INFLUENCE OF CASTRATION AND A HIGH PROTEIN DIET ON LOAD-MEDIATED HYPERTROPHY AND SKELETAL MUSCLE FORCE PRODUCTION IN RATS FOLLOWING IMMOBILIZATION

Ryan J. Viverette

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Approved by:

Erik Hanson

Alan Hayes

Eric Ryan

Zachary Kerr
ABSTRACT

Ryan J. Viverette: Influence of Castration and and a High Protein Diet on Load-Mediated Hypertrophy and Skeletal Muscle Force Production in Rats Following Immobilization (Under the direction of Erik Hanson)

The purpose of the present study to was to examine the effects of low testosterone on muscle mass and function in rats following 10 days of hindlimb immobilization and determine if a high protein diet enhances load-mediated hypertrophy. Average SOL CSA was significantly lower for immobilized vs. control legs at 0 (30.3%, p < 0.001) and 14 days of reloading (15.9%, p = 0.006). Significant differences in average soleus mass were present at all days of reloading (all p < 0.001). Immobilized SOL P₀ followed a similar pattern (all p < 0.001), and castrated animals showed lower P₀ at 14 days (p < 0.050). At present, functional overload in a testosterone-deprived state appears to reduce the regrowth of skeletal muscle size, suggesting that testosterone may play a role in load-mediated hypertrophy following immobilization. As well, a high protein diet did not result in enhanced load-mediated hypertrophy in castrated rats.
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CHAPTER I
INTRODUCTION

Basis for Study:

The loss of endogenous testosterone, as a result of normal aging or pharmacological intervention for prostate cancer therapy, has adverse effects on skeletal muscle mass, strength, function, and physical activity levels (Basaria et al., 2008). It is estimated that hypogonadism affects between 2.1 - 12.8% of adult males in the general population (Zarotsky et al., 2014). Primary hypogonadism is a result of a defect within the Leydig cells of the testes in males, while secondary hypogonadism occurs elsewhere, often within the hypothalamic-pituitary gonadal (HPG) axis. Androgen deprivation therapy (ADT) is a form of prostate cancer treatment that suppresses male sex hormone production to help slow the progression of prostate cancer by preventing the pituitary from releasing lutenizing hormone (LH) or through the use of antiandrogens antagonists (Sharifi et al., 2010). Regardless of the pathology, hypogonadism can induce changes similar to the effects of aging but at an accelerated rate (Bylow et al. 2007, Hanson et al., 2011 such as significant reductions in muscle mass, increases in fat mass (van Londen et al., 2008), and reduced bone mass (Basaria et al., 2002), and physical inactivity (Galvao et al., 2009).

Suppression of endogenous testosterone levels is also associated with a decrease in fractional muscle protein synthesis in younger men (Mauras et al., 1998) and older prostate cancer patients (Hanson et al., 2017). Resistance training may be a beneficial strategy to mitigate these losses, but studies examining the effects of resistance training to recover muscle atrophy and
function in men with hypogonadism show mixed results; with some studies showing attenuated regrowth (Kvorning et al., 2006; Nilsen et al., 2014, Winters-Stone et al. 2014), and other studies showing increases in muscle strength, muscle function, and lean mass (Hanson et al. 2013, Galvao et al. 2010). These mixed results in human research may due to a number of confounding factors influencing the responses, such as diet and physical activity levels. To better control for these factors and to investigate this relationship further, an animal model is a feasible alternative approach.

Animal models are a justifiable and potential alternative as they have demonstrated increased fat mass and decreased muscle mass similar results and behaviors to castration as humans do to hypogonadism. Eleven weeks of castration in aged rats showed increased fat mass, reduced muscle mass, strength, and bone mass compared to age- and weight-matched controls (Gentile et al., 2010). Young castrated rats also exhibit an attenuated weight gain, decreased muscle mass (Jiao et al., 2009), decreased food intake, and increased bone resorption (Borst & Conover 2006; Borst et al., 2007) compared to sham controls. Castration has also been shown to increase expression of ubiquitin ligases, markers of skeletal muscle proteolysis (Pires-Oliveira et al., 2010). Moreover, Ibebunjo et al. (2011) showed that castration results in almost complete cessation in voluntary wheel running that was restored to normal levels following testosterone replacement therapy.

Hindlimb immobilization is a model widely used to induce muscle atrophy that has been shown to significantly reduce muscle mass, muscle fiber size, muscle function, and body weight compared to control groups (Brown et al., 2001; Murphy et al., 2011; Childs et al., 2003; Morris et al., 2004?). Aged rats typically display less atrophy than younger rats, suggesting that older animals may already be experiencing significant reductions in muscle mass prior to the
immobilization period (Hepple et al., 2004; Blough et al., 2000). Following 10 days of immobilization and 15 days of reloading, young rats appear experienced more atrophy from immobilization and exhibited a trend towards increasing soleus mass while older rats lacked the ability to completely regrow the atrophied soleus (Childs et al., 2003; Morris et al., 2004). This diminished capacity for skeletal muscle regrowth in aged rats may be as a result of decreased growth factors, such as testosterone, as a result of aging. Heterochronic parabiosis (surgical pairing of young and old animals) restored physiologically normal levels of testosterone and showed improvements in muscle weight, and muscle fiber cross-sectional area compared to controls, indicating testosterone may have a critical role in mediating the improvements (Sinha et al., 2014). Castration results in loss of peak tetanic tension regardless of muscle fiber type or function, however, it does not exacerbate hindlimb unloading-related atrophy or peak tetanic tension (Brown et al. 2001). While aged rat skeletal muscle typically shows less atrophy as well as diminished capacity for skeletal muscle regrowth than young rats, some of which may be due to decreased testosterone (Sinha et al., 2014) due to aging, data are lacking that directly assess the role of testosterone on load-mediated hypertrophy.

Muscle mass is regulated by the balance between protein synthesis and degradation (Phillips and Van Loon, 2011), with hypogonadism (Mauras et al. 1998; Hanson et al. 2017) and immobilization (Cholewa et al. 2017) favoring an environment that promotes muscle loss. High protein diets are a common approach used to increase muscle protein synthesis and may be a beneficial strategy to help attenuate losses resulting from hypogonadism, immobilization, and decreased physical activity. For example, BCAA supplementation attenuates the increase in markers of skeletal muscle proteolysis (Jang et al., 2015; Maki et al. 2012) and to partly preserve specific transduction proteins that act as regulators of protein synthesis and cell growth in hindlimb
immobilized rats (Bajotto et al. 2011). Specifically, leucine supplementation has been shown to attenuate muscle mass loss in hindlimb immobilized young rats (Baptista et al., 2010) and to suppress reductions in muscle mass in the presence of an otherwise protein-free diet (Sugawara et al., 2009). To our knowledge there are currently no studies in rats that examine the ability of a high protein diet to overcome the detrimental effects resulting from castration, however leucine-rich diets have been shown to help increase muscle protein synthesis (Ventrucci et al., 2004), modulate proteasome subunits (Salomao et al., 2010), and attenuate decreases in lean body mass (Gomes-Marcondes et al., 2003) in tumor-bearing cachectic rats. The peripheral wasting of muscle mass associated with cachexia may be a result of increased protein degradation or decreased protein synthesis. The ability of high protein diets to mitigate losses in cachectic rats indicates there may be the ability to mitigate declines resulting from hypogonadism as well.

Therefore, the aims of this study were two-fold: 1) to examine the effects of testosterone status on muscle mass, force, and cross-sectional area during reloading following 10 days of immobilization and 2) to determine if a high protein diet attenuates immobilization- and castration-induced atrophy and enhances load-mediated hypertrophy. We hypothesized combined castration and immobilization will not act synergistically to induce greater atrophy than just immobilization alone and that suppression of endogenous testosterone will not attenuate adaptations to the reloading protocol. Further, a high protein diet will attenuate atrophy during the immobilization period and facilitate better adaptations to reloading compared to castrated mice with a traditional diet. The recovery of muscle mass, body weight, muscle force, and cross-sectional area will be similar between castrated and sham surgery rats.
Research Questions

RQ1: Will combined castration and immobilization act synergistically to induce greater muscle atrophy and loss of function than immobilization alone?

RQ2: Is the musculoskeletal function with reloading following immobilization influenced by testosterone levels?

RQ3: Does a high protein diet attenuate castration-and immobilization-induced atrophy and provide a greater anabolic stimulus for recovery during reloading?

Research Hypothesis

H1: Combined castration and immobilization will act synergistically to induce greater atrophy than just immobilization alone.

H2: Suppression of endogenous testosterone via orchidectomy will not attenuate adaptations to the reloading protocol.

H3: A high protein diet will attenuate atrophy during the immobilization period and facilitate better adaptations to reloading compared to castrated mice with a traditional diet.

Limitations

One limitation to this study is it is a retrospective analysis of previously collected data. Retrospective studies are vulnerable as the investigators have no control over exposure or outcome measures and must rely on accurate record keeping. Another limitation is the lack of a true control group. The non-immobilized contralateral limb served as the control for each animal, however as these rats are young they may still be growing as a part of normal development which potentially may have skewed the results (Murphy et al. 2011). However, pilot study data obtained specifically
from Sprague-Dawley rats (Krawiec et al. 2005) indicates that unilateral immobilization had no effect on the contralateral noncasted leg and served as the justification for this approach in the current study.

**Significance of Study**

It is well established that functional overload (i.e. strength training) elicits neural and morphological adaptations that may lead to increased muscle strength and muscle hypertrophy but relatively few studies have examined the hypertrophic response in the absence of testosterone. Moreover, many of these studies produce conflicting results. Protein and amino acid supplementation has been widely used as a nutritional countermeasure to increase muscle protein synthesis, as well as attenuate muscle protein degradation, but to our knowledge there are currently no studies in that examine the ability of a high protein diet to overcome the detriments resulting from hypogonadism and reduced activity/immobilization. Functional overload and combined protein supplementation may provide additional treatment options for those suffering from hypogonadism, as well as provide a potential therapy for prostate cancer patients undergoing androgen-deprivation therapy.
CHAPTER II
REVIEW OF LITERATURE

In this review, the effects of hypogonadism on skeletal muscle, body composition, and contractile properties of muscle of both humans and rats will be examined. It will also examine the use of hindlimb immobilization in rats as a model of muscle atrophy, and subsequent reloading as a model of functional overload (e.g. strength training). Finally, the potential anabolic effects of increased protein intake on recovery from muscle atrophy and castration will be examined. It has been established that functional overload facilitates neural and morphological adaptations that can lead to increased muscle force and hypertrophy in the eugonadal state; however studies present conflicting results as to the effectiveness of strength training in the hypogonadal state and none to our knowledge have included protein supplementation in patients at high risk for declines in musculoskeletal function.

Hypogonadism via Chemical Castration

It is estimated that hypogonadism affects between 2.1 - 12.8% of adult males in the general population (Zarotsky 2014). Primary hypogonadism is a result of a defect within the Leydig cells of the testes in males, while secondary hypogonadism occurs elsewhere, often within the hypothalamic-pituitary gonadal (HPG) axis. Androgen deprivation therapy (ADT) is a form of prostate cancer treatment that suppresses male sex hormone production in the body, namely testosterone, through preventing the pituitary from releasing lutenizing hormone (LH) or through
the use of antiandrogens antagonists that prevent activation of the androgen receptors (Sharifi 2010).

While ADT is an effective treatment for prostate cancer, it can have adverse effects on cancer patients in both the short-term and long-term. Hypogonadism results in the abrupt loss of endogenous testosterone which resembles the symptoms of aging but presents at an accelerated rate (Bylow et al. 2007, Hanson et al. 2011). Galvao et al. (2009) assessed the effects of 36 weeks of ADT on whole body and regional muscle, fat, and bone mass and physical activity in men with PCa. Their results showed that upper limb, lower limb, trunk, and whole-body lean mass decreased by [5.6 (0.6)%, 3.7 (0.5)%, 1.4 (0.5)% and 2.4 (0.4)% (p < 0.01) respectively], while fat mass increased in those respective areas [20.7 (3.3)%, 18.7 (2.7)%, 12.0 (2.5)% and 13.8 (2.3)% (p < 0.001)]. Hip, spine, whole-body, upper limb bone mineral density, and physical activity levels decreased as well but not lower limb density as a result of ADT. Van Londen et al. (2008) examined the long-term effects of ADT on body composition over a 2 year period. They showed that men undergoing acute ADT treatment (mean 3 months) had significant gains in body fat mass (1499.56±322.28 g after 12 months, 2167.15±676.45 g after 24 months) as well as losses in lean body mass (929.74±296.36 g after 12 months, 1785.81±501.31 g after 24 months) over the 2 year period. Men undergoing chronic ADT (mean 31 months) had smaller but still significant changes over the 2 year period. These results show that men undergoing ADT experience significant gains in body fat mass and losses in lean body mass and suggests that these changes are most pronounced at the initiation of ADT. Finally, Basaria et al. (2002) conducted a cross-sectional study to determine the effects of ADT on lean body mass, muscle strength, and bone mineral density. They showed that men on ADT had significantly lower total-body and lumbar spine bone mineral density compared to those not undergoing ADT (p = 0.02). As well, the ADT group had higher fat
mass (p = 0.0001) and significantly reduced upper body strength (p = 0.001) compared to the other groups. Suppression of endogenous testosterone levels is also associated with a decrease in fractional muscle protein synthesis in younger men (Mauras et al. 1998) and older prostate cancer patients (Hanson et al. 2017).

**Hypogonadism via Surgical Castration**

Animal models have demonstrated similar results to hypogonadism as humans. The most common method to induce hypogonadism in animal models is through orchidectomy, a procedure in which both testes are surgically removed, to remove endogenous testosterone production. Gentile et al. (2010) showed that 21 week old Sprague Dawley castrated rats develop increased fat mass (+15g), reduced muscle mass (-50g) and strength, as well as lower bone mass (-1.0g) as a result of castration when compared to age and weight-matched sham rats. 12 month old Wistar rats that were 15 weeks post-castration also had lower bone mineral content (-7.9%), bone mineral density (-5.7%) and fat-free-mass (-10.8%) compared to rats who underwent sham surgery (Vanderschueren et al. 2000). Borst et al. (2006) found that orchiectomized Fischer 344 male rats display many of the catabolic effects exhibited in humans with hypogonadism such as significant decreases in food intake, body weight, and muscle mass accompanied with increased adiposity and bone resorption when compared with Brown-Norway and Wistar rat strains. The orchidectomized F344 strain is thus found to be the best rat model for studying the androgenic pathway and the reversal of the effects of hypogonadism.

Hypogonadism not only affects body composition and skeletal muscle mass in animal models, it also affects skeletal muscle function as well. Harjola et al. (2000) examined whether testosterone administration (40mm silastic implant) or hypogonadism affects the recovery of muscle mass and myosin heavy chain (MHC) profile after 1 week of immobilization. Rats in the
recovery groups were allowed free cage activity for 2 weeks after immobilization. In all groups but the testosterone group the body masses after immobilization were lower than in the control group; however, gastrocnemius mass was significantly smaller in all groups after immobilization when compared to controls. Further, relative gastrocnemius mass was regained during the 2 week recovery period independent of testosterone status. These results suggest that the lack of testosterone does not significantly affect the recovery of muscle mass from immobilization using a reloading protocol. Brown et al. (2001) examined 6 month old Sprague-Dawley rats that underwent either sham or castration surgery, 2 weeks of recovery and then 2 weeks of hindlimb unloading to determine the effects of testosterone loss on skeletal muscle contractile properties and physical inactivity. They showed that body weight and muscle mass were similar in the castrated and sham groups, as well the ratio of peak tetanic tension to muscle mass was significantly reduced in the castrated group (p < 0.05). They concluded that orchidectomy results in loss of peak tetanic tension regardless of muscle fiber type or function but it does not exacerbate HLU-related atrophy and peak tetanic loss. Finally, removal of endogenous testosterone by orchidectomy and 7 days of recovery resulted in an almost complete cessation in voluntary wheel running but only a small decline in muscle mass in 4 month old C57Bl/6NTac mice. Testosterone replacement restored running behavior and muscle mass to normal levels (Ibebunjo et al. 2011).

**Hindlimb Immobilization**

Hindlimb immobilization of mouse and rat models using plaster casting techniques have widely been used a model to induce muscle atrophy. Hindlimb suspension is another technique used for limiting use, activity, or movement by restraining rats from hindlimbs or tails. Although both hindlimb suspension and hindlimb immobilization produce significant atrophy in skeletal muscles compared to controls, the amount of inactivity in each model differs. Specifically,
hindlimb suspension allows for unloaded isotonic contractions while immobilization restricts all muscle movement. In addition, both models produce significant atrophy in slow-twitch muscles but hindlimb suspension fails to produce significant atrophy in fast-twitch muscle in male rats (Bricout et al., 1999; Wineski et al., 2002) while hindlimb immobilization produces atrophy in fast-twitch muscle that approaches that of slow-twitch. Therefore in order to fully immobilize the hindlimb and investigate the recovery of both slow-twitch and fast-twitch muscles a hindlimb immobilization model will be used. Booth et al (1973), examined the effects of a hindlimb immobilization using 80 day old Sprague-Dawley rats. After 4 weeks of casting, significant reductions in body (-12%), gastrocnemius (-32%), soleus (-26%), and plantaris (-27%) weights of rats were shown compared to body weights and estimated muscle weights prior to casting. Murphy et al. (2011) showed 12 week old C57Bl/10 mice who underwent 2 weeks of hindlimb immobilization had reduced muscle mass (-27% and -28%, respectively) and CSA (-37% and -34%, respectively) of soleus and extensor digitorum longus muscles when compared to control mice. Chakravathy et al. (2000) found significant loss (-31-40%) in gastrocnemius mass after 10 days of hindlimb immobilization in aged FBN rats. Further, gastrocnemius muscle mass did not recover from atrophied values after either the first 3-week or later 9-week recovery periods. Booth et al (1977), examined the time course of muscle atrophy during 4 weeks of immobilization using female Wistar rats. Muscular atrophy was attenuated when a muscle is stretched (loaded) rather than shortened and about 60% of the total atrophy of the gastrocnemius and of the plantaris occurred within the first 10 days of immobilization. Finally, Lawler et al (2012), investigated the stress response during reloading after 4 weeks of hindlimb immobilization in 4 month old Sprague-Dawley rats. They showed soleus mass decreased (-55%) with HU and remained depressed (-41%) 7 days into reloading.
It is important to note that just as rat strains have differential responses to castration, aged rats respond differently than young rats to hindlimb immobilization. First, aged rats experience significant age-related atrophy when compared to younger rats prior to immobilization. Thomson et al. (2004) showed significant age-related atrophy in plantaris (-16%) and soleus (-14%) compared to control muscles. Hepple et al. (2004) examined young and late middle-aged male Fischer 344 x Brown Norway (FBN) hybrid rats to determine the effects of aging on gastrocnemius and soleus muscle. Significant age-related atrophy was noted in the gastrocnemius (-10%) and triceps surae (-9%) muscles. In addition, the duration of physical activity over a 70 hour period was 34% lower in late middle-aged FBN rats than the young FBN rats. A comparison of old (Morris et al., 2003) and young rats (Childs et al., 2003) showed old rats experienced less atrophy (19.2%) than young rats (38.5%) in response to 10 days of hindlimb immobilization. Finally, Zarzhevsky et al. (2000) examined the recovery from hindlimb immobilization in 24 month old female Wistar rats. They found 4 weeks of hindlimb immobilization caused reductions in wet weights of the gastrocnemius (-41%), plantaris (-47%), quadriceps (-44%) and soleus (-40%) of rats and after 4 weeks of reloading the muscles failed to return to the control weights. A comparison of a similar hindlimb immobilization and reloading in young (6 months old) rats showed immobilization resulted in atrophy [gastrocnemius (-58%), plantaris (-47%), quadriceps (-62%) and soleus (-47%)] compared to the aged rats and that reloading caused a substantial recovery in the hindlimb muscles with them reaching almost complete recovery to control levels. These studies suggest that aged rats experience significant reductions in muscle mass as a result of aging which results in aged rats experiencing less atrophy in response to immobilization compared to younger rats.
Strength Training to reduce effects of Hypogonadism

It has been established that strength training facilitates neural and morphological adaptations that can lead to increased muscle strength and muscle hypertrophy in the presence of endogenous testosterone and the eugonadal state (Kraemer et al. 2003, Tipton et al. 2001). However, studies examining the effects of resistance training to recover muscle atrophy and function in men with induced hypogonadism show mixed results. Certain groups show in hypogonadal men that strength training is associated with the preservation of lean mass but the regrowth is attenuated compared to controls. Kvornig et al. (2006), investigated the role of testosterone for increasing muscle mass and muscle strength in a group of young men with minor strength training experience. The subjects were randomized to treatment with the GnRH agonist goserelin or placebo subcutaneously every 4 weeks for 12 weeks. The goserelin group showed no changes in isometric knee extension strength after training, whereas the placebo group increased isometric knee strength (p = 0.05). Lean mass of the legs increased in the goserelin (0.37 ± 0.13 kg) and placebo groups (0.57 ± 0.30 kg) and body fat mass increased in the goserelin group (1.4 ± 1.0 kg) and decreased in the placebo (0.6 ± 1.2 kg) group. Winters-Stone et al. (2014) investigated whether 1 year of resistance training could reverse ADT-related declines in bone mineral density among prostate cancer patients. They showed no differences in hip or spine bone mineral density with the exception of L4 BMD which was preserved in the resistance training group (-0.4%) compared with a loss in the control group (-3.1%). Nilsen et al. (2015) examined the effects of a 16-week high-load strength training program on prostate cancer patients on ADT. They showed no statistically significant effect on total lean body mass but significant effects were found on lean body mass in the upper and lower extremities (0.49 kg, p < 0.01 and 0.15 kg, p < 0.05,
respectively). This data suggests that endogenous testosterone is of paramount importance to the adaptation to strength training.

Separate studies in men undergoing ADT show similar adaptations to strength training when compared healthy controls. Hanson et al (2013) examined the effects of 12 weeks of strength training on black men undergoing ADT for prostate cancer. Strength training significantly increased total body muscle mass (2.7%), thigh muscle volume (6.4%), power (17%), strength (28%), functional performance (20%), and muscle endurance (110%). Improved muscle function was associated with higher functional performance as well ($R^2 = 0.54$). Galvao et al. (2006) examined the effects of progressive resistance training in men receiving androgen deprivation therapy for prostate cancer. Their results showed that muscle strength (chest press, 40.5%; seated row, 41.9%; leg press, 96.3%; $p < 0.001$), muscle endurance (chest press, 114.9%; leg press, 167.1%; $p < 0.001$), and quadriceps muscle thickness increased (15.7%, $p < 0.05$) significantly after training. Significant improvements (+7-27%, $p < 0.05$) in measures of physical functioning and balance were shown as well. Alberga et al. (2011) examined the effects of age and ADT on body composition and fitness following a 24 weeks of resistance or aerobic exercise program in prostate cancer patients. For men over the age of 65 lean body mass decreased in the aerobic exercise and control groups but it was preserved in the resistance exercise group. The same response was noted in those undergoing ADT, as lean body mass decreased in aerobic exercise and control groups but not in the resistance exercise group. Galvao et al. (2010) examined the combined resistance and aerobic exercise program as a countermeasure to AST-related declines. They showed increases in total body lean mass (+1.2%), upper body lean mass (+3.2%) and lower body lean mass mass (+1.2%) as well as increases in muscle strength as well. This data indicates
that strength training may influence muscle hypertrophy and strength even in the absence of testosterone and could be an effective treatment for the adverse functional consequences of ADT.

Animal models present a unique challenge as they cannot undergo the same strength training protocols as humans. Therefore researchers use different strategies to achieve functional overload in animal models. One such strategy is through surgical ablation of synergistic muscle as this technique can be used to induce hypertrophy in intact muscle as the remaining muscle must compensate for the loss of the synergist. Blough et al. (2000) performed bilateral ablation of the gastrocnemius to functionally overload the fast-twitch plantaris for 8 weeks to examine the effects of old age on muscle plasticity. Plantaris wet weight, muscle CSA, average fiber CSA, and peak isometric tetanic tension were 44%, 42%, 40%, and 83% lower respectively in old (36 month) compared to young (6 month old) Fischer 344 x Brown Norway (FBN) hybrid rats. As well, Thomson et al., (2004) used unilateral gastrocnemius ablation in young (8 month) and old (30 month) Fischer 344 x Brown Norway hybrid rats to examine age-attenuated overload-induced hypertrophy. In fast-twitch plantaris muscles, percent hypertrophy with overload was significantly attenuated with age (9.7% in old v. 30.0% change in young). Surgical ablation is an invasive procedure induces muscle hypertrophy but is a rather extreme and therefore that may not best represent normal overload stimulus.

Another strategy to achieve functional overload is through the use of reloading following immobilization. This overload stimulus is achieved by allowing the rat to undergo normal activity after atrophy has been induced via immobilization or suspension. Reloading atrophied rat skeletal muscle through normal cage activity for has been shown to be sufficient to restore soleus muscle weight to control values (Litvinova et al., 2007; Fluck et al., 2003) and increase fiber CSA (Fluck et al., 2005) following 14 days of hindlimb suspension. Further, Childs et al. (2003) examined the
soleus muscle mass in young rats after 10 days of immobilization and 15 days of reloading through normal cage activity and found that younger rats experienced a trend towards increasing soleus mass with a 15% increase over the course of the experiment; while Morris et al. (2004) examined older rats after a similar immobilization protocol and found that they had attenuated regrowth. This model is also effective in achieving muscle hypertrophy but may be better suited for this study as it represents a non-invasive and realistic approach to achieving functional overload.

**High Protein Diets**

Muscle atrophy is characterized by a shift in the balance between protein synthesis and degradation. During limb immobilization, the rate of protein synthesis and degradation is affected through unloading of the tissue thereby triggering the ubiquitin proteasome system (UPS), specifically the muscle-specific E3 ligases atrogin-1 (Gomes et al. 2001) and MuRF-1 (Bodine et al. 2001) to carry out skeletal muscle proteolysis (Taillandier et al. 1996, Goldberg et al. 1996). Castration has also been shown to increase expression of atrogin-1 and MuRF-1, markers of skeletal muscle proteolysis (Pires-Oliveira et al., 2010). Studies in rat models show that BCAA supplementation attenuates the increase in atrogin-1 and MuRF1 mRNA driven by immobilization. Jang et al. (2015) investigated the effects of BCAA administration on muscle atrophy during growth in male Wistar rats. Rats underwent hindlimb suspension and half were given oral BCAA administration (600mg/kg) daily. After 14 days of hindlimb suspension, BCAA prevented a decrease in soleus muscle weight and attenuated atrogin-1 and MuRF1 mRNA expression. Maki et al. (2012) examined the effects of BCAA administration (600mg/kg) on hindlimb immobilization-induced atrophy in young male Sprague-Dawley rats. Hindlimb suspension significantly reduced soleus muscle weight and muscle fiber CSA and increased atrogin-1 and MuRF1. They showed that BCAA administration significantly attenuated the decrease in CSA and
attenuated the increases in atrogin-1 and MuRF1 in soleus muscles. As well, Bajotto et al. (2011) found that BCAA supplementation partly preserves specific transduction proteins, such as cyclin D1, that act as regulators of protein synthesis and cell growth, and reduced the loss myofibrillar proteins in the soleus of hindlimb immobilized rats.

Specifically, leucine supplementation has been shown to attenuate muscle mass loss in hindlimb immobilized young rats and suppress reductions in muscle mass in the presence of an otherwise protein-free diet. Baptista et al. (2009) assessed the effects of leucine supplementation on elements of ubiquitin-proteasome system (UPS) in rat skeletal muscle during immobilization. They showed that leucine supplementation attenuates soleus muscle mass loss driven by immobilization (p < 0.05). Leucine supplementation also abrogated the transient increase in MuRF1 and atrogin-1 gene expression during immobilization (p < 0.05). Sugawara et al. (2009) examined the effects of long-term leucine intake in dietary protein malnutrition on muscle protein synthesis and degradation. They showed that a reduction in muscle mass was suppressed by leucine supplementation in rats fed a protein-free diet for 7 days. Ubiquitin ligase mRNA (atrogin-1 and MuRF1) expression were not suppressed with leucine supplementation, but protein light chain 3 active form (LC3-II), an autophagy marker, expression was significantly decreased. These results suggest that BCAA and leucine supplementation attenuates muscle wasting induced by immobilization and may reduce markers of skeletal muscle protein degradation. However, no studies were found that examine the influence of a high protein diet and measures of muscle force, suggesting that there is a lack of data exploring this relationship.

In summary, the loss of endogenous testosterone, as a result of normal aging or pharmacological intervention for prostate cancer therapy, has adverse effects on skeletal muscle mass, strength, function, and physical activity levels in both humans and rats. Functional overload,
either as strength training in humans or synergistic ablation or immobilization with reloading in rodent models, has been used to help restore muscle mass and function following significant atrophy. However studies examining the ability of strength training to recover muscle atrophy and function in men with induced hypogonadism show mixed results. Combined castration and hindlimb immobilization has been used as a model to investigate the effects of hypogonadism and skeletal muscle atrophy in rats. As well, BCAA and leucine supplementation have been shown to help decrease the amount of protein degradation by attenuating increases in ligases of the ubiquitin-proteasome system in rats. However, to our knowledge there are currently no studies that examine the ability of a high-protein diet overcome detriments as a result of castration or the ability of combined reloading and a high-protein diet to reverse the effects of immobilization-induced atrophy. The results of this study will help elucidate the role of testosterone on load-mediated hypertrophy, as well as examine the ability of combined reloading and a high protein diet to help counteract the detrimental effects of castration and immobilization-induced atrophy in rats.
CHAPTER III

METHODOLOGY

*Animals.* All experiments and procedures were approved by the Animal Ethics Committee at Victoria University and in accordance with the Australian code of practice for the care and use of animals in scientific research. 110 postpubertal male Fischer 344 rats (~8 weeks, Animal Resource Centre, Canning Vale, WA, Australia) were housed in the Centre for Health Research and Education at Sunshine Hospital in pairs in a light- and climate-controlled room (12:12 hours of light and dark, 20-22°C). The F344 rat has previously been shown to be an appropriate model for the adverse effects of hypogonadism (Borst & Conover, 2006). Animals were allowed to acclimate for one week before being randomly allocated into castration + chow (N=32), castration + protein (N=23), or sham + chow groups (N=36). Testosterone levels were manipulated by performing a bilateral orchiectomy or sham surgery via a scrotal incision under sterile conditions (Jiao et al., 2009). Additional pain relief was administered via intraperitoneal injection 30 minutes prior to surgery (0.5mg/kg, Meloxicam, Therapon, Burwood, VIC, Australia). After surgery, animals recovered for one week.

*Experimental Protocols.* To induce muscle atrophy, the unilateral immobilisation of the right hindlimb was utilised for 10 days. Under 2-4% isoflurane anaesthesia, tape stirrups were attached to the top and bottom of the foot and the leg was then wrapped in cast padding and compression tape. The leg was immobilised by a thermoplastic splint (3’ Vet-lite casting material, Therapon, Burwood, VIC, Australia) attached to the outside of the leg. The splint was secured in place using
strapping tape with the foot in a neutral position. The splint was inspected and repaired daily, as required. After the immobilisation period, animals were anaesthetised and the splints were removed. The atrophied muscles were reloaded for 0, 6, or 14 days under normal cage activity before muscles were harvested. To establish a baseline group, 14 animals (7 castrated, 7 sham) were also sacrificed prior to immobilisation.

Diet. At the onset of immobilisation, animals were placed on two different diets. Castration + chow and sham + chow continued on the standard rodent growth diet (20% protein, AIN93G, Specialty Feeds, Glenn Forrest, WA, Australia) whereas the castrate + protein initiated a high protein diet (52% protein, SF00-252, Specialty Feeds, Glenn Forrest, WA, Australia) along with branched chain amino acids dissolved in their drinking water (~600mg/kg/day, Pro Performance RapidDrive BCAA 5000, General Nutrition Company, Pittsburgh, PA, USA). All groups maintained their respective diets for the remainder of the study.

Ex Vivo Contractions. After 0, 6, or 14 days of reloading, animals were anaesthetised with sodium pentobarbital (60mg/kg, Therapon, Burwood, VIC, Australia) via intraperitoneal injection. The EDL and SOL muscles from both legs were excised tendon to tendon and placed in custom-made organ baths (Zultek Engineering, Bayside, VIC, Australia) with Krebs Ringer solution at 30°C. Muscles were attached to a force transducer via hooks and optimal length (L₀) was established via a series of twitch contractions (pulse duration 0.2 msec). L₀ was the muscle length producing the maximal twitch contraction. Maximal isometric force (P₀) was established using a force frequency relationship via a series of supramaximal contractions (EDL: train duration 350 msec, 15 volts; SOL: train duration 500 msec, 12 volts) of increasing frequencies (10 – 200 Hz) separated by 3 mins of recovery. After completing the contractile experiments, the EDL and SOL were removed from the bath, blotted dried, and weighed on an analytical balance. The muscles were divided into
separate portions and either snap frozen in liquid nitrogen or mounted in embedding medium and frozen in isopentane for future analyses. The remaining plantaris and gastrocnemius muscles were then removed from both legs, weighed, and snap frozen in liquid nitrogen. Rats were euthanized via heart excision and any excess fat or vasculature was removed before the heart was weighed and frozen.

*Serum Analysis.* Serum samples were collected for each animal and stored at -80°C. Total testosterone was determined in duplicate via ELISA following the manufacturer instructions (Crystal Chem, Downers Grove, IL, USA).

*Muscle Cross Sectional Area.* The SOL muscle was cut transversely in a refrigerated cryostat (-20°C) and stained using haematoxylin and eosin to determine individual muscle fibre cross sectional area. Images were obtained on an automated scanning microscope and images were quantified using ImageJ software (version 1.49v; Rasband, 1997-2014).

*Statistical Analysis.* Analysis were done for muscle wet weights, soleus CSA, and soleus and EDL peak tension at 0 days, 6 days, and 14 days of reloading to determine specific differences between groups. Groups were compared using a two-way ANOVA (group, casting) with a Tukey HSD post hoc for to probe for specific differences within the main effects. The contralateral control limb serves as the internal control, thus all comparisons to the immobilized limb are made with the contralateral control. Data were analyzed using SPSS version 24.0 statistical software (SPSS Inc., Chicago, IL) and expressed as means ± SD. Statistical significance was determined at p < 0.05.
CHAPTER IV
RESULTS

Body Mass

![Graph showing body mass changes over time for different groups](image)

**Figure 1.** Body mass for sham + chow, castrate + chow, and castrate + protein groups beginning with surgery and continuing out to 14 days of reloading. Data are displayed as means ± SD.  
† Significant difference between sham + chow and castrate + protein groups.  
# Significant difference between sham + chow and castrate + chow groups.

**Body Weight.** There were significant group differences beginning at the pre-casting time point (p = 0.001) which persisted throughout the entire reloading period (p < 0.001) with both castration conditions exhibiting lower body weight than the sham animals.
Muscle Wet Weight

Figure 2. EDL muscle wet weight at baseline (prior to immobilization) and after 0 days, 6 days, or 14 days of reloading via normal cage ambulation.
* Significant difference from control limb p <0.05.
** Significant difference from control limb p < 0.001.

EDL. There were no significant group x cast interactions. Muscle wet weight was 10.6% and 10.4% lower in the immobilized vs. the contralateral limb at 0d and 6d (both p < 0.001; Figure 2), respectively. By 14 days there was no significant difference between the limbs. There were no significant group differences at any time point.
Figure 3. Gastrocnemius (GAST) muscle wet weight at baseline (prior to immobilization) and after 0 days, 6 days, or 14 days of reloading via normal cage ambulation.

* Significant difference from control limb p < 0.05
** Significant difference from control limb p < 0.001
† Significant difference between sham + chow and castrate + protein groups
# Significant difference between sham + chow and castrate + chow groups

**Gastrocnemius.** There were no significant group x cast interactions. Similar to EDL, casting reduced wet weight significant at 0 days and 6 days (26.6% and 24.6%, both p < 0.001; Figure 3) and was no longer statistically different at 14 days (p = 0.064). There was a trend for group differences at 14 days (p = 0.056) where both castration conditions exhibited lower muscle than the sham + chow group. No differences between castration + chow and castration + protein were found.
**Plantaris.** There were no significant group x cast interactions. Immobilization significantly reduced muscle wet weight at all time points (all $p < 0.05$; Figure 4), with the casted limb still being 4% less than control at 14 days. At baseline, castration reduced muscle weight ($p = 0.038$) relative to sham but group differences were not detected at any other time points.
Soleus. There were no significant group x cast interactions. Immobilization significantly reduced muscle wet weight at all time points (all \( p < 0.001 \); Figure 5), with only modest recovery being observed by 14 days. No group differences were present at baseline or 0 days, however sham + chow showed a trend to be greater than castration + protein at 6d (\( p = 0.067 \)) that was significant by 14d (\( p = 0.014 \)).
Figure 6. EDL peak muscle force at baseline (prior to immobilization) and after 0 days, 6 days, or 14 days of reloading via normal cage ambulation. SC = Sham + Chow, CC = Castrate + Chow, CP = Castrate + Protein.
* Significant difference from control limb p < 0.05
** Significant difference from control limb p < 0.001

Peak tension (force)

EDL. There were no significant group x cast interactions. Ten days of immobilization led to decreased maximal force production (20.8%, p = 0.007; Figure 6) that was also observed at 6 days (p = 0.003) but not 14 days. There was a significantly overall effect of group at 6 days (p = 0.034) on force production. However, post hoc analysis indicated only a trend for reduced force production in castration + protein compared to sham (p = 0.076) and castration + chow (p = 0.061).
Figure 7. SOL peak muscle force at baseline (prior to immobilization) and after 0 days, 6 days, or 14 days of reloading via normal cage ambulation. SC = Sham + Chow, CC = Castrate + Chow, CP = Castrate + Protein.
* Significant difference from control limb p < 0.05
** Significant difference from control limb p < 0.001
† Significant difference between sham + chow and castrate + protein groups.
# Significant difference between sham + chow and castrate + chow groups.

Soleus. The loss of force with immobilization was highly pronounced in soleus muscle, with large deficits being reported at all three recovery time points (all p < 0.001; Figure 7). Significant group differences were observed at 6 days (p = 0.050) and 14d (p = 0.009). Post hoc analysis revealed that sham force tended to be greater than castrate + protein (p = 0.063) at 6 days and this difference increased by 14 days with both castration groups demonstrating significantly less force than sham (both p < 0.05). There were no other group differences or significant interactions.
Figure 8. SOL cross-sectional area (CSA) at baseline (prior to immobilization) and after 0 days, 6 days, or 14 days of reloading via normal cage ambulation.

* Significant difference from control limb p < 0.05
** Significant difference from control limb p < 0.001

Soleus Cross-Sectional Area

Immobilization decreased SOL cross-sectional area (30.3%, p < 0.001: Figure 8) at 0d and while cross-sectional area remained reduced at 14 days, the deficit was smaller (15.9%, p = 0.006). Castration appeared to blunt the regrowth of the muscle fibers, but this difference did not reach significance. There was a trend for a weak group x cast interaction (p = 0.083), but this failed to reach significance as well.
Figure 9. Average food intake for sham + chow, castrate + chow, and castrate + protein groups beginning with 0 days of reloading and continuing out to 14 days of reloading.
† Significant difference between sham + chow and castrate + protein groups
# Significant difference between sham + chow and castrate + chow groups
& Significant difference between castrate + chow and castrate + protein groups

Average Food Intake

There were significant group differences present at all time points (all p < .001). Post hoc analysis showed the castrate + protein group consistently had lower food intake than sham + chow (p < .001 for all time points: Figure 9) and castrate + chow (p = .001 at 0d, p < .001 for 6d and 14d) for all time points, and sham + chow had greater food intake that castrate + chow (p < .001) at 14 days of reloading. Further analysis by adjusting average food weight intake into average caloric intake showed that castrate + protein consumed significantly less calories throughout compared to both castrate + chow (p = .002) and sham + chow (p < .001). However, when adjusting for protein intake in grams per day the castrate + protein group consumed significantly more protein throughout compared to castrate + chow and sham + chow groups (both p < .001).
**Figure 10.** Average water intake for sham + chow, castrate + chow, and castrate + protein groups beginning with 0 days of reloading and continuing out to 14 days of reloading.

† Significant difference between sham + chow and castrate + protein groups

& Significant difference between castrate + chow and castrate + protein groups

**Average Water Intake**

There were significant group differences shown at 0 days (p < .001: Figure 10) at and 6 days (p = .017) of reloading. Post hoc analysis revealed the castrate + protein group had greater water intake than sham + chow (p = .003) and castrate + chow (p < .001) at 0 days of reloading, and greater intake than castrate + chow (p = .013) at 6 days of reloading.
CHAPTER V
DISCUSSION

The loss of endogenous testosterone has adverse effects on skeletal muscle mass, strength, function, and physical activity levels. Functional overload (i.e. strength training) elicits adaptations that may attenuate or reverse these effects but relatively few studies have examined the hypertrophic response in the absence of testosterone. The current study showed that 10 days of hindlimb immobilization significantly reduced muscle wet weights, force, and CSA and these changes occurred independent of testosterone levels. During the 14 day reloading period, both castration groups displayed a blunted total body weight growth. Locally, significantly lower plantaris and gastrocnemius wet weight with 6 days of reloading, and diminished soleus peak tension after 14 days of reloading compared to sham animals. Contrary to some of our work in humans, functional overload in a testosterone-deprived state appears to attenuate the regrowth of skeletal muscle mass and recovery of peak tension, suggesting that testosterone may play a role in load-mediated hypertrophy following immobilization.

We hypothesized that castration combined with immobilization would act synergistically to induce greater atrophy than just immobilization alone, however this was not shown in the present study. Ten days of immobilization decreased soleus muscle weight by 34.8% in the castration + chow group, 33.8% in the castrate + protein group, and by 36.5% in the sham + chow group, with no differences between groups. These findings aligns with previous work from Childs et al. (2003)
which showed that 10 days of immobilization decreased soleus muscle weight by 38.5% in young rats. There were no significant group differences shown in EDL, gastrocnemius, plantaris or soleus muscle wet weight, soleus muscle CSA, or EDL and soleus peak tension at the conclusion of immobilization (0 days of reloading in Figures 1-4), indicating that castration did not act synergistically to induce greater atrophy and that immobilization appears to be the greater atrophy-inducing stimulus. This finding agrees with findings from an earlier study by Brown et al. (2001) where they showed that castration did not exacerbate hindlimb unloading-related atrophy on skeletal muscle contractile properties and physical inactivity. Immobilization also significantly reduced force and CSA. Interestingly, soleus appears to have been more affected as mass and peak tension both failed to recover compared to the control leg even with 14 days of reloading. This data conflicts with findings from Fluck et al. (2003) that showed that by 14 days of reloading, soleus mass had recovered to control values following 14 days of hindlimb suspension.

We hypothesized that muscle mass recovery after immobilization would occur independent of testosterone status. Contrary to our hypothesis, castration attenuated muscle mass recovery following both 6 and 14 days of reloading and protein supplementation did not augment the response. Both castration groups exhibited attenuated gastrocnemius mass recovery with 6 days of reloading (-12% lower than sham), and soleus mass of the castration + protein group remained was significantly lower than sham after 14 days of reloading (-17% lower than sham). Soleus muscle recovery at 6 days of reloading is similar to previous work (Childs et al., 2003), with 42% recovery in the current study vs. 37% recovery. We also hypothesized that musculoskeletal function would recover independent of testosterone status, however after 14 days of reloading soleus peak tension was still significantly lower in both castration groups compared to sham indicating that
testosterone may play a role in musculoskeletal function and mass recovery following immobilization.

The attenuated muscle mass recovery is in agreement with Kvorning et al. (2006) that found attenuated increases in lean mass following strength training in hypogonadal men. However, it should be noted the studies that show significant increases in lean mass with strength training, such as Hanson et al. (2013) and Nilsen et al. (2014), utilized relatively high intensity training protocols; with Hanson using a high-intensity protocol combined with heavy resistance at high volume which elicited near-maximal effort on all repetitions, and Nilsen using a daily undulating periodization model, with a linear progression in training volume through the intervention period. Therefore, the significant increases in lean mass in the absence of testosterone may be a result of higher intensity training protocols while those that show attenuated increases use lower intensity protocols. This may explain the attenuated increases in the present study as well, as the immobilization and subsequent reloading model is intended to act as an overload stimulus to induce muscle growth following atrophy. However, this model may not place a large enough load on the muscle to be considered high intensity and to induce significant atrophy as seen in Hanson et al. (2013) and Nilsen et al. (2014).

The lack of group differences after 14 days of reloading, in all muscles except the soleus, represent conflicting findings between muscle mass, CSA, and function within the present study. While it seems that muscle mass had recovered with 14 days of reloading, although at an attenuated rate, soleus cross-sectional area and peak tension failed to recover compared to the control leg in all groups even with 14 days of reloading. This is unexpected as muscle mass and cross-sectional area have been shown to be correlated (Kim et al., 2015; Hansen et al., 2007), therefore increases in one should typically exhibit similar increases in the other. We considered that the
immobilization protocol may have damaged the muscle tissue as immobilization been shown to increase oxidative stress which can cause muscle damage (Moylan et al., 2007; Powers et al., 2011), and as a result increased blood flow to damaged muscle. This means that at the time of muscle harvesting, edema could have accumulated within the tissue and increased muscle wet weight. However, if edema was accumulating within the tissue as a result of muscle damage centrally located nuclei would be seen indicating the presence of regenerating fibers, but none were found. Another possibility is that mass may be increasing as a result of these rats being young and therefore still developing into mature rats. An increase in muscle fiber length as a result of normal growth could increase muscle mass, but would not serve to increase muscle cross-sectional area or peak tension.

We hypothesized a high protein diet would attenuate atrophy during the immobilization period and facilitate better adaptations to reloading compared to castrated rats with a traditional diet, however this was not shown in the present study. In fact, castrate + protein group had significantly lower soleus peak tension and soleus mass after 14 days of recovery compared to the sham + chow groups, and the castrate + protein group exhibited a trend towards worse adaptations to reloading than the castrate + chow group in some instances. The standard chow diet (AIN93G) provides 17.5% of the total calories protein whereas the high protein diet (SF00-252) provides 45.83% of the total calories from protein. Castrate + protein consumed significantly more protein per day compared to the other groups by approximately 40-50%. However, the castrate + protein group consistently consumed significant less calories per day compared to both the castrate + chow (21-34%), and sham + chow (26-38%) groups. A lower daily caloric intake could stunt body weight and muscle regrowth as there is potentially a lack of energy available from the diet to promote growth. As well, a diet high in protein has been shown to increase satiety and suppress
hunger in humans (Martens et al., 2011; Marmonier et al., 2000) and rats (Maurer et al., 2009). This means that despite this group having a larger daily intake of protein compared to the other groups, they were likely eating less as a result of increased satiety from high protein diet. Moreover, increased protein intake increases thirst and increased water intake as result of this increased thirst may result in lowered food intake. Additionally, the high protein diet is composed of primarily casein, which has been shown to be less effective in promoting protein (or muscle) accretion when compared to whey (Pennings et al., 2011).

There are limitations to this study, one being that is it is a retrospective analysis of previously collected data. Retrospective studies are vulnerable as the investigators have no control over exposure or outcome measures. Another limitation is the lack of a true control group. The use of a non-immobilized contralateral limb served as the control for each animal in the present study, however this is somewhat controversial. In young mice who are still be growing, it was argued previously that unilateral immobilization may skew the results (Murphy et al. 2011). Other data from Krawiec et al. (2005) in rats indicates that unilateral immobilization had no effect on the contralateral non-casted leg. Lastly, immobilization induce muscle atrophy via inactivity that is reversed during by overloading the atrophied muscle via normal cage ambulation. As such, this stimulus may promote a muscular endurance phenotype rather than muscular strength and may not be an appropriate analog to study the effects of strength training. However, there are inherent limitations in studying the adaptions to functional overload in animals, given they cannot perform traditional strength training protocols that would be analogous to human exercise interventions.

In summary, our data suggests that testosterone influences the hypertrophic response as castrated rats exhibited attenuated muscle regrowth following immobilization compared to sham rats. As well, the deviation between the muscle weight and muscle function measures may be as a
result of increased muscle mass resulting from increased fiber length as opposed to increased muscle thickness. Further, the high protein diet did not attenuate immobilization-induced atrophy, nor did it facilitate greater adaptations to reloading in castrated rats compared to chow diet. This appears to be the result of decreased daily food intake (and lower total calories) from increased satiety and water intake as a result of the high protein diet. Future studies in rats using a high protein diet should aim to control for daily food intake and may explore possibility of a high protein diet composed of whey protein as opposed to casein.
REFERENCES


