Pre-clinical assessment of the selective androgen receptor modulator RAD140 to increase muscle mass and bone mineral density

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Abstract

Selective androgen receptor modulators (SARMs) are important hypertrophic molecules that pose as potential treatments for many types of myopathy and osteopathy. The aim of this study was to determine if the SARM RAD140 had additive effects on muscle hypertrophy when combined with the functional overloading (FO) of the plantaris muscle. Male, 24 Sprague-Dawley rats (226-250g, n=24 rats/group) where randomly selected into one of four treatment groups: (1) RAD140-FO, (2) RAD140-Control, (3) Vehicle-Control, or (4) Vehicle-FO. RAD140 groups received drug through drinking water and control groups received only vehicle (methylcellulose). Standard rat chow and water were provided *ad libitum*. Muscle weights of the triceps-surae group and muscle fiber cross-sectional area (CSA) were measured. Muscle weight analysis showed a marked increase in RAD140-FO groups but were not statistically different from the vehicle-FO group. CSA results indicated similar findings. The data presented here show the potential of RAD140 to stimulate muscle hypertrophy in participants. Further investigation with longer treatment duration with RAD140 and FO are required to gain a better insight into the physiological response of the muscle to the FO and SARMs treatment.

Table of Contents

General Research Question 12

Specific Aims 12

Hypothesis 12

Rationale 12

Chapter 2. Literature Review *14*

2.1 Aging and Functional Mobility 14

2.2 Aging and Ability to Hypertrophy 15

2.4 Models of Hypertrophy 17
2.4.1 Animal Models of Hypertrophy 17

2.5.1 Functional Overload and Skeletal Muscle Hypertrophy 19
2.5.2 Unilateral vs Bilateral 20
2.5.3 Muscle mass and Cross-sectional Area following Functional Overload 20

2.7 Improving Muscle Mass Prior to TJA 23

2.8 TJA Outcomes and State of Skeletal Muscle Leading to TJA 24

2.9 Selective Androgen Receptor Modulators 25

Chapter 3: Methodology and Experimental Design *28*

3.1 Institutional Animal Care Approval 28

3.2 Experimental Design 28
3.2.1 Animals 28
3.2.2 Treatments 29
Functional Overload Surgeries 29
Suture removal 30
3.2.3 Tissue Collection 30
Skeletal Muscle and Tibia Harvesting 30
Plantaris fiber cross-sectional area 30
3.2.5 Micro Computed Tomography 31
3.3 Limitations and Basic Assumptions 31
3.4 Delimitations 32
3.5 Statistical Analysis 33

Chapter 4 – Results *33*

4.1 Body Weight 33

4.2 Water Consumption 35
4.2.1 Average Daily Water Consumption by Treatment 35
4.2.2 RAD140 Dose Received 36

4.3 Functional Overload and RAD 140 Effects on Muscle Mass 37
4.3.1 Plantaris 37
4.3.2 Soleus 38

4.4 Functional Overload and RAD 140 Effects on Cross-Sectional Area 40

4.5 Micro Computed Tomography 42
4.5.1 Cortical Bone Analysis 42
4.5.2 Trabecular Bone Analysis 46

Chapter 5 – Discussion *51*
5.1 Water Consumption and Dose Effect **52**
5.2 Functional Overload and RAD140 Effects on Muscle Mass **52**
5.3 Functional Overload and RAD140 Effects on CSA **54**
5.4 Functional Overload and RAD 140 Effects on Bone Microarchitecture **55**
Chapter 6 – Conclusion *58*
Chapter 7 – Future Directions *58*
Chapter 9 – Bibliography *59*

Lay Summary

Faculty and students in the Department of Biology at Lakehead University are bound together by a common interest in explaining the diversity of life, the fit between form and function and the distribution and abundance of organisms. This research supplements the lack of data available for the physiological effects of the selective androgen receptor modulator (SARM) RAD140 on muscle hypertrophy in combination with a hypertrophy inducing exercise model. As humans age, our ability to build and repair muscle significantly decreases as we enter our 4^{th} and 5^{th} decades of life. For many people this can have serious health consequences as they age. Losing muscle strength can result in a sedentary lifestyle which has many negative outcomes such as increased chance of obesity, diabetes and cardiac diseases. Furthermore, losing strength will increase the individuals fall risk which can be very serious for older populations. While SARMs are a novel pharmaceutical that have yet to be cleared for human consumption, there is adequate evidence to suggest that SARMs may pose as a novel form of treatment for individuals experiencing difficulties with regaining, maintaining or building muscle mass and strength. As the frequency of total joint arthroplasty increases across the country, SARMs pose as a novel pharmaceutical addition to augment well recognized pre-habilitation and rehabilitation programs preparing for and following surgery. This potential treatment could also have a wide range of positive implementations for patients experiencing cancer cachexia, osteoporosis and more. Using a hypertrophy inducing model in combination with the SARM RAD140, this study suggests that RAD140 will positively augment the overall muscle size. This was evident through results found in this study that indicated increased muscle size in animals from the RAD140 treatment groups, compared to those in the vehicle groups. The results from this study may provide knowledge that is translatable from rats to humans, to add to the knowledge necessary for setting safe clinical trials for the future with RAD140. The SARM RAD140 has yet to be tested in a clinical setting for humans. However, based upon the results from this animal study and others using different SARMs, there is credible evidence to suggest this family of novel pharmaceuticals could have positive impacts on muscle hypertrophy and strength.

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Chapter 1 – Introduction

Statement of Problem

The prevalence of total joint arthroplasty (TJA) is rising across Canada in response to our aging population, rising rates of osteoarthritis and higher rates of obesity [21]. Individuals awaiting TJA would also benefit from an of improved quality of life prior to and following these operations for best possible surgical outcomes. To minimize the social and financial impact of this on society, there is a need for continually optimizing patients to further augment their functional recovery post operatively. Many people who suffer from osteoarthritis have decreased muscle mass and strength due to pain and disuse [1]. This can make it difficult to perform everyday tasks due to lack of strength and mobility. This challenge then often produces a generalized decrease in activity leading to significant disuse deconditioning [1]. Increasing these individual's physical strength preoperatively, will likely increase their mobility before surgery thus increase the likelihood of improved mobility and strength post TJA [2]. The impact of physical strength and mobility on a patients' confidence is significant. Increasing an individual's strength would allow for increased mobility while giving the patient a better sense of confidence allowing them to feel comfortable beginning rehabilitation exercises. Pre-habilitation has been proposed to improve pre-operative strength leading to improved rehabilitation outcomes [10]. By participating in physiotherapy before surgery with the intent of increasing the patient's strength, it is hypothesized to lead to improved quality of life before and after surgery while reducing time spent in hospital. However, the literature is conflicted with the efficacy of pre-habilitation. This is likely due to the disparate means of assessing the efficacy of the pre-habilitation. Furthermore, many individuals undergoing TJA are older and/or over-weight making the process of regaining muscle mass and strength difficult [3].

Recently, researchers have been exploring the use of non-steroidal selective androgen receptor modulators (SARMs) as a novel approach to androgen treatments. SARMs are a novel pharmaceutical molecule developed to have a high affinity to the androgen receptors (AR) within muscle and bone tissue while displaying minimal affinity

to AR within sex tissues such as the prostate or gonads. The AR is part of a large group of nuclear receptors in the body. This includes receptors that bind estrogens, progesterone, glucocorticoids, mineralocorticoids and androgens [17]. The AR is found in a wide array of tissues such as skeletal muscle, cardiac and smooth muscle as well as bone, prostate, seminal vesicles, male/female genitalia, liver, skin, sebaceous glands and the brain [18]. The AR has a DNA binding domain and ligand binding domain that is activated via testosterone and dihydrotesterone (DHT) through the 5α-reductase enzyme [19]. However, there are other hormones, growth factors and peptides capable of binding to the AR. To activate the AR, a ligand must bind to an available receptor located in the cytoplasm. The efficacy of a ligand (SARM or testosterone), in the activation process is based upon its ability to bind and replace the endogenous hormone typically activating the receptor [19]. When the AR is in an inactive state its binding region is occupied by heat shock proteins (HSPs), however in the presence of a ligand these HSPs are unfolded and make room for the ligand to form the AR-ligand complex. This allows the complex to translocate into the nucleus where it binds to an androgen response element (ARE) which further regulates gene expression [20]. Testosterone and SARMs are both ligands that produce anabolic responses on target tissues. Once bound, the ARE regulates expression of mRNA related to protein synthesis.

The purpose for the development of these novel pharmaceuticals is to combat conditions of skeletal muscle wasting while minimizing the negative side effects commonly associated with androgen treatments or other anabolic androgenic steroids. While testosterone poses as a promising solution to contest muscle wasting diseases such as seen in cancer cachexia [16], there are considerable downsides to the treatment as well. Since testosterone is an endogenous hormone it also effects sex tissues and can cause an enlargement in the prostate and seminal vesicles of men undergoing the treatment [28]. This may put individuals undergoing these treatments at an increased risk for the development of certain prostate related diseases. There are several different SARMs under investigation and some have shown promising results on muscle mass, bone mineral density and functional tests [25].

Previous studies have explored the use of classic steroids such as testosterone treatments for muscle wasting diseases [5]. However, testosterone supplementation is

associated with many negative side effects [6,28]. The common negative side effects from anabolic androgenic steroid use are not seen in SARMs patients which is where these novel pharmaceuticals make their case as the better alternative [7].

SARM RAD140 has been shown to be extremely tissue selective with a high affinity for the anabolic androgen receptor on skeletal muscle [8]. These factors make RAD140 an excellent candidate for possible future clinical use. Due to the lack of understanding surrounding these novel drugs, extensive research must be completed to determine the efficacy and safety of the SARM RAD140 prior to any human applications being possible.

Traditionally, exercise and physical therapy are best suited to increase muscle mass, strength and mobility. However, it is unknown if strength training programs for patients taking SARM's have an additive impact on muscle hypertrophy.

Significance of Study

There have been studies investigating the efficacy of prehabilitation therapy prior to TJA, however many of the findings are either inconclusive or greatly disputed [10]. If it can be demonstrated that RAD140 improves muscle growth and bone mineral density/microarchitecture in combination with a resistance exercise model, then this can be applied in a clinical setting with the intention of combining a prehabilitation training program with RAD140 supplementation treatments.

Functional overload (FO) is a model of resistance training inducing muscular hypertrophy in animals. This model works to achieve mechanical overload by chronically increasing the load on an animal's muscle group(s) by surgical intervention through removing key synergist muscle(s), leaving the remaining muscle(s) to maintain the animal's posture and mobility [23, 24]. The FO model, in combination with testosterone treatments, has proven to significantly increase muscle mass and cross-sectional area of myofibers. To date, there has not been any investigation into the use of the SARM RAD140 in combination with this model of resistance training thus this will be a novel aspect of our study design.

General Research Question

Does the combination of RAD140 and functional overload lead to increased muscle growth, bone mineral density and bone micro-architecture further than either treatment alone?

Specific Aims

1. Determine whether RAD140 works synergistically with FO to significantly increase muscle hypertrophy
2. Determine whether RAD140 will improve bone mineral density and micro-architecture

Hypothesis

The combination of RAD140 and functional overload of the gastrocnemius muscle will lead to an additive effect on increased plantaris muscle growth, and increased bone mineral density and bone micro-architecture in the tibia.

Rationale

Previous investigations into RAD140 have indicated that it can selectively enhance muscle growth while avoiding common side effects of other anabolic androgenic steroid treatments [8]. SARM treatments, like RAD140, have shown positive augmentation of muscle satellite cells (MSCs) following muscle injury [11, 4]. This process is a requirement for proper muscle regeneration and growth. Findings surrounding other SARMs have also demonstrated increases in bone mineral density, bone mineral content and increased muscle strength in rodent models [12, 13, 14, 15]. Further investigation into the role of these SARMs has found that they may be efficacious in increasing bone mineral density (BMD) in ovariectomized rat models [14, 22]. This was evident in a strength tensile test on femurs of rats treated with the SARM S-4 [26]. These rats were ovariectomized 90 days prior to treatment with the SARM thus reducing their BMD significantly before

intervention. The SARM treatment was able to increase the tensile strength of the bone back to the levels of rats who did not receive the surgical castration [26]. These findings suggest that the SARM S-4 can selectively bind to AR receptors related to BMD. Further investigation into the efficacy of SARMs on human populations is also underway. In a Phase-II clinical trial researchers investigated the SARM GTx-024 in treating cachexia in older men and women. This investigation included a functional test completed by participants in the study where individuals were required to perform a Stair Climb test. After 86 days, it was determined that individuals in the 3mg dose group had a significant improvement in Stair Climb scores compared to their baseline scores [27]. Additionally, there was a dose-dependent increase in total lean body mass which was statistically significant [27]. This increase in lean body mass caused by GTx-024 was not accompanied by any significant increases in other androgenic tissues demonstrating the SARMs efficacy in tissue selectivity [27]. A significant functional test result such as this demonstrates the clear clinical relevance of SARM treatments and their potential role in therapies. Based upon these findings, RAD140 may also have the potential to enhance musculature following synergist ablation resulting in functional overload while simultaneously increasing BMD. Investigating the effects of the SARM RAD140 on muscle hypertrophy and bone microarchitecture in the overloaded limb may help determine the clinical importance of this pharmaceutical for future clinical pre-habilitation purposes.

Scientific Name	**Common Name**
GTX-024	Enobosarm, MK2866, S22, Ostarine
S4	Andarine
LGD - 4033	Ligandrol
LGD - 3033	
LGD - 2226	
OPK - 88004	TT-701, LY-2452473
RAD140	Testolone
S-23	
YK11	Myostatin

Table 1. List of popular SARMs and their common names.

Chapter 2. Literature Review

2.1 Aging and Functional Mobility

As humans increase in age, skeletal muscle mass decreases and results in an associated loss of muscle strength. As age-related muscle atrophy progresses, individuals are at risk of developing reduced mobility, increased fall risk and decreased quality of life [32]. The average age in most countries is on the rise leading to a worldwide increase in the number of individuals suffering from age related muscle and its related complications [33]. Sarcopenia is defined as the age related, involuntary loss of skeletal muscle mass and strength [1]. This disease can affect people of all different age groups and health conditions, however, most commonly occurs in older populations. This disease can begin to develop as early as the 4th decade of life and causes a linear decline in skeletal muscle mass and strength [82]. The prevalence of sarcopenia can be anywhere from 1% to 29% in community-dwelling older adults, 14% to 33% in older adults in long-term care facilities, and ~10% in acute care facilities [83]. On average, it is estimated that 5–13% of elderly people aged 60–70 years old are affected by sarcopenia. The numbers increase to 11–50% for those aged 80 or above [84]. There are many factors that can increase or contribute to one's risk of developing age-related sarcopenia (Figure 1). However, the elderly is not the only population at risk of sarcopenia. Sarcopenia can also affect those with certain comorbidities such as diabetes, obesity, heart disease, cancer cachexia and more. Any individual with an underlying health condition preventing or hindering muscle growth increases one's risk of developing sarcopenia. This also includes lifestyle choices such as diet, exercise regime/activity level and drug/alcohol consumption.

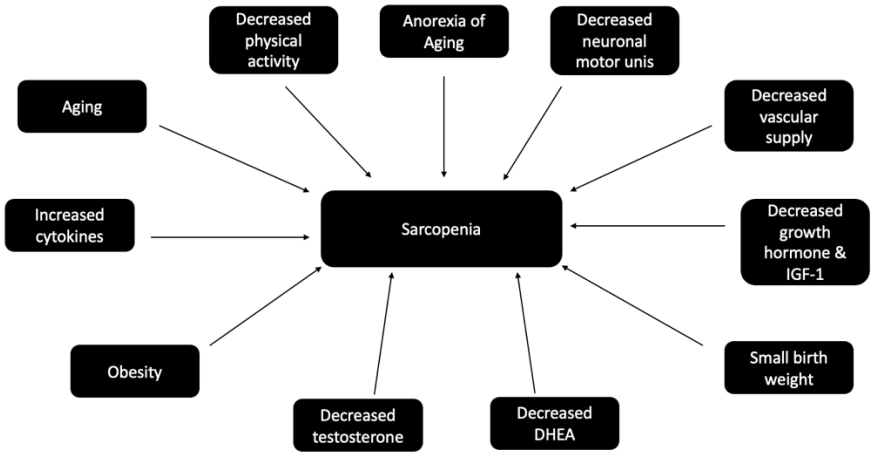

Figure 1. Factors involved in the pathogenesis of age-related sarcopenia.

Individuals suffering from muscle atrophy also experience diminished proprioception. Proprioception is vital for the body's ability to accurately detect and orient itself through space. As proprioception decreases, so does the body's ability to maintain posture and balance thus increasing fall risk [32]. Increased fall risk, lack of mobility and decreased proprioceptive results in an increasingly sedentary lifestyle in the elderly. Decreased mobility and an increased sedentary lifestyle raise the risk for the development of obesity [34]. As humans age, these factors play crucial roles in overall quality of life and although many of these clinical scenarios can be controlled, we still observe a high prevalence in our communities of advancing disability.

2.2 Aging and Ability to Hypertrophy

The physiological changes muscles undergo during the aging process is complex. With increased age comes a progressive loss in muscle mass due to atrophy within myofibers. This is a result of denervation along with a progressive decrease in the total number of myofibers [32]. There is also an associated decrease in force and power production due to the loss of these contractile motor units.

Other age-related changes within skeletal muscle relate to the vasculature supplying the muscle [32]. This may include a reduced blood supply, lower capillary density and

changes in vascular pathology [31,33]. Vascularity may be affected by many different factors, one important factor being exercise [32]. As many individuals increase in age, they tend to fall into sedentary lifestyles which in turn will cause a reduction in vascularity due to disuse.

Aging alters the skeletal muscles response to overload-induced growth for a few reasons. Along with the local physiological changes taking place within muscle as age progresses, there are also significant changes in hormonal regulation and the endocrine system which in turn reduces the individual's ability to regain/repair aging muscle tissue [35]. There is evidence showing an age-related decline in serum levels of growth hormone (GH) along with the associated decline in Insulin-like Growth Factor I (IGF-I) [36,37]. Investigations into the relationship between GH and aging have found that within study participants, between the 3rd and 9th decades, 55% of individuals had zero release of GH (<4ng/ml) [35]. Considering these trends in the reduction of GH production, in addition to the age associated increased rate of obesity, osteoarthritis and other sarcopenic diseases, it is easy to see the strong relationship between aging and a general tendency toward sarcopenia.

Hypertrophy

One of the most important features of skeletal muscle is its ability to adapt to increased mechanical loads by undergoing a process known as hypertrophy. Hypertrophy is an adaptation in muscle fiber that generates increased force output while the extracellular matrix (ECM) transmits these forces to tendons and bones surrounding the muscle [38]. The response from a muscle may depend on the type of injury or load specific exercise. Injury-dependent vs injury independent mechanisms of muscle adaptation will result from different forms of exercise [39]. Injury-independent mechanisms such as endurance exercise or concentric muscle contractions stimulate ECM production and remodeling but result in an insignificant amount of change to muscle fiber force output [39]. Injury-dependent mechanisms such as resistance exercise result in different outcomes. Resistance exercise, where a specific load is placed on the muscle during an eccentric contraction, results in damage to muscles fibres and the ECM. This damage then triggers an adaptive response from the muscle fibers and ECM. The areas that have been damaged are "broken

down" and typically replaced with an increased number of contractile and matrix proteins resulting in muscle hypertrophy and increased force production while also increasing the elastic energy storage capacity of the ECM [39 ,40].

2.4 Models of Hypertrophy

2.4.1 Animal Models of Hypertrophy

One of the primary advantages of animal models for the purpose of investigating muscle hypertrophy is the tight experimental control the investigator has when conducting the research. Many factors are difficult to control such as diet, sleep patterns and cooperability with exercise regimes when working with humans [41]. However, these factors are much easier to control when working with animal models, leaving much less room for external variables effecting outcomes. Additionally, animals are more homogenous than human participants and the studies can be easily randomized. These key factors allow animal studies to be much more sensitive and reproducible in terms of outcomes [41]. Another important advantage of animal studies over human studies is the ability to euthanize the animals and take full muscle samples after the study is completed. These samples allow for a full investigation into the underlying mechanisms of hypertrophy, where human muscle biopsies are often too small to gain full insight into the probable causes of hypertrophy [41].

There are a few animal hypertrophy models currently used for the purposes of investigating skeletal muscle hypertrophy in animals. These include, resistance training models, electrical stimulation models, compensatory overload models and chronic stretch models. Each one of these models has their own advantages and disadvantages. Resistance training models are useful for mimicking progressive resistive exercises (PRE), easily quantifiable and provide similar hypertrophic/functional outcomes when compared to PRE in humans [41]. However, there are also disadvantages associated with this model, for example the use of electric shock or food deprivation as incentive for compliance [41]. Along with the stress associated with the forced exercise. This may also have effects on growth of the animal if food is withheld due to lack of compliance.

Another animal model used for investigating skeletal muscle hypertrophy is electrical stimulation. Some advantages of this model include being able to have a contralateral control muscle, which reduces the number of animals needed for the study and reduces the number of animals that will be euthanized at the end of the study [60]. This method also does not require animal cooperation thus reducing another potential factor that may bias results. Finally, the electrical stimulation will activate all motor units in the muscle thus maximizing the potential results [60]. However, there are disadvantages as well. This specific model requires animals to be under anaesthesia during each treatment, meaning each animal will be anesthetized several times throughout the study before finally being euthanized [60]. Another disadvantage is the lack of physiological relevance with respect to the muscle contraction since it is involuntary [60].

Chronic stretch models are also beneficial when investigating muscle hypertrophy in animals [41,42]. The functional and hypertrophic outcomes from stretch models are well documented and allow for a contralateral control, once again reducing the number of animals required for the study. The model results in a large degree of hypertrophy in a short period of time. Some studies have demonstrated ~300% hypertrophy in only two weeks [42]. Since there is no surgical intervention, data collection can be done within minutes to hours after the treatment. This model does have its disadvantages, the overlying mechanisms to hypertrophy may not be applicable to the mechanisms in PRE. Additionally, it is also a chronic exercise model and therefore is not extremely physiologically relevant in determining physiologic mechanisms in human muscle hypertrophy.

Functional overload or compensatory overload is another common model used to investigate hypertrophy in animals. This model is useful as it allows for a contralateral control muscle, thus reducing the number of animals needed for the study. Following the surgical intervention there will be very limited interaction with the animals thus minimizing work for researchers. However, the largest advantage for these models is the rapid hypertrophy that takes place following the surgical intervention [43-45]. This allows investigators an opportunity to examine phenomena that would otherwise be very difficult to detect with models that result in only modest muscle hypertrophy. This model does have some disadvantages as well. Due to the invasive nature of surgery, any results obtained in

the first week are typically difficult to interpret because of inflammation around the wound and the pain that the animal may be experiencing.

2.5.1 Functional Overload and Skeletal Muscle Hypertrophy

Skeletal muscle hypertrophy can be accomplished in a variety of different ways, one of the most common and effective methods is known as functional overload. Most commonly, functional overload works to achieve this mechanical overload by chronically increasing the load on an animal's plantar flexion muscle group(s) through surgical intervention [24,43]. The FO model has shown extensive muscle hypertrophy in short periods of time making it an ideal method for investigating adaptive responses. Many studies have demonstrated that overload leads to an increase in muscle mass and cross-sectional area of muscle fibers. This induces an important change in the synthesis and degeneration of key proteins in the muscle regeneration process [24].

Functional overload can be accomplished in several ways. Functional overload through removal of key synergist muscles places the remaining musculature under a chronically increased load [44]. This is done by either severing, removing or denervating synergist muscle(s). This model can be used on a variety of different animal species; however, it is most studied in rats. Typical rodent FO models include the removal of the hind limb gastrocnemius muscle leaving the remaining soleus and plantaris muscles under increased tension. FO models have been used to investigate differences in muscle hypertrophy between muscle types, muscle hypertrophy during cancer cachexia, muscle hypertrophy with aging and effects of anabolic steroids on hypertrophy [45,46,47,48,71]. Depending on the aim of the study one surgical approach may be chosen over another. Depending on the aims of the research the soleus muscle be removed or severed to increase tension on the plantaris. The difference in these surgical procedures is very small but may result in different surgical outcomes. Completely removing a muscle (i.e., gastrocnemius or soleus) is an intrusive surgery that has an increased risk of complication, including damage to tissues like nerves, tendons, vasculature, or other adjacent muscles. This type of surgery would also require a longer healing stage following intervention and may increase the risks of infection due to a larger incision. Another option is to partially remove the

muscle by severing the distal portion of the muscle and the distal tendon allowing for only partial removal of the muscle. This surgical procedure is less invasive but still requires a larger incision and thus an increased risk of infection. The following option is much less invasive and requires a smaller incision. This involves a simple cut across the muscle at the distal tendon effectively removing its anchor to the bone and ability to contract with any force production. This incision is much smaller and the recovery time from this surgery is also shorter therefore minimizing the opportunity for infection.

2.5.2 Unilateral vs Bilateral

FO can be implemented in a bilateral or unilateral manner. Both methods have shown to increase muscle mass with no statistically significant differences between the two types of surgical intervention [24]. Researchers implementing a unilateral surgical method reported a mean increase in muscle mass of 46.8 ± 2.6% 14 days following FO surgery whereas, those researchers working with a bilateral surgical method report 52.3± 3.1% in the same period following surgery [24]. These insignificant differences between results speaks clearly to the advantage of using a unilateral surgical method. By doing so, researchers can reduce the number of animals required to accomplish the same goal thus minimizing the loss of life for the study.

2.5.3 Muscle mass and Cross-sectional Area following Functional Overload

As a muscle undergoes compensatory hypertrophy, there is an increase in cross-sectional area (CSA) [65]. The associated increase in muscle size is due to an increase in the size but not length of individual muscle fibers. When quantifying skeletal muscle hypertrophy, a good morphological measure is the associated change in CSA of the muscle of interest. Along with an increase in CSA, compensatory hypertrophy will result in an overall increase in total mass of the muscle. These measures are standard metrics for quantifying the level of skeletal muscle hypertrophy. The FO model has shown to induce

adequate compensatory hypertrophy and subsequently result in increased muscle mass and cross-sectional area [24].

2.5.4 Androgen Supplementation and Functional Overload Effects on Muscle Mass

FO has been investigated for its remarkable ability to induce extensive muscular hypertrophy over a short period of time. As interest in the field grew, researchers began to pair these hypertrophy driving models with androgen supplementation compounds. Some popular choices have been testosterone, trenbolone and nandrolone decanoate to name a few, along with other androgens/anabolic steroids. The combination of a FO model and androgen supplementation is of particular interest as both treatments have been individually proven to increase muscle hypertrophy [6, 7]. The interest into the amalgamation of these two treatments has led researchers to find evidence suggesting that the two treatments in combination may have an additive effect on muscle hypertrophy [77]. In a study completed by Lee et al, 2003, researchers investigated the combination of FO with nandrolone decanoate on aged and adult rats. Nandrolone decanoate was administered four separate times over the course of a 4-week study period [71]. FO occurred on the hindlimb soleus and plantaris muscles by ablation of the gastrocnemius at the beginning of the fourth week. Researchers found that adult rats treated with nandrolone decanoate and FO had a significant increase (35%) in soleus muscle mass following intervention compared to those who just received the FO treatment (27%) [71]. This is a good indication for the future of SARM research and future clinical interventions as it demonstrates some degree of additive effect between the two treatments.

2.6 Species Differences: Human vs Rodent

There are number of reasons that rodents present as an ideal model organisms compared to humans when investigating muscle hypertrophy. Rodents are far easier to work with as researchers can easily control variables that otherwise would be difficult in human based studies. When investigating muscle hypertrophy, it is well known that diet

and sleep may play crucial roles in muscle hypertrophy. Thus, being able to fully control the intake and sleep patterns of an animal gives the researchers the ability to remove a significant variable from the large equation surrounding muscle hypertrophy. Furthermore, the ability to control sleep patterns ensures that each subject is receiving the same amount of rest which can also play a factor in muscle regeneration [59]. Another key variable is controlling the amount of exercise each participant engages in. This can play a vital role in the outcomes of the research. Many human studies may require participants to complete a certain series of specific exercises, partially in a lab setting and then the rest on their own or may request that participants engage in no physical activity throughout the duration of the study. This is difficult to reliably control when working with human participants thus animals provide the better alternative for controlling all aspects of the study. Moreover, the ability to truly analyze findings is much more apparent when working with animals in contrast to humans. The ability to humanely euthanize and dissect animals enables the researchers to gain a full scope of view over the true mechanisms contributing to their findings. Human participant studies are limited to small muscle biopsies which are somewhat invasive and rely on the participants cooperation. This does not provide the research team with the ability to gain insight into the entire mechanism they are investigating. There are also limitations to the type of measurements that can be done with a small sample of a biopsy meant to represent the whole muscle meaning limitations of the types of assays that can be done with limited samples. The gold standard for muscle physiology research would be dissection and excision of the muscle which is simply not possible when working with humans. However, humans and animals are not the same physiologically so inferences based upon animal research cannot always be directly correlated to human physiological processes. On top of these differences, one other important aspect of animal research is the ability to use drugs otherwise not deemed safe, or have yet to be tested for safety, for human consumption. This is a key factor for many animal researchers (what makes this model often be referred to as pre-clinical) as they attempt to understand the efficacy of new pharmaceuticals and their potential future application for human studies.

2.7 Improving Muscle Mass Prior to TJA

The idea of increasing a patient's musculature and strength prior to a total joint replacement is becoming a common theme amongst many primary care physicians hoping to improve their patient's mobility and quality of life following surgical intervention. It has been proposed that being able to improve the structural integrity of the joint before surgical intervention will allow patients to have an improved quality of life following surgery. Researchers have done several clinical studies investigating this idea and have determined that cardiovascular fitness, strength training and flexibility exercises may all play a role in improving the surgical outcomes for a lower extremity TJA [54]. There are many factors that may affect the efficacy of this prehabilitation treatment. Studies that have failed to control for these variables have generated conflicting findings. Many individuals requiring lower extremity TJA are older and thus do not have the same potential to achieve a significant amount of muscle hypertrophy. Additionally, many of these individuals are also sedentary due to the lack of mobility and pain associated with their joints. This may exacerbate the atrophy of muscle in the areas of interest due to lack of use [58]. This lack of mobility may also coincide with weight gain further impeding their ability to use the joint in any meaningful type of exercise. Investigation into the types of prehabilitation that are most effective is still under way however, there have been strong cases made for the effects of strength training in combination with flexibility exercises [55]. Typical pre-habilitation for patients may be undergone 6-8 weeks before the surgery takes place. This timeline aligns with the suggested efficacy of SARM treatment perfectly as suggested duration of treatment is roughly 3month cycles [25]. The window for rehabilitation following surgery offers another ample opportunity for SARM treatment in combination with a physiotherapy regiment. Due to the wide array of scoring systems used to evaluate the effect of preoperative exercises on postoperative outcome, comparing findings across different studies can be difficult. A study completed by Calatayud et al. in 2017, described that high intensity strength training during the preoperative period reduced pain and improved lower limb muscle strength along with range of motion and functional task performance before surgical intervention. This in turn reduced the length of stay and resulted in a faster physical and functional recovery following total knee arthroplasty [56].

Similarly, another study completed in 2015 reported that preoperative progressive resistance training was an effective and safe intervention for improving postoperative functional performance and muscle strength but reported no patient improvements [57]. Currently there is no accepted gold standard for measuring the efficacy of prehabilitation but there certainly is a promising future for it in the field of preoperative care. Although findings are not always consistent across studies, when working with human subjects it can be difficult to control all aspects of your study parameters compared to working with animal subjects. Thus, as more interest is created in the area and more research is completed the results should become more consistent.

2.8 TJA Outcomes and State of Skeletal Muscle Leading to TJA

Investigation into the best outcomes surrounding TJA has been extensive over the last two decades. There is clear evidence that individuals with comorbidities experience worse outcomes then those who are free of chronic medical conditions. It is common practice for medical professionals to use the ASA physical status classification system to pre-determine a patient's physical fitness prior to surgical intervention [67]. The ASA physical status classification system is used to assess and communicate a patient's pre-anesthesia medical co-morbidities. It is often used in conjunction with other factors such as type of surgery, frailty and level of deconditioning [67]. For the best outcomes following TJA, a patient with an ASA scale that is 2 or lower is ideal [68]. However, due to the high rates of obesity, diabetes, osteoporosis, heart disease and other comorbidities in our populations, surgical teams often find themselves performing these procedures on patients with ASA scores of 3 and 4. It is well documented that these patients often experience worse outcomes due to their medical comorbidities [68].

Although there has been extensive research done on the effects of BMI and other comorbidities on surgical outcomes, there has not been equal interest in investigating the impact of muscle quality and strength on surgical results. Because muscle quality and strength are often determined by one's physical health parameters, by intentionally

intervening to improve muscle quality and strength there may be a profound impact on a patient's ability to achieve the best outcomes post TJA surgery.

Ideal outcomes following a TJA surgery are the goal of every surgical intervention. These include, no infection following surgery, short length of stay, lack of need to return for unscheduled follow ups, early return to work or full function, and pain relief [69]. While most patients who undergo TJA tend to experience similar results around 12 months post operation, there can be a significant difference between how long it takes for a patient to reach this point. This is where rehabilitation may play a role in providing optimal surgical outcomes fastest.

The speed of recovery may not seem important for all patients however, it can play an important role in many patients' recovery. For patients that are very old, recovering faster can be important as they may already be approaching end of life. Thus, getting to these recovery milestones quicker can provide these individuals with a better quality of life with the time they have left. Additionally, patients who must return to work would benefit from a faster recovery. Lastly, the longer patients suffer from pain prior to surgery, the higher their risk of developing dependencies on narcotics or alcohol becomes [70]. Thus, there is good reasoning behind continually augmenting the surgical process to lead to the best and quickest results for patients returning to normal life.

2.9 Selective Androgen Receptor Modulators

The last three decades have marked an important time for molecular advances in understanding the androgen receptor structure and function. Recently, the development of a novel family of nonsteroidal molecules with a selective affinity and specificity to the androgen receptor (AR) has changed AR therapy to date [49]. These are referred to as selective androgen receptor modulators (SARMs). There are many different molecules that fall under this category and there continues to be new developments of novel SARMs as research in the area deepens. These different molecules display different selective preferences for certain tissues or activities (i.e., trophic in muscle, strong or weak gonadotropin feedback) and a widely diverse ratio of activities in sex accessory tissues such as the prostate and seminal vesicles [18]. Due to the characteristics of this dynamic

family of nonsteroidal molecules, the role for SARMs in androgen therapy clearly has a bright future for potential future clinical applications.

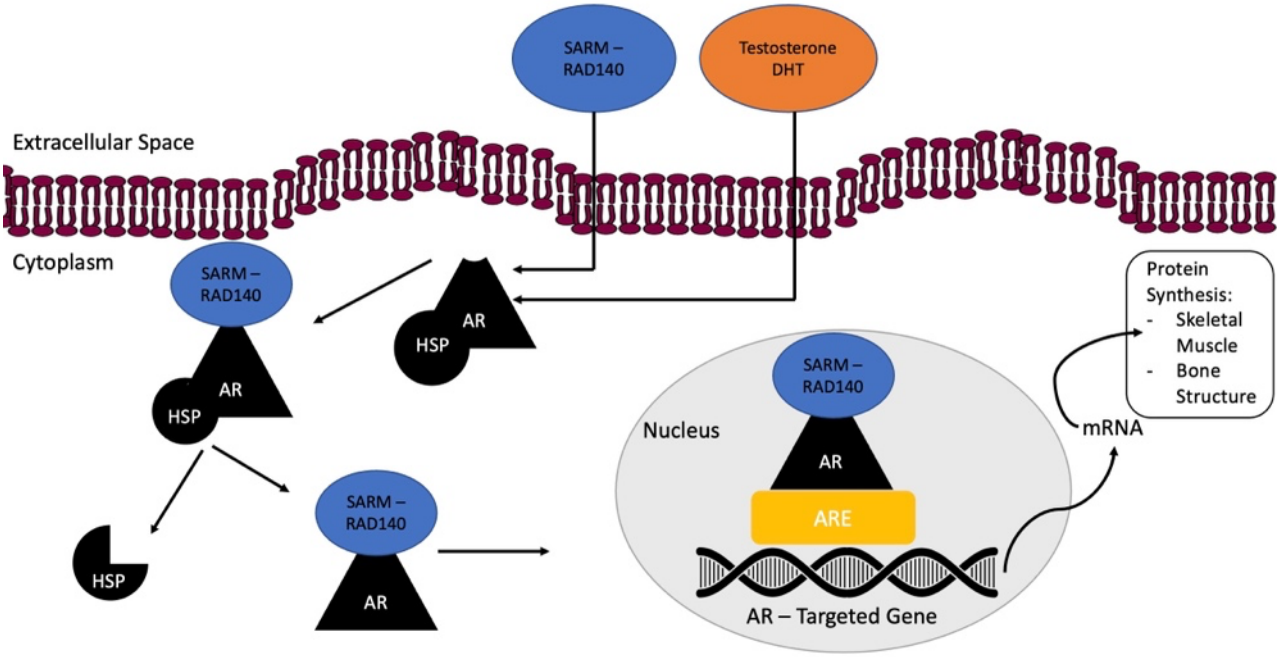

Figure 2. Mechanism of action of SARMs. Image adapted from *Haehling et al, 2020.*

The metabolism and impact of various forms of testosterone is complex. Testosterone, which is converted to dihydrotestosterone (DHT) in the prostate, produces cellular proliferation in many tissues [53]. DHT is a hormone made from testosterone in the prostate, testes and other tissues [53]. It plays a key role in development and maintenance of male sex characteristics [53]. SARMs are not substrates for 5α-reductase activity and thus do not affect either the activity of the enzyme or the normal metabolism of testosterone in its various forms [18]. Depending on the target for clinical use, SARMs may be designed to be active in distinct tissues [52]. For example, if the desired outcome is increased bone mineral density in older individuals with osteoporosis, then an anabolic SARM that clearly affects bone and muscle with less activity on prostate and sex tissues may be ideal [18]. This selectivity comes from the nature of the SARM molecule and its affinity for AR inside of muscle and bone tissue specifically. Variability in AR regulatory proteins in target tissues allow SARMs to selectively incite anabolic benefits while avoiding the issues associated with traditional androgen therapy [76]. The exact complex and mechanism allowing different SARMs to seemingly differentiate between ARs is an area requiring further research. There

are currently a few SARMs that have been investigated for the purposes of altering bone mineral density. Ostarine or GTx-024 is currently undergoing clinical trials to determine the efficacy of this SARM. Results found in a study investigating Ostarine using a menopausal osteoporosis rat model determined that the SARM significantly increased bone mineral density and volume at intermediate and high doses [62]. In a separate study, researchers investigating Ostarine in an ovariectomized rat model also determined that the SARM was able to improve bone healing while positively enhancing musculature and having minimal effects on sex tissues [63].

One SARM that has recently been investigated for the purpose of musculature enhancement is RAD140 (Testolone). Through investigation into several different molecular analogs, researchers were able to identify compound 7 (RAD140) [51]. RAD 140 showed a high stability ($t_{1/2} > 2$ h) in incubation with rat, monkey and human microsomes, with a good bioavailability in rats (F = 27 – 63%) and in monkeys (F= 65-75%) [51]. Additionally, it demonstrated an excellent affinity for the androgen receptor (Ki = 7 nM vs 29 nM for testosterone and 10 nM for DHT) and a good selectivity over other steroid hormone receptors with the closest off target receptor being progesterone (IC50 = 750 nM vs 0.2 nM for progesterone) [51]. The compound RAD 140 was further characterized through a series of *in vivo* assays to determine its oral efficacy on several parameters associated with androgenic activity. This was done in both young castrated and non-castrated rats to determine the effects through a multitude of endogenous androgenic signaling [51]. Using young, castrated rats provides a very sensitive *in vivo* assay for androgenic activity due to the lack of androgen signaling currently in the animal. There will be very little androgen signaling "superimposed" over the effects of the SARM allowing for a relatively clear picture of the mechanisms at play. It was determined that orally administered RAD 140 stimulated the levator ani muscle at a dose of 0.03 mg/kg and reached a level of efficacy equivalent to that of the sham-operated animal at 0.3 mg/kg [51]. In the same study, researchers investigated the effects of RAD140 in combination with testosterone propionate (TP) to determine if RAD140 acted as an antagonist towards TP effects on seminal vesicle and prostate stimulation. It was found that RAD140 caused a downward trend in TP release but was not statistically significant [51]. Based upon these findings, RAD 140 poses as a potent androgen agonist on the levator ani muscle but a weak partial

antagonist on the seminal vesicle and prostate in young, castrated rats. [51]. Since the young, castrated rat model is not a good reflection of true human adult male populations due to the low level of endogenous androgen, researchers also investigated the effects in a young male rat population that were not castrated. This provides a better picture for future clinical models and poses as a better pre-clinical model because the young rats have endogenous testosterone at reduced levels. The young male rats will display prostate sensitivity to an androgenic compound and allowing for a baseline stimulation that is more transferrable to the researchers target population. RAD140 increased the weight of the levator ani muscle more than that of the control at its lowest dose (0.1mg/kg) [51]. There was no stimulation of the prostate from RAD 140 until reaching the highest administered dose of 30mg/kg [51]. RAD 140 at a dose of .3mg/kg, displayed a similar muscle efficacy as TP at a dose of .5 mg/kg, however a dose of 30 mg/kg of RAD140 was required to approximate the effects on the prostate like that of 0.5 mg/kg of TP. [51]. These findings show that the SARM RAD140 efficacious in selective androgenic signaling making it a good candidate for potential future clinical trials.

Chapter 3: Methodology and Experimental Design

3.1 Institutional Animal Care Approval

All procedures were approved by Lakehead University Animal Care Committee and Georgia State University Institutional Animal Care and Use Committee.

3.2 Experimental Design

3.2.1 Animals

Male, Sprague-Dawley rats (226-250g, n=10 rats/group) were purchased from Charles River Laboratories and housed under a 12:12 light-dark cycle at room temperature in the Georgia State University animal facility which maintains a strict temperature and humidity range ideal for rodent housing. Animals were given one week to equilibrate to the new environment before starting the experiment. Rats were provided free access to food and water during this time. They were then randomly assigned to one of four groups (n =

10/group): Group 1: functional overload + vehicle (0.5% methylcellulose), Group 2: functional overload (FO) surgery + RAD140, Group 3: Control + Vehicle (0.5% methylcellulose) and Group 4: Control + RAD140. Functional overload surgery was unilateral, preserving the non-operated (NO) leg to serve as internal control for hypertrophy, which helps to minimize variability and also allows us to reduce the number of animals required. Skeletal muscle and bone samples were obtained after animals were euthanized. Animals were euthanized using CO_2 inhalation followed by removal of the diaphragm.

3.2.2 Treatments

RAD140 and functional overload surgeries were done at Georgia State University (GSU). RAD 140 was acquired through SelleckChem and shipped directly to the animal facility at GSU. Once rats are assigned to individual treatment groups FO was performed on one hindlimb, while the contralateral limb served as an internal control. Following surgical intervention, rats received their first dose of RAD140 or methylcellulose. The RAD140 groups received ~3mg/kg/day of RAD 140 in their drinking water for 2 consecutive weeks. The target dose of 3mg/kg/day was chosen because of the physiological relevance of the human equivalent dose (HED) [89]. Using the correction factor (K_m) of 6.2 and converting the rat dose to its human equivalent attained a dose of 0.48 mg/kg/day. In parallel subgroups of vehicle-treated, rats received 0.5% methylcellulose in their drinking water. Neither treatment affected the hydration status of the animals.

Functional Overload Surgeries

Unilateral, functional overload (FO) surgery was performed as described by (Otis et al., BMC Cancer 2007 7: 146). Rats were deeply anesthetized (isoflurane: 5% induction at 1000 ml/min of oxygen followed by a maintenance dose of 1-3% at 500 ml of oxygen) and a midline incision was made through the skin on the posterior aspect of the animal's lower leg. The distal tendon of the gastrocnemius muscle was isolated from the tendons of its major synergists (soleus and plantaris) and transected along with the distal portion of

the muscle. Removal was done with particular care, not to interrupt vasculature or innervation leading to the soleus or plantaris muscles. 3-0 Ethilon sutures were used to close the wound and were removed 10-14 days post-surgery if not already removed by the animal. The incision was treated with a topical antibiotic (Bacitracin). The animals were then closely monitored for sudden changes in health following the surgical procedure. Surgery occurred on one hind limb, preserving the NO leg to serve as an internal control for plantaris hypertrophy. All tissues were dissected 14 days after surgery.

Suture removal

Sutures were to be removed 10-14 days post-surgery using aseptic techniques and under anesthesia. However, all animals had removed the sutures themselves to remove the sutures before the end of the experiment. All incisions were monitored daily for infection or bleeding by animal facility staff or the research team.

3.2.3 Tissue Collection

Skeletal Muscle and Tibia Harvesting

The three hindlimb muscles of the posterior compartment (soleus, plantaris, gastrocnemius) were removed, trimmed free of connective tissues and fat, blotted dry and weighed, and flash frozen in isopentane cooled in liquid nitrogen until future analyses. Tibias were removed, length measurements taken and suspended in formalin before being shipped to the Central Animal Core Imaging and Transgenic Facilities at the University of Manitoba.

Plantaris fiber cross-sectional area

Plantaris muscles were removed, embedded in optimal cutting temperature compound (OCT), and immediately frozen in isopentane cooled in liquid nitrogen. The plantaris muscles were cut in 10 μm serial sections maintained at -20°C using a cryostat (Leica CM1520, Buffalo Grove, IL). Sections were then processed for hematoxylin and

eosin staining (H&E), dehydrated, mounted, and visualized at 20X with a Leica microscope. Cross-sectional areas of approximately 50 fibers per muscle were calculated using ImageJ software (NIH, Bethesda, MD). Data are represented as um^2 + SEM.

3.2.5 Micro Computed Tomography

Tibias were removed, suspended in formalin, and shipped to the Central Animal Core Imaging and Transgenic Facilities at the University of Manitoba. Using a MicroCT imager, the specimens were imaged, and the data was sent to Dr. Teja Guda from the San Antonio University for analysis. MicroCT generates high fidelity 3D models of solid material from which the outer layers can be virtually dissected or removed, revealing the inner structures [74]. This happens as radiographs of an object are taken from multiple angles, computed using an algorithm designed to reconstruct a stack of 2D x-ray projections (tomograms) into a 3D image [74]. MicroCT has drastically improved imaging standards as it can function at a resolution that can detect details down to 200nm (0.2 μm) [74]. Bone micro-architecture was evaluated by determining various structural parameters from the micro-CT such as trabecular bone volume, fractal dimension and trabecular bone pattern factor. Fractal dimension is a measurement of the fractal patterns (complexity) of the trabecular bone and can be used to describe the amount of space filled. Trabecular bone pattern factor is a 3-dimensional measurement of the orientation and connectedness of the trabeculae. Trabecular bone pattern factor shows positive values for concave structures and negative values for convex structures [61].

3.3 Limitations and Basic Assumptions

This study could not be performed on humans and therefore, a rat model was adopted to study the effects of RAD140 in combination with a resistance training model. Animal models often represent physiological treatments better than cell culture models. In this study all experiments used animals.

One limitation of our study design is that we monitored the weight of water bottles on each animal cage and then averaged the consumption across all rats in the cage. The decision to do this was made based on the social nature of rats. There is considerable evidence suggesting that rats who are not placed into group housing may experience detrimental physically and psychologically outcomes [78]. Therefore, it was in the best interest of our animals to put their needs for social interaction over the need to accurately determine individual water consumption for each animal. This is a limitation because of the possibility for some rats to ingest more or less of the RAD140 or vehicle compared to other rats.

Additionally, the expected mass of each rat upon arrival at the facility was 200-250g. Rats weighed before the procedure were roughly double the expected weight. This impacted the actual dose of RAD140 received in treatment groups due to our original dose calculation incorporating the expected weights. Furthermore, in hindsight it would have been beneficial to have measured daily drinking water before starting the intervention to gain a better idea of how much water the rats would drink. However, due to time constraints on the study this was not an option.

Based on information from the laboratory where the rats were acquired, it is assumed that they are to be pathogen free, however this was not verified.

3.4 Delimitations

FO is a model for mimicking resistance training in animals. However, it induces a chronic load on the muscle thus it is not a perfect representation of resistance training in humans. Due to the nature of the study, and the ethics surrounding the novel pharmaceutical agent being used, this model was selected as the appropriate model to determining the possible additive effects between a SARM and resistance training.

Image analysis was completed by the same person to maintain consistency and limit the amount of variation that comes with human error. This does not account for bias, but consistency in using one person is good practice and limited compounded human error. Additionally, the FO surgery was done by the same individual to maintain consistency and limit the amount of variation that is associated with human error.

Finally, the model we used for this study was on young healthy group of individuals with no induced muscle loss. This was not a true aging model with any underlying myopathies. However, it was necessary to determine the effects of RAD140 on young healthy individuals.

3.5 Statistical Analysis

Comparisons between treatment groups were done using a 2-way ANOVA mixed model for repeated measures comparing the internal control against the contralateral FO with an accepted α-error of 0.05 followed by a Fisher LSD post-hoc test [64]. Additional statistical tests such as a one-way ANOVA were completed in a similar way for some comparisons like body weight. Statistical analysis was completed using Graphpad Prism and R-Studio software's.

Chapter 4 – Results

4.1 Body Weight

A key indicating factor that our FO model functioned as designed is evident in our pre and post intervention bodyweights for each treatment group. Body weights were taken as soon as the rats arrived at the animal facility and were given ample time to acclimate. At the time of euthanasia, body weights were taken again and the two were compared. Body weights taken from the rats in the methylcellulose group were significantly higher

than their weights measured at the beginning of the study (p = 0.00114) (Figure 3).

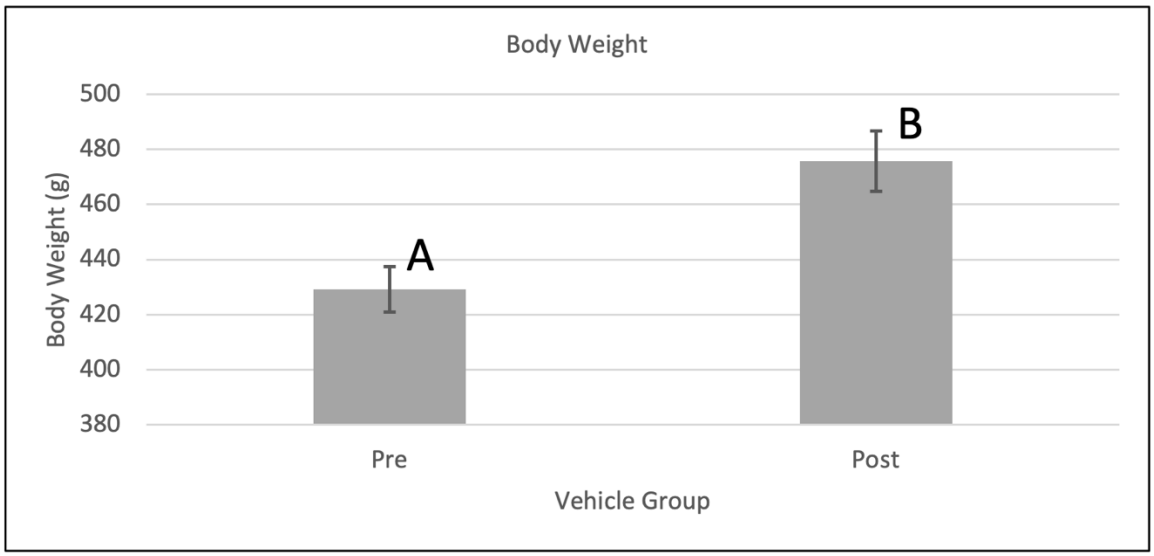

Figure 3. Average body weight (g) of vehicle groups pre and post intervention +/- SEM.

Similar to the methylcellulose group, the RAD140 rats were also weighed pre- and post-intervention. Body weights of rats in the RAD140 group were also significantly higher than their weights taken pre intervention (p = 0.0125) (Figure 4).

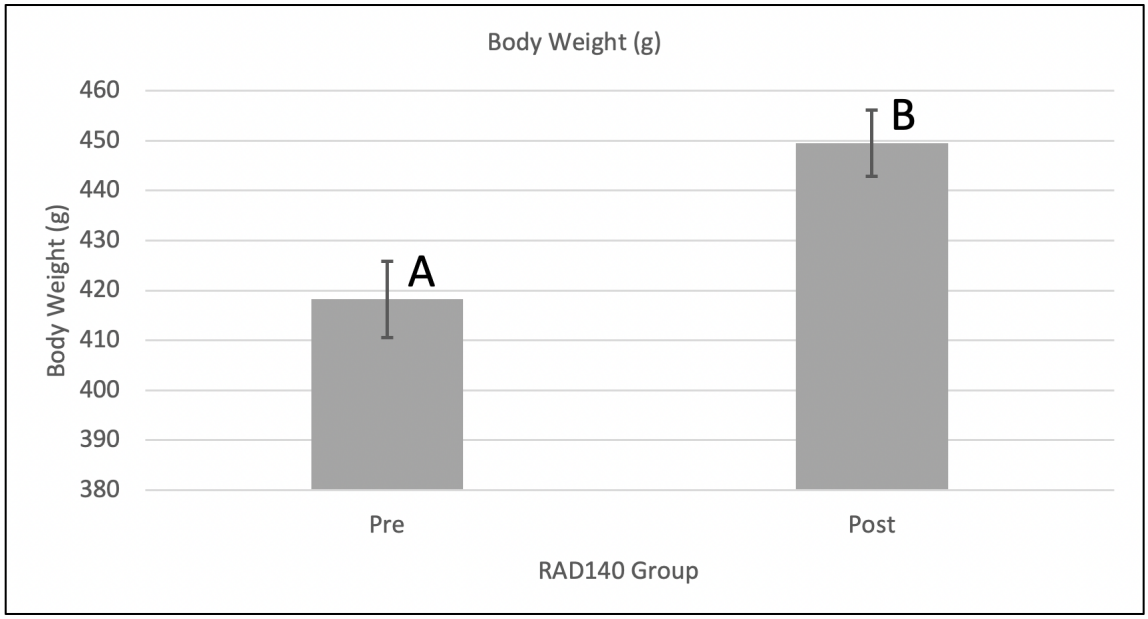

Figure 4. Average body weights of RAD140 group, pre/post intervention +/- SEM.

While it is important to determine significance within treatment groups to determine the effectiveness of the model, it is also imperative to compare between groups to determine any differences. Thus, a one-way ANOVA was done and determined that the post-intervention weights of both methyl cellulose group and RAD140 group were not statistically different ($p = 0.153$) (Figure 5).

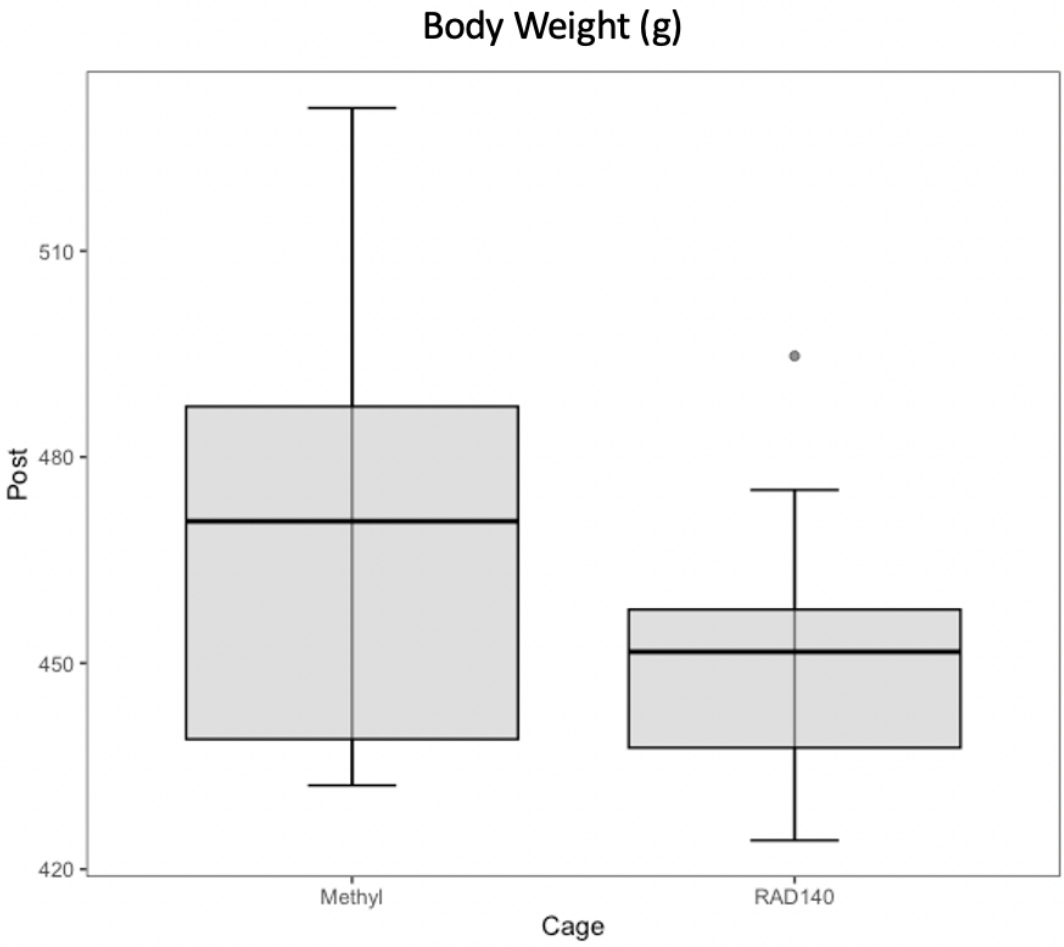

Figure 5. Boxplot illustrating the comparison of post intervention body weights of methyl cellulose and rad140 treatment groups +/- SEM. Neither group was statistically different.

4.2 Water Consumption

4.2.1 Average Daily Water Consumption by Treatment

Animals were pair-housed for health purposes and this should be considered when viewing these data Water weight measurements were taken each time the treatment water

needed to be filled. This occurred roughly 5 times per cage +/- one refill over the course of the 14-day study. A standard animal cage water bottle was weighed empty and then 500g of treatment solution was added. Measurements taken on a fill day were then used in conjunction with measurements taken on a refill day to determine the quantity of treatment water consumed in between. This was then averaged across the time span from the first measurement to give a daily average per cage. A one-way ANOVA indicated that the methyl cellulose group drank significantly more of the treatment water compared to the RAD140 group ($p = 0.0054$) (Figure 6).

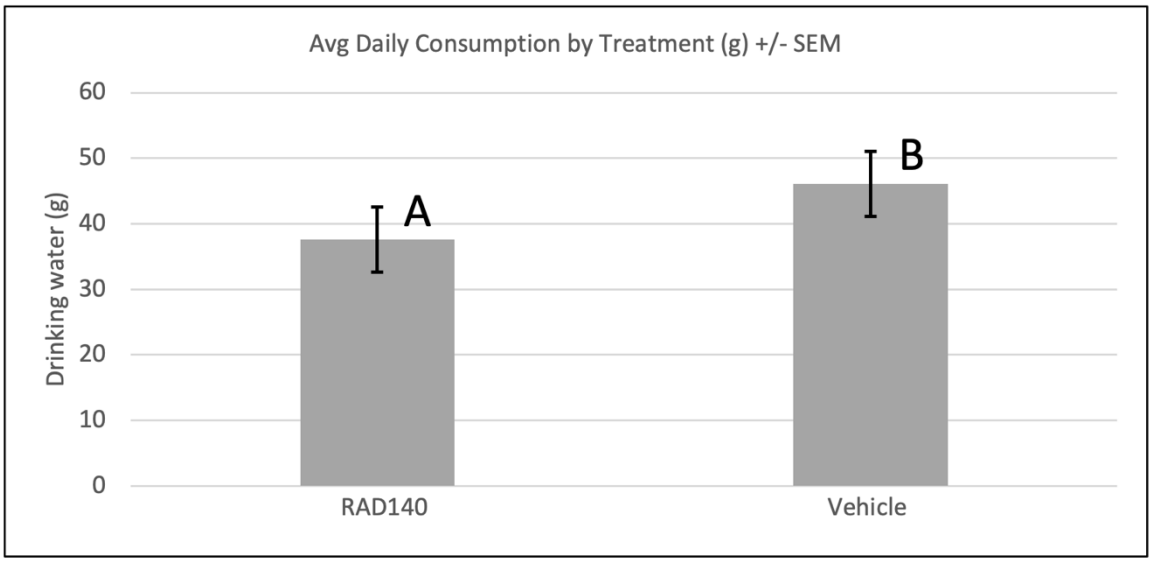

Figure 6. Average daily water consumption (g) per cage by treatment group +/- SEM.

4.2.2 RAD140 Dose Received

Using the average water consumption by cage and the average weight per cage estimated daily dose of RAD140 was calculated (mg/kg/day). Our original target dose was 3mg/kg/day; however, it is clear we did not reach our target. All RAD140 treatment animals received a dose between the levels of 1.8 and 2.5 mg/kg/day.

Cage	Avg Water Consumption(g)	Avg Pre/Post (kg)	Estimated Dose (mg/kg/Day)	HED Dose (mg/kg)

A	33.77	0.46	1.84	0.3
B	42.16	0.42	2.48	0.4
E	34.82	0.42	2.08	0.34
F	38.60	0.43	2.24	0.36
J	34.82	0.45	1.94	0.31

Table 2. Estimated daily RAD140 doses by cage (mg/kg/day) and the equivalent HED dose.

4.3 Functional Overload and RAD 140 Effects on Muscle Mass

4.3.1 Plantaris

The FO model increases plantaris hypertrophy by forcing the muscle to support in body weight due to the absence of the gastrocnemius muscle. Plantaris weights were collected at the time of euthanasia, blotted dry, weighed and then flash frozen (Figure 7). Following statistical analysis of the plantaris muscle compared across treatments, the plantaris muscle was significantly different ($p=0.000959$) across two of the four treatment groups. Using a FisherLSD post-hoc test, we determined that the vehicle-FO and RAD140-FO groups were significantly different from the vehicle-control and RAD140-control

groups.

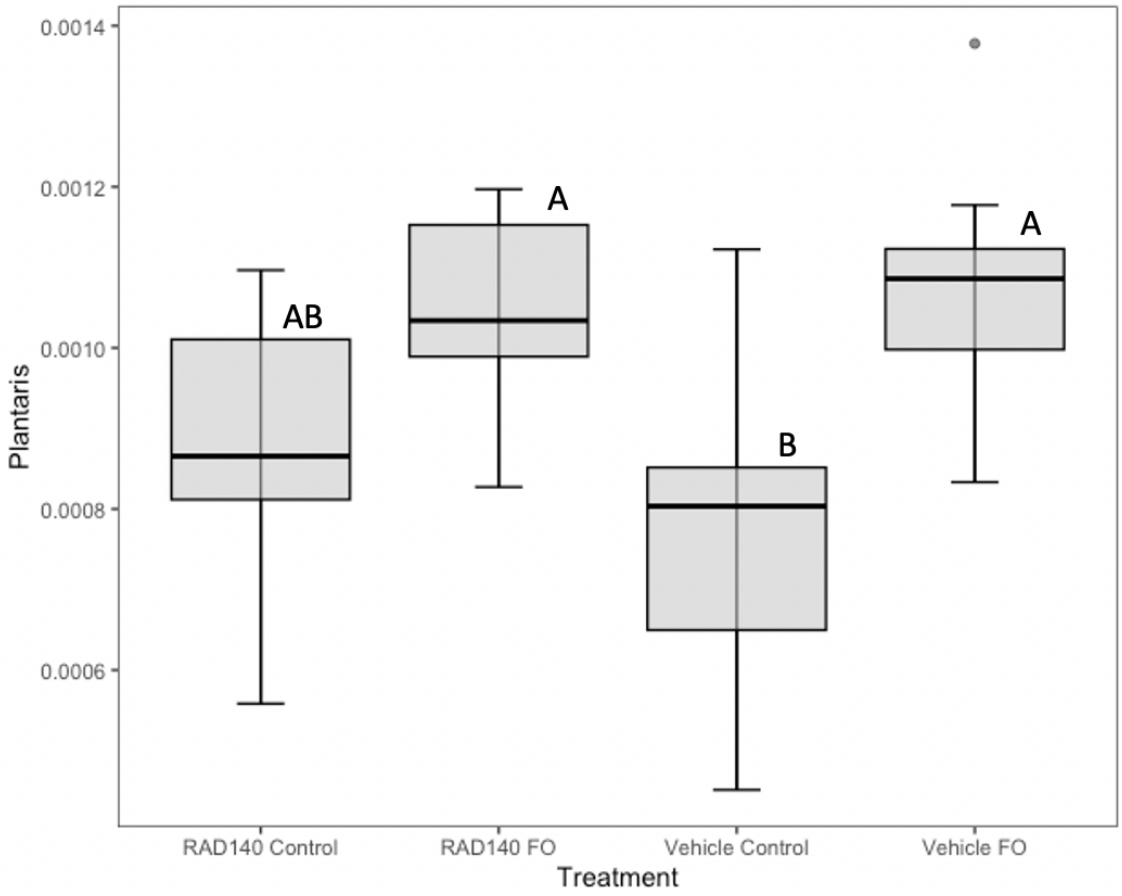

Figure 7. Average plantaris muscle mass (g) normalized to body weight by treatment group +/- SEM. Letters indicate significant differences in pairwise comparisons between treatment groups. RAD140-FO group and Vehicle-FO group were significantly different from the Vehicle-Control group. The RAD140-Control group was not significantly different from any treatment.

4.3.2 Soleus

The FO model was designed to maximize hypertrophy in the plantaris muscle; however, this model also creates additional load for the surrounding intact muscles of the triceps surae group. This includes the soleus muscle which was left intact during our surgical intervention. Following removal of the plantaris muscle, the soleus was also

removed, blotted dry and weighed. Muscle weights were then normalized to body and plotted in the form of a boxplot (Figure 8). Soleus muscle weights were significantly different ($p = 0.00376$). Using a FisherLSD post-hoc test we were able to determine that the vehicle-FO, RAD140-FO and RAD140-control were all significantly different from the vehicle-control but not from each other.

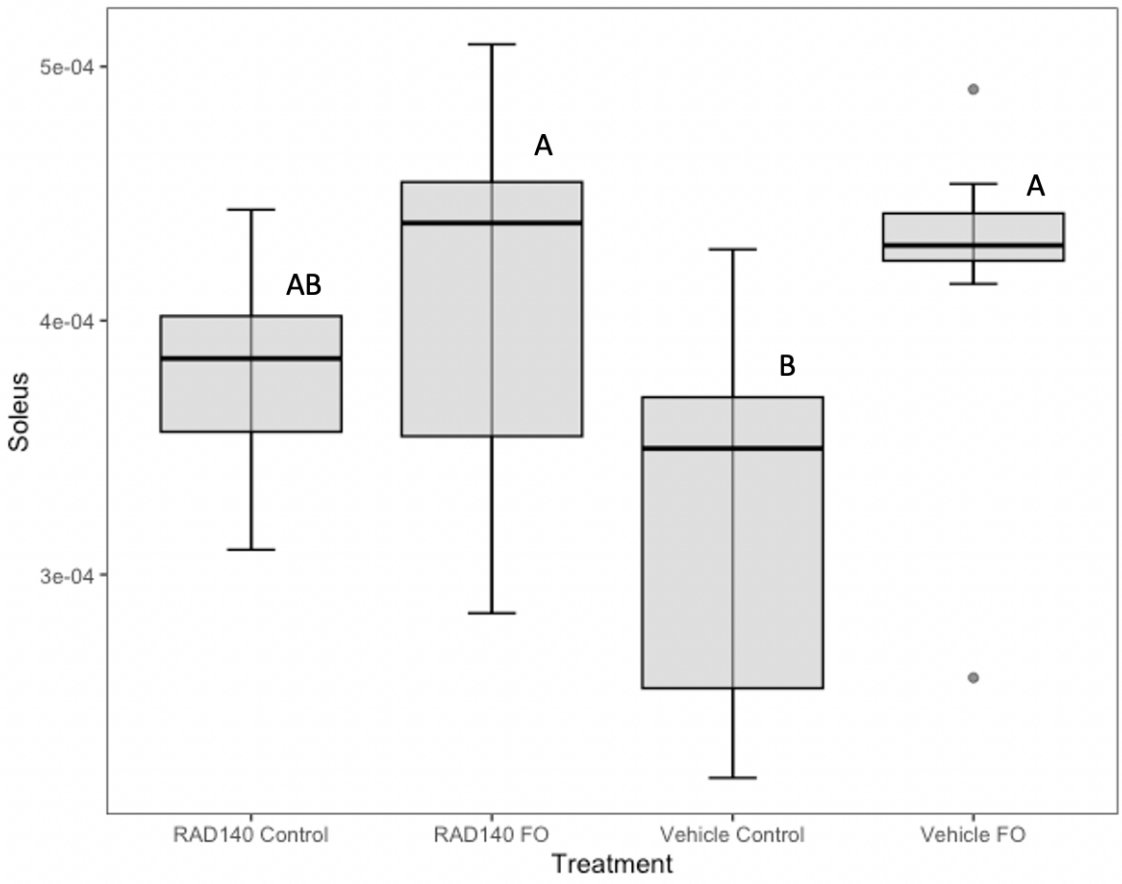

Figure 8. Boxplot illustrating the avg soleus muscle weights normalized to bodyweights across treatment groups +/-SEM. Letters indicate significant differences in pairwise comparisons between treatment groups. RAD140-FO group and Vehicle-FO group were significantly different from the Vehicle-Control group. The RAD140-Control group was not significantly different from any treatment.

4.4 Functional Overload and RAD 140 Effects on Cross-Sectional Area

Muscle hypertrophy can be gaged by increases in muscle-mass and cross-sectional area. Samples of the plantaris muscle were taken with a cryostat at the time of dissection for cross-sectional analysis. CSA treatment means and SEM were taken and plotted in a boxplot (Figure 9). Statistical analysis of the treatments indicated that that there was a significant difference between treatment groups ($p = 0.00384$). Further analysis using a Fisher post-hoc test indicated that the RAD140-FO and RAD140-Control were significantly different from the Vehicle-Control group (Figure 9).

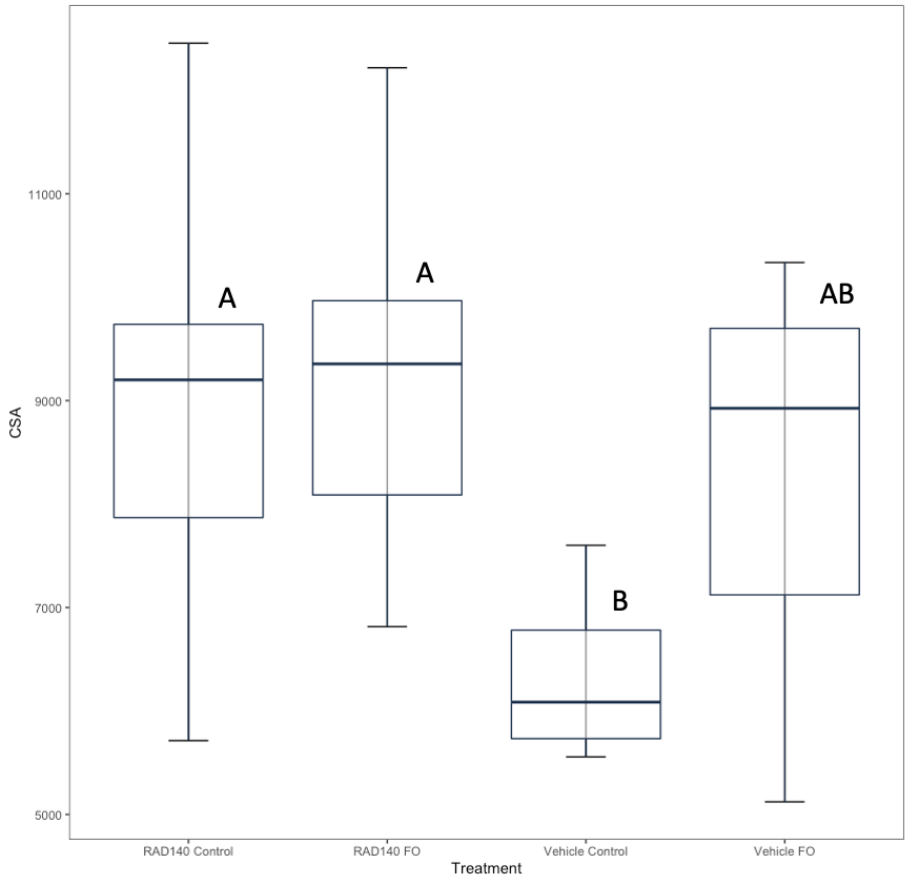

Figure 9. Boxplot illustrating the avg CSA (um^2) +/- SEM taken from plantaris muscle at the time of termination. Letters indicate significant differences in pairwise comparisons between treatment groups. RAD140-Control group and RAD140-FO group were

significantly different from the Vehicle-Control group. The Vehicle-FO group was not significantly different from any treatment.

Representative images for each CSA treatment group were also taken using a Leica CM1520 microscope and analyzed using ImageJ software (Figure 10A-D). The RAD140-FO group was not statistically significant when compared to the RAD140-Control group (Figure 9). It was statistically significant when compared to the vehicle-control group (Figure 9).

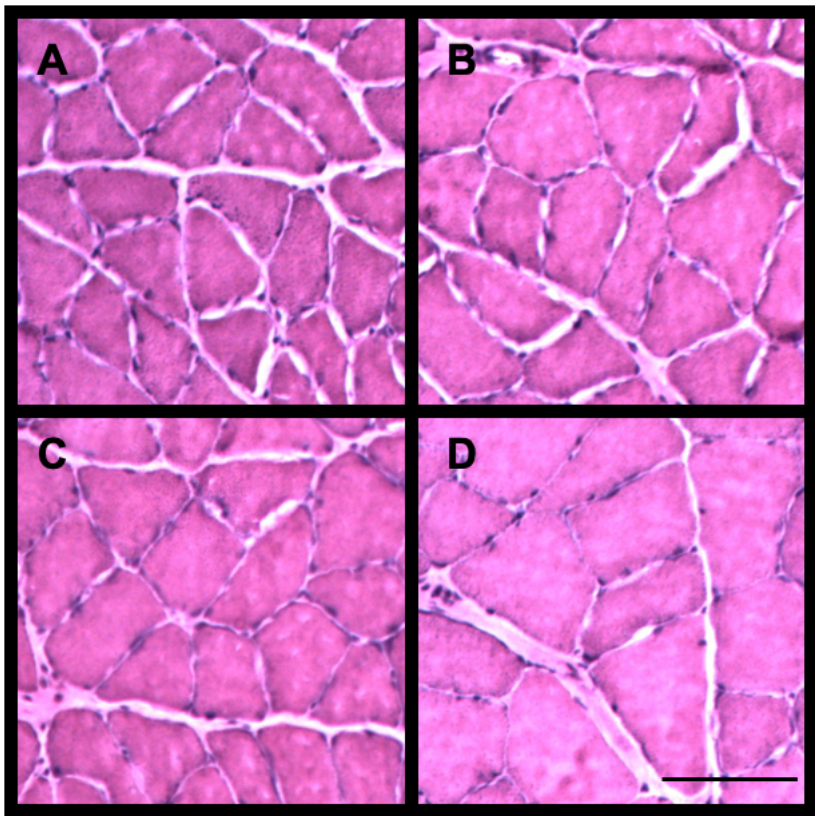

Figure 10. Representative histology of rat plantaris muscles. A, Vehicle-Control; B, Vehicle-FO; C, RAD140-Control; D, RAD140-FO. Mag bar in panel D = 100 µm.

4.5 Micro Computed Tomography

4.5.1 Cortical Bone Analysis

The FO model was designed to maximize hypertrophy in the triceps-surae muscle group; however, this model also provided the opportunity to examine the effects of RAD140 on the underlying bone tissue. The cortical polar moment of inertia (MMI), an excellent measure for the structural integrity of bone [79] was measured using microCT imaging on the distal metaphysis of the tibia then analyzed using PRISM software (Figure 11). There were no statistically significant trends found in the data (P= 0.9796).

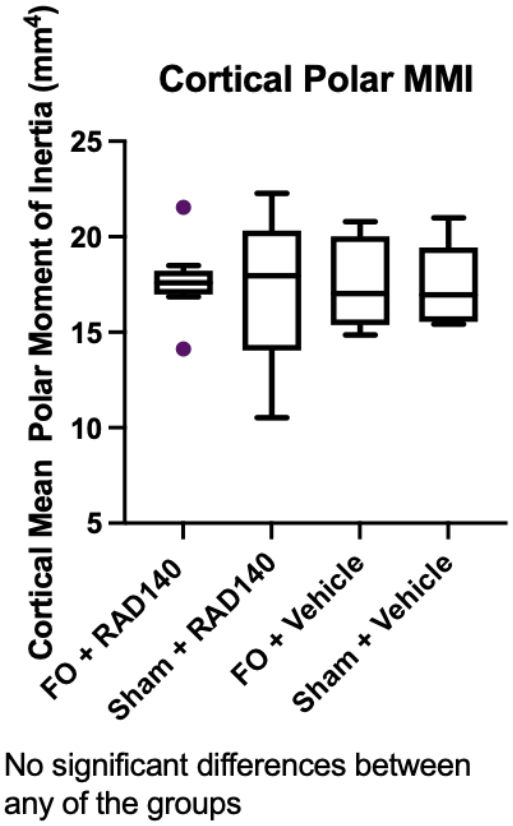

Figure 11. Boxplot illustrating the average Cortical Polar MMI +/- SEM for rat tibias across treatment groups. No significant differences between treatment groups.

Another important measure of cortical bone is cortical CS thickness which is a good representation of the mechanical properties of the cortical bone [81]. Cortical thickness was measured using microCT imaging on the distal metaphysis of the tibia then

analyzed using PRISM 9 software (Figure 12). There were no statistically significant findings (P= 0.4249).

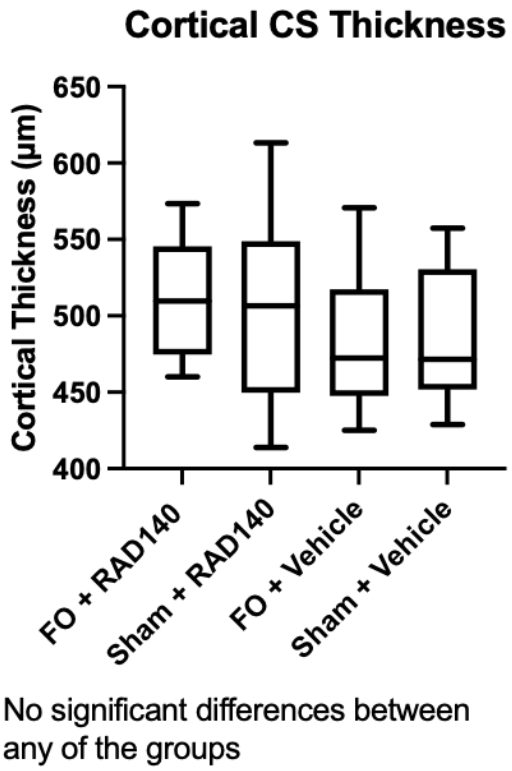

Figure 12. Boxplot illustrating the average Cortical CS Thickness +/- SEM for rat tibias across treatment groups. No significant differences between treatment groups.

Cortical CSA is another important indicator of the mechanical structure and health of cortical bone [81]. CSA was measured using microCT imaging on the distal metaphysis of the tibia then analyzed using PRISM 9 software (Figure 13). There were no statistically significant findings (P= 0.5838).

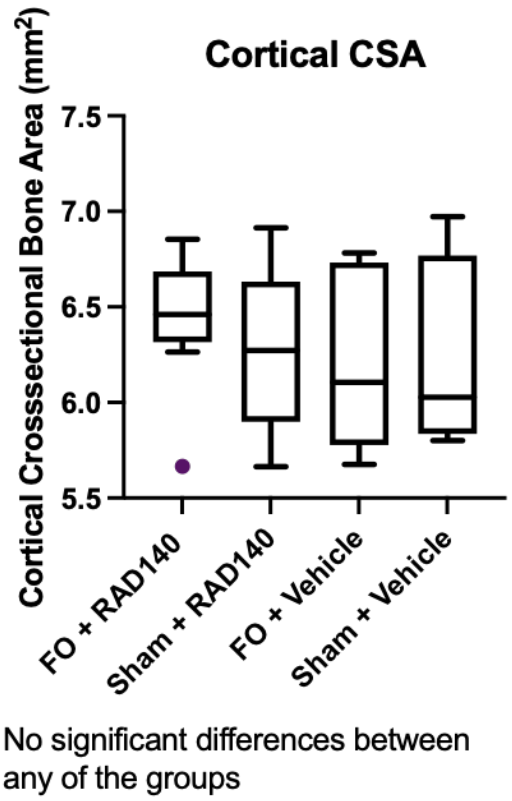

Figure 13. Boxplot illustrating the average Cortical CSA +/- SEM for rat tibias across treatment groups. No significant differences between treatment groups.

Cortical bone mineral density (BMD) may be used as a good indicator of pathology in bone tissue [81]. It was measured using microCT imaging on the distal metaphysis of the tibia then analyzed using PRISM 9 software (Figure 14). There were no statistically significant findings (P= 0.4025).

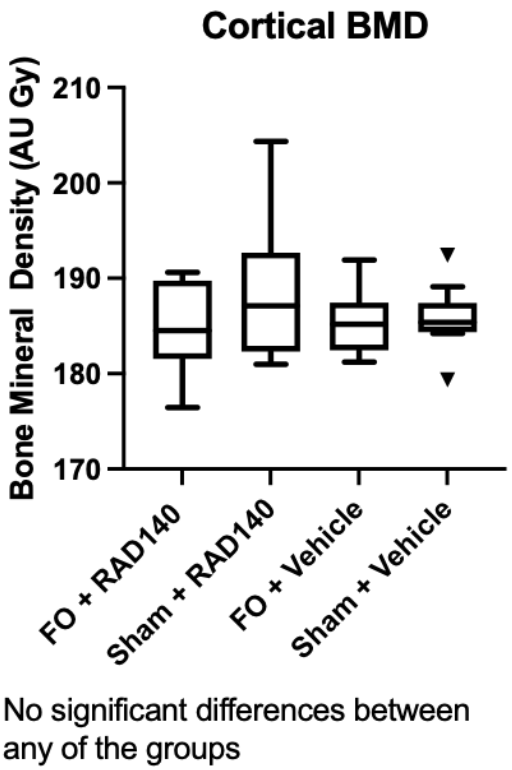

Figure 14. Boxplot illustrating the average Cortical BMD +/- SEM for rat tibias across treatment groups. No significant differences between treatment groups.

Representative microCT images of the distal metaphysis of the tibia for each treatment group were taken and visually analyzed for differences (Figure 15). Images were captured using a microCT imager and no obvious visual differences were evident between treatment groups.

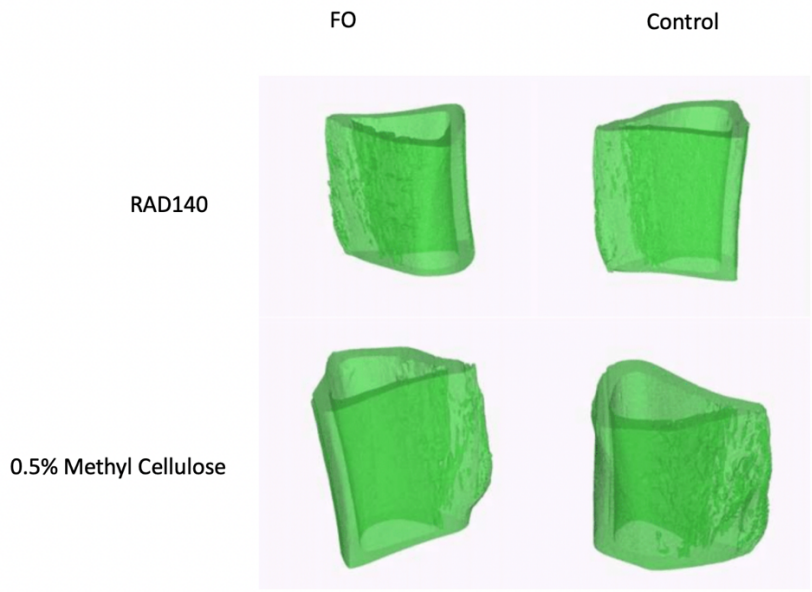

Figure 15. Representative microCT images of the cortical bone for each treatment group.

4.5.2 Trabecular Bone Analysis

The trabecular architectural data represents a region of bone extracted from the trabecular regions below the subchondral growth plate in the epiphysis of the tibia. Trabecular number is an important physiological measure as it represents the number of trabeculae present and gives insight into the amount of remodeling taking place inside of the trabecular bone tissue [81]. The trabecular number was measured using microCT imaging and analyzed using PRISM 9 software (Figure 16). There were no statistically significant findings between treatment groups ($p = 0.3761$).

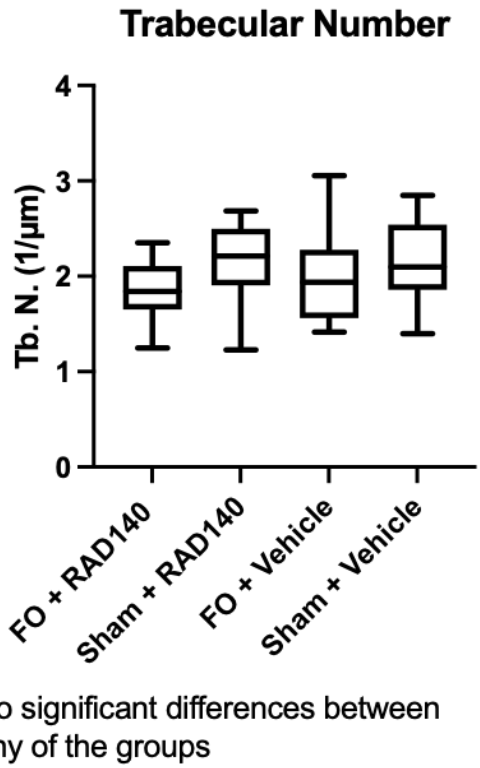

Figure 16. Boxplot illustrating the average Trabecular Number +/- SEM for rat tibias across treatment groups. No significant differences between treatment groups.

Trabecular bone volume% (BV%) is important indicator as it represents the overall size and connectivity of the trabecular bone [72,74]. Trabecular BV% was measured in the regions below the subchondral growth plate in the epiphysis of the tibia using microCT imaging and analyzed using PRISM 9 software (Figure 17). There were no statistically significant findings (p=0.5468).

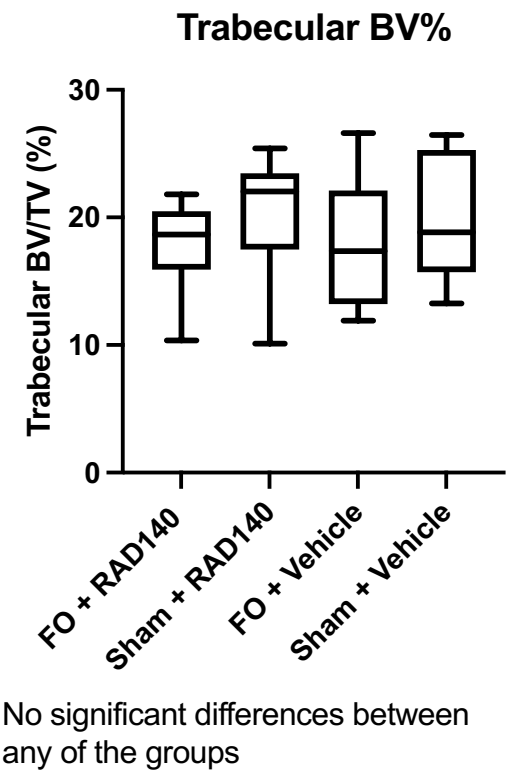

Figure 17. Boxplot illustrating the average Trabecular BV% +/- SEM for rat tibias across treatment groups. No significant differences between treatment groups.

Trabecular bone surface density is a measure of the ratio of segmented bone surface to the total volume of the region of interest [72]. Trabecular bone surface density was measured in the regions below the subchondral growth plate in the epiphysis of the tibia using microCT imaging and analyzed using PRISM 9 software (Figure 18). There were no significant findings (p=0.5336).

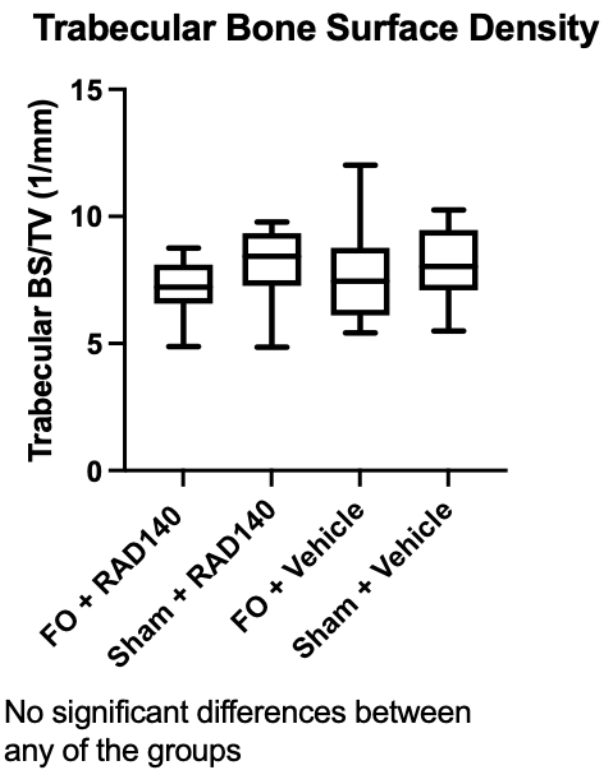

Figure 18. Boxplot illustrating the average Trabecular Bone Surface Density +/- SEM for rat tibias across treatment groups. No significant differences between treatment groups.

Trabecular pattern factor (TBPf) is the ratio of concave to convex surfaces of the bone pattern observed in two-dimensional sections representing a three-dimensional space [72,74]. TBPf was measured in the regions below the subchondral growth plate in the epiphysis of the tibia using microCT imaging and analyzed using PRISM 9 software (Figure 19). No significant results were found (p=0.3030).

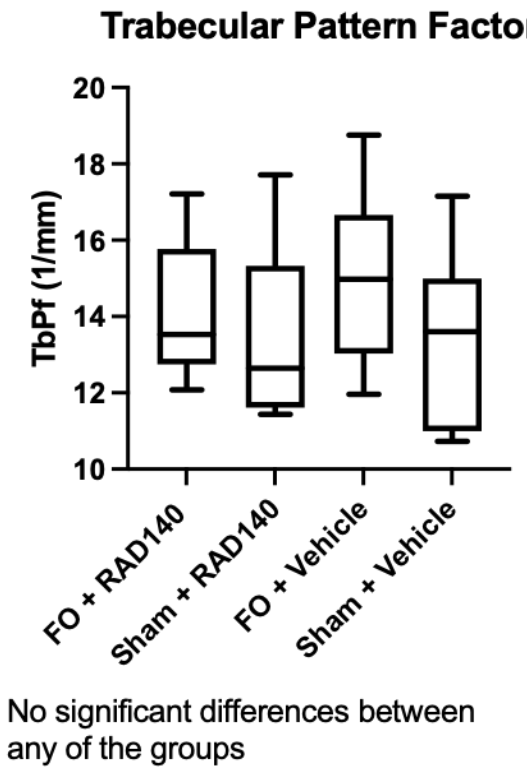

Figure 19. Boxplot illustrating the average Trabecular Pattern Factor +/- SEM for rat tibias across treatment groups. No significant differences between treatment groups.

Trabecular structural model index (SMI) is a measure used to quantify the number of rods and plates inside of trabecular bone [73]. Trabecular SMI was measured in the regions below the subchondral growth plate in the epiphysis of the tibia using microCT imaging and analyzed using PRISM 9 software (Figure 20). No significant results were found ($p=0.1096$).

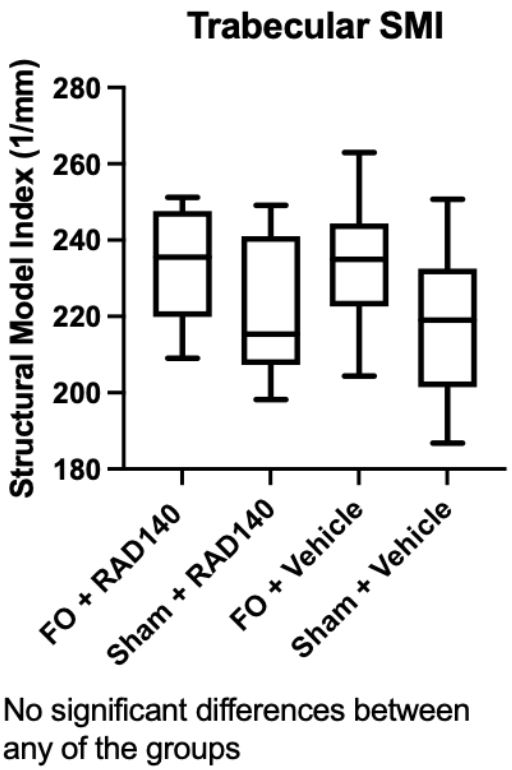

Figure 20. Boxplot illustrating the average Trabecular SMI +/- SEM for rat tibias across treatment groups. No significant differences between treatment groups.

Chapter 5 – Discussion

This study examined the effects of the selective androgen receptor modulator (SARM) RAD140 in combination with a functional overload (FO) model for hypertrophy. The assumption is that both RAD140 and FO will independently result in hypertrophy but in combination will result in a greater hypertrophic effect then any one treatment alone. While there has been some investigation into the efficacy and safety of other SARMs such as Ostarine (GTX-024), there has been little to no investigation into the SARM RAD140 for the purpose of increasing muscular hypertrophy. To investigate the potential hypertrophic benefits of RAD140, a rat model using a functionally overloaded hind limb was used. The outcomes from this study will be useful in determining potential clinical applications for RAD140 in humans. Based on the results from the muscle mass comparisons and CSA analysis, RAD140 causes increases in muscular hypertrophy in the

same magnitude that FO individually. However, the combination of the two creates no additive effect.

5.1 Water Consumption and Dose Effect

While it was expected that the rats would not consume identical amounts of water, it was assumed that they would consume an average quantity per day based upon their body weights [85]. Animals were ordered from Charles River Laboratories to Georgia State University animal facility and expected to be in the 200-250g weight range. To our surprise, animals grew much quicker than expected and weighed in the 400-450g range at the start of our experiment following their acclimation to the facility. The concentration of RAD140 in 0.5% methyl cellulose that was prepared for the treatment group was calculated based upon the assumption that our rats would be in the 200-250g range and designed to achieve a daily dose of ~3mg/kg. As a result of our animals weighing double their expected weights, our actual received daily doses were lower than what was intended (Table 2). Due to this unexpected change in animal weights prior to the surgical intervention our actual daily doses were in the range of 1.8-2.5mg/kg/day. This likely played into the lack of significant difference observed between the RAD140-control, RAD140-FO and Vehicle-FO groups.

Furthermore, water consumption was measured for both treatment group and determined that the rats preferred to drink the 0.5%methyl-cellulose solution over the RAD140 + 0.5% methyl cellulose (Figure 5). Since the rats drank significantly more of the vehicle compared to the RAD140 ($p = 0.0054$) we can assume that there may be attraction to the taste of the 0.5% methyl cellulose or that there may be a deterrent associated with the smell or taste of the RAD140 [86]. Although the vehicle group did consume more water on average per day, the range at which they were consuming the water was still within normal levels [85].

5.2 Functional Overload and RAD140 Effects on Muscle Mass

Functional overload (FO) is a surgical model designed to chronically load a muscle, resulting in hypertrophy for the remaining intact muscle or muscle group [23, 24]. This

surgical procedure forces the animal to carry the same load with fewer muscle groups, thus increasing the load on the individual muscle(s). While it was expected that the FO model would induce sufficient muscle hypertrophy, the effects of a combined treatment between a muscle hypertrophy inducing model and a SARM were unknown. While it was expected to have an additive effect, this was not found. Through statistically investigation of pre and post body weights, it was evident that each group had significantly increased from their pre intervention body mass ($p=0.00114$ – methyl, $p=0.0125$ – RAD140). However, the post intervention body masses for each treatment group were not statistically different from one another ($p=0.153$). Therefore, the combination of RAD140 and FO did not have an additive effect greater than that of RAD140 or FO alone on overall body weights of the animal's following intervention. Relative to pre intervention, the observed change in each group was not statistically different between groups. While we have no evidence of an additive effect between RAD140 and FO in overall body weight, it is possible that prolonging the study from 14 days to 21 days may have given the RAD140 enough time to be able to build up a larger effect on muscle mass. Although, SARMs are not yet clinically proven to be safe for human consumption, many people in the body building community have been taking this pharmaceutical for a few decades [90]. It is often agreed upon in this community that RAD140 can take up to 7-8 weeks in humans to show its full effects in muscle mass. While this is not scientifically proven, it leaves questions regarding whether the timeline of our study was long enough to see the maximum effectiveness of RAD140 in rats.

Further investigation into the individual muscles affected by the FO model revealed that FO and RAD140 both caused an increase in overall muscle mass of the plantaris muscle (Figure 7), compared to the vehicle control; however, these groups were not statistically significant different from one another. The RAD140-Control group was not statistically different from any other group. This may be due to the lack of time given for RAD140 to have its full effect in the muscle as animals were only treated for 14 days. It is also possible that the invasive and powerful nature of the FO treatment in causing immediate hypertrophy overpowered any smaller affects created from the RAD140 in such a short timeframe. We expect that if this timeline were to be extended that there would be a visible increase in the observed muscle mass produced from RAD140 alone. It is also possible that due to the nature of the plantaris muscle being the primary load baring muscle

in the triceps-surae group that the effect of the FO model may have masked the effect of the RAD140 in such a short time frame.

The plantaris muscle is not the only muscle group responsible for carrying the increased load in our FO model. Although it is the primary load baring muscle of the group, the soleus muscle also bares a certain percentage of the body weight and is also affected by the FO model. Investigation into the soleus muscle revealed that the RAD140-FO and vehicle-FO groups were statistically significant compared to the vehicle-control group ($p = 0.00376$) but were not statistically different from the RAD140-control group (Figure 8). Again, the same pattern arises as seen in the plantaris muscle. It is possible that due to the timeline of the intervention that the RAD140 groups were not given enough time to overcome the effects of the FO treatment, thus masking the true effectiveness of the SARM itself. As both processes occur simultaneously, it is possible that such a robust intervention like FO which has been proven to cause hypertrophy within a week of intervention [6, 7, 71, 77], that we cannot properly deduce the effect of RAD140 in the muscle. This may also come back to the timeline in which RAD140 is most effective. Further investigation into the timeline for RAD140 to act in its full capacity on muscle groups will be key to determining the efficacy of RAD140 in increasing muscle hypertrophy. From these findings, the FO model has significant effects on muscle hypertrophy in primary and secondary supporting muscles of the triceps-surae group. It is also clear that RAD140 is having some effect, to what degree is still to be determined. This will likely require a follow-up study with a prolonged timeline to determine where RAD140 peaks following administration.

5.3 Functional Overload and RAD140 Effects on CSA

We expected that the FO would increase CSA [87]. However, the additive effects of FO and RAD140 on the CSA of plantaris muscle fibers have not been investigated. The RAD140-FO group and RAD140-Control group were significantly different compared to the Vehicle-Control group (Figure 9). However, the two RAD140 groups were not statistically different from the Vehicle-FO treatment group. Additionally, the Vehicle-control group was not statistically different from the Vehicle-FO group (Figure 9). This

may be an indication that RAD140 in combination with FO treatment has elicited a larger physiological response then just the FO treatment alone. Although this cannot be for certain as the FO treatment alone did have a lower average CSA but was not statistically significant ($p=0.07880$). Potentially a larger sample size and longer study timeline may be able to deduce whether there is a real physiological difference between these two groups.

Representative histology of rat plantaris muscles were stained using H&E (Figure 10). Again, RAD140-FO group was only statistically significant when compared to the Vehicle-Control group (Figure 9). This is further indication that there is an interaction taking place; however, further investigation with larger sample size and longer treatment time would be beneficial in determining exactly what the relationship is between CSA and FO+RAD1140 treatments.

5.4 Functional Overload and RAD 140 Effects on Bone Microarchitecture

SARM's have been found to act on the AR's located primarily within muscle and bone. It is important to understand the full range of effects these novel pharmaceuticals may possess in creating positive change for osteopathy. The SARM RAD140 has previously been shown to be extremely active in the AR of muscle tissue. However, there is little to no information on its potential effects on the AR's inside of the cortical and trabecular bone tissues. Our model provided us the ample opportunity to collect this measure while also investigating our main hypothesis.

After thorough analysis of the tibia microCT data, there were no statistically significant results found between any of the four treatment groups for any of the measures. However, this is not overly surprising as the functional overload-SARM model created for the purposes of this study were not intended to create any pathological issue in the underlying bone tissue. Animals in the study were young, healthy individuals and are not good representations for a model looking to induce physiological change within bone tissue. We know this based upon the results of the Cortical Polar Moment of Inertia (MMI) graph (Figure 11) which is a good indication of the load carrying capacity of the bone [81]. This measurement considers the overall volume and density of the bone to determine the polar MMI. The polar MMI is inversely related to the stress created by torque pressure

acting on the bone [81]. Therefore, the higher the polar MMI a bone has the more force required to cause a fracture. Since there are no significant trends, it can be said that physiological homeostasis was not altered and therefore there are no pathological issues present in the underlying bone tissue.

Although there were no statistically significant findings, there were some noteworthy trends within the cortical data. An analysis of the cortical CS thickness (Figure 12) showed that the RAD140 groups had greater cortical thickness on average then did the methyl cellulose groups. Although it is not statistically significant, it appears as though the RAD140 treatment may be increasing cortical bone thickness irrespective of the FO treatment. Similarly, there were additional trends evident in the cortical bone data. For example, visible decreases between group averages in cortical CSA (Figure 13). This graph may be interpreted as a reduction in CSA when FO treatment is absent, implying that FO treatment is contributing to a larger cortical CSA. Additionally, it is possible to visualize a potential synergistic relationship between the RAD140 and FO treatments as the FO+RAD140 group had the largest cortical CSA on average (Figure 13). Lastly, cortical BMD was assessed and determined to have no significant findings between any treatment groups (Figure 14). This is not surprising to see based upon the other cortical bone results, small changes in BMD content may represent massive physiological change in the bone, which we did not see [88]. Representative mircoCT images illustrating the distal metaphysis of the tibia do not show any overly obvious change between treatment groups (Figure14).

Like the cortical bone findings, the trabecular bone analysis did not turn up any significant findings, however there were some notable trends in the data. The stability of trabecular bone is dependent on the amount of bone tissue but also the three-dimensional orientation and connectedness of the trabeculae, which can be summarized as the trabecular microarchitecture [74]. An important measure of trabecular bone is the total trabecular number (Figure 16). This is a measure of the average number of trabeculae per unit length [80] and is a good representation of bone remodeling [72]. There is an observable difference in the two FO groups average trabecular numbers compared to the control groups, however not statistically significant (Figure 16). When there is an observed decrease in trabecular number due to an added load or stress, it is common to see an

associated increase in the cortical bone thickness [74]. This is likely a homeostatic response within the bone to adjust to an increased stress by remodeling resources to be able to maintain structural integrity. Another relevant measure for trabecular bone is bone volume percentage (BV%) which is the ratio of segmented bone volume to the total volume of the region of interest (Figure 17) [80]. Results from the statistical analysis were not significant, however did illustrate a trend that was consistent among other measures. It appears that the FO treatment is affecting trabecular BV% in a way that is causing a slight decrease in bone volume. This is consistent with trends in the cortical CSA where FO treatments seemed to cause an increase in cortical CSA (Figure 13). In previous studies, researchers were able to establish that in three-dimensional bone tissue the relation of trabecular plates to rods can be illustrated in the ratio of concave to convex surfaces of the bone pattern observed in two-dimensional sections [74]. To be able to quantify the connectedness of these bone patterns, researchers further developed a histomorphometric parameter called Trabecular Bone Pattern Factor (TBPf) [74]. Similarly, to the BV% and CSA the FO treatment groups had higher TBPf's then the control groups (Figure 19). The same trend is also evident in the trabecular surface density results (Figure 18), where FO groups had lower average surface densities then the control groups. Lastly, the structural model index (SMI) of trabecular bone was also measured (Figure 20). Although, there were no significant findings there were similar trends observed in the other measures. The trabecular SMI is a method that has been used to determine the plate- or rod-like geometry of trabecular structures [73]. This method was developed in the early 1990s and has since been replaced with a more accurate representation for the measure known as mean ellipsoid factor (EF) [73]. Since this method for measuring trabecular geometry is no longer widely accepted, the SMI results should be interpreted lightly. Although, the findings align with trends evident in the other measures, FO groups exhibiting lower SMI numbers then the control groups. It is possible that this trend which is evident across almost all bone measures may be evidence of bone maintaining homeostasis by undergoing some degree of remodeling to account for an increased load.

Chapter 6 – Conclusion

As populations continue to age, the importance of understanding the impacts of sarcopenia and other age-related diseases will become even more important as many western societies are faced with aging populations. Furthermore, being able to provide an intervention that will improve the overall quality of life for every elderly person would be extremely valuable. This is one of many reasons why research into the effects of the SARM RAD140 in combination with FO treatment could be so beneficial for myopathy and osteopathy treatments in future generations. It was determined that the administration of RAD140 in combination with FO significantly increased muscle mass and CSA when compared to the vehicle-control groups. There were no significant findings in the tibia bone data. However, this was not an osteopathy model and thus did not elicit any pathological change to the bone. This is likely why there were no significant findings. The results from the muscle data seem to be sufficient evidence of an interaction that is taking place between treatments, further investigation into the exact mechanisms and extent of each treatments ability to produce hypertrophy in elderly populations is warranted.

Chapter 7 – Future Directions

From the conclusions we were able to make from data collected over the course of this study, there are several viable future directions for furthering the knowledge of SARMs treatments in humans and animals. We believe it will be important to implement this model for a longer period, extending the amount of time animals will receive RAD140. Much of the information regarding human consumption of RAD140 points to its effectiveness becoming most evident at the 7–8-week mark.

Further investigation into the ability of RAD140 to intact physiological change on underlying bone tissues within a well-established bone model is required to understand its ability to act on AR in bone tissue. The model used here was ideal for measuring hypertrophy in muscle and offered and ample opportunity for an observation of change in bone but was not intended to be an osteopathy model.

Another step to further study in this field would be implementing an FO model with SARM treatment in an aging model that better represents the reduced hormone profiles of aged individuals that might yield different results.

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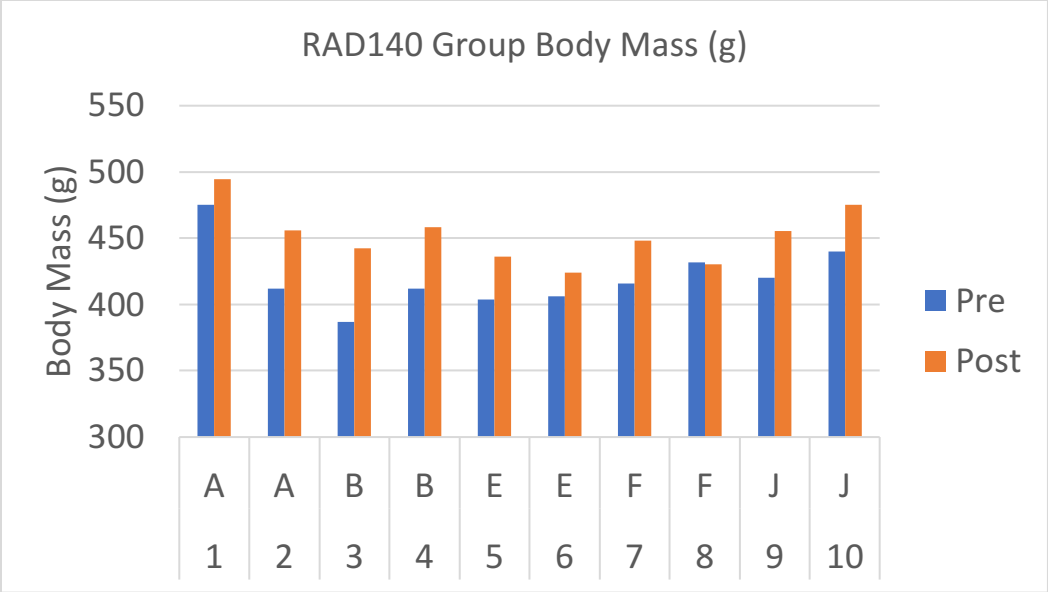
Abbreviations list

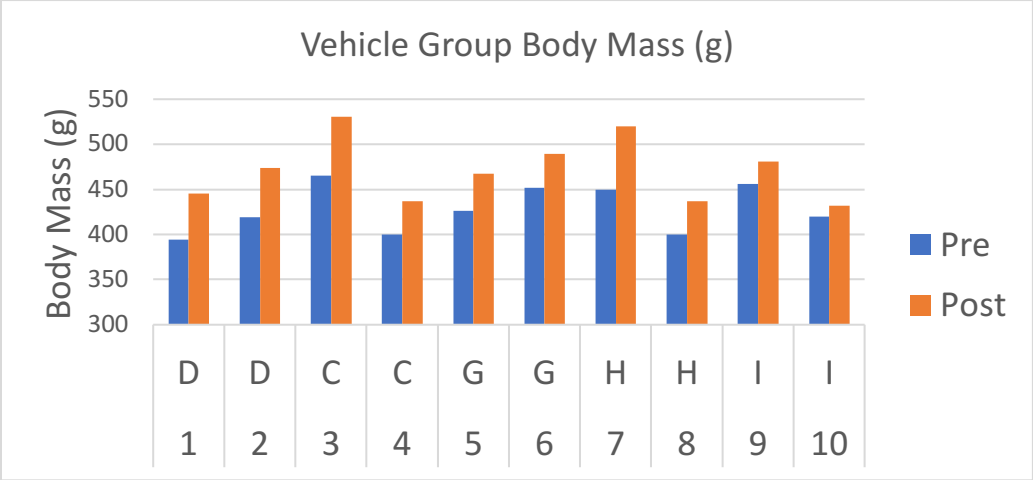
American Society of Anesthesiology score – ASA score
Analysis of Variance – ANOVA
Androgen Receptor – AR

Androgen Response Element - ARE
Bone Mineral Density - BMD
Bone volume Percent - BV%
Cross-sectional Area – CSA
Deoxyribonucleic Acid - DNA
Dihydrotesterone – DHT
Extracellular Matrix - ECM
Functional Overload - FO
Georgia State University - GSU
Growth Hormone - GH
Heat Shock Proteins - HSP
Human Equivalent Dose - HED
Insulin-like Growth Factor I - IGF-I
Messenger Ribonucleic acid - mRNA
Micro-computed tomography -MicroCT
Muscle Satellite Cells – MSC
Ribonucleic Acid - RNA
Selective Androgen Receptor Modulator - SARM
Structural Model Index -SMI
Total Joint Arthroplasty -TJA
Polar Moments of Inertia - MMI-Polar
Progressive Resistive Exercises – PRE

Chapter 10 - Appendix



Appendix A. RAD140 group individual body mass (g)



Appendix B. Methylcellulose group individual body mass(g)

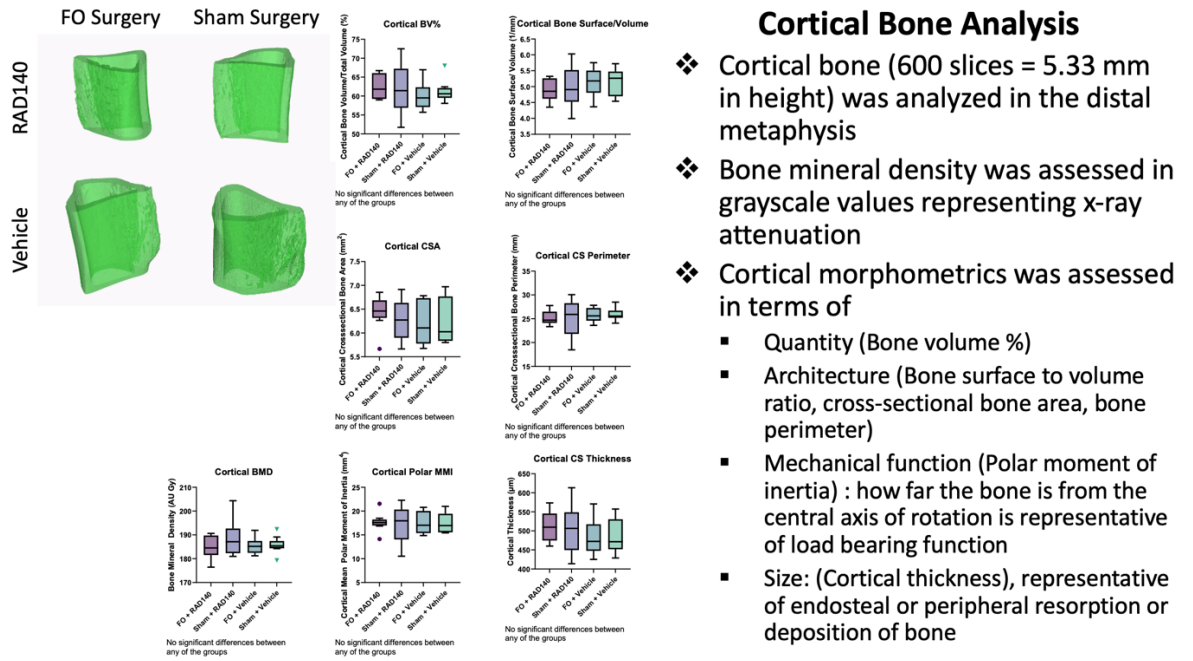
Treatment	diff	lwr	upr	p adj
RAD140 FO-RAD140 Control	0.00002852	-0.00004649	0.00010353	0.73666180

Vehicle Control-RAD140 Control	-0.00006302	-0.00013803	0.00001199	0.12603590
Vehicle FO-RAD140 Control	-0.00003797	-0.00003704	0.00011298	0.52985970
Vehicle Control-RAD140 FO	-0.00009154	-0.00016655	-0.00001653	0.01162730
Vehicle FO-RAD140 FO	0.00000946	-0.00006555	0.00008447	0.98630650
Vehicle FO-Vehicle Control	0.00010099	0.00002598	0.00017600	0.00468820

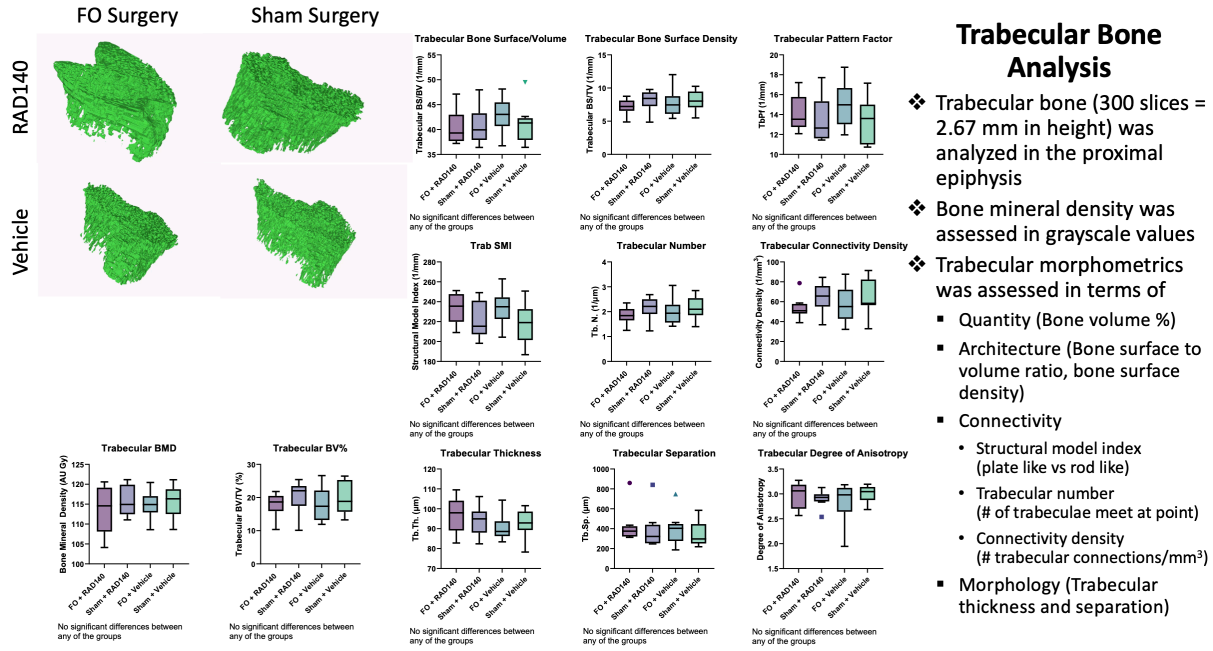
Appendix Table. Tukey HSD results for Soleus ~ Treatment

$Treatment	diff	lwr	upr	p adj
RAD140 FO-RAD140 Control	0.00017339	-0.00003030	0.00037708	0.11872180
Vehicle Control-RAD140 Control	-0.00008992	-0.00029361	0.00011378	0.63767790
Vehicle FO-RAD140 Control	0.00020075	-0.00000294	0.00040444	0.05463820
Vehicle Control-RAD140 FO	-0.00026331	-0.00046700	-0.00005961	0.00694270
Vehicle FO-RAD140 FO	0.00002736	-0.00017633	0.00023105	0.98353820
Vehicle FO-Vehicle Control	0.00029067	0.00008697	0.00049436	0.00256640

Appendix Table. TukeyHSD results for Plantaris ~ Treament



Appendix. Cortical Bone analysis summary.



Appendix. Trabecular Bone analysis summary.