The effects of furosemide on equine skeletal muscle satellite cell myogenesis and metabolism *in vitro*

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ACADEMIC ABSTRACT

Thoroughbred racehorses undergo strenuous exercise which often leads to the occurrence of exercise-induced pulmonary hemorrhage (EIPH), in which capillaries rupture within the alveoli in the lungs causing bleeding. Severe cases of EIPH lead to epistaxis and may result in fatality. Presently, the loop diuretic furosemide is the only medication approved to mitigate the effects of EIPH. Often regarded in the racing industry as "performance enhancing" due to 4% weight loss ensued by its diuretic effect, it is unknown what effects furosemide may have on muscle recovery. Therefore, the objective of this study was to determine the effects various doses of furosemide may have on equine satellite cell (eqSC) myogenesis and metabolism. Mitotic index was increased ($P<0.05$) as a result of treatment with 100 µg/mL furosemide, a 10-fold pharmacological dose, in comparison to vehicle, but was not different ($P>0.05$) compared to the physiological dose of 10 µg/mL furosemide. Average cell number decreased ($P<0.05$) in the excess furosemide group compared to all other groups. *Pax7* expression did not differ ($P>0.05$) between groups. Expression of the differentiation transcription factor *myogenin,* and embryonic sarcomeric myosin heavy chain decreased ($P<0.05$) when cells were treated with 100 µg/mL furosemide. Fusion index and myotube area decreased ($P<0.05$) as a result of treatment with excess furosemide. Glycogen concentration in myotubes was lower ($P<0.05$) following treatment with 100 µg/mL furosemide, while IGF-1 was unsuccessful in rescuing the effects of furosemide. Excess furosemide decreased expression of *muscle*

creatine kinase while increasing expression of *phosphoglucomutase 1*, *glycogen synthase 1*, and *glycogen branching enzyme 1* ($P<0.05$). Excess furosemide decreased basal oxygen consumption rate (OCR) and increased OCR after addition of oligomycin ($P<0.05$). Excess furosemide did not affect myotube glycolysis rates in vitro. In conclusion, furosemide inhibits muscle differentiation and oxidative metabolism in eqSCs.

KEY WORDS: EIPH, furosemide, skeletal muscle, satellite cells, myogenesis, metabolism

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GENERAL AUDIENCE ABSTRACT

Thoroughbred racehorses often bleed from the lungs as a result of high-intensity exercise. This condition can oftentimes be fatal depending on severity. Furosemide, is used in the industry to reduce blood pressure within the lungs during racing to prevent bleeding. Furosemide, a diuretic given four hours prior to a race, causes a horse to excrete up to 4% of its body weight. This effect of furosemide decreases the weight a horse must carry during a race, thus allowing the horse to run faster. Therefore, deemed as a performance enhancing drug due to its effects on the kidney, to our knowledge, no research has been conducted on what effects furosemide might have on muscle generation. High-intensity exercise causes massive muscle damage and therefore must be repaired to prepare for the next bout of exercise. Muscle generation is called myogenesis. Stem cells, or satellite cells, that lie within the muscle become activated, recognizing the need for muscle repair. Satellite cells divide, increasing in cell number and then fuse together, forming new muscle fibers. Satellite cells undergo different types of metabolism depending on their state of development. For example, proliferating cells require glucose for energy, while cells fusing together forming myotubes, require oxidative metabolism for long-lasting energy. Therefore, the objective of this study was to determine the effects furosemide might have on muscle formation and metabolism. The excess furosemide dose (100 μg/mL) decreased cell proliferation. The expression of regulatory factors responsible for forming myotubes at different stages of muscle development are decreased when cells

were treated with the defined excess furosemide dose. Furosemide decreased the ability of satellite cells to generate myotubes. Glycogen concentration was also decreased as a result of excess furosemide treatment. Gene expression of enzymes involved in glycogen synthesis were increased from treatment with our excess furosemide dose. No effect of furosemide was seen on glycolysis, whereas oxidative metabolism suffered as a result of treatment with excess furosemide. In conclusion, furosemide does indeed affect muscle generation and oxidative metabolism.

DEDICATION

To my grandmother, *Jean M. Southam* for being the toughest lady I know and for teaching me the meaning of true perserverance.

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CHAPTER I

LITERATURE REVIEW

The Equine Racing Industry

The impact horses made in United States history cannot go unrecognized. They provided transportation for mail carriers along the trails of the Pony Express in 1860, and braved the battlefields of the Korean War where Staff Sergeant Reckless transported ammunition to U.S. troops in 1953. The current, living Triple Crown winners, American Pharoah (2015), and Justify (2018), have relaunched the thrill of horse racing in the eyes of the public. The domestication and use of horses for travel, news, war, and sport, allowed pioneers to conquer the west and start farms to provide food for themselves. The Indians would have had to develop different hunting and war techniques, and individuals living through the Great Depression would have had almost no means of entertainment if it had not have been for the exhilarating match race between Seabiscuit and War Admiral. The outstanding service of the horse assembled the infrastructure of the United States and has since shaped the country's economy.

Still today, the equine industry has an immense economic impact on the United States. According to the annual National Economic Impact Study conducted by the American Horse Council Foundation in 2017, the equine industry directly contributed 50 billion USD to the economy, and created nearly 1 million jobs. When considering both direct and indirect effects on the economy, the equine industry generates a total of 122 billion USD, and creates 1.7 million jobs (American Horse Council Foundation, 2017). The indirect effects most likely include feed companies and pharmaceutical companies. As of 2017, 7.2 million horses reside in the United States with about 1.22 million, of

those horses involved in racing. The equine industry provides 38 billion USD in benefits, salaries, and hourly wages to employees (American Horse Council Foundation, 2017). In the National Summary of 2005, also published through the American Horse Council Foundation, 1.8 million people were listed as horse owners, with another 2 million people involved in the industry through relatives or volunteer positions.

Kentucky, known as the "horse capital of the world," is famous for the Kentucky Derby hosted at Churchill Downs in Louisville every first Saturday in May. The Breeder's Cup World Championships, scheduled to run at Churchill Downs in fall 2018, were hosted at Keeneland Race Course in Lexington in 2015. As reported in 2012, 54,000 Thoroughbreds resided in the state of Kentucky, making Thoroughbred horses about 22% of the state's equine population. However, the value of the Thoroughbreds equated to over 87% of total equine value in Kentucky (USDA, 2012), most likely due to the astronomical value of the stallions standing at stud and the elite value of the broodmares residing on breeding farms. For example, the stallion Tapit who stands at Gainesway Farm in Paris, Kentucky had a stud fee of $300,000 for the 2018 breeding season. American Pharoah, standing at Ashford Stud in Versailles, Kentucky, stood for $200,000 for the 2018 breeding season (Bloodhorse Stallion Registry). Profits quickly accumulate as stallions breed multiple mares per day. Sheikh Mohammed bin Rashid Al Maktoum, Prime Minister of the United Arab Emirates (UAE), spent over 19 million USD on 27 yearlings at the 2018 September Yearling Sale at Keeneland Race Course, with the sale topper being a colt by American Pharoah purchased for 2.2 million USD (Kenneland Sales Summaries). As of 2012, Thoroughbreds in the state of Kentucky included: 16,500 Broodmares; 600 stallions; 13,500 yearlings, weanlings and foals

combined; and 13,000 racing Thoroughbreds (USDA, 2012). In 2011 there were 13,000 full-time and part-time employees combined, with a total of over 200 million USD in payroll expenses (USDA, 2012). During this time, the racing industry was responsible for 15% of labor expenses and the breeding industry was accountable for 40% of labor expenses (USDA, 2012). Equine assets, including the horses themselves, amassed to a value of over 23 billion USD (USDA, 2012). Equine sales and operations income totaled approximately 1 billion USD in 2011 (USDA, 2012).

Thirty-two of the 50 U.S. states are directly affected by the Thoroughbred racing industry economically, as these 32 states host the country's race tracks. States largely involved in horse racing include: California, Kentucky, New York, Florida, Maryland, Pennsylvania, and Illinois. The Thoroughbred racing industry provides nearly 21% of the total equine contribution to the U.S. GDP, recognizing the racing industry as the largest sole entity to contribute (American Horse Council Foundation, 2005).

Issues that arise within the industry, must be explored and corrected in efforts to prevent the U.S. economy from being significantly impacted: an issue such as Exercise Induced Pulmonary Hemorrhage (EIPH) correlates to extensive economic loss (Gold et al., 2018). Not only is the U.S. affected by the prevalence of EIPH, other countries such as South Africa and Australia are impacted as well. These countries are heavily involved in the international realm of the Thoroughbred racing industry as stallions from the U.S. often shuttle to Australia, South Africa, and South America for the Southern Hemisphere breeding season. Therefore, EIPH is impacting racehorses worldwide and could in turn, affect international economies as EIPH continues to be a vast veterinary issue.

Exercise Induced Pulmonary Hemorrhage

Thoroughbred racehorses undergo respiratory stress during high-intensity exercise, leading to a condition called Exercise Induced Pulmonary Hemorrhage (EIPH). High-intensity exercise, such as a race, increases pulmonary vascular pressure which can cause capillaries in the alveoli of the lungs to rupture, and cause bleeding (West et al., 1993). Severe EIPH can manifest to the point at which blood travels rostral through the trachea, and exits the nares, defined as epistaxis.

Stress failure of the capillaries is largely due to the tremendous intervascular capillary pressure combined with extreme negative pressure due to inspiration (Gold et al., 2018). Vast increases in cardiac output and aerobic capacity sustained during intense exercise contribute to a higher risk of stress failure. During exercise, heart rate can climb to 240 bpm and cardiac output can surpass 750 ml/min*kg, which is extremely high (West et al., 1993). The VO_2 max of the equine lung is 180 ml/min*kg, more than double the VO_2 max in athletic humans. In fact, capillaries begin to rupture when pulmonary capillary pressure exceeds 100 mmHg at which capillary wall stress reaches over 800,000 dyn/cm^2 (West et al., 1993). The membrane surrounding the blood-gas barrier is only 0.3 μm - 0.6 μm thick allowing for easier gas diffusion, but also affirming that capillaries in the alveoli are extremely susceptible to rupture (Manohar and Goetz, 1996). A study conducted in 1993 investigated the alveoli and capillary microscopy of equine post-mortem with a known history of EIPH (West et al., 1993). Findings concluded that there were lesions in the capillary wall endothelium which led to the presence of macrophages, platelets, and white blood cells in that area, along with breaks in the alveolar epithelium leading to red blood cells and inflammatory markers in the alveolar space. Although the

capillary/alveolar breaks were present, they were very difficult to find and rather small. As soon as capillary pressure started to decrease, some of the breaks closed within three to six minutes post-exercise (West et al., 1993). Most lesions tend to appear in the dorsocaudal region of the lung, where blood flow is highest and vascular resistance is lowest (West et al., 1993; Ramzan, 2014). Breaks in the capillary walls lead to inflammation, remodeling and repair of the tissue, thereby increasing wall thickness and fibrosis. Wall thickness and fibrosis contribute to an additional increase in pulmonary capillary pressure, leading to increased probability of stress failure with the next EIPH episode (West et al., 1993). Other areas of the lung can also then become affected by chronic EIPH which could contribute to more severe episodes (Ramzan, 2014).

While the known cause of EIPH is the stress failure of capillaries, speculation suggests another cause of EIPH may be the physical locomotion of galloping itself (West et al., 1993). The diaphragm is positioned in an oblique fashion, from caudodorsal to cranioventral, therefore trapping the dorsocaudal region of the lungs between the ribs and the cranial section of the diaphragm (West et al., 1993). When a forelimb strikes the ground, shear waves cause the abdominal viscera to dislodge the diaphragm contributing to a dramatic drop in alveolar pressure (West et al., 1993; Ramzan, 2014; Newton et al., 2005). When a horse inspires, the diaphragm contracts inducing negative pressure which causes air to funnel into the lungs, therefore increasing pressure in the alveoli and expanding alveoli surface area (West et al., 1993). Resulting from inspiration, oxygen concentration is high in the alveoli and low within pulmonary capillaries. This gradient allows oxygen to diffuse easily across the blood-gas barrier and deliver adequate oxygen to the blood and tissues (Marlin and Nankervis, 2002). During normal exhalation,

alveolar pressure drops (becomes more positive), allowing air to exit the lungs. However, when the intestines collide into the diaphragm premature of exhalation, this forces air out of the dorsocaudal region of the lungs and thus causes a sudden drop in alveolar pressure. As the dorsocaudal alveoli entertain the most negative pressure in the lung, most inhaled air is expected to reach this region of the lung. However, the alveoli in the dorsocaudal region are of relatively larger size than alveoli elsewhere in the lungs, thus increasing overall lung volume and pressure upon inspiration. The expansive area in which the alveoli can stretch can increase the frequency of stress failure of capillaries in the lungs as it contributes to an increase in capillary permeability (West et al., 1993). A red blood cell measures approximately 5 μm in diameter, in comparison to the 0.5 μm thickness of the blood-gas barrier (Marlin and Nankervis, 2002).

When capillaries in the alveoli rupture, blood trickles into the lungs therefore impairing blood from reaching the tissues, resulting in a decrease of dissolved oxygen in the blood. This condition contributes to an early onset of fatigue, thus significantly weakening performance (Hinchcliff et al., 2005; Morley et al., 2015). Since horses are unable to breathe through their mouths, the only way for them to receive adequate oxygen is through the nostrils (West et al., 1993). Airway obstructions cause resistance, further limiting the athletic capability of the racehorse (Marlin and Nankervis, 2002).

Detection of EIPH

EIPH can be detected with tracheobronchoscopy (TBE) and bronchoalveolar lavage (BAL). Sometimes EIPH can be confirmed with both methods of detection (Gold et al., 2018). However, South Africa does not utilize the TBE or BAL method of

detection due to time and cost restraints, suggesting their only method of detection is physically visualizing epistaxis episodes (Weideman et al., 2004). This may contribute to underreporting of EIPH in South Africa. EIPH is most often detected with the TBE method in which a fiber-optic endoscope is inserted into one of the nares and is further passed through the upper airway, through the trachea, and down to the carina, or tracheal bifurcation. The severity of the condition is graded on a scale of 0 to 4, with a grade of 0 given when no blood is detected in the airway from the nares to the tracheal bifurcation. Grade 1 is described as no more than a couple of flecks of blood detected or no more than two very small streams of blood present. Grade 2 signifies a long stream of blood detected or two short streams, but covering less than one-third of the circumference of the trachea. Grade 3 is described as more than a few streams of blood seen in the trachea, covering more than one-third of the circumference, without blood pooling at the thoracic inlet. Grade 4 denotes an immense amount of blood covering the circumference of the trachea, with blood pooling at the thoracic inlet. Evidence of epistaxis would also be listed as grade 4 EIPH (Gold et al., 2018; Hinchcliff et al., 2005; Morley et al., 2015; Ramzan, 2014). The BAL detection method is described as instilling an isotonic fluid into one side of the lung to wash and remove various blood cells so they can be counted to aid in determination of EIPH grade (Gold et al., 2018). The BAL method of detection is the most effective in diagnosing EIPH. However, the BAL method is rarely used due to the need for sedation and laboratory analysis of the sample (Ramzan, 2014).

The TBE and BAL detection methods were used to analyze the prevalence of EIPH in barrel racing Quarter horses (Gold et al., 2018). If one of the bronchi contained more blood than the other, the fiberoptic endoscope was advanced farther into the

bronchus until wedged in place. Once the endoscope was situated, 300 mL of PBS was instilled into the lung and then aspirated to wash out alveolar fluid containing red blood cells. Afterwards, red blood cell counts were performed. This method was used in conjunction with TBE results to determine EIPH severity (Gold et al., 2018). In contrast to the above, a racehorse veterinary manual suggests that the use of only 10-30 mL of 0.9% Saline would be enough to obtain red blood cells present in the airway, but would not always result in a representative sample of the entire airway (Ramzan, 2014). Although not universally recognized, the authors of the study decided to establish that a red blood cell count of more than 1,000 cells/μL was considered a positive test for EIPH (Gold et al., 2018). BAL results revealed that 66% of horses in the study tested positive for EIPH. TBE results confirmed that 54% of horses proved positive for EIPH (grades 1-4). Specifically, 37% of horses were considered an EIPH grade 1, 39% were grade 2, 18% were grade 3, and 6% were grade 4. Due to discrepancies of the percentages in horses considered positive for EIPH in the study, data suggest that new methods of detection need to be discovered, or perhaps a different threshold number of red blood cells in alveolar fluid need to be universally established. Some horses tested negative for EIPH with one method of detection whereas they were considered EIPH positive with the other method of detection (Gold et al., 2018). Due to increased tidal volumes ensued from a race or other high-intensity exercise, blood resulting from EIPH moving rostral decreases the incidence of a false negative EIPH diagnosis during TBE detection (Hinchcliff et al., 2005). The optimum time to detect EIPH is between 60-120 min post-exercise, unless epistaxis occurs prior to this time (Manohar and Goetz, 1996; Hinchcliff et al., 2005; Morley et al., 2015). In contrast, one source recommended performing the

endoscopy within 30 min post-exercise to observe the most representative sample of blood in the trachea (Ramzan, 2014).

Incidence of EIPH

EIPH is a very common condition found in the Thoroughbred breed and racing industry worldwide (Hinchcliff et al., 2005). Between 50-80% of Thoroughbred racehorses bleed to various degrees (Morley et al., 2015; Hinchcliff et al., 2005; Manohar and Goetz, 1996). Another source suggests that all Thoroughbreds bleed from the time they begin galloping on the track (West et al., 1993). Excluding musculoskeletal breakdowns, 35-60% of track fatalities are caused by severe cases of EIPH as determined with post-mortem necropsies (Lyle et al., 2011; Morley et al., 2015). In another study that took place from 2000 to 2013, a total of 628 fatalities occurred in 6,727 Thoroughbred racehorses that experienced 7,993 veterinary events (Rosanowski et al., 2017). While most of these fatalities were ascribed to skeletal and cardiac injuries, 100% of respiratory fatalities recorded, resulted from epistaxis (Rosanowski et al., 2017). Twenty-one percent of veterinary events over the 14-year period were attributed to respiratory conditions, most of which were due to epistaxis. In comparing the results from a study conducted between 1996 and 1998 to another study conducted between 2000 and 2013, the incidence of epistaxis in flat racing increased 378% (Rosanowski et al., 2017; Williams et al., 2001; Newton et al., 2005). While epistaxis occurs in 2-3% of racehorses, some blood exiting the nostrils may originate from the nares, not from the alveoli (Ramzan, 2014). Study results indicated that as a racehorse increases in age, they succumb to less veterinary events compared to younger racehorses, but the opposite is true for epistaxis

and resulting fatality; older racehorses are more likely to attain epistaxis and perish (Rosanowski et al., 2017). To be specific, racehorses of at least 4 years of age are more likely to develop epistaxis (Ramzan, 2014). In contrast, another study stated that the incidence of EIPH does not increase as the age of the race horse increases (Manhoar and Goetz, 1996). The incidence of bleeding is believed to be only 3% (West et al., 1993). However, this incidence rate is likely due to inadequate methods of detection such as relying on the visual observation of an epistaxis episode, rather than utilizing other methods of detection, such as BAL or TBE that can identify the presence of EIPH sooner. A South African study reported that in 1950, the incidence of epistaxis was 1.2%, but has since raised to 2.1% in 2003. In 1998, the incidence of epistaxis in Korea was 13.5%. Once a horse suffers from one episode of epistaxis, the probability of recurrence is over 30%. Rest and treatment do not curtail recurrence (Ramzan, 2014). Since the percentage of epistaxis is increasing worldwide, perhaps the Thoroughbred breed has weakened and can no longer tolerate the stress of high intensity exercise (Weideman et al., 2004).

EIPH Affects Performance

Cardiac and respiratory conditions contribute to poor performance and causes a racehorse to retire (Rosanowski et al., 2017). EIPH and epistaxis, the more severe form of the condition, significantly impair performance on the race track, therefore contributing to exercise intolerance (Morley et al., 2015; Hinchcliff et al., 2005; Weideman et al., 2004; Manohar and Goetz, 1996; Ramzan, 2014; Gold et al., 2018). However, some suggest that EIPH is indicative of superior performance ability as the race horse can make a greater effort during exercise to push themselves through symptoms of the condition,

although there is no scientific evidence supporting this theory (Hinchcliff et al., 2005). EIPH severity may worsen over time. Thoroughbreds most affected by EIPH in terms of well-being and performance may warrant retirement (Ramzan, 2014).

Several studies have analyzed the impact EIPH has on Thoroughbred performance. Tested performance indicators include: Career race earnings, the distance the horse finished behind the winner of the current race, as well as finishing position of the horse (Hinchcliff et al., 2005; Morley et al., 2015). In a study performed in Australia, 744 Thoroughbreds ranging from ages 2-10 years old were examined for the incidence of EIPH and how the severity of the condition related to racing performance. Results revealed that: 170/744 (about 23%) finished in the top three positions (first, second, and third) with 54 of the 170 horses (7.3%) finishing first. Upon examination of the horses for presence of blood in the trachea, the TBE method of detection found that 412/744 horses (55.3%) had evidence of blood in the trachea showing various degrees of EIPH. Almost 37% of horses examined were classified as having grade 1 EIPH, whereas almost 2% of horses were classified as having grade 4 EIPH. Thoroughbreds that received a TBE EIPH grade of 0 or 1 were 1.8 times more likely to finish a race in the top three positions, and 4.0 times more likely to win a race compared with horses who received an EIPH score of 2 through 4. Although, horses that received a score of 0 were no more likely to win a race when collectively compared to EIPH scores 1 through 4. There was no difference in performance seen between horses who received an EIPH score of 0 compared to an EIPH score of 1. There was a significant difference between horses whom received an EIPH score of 1 or higher compared to horses who had no evidence of EIPH. In fact, distance behind the winner was correlated to the severity of EIPH the horse exhibited; the more

severe the EIPH condition, the farther the distance the horse was behind the winner of the race. Horses who received an EIPH score of 0 or 1 had more than three times more race earnings when compared to horses assigned an EIPH score of 2 or higher. In conclusion, an EIPH score of 2, 3, or 4 affects performance, whereas EIPH scores of 0 and 1 do not affect performance. The results from this study indicate that the increased volume of blood in the trachea, corresponding to increased severity of EIPH, is responsible for affecting the performance of race horses. In fact, it takes a minimum of about 200 mL of blood in the airway to decrease oxygen consumption of the alveoli, leading to a decreased concentration of dissolved oxygen in the blood, contributing to fatigue, and ultimately causing a decrease in performance ability (Hinchcliff et al., 2005; Ramzan, 2014).

Researchers in South Africa also performed a study analyzing EIPH severity and race performance (Morley et al., 2015). This study focused on elevation of the race tracks and track surface conditions in relation to EIPH severity. Researchers involved in this study performed TBE examinations on Thoroughbreds no more than two hours after a race. Results indicated that horses negative for EIPH were about 2.3 times more likely to win a race compared to horses positive for the condition (EIPH grades 1-4). When further analyzed, horses negative for EIPH (grade 0) were about 2 times more likely to win a race compared to those with EIPH grades 1 through 3, and more than 6 times more likely to win a race compared to horses with grade 4 EIPH. Researchers found no significant difference in finishing position when comparing a horse without EIPH to those positive for EIPH. Horses positive for EIPH were typically at least one length behind the winner. A trend suggested the more severe the EIPH, the more lengths were between the winner and the horse with EIPH. For example: if a horse had an EIPH score of 0 and it was not

the winner of the race, the horse would only be behind the winner by about 2 lengths, whereas the horse with EIPH scores of 1 through 3 finished about three lengths behind the winner. Horses with grade 4 EIPH finished roughly five lengths behind the winner. Horses who showed no evidence of EIPH after the race were more likely to have higher career earnings than those with EIPH scores of 1 or greater (Morley et al., 2015). This finding agrees with that of the Australian study (Hinchcliff et al., 2005). There was no effect on the occurrence of EIPH when compared to track elevation or track surface (Morley et al., 2015). In contrast, a study performed in the United Kingdom analyzing incidence of injuries concluded that track surface does impact epistaxis frequency (Rosanowski et al., 2017). Specifically, all-weather tracks, and firm turf tracks led to higher incidence of epistaxis compared to other track surfaces (Rosanowski et al., 2017; Ramzan, 2014). According to one source, epistaxis is more likely to occur in the fall and winter months (Ramzan, 2014). Authors of the study conducted in South Africa suggested that EIPH only limits Thoroughbred racehorses from performing at their highest potential, but the condition does not impair performance altogether (Morley et al., 2015). Horses in these studies were prohibited from taking any race-day medications that might conflict with how EIPH effects performance in the Thoroughbred athlete (Morley et al., 2015; Hinchcliff et al., 2005).

Prevention of EIPH

EIPH is a concern in Thoroughbred racing. It is necessary to protect the welfare of race horse and ensure that public perception of the industry is not skewed (Morley et al., 2015). Since the racing industry contributes significantly to the economy, and the

occurrence of epistaxis is very costly to the industry (Weideman et al., 2004), the issue of EIPH cannot be ignored by industry leaders. Therefore, the industry must find a way to prevent this condition.

Since capillary walls are only 0.3 μm - 0.6 μm thick, a possible way to prevent the occurrence of EIPH is to strengthen capillary walls. One of the components of the extracellular matrix of capillary walls is Type IV Collagen. If there is a way to either increase the strength of the collagen already present in the capillary walls or increase the concentration of collagen to this area, EIPH occurrence may be reduced (West et al., 1993). However, thickening the blood-gas barrier will prevent oxygen from easily diffusing across the membrane into the capillaries, therefore decreasing the concentration of oxygen in the blood, contributing to an early onset of fatigue (West et al., 1993). Thus, EIPH may be prevented through decreasing stress failure of capillaries, but performance may not be enhanced. Another way to possibly prevent the occurrence of EIPH is to repair any upper airway restrictions such as recurrent laryngeal neuropathy, otherwise known as "roaring." Restricting air from entering the lungs increases alveolar pressure and results in a decrease of dissolved oxygen in the blood, hindering performance. Such a restriction of the upper airway can be repaired with a tie-back surgery in which the arytenoids of the larynx are kept from obstructing the airway (West et al., 1993; Ramzan, 2014).

Strong evidence indicates that epistaxis has some genetic merit. Therefore, it may be possible to prevent the occurrence of the condition by actively working to select sires for breeding who showed no evidence of the condition on the race track. An international database could be formed that contains pedigree, bleeder history information, and

estimated breeding value of potential sires to confirm that epistaxis is not passed to the progeny. The issue with this idea is that true breeding value cannot fully be established until the sire is about ten years old. Typically, a potential sire races for about five years, then goes to stud and breeds mares who foal out the following spring. The progeny might not start on the track until they are at least two or three years old. By this time, the stallion has already bred hundreds of mares and the breeding value would no longer matter. True breeding value of the stallion can only be determined with enough progeny of racing age. South Africa makes great effort in eliminating epistaxis. If a horse shows signs of epistaxis while in training or in a race, the horse is suspended from racing for three to six months during which the horse has time to heal (Weideman et al., 2004). Rest and the use of non-steroidal anti-inflammatory drugs may aid in healing from a severe epistaxis episode (Ramzan, 2014). If after this time, the horse returns to the track and continues to show evidence of epistaxis, the animal is banned from racing and forced to find another career, therefore protecting the welfare of the horse itself, the Thoroughbred breed, and also the racing industry.

South Africa allows horses to have medication to prevent epistaxis during training. However, the country does not allow the use of any race-day medications (Weideman et al., 2004). Possible treatments for EIPH include: Herbal diuretics, hemostatic drugs, vasodilators, bioflavonoids, Vitamin C, and Omega-3 Fatty Acids; with the most effective treatments being Furosemide and the FLAIR nasal strip (Ramzan, 2014). Although South Africa and Australia have banned the use of Furosemide (Lasix) on race-day, this medication is legal on race-day in the United States.

Furosemide

Furosemide, a therapeutic medication often referred to in the Thoroughbred racing industry under the trade name Lasix, is a high-ceiling loop diuretic commonly used in the United States to reduce the prevalence and severity of EIPH in racehorses (Abbott and Kovacic, 2008; Soma et al., 1985; Vengust et al., 2011). Currently, furosemide is the only race-day medication permitted in the United States and Canada (Gross et al., 1999) and has been used in the industry for over 40 years (Beyer, 2012). In fact, Northern Dancer, a prominent stallion highlighted in pedigrees of many competitive racehorses today, received furosemide before a race in 1964, though no regulations of the drug's use were yet in place. Today, more than 90% of all racehorses in the United States run while under the influence of furosemide, with about 400,000 doses of the drug administered per year (Hinchcliff et al., 2009; Ross, 2014). If a horse runs a race after receiving Lasix, it must be indicated on the racing program to the public with an "L" next to the name of the horse. If a horse is running under Lasix for the first time, an "L1" will be listed after the horse's name. Doses between 250 – 500 mg, or a dosage between 0.5 – 1.0 mg/kg respectively, are permitted in the United States to prevent EIPH in racehorses (Soma and Uboh, 1998).

The drug's swift onset and short half-life allow the Thoroughbred racehorse to lose up to 4% of its body weight when administered 500 mg of furosemide intravenously, 4 h prior to post-time (Abbott and Kovacic, 2008; Gross et al., 1999). The extent of furosemide's diuretic effect depends on the dose administered, suggesting if a dose of 250 mg is administered intravenously, only 2% of body weight would be lost (Vengust et al., 2011). Urine volume increases as the administered dose increases (Soma and Uboh,

1998). In the blood, 90-95% of furosemide is protein bound, while the plasma concentration of free, unbound furosemide is inversely related to the concentration of plasma albumin (Abbott and Kovacic, 2008; Cadwallader et al., 2010). Therefore, the diuretic effect of furosemide is not dependent on plasma concentration, but rather dependent on how much of the drug makes it to the tubular lumen in the Loop of Henle (Abbott and Kovacic, 2008). Diuresis occurs within a few minutes post-injection and continues until about 1-2 h prior to post-time (Soma and Uboh, 1998). Within the first hour of diuresis, a horse can excrete anywhere from 10-15 L of urine, the amount usually excreted in a 24 h period (Ross, 2014). Hay and water are typically withheld during this time to ensure the racehorse does not re-gain lost weight before racing (Hinchcliff et al., 2009; Ross, 2014). Intense diuresis leads to massive electrolyte depletion, including: potassium, sodium, chloride, magnesium, and calcium (Abbott and Kovacic, 2008).

Free, unbound furosemide is filtered through the glomerulus within the Bowman's Capsule of the kidney and available to inhibit the luminal $Na^+/K^+/2Cl^-$ (NKCC2) co-transporter, thereby contributing to the rapid onset of diuresis (Abbott and Kovacic, 2008). Furosemide previously bound in the vascular compartment by binding proteins enters the renal tubular cells via the Organic Anion Transporters, OAT3 and OAT1. The drug then travels into the tubular lumen, specifically into the proximal renal tubule of the nephron, over time, allowing a prolonged inhibition of the NKCC2 symporter, thus sustaining the diuretic effect (Abbott and Kovacic, 2008; Soma and Uboh, 1998). Furosemide acts on the thick ascending Loop of Henle within the nephron of the kidney to inhibit the luminal NKCC2 co-transporter. Furosemide blocks the second chloride binding site on the NKCC2 co-transporter needed to reabsorb electrolytes from

the urine, thereby contributing to an increase in dilute urine and electrolyte excretion (Abbott and Kovacic, 2008; Cadwallader et al., 2010; Soma and Uboh, 1998). Therefore, the delivery of chloride to the NKCC2 co-transporter is the rate-limiting step to reabsorb electrolytes from the tubular lumen, as all binding sites must be occupied to translocate ions across the membrane (Abbott and Kovacic, 2008). Within the first 24 h, the body eliminates 85% of furosemide through the urine. About 50% of the drug is secreted from the OAT transporters unchanged, and the other 50% is metabolized or inactivated through glucuronidation in the kidney (Abbott and Kovacic, 2008; Ho and Power, 2010).

Although the diuretic effect of furosemide is mediated through the NKCC2 co-transporter, furosemide also affects the NKCC1 co-transporter and the KCC transporters. NKCC1 co-transporters have been identified in the heart, colon, brain, stomach, smooth muscle, neurons, fibroblasts, epithelia, lungs, vascular endothelium, kidney, red blood cells, and skeletal muscle. In the kidney, the secretory NKCC1 co-transporter can be found on the basolateral portion of the tubular cells, whereas the absorptive NKCC2 co-transporter is primarily found on the apical portion of the tubular cells. Although similar in their amino acid sequence, NKCC1 co-transporters have 80 additional amino acids attached to its "A," or amino, terminus. Similar to NKCC co-transporters in sequence homology of 25%, K^+/Cl^- co-transporters (KCC) are also inhibited by loop diuretics. In fact, compared to the loop diuretic butanemide, furosemide has a higher binding affinity for KCC transporters, than for NKCC co-transporters. KCC co-transporters are found in the liver, red blood cells, epithelia, spleen, colon, brain, kidney, and heart. Four isoforms of KCC co-transporters have been identified that are sensitive to furosemide: KCC1, KCC2, KCC3, and KCC4 (Abbott and Kovacic, 2008).

Effects of furosemide

Strenuous exercise triggers roughly 4% of cardiac output to move from the pulmonary vasculature into the pulmonary interstitial space; such events are regulated by erythrocytes. Erythrocytes contain a regulatory mechanism called the "chloride shift" that moves fluid across the cell membrane of the red blood cell, and deposits fluid into the interstitial space. Furosemide inhibits the chloride shift, reducing the amount of fluid in the interstitial space, therefore reducing pressure and bleeding in the lungs (Vengust et al., 2011). The diuretic action of furosemide contributes to a lower circulating volume of blood and has several beneficial effects that help alleviate symptoms of EIPH (West et al., 1993). Following diuresis, plasma volume of the blood decreases. This action contributes to a decrease in blood pressure within the pulmonary artery and transmural hydrostatic pressures within the pulmonary capillaries, thereby reducing the incidence of stress failure. As a result of the decrease in plasma volume of the blood, right atrial pressure is also decreased (Vengust et al., 2011; West et al., 1993). Filling pressure of the left ventricle within the heart is decreased as an effect of furosemide administration, subsequently contributing to a decrease in cardiac output (Abbott and Kovacic, 2008; Cadwallader et al., 2010; Soma and Uboh, 1998). Furosemide also promotes pulmonary vasodilation as well as dilation of the bronchioles in the lungs, therefore making strenuous exercise more tolerable for the racehorse (Vengust et al., 2011). Although the diuretic effects of furosemide occur within minutes post-administration, pulmonary arterial pressures are not altered when furosemide is administered only one hour prior to exercise. Rather, pulmonary changes have only been noticed when furosemide is administered 2, 3, or 4 h prior to strenuous exercise (Kynch et al., 2018). The

predominantly venous vasodilatory mechanism may be due to a high concentration of NKCC1 co-transporters found in veins versus arteries. This vasodilatory effect of furosemide can be inhibited by the non-steroidal anti-inflammatory drug (NSAID) Flunixin (Banamine®), but not with the NSAID Phenylbutazone (Abbott and Kovacic, 2008; Soma and Uboh, 1998).

After the administration of furosemide, in resting horses, cardiac output and stroke volume decrease, whereas packed cell volume, heart rate, urine production, and peripheral venous dilation increase (Soma and Uboh, 1998). Peripheral vasodilation decreases the quantity of blood in central circulation, contributing to a reduction in stroke volume and ventricular filling, and may allow more time for heat to escape the body (Naylor et al., 1993). While in exercise, increased blood flow to peripheral areas of the body may provide more oxygen to surrounding muscle and connective tissues. This may decrease the overall expenditure of energy as measurements of mixed venous oxygen decreased after the administration of furosemide (Soma and Uboh, 1998).

Efficacy of furosemide

Concern in the racing industry has increased regarding the efficacy of furosemide to attenuate EIPH (Gross et al., 1999). In a study performed in South Africa under normal racing conditions, Thoroughbred racehorses were either administered 500 mg of furosemide or saline intravenously 4 h prior to post time, and were restricted from eating hay and water until their subsequent turf races. TBEs were performed after racing where EIPH scores of 0-4 were assigned. Racehorses who did not receive furosemide prior to racing were about 3-4 times more likely to develop EIPH with a severity of 1 or greater

and were about 7-11 times more likely to develop EIPH with a severity of 2 or greater. Roughly 80% of horses who received the saline solution control developed EIPH with a severity score of 1 or greater, while only about 55% of horses who received furosemide 4 h prior to racing developed EIPH with a severity score of 1 or greater. Approximately 68% of racehorses who received a prerace administration of furosemide had a one-grade reduction in their EIPH severity scores when compared to those who received saline, concluding that furosemide does indeed aid in mitigating EIPH (Hinchcliff et al., 2009; Kynch et al., 2018).

Efficacy of furosemide is dose-dependent in that a low dose of 250 mg may not affect pulmonary pressures and therefore not reduce the incidence of EIPH in Thoroughbred racehorses. A study investigating the effects of trans-vascular fluid fluxes after the 250 mg IV administration of furosemide 4 h before exercise discovered no significant difference between the furosemide treatment and the control. Since no effect was found in this study, the authors concluded that other beneficial effects of furosemide may contribute to the reduced pulmonary pressures that could ultimately reduce the incidence of EIPH (Vengust et al., 2011).

EIPH does not affect every horse in the same way nor is furosemide always able to fully mitigate EIPH (Soma et al., 1985). It is possible that furosemide may not be able to reduce transmural pressures enough to prevent capillary hemorrhage (Soma and Uboh, 1998). In a study performed at the Breeder's Cup World Championships 55 horses were endoscopically examined following their racing performance. Seventy-one percent of horses who received administration of furosemide prior to racing had evidence of EIPH, while only 37% who participated in the Lasix ban for 2-year-olds, therefore running

medication free, revealed any evidence of EIPH (Voss, 2013). Racing jurisdictions in which these horses train must approve a Lasix regime for the racehorse as track veterinarians must confirm evidence of EIPH for race-day Lasix to be permitted. Therefore, it is to be expected that horses who have received permission to be administered race-day Lasix have had some evidence of EIPH in the past. A typical reduction of one severity grade (Hinchcliff et al., 2009) is seen in horses treated with Lasix in effort to attenuate the effects of EIPH, making racing safer for the horse.

Strenuous exercise contributes to an increase in capillary pressures above 100 mmHg. A dose of 500 mg (1.0 mg/kg) of furosemide IV is enough to cause a decrease in pulmonary pressures by about 7-10 mmHg when a horse runs at speeds over 95% VO_2 max. However, when a horse is administered furosemide IV at a dose of 1000 mg (2.0 mg/kg), a pressure change of about 13 mmHg occurs (Soma and Uboh, 1998). Typically, transmural pressures lower than 75 mmHg do not result in EIPH. After the use of furosemide, 64% of horses have a reduction in blood seen in the trachea upon TBE examination (Soma and Uboh, 1998). Furosemide may not inhibit bleeding from capillaries in the alveoli, but may slow the rostral progression of blood in the trachea, resulting in a lower EIPH score as TBE examinations only typically extend to the tracheal bifurcation (Hinchcliff et al., 2009).

Furosemide enhances race performance

Furosemide has often been regarded as a performance enhancing drug in Thoroughbred racing. Although a legal medication, the drug's diuretic action contributes to the consideration that rapid weight loss may be the true mechanism for enhancing

performance; rather than the mitigation of EIPH symptoms (Soma and Uboh, 1998). Racehorses receiving furosemide have faster race times (Gross et al., 1999). A study examining furosemide and its effects on racing performance on dirt tracks in the United States and Canada analyzed sex, age, and race distance as it pertains to performance enhancement (Gross et al., 1999). In a study examining the correlation between furosemide administration and performance, 16,761 horses (74%) received furosemide before racing (Gross et al., 1999). When grouped within sex, 71.7% of fillies or mares, 66% intact colts, and 78.3% geldings received furosemide (Gross et al., 1999). Results suggested that colts benefitted most from effects of furosemide as their racing times were faster. However, when analyzed within sex, racing following furosemide administration proved effective in reducing race times in every gender compared to racing without furosemide administration (Gross et al., 1999). In another study, geldings benefitted most by the administration of furosemide, while colts produced no reduction in race times with furosemide administration (Soma and Uboh, 1998). Horses under the age of 7 years had faster race times following furosemide administration, compared to older horses (Gross et al., 1999). However, older horses still at the track tend to be geldings as colts go out to stud following their peak performance on the track and most mares retire to become broodmares before the age of seven. Thoroughbreds receiving furosemide before racing are 1.2 times more likely to cross the finish line in one of the top three positions (win – place – show) and 1.4 times more likely to finish the race first compared to those not receiving furosemide. Almost 76% of racehorses in the study who received furosemide increased their earnings. Possible explanations of performance enhancement induced by furosemide include: a reduction in body weight resulting from diuresis; bronchodilation;

reduction in the severity of EIPH; and furosemide-induced metabolic alkalosis (although not currently proven in horses) (Gross at al., 1999). Metabolic alkalosis is the idea that by increasing the pH of the blood before exercise, the body will take longer to fatigue as lactic acid accumulation (although not a sole indicator of fatigue) resulting from exercise will require more time to increase. It has been reported that the rate and time to fatigue remains unchanged with or without the administration of furosemide (Soma and Uboh, 1998; Vengust et al., 2011). However, at maximum oxygen consumption, it is possible for plasma lactate concentrations to decrease due to lost weight during diuresis. Standardbreds exercised for two minutes on a treadmill at 120% VO_2 max decreased the rate of lactic acid accumulation (Soma and Uboh, 1998). Furosemide does not amend the aerobic capacity of running, but reduces the energy required to maintain exercise capacity at high speeds, such speeds achieved during a race (Hinchcliff et al., 1996). Furosemide also decreases the rate of carbon dioxide accumulation, increases the oxygen consumption rate, and decreases the oxygen deficit. The performance enhancing diuretic effect of furosemide disappears when lost weight is carried again during racing (Gross et al., 1999; Hinchcliff et al., 1996; Soma and Uboh, 1998).

Interestingly, furosemide seems to be more effective in preventing EIPH and enhancing performance in horses of higher value. Horses worth more than $10,000 seemed to have better finishing position and faster race times compared to those worth less than $10,000 yielding slower race times and subsequently worse finishing positions (Soma et al., 1985). Race times for colts, geldings and mares were faster when administered pre-race furosemide, even though no evidence of EIPH was detected. In

fact, race times were faster by up to 1.09 s, a distance equal to approximately 5.5 lengths, a span surely capable of manipulating a race's outcome (Gross et al., 1999).

Furosemide as a masking agent

Furosemide is often regarded as a masking agent in the Thoroughbred racing industry. The World Anti-Doping Agency, an agency that works alongside the International Olympic Committee, banned the use of diuretics in sport as these agents massively dilute urine, rendering prohibited substances undetectable. Thus, all diuretics are classified as masking agents (Cadwallader et al., 2010).

Furosemide can mask the detection of other prohibited substances used within days or hours prior to testing before a race, making it hard to regulate. In fact, some NSAIDs can alter the renal effects of furosemide (Soma and Uboh, 1998). For example, in humans, COX-1 and COX-2 inhibitors impede the diuretic effect of furosemide. However, diuretic effects of furosemide are not altered with the co-administration of Meloxicam (Abbott and Kovacic, 2008). In equine, Phenylbutazone administered in combination with furosemide can contribute to a decrease in pulmonary resistance, but also a decrease of diuresis by about 40%. However, this combination does not change plasma concentration as it requires a 50% change in urinary clearance to alter the plasma concentration of the drugs in the blood. The concomitant treatment of the cyclooxygenase (COX) inhibitors phenylbutazone and flunixin (Banamine®), completely inhibit the pulmonary benefits induced by furosemide (Soma and Uboh, 1998).

Specific gravity is the ratio of urine density to water density. It is used as a measure to help determine the concentration of solutes in the urine. If the specific gravity

of urine is determined to be below the value of 1.010, it is suspected that a prohibited substance along with a higher dosage of furosemide, was administered to mask the presence of an illegal substance in a racehorse (Soma and Uboh, 1998).

Furosemide affects other organs

Although the kidney is furosemide's target organ, the drug also effects several other organs (Abbott and Kovacic, 2008). Furosemide can cause necrosis of the liver. In mice, furosemide is converted to an arylating, toxic metabolite by microsomal enzymes in the liver. The toxic metabolite binds covalently to hepatic macromolecules causing liver necrosis. Mice were given furosemide diluted in 0.9% saline intraperitoneally. After the mice were sacrificed, liver necrosis was graded in a range of 0-4, with 0 signifying no necrosis, to 4, equaling more than 50% hepatocyte necrosis. Furosemide-induced necrosis is dose-dependent in mice. At an administered dose of 200 mg/kg, necrosis was observed in only 16% of mice, but as the dose reached 400 mg/kg, 92% of mice had liver necrosis, with 60% of necrotic livers labelled with severity between 2 and 4. The amount of furosemide metabolites covalently bound to the liver directly correlated to the amount of liver necrosis. Only trace amounts of furosemide metabolites were covalently bound to the Sartorius muscle 24 h post-treatment. It was determined that the furan ring portion of the furosemide molecule is responsible for hepatic necrosis. The dose at which hepatic necrosis would occur in mice is beyond the furosemide dose required for maximal diuresis (Mitchell et al., 1976).

Furosemide affects a horse's heart rate during submaximal exercise. In a study comparing the effects of dehydration on exercise, heart rate was lower in both the

furosemide treatment group (when administered 4 h prior to exercise) and the water with-holding dehydrated treatment group (no water 30 h prior to exercise) at the beginning of the 40 min exercise (Naylor et al., 1993). The hydrated control group's heart rate was lower than the water with-holding dehydrated group at the end of the exercise period. During certain portions of exercise, stroke volume was significantly lower in the furosemide group compared to the hydrated control group. In the first 10 min of exercise, the temperature within the mid-gluteal muscle was significantly higher in the water with-holding dehydrated group, whereas at 40 min of exercise, mid-gluteal muscle temperatures were significantly higher in the furosemide treated group. Rectal temperature was higher in the water with-holding group compared to the control group after 40 min of exercise. Temperatures inside the carotid artery and pulmonary artery were significantly higher in both dehydration treatment groups relative to the control. Interestingly, subcutaneous temperature was lower in the furosemide treated group when compared to the control between 10 and 20 min of exercise. Temperature of the superficial thoracic veins were lower in the furosemide treated group compared to the control group. Horses treated with furosemide prior to exercise sweated less than the control, as the horses in the control group had considerably more sweat than any other group. Overall, the horses in the two dehydrated groups either produced more internal heat, or were unable to dissipate the heat produced, compared to horses in the hydrated control group. In both the dehydration treatments, temperatures within the mid-gluteal muscle increased faster than the control, and the muscle maintained this temperature until 20 min post-exercise. Thus, this suggests prolonged time for heat removal when

succumbed to dehydration, and that furosemide affects recovery post-exercise (Naylor et al., 1993).

Aside from fluid and electrolyte imbalances, furosemide can affect other areas of the body. Furosemide can decrease plasma levels of high-density lipoprotein (HDL) cholesterol and increase plasma levels of low-density lipoprotein (LDL) cholesterol and triglycerides. Furosemide can contribute to ototoxicity, or hearing impairment of the inner ear, and can adversely interact with several other drugs including sulphonylureas and propranolol. In humans, furosemide use followed by exercise can lead to heart attack, irregular heartbeat, major exhaustion, and death (Cadwallader et al., 2010). Furosemide can also lead to iodide depletion, a nutrient essential for neonatal growth. Furosemide can inhibit mucociliary function through the reductions of mucosal secretions as a result of dehydration. In rare cases, furosemide can contribute to acute pancreatitis in humans (Abbott and Kovacic, 2008).

Furosemide may cause a delay in wound healing as a study performed in rats revealed a 29% slower healing rate of a corneal laceration compared to the control (Abbott and Kovacic, 2008). In rats, furosemide has been found to inhibit myometrial contractions. Infertility can arise in mice if the animals are deficient in NKCC1 co-transporters. Furosemide can extend the half-life of T4 and T3, signaling a negative feedback response on the anterior pituitary. This leads to the reduction in TSH secretion from the brain, making it seem as though a patient is hypothyroid (Abbott and Kovacic, 2008).

Furosemide provides many beneficial effects as well. It can help reduce epileptic episodes therefore decreasing the occurrence of seizures, as it contains anticonvulsant

properties. Furosemide is lipid soluble and can readily cross the blood-brain-barrier. Furosemide inhibits NKCC1 and KCC2 co-transporters found in the brain that maintain intracellular chloride concentrations in neurons, therefore inhibiting seizures caused by cell swelling. Inhibition of NKCC1 found on choroid plexus epithelium may reduce cerebral spinal fluid production. Furosemide also has antioxidant properties that help reduce free radicals in the body, promoting mitochondrial health (Abbott and Kovacic, 2008).

The Lasix controversy

The use of race-day furosemide in the Thoroughbred racing industry has been under debate for the past decade. First introduced to the industry in the mid-1970s, the drug was legalized for race-day use in all US racing jurisdictions by 1995 (Beyer, 2012; Hegarty, 2012). The United States and Canada are the only countries that allow the use of furosemide on race-day as a treatment for EIPH. Countries including South Africa, Japan, United Kingdom, and Australia allow the use of furosemide in training, but not on race-day, whereas Hong Kong neither allows it during training or on race-day (Zorn, 2011). As previously mentioned, many penalties are in place for horses that exhibit signs of EIPH. Since the United States and Canada allow the use of furosemide to attenuate symptoms of EIPH on race-day, the integrity of horse racing in North America remains in question, given that the use of any race-day medications are banned internationally (Kynch et al., 2018).

However, not only is the use of furosemide controversial amongst industry officials, but it is also controversial in the eyes of the public (Soma and Uboh, 1998).

Managers within the Thoroughbred racing industry claim the use of race-day medications will lead to the demise of horse racing in North America. The public often perceives horse racing as a greedy, powerful industry who overbreeds mares, dopes horses with all sorts of prohibited medications, and runs horses into the ground. To the public, it often seems racing is all about the money, not about the horses and their performance as athletes. Conversely, from 2001-2010, the number of furosemide residue violations dropped 33%. In 2010, no prohibited substances were detected in 99.5% of the 320,000 samples tested (Larkin, 2011).

Industry leaders fear the longevity of the racing industry is threatened due to race-day furosemide use. Young fan base is shrinking; betting totals have dropped and the number of foals born per year have declined. However, veterinarians tend to be in support of administering the drug on race day as it proves effective in reducing EIPH severity. Despite its efficacy, Breeder's Cup World Championship officials moved to ban race-day furosemide in 2-year old Juvenile races in 2012, and in all races in 2013 (Larkin, 2011). However, this action yielded a small race-field as trainers and owners boycotted the furosemide ban, resulting in a lifted ban at the Breeder's Cup World Championships in 2013. Breeder's Cup officials believed the furosemide ban would be very proactive in getting North America on-board with medication-free racing. Since horses are brought from all over the world to run in these high-class races, following international racing guidelines would deem appropriate (Beyer, 2012). The Kentucky Horse Racing Commission even prohibited the use of race-day furosemide in 2-year-old stakes races in 2014, with the subsequent understanding that when these horses turned 3 years of age, they would not be allowed to race on furosemide as well. Eventually the hope was to

phase out the use of race-day furosemide in all stakes races in 2016. Splitting the industry into two sides, the Commission failed to pass this rule. Organizations such as the Breeder's Cup, Jockey Club, and the Thoroughbred Breeders and Owners Association supported the ban on race-day furosemide while the opposition of the furosemide phase-out included a conglomerate of trainers and owners in the industry. However, John Philips, the owner of Darby Dan Farm who once supported the use of furosemide on race-day and whom has since voted to prohibit the drug, said that the public has failed to accept the drug and recognize its intended use, to benefit the health of the horse by mitigating effects of EIPH (Hegarty, 2012).

In 2018, the Jockey Club reported that some trainers who decided to run some of their horses without the use of race-day furosemide were still able to win races. Out of almost 280,000 starts, approximately 10,000 starts were made of horses who had not received Lasix (Gorajec, 2019). Trainers Kenneth McPeek with 19 wins, and Larry Rivelli with 18 wins, were among top trainers last year with the greatest number of wins without the use of Lasix. Of the races won last year without the use of Lasix, 54% were Maiden races, 32.5% were Claiming races, 7.6% Allowance races, and only 5.6% Stakes races (Gorajec, 2019). Typically, better racehorses qualify for more prestigious races (Group 1/Grade 1, or Stakes), and race against stronger competitors. Maiden races tend to be very difficult for handicappers to predict the winner as these horses have never won a race. Multiple studies expressed that Lasix tended to benefit better performing horses (faster horses) running in the higher-level races, than the lower level horses (slower horses) running in maiden, claiming, and allowance races (Kynch et al., 2018; Soma et al., 1985).

In racing jurisdictions in the United States, when a racehorse exhibits signs of EIPH, they are added to a list of known bleeders and approved by the state's Jockey Club to be on a furosemide regimen (Gross et al., 1999; Soma and Uboh, 1998). According to the National Thoroughbred Racing Association Safety and Integrity Alliance (NTRASIA) Code of Standards (2018), an official veterinarian must approve the use of furosemide for the racehorse and the proper forms must be completed so the public can be notified that the horse was added to the furosemide list. Furosemide must be administered on track grounds and only furosemide can be given on race-day to prevent EIPH. Detention barns may be used to house racehorses 4 h prior to post-time to ensure medication compliance (NTRASIA, 2018). When blood and urine are collected post-race, the concentration of furosemide in serum or plasma cannot exceed 100 ng/mL and the specific gravity of the urine cannot be less than 1.010 (NTRASIA, 2018; Racing Medication & Testing Consortium).

In effort to address race-day use while still providing therapeutic effects preventing EIPH, a study was performed comparing the efficacy of furosemide in attenuating the effects of EIPH when administered 4 h versus 24 h prior to a simulated race. The authors believed that furosemide administered 24 h in advance may still provide beneficial effects in preventing EIPH. Horses received 250 mg of furosemide or saline control intravenously 4 h and 24 h before racing against one other horse. Access to water was restricted for the horses who were administered treatment 4 h before racing, whereas horses racing 24 h after treatment administration were allowed 2.5 L of water every four hours up until 4 h prior to post time. EIPH severity was determined with the use of an endoscopic examination, bronchoalveolar lavage (BAL), and serum blood

samples. Serum was collected to test levels of lactate, hemoglobin, and various electrolytes (Na^+, K^+, Ca^+, Cl^{2-}, and HCO_3). Post-race examinations revealed that 93% of horses who received furosemide 4 h prior to post time had an EIPH score of 0, whereas only 60% of horses who received furosemide 24 h prior to post time had an EIPH score of 0. Of the horses who had received saline placebo, 69% had an EIPH severity score of 0 post-exercise. No differences were seen between the saline treatment and furosemide treatments within administration time, but there was a significant difference in EIPH videoendoscope scores when comparing furosemide treatments administered 4 h and 24 h prior to post-time. Red blood cell counts from the BAL tended to be lower when horses were administered furosemide 4 h prior to post-time, compared to horses who received furosemide 24 h before post-time. Overall, furosemide administered 4 h prior to post-time seemed to be more effective in reducing EIPH severity. However, prior to the performed study, it was determined that about 74% of all horses included in the sample previously showed no evidence of EIPH. Small sample size and lack of EIPH diversity within the sample limited this study as more horses with varying backgrounds of EIPH would yield a stronger, more conclusive result (Kynch et al., 2018).

Furosemide use may also affect bone density. In humans, several different studies have investigated the fracture risk of patients who use loop diuretics. Although the studies yielded confounding results, patients who use loop diuretics may be at a greater risk of hip fractures, as a 5% decrease of bone density was detected in the hip in users of loop diuretics. As furosemide inhibits the $Na^+/K^+/Cl^{2-}$ co-transporter in the ascending Loop of Henle, it also inhibits the re-uptake of calcium from the urine, leading to a condition called hypercalciurea. Bone resorption due to the administration of furosemide,

may cause weakening of the bones, leading to an increased risk of fracture (Rejnmark et al., 2006). Although these studies were performed in humans, the same effects may also be present in racehorses with long-term use of furosemide.

Considering recent reports, Lasix's use in the North American Thoroughbred Racing Industry has indeed been threatened. From December 26th, 2018, to March 5th, 2019, 22 equine fatalities have been reported at Santa Anita Park, located in Arcadia, California, as a result of breakdowns during training workouts or during racing (Balan, 2019; Paulick, 2019). As these breakdowns are a true anomaly, the safety and welfare of racehorses and jockeys is in question for many followers of the sport. Believed to be the fault of the use of furosemide in the industry, regulations set forth by The Stronach Group, who owns Santa Anita Park, Gulfstream Park, Laurel Park, Pimlico Race Course, and Golden Gate Fields, have amended regulations on training and medication practices at both Santa Anita Park and Golden Gate Fields (Paulick, 2019). After receiving much scrutiny from California Thoroughbred Trainers and Thoroughbred Owners of California regarding these changes, the International Federation of Horseracing Authorities (IFHA) Chairman, Louis Romanet, commended the action The Stronach Group chose and encouraged all racing jurisdictions in North America to follow suit (Paulick, 2019; Paulick Report Press Release, 2019). All horses currently receiving the therapeutic medication Lasix can continue to race under the influence of the drug, but at only half the currently allowed dose. Therefore, instead of receiving a 500 mg dose of Lasix, horses may now only receive a maximum dose of 250 mg (Paulick Report Press Release, 2019), a dose previously discussed that does not seem to have any effect on mitigating EIPH (Vengust et al., 2011). In 2020 and thereafter, 2-year-old horses will not be allowed to

race under the influence of the therapeutic medication furosemide, and in following years, horse racing will be considered medication-free.

Racing medication reform may soon occur on a national level as Representative Andy Barr (KY) reintroduced legislation to the House of Representatives on March 14th. The 2019 Horse Racing Integrity Act, supported by The Coalition for Horse Racing Integrity which comprises The Jockey Club, the Thoroughbred Owners and Breeders Association and welfare organizations, promotes the establishment of an independent, anti-doping authority. This nationwide authority would be responsible for creating uniform medication rules in the racing industry, rather than relying on state jurisdictions (Angst, 2019). More research regarding the effects of furosemide in racehorses may aid in creating more uniform rules of the therapeutic medication in the industry.

Muscle

The primary functions of muscle include locomotion, power for intense exercise, and stabilization. The collaborative effort of the equine skeletal and muscular systems allows the horse to engage in intense exercise, displaying its athleticism. Equine have considerably more muscle mass compared to livestock, or meat-producing animals. Muscle mass comprises 30-40% of meat-producing animals, much less than equine, consisting of roughly 50-55% muscle (McGowan and Hyytiainen, 2017). The Thoroughbred has more muscle mass in its hind end compared to most other breeds. The hind limb muscles which provide the horse with the most power include: the mid-gluteal muscle, the biceps femoris, and the longissimus dorsi (McGowan and Hyytiainen, 2017).

Training, exercise, and breed type provides equine with the fundamental structure required to become successful athletes. This structure is largely based on fiber type composition of the muscle. However, fiber type of muscle is also largely dependent on metabolic properties and contractile ability (Lopez-Rivero et al., 1989). These fiber types include: A) slow-twitch, oxidative type I; B) fast-twitch, oxidative and fatigue-resistant type IIA; C) fast-twitch glycolytic type IIB; and D) fast-twitch oxidative/glycolytic mixed-type IIX. Fiber type is determined with muscle histology and Myosin Heavy Chain (MHC) isoforms (McGowan and Hyytiainen, 2017). Most muscles are composed of mixed fiber types (Hyytiainen et al., 2014). Structural composition and metabolism of muscle fibers allow the animal to perform a specific type of exercise. For example, certain muscles in Arabians consist of mostly type I (slow twitch, highly oxidative) myofibers, allowing this breed to excel in endurance races since immense stamina is necessary to travel 100 mi within 24 h. Type I fibers contain more mitochondria, providing adequate energy for sustained exercise. Thoroughbred racehorses contain higher quantities of type IIA myofibers in hind limb muscles as they confront intense speeds and rapid acceleration since flat races only last 2 min, although fibers are also fatigue-resistant as extreme speeds must be sustained for the full exercise period (Hyytiainen et al., 2014). The hind limbs of quarter horses contain mostly type IIX fibers as only a short, fast, 0.25 mi surge of energy is necessary to compete in a sprint race (Hyytiainen et al., 2014). Type IIX myofibers rely mostly on glycolytic metabolism as opposed to oxidative metabolism. Energy must be produced rapidly to sustain the short bout of exercise required in a sprint race (Hyytiainen et al., 2014).

The mid-gluteal muscle is comprised of 85-90% fast-twitch type IIA or IIX myofibers (McGowan and Hyytiainen, 2017) and is involved in hind limb propulsion and locomotion (Lopez-Rivero et al., 1989). Interestingly, within the mid-gluteal muscle, the fiber type changes. The superficial section of the mid-gluteal muscle contains more type II fibers compared to the deeper portions of the muscle, which contain a greater proportion of type I fibers (Hyytiainen et al., 2014).

A study examined fiber types found in the right mid-gluteal muscle of 55 horses of three breeds: Arabians (10), Thoroughbreds (20), and Andalusians (25) (Lopez-Rivero et al., 1989). The horses studied ranged from 5 to 12 years of age with varying sex. Muscle samples were obtained on the ventral portion of the mid-gluteal muscle using a biopsy needle at a sample depth of 5 cm. Fiber types were classified as type I, IIA, and IIB and were stained in accordance with m-ATPase activity, indicative of the oxidative capacity of the muscle. Type IIB fibers stained grey in color, type IIA fibers stained white, and type I fibers stained black. Intensity of the stain was also determined. The researchers observed a significant difference between the percentage of type I and type II fibers found in the mid-gluteal muscle. Surprisingly, the mid-gluteal muscle in Andalusians consisted of roughly 35% type I fibers compared to 30% type I fibers in Thoroughbreds and 24% type I fibers in Arabians at the sample depth of 5 cm (Lopez-Rivero et al., 1989). Thoroughbreds exhibited a higher percentage of type IIA fibers in the mid-gluteal muscle compared to the other breeds in the study. In addition, the differences in the percentage of type IIB muscles were not significant between the breeds (Lopez-Rivero et al., 1989). Irregularities of fiber type composition reported in the mid-gluteal muscle correlates with sample depth and therefore suggests that sample depth and

location should be precise when comparing fiber types of muscles amongst different breeds. Thoroughbreds are best suited for racing at distances exceeding 0.5 mi as type IIA fiber type provides quick muscle contraction and resistance to fatigue (Lopez-Rivero et al., 1989).

Another study analyzed fiber type prevalence in various equine muscles, and compared them to myonuclear domain, defined as the amount of cytoplasm per one myonuclei (Kawai et al., 2010). Three male Thoroughbreds between the ages of 3 to 5 years old, were euthanized and the vastus lateralis, soleus, masseter, diaphragm, gluteus medius, pectoralis profundus, and longissimus lumborum muscles were extracted. A 1 cm^3 block of muscle was excised from the superficial portions of extracted whole muscles for analysis. Cross-sections of the muscles were made and stained with antibodies that react with type I, type IIA, and type IIX fibers. Results of the study revealed that approximately 44% of the gluteus medius muscle is comprised of type IIX fibers (Kawai et al., 2010). Phosphofructokinase (PFK) activity in these muscles was also measured, with the gluteus medius muscle being one of the two muscles that exhibited the highest PFK activity. These data suggest increased anaerobic metabolism occurs in this muscle, although no relationship between myonuclear domain and metabolism were found. Smaller myonuclear domain is correlated with slow-twitch muscle fibers, and a larger myonuclear domain is correlated with fast-twitch muscle fibers (Kawai et al., 2010). Fiber type of each muscle directly correlates to training and performance ability of the animal.

Muscle Injury

Exercise increases the risk of injury in any athlete. Racehorses typically train six days a week although not always at such high speeds as those achieved during races. Many training regimens start with strength training, followed by stamina or endurance training, and lastly focus on speed training (Marlin and Nankervis, 2002; McGowan and Hyytiainen, 2017). Racehorses can reach speeds of roughly 40 mph during a race (McGowan and Hyytiainen, 2017). However, such intense exercise leads to increased likelihood of injury. Musculoskeletal injuries are the most common type of fatal injury that occur on the race track on race-day (Rosanowski et al., 2017).

Exercise is classified as either concentric or eccentric. Concentric exercise is the shortening of the muscle during contraction and eccentric exercise is the stretch, or extension, of the muscle during contraction (Proske and Morgan, 2001). Eccentric exercise, often associated with intense exercise and increased muscle stress, leads to muscle soreness. As Thoroughbred racehorses run, they undergo both concentric and eccentric contractions. The onset of soreness usually occurs several hours after exercise and peaks at 48 h post-exercise. Muscle soreness indicates muscle damage, possibly from damage of the excitation-contraction coupling mechanism and ruptured sarcomeres. Swelling, which peaks 24 h post-exercise, contributes to stiffness and soreness of the muscle post-exercise (Proske and Morgan, 2001).

Muscle damage can be mitigated with increased frequency of training, leading to muscle adaptation. Repeating exercise a week after the initial onset of muscle damage, diminishes muscle soreness, indicating a decrease in muscle damage. Researchers speculate that a decrease in muscle soreness and damage as a result of adaptation

contributes to an increase in the number of muscle sarcomeres (Proske and Morgan, 2001). A sarcomere is the functional unit of the muscle and is composed of repeating thick, myosin filaments and thin, actin filaments (Holash and MacIntosh, 2019). In a study in which rats ran downhill for one week, therefore performing eccentric exercise, researchers observed an increase in the number of sarcomeres in myofibers compared to rats who ran uphill, an example of concentric exercise (Lynn et al., 1998). Increased numbers of sarcomeres may contribute to less muscle damage as training continues and muscle grows. Training including only concentric exercise may lead to a loss of sarcomeres in the muscle fiber, therefore succumbing the muscle to an increased risk of damage when confronted with eccentric exercise (Proske and Morgan, 2001).

Muscle Satellite Cells

When muscle damage occurs, such damage can be repaired with undifferentiated progenitor cells located adjacent to the muscle, known as satellite cells, or rather, muscle stem cells. Located directly next to an existing myofiber and situated in-between the sarcolemma and underneath the basal lamina, muscle satellite cells exist at birth and stem from the mesoderm/dermamyotome layer during embryonic development (Abou-Khalil et al., 2015; Almada and Wagers, 2016; Dhawan and Rando, 2005; Dumont et al., 2015). Muscle stem cells are unipotent in that they can regenerate the tissue type in which they reside (Markoski, 2016; Seale et al., 2001). Satellite cells participate in regenerating damaged or diseased skeletal muscle and aid in hypertrophy, or postnatal muscle growth (Dhawan and Rando, 2005), although their requirement for hypertrophy post-satellite cell depletion, followed by overload, is currently under debate (Enger et al., 2016; McCarthy

et al., 2011; Enger et al., 2017; McCarthy et al., 2017). A study performed in mice suggested that satellite cell depleted muscle can initially undergo hypertrophy that was subjected to increased muscular stress. However, for the ability of the muscle to undergo continued adaptation to stress, satellite cells must be present (Mackey et al., 2016; McCarthy et al., 2011; Fry et al., 2014). Satellite cell depleted muscle and continued muscle stress cause the increase of fibrotic tissue, therefore compromising muscle function (Mackey et al., 2016). Thus, muscle regeneration is impaired without satellite cells present (Almada and Wagers, 2016).

Only 2 to 10% of skeletal muscle is comprised of muscle satellite cells, or roughly 200,000 to 1,000,000 cells per gram of muscle tissue (Dumont et al., 2015). Aside from satellite cells, muscle is also comprised of myonuclei, the differentiated daughter cell of the parent, undifferentiated muscle satellite cells that have fused into the myofiber. The universal indicator of muscle satellite cells is the paired-box protein/transcription factor Pax7 (Almada and Wagers, 2016; Dumont et al., 2015). More specifically, Pax7 and Pax3 are both universal indicators of muscle satellite cells, however, Pax3 is only present in satellite cells of specific muscles (Dhawan and Rando, 2005). Therefore, when determining the presence of satellite cells in laboratory procedures, the use of a Pax7 antibody is more prevalent.

Not only are muscle satellite cells responsible for the regeneration of skeletal muscle, satellite cells also aid in the repair of bone. However, in order for satellite cells to contribute to repair of neighboring bone, adjacent tissues must touch causing activation of the satellite cells. A scaffold placed between the damaged bone and the adjacent skeletal muscle impaired bone regeneration (Abou-Khalil et al., 2015). Skeletal muscle

recognizes the need for bone repair and thus causes the release of growth factors naturally produced in skeletal muscle, such as Fibroblast Growth Factor 2 (FGF2) and Insulin-like Growth Factor-1 (IGF-1). These growth factors are essential for bone regeneration. A study performed in mice demonstrated the essential nature of satellite cells for bone repair. Muscle satellite cell null mice ($Pax7^{-/-}$) and ablated satellite cell mice ($Pax7^{-/-}Cre^{+}$) models caused severely impaired bone regeneration when compared to mice that expressed $Pax7^{+}$ satellite cells (Abou-Khalil et al., 2015).

The environment in which the satellite cells reside, hereby known as the niche, is very important to the regenerative capacity of muscle. The cell adhesion molecule, M-Cadherin expressed within the satellite cell, communicates with the basal lamina to detect any extrinsic signals or a disturbance in the cells environment that would lead to the cells activation and subsequent repair of the damaged muscle tissue (Dhawan and Rando, 2005). The satellite cell niche is very diverse in that it includes: macrophages, fibro-adipogenic progenitors, growth factors, myofibers, and various extracellular matrix proteins (Almada and Wagers, 2016). These various niche components are responsible for maintaining myogenic identity of the satellite cells as well as providing an aid in the regenerative ability of muscle (Almada and Wagers, 2016).

The non-steroidal anti-inflammatory drug (NSAID) Ibuprofen promotes muscle regeneration and repair post-damage. A study conducted in Copenhagen involved 32 men who consumed 1200 mg of Ibuprofen per day, 2 weeks prior to an induced electrically-stimulated injury, an example of eccentric exercise (Mackey et al., 2016). The subjects continued the NSAID treatment 4 weeks post-injury and reported an increased rate of muscle repair compared to those who did not consume Ibuprofen. Muscle biopsies were

isolated at 2.5 h, 2 d, 7 d, and 30 d post-injury, from the vastus lateralis muscles. Blood was drawn to determine plasma lactate dehydrogenase (LDH) and creatine kinase (CK) levels, plasma myoglobin (MB), and plasma concentration of the circulating Ibuprofen levels in the body. Blood was sampled at -14 d, 0 d, 2.5 h, 2 d, 7 d, and 30 d post-induced muscle damage. Vastus lateralis muscle biopsies were taken from the control leg and the electrically-stimulated leg at 0 d, 2 d, 7 d, and 30 d. Immunohistochemistry and Real-Time PCR were performed. NSAIDs modify Notch signaling within the cell, which is important for maintaining a pool of quiescent satellite cells within the muscle. Satellite cell content increased in those subjects in the Ibuprofen treated group as Notch signaling contributed to the activation of satellite cells, leading to a faster rate of repair compared to cells in the placebo group. In the Ibuprofen treated group, there was an increase in Notch-positive satellite cells found in the muscle after 2 d post-induced muscle damage, contributing to the increase in number of satellite cells on day 7 post-damage. In fact, the whole time-course of repair was shifted, overall regenerating the muscle faster than in the placebo group (Mackey et al., 2016).

Quiescence

When satellite cells are not actively regenerating or repairing muscle, the cells are in a non-dividing, or "maintained," state within the uninjured muscle, otherwise known as quiescence. Satellite cells are primarily in a state of quiescence and are only activated upon *in vivo* muscle damage or *in vitro* satellite cell isolation (Dhawan and Rando, 2005). Although these cells appear inactive or dormant, they are strictly regulated by the Notch signaling pathway, which promotes quiescence and therefore inhibits proliferation of the

muscle progenitor cells. The expression of approximately 500 genes promotes satellite cell quiescence (Dumont et al., 2015). Notch signaling helps determine cell fate, thus maintaining cell myogenic lineage (Dumont et al., 2015). Satellite cells still undergo mechanisms of metabolism while in this quiescent state (Almada and Wagers, 2016). Adult quiescent satellite cells express Pax7, but do not express the myogenic regulatory factor, MyoD, which would allow entrance into the cell cycle to begin the process of proliferation and subsequent myogenic commitment (Almada and Wagers, 2016; Dhawan and Rando, 2005; Dumont et al., 2015).

Developing embryonic tissues express the transcription factors Pax3 and Pax7. For example, satellite cells of specific muscles in young mice express Pax3, and Pax7 is expressed in the mouse limb during development. In development, these regulatory transcription factors define myogenic progenitor cells that then form post-natal muscle tissue in the mouse (Dhawan and Rando, 2005).

In addition to Pax7, adult quiescent satellite cells express the extracellular matrix proteins syndecan-3 and syndecan-4, the cell surface marker CD34, and the previously described M-Cadherin. The protein myostatin may also play a role in the promotion of quiescence as mice lacking myostatin exhibited an increase in proliferating satellite cells. The expression of such proteins aid in the identification of satellite cells *in vitro* and aid in maintaining myogenic identity. Desmin, a muscle specific protein, can also help maintain myogenic identity (Dhawan and Rando, 2005). In the cell cycle, the quiescent state is labeled as the G_0 phase and is ready for activation in response to injury (Dumont et al., 2015).

Activation

Muscle damage, injury, or the process of satellite cell isolation causes the activation of satellite cells to regenerate muscle tissue. However, before satellite cells are fully activated, the cells are "primed" for activation in the G_{Alert} phase before entering the G_1 phase of the cell cycle. In this G_{Alert} phase, the cells start to upregulate genes required for entrance into the cell cycle but do not incorporate the thymidine analogs, bromodeoxyuridine (BrdU) or 5-ethynyl-2'-deoxyuridine (EdU), as DNA replication has not yet occurred. The cells begin to enlarge as metabolic activity increases within the cell and more adenosine tri-phosphate (ATP) is produced. As the first division of the satellite cell takes the longest to occur, the G_{Alert} phase increases the rate of initial division when compared to satellite cells in the quiescent G_0 phase. The tight regulation of the G_0 phase by the Notch signaling pathway decreases the efficiency of regeneration compared to the satellite cells in the G_{Alert} phase (Dumont et al., 2015).

The release of various growth factors after injury also causes activation of satellite cells for muscle regeneration through signaling pathways. Hepatocyte Growth Factor (HGF) released at the site of injury causes nearby cells to activate the mTOR signaling pathway, transitioning the cells from the quiescent G_0 phase to the G_{Alert} phase (Dhawan and Rando, 2005; Dumont et al., 2015). This transition to the G_{Alert} phase allows the cells to ultimately prepare for activation, and subsequent proliferation and differentiation. Fibroblast Growth Factor 2 (FGF2) released from the site of injury, activates MAPK signaling pathways within the satellite cell allowing the cells to progress through the cell cycle (Dhawan and Rando, 2005; Dumont et al., 2015). Insulin-like Growth Factor (IGF-1) causes satellite cells to enter the cell cycle through the downregulation of the

transcription factor FOXO1, responsible for the maintenance of quiescence (Dumont et al., 2015).

Satellite cells are in the G_1 phase when they are fully activated, and are hereby referred to as myoblasts (Dhawan and Rando, 2005; Dumont et al., 2015). Proteins expressed in quiescent satellite cells such as: CXCR4, syndecan-4, syndecan-3, and c-met are involved in signaling pathways responsible for activation and proliferation (Dhawan and Rando, 2005). The extracellular matrix component, Decorin, also plays a role in the activation of satellite cells (Dumont et al., 2015). An increase in the expression of Myogenic Determination Protein (MyoD) and Myogenic Factor 5 (MYF5) contributes to the activation of satellite cells (Almada and Wagers, 2016; Dhawan and Rando, 2005) and satellite cell lineage progression is determined, advancing to myoblast formation.

Proliferation

Myoblasts rapidly proliferate once they are fully activated due to the increased expression of the Notch signaling inhibitor, Numb (Dhawan and Rando, 2005). Platelet-derived growth factor (PDGF) released from blood platelets during the clotting process in the injured tissue contributes to the proliferation of satellite cells (Cooper, 2000). Satellite cells proliferate and undergo cell division, or mitosis, where the DNA can divide either symmetrically or asymmetrically. The expression of myogenic regulatory factors, or MRFs, such as MyoD, Myogenin (Myog), and Myf5, revealed that a cell can undergo an asymmetric cellular division (Dumont et al., 2015).

When a myoblast is fully activated and thus ready to proliferate, the cell enters the G_1 phase of the cell cycle. The cell spends most of its time within this phase in the

proliferation process, as this is where the cell primarily grows. At the end of the G_1 phase, the cell must pass through the first checkpoint, set in place to ensure damaged DNA will not replicate in the next phase. If damaged DNA is found at the checkpoint, the protein p53 arrests the cell cycle and the damaged DNA is allowed to repair (Cooper, 2000).

After passing through the first cell cycle checkpoint, the cell then proceeds through the S phase, where DNA replicates (Cooper, 2000). During the S-phase the thymidine analogues, EdU or BrdU can be detected as the cell incorporates these markers while replicating DNA (Cooper, 2000). After the S phase, the cell progresses through the G_2 phase, where the cell prepares for mitosis. The cell then progresses through the second checkpoint which ensures all DNA has successfully replicated. If this process is performed erroneously, the cell will undergo cell cycle arrest and will remain in the G_2 phase until the DNA can be correctly replicated. Damaged or incomplete genetic material must be prevented from being passed on to daughter cells at the time of cell division. Only then will the cell undergo the process of active cell division, or mitosis, otherwise known as the M phase. Human cells typically require about 24 h to complete a cell proliferation cycle, whereas the bacteria yeast takes approximately 30 min to complete a cycle (Cooper, 2000). Once the satellite cell completes an entire proliferation cycle, the daughter cells exit the cell cycle and either return to a quiescent state, thus participating in the self-renewal of the satellite cell pool, or exit the cell cycle and proceed through myogenesis.

Return to Quiescence

In addition to muscle repair, muscle satellite cells replenish the satellite cell pool. This replenishment ensures that muscle is continually able to regenerate upon multiple bouts of muscle damage as the number of muscle satellite cells remains constant (Almada and Wagers, 2016; Dhawan and Rando, 2005; Dumont et al., 2015). Muscle satellite cells are directly involved in cell fate as some satellite cells enter the cell cycle and progress through myogenesis, while other satellite cells are responsible for self-renewal of the stem cell population (Almada and Wagers, 2016; Dumont et al., 2015). Intrinsic and extrinsic signals aid in cell fate determination. An imbalance or dysfunction of these signals, such as that caused by aging, decreases the ability of muscle satellite cells to self-renew as their focus favors towards myogenic progression and repair of muscle rather than self-renewal. The inability of the cells to renew leads to muscle atrophy, a condition commonly seen in aging and muscle deconditioning (Dumont et al., 2015). Therefore, quiescence is a reversible process, allowing for the replenishment of the satellite cell pool (Dumont et al., 2015).

Satellite cell division is dependent on the expression of cell fate determinants (Almada and Wagers, 2016; Dumont et al., 2015). Typically, if a satellite cell undergoes symmetric division during the M phase, both daughter cells produced as a result of cytokinesis, will contain transcriptional factors that enable them to replenish the satellite cell pool. If an asymmetric division occurs, one daughter cell will most likely participate in self-renewal of the quiescent satellite cell pool, while the remaining, committed daughter cell will proceed through the myogenic process to contribute to regeneration and repair of the damaged muscle (Dumont et al., 2015). The committed daughter cell that

proceeds through myogenic lineage is Myf5-positive and thus Numb, the Notch signaling pathway inhibitor, is upregulated. Whereas, the non-committed daughter cell that replenishes the satellite cell pool is Myf5-negative and the Notch signaling pathway is upregulated (Almada and Wagers, 2016; Dumont et al., 2015). Pax7 expression in the two asymmetric daughter cells determines cell fate as Pax7 activates the subsequent expression of Myf5, and thus the ability of the daughter cells to proceed through myogenic lineage progression (Dumont et al. 2015). MyoD and Myog were also distributed in daughter cells thus contributing to cell fate. For example, $Pax7^{+}MyoD^{-}$ cells contribute to self-renewal of the satellite cell pool, whereas $Pax7^{-}MyoD^{+}$ daughter cells commit the cells to proceed through myogenesis. Similarly, $Pax7^{+}Myog^{-}$ daughter cells also contribute to self-renewal of the satellite cell pool and $Pax7^{-}Myog^{+}$ daughter cells proceed through myogenesis (Dumont et al., 2015). The type of division satellite cells partake in directly allows them to contribute to the needs of the muscle. Symmetric divisions of template DNA in daughter cells promote self-renewal of the satellite cell pool. Asymmetric template DNA divisions lead to one cell retaining its $Pax7^{+}$ expression, thus leading to the renewal of the satellite cell pool. Meanwhile, the other cell contains the replicated/newly synthesized DNA which expresses myogenic transcription factors that lead to myogenic lineage commitment, helping the muscle repair detected damage (Dumont et al., 2015).

Differentiation

Myoblasts exit the cell cycle, or undergo mitotic arrest (Feghali et al., 1992), and have the capability to progress through terminal myogenic lineage as the myogenic

regulatory factors, MyoD, Myogenin, and MRF-4 gene, expression increases (Dhawan and Rando, 2005). When myoblasts become confluent, they align and fuse, creating multinucleated myotubes. As more and more myotubes fuse together, myofibers arise, which contribute to regeneration and the repair of muscle both *in vivo* and *in vitro* (Almada and Wagers, 2016). These myofibers will continue to increase in size and adopt contractile properties once they mature (Feghali et al., 1992). During differentiation, sarcomeric proteins and membrane proteins are also expressed as these proteins are required to build a functional muscle (Feghali et al., 1992). Genes that also encode acetylcholine receptors, creatine kinase, and myosin heavy chain increase as these components are associated with mature, functional muscle (Dhawan and Rando, 2005).

As myofibers develop contractile proteins, various fiber types arise due to the expressed sarcomeric protein isoforms and the metabolic activity of myosin heavy chain (Feghali et al., 1992). The expression of these sarcomeric protein isoforms depend on location of the muscle, the development period of the new fiber, and the work the fiber expects to perform. In addition, other extrinsic factors in the body can also influence the sarcomeric isoform expression. The sarcomere contracts allowing the muscle to contract, and relaxes, allowing the muscle to relax.

Myosin heavy chain isoforms also aid in the determination of the developmental stage of the muscle, such as in the classification of embryonic, perinatal, or adult myofibers (Feghali et al., 1992). Embryonic myosin heavy chain isoforms are prominent in all stages of muscle development. Therefore, the embryonic sarcomeric protein antibody, MF-20, is commonly used as a marker of differentiation *in vitro* to assess myotubular development (Feghali et al., 1992). Fiber types represented in mature muscle

are pre-determined based on different lineages of myoblasts responsible for generating either fast, slow, or mixed fiber type (Feghali et al., 1992).

Embryonic myosin isoforms are expressed during embryonic development, and may later re-express during muscle regeneration post-injury. Such embryonic myosin isoforms are expressed 2-3 d post-injury until roughly 2-3 weeks post-injury, when adult myosin isoforms begin to be expressed and fiber type is clearly determined (Schiaffino et al., 2015). These embryonic myosins indicate muscle regeneration and provide structures that aid in the formation of myofibrils during myogenesis. The gene responsible for the expression of embryonic myosin heavy chain is MYH3, which is regulated by the myogenic regulatory factors MyoD and Myf5 (Schiaffino et al., 2015). This process of differentiation and fusion allows the formation of functional, contractile skeletal muscle (Feghali et al., 1992).

Insulin-like Growth Factor (IGF-1)

Insulin-like Growth Factor-1 (IGF-1) promotes growth and repair of skeletal muscle after injury (Pyne et al., 2018). This growth factor has affinity for Insulin receptors, IGF-1 receptors, as well as IGF-2 receptors located on the cell membrane. In L6A1 myoblasts, IGF-1 is responsible for myogenic differentiation through contributing to increased expression of the Myogenin gene (Florini et al, 1991). Other myogenic regulatory factors such as MyoD, Myf5, and Mrf4 are either not expressed or undetectable in differentiating cells in the L6A1 cell line. Therefore, IGF-1 is directly correlated to increasing the mRNA expression of Myogenin (Florini et al., 1991).

Myogenin is universally known as a regulatory marker of late stage differentiation (Florini et al., 1991).

Septic rats were utilized in a study in which IGF-1 stimulated protein synthesis in the gastrocnemius muscle (Vary, 2006). Sepsis is a model for protein wasting since around 70% of protein loss comes from skeletal muscle; protein degradation is greater than protein synthesis (Vary, 2006). IGF-1 increased the rate of protein translation and therefore contributed to increased protein synthesis in the gastrocnemius muscle. IGF-1 activated the mammalian target of rapamycin (mTOR), a kinase involved in the protein synthesis pathway, whereas sepsis decreases activation of mTOR, leading to a decrease in protein synthesis (Vary, 2006).

In equine, IGF-1 does not lead to an increase in proliferation of equine muscle satellite cells, nor lead to an increase in fusion of myotubes *in vitro*, a marker of differentiation. However, IGF-1 does contribute to an increase in protein synthesis evidenced when phosphorylation of Akt visually increased when examined on Western blot (Pyne et al., 2018).

Glycogen Synthesis

The body's main source of energy, glucose, is stored in the body as glycogen, a branched polysaccharide that consists of numerous glucose molecules. Glycogen synthesis primarily occurs in skeletal muscle and can be stored in either the liver or skeletal muscle (DeFronzo and Tripathy, 2009). In fact, horses with a low concentration of glycogen in their muscles tend to fatigue more rapidly, because low intensity exercise cannot be sustained for long periods of time without adequate energy reservoirs (Davie et

al., 1999; Waller et al., 2009a). Low muscle glycogen concentrations are known to limit exercise capacity (Davie et al., 1999). High intensity exercise increases lactate and ammonia concentration, contributors of fatigue (Davie et al., 1999).

A study was performed in which exercise intensity and muscle glycogen concentration was compared to onset of fatigue in Thoroughbreds (Davie et al., 1999). Six geldings underwent two exercise protocols. The first exercise protocol was of low intensity and long duration, while the second protocol consisted of short bouts of high intensity exercise. Geldings in the control group did not undergo any exercise prior to the exercise test. These protocols were designed by the investigators to deplete the glycogen present in the middle gluteal muscle before undergoing an exercise test 5 h post-depletion. Glycogen depleted 20-30% in treatment groups who exercised prior to the exercise test (Davie et al., 1999). Geldings who participated in glycogen depleting exercise prior to the treadmill exercise test fatigued quicker when compared to geldings in the control group who had not participated in either glycogen-depleting protocol. Slow-twitch muscle fibers were found to be more depleted of glycogen when undergoing low intensity, long duration exercise. Fast-twitch muscle fibers were more affected by depletion of glycogen when undergoing high intensity, short duration exercise. Although the concentration of muscle glycogen was significantly lower in horses who underwent glycogen-depletive exercise compared to the control, no difference appeared in glycogen concentration between these two treatment groups. In humans, onset of fatigue and exhaustion highly correlates to glycogen concentration. However, glycogen stores may not be the only contributor to fatigue as other metabolic and cardiovascular responses may also ensue due to exercise, such as an increase in core temperature and the

accumulation of ammonia (Davie et al., 1999). Although researchers in this study were able to achieve glycogen depletion of 20-30%, such amounts did not contribute to increased glycolysis rates, even though plasma glucose concentration increased in the blood gradually over time in all groups (Davie et al., 1999).

Both long duration with moderate intensity and short duration with high intensity exercise lead to dehydration. Exercise also diminishes electrolytes and glycogen in skeletal muscle (Waller et al., 2009a). Rehydration has been speculated to contribute to an increased rate of glycogen re-synthesis. In comparison to humans and rats, the rate of glycogen synthesis is slower in horses because they require between 48-72 h to fully replenish muscle glycogen reserves, whereas humans require less than 24 h and rats require about 2.5 h (Waller et al., 2009a; Waller et al., 2009b). Feeding glucose to horses in efforts to replenish glycogen at a faster rate has not been successful due to the sensitivity of horses to large quantities of carbohydrates as carbohydrates are suspected to contribute to colic and laminitis. Hydration with water, which does not contain electrolytes, does not lead to exercise recovery as electrolytes are required to maintain osmotic balance in extracellular and intracellular areas (Waller et al., 2009a). Glycogen replenishment increases post-exercise with intravenous infusion of saline (Waller et al., 2009a). Although not assessed in horses, replenishment of water and potassium may contribute to increased rates of glycogen synthesis as both potassium and water are released when glycogen is utilized and muscle undergoes contraction (Waller et al., 2009a). Therefore, the evaluation of post-exercise hydration rates in horses may correlate to faster post-exercise recovery and quicker rates of glycogen synthesis (Waller et al., 2009a).

A study with six Standardbred horses analyzed the rate of exercise recovery as a result of rehydration with an electrolyte solution (Waller et al., 2009a). Muscle biopsies were extracted from the gluteus medius muscle and blood samples were drawn after horses participated in an exercise test simulating a three-day event. Post-exercise, horses received their regular hay and grain diet and water was provided ad libitum. In addition, horses in the treatment group received an electrolyte solution via the nasogastric tube. Electrolyte supplementation increased the rate of glycogen re-synthesis as exhibited with no difference in muscle glycogen concentration at 4 h post-exercise compared to glycogen concentrations prior to exercise. Glycogen concentration at 24 h in the electrolyte supplemented group slightly exceeded concentrations exhibited before exercise. The exercise test was successful at depleting glycogen around 20% and exercise protocols were chosen to minimize injury (Waller et al., 2009a). In comparison, at 4 h post-exercise, glycogen concentration in the control group remained below the values exhibited pre-exercise. Supplementation of electrolytes increased intracellular fluid volume by 18%, in comparison to 4% in the control group. Electrolyte supplementation contributed to total rehydration as exhibited by a pre-exercise value of plasma protein concentration (Waller et al., 2009a). No thirst response is triggered in dehydrated horses as it is in humans, as electrolytes lost in sweat are hypertonic in comparison to plasma, resulting in dehydration that is hypotonic (Waller et al., 2009a). In equine, intracellular water and potassium are preserved in muscle despite overall dehydration and glycogen depletion. Of the 8 L of sweat lost from the horses, total body percentages of potassium (K^+), sodium (Na^+), and chloride (Cl^-) equaled roughly 1%, 4%, and 8%, respectively (Waller et al., 2009a). Muscle electrolytes were not significantly different between the

treatment group and the control. Exercise promotes glucose uptake in the muscle (Waller et al., 2009a).

Acetate, a volatile fatty acid produced and absorbed from the large intestine in hindgut fermentation, is the main energy source used by equine skeletal muscle at rest that may also contribute to glycogen synthesis (Waller et al., 2009b). Acetate generates between 30-40% of energy required for maintenance metabolism (Waller et al., 2009b). Acetate and glucose are the main energy sources used by hind limb muscles of equine. Acetate is converted to Acetyl-coA, (and acetylcarnitine) which enters the tricarboxylic acid (TCA) cycle and subsequently generates CO_2 and water, and aids in the generation of adenosine tri-phosphate (ATP) in the mitochondria. In rats, acetate and glucose supplementation *in-vitro* stimulated glycogen synthase activity, therefore contributing to glycogen synthesis, unlike supplementation with only glucose (Waller et al., 2009b). In pigs, skeletal muscle glucose uptake and glycogen increased as greater quantities of acetate were fed (Waller et al., 2009b).

Nine horses participated in a study in which acetate was administered via nasogastric tube post-exercise to determine the effects acetate might have on glycogen synthesis in skeletal muscle (Waller et al., 2009b). Horses underwent exercise on a high-speed treadmill in efforts to deplete muscle glycogen content and induce sweat loss of 8-10 L. The exercise was meant to simulate an endurance test and speed typically seen in horses during a three-day event. Horses were fed grain, hay, and allowed free access to both water and a salt block. Muscle biopsies were extracted from the gluteus medius and venous blood was collected. Horses in the treatment group received 8 L of acetate-acetic acid solution via a nasogastric tube, and horses in the control group were only given hay

and grain (Waller et al., 2009b). The exercise protocol, chosen to minimize musculoskeletal injury, decreased muscle glycogen content by about 21% in the treatment group and by about 17% in the control group (Waller et al., 2009b). Acetate was supplemented as an alternative energy source in efforts to "spare" glucose for glycogen re-synthesis. Plasma acetate concentration was increased as a result of treatment. acetyl coA and acetylcarnitine concentrations were increased in skeletal muscle by 4 h post-treatment/exercise in both treatments, suggesting successful tissue absorption of the supplement. However, these concentrations were significantly increased in the acetate treatment group. As a result of acetate supplementation, glycogen synthesis was enhanced during the first 4 h post-exercise when compared to the control group. However, the groups were not different 24 h post-exercise and muscle glycogen concentrations were fully restored to pre-exercise values (Waller et al., 2009b). In fact, glycogen concentration was fully restored 4 h post-exercise in skeletal muscle in horses supplemented with acetate-acetic acid solution. The treatment revealed no effect on glucose metabolism intermediates, in the gluteus medius muscle (Waller et al., 2009b). Therefore, acetate can be supplemented post-exercise to aid in the re-synthesis of glycogen and muscle recovery (Waller et al., 2009b). The rate at which horses re-synthesized muscle glycogen was similar to maximal rates achieved in glycogen-depleted horses following intravenous glucose infusion (Waller et al., 2009b). Post-exercise recovery supplementation with acetate was successful in sparing glucose for glycogenesis. Acetate increased concentrations of acetyl-coA and acetylcarnitine in the muscle, suggesting acetate was used as the primary source of oxidation in the TCA cycle and subsequent ATP production (Waller et al., 2009b). Acetate supplementation may

possibly cause a decrease in Phosphofructokinase (PFK) activity, leading to inhibition of glycolysis, thus allowing glycogen synthesis to occur as it does in rats (Waller et al., 2009b). Overall, acetate supplementation increased the rate of glycogen replenishment in horses after glycogen-depleting exercise (Waller et al., 2009b).

It was unknown whether the supplementation of glucose, either orally or intravenously, could increase glycogen synthesis, and decrease post-exercise recovery after glycogen-depleting exercise. Seven horses participated in a study in which muscle glycogen depleted by 50% following treadmill exercise (Goer et al., 2006). Post-exercise, horses were supplemented with 1) oral glucose, 2) a bolus of intravenous glucose, or 3) not supplemented with glucose, the control group. Blood samples were collected to measure insulin and glucose concentrations before exercise and 6 h post-exercise (Goer et al., 2006). Muscle biopsies were also collected before exercise, 3 h post-exercise, and again at 6 h post-exercise to examine glycogen content and glycogen synthase enzyme activity (Goer et al., 2006). Average plasma concentration and blood serum insulin concentrations significantly increased in both the oral and intravenous routes compared to the control group (Goer et al., 2006). Immediately following exercise, glycogen content remained the same between the three groups, concluding that glycogen was successfully depleted in all treatment groups. Glycogen storage rates significantly increased with intravenous glucose supplementation compared to both the oral glucose treatment group and the control group at both 3 h and 6 h post-exercise (Goer et al., 2006). Glycogen content significantly increased in the treatment group supplemented with intravenous glucose compared to both the oral and control groups at 6 h post-exercise (Goer et al., 2006). In addition, glycogen synthase activity significantly increased in the intravenous

glucose treatment group compared to both of the other treatment groups at 3 h post-exercise (Goer et al., 2006). In conclusion, intravenous administration of glucose at a concentration of 3 g/kg, enhanced glycogen synthesis and storage 6 h post-exercise and may contribute to faster post-exercise recovery (Goer et al., 2006).

Disease states can affect how the body stores and metabolizes energy. Insulin resistance is the lack of sensitivity of tissues such as adipose, liver, and skeletal muscle, in response to insulin release from the pancreas and can often lead to Type 2 diabetes (DeFronzo and Tripathy, 2009; Jimenez-Chillaron et al., 2002). When blood glucose is high, beta-cells in the pancreas release insulin. If glucose uptake is impaired in the body, more insulin is released from the pancreas trying to overcome the impairment. As a result, the number of insulin receptors decreases, therefore exacerbating the insulin resistance disorder and leading to a loss of beta-cell function (DeFronzo and Tripathy, 2009). Skeletal muscle is the predominant site of glucose uptake in the body, as 80-90% of glucose is absorbed into skeletal muscle (DeFronzo and Tripathy, 2009). Insulin resistance occurs as an early indicator of Type 2 diabetes and impairs glycogen synthesis through reduced activity of glycogen synthase enzyme, the rate limiting step of glycogen synthesis (DeFronzo and Tripathy, 2009). Insulin regulates enzymes that are responsible for metabolizing glucose upon entering the cell via the GLUT4 transporter (DeFronzo and Tripathy, 2009). Insulin signaling leads to a cascade of phosphorylation and dephosphorylation reactions. For example, insulin binds to the insulin receptor, leading to the activation of three tyrosine kinases on the intracellular component of the receptor. Intracellular signaling leads to the eventual activation of Phosphatidylinositol-kinase (PI3-K) and downstream activation of Protein Kinase B, also known as Akt (DeFronzo

and Tripathy, 2009). After which, the GLUT4 transporter is translocated from the cytoplasm to the sarcolemma, supporting glucose uptake. The balance in this signaling pathway is essential for the maintenance of glucose metabolism in the muscle in response to insulin (DeFronzo and Tripathy, 2009). Insulin resistance is characterized by a decrease in PI3-K activity, subsequently leading to a decrease in glucose uptake and glycogen synthesis (DeFronzo and Tripathy, 2009). Muscle glucose homeostasis depends on tissue sensitivity to insulin.

A model for Type 2 diabetes and obesity is the Zucker Diabetic Fatty (ZDF) rat of which both males and females are insulin resistant (Jimenez-Chillaron et al., 2002). This model contains a genetic mutation for the leptin receptor, the hormone secreted from the stomach that signals to the brain that it is "full." In this study, rats were genetically transferred with glucokinase. In this particular animal model, glucose uptake is impaired in the soleus, the gastrocnemius, and the diaphragm muscles (Jimenez-Chillaron et al., 2002). However, glucose uptake impairment was not a result of a decrease in GLUT4 transporters (Jimenez-Chillaron et al., 2002). Instead, the glucose uptake impairment was contributed by the auto-inhibition of hexokinase II by its product, glucose-6-phosphate. ZDF rats expressing glucokinase (GK) increased GLUT4 transporter content, which therefore increased glucose uptake. In GK-expressing rats, HKII protein increased by 3-fold as seen on Western blot (Jimenez-Chillaron et al., 2002). In these same animals, HKII activity increased by 140% in comparison to the controls (Jimenez-Chillaron et al., 2002). GLUT4 transporter protein increased by 4-fold in comparison to controls, while content of GLUT1 and activity of HKI were unchanged (Jimenez-Chillaron et al., 2002).

Another disease state that affects glucose storage in skeletal muscle is a condition commonly seen in Quarter horses, called Polysaccharide Storage Myopathy, or PSSM (Annandale et al., 2004). Horses with this disease are characterized as having stiff muscles, muscle fasciculations, cramping, and hesitation to move after partaking in light exercise (Annandale et al., 2004). This disease state enhances glycogen storage secondary to an increase in glucose transporters at the cell membrane, such as GLUT 4, and to a lesser extent, GLUT1 (Annandale et al., 2004). Gluteus medius muscle biopsies were isolated from eleven horses with PSSM (Annandale et al., 2004). The objective of the study was to determine if horses with PSSM had higher muscle glycogen concentrations than horses without PSSM. In fact, horses with PSSM had 1.8 times more glycogen in their muscles than control horses (Annandale et al., 2004). However, no differences existed with regard to presence of GLUT4 transporter between the groups, nor in glucose 6-phosphate concentrations or glycogen branching enzyme activity (Annandale et al., 2004). Significantly fewer GLUT1 transporters were present in PSSM muscle compared to the control group (Annandale et al., 2004). The GLUT1 transporter is responsible for glucose uptake independent of insulin. Signaling pathways responsible for glucose uptake in the muscle activate in response to the presence of insulin and exercise. The activation of these signaling pathways causes GLUT4 transporters to move from the vesicles within the cytoplasm to the sarcolemma where glucose can be absorbed. Mice with increased expression of GLUT4 transporters revealed a higher concentration of glycogen in their muscles whereas horses with PSSM did not (Annandale et al., 2004). Although insulin receptors in PSSM horses were present in the same proportions as in the control horses, activity of the receptors, or perhaps signaling pathways involved in GLUT4 translocation,

could be compromised in horses with PSSM. Similarly to horses with PSSM, horses with a PFK enzyme deficiency exhibit increased concentrations of glucose-6-phosphate activity, leading to an increase in glycogen synthesis. As a result, glycogen synthase enzyme is more active than glycogen branching enzyme, leading to the formation of a poorly branched glycogen molecule (Annandale et al., 2004). This imbalance of enzymes within the muscle results in a disruption of metabolism. For example, mice who overexpress glycogen synthase enzyme and have increased expression of GLUT1 suffer from muscle wasting disorders (Annandale et al., 2004).

Cellular Metabolism

Adaptable energy metabolism allows cells to undergo certain functions such as the maintenance of quiescence, proliferation, self-renewal of the satellite cell pool, and differentiation. Thus, adaptable energy metabolism allows cells to determine their fate (Folmes et al., 2012). Substrates such as glucose, amino acids, and fatty acids provide the energy that satellite cells need to survive an always changing environment (Folmes et al., 2012). In fact, the ability of satellite cells to adapt to certain types of energy metabolism provides cells with specific behavior and cellular functions that define satellite cells as "plastic," or versatile (Folmes et al., 2012). A balance of catabolism and anabolism is used to maintain cell function and fate. Catabolism is the process of breaking down substrates to produce energy. Alternatively, anabolism is the construction of substrates from its precursors (Folmes et al., 2012).

Proliferating cells with a sufficient supply of glucose mainly utilize glycolysis for energy generation (Folmes et al., 2012; Vander Heiden et al., 2009). The PI3K signaling

pathway regulates glucose uptake via hexokinase, regulates cell growth, and can increase the expression of glucose transporters (Vander Heiden et al., 2009). Glycolysis breaks down glucose molecules to generate ATP quickly for this anaerobic process to occur. Since glucose is catabolized to glucose-6-phosphate, some of these metabolites are shunted over to the Pentose Phosphate Pathway (PPP), where NADPH and ribose-5-phosphate are produced (Folmes et al., 2012; Pratt and Cornely, 2011). NADPH is an antioxidant that abolishes reactive oxygen species (ROS), and ribose-5-phosphate is a building block of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Pratt and Cornely, 2011). Although the PPP does not produce or consume ATP, the PPP is still essential in cellular replication as DNA and RNA are newly synthesized in the daughter cells within the cell cycle (Folmes et al., 2012; Pratt and Cornely, 2011). Therefore, proliferating cells require the fast production of ATP to sustain demands for nucleotides, amino acids, and lipids required for the formation of newly synthesized cells (Vander Heiden et al., 2009). In order for cellular lifespan to increase, the expression of glycolytic enzymes must also increase (Folmes et al., 2012). If glucose is abundant in the cellular environment, ATP generation can exceed such levels as those generated in oxidative phosphorylation (Folmes et al., 2012). Inhibiting the process of glycolysis, therefore halting ATP production, promotes cell death (Folmes et al., 2012: Vander Heiden et al., 2009).

The master metabolic regulator, STAT3 controls the switching of metabolism from non-oxidative to oxidative and vice versa (Folmes et al., 2012). The transcription factor, OCT4 shares many similarities with STAT3 and may also help regulate cellular metabolism between glycolysis and aerobic respiration (Folmes et al., 2012). Within the

OCT family of transcription factors, OCT1 absence can cause cellular metabolism to switch from glycolysis to mitochondrial oxidative phosphorylation (Folmes et al., 2012). Energy metabolism is regulated in efforts to promote cellular homeostasis (Folmes et al., 2012).

Once muscle satellite cells have differentiated, glycolytic metabolism is no longer required, and cells therefore switch their metabolic focus to mitochondrial oxidative metabolism (Folmes et al., 2012; Vander Heiden et al., 2009). Differentiated cells no longer proliferate, so their metabolic focus is turned into sustainable long-lasting energy that can be used for specific functions, such as muscle contraction (Folmes et al., 2012). In differentiated cells, substrates undergo complete oxidation through the TCA cycle. A transfer of reduced cofactors occurs in the electron transport chain, and ATP is produced as a result of oxidative phosphorylation (Folmes et al., 2012). Although aerobic respiration requires more time to produce ATP, the yield is significantly higher than the ATP for glycolysis. Aerobic respiration produces between 36 – 38 net ATP per one molecule of glucose, and anaerobic respiration produces only 2 net ATP per one molecule of glucose (Folmes et al., 2012; Vander Heiden et al., 2009). Satellite cells begin to differentiate in response to an increase in mitochondrial DNA (mtDNA), increased expression of TCA cycle enzymes, as well as increased presence of electron transport chain subunits. ATP can therefore be produced more efficiently than anaerobic respiration and sustain the differentiating satellite cells over a period of time (Folmes et al., 2012). Therefore, as satellite cells proliferate, they undergo glycolytic metabolism and oxidative metabolism is inhibited. In contrast, when satellite cells differentiate,

oxidative metabolism is promoted and glycolytic metabolism is suppressed (Folmes et al., 2012).

Summary

Intense exercise often damages muscle. In response, muscle undergoes repair in which functional, contractile muscle is formed. To begin the repair process, satellite cells activate, entering the cell cycle where they grow and divide and after which can either proceed through myogenesis to generate muscle, or return to a state of quiescence therefore renewing the satellite cell pool. Satellite cell pool replenishment allows the muscle to retain its regenerative capacity. Myogenic regulatory factors are expressed during myogenesis are responsible for determining the fate of the satellite cell through the upregulation or downregulation of terminal differentiation factors.

Glucose is required for satellite cell proliferation. Glucose is an energy source that is stored in the body as glycogen. The synthesis of glycogen depends on gene expression, protein translation, and enzymatic activity involved in the glycogen synthesis pathway. Glycogen branching enzyme and glycogen synthase work concurrently to form the multi-branched polymer of glucose. As satellite cells differentiate, reliance on oxidative phosphorylation increases in order to meet energy demands. Sustained changes in homeostatic metabolism can greatly alter the cell's ability to respond to stress.

Cellular metabolism is often affected by pharmaceuticals. Furosemide, the diuretic commonly used to treat and manage EIPH, has recently been under scrutiny in the Thoroughbred racing industry. Followers of the sport are concerned the drug may be causing the dramatic increase in musculoskeletal related fatalities such as those reported

at Santa Anita Racetrack earlier this year. Although proven to be effective at reducing the severity of EIPH, the drug is regarded as a "performance-enhancing" drug. Therefore, it is uncertain exactly how the drug enhances performance. It may be due to the short-term weight loss that occurs a few hours before racing, or through an undiscovered muscular stimulatory effect. The research outlined hereafter describes the effects furosemide has on the recovery, or regeneration, of the damaged muscle post-exercise. Although thought not to impact muscle due to the drug's short-term effects, furosemide does indeed impact myogenesis, delaying muscle recovery.

MATERIALS AND METHODS

Animals

Twelve Thoroughbred geldings (6.75 y ± 0.54) were used as donors for skeletal muscle satellite cell isolations. Horses were housed in dry lot paddocks and fed a forage-based diet that met or exceeded their requirements to total digestible nutrients. All minor surgical techniques were approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee.

Equine Muscle Biopsies

Geldings were weighed on a Tru-Test EW5 scale connected to a RadioShack 3AMP Regulated Power Supply prior to taking biopsies to ensure adequate dosages of sedation were administered. Geldings were sedated with either XylaMed™ 100 mg/mL (Xylazine, 0.5 mL/100 lbs BW) or Dormosedan® 10 mg/mL (detomidine hydrochloride, 0.2-0.4 mL/220 lbs BW) intravenously in either the left or right jugular vein. Once sedated, an approximate 10 cm x 7 cm rectangle of hair was clipped with electronic clippers on the top of the rump, superficial to either the left or right mid-gluteal muscle of the horse. The shaved area was surgically prepared, first with 70% Ethanol (EtOH) soaked gauze sponges in a circular fashion, starting in the middle of the shaved patch, then working outward to ensure dirt was not re-introduced to the area. The shaved area was scrubbed next with 2% Chlorohexidine Gluconate (Vetoquinol USA, Inc.), diluted with ddH_2O soaked gauze for five minutes to sterilize the area. A final scrub with 70% EtOH was performed, once again in a circular fashion, to remove the chlorohexidine soap from the horse's skin and remove any dirt from the area. Two milliliters of 2% Lidocaine (VetOne®) was administered subcutaneously (SQ) at the biopsy site prior to the creation

of a small incision site through the skin and muscle fascia. The VACORA® Vacuum assisted biopsy system (BARD Peripheral Vascular, Inc., Tempe, AZ) equipped with a 10 gauge (g) needle was inserted through the incision at a 45^{o} angle with the needle tip pointing caudal to the barrel of the horse. The muscle biopsy (~200 mg) was extracted and transferred into a 15 mL Falcon conical tube containing 5 mL of sterile PBS supplemented with 5% penicillin and streptomycin (Gibco), 5% Fetal Bovine Serum (FBS, HyClone Lot no. AB1012), 0.2% Gentamicin (Gibco) and is hereby referred to as treated PBS. The biopsy retrieval process was repeated for a total of two specimen collections, with the second specimen isolated more cranial than the first within the gluteal muscle.

The incision was cleaned by applying pressure with gauze soaked in 70% EtOH, followed by the application of a small globule of Triple Antibiotic Ointment on dry gauze. Horses were monitored twice daily for seven days for signs of infection.

Equine Satellite Cell Isolation

Biopsies were washed three times with treated PBS to remove blood and extraneous debris. The sample was dissected free of visible connective tissue. The muscle was minced into small pieces, weighed and digested with protease (P5147, Sigma; 1 mg/1 mL in PBS) at a final concentration of 1 g tissue/10 mL enzyme. Digestions were performed in a 37^{o}C water bath for 40 min with gentle agitation every 15 min. The tissue slurry was passed through a 70 μm Falcon Nylon Cell Strainer (Corning, Corning, NY) atop a 50 mL Falcon conical tube. The muscle fragments were washed with 5 mL of treated PBS and collected into a sterile centrifuge tube. Muscle fragments were vortexed in treated PBS for 2-3 min to liberate the satellite cells from the fibers. Cells were

collected by passage through a 40 μm Falcon Nylon Cell Strainer and centrifugation (800 X g, 5 min). Cells were cultured on entactin-collagen IV-laminin (ECL, EMD Millipore) coated 100 mm tissue culture ware (Corning, NY) in growth media (GM) comprised of high glucose Dulbecco's modified Eagle media supplemented with 20% FBS, 5% P/S, 0.2% Gentamicin, and 4 ng/mL rhFGF2 (Gibco) at 37°C and 5.0% CO_2. Media was exchanged every 48 h and cells were passaged at 50% confluency by physical removal (scraping) from the substratum. Passaged satellite cells were used immediately or stored frozen in GM containing 10% Dimethyl Sulfoxide (DMSO) in the vapor phase of liquid nitrogen.

Furosemide Dosage Determination

Blood volume in the horse is approximately 10% of total body weight (Marlin and Nankervis, 2002). Furosemide (Lasix, VetOne®) is administered at a concentration of 1.0 mg/kg of body weight. Using these values, maximal systemic concentrations of furosemide were calculated as 10 μg/mL. An off-label excess concentration was used at 100 μg/mL furosemide.

Proliferation Assay

Satellite cells were seeded at a density of 5,000 cells/well in ECL coated 24-well Costar® plates (Costar Incorporated, Corning, NY), with duplicate wells per treatment. Cells were cultured in high glucose DMEM supplemented with 1% FBS, 1% P/S, 0.2% Gentamicin and either 0, 10, or 100 μg/mL of furosemide for 24 h with 10 μM EdU supplemented to the media 2-h prior to assay termination. Cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature and permeabilized with 0.5% Triton X-100 in PBS (Fisher BioReagents, New Plains, New Jersey) at room temperature

for 20 min. The cells were washed twice with 3% Bovine Serum Albumin (BSA; Fisher BioReagents, New Plains, New Jersey) in PBS. Click-iT® reaction cocktail was added to each well and the plate was incubated for 30 min at room temperature while protected from light. The cells were washed once with 3% BSA in PBS and a final wash with PBS. Hoechst 33342 secondary antibody (5 μg/mL) was added to the wells and incubated for 30 min at room temperature, while protected from light. Cells were then washed twice with PBS and visualized with a Nikon Eclipse TS100 epi-fluorescent microscope (Nikon Imaging Corp., Melville, NY) connected to a CoolSNAP HQ2 Camera (Photometrics, Tuscon, AZ) with shutter speed and image capture controlled with NIS-Elements AR Ver4.13.00 software (Nikon). Percent EdU positive cells and total cell number were obtained using the automated counting feature within the HCA Jobs Explorer menu in the NIS-Elements AR Ver4.13.00 software (Nikon). Twenty images were quantified per well, with duplicate wells averaged.

Early and Mid-Differentiation Assays (Pax7, Myogenin)

Equine satellite cells were seeded into 24-well Costar® cell culture treated plates (Corning, Incorporated, Corning, NY) at a density of 10,000 cells/well. When cells reached 40-50% confluence, they were treated with low glucose DMEM (Gibco) supplemented with 1% FBS, 1% P/S, 0.2% Gentamicin and either 0, 10, or 100 μg/mL furosemide (VetOne®) for 48 h. After 48 h in treatment, cells were washed with PBS and fixed with 4% PFA for 15 min at room temperature. Cells were permeabilized in 3% BSA and 0.1% Triton X-100 in PBS for 20 min at room temperature. Cells were washed three times with PBS and incubated overnight at 4° C in either Pax7 or myogenin hybridoma supernatant diluted 1:1 with PBS and 0.1% Triton X-100. The following day,

cells were washed with PBS and incubated with goat-anti-mouse IgG AlexaFluor568 secondary antibody (1:200) and Hoechst 33342 secondary antibody (1:1000) for 1 h at room temperature while protected from light. After extensive washes with PBS, representative photomicroscopic images were captured using a Nikon Eclipse TS100 epi-fluorescent microscope (Nikon, Melville, NY) connected to a CoolSNAP HQ2 camera (Photometrics, Tuscon, AZ) with shutter speed controlled by NIS Elements software. Percent Pax7 positive cells were calculated as the number of Pax7 (red) cells divided by total cell number as identified with Hoechst 33342 (blue) in the image. Eight images were counted per well and duplicates for each treatment were averaged. Percent myogenin positive cells were calculated as the number of myogenin positive cells (red) divided by the total cell number in the image as indicated with Hoechst 33342 (blue). Twenty images were quantified per well using the HCA Jobs Explorer feature of NIS Elements, and duplicate wells were averaged.

Late differentiation assay (MF20)

Equine satellite cells were seeded at a density of 35,000 cells/well into a 12-well Costar® cell culture treated plate (Corning, Inc., Corning, NY) coated with ECL. Cells were grown to 70-80% confluence and treated with low glucose DMEM supplemented with 1% FBS, 1% P/S, 0.2% Gentamicin and either 0, 10, or 100 µg/mL furosemide (VetOne®) in the presence or absence of 100 ng/mL recombinant human IGF-1 (R&D Systems, Minneapolis, MN). Cells were then incubated at 37°C with 5% CO_2 for 48 h followed by fixation with acidified formalin (AFA; equal parts ethanol, glacial acetic acid and ddH_2O) for 8 min. Cells were washed 3 times with PBS followed by permeabilization with 0.5% Triton X-100 and 1% BSA in PBS for 20 min at room

temperature. Cells were incubated with undiluted MF20 primary antibody hybridoma supernatant overnight at 4°C. The following day, cells were washed with PBS and incubated with goat-anti-mouse IgG AlexaFluor568 (1:200) and Hoechst 33342 (1:1000), in 0.5% Triton X-100, 1% BSA in PBS for 1 h. Cells were washed with 1x PBS and representative images were acquired with a Nikon Eclipse TS100 epi-fluorescent microscope (Nikon Imaging Corp., Melville, NY) connected to a CoolSNAP HQ2 camera (Photometrics, Tuscon, AZ). Eight images per well were used for calculation of a fusion index defined as the number of myonuclei (blue) within a myotube (red) divided by the total number of myonuclei in the field of view (all blue), multiplied by 100. The definition of a myotube was greater or equal to three myonuclei fused into one myotube. Myotube area (%), was defined as the area of the myotube (Binary Area) divided by the area of the field of view (Measured Area) multiplied by 100.

Glycogen Assay

Equine satellite cells were seeded at a density of 60,000 cells/well into 6-well Costar® cell culture treated plates (Corning, Inc., Corning, NY). Once cells were about 80% confluent, they were treated with low glucose DMEM supplemented with 1% FBS, 1% P/S, and 0.2% Gentamicin with either 0, 10, or 100 µg/mL furosemide (VetOne®) ± 100 ng/µL rhIGF-1 (R&D Systems, Inc., Minneapolis, MN) for 48 h. Cells were washed three times with PBS and scraped into 200 µL of ddH_2O. Samples were sonicated for 15 s at 30% amplitude (QSonica Sonicators) and heated for 10 min at 95°C in a Multi Blok Heater (Thermo Scientific) to denature enzymes in the samples. Lysates were centrifuged at 18,000 x g for 10 min at 4°C and the supernatant was collected in a fresh microcentrifuge tube.

A Glycogen Colorimetric/Fluorometric Assay Kit (BioVision Incorporated, Milpitas CA) was used to measure glycogen concentration in the samples per manufacturer instructions. Absorbance was detected at a wavelength of 570 nm using a multi-well spectrophotometer. Sample Glycogen concentration (C, μg/μL) was calculated as B (μg of glycogen from the Standard Curve)/V (μL of sample volume added into the reaction well) X D (sample dilution factor) after the subtraction of the 0 glycogen standard from all readings.

RNA Isolation

Satellite cells were then seeded into 6-well Costar® cell culture treated plates (Corning Inc., Corning, NY) at a density of 60,000 cells/well with duplicate wells per treatment. When cells reached a confluence of about 80%, cells were treated for 48 h with low glucose DMEM (Gibco) supplemented with 1% FBS, 1% P/S, 0.2% Gentamicin, and either 0, 10, or 100 μg/mL furosemide (VetOne®). At the end of the treatment, cells were then washed with PBS (Gibco) and total RNA was extracted at room temperature with 1 mL/well TRIzol® Reagent (Ambion by Life Technologies, Carlsbad, CA). Lysates in TRIzol Reagent were frozen in microcentrifuge tubes at -80^{o}C until RNA could be purified. Total RNA was purified using a PureLink® RNA Mini Kit (Invitrogen by Thermo Fisher Scientific, Carlsbad, CA) per manufacturer instructions. Total RNA concentration was measured with a NanoDrop® Spectrophotometer.

Reverse transcription and PCR

Total RNA samples were diluted to a concentration of 50 ng/μL and an RNA pool was made that contained an equal volume of diluted RNA from each sample. The RNA pool served as the negative control for each gene. For the DNase step, 2 μL of 10x DNase

Buffer, 6 µL of RNA (50 ng/µL), 11 µL RNase-free H_2O, and 1 µL of RO1 DNase enzyme (Ambion) were combined to achieve a total volume of 20 µL. Samples were incubated in a Realplex4 Mastercycler epgradient S (Eppendorf) as follows: 37°C for 30 min, 75°C for 10 min, 4°C hold.

The reverse transcription reaction was prepared in MicroAmp Reaction Tubes with Caps (Assorted colors; Life Technologies) with the High Capacity cDNA Reverse Transcription Kit (200 rxns) (Thermo Fisher Scientific) as follows: 3 µL 10x RT Buffer, 3 µL 10x Random Primers, 1.5 µL 25x dNTPs, 1.5 µL RNAse Free H_2O, 1 µL Reverse Transcriptase Enzyme, and 20 µL of RNA (from the DNAse step). Two tubes of RT were prepared per sample to ensure gene expression from all six genes could be analyzed. The RT reaction was performed in the Mastercycler personal (Eppendorf) with the following protocol: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and 4°C hold.

Quantitative PCR was performed using a master mix prepared for each gene containing 10 µM of Forward Primer, 10 µM of Reverse Primer, Sterile H_2O, and 2x *Power*SYBR Green PCR Master Mix (Thermo Fisher Scientific). Reactions were run in triplicate for each gene with 18 µL of master mix and 2 µL of RT product (20 ng RNA) per well of the Eppendorf twin.tec real time PCR 96, unskirted plate. The plate was sealed with MicroAmp Optical Adhesive Film (Thermo Fisher Scientific, Carlsbad, CA), centrifuged in the Perfect SpinP, and then placed into the Realplex4 Mastercycler epgradient S (Eppendorf). A template of the plate was made and the assay was saved in the Mastercycler ep Realplex database. The program consisted of: 1 cycle at 95°C for 10 min; 40 cycles at 95°C for 15 s and 55-60°C for 1 min; then a melting curve analysis. Analysis was recorded in Ct (cycle threshold) values. Data were analyzed using the ΔΔCt

method ($\Delta\Delta Ct = \Delta Ct^{\text{gene of interest}} - \Delta Ct^{\text{housekeeping gene}}$) and relative expression graphically visualized with $2^{-\Delta\Delta Ct}$ method (fold change).

Glycolysis Stress Test

Extracellular acidification rate (ECAR) of eqSC was measured using the XF extracellular flux analyzer (Agilent, Seahorse Bioscience). Cells were seeded equally into XF24 V7 cell culture plates at a density of 50,000 cells per well. Cells were incubated for 48 h in growth medium (DMEM, Gibco) until cells reached 80% confluence when treatment media, low glucose DMEM containing 1% FBS, 1% Pen/Strep, 0.2% Gentamicin and 0, 10, or 100 µg/mL furosemide, was exchanged. After incubation of treatment media for 40 h, media was again exchanged with serum-free, bicarbonate-free, Seahorse base medium (Agilent, Seahorse Bioscience) containing 100 µL of 200 mM Glutamine and incubated in a non-CO_2 incubator (Agilent, Seahorse Bioscience) for 1 h. The XF24 V7 microplate was loaded into the Seahorse Bioanalyzer (Agilent, Seahorse Bioscience) and the experiment proceeded. The experiment consisted of 3-min mixing, 2-min wait, and 3-minute measurement cycle. ECAR was measured under basal conditions in the presence of glucose (10 mM/well, Fisher), the F_1F_0ATPase inhibitor oligomycin (1.25 µM/well, Sigma), and the glycolysis inhibitor 2-deoxyglucose (50 mM/well, Sigma). All experiments were performed at 37^{o}C. Data presented are the mean ± SEM from six independent experiments performed in replicates of three and normalized to protein content (BCA Assay, ThermoFisher).

Calculations encompassing the interpretation of glycolytic metabolism include non-glycolytic acidification, glycolysis, glycolytic capacity, and glycolytic reserve. Non-glycolytic acidification is calculated as the average ECAR measurements prior to the

addition of glucose in the medium. Glycolysis was calculated as the (average ECAR measurement post-glucose injection before the addition of oligomycin) – (non-glycolytic acidification). Glycolytic capacity was defined as the (maximum ECAR measurement post-oligomycin injection, before 2-DG was added) – (non-glycolytic acidification). Lastly, glycolytic reserve was calculated as (glycolytic capacity) – (glycolysis) (Divakaruni et al., 2014).

Mitochondria Stress Test

Oxygen consumption rate (OCR) of eqSCs was determined using an XF extracellular flux analyzer (Agilent, Seahorse Bioscience). Cells were seeded equally into XF24 V7 cell culture microplates at a density of 50,000 cells per well and incubated for 48 h in growth medium (DMEM, Gibco) until cells reached 80% confluence when treatment media, as previously defined, (0, 10, or 100 µg/mL furosemide) was exchanged. After the incubation of treatment media for 40 h, media was again replaced for serum-free, bicarbonate-free, DMEM medium containing L-glutamine and sodium pyruvate. Cells were incubated for 1 h prior to assay in a non-CO_2 incubator (Agilent, Seahorse Bioscience). The XF24 V7 cell culture microplate was loaded into the Seahorse Bioanalyzer (Agilent, Seahorse Bioscience) and the experiment proceeded. Experiments consisted of 3-minute mixing, 2-minute wait, 3-minute measurement cycles. OCR was measured under basal conditions in the presence of the F_1F_0ATPase inhibitor oligomycin (0.5 µmol/L, Sigma), the mitochondria uncoupler carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP, 0.6 µmol/L, Sigma), and the complex I and III inhibitors Rotenone/Anti-mycin A, respectively (1 µM each/well, Sigma). All experiments were performed at 37^{o}C. Data presented are the mean ± SEM from six

independent experiments performed in replicates of seven and normalized to protein content (BCA Assay, ThermoFisher).

Calculations surrounding the interpretation of assay parameters include basal respiration, proton leak-linked respiration, ATP-linked respiration, maximal respiration, reserve respiratory capacity, and non-mitochondrial respiration. Non-mitochondrial respiration was defined as the minimum measurement after the Rotenone/Anti-mycin A injection. Basal respiration was calculated as (the average OCR measurements prior to injections) – (non-mitochondrial respiration). Proton-leaked respiration was calculated as follows: (the minimum OCR measurement post-Oligomycin injection before the injection of FCCP) – (non-mitochondrial respiration). ATP-linked respiration was calculated as (average basal respiration) – (the value calculated for proton leak). Maximal respiration was defined as (the maximum OCR measurement after the injection of FCCP) – (non-mitochondrial respiration). Reserve respiratory capacity was calculated as (maximal respiration) – (basal respiration). Lastly, coupling efficiency was calculated as (ATP-linked respiration/basal respiration) X 100. Calculations were defined in Divakaruni and colleagues (2014).

Statistical Analysis

Data were analyzed using SAS Enterprise Guide 6.0 software (Cary, NC) and visually graphed using GraphPad Prism 6.0 software (La Jolla, CA). A one-way ANOVA (GLM procedure) was performed for assays when furosemide dosages were compared. In assays where furosemide dosages were compared to rhIGF-1, the GLM procedure with an interaction code was used to analyze data. A 95% confidence interval was set,

therefore alpha = 0.05. Data were considered significantly different when $P < 0.05$. No tendencies were considered. The mean ± SEM were represented graphically.

RESULTS

Initial visualization of eqSCs suggested that furosemide may affect cell confluency. Thus, an EdU incorporation assay was performed to measure cell proliferation. Results indicated that the percentage of EdU positive cells (mitotic index) was increased ($P < 0.05$) in cells treated with 100 μg/mL furosemide by comparison to control. The greater percentage of EdU containing cells was a reflection of fewer total cells ($P \leq 0.05$) in the cultures supplemented with the greater dose of furosemide. No differences ($P > 0.05$) in the mitotic index (Fig. 1A) or total cell number (Fig. 1B) between 0 and 10 μg/mL furosemide treatment were found.

Pax7, a lineage marker of satellite cells, is expressed during early to late proliferation stages of myogenesis. Immunocytochemistry was performed to determine if Pax7 expression and localization in eqSCs was affected by furosemide. Supplementation of subconfluent eqSCs with 0, 10 or 100 μg/mL furosemide for 24 h did not alter subcellular localization (Fig. 2A). Total and Pax7 immunopositive nuclei were enumerated in the control and furosemide treated cells. Results demonstrate that the percentage of Pax7 positive cells was not affected ($P > 0.05$) by treatment with furosemide (Fig. 2B).

Nearly 80% of the subconfluent eqSC were Pax7 immunopositive with the molecular identity of the remaining myoblasts unknown. Myogenin is expressed in myoblasts immediately prior to differentiation and fusion. Equine SCs were

immunostained for myogenin expression and localization following treatment for 48 h with 0, 10 or 100 μg/mL furosemide. In all instances, myogenin was localized to the nucleus (Fig. 3A). Enumeration of total and myogenin immunopositive cells demonstrated a reduction in the percentage of myogenin expressing cells treated with 100 μg/mL furosemide compared to all other groups ($P < 0.05$; Fig. 3B). No decrease in the percentage of myogenin positive cells was seen when comparing 10 μg/mL furosemide to the control ($P > 0.05$).

Confluent eqSCs were cultured with 0, 10 or 100 μg/mL furosemide for 48 h followed by fixation and immunostaining for sarcomeric MyHC (MF20). Although all cultures contained some amount of MyHC, there was a visual reduction in cultured eqSCs treated with 100 μg/mL furosemide compared to the 0 μg/mL and 10 μg/mL furosemide groups (Fig. 3C). A fusion index was calculated as the percentage of myonuclei contained within a myotube. Cultures treated with 100 μg/mL furosemide failed ($P < 0.05$) to fuse at levels comparable to control (Fig. 3D). No difference ($P > 0.05$) in the fusion index of 0 and 10 μg/mL furosemide treated eqSCs was noted. Myotube area (%), defined as the binary area of the myotube divided by the measured area of the field of view, was decreased ($P < 0.05$) for the 100 μg/mL furosemide group compared to the control (Fig. 3E). Again, the myotube area did not differ ($P > 0.05$) between the 0 μg/mL and 10 μg/mL furosemide treated groups.

Insulin-like growth factor (IGF-1) stimulates differentiation and myotube protein synthesis in other species (Barton et al., 2012; Ge et al., 2013; Gonçalves et al., 2019; Song et al., 2019; Zou et al., 2018). The growth factor was tested for its ability to rescue the fusion defect in eqSCs treated with 100 μg/mL furosemide for 48 h. Cells were fixed

and immunostained for MyHC and morphometric measures calculated, as described above. Large myotubes formed in 0 and 10 μg/mL furosemide treated groups irrespective of IGF-1 inclusion. Cells cultured with 100 μg/mL furosemide remained mononucleated in the presence or absence of IGF-1 (Fig 4A). Percent fusion was less ($P < 0.05$) in the group treated with 100 μg/mL furosemide when compared to the control and the 10 μg/mL furosemide group (Fig. 4B). Percent myotube area was also less ($P < 0.05$) in the group treated with 100 μg/mL furosemide compared to all other groups (Fig. 4C). Supplementation of eqSCs with IGF-1 failed to affect the fusion incidence or myofiber area for any treatment group.

The metabolic profile of eqSC myotubes was assessed in the presence and absence of furosemide. As a first step, expression of genes associated with glycogen synthesis were examined (Fig 5B). Confluent eqSCs were treated for 48 h with 0, 10 or 100 μg/mL furosemide followed by total RNA isolation. Gene expression levels of *hexokinase II* (*HKII*), the enzyme responsible for reversible conversion of glucose to glucose-6-phosphate, did not differ in the 10 or 100 μg/mL furosemide treatment group by comparison to control ($P > 0.05$). By contrast, 100 μg/mL furosemide increased ($P < 0.05$) expression of *phosphoglucomutase* (*PGM*), *glycogen synthase 1* (*GYS1*) and *glycogen branching enzyme* (*GBE*) in eqSC myotubes. The higher dose of diuretic also suppressed expression of *creatine kinase M* (*CKM*) in myotubes. Treatment of myotubes with 10 μg/mL furosemide did not alter ($P > 0.05$) expression of *CKM*, *PGM* or *GBE* but did increase ($P < 0.05$) expression of *GYS1*.

Greater amounts of glycogen synthesis enzymes may increase the levels of glycogen found with the cells. Thus, confluent eqSCs were treated as described

throughout and total glycogen content measured. As shown in Fig 5A, glycogen content was substantially lower ($P < 0.05$) in eqSC treated with 100 μg/mL furosemide by comparison to either 0 or 10 μg/mL furosemide treatment groups.

The disparate results of low glycogen content coupled with greater concentrations of glycogen synthetic enzyme transcripts was further evaluated by metabolic profiling. The glycolysis stress test revealed that ECAR was unaltered with the addition of inhibitors intended to test the glycolytic capacity and glycolytic reserve of eqSCs ($P > 0.05$; Fig. 6A; Table 2). The mitochondrial respiration assay revealed that OCR was decreased at the basal level in eqSCs treated with 100 μg/mL furosemide compared to both 0 and 10 μg/mL furosemide treated groups ($P < 0.05$). The higher dose of furosemide also increased OCR upon the addition of oligomycin ($P < 0.05$). The addition of FCCP and Rotenone/Antimycin-A did not impact OCR in any of the groups ($P > 0.05$; Fig 6B). The "off label" furosemide dose decreased basal OCR ($P < 0.05$), decreased ATP-linked respiration ($P < 0.05$), increased reserve capacity and proton leak-linked respiration ($P < 0.05$), and greatly decreased coupling efficiency ($P < 0.05$) compared to 0 and 10 μg/mL furosemide groups (Table 1). In addition, treatment with 10 μg/mL furosemide increased reserve capacity of eqSCs although not to the same extent as 100 μg/mL furosemide, but did increase maximal respiration to the same degree as cells treated with 100 μg/mL furosemide (Table 1). Furosemide did not alter non-mitochondrial respiration ($P > 0.05$).

DISCUSSION

Much is known about furosemide and its effects on the kidney, the target organ. Labeled in the horse racing industry as "performance-enhancing," minimal information is known about how furosemide might affect muscle recovery in the horse or any of its long-term effects. Our physiological dose of furosemide was determined with the consideration that 10 µg/mL furosemide would be the maximum concentration of furosemide seen in circulation and the most the muscle would ever encounter per manufacturer dosage recommendations of 1.0 mg/kg of body weight. Our off-label dose of 100 µg/mL furosemide was developed to represent industry speculation that furosemide may be administered in greater dosages than reported to racing stewards in effort to further decrease the weight a horse carries during a race or to mask prohibited drugs.

Muscle often undergoes microdamage as a result of high-intensity exercise, such as that sustained during a race. Muscle repair begins with the activation and proliferation of satellite cells followed by myogenic lineage progression, leading to the creation of functional, contractile myofibers. Although mitotic index did not differ amongst the treatment groups in the present study, average cell number decreased in the off-label furosemide group. Perhaps this suggests the incorporation of EdU during DNA replication, with the arrest of the cell cycle before cytokinesis occurred, as satellite cell proliferation was halted. The tumor suppressor protein, p53 recognized DNA damage or stress and prevented cellular growth through the arrest of the cell cycle (Fernandez-Salas et al., 2002). This tumor suppressor protein upregulates the genes GADD45 and p21/WAF1 that arrest the cell cycle at the G1 checkpoint, as well as the gene 14-3-3 σ

responsible for cell cycle arrest at the G2/M checkpoint (Fernandez-Salas et al., 2002). EdU incorporation into the newly synthesized DNA suggests the cell cycle progressed past the G1 checkpoint and was instead arrested at the G2/M checkpoint before cell division could occur, decreasing cell number in the 100 µg/mL furosemide group.

Upregulation of p53 due to DNA damage and cellular stress can also affect genes in the mitochondria typically regulated by p53 or in the p53-family. CLIC4, also termed mtCLIC, is a chloride intracellular channel and is located on the inner membrane of the mitochondria (Fernandez-Salas et al., 2002; Suh et al., 2004). This protein is upregulated in response to an upregulation of p53. The upregulation of mtCLIC, perhaps in combination with the pro-apoptotic gene of the outer mitochondrial membrane *Bax*, initiates apoptosis, a highly controlled process of programmed cell death. Increased expression of mtCLIC changes mitochondria function such as altering mitochondrial membrane permeabilization and mitochondrial membrane potential, depletion of ATP and ADP, the production of reactive oxygen species (ROS), changes in pH, the release of cytochrome c, and the activation of caspases involved in apoptosis. As mtCLIC may also act as a regulator of ion flux across the inner mitochondrial membrane (Fernandez-Salas et al., 2002), it is possible that furosemide may inhibit these chloride channels, possibly contributing to a supplementary upregulation of mtCLIC, further exacerbating apoptosis.

Oxygen consumption was decreased during the basal state as there was a decreased ability for ATP generation due to an increase in proton leak, confirmed with the addition of oligomycin and FCCP. In addition, oxygen consumption in this state may have also been compromised due to the upregulation of p53 and mtCLIC initiating the

process of apoptosis (Fernandez-Salas et al., 2002). Proton leak may be indicative of an alteration in mitochondrial membrane potential ($\Delta\psi_m$; Zorova et al., 2018).

Decrease in ion transport, as a result of treatment with furosemide, could also alter $\Delta\psi_m$ (Buckman and Reynolds, 2001). While it is normal for the mitochondria to experience small fluctuations, or oscillations in membrane potential for a short duration of time, a sustained change in membrane potential could lead to the opening of the Permeability Transition Pore (PTP; Zorov et al., 2000). This, in turn, can lead to either (1) mitochondrial swelling and subsequent rupture causing apoptosis, or (2) the release of cytochrome c, also resulting in cell death (Juhaszova et al., 2004; Zorova et al., 2018). This process may explain why the addition of oligomycin increased oxygen consumption post-treatment with 100 µg/mL furosemide. Changes in pH and ion transport all disrupt the homeostatic $\Delta\psi_m$ (Zorova et al., 2018).

As the cell experiences oxidative damage and prolonged oxidative stress, reactive oxygen species (ROS) may be produced which would further exacerbate the dysfunctional status of the cell. Thus, the positive feedback mechanism of ROS-induced ROS release where ROS acts as a secondary messenger to send a signal to neighboring cells to generate ROS, further results in cellular injury (Zorov et al., 2006).

Interestingly, with the addition of the mitochondrial intermembrane uncoupler FCCP, the oxygen consumption rate increased only slightly in the 100 µg/mL furosemide group compared to oxygen consumption with the addition of the oligomycin. In fact, FCCP eliminates membrane potential altogether through massive proton leak and often serves as a positive control in experiments using fluorescent dye to detect changes in membrane potential (Keck et al., 2018; Simula et al., 2018; Zorov et al., 2000). The

addition of Rotenone/Antimycin A inhibits Complex I and III, respectively, completely arresting the electron transport chain and allowing for the measurement of non-mitochondrial respiration. Complexes I and III are the major producers of ROS (Zorov et al., 2000).

Maximal respiration was increased in the mitochondria as a result of treatment with both 10 and 100 μg/mL furosemide. However, coupling efficiency was drastically decreased in the 100 μg/mL furosemide group. Therefore, even though the mitochondria were operating at maximum capacity, no ATP was being produced, confirming the probability of impending apoptosis. In the 10 μg/mL furosemide group, coupling efficiency was not different than the control, suggesting that while mitochondria were operating at full capacity, ATP production was not compromised. Thus, maximal respiration was likely increased in the physiological dose of furosemide due to an increased demand for cellular energy as a result of treatment. As cellular energy demand increased, the TCA cycle worked rapidly to produce the cofactors NADH and $FADH_2$ necessary for the function of the electron transport chain. Therefore, an increase in maximal respiration could be contributed to an increased production of such cofactors, which may ultimately be due to hyperactivity of the TCA cycle. Such hyperactivity of the TCA cycle can be attributed to an increase in intermediates or the increased activity of TCA cycle enzymes. An alteration of the TCA cycle can be detected with DNA microarrays to examine gene epression of the eight enzymes involved in the cycle as described in McCammon and colleagues (2003). Enzymatic activity within the TCA cycle can be detected with three separate enzymatic assays that detect changes in activity amongst all eight enzymes in the TCA cycle (Goncalves et al., 2010), and the

concentration of TCA cycle intermediates can be detected with liquid chromatography tandem mass spectrometry (LC-MS/MS) as initially developed by Kadhi and colleagues (2017). Metabolic profiling of the TCA cycle would provide additional knowledge as to how furosemide affects maximal respiration.

However, L-Glutamine was supplmented in the non-glucose medium in both the glycolysis and mitochondria stress tests. L-Glutamine is an alternative energy source for cells and is necessary for proliferation and cell viability. Although exogenous L-Glutamine was added to the medium in efforts to maintain cellular health during such assays, it is possible that it affected the metabolic activity of the TCA cycle by promoting the conversion of L-Glutamine to Glutamate. This would ultimately lead to the formation of the TCA cycle intermediate, alpha-ketoglutarate. An increase in cycle intermediates could allow the production of additional NADH and $FADH_2$. An increase in these TCA cycle products may contribute to an increase in maximal respiration in the mitochondria.

The concentration of L-Glutamine present in both metabolism assays (200 mM) was consistent with the concentration used in both high and low DMEM base mediums with GlutaMAX^{TM} supplement (Gibco) used in all assays in this study. Therefore, it is possible that the supplemented L-Glutamine was not enough to alter metabolism, concluding that furosemide may indeed affect TCA metabolism. Nevertheless, further assays will need to be performed in order to confirm the effects of furosemide on the TCA cycle.

In addition to maximal respiration, reserve capacity of the mitochondria was increased in both the physiological and excess doses. Reserve capacity was calculated as (maximal respiration) – (basal respiration) as defined in Divakaruni and colleagues

(2014). Since maximal respiration was increased in both treatment groups compared to the control, it is sensible that reserve capacity would also be increased.

In regards to the early differentiation assay, the expression of *Pax7*, the universal marker of satellite cells, was unchanged in comparison to all treatment groups. This suggested that the quantity of satellite cells remained the same in culture regardless of the increase in cell number in the control and physiological furosemide groups. However, expression of the intermediate differentiation marker, *Myog*, decreased in the excess furosemide treatment group as DNA damage most likely prompted the arrest of the cell cycle, restricting the emergence of any sarcomeric markers. As expected, since late differentiation was hijacked in the 100 µg/mL furosemide group, expression of myosin heavy chain was also decreased. Myotube formation was suppressed as a result of treatment with 100 µg/mL furosemide for 48 h.

Our results are consistent with another study in which murine C2C12 myoblasts were treated with either 0.3 µM, 3 µM, or 30 µM furosemide for both 48 h and 96 h *in-vitro* (Mandai et al., 2017). All doses of furosemide at both 48 h and 96 h post-treatment resulted in a significant decrease of Myogenin gene expression compared to the control. Treatment of C2C12 myoblasts of 3 µm furosemide after 96 h resulted in a significant reduction of both fusion index and myotube diameter compared to controls (Mandai et al., 2017). Gene expression of myosin heavy chain (*MHC*) as well as *MyoD* was significantly decreased in all doses of furosemide at 48 h and 96 h, respectively compared to the control (Mandai et al., 2017).

The NKCC1 co-transporter is responsible for regulating membrane currents, such as chloride conductance, and membrane potential. Furosemide inhibits this co-

transporter, which is abundant in skeletal muscle, and essential for myogenesis (Mandai et al., 2017). Western blot revealed an increase in both total and phospho-NKCC1 expression as murine myoblasts differentiated into myotubes *in vitro*, as well as in hypertrophic muscles *in vivo* (Mandai et al., 2017). Chloride conductance, controlled by NKCC1, stimulates Ca^{2+} signaling which is responsible for calcineurin activation which, in turn, upregulates MyoD and Myogenin. Furosemide decreases intracellular calcium concentrations through inhibition of NKCC1 (Mandai et al., 2017). Therefore, furosemide is concluded to inhibit muscle differentiation.

While proving the presence or absence of NKCC1 in equine muscle would have provided a stronger argument as to why furosemide inhibited myogenesis, a proper immunoblotting antibody was unable to be found. The T4 antibody, as mentioned in Mandai and colleagues, (2017; tNKCC1; Developmental Studies Hybridoma Bank, Univeristy of Iowa, Iowa City, IA), did not appear at the correct size (kDa) when using the appropriate gel gradient pore size (data not shown). Perhaps the development of a similar antibody for p/tNKCC1 would be useful in confirming the presence of this co-transporter in equine muscle. If possible, future studies should confirm NKCC1 inhibition with furosemide in eqSCs *in-vitro* as well as *in-vivo*. Such information would serve useful in the racing industry to determine the dose of furosemide that may affect the racehorse's ability to recover after the occurrence of muscle microdamage as a result of intense exercise.

Furosemide affected glycogen concentration. Due to the lack of myotube formation in the excess furosemide treatment group, it was expected that glycogen concentration would decrease compared to all other groups. Furosemide has been proven

to impair glucose uptake in rats at a concentration of 0.5 mmol/L and at higher concentrations (6.0 mmol/L), glycogen synthesis is inhibited (Dimitriadis et al., 1988). In skeletal muscle, Protein Kinase B (Akt) signals for the GLUT4 transporter to translocate to the cell membrane so glucose can be stored in the cell in its branched form, glycogen (Annandale et al., 2004; DeFronzo and Tripathy, 2009). In cells affected by furosemide, GLUT4 translocation to the cell membrane is inhibited, ultimately decreasing the ability of glucose uptake by the cell and the ability to synthesize glycogen (Dimitriadis et al., 1988, Annandale et al., 2004). Inhibition of GLUT4 translocation to the cell membrane as well as a decrease in myotube formation may explain why cells treated with the excess furosemide dose have reduced glycogen concentration.

RT-qPCR was performed on *HKII*, *PGM1*, *GYS1*, and *GBE1* to determine if gene expression of glycogen synthesis enzymes were altered due to effects of furosemide. As *CKM* is a marker of differentiation, it is sensible that gene expression was decreased in the 100 μg/mL furosemide group as myotube formation was decreased. The glucokinase isoform *HKII* has a high affinity for glucose and is most prevalent in skeletal muscle (Jimenez-Chillaron et al., 2002; Zois and Harris, 2016). Gene expression of *HKII* remained unchanged when cells were treated with furosemide, possibly because this reaction within glycogenesis is reversible (Zois and Harris, 2016). However, gene expression of Phosphoglucomutase-1 (*PGM1*), Glycogen Synthase-1 (*GYS1*), and Glycogen Branching Enzyme (*GBE1*) were increased as a result of 48 h treatment with furosemide. It is possible that furosemide temporarily caused an increase in AMP-kinase (AMPK) activation which contributed to decreased glycogen synthesis and suppressed cellular growth (Bhanot et al., 2016). Perhaps as furosemide was metabolized, its effects

decreased over time and by 48 h post-treatment was unable to sustain AMPK activation, therefore contributing to increased expression of *PGM1*, *GYS1*, and *GBE1*. AMPK and glycogen synthesis have an inverse relationship as AMPK is a regulator of the rate-limiting *GYS1* through phosphorylation at S8, which inhibits glycogen synthesis (Bhanot et al., 2016). *GYS1* and *GBE1* work concurrently to increase glycogen synthesis. Increased expression of enzymes associated with glycogen synthesis contributes to poor prognosis in patients diagnosed with acute myeloid leukemia (AML), a disease associated with the activation of tyrosine kinases. In fact, high expression of enzymes associated with glycogen synthesis are often also associated with other types of malignant cancers, such as lung adenocarcinoma and kidney clear cell carcinoma (Bhanot et al., 2016). Activation of AMPK also resulted in a decrease in glucose uptake in KU812 cells (Bhanot et al., 2016).

With the understanding that IGF-1 increased muscle growth by promoting protein synthesis and myotube differentiation in both bovine and murine models (Barton et al., 2012; Ge et al., 2013; Gonçalves et al., 2019; Song et al., 2019; Zou et al., 2018), it was anticipated that combined treatment would rescue the muscle from the effects of furosemide and allow differentiation to occur once again. Our results were opposite of our expectations. IGF-1 did not rescue muscle from the effects of furosemide.

The administration of excess furosemide does indeed inhibit myogenesis and subsequently muscular recovery as a result of microdamage. This 100 µg/mL dose alters oxidative metabolism, inhibits myogenesis, arrests the cell cycle, decreases glycogen concentration, and upregulates glycogenesis enzymes. Therefore, although industry speculation ascribes the use of increased doses of furosemide to increase the diuretic

weight loss effect or mask the presence of prohibited drugs, it may in fact slow race recovery and contribute to a decrease in overall performance.

In conclusion, furosemide administered at physiological doses (10 μg/mL) does not inhibit satellite cell myogenesis, but may increase maximal respiration in the mitochondria. This increase in respiration may translate to an increase of performance ability *in vivo*, thereby confirming furosemide as a performancing enhancing drug in addition to weight loss ensued by its diuretic effect. Of course, *in vivo* experiments must be performed to determine the possible cause of the increased respiration in the mitochondria and to define possible performance ability enhancements. However, a racehorse's overall performance relies on multiple factors such as: training program, nutrition, pedigree, EIPH severity, muscle conditioning, environment, and cardiac output. Obtaining more information about furosemide will allow the racing industry to more accurately regulate its use. Industry discussions currently focus on establishing a federal entity to govern over medictions used in the industry as a result of the numerous breakdowns at Santa Anita track this past year. Although these breakdowns may not be directly attributed to furosemide usage, such discussions are important to preserve the integrity of the racing industry and keep the sport alive.

However, numerous trade-offs must be considered. The quantity of severe EIPH cases may increase if furosemide is banned, leading to the increase of animal welfare concerns. In addition, the integrity of the industry may be at stake if furosemide usage continues to be legal. The perfect balance of all three matters is crucial to the survival of Thoroughbred racing in the United States.

Table 2.1. Oxygen consumption rates, pmol O_2/min/µg protein

	Furosemide, µg/mL		
	0	10	100
Basal oxygen consumption	27.42 ± 3.50[a]	27.78 ± 2.74[a]	17.61 ± 2.47[b]
ATP-linked respiration	13.91 ± 2.00[a]	11.30 ± 1.02[a]	-11.41 ± 2.06[b]
Reserve capacity	-4.44 ± 3.49[a]	9.89 ± 1.65[b]	20.10 ± 2.21[c]
Proton leak-linked respiration	13.51 ± 1.62[a]	16.49 ± 2.33[a]	29.02 ± 3.40[b]
Maximal respiration	22.98 ± 2.60[a]	37.67 ± 3.97[b]	37.70 ± 3.62[b]
Nonmitochondrial respiration	5.49 ± 1.44[a]	4.50 ± 1.78[a]	6.04 ± 0.90[a]
Coupling efficiency (ATP-linked/basal) x 100	49.52 ± 3.40[a]	41.80 ± 3.94[a]	-73.31 ± 20.61[b]

Table 2.2. Extracellular acidification rates, mpH/min

	Furosemide, µg/mL		
	0	10	100
Non-glycolytic acidification	0.528 ± 0.790	0.114 ± 0.465	-0.179 ± 0.606
Glycolysis	7.994 ± 0.828	7.988 ± 0.965	10.182 ± 1.350
Glycolytic capacity	7.762 ± 0.879	7.526 ± 0.833	8.501 ± 0.971
Glycolytic reserve	-0.232 ± 0.671	-0.462 ± 0.160	-1.681 ± 0.847

Figure 2.1.

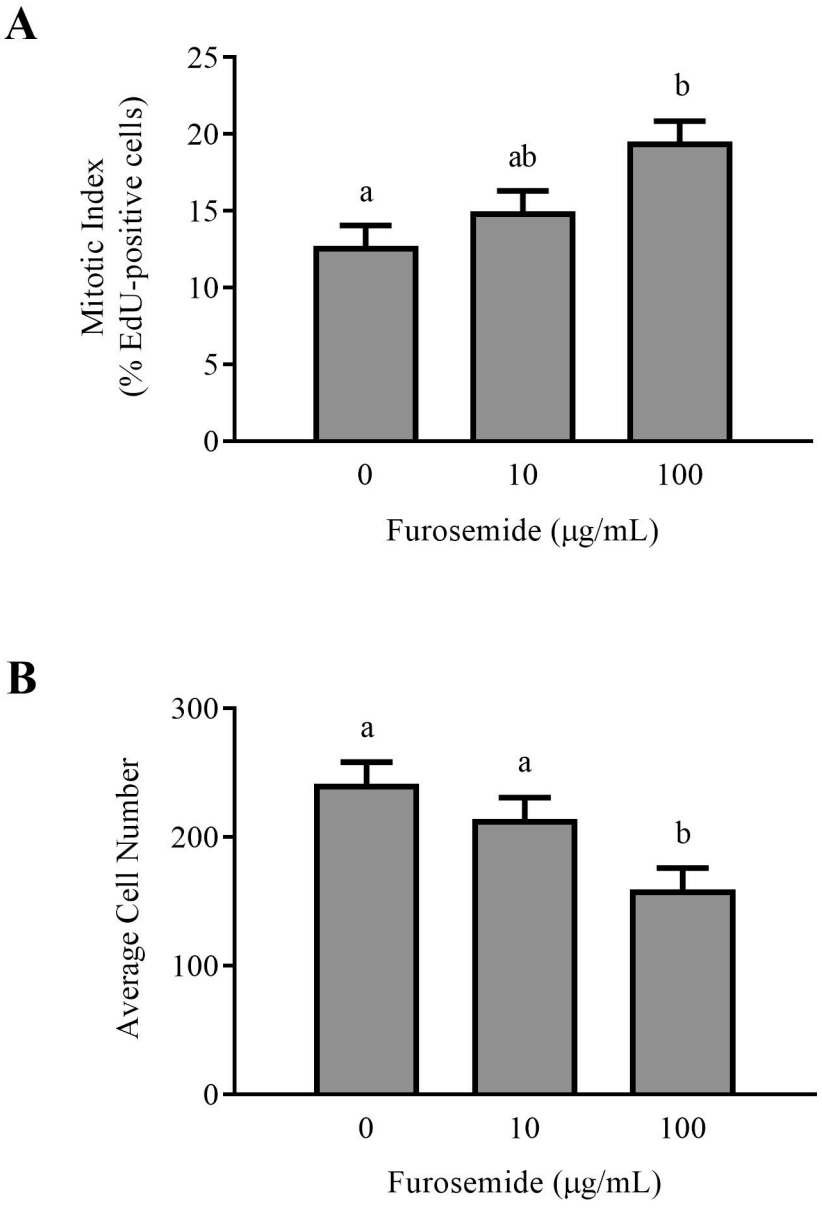

A
Mitotic Index
(% EdU-positive cells)
25
20
15
10
5
0
a
ab
b
0
10
100
Furosemide (μg/mL)
B
Average Cell Number
300
200
100
0
a
a
b
0
10
100
Furosemide (μg/mL)

Figure 2.1. Effects of furosemide on equine satellite cell proliferation *in vitro*. Satellite cell cultures were treated with either 0, 10, or 100 µg/mL furosemide for 24 hours, then pulsed with EdU two hours prior to fixation with 4% Paraformaldehyde and cell number counted. Results are expressed as mean ± SEM. (A) Percent EdU was the same for 0 and 10 µg/mL furosemide groups ($P>0.05$) and 10 and 100 µg/mL groups ($P>0.05$), whereas 100 µg/mL furosemide was increased compared to 0 µg/mL furosemide ($P<0.05$). (B) Average cell number was counted using NIS Elements software. There was a decrease in cell number in the 100 µg/mL furosemide group ($P<0.05$) compared to all other groups ($P>0.05$).

Figure 2.2.

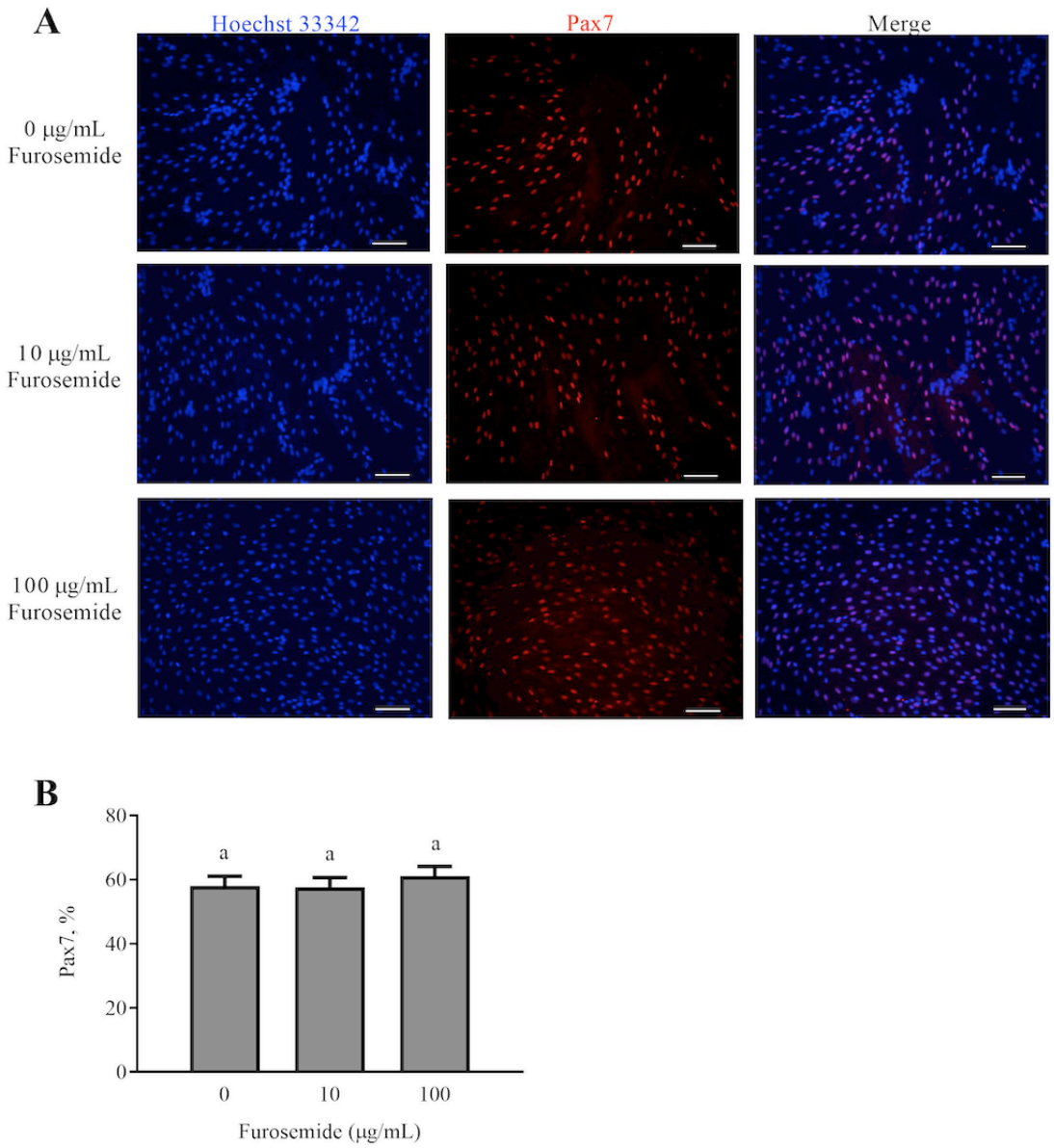
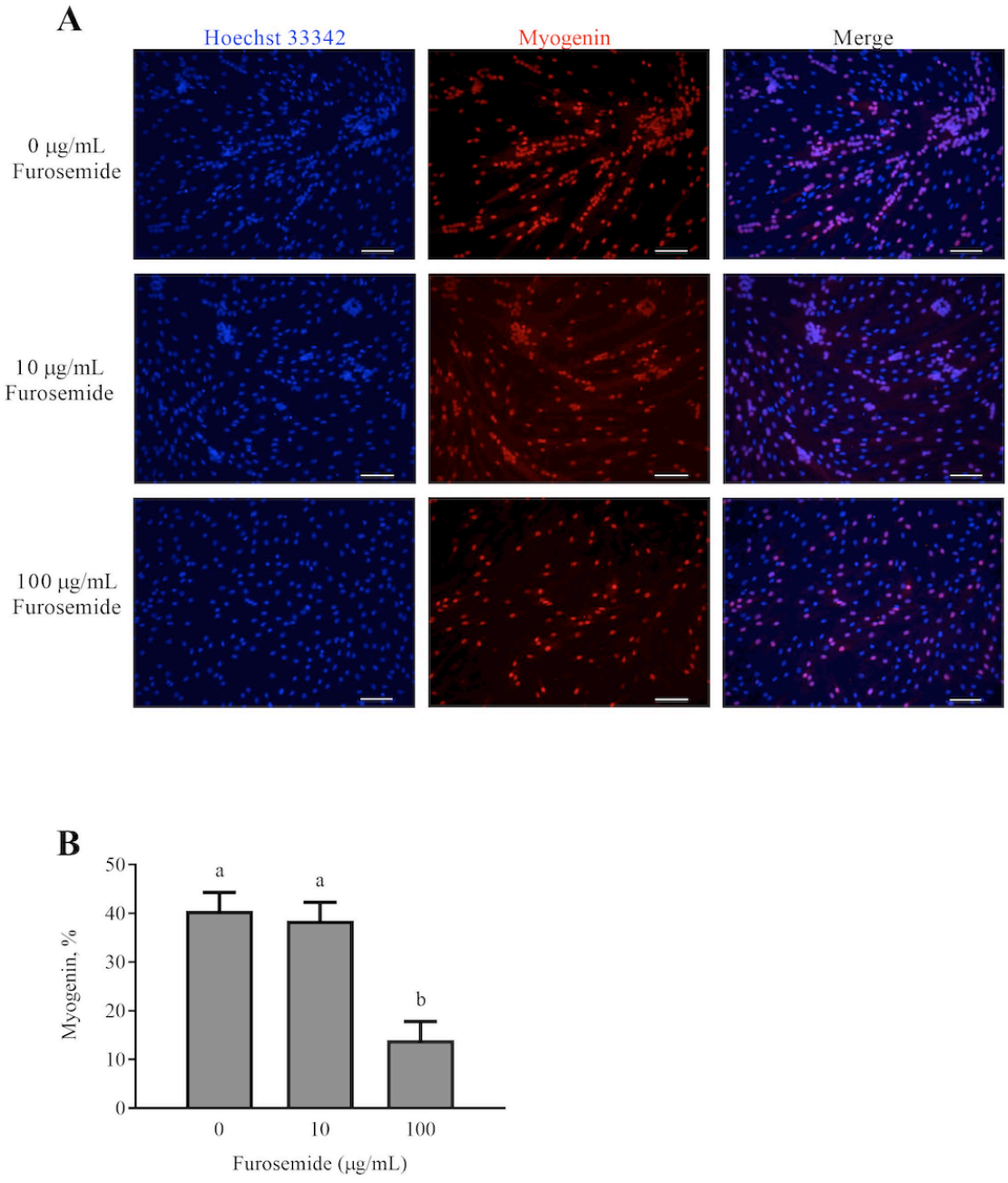

A
Hoechst 33342
Pax7
Merge
0 µg/mL Furosemide
10 µg/mL Furosemide
100 µg/mL Furosemide
B
Pax7, %
80
60
40
20
0
a
a
a
0
10
100
Furosemide (µg/mL)

Figure 2.2. Equine satellite cells stained with Pax7 primary antibody and counter-stained with fluorescent secondary antibodies Hoechst 33342 and goat-anti-mouse IgG 568. Results are expressed as mean ± SEM. (A) Immunocytochemistry images (10x objective) of 0, 10, 100 µg/mL furosemide. Images of Hoechst 33342 and Pax7 are displayed separately in the first two columns, respectively, followed by the merged image in the third column. Image brightness was adjusted for publication purposes. (B) Quantification of the images displayed in A. There was no difference in the percentage of Pax7(+) cells between the groups ($P>0.05$).

Figure 2.3.


A
Hoechst 33342
Myogenin
Merge
0 µg/mL Furosemide
10 µg/mL Furosemide
100 µg/mL Furosemide
B
Myogenin, %
50
40
30
20
10
0
a
a
b
0
10
100
Furosemide (µg/mL)

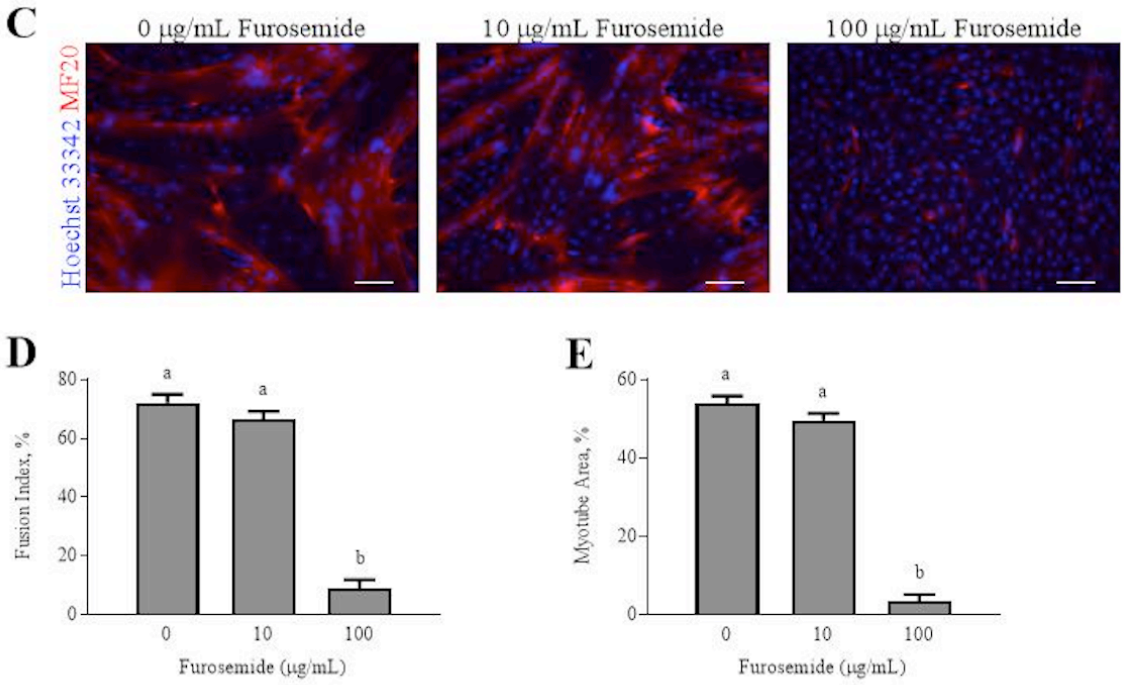
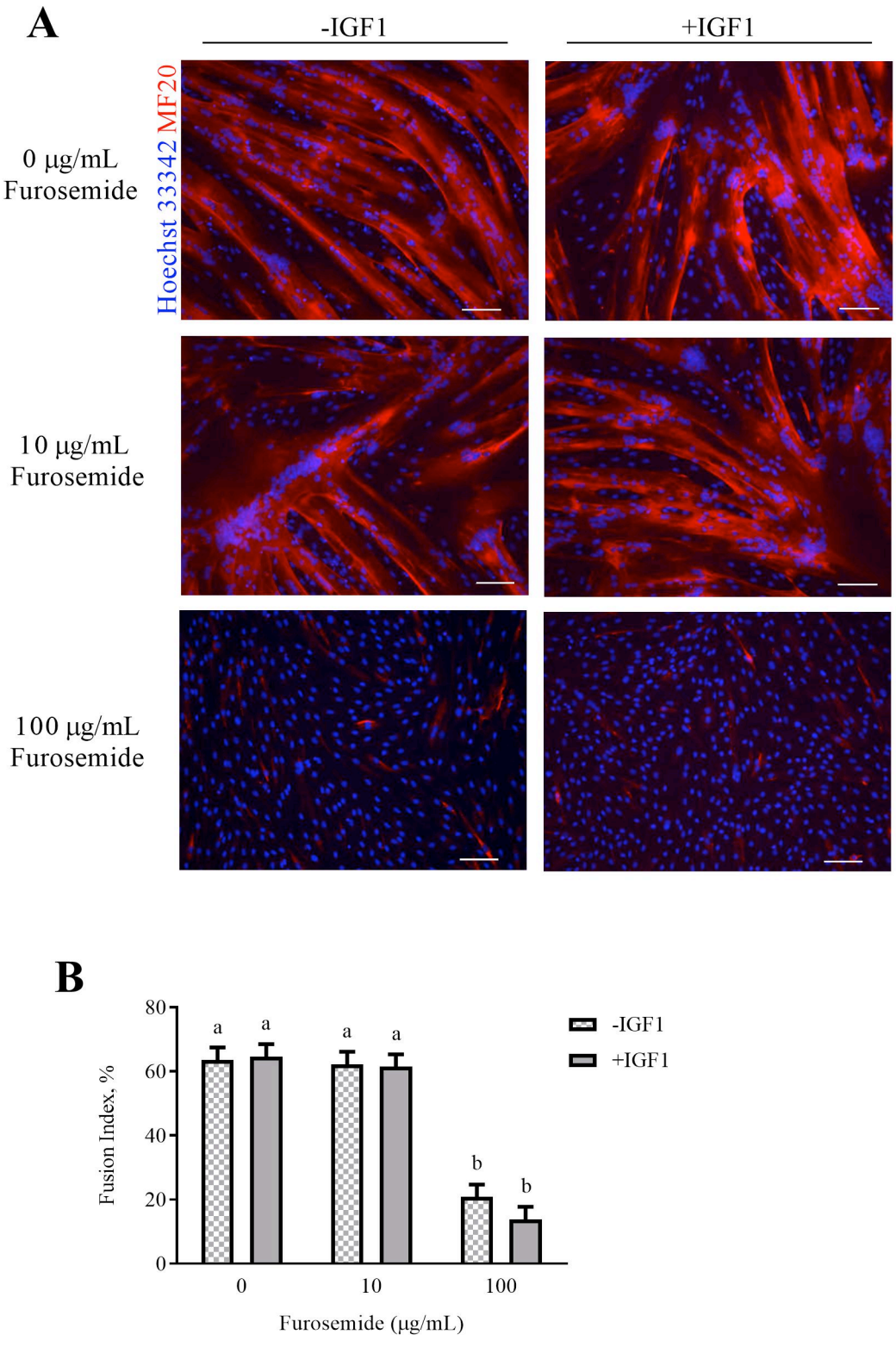

C
0 μg/mL Furosemide
10 μg/mL Furosemide
100 μg/mL Furosemide
Hoechst 33342 MF20
D
Fusion Index, %
80
60
40
20
0
a
a
b
0
10
100
Furosemide (μg/mL)
E
Myotube Area, %
60
40
20
0
a
a
b
0
10
100
Furosemide (μg/mL)

Figure 2.3. Equine satellite cells stained with Myogenin or MF20 primary antibody and counter-stained with fluorescent secondary antibodies Hoechst 33342 and goat-anti-mouse IgG 568. Results are expressed as mean ± SEM. (A) Immunocytochemistry images of 0, 10, or 100 µg/mL furosemide. Images of Hoechst 33342 and Myogenin are displayed separately in the first two columns, respectively, followed by the merged image in the third column. Image brightness was adjusted for publication purposes. (B) Quantification of the images displayed in A. No difference was seen in the percentage of Myogenin positive cells between 0 and 10 µg/mL furosemide ($P>0.05$). There was a significant difference in the percentage of Myogenin positive cells with 100 µg/mL furosemide compared to all other groups ($P<0.05$). (C) Immunohistochemistry images of 0, 10, or 100 µg/mL furosemide stained with MF20. Only merged images shown. Image brightness was adjusted for publication. (D) Percent fusion of the images in C. The fusion index was calculated as the number of myonuclei in the myotube divided by the total number of myonuclei in the image. The 100 µg/mL furosemide group was significantly different when compared to all other groups ($P<0.05$). (E) Percent myotube area of the images in C. Myotube area was calculated as the area of the myotube divided by the area of the image. The 100 µg/mL furosemide group was significantly different compared to all other groups ($P<0.05$).

Figure 2.4.


A
-IGF1
+IGF1
Hoechst 33342 MF20
0 µg/mL Furosemide
10 µg/mL Furosemide
100 µg/mL Furosemide
B
Fusion Index, %
80
60
40
20
0
a
a
a
a
b
b
0
10
100
Furosemide (µg/mL)
-IGF1
+IGF1

C

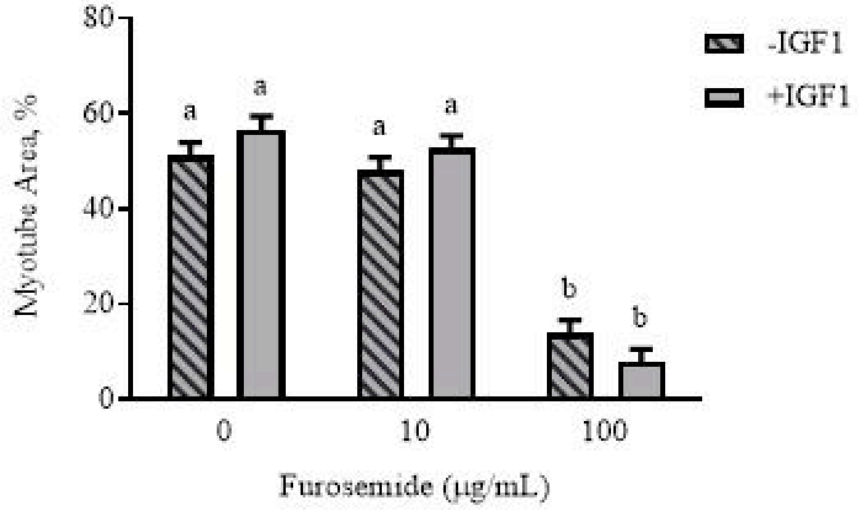

80
60
40
20
0
Myotube Area, %
a
a
a
a
b
b
0
10
100
Furosemide (μg/mL)
-IGF1
+IGF1

Figure 2.4. Equine satellite cells were grown to confluence and treated with either 0, 10, or 100 µg/mL furosemide ± 100 ng/µL IGF1 for 48 hours. Cells were then fixed with AFA and stained for MF20 primary antibody overnight at 4^{o}C, then counter-stained the following day with secondary antibodies Hoechst 33342 and goat-anti-mouse IgG 568. Images were captured with a fluorescent microscope and NIS Elements software. Results are expressed as ± SEM. (A) Images stained for MF20. Image brightness was adjusted for publication. (B) Percent fusion of myotubes was calculated as the number of myonuclei in the myotube divided by the total number of myonuclei in the field of view. There was a decrease of fusion in the 100 µg/mL furosemide ± 100 ng/µL IGF1 compared to all other groups as indicated by the different letter ($P<0.05$). The 100 µg/mL furosemide ± 100 ng/µL IGF1 groups were the same as indicated with the same letter ($P>0.05$). (C) Percent myotube area was calculated as the area of the myotube divided by the area of the image. There was a decrease in the myotube area in the 100 µg/mL furosemide ± 100 ng/µL IGF1 groups compared to all other groups as indicated with the different letter ($P<0.05$). The 100 µg/mL furosmide ± 100 ng/µL IGF1 groups were the same as indicated by the same letter ($P>0.05$).

Figure 2.5.

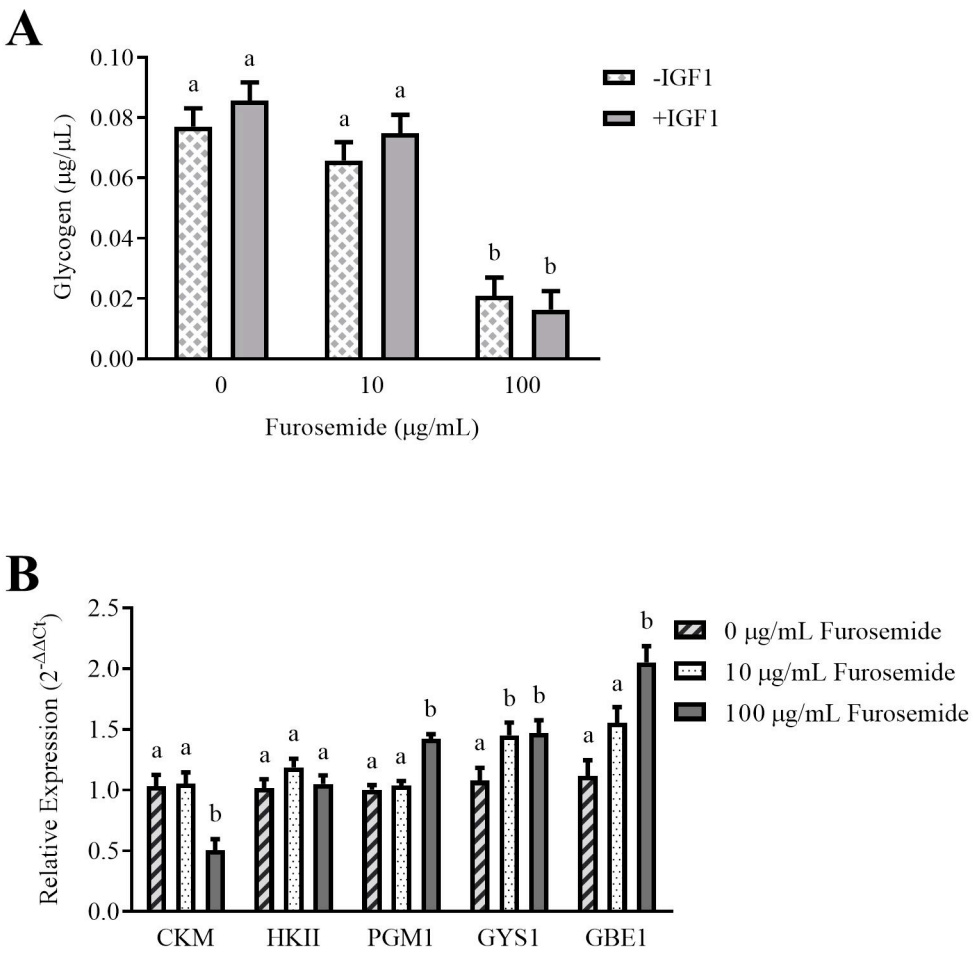
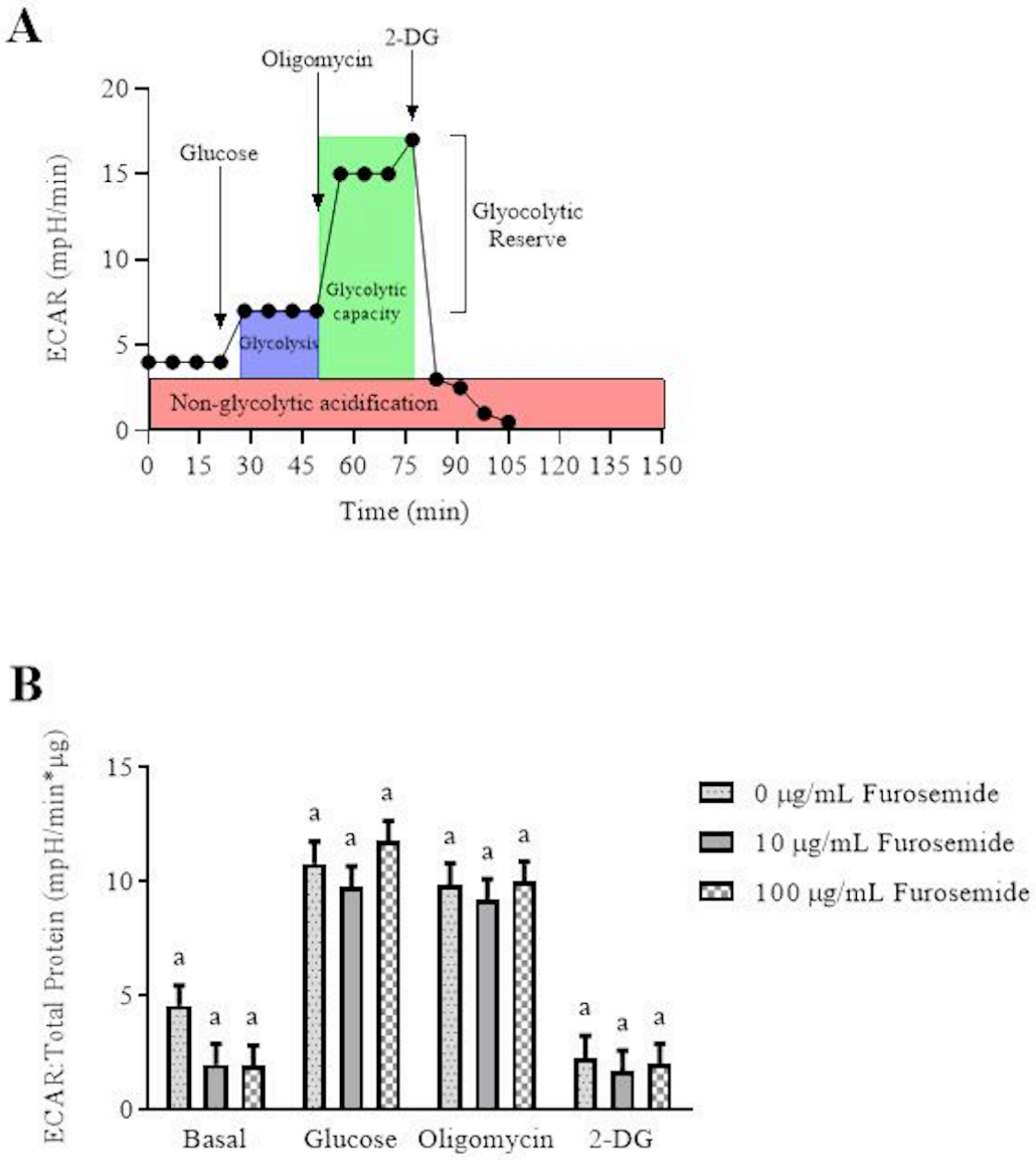
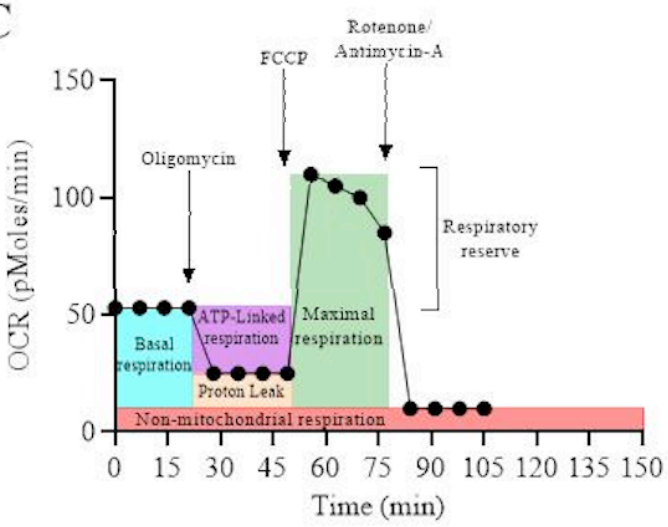

A
Glycogen (µg/µL)
0.10
0.08
0.06
0.04
0.02
0.00
a
a
a
a
b
b
0
10
100
Furosemide (µg/mL)
-IGF1
+IGF1
B
Relative Expression ($2^{-\Delta\Delta Ct}$)
2.5
2.0
1.5
1.0
0.5
0.0
a a b
a a a
a a b
a b b
a a b
CKM
HKII
PGM1
GYS1
GBE1
0 µg/mL Furosemide
10 µg/mL Furosemide
100 µg/mL Furosemide

Figure 2.5. Effects of furosemide on glycogen concentration and the glycogen synthesis pathway. Results are expressed as mean ± SEM. Significant differences are denoted by different letters. (A) Glycogen concentration was decreased in the 100 μg/mL furosmide group compared to all other groups ($P<0.05$). No effect of IGF1 was seen between any of the groups ($P>0.05$). (B) Gene expression of enzymes involved in glycogen synthesis pathway. Data were analyzed within gene. Cells were treated with either 0, 10, or 100 μg/mL furosmide for 48 hours. RT-qPCR was performed. A downregulation of CKM expression was seen in cells treated with 100 μg/mL furosmide compared to all other groups ($P<0.05$). Gene expression of HKII was the same ($P>0.05$) between all groups. There was an upregulation in expression of PGM1 in the 100 μg/mL furosemide group compared to all other groups ($P<0.05$). Expression of GYS1 was increased in the 10 and 100 μg/mL furosemide treated groups ($P<0.05$ and $P<0.05$, respectively) compared to the control. However, expression of GYS1 did not differ between 10 and 100 μg/mL furosemide ($P>0.05$). Finally, gene expression of GBE1 was upregulated in the 100 μg/mL furosemide treated group compared to all other groups ($P<0.05$). No tendencies were considered between the control and the 10 μg/mL furosmide treated group ($P>0.05$).

Figure 2.6.


A
Oligomycin
2-DG
Glucose
Glyocolytic Reserve
Glycolytic capacity
Glycolysis
Non-glycolytic acidification
ECAR (mpH/min)
Time (min)
B
ECAR:Total Protein (mpH/min*µg)
0 µg/mL Furosemide
10 µg/mL Furosemide
100 µg/mL Furosemide
Basal
Glucose
Oligomycin
2-DG

C


Rotenone/
Antimycin-A
FCCP
Oligomycin
Respiratory
reserve
ATP-Linked
respiration
Maximal
respiration
Basal
respiration
Proton Leak
Non-mitochondrial respiration
OCR (pMoles/min)
Time (min)
0
50
100
150
0 15 30 45 60 75 90 105 120 135 150

D

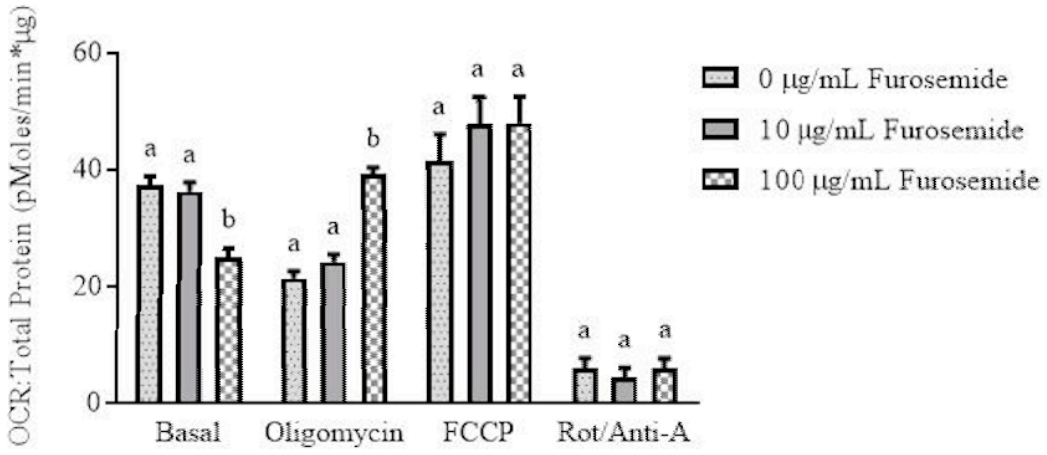

OCR:Total Protein (pMoles/min*µg)
0
20
40
60
a a b
a a b
a a a
a a a
Basal
Oligomycin
FCCP
Rot/Anti-A
0 µg/mL Furosemide
10 µg/mL Furosemide
100 µg/mL Furosemide

Figure 2.6. Metabolism of furosemide *in vitro* after a 40 hour treatment with 0 µg/mL furosemide, 10 µg/mL furosemide, or 100 µg/mL furosemide. Results are expressed as mean ± SEM. (A) Glycolysis stress test adapted from Pelletier et al., 2014. (B) Glycolysis stress test (*n=6*). No differences were seen in ECAR: Total protein amongst any of the treatments with the addition of glucose, oligomycin, or 2-DG (*P>0.05*). (C) Mitochondria stress test adapted from Pelletier et al., 2014. (D) Mitochondria stress test (*n=6*). OCR:Total protein was decreased in the 100 µg/mL furosemide treatment when basal OCR measurements were acquired before the addition of substrates (*P<0.05*). When Oligomycin was added, OCR: Total protein increased in the 100 µg/mL furosemide treatment group (*P<0.05*). No differences were seen between treatments with addition of FCCP or Rotenone/Antimycin-A (*P>0.05*).

CHAPTER II

Conclusions and future directions

Scrutiny of the racing industry has prompted recent promotion of federal drug oversight by the United States Anti-Doping Agency. The more information learned regarding the effects of furosemide on Thoroughbred performance, the more effectively the drug may be regulated. Until the present study, it was unknown what effects furosemide might have on equine muscle recovery.

Furosemide, given in excess amounts, causes cellular growth arrest. This further leads to an inability for muscle to differentiate and synthesize contractile myofibers, reflecting a decrease in glycogen content. Increased gene expression of glycogen synthesis enzymes, may have resulted from the eventual inactivation of AMPK which may have only promoted glycogen synthesis towards the end of the 48 h treatment (Bhanot et al., 2015). However, even though gene expression of the glycogen synthesis enzymes increased, this does not translate to enzyme activity. Further enzyme kinetic assays must be performed in order to assess why gene expression was increased in eqSCs after treatment with 100 µg/mL furosemide.

Oxidative stress caused increased proton leak which may be further detected with fluorescent dyes used to detect oscillations in mitochondrial membrane potential, which may consequently lead to apoptosis (Keck et al., 2018; Simula et al., 2018). Pro-apoptotic markers such as increases of caspases and detection of cytochrome c release require detection for programmed cell death to be considered as an effect of furosemide (Zorov et al., 2000). Detection of p53 and mtCLIC protein can be achieved through western blot (Fernandez-Salas et al., 2002).

The physiological dose of furosemide increases maximal respiration *in vitro*. This may translate to an increase in performance ability of the racehorse, however, *in vivo* experiments must be performed in order to validate this finding. An upregulation of TCA cycle enzymes, or an increase in enzyme kinetics may have contriibuted to an increase in maximal respiration seen in the mitochondria stress test, as well as an an increase in TCA cycle intermediates. In order to get a complete understanding of cellular metabolism as influenced by furosemide, additional assays are required to determine alterations in TCA cycle function.

Although our *in-vitro* study allows for a controlled environment in which no other bodily factors may influence growth of the eqSCs, the importance of *in-vivo* experiments cannot be overlooked. *In-vivo* analyses may provide more information regarding other factors that may influence the behavior of furosemide in the muscle. While it may not be ethical to subject animals to our defined excess dose of furosemide, a concentration surpassing our said physiological dose may be useful in determining the true effects of the diuretic, while also considering industry speculation.

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