressors within each category.

Skeletal Muscle Mitochondrial Sensitivity

For the purpose of this review, we defined skeletal muscle mitochondrial sensitivity as any changes that occur in mitochondrial physiology, i.e., mitochondrial content or mitochondrial respiratory function. These measures of mitochondrial physiology provide

direct assessments of mitochondrial health and make ideal metrics to assess whether mitochondria are sensitive to muscle injury. Changes in mitochondrial content is best assessed via mitochondrial enzyme activities (7). In general, mitochondrial enzyme activities reflect either an increase in energy demand within the muscle cell stimulating mitochondrial biogenesis or, conversely, a reduction in mitochondrial content due to damage and/or disease. Mitochondrial respiratory function is perhaps the most physiologically relevant marker of mitochondrial health considering that mitochondrial respiration accounts for approximately 90% of total oxygen consumption (8). Skeletal muscle mitochondrial function can be directly assessed using high-resolution respirometry to measure mitochondrial oxygen consumption in either live isolated mitochondria or permeabilized muscle fibers. One important note about mitochondrial function assessments is that it is highly dependent on mitochondrial content, specifically, gains or losses in mitochondrial function may be mirrored by gains and losses in mitochondrial content. That being said, mitochondrial dysfunction, a specific dysfunction in the mitochondrial respiratory chain irrespective of mitochondrial content, could be attributed to a number of factors within the mitochondria such as lower enzyme function or quantity, altered redox and polarization states, disrupted ion gradients, or damaged mitochondrial membranes. Many other changes within the mitochondria can result from a reaction to muscle injury. Changes in membrane potential, calcium absorption, oxidative status, mitochondrial DNA number, or reactive oxygen species production are some of the additional mitochondrial reactions that can happen in response to muscle stress. Overall, mitochondrial sensitivity to a muscle stressor can be evaluated by assessing changes in mitochondrial function.

Skeletal Muscle Trauma

Any major trauma to the skeletal muscle will cause dramatic functional disruptions and place an enormous stress load on the muscle. For this section, we have classified three traumatic skeletal muscle injuries that cause significant skeletal muscle stress indicated by a loss in muscle strength, and we will highlight mitochondria changes in response to these skeletal muscle injuries.

Burn Injury

Burn injuries are known to cause chronic muscle strength loss (15-20% compared to controls), muscle wasting, and metabolic disruptions (9, 10). The extent of burn injury is a factor of exposure duration and the temperature. Severe burns that exceed 30% of total body surface area trigger a systemic inflammatory response that can affect tissue both local and distant to the burn site. Following a severe burn, blood serum concentrations of inflammatory cytokines are dramatically elevated in burn patients compared to controls (11). In the areas local to the burn injury, tissue directly affected by the burn becomes necrotic while surrounding tissues are subject to damage from reduced tissue perfusion mediated by inflammatory factors (12). Skeletal muscle directly adjacent and distant to a burn has compromised cell membranes indicated by altered polarization states due to ion imbalances within the cells (12).

All of these stressors following a burn injury have major consequences on the skeletal muscle mitochondria. Indeed, investigations of mitochondria in the tissue localized to the burn site reveal that mitochondrial function is dramatically diminished acutely after

the injury. Three hours after the burn the mitochondria respiration is already diminished respiration, while twenty-four hours after the burn, mitochondrial respiration is nearly zero (13). Remodeling of the mitochondrial network begins soon after the burn injury, as mitochondrial function starts to recover by day 4-10 following burn (13). A common side effect of burn injury is elevated whole-body metabolism caused by hyper-mitochondrial function, which has been known to persist up to a year after the initial burn (14). Paradoxically, after the initial impairment within the first 7-10 days, mitochondrial function exceeds pre-burn functional levels, and the extent to which the function exceeds baseline levels is determined by the burn severity (14). This hyper-mitochondrial function following burn is caused by mitochondrial uncoupling, meaning that the oxygen consumed by the mitochondrial is not coupled to ATP production (15).

In addition to changes in mitochondrial function, mitochondrial content is also altered following burn injuries. Porter et al. (15), analyzed muscle biopsies from burn patients approximately 7 and 21 days post burn. Compared to controls, burn patients had diminished mitochondrial content and mitochondrial respiratory function at both time points. These data indicate that the mitochondria are sensitive to burn injury indicated by initial reductions in content and function, followed by hyper-mitochondrial function and ROS due to uncoupling.

Ischemia reperfusion injury

Ischemia reperfusion injury (IR) is an extremity trauma that most commonly results from surgical or emergency tourniquet application. IR can be defined as any event resulting in a complete restriction of blood flow to an extremity followed by a period of

reoxgyenation via restoration of blood flow. The length of ischemia will determine the extent of the injury. Generally, 3-6 hours of ischemia is sufficient to cause significant muscle injury (16, 17), but muscle damage has been reported with as little as 1 hour of ischemia (18). Typically, skeletal muscle damage from lengthy ischemia is caused by depletion of ATP stores due to lack of oxygen for mitochondrial respiration. With the loss in energy stores, the muscle cell will become necrotic. The damage during the ischemic portion of IR is compounded by a secondary injury that occurs during the reperfusion (19). Restoration of blood flow after extended ischemia leads to edema, inflammation, and an uncontrolled bolus of reactive oxygen species, which further perpetuates the damage caused by IR.

Skeletal muscle mitochondria function is dramatically impaired following IR, indicating that mitochondria are sensitive to burn-induced muscle stress. Mitochondrial function is impaired following IR. Three hours of ischemia and 2 hours of reperfusion lead to mitochondrial respiratory dysfunction to an uninjured contralateral control limb (20-22). Mitochondrial damage from IR occur rapidly after reperfusion. Thaveau et al. found that 5 hours of ischemia followed by just 5 minutes of reperfusion impaired mitochondrial respiratory function and the activities of complexes I, II, and IV (23, 24). However, it should be noted that mitochondrial dysfunction after IR could be the result of the ischemia itself rather than the reperfusion. Brandão et al. (25) demonstrated that skeletal muscle mitochondria harvested immediately after 5 hours of ischemia had significantly reduced respiratory function as well as a reduction in membrane potential. These decrements in mitochondrial function may persist long after the IR injury. Just 1 hour of ischemia led to reductions in mitochondrial proteins 7 and 14 days after the injury, suggesting that

mitochondrial function is initially impacted, with decrements in mitochondrial function coming long after the injury. In cardiac muscle, 25 mins of ischemia followed by 30 mins of reperfusion resulted in mitochondrial respiration dysfunction, but mitochondrial content, measured by citrate synthase activity, was not immediately impacted by the IR (26).

One of the cause for the reduction in mitochondrial function following IR is that mitochondrial permeability transition pore (MPTP) is opened during reperfusion. Following 3 hours of ischemia and 2 hours of reperfusion, the mitochondrial calcium retention capacity, a measure of the resistance to the MPTP to open, was lower in the IR injured limb compared to the contralateral control limb. Generally, opening of the MPTP is induced by mitochondrial stress from excessive Ca2+ absorption, ROS, alkalosis, and mitochondrial membrane depolarization (27). Reperfusion-induced opening of the MPTP is likely caused by the increase in mitochondrial ROS production during the reperfusion phase of IR (22, 27-29). With the opening of this pore, the mitochondria become permeable to small molecules, which causes mitochondrial absorption of water and subsequent swelling. Once open, the mitochondrial membrane potential diminishes leading to uncoupled respiration and additional ROS production. In addition, the mitochondria release Ca2+ stores and other pro-apototic factors that can further perpetuate mitochondrial dysfunction and eventually lead to cell death (20).

Volumetric Muscle Loss Injury

Volumetric muscle loss (VML) is characterized by the removal of a large volume of muscle tissue from the body. The largest impact of VML is obviously the frank loss of tissue from the muscle body, but secondary to the void created by the VML is the trauma

generated in the adjacent remaining muscle tissue where muscle fibers, nerves, and blood vessels are severed. A prolonged inflammatory response within the adjacent tissue ensues, further perpetuating the damage induced by the injury.

Very little information is available regarding the effect of VML on mitochondrial function. In fact, to our knowledge, only our lab has directly assessed the impact of VML on mitochondrial content and function (Greising et al. 2018). We found that 1 month after injury when compared to controls, a unilateral VML injury causes a mild reduction in mitochondrial function in both the injured limb as well as the uninjured limb suggesting that VML induces mild systemic mitochondrial dysfunction. This change in mitochondrial function occurred independently of mitochondrial content. Given the systemic nature of the dysfunction, it is possible that this response is linked to the prolonged systemic inflammation following VML. This type of inflammation-linked mitochondrial dysfunction following VML is not unreasonable considering the mitochondrial dysfunction and elevation inflammatory profile in burn victims. Overall, the little information that does exist concerning the impact of VML on mitochondria suggests that the mitochondria of the remaining muscle are sensitive to such trauma.

Contraction Induced Injury

An eccentric contraction is defined as forced lengthening of the muscle while the muscle is actively producing tension. During an eccentric contraction, muscle fibers are placed under a high stress load, and for some of the fibers this results in a structurally compromised sarcolemma. The number of eccentric contractions performed determines the severity of the injury, as a greater number of fibers are injured. Eccentric contractions result

in immediate loss in muscle strength, caused by sarcolemma damage and an uncontrolled influx of Ca^{2+} into the cell. Aberrant intercellular Ca^{2+} levels initiate intrinsic degradative processes that begin to breakdown damaged tissue. Furthermore, elevated levels of Ca^{2+} can lead to mitochondrial absorption of Ca^{2+}, which can trigger apoptosis. Within hours after the injury, invasion of inflammatory cells to the injury site contribute to the multi-day process of clearing post-injury debris.

Skeletal muscle mitochondria appear to be sensitive to eccentric contraction induced injury, although the few studies that have directly addressed mitochondrial function after eccentric exercise have shown mixed results. Magalhães et al. evaluated skeletal muscle mitochondrial function in mice after 120 minutes of downhill running and found that mitochondrial respiratory function was impaired immediately after injury (30). Mitochondrial enzyme activities of complexes I, II, and V were all assessed, but only complex V activity was reported to be lower immediately after the downhill running. These mitochondrial changes were only transient as all alterations in mitochondrial function were found to have resolved by 48 hours after downhill running. Similarly, rats that were subjected to 90 minutes of downhill running had impaired mitochondrial respiration rates, but unlike the previous study, mitochondrial respiration was still impaired 48 hours after injury (31). On the other hand, mitochondrial respiration was not impaired when, in a experimentally similar but separate study, rats were subjected to a 90-minute bout of downhill. It should be noted, however, that there was a trend for a difference in mitochondrial respiration ($P=0.09$) between control and exercised rats (32). Another study assessed mitochondrial respiration function in humans after 30 minutes of eccentric exercise (33). Muscle biopsies were collected prior to exercise, and immediately, 2, and 4

days after exercise, and no differences were found in mitochondrial respiratory activity at any of the timepoints. The disagreement in between the previously mentioned studies could be due to a number of reasons including distinct responses to eccentric exercise between humans and rodents, duration of eccentric exercise, or the timepoint at which mitochondrial function was measured.

While it is unclear whether eccentric exercise has a significant impact on mitochondrial respiratory function, studies reporting alterations in mitochondrial Ca^{2+} homeostasis are in better agreement. After eccentric exercise, the sarcolemma integrity is compromised (34) leading to a substantial Ca^{2+} influx into the cell. In an attempt to regulate the Ca^{2+} levels, the mitochondria start absorbing excess Ca^{2+} (35). When inter-mitochondrial Ca^{2+} levels exceed a certain threshold, the mitochondrial permeability transition pore opens and causes mitochondrial respiratory dysfunction (36-38), mitochondrial membrane depolarization (39), ROS production (40), and release of pro-apoptotic factors (41). Immediately after downhill running, mitochondria showed signs of susceptibility to MPTP opening and Ca2+ absorption (30). Mitochondrial Ca2+ concentration was elevated even after 48 hrs after eccentric contractions indicating prolonged effects of eccentric exercise on mitochondria (32). The prolonged elevated mitochondrial Ca^{2+} levels could contribute to the eccentric exercise-induced muscle damage. Duan et al. (35) explored the relationship between severity of eccentric contraction induced injury and mitochondrial Ca^{2+} levels and demonstrated that elevated mitochondrial Ca^{2+} levels after eccentric contraction induced injury was associated with fewer numbers of intact muscle fibers. Mitochondrial absorption of Ca2+ does not always have a detrimental effect. In fact, Ca2+ is important for regulation of mitochondrial

respiration (42, 43) and the mitochondria themselves may mediate plasma membrane repair in a Ca2+ dependent manner (1, 44). Overall, altered mitochondrial Ca^{2+} homeostasis after eccentric contractions provides evidence that mitochondria are sensitive to eccentric contraction induced injury.

Inflammation could potentially cause mitochondrial damage, but there is currently no direct evidence to support this claim. Inflammation following muscle injury is necessary for initiation and guidance of the satellite cell-mediated repair (45). However, it is plausible that a widespread and uncontrolled inflammatory response could potentially cause damage to adjacent fibers that were previously uninjured (46, 47). That being said, there is minimal evidence for secondary force loss following injury, suggesting that inflammation may not cause exacerbate post-injury muscle cells damage.

Mechanical and Myotoxic Muscle Injuries

For this category of muscle injuries, we selected injuries that result in rapid and extensive degeneration of muscle fibers and near immediate strength loss (48). These muscle injuries are most commonly used as models to first induce and then study muscle regeneration (48, 49). This category of muscle injuries can be broken down into two distinct subcategories of muscle injuries: mechanical injuries (i.e. freeze, crush, laceration) and myotoxic injuries (cardiotoxin, bupivacaine, barium chloride). Each injury model involves directly compromising the plasma membrane, but the exact mechanism varies from direct mechanical destruction (i.e. freeze, crush, laceration) to toxin-mediated increases in membrane permeability (cardiotoxin, bupivacaine, barium chloride). A dysfunctional plasma membrane leads to depolarization and loss of ion gradients,

specifically Ca2+, which can remain at elevated levels for extended periods of time inducing apoptosis and eventually necrosis. Mechanical and toxic muscle injuries produce extensive necrosis throughout the muscle, which is followed by a large, multi-day inflammatory response that triggers satellite cell-mediated regeneration (37). With all of the extreme damage brought about by these injuries, it comes as no surprise that the mitochondrial are also severely impacted.

Toxin mediated cell death causes dramatic loss in mitochondrial content and function that coincides with widespread muscle cell death. Bupivacaine is a local anesthetic that induces rapid degeneration of muscle fibers via plasma membrane lysis if injected directly into the muscle (50). Three days after a unilateral bupivacaine injection, mitochondrial function was less than 20% of the uninjured contralateral limb (51). The reductions in mitochondrial respiration were likely due to the nearly complete destruction of the mitochondrial network after the injury, as mitochondrial content was only 20% of the control limb at day 3. The muscle fiber degeneration that follows bupivacaine injection is largely mediated by elevated intracellular Ca2+ (52). Bupivacaine causes sarcoplasmic reticulum dysfunction (50) and an uncontrolled Ca2+ influx into the cell (53) which triggers opening of the mitochondrial permeability transition pore and subsequent cell death (52). Cardiotoxin injury, similar to bupivacaine, operates by causing intercellular Ca2+ disruptions. Specifically, cardiotoxin facilitates Ca2+ transport into the cell resulting in rapid depolarization and cell death (54). Mitochondria also suffer from cardiotoxin's destructive nature. Muscle mitochondrial proteins (CoxIV and cytochrome c) started to decrease within 1 day of cardiotoxin injection and reached less than 50% of uninjured muscle around 3-7 days before starting to recover (18). In a similar injury model to

cardiotoxin, citrate synthase activity values were reported to be less than 10% at days 2 and 4 after injection of notexin. Mitochondrial function and content, characterized by a host of mitochondrial enzyme activities, was still depressed at 14 days after cardiotoxin injury, suggesting that recovery after injury is slow (55). Indeed, the complete recovery of mitochondrial content after notexin-induced injury took 40+ days to return to baseline. Overall, mitochondria undergo a dramatic decrease in content and function following injection of various muscle toxins indicating that mitochondria are sensitive to toxin-induced muscle injuries.

Unlike other injuries where damage to the mitochondrial network is a secondary consequence, i.e. altered Ca2+ homeostasis, ROS production, and inflammation, physical/mechanical injuries directly disturb and destroy muscle cell membranes as well as the mitochondrial network. Freeze injury is a very severe injury model that causes rapid and widespread damage to all structures at the injury site including mitochondria (49). Mitochondrial content as measured by citrate synthase was approximately 20% of control by day 1 and nearly 0% of control by day 3, indicating that freeze injury causes almost complete destruction of the mitochondrial network. Similar to freeze injury, crush injury causes extreme muscle damage by directly compromising muscle membrane structure (56). Also, similar to freeze injury, crush injury results in severe mitochondrial damage that almost completely destroys the mitochondrial network. Muscle mitochondrial content is less than 15% of control within the first 10 days after crush injury and this deficit is persistent, as the mitochondrial content is still not fully recovered even by day 56. Overall, mitochondria content is dramatically diminished following mechanical injury,

demonstrating that muscle mitochondria are sensitive to extreme stresses exerted on the muscle.

Summary

The goal of the first section of this review was to discuss whether mitochondria are sensitive to muscle injury. In this section, we presented a number of distinct muscle injuries that result in impaired mitochondrial function and content indicating that mitochondria are indeed sensitive and susceptible to muscle damage. Mitochondrial sensitivity to muscle injury appears to be largely dictated by altered Ca2+ homeostasis, although direct damage to the mitochondrial network from the injury itself is also a contributor. The inflammatory response that is common among all muscle injuries, could also direct mitochondrial sensitivity to muscle damage. In the following section of this review, we will discuss the interaction between mitochondria and inflammation.

Overview of Mitochondrial Response to Skeletal Muscle Injury

Although there are many different types of muscle injuries and each have various means of inducing muscle damage, one commonality among all injuries is the inflammatory response that ensues. Inflammation after muscle damage is necessary for skeletal muscle repair/regeneration after injury, and a growing body of evidence suggests that mitochondria could be important contributors to this inflammatory response. In this section of the review, we will first discuss the ways in which mitochondria can contribute to and potentially initiators of the inflammatory response after muscle injury. Then we will explore the ways in which inflammation can impact, either directly or indirectly, muscle

mitochondria. Overall, this section should clearly describe the mitochondrial response to muscle injury

Mitochondria as Signaling Centers for Inflammation

Over the last 20 years, research has revealed that mitochondria are far more than bioenergetic organelles, rather mitochondria have emerged as active signaling centers that communicate with the cell and help direct cellular function. Mitochondria can communicate with the cell via release of physical signals (i.e. ROS, cytochrome c, Krebs Cycle metabolites, DNA and ATP), or by deviating from their routine functions (i.e. reduced ATP production, or regulating intracellular Ca2+ levels) (4). Over the years, mitochondrial-derived signals have been discovered to play both direct and indirect roles in routine cell function such as cell proliferation, differentiation, and adaptation. Furthermore, a new body of evidence is growing around the involvement of mitochondria in inflammation (5). Signals from the mitochondria, known as mitochondrial-derived damage-associated molecular patterns (mtDAMPs) could help to coordinate inflammatory responses to cellular stress (57).

Mitochondrial-Derived DAMPs

DAMPs are molecules released in response to cell damage or death that can initiate inflammatory responses through direct communication with the innate immune system (58). DAMPs can be derived from multiple cellular sources including the nucleus, endoplasmic reticulum, cytosol, and plasma membrane, but for the purpose of this review, we will focus strictly on mitochondrial-derived DAMPs (59). Stressed, damaged, or

dysfunctional mitochondria can release multiple signaling factors that can modulate cell function. Most notably, mitochondria control cell apoptosis through the stress-induced formation of a pore in the outer mitochondrial membrane which results in cytochrome c release and subsequent caspase-mediated cell death. Moreover, recent evidence suggests that mitochondria can not only dictate cellular apoptosis, but inflammatory cell recruitment as well. Specific mtDAMPs have been shown to directly activate the NLRP3 inflammasome, which is responsible for perpetuating cell inflammation.

Mitochondrial Activation of NLRP3 Inflammasome

Inflammasomes are intracellular multiprotein complexes that sense pathogens and initiate inflammatory responses (60). Inflammasomes can be activated by pathogens or by intracellular signals such as DAMPs. Upon activation, inflammasomes promote production and maturation of proinflammatory cytokines such as IL-1β and IL-18 and can induce inflammation mediated cell death termed pyroptosis (60, 61). There are many individual inflammasomes, each with a unique protein complex structure and function. The NLRP3 inflammasome is specifically responsible for promoting production of IL-1β, and mtDAMPs have been shown to either directly or indirectly activate this inflammasome (62, 63). We have summarized NLRP3-specific mtDAMPs and their proposed mechanism of activation in Table 1. In addition to secreting molecules that activate NLRP3, the mitochondrial outer membrane may also serve as a signaling platform for the NLRP3 complex as well as MAVS, a key inflammatory signaling protein. In summary, mitochondria can help coordinate inflammation in response to cell stress through the

release mtDAMPs which, through activation the NLRP3 inflammasome, initiate and propagate inflammatory signaling.

Potential Role of Mitochondrial-Mediated Inflammation after Skeletal Muscle Injury

Inflammation is a common characteristic of all skeletal muscle injuries. Following muscle injury, happens and signals for the recruitment of inflammatory cells. Inflammation is a self-propagating system, as recruited inflammatory cells localized to the injury site recruit additional cells via secretion of proinflammatory factors. Given the role that mitochondria play in activation of the NLRP3 inflammasome, we postulate that injury-induced mitochondrial stress contribute the inflammatory cell recruitment process after injury via NLRP3-mediated production of IL-1β (64)._As previously discussed, mitochondria are sensitive to skeletal muscle injury, in that they present with dysfunction or loss of content after a muscle injury. This mitochondrial stress is likely sufficient to produce mtDAMPs and activation of NLRP3. For instance, mitochondrial DNA (mtDNA), which is released from compromised mitochondria (65), has been shown to directly activate the NLRP3 inflammasome (66, 67). Similarly, NLRP3 can be activated by cardiolipin, a mitochondrial membrane specific lipid that is mobilized from mitochondrial destabilization (68). Other mitochondrial-derived signals such as ROS or alterations in cytosol ATP or NAD^+ levels could induce NLRP3 activation following skeletal muscle injury and mitochondrial damage (62, 69). Overall, mitochondria are sensitive to and directly impacted by skeletal muscle injury, and the mitochondrial response to this injury (i.e. mtDAMPs) could be one of the earliest initiators of the critical inflammatory response that follows muscle injury.

The Impact of Inflammatory Cytokines on Mitochondria

While mito-DAMPs provide strong evidence for a mitochondrial contribution to inflammation, it is unclear whether the inflammatory response itself either directly or indirectly acts on mitochondria. To better understand the cytokine mediated inflammatory response caused by skeletal muscle injury, we conducted a systematic review of gene array data across three injury types (Table 1). Upon analysis, Interleukin-6 (IL-6), chemokine (C-C) motif ligand 2 (MCP-1), Interleukin-1β (IL-1β), and chemokine (C-X-C motif) ligand 1 (CXCL1) and were found to be significantly elevated in their respective studies across all three injuries (48) (Greising et al. 2018). We chose to focus on these four cytokines based on their common expression patterns across the three distinct muscle injuries. We hypothesize that mitochondrial structure and function is responsive to the four inflammatory cytokines, and we speculate on the downstream consequences in terms of further amplification or attenuation of molecular signaling within the skeletal muscle fiber. Table 1 summarizes 13 common inflammation genes presented as fold change in gene expression vs. control following injury. Warren et. al (48) freeze injury and eccentric contraction injury data was collected across 6-hour plus 1, 3, and 7-day time points post-injury, while Greising et. al VML injury data was collected across 3, 7, and 14-day time points post-injury-1.

Interleukin-6

IL-6 is a well-known pleiotropic pro-inflammatory cytokine secreted by T cells and macrophages in response to infection and tissue injury. Systemic elevation of IL-6 has been known to have detrimental effects on skeletal muscle such as muscle atrophy (70), but the

effects of IL-6 on skeletal muscle mitochondria are less established. Studies utilizing IL-6 overexpression (71, 72) and IL-6 receptor antibody administration (71) show a correlation between IL-6 levels and muscle mitochondrial dysfunction. At the transcriptional level, over-expression of IL-6 attenuated mitochondrial biogenesis signaling via reduced PGC-1α mRNA expression (71). Overabundant IL-6 levels also altered mitochondrial dynamics as shown by decreased mitochondrial fusion proteins Mfn1 and Mfn2, and increased mitochondrial fission protein FIS1 (71). This is in agreement with a study on the effects of supplemental IL-6 treatment on human myoblasts that demonstrated a consistent reduction in mitochondrial fusion based on Mfn2 protein levels (72). In contrast, IL-6 inhibition via IL-6 receptor antibody treatment rescues the loss of mitochondrial content and biogenesis in mice susceptible to cachectic muscle wasting (71). These studies support the position that IL-6 mediates mitochondrial network size and content in skeletal muscle.

One possible mechanism by which IL-6 impacts mitochondria structure and possibly function is via increased STAT3 activation (71-74). While IL-6 has a well-characterized role as an activator of the intracellular signaling JAK/STAT pathway involved in basal cellular respiration (73), further evaluation (74) suggests a more nuanced role of JAK signaling in mitochondrial function. Specifically, JAK signaling is involved in a critical trigger point of STAT3 phosphorylation and subsequent mitochondrial import that increases ROS generation through alterations in electron transport chain (ETC) function (74). However, STAT3 attenuates ROS generation in hypoxic conditions (74) and STAT3 null cells display decreased ETC activity. The contrary roles of STAT3 may depend on its accumulation in the mitochondria versus its role as a transcription factor (75). In summary, IL-6 may mediate mitochondrial structure and function via mitochondrial-

targeted STAT3, though it appears the relationship is context dependent and normal ETC function may require an optimal level of STAT3 accumulation in the mitochondria.

Interleukin-1β and Chemokine (C-X-C motif) Ligand 1

IL-1β is an inflammasome-dependent cytokine that functions as an endogenous pyrogen and mediator of the inflammatory response via promotion of cytokine secretion. IL-1β production requires two steps: first, inflammatory signals must stimulate transcription and synthesis of pro-IL-1β, followed by a separate signal (commonly the NLRP3 inflammasome) that activates CASP1 to proteolytically cleave pro-IL-1β into its mature and secretable form (76, 77). Given the role of mitochondria in activating the NLRP3 inflammasome, it is not unlikely that IL-1β could directly interact with the mitochondria. Indeed, hypersecretion of IL-1β, mimicked via isoprenoid-deficient monocytes, demonstrated increased mitochondrial transmembrane potential, reduced mitochondrial stability measured by MitoTracker fluorescence, accumulation of damaged mitochondria due to impaired mitophagy, and reduced antioxidant capacity (measured with normalized ROS production values) (78). Consistent with these findings, IL-1β receptor gene knockout (IL-1R1$^{-/-}$) in diabetic mice demonstrated attenuated mtDNA damage, mitochondrial superoxide production (e.g. ROS), and cytochrome c leakage compared to WT diabetic mice (79). This evidence suggests that IL-1β directly impairs healthy mitochondrial function; however, the relevance of this relationship specifically in the context of skeletal muscle stress needs to be investigated.

CXCL1 is a small cytokine belonging to the CXC family involved primarily in neutrophil attraction (80). To our knowledge, there have not been any direct studies on the relationship between CXCL1 levels and skeletal muscle mitochondria. Interestingly, models of acute arthritis (81), dermatitis (64), and group B streptococcus (82) have found IL-1β to induce production of CXC chemokines, including CXCL1, which suggests the existence of a "IL-1β /CXCL1/2/ Neutrophil Axis" responsible for a potential feedforward relationship between IL-1β and CXCL1. Further evidence for this feedforward relationship comes from IL-1β deficient mice, which have severely deficient CXCL1/2 production and neutrophil levels, Conversely, Ritzman *et al.* found that CXCL1 mediates neutrophil recruitment independent of IL-1β in a lyme arthritis/carditis model (83). Thus, it is unclear in which circumstances IL-1β-induced production of CXCL1 exists. Our analysis of muscle injuries revealed highly correlated levels of CXCL1 and IL-1β, which suggests a that the feedforward relationship may exist in the context of skeletal muscle stress.

Chemokine (C-C motif) Ligand 2

CCL2, also commonly referred to as monocyte chemoattractant protein 1 (MCP-1), is a small cytokine in the CC family that exhibits chemotactic activity for monocytes and macrophages to sites of inflammation (84). Importantly, skeletal muscle injury studies utilizing MCP-1 knockout mice (85) have established that MCP-1 expression is necessary for macrophage recruitment and repair of acute skeletal muscle injury. However, Zhang et al. (86) found MCP-1 administration to have a pathogenic role beyond chemotaxis, including direct mitochondrial damage and pro-apoptotic effects mediated by the p53-dependent mitochondrial pathway (86). Specifically, human umbilical vein epithelial cells

treated with MCP-1 had decreased mitochondrial mass, greater release of cytochrome c (a mitochondrial derived activator of apoptosis), reduced transmembrane potential, and upregulated p53, suggesting MCP-1 over-expression may be detrimental to the mitochondria (86).

While the specific interaction of MCP-1 in the skeletal muscle mitochondria is not known, it is possible that an IL-6/MCP-1 positive feedback loop reported in vascular inflammation studies (87, 88) may exist in the context of skeletal muscle stress. Activation of IL-6 production stimulates local monocyte recruitment and thereby induce MCP-1 expression, which appears to coordinate stress-induced production of additional IL-6 (87, 88). This hypothesis is supported by evidence of reduced IL-6 found in MCP-1 null cells compared to normal hepatocytes. In the context of skeletal muscle injury, an amplification loop between MCP-1 and IL-6 would further exacerbate the inflammatory response acting on the mitochondria as described above in the previous IL-6 section. Notably, a study on neurite growth found CCL2 overexpression to operate via a STAT3 dependent mechanism, which supports the proposed link between MCP-1 and IL-6 (89). Overall, the IL-6/MCP-1 positive feedback loop poses as a potential mechanism for exacerbating the inflammatory response which could directly impact mitochondrial function and recovery from injury.

Conclusion

In this review, we conclude that mitochondria are sensitive to skeletal muscle stress in that they undergo alterations in network size and function in response to various muscle injuries. Moreover, upon sensing stress, mitochondria are power signaling centers that are capable of reacting to stress by secreting proapoptotic and proinflammatory signals.

Through this stress-induced signaling, mitochondrial can contribute to coordination of the inflammatory response after injury. Overall, mitochondria are sensitive cellular detectors of skeletal muscle injury and potentially may be one of the earliest initiators of post-injury skeletal muscle inflammation.

References

1. Horn A, Van der Meulen JH, Defour A, Hogarth M, Sreetama SC, Reed A, Scheffer L, Chandel NS, Jaiswal JK. Mitochondrial redox signaling enables repair of injured skeletal muscle cells. Sci Signal. 2017;10(495). Epub 2017/09/07. doi: 10.1126/scisignal.aaj1978. PubMed PMID: 28874604.

2. Sharma N, Medikayala S, Defour A, Rayavarapu S, Brown KJ, Hathout Y, Jaiswal JK. Use of quantitative membrane proteomics identifies a novel role of mitochondria in healing injured muscles. J Biol Chem. 2012;287(36):30455-67. Epub 2012/07/11. doi: 10.1074/jbc.M112.354415. PubMed PMID: 22778268; PMCID: PMC3436295.

3. Bhola PD, Letai A. Mitochondria-Judges and Executioners of Cell Death Sentences. Mol Cell. 2016;61(5):695-704. Epub 2016/03/05. doi: 10.1016/j.molcel.2016.02.019. PubMed PMID: 26942674; PMCID: PMC4806554.

4. Chandel NS. Mitochondria as signaling organelles. BMC Biol. 2014;12:34. Epub 2014/06/03. doi: 10.1186/1741-7007-12-34. PubMed PMID: 24884669; PMCID: PMC4035690.

5. Dela Cruz CS, Kang MJ. Mitochondrial dysfunction and damage associated molecular patterns (DAMPs) in chronic inflammatory diseases. Mitochondrion. 2017. Epub 2017/12/10. doi: 10.1016/j.mito.2017.12.001. PubMed PMID: 29221810.

6. West AP, Khoury-Hanold W, Staron M, Tal MC, Pineda CM, Lang SM, Bestwick M, Duguay BA, Raimundo N, MacDuff DA, Kaech SM, Smiley JR, Means RE, Iwasaki A, Shadel GS. Mitochondrial DNA stress primes the antiviral innate immune response. Nature. 2015;520(7548):553-7. Epub 2015/02/03. doi: 10.1038/nature14156. PubMed PMID: 25642965; PMCID: PMC4409480.

7. Larsen S, Nielsen J, Hansen CN, Nielsen LB, Wibrand F, Stride N, Schroder HD, Boushel R, Helge JW, Dela F, Hey-Mogensen M. Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. J Physiol. 2012;590(14):3349-60. Epub 2012/05/16. doi: 10.1113/jphysiol.2012.230185. PubMed PMID: 22586215; PMCID: PMC3459047.

8. Rolfe DF, Brown GC. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiol Rev. 1997;77(3):731-58. Epub 1997/07/01. doi: 10.1152/physrev.1997.77.3.731. PubMed PMID: 9234964.

9. Ibebunjo C, Martyn J. Disparate dysfunction of skeletal muscles located near and distant from burn site in the rat. Muscle Nerve. 2001;24(10):1283-94. Epub 2001/09/20. PubMed PMID: 11562907.

10. St-Pierre DM, Choiniere M, Forget R, Garrel DR. Muscle strength in individuals with healed burns. Arch Phys Med Rehabil. 1998;79(2):155-61. Epub 1998/02/25. PubMed PMID: 9473996.

11. Merritt EK, Cross JM, Bamman MM. Inflammatory and protein metabolism signaling responses in human skeletal muscle after burn injury. J Burn Care Res. 2012;33(2):291-7. Epub 2011/11/15. doi: 10.1097/BCR.0b013e3182331e4b. PubMed PMID: 22079905; PMCID: PMC3292697.

12. Kaddoura I, Abu-Sittah G, Ibrahim A, Karamanoukian R, Papazian N. Burn injury: review of pathophysiology and therapeutic modalities in major burns. Ann Burns Fire Disasters. 2017;30(2):95-102. Epub 2017/10/13. PubMed PMID: 29021720; PMCID: PMC5627559.

13. Porter C, Herndon DN, Bhattarai N, Ogunbileje JO, Szczesny B, Szabo C, Toliver-

Kinsky T, Sidossis LS. Differential acute and chronic effects of burn trauma on murine skeletal muscle bioenergetics. Burns. 2016;42(1):112-22. Epub 2015/12/01. doi: 10.1016/j.burns.2015.10.026. PubMed PMID: 26615714; PMCID: PMC4724536.

14. Rontoyanni VG, Malagaris I, Herndon DN, Rivas E, Capek KD, Delgadillo AD, Bhattarai N, Elizondo A, Voigt CD, Finnerty CC, Suman OE, Porter C. Skeletal Muscle Mitochondrial Function is Determined by Burn Severity, Sex and Sepsis, and is Associated with Glucose Metabolism and Functional Capacity in Burned Children. Shock. 2017. Epub 2017/12/06. doi: 10.1097/SHK.0000000000001074. PubMed PMID: 29206761.

15. Porter C, Herndon DN, Borsheim E, Chao T, Reidy PT, Borack MS, Rasmussen BB, Chondronikola M, Saraf MK, Sidossis LS. Uncoupled skeletal muscle mitochondria contribute to hypermetabolism in severely burned adults. Am J Physiol Endocrinol Metab. 2014;307(5):E462-7. Epub 2014/07/31. doi: 10.1152/ajpendo.00206.2014. PubMed PMID: 25074988; PMCID: PMC4154069.

16. Martou G, O'Blenes CA, Huang N, McAllister SE, Neligan PC, Ashrafpour H, Pang CY, Lipa JE. Development of an in vitro model for study of the efficacy of ischemic preconditioning in human skeletal muscle against ischemia-reperfusion injury. J Appl Physiol (1985). 2006;101(5):1335-42. Epub 2006/10/18. doi: 10.1152/japplphysiol.00278.2006. PubMed PMID: 17043328.

17. Paradis S, Charles AL, Meyer A, Lejay A, Scholey JW, Chakfe N, Zoll J, Geny B. Chronology of mitochondrial and cellular events during skeletal muscle ischemia-reperfusion. Am J Physiol Cell Physiol. 2016;310(11):C968-82. Epub 2016/04/15. doi: 10.1152/ajpcell.00356.2015. PubMed PMID: 27076618; PMCID: PMC4935201.

18. Call JA, Wilson RJ, Laker RC, Zhang M, Kundu M, Yan Z. Ulk1-mediated

autophagy plays an essential role in mitochondrial remodeling and functional regeneration of skeletal muscle. Am J Physiol Cell Physiol. 2017;312(6):C724-C32. Epub 2017/03/31. doi: 10.1152/ajpcell.00348.2016. PubMed PMID: 28356270; PMCID: PMC5494591.

19. Blaisdell FW. The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome: a review. Cardiovasc Surg. 2002;10(6):620-30. Epub 2002/11/28. PubMed PMID: 12453699.

20. Mansour Z, Bouitbir J, Charles AL, Talha S, Kindo M, Pottecher J, Zoll J, Geny B. Remote and local ischemic preconditioning equivalently protects rat skeletal muscle mitochondrial function during experimental aortic cross-clamping. J Vasc Surg. 2012;55(2):497-505 e1. Epub 2011/11/08. doi: 10.1016/j.jvs.2011.07.084. PubMed PMID: 22056287.

21. Pottecher J, Kindo M, Chamaraux-Tran TN, Charles AL, Lejay A, Kemmel V, Vogel T, Chakfe N, Zoll J, Diemunsch P, Geny B. Skeletal muscle ischemia-reperfusion injury and cyclosporine A in the aging rat. Fundam Clin Pharmacol. 2016;30(3):216-25. Epub 2016/01/21. doi: 10.1111/fcp.12180. PubMed PMID: 26787364.

22. Talha S, Bouitbir J, Charles AL, Zoll J, Goette-Di Marco P, Meziani F, Piquard F, Geny B. Pretreatment with brain natriuretic peptide reduces skeletal muscle mitochondrial dysfunction and oxidative stress after ischemia-reperfusion. J Appl Physiol (1985). 2013;114(2):172-9. Epub 2012/10/30. doi: 10.1152/japplphysiol.00239.2012. PubMed PMID: 23104692.

23. Thaveau F, Zoll J, Bouitbir J, N'Guessan B, Plobner P, Chakfe N, Kretz JG, Richard R, Piquard F, Geny B. Effect of chronic pre-treatment with angiotensin converting enzyme inhibition on skeletal muscle mitochondrial recovery after ischemia/reperfusion. Fundam

Clin Pharmacol. 2010;24(3):333-40. Epub 2009/08/18. doi: 10.1111/j.1472-8206.2009.00763.x. PubMed PMID: 19682081.

24. Thaveau F, Zoll J, Rouyer O, Chafke N, Kretz JG, Piquard F, Geny B. Ischemic preconditioning specifically restores complexes I and II activities of the mitochondrial respiratory chain in ischemic skeletal muscle. J Vasc Surg. 2007;46(3):541-7; discussion 7. Epub 2007/09/11. doi: 10.1016/j.jvs.2007.04.075. PubMed PMID: 17826242.

25. Brandao ML, Roselino JE, Piccinato CE, Cherri J. Mitochondrial alterations in skeletal muscle submitted to total ischemia. J Surg Res. 2003;110(1):235-40. Epub 2003/04/17. PubMed PMID: 12697407.

26. Tompkins AJ, Burwell LS, Digerness SB, Zaragoza C, Holman WL, Brookes PS. Mitochondrial dysfunction in cardiac ischemia-reperfusion injury: ROS from complex I, without inhibition. Biochim Biophys Acta. 2006;1762(2):223-31. Epub 2005/11/10. doi: 10.1016/j.bbadis.2005.10.001. PubMed PMID: 16278076.

27. Honda HM, Korge P, Weiss JN. Mitochondria and ischemia/reperfusion injury. Ann N Y Acad Sci. 2005;1047:248-58. Epub 2005/08/12. doi: 10.1196/annals.1341.022. PubMed PMID: 16093501.

28. Clarke SJ, Khaliulin I, Das M, Parker JE, Heesom KJ, Halestrap AP. Inhibition of mitochondrial permeability transition pore opening by ischemic preconditioning is probably mediated by reduction of oxidative stress rather than mitochondrial protein phosphorylation. Circ Res. 2008;102(9):1082-90. Epub 2008/03/22. doi: 10.1161/CIRCRESAHA.107.167072. PubMed PMID: 18356542; PMCID: PMC2629616.

29. Lejay A, Meyer A, Schlagowski AI, Charles AL, Singh F, Bouitbir J, Pottecher J, Chakfe N, Zoll J, Geny B. Mitochondria: mitochondrial participation in ischemia-

reperfusion injury in skeletal muscle. Int J Biochem Cell Biol. 2014;50:101-5. Epub 2014/03/04. doi: 10.1016/j.biocel.2014.02.013. PubMed PMID: 24582887.

30. Magalhaes J, Fraga M, Lumini-Oliveira J, Goncalves I, Costa M, Ferreira R, Oliveira PJ, Ascensao A. Eccentric exercise transiently affects mice skeletal muscle mitochondrial function. Appl Physiol Nutr Metab. 2013;38(4):401-9. Epub 2013/05/30. doi: 10.1139/apnm-2012-0226. PubMed PMID: 23713533.

31. Rattray B, Caillaud C, Ruell PA, Thompson MW. Heat exposure does not alter eccentric exercise-induced increases in mitochondrial calcium and respiratory dysfunction. Eur J Appl Physiol. 2011;111(11):2813-21. Epub 2011/03/19. doi: 10.1007/s00421-011-1913-4. PubMed PMID: 21416144.

32. Rattray B, Thompson M, Ruell P, Caillaud C. Specific training improves skeletal muscle mitochondrial calcium homeostasis after eccentric exercise. Eur J Appl Physiol. 2013;113(2):427-36. Epub 2012/07/11. doi: 10.1007/s00421-012-2446-1. PubMed PMID: 22777498.

33. Kasperek GJ, Snider RD. Effect of eccentric exercise on muscle protein degradation. Prog Clin Biol Res. 1985;180:611-3. Epub 1985/01/01. PubMed PMID: 3162169.

34. Armstrong RB, Warren GL, Warren JA. Mechanisms of exercise-induced muscle fibre injury. Sports Med. 1991;12(3):184-207. Epub 1991/09/01. PubMed PMID: 1784873.

35. Duan C, Delp MD, Hayes DA, Delp PD, Armstrong RB. Rat skeletal muscle mitochondrial [Ca2+] and injury from downhill walking. J Appl Physiol (1985). 1990;68(3):1241-51. Epub 1990/03/01. doi: 10.1152/jappl.1990.68.3.1241. PubMed PMID: 2111311.

36. Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am J Physiol Cell Physiol. 2004;287(4):C817-33. Epub 2004/09/10. doi: 10.1152/ajpcell.00139.2004. PubMed PMID: 15355853.

37. Gunter TE, Yule DI, Gunter KK, Eliseev RA, Salter JD. Calcium and mitochondria. FEBS Lett. 2004;567(1):96-102. Epub 2004/05/29. doi: 10.1016/j.febslet.2004.03.071. PubMed PMID: 15165900.

38. Szabadkai G, Duchen MR. Mitochondria: the hub of cellular Ca2+ signaling. Physiol (Bethesda). 2008;23:84-94. Epub 2008/04/11. doi: 10.1152/physiol.00046.2007. PubMed PMID: 18400691.

39. Miyamoto S, Howes AL, Adams JW, Dorn GW, 2nd, Brown JH. Ca2+ dysregulation induces mitochondrial depolarization and apoptosis: role of Na+/Ca2+ exchanger and AKT. J Biol Chem. 2005;280(46):38505-12. Epub 2005/08/03. doi: 10.1074/jbc.M505223200. PubMed PMID: 16061478.

40. Batandier C, Leverve X, Fontaine E. Opening of the mitochondrial permeability transition pore induces reactive oxygen species production at the level of the respiratory chain complex I. J Biol Chem. 2004;279(17):17197-204. Epub 2004/02/14. doi: 10.1074/jbc.M310329200. PubMed PMID: 14963044.

41. Kroemer G, Reed JC. Mitochondrial control of cell death. Nat Med. 2000;6(5):513-9. Epub 2000/05/10. doi: 10.1038/74994. PubMed PMID: 10802706.

42. Fink BD, Bai F, Yu L, Sivitz WI. Regulation of ATP production: dependence on calcium concentration and respiratory state. Am J Physiol Cell Physiol. 2017;313(2):C146-C53. Epub 2017/05/19. doi: 10.1152/ajpcell.00086.2017. PubMed PMID: 28515085.

43. Kavanagh NI, Ainscow EK, Brand MD. Calcium regulation of oxidative

phosphorylation in rat skeletal muscle mitochondria. Biochim Biophys Acta. 2000;1457(1-2):57-70. Epub 2000/02/29. PubMed PMID: 10692550.

44. Vila MC, Rayavarapu S, Hogarth MW, Van der Meulen JH, Horn A, Defour A, Takeda S, Brown KJ, Hathout Y, Nagaraju K, Jaiswal JK. Mitochondria mediate cell membrane repair and contribute to Duchenne muscular dystrophy. Cell Death Differ. 2017;24(2):330-42. Epub 2016/11/12. doi: 10.1038/cdd.2016.127. PubMed PMID: 27834955; PMCID: PMC5299714.

45. Peake JM, Neubauer O, Della Gatta PA, Nosaka K. Muscle damage and inflammation during recovery from exercise. J Appl Physiol (1985). 2017;122(3):559-70. Epub 2016/12/31. doi: 10.1152/japplphysiol.00971.2016. PubMed PMID: 28035017.

46. Pizza FX, McLoughlin TJ, McGregor SJ, Calomeni EP, Gunning WT. Neutrophils injure cultured skeletal myotubes. Am J Physiol Cell Physiol. 2001;281(1):C335-41. Epub 2001/06/13. doi: 10.1152/ajpcell.2001.281.1.C335. PubMed PMID: 11401857.

47. Toumi H, Best TM. The inflammatory response: friend or enemy for muscle injury? Br J Sports Med. 2003;37(4):284-6. Epub 2003/08/02. PubMed PMID: 12893707; PMCID: PMC1724680.

48. Warren GL, Summan M, Gao X, Chapman R, Hulderman T, Simeonova PP. Mechanisms of skeletal muscle injury and repair revealed by gene expression studies in mouse models. J Physiol. 2007;582(Pt 2):825-41. Epub 2007/05/05. doi: 10.1113/jphysiol.2007.132373. PubMed PMID: 17478534; PMCID: PMC2075314.

49. Hardy D, Besnard A, Latil M, Jouvion G, Briand D, Thepenier C, Pascal Q, Guguin A, Gayraud-Morel B, Cavaillon JM, Tajbakhsh S, Rocheteau P, Chretien F. Comparative Study of Injury Models for Studying Muscle Regeneration in Mice. PLoS ONE.

2016;11(1):e0147198. Epub 2016/01/26. doi: 10.1371/journal.pone.0147198. PubMed PMID: 26807982; PMCID: PMC4726569.

50. Nonaka I, Takagi A, Ishiura S, Nakase H, Sugita H. Pathophysiology of muscle fiber necrosis induced by bupivacaine hydrochloride (Marcaine). Acta Neuropathol. 1983;60(3-4):167-74. Epub 1983/01/01. PubMed PMID: 6613529.

51. Duguez S, Feasson L, Denis C, Freyssenet D. Mitochondrial biogenesis during skeletal muscle regeneration. Am J Physiol Endocrinol Metab. 2002;282(4):E802-9. Epub 2002/03/08. doi: 10.1152/ajpendo.00343.2001. PubMed PMID: 11882500.

52. Irwin W, Fontaine E, Agnolucci L, Penzo D, Betto R, Bortolotto S, Reggiani C, Salviati G, Bernardi P. Bupivacaine myotoxicity is mediated by mitochondria. J Biol Chem. 2002;277(14):12221-7. Epub 2002/01/16. doi: 10.1074/jbc.M108938200. PubMed PMID: 11790774.

53. Steer JH, Mastaglia FL, Papadimitriou JM, Van Bruggen I. Bupivacaine-induced muscle injury. The role of extracellular calcium. J Neurol Sci. 1986;73(2):205-17. Epub 1986/04/01. PubMed PMID: 3701376.

54. Chang CC, Wei JW, Chuang ST, Lee CY. Are the blockade of nerve conduction and depolarization of skeletal muscle induced by cobra venom due to phospholipase A, neurotoxin or cardiotoxin? Taiwan Yi Xue Hui Za Zhi. 1972;71(6):323-7. Epub 1972/06/28. PubMed PMID: 4512907.

55. Nichenko AS, Southern WM, Atuan M, Luan J, Peissig KB, Foltz SJ, Beedle AM, Warren GL, Call JA. Mitochondrial maintenance via autophagy contributes to functional skeletal muscle regeneration and remodeling. Am J Physiol Cell Physiol. 2016;311(2):C190-200. Epub 2016/06/10. doi: 10.1152/ajpcell.00066.2016. PubMed

PMID: 27281480.

56. Dobek GL, Fulkerson ND, Nicholas J, Schneider BS. Mouse model of muscle crush injury of the legs. Comp Med. 2013;63(3):227-32. Epub 2013/06/14. PubMed PMID: 23759525; PMCID: PMC3690428.

57. Krysko DV, Agostinis P, Krysko O, Garg AD, Bachert C, Lambrecht BN, Vandenabeele P. Emerging role of damage-associated molecular patterns derived from mitochondria in inflammation. Trends Immunol. 2011;32(4):157-64. Epub 2011/02/22. doi: 10.1016/j.it.2011.01.005. PubMed PMID: 21334975.

58. Tang D, Kang R, Coyne CB, Zeh HJ, Lotze MT. PAMPs and DAMPs: signal 0s that spur autophagy and immunity. Immunol Rev. 2012;249(1):158-75. Epub 2012/08/15. doi: 10.1111/j.1600-065X.2012.01146.x. PubMed PMID: 22889221; PMCID: PMC3662247.

59. Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, Brohi K, Itagaki K, Hauser CJ. Circulating mitochondrial DAMPs cause inflammatory responses to injury. Nature. 2010;464(7285):104-7. Epub 2010/03/06. doi: 10.1038/nature08780. PubMed PMID: 20203610; PMCID: PMC2843437.

60. Sharma D, Kanneganti TD. The cell biology of inflammasomes: Mechanisms of inflammasome activation and regulation. J Cell Biol. 2016;213(6):617-29. Epub 2016/06/22. doi: 10.1083/jcb.201602089. PubMed PMID: 27325789; PMCID: PMC4915194.

61. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell. 2002;10(2):417-26. Epub 2002/08/23. PubMed PMID: 12191486.

62. Gurung P, Lukens JR, Kanneganti TD. Mitochondria: diversity in the regulation of the NLRP3 inflammasome. Trends Mol Med. 2015;21(3):193-201. Epub 2014/12/17. doi: 10.1016/j.molmed.2014.11.008. PubMed PMID: 25500014; PMCID: PMC4352396.

63. Mills EL, Kelly B, O'Neill LAJ. Mitochondria are the powerhouses of immunity. Nat Immunol. 2017;18(5):488-98. Epub 2017/04/19. doi: 10.1038/ni.3704. PubMed PMID: 28418387.

64. Miller LS, Pietras EM, Uricchio LH, Hirano K, Rao S, Lin H, O'Connell RM, Iwakura Y, Cheung AL, Cheng G, Modlin RL. Inflammasome-mediated production of IL-1beta is required for neutrophil recruitment against Staphylococcus aureus in vivo. J Immunol. 2007;179(10):6933-42. Epub 2007/11/06. PubMed PMID: 17982084.

65. Patrushev M, Kasymov V, Patrusheva V, Ushakova T, Gogvadze V, Gaziev A. Mitochondrial permeability transition triggers the release of mtDNA fragments. Cell Mol Life Sci. 2004;61(24):3100-3. Epub 2004/12/08. doi: 10.1007/s00018-004-4424-1. PubMed PMID: 15583871.

66. Nakahira K, Haspel JA, Rathinam VA, Lee SJ, Dolinay T, Lam HC, Englert JA, Rabinovitch M, Cernadas M, Kim HP, Fitzgerald KA, Ryter SW, Choi AM. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat Immunol. 2011;12(3):222-30. Epub 2010/12/15. doi: 10.1038/ni.1980. PubMed PMID: 21151103; PMCID: PMC3079381.

67. West AP, Shadel GS. Mitochondrial DNA in innate immune responses and inflammatory pathology. Nat Rev Immunol. 2017;17(6):363-75. Epub 2017/04/11. doi: 10.1038/nri.2017.21. PubMed PMID: 28393922.

68. Iyer SS, He Q, Janczy JR, Elliott EI, Zhong Z, Olivier AK, Sadler JJ, Knepper-

Adrian V, Han R, Qiao L, Eisenbarth SC, Nauseef WM, Cassel SL, Sutterwala FS. Mitochondrial cardiolipin is required for Nlrp3 inflammasome activation. Immunity. 2013;39(2):311-23. Epub 2013/08/21. doi: 10.1016/j.immuni.2013.08.001. PubMed PMID: 23954133; PMCID: PMC3779285.

69. Misawa T, Takahama M, Kozaki T, Lee H, Zou J, Saitoh T, Akira S. Microtubule-driven spatial arrangement of mitochondria promotes activation of the NLRP3 inflammasome. Nat Immunol. 2013;14(5):454-60. Epub 2013/03/19. doi: 10.1038/ni.2550. PubMed PMID: 23502856.

70. Merritt EK, Thalacker-Mercer A, Cross JM, Windham ST, Thomas SJ, Bamman MM. Increased expression of atrogenes and TWEAK family members after severe burn injury in nonburned human skeletal muscle. J Burn Care Res. 2013;34(5):e297-304. Epub 2013/07/03. doi: 10.1097/BCR.0b013e31827a2a9c. PubMed PMID: 23816995; PMCID: PMC3770758.

71. White JP, Puppa MJ, Sato S, Gao S, Price RL, Baynes JW, Kostek MC, Matesic LE, Carson JA. IL-6 regulation on skeletal muscle mitochondrial remodeling during cancer cachexia in the ApcMin/+ mouse. Skelet Muscle. 2012;2:14. Epub 2012/07/10. doi: 10.1186/2044-5040-2-14. PubMed PMID: 22769563; PMCID: PMC3431229.

72. Bach D, Naon D, Pich S, Soriano FX, Vega N, Rieusset J, Laville M, Guillet C, Boirie Y, Wallberg-Henriksson H, Manco M, Calvani M, Castagneto M, Palacin M, Mingrone G, Zierath JR, Vidal H, Zorzano A. Expression of Mfn2, the Charcot-Marie-Tooth neuropathy type 2A gene, in human skeletal muscle: effects of type 2 diabetes, obesity, weight loss, and the regulatory role of tumor necrosis factor alpha and interleukin-6. Diabetes. 2005;54(9):2685-93. Epub 2005/08/27. PubMed PMID: 16123358.

73. Carson JA, Hardee JP, VanderVeen BN. The emerging role of skeletal muscle oxidative metabolism as a biological target and cellular regulator of cancer-induced muscle wasting. Semin Cell Dev Biol. 2016;54:53-67. Epub 2015/11/26. doi: 10.1016/j.semcdb.2015.11.005. PubMed PMID: 26593326; PMCID: PMC4867246.

74. Smith IJ, Godinez GL, Singh BK, McCaughey KM, Alcantara RR, Gururaja T, Ho MS, Nguyen HN, Friera AM, White KA, McLaughlin JR, Hansen D, Romero JM, Baltgalvis KA, Claypool MD, Li W, Lang W, Yam GC, Gelman MS, Ding R, Yung SL, Creger DP, Chen Y, Singh R, Smuder AJ, Wiggs MP, Kwon OS, Sollanek KJ, Powers SK, Masuda ES, Taylor VC, Payan DG, Kinoshita T, Kinsella TM. Inhibition of Janus kinase signaling during controlled mechanical ventilation prevents ventilation-induced diaphragm dysfunction. FASEB J. 2014;28(7):2790-803. Epub 2014/03/29. doi: 10.1096/fj.13-244210. PubMed PMID: 24671708; PMCID: PMC4062832.

75. Wegrzyn J, Potla R, Chwae YJ, Sepuri NB, Zhang Q, Koeck T, Derecka M, Szczepanek K, Szelag M, Gornicka A, Moh A, Moghaddas S, Chen Q, Bobbili S, Cichy J, Dulak J, Baker DP, Wolfman A, Stuehr D, Hassan MO, Fu XY, Avadhani N, Drake JI, Fawcett P, Lesnefsky EJ, Larner AC. Function of mitochondrial Stat3 in cellular respiration. Science. 2009;323(5915):793-7. Epub 2009/01/10. doi: 10.1126/science.1164551. PubMed PMID: 19131594; PMCID: PMC2758306.

76. Hoffman HM, Wanderer AA. Inflammasome and IL-1beta-mediated disorders. Curr Allergy Asthma Rep. 2010;10(4):229-35. Epub 2010/04/29. doi: 10.1007/s11882-010-0109-z. PubMed PMID: 20425006; PMCID: PMC2892083.

77. Liu W, Yin Y, Zhou Z, He M, Dai Y. OxLDL-induced IL-1 beta secretion promoting foam cells formation was mainly via CD36 mediated ROS production leading

to NLRP3 inflammasome activation. Inflamm Res. 2014;63(1):33-43. Epub 2013/10/15. doi: 10.1007/s00011-013-0667-3. PubMed PMID: 24121974.

78. van der Burgh R, Nijhuis L, Pervolaraki K, Compeer EB, Jongeneel LH, van Gijn M, Coffer PJ, Murphy MP, Mastroberardino PG, Frenkel J, Boes M. Defects in mitochondrial clearance predispose human monocytes to interleukin-1beta hypersecretion. J Biol Chem. 2014;289(8):5000-12. Epub 2013/12/21. doi: 10.1074/jbc.M113.536920. PubMed PMID: 24356959; PMCID: PMC3931060.

79. Kowluru RA, Mohammad G, Santos JM, Tewari S, Zhong Q. Interleukin-1beta and mitochondria damage, and the development of diabetic retinopathy. J Ocul Biol Dis Infor. 2011;4(1-2):3-9. Epub 2011/06/01. doi: 10.1007/s12177-011-9074-6. PubMed PMID: 23272269; PMCID: PMC3342404.

80. Moser B, Clark-Lewis I, Zwahlen R, Baggiolini M. Neutrophil-activating properties of the melanoma growth-stimulatory activity. J Exp Med. 1990;171(5):1797-802. Epub 1990/05/01. PubMed PMID: 2185333; PMCID: PMC2187876.

81. Matsukawa A, Yoshimura T, Maeda T, Takahashi T, Ohkawara S, Yoshinaga M. Analysis of the cytokine network among tumor necrosis factor alpha, interleukin-1beta, interleukin-8, and interleukin-1 receptor antagonist in monosodium urate crystal-induced rabbit arthritis. Lab Invest. 1998;78(5):559-69. Epub 1998/05/30. PubMed PMID: 9605181.

82. Biondo C, Mancuso G, Midiri A, Signorino G, Domina M, Lanza Cariccio V, Mohammadi N, Venza M, Venza I, Teti G, Beninati C. The interleukin-1beta/CXCL1/2/neutrophil axis mediates host protection against group B streptococcal infection. Infect Immun. 2014;82(11):4508-17. Epub 2014/08/13. doi: 10.1128/IAI.02104-

14. PubMed PMID: 25114117; PMCID: PMC4249330.

83. Ritzman AM, Hughes-Hanks JM, Blaho VA, Wax LE, Mitchell WJ, Brown CR. The chemokine receptor CXCR2 ligand KC (CXCL1) mediates neutrophil recruitment and is critical for development of experimental Lyme arthritis and carditis. Infect Immun. 2010;78(11):4593-600. Epub 2010/09/09. doi: 10.1128/IAI.00798-10. PubMed PMID: 20823213; PMCID: PMC2976349.

84. Lu H, Huang D, Ransohoff RM, Zhou L. Acute skeletal muscle injury: CCL2 expression by both monocytes and injured muscle is required for repair. FASEB J. 2011;25(10):3344-55. Epub 2011/06/24. doi: 10.1096/fj.10-178939. PubMed PMID: 21697550; PMCID: PMC3177578.

85. Shireman PK, Contreras-Shannon V, Ochoa O, Karia BP, Michalek JE, McManus LM. MCP-1 deficiency causes altered inflammation with impaired skeletal muscle regeneration. J Leukoc Biol. 2007;81(3):775-85. Epub 2006/12/01. doi: 10.1189/jlb.0506356. PubMed PMID: 17135576.

86. Zhang X, Liu X, Shang H, Xu Y, Qian M. Monocyte chemoattractant protein-1 induces endothelial cell apoptosis in vitro through a p53-dependent mitochondrial pathway. Acta Biochim Biophys Sin (Shanghai). 2011;43(10):787-95. Epub 2011/08/24. doi: 10.1093/abbs/gmr072. PubMed PMID: 21859809.

87. Tieu BC, Lee C, Sun H, Lejeune W, Recinos A, 3rd, Ju X, Spratt H, Guo DC, Milewicz D, Tilton RG, Brasier AR. An adventitial IL-6/MCP1 amplification loop accelerates macrophage-mediated vascular inflammation leading to aortic dissection in mice. J Clin Invest. 2009;119(12):3637-51. Epub 2009/11/19. doi: 10.1172/JCI38308. PubMed PMID: 19920349; PMCID: PMC2786788.

88. Ziraldo C, Vodovotz Y, Namas RA, Almahmoud K, Tapias V, Mi Q, Barclay D, Jefferson BS, Chen G, Billiar TR, Zamora R. Central role for MCP-1/CCL2 in injury-induced inflammation revealed by in vitro, in silico, and clinical studies. PLoS ONE. 2013;8(12):e79804. Epub 2013/12/07. doi: 10.1371/journal.pone.0079804. PubMed PMID: 24312451; PMCID: PMC3849193.

89. Niemi JP, DeFrancesco-Lisowitz A, Cregg JM, Howarth M, Zigmond RE. Overexpression of the monocyte chemokine CCL2 in dorsal root ganglion neurons causes a conditioning-like increase in neurite outgrowth and does so via a STAT3 dependent mechanism. Exp Neurol. 2016;275 Pt 1:25-37. Epub 2015/10/04. doi: 10.1016/j.expneurol.2015.09.018. PubMed PMID: 26431741; PMCID: PMC4688065.

Figure Legends

Figure 2.1: The relationship between skeletal muscle injury, mitochondrial stress, and inflammation. Skeletal muscle stress triggers a stress response in the mitochondria that stimulates release of mitoDAMPs from the mitochondria. Release of mitoDAMPs have been associated with activation of the NLRP3 inflammasome, which is involved in IL-1β activation and a neutrophil-mediated inflammatory response. The mitoDAMP-initiated inflammatory response may also affect mitochondrial function, which could potentially create a positive feedback loop further exacerbating the mitochondrial stress response. This figure was generated using Smart Servier Medical Art (www.smart.servier.com).

Table 1 - Inflammatory Gene Response to Various Skeletal Muscle Injuries

Gene	Name	VML 3 day	VML 7 day	VML 14 day	ECC 6 hr	ECC 1 day	ECC 3 day	ECC 7 day	FRZ 6 hr	FRZ 1 day	FRZ 3 day	FRZ 7 day
Cxcl10	**chemokine (C-X-C motif) ligand 10**	62.4	17.8	12.7	2.5	2.0	2.0	1.9	1.2	1.0	1.5	1.0
Cxcr3	**chemokine (C-X-C motif) receptor 3**	43.8	20.1	16.1	2	2.0	1.9	2.0	1.0	0.9	0.9	1.0
Ccl2	**chemokine (C-C motif) ligand 2**	336.9	13.2	3.9	5.7	6.0	6.6	1.9	7.5	14.2	5.6	1.1
Ccl5	**chemokine (C-C motif) ligand 5**	32.4	30.8	34.7	2	2.0	2.0	2.0	1.0	1.0	1.2	1.2
Cxcl1	**chemokine (C-X-C motif) ligand 1**	144.9	10.9	2.3	3.6	2.1	2.0	2.0	4.6	1.8	1.0	1.0
Ifng	**interferon gamma**	10.3	6.2	15.1	2	2.0	1.9	1.9	1.0	0.9	0.9	1.0
IL-1a	**interleukin 1 alpha**	12.3	4.7	2.0	1.9	1.9	1.9	1.9	1.0	0.9	0.8	0.9
IL-1b	**interleukin 1 beta**	252.2	46.5	5.1	2.5	2.0	1.9	1.9	2.8	1.8	1.1	1.0
IL-4	**interleukin 4**	5.5	4.3	2.0	1.9	2.0	2.0	1.9	1.0	0.9	0.9	1.0
IL-6	**interleukin 6**	104.8	17.0	1.9	2.9	2.1	2.0	1.9	3.1	1.3	1.0	1.0
IL-10	**interleukin 10**	167.5	18.5	14.7	1.9	19.0	1.9	1.9	1.0	0.9	0.9	0.9
Tgfb1	**transforming growth factor, beta 1**	38.7	10.4	7.9	2.0	2.0	2.0	2.0	1.1	1.1	1.2	1.2
Tnf	**tumor necrosis factor**	95.2	11.2	6.9	1.9	1.9	1.9	1.9	1.0	0.9	0.8	0.9

Note: Inflammation gene array markers presented as fold change in gene expression vs. control following injury. Highlighted genes were selected for analysis. Warren et al. freeze injury and Greising et al. eccentric contraction injury data were collected at 6 hours, 1, 3, and 7-day time points post injury, while Call et al. VML injury data was collected at 3, 7, and 14-day time points. VML: Volumetric Muscle Loss injury, ECC: Eccentric Contraction Induced Injury, FRZ: Freeze Injury

Table 2 – *Effects of Inflammatory Cytokines on Mitochondria*

Cytokine	Direction	Reference
IL-6		
Mitophagy	↑	[71]
Biogenesis (PGC-1a)	↓	[71]
ROS	↑	[71]
Fusion (Mfn1 and Mfn2)	↓	[71,72]
Fission (FIS1)	↑	[71]
IL-1b		
Mitophagy	↓	[78]
ROS	↑	[79]
ETC dysfunction	↑	[78]
CCL2		
ETC dysfunction	↑	[86]

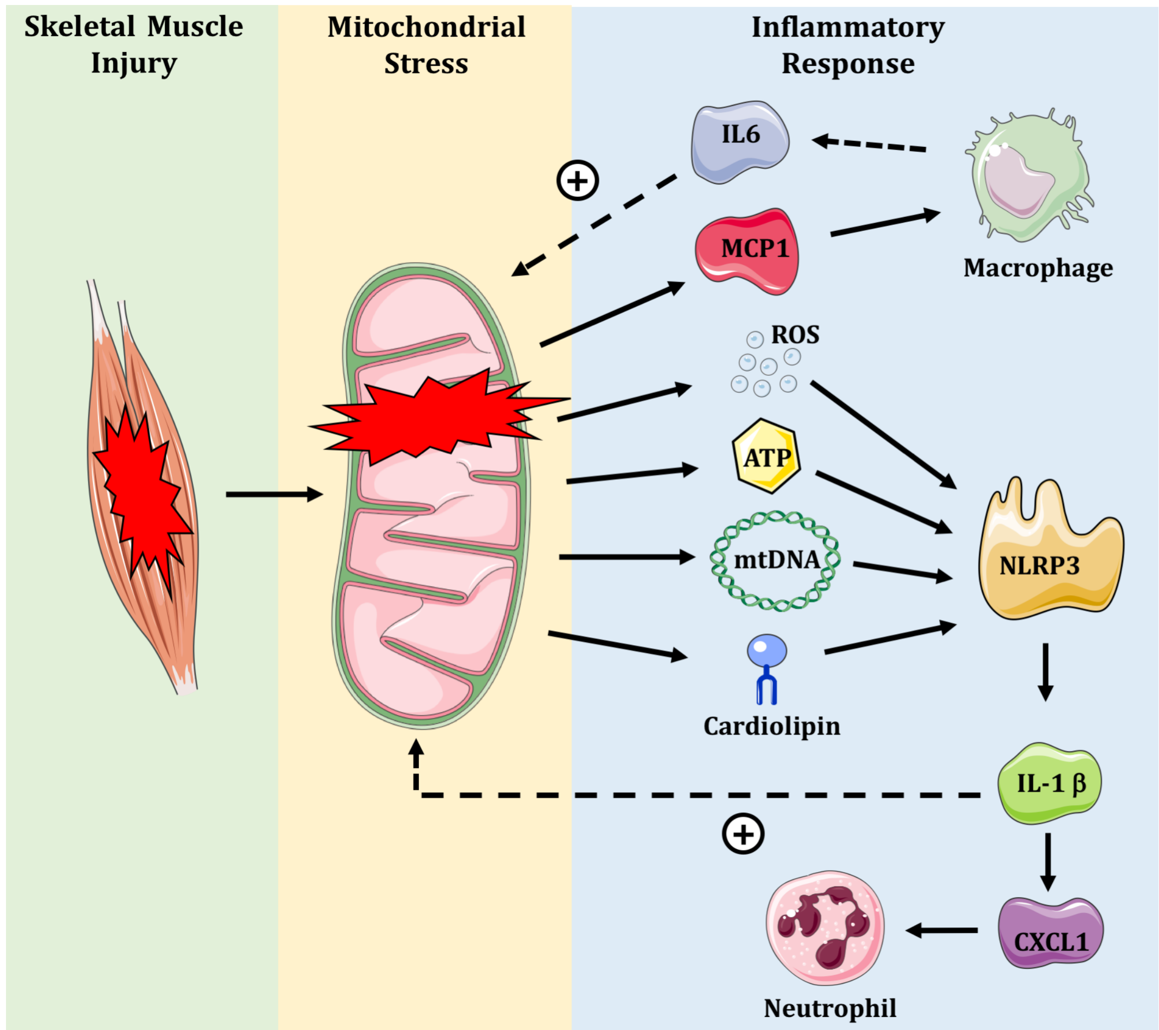

Figure 2.1

CHAPTER 3

PGC-1α OVEREXPRESSION CORRECTS IMPAIRED MITOCHONDRIAL DYSFUNCTION AND BIOGENESIS AND PARTIALLY RESCUES STRENGTH FOLLOWING VOLUMETRIC MUSCLE LOSS[1]

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Abstract

Volumetric muscle loss (VML) is characterized by a large volume of muscle tissue being removed from the body due to surgery or severe trauma. The remaining muscle after VML has poor function and unknown effects on oxidative capacity and adaptive potential during physical rehabilitation. We sought to investigate the oxidative capacity and plasticity of the remaining skeletal muscle after VML injury. We found that VML mitochondrial respiratory function at 3 and 7 days post injury was severely impaired (38% and 53% of control, $P<0.001$). In an attempt to correct these mitochondrial deficits, we subjected VML injured mice to voluntary wheel running (WR). After 4 weeks of WR, mitochondrial function was ~23% greater in the uninjured limb of VML+WR mice compared to VML without WR, but mitochondrial function in the injured limb of VML+WR mice was not different from VML alone, suggesting VML injury prevents oxidative adaptations to exercise ($P<0.001$). To determine if a faulty oxidative signaling cascade (i.e., PGC-1α) was responsible for impaired metabolic adaptation in the VML-injured muscle, direct muscle activation via sciatic nerve electrical stimulation was used to initiate oxidative gene transcription in control (CON) and VML mice. The stimulated muscle in CON mice had ~4-fold greater PGC-1α gene expression than the unstimulated muscle; however, there was no effect of stimulation on PGC-1α expression in VML mice (Interaction: $P<0.001$) suggesting VML injury attenuates oxidative gene regulation. PGC-1α (CMV promoter) transfection was performed to bypass endogenous PGC-1α activation pathways. Forced expression of PGC-1α resulted in ~33% and ~31% greater mitochondrial function in CON+ PGC-1α and VML+ PGC-1α mice, respectively, compared to VML alone

(P<0.001), and VML+ PGC-1α mice had 47% greater muscle strength than VML alone (P<0.001). In conclusion, PGC-1α activation is the limiting factor impairing oxidative plasticity in VML-injured muscle; and improving oxidative capacity of the remaining muscle after VML injury improves recovery of strength.

Introduction

Oxidative capacity is a cornerstone of skeletal muscle health, and for the past 40 years we have known that the most robust adaptation to regularly scheduled physical activity (i.e., exercise training) is an increase in oxidative capacity (1, 2). Improvements in muscle oxidative capacity are made possible with exercise training through adaptations affecting the density and function of the intramuscular mitochondrial network. The signaling pathways that initiate and coordinate mitochondrial improvements with exercise are very complex, but advancements in molecular biology in the last two decades have revealed many of the key players involved. Most notably, the transcription factor PGC-1a is considered a critical molecular modulator of skeletal muscle oxidative plasticity because it regulates gene expression patterns for mitochondrial, vascular, and motor-neuron associated adaptations with exercise training (3-5). Expansion of the vascular bed and mitochondrial network with exercise training enhances the functional capacity of the muscle (e.g., fatigue resistance), and in general this type of acclimation is considered beneficial for human performance and health (6, 7).

Large-scale skeletal muscle trauma, such as volumetric muscle loss (VML) injury, is unique in that: 1) the loss of muscle function (i.e., contractility) exceeds the loss of tissue mass, and 2) the muscle does not fully recovery strength (8). The permanent loss of muscle function leaves patients with lifelong disability (9), and there are currently no guidelines for physical rehabilitation to help correct the functional deficits. Furthermore, the extent to which the remaining skeletal muscle can adapt to rehabilitation is unclear. We recently employed a multi-muscle VML injury in the mouse model to investigate the contractile

plasticity of the remaining muscle with early rehabilitation intervention using electrical stimulation. We reported modest gains in muscle strength indicating contractile plasticity can be accomplished; however, we were most intrigued by a finding that suggested VML injury produced systemic and detrimental effects on muscle oxidative capacity. Specifically, the mitochondrial respiration rates of muscle fibers analyzed from the injured and uninjured muscles of VML-injured mice were less than the mitochondrial respiration rates of muscle fibers from completely injury naïve mice. Systemic reductions in skeletal muscle oxidative capacity are associated with an increased risk for a host of disorders such as diabetes (10) and cardiovascular disease (11-13) and therefore can have major ramifications on whole body health. The overall goal of this project was to determine the oxidative capacity and plasticity of the remaining muscle after VML injury. We hypothesized that VML injury would adversely affect oxidative capacity of the remaining muscle, but early intervention with physical rehabilitation would lead to corrective oxidative adaptations in the VML injured muscle.

Methods

Experimental Design

C57BL/6 mice were housed at 20-23°C on a 12:12-h light-dark cycle, with food and water provided ad libitum. For study 1, 9-week-old mice (n=6) were randomized to either unilateral VML+3 days) or unilateral VML+7 days. For study 2, 9-week-old mice (n=5-7) were randomized to unilateral VML alone (VML), unilateral VML + 4wks of wheel running (VML+WR), and uninjured control + 4wks of wheel running (CON+WR). WR mice were given access to wheels 72hrs after VML injury. For study 3, 9-week-old mice

(n=4-5) were randomized to unilateral VML + empty vector (i.e. saline) (VML), bilateral VML + PGC-1a plasmid (VML+PGC-1a), and control + PGC-1a plasmid (CON+PGC-1a). All outcome measures were also collected in in an uninjured, untreated control group (CON, n=7) and was used as a reference group across all three studies. For study 3, electroporation and PGC-1a plasmid transfection were conducted at the time of VML injury. To determine if WR affected muscle and mitochondrial function following VML injury, plantarflexor peak isometric strength and gastrocnemius (GAS) mitochondrial respiration were measured after 3 and 7 days (study 1), 4 weeks of WR (study 2), and after 4 weeks of PGC-1a overexpression (study 3) in mice with and without VML injury. Immediately after assessment of peak strength, the GAS muscle was harvested and prepared for mitochondrial respiration measurements and mitochondrial enzyme assays. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Georgia.

VML injury

VML injury was conducted on the posterior compartment of anesthetized (isoflurane 1.5 – 2.0%) mice as previously described (14). Briefly, 30 mins prior to surgery, all mice received administration of buprenorphine-SR (1.2 mg/kg; s.c.) for pain management. Blunt dissection was used to remove skin, fascia, and hamstrings to expose the gastrocnemius muscle. A small metal plate was inserted behind the gastrocnemius and soleus muscles and a 4mm biopsy punch was used to remove ~20% of muscle volume from the center of the gastrocnemius muscle. Following the VML, the incision was sutured closed (6-0 silk).

Wheel running

VML+WR and CON+WR mice were housed individually and given free access to a running wheel (Columbus Instruments, Columbus, Ohio), while sedentary VML mice were housed in a standard mouse cage without access to a running wheel. Daily running totals were calculated from wheel revolutions collected at 5 min intervals.

Active and Passive Torque and Fatigue

In vivo peak isometric torque of the ankle plantarflexors was assessed as previously described (14, 15). Briefly, anesthesia was induced using an induction chamber and 5% isoflurane in oxygen. Anesthesia was maintained using 1.5% isoflurane at an oxygen flow rate of 0.4L/min. The left hindlimb was depilated and aseptically prepared and the foot placed in a foot-plate attached to a servomotor (Model 300C-LR; Aurora Scientific, Aurora, Ontario, Canada). The left peroneal nerve was severed and platinum-iridium needle electrodes (Model E2-12; Grass Technologies, West Warwick, RI) were placed on either side of the sciatic nerve to elicit contraction of the plantarflexor muscles. Peak isometric torque was defined as the greatest torque measured during a 200-ms stimulation using 1-ms square-wave pulses at 300 Hz and increasing amperage 0.6 to 2.0 mA (models S48 and SIU5; Grass Technologies). To account for differences in body size among mice, torques (mN●m) was normalized by body mass (kg). Fatigability of the plantarflexors muscles was assessed as previously described (15). Briefly, 120 submaximal isometric contractions were performed in 2 min using 330 ms stimulations at 50 Hz.

Acute stim

To assess the integrity of mitochondrial biogenesis signaling in VML injured muscle, a 30 min in vivo electrical stimulation protocol was used to simulate an acute bout of exercise. The stimulation protocol was performed two weeks after VML injury on the left limb of 11-week-old uninjured control mice (n=6) and bilateral VML injured mice (n=6). Electrical stimulation was used instead of voluntary exercise to ensure activation of the injured gastrocnemius muscles. Prior to the start of the stimulation protocol, platinum-iridium needle electrodes were placed around the sciatic nerve of anesthetized (isoflurane 1.5 – 2.0%) mice and electrical current was optimized for peak torque generation. The electrical stimulation protocol consisted of 10 sets of 1800 contractions (parameters: pulse frequency =100, pulse width=0.1, pulses per train = 1, train frequency = 10hz) conducted over 30 mins. At the end of the protocol, gastrocnemius muscles were quickly harvested, flash frozen in liquid nitrogen, and stored at -80°C for later qRT-PCR analysis.

Mitochondrial Respiration Assay

Immediately following sacrifice, the medial and lateral gastrocnemius muscles from uninjured and injured limbs were dissected on a chilled aluminum block in 4°C buffer X containing 7.23mM K_2EGTA, 2.77mM Ca K_2EGTA, 20mM imidazole, 20mM taurine, 5.7mM ATP, 14.3mM PCr, 6.56mM $MgCl_2$-$6H_2O$, 50mM k-MES. Muscles were carefully dissected <1mg bundles of muscle fibers as reported by Kuznetsov et al. (16). Fiber bundles were permeabilized via an incubation (i.e. rocking) in buffer X and saponin (50 µg/ml) at 4°C for 30 mins. Following permeabilization, muscle fiber bundles were rinsed for 15 minutes in buffer Z (105mM k-MES, 30mM KCl, 10mM KH_2PO_4, 5mM $MgCl_2$, 0.5 mg/ml

BSA, 1mM EGTA) at 4°C. All respiration measurements were performed using a Clark-type electrode (Oxygraph Plus System, Hansatech Instruments, UK) at 25° C. Prior to each experiment, the electrode was calibrated according to the manufacturer's instructions and 1 ml of oxygen infused buffer Z was added to the chamber. Muscle fiber bundles were weighed (~2.5 mg for all samples) and added to the chamber. State 4 respiration (leak respiration in the absence of ADP) was initiated by the addition of glutamate (10mM) and malate (5mM). State 3 respiration (respiration coupled to ATP synthesis) was initiated by the addition of ADP (2.5mM) and succinate (10mM). Cytochrome *c* (10µM) was added to measure the integrity of the outer mitochondrial membrane. State 3 uncoupled respiration (respiration uncoupled from ATP synthesis) was initiated by the addition of FCCP (0.5µM). Mitochondrial respiration was terminated by the addition of cyanide (250 mM). Respiration rates were expressed relative to the mg of tissue loaded into each oxygraph chamber as well as to citrate synthase activity to account for differences in mitochondrial content between samples.

Mitochondrial Enzyme assays

Approximately 20mg of gastrocnemius muscle was homogenized in ~800ul of 33mM phosphate buffer (pH 7.0). Citrate synthase (CS), β-hydroxy acyl-CoA dehydrogenase (β-HAD), and succinate dehydrogenase activity (SDH) were assessed as previously described (15, 17). Enzyme activities were normalized to mg of tissue in the sample homogenate.

Plasmid and Transfection

The GFP-PGC1 plasmid expressing eGFP-tagged mouse PGC1a was acquired from Addgene (#4) (18). For in vivo eletroporation, GFP-PGC1 plasmid was prepared by cesium chloride density-gradient centrifugation and isopropanol precipitation as previous reported (19). In vivo eletroporation of mouse gastrocnemius muscles was performed as previously reported (20). Briefly, 20 μl of GFP-PGC1 plasmid (concentration = 2.8 μg/μL) was injected at 2 sites: medial and lateral GAS. Electroporation was conducted on an BTX ECM 830 electroporation system equipped with 5 mm 2-needle arrays. The following settings of the electronic pulses were use: LV = 500V/99msec, set voltage = 100 V, set pulse length = 50 msec, set number of pulses = 3 pulses. When 3 pulses were done, the 2-needle array was reversed, and 3 addition pulses were applied to the muscle with the above settings.

Gene Expression

cDNA generation and qRT-PCR was conducted as previously described (15). The following sequence-specific primer was used to assess mRNA levels for *PGC-1α*, (For: 5'-AGC CGT GAC CAC TGA CAA CGA G-3'; Rev: 5'-GCT GCA TGG TTC TGA GTG CTA AG-3'). NormFinder Software (Andersen et al. 2004, PMID:15289330) was used to identify the most stable reference gene between 18s, Hprt, and Hsp90. Hprt (For: 5'-TCAACGGGGGACATAAAAGT-3'; Rev: 5'-TGCATTGTTTTACCAGTGTCAA-3') was identified as the most stable gene in this VML model and therefore was the reference gene of choice for this analysis. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Micro-CT Angiography

A subset of 8-week old C57Bl/6 mice were randomly assigned to either VML alone or VML plus 4 weeks of wheel running therapy (VML + WR). Unilateral VML injury was performed on the posterior compartment of all mice. Four weeks after injury, micro-CT angiography was used to quantitatively evaluate hind limb vasculature as previously reported (21, 22). Briefly, after animal euthanasia, the vasculature was cleared with 0.9% saline, perfusion fixed with 10% neutral buffered formalin, rinsed again with saline, and injected with Microfil contrast agent (MV-122, Flow Tech Inc.). Samples were stored at 4 °C overnight to allow for polymerization of the contrast agent. Hind limbs were harvested and stored in PBS at 4 °C until imaging.

Samples were oriented with long axis of the tibia extending in the z-direction for micro-CT scanning (µCT50, Scanco Medical). Scans were performed on the lower leg with an applied electric potential of 55 kVp, a current of 145 µA, and an isometric voxel size of 20 µm. After automated reconstruction to 2D slice tomograms, contouring was performed on slices to mark a total muscle volume of analysis that excluded bones and only selected the musculature in the posterior compartment of the lower hindlimb. A global X-ray attenuation threshold was applied for segmentation of Microfil perfused vasculature, and a Gaussian low-pass filter was used for smoothing and noise suppression. This produced 3D images and volumetric quantifications (using direct distance transformation methods included in Scanco software) for vascular anatomy with the outcome measure of vascular volume normalized to total volume.

2-Photon Scanning Microscopy

Unilateral VML injury was performed on the left tibialis anterior (TA) muscle of 8-week old C57Bl/6 mice ubiquitously expressing mitochondrial Dendra2 green/red photoswitchable monomeric fluorescent protein (Jackson Laboratory). We elected to use the TA for the imaging rather than the GAS because of its accessibility and lack of pennation. Imaging was performed immediately, 3, 7, and 28 days after VML injury, and the contralateral limb was imaged at each timepoint as a control.

Prior to imaging, the TA was extracted, placed in Buffer X (see Methods: Mitochondrial Respiration), and secured to a dissection gel with pins. 2-photon microscopy was used to characterize the mitochondrial network of the muscle fibers remaining after VML. Our 2-photon microscope is a homebuilt system explained in (23). We used a Ti:Saphire laser, with 840nm and 940nm wavelength for excitation of the inherent Denra2 fluorescent protein of the mouse. For collection of fluorescent light, we used a 525/50nm filter. For each muscle, we imaged within an area of approximately 1.0mm^2 in the proximity of the injury site. The ROIs for the injured muscle were determined prior to imaging with the goal to image fibers directly adjacent to the injury site. Within each ROI, we collected multiple z-stacks ($n>10$), with resolution and pixel size small enough to satisfy the Nyquist sampling theorem, to characterize mitochondrial structure and organization with and without VML.

To analyze the mitochondrial network organization, we developed an angular Fourier filtering (AFF) method. Fourier transform is a method to describe spatial or temporal information through a complete set of sine and cosine waves. Transforming an

image from the spatial domain to the Fourier domain, converts it to an array of weighted coefficients that include information such as periodicity and angle of the features in the image. Our method uses a wedge filter ψ (figure x) with a gaussian profile (w.r.t. φ in cylindrical coordinates ρ,φ) to collect information about features at an angle α:

$$\Psi(\rho,\varphi,\alpha) = \exp\left(\frac{-(\varphi-\alpha)^2}{2\sigma^2}\right), \tag{1.1}$$

where σ is the standard deviation of gaussian filter (fig. 1 E). We then calculate the 2D Fourier transform of each image *i* at depth *z*:

$$I(\rho,\varphi,z) = \mathrm{F}\left\{i\left(\vec{r}\right)\right\}(z) \rightarrow r^2 = x^2 + y^2. \tag{1.2}$$

We rotate the filter from 0 to 180 degrees for each optical sectioned image at depth z to produce a histogram of the angle of alignment and periodicity of the structure. Each point in the generated 2D histogram AFF is calculated using:

$$AFF(z,\alpha) = \frac{\iint \left(\left|I(\rho,\varphi,z)\right| \times \Psi(\rho,\varphi,\alpha) \times M(\rho,\varphi)\right)^2 d\vec{\rho} d\varphi}{\iint \left(\left|I(\rho,\varphi,z)\right| \times M(\rho,\varphi)\right)^2 d\vec{\rho} d\varphi}, \tag{1.3}$$

where M is a mask determined by the numerical aperture of the microscope *NA* and the wavelength of the emitted light λ,

$$M(\rho,\varphi) = \begin{cases} 1 & \rho < (2NA/\lambda) \\ 0 & \textit{otherwise.} \end{cases} \tag{1.4}$$

An intact mitochondrial network has a very periodic structure, which makes AFF an ideal analysis, as it will detect 2 dominant angles of alignment in uninjured muscle: 1) mitochondrial network alignment that is parallel to muscle fiber orientation and 2) mitochondrial network alignment that is perpendicular to muscle fiber orientation. We applied AFF to each frame from a Z-stack to produce 3D mesh plots (Fig. 3.2 C). The magnitude of the peaks within the 3D mesh plots represents the strength of alignment of mitochondrial structures to a particular angle. To quantify the organization of mitochondrial structures we calculated the ratio of peak alignment strength of a given z-stack and its average alignment strength across the entire z-stack (Fig. 3.2 D), which we call alignment ratio (AR):

$$AR = \frac{\max\left(AFF\left(z,\alpha\right)\right)}{avg\left(AFF\left(z,\alpha\right)\right)} - 1. \qquad (1.5)$$

Statistical Analysis

Data are presented in the results as mean ± SD. A multi-factor repeated measures analysis of variance (ANOVA) was used to analyze assessments wheel running performance, where the between-subject factors were control and VML and the repeated measure factor was time. A multi-factor repeated measures ANOVA was also used to analyze data from the acute VML study, where the between-subject factors were day 3 and day 7 and the within-subject factors were injured and uninjured limbs. Gene expression data was analyzed using nonparametric tests in REST 2009 Software (M. Pfaffl, Technical University Munich, and QIAGEN). A two-way ANOVA was used to analyze torque in studies 2 and 3 as well as fatigue resistance after PGC-1α transfection, where the between-subject factors were group

(control and VML) and treatment (WR and no WR or PGC-1α and no PGC-1α). All other data were analyzed using ANOVA. All data were required to pass normality (Shapiro-Wilk) and equal variance tests (Brown-Forsythe *F* test) before proceeding with the ANOVA. Differences among groups are only reported where significant interactions were observed and subsequently tested with Tukey's *post hoc* test using JMP statistical software (SAS, Cary, NC). Group main effects are reported where significant interactions were not observed. An α level of 0.05 was used for all analyses.

Results

Study 1: Effect of VML on injured muscle function and mass acutely after injury

To determine the impact of VML on muscle function acutely after injury, we performed in vivo functional analysis of plantarflexor muscles at 3 and 7 days post VML. VML mice were 86% and 76% weaker than uninjured control mice at 3 and 7 days after injury, respectively. (Fig. 3.1A). GAS mass at day three was 101% of the uninjured limb despite removal of nearly 15% of the total muscle volume 3 days prior indicating the presence of severe edema in the injured limb. By day 7, however, the GAS mass was down to 84% of the uninjured limb, suggesting that the edema in the injured limb had subsided (Fig. 3.1C).

Study 1: Effect of VML on mitochondrial function

To determine the extent to which VML affects muscle oxidative capacity acutely after injury, we assessed mitochondrial function and content at 3 and 7 days post VML. Mitochondrial function was 62% and 47% lower than the uninjured limb at 3 and 7 days

post injury, respectively (Fig. 3.1 D). By day 7, mitochondrial function was 27% greater than on day 3 (Interaction: P=0.016, Fig. 3.1 D). CS and SDH (proxies for mitochondrial content) in VML injured limbs were 6% and 25% lower than the uninjured contralateral limbs, respectively (Main effect of injury, CS and SDH: P<0.001, Fig. 3.1 E,G). Normalizing mitochondrial function to mitochondrial content (i.e. CS activity) only accounted for ~5-10% of the total deficit in respiration, which is not surprising considering the relatively minor effect (6%) of VML on CS activity (P=0.008, Fig. 3.1F). To assess the hypothetical broad impact of the mitochondrial deficits on the whole body, we extrapolated the respiration rate for 1 mg of muscle tissue to the mass of the entire GAS. Similar to the other mitochondrial metrics, this measure of whole muscle oxygen consumption was 39% and 50% lower at 3 and 7 days post injury compared to contralateral controls (Fig. 3.1H). Together, these data suggest that VML injury has an adverse effects on muscle mitochondria.

Study 1: Effect of VML on mitochondrial organization

To assess whether mitochondrial dysfunction was associated with mitochondrial network disorganization after VML, we imaged the mitochondrial network around the injury site at immediately, 3, 7, and 28 days post injury. Mitochondrial network organization was 48% and 31% lower than control at 3 and 7 days post injury, respectively (P<0.001, Fig. 3.2C-D and Fig. 3.3). Immediately after injury, mitochondrial organization was only 23% lower than the control (P<0.001) suggesting that the further deterioration of the mitochondrial network at later time points (i.e. 3 and 7 days) was due to an indirect impact of VML on the remaining muscle such as inflammation. This notion is further

supported by the qualitative observation of what appeared to be mononuclear inflammatory cells at the injury site 3 and 7 days post injury (Fig. 3.2 A-B and Fig. 3.3). By day 28, the mitochondrial network organization was 40% higher than day 7, but not any higher than day 3 (Fig. 3.2D). Qualitatively assessment of 3D mitochondrial network reconstructions at day 28 shows more disorganization in the arrangement of muscle fibers than mitochondrial network, which may cause our analysis to underestimate the mitochondrial improvements (Fig. 3.3). To assess the extent of the impact of VML injury on mitochondrial disorganization across the entire muscle, we imaged 7 days post injury at various distances away from the injury site. Our qualitative results suggest that the greater the distance from the injury site, the more organized the mitochondrial network (Fig. 3.4). That being said, even at 2mm away from the injury site, there was still qualitative evidence of an inflammatory cell presence, suggesting that the impact of VML extends well beyond the bounds of the injury. Together, this data indicates that the mitochondrial network is severely disrupted following VML injury, and that this disruption is potentially mediated by a widespread inflammatory response.

Study 2: Effect of voluntary wheel running on muscle mass and function

To determine the therapeutic impact of WR on muscle function after VML, plantarflexor contractility was assessed prior to sacrifice. VML mice were substantially weaker (~62%) than CON and CON+WR mice, but after 4 weeks of WR therapy, VML+WR mice were moderately stronger (38% greater) than VML mice, suggesting that WR therapy was beneficial for muscle function (Interaction: P=0.017, Fig. 3.5A). However, even though the remaining muscle in VML+WR mice was stronger after 4 weeks

of WR, it was still ~44% weaker than uninjured control mice (Fig. 3.5A). Four weeks after the injury, the GAS mass in VML mice was 20% lower than the uninjured contralateral control, indicating that the ~20mg muscle defect removed during the injury had not been replaced (Fig. 3.5C). Four weeks of wheel running had no effect on GAS mass, as the GAS mass of VML+WR mice was still ~20% lower the uninjured contralateral limbs (Fig. 3.5C). To account for the smaller muscle mass, we normalized the torque by the plantarflexor mass and found that the strength deficits in VML and VML+WR mice were still present (VML: 54% lower than control, VML+WR: 32% lower than control, Interaction: P=0.045, Fig 3.5B). VML injury and wheel running therapy had no effect on body mass (P=0.126). Daily wheel running distance was recorded to determine if VML mice would run to the same extent as uninjured controls. Despite being injured, VML mice did not run less distance than control mice (P=0.541, Fig. 3.5D). Together, these data suggest that WR can partially restore muscle function after VML, despite the GAS muscle mass not recovering following injury.

Study 2: Effect of voluntary wheel running on VML injured muscle mitochondria

To determine whether VML injury impacts the metabolic plasticity of the remaining muscle, we assessed mitochondrial respiratory function in permeabilized fibers as well as various mitochondrial specific enzymes 4 weeks after VML and VML+WR. Significant ANOVAs were observed for mitochondrial function (P<0.001, Fig. 3.5E), CS activity (P<0.001, Fig. 3.5F), and SDH activity (P<0.001, Fig. 3.5H). Compared to CON, mitochondrial function, CS activity, and SDH activity were not impaired in VML injured muscle 4 weeks after injury suggesting that VML injury does not cause chronic metabolic

dysfunction in the remaining muscle. Mitochondrial function and SDH activity were greater in CON+WR mice compared to CON, VML injured, and VML uninjured mice, confirming that 4 weeks of WR is sufficient to produce oxidative adaptations ($P<0.05$, Fig. 3.5E, H). Interestingly, the uninjured limb and not the injured limb of VML+WR mice had 26% greater mitochondrial function than the injured limb of VML mice suggesting that the injured limb did not undergo mitochondrial adaptations to WR (Fig. 3.5E). In contrast, CS activity was actually greater only in the injured limb of VML+WR mice compared to CON+WR mice and the injured and uninjured limbs of VML mice (Fig. 3.5F). Together, these data suggest that mitochondrial content is elevated in the VML injured limb after WR, but that the respiratory function of those mitochondria is impaired. To determine if WR induced changes in mitochondrial function were the result of changes in mitochondrial content, we normalized mitochondrial function to CS activity (Fig. 3.5G). Normalization of mitochondrial function further supported mitochondrial dysfunction in the injured limb of VML+WR mice as mitochondrial function was 41% lower than CON+WR and 42% lower than the uninjured limb of VML mice. All other differences between groups in mitochondrial function were eliminated after normalization, indicating that mitochondrial WR adaptations in VML+WR and uninjured limb of VML+WR mice were due to changes in mitochondrial content Fig. 3.5G). Calculation of whole muscle oxygen consumption reflected a lack of mitochondrial adaptations to WR in the VML injured limb. Interestingly, this measure also revealed the potential for an oxidative deficiency in the VML injured limb compared to control, which is not surprising considering that the mitochondrial deficit is being compounded by the reduction in muscle mass ($P<0.001$, Fig. 3.5I).

Study 2: Effect of VML on muscle vasculature

Poor vascular perfusion is a potential cause of impaired oxidative plasticity, so we imaged injured and uninjured vasculature in the posterior compartment of sedentary and WR trained mice. Surprisingly, we found a main effect for VML injured limbs having higher vessel volume compared to uninjured control limbs independent of WR (P=0.007) (Fig. 3.6A,B). This data suggests that limitations in vascular perfusion are probably not contributing to the impaired oxidative plasticity after VML.

Study 2: Effect of VML on muscle mitochondrial biogenesis capacity

Impaired mitochondrial biogenesis is a potential explanation for the lack of mitochondrial adaptations in the injured limb of VML+WR mice. To explore whether mitochondrial biogenesis was impaired in VML injured muscle, we subjected uninjured control mice and VML injured mice to a unilateral 30-minute electrical stimulation protocol to stimulate mitochondrial biogenesis after VML injury. Initial torque production was 57% lower in VML compared to control mice, but all mice began to fatigue shortly after initiation of the stimulation protocol. After several minutes, both VML and control mice plateaued at substantially lower but similar torque values. Despite the VML mice being initially weaker (P<0.001, Fig. 3.7A,B), the torque x time integral calculated over the course of the stimulation protocol was not different between VML and control mice (P=0.733, 3.7C), suggesting that both groups of mice received a similar exercise stimulus. To assess mitochondrial biogenesis capacity, PGC-1α gene expression, a well-established marker of mitochondrial biogenesis, was measured in both the stimulated and unstimulated limbs of control and VML mice. There was a significant interaction between group and limb for PGC-1α expression (P<0.001, Fig 3.7D). The stimulated limb of control mice had

4-fold greater PGC-1α expression compared to the unstimulated limb, indicating a robust stimulus for mitochondrial biogenesis following the acute bout of exercise. However, PGC-1α expression in the stimulated limb of the VML mice was not significantly different from the unstimulated limb, suggesting that VML injury impairs oxidative gene expression (Fig. 3.7D). Together, these data suggest that VML injured muscle has impaired mitochondrial biogenesis, which is potentially leading to poor mitochondrial adaptations in the injured muscle of VML+WR mice.

Study: Effect of PGC-1α overexpression on VML injured muscle mitochondria

Given the apparent impaired mitochondrial biogenesis in VML injured muscle, we next wanted to test whether it was possible to improve the oxidative capacity of the remaining muscle. To accomplish this, we bypassed endogenous PGC-1α activation pathways via forced expression of PGC-1α. Four weeks after PGC-1α transfection, CON+PGC-1α mice and VML+ PGC-1α mice had 26% and 33% greater mitochondrial respiratory function compared to non-PGC-1α transfected mice ($P<0.001$, Fig. 3.8A,B). Similarly, SDH activity was 69% and 48% elevated in CON+ PGC-1α and VML+ PGC-1α mice, respectively, compared to the injured limb of VML mice ($P<0.001$, Fig. 3.8C). CS activity was also greater in VML+ PGC-1α compared to the injured limb of VML mice ($P=0.006$, Fig. 3.8D). CS activity had relatively small differences between groups, and as a result, when mitochondrial function was normalized to CS, many of the same group differences were present (Fig 3.8E). A two-way ANOVA yielded a main effect of group (VML and Control) ($P=0.014$) and treatment (PGC-1α transfection) ($P=0.014$) for muscle fatigue resistance (Fig. 3.8F). A main effect of group was found for muscle fatigue

resistance where CON mice were 43% higher than VML mice (P=0.014). Additionally, a main effect of treatment revealed PGC-1α overexpression mice had 42% greater fatigue resistance than untreated mice (P=0.014). Our calculated measure of whole muscle oxygen consumption again revealed a potentially large deficit (45% of control) in oxidative capacity of VML injured muscle (Fig. 3.8G). PGC-1α overexpression rescued some muscle oxidative capacity, but because the deficit is compounded by a lower GAS mass, the deficit could not be completely restored. Together these data demonstrate that PGC-1α overexpression was sufficient to overcome the impaired mitochondrial biogenesis in VML injured muscle and improve the oxidative capacity.

Effect of PGC-1α overexpression on muscle function after VML injury

To determine if enhancing oxidative capacity of the remaining muscle via PGC-1α overexpression would improve functional recovery after VML injury, we assessed GAS mass and plantarflexor contractility 4 weeks after VML injury and PGC-1α transfection. Plantarflexor strength in VML mice was on average ~65% lower than CON and CON+PGC-1α mice (P<0.001, Fig 3.8H). VML+ PGC-1α mice were 38% stronger than VML mice and only ~45% weaker than CON+PGC-1α mice, suggesting that PGC-1α overexpression partially rescued muscle function in VML injured muscle (P=0.007). GAS mass was 61% lower in the injured limb of VML mice compared to the uninjured limb of VML mice (P<0.001, Fig. 3.8J). PGC-1α overexpression did not alter this deficit, as the GAS mass was still 40% lower in VML+ PGC-1α mice compared to the uninjured limb of VML mice (P<0.001). After accounting for the smaller muscle mass in VML mice, VML+PGC-1α mice were 27% stronger than VML mice, only slightly weaker (13%) than CON+PGC-1α mice, and not different from CON mice, suggesting that PGC-1α

overexpression rescued VML injured muscle function (Interaction: $P<0.035$), Fig. 3.8I). Overall, these data demonstrate that PGC-1α overexpression in VML injured muscle is associated with enhanced recovery of muscle function.

Discussion

A core principle of physical rehabilitation is the assumption that the body tissues, specifically the skeletal muscle, are plastic and capable of being remodeled in response to an intervention. Without the ability to remodel and adapt, physical rehabilitation interventions are pointless endeavors. VML is an obvious candidate for rehabilitative therapy, but the plasticity of the remaining muscle is not well characterized, and as a result, there are no post-VML standard of care guidelines. We and others have previously demonstrated that the remaining muscle is plastic with regards to contractile function following early interventions (14, 24), but the oxidative plasticity of the remaining muscle has yet to be fully explored. More research is necessary to find the optimal type (endurance vs. resistance vs. combination) and timing (early vs. late) of rehabilitation therapy for individuals with VML. With this study, we sought to determine the plasticity of the remaining muscle after VML, specifically pertaining to the oxidative plasticity with the hope that we could inform future guidelines of post-VML rehabilitation.

VML Impairs Oxidative Plasticity

Oxidative plasticity is necessary for rehabilitation induced remodeling of muscle oxidative capacity, and in this study, we found that VML potentially impairs the oxidative plasticity of the remaining muscle. We initially hypothesized that inadequate vascular

perfusion was limiting oxidative adaptations because delivery of oxygen and fuel to the muscle is critical to oxidative function. Contrary to our hypothesis, we found increased vessel density around the injury site suggesting that vascular perfusion was likely not a contributor. We next hypothesized that VML could alter signaling pathways associated with mitochondrial biogenesis. Exercise-induced mitochondrial biogenesis is activated through several signals including increased intramuscular Ca^{2+} levels, activation of the cellular energy sensor AMPK, and contraction induced ROS production (25). All of these signals work together to increase PGC-1α gene expression (26), which we found to be blunted after exercise in VML injured muscle. This data suggests that VML alters the mitochondrial biogenesis signaling pathway. However, after overexpression of PGC-1α, the oxidative capacity of the muscle was found to be greater in VML injured muscle, suggesting that the mitochondrial biogenesis signaling pathway was not compromised.

If VML injury does not blunt oxidative adaptations through limited vascular perfusion or altered mitochondrial biogenesis signaling, then perhaps altered muscle activation after VML plays an important role. Indeed, Beltran et al. reported that ~20% of VML patients suffer motor neuron injuries (27). Motor unit recruitment is necessary for muscle contraction, breakdown of ATP, and by extension, mitochondrial activation and adaptation. Thus, reduced motor unit recruitment caused by motor neuron axotomy from VML could potentially explain the lack of oxidative adaptations to WR therapy as well as blunted exercise-induced PGC-1α gene expression. Further research is needed to fully elucidate the reason for blunted oxidative adaptations in VML injured muscle, but no

matter the mechanism, impaired oxidative plasticity is a critical problem for whole muscle function.

In order to appreciate the broad implications of the mitochondrial respiration deficits after VML, we assumed the mitochondrial deficit was muscle wide and calculated the hypothetical oxygen consumption rate of the entire muscle. The oxidative deficit in the injured muscle was compounded due to the smaller muscle mass after injury, revealing that whole-body oxidative capacity could potentially be greatly reduced in patients with VML. Given that reduced oxidative capacity has been implicated in numerous diseases such as aging (28), cardiovascular disease (29), and diabetes (30), it is critical to correct oxidative deficits in VML injured muscle to prevent long term health complications in patients with VML. With this in mind, we overexpressed PGC-1α in to determine if forcing an increase in oxidative capacity was possible and if so, what effects would it have on muscle function after VML. Intriguingly, we found that 4 weeks of PGC-1α overexpression in VML injured muscle almost completely rescued muscle strength normalized to muscle mass (Fig. 3.8I). This data indicates that PGC-1α plays an important role in recovery of muscle function after VML, but it remains to be seen exactly how PGC-1α overexpression leads to greater strength.

There are several possible ways that PGC-1α overexpression could strengthen VML injured muscle. First, mitochondria have been shown to aid in muscle fiber sarcolemmal repair after injury (31, 32), meaning that myofibers with more mitochondria (i.e. PGC-1α overexpression) may be more resistant to cell death after injury. Second, PGC-1α overexpression has been shown to enhance pre- and post- synaptic NMJ

morphology and function, which could partially compensate for motor neuron damage caused by VML (3). Third, PGC-1α overexpression has been shown to prevent muscle atrophy, which is a common side effect of VML, by inhibiting FoxO3 signaling (33). Overall, our data shows that PGC-1α overexpression and the resulting increases in oxidative capacity are associated with greater muscle strength after VML injury. However, the exact mechanism by which PGC-1α overexpression causes greater muscle strength remains unclear and should be a focus of future research.

Mitochondrial Role in VML Pathophysiology

One of the reasons that rehabilitation guidelines do not exist for patients with VML is that the pathophysiology of VML is poorly understood. In this study, we investigated mitochondrial function, which up to this point, was an uncharacterized area of VML pathophysiology. We found that VML severely impacts mitochondrial organization and function acutely after injury, which is not surprising considering the trauma inflicted on the muscle fibers immediate to the injury area. Disrupted intracellular Ca^{2+} homeostasis is the potential mechanism for the mitochondrial impairments following VML, as Ca^{2+}-induced mitochondrial dysfunction has been well documented after other severe skeletal muscle injuries (34-36). After mechanical muscle damage, such as VML where myofibers are completely severed, sarcolemmal integrity is compromised resulting in Ca^{2+} flooding into the cell, which the mitochondria try to correct by absorbing some of the Ca^{2+}. Excessive mitochondrial Ca^{2+} absorption leads to declines in mitochondrial respiration (37), opening of the mitochondrial permeability transition pore (38), and release of proinflammatory (39) and proapoptotic factors (40). Overall, the mitochondrial

impairments that we are reporting are potentially caused by Ca^{2+} overload within the muscle cell, which likely contributes to the extensive cell death of severed myofibers after VML.

Normally, inflammation after muscle injuries is important for initiating recovery of muscle function, but inflammation after VML may actually exacerbate the injury (41, 42). Our 2-photon scanning microscopy experiments provide semi-quantitative evidence that suggests the effects of VML injury on mitochondrial organization extend well beyond the borders of the injury (Fig 3.3 and 3.4). This widespread effect of VML injury could be due to the excessive inflammatory response that occurs after the injury. Generally, following a muscle injury, the inflammatory response resolves within 3-7 days (43, 44), however, in VML, inflammation is prolonged, potentially lasting for several weeks (41, 42). Chronic inflammation of this nature has been linked to mitochondrial dysfunction (45) and impaired muscle regeneration (46, 47), supporting the notion that the inflammatory response after VML may injure myofibers that otherwise would be unaffected by the injury.

Technical Considerations

Several technical details should be considered when interpreting our results, specifically pertaining to the mitochondrial function assay. Our 2-photon data indicates that 7 days after injury mitochondrial disorganization is more severe than day 7 mitochondrial respiratory dysfunction, where day 7 seems to be recovering after injury. A possible explanation for this discrepancy is a selection bias at day 7 where we can only select higher functioning fibers due to the complete disintegration of other fibers closer (i.e. the fiber pool probably used for selection on day 3) to the injury. Thus, we may be

underestimating the mitochondrial respiratory dysfunction at day 7 and possibly day 28 post injury. We could also be underestimating the impact of VML on mitochondrial content, as the CS assay was performed on a muscle homogenate that comprised of the entire GAS and not the muscle fiber bundles used for the mitochondrial respiration assay. As a result, CS values may reflect mitochondrial contributions from inflammatory cells as well as muscle. This information is especially relevant to interpretation of the mitochondrial respiration rates normalized to CS. Overall, our results indicate that VML causes mitochondrial dysfunction and loss of content, but because of inhomogeneities in the injury level across the muscle, these measures may be underestimating the true impact of VML on the remaining muscle. Finally, we would like to note that our mitochondrial organization analysis cannot account for disorganization among of muscle fibers. Immediately after the injury as well as 28 days post injury, muscle fibers were not as organized as the control, while the mitochondrial network within the fibers was seemingly intact and well organized. This effect will likely cause our analysis to underestimate mitochondrial network organization.

Considerations for Future VML Treatments

Current research on treatments for VML focus primarily on two areas: 1) designing structured physical therapy programs that strengthen the remaining muscle (48) and 2) designing physical constructs fill the void and facilitate regrowth of muscle in the wound area (41, 48-52). This study has uncovered an important aspect of VML pathophysiology, namely mitochondrial dysfunction, that we believe can provide several novel contributions to the both areas of VML treatment research. 1) Our PGC-1α overexpression experiments

describe a potential important role for mitochondria in the recovery of muscle strength after VML. Thus, we propose that rehabilitation programs should combine resistance exercise with endurance exercise to potentially benefit from synergistic adaptations of muscle strength. 2) In this study, for the first time, we have identified mitochondrial network disorganization and dysfunction as muscular complications caused by VML injury. These complications, in addition to other factors like limited vascular perfusion, motor neuron axotomy, and excessive inflammation, will likely create a hostile environment that should be accounted for during the development and implementation of physical constructs for treatment of VML. In conclusion, we believe that our study can provide valuable insight for development and optimization of future VML treatment strategies.

Conclusion

Collectively, our results indicate that VML injury causes mitochondrial dysfunction and diminished oxidative plasticity in the remaining muscle, which is not good news for patients with VML. However, the good news is that forcibly increasing oxidative capacity in the remaining muscle is associated with moderate improvements in muscle strength. This finding provides rationale for designing rehabilitation programs that combine both resistance and endurance exercises with the hope of maximizing the functional recovery of the remaining muscle.

References

1. Gollnick PD, Armstrong RB, Saltin B, Saubert CWt, Sembrowich WL, Shepherd RE. Effect of training on enzyme activity and fiber composition of human skeletal muscle. J Appl Physiol. 1973;34(1):107-11. Epub 1973/01/01. doi: 10.1152/jappl.1973.34.1.107. PubMed PMID: 4348914.

2. Ingjer F. Effects of endurance training on muscle fibre ATP-ase activity, capillary supply and mitochondrial content in man. J Physiol. 1979;294:419-32. Epub 1979/09/01. PubMed PMID: 159945; PMCID: PMC1280565.

3. Arnold AS, Gill J, Christe M, Ruiz R, McGuirk S, St-Pierre J, Tabares L, Handschin C. Morphological and functional remodelling of the neuromuscular junction by skeletal muscle PGC-1alpha. Nat Commun. 2014;5:3569. Epub 2014/04/02. doi: 10.1038/ncomms4569. PubMed PMID: 24686533; PMCID: PMC4846352.

4. Fernandez-Marcos PJ, Auwerx J. Regulation of PGC-1alpha, a nodal regulator of mitochondrial biogenesis. Am J Clin Nutr. 2011;93(4):884S-90. Epub 2011/02/04. doi: 10.3945/ajcn.110.001917. PubMed PMID: 21289221; PMCID: PMC3057551.

5. Rowe GC, Raghuram S, Jang C, Nagy JA, Patten IS, Goyal A, Chan MC, Liu LX, Jiang A, Spokes KC, Beeler D, Dvorak H, Aird WC, Arany Z. PGC-1alpha induces SPP1 to activate macrophages and orchestrate functional angiogenesis in skeletal muscle. Circ Res. 2014;115(5):504-17. Epub 2014/07/11. doi: 10.1161/CIRCRESAHA.115.303829. PubMed PMID: 25009290; PMCID: PMC4524357.

6. Egan B, Zierath JR. Exercise metabolism and the molecular regulation of skeletal muscle adaptation. Cell Metab. 2013;17(2):162-84. Epub 2013/02/12. doi: 10.1016/j.cmet.2012.12.012. PubMed PMID: 23395166.

7. Haskell WL, Lee IM, Pate RR, Powell KE, Blair SN, Franklin BA, Macera CA, Heath GW, Thompson PD, Bauman A. Physical activity and public health: updated recommendation for adults from the American College of Sports Medicine and the American Heart Association. Med Sci Sports Exerc. 2007;39(8):1423-34. Epub 2007/09/01. doi: 10.1249/mss.0b013e3180616b27. PubMed PMID: 17762377.

8. Corona BT, Wenke JC, Ward CL. Pathophysiology of Volumetric Muscle Loss Injury. Cells Tissues Organs. 2016;202(3-4):180-8. Epub 2016/11/09. doi: 10.1159/000443925. PubMed PMID: 27825160.

9. Corona BT, Rivera JC, Owens JG, Wenke JC, Rathbone CR. Volumetric muscle loss leads to permanent disability following extremity trauma. J Rehabil Res Dev. 2015;52(7):785-92. Epub 2016/01/09. doi: 10.1682/JRRD.2014.07.0165. PubMed PMID: 26745661.

10. Lowell BB, Shulman GI. Mitochondrial dysfunction and type 2 diabetes. Science. 2005;307(5708):384-7. Epub 2005/01/22. doi: 10.1126/science.1104343. PubMed PMID: 15662004.

11. Ballinger SW. Mitochondrial dysfunction in cardiovascular disease. Free Radic Biol Med. 2005;38(10):1278-95. Epub 2005/04/28. doi: 10.1016/j.freeradbiomed.2005.02.014. PubMed PMID: 15855047.

12. Chistiakov DA, Shkurat TP, Melnichenko AA, Grechko AV, Orekhov AN. The role of mitochondrial dysfunction in cardiovascular disease: a brief review. Ann Med. 2018;50(2):121-7. Epub 2017/12/15. doi: 10.1080/07853890.2017.1417631. PubMed PMID: 29237304.

13. Madamanchi NR, Runge MS. Mitochondrial dysfunction in atherosclerosis. Circ Res. 2007;100(4):460-73. Epub 2007/03/03. doi: 10.1161/01.RES.0000258450.44413.96. PubMed PMID: 17332437.

14. Greising SM, Warren, G. L., Southern, W. M., Nichenko, A. S., Qualls, A. E., Corona, B. E., Call, J. , A. Early rehabilitation for volumetric muscle loss injury augments endogenous regenerative aspects of muscle strength and oxidative capacity. BMC Musculoskeletal Disorders. 2018.

15. Southern WM, Nichenko AS, Shill DD, Spencer CC, Jenkins NT, McCully KK, Call JA. Skeletal muscle metabolic adaptations to endurance exercise training are attainable in mice with simvastatin treatment. PLoS ONE. 2017;12(2):e0172551. Epub 2017/02/17. doi: 10.1371/journal.pone.0172551. PubMed PMID: 28207880; PMCID: PMC5313210.

16. Kuznetsov AV, Veksler V, Gellerich FN, Saks V, Margreiter R, Kunz WS. Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. Nat Protoc. 2008;3(6):965-76. Epub 2008/06/10. doi: 10.1038/nprot.2008.61. PubMed PMID: 18536644.

17. Nichenko AS, Southern WM, Atuan M, Luan J, Peissig KB, Foltz SJ, Beedle AM, Warren GL, Call JA. Mitochondrial maintenance via autophagy contributes to functional skeletal muscle regeneration and remodeling. Am J Physiol Cell Physiol. 2016;311(2):C190-200. Epub 2016/06/10. doi: 10.1152/ajpcell.00066.2016. PubMed PMID: 27281480.

18. Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell. 1998;92(6):829-39. Epub 1998/04/07. PubMed PMID: 9529258.

19. Little RV, Brubaker RR. Characterization of deoxyribonucleic acid from Yersinia pestis by ethidium bromide-cesium chloride density gradient centrifugation. Infect Immun. 1972;5(4):630-1. Epub 1972/04/01. PubMed PMID: 4636787; PMCID: PMC422416.

20. Aihara H, Miyazaki J. Gene transfer into muscle by electroporation in vivo. Nat Biotechnol. 1998;16(9):867-70. Epub 1998/09/22. doi: 10.1038/nbt0998-867. PubMed PMID: 9743122.

21. Boerckel JD, Uhrig BA, Willett NJ, Huebsch N, Guldberg RE. Mechanical regulation of vascular growth and tissue regeneration in vivo. Proc Natl Acad Sci U S A. 2011;108(37):E674-80. Epub 2011/08/31. doi: 10.1073/pnas.1107019108. PubMed PMID: 21876139; PMCID: PMC3174614.

22. Duvall CL, Taylor WR, Weiss D, Guldberg RE. Quantitative microcomputed tomography analysis of collateral vessel development after ischemic injury. Am J Physiol Heart Circ Physiol. 2004;287(1):H302-10. Epub 2004/03/16. doi: 10.1152/ajpheart.00928.2003. PubMed PMID: 15016633.

23. Tehrani KF, Pendleton EG, Southern WM, Call JA, Mortensen LJ. Two-photon deep-tissue spatially resolved mitochondrial imaging using membrane potential fluorescence fluctuations. Biomedical Optics Express. 2018;9(1):254-9. doi: 10.1364/BOE.9.000254.

24. Aurora A, Garg K, Corona BT, Walters TJ. Physical rehabilitation improves muscle function following volumetric muscle loss injury. BMC Sports Sci Med Rehabil.

2014;6(1):41. Epub 2015/01/20. doi: 10.1186/2052-1847-6-41. PubMed PMID: 25598983; PMCID: PMC4297368.

25. Ljubicic V, Joseph AM, Saleem A, Uguccioni G, Collu-Marchese M, Lai RY, Nguyen LM, Hood DA. Transcriptional and post-transcriptional regulation of mitochondrial biogenesis in skeletal muscle: effects of exercise and aging. Biochim Biophys Acta. 2010;1800(3):223-34. Epub 2009/08/18. doi: 10.1016/j.bbagen.2009.07.031. PubMed PMID: 19682549.

26. Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O, Michael LF, Puigserver P, Isotani E, Olson EN, Lowell BB, Bassel-Duby R, Spiegelman BM. Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. Nature. 2002;418(6899):797-801. Epub 2002/08/16. doi: 10.1038/nature00904. PubMed PMID: 12181572.

27. Beltran MJ, Burns TC, Eckel TT, Potter BK, Wenke JC, Hsu JR, Skeletal Trauma Research C. Fate of combat nerve injury. J Orthop Trauma. 2012;26(11):e198-203. Epub 2012/03/23. doi: 10.1097/BOT.0b013e31823f000e. PubMed PMID: 22437422.

28. Short KR, Bigelow ML, Kahl J, Singh R, Coenen-Schimke J, Raghavakaimal S, Nair KS. Decline in skeletal muscle mitochondrial function with aging in humans. Proc Natl Acad Sci U S A. 2005;102(15):5618-23. Epub 2005/04/01. doi: 10.1073/pnas.0501559102. PubMed PMID: 15800038; PMCID: PMC556267.

29. Marzetti E, Csiszar A, Dutta D, Balagopal G, Calvani R, Leeuwenburgh C. Role of mitochondrial dysfunction and altered autophagy in cardiovascular aging and disease: from mechanisms to therapeutics. Am J Physiol Heart Circ Physiol. 2013;305(4):H459-76. Epub

2013/06/12. doi: 10.1152/ajpheart.00936.2012. PubMed PMID: 23748424; PMCID: PMC3891249.

30. Goodpaster BH. Mitochondrial deficiency is associated with insulin resistance. Diabetes. 2013;62(4):1032-5. Epub 2013/03/23. doi: 10.2337/db12-1612. PubMed PMID: 23520282; PMCID: PMC3609595.

31. Horn A, Van der Meulen JH, Defour A, Hogarth M, Sreetama SC, Reed A, Scheffer L, Chandel NS, Jaiswal JK. Mitochondrial redox signaling enables repair of injured skeletal muscle cells. Sci Signal. 2017;10(495). Epub 2017/09/07. doi: 10.1126/scisignal.aaj1978. PubMed PMID: 28874604.

32. Vila MC, Rayavarapu S, Hogarth MW, Van der Meulen JH, Horn A, Defour A, Takeda S, Brown KJ, Hathout Y, Nagaraju K, Jaiswal JK. Mitochondria mediate cell membrane repair and contribute to Duchenne muscular dystrophy. Cell Death Differ. 2017;24(2):330-42. Epub 2016/11/12. doi: 10.1038/cdd.2016.127. PubMed PMID: 27834955; PMCID: PMC5299714.

33. Sandri M, Lin J, Handschin C, Yang W, Arany ZP, Lecker SH, Goldberg AL, Spiegelman BM. PGC-1alpha protects skeletal muscle from atrophy by suppressing FoxO3 action and atrophy-specific gene transcription. Proc Natl Acad Sci U S A. 2006;103(44):16260-5. Epub 2006/10/21. doi: 10.1073/pnas.0607795103. PubMed PMID: 17053067; PMCID: PMC1637570.

34. Brandao ML, Roselino JE, Piccinato CE, Cherri J. Mitochondrial alterations in skeletal muscle submitted to total ischemia. J Surg Res. 2003;110(1):235-40. Epub 2003/04/17. PubMed PMID: 12697407.

35. Porter C, Herndon DN, Bhattarai N, Ogunbileje JO, Szczesny B, Szabo C, Toliver-Kinsky T, Sidossis LS. Differential acute and chronic effects of burn trauma on murine skeletal muscle bioenergetics. Burns. 2016;42(1):112-22. Epub 2015/12/01. doi: 10.1016/j.burns.2015.10.026. PubMed PMID: 26615714; PMCID: PMC4724536.

36. Wagatsuma A, Kotake N, Yamada S. Muscle regeneration occurs to coincide with mitochondrial biogenesis. Mol Cell Biochem. 2011;349(1-2):139-47. Epub 2010/11/27. doi: 10.1007/s11010-010-0668-2. PubMed PMID: 21110070.

37. Fink BD, Bai F, Yu L, Sivitz WI. Regulation of ATP production: dependence on calcium concentration and respiratory state. Am J Physiol Cell Physiol. 2017;313(2):C146-C53. Epub 2017/05/19. doi: 10.1152/ajpcell.00086.2017. PubMed PMID: 28515085.

38. Haworth RA, Hunter DR. The Ca2+-induced membrane transition in mitochondria. II. Nature of the Ca2+ trigger site. Arch Biochem Biophys. 1979;195(2):460-7. Epub 1979/07/01. PubMed PMID: 38751.

39. Gurung P, Lukens JR, Kanneganti TD. Mitochondria: diversity in the regulation of the NLRP3 inflammasome. Trends Mol Med. 2015;21(3):193-201. Epub 2014/12/17. doi: 10.1016/j.molmed.2014.11.008. PubMed PMID: 25500014; PMCID: PMC4352396.

40. Bhola PD, Letai A. Mitochondria-Judges and Executioners of Cell Death Sentences. Mol Cell. 2016;61(5):695-704. Epub 2016/03/05. doi: 10.1016/j.molcel.2016.02.019. PubMed PMID: 26942674; PMCID: PMC4806554.

41. Aguilar CA, Greising SM, Watts A, Goldman SM, Peragallo C, Zook C, Larouche J, Corona BT. Multiscale analysis of a regenerative therapy for treatment of volumetric muscle loss injury. Cell Death Discov. 2018;4:33. Epub 2018/03/14. doi: 10.1038/s41420-018-0027-8. PubMed PMID: 29531830; PMCID: PMC5841404.

42. Garg K, Ward CL, Corona BT. Asynchronous inflammation and myogenic cell migration limit muscle tissue regeneration mediated by a cellular scaffolds. Inflamm Cell Signal. 2014;1(4). Epub 2014/01/01. doi: 10.14800/ics.530. PubMed PMID: 26949720; PMCID: PMC4776328.

43. Szelenyi ER, Urso ML. Time-course analysis of injured skeletal muscle suggests a critical involvement of ERK1/2 signaling in the acute inflammatory response. Muscle Nerve. 2012;45(4):552-61. Epub 2012/03/21. doi: 10.1002/mus.22323. PubMed PMID: 22431089.

44. Warren GL, Summan M, Gao X, Chapman R, Hulderman T, Simeonova PP. Mechanisms of skeletal muscle injury and repair revealed by gene expression studies in mouse models. J Physiol. 2007;582(Pt 2):825-41. Epub 2007/05/05. doi: 10.1113/jphysiol.2007.132373. PubMed PMID: 17478534; PMCID: PMC2075314.

45. VanderVeen BN, Fix DK, Carson JA. Disrupted Skeletal Muscle Mitochondrial Dynamics, Mitophagy, and Biogenesis during Cancer Cachexia: A Role for Inflammation. Oxid Med Cell Longev. 2017;2017:3292087. Epub 2017/08/09. doi: 10.1155/2017/3292087. PubMed PMID: 28785374; PMCID: PMC5530417.

46. Naito AT, Sumida T, Nomura S, Liu ML, Higo T, Nakagawa A, Okada K, Sakai T, Hashimoto A, Hara Y, Shimizu I, Zhu W, Toko H, Katada A, Akazawa H, Oka T, Lee JK, Minamino T, Nagai T, Walsh K, Kikuchi A, Matsumoto M, Botto M, Shiojima I, Komuro I. Complement C1q activates canonical Wnt signaling and promotes aging-related phenotypes. Cell. 2012;149(6):1298-313. Epub 2012/06/12. doi: 10.1016/j.cell.2012.03.047. PubMed PMID: 22682250; PMCID: PMC3529917.

47. Perandini LA, Chimin P, Lutkemeyer DDS, Camara NOS. Chronic inflammation in skeletal muscle impairs satellite cells function during regeneration: can physical exercise restore the satellite cell niche? FEBS J. 2018. Epub 2018/02/24. doi: 10.1111/febs.14417. PubMed PMID: 29473995.

48. Dziki J, Badylak S, Yabroudi M, Sicari B, Ambrosio F, Stearns K, Turner N, Wyse A, Boninger ML, Brown EHP, Rubin JP. An acellular biologic scaffold treatment for volumetric muscle loss: results of a 13-patient cohort study. NPJ Regen Med. 2016;1:16008. Epub 2016/07/21. doi: 10.1038/npjregenmed.2016.8. PubMed PMID: 29302336; PMCID: PMC5744714.

49. Corona BT, Wu X, Ward CL, McDaniel JS, Rathbone CR, Walters TJ. The promotion of a functional fibrosis in skeletal muscle with volumetric muscle loss injury following the transplantation of muscle-ECM. Biomaterials. 2013;34(13):3324-35. Epub 2013/02/07. doi: 10.1016/j.biomaterials.2013.01.061. PubMed PMID: 23384793.

50. Kim JH, Ko IK, Atala A, Yoo JJ. Progressive Muscle Cell Delivery as a Solution for Volumetric Muscle Defect Repair. Sci Rep. 2016;6:38754. Epub 2016/12/08. doi: 10.1038/srep38754. PubMed PMID: 27924941; PMCID: PMC5141432.

51. McClure MJ, Cohen DJ, Ramey AN, Bivens CB, Mallu S, Isaacs JE, Imming E, Huang YC, Sunwoo M, Schwartz Z, Boyan BD. Decellularized Muscle Supports New Muscle Fibers and Improves Function Following Volumetric Injury. Tissue Eng Part A. 2018. Epub 2018/02/13. doi: 10.1089/ten.TEA.2017.0386. PubMed PMID: 29431032.

52. Quarta M, Cromie M, Chacon R, Blonigan J, Garcia V, Akimenko I, Hamer M, Paine P, Stok M, Shrager JB, Rando TA. Bioengineered constructs combined with exercise enhance stem cell-mediated treatment of volumetric muscle loss. Nat Commun.

2017;8:15613. Epub 2017/06/21. doi: 10.1038/ncomms15613. PubMed PMID: 28631758; PMCID: PMC5481841.

Figure Legends

Figure 3.1: The effects of VML on plantarflexor muscle strength and oxidative capacity at 3 and 7 days after injury. A) Peak isometric torque normalized to body mass. B) Peak isometric torque normalized to plantarflexor mass. C) GAS mass presented as percent of the uninjured contralateral control limb. D) Mitochondrial respiratory function normalized by grams wet weight of permeabilized muscle fibers ($n \geq 15$ permeabilized fiber bundles from n=4 mice for each condition). E) Citrate synthase enzyme activity; $P<0.001$ for main effect of limb ($n \geq 8$ replicates from n=4 mice for each condition). F) Mitochondrial respiratory function normalized to citrate synthase enzyme activity. G) Succinate dehydrogenase activity; $P<0.001$ for main effect of limb ($n \geq 12$ replicates from n=4 mice for each condition). H) Extrapolation of mitochondrial respiration rates (panel D) to entire muscle mass (panel C). *$P<0.05$

Figure 3.2: The effects of VML on muscle mitochondrial network organization. A) Schematics showing regions of interest and representative 2D images from control and injured TA muscles. B) Representative 3D reconstruction of mitochondrial network near VML injury site from control and injured TA muscles. C) Representative 3D surface plots showing angle and alignment strength of the 3D mitochondrial network depicted in panel B. D) Quantification of mitochondrial network organization (peak alignment/average alignment) in control and injured muscle at various time points after injury ($n>10$ z-stacks from n=1-2 mice for each time point). *$P<0.05$

Figure 3.3: The effects of VML on mitochondrial organization in control and injured muscle at various time points after injury. Representative 3D reconstructions of mitochondrial networks from control (A), and VML injured muscle. VML injured muscle was imaged immediately (B), 3 days after injury (C), 7 days after injury (D), and 28 days after injury.

Figure 3.4: The effects of VML on mitochondrial organization at various distances from the injury site. Representative 3D reconstruction of mitochondrial networks from injured muscle at 0mm (A), <0.5mm (B), 1.5mm (C), and 2mm (D) away from the injury site.

Figure 3.5: The effects of VML and voluntary wheel running on muscle strength and oxidative capacity 4 weeks post injury. A) Peak isometric torque normalized to body mass. B) Peak isometric torque normalized to plantarflexor mass. C) GAS mass presented as percent of the uninjured contralateral control limb. D) Wheel running distance of control and VML injured mice. E) Mitochondrial respiratory function normalized by grams wet weight of permeabilized muscle fibers ($n\geq12$ permeabilized fiber bundles from $n=6$ mice for each condition). F) Citrate synthase enzyme activity, ($n\geq12$ replicates from $n=6$ mice for each condition). G) Mitochondrial respiratory function normalized to citrate synthase enzyme activity. H) Succinate dehydrogenase enzyme activity, ($n\geq18$ replicates from $n=6$ mice for each condition). I) Extrapolation of mitochondrial respiration rates (panel E) to entire muscle mass (panel C). $*P<0.05$

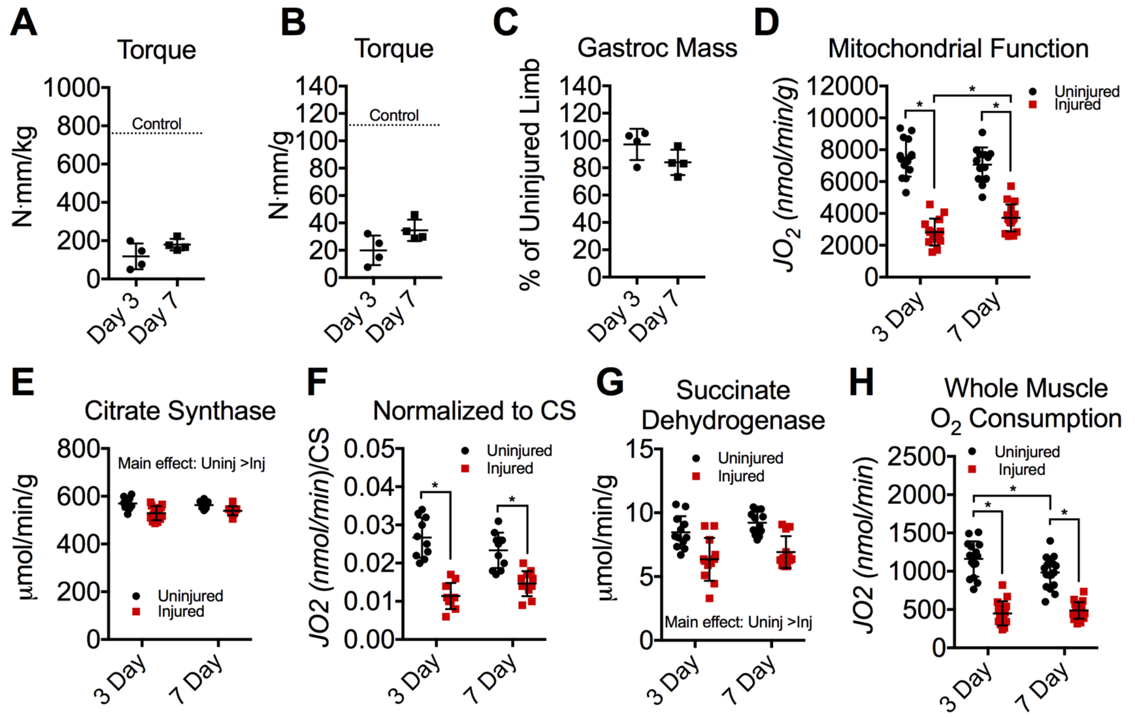
Figure 3.6: The effect of VML on muscle vasculature. A) Representative 3D reconstructions with vessel diameter mapping of vasculature in the posterior

compartment of VML and VML+WR mice. B) Quantification of blood vessel volume normalized to total volume; P=0.007 for main effect of muscle injury.

Figure 3.7: The effect of VML on mitochondrial biogenesis signaling after exercise. A) Muscle torque normalized to body mass prior to starting stimulation protocol. B) Torque production for control and VML injured mice throughout the stimulation protocol. C) Calculated area under the curves shown in panel B for control and VML injured mice. D) PGC-1α gene expression 3 hours after stimulation protocol for stimulated and non-stimulated limbs of control and VML injured mice. *P<0.05, **indicates significantly different from all, P<0.05

Figure 3.8: Effect of PGC-1α overexpression on oxidative capacity and plantarflexor muscle strength 4 weeks after VML injury. A) Representative images of GAS muscles with and without PGC-1α overexpression showing increased red hue in PGC-1α muscle. B) Mitochondrial respiratory function normalized by grams wet weight of permeabilized muscle fibers (n≥15 permeabilized fiber bundles from n=5 mice for each condition). C) Succinate dehydrogenase enzyme activity, (n≥18 replicates from n=5 mice for each condition). D) Citrate synthase enzyme activity, (n≥12 replicates from n=6 mice for each condition). E) Mitochondrial respiratory function normalized to citrate synthase enzyme activity. F) Plantarflexor torque loss following a fatiguing bout of 120 contractions; P=0.014 for both main effects of PGC-1α and group. G) Extrapolation of mitochondrial respiration rates (panel B) to entire muscle mass (panel J). H) Peak isometric torque normalized to body mass. I) Peak isometric

torque normalized to plantarflexor mass. J) GAS mass presented as percent of the uninjured contralateral control limb. *P<0.05



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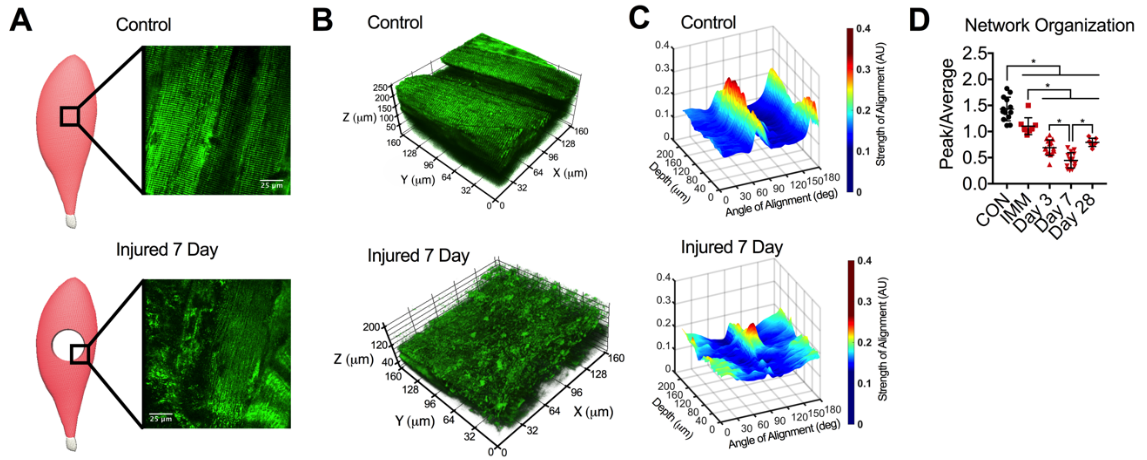
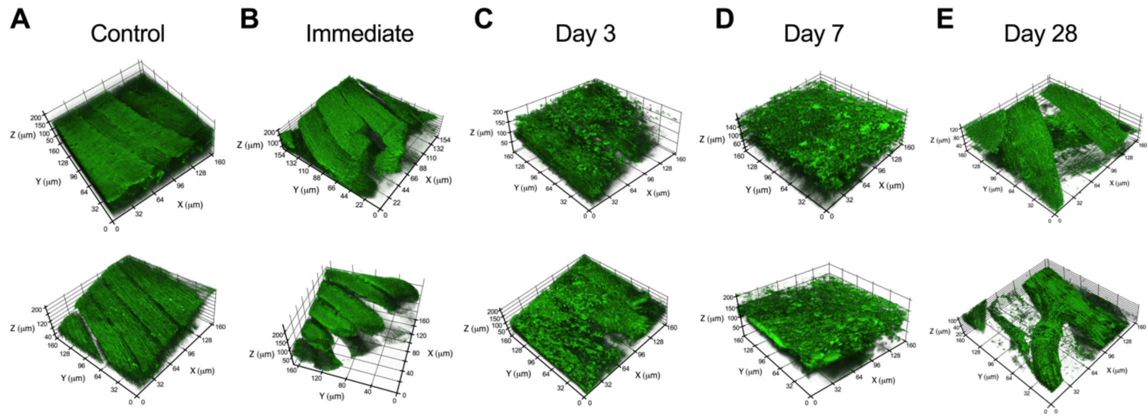


Figure 3.2



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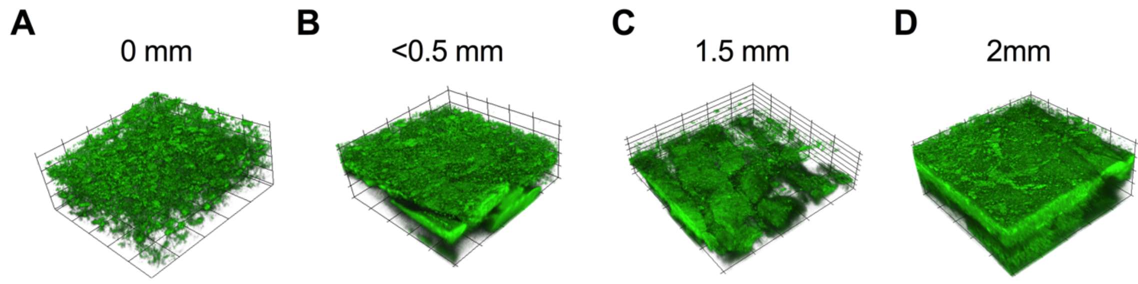

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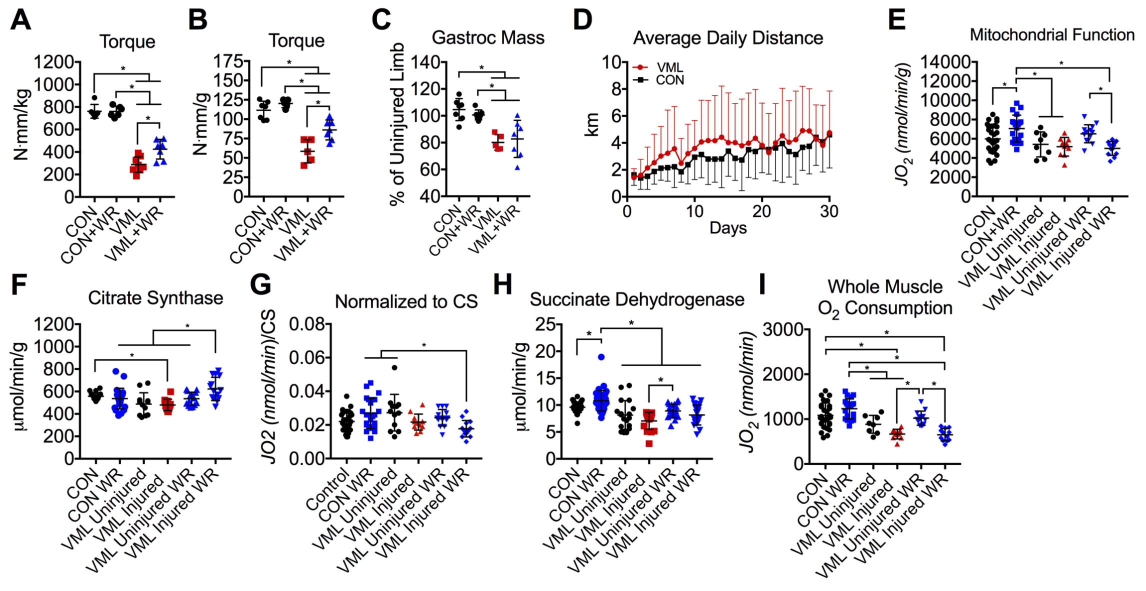

Figure 3.5

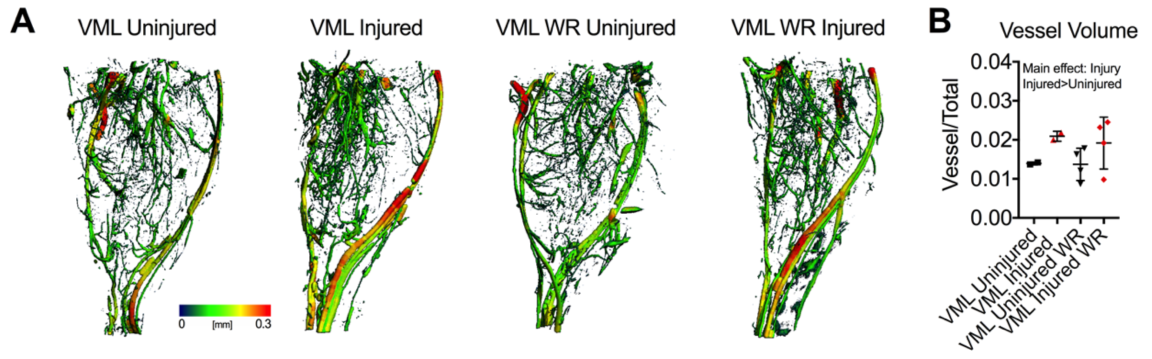

Figure 3.6

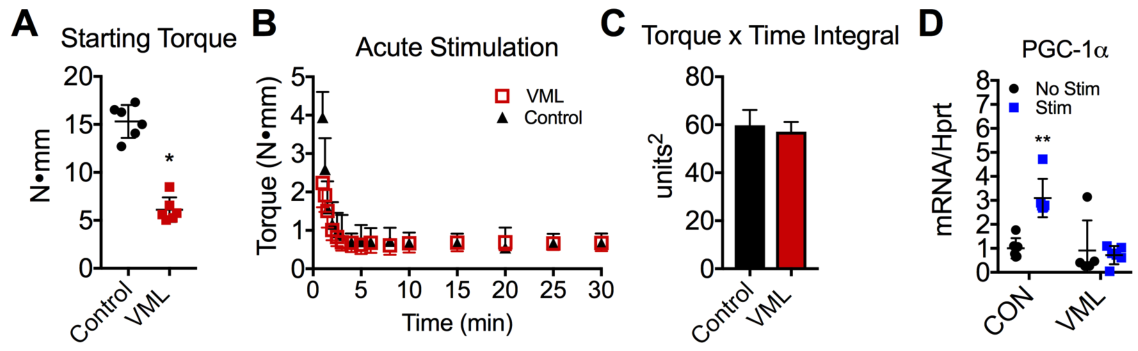

Figure 3.7

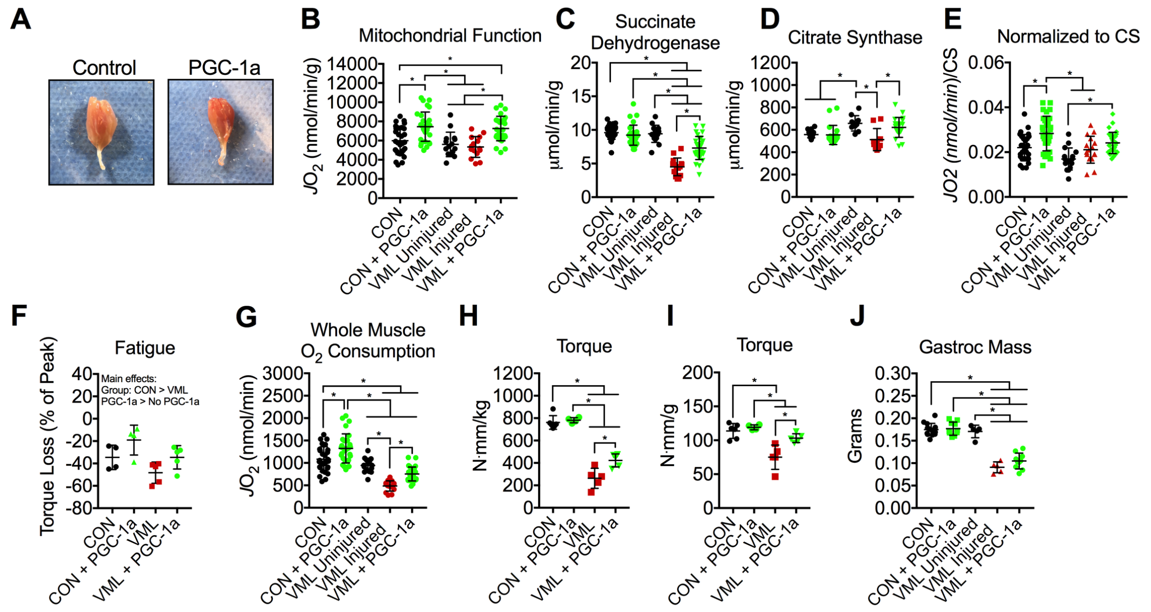

Figure 3.8

CHAPTER 4

DISSCUSSION AND CONCLUSIONS

Identifying and understanding mechanisms underlying the cause of a disease is one of the first steps for developing effective treatments specific to that disease. Similarly, we must fully understand the pathophysiology of volumetric muscle loss (VML) injury so that we can design appropriate and specific treatment strategies for patients with VML. Currently, there are no standard guidelines of care for patients with VML, which is largely due to the fact that every injury is unique and therefore needs unique treatment. This problem is further compounded by the fact that VML pathophysiology is poorly understood (3), which makes designing broad but effective treatments an extremely challenging task. In the present study, we have 1) identified mitochondrial dysfunction as an element of VML pathophysiology, and 2) identified a role for the mitochondria in recovery of muscle function after VML. We believe that this research provides valuable insight into VML pathophysiology and can inform future post-VML treatment strategies.

Directions for Future Research

This body of research extensively explored a novel area of VML pathophysiology, namely oxidative capacity and plasticity of the remaining muscle. As is common with research, extensive exploration of one area in the hopes of answering one question, often yields multiple new questions. Upon reflection of this work, three new important questions arise: 1) What is the mechanism by which VML injury causes such severe mitochondrial disturbances? 2) What is the mechanism by which VML injury impairs oxidative plasticity

of the remaining muscle? 3) What is the mechanism by which PGC-1α overexpression leads to greater in strength in the remaining muscle? Answering these three questions would provide important mechanistic insight into VML pathophysiology and potential treatment strategies.

<u>Question 1: What is the mechanism by which VML injury causes such severe mitochondrial disturbances?</u>

In this study, we reported that VML caused severe mitochondrial dysfunction and disorganization early after the injury that may still persist at 4 weeks post injury. While we did not specifically address the mechanism for the mitochondrial dysfunction it is likely multifaceted and dependent on the proximity of the mitochondrial network to the injury site. Myofibers that were directly impacted by the VML injury, i.e. severed, most likely suffered from loss of Ca^{2+} homeostasis due to complete loss of sarcolemma structural integrity. However, our 2-photon data suggested that the myofibers and mitochondria directly adjacent to the injury site were not the only casualties of the injury, as mitochondrial organization was still disrupted several hundred microns away from the edge of the VML. This is further supported by the mitochondrial dysfunction assay which is requires ~15 milligrams of muscle fibers to perform. While we selected myofibers from regions directly adjacent to the injury site, it is likely that only a fraction of the selected fibers suffered direct mechanical damage from VML injury, which supports the notion that VML-induced mitochondrial damage extends well beyond the bounds of the injury site.

Considering that inflammation has been previously implicated in mitochondrial dysfunction (17), it is likely that inflammation is a contributor to the VML-induced mitochondrial dysfunction. The inflammatory response after VML is excessive and

prolonged as it does not completely resolve even weeks after the injury (18, 19), which is in contrast to other injuries where the inflammatory response typically resolves within 3-7 days (20, 21). Furthermore, the inflammatory response after VML also extends beyond the injury site as our 2-photon data revealed what appeared to be blanket of mononuclear inflammatory cells distributed all across the muscle early after the injury. Together, this data suggests that inflammation is a type of indirect VML injury that causes mitochondrial dysfunction in myofibers that would otherwise be unharmed by the VML. Future research should determine if modulation of the inflammatory response after VML would preserve mitochondrial function in muscle fibers not mechanically impacted by VML.

<u>Question 2: What is the mechanism by which VML injury impairs oxidative plasticity of the remaining muscle?</u>

In this study, we reported that VML injured muscle did not adapt to 4 weeks of voluntary wheel running, which suggested that VML injury potentially limits oxidative plasticity of the affected muscle. In an attempt to tease out a mechanism for how VML injury would impair oxidative adaptations, we performed several experiments, the first of which focused on muscle vasculature and the second focused on mitochondrial biogenesis. The first experiment, demonstrated that muscle vessel volume was actually higher after VML, which put to rest our hypothesis that blood perfusion deficiencies after VML could limit adaptations to exercise. On the other hand, when we examined mitochondrial biogenesis, we found that PGC-1α expression did not increase after exercise in VML injured muscle, which suggests that VML injury either somehow disrupts the mitochondrial biogenesis pathway. Given that vascular perfusion is not an issue, there are three other candidate explanations for how VML injury would disrupt mitochondrial

adaptations to exercise.

The first possible explanation of how VML could limit oxidative adaptations to exercise is through direct disruptions in the mitochondrial biogenesis signaling pathway. Considering that we found blunted PGC-1α expression after exercise and increased oxidative capacity after PGC-1α overexpression in VML injury muscle, it is logical to assume that if VML was affecting mitochondrial biogenesis signaling it would be upstream of PGC-1α. Increased PGC-1α expression, and by extension mitochondrial biogenesis, is initiated by several main signals that include, increased intracellular Ca^{2+}, contraction induced ROS production, and activation of AMPK (22). Besides our PGC-1α overexpression experiments, we do not have any data that specifically examines the effect of VML on these upstream activators of PGC-1α, therefore, we can only speculate on how VML would affect mitochondrial biogenesis. Logically, VML-induced disruption of PGC-1α activators is unlikely because intramuscular disturbances after VML reflect more of a pro- rather an anti-mitochondrial biogenesis environment (i.e. increased Ca^{2+}, inflammation-induced ROS production, and mitochondrial dysfunction-induced AMPK activation). Obviously, this seemingly pro-mitochondrial biogenesis environment does not actually translate to mitochondrial biogenesis, but rather initiating cell survival or death pathways (i.e. mitochondrial permeability transition pore opening from mitochondrial Ca^{2+} overload, apoptosis, or necrosis). Overall, there is not an obvious reason to believe that VML would disturb molecular activators and signaling pathways upstream of PGC-1α.

The second possible explanation of how mitochondrial biogenesis could limit oxidative adaptations is biomechanical alterations of the hindlimb. In order for mitochondrial adaptations to exercise to take place, muscle contraction must occur (23). In

this study, the VML injury could have restricted muscle movement to the point where the muscle is rendered useless. If that was the case, the muscle would not have been used during wheel running and the mitochondria, even though all signaling pathways were intact, would not have adapted. In the present study, we did not directly assess hindlimb biomechanics in VML injured mice, but indirect evidence points to the notion that both the injured and the uninjured hindlimbs were used during voluntary wheel running. First, VML injured mice ran the same distance as uninjured mice suggesting that they were not hindered by major biomechanical issues. The second and most convincing piece of evidence that the injured limb was activated during wheel running is that muscle strength was greater in VML injured muscle compared to controls after wheel running. Overall, biomechanical abnormalities probably did not play a large role in limiting oxidative adaptations to exercise in VML injured muscle.

The third possible explanation of how mitochondrial biogenesis could limit oxidative adaptations is lack of muscle activation due to motor neuron axotomy. Previous studies have reported that around 20% of VML patients suffer motor neuron injuries (24), which undoubtedly contributes to the significant strength deficits observed after VML (25). Muscle activation is necessary for mitochondrial activation and adaptation to exercise, so it is plausible that muscle fibers around the injury site (i.e. muscle fibers selected for mitochondrial respiration analysis) were not activated during wheel running and did not adapt to the exercise. In conclusion, we have ruled out vascular limitations, alterations in mitochondrial biogenesis signaling, and biomechanical abnormalities as potential mechanisms by which VML impairs oxidative adaptations. Rather, lack of muscle activation from damage to motor neurons appears to be the most reasonable explanation.

To test this hypothesis, future studies could attempt to stimulate mitochondrial biogenesis by directly depolarizing VML injured muscle thereby bypassing motor neuron damage.

Question 3: What is the mechanism by which PGC-1α overexpression leads to greater in strength in the remaining muscle?

In this study, we used plasmid transfection to overexpress PGC-1α in VML injured muscle so that we could 1) determine if the mitochondrial biogenesis signaling pathway was intact downstream of PGC-1α, and 2) to determine if increasing the oxidative capacity of the VML injured muscle is of any benefit to recovery of muscle function. Oxidative capacity of VML injured muscle was indeed greater after PGC-1α overexpression indicating that mitochondrial biogenesis signaling downstream of PGC-1α was fully functioning. We also found that muscle strength was greater in the VML injured muscle after PGC-1α overexpression. In fact, when strength from VML injured muscle was normalized to muscle size, the strength deficit was almost completely abolished. This data suggests that mitochondria play an important role in the recovery of muscle function and should be the incorporated into VML treatment strategies.

There are a number of ways by which PGC-1α overexpression could elicit strength improvements in VML injured muscle. The first of which is by mitochondrial mediated membrane repair. Recently, a role has been described for mitochondria in mediating muscle fiber sarcolemmal repair after injury (26, 27). Considering that VML injury involves severe mechanical damage to the sarcolemma, we postulate that bolstering mitochondria in myofibers after VML injury could provide resistance to cell death via enhanced sarcolemmal repair. Essentially, greater resistance to cell death after VML would

effectively increase the number of myofibers that survive after VML and possibly reduce the number of fibers affected by inflammation, which could potentially lead to better strength outcomes 4 weeks after injury. A second way in which PGC-1α overexpression could lead to higher muscle strength after VML, is through neuromuscular junction (NMJ) modulation. PGC-1α overexpression has been shown to enhance pre- and post- synaptic NMJ morphology and function in a similar fashion to exercise training (28). Considering that motor neuron damage is a common effect of VML (24), enhancing NMJ function after VML may help to partially compensate for this shortcoming leading to an overall stronger muscle. Lastly, PGC-1α overexpression may help to prevent muscle atrophy, which is common after VML (29). PGC-1α overexpression has been shown to inhibit muscle atrophy by blocking FoxO3 signaling (30). Given that atrophied muscles tend to be weaker, PGC-1α-mediated prevention of atrophy could be an important factor in VML injured muscle producing more torque. However, it should be noted, that analysis of gastrocnemius mass 4 weeks after injury did not reveal 1) any signs of muscle atrophy in the VML injured limb and 2) any signs of PGC-1α-mediated prevention of atrophy, i.e. VML+ PGC-1α mass was not different from VML alone. In conclusion, our results clearly demonstrate that PGC-1α overexpression leads to greater muscle strength after VML, but it is not clear whether this is due to enhancements in mitochondria, NMJ function, or anti-atrophy signaling.

Implications for Clinical Practice

This study presents several novel findings regarding VML pathology that are relevant to clinical practice as it relates to treatment of VML. First, we demonstrated that oxidative capacity is severely reduced acutely after VML and, although to a much lesser

extent, this effect may still be present 4 weeks after the injury. Mitochondrial dysfunction can lead to a number of complications within the muscle, that if left untreated, could lead to a number of metabolic related diseases such as diabetes (31). In light of this information, we propose that future rehabilitative guidelines should contain objectives that specifically aim to improve the oxidative capacity of the remaining muscle. Secondly, we demonstrated that oxidative plasticity of VML injured muscle is impaired, which could limit the efficacy of mitochondrial specific rehabilitation programs. We believe that the most reasonable explanation for VML limiting oxidative plasticity is lack of muscle activation due to motor neuron damage. Therefore, we propose that current physical therapy guidelines for rescuing muscle function after nerve damage be combined with endurance type exercises that would help to promote motor neuron innervation and improve oxidative capacity of the remaining muscle.

References

1. Sicari BM, Rubin JP, Dearth CL, Wolf MT, Ambrosio F, Boninger M, Turner NJ, Weber DJ, Simpson TW, Wyse A, Brown EH, Dziki JL, Fisher LE, Brown S, Badylak SF. An acellular biologic scaffold promotes skeletal muscle formation in mice and humans with volumetric muscle loss. Sci Transl Med. 2014;6(234):234ra58. doi: 10.1126/scitranslmed.3008085. PubMed PMID: 24786326.

2. Owens BD, Kragh JF, Jr., Macaitis J, Svoboda SJ, Wenke JC. Characterization of extremity wounds in Operation Iraqi Freedom and Operation Enduring Freedom. J Orthop Trauma. 2007;21(4):254-7. doi: 10.1097/BOT.0b013e31802f78fb. PubMed PMID: 17414553.

3. Corona BT, Wenke JC, Ward CL. Pathophysiology of Volumetric Muscle Loss Injury. Cells Tissues Organs. 2016;202(3-4):180-8. doi: 10.1159/000443925. PubMed PMID: 27825160.

4. Garg K, Ward CL, Hurtgen BJ, Wilken JM, Stinner DJ, Wenke JC, Owens JG, Corona BT. Volumetric muscle loss: persistent functional deficits beyond frank loss of tissue. J Orthop Res. 2015;33(1):40-6. doi: 10.1002/jor.22730. PubMed PMID: 25231205.

5. Mase VJ, Jr., Hsu JR, Wolf SE, Wenke JC, Baer DG, Owens J, Badylak SF, Walters TJ. Clinical application of an acellular biologic scaffold for surgical repair of a large, traumatic quadriceps femoris muscle defect. Orthopedics. 2010;33(7):511. doi: 10.3928/01477447-20100526-24. PubMed PMID: 20608620.

6. Dziki J, Badylak S, Yabroudi M, Sicari B, Ambrosio F, Stearns K, Turner N, Wyse A, Boninger ML, Brown EHP, Rubin JP. An acellular biologic scaffold treatment for

volumetric muscle loss: results of a 13-patient cohort study. Npj Regenerative Medicine. 2016;1:16008. doi: 10.1038/npjregenmed.2016.8.

7. Corona BT, Rivera JC, Owens JG, Wenke JC, Rathbone CR. Volumetric muscle loss leads to permanent disability following extremity trauma. J Rehabil Res Dev. 2015;52(7):785-92. doi: 10.1682/JRRD.2014.07.0165. PubMed PMID: 26745661.

8. Li MT, Willett NJ, Uhrig BA, Guldberg RE, Warren GL. Functional analysis of limb recovery following autograft treatment of volumetric muscle loss in the quadriceps femoris. J Biomech. 2014;47(9):2013-21. doi: 10.1016/j.jbiomech.2013.10.057. PubMed PMID: 24280565; PMCID: PMC4017006.

9. Corona BT, Wu X, Ward CL, McDaniel JS, Rathbone CR, Walters TJ. The promotion of a functional fibrosis in skeletal muscle with volumetric muscle loss injury following the transplantation of muscle-ECM. Biomaterials. 2013;34(13):3324-35. doi: 10.1016/j.biomaterials.2013.01.061. PubMed PMID: 23384793.

10. Willett NJ, Li MT, Uhrig BA, Boerckel JD, Huebsch N, Lundgren TL, Warren GL, Guldberg RE. Attenuated human bone morphogenetic protein-2-mediated bone regeneration in a rat model of composite bone and muscle injury. Tissue Eng Part C Methods. 2013;19(4):316-25. doi: 10.1089/ten.TEC.2012.0290. PubMed PMID: 22992043; PMCID: PMC3583252.

11. Chen XK, Walters TJ. Muscle-derived decellularised extracellular matrix improves functional recovery in a rat latissimus dorsi muscle defect model. J Plast Reconstr Aesthet Surg. 2013;66(12):1750-8. doi: 10.1016/j.bjps.2013.07.037. PubMed PMID: 24007646.

12. Ingalls CP, Warren GL, Armstrong RB. Dissociation of force production from MHC and actin contents in muscles injured by eccentric contractions. J Muscle Res Cell Motil. 1998;19(3):215-24. PubMed PMID: 9583362.

13. Warren GL, Hulderman T, Mishra D, Gao X, Millecchia L, O'Farrell L, Kuziel WA, Simeonova PP. Chemokine receptor CCR2 involvement in skeletal muscle regeneration. FASEB J. 2005;19(3):413-5. doi: 10.1096/fj.04-2421fje. PubMed PMID: 15601671.

14. Corona BT, Henderson BE, Ward CL, Greising SM. Contribution of minced muscle graft progenitor cells to muscle fiber formation after volumetric muscle loss injury in wild-type and immune deficient mice. Physiol Rep. 2017;5(7). doi: 10.14814/phy2.13249. PubMed PMID: 28400501; PMCID: PMC5392532.

15. Garg K, Ward CL, Rathbone CR, Corona BT. Transplantation of devitalized muscle scaffolds is insufficient for appreciable de novo muscle fiber regeneration after volumetric muscle loss injury. Cell Tissue Res. 2014;358(3):857-73. doi: 10.1007/s00441-014-2006-6. PubMed PMID: 25300647.

16. Kim JH, Ko IK, Atala A, Yoo JJ. Progressive Muscle Cell Delivery as a Solution for Volumetric Muscle Defect Repair. Sci Rep. 2016;6:38754. doi: 10.1038/srep38754. PubMed PMID: 27924941; PMCID: PMC5141432.

17. VanderVeen BN, Fix DK, Carson JA. Disrupted Skeletal Muscle Mitochondrial Dynamics, Mitophagy, and Biogenesis during Cancer Cachexia: A Role for Inflammation. Oxid Med Cell Longev. 2017;2017:3292087. Epub 2017/08/09. doi: 10.1155/2017/3292087. PubMed PMID: 28785374; PMCID: PMC5530417.

18. Aguilar CA, Greising SM, Watts A, Goldman SM, Peragallo C, Zook C, Larouche J, Corona BT. Multiscale analysis of a regenerative therapy for treatment of volumetric muscle loss injury. Cell Death Discov. 2018;4:33. Epub 2018/03/14. doi: 10.1038/s41420-018-0027-8. PubMed PMID: 29531830; PMCID: PMC5841404.

19. Garg K, Ward CL, Corona BT. Asynchronous inflammation and myogenic cell migration limit muscle tissue regeneration mediated by a cellular scaffolds. Inflamm Cell Signal. 2014;1(4). Epub 2014/01/01. doi: 10.14800/ics.530. PubMed PMID: 26949720; PMCID: PMC4776328.

20. Szelenyi ER, Urso ML. Time-course analysis of injured skeletal muscle suggests a critical involvement of ERK1/2 signaling in the acute inflammatory response. Muscle Nerve. 2012;45(4):552-61. Epub 2012/03/21. doi: 10.1002/mus.22323. PubMed PMID: 22431089.

21. Warren GL, Summan M, Gao X, Chapman R, Hulderman T, Simeonova PP. Mechanisms of skeletal muscle injury and repair revealed by gene expression studies in mouse models. J Physiol. 2007;582(Pt 2):825-41. Epub 2007/05/05. doi: 10.1113/jphysiol.2007.132373. PubMed PMID: 17478534; PMCID: PMC2075314.

22. Ljubicic V, Joseph AM, Saleem A, Uguccioni G, Collu-Marchese M, Lai RY, Nguyen LM, Hood DA. Transcriptional and post-transcriptional regulation of mitochondrial biogenesis in skeletal muscle: effects of exercise and aging. Biochim Biophys Acta. 2010;1800(3):223-34. Epub 2009/08/18. doi: 10.1016/j.bbagen.2009.07.031. PubMed PMID: 19682549.

23. Hood DA. Invited Review: contractile activity-induced mitochondrial biogenesis in skeletal muscle. J Appl Physiol (1985). 2001;90(3):1137-57. Epub 2001/02/22. doi: 10.1152/jappl.2001.90.3.1137. PubMed PMID: 11181630.

24. Beltran MJ, Burns TC, Eckel TT, Potter BK, Wenke JC, Hsu JR, Skeletal Trauma Research C. Fate of combat nerve injury. J Orthop Trauma. 2012;26(11):e198-203. Epub 2012/03/23. doi: 10.1097/BOT.0b013e31823f000e. PubMed PMID: 22437422.

25. Corona BT, Flanagan KE, Brininger CM, Goldman SM, Call JA, Greising SM. Impact of volumetric muscle loss injury on persistent motoneuron axotomy. Muscle Nerve. 2018;57(5):799-807. Epub 2017/11/17. doi: 10.1002/mus.26016. PubMed PMID: 29144551.

26. Horn A, Van der Meulen JH, Defour A, Hogarth M, Sreetama SC, Reed A, Scheffer L, Chandel NS, Jaiswal JK. Mitochondrial redox signaling enables repair of injured skeletal muscle cells. Sci Signal. 2017;10(495). Epub 2017/09/07. doi: 10.1126/scisignal.aaj1978. PubMed PMID: 28874604.

27. Vila MC, Rayavarapu S, Hogarth MW, Van der Meulen JH, Horn A, Defour A, Takeda S, Brown KJ, Hathout Y, Nagaraju K, Jaiswal JK. Mitochondria mediate cell membrane repair and contribute to Duchenne muscular dystrophy. Cell Death Differ. 2017;24(2):330-42. Epub 2016/11/12. doi: 10.1038/cdd.2016.127. PubMed PMID: 27834955; PMCID: PMC5299714.

28. Arnold AS, Gill J, Christe M, Ruiz R, McGuirk S, St-Pierre J, Tabares L, Handschin C. Morphological and functional remodelling of the neuromuscular junction by skeletal muscle PGC-1alpha. Nat Commun. 2014;5:3569. Epub 2014/04/02. doi: 10.1038/ncomms4569. PubMed PMID: 24686533; PMCID: PMC4846352.

29. Dziki J, Badylak S, Yabroudi M, Sicari B, Ambrosio F, Stearns K, Turner N, Wyse A, Boninger ML, Brown EHP, Rubin JP. An acellular biologic scaffold treatment for volumetric muscle loss: results of a 13-patient cohort study. NPJ Regen Med. 2016;1:16008. Epub 2016/07/21. doi: 10.1038/npjregenmed.2016.8. PubMed PMID: 29302336; PMCID: PMC5744714.

30. Sandri M, Lin J, Handschin C, Yang W, Arany ZP, Lecker SH, Goldberg AL, Spiegelman BM. PGC-1alpha protects skeletal muscle from atrophy by suppressing FoxO3 action and atrophy-specific gene transcription. Proc Natl Acad Sci U S A. 2006;103(44):16260-5. Epub 2006/10/21. doi: 10.1073/pnas.0607795103. PubMed PMID: 17053067; PMCID: PMC1637570.

31. Goodpaster BH. Mitochondrial deficiency is associated with insulin resistance. Diabetes. 2013;62(4):1032-5. Epub 2013/03/23. doi: 10.2337/db12-1612. PubMed PMID: 23520282; PMCID: PMC3609595.