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The Comparison of Endothelial Permeability at 48 Hours Post Skeletal Muscle Injury in Male and Female Mice at Various Ages

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Submitted to the Faculty of Ursinus College in fulfillment of the requirements for Honors in the Health and Exercise Physiology department.

Introduction

Research regarding skeletal muscle injury is a growing interest especially for athletes. Most athletes want to be able to return fully to their sport as soon as possible after an injury as well as be fully healed. Although many athletic injuries include tendon, ligament, and bone injuries, muscle injuries are among the most common accounting for up to 55% of sports injuries (Guillodo 2009). Because it is unethical to create an identical injury in humans, our study used mice. There are a variety of ways to injure a mouse's muscle including myotoxic agents, chemical injury, or physical injuries (Hardy 2016). Our study uses chemical injury with barium chloride injected into the tibialis anterior (TA) because it can be consistently administered and it is the most commonly used method of skeletal muscle injury in mouse research (Hardy 2016). We also inject a control of NaCl in the TA in the opposite leg to show that the needle injecting the substances is not causing enough of an injury to impact the endothelial permeability of the muscle.

Currently in scientific research there is an imbalance of research done between male and female subjects. There is a growing need to understand the differences in disease progression and healing in females. We are specifically interested in studying muscle injury and healing. A study done using human subjects found that males tend to have higher expression of genes encoding mitochondrial proteins, ribosomal proteins, and some translation initiation factors, while females have a greater expression of growth factor pathways that regulate muscle mass (Welle 2008). One of the growth factor pathways that are more highly expressed in females inhibits insulin-like growth factor (IGF), which plays a large role in the regeneration of skeletal muscle (Welle 2008,

Vignaud 2010). Studies show that macrophage-produced IGF-1 helps directly regulate muscle regeneration by expanding satellite cell numbers while also regulating macrophages to change to the M2 phenotype which promotes muscle growth, regeneration and recruitment of monocytes (Tidball 2015, Chazaud 2009, Ruffell 2009, Tidball 2006, McLennan 1996).

Sex differences were also identified during the process of inflammation. In order to measure inflammation and injury, researchers measure the vascular permeability of the injured area. Permeability is how easily or how many cells and proteins can pass through the blood vessel walls. It is an important factor in the healing process as it is what allows healing and regeneration factors as well as multiple types of cells to reach the injured area to clean out the dead and injured cells as well as begin regenerating the muscle tissue. Our preliminary unpublished studies show that endothelial permeability peaks at three times post skeletal muscle injury; 30 minutes, 48 hours, and 7 days. The 30-minute peak is when neutrophils infiltrate to begin clearing out the dead and injured cells. The 48-hour peak is when macrophages infiltrate to continue clearing out waste and begin regeneration (Nicholas 2014). The 7-day peak is around when angiogenesis, or the formation of new blood vessels, begins. Our focus is on macrophages, which are an important leukocyte in the regeneration of skeletal muscle. A study showed that macrophages present 2 to 4 days post skeletal muscle injury affected muscle fiber membrane repair which increased muscle use, repair, growth, and regeneration (Nicholas 2014, Tidball 2006, Tidball 2011).

Due to the variety of gene expression differences, we do not expect males and females to heal exactly the same, or have an identical inflammatory response. The main

goal of this study was to identify the endothelial permeability differences between males and females at different ages. The three ages we studied were one month, two months, and three months which are loosely equivalent to childhood, adolescence, and adulthood in humans, as the mice are still growing at one and two months of age and start reproducing around 3 months. Our present data tested the hypothesis that the female mice will show more permeability at 48 hours post injury than the male mice at all three ages.

Methods

Animals: Male (n=24) and female (n=24) Swiss Webster mice ages one month old, two months old, three months old (regular, castrated, and ovariectemized), 6 months old, and 12 months old (n=4 per group) were used in this study. Castration and ovariectomy were performed at 8 weeks at Charles River Labs.

Muscle Injury Model: Each mouse was weighed in milligrams to calculate how much injection each mouse received based off of their body weight divided by 12. Each mouse was injected with this amount of NaCl as a control in the right tibialis anterior (TA) and BaCl₂ to cause a chemical injury in the left TA. The control injection is used to show that the puncture wound from the injection is not what is causing the injury to the muscle or the enhanced permeability.

Assessment of Permeability: Two days post injection, mice were injected with 200ul of Evan's Blue dye (EB) into the lateral tail vein (Wang 2015). After 30 minutes, mice were sacrificed and both TA muscles were removed and weighed. Muscles were placed in 200ml of formamide overnight to extravasate the Evan's blue from the muscle. The

absorbance of the formamide solution was measured on an Accuris Instruments SmartReader™ 96 at 630nm.

Statistical Analysis: A two-way ANOVA was done to compare weights, absorbance measurements, and nanograms of EB per mg of muscle weight for males and females as well as NaCl treated muscles to BaCl₂ treated muscles.

Results

At 4, 8, and 12 weeks and at 6 months and 1 year of age, baseline permeability is not significantly different between males and females suggesting baseline muscle permeability is similar between the sexes up to a year of age (Fig. 1). In both sexes, injection of BaCl₂ elevated Evan's blue entry into the muscle at all ages investigated (Fig. 1). Because BaCl₂ injection results in a chemical injury to the muscle this was an expected finding. BaCl₂ injection in males resulted in significantly higher permeability than in females at 4 weeks (Fig. 1A). Because the amount of BaCl₂ injected was equalized to weight, this may suggest that males have a greater inflammatory response at younger ages. In contrast, BaCl₂ injection in females resulted in significantly higher permeability than in males at 1 year (Fig. 1E). Additionally, there was a trend in females that permeability following injury was greater with age. Evan's blue extraction was significantly greater at 12, 26 and 52 weeks as compared to 4 and 8 weeks in females (Fig. 2A). The opposite trend is observed in males with permeability following injury decreasing with age. Evan's blue extraction was significantly reduced at 8, 26 and 52 weeks as compared to 4 week old males (Fig. 2B). This suggests the inflammatory response may be different between the sexes as they age.

Baseline muscle permeability is not significantly different between castrated and uncastrated mice, or ovariectomized and unovariectomized mice, respectively (Fig. 3). Injection of BaCl₂ elevated Evan's blue entry into the muscle in the castrated, uncastrated, ovariectomized and unovariectomized groups (Fig. 3). No significant differences were found between BaCl₂ injected TAs of castrated or uncastrated mice (Fig. 3B). In addition, no significant differences were found between BaCl₂ injected TAs of ovariectomized or unovariectomized mice (Fig. 3A). This suggests that gonadal hormones do not play a role in permeability differences in mice 12 weeks of age.

Discussion

There are three types of capillaries that make up capillary beds, each varying in permeability and EB absorption. Sinusoidal capillaries are the most permeable and absorb the most EB, fenestrated capillaries are somewhat permeable and absorb some EB, and continuous capillaries are the least permeable and do not absorb much EB (Carpenter 2009). Skeletal muscles contain continuous capillaries; therefore, a healthy skeletal muscle is not very permeable. We injected the TA's with either NaCl or BaCl₂ as a control or to cause a chemical injury respectively. Consistently across all ages and both sexes we saw significantly increased permeability in the BaCl₂ treated (injured) muscles compared to the NaCl treated (control) muscles in the same mouse. This shows that the BaCl₂ treated muscle was more injured than the NaCl treated muscle. The permeability is what allows inflammation in the injured area, however inflammation may not be a bad thing as it allows cells, proteins, and regeneration factors to come to the injured muscle.

We compared endothelial permeability at 48 hours post skeletal muscle injury in male and female mice at 1 month, 2 months, 3, 6 and 12 months of age. We hypothesized that the female mice would show more permeability at 48 hours post injury than the male mice at all ages. Our findings were more complicated as permeability following injury trends down with age in females, and up with age in males. These findings could be beneficial for medical professionals to tailor an injured athlete's treatment to their age and sex since there are permeability and therefore possibly inflammation differences.

Despite the fact we see sex differences in permeability after injury, eliminating gonadal hormones did not change these findings. However, we did look at 12 weeks of age, a time point where we did not see significant differences between evan's blue extraction following injury between the sexes. The castration and ovariectomy procedures cannot be performed earlier and would be unlikely to make a difference as the mice would not have yet reached sexual maturity. However, we do plan on repeating these experiments at 1 year of age to see if sex hormones play a role in the permeability differences we see as mice age.

In conclusion, we found that in all sexes and ages BaCl_2 treated muscles were more permeable, therefore more injured, than the NaCl treated muscles. We also concluded that the inflammatory response becomes more robust as females age and less robust as males age in response to muscle injury. This data should be considered when treating muscle injury.

Prior to the COVID-19 pandemic we planned to further this research by examining the concentration of wls in injured and uninjured muscles. Wls is a messenger protein

that helps release wnts in macrophages from the golgi apparatus to the cell membrane. We used liquid nitrogen to freeze the muscles at 48 hours post injury that we planned to perform western blots with. We currently have an antibody in the lab for wls western blots, however we have not used it yet and cannot confirm that it will work properly. If western blots did not work, we had planned to use polymerase chain reactions (PCR) to measure the amount of wls signaling in each of the muscles. This methodology has been established in the lab.

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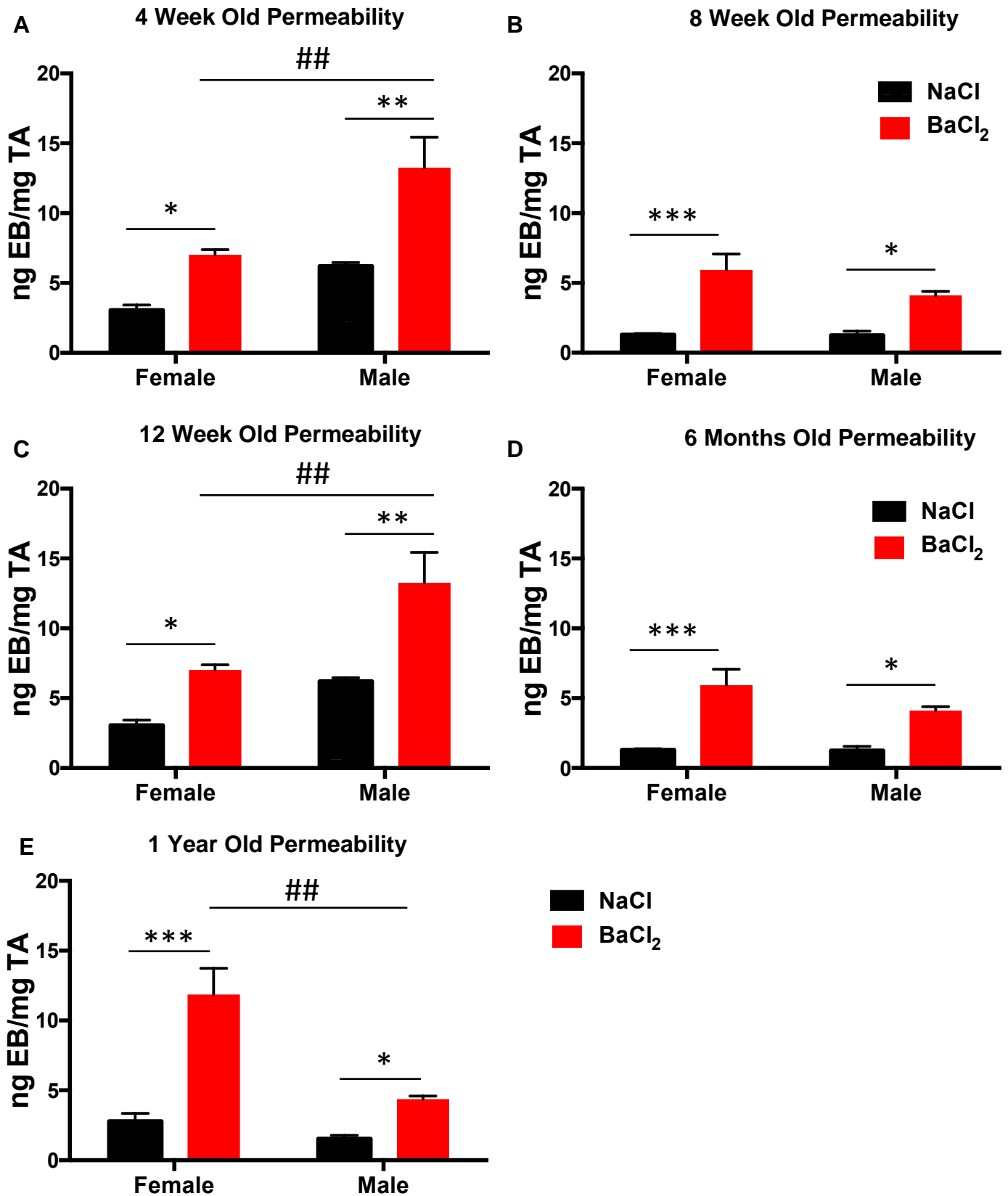


Figure 1. Absorbance of Evans Blue Compared to Weight of Tibialis Anterior. The black bars represent NaCl treated muscles and the red bars represent BaCl₂ treated muscles. (A) 4 week old comparison (B) 8 week old comparison (C) 12 week old comparison (D) 6 month old comparison (E) 1 year old comparison. *=p<0.05, **, ##=p<0.01, ***=p<0.001, ****=p<0.0001

Female Permeability Changes

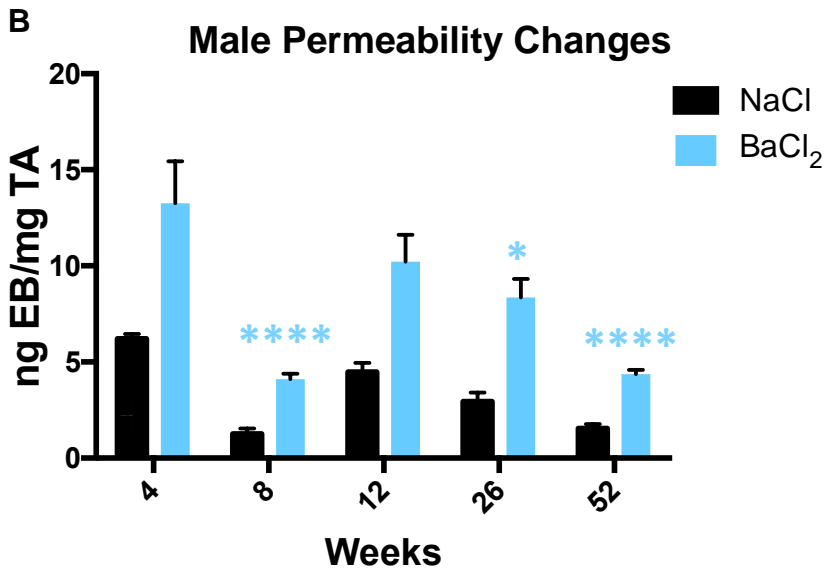
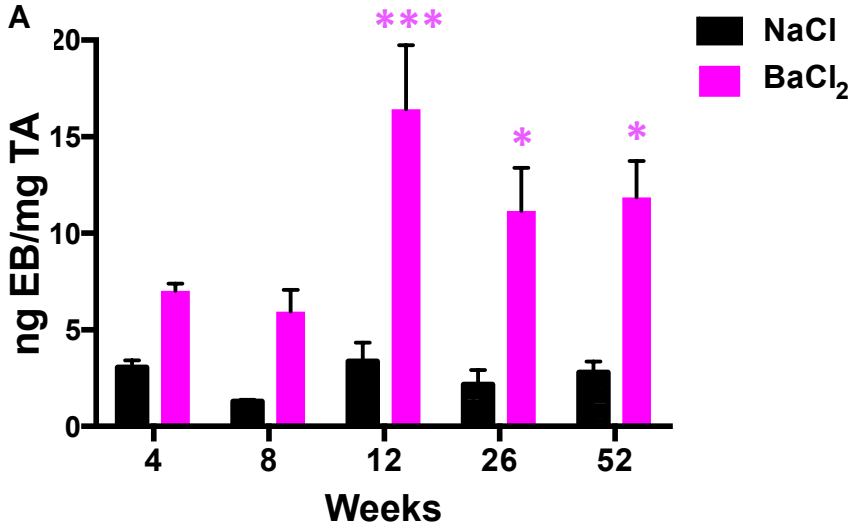


Figure 2. Absorbance of Evans Blue Compared to Weight of Tibialis Anterior with age. The black bars represent NaCl treated muscles and the pink/blue bars represent BaCl₂ treated muscles. (A) female comparison (B) male comparison *= $p < 0.05$, ***= $p < 0.001$, ****= $p < 0.0001$ vs. 4 week BaCl₂ injected TAs.

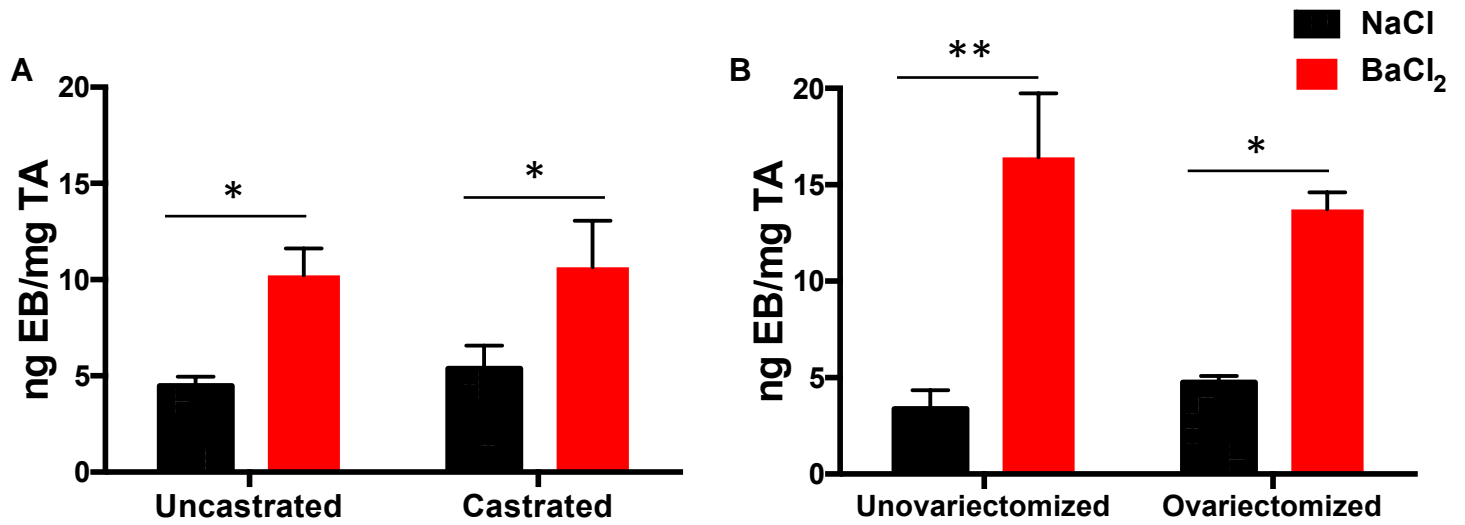


Figure 3. Absorbance of Evans Blue Compared to Weight of Tibialis Anterior in Castrated and Ovariectomized Mice. The black bars represent NaCl treated muscles and the red bars represent BaCl₂ treated muscles. (A) Permeability differences in uncastrated vs. castrated mice. (B) Permeability differences between ovariectomized and unovariectomized mice. *=p<0.05, **, =p<0.01