EFFECTS OF ESSENTIAL AMINO ACID SUPPLEMENTATION AND BLOOD FLOW RESTRICTION TRAINING ON MYONUCLEAR ACCRETION IN SINGLE MUSCLE FIBERS

by

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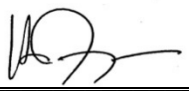
Approved: 
Hans C. Dreyer, Ph.D.

Healthy muscle is characterized in part by its ability to recover from atrophy after surgery, bedrest, or immobilization. The goal of this thesis is to determine if the combined effects of essential amino acid (EAA) supplementation and blood flow restriction (BFR) exercise would upregulate muscle resilience by stimulating muscle stem cell (satellite cell) proliferation to increase myonuclear numbers.

Healthy (22±2) male and female (n = 4 and n = 3, respectively) subjects were randomized to ingest 23 grams of EAA or placebo 3x/day for 7 consecutive days. On days 2, 4, and 6, BFR exercise was performed on the right leg only, followed by supplement (EAA or placebo) ingestion. Bilateral biopsies were obtained on day 8 to determine the impact of supplement alone (left) versus supplement with BFR exercise (right). Single fibers were analyzed using immunohistochemistry to quantify myonuclei (DAPI) per fiber type (MyHC 1). Analysis was performed in a blinded manner (treatment condition) using ImageJ software.

We did not have a large enough sample size to produce significant results. We will be presenting the raw data from the analyses we have performed this far, and plan to continue the study into next year in hopes of producing significant results thanks to a larger sample size.

Acknowledgements

I would first like to thank the Clark Honors College for being such a wonderful institution here at the University of Oregon. The thesis process is what led me to my current research lab, as well as to my post-graduation and career plans of continuing to do research on muscle cells. Beyond the thesis, the CHC has introduced me to many wonderful people and has really shaped who I am as a person, student, and researcher today. I would like to specifically thank Dr. Angela Rovak, who has been my advisor, professor, and supervisor during my time in the CHC. Thank you for always being there for me, and for helping me see in you the person that I hope to someday be myself.

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Introduction

Healthy muscle is central to maintaining human health across the lifespan. Skeletal muscle makes up approximately 40% of total body weight and contains 50% to 75% of all body proteins.[1] In this context, skeletal muscle can be viewed as a storage medium of amino acids, the building blocks of protein, and much like fatty acids that are stored as triglycerides and sugars that are stored as glycogen these substrates are constantly being broken down and resynthesized. This dynamic cycle of breaking down and building back of proteins, fats, and sugar is under continuous micro- and macro-adjustments incorporating cues from hormones, mechanical and physiological forces, and the environment—including exposure to light and dark.

The musculoskeletal system is a dynamic tissue made up of contractile fibers, connective tissues, blood vessels, nerves, and immune cells. In all, approximately 10 different cell 'types' reside within muscle tissues.[2] The central nature of the muscular system to so many of the body's most critical functions makes it an area that has been extensively researched, but that also constantly gives rise to new questions about how our muscles work and how we can better prepare them for recovery after injury, atrophy, or disuse. Our work to better understand skeletal muscles will hopefully lead to better outcomes for a wide range of people, from elderly individuals recovering after surgery to young athletes bouncing back from injury.

Our research group focuses on using treatment methods and techniques designed to prevent muscle loss and accelerate the return of mass and strength following atrophy in humans. Muscle disuse occurs with illness, injury, following surgery, and during bed rest or immobilization. In each instance, including in healthy adults, this leads to muscle atrophy, reduced strength, increased fatigue, impaired insulin sensitivity, and anabolic resistance to protein ingestion.[3-8] Muscle disuse atrophy occurs rapidly, with the rate of muscle loss being

greatest in the first few days; acute loss has significant clinical implications because the average length-of-stay in hospital is <7 days. [8-11] In the past, our research group has focused specifically on elderly total knee arthroscopy (TKA) patients for this reason. Thus, identifying interventions that prepares muscle for atrophy-associated life events will have an immediate and broad clinical impact.

Muscle atrophy from surgery, bed rest, or immobilization is now viewed as a significant contributor to sarcopenia in older adults' because each time atrophy occurs, recovery is incomplete.[12,13] Sarcopenia is defined as "a progressive and generalized skeletal muscle disorder implicating accelerated loss of muscle mass, strength, and function" and is generally associated with aging.[14] In the U.S. mitigating sarcopenia by as little as 10% can save an estimated $1.1 billion per year.[15] Hospital costs for persons with low muscle mass in 2020 were estimated at $40.4 billion.[16] Globally adults ≥65 years are projected to double from 703 million to 1.5 billion by 2050, indicating a need for active research into this issue.[17]

This thesis project is specifically designed to test a model of combined therapy that will directly address this problem, and a central feature of our approach hinges on a concept referred to as "muscle memory." (**Figure** 1) Muscle memory describes a previously resistance trained muscle re-acquiring strength and size following atrophy faster than an untrained muscle. Muscles are one of the only cell types that is multi-nucleated, which contributes to their unique function. At the cellular level, the "memory" is stimulated by these myonuclei, gained during resistance training, being immediately available at the start of recovery to re-synthesize lost proteins and accelerate the *full* return of muscle cross-section and strength.

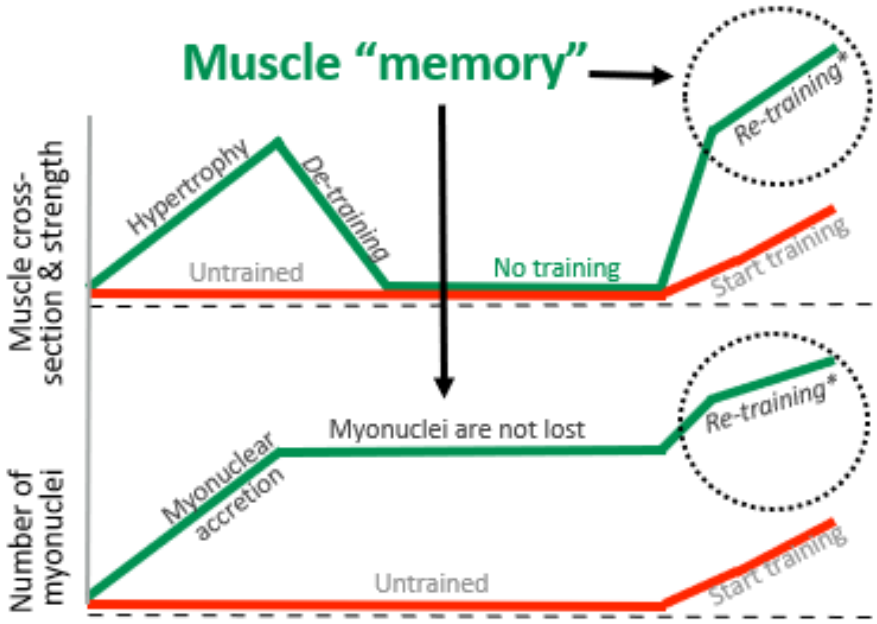

Figure 1: Concept of Muscle Memory

Diagram depicting the concept of muscle memory, in which myonuclear accretion allows a previously trained fiber to recover more quickly after detraining.

Adapted from: Gundersen 2016[18]

In many instances, especially in older adults, the recovery of mass and strength is incomplete even under ideal circumstances. Thus, increasing myonuclei prior to atrophy will maximize recovery—like planting seeds of DNA to promote re-growth. We have two independent treatment modalities that can stimulate muscle resident stem cells (satellite cells) to produce these new nuclei. First, low-load blood flow restriction (BFR) exercise, performed at 20% of a 1-repetition maximum (1RM), allows older adults, including those with painful osteoarthritic knees to achieve gains in strength and mass identical to traditional *'heavy'* resistance exercise (heavy refers to the loads being at or above 70% of 1RM). Second, is essential amino acid supplementation (EAAs), which we have shown in two clinical trials

reduces atrophy and accelerates the return of functional mobility.[19,20] Interestingly, each modality (EAA & BFR) stimulates satellite (stem) cells, and our preliminary results show that this translates to increased myonuclei and, importantly, performance.

Our long-term goal is to understand how myonuclear accretion can be manipulated for pre-habilitation purposes to better prepare muscle before atrophy occurs. The objective here, which is our next step in pursuit of that goal, is to determine the factors that regulate muscle resilience with a focus on myonuclei accretion and to determine mechanisms of action. Our central hypothesis is that the combined effects of EAA supplementation and BFR exercise are additive in stimulating myonuclear accretion and promoting muscle resilience as measured by increases in muscle cross-section and strength both at the whole muscle (quadriceps) and single fiber level. Our hypothesis has been formulated on the basis of our previous clinical work, mentioned above, highlighting our novel findings that EAAs have an impact on satellite cells associated with both Type I and II fibers, and have an impact on resident macrophage populations in favor of EAA (vs. placebo). Relative to this proposal, secondary analysis revealed increases in central and myonuclei, providing proof-of-principal that EAAs promote resilience. Increasing the EAA dose to 3x/day for 7 days lead to similar increases in satellite cells (in Type I and II fibers), central nuclei, and a trend ($p=0.062$) for more myonuclei vs. placebo, in four young adults. Thus, our published data point to EAAs contributing to muscle resilience and both our preliminary and published data suggest that the mechanism(s) involve satellite cells and myonuclei as being a key component; what is not known, however, is the magnitude and persistence of effect.

Overall, this work advances our mechanistic understanding of myonuclear accretion—using a combination therapy that is well tolerated, easy to use, and broadly applicable. With

respect to expected outcomes, this work will help to identify key pathways modulating myonuclear accretion and contributions from satellite cell activation on muscle resilience. Second, this body of work seeks to help identify mechanisms responsible for muscle resilience that can be exploited by future interventions, to mimic or enhance muscle resilience and recovery. Last, this work will help establish therapeutic modalities that target muscle resilience by promoting nuclear accretion in men and women and promote human health across the lifespan.

In this honors thesis, we aimed to induce elevated levels of myonuclei in our subjects using EAA supplementation as well as BFR strength training. By testing these interventions in our study participants, we gleaned valuable information about how EAA supplementation and BFR training work both separately and in tandem to influence myonuclear accretion in single muscle fibers. These findings can be used to develop better procedures for elderly patients both pre- and post-surgery, as well as for a wide range of individuals that want to increase their muscles' capacity to respond to atrophy.

Background

Skeletal muscle structure and function

The fibrous proteins actin (a thin filament) and myosin (a thick filament) make up skeletal muscle, and together they use adenosine triphosphate (ATP) to cause muscle contractions.[21] Contractions are the result of ATP hydrolysis; this conformational change of ATP into ADP allows the myosin head domain to bind to actin, sliding the actin filament along the myosin filament and shortening the muscle fiber. These thin and thick filaments come together to form a single muscle fiber, bundles of which group together to form fascicles. Groups of fascicles are what form whole muscles. (**Figure 2**)

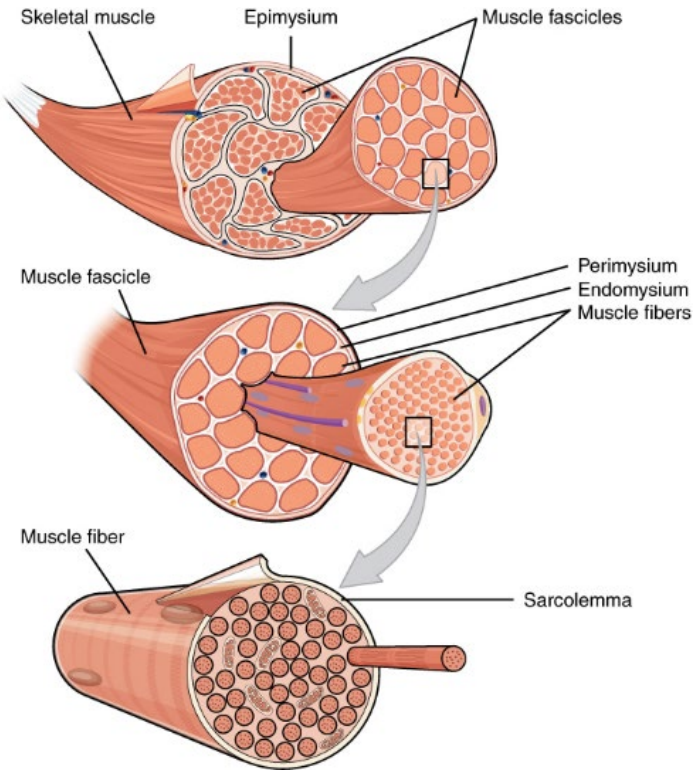

Figure 2: Skeletal Muscle Structure

Source: Lumen Learning[22]

Muscles fibers are characterized as either fast twitch (type II) or slow twitch (type 1). These speeds refer to the speed of contraction, or how quickly the actin and myosin slide past each other to shorten the fiber.[21] Type II fibers are used for short, explosive movements such as power lifting and sprinting, while type I fibers are used for endurance exercises such as distance running and cycling. The ratio of type I to type II fibers, and the amount of each fiber type present in a certain muscle, may change and adapt with the type of exercise that muscle is doing, allowing for greater plasticity of the muscular system.[23]

Each individual muscle fiber is innervated by a singular motor neuron at the neuromuscular junction; neurons transfer electrical signals from the somatic motor division of the nervous system to the muscle fibers, letting them know when to contract (i.e., activate the actin and myosin filaments that drive muscle contractions). These muscle contractions are what enable us to walk, eat, breathe, maintain our posture, stabilize our joints, and do so many of the things that allow us to be functioning, independent members of society.

Muscle atrophy, sarcopenia, and total knee arthroscopies

The incredible adaptability of the muscular system is one of its greatest advantages; this does mean, however, that muscles respond to disuse as much as they respond to training. Atrophy can occur in all tissues within the body, and is defined as "a decrease in the size of a tissue or organ due to cellular shrinkage; the decrease in cell size is caused by the loss of organelles, cytoplasm, and proteins."[24] In muscle specifically, proteolytic systems (systems that break down proteins and peptides into amino acids using enzymes) are activated in response to atrophy, removing contractile organelles and proteins that result in muscle fiber shrinkage.[24]

Two main systems regulate muscle atrophy: the ubiquitin-proteasome system and the autophagy-lysosome systems. The ubiquitin-proteasome system involves three main classes of

enzymes: E1, E2, and E3. Ubiquitin, a single-chain polypeptide involved in the degradation of proteins, is activated by E1 enzymes after the cleavage of ATP. It is next moved to the E2 enzymes, and then E3 enzymes bind to both the E2 enzyme and the protein substrate that is to be degraded, transferring the ubiquitin from enzyme to the substrate. Once the substrate is polyubiquitylated, it is docked to the proteasome, a protein complex containing proteases that will break down the substrate tagged by ubiquitin.[24]

The autophagy-lysosome system involves the transfer of autophagic cargo to lysosomes, a cellular organelle that contains digestive enzymes and is used to break down proteins. Autophagy is literally "self-eating," which is exactly what the cell does as it uses its own organelles to degrade other parts of itself. There are three different mechanisms for delivering autophagic cargo to lysosomes: macroautophagy, in which ubiquitin-labeled cargo is isolated within a membrane called the autophagosome and then transferred to the lysosome; microautophagy, in which small portions of the cytoplasm are directly absorbed by the lysosome; and chaperone-mediated autophagy (CMA), in which damaged proteins expose a specific amino acid sequence that results in the chaperone protein delivering them to the lysosome through interactions with Lamp2a receptors.[24] It is important to remember that while autophagy is the breakdown of proteins and can result in muscle atrophy, it is still a crucial waste-removal process that is required for healthy cellular function throughout the body.

In these ways, the ubiquitin-proteasome and the autophagy-lysosome systems, which under normal conditions facilitate turnover of older 'damaged' proteins with no net loss, result in muscle atrophy under pathophysiological conditions such as disuse or denervation. These degradative processes are triggered by changes in muscle activity: the less a muscle is used, the more upregulated these systems become, while increased use of a muscle will downregulate

these systems. The maintenance of muscle size is a delicate balance between protein synthesis and protein breakdown, which can lead to detrimental outcomes if unregulated. (**Figure 3**)

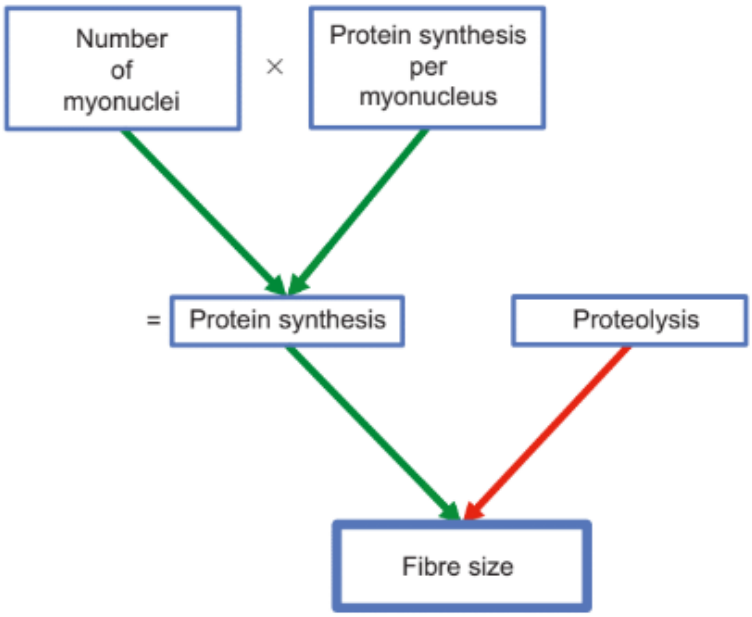

Figure 3: Contributors to Muscle Fiber Size

Source: Gundersen 2016[18]

Sarcopenia

Sarcopenia is a case in which that delicate balance between synthesis and breakdown has been dysregulated. As described in the introduction, sarcopenia is a progressive muscle disorder associated with aging that results in the loss of muscle mass, strength, and function. Muscle mass decreases at an annual rate of 1-2% after the age of 50, and muscle strength decreases 1.5% annually between the ages of 50 and 60, increasing to 3% per year after the age of 60 is reached.[25] These changes in muscle mass and strength lead to increased risk of falls, which can be especially dangerous for elderly patients. Not only can the fall itself result in injuries, but those suffering from sarcopenia will struggle to ever return to baseline muscle health after atrophy or damage has occurred. Pathophysiological changes involved in sarcopenia include

denervation of muscle fibers, net conversion of type II fibers to type I fibers, and the decline of muscle stem cells, or satellite cells. Altogether, these issues impact the ability of elderly muscle to respond to damage and disuse, leading to accelerated muscle atrophy in older populations. (**Figure 4**)

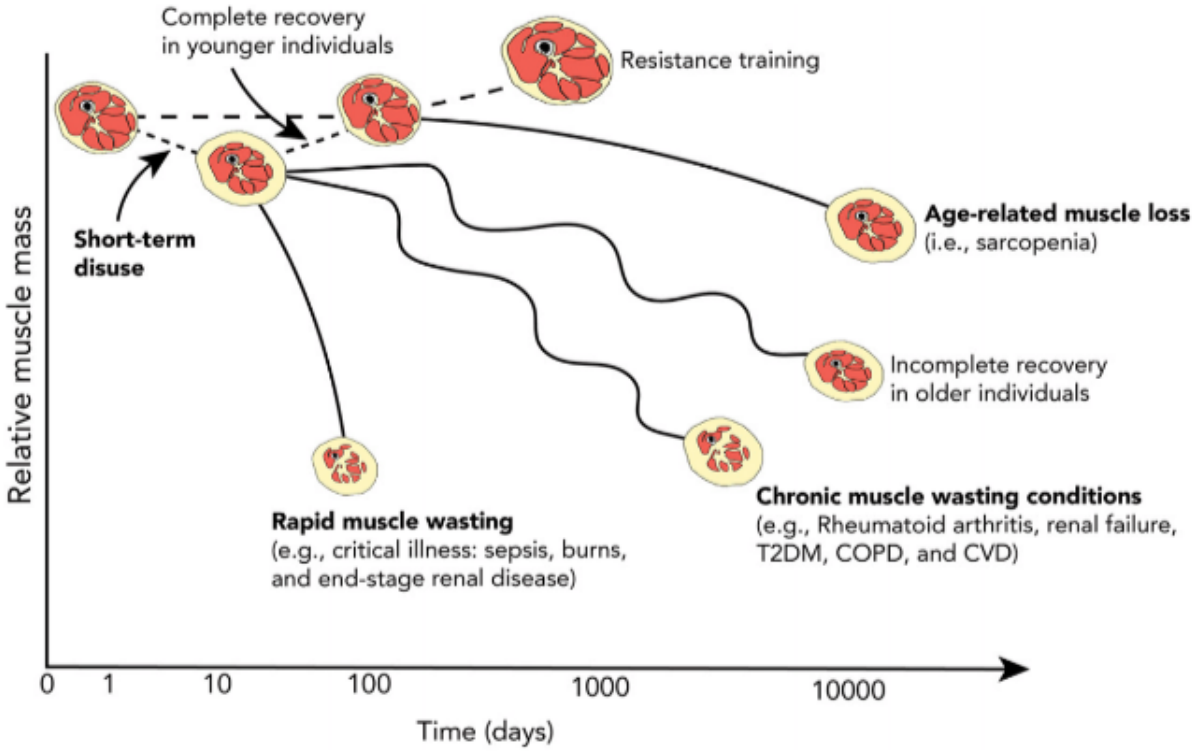

Figure 4: Timeline of Skeletal Muscle Atrophy Due to Different Underlying Causes

Source: Nunes et al. 2022[13]

Total Knee Arthroscopies (TKAs)

As a muscle physiology lab, our primary objective revolves around investigating the physiological aspects of damaged muscle and devising effective strategies to mitigate associated complications. Specifically, our focus has primarily centered on a specific subgroup of elderly individuals who undergo a prevalent surgical intervention known as total knee arthroscopy (TKA). In the past, we have focused on one elderly population in particular: those undergoing

total knee arthroscopies, or TKAs. This surgical procedure involves the complete replacement of the knee joint and is the most effective intervention to eliminate chronic knee pain, for example from osteoarthritis. Despite its well-established nature, TKAs are still accompanied by unwanted detrimental effects on the surrounding musculature, in particular muscle atrophy and strength loss. Additionally, the large overlap in patients suffering from sarcopenia and those having TKAs makes this demographic a particularly pertinent cohort for our research. The hope is that by implementing our results-based therapies, these patients will spend less time in the hospital, recover more quickly, and, more importantly, recover more completely—helping to improve quality of life at the end of the lifespan.

Clinical trials previously completed by our research group have shown that supplementation with EAAs results in attenuation of muscle atrophy is TKA patients.[19] In fact, those receiving the EAA supplement had a 2- to 4.6-fold reduction in muscle atrophy when compared to the placebo group 6 weeks post-surgery.[19] This was quantified using an MRI to assess muscle and adipose tissue volume, as well as muscle and functional strength testing, meaning that any mechanistic outcomes relating to satellite cells and myonuclei within the actual muscle fibers have not been investigated.

This thesis aims to remedy this by looking directly at myonuclear numbers as well as fiber size and type. Additionally, we tested the effects of BFR training, which had not been included in previous studies. Although this research was not directly done on the target population of TKA patients, it is necessary to first test these other aspects of treatment in a more accessible treatment group before applying them to a larger clinical trial, involving elderly TKA patients.

Satellite cells and myonuclei

Satellite cells are defined as "mononucleated stem cells with myogenic potential located under the basal lamina of myofibers [muscle fibers] but possessing their own plasma membrane, distinct from the plasma membrane of the myofibers."[26] These special cells have been generally accepted as having a central role in muscle regeneration, allowing the muscle to repair and grow. In adult skeletal muscle, satellite cells are normally mitotically quiescent until called upon: muscle damage will wake up these sleeping cells and cause them to undergo divisions resulting in either undifferentiated cells that will replenish the bank of satellite cells, or differentiated myoblasts that will form new muscle fibers.[26] In this way, muscles are able to not only repair themselves in response to damage, but also hypertrophy beyond their original size in response to training or overuse of the muscle.

Part of this important role of myogenesis is contributing myonuclei to the muscle fiber. Myonuclei are nuclei specific to muscle tissue, and are usually located at the periphery of muscle fibers in the space between contractile myofibrils and the cell membrane.[27] The satellite cells that differentiate into myoblasts and fuse with existing muscle fibers result in new myonuclei; it has been generally accepted that fusion of satellite cells is the main (and only) contributor of myonuclei to muscle fibers, because myonuclei were understood to be post-mitotic.[27,28] Recent research in mice has shown, however, that myonuclei are actually *not* post-mitotic, and may be duplicating their DNA through endoreplication.[29] These findings open another avenue for DNA to be added to the existing muscle fiber—and in this case not by increasing the number of myonuclei.[29]

Muscle Memory and Myonuclear Domain

The number of myonuclei plays a critical role in determining the functional capacity of

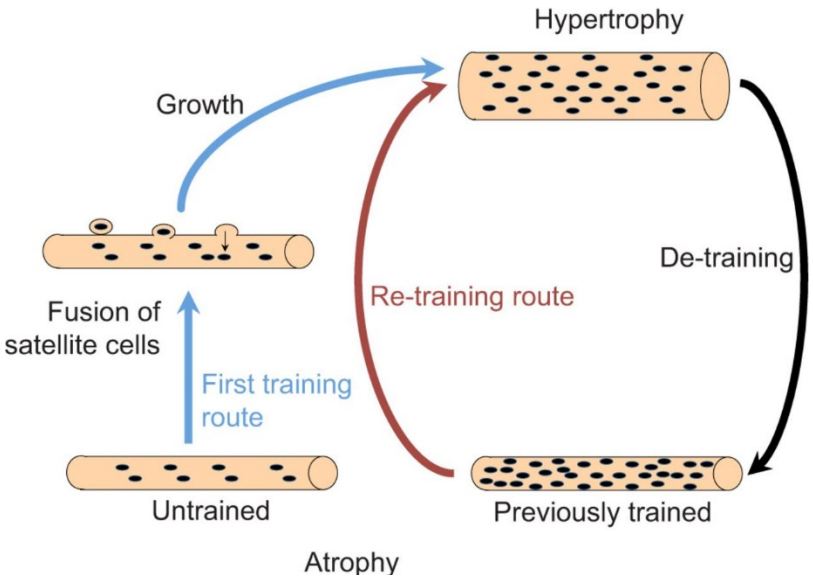

Figure 5: Model of Muscle Resilience through Myonuclei

Source: Gundersen 2016[18]

muscle fibers as it directly influences the DNA available for gene transcription.[28] Additionally, there is evidence that myonuclei that are added to the muscle fiber during hypertrophy will persist in the muscle and not be lost during atrophy, accelerating muscle recovery. which may be key in promoting full recovery of muscle mass and strength after surgery in older adults, for whom recovery of strength and mass is incomplete.[13,30] (**Figure 4, Figure 5**) The increased speed of recovery stemming from having more nuclei at the start of recovery has been described as "muscle memory," which means that "even after short-term environmental stimuli, skeletal muscle can retain molecular information in order to be primed for future plasticity following encounters with the same stimulus."[31] (**Figure 1**) For this reason, increasing myonuclear numbers could be a beneficial way to help prepare muscles for trauma, damage, or

immobilization. In older adults, having more nuclei before surgery and the subsequent atrophy may be the difference between partial and full recovery.

This goes hand in hand with the myonuclear domain theory, which was first proposed by Cheek *et al.* in 1985.[32] According to this concept, each myonucleus is responsible for regulating a certain amount of muscle fiber cytoplasm through gene expression and protein production. As myonuclear density increases, the myonuclear domain diminishes; alternatively, if myonuclei are removed from the fiber, the remaining myonuclei are responsible for a greater domain. Muscles that increase their domain also exhibit a loss of force, emphasizing the importance of myonuclei.[26] Both myonuclei and the satellite cells they stem from are crucial for muscle regeneration, repair, and growth, contributing the necessary DNA transcripts that will determine which, and how many, proteins are made and circulated throughout the muscle fiber.

Muscle regeneration, regrowth, and hypertrophy through satellite cells and myonuclei

The full function of satellite cells, and the pathways through which they function, are incredibly complex and still debated within the field. For example, in their 2011 study entitled, "An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration," Lepper *et al.* determined that "elimination of Pax7+ cells [satellite cells] completely blocks regenerative myogenesis."[33] Conversely, a 2012 study by Jackson *et al.* entitled, "Satellite cell depletion does not inhibit adult skeletal muscle regrowth following unloading-induced atrophy" found that "myonuclear domain size was reduced following suspension due to decreased cytoplasmic volume and was completely restored following reloading, independent of the presence of satellite cells."[34]

Although at first glance these findings may seem to contradict each other, upon further inspection they suggest that satellite cells may be needed following muscle damage, but not

required following recovery from muscle atrophy. This exemplifies the multitude of responses that satellite cells are involved in, each different depending on what kind of changes are occurring within the muscle.

Muscle regeneration and repair following injury

Following acute muscle injury, the process of muscle regeneration begins and occurs in three phases: distraction, repair, and remodeling.[28] First, injured muscle cells undergo necrosis in response to the trauma that they have undergone, releasing their cellular contents into the extracellular space through damaged membranes. This triggers the recruitment of a host of immune cells, which take on tasks such as clearing damaged muscle fibers and attracting satellite cells to the site of damage.[35] Upon activation, satellite cells proliferate and differentiate into new muscle fibers, which then mature and undergo a remodeling process as they are incorporated into the existing muscle.[35] New myonuclei are also contributed to the fiber upon satellite cell fusion.

In the case of acute muscle injury, it appears that Pax7+ cells (muscle satellite cells) are indeed required to repair and regenerate the damaged muscle. Pax7 is a known marker of satellite cells. To test this, Lepper *et al.* eliminated Pax7+ cells *in vivo* by inducing expression of the diphtheria toxin, and subjected the tibialis anterior (TA) muscles of mice to injury through injection of cardiotoxin.[33] They found that in "control animals, muscle regeneration was clearly evident 5 days after injury, as demonstrated by the presence of small myofibers with centrally located nuclei… By contrast, none was detected in the injured area of the TA muscle of tmx-treated [tamoxifen] mice [mice without satellite cells],"[33] providing strong support for regeneration to be reliant on the presence of satellite cells. For example, the authors conclude, "in the absence of Pax7+ cells, there is a complete lack of myogenic differentiation during the

initial wave of muscle regeneration," emphasizing the central role of satellite cells following muscle injury.[33]

Muscle regrowth following atrophy

Muscle atrophy is accompanied by decreased protein synthesis and increased protein degradation. The regrowth of atrophied muscle comes with increased protein synthesis, and interestingly, also an increase in protein degradation, probably in order to clear out unusable, damaged, or old proteins.[35] Although it was previously accepted that regrowth must come with satellite cell and myonuclear proliferation, as it does in response to muscle damage, recent research has disputed this fact, implying that the response of muscle to atrophy is distinct from other models of muscle responses.

In a study published in 2012, Jackson *et al.* showed, using a mouse model, that muscle was able to fully regrow following atrophy regardless of the presence or absence of satellite cells, indicating that "satellite cells are not required for muscle regrowth following atrophy and that, instead, the myonuclear domain size changes as myofibers adapt."[34] This was supported by the fact that myonuclear number did not decrease during the atrophy period, meaning that when regrowth occurred, the existing myonuclei were able to suport the elevated need for new proteins for the increasing muscle mass without requiring any additional myonuclei or satellite cell fusion. These findings demonstrate the distinct response of skeletal muscle to atrophy as it returns to baseline mass and strength, as opposed to situations of muscle damage or hypertrophy.

Muscle hypertrophy

Muscle hypertrophy is another, separate process as it involves increasing muscle strength and mass beyond baseline levels. Hypertrophy is usually characterized by satellite cell fusion and myonuclear accretion in response to training, similar to the response of muscle to damage. In this

case, however, satellite cell fusion and myonuclear increases allow the muscle to grow, rather than simply repairing a damaged muscle fiber. The 2013 study done by Fry *et al.* supports the idea that satellite cells mediate muscle hypertrophy, finding that satellite cell-depleted muscle had attenuated myonuclear accretion, fiber regeneration, and muscle hypertrophy, as well as decreased whole-muscle function.[36]

Essential amino acids and blood flow restriction

Although not fully understood, the mechanisms behind muscle repair, regrowth, and hypertrophy are well-enough characterized that researchers have begun to question how they can best influence these pathways in order to get the desired outcomes. This thesis focuses on two potential strategies, looking at them both separately and in tandem to dissect what impact they may be having on muscle: EAA supplementation and BFR training.

Essential amino acid supplementation

Amino acids are the building blocks of proteins, which carry out the majority of functions throughout the entire body, including skeletal muscle. As components of proteins, and free-form within the body, amino acids have three main functions: nutritional, sensory, and biological regulation.[37] Essential amino acids (EAAs) are especially crucial as the body cannot synthesize them itself; instead, they must come from the diet.

EAAs are primarily used to synthesize proteins and as an energy source within skeletal muscle. Additionally, they have the important role of stimulating protein synthesis by activating translation.[37] They also activate the mTORC1 pathway, which inhibits autophagy and promotes cell growth. For these reasons, it has been concluded that supplementation with EAAs may work to suppress atrophy and upregulate muscle regrowth and hypertrophy.

As mentioned earlier, the principal investigator in our lab performed a study in 2013 on elderly TKA patients to test the efficacy of EAA supplementation as it pertains to muscle atrophy. Results from this study showed attenuated muscle atrophy in patients receiving EAAs as compared to the placebo, and although they did experience some muscle atrophy, the treatment group also exhibited greater functional mobility after surgery.[18] A follow up trial was also performed in 2018 that replicated these findings, showing that the EAA group had significantly less atrophy in both their quadriceps and hamstrings as compared to the placebo group.[19]

These findings indicate that EAA supplementation does in fact attenuate muscle atrophy. Our goal with this thesis was to determine whether they may be doing this by increasing myonuclei within the muscle fibers, thereby decreasing the myonuclear domain and upregulating protein synthesis. A better understanding of exactly *how* EAAs attenuate muscle atrophy will contribute to our knowledge of how muscles work and respond to amino acids.

Blood flow restricted strength training

Strength training is a very effective way to build muscle and prevent atrophy, but may not be accessible for elderly patients who are struggling with their health in various aspects. Therefore, finding ways to make smaller amounts of strength training more effective could be a helpful treatment option for elderly patients looking to increase their muscle mass.

Studies have shown that BFR exercise stimulates mTORC1 signaling and muscle protein synthesis in older men, and "may enhance muscle rehabilitation to counteract sarcopenia."[38] One study done in intensive care units found that BFR "seems to be a valid strategy to reduce the magnitude of the rate of muscle wasting,"[39] while another study found that BFR resulted in "increases in several measures of self-reported function… ROM [range of motion]… and reductions in knee joint pain" when compared to traditional resistance training.[40] Evidence shows

that incorporating BFR could be a way to make smaller amounts of strength training just as effective as larger amounts of traditional strength training. We wanted to gauge the effects of this treatment on myonuclei in single muscle fibers, and to see the combined effects of this treatment with EAA supplementation.

Single fiber approach

Much of the previous research into satellite cells and nuclei has been done cross-sectionally (**Figure 6**). With this approach, the muscle fibers are oriented perpendicularly, and a thin cross-sectional slice (7µm to 10µm) is taken from the tissue. This allows for a sampling of

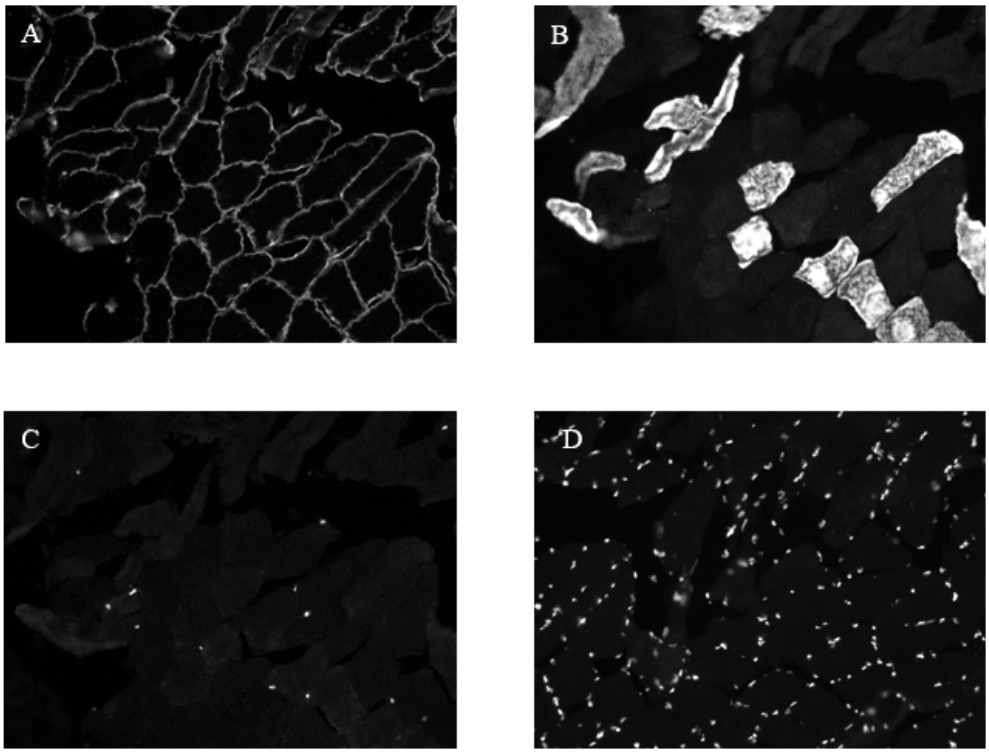

Figure 6: **Cross-sectional Images**

Images depicting the cross-sectional approach. A thin slice is taken through the whole tissue sample, so many cells can be seen top-down at the same time. A) Staining for Laminin B) staining for fiber type (MYHC) C) Staining for satellite cells (Pax7+) D) Staining for myonuclei (DAPI)

many cells – 200 or more for each biopsy timepoint – and greatly increases the number of

individual muscle cells that can be included in the statistical analysis and, therefore, improves dramatically, the confidence in our results. Using immunohistochemistry, nuclei and satellite cells can then be counted around the perimeter of each muscle cell, and fiber type and cross-sectional area of each cell can be determined from cross-sectional analysis as well. The disadvantage of this approach, however, is that it only shows a very thin (approximately 7-8 micrometers) slice of each cell, making it difficult to characterize each muscle cell as a whole. This is important for our purposes as information about myonuclear number, shape, and size may be lost as the view is top-down on to each cell rather than longitudinal.

To overcome this problem, we employ the single fiber approach (described in detail in the Methods section, above), which provides significantly more information about a single muscle fiber (**Figure 7**). The disadvantage here is that less information is gleaned about the muscle tissue as a whole, as work is done on one muscle fiber at a time. The tradeoff is that on a

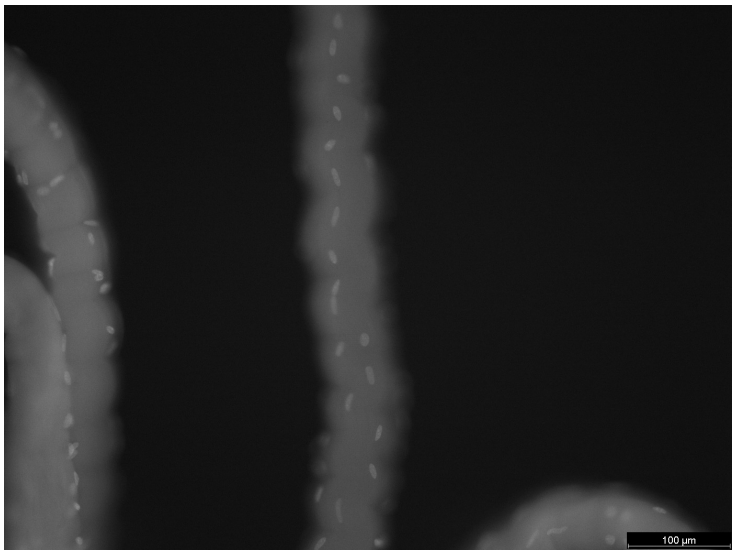

Figure 7: Single Fiber Image

Image showing the single fiber approach at 20x magnification. We can see the shape of the fiber, size and shape of myonuclei, and where myonuclei are located along the fiber.

per fiber basis, the single fiber approach yields superior data and, critically, a true readout of our treatments when our question is, “how many nuclei does each muscle fiber have?” Instead of looking at many fibers top-down, the single fiber approach looks at one entire fiber longitudinally, giving us much more information about the fiber itself. This allows us to better characterize things like shape of the fiber, size and shape of the myonuclei, and where the myonuclei are located along the muscle fiber.

It is also a very useful way to corroborate cross-sectional findings, making sure that the number of myonuclei per millimeter of muscle fiber is exactly accurate. For the cross-sectional approach, that kind of measurement would have to be extrapolated from the thin slices of cells being analyzed. Because one portion of our lab is working on this data cross-sectionally, this thesis used the single fiber approach in order to improve our accuracy in nuclei counts.

Methods

Study Design

Healthy (22±2 years) male and female (N = 4 and 3, respectively) subjects were randomized to ingest 23 grams of EAA or Placebo 3x/day for 7 consecutive days. On days 2, 4, and 6, a single leg (right leg) BFR exercise was performed followed by supplement (EAA or Placebo) for that day. This resulted in 4 treatment groups total: placebo only (left leg), placebo + BFR (right leg), EAA only (left leg), and EAA + BFR (right leg). In this way, we were able to test each treatment both alone and in combination, along with a complete control that received no treatment at all (placebo only). Bilateral biopsies were obtained on day 8 to determine the impact of supplement (left) vs. EAA/Placebo+BFR (right). (**Figure 8**)

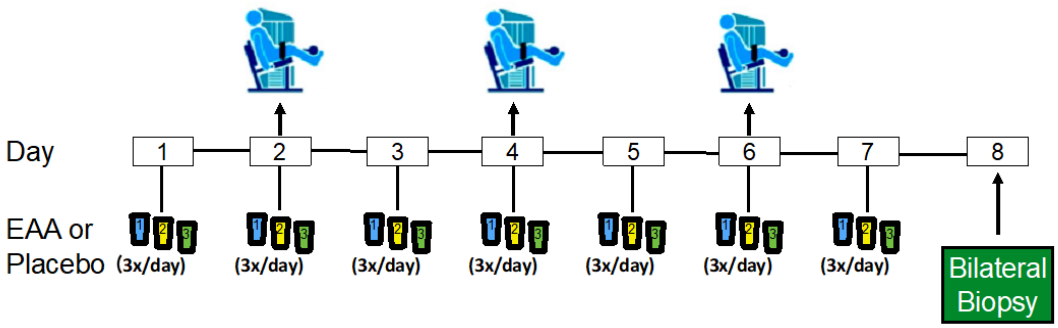

Figure 8: Study Schematic

Schematic depicting the protocol for subjects across 8 days of the study.

Subjects were asked to consume a supplement (EAA or placebo) 3 times per day for 7 days: in the morning before breakfast, at noon before lunch, and in the evening before dinner. On days 2, 4, and 6, subjects came into the lab in the morning before their first supplement dose and performed BFR exercise on their right leg only. The sets and repetitions for the BFR exercise

were set at 20% of the subject's one-repetition maximum (1RM), and they performed 4 sets of 30, 15, 15, and 15 seated leg extensions. Each set was separated by 60 seconds of rest. The BFR tourniquet was inflated during the entire exercise, about 4-6 minutes total. Individuals of child bearing potential were also asked to take a urine pregnancy test on days 1 and 8.

On day 8 of the study, the subjects came in for a bilateral biopsy. Part of the sample gained from the biopsy went to cross-sectional analysis, while another piece went to single fiber analysis, the process of which is described here.

Tissue acquisition (biopsy)

The skin of the mid-thigh is shaved and sterilized with chlorhexidine gluconate (Chloraprep®), then overlaid with a sterile, fenestrated drape. The biopsy region is then anesthetized with 6-10cc of 1% lidocaine HCL injected subcutaneously and down to the level of the fascia; wait 2-3 minutes for the lidocaine to take effect. Make a small (5-6mm) incision through the skin and down to the level of fascia in the anesthetized region using a sterile disposable Feather point #11 scalpel. Insert a sterile biopsy needle (5mm internal diameter) into the skin, through the fascia, and into the muscle belly. Draw the inner cannula out and apply suction using a 60cc syringe attached to the end of the inner cannula biopsy needle to create negative pressure. After the muscle tissue section (100-200mg) is drawn into the Bergstrom cannula via suction and cut with the razor edge of the inner cannula, withdraw the biopsy needle. The muscle tissue should be visible in the window of the Bergstrom cannula and can then be forced out by applying positive pressure to the 60cc syringe. Collect the muscle tissue in a sterile cover pad (do not use gauze).

After the muscle tissue has been collected, place direct pressure on the biopsy site for approximately 10 minutes to stop the bleeding. After bleeding has stopped, use Benzoin tincture

to encircle the incision site in a 6 cm diameter around the site. Use three individual steri-strips to close the incision, either parallel to each other or in an asterisk formation. Place a small square (3cm by 3cm) of gauze on top of the steri-strips and place a Tegaderm over the top of the gauze.

Sample preparation

Place a portion of the muscle biopsy (15-20 mg) into an Eppendorf tube filled with ~700 µm of 4% paraformaldehyde (PFA). Incubate the sample in 4% PFA for 2 days at room temperature to achieve fixation. Make sure the entire sample is submerged in the PFA. After fixation, samples should be placed in 1x PBS in a 0.65ml microcentrifuge tube labeled with narrow gauge tape until muscle can be processed.

Sample processing

When ready to process the samples, place the muscle in a 3cm diameter glass saucer with the volume of PBS from the vial to keep samples from drying during separation. The goal is to strip the muscle biopsy into smaller bundles of muscle fibers. To strip bundles, peel strips of muscle bundles from the muscle (much like peeling off a band aid). These bundles should be about 30 fibers per diameter. Once the muscle is pulled apart into many small bundles, transfer to a 1.5ml Eppendorf tube. Submerge the muscle with an adequate amount of 40% NaOH (about 700-800 µm — you want enough fluid to fully submerge the sample, but also leave some space for the fluid to "slosh" around to facilitate NaOH interaction with the bundles) and incubate on the "Ferris wheel" for 2 hours at a moderate speed to digest the connective tissue between the fibers.

After NaOH treatment, pour the contents of the tube through a 40-100 µm mesh strainer to remove NaOH. Wash the fibers in the strainer with PBS (transfer pipettes can be used for this process). Try to remove as much of the NaOH as possible. Wash with large volumes of 1x PBS

(phosphate buffered saline) to restore pH (do at least 10-12 washes). Using flat head tweezers, grab all the fibers (check under the microscope to make sure all are collected) and add them to the Eppendorf tube with 300 µm 1x PBS. With a 200 µm pipette, triturate the fibers until they fall apart into full length individual fibers (or sometimes very small bundles). This usually takes 2-3 minutes. You will probably have to cut the tip of the pipette tip to allow the muscle to move in and out of the tip, but don't cut too much off or it will be a weak trituration.

Staining

Following trituration of fibers, permeabilize the fibers in 1% Triton-x100 in PBS (e.g. 10 milliliters PBS, 100 µm Triton) for 10min on Ferris wheel (make sure sufficient volume is added for good fluid action, about 700 µm). Then centrifuge fibers at 13000g for 2 minutes to "pellet" fibers. Afterwards, remove as much supernatant (PBS/Triton) as possible without disturbing the fiber pellet.

Now add the primary antibody to approximately 200 µm of PBS. Use 1° Ab MyHC, Sigma #M8421 with a ratio of 1:200 in PBS (the final volume will be approximately 200 µm). Mix by pipetting, then place the tube on the Ferris wheel for 90 min at room temperature. Gently agitate with flicking every 20 min or so, as fibers tend to sink and pool in the bottom of the tube.

After 90 minutes, remove antibody/PBS and wash the tissue 3 times for 3 minutes in PBS + 1% Triton. Centrifuge after each wash and remove supernatant before performing the next wash. Following the last wash, remove supernatant and transfer tissue to a new tube containing 250 µm PBS + 1% Triton. Add the secondary antibody using the ratio of 1:500. Use 2° Ab MyHC I, Gt anti-Ms IgG1, Alexa Fluor 555 (Invitrogen, CatA-21127). Place the tube on the Ferris wheel and gently agitate with flicking every 20 minutes for 60 minutes at room temperature.

After 60 minutes, remove antibody/PBS and wash the tissue 3 times for 3 minutes in PBS + 1% Triton. Centrifuge after each wash and remove supernatant before performing the next wash. Following the last wash, remove supernatant and transfer tissue to a new tube with 50 µm. Add 10 µm DAPI (ThermoFisher#D1306, dilute (100µM) DAPI stock, 1:10,000 in PBS), mix by pipetting, and incubate for 5 min. After incubation, dispense a few droplets of muscle fiber plus DAPI onto a slide. Add a drop of Vectashield Mounting Medium (WITHOUT DAPI) and add glass cover slip. Gently wipe off edges if it is too wet. Take pictures.

Image Acquisition

Starting at 10x magnification, locate the straightest fibers that have the least damage (tearing and fraying can occur during sample processing) and the smallest amount of overlap with other fibers on the slide. The goal is to image 10 fibers on each slide, approximately 5 type I fibers and 5 type II fibers. This can be challenging as the ratio of type I to type II fibers are not always equal within a sample.

Once a desired fiber has been located, image fiber at 20x magnification for MyHC and DAPI. Choose one end of the fiber to start at, then move the slide to image the next section of fiber, and so on until the other end of the fiber is reached. Use the CHE channel to visualize the MyHC stain (fiber type), and focus the image on the muscle fiber before taking the picture. For DAPI (myonuclei), use the YFP channel on the microscope. The most important part of imaging with the DAPI stain is making sure that all myonuclei are included within at least one of the images. First, focus all the way out, so that the fiber is blurry and no nuclei are visible. Then, slowly focus inwards until the edges of the first nuclei become crisp and are in focus. These first nuclei are those closest to you, on the top of the muscle fiber. Take a picture of this layer of nuclei. Then, slowly focus in further until a new set of nuclei are in focus. This is the middle of

the muscle fiber. Finally, focus all the way down to visualize the nuclei on the back of the muscle fiber. Take a picture each time a new set of nuclei are focused on. All the nuclei may not be able to be visualized in just three images, depending on the different focal planes, so take as many images with different focuses as needed. In this way, we can make sure we are imaging all the nuclei on the muscle fiber, rather than just those on the top layer of the fiber. Once you have MyHC and DAPI images for a total of 10 muscle fibers, save images to the correct file, turn off the microscope and mercury lamp, and place the slide back where it was found. (**Figure 9**)

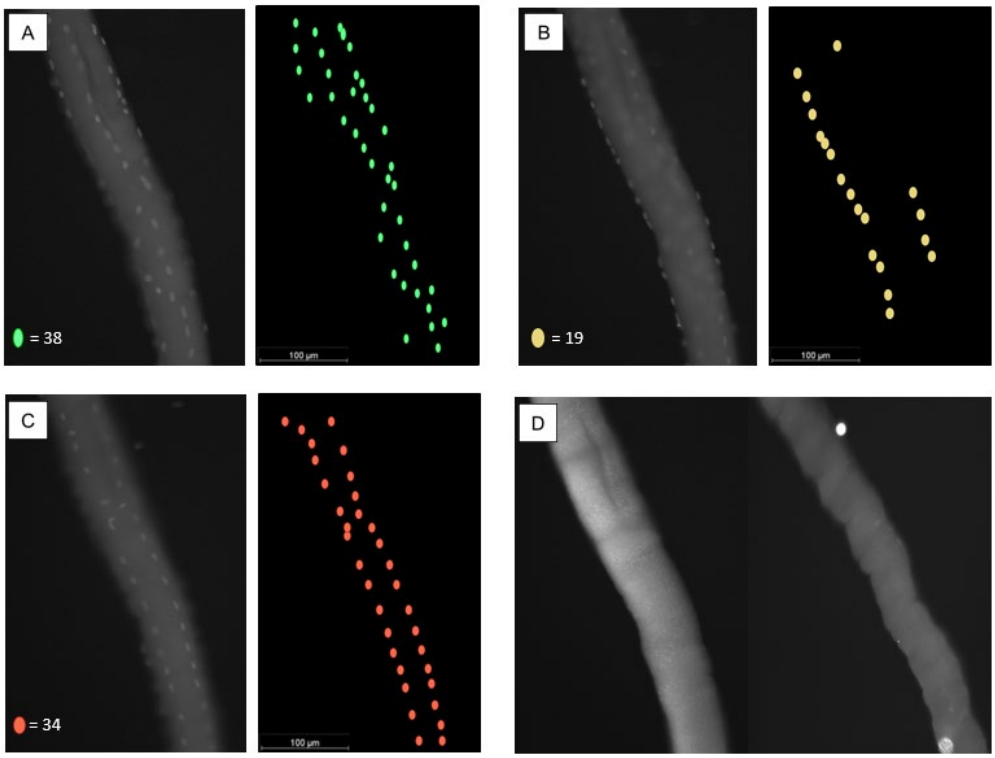

Figure 9: Single Fiber Approach at 20x Magnification

Images showing top **(A),** middle **(B),** and bottom (**C**) layers of one muscle fiber, with the DAPI stain highlighting the distinct myonuclei on each layer. Colored cartoons define each distinct layer of myonuclei within the muscle fiber. (**D**) Depicts MYHC stain for fiber type, showing an example of a type I fiber on the left and a type II fiber on the right.

Data Analysis

22_SF_EAABFR Data

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L32

	22_SF_EAABFR_01_Bx08R	MyHC+	#1 Myonuclei	#2 Myonuclei	#3 Myonuclei	Total Myonuclei	Length (um)	Radius (um)	Volume (um^3)	Domain (um)	NMJ	Total Length(um	Total myonuclei
2	Fiber 1 set 1	1	41	16	33	90	5.31E+02	4.53E+01	3.41E+06	3.78E+04	NMJ in this set		
3	Fiber 1 set 2	1	21	30	31	82	5.73E+02	4.81E+01	4.17E+06	5.08E+04	0.00E+00		
4	Fiber 1 set 3	1	45	26	51	122	7.40E+02	5.34E+01	6.62E+06	5.43E+04	0.00E+00		
5	Fiber 1 set 4	1	32	24	54	110	6.47E+02	5.45E+01	6.04E+06	5.49E+04	0.00E+00		
6	Fiber 1 set 5	1	46	25	32	103	6.20E+02	4.96E+01	4.80E+06	4.66E+04	0.00E+00		
7	Fiber 1 set 6	1	42	19	53	114	6.13E+02	5.65E+01	6.15E+06	5.39E+04	0.00E+00		
8	Fiber 1 set 7	1	46	23	65	134	5.65E+02	4.93E+01	4.30E+06	3.21E+04	0.00E+00		
9	Fiber 1 set 8	1	38	14	47	99	5.36E+02	4.65E+01	3.64E+06	3.67E+04	0.00E+00		
10	Fiber 1 set 9	1	33	14	27	74	4.88E+02	5.04E+01	3.89E+06	5.26E+04	0.00E+00	5.31E+03	928
11	Fiber 2 set 1	1	68	25	69	162	9.52E+02	5.33E+01	8.50E+06	5.25E+04	0.00E+00		
12	Fiber 2 set 2	1	44	18	48	110	6.42E+02	42.38	3.62E+06	3.29E+04	0.00E+00		
13	Fiber 2 set 3	1	46	18	48	112	5.52E+02	54.5	5.15E+06	4.60E+04	0.00E+00		
14	Fiber 2 set 4	1	64	16	55	135	8.01E+02	50.35	6.38E+06	4.73E+04	0.00E+00		
15	Fiber 2 set 5	1	47	21	44	112	6.12E+02	47.75	4.38E+06	3.91E+04	0.00E+00		
16	Fiber 2 set 6	1	25	12	34	71	3.97E+02	44.13	2.43E+06	3.42E+04	0.00E+00	3.96E+03	702
17	Fiber 3 set 1	1	57	12	52	121	7.76E+02	55.5	7.51E+06	6.21E+04	0.00E+00		
18	Fiber 3 set 2	1	53	15	42	110	7.22E+02	56	7.12E+06	6.47E+04	0.00E+00		
19	Fiber 3 set 3	1	62	15	40	117	8.23E+02	58.38	8.81E+06	7.53E+04	0.00E+00		
20	Fiber 3 set 4	1	45	13	46	104	7.73E+02	49.25	5.89E+06	5.66E+04	0.00E+00		
21	Fiber 3 set 5	1	29	11	29	69	5.73E+02	45.25	3.69E+06	5.35E+04	0.00E+00		
22	Fiber 3 set 6	1	45	22	38	105	8.03E+02	45.88	5.31E+06	5.06E+04	0.00E+00		
23	Fiber 3 set 7	1	27	16	30	73	5.25E+02	49.5	4.04E+06	5.53E+04	0.00E+00		
24	Fiber 3 set 8	1	27	13	36	76	4.88E+02	50.38	3.89E+06	5.12E+04	0.00E+00	5.48E+03	775

Figure 10: Spreadsheet Set Up for Data Analysis

Example of what the spreadsheet should look like for data analysis

We use the Fiji software from ImageJ to analyze our images. First, in the appropriate column, (**Figure 10**) record whether or not MyHC stain is present: 1 for present, 0 for not present. Type I fibers will have MyHC present. This can be seen in the image taken for the MyHC stain (**Figure 9D**).

Next, make sure the units are correct in Fiji. Go to analyze > set scale and make sure the unit of length is set to µm. This will ensure that it gives you the measurements in µminstead of pixels.

Download the DAPI images for Fiber 1 set 1. Drag and drop them into Fiji. Pull up the second DAPI image. This is (usually) the image that will have the clearest borders for the muscle fiber. Using the segmented line tool (right click the straight-line tool for options), follow the contours of the muscle fiber through the center of the fiber. Double click where you want the last segment to finish to complete the line. Once the line is drawn through the center of the fiber, go

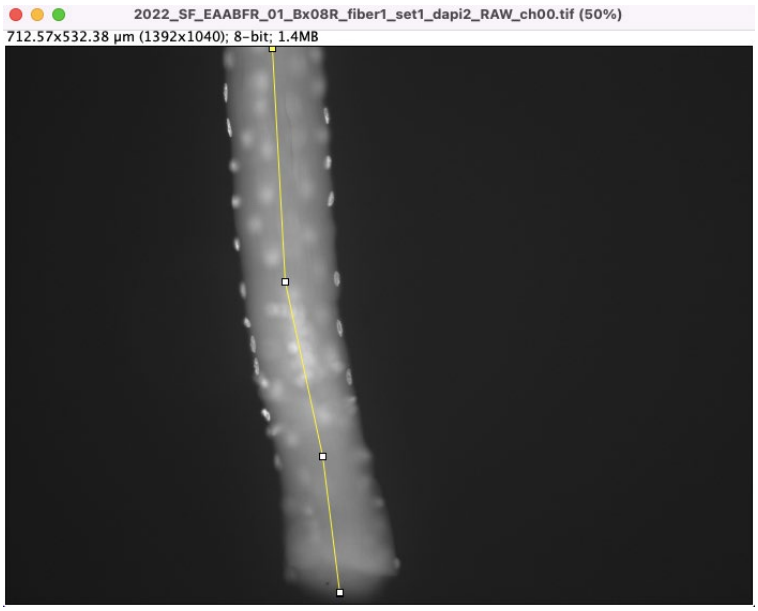

Figure 11: Measuring Length of Fiber Segment in Fiji

Example of what measuring the length of one fiber segment looks like in Fiji, using the segmented line tool

to analyze > measure to get the length of the fiber in µm. Record the length in the appropriate column on the spreadsheet. Then, switch to using the straight-line tool (right click the straight-line tool for options). Create 4 lines measuring the diameter of the fiber, each one evenly spaced to get an average diameter of the fiber. Each time you draw a line, go to analyze > measure to record that diameter. Once you have 4 diameters, add them up and divide by 4 to get the average. Round to the nearest whole number for this measurement. Divide that average in half to obtain the average radius of that fiber, and then record the radius in the appropriate column on the spreadsheet. Always take a screenshot of the measurements so they can be double checked if needed. Using the length and radius, find the volume (volume of a cylinder = pi(radius^2) (length)) of that fiber segment and record in the appropriate column.

Now begin counting myonuclei. Pull up the dapi1 image and select the multi-point tool. Click on each nucleus that is in focus. Place all three images side by side, so that if you are unsure about a nucleus you can look at the other images to see if it is present there as well. Each

image should have its own unique set of nuclei. Once you have counted up all the nuclei on the image using the multi-point tool, record in the column labeled "#1 Myonuclei." Then move on to the next DAPI images, repeating the steps to count up and record nuclei. Remember to take a screenshot of each image with your counts on it, so that they can be double checked if needed.

Sum all the nuclei counts to obtain total myonuclei in that segment of fiber. Record. Divide the volume of that fiber segment by the total number of myonuclei in the segment to obtain the domain, and record that value in the appropriate column.

Repeat this process of measuring and counting for the rest of the fiber segments until the entire fiber has been analyzed. Then fill in the columns for total length, total myonuclei, and myonuclei/mm of muscle fiber.

*Protocols taken from Dreyer Lab, Pacific 107, University of Oregon

Results

Currently, 7 subjects have completed this study — 4 in the group receiving EAAs, and 3 in the placebo group. Of the EAA group, 1 subject was female and 3 subjects were male; in the placebo group, 2 subjects were female and 1 subject was male. The mean age in both groups was 22 with a standard deviation of 2 years. In the EAA group, the mean height was 178 cm, the mean weight was 80 kg, and the mean BMI was 25. In the placebo group, the mean height was 165 cm, the mean weight was 65 kg, and the mean BMI was 24. **Table 1** depicts the subject demographics.

Table 1. Subject Demographics

	Age	*Height (cm)*	*Weight (kg)*	*BMI*
EAA (n = 4)	22 ± 2	178 ± 9	80 ± 14	25 ± 3
Placebo (n = 3)	22 ± 2	165 ± 16	65 ± 12	24 ± 2

Table 1: Subject Demographics

Mean reported with standard deviation.

Due to the small number of subjects, we are currently underpowered and will perform statistical analysis when the study is complete. **Tables 2** and **3** depict the preliminary data we have gathered from the 7 subjects that have completed the study so far. **Table 2** depicts the cross-sectional area of the fibers in micrometers squared, categorized by fiber type and treatment group. In the left leg (no BFR), both type I and type II fibers in the EAA group had larger cross-sectional areas on average than in the placebo group. In the right leg (BFR), the EAA group had

Table 2. Cross-sectional Area		
Left leg (EAA or placebo only)		
	Type I CSA (um^2)	*Type II CSA (um^2)*
EAA only (n = 4)	8,863 ± 889	10,868 ± 598
Placebo only (n = 3)	5,814 ± NA	8,050 ± 2,886
Right leg (EAA + BFR or placebo + BFR)		
	Type I CSA (um^2)	*Type II CSA (um^2)*
EAA + BFR (n = 4)	7,572 ± 662	9,757 ± 1,099
Placebo + BFR (n = 3)	8,687 ± 2,385	8,206 ± 2,418

Table 2: Cross-sectional Area

Mean reported with standard deviation.

smaller cross-sectional areas on average in type I fibers, but larger cross-sectional areas on average in type II fibers as compared to the placebo group.

Table 3 depicts, on average, the myonuclei per fiber, the domain (in micrometers), the surface area (in micrometers squared), and the surface area per myonucleus. This table is also categorized by fiber type and treatment group.

Table 3. Myonuclei, Domain, and Surface Area			
Placebo (left)		***Placebo + BFR (right)***	
Type I	Type II	Type I	Type II
Myonuclei per Fiber			
194 ± 8	201 ± 12	179 ± 0	205 ± 105
Domain (um)			
32,752 ± 0	46,299 ± 14,395	45,650 ± 12,747	51,874 ± 10,743
Surface Area (um^2)			
144,382 ± 0	166,893 ± 39,500	179,513 ± 10,557	173,452 ± 21,070
Surface Area per Myonucleus			
1,579 ± 0	2,010 ± 0	1,702 ± 257	2,098 ± 143
EAA (left)		***EAA + BFR (right)***	
Type I	*Type II*	*Type I*	*Type II*
Myonuclei per Fiber			
172 ± 7	189 ± 23	194 ± 18	163 ± 12
Domain (um)			
45,925 ± 3,956	55,757 ± 4,785	44,941 ± 2,662	53,050 ± 3,542
Surface Area (um2)			
184,876 ± 15,348	196,914 ± 1,272	165,586 ± 15,428	194,230 ± 9,269
Surface Area per Myonucleus			
1,612 ± 185	1,872 ± 120	1,845 ± 44	1,942 ± 164

Table 3: Myonuclei, Domain, and Surface Area

Mean reported with standard deviation.

Discussion

Limitations

The primary limitation of this study is the small sample size, which limits our ability to conduct meaningful statistical analyses on the data collected from the participants who have completed the study thus far. In order to obtain conclusive answers to our research questions, we intend to continue the study throughout the upcoming academic year in order to gather a larger dataset that will enable us to perform rigorous statistical analyses.

Another limitation of this study pertains to the reliance on participants to strictly adhere to the established protocol. It is challenging to ensure consistent compliance with the study guidelines, including the timing and dosage of the supplement intake. Moreover, external factors beyond the study's control, such as subjects' dietary habits, exercise routines, or alcohol consumption, may have influenced the experimental measurements we were assessing.

Overall, these limitations should be considered when interpreting the findings of this study, as they may impact the generalizability and reliability of the results. Future research endeavors should aim to address these limitations to enhance the validity and comprehensiveness of the investigation.

Conclusions

At present, the data obtained from this study does not allow for any definitive conclusions to be drawn. We will be able to perform statistical analyses once more subjects have been progressed through the study and we have more data to analyze. However, it is worth noting that this study has successfully implemented a single fiber protocol within the laboratory setting, facilitating the continuity of this study and its potential application in other research

domains. Future research will focus on myonuclear shape, size, and location (clustering to neuromuscular and myotendinous junctions), which can be seen clearly in **Figure 12**. We will also use spatial transcriptomics to gain further insights into mechanisms of action. Our long-term goal is to identify interventions that prepare muscle before atrophy-associated life events from older adults with arthritic knees to high-end athletes for in-season preventative maintenance.

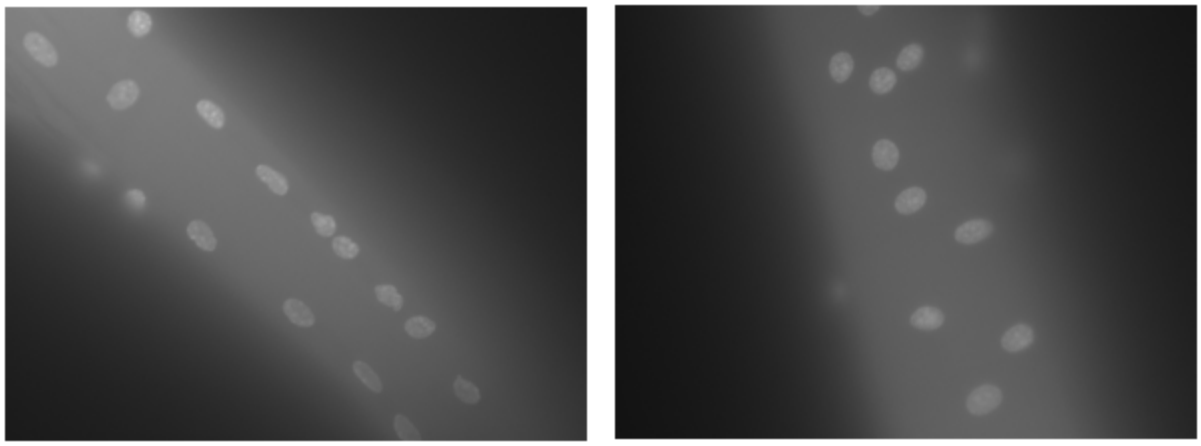

Figure 12: DAPI Stain at 60x Magnification

Images of the DAPI stain taken at 60x magnification, in order to highlight myonuclei. Differences in size, shape, and location can be seen between nuclei, which we plan to further investigate in future studies.

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