2-13-2023

EFFECTS OF TRIPTORELIN ON GLOBAL DEOXYRIBONUCLEIC ACID METHYLATION IN SKELETAL MUSCLE IN MALE ADOLESCENT RATS

Max Boland
University of Northern Colorado, bola7456@bears.unco.edu

UNIVERSITY OF NORTHERN COLORADO

Greeley, Colorado

The Graduate School

EFFECTS OF TRIPTORELIN ON GLOBAL DEOXYRIBONUCLEIC ACID METHYLATION IN SKELETAL MUSCLE IN MALE ADOLESCENT RATS

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Master of Science

Max Boland

Natural and Health Sciences
School of Sport and Exercise Science
Exercise Physiology

May 2023

This Thesis by: Max Boland

Entitled: *Effects of Triptorelin on Global Deoxyribonucleic Acid Methylation in Skeletal Muscle in Male Adolescent Rats*

has been approved as meeting the requirement for the Degree of Master of Science in College of Natural and Health Sciences in School of Sport and Exercise Science, Program of Exercise Physiology

Accepted by the Thesis Committee:

_____

David S. Hydock, Ph.D., Chair

_____

Yuyan Han, Ph.D., Committee Member

Accepted by the Graduate School

_____

Jeri-Anne Lyons, Ph.D.
Dean of the Graduate School
Associate Vice President for Research

ABSTRACT

Boland, Max. *Effects of Triptorelin on Global Deoxyribonucleic Acid Methylation in Skeletal Muscle in Male Rats.* Unpublished Master of Science Thesis, University of Northern Colorado, 2023.

The purpose of this study was to investigate how treatment with triptorelin, a gonadotropin releasing hormone agonist, to block puberty in adolescent male rats affects their epigenetic regulation via global DNA methylation changes in skeletal muscle and to determine if physical activity has an effect on DNA methylation status during treatment. Twenty-four male, Sprague-Dewey rats were randomly assigned to different groups: 12 treated with 1 μg of triptorelin daily (PB group) while the other 12 received an equivalent volume of saline as a placebo (control, CON) for four weeks. In order to determine the interaction with physical activity, six PB rats and six CON rats were housed in cages with running wheels so they could be physically active (WR), while the other 12 rats were housed in cages without wheels as the sedentary group (SED). There were, therefore, four total groups (CON + SED, CON + WR, PB + SED, PB + WR). Following the treatment period, rats were euthanized, and the right soleus muscle was collected to isolate its DNA and analyze for global DNA methylation level using a MethylFlash Methylated DNA 5-mC Quantification Kit from Epigentek (Farmingdale, New York, U.S.). No significant drug nor physical activity effects were found on DNA methylation status, weight, weight gain, nor soleus weight. There were also no significant interactions for all parameters. Potential explanations are differentially methylated CpG islands canceling each other out, the short length of the treatment period, or the type of physical activity performed. The

results do not support the hypothesis that triptorelin or physical activity alter global DNA methylation.

TABLE OF CONTENTS

CHAPTER

I. INTRODUCTION……...……... 1
Purpose of the Study……. 3
Research Hypotheses……. 3

II. REVIEW OF LITERATURE……. 4
Triptorelin……. 4
Gender Dysphoria and Treatment……. 4
Epigenetics…… 6
Cardiovascular Disease and Epigenetics……... 7
Skeletal Muscle and Epigenetics……... 9
Conclusion……. 11

III. METHODS……. 13
Animal Care……. 13
Triptorelin Treatment……... 13
Euthanization……...…… 14
Homogenization and Deoxyribonucleic Acid Isolation……...… 14
Deoxyribonucleic Acid Methylation Analysis……..... 15
Statistical Analysis……... 16

IV. RESULTS…… 17

V. DISCUSSION……. 21
Findings……. 21
Conclusion……. 23

REFERENCES…… 25

APPENDIX
A. Institutional Animal Care and Use Committee Approval……... 32

LIST OF TABLES

Table

1. Descriptive Statistics for Weights by Group……. 17

LIST OF FIGURES

Figure

1. Mean Final Body Weight (g) for Each Group…….. 18
2. Mean Weight Gain (g) Over the Treatment Period for Each Group…… 18
3. Mean Soleus Muscle Weight (g) for Each Group…… 19
4. Mean Values of Methylation Levels for Each Group with One Outlier Present…. 20

CHAPTER I

INTRODUCTION

Triptorelin is a gonadotropin-releasing hormone agonist (GnRHa) used to inhibit the production of sex hormones, such as testosterone and estrogen, from the gonads (Shumer et al., 2016). It works by mimicking gonadotropin-releasing hormone (GnRH) and binding to the receptors on the anterior pituitary causing an initial increase of gonadotropins, such as follicle-stimulating hormone (FSH) and lutenizing hormone (LH), followed by strong downregulation of the receptors resulting in no more stimulation to produce gonadotropins (Keating, 2010). Goserelin, leuprolide, and nafarelin are other types of GnRHa that are commonly used in clinical practice (Magon, 2011). However, triptorelin is slightly more potent and lowers testosterone/estrogen to a greater degree compared to the others (Shim et al., 2019). Recently, these drugs have been used to treat adolescents with gender dysphoria (Shumer et al., 2016). Gender dysphoria is a term used to describe those who feel a disconnect between their biologically assigned sex and their identified gender (Garg et al., 2022). Gonadotropin-releasing hormone agonist is a useful treatment for adolescents that feel this way because its effects are reversible and would allow for them to discontinue treatment, which would result in sex hormone production starting again (Keating, 2010; Shumer et al., 2016).

According to Kreukels and Cohen-Kettenis (2011), adolescents receiving treatment with GnRHa have reported positive psychological effects in response to treatment; however, some studies have shown some potentially negative physical side effects, such as increased fat mass (Kvorning et al., 2006; Schagen et al., 2016), decreased lean mass percentage (Schagen et al.,

2016), and decreased bone mineral density (Bangalore Krishna et al., 2019). These are short-term symptoms that could start to present a long-term risk of obesity and/or cardiovascular disease. Testosterone and estrogen have both been shown to be important for healthy cardiovascular function in men and women, respectively (Faubion et al., 2015; Pastuszak et al., 2017). Therefore, blocking the production of these sex hormones could potentially be directly linked to an increased risk of cardiovascular disease. It is possible that this increased risk could be reversed by increasing physical activity in those who are undergoing treatment. Treatment with GnRHa has been shown to decrease physical activity levels in adult rats (Hydock, 2022). Ensuring that physical activity requirements are still being met, therefore, could mitigate the risk of cardiovascular disease since there is a very strong association between amount of physical activity and cardiovascular disease (Benjamin et al., 2019).

Epigenetics is the mechanism of how gene expression is regulated due to modifications, such as deoxyribonucleic acid (DNA) methylation, made to the DNA that do not change the DNA bases (Corella & Ordovás, 2017). Observing epigenetic changes in the DNA could provide an effective way to monitor how environmental factors, such as GnRHa treatment and physical activity, could be affecting long-term cardiovascular health in a short period of time. Studies have shown that individuals with coronary artery disease or previous myocardial infarction express higher levels of global DNA methylation compared to those with no history of cardiovascular disease (Kim et al., 2010; Sharma et al., 2008). Altered levels of DNA methylation are also seen in response to poor diet, smoking, and pollution which are also known to elevate cardiovascular disease risk (Ordovás & Smith, 2010). In contrast, exercise has been shown to lower levels of global DNA methylation after a single bout of exercise (Barrès et al., 2012). This change in methylation status can also be seen in the long term in response to chronic

exercise training (Lindholm et al., 2014) and remains lower compared to baseline throughout a period of detraining (Seaborne et al., 2018). Therefore, exercise could be an effective tool to restore global methylation levels in individuals who express elevated levels. This thought is supported by Denham et al. (2015), who found that exercise training caused DNA methylation changes at known cardiovascular physiology gene sites as well as improved cardiorespiratory function.

Purpose of the Study

Therefore, the primary purpose of this study was to investigate how treatment with GnRHa in adolescent male rats affects their epigenetic regulation via DNA methylation changes in skeletal muscle. The secondary purpose of this study was to determine the effect of increased physical activity on DNA methylation during GnRHa treatment.

Research Hypotheses

The first hypothesis is that triptorelin treatment will increase global DNA methylation levels in skeletal muscle due to lack of testosterone availability. The second hypothesis is that increased physical activity via wheel running will lower global DNA methylation levels compared to rats who are sedentary (i.e., lack of a running wheel in cage).

H1 Triptorelin will cause the global methylation status in the soleus muscle to be elevated compared to the control group.

H2 Physical activity will lower the global methylation status of the soleus muscle.

CHAPTER II

REVIEW OF LITERATURE

Triptorelin

Triptorelin is a GnRHa that was approved for medical use in 1986 (Fischer & Ganellin, 2006). It was originally used for the treatment of androgen-dependent prostate cancer as well as central precocious puberty. However, in the late 1990s, it began to be used to treat adolescents with gender dysphoria (Shumer et al., 2016). Gonadotropin releasing hormone works by binding to G-protein coupled receptors in the anterior pituitary and stimulating the release of FSH and LH which then travel to the gonads to stimulate testosterone or estrogen production in the testes or ovaries, respectively (Shumer et al., 2016). Triptorelin, in turn, mimics GnRH by binding to the same receptor causing it to eventually be down regulated (Keating, 2010). Down regulation of the receptor occurs due to it having a higher affinity for triptorelin compared to GnRH as well as triptorelin taking longer to degrade when compared to GnRH (Keating, 2010). This means that there is an initial increase of FSH and LH before the pituitary enters a refractory period and becomes desensitized/down regulated resulting in a loss of FSH and LH secretion. Therefore, there is little to no testosterone or estrogen produced, and secondary sex characteristics are not developed. This is the primary reason it is used as a treatment for gender dysphoria in adolescents.

Gender Dysphoria and Treatment

Gender dysphoria is when an individual's gender identity does not match their biologically assigned gender (Garg et al., 2022). This can occur as early as childhood; however,

it is suggested that many young children with gender dysphoria do not continue to experience this incongruence into adolescence or adulthood (Shumer et al., 2016). Treatment with hormone therapy and/or sex change surgery is too permanent and would not allow for a change of mind later in life; however, the effects of triptorelin are reversible once treatment is discontinued (Keating, 2010; Shumer et al., 2016). This makes it a logical treatment for adolescents with gender dysphoria in the event their decision changes later in life.

Treatment with GnRHa has shown to have positive psychological effects in adolescents with gender dysphoria (Kreukels & Cohen-Kettenis, 2011). Although, some studies have shown some potentially adverse physical side effects, such as increased fat mass (Kvorning et al., 2006; Schagen et al., 2016), decreased lean mass percentage (Schagen et al., 2016), and decreased bone mineral density (Bangalore Krishna et al., 2019). These effects are easy to observe/measure after only a short period of treatment with triptorelin, but little research has been done on the potential long-term effects. For example, these changes in body composition are trending toward becoming overweight or obese, which indicates increased risk of cardiovascular disease (Benjamin et al., 2019). It has been shown that the lack of testosterone correlates with an increase in biomarkers for cardiovascular disease (Pastuszak et al., 2017), and estrogen is potentially protective of cardiovascular function (Faubion et al., 2015) supporting the potential risk for GnRHa treatment increasing cardiovascular disease risk. In adults, this treatment regimen would include supplementation of testosterone for transmen and estrogen for transwomen which could potentially offset the increased risk due to lack of key sex hormones. However, in adolescents, no alternate sex hormone replacement is given, leaving them with a lack of both estrogen and testosterone. This could have an effect on the individual's DNA

methylation status as testosterone levels have shown to be linked to methylation levels (Moore et al., 2020).

A potential mechanism for the shift in body composition following GnRHa administration is a lower resting energy expenditure due to decreased tonic sympathetic nerve stimulation on skeletal muscles (Day et al., 2011). This helps explain the loss of lean mass from decreased use/activation and the increased fat mass from less fat being metabolized by the muscles. Treatment with GnRHa has also been shown to decrease physical activity levels (Hydock, 2022), which could potentially explain body composition shifts. This would also present a well known increase in cardiovascular disease risk. There is a very strong association between amount of physical activity and cardiovascular disease, and this association is independent of body mass index (Benjamin et al., 2019). This means that treatment with GnRHa could increase risk of cardiovascular disease due to increased body mass, lack of sex hormones, and/or decreased physical activity. One way to determine if there is a change in cardiovascular disease risk without having to wait for the long-term consequences to present themselves is to look for alterations in the epigenetic profile of an individual's DNA. Muscle tissue would be a good tissue to observe because it is a very metabolic tissue that is highly affected by physical activity.

Epigenetics

Epigenetics is the mechanism of how gene expression is regulated due to modifications made to the DNA that do not change the DNA bases (Corella & Ordovás, 2017). Some examples of epigenetic regulations are DNA methylation, histone modification, and regulation by microRNA. One of the most studied and understood types of epigenetic regulation is the methylation and demethylation of DNA (Corella & Ordovás, 2017). A methylation occurs when

a cytosine is converted to a 5-methylcytosine by covalently bonding a methyl group to the C-5 carbon in the ring (Corella & Ordovás, 2017). According to Corella and Ordovás (2017), this addition is done by DNA methyltransferases, some of which work to maintain current 5-methylcytosines (DNMT1) while others create new ones (DNMT3A and DNMT3B). According to Mazzio and Soliman (2012), these methylations typically take place at a cytosine-phosphate-guanine dinucleotide (CpG). These CpGs are heavily concentrated in the region between the promoter and the transcription site with more than 60% of the base pairs being CpGs (Corella & Ordovás, 2017). When a CpG is methylated, the result is a decreased expression of the gene encoded downstream (Mazzio & Soliman, 2012). There are a few different mechanisms for this inhibition of expression, such as directly blocking transcription initiation complexes from binding to promoter regions, inhibiting RNA polymerase, and recruiting transcriptional repressor complexes (Mazzio & Soliman, 2012).

Unlike the base pair sequence of DNA, the epigenetic make-up is not uniform across all cell types and can change in cells throughout life (Corella & Ordovás, 2017; Mazzio & Soliman, 2012). There are multiple external factors that can cause both short- and long-term changes in DNA's methylation status, such as diet, exercise, smoking, chemical exposure, etc. (Hamilton, 2011).

Cardiovascular Disease and Epigenetics

Epigenetic profiles could be useful in determining cardiovascular disease risk. Certain levels of DNA methylation at specific genes are associated with elevated cardiovascular disease risk (Kalea et al., 2018). A study conducted by Sharma et al. (2008) tested 137 coronary artery disease (CAD) patients and 150 healthy individuals for global methylation levels and found that CAD patients showed significantly higher levels of DNA methylation compared to healthy

individuals. They also noted an independent association between CAD and hypermethylation. These findings were confirmed by Kim et al. (2010) who found that individuals with previous myocardial infarction, stroke, hypertension, and diabetes expressed elevated levels of global DNA methylation compared to individuals with no history of cardiovascular disease. Changes in methylation status are also seen in response to poor diet, smoking, and pollution which are also known to elevate cardiovascular disease risk (Ordovás & Smith, 2010). Metabolic syndrome is also independently related to altered DNA methylation, but unlike cardiovascular disease, the DNA is hypomethylated in those with metabolic syndrome (Luttmer et al., 2013). However, a study by Akinyemiju et al. (2018) found two specific gene sites that were consistently hypermethylated in association with metabolic syndrome.

These epigenetic markers can be just as useful in finding ways to treat and prevent cardiovascular disease by seeking out dietary and environmental factors that cause opposing methylation changes of specific genes shown to increase cardiovascular and metabolic disease risk (Muka et al., 2016). One potential factor to induce positive epigenetic changes that would reduce the risk of cardiovascular disease is exercise (Grazioli et al., 2017). A study by Denham et al. (2015) found that exercise training caused DNA methylation changes at known cardiovascular physiology gene sites as well as improved cardiorespiratory function. They also noted a slight decrease in global DNA methylation in leukocytes following exercise training. This suggests that problematic epigenetic profiles have the potential to be corrected with physical activity/exercise and that DNA methylation could be used as a measure for how effective a certain exercise prescription is at reducing the risk of cardiovascular and/or metabolic disease.

Skeletal Muscle and Epigenetics

Skeletal muscle tissue is highly affected by exercise and therefore should show a high level of exercise-induced modification at the epigenetic level. A study done by Barrès et al. (2012) took a muscle biopsy from 14 participants before and after a cycle ergometer VO_{2peak} test. DNA was extracted from these muscle biopsies and tested for levels of global methylation. They found that global methylation was significantly lower following the VO_{2peak} test, showing exercise causes overall hypomethylation. This same study also wanted to investigate whether this change was intrinsic to the muscle cell or mediated by an external neural or hormonal factor. To do this, they removed the soleus muscle from female mice and stimulated them to contract *ex vivo*. They found that after 45 minutes of contraction, methylation of the promoter region of select genes was lower than before stimulation started. This shows that the hypomethylation of the DNA in skeletal muscle following exercise is an intrinsically controlled adaptation. This makes sense for an acute bout of exercise because it is also known that mRNA of specific genes increases to assist in the beneficial adaptation of the skeletal muscle to tolerate exercise-induced stress. However, it does not address long-term changes in global methylation in response to chronic exercise.

A study done by Lindholm et al. (2014) investigated how the DNA methylation of skeletal muscle is affected by chronic exercise. Twenty-three sedentary volunteers completed a three-month unilateral training program consisting of one-legged knee extension workouts four times a week. Muscle biopsies were taken from the trained and untrained leg, both before and after the training program, to be tested for global methylation as well as methylation at specific gene sites. They found that there was no difference between the global methylation levels between legs (untrained vs. trained) either before or after the training program as well as no

training effect (before vs. after training) in the trained leg. However, when looking at specific genes, it was shown that over 5,000 sites had methylation changes that occurred between pre- and post-training in the trained leg. The results of the global methylation test hide these changes because some genes, such as the ones that control structural modeling and glucose metabolism, were methylated while other genes, such as the ones that control inflammatory/immunological processes, were demethylated so that there was no change in overall methylation globally. The authors suggested these results could be due to a potential negative feedback loop occurring after exercise has already caused morphological changes or an incompletely understood phenomena that uses DNA methylation to inhibit the transcription of an inhibitory RNA molecule. Regardless, this study suggests that the long-term effects of exercise are associated with changes in DNA methylation in response to long-term exercise. The logical next step would be to address how the DNA methylation of skeletal muscle is affected by the discontinuance of exercise.

Studies have shown that adaptations in response to exercise take less time in those who have been previously exposed to chronic exercise despite being sedentary for a length of time. Seaborne et al. (2018) wanted to determine if these accelerated adaptations to “retraining” were due to persisting DNA methylation changes that lasted throughout a period of detraining. In order to test this hypothesis, eight untrained males underwent a seven-week resistance training program (loading) followed by seven weeks of detraining (unloading) and then completed the seven-week program again (reloading). Muscle biopsies were taken from the vastus lateralis before and after loading and before and after reloading. Lean body mass and quadricep strength were also recorded at these times. They found that compared to baseline, DNA was hypomethylated after loading, unloading, and reloading despite a loss of lean body mass after unloading. This shows that exercise-induced epigenetic changes were partially maintained even

when the chronic exposure to exercise was discontinued/interrupted. They also noted that hypomethylation of DNA was greater after reloading compared to the initial loading, and lean mass also followed this trend. This suggests that not only are the epigenetic modifications long-lasting, but they are also easier to induce after previous exercise training.

Conclusion

Triptorelin is a GnRHa that can be used to treat adolescents with gender dysphoria (Shumer et al., 2016). It works by binding the same receptor as GnRH in the anterior pituitary and down regulating it so that there is eventually a loss of FSH and LH production (Keating, 2010). This results in a loss of testosterone/estrogen and the suppression of secondary sex characteristics. Gonadotropin releasing hormone agonist has shown to have beneficial psychological outcomes for adolescents with gender dysphoria (Kreukels & Cohen-Kettenis, 2011), but has also shown some potential negative physiological effects such as increased fat mass (Kvorning et al., 2006) and decreased lean mass (Schagen et al., 2016). These changes in body composition trend toward obesity indicating increased risk of cardiovascular disease (Benjamin et al., 2019). The risk of cardiovascular disease could potentially be increased as a result of a lack of testosterone and/or estrogen which are both shown to be important to cardiovascular health (Faubion et al., 2015; Pastuszak et al., 2017). Monitoring changes in cardiovascular health could be done by observing an individual's epigenetic alterations. Epigenetics regulate gene expression by modifying DNA without changing the DNA sequence (Corella & Ordovás, 2017). DNA methylation is a type of epigenetic modification that "turns off" a gene when methyl groups are bound (Mazzio & Soliman, 2012), and increased levels of global DNA methylation could be indicative of cardiovascular disease (Kim et al., 2010; Sharma et al., 2008). These negative changes in DNA methylation could be reversed by exercise training

(Denham et al., 2015). Global DNA methylation in skeletal muscle is decreased following a bout of exercise (Barrès et al., 2012). Exercise training also causes hypomethylation in skeletal muscle long after training ends (Seaborne et al., 2018). This study sought to determine if treatment with triptorelin in adolescent rats caused epigenetic changes via global DNA methylation that could potentially be associated with increased cardiovascular and/or metabolic disease risk. It also investigated if these changes are reversed/attenuated by physical activity.

CHAPTER III

METHODS

Animal Care

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Northern Colorado and were in compliance with the Animal Welfare Act. Prepubescent (3-week-old) male, Sprague-Dawley rats (n = 24) were purchased from Envigo (Indianapolis, Indiana, U.S.) in order to investigate how treatment with GnRHa affects their epigenetic regulation via DNA methylation changes in skeletal muscle. These rats were maintained on a 12:12 hour light: dark cycle and provided standard rodent chow and water *ad libitum*. Following an acclimatization period of one week, rats were randomly assigned to either receive puberty blocker treatment with triptorelin (PB) or be a control (CON). Both groups were again assigned to two additional groups--one group having a running wheel in the cage to allow for increased physical activity (WR) and the other group having an empty cage as the sedentary condition (SED). This allowed for a total of four different groups of rats: CON + SED (n = 6), CON + WR (n = 6), PB + SED (n = 6), and PB + WR (n = 6).

Triptorelin Treatment

A triptorelin solution was made by dissolving 10 mg of triptorelin into 10 mL of sterile water. The PB group was injected with 100 μL, this triptorelin solution dosing them with 100 μg of triptorelin, while the CON group was injected with 100 μL of a saline solution (0.9% NaCl) as a placebo. These injections were administered subcutaneously by pinching the skin on the back of the neck/shoulder area and inserting the syringe needle into the scruff. Injections were

administered daily at 0800 in the morning for 28 days. The rats were also weighed weekly every Tuesday at 0800 in the morning.

Euthanization

On the 29th day starting at 0800 in the morning, rats were euthanized via intraperitoneal injection with sodium pentobarbital (50 mg/kg) and heparin to prevent blood clotting. Once there was no response to the tail being pinched, the dissection was started. Once rats were confirmed as anesthetized, their hearts were removed to ensure death. The right soleus muscles were then surgically removed, weighed on a scale, and snapped frozen in liquid nitrogen. DNA from the right soleus muscles was later isolated and analyzed for global DNA methylation. Global DNA methylation was analyzed using a MethylFlash Methylated DNA 5-mC Quantification Kit from Epigentek (Farmingdale, New York, U.S.).

Homogenization and Deoxyribonucleic Acid Isolation

DNA was isolated using a QIAamp DNA kit from QIAGEN (Germantown, Maryland, U.S.). Ten to 20 mg of soleus muscle tissue was isolated and broken up mechanically via mortar and pestle before being transferred to a 1.5 ml microcentrifuge tube. Crushed tissue sample was mixed with 180 µl of tissue lysis buffer (buffer ATL) and 20 µl of proteinase K and then vortexed for 15 seconds. The 1.5 ml tubes were then incubated at 56℃ for two hours until completely lysed. The solution was mixed with 200 µl of lysis buffer (buffer AL), vortexed for 15 seconds, mixed with 200 µl of 100% ethanol, and again vortexed for 15 seconds. After incubating at room temperature for five minutes, the solution was transferred to a MinElute spin column with a 2 ml collection tube and centrifuged for one minute at 8,000 rpm. The column was removed and placed into a clean collection tube before adding 500 µl of wash buffer (buffer AW1) and centrifuged again for one minute at 8,000 rpm. The column was again placed into a

fresh collection tube and then 500 µl of a second wash buffer (buffer AW2) to remove any salts. The solution was once again centrifuged at 8,000 rpm for one minute. Once more, the column was put in a fresh collection tube and centrifuged at 14,000 rpm for three minutes to dry the membrane in the column. The column was then placed into a 1.5 ml microcentrifuge tube before adding 50 µl of elution buffer (buffer AE) to elute the DNA. After incubating at room temperature for one minute, the solution was centrifuged one last time at 14,000 rpm for one minute.

Concentration of DNA in the solution was tested using a Thermo Scientific NanoDrop 2000 Spectrophotometer (Waltham, Massachusetts, U.S.). The machine was calibrated by placing 1 µL of buffer AE on the pedestal and running a blank on the program. Following calibration, 1 µL of each sample was placed on the pedestal individually and tested for DNA concentration. Samples were diluted to 60 ng/µL using the buffer AE and retested to ensure appropriate concentration for the DNA methylation assay.

Deoxyribonucleic Acid Methylation Analysis

Global DNA methylation was assessed using a MethylFlash Methylated DNA 5-mC Quantification Kit from Epigentek (Farmingdale, New York, U.S.). The first two columns of a 96-well plate were filled with prepared solutions with known levels of methylation (0%, 0.1%, 0.2%, 0.5%, 1%, 2%, and 5%) with each concentration taking up two wells. The remaining wells were filled with 1 µL of the isolated DNA solution (two wells for each sample) as well as 100 µL of binding solution to bind the DNA to the wells. The wells were covered with parafilm and put in an incubator at 37°C for 60 minutes. Following the incubation period, the wells were emptied and washed with 150 µL of a diluted wash buffer three times. Then, 50 µL of 5-mC detection complex solution was added to each well. The wells were again covered in parafilm and left to

incubate at room temperature for 50 minutes. Next, the wells were again emptied and washed with 150 μL of a diluted wash buffer five times. Finally, 100 μL of developer solution was added to each well to begin the color reaction. Once the wells containing the 5% methylation solution turned a deep blue (4-5 minutes), 100 μL of stop solution was added to each well to stop the color reaction from continuing. Then the plate was immediately read at 450 nm using a microplate reader.

The level of methylation was calculated by using the optical density (OD) from the microplate reader. The OD for each sample, including the known methylation solutions, was found by averaging the readings of the two wells containing identical samples. The OD for the known methylation solutions was plotted on a scatter plot, and a linear best fit line was created. The slope of this line was used to calculate the methylation percentage (5-mC%) of each DNA sample via the equation, where S stands for the amount of input sample DNA in ng:

$$5\text{-mC\%} = (\text{Sample OD} - 0\%\ \text{methylation OD}) / (\text{Slope} * S) * 100\%$$

Statistical Analysis

Data are presented as a mean ± standard error of the mean (SEM) and are reported for each group for change in bodyweight, right soleus weight, and global DNA methylation level. A two-way analysis of variance (ANOVA) was used to determine significant main drug effect as well as significant main physical activity effect. It was also used to determine interactions between the groups (drugs x activity). Significance was set at $\alpha = 0.05$.

CHAPTER IV

RESULTS

The primary purpose of this study was to investigate how treatment with triptorelin in adolescent male rats affects their epigenetic regulation in skeletal muscle. Descriptive statistics for body weight, weight gain, soleus muscle weight, and relative muscle-to-body weight ratio are shown in Table 1. There was no significant drug effect ($p = 0.168$) nor physical activity effect ($p = 0.828$) on final body weight (Figure 1). There was also no significant interaction (drug*physical activity, $p = 0.255$). Weight gained was assessed by taking the rats' last recorded body weight and subtracting their initial body weight at the beginning of the study. There was no significant drug effect ($p = 0.155$) nor physical activity effect ($p = 0.143$) on weight gain (Figure 2). There was also no significant interaction ($p = 0.430$). The PB + SED group tended to have the most weight gain, while the CON + WR group trended toward the least (Figure 2).

Table 1

Descriptive Statistics for Weights by Group

Group	Body Weight (g)	Δ Weight (g)	Soleus Weight (mg)	Soleus/ Body Weight %
CON + SED	250.0 ± 11.48	164.67 ± 6.66	86.7 ± 8.08	0.035
CON + WR	262.67 ± 9.47	140.33 ± 13.03	96.8 ± 8.16	0.037
PB + SED	273.67 ± 8.97	171.67 ± 10.31	89.6 ± 5.44	0.033
PB + WR	265.0 ± 5.41	164.17 ± 10.76	102.7 ± 14.72	0.039

Figure 1

Mean Final Body Weight (g) for Each Group

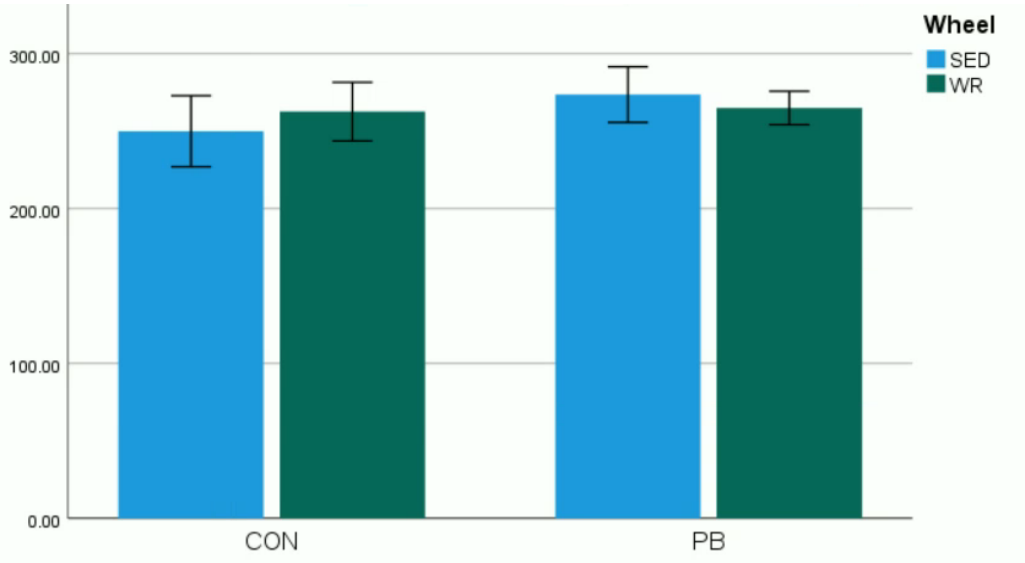
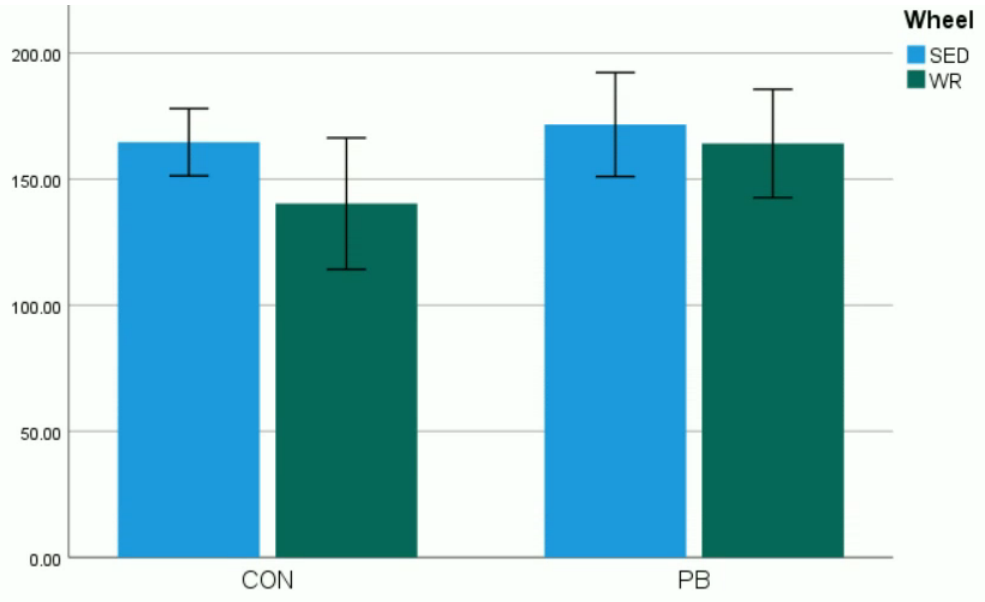


Figure 2

Mean Weight Gain (g) Over the Treatment Period for Each Group

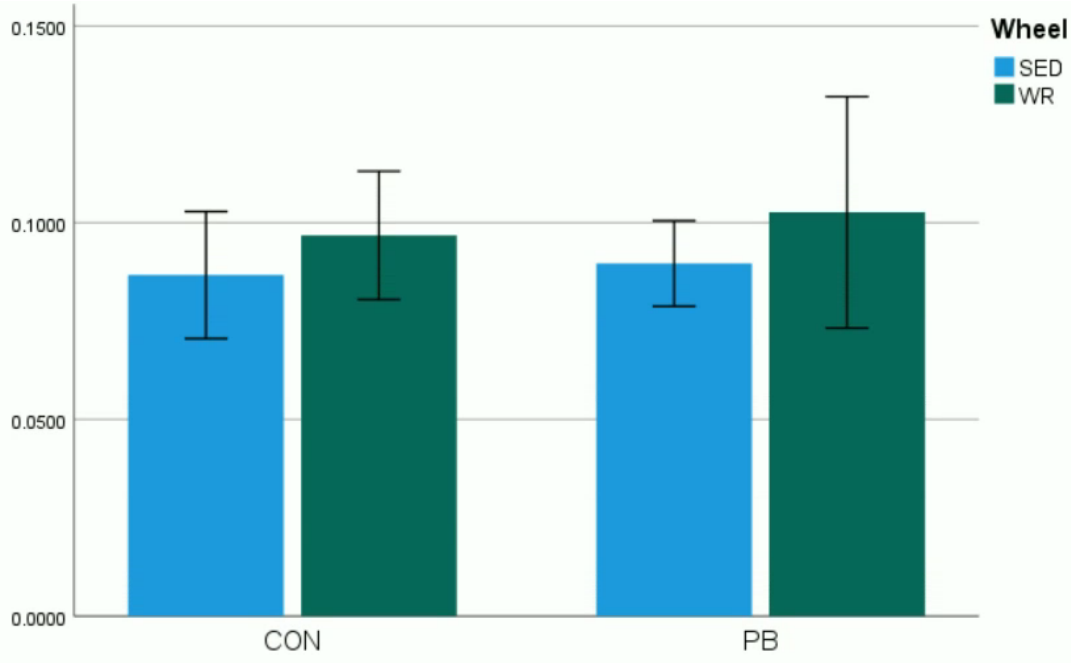


There was no significant drug effect ($p = 0.656$) nor physical activity effect ($p = 0.249$) on soleus weight (Figure 3). There was also no significant interaction ($p = 0.882$). The group

trending toward having the biggest soleus muscle by weight was the PB + WR group, while the group trending toward having the smallest was the CON + SED group (Figure 3). While body composition was not directly measured, a ratio of the right soleus weight to total body weight was used to compare relative muscle mass between groups (Table 1).

Figure 3

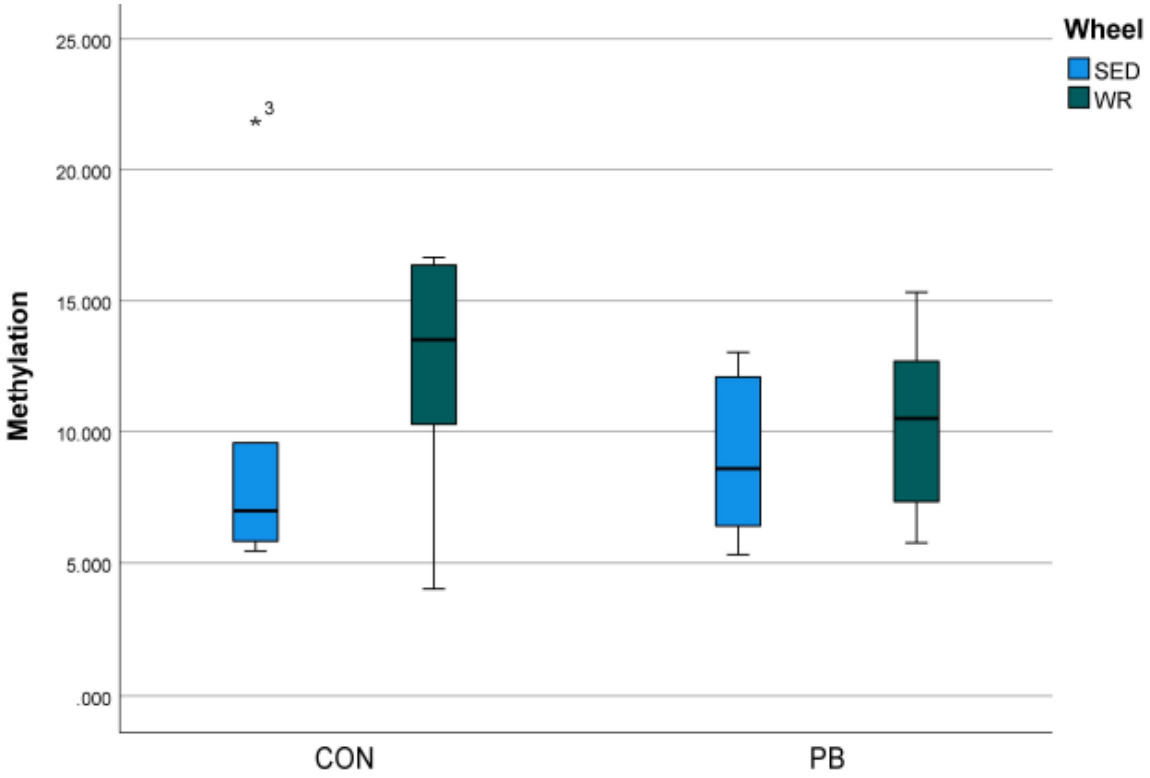
Mean Soleus Muscle Weight (g) for Each Group



There was no significant drug effect on DNA methylation status ($p = 0.570$), nor was there a significant effect of physical activity ($p = 0.312$). There was also no significant interaction between drug and physical activity ($p = 0.730$) on DNA methylation status (see Figure 4). The group trending toward the lowest methylation level, with the exception of one outlier, was the CON + SED group; while the group trending toward the highest level of methylation was the CON + WR group (Figure 4).

Figure 4

Mean Values of Methylation Levels for Each Group with One Outlier Present



CHAPTER V

DISCUSSION

Findings

The purpose of this study was to investigate how treatment with triptorelin in adolescent male rats affects their epigenetic regulation via global DNA methylation changes in skeletal muscle and to determine if physical activity has an effect on DNA methylation status during treatment. The main finding of this study was that there were no significant effects by drug nor physical activity on DNA methylation levels. While nonsignificant, the average methylation of both SED groups trended lower than that of both WR groups. This does not support the hypothesis (H2) that physical activity lowers methylation status of skeletal muscle. This could be due to methylation of certain genes, such as those controlling metabolism and structural remodeling, out pacing the demethylation of other genes (Lindholm et al., 2014). Seaborne et al. (2018) found that numerous CpG islands were differentially methylated after seven weeks of resistance training, with some being hypomethylated and others being hypermethylated. It is possible that with more time, the hypomethylated genes would surpass the hypermethylated genes causing the global methylation of the DNA to decrease overall. There could also be differences due to exercise modality and/or intensity. Machado et al. (2021) observed a correlation between the training volume and level of increase in global DNA methylation in lymphocytes. They also found that regular exercise increases global methylation in lymphocytes which supports the findings of the current study (Machado et al., 2021).

There was no significant drug effect or physical activity effect on weight gain nor right soleus weight. Total weight gain might be lower than expected due to increased fat mass being paired with decreased muscle mass. A study done by Lee et al. (2005) found that males with prostate cancer being treated with GnRHa showed an increase in fat mass and a decrease in lean mass during their first year of treatment. These results were also validated for transwomen undergoing cross-sex hormone therapy (Klaver et al., 2018). Increased fat mass could be a result of a lack of testosterone which would normally help to stop the storage of fatty acids (Santosa et al., 2017). However, despite being nonsignificant, both PB groups tended to greater weight gain compared to their relative CON groups. It is possible that a longer duration of treatment with GnRHa would have allowed this trend to continue to the point that it would be significant. Schagen et al. (2016) found that weight and body mass index significantly increased in adolescent boys and girls after one year of treatment with triptorelin as well as increased fat mass and decreased lean mass percentage.

Physical activity is proven to be effective in reducing fat mass as well as increasing muscle mass (Hernández-Reyes et al., 2019). Therefore, pairing the GnRHa treatment with wheel running should help attenuate fat mass gain and muscle mass loss. In this study, both WR groups tended to less weight gain and larger soleus muscles compared to both SED groups. This supports the hypothesis that physical activity could be an effective tool to counteract the negative effects of GnRHa treatment. Comparing the soleus weight to the final body weight between each group shows that both WR groups also tended to having larger solei relative to body weight, indicating a lower body fat percentage. This suggests that even if treatment with triptorelin is causing increased fat mass, then the addition of exercise is helping to at least maintain a healthier overall body composition.

One limitation of this study was the length of the experimental protocol. The rats were four weeks old when injections started and eight weeks old at the time of sacrifice. These ages are equivalent to a human starting injections around 10 years old and ending around 14 years old, or in terms of development starting just prior to the peripubertal stage and ending just after this stage (Ghasemi et al., 2021). It would be more beneficial to extend the treatment period to eight weeks. This would allow more time for the triptorelin to present long-term changes as well as represent a treatment period that would last into adulthood (18 to 25 years of age in a human) allowing the rat to be fully passed the typical stage of sexual development and into the adulthood development stage (Ghasemi et al., 2021). Another limitation was the small number of samples observed for each group.

More research is needed to fully assess safety and long-term effects of GnRHa treatment in adolescents. The DNA methylation changes in tissues other than skeletal muscle, such as cardiac muscle, liver, and immune cells, should be observed because these tissues play important roles in cardiovascular and metabolic health and are also highly affected by exercise. Other studies could also be done comparing different types, lengths, and frequencies of exercise along with GnRHa treatment in adolescents to determine which combination of these factors would be the most efficient in maintaining healthy levels of DNA methylation. Lastly, studies could be done looking at which specific genes are being hyper- and hypomethylated in response to GnRHa treatment with and without physical activity.

Conclusion

In conclusion, the main finding of this study was that there were no significant drug nor physical activity effects on any of the observed variables (weight, weight gain, soleus weight, and soleus DNA methylation level). There were also no significant interactions between drug

and physical activity on these observed variables. This is potentially due to differentially methylated CpG islands canceling each other out, the short length of the treatment period, or the type of physical activity performed. Both PB groups tended to have higher body weights and tended to gain more weight throughout the treatment period compared to both CON groups, which could be indicative of increased fat mass. Future research should be done investigating DNA methylation changes in other tissues and using other types of physical activity/exercise.

REFERENCES

Akinyemiju, T., Do, A. N., Patki, A., Aslibekyan, S., Zhi, D., Hidalgo, B., Tiwari, H. K., Absher, D., Geng, X., Arnett, D. K., & Irvin, M. R. (2018). Epigenome-wide association study of metabolic syndrome in African-American adults. *Clinical Epigenetics, 10*, 49. https://doi.org/10.1186/s13148-018-0483-2

Bangalore Krishna, K., Fuqua, J. S., Rogol, A. D., Klein, K. O., Popovic, J., Houk, C. P., Charmandari, E., Lee, P. A., Freire, A. V., Ropelato, M. G., Yazid Jalaludin, M., Mbogo, J., Kanaka-Gantenbein, C., Luo, X., Eugster, E. A., Klein, K. O., Vogiatzi, M. G., Reifschneider, K., Bamba, V., . . . Medina Bravo, P. G. (2019). Use of gonadotropin-releasing hormone analogs in children: Update by an international consortium. *Hormone Research in Paediatrics, 91*(6), 357–372. https://doi.org/10.1159/000501336

Barrès, R., Yan, J., Egan, B., Treebak, J. T., Rasmussen, M., Fritz, T., Caidahl, K., Krook, A., O'Gorman, D. J., & Zierath, J. R. (2012). Acute exercise remodels promoter methylation in human skeletal muscle. *Cell Metabolism, 15*(3), 405–411. https://doi.org/10.1016/j.cmet.2012.01.001

Benjamin, E. J., Muntner, P., Alonso, A., Bittencourt, M. S., Callaway, C. W., Carson, A. P., Chamberlain, A. M., Chang, A. R., Cheng, S., Das, S. R., Delling, F. N., Djousse, L., Elkind, M., Ferguson, J. F., Fornage, M., Jordan, L. C., Khan, S. S., Kissela, B. M., Knutson, K. L., . . . American Heart Association Council on Epidemiology and Prevention Statistics Committee and Stroke Statistics. (2019). Heart disease and stroke statistics-2019 update: A report from the American Heart Association. *Circulation, 139*(10), e56–e528. https://doi.org/10.1161/CIR.0000000000000659

Corella, D., & Ordovás, J. M. (2017). Basic concepts in molecular biology related to genetics and epigenetics. *Revista Espanola de Cardiologia (English ed.), 70*(9), 744–753. https://doi.org/10.1016/j.rec.2017.05.011

Day, D. S., Gozansky, W. S., Bell, C., & Kohrt, W. M. (2011). Acute sex hormone suppression reduces skeletal muscle sympathetic nerve activity. *Clinical Autonomic Research, 21*(5), 339–345. https://doi.org/10.1007/s10286-011-0127-5

Denham, J., O'Brien, B. J., Marques, F. Z., & Charchar, F. J. (2015). Changes in the leukocyte methylome and its effect on cardiovascular-related genes after exercise. *Journal of Applied Physiology, 118*(4), 475–488. https://doi.org/10.1152/japplphysiol.00878.2014

Faubion, S. S., Kuhle, C. L., Shuster, L. T., & Rocca, W. A. (2015). Long-term health consequences of premature or early menopause and considerations for management. *Climacteric : The Journal of the International Menopause Society, 18*(4), 483–491. https://doi.org/10.3109/13697137.2015.1020484

Fischer, J., & Ganellin, C. R. (2006). Gonadotropin releasing hormone analogues . In *Analogue-based drug discovery* (p. 514). Wiley-VCH.

Garg, G., Elshimy, G., & Marwaha, R. (2022). Gender dysphoria. In *StatPearls*. StatPearls Publishing.

Ghasemi, A., Jeddi, S., & Kashfi, K. (2021). The laboratory rat: Age and body weight matter. *EXCLI Journal, 20*, 1431–1445. https://doi.org/10.17179/excli2021-4072

Grazioli, E., Dimauro, I., Mercatelli, N., Wang, G., Pitsiladis, Y., Di Luigi, L., & Caporossi, D. (2017). Physical activity in the prevention of human diseases: Role of epigenetic modifications. *BMC Genomics, 18*(S8), https://doi.org/10.1186/s12864-017-4193-5

Hamilton, J. P. (2011). Epigenetics: Principles and practice. *Digestive Diseases (Basel, Switzerland), 29*(2), 130–135. https://doi.org/10.1159/000323874

Hernández-Reyes, A., Cámara-Martos, F., Molina-Luque, R., Romero-Saldaña, M., Molina-Recio, G., & Moreno-Rojas, R. (2019). Changes in body composition with a hypocaloric diet combined with sedentary, moderate and high-intense physical activity: A randomized controlled trial. *BMC Women's Health, 19*(1), 167. https://doi.org/10.1186/s12905-019-0864-5

Hydock, D. S. (2022). Sex hormone suppression and physical activity: Possible implications for transgender individuals. *Transgender Health, 7*(1), 43–51. https://doi.org/10.1089/trgh.2020.0073

Kalea, A. Z., Drosatos, K., & Buxton, J. L. (2018). Nutriepigenetics and cardiovascular disease. *Current Opinion in Clinical Nutrition and Metabolic Care, 21*(4), 252–259. https://doi.org/10.1097/MCO.0000000000000477

Keating, G. M. (2010). Triptorelin embonate (6-month formulation). *Drugs, 70*(3), 347–353. https://doi.org/10.2165/11202230-000000000-00000

Kim, M., Long, T. I., Arakawa, K., Wang, R., Yu, M. C., & Laird, P. W. (2010). DNA methylation as a biomarker for cardiovascular disease risk. *PloS One, 5*(3), e9692. https://doi.org/10.1371/journal.pone.0009692

Klaver, M., de Blok, C., Wiepjes, C. M., Nota, N. M., Dekker, M., de Mutsert, R., Schreiner, T., Fisher, A. D., T'Sjoen, G., & den Heijer, M. (2018). Changes in regional body fat, lean body mass and body shape in trans persons using cross-sex hormonal therapy: Results from a multicenter prospective study. *European Journal of Endocrinology, 178*(2), 163–171. https://doi.org/10.1530/EJE-17-0496

Kreukels, B. P., & Cohen-Kettenis, P. T. (2011). Puberty suppression in gender identity disorder: The Amsterdam experience. *Nature Reviews. Endocrinology, 7*(8), 466–472. https://doi.org/10.1038/nrendo.2011.78

Kvorning, T., Andersen, M., Brixen, K., & Madsen, K. (2006). Suppression of endogenous testosterone production attenuates the response to strength training: A randomized, placebo-controlled, and blinded intervention study. *American Journal of Physiology-Endocrinology and Metabolism, 291*(6), https://doi.org/10.1152/ajpendo.00143.2006

Lee, H., McGovern, K., Finkelstein, J. S., & Smith, M. R. (2005). Changes in bone mineral density and body composition during initial and long-term gonadotropin-releasing hormone agonist treatment for prostate carcinoma. *Cancer, 104*(8), 1633–1637. https://doi.org/10.1002/cncr.21381

Lindholm, M. E., Marabita, F., Gomez-Cabrero, D., Rundqvist, H., Ekström, T. J., Tegnér, J., & Sundberg, C. J. (2014). An integrative analysis reveals coordinated reprogramming of the epigenome and the transcriptome in human skeletal muscle after training. *Epigenetics, 9*(12), 1557–1569. https://doi.org/10.4161/15592294.2014.982445

Luttmer, R., Spijkerman, A. M., Kok, R. M., Jakobs, C., Blom, H. J., Serne, E. H., Dekker, J. M., & Smulders, Y. M. (2013). Metabolic syndrome components are associated with DNA hypomethylation. *Obesity Research and Clinical Practice, 7*(2), e106–e115. https://doi.org/10.1016/j.orcp.2012.06.001

Machado, O., Diniz, V., Passos, M., de Oliveira, H. H., Santos-Oliveira, L. C., Alecrim, A. L., Bertola Lobato, T., Manoel, R., Correa, I., Silva, E. B., de Oliveira Poma, S., Mendes de Almeida, M., Pithon-Curi, T. C., Diniz, S., Levada-Pires, A. C., Curi, R., Masi, L. N., Hirabara, S. M., & Gorjão, R. (2021). Physical exercise increases global and gene-specific (interleukin-17 and interferon-γ) DNA methylation in lymphocytes from aged women. *Experimental Physiology, 106*(9), 1878–1885. https://doi.org/10.1113/EP089673

Magon, N. (2011). Gonadotropin releasing hormone agonists: Expanding vistas. *Indian Journal of Endocrinology and Metabolism*, *15*(4), 261–267. https://doi.org/10.4103/2230-8210.85575

Mazzio, E. A., & Soliman, K. F. (2012). Basic concepts of epigenetics: impact of environmental signals on gene expression. *Epigenetics, 7*(2), 119–130. https://doi.org/10.4161/epi.7.2.18764

Moore, S. R., Humphreys, K. L., Colich, N. L., Davis, E. G., Lin, D., MacIsaac, J. L., Kobor, M. S., & Gotlib, I. H. (2020). Distinctions between sex and time in patterns of DNA methylation across puberty. BMC genomics, *21*(1), 389. https://doi.org/10.1186/s12864-020-06789-3

Muka, T., Koromani, F., Portilla, E., O'Connor, A., Bramer, W. M., Troup, J., Chowdhury, R., Dehghan, A., & Franco, O. H. (2016). The role of epigenetic modifications in cardiovascular disease: A systematic review. *International Journal of Cardiology, 212*, 174–183. https://doi.org/10.1016/j.ijcard.2016.03.062

Ordovás, J. M., & Smith, C. E. (2010). Epigenetics and cardiovascular disease. Nature reviews. *Cardiology, 7*(9), 510–519. https://doi.org/10.1038/nrcardio.2010.104

Pastuszak, A. W., Kohn, T. P., Estis, J., & Lipshultz, L. I. (2017). Low plasma testosterone is associated with elevated cardiovascular disease biomarkers. *The Journal of Sexual Medicine, 14*(9), 1095–1103. https://doi.org/10.1016/j.jsxm.2017.06.015

Santosa, S., Bush, N. C., & Jensen, M. D. (2017). Acute testosterone deficiency alters adipose tissue fatty acid storage. *The Journal of Clinical Endocrinology and Metabolism, 102*(8), 3056–3064. https://doi.org/10.1210/jc.2017-00757

Schagen, S. E., Cohen-Kettenis, P. T., Delemarre-van de Waal, H. A., & Hannema, S. E. (2016). Efficacy and safety of gonadotropin-releasing hormone agonist treatment to suppress puberty in gender dysphoric adolescents. *The Journal of Sexual Medicine, 13*(7), 1125–1132. https://doi.org/10.1016/j.jsxm.2016.05.004

Seaborne, R. A., Strauss, J., Cocks, M., Shepherd, S., O'Brien, T. D., van Someren, K. A., Bell, P. G., Murgatroyd, C., Morton, J. P., Stewart, C. E., & Sharples, A. P. (2018). Human skeletal muscle possesses an epigenetic memory of hypertrophy. *Scientific Reports, 8*(1), 1898. https://doi.org/10.1038/s41598-018-20287-3

Sharma, P., Kumar, J., Garg, G., Kumar, A., Patowary, A., Karthikeyan, G., Ramakrishnan, L., Brahmachari, V., & Sengupta, S. (2008). Detection of altered global DNA methylation in coronary artery disease patients. *DNA and Cell Biology, 27*(7), 357–365. https://doi.org/10.1089/dna.2007.0694

Shim, M., Bang, W. J., Oh, C. Y., Lee, Y. S., & Cho, J. S. (2019). Effectiveness of three different luteinizing hormone-releasing hormone agonists in the chemical castration of patients with prostate cancer: Goserelin versus triptorelin versus leuprolide. *Investigative and Clinical Urology, 60*(4), 244–250. https://doi.org/10.4111/icu.2019.60.4.244

Shumer, D. E., Nokoff, N. J., & Spack, N. P. (2016). Advances in the care of transgender children and adolescents. *Advances in Pediatrics, 63*(1), 79–102. https://doi.org/10.1016/j.yapd.2016.04.018

APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

UNIVERSITY OF
NORTHERN COLORADO

Institutional Animal Care and Use Committee

IACUC Memorandum

To: David Hydock

From: Laura Martin, Director of Compliance and Operations

CC: IACUC Files

Date: February 19, 2020

Re: IACUC Protocol Approval, 2001D-DH-R-23

The UNC IACUC has completed a final review of your protocol "Sex Hormone Manipulation, Physical Activity, and Health".

The protocol review was based on the requirements of Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training; the Public Health Policy on Humane Care and Use of Laboratory Animals; and the USDA Animal Welfare Act and Regulations. Based on the review, the IACUC has determined that all review criteria have been adequately addressed. The PI/PD is approved to perform the experiments or procedures as described in the identified protocol as submitted to the Committee. This protocol has been assigned the following number 2001D-DH-R-23.

The next annual review will be due before February 19, 2021.

Sincerely,

Laura W. Martin
Director of Compliance and Operations
Animal Care and Use Program