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The Effects of VML on Satellite Cell Mechanics Distal to the Defect Site

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The Effects of VML on Satellite Cell Mechanics Distal to the Defect Site

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Table of Contents

Chapter 1: Review of Literature

Review of Literature

Civilians and military personnel are susceptible to traumatic muscle injury. Whether it be due to automobile accidents, explosive devices or tumor removal, severe muscle trauma often results in permanent loss of function. The perpetuation of the damage is mostly due to the impotence of skeletal muscle to regenerate independently. Regeneration of skeletal muscle is directed by satellite cells, or muscle stem cells, and activity of this regeneration can be monitored by tracking specific protein markers. Pax7 is detected when the satellite cell is dormant, whereas MyoD, Ki67, and MyoG are detected when satellite cells are proliferating and differentiation. However, volumetric muscle loss, VML, results in the bereavement of satellite cells which contributes to improper regeneration. Exercise seems to increase proliferation and differentiation of satellite cells by activating upstream regulators (HGF, IGF-1, FGF, etc.) to augment cell activation. This continues to be a significant area of research as the interactions of exercise and autologous muscle grafts affecting cell signaling and functional regeneration are still unknown. This literature review will give an overview of volumetric muscle loss and the prevailing models to study regeneration in skeletal muscle. The review will be divided into three sections: (1) skeletal muscle physiology, (2) satellite cell markers for activation, and (3) current models to study VML. The physiology of skeletal muscles will focus on the ability of skeletal muscles to regenerate, and how they utilize satellite cells to introduce new myofibers. After exercise, skeletal muscle activates precursor regulators of such satellite cells (IGF-1, HGF, etc.), and certain proteins are emphasized during proliferation and differentiation (MyoD, MyoG, and Ki67). The satellite cell section of this review will focus the known relationships between Pax7, MyoD, MyoG, and Ki67 and satellite cell activation. Lastly, since exercise has been shown to improve satellite cell activation, the models explained will be specifically related to the effects of exercise on VML regeneration in skeletal muscle.

Skeletal muscle physiology

Skeletal muscle is responsible to skeletal movements. The loss of significant volume of this type of muscle would be debilitating. The muscle fibers are filled with many nuclei and are all characterized by being post-mitotic. Post-mitotic nuclei are incapable of dividing and creating new cells, and therefore require the aid of other stem cells. In muscles, these are called satellite cells, which will be explained more later in the review.

Basic Anatomy

In the human body, skeletal muscles make up about 40% of the total body weight and accounts from 30-50% of protein turnover for the whole body [5]. Skeletal muscle cells, or fibers, are multinucleated, post-mitotic cells which are often in bundles called fascicles. Muscles vary significantly in size throughout the system and range in force capabilities. The bundles of muscle fibers, or fascicles, are surrounded by a protective layer of connective tissue called the perimysium. Surrounding the muscle fiber is a plasma membrane called the sarcolemma which consists of the muscle fiber itself and sarcoplasm. The functional contractile unit of the muscle is the sarcomere which consists of the myofilaments: actin and myosin. The muscle function via a motor unit. The motor unit is composed of a motor neuron and the muscle fiber it innervates. The muscle is formed by many motor units assembled in such a way to fit the functional demand of that muscle [14].

Cell Cycle

A typical cell undergoes a cycle that allows it to regenerate and repair itself. The cell cycle begins with the M phase which constitutes mitosis and followed by cytosis. This phase is followed by G1 phase in which the cell begins to grow. The S phase follows and here is where the replication of DNA takes place. Lastly, the G2 phase is clear when the synthesis of DNA is

complete and there is protein synthesized to prepare for beginning the cycle over again. Muscle cells cannot engage in the cell cycle on their own. Once a cell is differentiated into a myofiber, it is post-mitotic and therefore cannot regenerate on its own. It would seem that this could be detrimental to the body and that any damage would lead to severe degradation; however, satellite cells are muscle stem cells that are able to provide the extra assistance that the damaged muscle requires.

Hypertrophy and Atrophy

Skeletal muscle mass is dependent on the difference in rates of protein synthesis and protein degradation. Muscle hypertrophy is how muscles grow in size, of increase in muscle mass. Muscle hypertrophy happens when the rate of protein synthesis is greater than the rate of protein degradation [14]. Hypertrophy appears during development and as a response to overload of muscles (i.e. strength training). Skeletal muscle growth is determined by satellite cell integration. As mentioned above, myofibers are post-mitotic and therefore require the introduction of additional nuclei. Those additional nuclei are provided by the fusion of differentiated satellite cells, or muscle stem cells [4].

Whereas atrophy is a decrease in muscle mass, which occurs when protein degradation rates are greater than synthesis rates [14]. Atrophy can be induced by a number of things from physical inactivity, cancer cachexia, starvation and to aging [14]. Skeletal muscle degradation can be very detrimental as they play an important role in the actions of converting chemical energy into energy of movement, sustaining posture, and aids in an individual's ability to be functionally independent [5]. Additionally, skeletal muscles store amino acids and carbohydrate sources, as well as produce heat to maintain homeostatic temperature [5].

Skeletal Muscle Regeneration

Skeletal muscle regeneration occurs in three stages: 1) inflammatory response, 2) satellite cell activation, proliferation and differentiation, and 3) maturation of new muscle cells [20]. The sarcolemma of the myofiber is damaged when the muscle degenerates which leads to an increase in permeability. This response can be expressed by the increased plasma levels of creatine kinase and other muscle proteins [20]. Neutrophils, a leukocyte, are the first inflammatory cells to enter the cell that phagocytize the muscular necrosis [20]. Following the inflammatory response and degradation, satellite cells begin to be activated. Satellite cells are discussed more extensively below, but in short, they provide additional nuclei for the muscle fiber. The satellite cells are differentiated into committed myofibers and can contribute to the growth, repair and healing of muscle fibers post-injury [20].

Satellite cell physiology and transcription factors

In adult skeletal muscle, satellite cells remain dormant, or quiescent, until injury and are then activated and proliferate [4]. The up or down regulation of certain transcription factors can illustrate the activity of satellite cells. As previously mentioned, skeletal muscle regeneration is made possible by the proliferation and introduction of satellite cells into the myofiber.

Satellite cells, a muscle stem cell

Satellite cells are a specialized type of stem cells that are specific to skeletal muscle. They are found between the basal lamina and the sarcolemma of muscle fibers. Stem cells are undifferentiated, meaning that they can become any specific cell, and they possess high proliferative qualities, meaning that they can engage in continuous regeneration [1]. Subsequently, muscle stem cells are unquestionably required for skeletal muscle regeneration. These cells contribute greatly to muscle growth, repair, and regeneration [5]. It is important to

recall from above that skeletal myofibers are post-mitotic. Post-mitotic cells are unable to proliferate on their own and therefore require the introduction of other nuclei, from satellite cells, to maintain the cell function post-injury. Studies show that satellite cells are undoubtedly necessary for injury-induced muscle regeneration [8, 13]. When activated by myogenic factors, these satellite cells can proliferate into new muscle fibers to aid in muscle repair [5].

Quiescence

Satellite cells, as mentioned above, reside in the space between the basement membrane and sarcolemma of the muscle fibers. They require specific proteins to be activated post-injury, as they are typically in a quiescent, or dormant state. Quiescent satellite cells express high levels of Pax7 [2]. Pax7 is able to activate transcription but is known to be a poor transcriptional activator [21]. Typically, Pax7 is expressed only in quiescent cells and decreases in expression as the cell enters activation. However, a study interestingly found that some satellite cells continue to express Pax7 and downregulate MyoD, an activation factor, which propels the satellite cell back into a quiescent-like state [21]. Zammit et. al (2006) discussed previous studies implicating that Pax7 plays a role in satellite cell survival and maintenance of quiescence.

Activation and proliferation

As a result of muscle damage, MyoD is expressed which establishes activation. Then, Ki67 is expressed which initiates proliferation [17]. MyoD plus Ki67 plus the satellite cell begin to proliferate to produce myoblasts. Satellite cells are cued to enter the proliferation stage once exposed to signals of a damaged environment [20]. The damaged environment does not have to be localized; damage at one end of the muscle prompts activation of all the satellite cells along that same muscle fiber and stimulates the migration of the cells to the damaged site [15]. $MyoD^{-/-}$

mice have been studied to display reduced muscle mass, as well as impaired muscle regeneration [9].

Differentiation

Satellite cells that are committed myoblasts express high levels of MyoG and low levels of Pax7 and these MyoG-expressing cells go on to create myofibers [17]. Differentiation is required to prompt the satellite cell into the characteristics of muscle cells. Without differentiation, the satellite cell has been shown to reenter a quiescent-like state [21]. Myogenin is crucial for the cell as it mediates the terminal differentiation so the cell can exit the cycle and is also greatly responsible for muscle fiber characteristics [7]. Once the cell enters differentiation, the cell is able to fuse with the existing myofibers and aid in muscle repair, growth, etc.

Models to study VML and exercise

Volumetric Muscle Loss (VML)

VML is defined as traumatic or surgical loss of muscle that will result in an impairment in function [19]. VML has been shown to result in the loss of satellite cells thus contributing to improper muscle regeneration. In medicine, the standard of care to treat VML is to us autologous muscle transfer and the use of physical therapy exercises [19]. This is obviously not an effective form of treatment for an injury with significant VML and requires high technical skills. Modeling this type of injury poses a difficult problem. An animal model that is ethical and clinically relevant has been attempted by a variety of researchers to accurately depict VML. Wu et. al (2012) describes the requirements of such an animal model that will reproduce the characteristics of volumetric muscle loss in rats: 1) involve an injury that is worse than the rat's

known self-regeneration capacity, 2) allow for a standard and accurate functional assessment, 3) able to remain stable to accommodate long-term studies.

VML Surgery and repair using autologous grafting

The surgical procedure used is as follows: once the skin was separated from the fascia, a blunt medium-sized hemostat was used to separate the EDL from the TA and a flat spatula was used to flatten the TA [19]. The defect was introduced by scoring the TA with two horizontal incisions and two longitudinal incisions and then excised from the tissue [19]. The defect should be ~20% of the weight of the TA weight. Wu et al (2012) determined that a defect weight ~20% of the TA weight was the most effective that ensured the return of blood vessels and nerves without damaging the distal tendon. To estimate the weight of the TA muscle, Wu et al (2012) used the regression equation: y=0.0017*body weight - 0.0716. Incorporation of autologous muscle grafting improves the regenerative response by promoting *de novo* muscle fiber regeneration and functional recovery. This is possibly due to the introduction of new satellite cells [18]. Autologous muscle grafting helps the muscle repair and promotes myogenesis.

Exercise affecting VML

Exercise has been shown to increase activation of satellite cells by stimulating upstream regulators of these cells. Damaged muscles subjected to exercise were analyzed by Quarta et. al (2017), and the muscles from the non-exercised mice were significantly smaller in mass that the muscles from exercised mice. The exercised mice also showed increased force production and illustrated that exercise was capable of reestablishing the muscle forces damaged by VML injuries [12]. It was determined that exercise improved the recovery of VML by decreasing the effects of fibrosis and enhancing vascularization [12].

Conclusion of Review

Current research supports the ideas that satellite cells help repair skeletal muscles as that tissue is post-mitotic [1]. The expression of Pax7 highlights that the satellite cells are in their dormant, or quiescent state [21], the expressions of MyoD and Ki67 shows that the cell is in the activation and differentiation stage [17], and once myogenin is expressed, you know that the satellite cell is a committed myoblast and will potentially fuse to the myofiber [17]. Autologous muscle grafting has shown that the incorporation of healthy muscle tissue can aid in the regeneration process by providing additional myoconductive biomaterial [18]. Exercise has also been shown to promote VML recovery. Exercise stimulates regulators of satellite cells which will further increase activation of these cells. However, how exercise and autologous muscle grafting interact to prompt functional recovery in the skeletal muscle is still unknown.

Chapter 2- Purpose and Hypothesis

Purpose

The purpose of this study was to evaluate the effects of autologous muscle grafting and exercise on satellite cell mechanics distal to the defect site.

Hypothesis

I predicted that the exercised rats post VML injury will recover better than sedentary rats which will be evidenced by increased satellite cell activity distal to the defect site.

Chapter 3-Manuscript

The Effects of VML on Satellite Cell Mechanics Distal to the Defect Site

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Abstract

Background: Volumetric muscle loss (VML) is defined as a permanent loss of muscle tissue and function. Recent studies have used biomedical interventions such as decellularized matrices and autologous repair to enhance muscle regeneration of these injuries. Furthermore, exercise has also been shown to aid in the regenerative response. Biomedical interventions and physical activity help to attenuate severe fibrosis as well as encourage satellite cell activation in VML models. However, it is not known whether these interventions affect muscle tissue distal to the injury site. **Purpose:** The purpose of this study was to evaluate the effects of autologous muscle grafting and exercise on satellite cell mechanics distal to the defect site. **Methodology:** Male, Sprague-Dawley rats were randomly assigned to either exercised (wheel access, WA) or sedentary (cage access, CA) groups and were further divided into 2 and 8 week groups (2WA, 8WA, 2CA, 8CA). A muscle biopsy punch was utilized to cause a defect in the left tibialis anterior (TA) equivalent to 20% of the TA by weight. The defect tissue was then sutured into the site, and the experimental groups continued their respective activities. The TA was harvested 2 and 8 weeks post-VML. Uninjured TA tissue of the VML muscle was isolated, RNA was extracted, cDNA synthesized, and quantitative PCR was performed. Quantitative PCR determined the gene expression of IGF-1, MyoD, MyoG, Ki67, and TGF-β1. **Results:** There was no significant difference in the BW at surgery ($p>0.05$). There was a main effect of exercise to decrease terminal BW by ~6% and ~5% in the 2-week and 8-week rats, respectively ($p<0.05$). There was a main effect of time to increase BW ~7% and ~8% in the sedentary and exercise rats, respectively ($p<0.05$). The % BW difference saw similar results as terminal BW with main effects of time and exercise to increase (~ 42-80%) and decrease (~22-33%) the % body weight difference, respectively. There was a main effect of time to decrease Ki67 mRNA expression ($p<0.05$). There were no significant differences in mRNA abundance of MyoD, MyoG, IGF-1 or

TGF-β1. **Discussion:** Based on the current findings, there are no indications that satellite cell activation is initiated in uninjured tissue distal to the injury 2- and 8- weeks post-injury. However, acute elevation of Ki67 may indicate proliferation of cells other than satellite cells such as fibroblasts which have been shown to be rapidly increased in the presence of VML injuries.

Introduction

Military personnel are vulnerable to severe trauma from explosive devices and weapons. Additionally, civilians are also at risk for traumatic muscle injury due to factors such as tumor removal or automobile accidents. 2010 data shows that upwards of $400 billion is spent yearly in care of traumatic injuries in the United States [3]. Among all extremity injuries, 53% are the result of penetrating soft-tissue wounds which significantly damage muscle tissue and their ability to regenerate [11]. VML is characterized by the loss of tissue and function of skeletal muscle by surgical means, trauma, or lab-induced damage and often results in permanent, functional loss of force. One possible reason for the severity of this type of injury is the improper ability for muscles to regenerate.

Proper regeneration is regulated by skeletal muscle stem cells termed satellite cells. Upon injury, satellite cells proliferate and differentiate into myotubes which fuse into the damaged myofibers. Dormant satellite cells express Pax7, proliferating and differentiating satellite cells express MyoD/Ki67 and MyoG [16]. However, VML has been shown to result in the loss of satellite cells thus contributing to improper muscle regeneration. Incorporation of autologous muscle grafts improves the regenerative response by promoting *de novo* muscle fiber regeneration and functional recovery possibly due to the incorporation of new satellite cells [18].

Exercise has also been shown to be effective within a VML model to enhance satellite cell activation and improve muscle regeneration. Exercise activates upstream regulators of satellite cells (i.e. HGF, IGF-1, FGF, etc.) thereby increasing activation, proliferation and differentiation of these cells. Additionally, exercise promotes damage of the ECM and basement membrane further improving satellite cells' activation and migration. How exercise and incorporation of autologous muscle grafts interact to affect cellular signaling and functional recovery within skeletal muscle is unknown.

This research is the first paper to look at the effect of autologous muscle grafting combined with exercise in the regeneration distal to the VML damage. We chose to look at the distal site because it is the site where the most functional effect would be apparent. The distal end of the muscle is the point of attachment and where the damage would have the most obvious consequence. Schultz et. al. (1985) interestingly described that the damaged environment does not have to be widespread to show regeneration throughout the muscle fiber. Damage at one end of the muscle prompted activation of all the satellite cells along that same muscle fiber and stimulated the migration of the cells to the damaged site [15]. Although it was already known that the satellite cells are stimulated along the length of the muscle fiber post-VML, it was unknown if exercise could prompt improved regeneration compared to a sedentary activity. Our research was looking at how exercise either helps promote or has no effect on the satellite cell activity at the site distal to the area subjected to VML.

Methods

Animals

In this study, we used 24 Sprague-Dawley rats (3-4 months old). Throughout the duration of the study, the rats were permitted access to normal chow and water as they wished.

The rats were in individual cages and placed in a climate-controlled room with an automatic 12-12 hr light/dark cycle. Once the rats reach the needed mass of 300g, their running wheels were unlocked, and the rats were allowed ad libitum wheel access for 72 hours. This allowed for an assessment of baseline running distance. After 72 hours unlimited access to the wheel, the wheels were locked with at least seven days between this time and the VML surgery to forestall any activity benefits. The average distance ran by the rats during the last 24 hours of the three-day course was recorded as the baseline activity.

At the end of the one-week acclimation period following wheel lock, rats underwent VML surgery. Pain management was accomplished via intermittent injections of buprenorphine and administration of Carprofen as conducted in a previous study done by this laboratory [6]. One-week post-surgery, wheels either remain locked (Cage Activity, CA) or unlocked (Wheel Activity, WA) for a period of either one week or seven weeks. Two-weeks post-surgery, tissue harvest will be conducted on 12 rats (n=6 CA, n=6 WA). Eight-weeks post-surgery, tissue harvest was conducted on 12 rats (n=6 CA, n=6 WA). Twenty-four hours before tissue harvest, wheels were locked on the WA rats in order to avoid acute variations in exercise between rats.

VML Surgery

Rats were anaesthetized by inhalation of isoflurane (2% in oxygen) during surgery. A 1-2 cm longitudinal incision was made over the belly of the tibialis anterior (TA) cutting through the deep fascia to expose the belly of the TA. Next, a muscle biopsy punch (approximately 8mm x 3mm) was used to cause a defect equaling approximately 20% of the TA by weight. The weight of the defect was determined using a regression equation based on the rat's body weight as defined by [19]. Once the defect was removed, it was weighed and replaced in the defect site. Polypropylene sutures were used to anchor the defect to the intact TA tissue

using two sutures on both the distal and proximal ends of the defect. Vicryl thread was used to suture the fascia and then the cutaneous layers together to completely close the wound.

Electrophysiology

The following protocol for electrophysiology was adopted from several sources [2, 10]. Rats were anaesthetized by inhalation of isoflurane (2% in oxygen). A transverse incision was made on the proximal, anterior lower limb which exposed the distal tendons of the TA, extensor digitalis longus (EDL) and extensor hallucis longus (EHL). Tenotomy of the EDL and EHL tendons was conducted leaving the TA tendon intact and isolated. The ankle and pad of the foot was secured onto the platform of the electrophysiology machine using surgical tape. Two probes were inserted as described in [10] to adequately stimulate the peroneal nerve which innervates the TA. Once the leg and probes were secured, 100Hz at a 400ms pulse were delivered to induce adequate peak tension. Five measurements were taken with 60 seconds of rest allowed in between each measurement.

Tissue Harvest

The TA was extracted from each leg and weighed. The muscle tissue of the left TA (VML) that is unaffected by the VML injury was collected for gene expression analysis. This tissue was immediately flash frozen in liquid nitrogen and stored in -80°C. Rats were euthanized via either carbon-dioxide asphyxiation or exsanguination by removal of the heart. All collected tissue was stored in -80°C until it was processed for analyses.

RNA isolation, cDNA synthesis, and quantitative real time polymerase chain reaction (RT-qPCR)

RNA was extracted with Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was isolated using the Purelink mRNA mini kit (Thermo Fisher Scientific, Waltham,

MA, USA). cDNA was reversed transcribed from 1 µg of total RNA using the Superscript Vilo cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) for a final result of a 1:20 ratio of RNA to total volume. That final volume was then brought to a 1:100 dilution factor. Real-time PCR was performed, and results were analyzed by using the StepOne Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was amplified in a 25 µL reaction containing appropriate probes (18s, MyoD, MyoG, IGF-1, TGF-β1, and Ki67) and Taqman Gene Expression Mastermix (Thermo Fisher Scientific). Samples were incubated at 95°C for 4 minutes, followed by 40 cycles of denaturation, annealing and extension at 95°C, 55°C and 72°C, respectively. TaqMan fluorescence was measured at the end of the extension step each cycle. Cycle Threshold (Ct) was determined, and the ΔCt value will calculated as the difference between the Ct value and the 18s Ct value. Final quantification of gene expression was calculated using the ΔΔCt method Ct = [ΔCt(calibrator) – ΔCt(sample)]. Relative quantification was then calculated as $2^{-\Delta\Delta Ct}$.

Statistical Analysis

All data was analyzed using Statistical Package for the Social Sciences (SPSS version 22.0, Armonk, NY). Results are reported as mean ± SEM. A two-way ANOVA was performed to analyze main effects of physical activity and time and if there are any interactions between the variables. When a significant interaction was detected, differences among individual means were assessed using Fisher's LSD post-hoc analysis. Statistical significance was set at $p \leq 0.05$.

Results

Morphological data

Morphological data is presented in Table 1. There were no significant differences for body weight at the time of surgery. There was a main effect of exercise to decrease terminal BW by ~6% and ~5% in the 2- and 8-week groups, respectively (Table 1, $p<0.05$). There was a main effect of time to increase terminal BW by ~7% and ~8% in the sedentary and exercise groups, respectively (Table 1, $p<0.05$). A main effect of time to increase percent change of body weight from baseline body weight was seen and a main effect of exercise to decrease body weight compared to the sedentary group within the same time point (Table 1, $p<0.05$). There were no significant differences for the TA defect mass or the LTA mass (Table 1, $p>0.05$). There was no significant difference when comparing the LTA mass as a percent of RTA (Figure 1, $p>0.05$). However, the LTA mass was only able to reach 88.1 ± 6.0 %, 89.6 ± 5.8 %, 85.7 ± 6.8 %, and 81.9 ± 4.9% of the RTA mass for the 2-week sedentary, 2-week exercise, 8-week sedentary, and 8-week exercise groups, respectively (Table 1).

Markers of proliferation and differentiation

Within the 2-week group, there were no differences of mRNA abundance in the exercised and sedentary groups for myoD (Figure 2A, $p>0.05$). Additionally, within the 8-week group, there were no differences of mRNA abundance in the exercised and sedentary groups for myoD (Figure 2A, $p>0.05$). Within the 2-week group, there were no differences of mRNA abundance in the exercised and sedentary groups for myogenin (Figure 2B, $p>0.05$). Within the 8-week group, there were no differences of mRNA abundance in the exercised and sedentary groups for myogenin (Figure 2B, $p>0.05$). There was a main effect of time between the 2- and 8-week groups to decrease mRNA abundance of Ki67 (Figure 3A, $p<0.05$). However, there was no

difference within the time groups of exercise vs sedentary activity on mRNA abundance of Ki67 (Figure 3A, $p>0.05$). Within the 2-week group, there were no differences of mRNA abundance in the exercised and sedentary groups for TGF-β1 (Figure 3B, $p>0.05$). Within the 8-week group, there were no differences of mRNA abundance in the exercised and sedentary groups for TGF-β1 (Figure 3B, $p>0.05$). Within the 2-week group, there were no differences of mRNA abundance in the exercised and sedentary groups for IGF-1 (Figure 3C, $p>0.05$). Within the 8-week group, there were no differences of mRNA abundance in the exercised and sedentary groups for IGF-1 (Figure 3C, $p>0.05$).

Discussion

This is the first paper to look at the effects of exercise combined with autologous muscle grafting on markers of cell proliferation and differentiation distal to the defect site. Earlier research supports that exercise shows improved satellite cell activity at the site of VML, however it remains unknown if exercise affects satellite cell/myoblast activity distal to the injury site [12]. Quarta et. al (2017) discovered that at the damaged site of the muscle, exercise improved the recovery of VML by improving vascularization and decreasing the effects of fibrosis. In that study, they also found that the sedentary rats, had significantly smaller muscle mass and much less force production [12]. It was determined that exercise alone was capable of regenerating the muscle post-VML [12].

Although we know from previous research that exercise has a significant effect on satellite cell activity at the damaged site, this study analyzed how that carries over to tissue distally. The distal tissue is of more importance to researchers than the proximal area because the distal site is where the muscle attaches to the functional unit. If there were to be extensive damage in the muscle, it would be more detectable in the overall function of the muscle in the

distal area. We found that the exercise intervention had no effect on proliferation and differentiation of satellite cells. Interestingly, this contradicts what happens at the defect site and shows that the effect of exercise on proliferation of cell markers must be a localized event. When the muscle tissue is exposed to damage, the satellite cells within the muscle are cued to proliferate and we know from past studies that VML affects the muscle's ability to regenerate and repair which was illustrated by a decrease in myoD expression post-VML [6, 20]. MyoD is a known myogenic regulatory factor for the differentiation of satellite cells and is necessary to allow regeneration to occur [17]. MyoD mRNA abundance is seen when the satellite cell is entering the activation stage of the cell cycle, and myogenin is in abundance during the differentiation stage [17]. Myogenin is responsible for mediating the satellite cell out of the cell cycle and fusing the cell with existing muscle fibers [7]. Myogenin is also important in establishing the muscle fiber characteristics once the satellite cell has combined into the muscle [7]. Additionally, earlier research shows that as a result of muscle damage, satellite cells begin to activate and proliferate as note by the expression of Ki67 [20]. We saw, as shown in Figure 3A, a decrease in Ki67 overtime which would indicate an overall decline in cell proliferation at the defect site. Although, Ki67 is a known proliferative marker, it is not satellite cell specific, so it is crucial to look at other regulatory factors known to be directly related to satellite cell differentiation, such as MyoD and myogenin mentioned above [6, 7, 17, 20]. Therefore, with the data showing no differences in the known satellite cell markers, we predicted that the proliferation of Ki67 in the earlier time point was likely due to other cells, such as fibroblasts, and not satellite cells. This shows that exercise seemed to have no effect on the regenerative capacity of the skeletal muscle located distal to the defect site. As mentioned in earlier research, this doesn't mean that the muscle distal to the VML site is not contributing to the regeneration

[15]. However, it does imply that the capacity of the distal site to aid in recovery may be capped at a certain point because the intervention of exercise was not significant in improving the migration of satellite cells.

Limitations of this study are as follows. There were small group sizes, and that contributed to the resulting high standard errors in the data. Increasing the number of samples would have helped get more precise data and more definite conclusions. Additionally, we used only young male rats to decrease the possible confounding variables, but in future research it could be important to research female rats and rats of different ages. There was also a lack of a baseline control to compare the experimental rats to. There were internal controls within each rat to compare non-VML to VML tissue, however it could be beneficial to see if autologous tissue grafting was effective by having a control with no tissue grafting performed. It would be important to see rats that just had VML performed on them so we could see how exercise and autologous muscle grafting affects the abundance of proliferation and differentiation markers.

Research that still needs to be done is seeing the effect of different types of exercise on satellite cell activation. It could be possible that the wheel activity of the rats in this study was insufficient to stimulate the proliferation of satellite cells and looking at different types of exercise would be important. In addition to the different types of exercise, it would be important to harvest tissue at different distances from the defect site. This would help researchers understand at what point the muscle tissue stops recruiting satellite cells for regeneration which could be useful in human studies as well.

In conclusion, the data shows that the effects of exercise on regeneration is a localized event as illustrated by the lack of a difference in cell proliferation markers. Volumetric muscle

loss affects civilians and military personnel by permanently damaging the muscle tissue and its ability to regenerate. This paper stands alone in the field by being the first study to analyze the tissue distal to VML damage and will continue to show importance to future researchers.

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Table 1

	2 Week		8 Week	
	Sedentary	**Exercise**	**Sedentary**	**Exercise**
TA Defect (mg)	103.5 ± 5.0	104.2 ± 6.1	104.1 ± 4.6	98.6 ± 3.2
LTA Mass (g)	0.56 ± 0.0	0.55 ± 0.0	0.57 ± 0.02	0.59 ± 0.05
LTA Mass % RTA	88.1 ± 6.0	89.6 ± 5.8	85.7 ± 6.8	81.9 ± 4.9

Figure Legends

Table 1. Tibialis anterior defect mass, left tibialis anterior mass, and left tibialis anterior mass as a percent of the right tibialis anterior muscle mass for 2 weeks post-VML and 8 weeks post-VML sedentary vs exercise. Values are means ± SEM.

Figure 1: Percent body weight difference. Main effect of time indicated by “ME Time”. Main effect of exercise indicated by “ME Exercise”. $P<0.05$. n=6 per group. Data are means ± SEM.

Figure 2: mRNA abundance (normalized to 18s) of myogenic regulatory factors A) myogenin mRNA abundance. B) myoD mRNA abundance. $P<0.05$. n=6 per group. Data are means ± SEM.

Figure 3: mRNA abundance (normalized to 18s) of cell proliferation markers A) Ki67 mRNA abundance. B) TGF-β1 mRNA abundance. C) IGF-1 mRNA abundance. Main effect of time indicated by “ME time”. Main effect of exercise indicated by “ME exercise”. $P<0.05$. n=6 per group. Data are means ± SEM.

Figure 1. Percent body weight difference

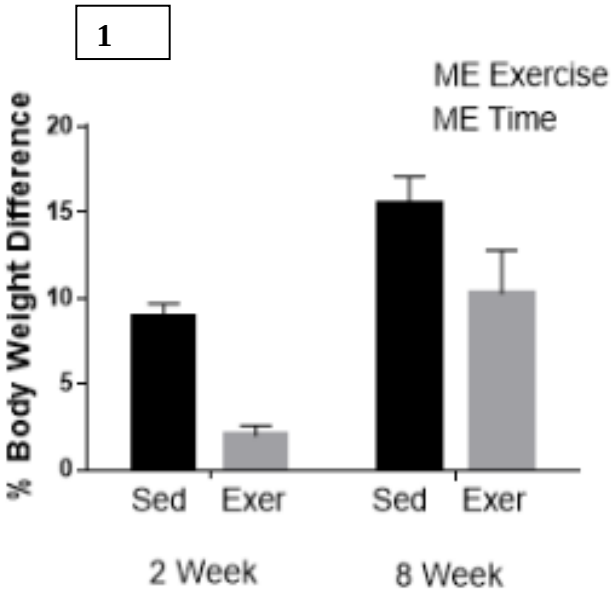
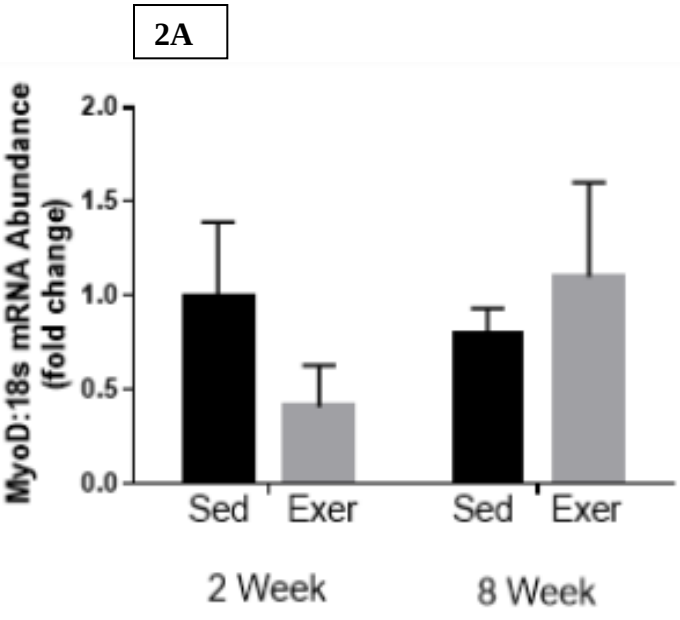


Figure 2. Myogenic regulatory factors



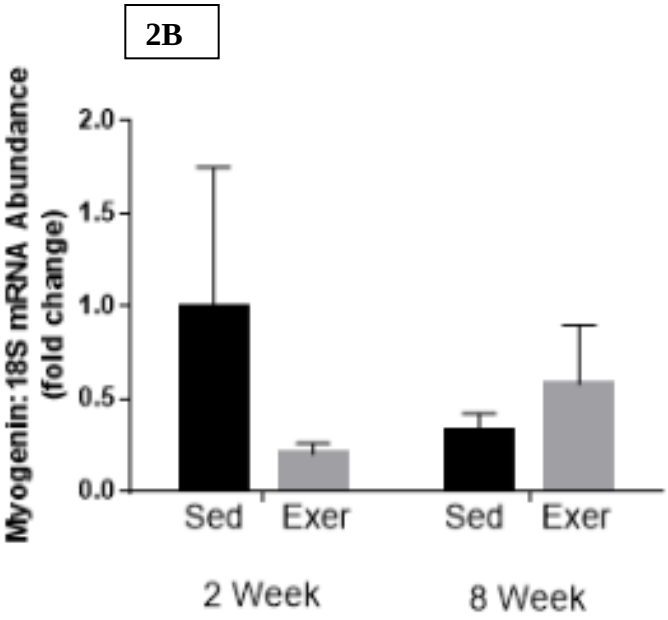


Figure 3. Cell proliferative markers