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The Effect of Estrogen on GDNF Production of Skeletal Muscle In Vitro

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Abstract

As life expectancy continues to rise, the number of people over age 65 in the U.S. is increasing. An aging population brings with it impacts on the national health system and associated economic expenditures. As individuals age, the skeletomuscular system suffers reduced function due to disease or conditions such as sarcopenia. Sarcopenia is characterized by muscular atrophy resulting from reduced muscle innervation. Exercise has been linked to prevention of sarcopenia, a suspected result of increased production of glial cell line-derived neurotrophic factor (GDNF). GDNF promotes neuronal survival, regeneration, and the formation of neuromuscular junctions. Therefore, treatments like exercise that increase GDNF expression by skeletal muscle cells could be used as possible preventative measures for sarcopenia. It is well known that skeletal muscle structure differs between the sexes, and prior studies have shown that males have a higher incidence of sarcopenia compared to females. This raises the possibility that differences in sex hormones could be responsible for reduced muscular atrophy observed in females. Estrogen is one such hormone that is important for sexual development in females (although it is produced in low levels by male gonads). It is currently unclear if estrogen is responsible for neuroprotection in female skeletal muscle. The aim of this study was to observe the effects of E2 (estradiol) estrogen treatments on levels of GDNF production in C_2C_{12} skeletal muscle cells. To test this, we treated murine myotubes with estradiol in vitro and quantified GDNF protein levels using an enzyme-linked immunosorbent assay (ELISA). The results of this study suggest that estradiol treatments of 5 nM and 100 nM lead to decreased GDNF production 4 hours after treatment. When compared to the control, the 5 nM estradiol treated cells produced an increase in GDNF at 48 hours. Our data indicate that chronic estradiol exposure could increase GDNF production in skeletal muscle in vitro, highlighting a potential mechanism by which estrogen could lead to the reduced incidence of sarcopenia in females.

Introduction

Figure 1. Graph displaying the loss of muscle mass with age (Batsis & Buscemi, 2011).

Significance: It is evident that with an advancing life expectancy, the elderly population is continuously growing in developed countries. By the year 2050, it is expected that 25% of the population will be 65 years of age or older (Chen et al., 2009). With aging, the musculoskeletal system undergoes detrimental changes, including decreased muscle and bone strength (Boros & Fremont, 2017). As a result, the elderly are more prone to fractures and skeletal disease such as arthritis. Not only are the elderly experiencing a lower quality of life, but these effects also lead to economic expenses. In 1992, the elderly incurred the largest share of medical costs due to musculoskeletal conditions of all age groups at about 34% of the total cost. Along with this, fractures accounted for 36.9% of health care expenditures nationally (Hamerman, 1997). Namely, a change in the musculoskeletal system with aging that accounts for loss of muscle mass, or muscular atrophy, is sarcopenia. This condition results from a loss of contact between motor neurons and skeletal muscle at the neuromuscular junction (Larsson et al., 2019). The nervous system serves an important role in muscle maintenance, however, the nervous system can also change over time with aging and experience, which is known as neuroplasticity. Recent studies have made efforts to try to understand factors that regulate neuroplasticity, although mechanisms vary across individuals and throughout life. Factors including age and sex contribute to this variability (Voss et al., 2017).

The motor-nervous system: Also known as the peripheral nervous system, the motor-nervous system is composed of motor neurons and the skeletal muscles they innervate. These specialized types of neuronal cells integrate signals from the brain and sensory systems to control muscular movement (Swetenburg et al., 2017). The motor neuron fibers have an origin in the ventral horn of the spinal cord, and end at the neuromuscular junctions (NMJ) of skeletal muscles (Patel, 2015). The NMJ consists of a nerve terminal and a postsynaptic muscle cell, with the small space between them known as a synapse. Ions, neurotransmitters, peptides and other signal molecules are exocytosed from the nerve terminal, and act upon cell receptors on the postsynaptic cell. The postsynaptic muscle fiber can then respond to the signals provided by the nerve cell (Engel, 2008). Our study focuses on effects in non-innervated skeletal muscle cells, as opposed to effects in presynaptic motor neurons.

Glial cell line-derived neurotrophic factor (GDNF): GDNF is a neurotrophic factor for somatic motor neurons, and is a candidate for nervous system regeneration (Storer et al., 2003). GDNF promotes motor neuron survival (Lin et al., 1993), and has been found to be produced in skeletal muscle with colocalization at the NMJ (Suzuki et al., 1998). Previous studies from our lab have shown that GDNF content is affected by involuntary exercise in vivo (McCullough et al., 2011), where higher levels were associated with a 2-week running regimen in soleus muscle.

Alternatively, low levels of GDNF were associated with compromised structure and function at the NMJ (Lie & Weis, 1998). In a study published in 2014, it was found that GDNF content from C_2C_{12} myotubes could be affected by electrical stimulation. In a study done with electrical stimulation of C_2C_{12} cells in cell culture, it was found that GDNF expression can be changed with treatment (Vianney et al., 2014).

Sex comparison of skeletal muscle: Several studies have analyzed the differences between skeletal muscle depending on sex, drawing clear sex-dependent distinctions between fatigability (Semmler et al., 1999) and fiber-type (Haizlip et al., 2015). Furthermore, additional studies indicate that the incidence of sarcopenia may be dependent on sex, where men have a higher prevalence of sarcopenia than women (with adjustments for a number of factors, including weight) (Figure 2) (Mata Diz et al., 2015). An additional study by Khongsri et al. found similar results displaying the male population with a higher prevalence of sarcopenia in comparison to the female population when looking at those of 60-69 and 80+ years of age (2016). Along with this, muscle degeneration and loss of muscle strength has been found to occur faster in males than in females, specifically those of 75 years or older (Du et al., 2019).

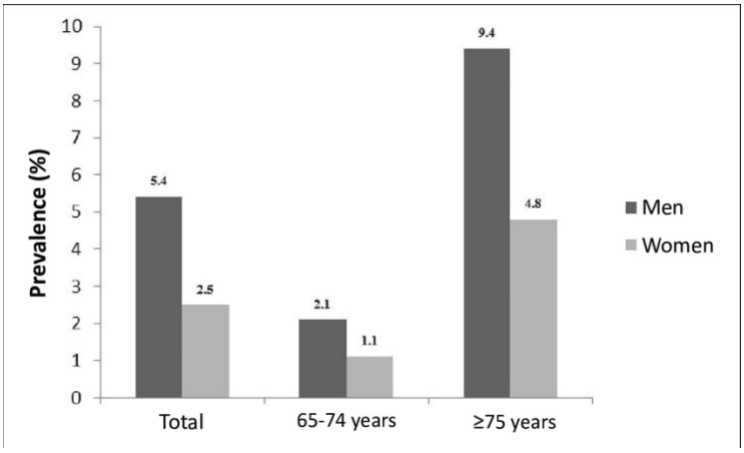

Figure 2. The male population faces a higher prevalence of sarcopenia in comparison to their female counterparts (Mata Diz et al., 2015).

Estrogen: Estrogen is a hormone that interacts with the female body to regulate a woman's menstrual cycle and reproductive development. This hormone is secreted by the ovaries, placenta, and in the peripheral conversion tissues in women. Estrogen levels within the human bloodstream cycle throughout the menstrual cycle and decline with age (Reed & Carr, 2018). Estrogen interacts with estrogen receptors found on organs of the female reproductive system and skeletal muscle, amongst others. Throughout menopause, women lose most of their estrogen, with continued loss at each year of the process. On another note, it is present at low levels within the male body as a vital player in male fertility and reproductive organ development. Estrogen in males is found at low levels within the bloodstream, and higher levels in male reproductive serums. Thus, sex is in part determined by quantitative comparisons of estrogen and the primary male sex hormone, testosterone (Hess et al., 1997).

Estrogen is found in three major forms: estrone (E1), estradiol (E2), and estriol (E3). In this study, estradiol was used. This form of estrogen was chosen due to its potency, and its relevance to menopause. During menopause, the primary estrogen changes to E1 from E2. Due to our focus on sarcopenia, which occurs during or after menopause, E2 was selected as the experimental estrogen (Cleveland Clinic). Several studies have investigated the role of ovarian hormones on skeletal muscle contractility, including that from Moran et al. which found that estradiol replacement restored the decline in muscle contractility and myosin function in vivo in post-

ovariectomy rats (2007). In a study from 2006, the authors found estrogen to be a regulator of skeletal muscle fiber growth during recovery from disuse in female rats. In addition, estrogen treatments were found to have direct effects on myofiber size post-injury of skeletal muscle (McClung et al.).

Rationale: Previous work in Dr. Spitsbergen's laboratory has shown that exercise increased GDNF protein content and end plate area in skeletal muscle (Gyrokos et al., 2014). Along with this, GDNF has been found to increase the number of motor neurons innervating neuromuscular junctions, meaning that the amount of GDNF in skeletal muscle can regulate the number of neuromuscular junctions formed (Nyugen et al., 1998). Therefore, an increase in levels of GDNF expression in skeletal muscle due to exercise provides a possible explanation as to why exercise has been seen to reverse and prevent sarcopenia in aging adults. Alternatives to exercise that increase the amount of GDNF produced by skeletal muscle could provide further options of sarcopenia prevention and treatment. The studies presented displayed clear distinctions between skeletal muscle found in males and females, which could be due to the female sex hormone, estrogen. Along with this, the loss of estrogen is related to the decline in muscle strength and mass (Horstman et al., 2012). Supplemental studies presented here suggest estrogen interacts with skeletal muscle within the female body, leading to speculation of its role in muscular degeneration and possible regeneration. Due to the correlations between estrogen, muscle mass preservation, and GDNF expression, I hypothesized that altering levels of estrogen in skeletal muscle cultures would alter levels of GDNF expression. To assess whether estrogen could have an effect on GDNF expression, I treated C_2C_{12} murine skeletal muscle cells with various estradiol concentrations. Enzyme-linked immunosorbent assay (ELISA) was then used to quantify levels of GDNF expression. The results of this study support the hypothesis that chronic estradiol treatments could increase GDNF production by skeletal muscle cells in vitro. If the estradiol treatments lead to changes in GDNF production in the skeletal muscle cells, these results could provide a possible explanation as to why females experience sarcopenia at lower rates in the human population.

Materials and methods

Cell Culture: C_2C_{12} mouse myoblasts of an adult C3H mouse were obtained from the American Type Culture Collection (ATCC). Handling procedures from the ATCC were adhered to. Cells were maintained in growth medium that contained Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum and 1% antibiotic antimycotic solution. Routine culture was performed in 100 mm CytoOne plates. Experimental cultures were performed in 6-well plates to confluence and allowed to differentiate. The cells were maintained so that experiments were performed during passages 7 and 8. Plated cells were stored in a 37-degree Celsius incubator with 5% CO_2.

Estrogen treatment: For this experiment, estradiol concentrations of 0 nM, 1 nM, 5 nM, 10 nM and 100 nM were added to different samples of C_2C_{12} skeletal muscle cells in vitro. The 0 nM treatment acted as the control. The concentrations were varied within a relevant range to physiological estrogen concentration. The vehicle for estradiol was ethanol, which was present at such low concentrations that no vehicle control was necessary.

To add the estradiol, 2 microliters of the estrogen solution were pipetted into each well with 2 mL of growth media. Each 6-well plate was designated to a concentration of 0 nM, 1 nM, 5 nM, 10 nM and 100 nM of estradiol. Each column in the plate was designated to a time interval of 4 hours, 24 hours, or 48 hours.

Samples of the media (1 mL) and the cells were taken separately after 4 hours of adding the estradiol, 24 hours after, and 48 hours after. The scraped cells were stored in 1 mL sample buffer solution. To scrape cells, a standard cell scraper was used. The 4-hour time was used to study short-term effects, whereas the 24- and 48-hour samples were utilized to evaluate long-term effects.

GDNF ELISA (Enzyme-linked immunosorbent assay): The standard ELISA protocol was performed following R&D Systems MAB212 manufacturer specifications.

Statistical analysis: Differences between means were examined using Student's t-test assuming equal standard deviations.

Results

Previous studies have displayed a correlation between a decline in estrogen and loss of muscle mass (Horstman et al., 2012). Here, we sought to determine if GDNF expression by skeletal muscle could be affected by estrogen treatments. First, routine cell culture was performed with C_2C_{12} murine skeletal muscle cells. Estradiol treatments of varying concentrations were performed, and samples of both the cells and the media in which they were incubating were obtained in intervals. ELISA was utilized to quantify the GDNF content present in samples. Statistical analysis was performed with ELISA results to analyze GDNF content in the cells and the media with varying estradiol treatments. The experiment had a sample size of n=4. Results displayed significant changes in GDNF production of skeletal muscle cells treated with 5 nM and 100 nM estradiol solutions.

Figure 3 displays data from a representative concentration showing a comparison of GDNF content between the cell and media samples. The cells were collected 4 hours after a 1nM estradiol treatment. The results show that the majority of GDNF produced is retained within cells and not found in incubating media. Due to undetectable GDNF levels in the media samples by the ELISA, the cells alone were further analyzed.

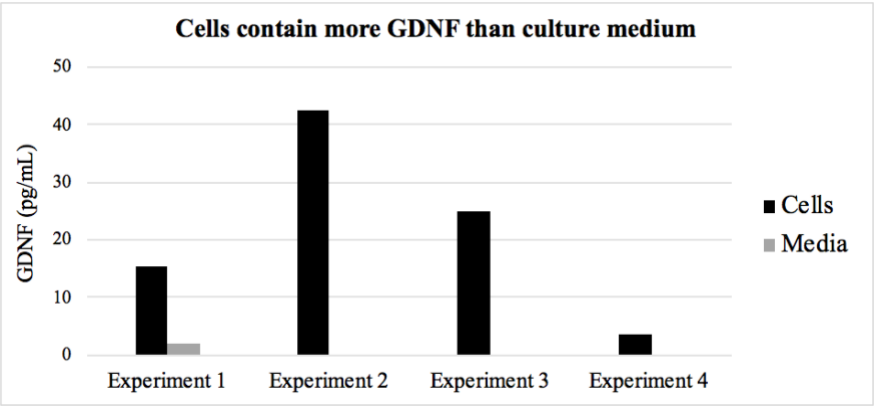

Figure 3. Changes in cellular GDNF content (pg/mL) following estradiol treatment. C_2C_{12} skeletal muscle cells were grown in cell culture and treated with 1 nM estradiol solutions. Cell and media samples were collected 4 hours after treatment. ELISA was used to quantify GDNF concentration in the samples. Cells collected 4 hours after 1 nM treatment retained GDNF produced. 3 out of 4 experiments show 0 pg/mL GDNF concentration in media.

Figure 4 displays the average GDNF concentration (pg/mL) found in the cell samples collected 4 hours after treatment. These results show that the cells treated with concentrations of 5 nM and 100 nM of estradiol produced a statistically significant decreased amount of GDNF in comparison to the control at this time interval.

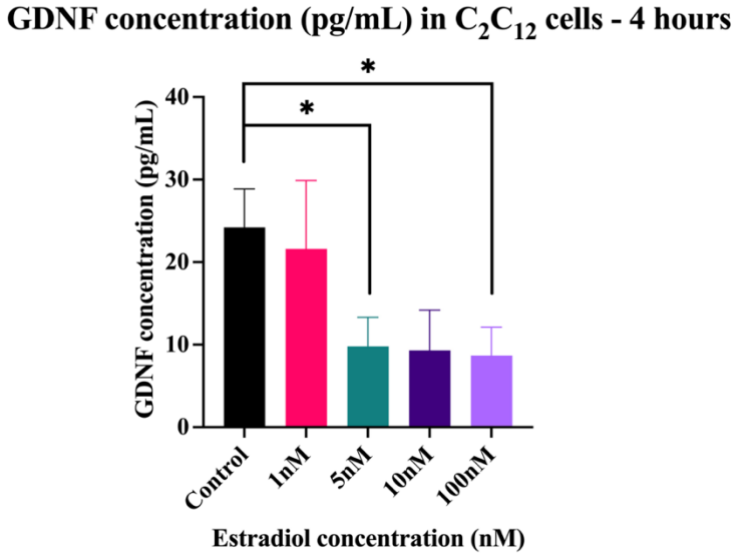

Figure 4. GDNF content (pg/mL) found in cell samples collected 4 hours after treatment. Myotubes in cell culture were treated with varying estradiol doses and samples were collected 4 hours later. GDNF production was quantified using ELISA. T-test analysis was performed following mean calculations of GDNF content for n=4. Significant results are represented with an asterisk (*), indicating a p-value < 0.05. Data shown represent mean +/- standard error of the mean. Results show a significant decrease in GDNF concentration in cells treated with 5 nM and 100 nM estradiol solutions in comparison to the control.

Figure 5 displays the results of average GDNF content measured in the cell samples collected 24 hours after the various estradiol treatments. Statistical analysis produced no significant data.

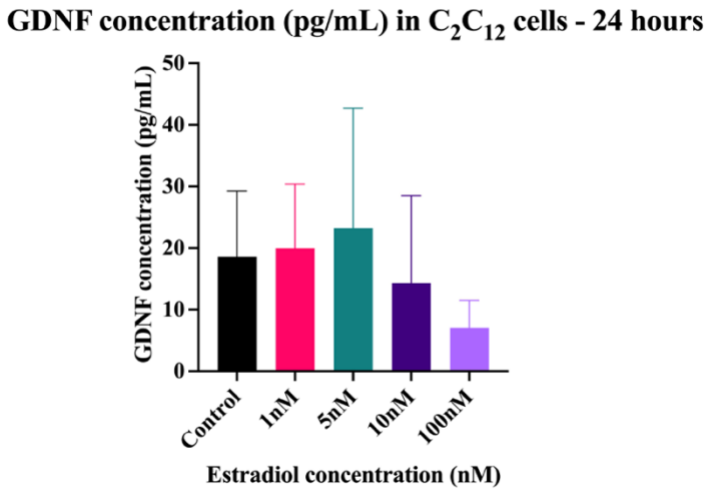

Figure 5. GDNF concentration (pg/mL) in cell samples collected 24 hours after treatment. Murine skeletal muscle cells in culture were treated with varying estradiol concentrations and samples were collected 24 hours after. ELISA was used to quantify GDNF in samples. T-test analysis was performed following mean calculations of GDNF content for n=4. Significant results are represented with an asterisk (*), indicating a p-value < 0.05. Data shown represent mean +/- standard error of the mean. Results showed no significance.

Figure 6 shows the results of average GDNF content found in the cell samples collected 48 hours after estradiol treatment. The results display a significant increase in GDNF concentration found in cells treated with 5 nM estradiol in comparison to the control.

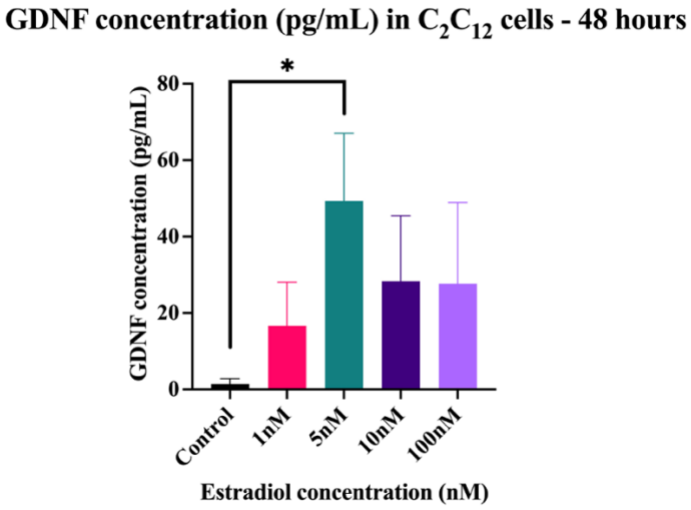

Figure 6. GDNF concentration (pg/mL) found in cell samples collected 48 hours after estradiol treatments. C_2C_{12} skeletal myotubes were treated with varying estradiol concentrated solutions and cellular samples were collected 48 hours later. GDNF concentration was quantified with ELISA. T-test analysis was performed following mean calculations of GDNF content for n=4. Significant results are represented with an asterisk (*), indicating a p-value < 0.05. Data shown represent mean +/- standard error of the mean. Results show a significant increase in GDNF concentration in cells treated with 5 nM in comparison to the control.

Figure 7 represents a comparison between the cells treated with 5nM and the control using average GDNF concentration in the samples at each time interval. The results show a significant difference in GDNF production between the treated and untreated cells at 4 hours and 48 hours after treatment. At 4 hours, the treated cells appeared to produce a decrease in GDNF production, whereas at 48 hours there was an apparent increase.

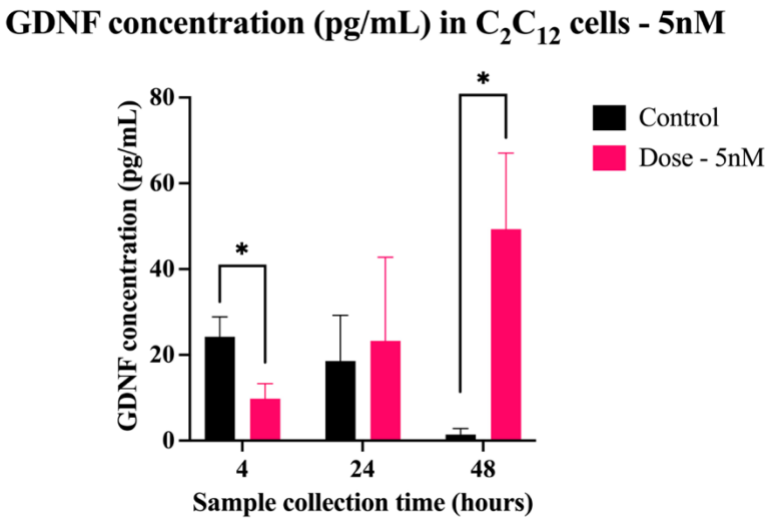

Figure 7. GDNF concentration (pg/mL) for cells treated with 5 nM estradiol compared to the control.
Skeletal muscle cells were treated with 5 nM estradiol in vitro, and samples were collected at various time intervals after treatment. Control cells were not treated with estradiol. Statistical analysis was done using a t-test to compare 5nM treated cells with the control. Asterisks (*) indicate significance, or a p-value < 0.05. Data shown represent mean +/- standard error of the mean. The results show significant differences in GDNF produced between treated and untreated cells at 4 hours and 48 hours after treatment. No significance was found at 24 hours.

Figure 8 represents a comparison between the cells treated with 100nM and the control using average GDNF concentration in the samples at each time interval. The results suggest a significant decrease in GDNF production between the treated and untreated cells at 4 hours. Although there is no significant difference in the GDNF produced at 48 hours, preliminary data suggests a trend towards increased GDNF production by treated cells at this concentration.

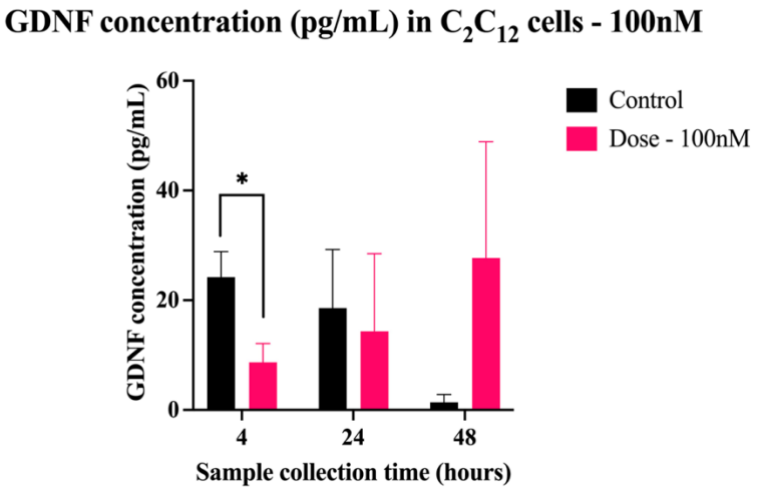

Figure 8. GDNF concentration (pg/mL) for cells treated with 100 nM estradiol compared to the control. Skeletal muscle cells were treated with 100 nM estradiol in vitro, and samples were collected at various time intervals after treatment. Control cells were not treated with estradiol. Statistical analysis was done using a t-test to compare 100nM treated cells with the control. Asterisks (*) indicate significance, or a p-value < 0.05. Data shown represent mean +/- standard error of the mean. The results show significant differences in GDNF produced between treated and untreated cells at 4 hours after treatment. No significance was found at 24 and 48 hours.

Discussion

The primary goal of the present study was to determine the effects of treatment with estradiol on GDNF expression in C_2C_{12} skeletal muscle myotubes in cell culture. To do this, C_2C_{12} cells were treated with estradiol at various concentrations and samples were taken at specific time intervals. ELISA assays were used to quantify GDNF protein concentration in the cells and in the media samples after treatment. The results of this study suggest that: 1.) the majority of GDNF produced is found within cells with little being released in medium, 2.) estradiol treatment with 5nM and 100nM appeared to cause a decrease in GDNF production and 3.) cells collected 4 hours after a 5 nM estradiol treatment produced an increased amount of GDNF in comparison to the control.

The results from this present study are consistent with those produced in a study investigating the effects of electrical stimulation on GDNF production (Vianney et al., 2014). First, both studies found that treated myotubes retained the GDNF they produced. Here, this was found specifically in cells sampled 4 hours after treatment, independent of the estradiol concentration added. These results suggest stored GDNF could be secreted based upon a system of demand. In an *in vivo* model, this could be due to a need for GDNF by spinal motor neurons. The *in vitro* model used here may not have allowed for this demand to be met, therefore no GDNF secretion was detected. Second, both studies showed differences in GDNF production with acute and chronic treatments. Acute treatments displayed inhibitory effects on GDNF production, whereas chronic treatments suggested stimulation of GDNF production in cell samples. Specifically in this study, these differences were seen in 5 nM and 100 nM estradiol treatments acutely, and 5 nM estradiol treatments alone chronically. These results suggest that estradiol exposure may cause changes in skeletal muscle cells that alters GDNF production, which changes from inhibition to stimulation over time.

Naturally, estradiol is found in human blood at concentrations of 0.11 nM - 1.47 nM in premenopausal women, and 0 nM - 0.11 nM in postmenopausal women (University of Rochester Medical Center). Therefore, the 5nM estradiol treatment used in this study is in the range of physiologically relevant and could represent the concentration found at estradiol receptors in skeletal tissue.

In summary, the results of this study showed that GDNF produced by myotubes was not secreted into the media, and that chronic exposure to 5 nM estradiol treatments lead to increased GDNF production by these cells. Acute exposure of 5 nM and 100 nM estradiol treatments appeared to have decreased GDNF production by the cells. The results support the hypothesis that chronic estradiol treatments could increase GDNF production by skeletal muscle cells in vitro. If estradiol treatments increased GDNF production, this may provide an explanation as to why females experience sarcopenia resistance in comparison to their male counterparts. Additionally, these results may suggest that an estradiol therapy could help relieve sarcopenia symptoms.

Based upon observations of cell differentiation during estrogen treatments, future studies of importance would be those that investigate the role of estrogen in development of skeletal muscle cells. Additionally, if time permitted, a real-time polymerase chain reaction (PCR) for mRNA quantitation would allow for information to be given about the presence and type of estrogen receptors in the skeletal muscle cells tested. Along with this, a replication of this study with progesterone would provide a better understanding of what hormones impact GDNF in skeletal muscle cells. Lastly, an experimental design that would allow for longer-term studies of the effects of the 100 nM estradiol treatment would allow for a better understanding of the effects of estradiol on skeletal muscle with time.

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