The effects of hyaluronic acid and exercise on equine skeletal muscle

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SCIENTIFIC ABSTRACT

Unaccustomed, strenuous exercise can cause skeletal muscle damage that subsequently induces an acute inflammatory response in the tissue which is marked by an infiltration of leukocytes into the damaged muscle. To try and suppress the initial pro-inflammatory response in skeletal muscle of horses performing a single exercise stress test, a commercial sodium hyaluronate (HA) treatment was administered and tested for anti-inflammatory properties. Unfit, adult Thoroughbreds were intravenously injected three times with HA or received no injection at all (CON) over a 3-week period before performing a single submaximal exercise test. Gluteal muscle biopsies were collected before and 1 h after the completion of exercise for RNA-Seq and staining. The results indicated that HA treatment in horses down regulated genes associated with lymphocyte activation and cytokine production (*Il17RA, OSCAR, LYL1, TLR1, TLR2, TLR8, TLR10*) but did not irreversibly down regulate these genes with the addition of exercise. Exercise as a stressor did cause an acute inflammatory response in muscle which was seen through global expression of macrophage and neutrophil surface markers (*NCF2, ELANE, CD168I)*. These results determine that HA treatment does act as an anti-inflammatory in equine skeletal muscle but does not possess prolonged effects with the initiation of inflammation.

GENERAL AUDIENCE ABSTRACT

Horses subjected to an unaccustomed increase in exercise intensity can experience damage and subsequent acute inflammation within the skeletal muscle tissue that may hinder the performance of the horse by causing muscle swelling and soreness. Hyaluronic acid treatment may suppress this exercise-induced inflammatory response by acting as an anti-inflammatory in the muscle. Adult Thoroughbred horses were injected intravenously with a commercial sodium hyaluronate treatment in the weeks prior to performing an exercise stress test. Muscle biopsy samples were obtained before and after the exercise stress test was performed. The results indicate that horses receiving the hyaluronic acid treatment had decreased expression of inflammatory genes within skeletal muscle, but no genes remained suppressed after the induction of inflammation through exercise. These results demonstrate that hyaluronic acid treatment does act as an anti-inflammatory in skeletal muscle tissue but does not have long-term suppressive effects when inflammation does occur.

DEDICATION

To my mom, dad, and papa for supporting my academic and horsey ambitions.

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CHAPTER 1

Literature Review

Introduction

Horses are considered one of the world's greatest athletes. With their 700 skeletal muscle groups that contain a greater proportion of type II fibers compared to other species and high aerobic capacity that allows them to increase oxygen uptake by a factor of 60, horses have often been utilized to run at high speeds for sport (Art and Lekeux, 2005). Developing optimal performance in the Thoroughbred relies on understanding and assessing training and conditioning that can alter genetic expressions and affect physical parameters. Exercise exerts stress that causes muscle fiber damage and results in metabolic and inflammatory changes in skeletal muscle (Markus et al., 2021). With inflammation negatively impacting the horse by generating pain and clinical lameness, anti-inflammatory drugs have become popular in veterinary medicine (Knych, 2017). Within this review the response to exercise in equine skeletal muscle and associated inflammation and countermeasures will be discussed.

Exercise in horses

Exercise's effect on body systems

Exercise involves repetitive skeletal muscle myofibril contractions that allow for fast and powerful movements across the body and results in the stimulation of specific body systems from a normal resting state. The easiest and quickest changes to the body seen in horses that are stimulated by exercise is an increase in the functionality of the cardiovascular and respiratory systems which can be characteristically identified through an increase in heart rate and respiratory minute volume by 4-fold and 47-fold resting values respectively during a single

session standardized exercise test (Weber et al., 1987; Butler et al., 1993). Specialized equine training programs lasting of 6-weeks or more optimize efficiencies of respiratory and cardiovascular systems through an increase in maximal oxygen consumption (VO_{2max}) and cardiac output (CO; Evans and Rose, 1988; Foreman et al., 1990). It has clearly been validated that fit horses present contrasting physical and metabolic measurements as a response to exercise compared to unfit horses, and for the purpose of this study an unfit horse is considered to be a horse that has had no training within at least a 2-week period from the cessation of previous work (Guy and Snow, 1977; Tyler et al., 1996; Tyler et al., 1998; Mukai et al., 2006).

Both submaximal exercise testing and running to fatigue have shown to achieve metabolic changes. More specifically, changes in muscle energy metabolism. During intense and prolonged exercise that limits oxygen availability to tissues, the muscle becomes reliant on an increased rate of adenosine triphosphate (ATP) synthetization to power continuous muscle contraction, and equine skeletal muscle utilize glycogen stores during anaerobic metabolism for this energy production (Waller and Lindinger, 2010). Both consecutive multi-day exercise (Lacombe et al., 1999) and single bout submaximal exercise testing (Davie et al., 1999) in Thoroughbreds and Standardbreds show depleted gluteal muscle glycogen in post-exercise biopsy samples compared to pre-exercise biopsy samples (Davie et al., 1999; Lacombe et al., 1999). A recent study also quantified the muscle glycogen of gluteus medius (GL) and semitendinosus (ST) skeletal muscle in horses through a noninvasive ultrasound imaging technique employed in human sports medicine and saw an overall decrease in glycogen muscle stores after steady-state treadmill exercise lasting from 2-20 min (Tabozzi et al., 2021). Collectively, this is indicative that strenuous exercise leading to an oxygen deficit in the muscle systems plays a role in increasing muscle glycogen metabolism in the horse.

In correlation with glycogen metabolism, the accumulation of lactate in the blood and muscle occurs during anaerobic respiration induced by strenuous exercise as it is a byproduct of glycolysis. The production of lactate is not exclusive to anaerobic metabolism and can occur both during an ATP and oxygen deficit (Rabinowitz and Enerbäck, 2020). The buildup of lactate in the muscle has been observed in horses with and without previous training when performing various exercise tests of moderate to high levels of intensity or 10-12 m/s (Harris et al., 1991; Evans et al., 1993; Davie and Evans, 2000). In a normal aerobic state, the process of glycolysis breaks down glucose into pyruvate which is used to produce ATP in the Krebs Cycle. However, when oxygen availability in a cell becomes limited and energy starts to be produced anaerobically, the pyruvate is converted to lactate by lactate dehydrogenase and transported out of the cell into the blood via monocarboxylate transporters. In relation to this, an increase in skeletal muscle lactate starting at 40 mmol/kg is typically coupled with a decrease in the availability of muscular ATP up to 47% (Harris et al., 1991).

The overall response to exercise by the body is dependent on factors such as age and current fitness level, but all changes are correlated with variations in genetic expression. Differentially expressed genes (DEGs) are genes that differ in read counts within a sample under two different conditions. Through various analysis methods, the response to exercise has been analyzed in the Thoroughbred (McGivney et al., 2010; Park et al., 2012; Kim et al., 2013; Jang et al., 2017; Lee et al., 2019; Klein et al., 2020). Both 30 min trot sets and exercise to exhaustion in which the horse is no longer able to maintain position on the treadmill causes up regulation of *IL6* and *IL8* in equine gluteal and triceps brachii muscle biopsies taken 1-3 hours post exercise (Kim et al., 2013; Lee et al., 2019; Johnson et al., 2023). These studies often examine conditioned horses that have been in a several week training program while the gene response

after exercise in unfit horses has been less described making the analysis of gene expression in unfit horses after exercise significantly relevant to the literature.

The skeletal muscle response to training involves adaptations that alter the structure and chemistry of the tissue to better withstand the physical stressors that exercise inflicts on the body (Kim et al., 2005). Some adaptive responses to exercise training in sport horse muscle involve alterations in fiber types, an increase in mitochondrial volume, and an increase in oxidative capacity (Tyler et al., 1998; Lindner et al., 2013; Latham et al., 2021). When the equine muscle endures contraction rates associated with exercise of increasing intensity an unaccustomed need for power and speed encourages an increase in type IIa myofibers which have a greater oxidative capacity and mitochondrial density than type IIb (Kim et al., 2005; Lindner et al., 2013; Latham et al., 2021). As for increases in mitochondrial volume in equine skeletal muscle tissue in response to training, it has been observed to be directly correlated with increases in oxidative capacity of the tissue and longer running times throughout the duration of the training program (Tyler et al., 1998; Latham et al., 2021). In avoidance of continual harmful stressors on the musculoskeletal system these training adaptations in the horse can be considered countermeasures to muscle damage.

Exercise causes muscle damage

Muscle damage is defined as a disruption in the fascicles of muscle tissue in which repair is necessary, and skeletal muscle injury can range from a grade 1 sprain to a complete tear that requires full muscle regeneration (Laumonier and Menetrey, 2016). Specifically, within the sarcomere, the basic contractile unit of muscle fibers, an increase in either load or rate of muscle contraction can cause overstretching of the sarcomere which results in ultrastructural damage (Proske and Morgan, 2001). This mechanical damage that can come from repeated contractions

and unaccustomed loading are a loss of contractile ability and muscle soreness which has been correlated to actual damage of the muscular structure and not a product of exercise fatigue which was first proven in humans (Hough, 1900). In horses, ultrastructural damage of the myofiber membrane after exercise has been identified through scintigraphy where abnormal distribution and uptake of the radioisotope is observed in and around the muscle of sport horses who were experiencing subtle discomfort after a high-speed treadmill lameness evaluation (Hornof and Koblik, 1991; Morris et al., 1991).

Proposed mechanisms contributing to exercise-induced muscle damage have been studied in the rat and mouse. Rats forced to run repetitive sets on a downhill treadmill experienced disorganization of myofilaments, disruptions in Z-line streaming, and degradation of the basement membrane characterized by a loss and transcriptional suppression of collagen IV (Armstrong et al., 1983; Koskinen et al., 2001; Kanazawa et al., 2021; Kanazawa et al., 2023). Comparable observations in Z-line streaming have been noted in the mouse along with an exorbitant influx of Ca^{2+} into the intracellular space when mouse skeletal muscle fibers were stretched manually using a force transducer (Balnave and Allen, 1995; Balnave et al., 1997; Zhang et al., 2012). It is predicted that this increase in resting Ca^{2+} after exercise is because of exercise-induced damage to the sarcolemma membrane or failure of the Ca^{2+} ATPase pump in reabsorbing Ca^{2+} (Ebbeling and Clarkson, 1989; Balnave and Allen, 1995). These observations have not been examined in the horse, but it can be speculated that muscle fiber destruction because of exercise is associated with structural deformities of the membrane and sarcomere components.

Blood plasma molecular markers can be measured to determine skeletal muscle damage such as serum creatine kinase (CK) and aspartate aminotransferase (AST). Both enzymes are

detectable within the sarcoplasm of muscle cells, and serum muscle enzyme levels are significantly elevated after unaccustomed training that stresses muscle tissue in racehorses due to disruption in sarcolemma diffusion (Mack et al., 2014). However, CK and AST are not muscle or species specific, most notably with increased serum AST being linked to liver damage in humans (Panteghini, 1990). Nonetheless, studies have validated these two measurements as being indicators of exercise-induced muscle damage in Thoroughbred horses with experimental protocols that normalize data to age, sex, fitness level, intensity of exercise, and training (Harris et al., 1990; Harris et al., 1998).

The measuring of serum CK and AST levels as a way to detect muscle membrane destruction in equines was largely in part to the clinical study of exertional rhabdomyolysis in exercising horses (Valberg et al., 1993; MacLeay et al., 1999; McKenzie et al., 2004). Exertional rhabdomyolysis is a muscular disorder that lowers the threshold for muscle and results in the secretion of cellular contents into the bloodstream after a bout of strenuous exercise (Scalco et al., 2016). While this severe myopathy is an extreme case of exercise-induced muscle damage, it gives insight to what can be observed and quantified when muscle damage does occur. Interestingly, the expression of the *CK-M* gene is the most highly expressed gene in equine skeletal muscle in association with muscle contraction and energy metabolism, and skeletal muscle tissue has the highest expression rate of *CK-M* out of all other body tissues in the horse (McGivney et al., 2010; Do et al., 2015).

Exercise-induced stress factors such as oxidative, heat, hypoxic, and hormone stressors acting on muscle groups can also cause subsequent muscle fiber damage in the horse (Hodgson et al., 1994; Okabe et al., 2017; Kim et al., 2021). Damage is a necessary part of exercise to allow for the adaptation of skeletal muscle for further and amplified stimulus, and severity of

damage and rate of recovery post-exercise is based on characteristics such as sex, age, fitness level, and genetics of the individual (Markus et al., 2021). Oxidative stress in particular can cause DNA damage as well as damage to myofiber membranes through the accumulation of reactive oxygen species which are oxygen containing molecules that are released with repeated muscle contractions that stimulate nitric oxidase synthesis and *NADPH oxidase* expression (Valko et al., 2007; Steinbacher and Eckl, 2015). However, antioxidant supplements such as dietary selenium have been known to reduce oxidative activity in equine skeletal muscle by balancing the oxidative to antioxidative ratio and mitigating lipid peroxidation (White et al., 2016; Williams, 2016; White and Warren, 2017).

Damage induces inflammation

One cause of inflammation in muscle is exercise-induced muscle damage, and the inflammatory response related to exercise is different from a trauma-induced systemic inflammatory response which increases the body's susceptibility to infection which is observed across species (Fehrenbach and Schneider, 2006). Inflammation as a result of exercise is an acute response meaning it remains localized to the affected tissue and resolves quickly. Out of the metabolic stressors previously discussed, oxidative stress from anaerobic metabolism is known to be one of the leading causes of muscle damage and inflammation within the horse after exercise (Lee et al., 2019; Kim et al., 2021). The addition of H_2O_2 in skeletal muscle cell cultures to stimulate oxidative stress has resulted in comparable expression levels for *IL6, IL8, CXCL6, ADAMTS4, HSPA6,* and *SELE* as seen in muscle tissue biopsies undergoing exercise-induced inflammation (Kim et al., 2021).

Muscle inflammation is characterized by a cohort of innate immune cells at the site of muscle damage. Leukocytes involved in inflammation are derived from bone marrow and travel

systemically to the affected muscle tissue where they act as inflammatory mediators while other cell types that are native to the muscle such as resident macrophages secrete additional inflammatory chemokines (Tidball, 1995). Leukocytes are responsible for the soreness and occasional swelling experienced in the skeletal muscle the day after exercise and can be used to measure the level of systemic inflammation after a bout of exercise. Single treadmill exercise tests to fatigue have resulted in pro-inflammatory cytokine mRNA levels of *TNF-α* and *IL1β* detectable and elevated up to 120-fold in the blood immediately after the cessation of exercise in fit and unfit Thoroughbred and Standardbred horses (Donovan et al., 2007; Liburt et al., 2010). Direct mRNA measurements of gluteal muscle tissue in Standardbred horses after exercise-induced fatigue showed a paralleled increase in expression of *TNF-α* within the muscle itself along with a novel 10-fold increase in *IL6* which was not evident in blood (Liburt et al., 2010).

Complete muscle repair cannot occur until the damaged area has gone through a cycle of localized inflammation that stimulates myogenesis. The act of inflammation and infiltration of immune cells into the muscle stem cell niche promotes satellite cell proliferation; however, inflammatory macrophages, a molecular mediator upregulated during the inflammatory response after exercise, have been described to inhibit the progression of the satellite cell life cycle after muscle injury (Saclier et al., 2013; Fu et al., 2015). Satellite cells are the drivers of muscle repair and regeneration, and when activated from a quiescent state, ultimately fuse to existing muscle fibers or fuse with each other to make new fibers depending on the extent of damage. A mouse study examined the effects of inflammatory macrophage depletion in injured muscle through inhibiting the binding of macrophage colony-stimulating factor (M-CSF) via the use of an injected antibody (AFS98) and found it to hinder satellite cell proliferation and differentiation

which delays myofiber repair (Segawa et al., 2008). Proliferation is the expansion of the satellite cell population after activation, and differentiation is when the cells specify to specific roles where in this case satellite cells differentiate into myocytes. An *in vitro* satellite cell study conducted co-culture experiments with human satellite cells seeded with inflammatory macrophages and saw an initiation of proliferation but an inhibition of differentiation of the satellite cells (Arnold et al., 2007).

During the time course of inflammation after exercise-induced injury in unfit horses who were ran to exhaustion, satellite cell numbers significantly increased in fibers at 1 and 2 weeks after running along with increased expression of cytokine *IL-6* mRNA 3 h post exercise in the gluteal muscle (Kawai et al., 2013). Similar results were witnessed in the rat where after acute resistance training, *IL-6* expression and satellite cell proliferation increased in an associated manner (Begue et al., 2013). Mice who had IL-6 deficiencies and mice who were ablated of IL-6 experienced decreased satellite cell proliferation and subsequently a lack of muscle hypertrophy during repair from muscle overload (Serrano et al., 2008; Washington et al., 2011). This is indicative that IL-6 contributes to myogenesis and muscle hypertrophy when stimulated by muscle exertion.

Evidence also exists to support the notion that exercise training can reduce the inflammatory response in fit individuals. Firstly, exercise training increases overall satellite cell abundance and mitochondrial function which decreases the muscle's susceptibility to damage which reduces the need for inflammation (Latham et al., 2021). At rest, racing Thoroughbreds trained daily had a greater baseline expression of cytokines such as *IL-6*, *IL-8*, and *IL-1β* in the blood suggesting training causes genetic adaptation to allow for a quicker inflammatory response to reduce recovery time (Cappelli et al., 2013). In humans, frequent strenuous exercise has been

suggested to act as an anti-inflammatory through the reduction of peripheral blood inflammatory TH1 cells, which produce pro-inflammatory IFN-y, and increase of anti-inflammatory cytokine IL-4 from TH2 cells (Steensberg et al., 2001; Shaw et al., 2018).

Inflammation in muscle

Timeline of inflammation

Most often studied in humans and mice, the immediate inflammatory response to skeletal muscle injury is increased blood flow to the affected area. Neutrophils infiltrate the basal lamina from the blood 1-3 h after injury (Oishi and Manabe, 2018). Resident macrophages also secrete chemokines at this time. This is the initiation of the pro-inflammatory phase. In humans, neutrophil numbers will peak 12-24 h post injury and recruit monocytes, a process common across species, that differentiate into pro-inflammatory M1 macrophages 24-48 h post injury (Arnold et al., 2007). Macrophage numbers will increase for 48 h after injury and participate in efferocytosis to clean up apoptotic tissue debris which can be detected through DNA fragmentation (Quadrilatero et al., 2011). The act of efferocytosis causes a change of the macrophage phenotype from a pro-inflammatory M1 to anti-inflammatory M2 phenotype at day 2-4 post injury in people (St. Pierre and Tidball, 1994; Arnold et al., 2007). These M2 macrophages produce anti-inflammatory cytokines such as IL-10. This marks the beginning of the restorative phase.

Inflammation is required for skeletal muscle repair as inflammatory molecules clear apoptotic tissue and release regenerative signals that alter the stem cell niche. Once the restorative phase is initiated 2-4 days after injury coinciding with the introduction of anti-inflammatory mediators in humans, muscle repair through myogenesis can start (Oishi and

Manabe, 2018). New tissue formation begins 2-10 days after injury and maturation starts 2-3 weeks post injury with complete muscle regeneration after 1 month in humans (Baghdadi and Tajbakhsh, 2018; Oishi and Manabe, 2018). However, factors such as scar tissue and a disruption in the inflammatory process can prolong the repair period. A similar time course of inflammatory markers is expected in the horse (Figure 1).

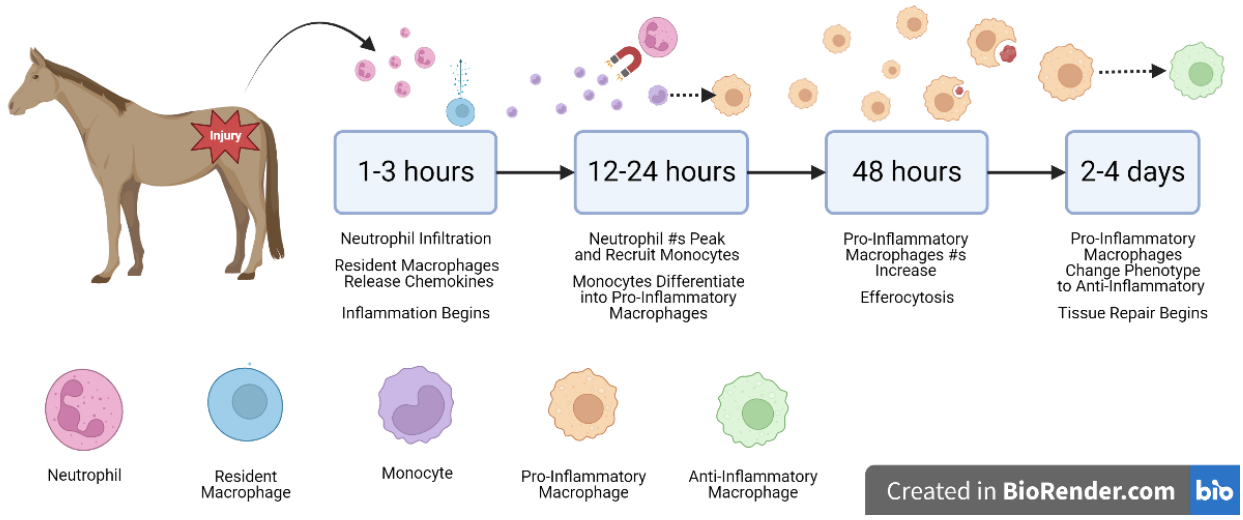

Figure 1 Timeline of muscle inflammation

Neutrophils are the first leukocytes to appear at the site of injury and initiate inflammation. Neutrophils recruit monocytes which differentiate into pro-inflammatory macrophages that participate in efferocytosis to clean up tissue debris. Tissue repair begins with the act of efferocytosis as the pro-inflammatory macrophages become anti-inflammatory.

Mechanical damage of the human muscle fiber is detectable immediately post exercise by examining the disruption in the banding patterns of myofibrils (Peake et al., 2017). Disruptions in Z-line streaming within the sarcomeres of human skeletal muscle tissue peak around 1-3 days post intense eccentric exercise, and generally don't start returning to a normal pattern until 6-8

days post exercise (Friden et al., 1983; Yu et al., 2004; Peake et al., 2017). The peak in Z-line streaming disruption occurs in a corresponding time frame with the infiltration of inflammatory leukocytes and differentiation of monocytes into macrophages. This is suggestive that these cell types participate in a signaling cascade that may exacerbate damage once exercise is over.

Pro-inflammatory to anti-inflammatory transition

The most prevalent mediators in the pro-inflammatory to anti-inflammatory transition are macrophages. The Ly6CHi (M1) and Ly6CLo (M2) macrophages have roles in facilitating different physiological responses, and M2 macrophages are phenotypic variants of M1 macrophages. M1 macrophages derived from bone marrow procured monocytes through differentiation are responsible for the efferocytosis of necrotic tissue debris that accumulated from damaged myofibers and secrete inflammatory molecules such as TNF-α, IL-β, and TGF-β which can exacerbate damage (Li et al., 2022). The process of efferocytosis, as well as IL-10 stimulation, contributes to the phenotypic switch of M1 to M2 macrophages (Deng et al., 2012; Ip et al., 2017; Li et al., 2022).

Hyaluronic acid may be another contributor to the phenotypic change of macrophages from M1 to M2 during inflammation. This was observed in the synovium of human osteoarthritic patients injected with high molecular weight hyaluronic acid (HMW-HA) and through the isolation and culturing of synovium originated macrophages with and without HMW-HA (Lee et al., 2021). The presence of HA in macrophage cell cultures showed a decrease in the M1 macrophage phenotype and an increase in the M2 macrophage phenotype through inhibiting the expression of *GRP78* which is a molecule involved in protein folding (Lee et al., 2021). In other parts of the body, CD44 molecules, the primary receptor for HA, have been detected in high quantities on the cell surface of alveolar macrophages. On these macrophages HA has been

documented to assist the IL-4 cytokine in reaching murine M1 macrophages through its high affinity to the CD44 receptors (Shahbazi et al., 2018). IL-4 and IL-13 are two inflammatory molecules that have been known to trigger M2 macrophage polarization. A potential reasoning behind HA's anti-inflammatory capabilities could be in its ability to assist in M1 macrophage polarization.

Molecular mediators of inflammation

The induction of inflammation from subsequent exercise-induced muscle damage involves the accumulation of cytokines that play different roles in mediating the inflammatory response with specific molecules being associated with pro-inflammation and others carrying out anti-inflammatory tasks. As skeletal muscle has been identified as an endocrine organ, cytokines specifically released from muscle cells during contraction are called myokines (Pedersen et al., 2007). Other cytokines are secreted from neutrophils, monocytes, and monocyte derived macrophages that originate from bone marrow and reach the skeletal muscle tissue via the circulatory system and extravasate from blood across the endothelium.

Interleukin-8 (IL-8) is a CXC chemokine also referred to as CXCL8. The chemokine is a protein that promotes cell migration through chemotaxis of white blood cells such as neutrophils during an immune response and has been linked to angiogenesis in humans (Pedersen et al., 2007; Cui et al., 2017). IL-1 and TNF-α are pro-inflammatory cytokines that promote the production of IL-8 by monocytes and macrophages during an injury or cardiotoxin injection that causes inflammation to promote recovery (Long et al., 2016; Kumar et al., 2022). When left unchecked, elevated levels of IL-8 can lead to formation of inflammatory diseases such as rheumatoid arthritis, atherosclerosis, and diabetic nephropathy which has been investigated in people and rabbits (Podolin et al., 2002; Qin et al., 2013; Cui et al., 2017). Across several

species, after a bout of exhaustive exercise IL-8 is expressed in skeletal muscle supporting the belief that IL-8 is also a myokine (Pedersen et al., 2007; Lee et al., 2019). In Thoroughbred horses, qPCR determined that there was a 75-fold increase in IL-8 mRNA expression in skeletal muscle immediately following 30 min of trot exercise on a treadmill (Lee et al., 2019). Two receptors for IL-8, CXCR1 and CXCR2, are G protein-coupled receptors that also antagonistically bind to G31P which initiates an anti-inflammatory signaling cascade that inhibits neutrophil migration and lessens the inflammatory side effects of diseases such as diabetes, obesity, and ulcerative colitis in mice (Qin et al., 2013; Cui et al., 2017; Walana et al., 2018).

Another myokine, Interleukin-6 (IL-6), is a cytokine that has both pro-inflammatory and anti-inflammatory properties based on the origin of secretion. IL-6 was one of the first cytokines to be classified as a myokine (Pedersen and Febbraio, 2008). Muscle-derived IL-6 is secreted during muscle contraction and becomes systemic to promote immune and metabolic responses (Pedersen et al., 2001). As an anti-inflammatory mediator in humans, the increased expression of *IL-6* from skeletal muscle during exercise acts as a suppressor of *TNF-α*, and repetitive short bouts of exercise leads to a greater increase in plasma IL-6 than prolonged or single bouts of exercise in humans (Ronsen et al., 2002; Steensberg et al., 2002; Starkie et al., 2003; Hennigar et al., 2017). The differences in the increase in IL-6 production from contracting muscle in humans is based on the duration and repetitiveness of exercise and may be related to muscle glycogen concentrations (Keller et al., 2001; Steensberg et al., 2001; Ronsen et al., 2002). Single bouts of exercise deplete muscle glycogen content, and low pre-exercise glycogen levels promote IL-6 production in a pro-inflammatory manner (Steensberg et al., 2001; Pedersen et al., 2007).

Interleukin-1 beta (IL-1β) is a pro-inflammatory cytokine that is upregulated during an initial inflammatory response and is typically categorized as an immune defense to injury or infection (Chaweewannakorn et al., 2018). Macrophages and neutrophils are innate immune cells that release IL-1β as well as enlist other mediators when signaled to by inflammatory stimuli in the surrounding damaged tissue (Duque and Descoteaux, 2014). IL-1β temporarily augments damage during acute skeletal muscle injury due to overexpression in the tissue which can lead to chronic inflammation and result in loss of muscle function (Lopez-Castejon and Brough, 2011). In order for IL-1β to be produced and activated, inflammasomes must activate Caspase 1 which converts pro-IL-1β to active IL-1β. Interleukin-1β signaling occurs through interleukin-1 type 1 receptor (IL-1R1) which activates the interleukin-1 receptor accessory protein (IL-1RAcP) that is required to assist in intracellular signaling by forming a receptor complex at the cell membrane (Agostini et al., 2004; Ren and Torres, 2009; Fields et al., 2019). A second IL-1β receptor exists, interleuking-1 type 1 receptor (IL-1R2), but does not activate a signaling pathway as it is functionally inactive (Ren and Torres, 2009). This signaling transduction can be inhibited by the attachment of interleukin-1 receptor antagonist (IL-1Ra) to IL-1R (Dinarello, 1991).

Interleukin-10 (IL-10) is an anti-inflammatory cytokine that is up regulated after strenuous exercise and functions to suppress pro-inflammatory factors. The interleukin-10 receptor (IL-10R) is present on the cell surface of monocytes and macrophages, and when IL-10 binds to its receptor the Jak-STAT signaling pathway is activated (Ouyang et al., 2011). This causes the phosphorylation of the downstream transcription factor and signal transducer, STAT3. The expression of pro-inflammatory genes present in monocytes and macrophages are then inhibited (Ouyang et al., 2011; Islam et al., 2021). Ablation of *STAT3* in mice suffering from endotoxin shock exhibit increased inflammation when compared to WT $STAT3^{+/-}$ mice because

pro-inflammatory monocytes and macrophages are no longer receptive to IL-10 (Takeda et al., 1999). In mice with age-associated and obesity-mediated insulin resistance, transgenic overexpression of *IL-10* results in improved skeletal muscle insulin sensitivity (Hong et al., 2009; Dagdeviren et al., 2016; Dagdeviren et al., 2017).

Nuclear factor-kappa B (NF-kB) is a ubiquitous transcription factor that is up-regulated by TNF-α. Upon exercise that causes subsequent muscle injury, the initiation of an exercise-induced inflammatory response has been reported to stimulate the systemic and localized muscle expression of TNF-α in equines (Van Den Boom et al., 2004; Donovan et al., 2007; Liburt et al., 2010; Hale et al., 2023). In fit Thoroughbred and Standardbred horses performing a single bout of incremental treadmill exercise to fatigue, serum TNF-α values were significantly elevated from baseline at 0-1 h after completion of exercise (Donovan et al., 2007; Hale et al., 2023). In unfit Standardbreds, a similar time response of TNF-α mRNA expression was measured in skeletal muscle, but contrary to fit horses, blood serum values did not peak until 6 h after completion of exercise (Liburt et al., 2010). The upregulation of *TNF-α* as part of the initial inflammatory response contributes to muscle atrophy by activating NF-kB which represses the transcription of *myoblast determination protein* (*MyoD*; Shirakawa et al., 2022). MyoD is a myogenic regulatory factor found within skeletal muscle that is responsible for the differentiation of satellite cells following muscle injury to allow for repair and regeneration of myofibers (Yamamoto et al., 2018). It has been validated using C2C12 cell cultures that in an inflammatory state representative of the introduction of TNF-α into the media, NF-kB increases expression and inversely suppresses myogenic differentiation (Langen et al., 2001).

Transforming growth factor beta (TGF-β) is a superfamily of cytokines consisting of three different isoforms and respond to two functionally active receptors for signaling, TGFβR1

and TGFβR2 (Gumucio et al., 2015; Ismaeel et al., 2019). During exercise, TGF-β functions to promote fibrosis through extracellular matrix remodeling through pro-inflammatory signaling that can inhibit muscle regeneration (Kim and Lee, 2017). TGF-β is not exclusive to the muscle tissue and has been identified as an exercise-induced adipokine in mice as well (Takahashi et al., 2019). Interestingly, the SMAD signaling pathway of TGF-β1 contributed to the synthesis of HA in hGL cells through increasing the expression of *has2*, the protein responsible for HA synthesis, and blocking the binding of HA to its receptor, CD44, inhibited TGF-β receptor trafficking (Ito et al., 2004; Wang et al., 2019). The suppression of TGF-β has resulted in both improved and diminished muscle repair after injury. Inhibition of TGF-β1 through the blocker protein, suramin, impaired the onset of fibrosis through the reduction of muscle injury in mdx mice which was validated through a decrease in creatine kinase in the skeletal muscle (Taniguti et al., 2011). Contrastingly, suppression of TGF-β in mice during muscle lengthening exercise resulted in impaired muscle force production 21 d after injury (Gumucio et al., 2015).

Hyaluronic acid as an anti-inflammatory

Hyaluronic acid

Hyaluronic acid (HA), alternatively referred to as hyaluronan or hyaluronate, is a glycosaminoglycan non-sulfated polysaccharide that is composed of N-acetylglucosamine and glucuronic acid. Discovered in 1934 by Karl Meyer and John Palmer while investigating the vitreous humor of cattle eyes, HA was first speculated to contribute to disease formations such as glaucoma (Meyer and Palmer, 1934). Now, HA has been determined to be an important structural component of the extracellular matrix (ECM) of connective tissue in vertebrates, and the study of this substance has evolved to focus on its role in tissue repair and inflammatory responses (Marinho et al., 2021).

A high concentration of HA can be found within the synovial cavity of joints where it is synthesized by synoviocytes and chondrocytes and functions to reduce friction during movement by lubricating the articular cartilage on the ends of bones (Gupta et al., 2019). A decrease in the molecular weight of HA within the synovial fluid of articular cartilage has largely been linked to degenerative joint diseases in horses (Caron, 2005). In the ECM of other tissues, HA has been documented to increase cell proliferation, differentiation, adhesion, and cellular migration (Solis et al., 2012; Dicker et al., 2014; Nevi et al., 2019). Recent discoveries have pointed towards the necessity of HA within the ECM of skeletal muscle tissue to help mitigate inflammation and allow repair signals to initiate muscle recovery after injury in mice (Nakka et al., 2022).

The transcriptional regulation of HA is maintained through the *JMJD3* gene acting on the has2 enzyme through histone demethylation (Nakka et al., 2022). Hyaluronic acid synthase, has2, is the inner plasma membrane protein that produces hyaluronic acid, and hyaluronidase is the depolymerizing enzyme that degrades hyaluronic acid (Kobayashi et al., 2020). Specific cell surface receptors affiliated with HA are CD44 and RHAMM. CD44 is a cell-surface glycoprotein and HA contains at least 3 sites that bind to CD44 through low-affinity hydrogen bonds (Yang et al., 1994; Liao et al., 1995). The receptor for hyaluronan-mediated motility (RHAMM) is another HA receptor. In a study using CD44 knockout mice, it was determined that the RHAMM receptor can perform the same functions as CD44 (Nedvetzki et al., 2004). However, CD44 is the dominant receptor and RHAMM typically only becomes significant when there is an accumulation of HA within the ECM due to a loss of CD44.

Use of hyaluronic acid in horses and people

Recombinant HA is a popular supplement marketed in recovery and regenerative medicine. For example, to combat a loss in concentration of molecular HA within equine joints,

therapeutic doses of HA have been administered through both intravenous and intra-articular injections (Rydell et al., 1970; Popot et al., 2004; Johnston et al., 2020). Administrative exogenous HA is typically marketed as sodium hyaluronate which is the sodium salt version of hyaluronic acid. Sodium hyaluronate has a smaller molecular size than hyaluronic acid giving it a higher stability and ability to penetrate the skin deeper, but they are relatively interchangeable.

Following completion of a flat race in Standardbred and Thoroughbred horses, the concentration of HA within the plasma of these fit horses is 89 ng/mL with the endogenous production rate per a day being 33-164 mg per horse (Popot et al., 2004). Intravenous injections of HA increase serum values for 3 h before returning to baseline with the half-life lasting 43 ± 29 mins in horses, and intra-articular joint injections returning to baseline within the synovial fluid at 24 h post injection. The injection of HA into joints serves to treat degenerative joint diseases such as osteoarthritis in horses and people. In horses, degenerative joint diseases are associated with synovial inflammation that results in reduction of HA concentration and synovial fluid viscosity and is accompanied by clinical signs of inflammation of the joint such as visible lameness and joint effusion (Watkins et al., 2021).

Intra-articular injections of 20 mg sodium hyaluronate resulted in improved lameness of over 75% of treated racing Thoroughbreds (Åsheim and Lindblad, 1976). The anti-inflammatory properties of HA in treatment of human arthritis have indicated a significant suppression of prostaglandin E2 (PGE2) *in vitro* with cultured human synovial cells (Yasui et al., 1992). Human U937 macrophages cultured with lipopolysaccharide (LPS) also exhibited production of TNF-α, IL-1β, and IL-6 that was inhibited by the addition of HA (Yasuda, 2007). However, the effects of HA have been determined to be dosage- and molecular weight-dependent. There are two classifications of HA in regard to molecular weight, low molecular weight (LMW) and high

molecular weight (HMW) -HA. In horses, LMW-HA had a greater effect on inhibiting the release of cytokines during LPS-induced synovitis as well as slowed the further breakdown of endogenous synovial fluid HA (Neuenschwander et al., 2019) while HMW-HA suppressed inflammation in human patients with diagnosed rheumatoid arthritis (Goto et al., 1993). Intra-articular injections of cortisone is alternatively another common way to treat osteoarthritis, but HA in combination with cortisone has shown greater improvement in joint inflammation than cortisone alone in horses suffering from arthritis (Rydell et al., 1970).

Hyaluronic acid is popularly used in tissue engineering to aid in wound healing through rejuvenation and regeneration of the dermal layers in humans and other animals (Price et al., 2005; Neuman et al., 2015). HA has been utilized in wound scaffolds consisting of collagen and epidermal growth factor (EGF) to make wound dressing that promotes epithelial regeneration (Kondo and Kuroyanagi, 2012). HA and collagen sponge matrix without the addition of EGF have been observed to help promote granulation tissue formation of epidermal wounds (Kubo and Kuroyanagi, 2003; Kondo and Kuroyanagi, 2012). However, HA combined with EGF has consistently elevated healing rates over a 20-day period than wound scaffolds not containing HA and EGF (Su et al., 2014).

Implications of suppressing inflammation

Suppressing inflammation has a range of benefits for human and equine athletes alike as the use of non-steroidal anti-inflammatory drugs (NSAIDs) can mitigate musculoskeletal pain (MacHado et al., 2021). NSAIDs are over the counter or prescribed drugs that can be administered topically, orally, or through injection in a dose-dependent manner. Most NSAIDs act as cyclooxygenase (COX) inhibitors to suppress the production of prostaglandins (PG) by

COX-1 and COX-2 isoenzymes (Vane and Botting, 1998). COX-2 is the isoenzyme that is stimulated during pro-inflammation and contributes to pain and swelling through PG production; contrastingly, COX-1 served to support normal gastric and kidney function (Simon, 1999). While inhibiting the inflammatory effects of COX-2 through NSAID treatment has been proven beneficial in improving the short-term well-being of the horse (Orsini et al., 2012), adverse effects have been reported across various species through *in vivo* and *in vitro* experimentation (MacKay et al., 1983; Tanaka et al., 2002).

Early COX inhibitors worked to suppress both isoenzymes which led to adverse effects of gastrointestinal upset and renal dysfunction when trying to extinguish COX-2 derived inflammatory discomfort in rats (Tanaka et al., 2002; Takeuchi et al., 2003; Barkin and Buvanendran, 2004). This was because beneficial PGs from COX-1 were also being suppressed. In horses, a frequent NSAID and classified COX inhibitor used by owners and trainers is phenylbutazone. Horses dosed intravenously with a large concentration (>8 mg/kg) of phenylbutazone measured significant loss of blood neutrophil concentration, and fatal toxicity associated with administration of >15 mg/kg of phenylbutazone caused gastrointestinal ulcerations and renal necrosis (MacKay et al., 1983). Unexpected consequences in suppressing inflammation such as this led to drug development aimed at creating selective COX-2 inhibitors (Meade et al., 1993; Gierse et al., 1996). This gives insight to some of the unexpected implications that suppressing inflammation may have.

Ibuprofen and Aspirin, common NSAIDs and COX inhibitors used by humans, have also reportedly delayed muscle repair after injury (Cheung and Tidball, 2003; Dearth et al., 2016). Ibuprofen reduces muscle damage through a decreased concentration of necrotic tissue when given starting at 8 h before exercise-induced muscle damage in rats (Cheung and Tidball, 2003).

Aspirin suppressed myogenesis and ECM remodeling as well as reduced the presence of the M2 macrophage phenotype within injured rat skeletal muscle (Dearth et al., 2016). Delayed muscle repair through suppressed inflammation is correlated with decreased numbers of satellite cells in human athletes (Mackey et al., 2007; Mikkelsen et al., 2009). Men performing eccentric leg contraction with continuous NSAID infusion for 7.5 h had suppressed exercise-induced increased numbers of satellite cells 8 days after exercise (Mikkelsen et al., 2009). In a similar manner, male athletes receiving 100 mg of indomethacin daily starting at 4 days prior to exercise-induced muscle injury through 8 days after exercise had fewer satellite cells in the injured tissue (Mackey et al., 2007).

Corticosteroids are another classification of drug that can work as anti-inflammatories (Barnes, 2006). Rats suffering from muscle contusion injury and administered a corticosteroid had improved function and repair 2 days after injury compared to control but began to suffer from muscle weakness at day 7 after injury and continued to regress until day 14 with visibly noticeable myofiber disruption (Beiner et al., 1999a). Porcine models administered dexamethasone, a corticosteroid, and subsequently recovering from bupivacaine hydrochloride-induced muscle injury suffered from a prolonged period of necrosis in the skeletal muscle tissue due to a decrease in M1 macrophages that further delayed myogenesis (Otrocka-Domagała et al., 2019). A similar study in rats given dexamethasone injections immediately after a single eccentric contraction that caused muscle damage resulted in reduced inflammatory factors, IL-1β and TGF-β1, and did not experience reduction in repair and recovery during the 3 weeks of further monitoring (Hakim et al., 2005). Analyzing these two studies and comparing the outcomes leads to speculation of the time sensitive effects of

administering anti-inflammatories for inflammation of the skeletal muscle and the insinuation it can have on inhibiting timely and proper muscle repair.

When the actual leukocytes of inflammation are diminished during an acute immune response to the muscle, further delay in muscle repair is observed in mice (Summan et al., 2006; Arnold et al., 2007; Segawa et al., 2008). When monocytes are depleted in muscle tissue of mice through liposomal clodronate injections that stimulate monocyte apoptosis, there is a delay in removal of necrotic myofibers (Arnold et al., 2007). Likewise, when monocytes are diminished in mouse skeletal muscle through a diphtheria toxin injection, a delay in removal of necrotic myofibers and an accumulation of adipocytes in the muscle tissue is also observed (Arnold et al., 2007). When macrophages are targeted through an injectable antibody that acts to suppress function, an increased occurrence of skeletal muscle fibrosis occurs in mice (Segawa et al., 2008). Transforming growth factor beta is the protein responsible for fibrosis by signaling for ECM remodeling through altered collagen deposition. Inhibiting TGF-β during a pro-inflammatory response after muscle injury in mice has improved muscle repair in the short-term (Chan et al., 2005; Taniguti et al., 2011b; Kim and Lee, 2017) but reduced muscle strength in the long-term (Gumucio et al., 2013; Kim and Lee, 2017).

Summary

The benefits of HA injections to joint health is established. The use of the glycoprotein as an anti-inflammatory may negatively impact the initial phase of muscle repair. The hypothesis that intravenous hyaluronan treatment will find ways to integrate into the basal lamina of skeletal muscle and act as an anti-inflammatory by suppressing inflammatory genes was tested in unfit, adult horses.

CHAPTER 2

Materials and Methods

All animal protocols and procedures were reviewed and approved by the Virginia Tech Institutional Animal Care and Use Committee (22-170).

Animal care and groupings

Twelve mature unfit Thoroughbred geldings with a mean age of 6.83 ± 2.04 yrs and body weight (BW) of 547 ± 40 kg were housed in groups of 3 in dry lots of equal size (0.2 ha) with ad libitum access to water and minerals. Horses received mixed grass hay at 1.5% of BW and were fed a commercial feed (2.2 kg, Ultium Competition, Land O'Lakes, Arden Hills, MC) twice daily. Horses were blocked into 6 pairs based on age, weight, and body condition score, and one horse was randomly assigned to control or treatment from the pair. Experimental groups were evenly split (n = 6/group).

Treatment and exercise

Horses (n = 6) were injected IV with 40 mg of sodium hyaluronate (HA; Legend, Boehringer Ingelheim, Duluth, GA) each week for 3 weeks, according to the manufacturer's recommendations. Control horses (CON) received no intravenous injection of any kind. Two weeks after the final injection (d 35 of the experiment), all horses were fitted with a heart rate monitor (Polar Equine H10, Kempele, Finland) prior to performing a submaximal exercise test on a free run exerciser (EquiGym LLC, Paris, Kentucky). Exercise test parameters consisted of 6 min of walk (2 m/s), 14 min of trot (4 m/s), and 10 min of canter (8 m/s) for 4 repetitions with a change of direction between reps.

Muscle biopsies and tissue preparation

Skeletal muscle biopsies were taken from the gluteus medius (GM) of all horses from alternating limbs on d 0 prior to the first Legend injection, d 34, and d 35 one hour after completion of the submaximal exercise test. Horses were sedated with 50 mg of xylazine (1 mg/kg; Bimeda-MTC Animal Health, Cambridge, ON) and the hair above the center of the GM was shaved and cleaned with chlorohexidine (Nolvasan scrub, Zoetis, Kalamazoo, MI) and 70% ethanol [v/v] in water. The biopsy site was anesthetized with a subcutaneous injection of 2% lidocaine (VetOne, Biose, ID) and a surgical scalpel blade was used to pierce the skin. A 10-gauge vacuum-assisted biopsy needle (EleVation, BD Biosciences, Franklin Lakes, NJ) was inserted into the initial incision perpendicular to the muscle and 5 cm deep. The needle was rotated 90 degrees three times within the biopsy site to collect a total of 2 skeletal muscle tissue samples. Tissue samples were washed with phosphate buffered saline (PBS) prior to embedding in optimal cutting temperature media (OCT; Thermofisher, Waltham, MA) or flash freezing in liquid nitrogen. All samples were stored at -80°C until further processing.

Muscle cryosectioning and staining

Cryosections (15 uM) affixed to glass slides were washed three times with PBS and blocked with 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature. Following a brief rinse with PBS, tissue sections were incubated at room temperature with a biotinylated recombinant hyaluronan binding protein (250 ug/mL; AMS.HKD-BC41; AMSBIO, Cambridge, MA) reconstituted in sterile water and diluted (10 ug/mL) in 1x PBS for 1 h. Sections were washed and stained with Streptavidin Alexa Fluor 568 conjugate (2 ug/mL), Wheat Germ Agglutinin Oregon Green™ 488 conjugate (2.5 ug/mL), and Hoechst 33342 (1 ug/mL) in 1x PBS for 1 h covered at room temperature. Slides were mounted with 10% glycerol and stored at 4°C until imaging.

Epifluorescent imaging

Representative microscope images (n = 12) of stained skeletal muscle cryosections (n = 2 per horse) were captured at 100 or 200-fold magnification using an epifluorescent microscope (ECHO Revolve, San Diego, CA). Digital images were annotated using the manufacturer supplied software. Total fluorescence was measured by taking the average fluorescence intensity and multiplying it by the area being measured. Ratios of HABP-AlexaFluor594:WGA-AlexaFluor488, HABP-AlexaFluor594:Hoescht33324, and WGA-AlexaFluor488:Hoescht33324 were calculated. Images at 10x objective were used to measure average cross-sectional area of muscle fibers and the width of the WGA-AlexaFluor488 (green) and HABP-AlexaFluor594 (red) fluorescent bands which are representative of the basal lamina (BL) and HA. Cross-sectional area was measured by outlining the fibers (n = 5 per image: n = 15 per section; n = 30 per horse). Width of the BL was measured by measuring the distance of the green fluorescent band between two fibers (n = 8 measurements per fiber, n = 5 fibers per image; n = 24 measurements per section; n = 48 measurements per horse). Relative distribution of HA within the BL was measured by measuring the distance of the red fluorescent band between two fibers (n = 8 measurements per fiber; n = 5 fibers per image; n = 24 measurements per section; n = 48 measurements per section).

Total RNA isolation

Approximately 500 mg of frozen skeletal muscle tissue was homogenized on ice in 10 mL of TRIzol (Invitrogen, Carlsbad, CA) using a handheld Polytron homogenizer (POLYTRON® PT 1200 E, Malters, Switzlerland) for 1 min at 60% of maximum speed. Homogenates were incubated on ice for 5 min and homogenized a second time at the same time and speed. Homogenized tissue was incubated at room temperature for 20 min prior to the

addition of chloroform (20% final volume). Tubes were shaken vigorously and incubated at room temperature for another 5 min. Samples were centrifuged at 3220 x g for 56 min in a swing bucket rotor at 22°C (Centrifuge 5810 R, Eppendorf, Enfield, CT). The aqueous layer was retained, and the lysate was extracted with chloroform. The combined aqueous samples were precipitated by addition of an equal volume of isopropanol (Thermofisher, Waltham, MA) overnight at 4°C. Total RNA was collected by centrifugation at 3220 x g for 56 min, washed with 70% ethanol [v/v] in water, and resuspended in sterile water. Total RNA was stored at -80°C until use.

RNA-Sequencing and bioinformatics

One pre and one post exercise RNA isolate sample for each horse was sent to Novogene for RNA-sequencing. Samples were sequenced by Illumina software and turned into raw reads by CASAVA base recognition and stored in FASTQ(fq) format files. Data quality control was performed by calculating the GC content distribution and the error rate of each base by the Phred score to check for sequencing error rate and base quality. The raw reads are then filtered to remove reads with adaptor contamination, uncertain nucleotides that make up more than 10% of the read ($N > 10\%$), and when nucleotides with a base quality less than 5 ($BQ < 5$) make up more than 50% of the read. Sequence alignments are performed between the sample and the reference genome using (Equus caballus) HISAT2. Results are mapped to specific locations on the reference genome and classified as exons, introns, or intergenic regions. Gene expression level is then estimated by the number of mapped reads using the expected number of fragments per kilobase of transcript sequence per million base pairs sequenced (FPKM). Biological replicates are done to determine a correlation coefficient between gene expression levels of samples where a Pearson correlation coefficient greater than 0.92 ($PCC > 0.92$) and R^2 value greater than 0.2 (R^2

> 0.8) are sufficient. Differential gene expression (DEG) analysis is then performed. Raw read counts are normalized, and a P-value ($P < 0.05$) is calculated for each DEG under different experimental conditions. A Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis is performed using the DEGs to determine significantly ($P < 0.05$) associated pathways.

Chapter 3

Results

Libraries were constructed from total RNA isolated before and after exercise from both CON and HA treated horses. The average total reads for the samples was approximately 49-million and ranged from 40,936,546 to 77,200,234 bp. More than 85% of the sequencing reads mapped to the *Equus caballus* genome with 75.2 ± 0.28% mapping to exons and 8.5 ± 0.20% mapping to introns. The remaining 16.3 ± 0.19% of sequencing reads represent intergenic regions.

Transcript abundance between CON and HA were compared to evaluate differential gene expression (DEG). Genes with fewer than 1 count per million reads in 3 or more of the samples were removed from the dataset. A total of 11,029 genes were quantified and used to construct a Venn diagram that demonstrates that 10,428, the majority, of the genes are common between sample groups with 155 genes being unique to HA and 446 genes being unique to CON (Figure 2).

Gene ontology (GO) terms were assigned to the DEGs and analyzed for clusters of genes segregated by known functions. It was determined that the greatest number of DEGs were associated with T-cells and the inflammatory response (Figure 3). Specific genes within the lymphocyte activation and cytokine production GO families that were down regulated by HA treatment included *IL17RA, OSCAR, LYL1,* and *TLR1, 2, 8,* and *10*. Because the initial post-exercise response requires acute inflammation, a heat map comparing pre- and post-exercise reads per kilobase million (RPKM) for selected DEGs (Table 1) within the GO cluster was constructed to visualize the response to exercise (Figure 4). The map demonstrates a global

increase in expression for the majority of the genes associated with inflammation with different magnitudes of responses being apparent amongst horses following the stressor test. These results indicate that the majority of the inflammatory genes are not irreversibly down regulated.

Selected genes were used as indictive markers for neutrophils, macrophages, and dendritic cells and examined for their relative expression during the acute inflammatory period post-exercise (Figure 5). As previously described, neutrophil invasion occurred in the post-exercise period at 1 h as indicated by increased expression of *NCF2* and *ELANE* ($P = 0.05$ and $P = 0.07$, respectively). The macrophages expressed gene, *CD168*, increased ($P < 0.01$) with exercise, but no change was observed ($P > 0.05$) in the dendritic cell marker, *Flt3*.

These results indicate that HA suppressed inflammation prior to exercise suggesting a direct effect in the muscle tissue. It was determined using HABP that HA was localized to the basal lamina of muscle fibers (Figure 6A). However, the administration of HA did not alter the relative quantity of HA within the extracellular matrix (ECM) of the muscle fibers either before or after exercise (Figure 6B). While no effect was seen as a result of HA administration, Exercise tended to increase ($P = 0.10$) the size of the ECM (Figure 6C). In coincidence with the larger size, a greater abundance of genes involved with matrix remodeling was detected and included *CTGF, TGF-β1, MMP9,* and *Col4A1* (Figure 7).

CHAPTER 4

Discussion

Hyaluronic acid (HA) is a naturally occurring glycosaminoglycan that when supplemented into the body has proven to have anti-inflammatory effects and improve lameness in the articular capsule of equine and human joints with osteoarthritis (Rydell et al., 1970; Åsheim and Lindblad, 1976; Neuenschwander et al., 2019). In the human, HA acts as an anti-inflammatory by reducing IL-6, IL-1β, and TNF-α production through CD44 receptor interaction within synovial fluid (Yasuda, 2007; Neuenschwander et al., 2019). While HA treatment for degenerative joint diseases has been validated in the human and horse, a recent study in mice speculated the necessity of HA within the basal lamina of skeletal muscle fibers after injury for repair and regenerative signaling through extracellular matrix (ECM) remodeling that acts as a protective mechanism during inflammation (Nakka et al., 2022).

Intravenous injections of HA showed anti-inflammatory characteristics in the skeletal muscle of Thoroughbred horses through the down regulation of *IL17RA, OSCAR, LYL1,* and *TLR1, 2, 8,* and *10* and a suggested inhibition of lymphocyte activation and cytokine production. An objective of this study was to determine if exogenous HA could find ways to integrate into the basal lamina of skeletal muscle. It is apparent that HA did initiate changes in myofiber gene expression through the 11,209 DEGs identified between HA and CON sample groups; however, there was no relative increase in the amount of HA within the basal lamina. A possible implication for this is HA may be acting up stream of skeletal muscle on inflammatory innate immune cells such as neutrophils and monocytes/macrophages that are originating from bone marrow. For example, the toll-like receptor pathway is integral in cytokine and chemokine production, and inhibition of this process in immune cells has proven beneficial in limiting

inflammation associated with inflammatory diseases such as sepsis, asthma, inflammatory bowel disease (Gao et al., 2017). It has been previously validated that CD44 proteins, the dominant receptor for HA, are on neutrophil, monocyte, and macrophage cell types (Smadja-Joffe et al., 1996). *LYL1*, a gene shown to be down regulated by HA, produces a cell surface protein found on primitive macrophage progenitors (M0). Ablation of *LYL1* in hematopoietic stem cells (HSC) leads to apoptosis of macrophage progenitors and reduces HSC function (Wang et al., 2021). In mice devoid of HA synthesis through knockout of hyaluronan synthase genes in bone marrow, hematopoiesis and chemotaxis of HSCs was inhibited which indicates HA has some effect on the initiation of the inflammatory innate immune response (Goncharova et al., 2012). HA treatment has also been thought to act as an anti-inflammatory through instigating the polarization of macrophages from a pro-inflammatory M1 to anti-inflammatory M2 phenotype (Shahbazi et al., 2018; Lee et al., 2021). However, no gene markers for M2 macrophages were differentially expressed with HA treatment suggesting that HA possibly works as an anti-inflammatory through the inhibition of macrophage maturation into an anti-inflammatory state rather than promoting the switch from a pro-inflammatory to anti-inflammatory state.

From the results it can be determined that HA does suppress basal pro-inflammatory genes, but they are not irreversibly down regulated after the initiation of exercise-induced inflammation. At 1 h post-exercise, infiltration of neutrophils and macrophages to the damaged skeletal muscle tissue is apparent, but no difference in previously suppressed myeloid cell surface markers *IL17RA, OSCAR,* and *LYL1* post-exercise expression levels indicates that HA does not have a long-term effect. This offers no benefit to the performance of the horse in terms of reducing discomfort such as pain and muscle stiffness that is associated with muscle damage and subsequent inflammation. However, other anti-inflammatory drugs that have been validated

to irreversibly down regulate inflammatory genes consequently also delay and/or incapacitate muscle repair. Inflammation is necessary for the initiation and progression of myogenesis (Paulsen et al., 2012). Rats suffering from muscle contusion injury and administered a corticosteroid, a class of anti-inflammatory drug, had improved function and repair 2 days after injury compared to control but began to suffer from muscle weakness at day 7 after injury and continued to regress until day 14 with visibly noticeable myofiber disruption (Beiner et al., 1999). When macrophages are targeted directly through an injectable antibody that acts to suppress function, an increased occurrence of skeletal muscle fibrosis occurs in mice (Segawa et al., 2008). Tumor growth factor beta (TGF-β) is the protein responsible for fibrosis by signaling for ECM remodeling through altered collagen deposition. Inhibiting TGF-β during a pro-inflammatory response after muscle injury in mice has improved muscle repair in the short-term (Chan et al., 2005; Taniguti et al., 2011; Kim and Lee, 2017) but reduced muscle strength in the long-term (Gumucio et al., 2013; Kim and Lee, 2017). Within this study, HA treatment did not work to inhibit the initial inflammatory response by maintaining long term suppressive effects giving HA the benefit of not disrupting the skeletal muscle repair process.

Damage to skeletal muscle tissue causes an up regulation of genes associated with myofiber ECM remodeling (Zhang et al., 2021). In mice suffering from muscular dystrophy, a chronic inflammatory disease of skeletal muscle, greater than 75% of genes related to muscle structure and ECM remodeling were up regulated when RT-PCR analysis was performed (Marotta et al., 2009). As mentioned previously, TGF-β functions to signal for increased collagen deposition within the ECM to promote fibrosis. Inhibition of TGF-β1 through the blocker protein, suramin, impaired the onset of fibrosis through the reduction of muscle injury in mdx mice which was validated through a decrease in creatine kinase in the skeletal muscle (Taniguti

et al., 2011). Connective tissue growth factor (CTGF) is a fibrotic protein that's synthesis by myoblasts is stimulated by TGF-β (Vial et al., 2008). *TGFB1* along with *COL4A1* and *CTGF* were up regulated in equine skeletal muscle with exercise which is speculated to correlate with the relative increase in basal lamina thickness. In rats performing down-hill running, disturbances in the basal lamina were attributed to a loss of collagen IV proteins and decrease in overall thickness of the ECM (Kanazawa et al., 2021; Kanazawa et al., 2023) which is contrasting to the idea that collagen IV synthesis should be encouraged through exercise which has previously been reported in rats (Koskinen et al., 2002). However, *MMP9* a gene that encodes a type IV collagen degradation protein was upregulated after the experimental stress test. A study utilizing another rat model produced similar results where Col4 protein synthesis was elevated along with an observed increase in MMP9 after an acute damaging exercise, and they concluded that MMP degradation was being inhibited by TIMP proteins which allowed for the net increase of Col4 (Koskinen et al., 2002). *TIMP4* was a DEG measured to be up regulated after exercise in our horses; however, an assay would need to be performed to determine the actual possible loss or gain of Col4A1 within the myofiber membrane and further confirm if an increase in basal lamina thickness is attributed to a net increase in *COL4A1* gene expression or is MMP9 potentially working to degrade this protein.

Previous work distinguishing HA's role in inflammation detected decreased levels of IL-6, IL-1β, and TNF-α in the synovial fluid of humans diagnosed with osteoarthritis after HA injection (Yasuda, 2007; Neuenschwander et al., 2019). Alternatively, our results reported a suppression related to neutrophil and macrophage maturation and possibly inhibition of cytokine production through down regulation of toll-like receptors. A possible implication behind this difference is in previous studies, HA is given during an already active inflammatory state.

Therefore, the production of cytokines is already occurring. Exercise-induced fatigue in the horse has shown increased expression in *TNF-α* and *IL6* (Liburt et al., 2010). To determine if active inflammation at the time of injections improves HA's ability to suppress the inflammatory environment in skeletal muscle, a fourth intravenous injection should be given immediately after exercise, or to stay within the manufacturer's dosage recommendations, the third injection could be given immediately after completion of exercise. This would give insight to the possible time sensitive effects that HA has as an anti-inflammatory. Evidence also already exists that fit horses that are part of an exercise training program form a more adaptive muscle that can better withstand the stressors of exercise (Cappelli et al., 2013; Latham et al., 2021). At rest, racing Thoroughbreds trained daily have a greater baseline expression of *IL-6, IL-8*, and *IL-1β* in the blood suggesting training causes genetic adaptations to allow for a quicker inflammatory response and reduce recovery time (Cappelli et al., 2013). As unfit horses were used for this study, it is unknown whether trained horses would illicit the same receptiveness to HA treatment. Future research should aim to examine the anti-inflammatory capacity of HA through a replicate study where the horses endure an eight-week training program before performing a single stress test.

Figure 2

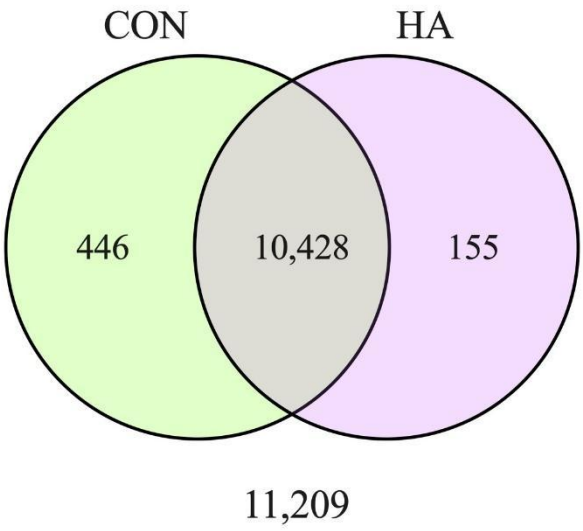

Figure 2. Venn diagram of DEGs in equine skeletal muscle following HA injection.

DEGs were identified between CON and HA treatment groups. The majority of genes are common between groups. 155 genes were unique to HA and 446 genes were unique to CON.

Figure 3

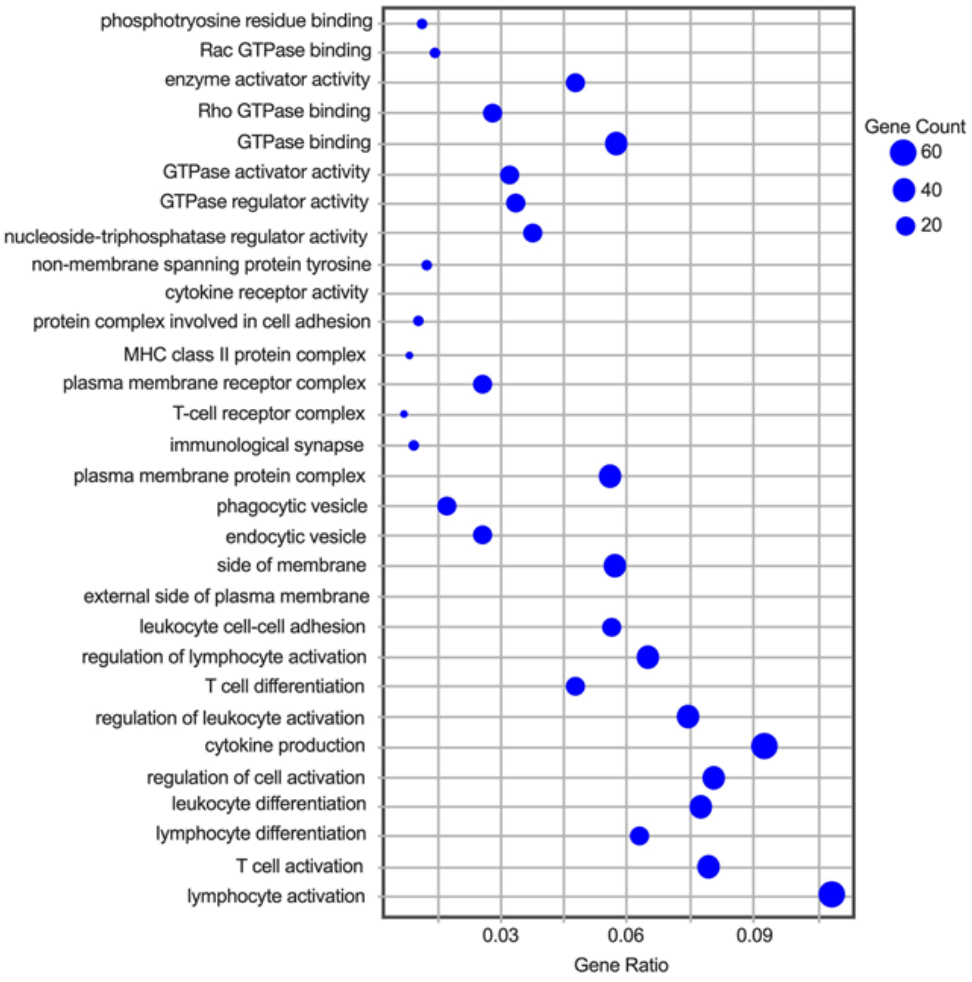

Figure 3. Gene ontology (GO) terms associated with inflammatory DEGs affected by HA treatment.

Enrichment analysis was performed with ClusterProfiler and the GO terms with the greatest numbers of DEGs are shown. The gene count histogram is scaled to size with the largest blue dots containing at least 60 DEGs and the smallest dots containing fewer than 20 DEGs per GO term.

Table 1. Selected DEGs associated with inflammation at 1 h post-exercise.

Gene Name	Gene Description
FCERG1	Equus caballus Fc fragment of IgE receptor Ig
DOCK2	Dedicator of cytokinesis 2
LCP1	Lymphocyte cytosolic protein 1
IRF8	Interferon regulatory factor 8
SEMA4A	Semaphorin 4A
CD5	CD5 molecule
SASH3	SAM and SH3 domain containing 3
PREX1	Phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1
JAML	Junction adhesion molecule like
HES1	Hes family bHLH transcription factor 1
FGR	FGR proto-oncogene, Src family tyrosine kinase
ELF4	E74 like ETS transcription factor 4
OSCAR	Osteoclast associated, immunoglobulin-like receptor
IL17RA	Interleukin 17 receptor A
SEMA7A	Semaphorin 7A
ID1	Inhibitor of DNA binding 1, HLH protein
TCF7	Transcription factor 7
CD22	CD22 molecule
LYL1	LYL1, basic helix-loop-helix family member
LCK	LCK proto-oncogene, Src family tyrosine kinase
MYD88	MYD88, innate immune signal transduction adaptor
IL27RA	Interleukin 27 receptor subunit alpha
CCR7	C-C motif chemokine receptor 7
GPR183	Equus caballus G protein-coupled receptor 183
CD47	CD47
CASP1	Caspase 1
LTB	Lymphotoxin beta
CD4	CD4 molecule
IL18	Equus caballus interleukin 18
TREM173	triggering receptor expressed on myeloid cells 1

Figure 4

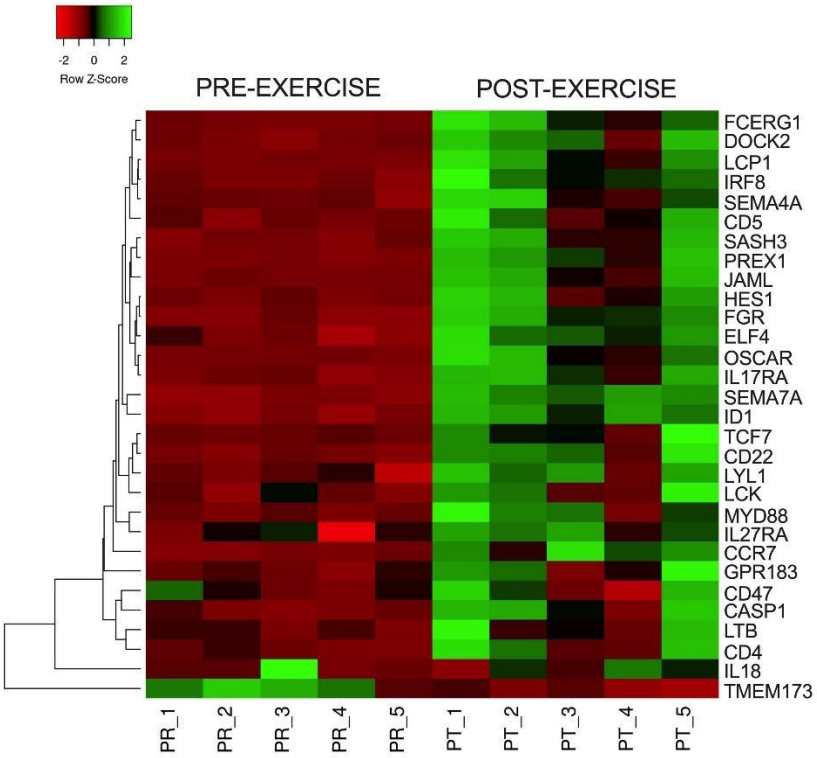

Figure 4. Inflammatory DEGs downregulated by HA are not refractile to exercise-induced stress.

A heat map was constructed comparing pre- and post-exercise reads per kilobase million (RPKM) for selected DEGs (right column) in HA treated horses. Row Z-scores range from -2-fold lower (red) to +2-fold greater (green). Down-regulated DEGs increase in response to exercise indicating no detrimental effects of HA on the acute inflammatory response post exercise.

Figure 5

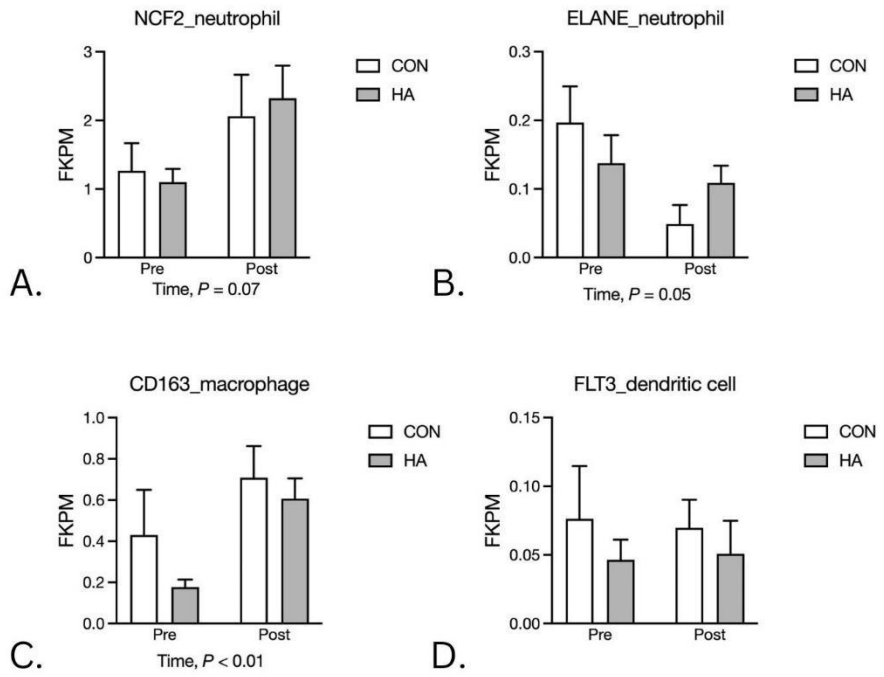

Figure 5. Neutrophil and macrophage invasion was apparent in the muscle tissue at 1 h post-exercise.

Gene expression in Fragments Per Kilobase of transcript per Million mapped reads (FKPM) for neutrophil markers *NCF2* (A; $P = 0.05$) and *ELANE* (B; $P = 0.07$), and the macrophage marker, *CD168* (C; $P < 0.01$) was increased following submaximal exercise. No change in dendritic cell marker *FLT3* ($P > 0.05$) was detected (D). No effect of HA was observed.

Figure 6

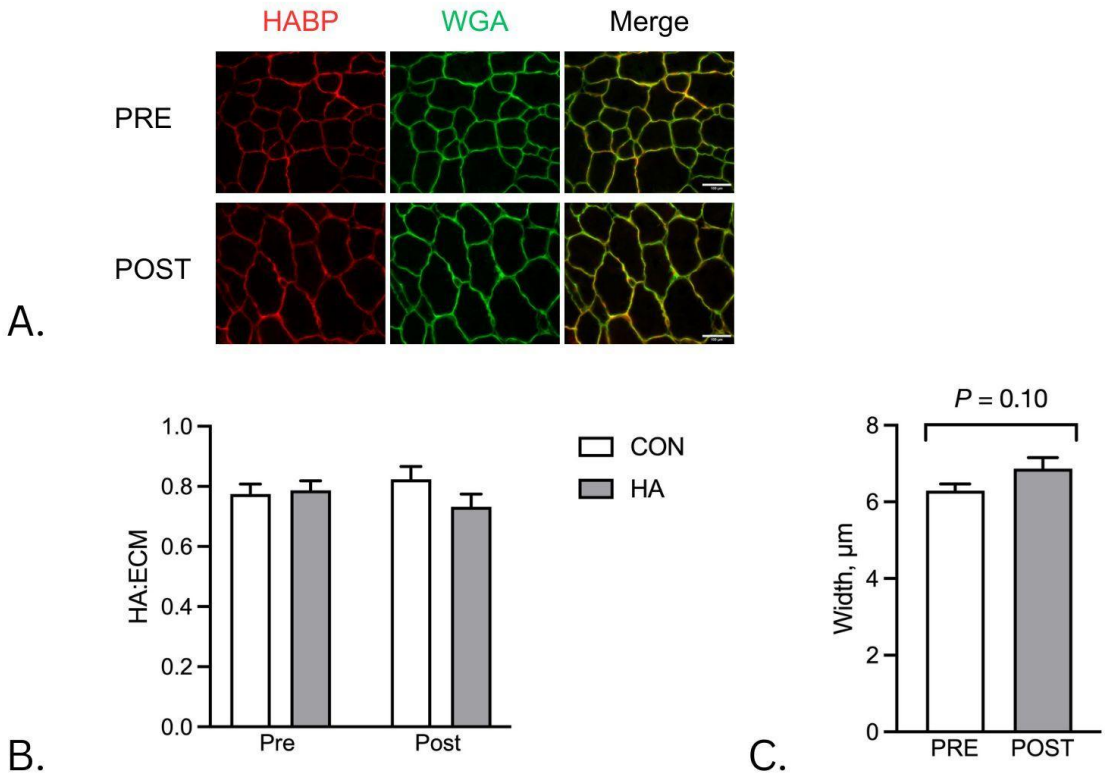

Figure 6. Exercise affects the structure of the basal lamina.

HA was localized to the basal lamina (WGA) of muscle fibers with HABP (A). The ratio of HABP:WGA was calculated and used as an estimate of basal lamina composition (HA:ECM) (B). The width of the basal lamina was measured throughout the GM samples of HA and CON horses before and after exercise (C). HA administration did not alter the relative quantity of HA within the ECM of muscle fibers before or after exercise. Exercise tended to increase ($P = 0.10$) the size of the ECM.

Figure 7

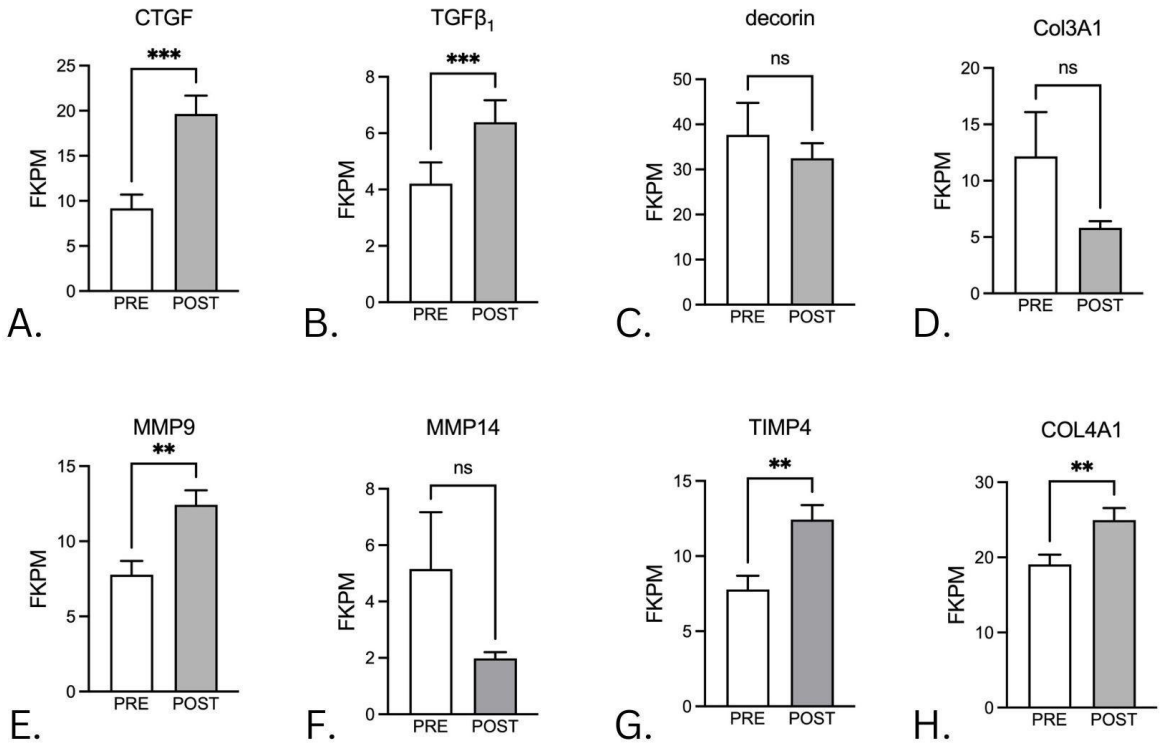

Figure 7. Genes associated with ECM remodeling are up regulated by exercise.

Select growth factor (CTGF, TGFβ_1, decorin), ECM proteases (MMP9, MMP14, TIMP4) and collagens (Col3A1, Col4A1) involved with remodeling were examined before (PRE) and 1 h after (POST) exercise. Expression values in Fragments Per Kilobase of transcript per Million mapped reads (FPKM) for CTGF, TGFβ_1, MMP9. TIMP4 and Col4A1are greater ($P < 0.01$) following exercise. ** denotes significance at $P < 0.01$; *** denotes significance at $P < 0.001$.

CHAPTER 5

Conclusions and future directions

Anti-inflammatory drugs help equine and human athletes by mitigating pain that otherwise hinders optimal performance. However, there are many supplements that are under doping regulations as they are abused and used as performance enhancers before a competition. While many non-steroidal anti-inflammatory drugs (NSAIDs), such as banamine and phenylbutazone (bute), have already been extensively studied and given dosage restrictions and withdrawal periods for equine related competitions, new athletic supplements are constantly emerging. Currently, little is known about the effects of hyaluronic acid (HA) as an anti-inflammatory regulator in equine skeletal muscle.

Unaccustomed, strenuous exercise does induce an acute inflammatory response in muscle tissue by upregulating inflammatory factors 1 h after the completion of exercise in Thoroughbred horses. It is unknown whether trained horses or a shorter exercise test would illicit the same response as it was not an objective of this thesis. However, evidence already exists that equine exercise training allows for the formation of a more adaptive muscle that can better withstand the stressors of exercise (Cappelli et al., 2013; Latham et al., 2021). A replicate study would be beneficial after an eight-week training period followed by the same treatment and submaximal exercise test to determine if a reduced inflammatory response in the muscle tissue is observed.

While exercise is important to improve performance parameters, the only way to continue to make these improvements is by increasing the exertion required from the muscle by scaling up the load or length of the workout which ultimately causes muscle damage. Recovery from acute muscle damage can be an uncomfortable and painful process due to the localized inflammation

that can hinder the ability to perform for up to four days. This study goes beyond the already published literature by offering a novel approach to diminishing skeletal muscle inflammation in horses. Preemptively, HA treatment suppresses inflammatory genes in the skeletal muscle tissue of Thoroughbred horses before a workout intended to cause muscle damage and subsequent inflammation is performed. However, once the muscle is declared to be in an initial inflammatory state, HA no longer has a suppressive effect. Previous work distinguishing HA's roles in inflammation detected decreased levels of IL-6, IL-1β, and TNF-α in the synovial fluid of osteoarthritis patients after HA injection where inflammation is already present (Yasuda, 2007; Neuenschwander et al., 2019). Our study did not produce similar results, and instead there were down regulations of inflammatory macrophage and neutrophil markers *NCF2, ELANE*, and *CD168*. To determine if active inflammation at the time of injections improves HA's ability to suppress the inflammatory environment in skeletal muscle, a fourth intravenous injection should be given immediately after exercise. Alternatively, to stay within the manufacturers' dosage recommendations, the third injection could be given immediately after completion of exercise. This would give insight to the time sensitive effects that HA has as an anti-inflammatory.

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