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Examining Permeability and Contractility of Skeletal Muscle in Castrated Male Mice

Evan Scott
Ursinus College, evscott@ursinus.edu

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INTRODUCTION

The widespread health benefits of regular physical activity are well-established. Exercise has been shown to limit the incidence and improve the symptoms of cardiovascular disease, dementia and diabetes, to name a few. To prevent disease and promote a healthy lifestyle, exercise is often prescribed. Skeletal muscles are required for voluntary movement of the body. Injuries or damage to skeletal muscle can compromise this. Immediately following muscle injury, an inflammatory response occurs. Inflammation is characterized by redness, heat, pain and swelling. The importance of the inflammatory response to efficient and timely healing is becoming more recognized. For this reason, our lab has begun to look at the effects of temperature therapy on different parameters of muscle injury and healing.

Temperature therapies are commonly used to alter pain and the inflammatory response following injury. Temperature affects one of the initial aspects of skeletal muscle healing, increased vascular permeability. Vascular permeability is an important component of inflammation and consists of opening junctions between endothelial cells to allow plasma proteins and inflammatory cells to the site of injury. Previous data accumulated from the Ursinus College Skeletal Muscle Lab suggests that vascular permeability following thermotherapy is different between the sexes and will be discussed in the previous findings section.

Most research studies focus on the male sex to eliminate the contributions of the difficult to interpret female cycle. To date, there have not been any studies analyzing permeability differences following thermotherapy between male and female sexes. In addition, it is currently unclear how increased vascular permeability affects skeletal muscle healing. Thermotherapy is often implemented for pain reduction, but it also increases blood flow to the damaged area. Studies suggest an increase in permeability following heat therapy in male mice. (Song, 1978)

(Song, Kang, Rhee, & Levitt, 1980) These studies utilized an in vivo assay of Iodine labeled human serum albumin in order to compare vascular permeability. Similar studies in female mice do not exist.

While there are no studies investigating the cause of permeability differences between sexes, one possible explanation could be differences in the sex hormones, testosterone and estrogen. A previous study suggests estrogen may assist in inflammatory processes following skeletal muscle injury in rats. Using a myeloperoxidase assay, researchers found decreased inflammatory cell invasion in ovariectomized female rats after ischemia-reperfusion injury (Stupka & Tiidus, 2001). A different study showed an increase in vascular permeability following localized estrogen injections in mice (Lindhe, 1967). Similar studies have found that the inflammatory protein interleukin-6 production is decreased in the presence of testosterone, suggesting that testosterone may inhibit inflammation in humans (Maggio, et al., 2006) (O'Connor, Motivala, Valladares, Olmstead, & Irwin, 2006). To summarize, it is unclear if sex hormones affect vascular permeability following muscle injury.

We designed an experiment to determine if testosterone affected permeability following muscle injury in male mice. In addition, we planned to examine the effects of testosterone on muscle healing and functionality after injury, utilizing contractile force measures to compare muscle strength following completed muscle repair both with and without thermotherapy. The goal of this study was to analyze the effects of a lack of testosterone on vascular permeability following thermotherapy treatment as well as investigate the effects of thermotherapy on muscular force development following injury. However, due to unforeseen circumstances, the results for this experiment are limited. This paper will focus more on the methods and future plans of the experiment should it be conducted in full.

PREVIOUS FINDINGS

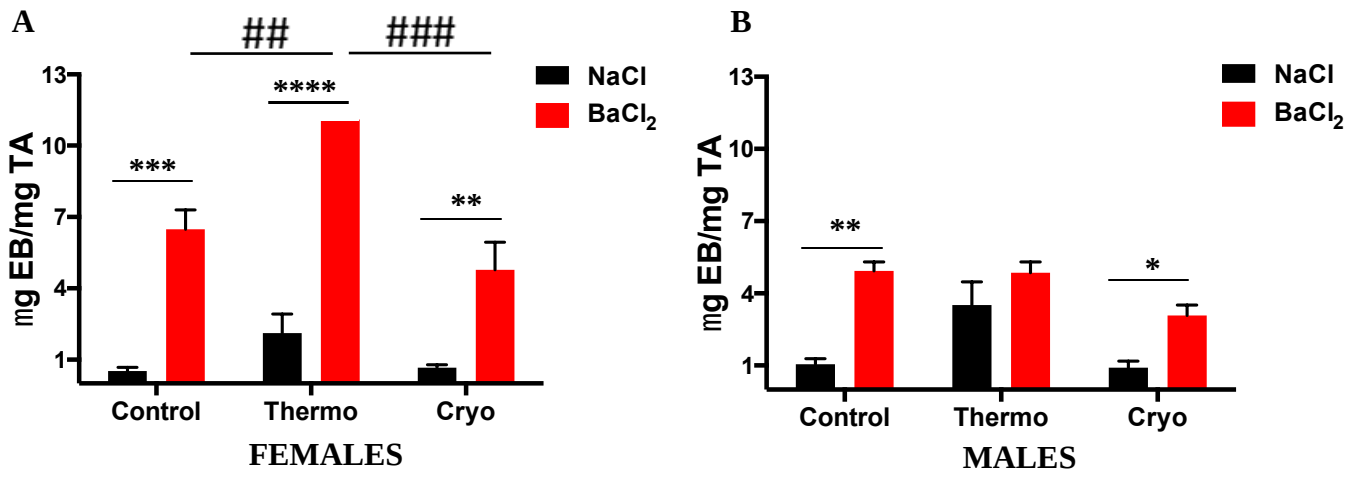

Figure 1: Evan's blue extraction relative to muscle size. Injured and non-injured muscle tissue was compared to determine the effects of temperature treatment on the amount of evan's blue extracted per muscle weight. A (females). The evan's blue extracted was significantly greater in $BaCl_2$ injected TAs in all three groups. B (males). The evan's blue extracted was significantly greater in $BaCl_2$ injected TAs in both control and cryotherapy treated groups, but not in the thermotherapy group. All statistical differences are indicated. *$p < 0.05$, **. ##p < 0.01, ***, ###$p < 0.001$, ****p<0.0001.

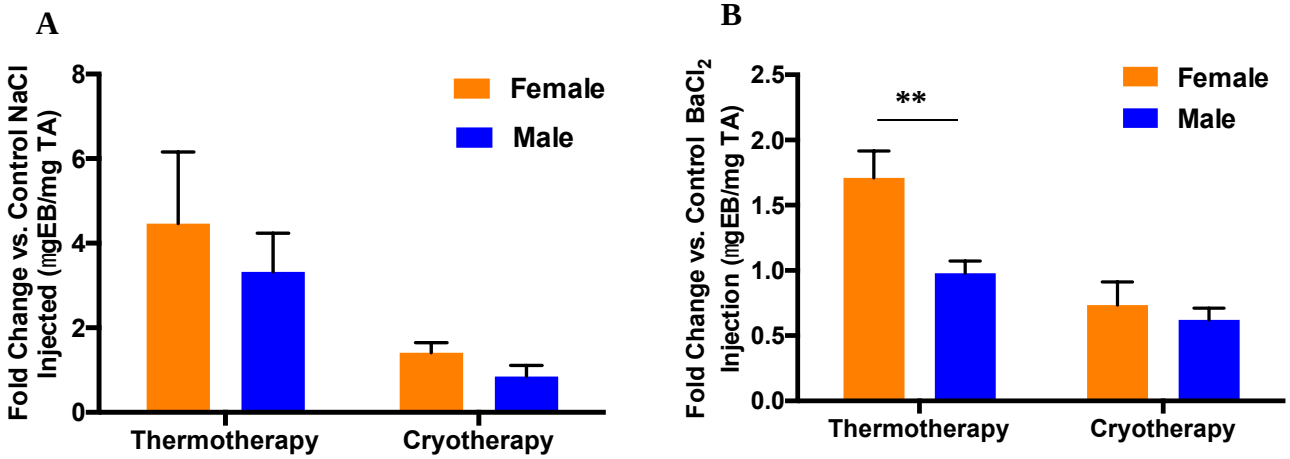

Figure 2: Fold change differences in evan's blue extraction. A. Fold Change vs. Control NaCl treated muscles. Thermotherapy and cryotherapy injured groups were normalized to the control group to determine sex differences in the effects of temperature treatment on permeability in NaCl injected muscles. B. Fold Change vs. Control $BaCl_2$ treated muscles. Thermotherapy and cryotherapy injured groups were normalized to the control group to determine sex differences in the effects of temperature treatment on permeability in $BaCl_2$ injected muscles. Standard deviation error bars, **p < 0.01. Image courtesy of Brittany Gasser.

Previous data from our lab showed permeability differences between males and females following thermotherapy treatments Permeability was greater between NaCl and BaCl2 groups in all groups except thermotherapy treated males. Thermotherapy treatment alone increased evan's blue extraction in males (Fig. 1B). Thermotherapy following injury significantly elevated permeability in females. In fact, thermotherapy following injury was increased 1.7 fold as compared to males. (Fig. 2B). In addition, cryotherapy did not significantly limit permeability in males or females following injury.

METHODS AND MATERIALS

Mice: There were to be 32 total mice used during the experiment: sixteen 12-week old castrated and sixteen 12-week old uncastrated mice. The castration was completed at Charles River at 4 weeks of age. Because each round of the experiment is time consuming and animals are undergoing thermotherapy for 20 minutes every 4 hours during the wake cycle, we divided the experiment into four parts: Part 1 was completed before spring break and Parts 2-4 were to be completed in the two weeks following spring break.

1) 4 castrated mice that underwent thermotherapy treatment; 4 castrated mice that underwent anesthesia in place of thermotherapy treatment
2) 4 non-castrated mice that underwent thermotherapy treatment; 4 non-castrated mice that underwent anesthesia in place of thermotherapy treatment
3) Repeat; 4 castrated mice that underwent thermotherapy treatment; 4 castrated mice that underwent anesthesia in place of thermotherapy treatment
4) Repeat; 4 non-castrated mice that underwent thermotherapy treatment; 4 non-castrated mice that underwent anesthesia in place of thermotherapy treatment

Chemically Induced Injury Procedures: All mice were anesthetized for intra-muscular injection via the Tibalis Anterior (TA). 25 µL of Barium Chloride ($BaCl_2$) was injected into the right tibialis anterior to induce chemical muscle injury. The left TA was injected with 25µL of 0.09% sodium chloride (NaCl), serving as the control injection.

Figure 3. Mouse undergoing thermotherapy treatment. Photo courtesy of Emily Megill

Thermotherapy Treatment: Mice were anesthetized and placed stomach down under the nose cone for the duration of the treatment. The legs of the mouse were placed on top of a heating pad. The feet were taped down, and the heating pad attached to a heat therapy pump (Androit Medical Systems HTP-1500) was folded over on top of the legs (Fig. 3). Both ends of the heating pad were held by a clip to ensure the tibias were maintained at a constant temperature of 41°C for each 20 minute sessions. After each heating interval, the mice were placed back into the cage. Thermotherapy treatments were repeated every 4 hours from 8AM to 8PM (8AM, 12PM, 4PM, 8PM) over a 36 hour period. Mice that did not undergo thermotherapy were anesthetized for the 20-minute sessions every 4 hours from 8AM to 8PM (8AM, 12PM, 4PM, 8PM) over a 36 hour period.

Evan's Blue Permeability Assay: At the end of this 36-hour period, the mice were injected via the tail vain with Evan's Blue dye. After 20 minutes, the mice were terminated and both tibialis anterior of each mouse were extracted. Each muscle was placed in a labeled collection tube containing 100µL of formamide. The tubes were subsequently placed 4°C for 24 hours to extract Evans blue from the muscle. The tibialis anteriors were removed and the remaining solution was spun in a microcentrifuge at 1500 RPMs. The absorbance of 100µl of Evan's Blue formamide solution at wavelength of 630nm was measured.

ELISA Testosterone Test: Blood testosterone levels of the castrated and uncastrated mice were to be assessed using an ELISA in order to confirm relative testosterone levels. Blood was to be extracted from the plural cavity following mouse termination. I practiced this technique on approximately 5 mice and was able to collect about 1mL of blood each time. This is a large enough volume to be used for the ELISA.

RESULTS AND DISCUSSION

Following injury to skeletal muscle, the local application of controlled temperature is the most commonly utilized non-invasive treatment (Lee, Choi, & Mcanulty, 2018). However, it remains unclear how different genders may be affected by thermotherapy differently. Data collected in the present study examined how a lack of testosterone may influence permeability following skeletal muscle injury. The average absorbance of Evan's blue dye was significantly elevated after chemical injury ($BaCl_2$) in the castrated mice that underwent control anesthetization (Fig. 4). Evan's blue dye absorbance was not significantly different in the castrated mice between the chemically injured ($BaCl_2$) mice that underwent thermotherapy, or the NaCl injected control mice that underwent anesthetization (Fig.4). It should be noted that

one mouse was removed from the data due to a lack of Evan's blue perfusion, which was an indication the muscle was probably not properly injured.

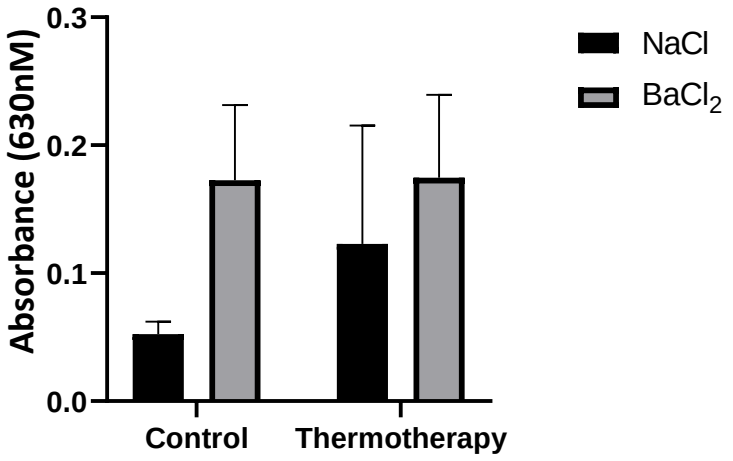

Figure 4. Average absorbance (nm) in isolated tibialis anterior muscles. n= 7, 3 thermotherapy, 4 control. Standard deviation error bars.

No significant differences were observed in the muscle weights between groups (Fig. 5). In the both the castrated anesthetized group and the castrated thermotherapy treated group, significant differences were observed in the amount μgEB/mgTA extracted following chemical injury with $BaCl_2$ (Fig. 6). This indicates that in both treatments, $BaCl_2$ injury elevated permeability. In addition, in castrated mice thermotherapy had no significant effect on permeability when control NaCl injection. Also, even though the data showed a trend, there was no significant difference in the permeability of the $BaCl_2$ groups whether just anesthetized or thermotherapy treated. This is opposite to our previous data in uncastrated mice where thermotherapy alone elevated permeability and further injury did not significantly increase permeability further. This suggests testosterone may play a role in the permeability induced by thermotherapy.

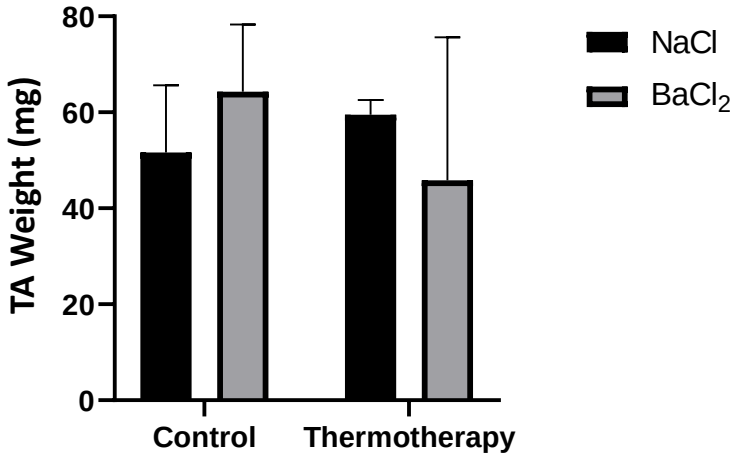

Figure 5. Average weight of TAs following extraction. n=7, 3 Thermotherapy, 4 Control. Standard deviation error bars.

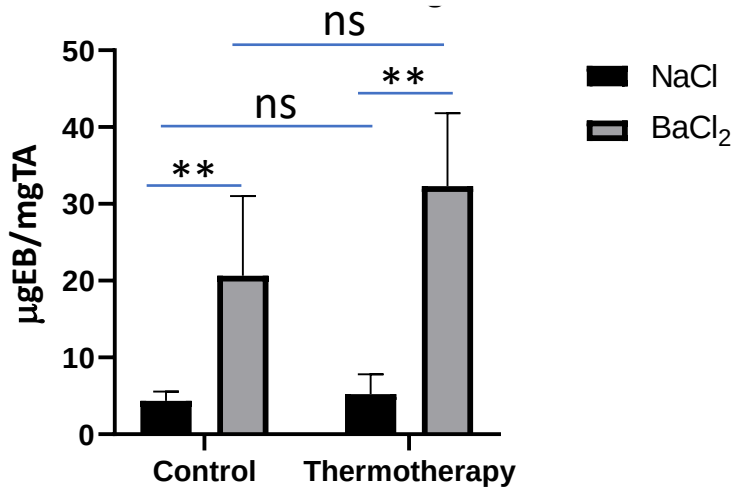

Figure 6. Average absorbance per mg of Tibialis Anterior. n=7, 3 Thermotherapy, 4 Control. Standard deviation error bars.

LIMITATIONS

When comparing the data collected to previous data from the UC skeletal muscle lab, the average absorption of the castrated mice was greater in both the control and thermotherapy treatment groups than the uncastrated male and female mice groups. Our control values are usually around 1 µgEB/mgTA for NaCl injection and between 5-10µgEB/mgTA for $BaCl_2$ injection. The values in this experiment were 2-3 fold higher in both NaCl and $BaCl_2$ injected

TAs. This could be due to the fact the injury was performed by different researchers. Therefore, the validity of this data will need to be confirmed by repeating these experiments.

CONCLUSION

Recreation of the data and further analysis is a necessary step in moving forward following this experiment. Due to the discrepancies in results as well as unforeseen circumstances, much of the data collected is inadequate for drawing conclusions. Utilization of a force transducer and specific setup techniques were not possible. As a result, the thorough completion of this experiment is required to shed light on the effects of testosterone on skeletal muscle repair and subsequent contractility.

ACKNOWLEDGMENTS

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