INFLUENCE OF LONG-TERM FUROSEMIDE USE ON BONE MINERAL CONTENT, BONE METABOLISM MARKERS, AND WEIGHT LOSS IN HORSES

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To Francisco and my family for their love, support, and encouragement and to my
professors for giving me a chance.

ABSTRACT

Furosemide is a diuretic commonly used to reduce the incidence of exercise induced pulmonary hemorrhage (EIPH) in racehorses. Previous research suggests furosemide negatively influences calcium balance and may have long-term implications for bone health. In this study, furosemide use was evaluated over 56d for effect on bone mineral content (BMC), bone metabolism markers osteocalcin (OC) and pyridinoline cross-links (PYD) and weight loss post administration. No treatment effects were observed for BMC, but there was a period effect across all bone cortices ($p<0.0001$). OC showed no difference between groups ($P=0.26$) or days ($P=0.25$). PYD tended to be lower in FUR ($P=0.0584$) and exhibited a day effect ($P<0.0001$). Body weight change indicated day by time ($P=0.0001$), treatment by time ($P<0.0001$), and day by treatment ($P<0.0001$) interactions. While there was no treatment effect on BMC, the trend toward lower PYD in FUR may warrant further investigation with different times and imaging techniques.

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CHAPTER I: LITERATURE REVIEW

Introduction

Horse racing is a sport that spans many centuries and continents from the United Kingdom to the United States to Australia. Today, Thoroughbred and Quarter Horse racing draw huge crowds and significant amounts of money, both in betting and horse sales. In America alone, gross sales at auction totaled $940,874,621 in 2014 and pari-mutuel handle brought over $10,522,000,000, according to the Jockey Club Fact Book Index. In this industry, lameness and injury of a horse are the most common forms of economic loss, either through loss in training time or inability to race and earn money. Catastrophic bone failure, while not the most common, is the most devastating and generally creates a poor public image for racing. Much research has been done on risk factors and strengthening bone to avoid such sudden breakdown. Certain drugs permitted in racing, including furosemide, commonly used to reduce problems associated with Exercise Induced Pulmonary Hemorrhage, may contribute to decreased bone strength. Short-term effects of furosemide have been studied on various systems, but little is known about its long term impact on horses.

Injuries on the Track

Most of the injuries that occur in horse racing are musculoskeletal injuries of varying degrees (Stover, 2003). Some minor injuries may only postpone a horse's training while others may end a horse's career or life. Catastrophic bone failure is one of the leading reasons for euthanasia on the track (Johnson et al, 1994; Stover, 2003) and

probably one of the most devastating to the sport. Many fans remember the tragic stories of Ruffian, who snapped her sesamoid bones during a match race, Eight Belles, who collapsed shortly after crossing the finish line in the 134th Kentucky Derby with two broken front legs, and Barbaro, the Triple Crown hopeful whose dreams were ended when he sustained multiple fractures in his right hind leg.

These unfortunate events have increased research interests addressing how to heal and prevent these injuries with many studies focusing on possible risk factors for fatal injuries. Johnson et al. (1994) studied the causes of death of racehorses over a two-year period and found musculoskeletal injuries comprise nearly 83% of fatalities in Thoroughbreds on the track with 85% of those injuries being bone fractures. In this study, fatal injuries occurred evenly between races and training, showing there appeared to be a point where the horse's bone was weakened and failed after passing a certain threshold. Stover (2003) had similar findings and listed exercise as a risk factor in fatal injuries. An earlier study found that risk for injuries increased 4.2 times after horses exceeded a certain exercise intensity threshold (Estberg et al., 1998).

In the Jockey Club's most recent release of the Equine Injury Database Statistics, fatalities and number of starts are listed and divided among track surface, distance, and age of horses over a six-year period, from 2009 to 2014. Fatal injuries occur in an average of 1.91 horses per 1000 starts. The greatest fatalities per start occurred in races shorter than 6 furlongs, in horses over the age of four, and on dirt tracks. In shorter races, greater speeds are reached and therefore increase strain on the legs, which could explain the findings related to more fatalities in shorter races. Older horses are also more likely to

have previous injuries that weaken the bone as well as less elastic bone, and both of these factors could result in fracture. Dirt tracks are associated with increased incidence of injury in most states, but there are other confounding variables in states where injuries occur less often on dirt (Stover, 2003). Bone fatigue has been cited as a potential risk factor for catastrophic bone injuries (Beisser et al, 2014; Martig et al, 2014), but more research is needed to determine fatigue factors of bone in racehorses.

Little is known about how to prevent such catastrophic injuries and other non-catastrophic injuries. In a survey of non-fatal injury occurrence in California racehorses, Hill et al. (2015) found that non-fatal injuries occurred 17 times more frequently than fatal injuries, and the most common injury was fracture. Previously, a common injury that plagued racehorses, especially those in the beginning of their training, was a condition called bucked shins. Veterinarians and researchers now consider this condition to be a fatigue injury of the third metacarpal bone (Nunamaker, 2000). While up to 70% of Thoroughbreds develop bucked shins, around 12% of those horses will develop a stress fracture within a year (Nunamaker, 2000). Working a horse over short distances at very high speed, known as breezing, is indicated in preventing or reducing this condition while long gallops appear to increase incidence of this injury (Boston and Nunamaker, 2000). As a bone fatigue injury, bucked shins is not always apparent and sometimes requires specialized diagnostics to measure the bone and its possible damage.

Bone

Bone is a much more dynamic system than most realize. It protects internal organs, provides structure and movement, and serves as the largest mineral store in the body. Bones consist of a small organic matrix, mostly collagen, to provide flexibility and an insoluble salt of calcium and phosphorus, called hydroxyapatite, for rigidity and strength. Nearly all of the horse's calcium is stored in bone. As the horse grows, cartilage in the growth plate goes through mineralization, slowly transforming into hydroxyapatite, and expands in length and size. This process is called modeling. The growth plate usually lies between the epiphysis and metaphysis of the bone, and as it expands, bone is simultaneously resorbed from other areas.

While long bone growth plates, like those in the third metacarpal bone, are usually fused by 3.5 years in horses, bone is constantly adapting to suit the needs of the horse. Remodeling occurs when physical stress causes microdamage in the bone. Osteocytes near the microdamage undergo apoptosis and release growth factors and prostaglandins to signal the need for repair. Through the osteoclasts and osteoblasts, the bone matrix is resorbed from other areas and regenerated at points where the most strain occurs. Unlike in growth, the resorption of the bone matrix and hydroxyapatite and the mineralization and strengthening of weak areas may not happen at the same time or in the same location. Once bone is resorbed for remodeling, it may take months before the bone is fully repaired or strengthened (Firth, 2006), thus suggesting that a bone remodeling may in fact be weaker before it is stronger. Stress and risk for microdamage increases

with gait and speed (Biewener et al., 1983; Boston and Nunamaker, 2000) and the greatest strain is on the metacarpal bones in the legs.

Numerous studies (Hoekstra et al., 1999; Bell et al., 2001; Nielsen et al., 2002; Hiney et al., 2004) show the negative effects of stalling on bone mineral content in horses. When horses transition from pasture to stall, bone mineral content decreases dramatically. At pasture, horses have access to free choice exercise and can easily stress bone enough to maintain or increase their bone mineral content. Stalling horses limits their movement dramatically and typically causes their body to resorb much of the calcium and bone matrix from lack of use. Many studies have used this adaptation to prevent confounding factors and determine methods that can maintain or even increase bone mineral content (Nielsen et al., 2002; Hoekstra et al., 1999). Usually, long term stalling decreases total RBAE but can affect also behavior of young horses who may find other ways to exercise and relieve their boredom (Hiney et al., 2004). After 28 days of confinement, Bell et al. (2001) found an increase in total RBAE of stalled weanlings that correlated with increased activity in the stall, primarily through the action of pawing. This study also found that daily access to pasture was enough to prevent the loss of bone mineral content from stalling.

In a review by Nielsen and Spooner (2007), exercise appeared to have a greater effect on bone quality than nutrition. Using biochemical markers reported by a series of studies, this review found that two out of seven nutrition-based studies showed trends for increased bone quality and osteocalcin, but six out of eight exercise-based studies showed differences in osteocalcin and bone quality. Looking at these nutritional factors, mineral

balance seems to be the most important factor. Feeding a very high mineral ration may help horses in training retain more calcium and increase total RBAE (Nielsen et al., 1997). However, one study (Nielsen et al., 1998) showed that when horses were initially brought into training, calcium retention was negative, and bone mineral content declined until day 56, in spite of an increase in calcium intake from non-training to training periods.

While it has been suggested that bone needs high intensity exercise like galloping to strengthen (Boston and Nunamaker, 2000; Nunamaker, 2002; Firth, 2006), trotting with supplemental weight provided enough strain to increase bone mineral content over non-weighted controls, suggesting a role of over-strain as opposed to pure speed (Nielsen et al. (2002). Short bouts of exercise at a fast speed showed improvement in bone mineral content in as little as eight weeks (Hiney, 2004). However, bone experiences the greatest change to novel stimuli and can return to a maintenance state after long-term exposure to the same exercise (Firth, 2006), often referred to as reaching a new steady-state. Thus, exercise protocols that may initially strengthen bone may later maintain bone without further increase in strength. That "steady state" point is likely a state uniquely suited to the stress being applied.

Mechanisms for Measuring Bone

Many non-invasive measures of bone formation and degradation have been established (Lepage, 2001). Some of the most heavily used methods include biochemical markers and radiographic bone aluminum equivalencies (RBAE). Researchers have used

biochemical markers in horses since the development of similar assays in humans in the 1980s. The original tests required urine collections, which can prove difficult and labor intensive with horses. When blood tests became available, use in equine research increased (Lepage, 2001).

Biochemical markers are proteins believed to be released by bone into the bloodstream during formation, like osteocalcin, or resorption, like deoxypyridinoline, pyridinoline, carboxy-terminal PYD crosslinked telopeptides of type I collagen (Lepage, 2001). Two of the most commonly used biochemical markers for bone, and those with the strongest correlation to bone metabolism, are osteocalcin and pyridinoline cross-links. Osteocalcin (OC) or bone gla-protein is a small, noncollagenous protein synthesized by osteoblasts in bone formation (Lepage, 2001) and comprises the majority of protein in bone (Zoch et al., 2015). Osteocalcin can be either carboxylated or uncarboxylated in posttranslational processing (Zoch et al., 2015), and this processing determines its role in the body. Carboxylated OC binds tightly with the hydroxyapatite in bone, but under- or uncarboxylated OC is secreted into the blood (Zoch et al., 2015). Increased levels of circulating OC indicate increased osteoblast or bone activity, such as during growth, and horses show decreasing levels of osteocalcin after 3.5 yearswhen horses are typically finished growing (Lepage, 1989). Pyridinoline cross-links (PYD) are cross-linking amino acids that help to stabilize the collagenous bone matrix (Lepage, 2001). Bone releases PYD into blood during degradation of the bone matrix. In a comparison to pasture-reared horses, stalled horses had lower OC concentrations and greater concentration of deoxypyridinoline, another marker of bone degradation (Hoekstra et al.,

1999). Thus, OC and PYD are useful together to determine changes in both rate of formation and resorption. However, the rate of clearance of these markers have not been determined, and concentrations do not reflect bone structure or strength (Lepage, 2001).

Radiographic Bone Aluminum Equivalencies (RBAEs) are used to measure bone mineral content radiographically (Meakim et al., 1981). This technique uses an aluminum step wedge as a reference standard in radiographs of the third metacarpal, usually in the left foreleg, in horses. Area of mineralization is then compared to the wedge, and amount of mineralization is calculated. Though this technique only measures mineral content and not actual bone density, it is considered that greater bone mineral content indicates a stronger, denser bone. With the recent development of digital radiography, software packages have been developed and tested to determine RBAE values (O'Connor-Robison and Nielsen, 2013). However, his technique is limited in its ability to measure changes in bone mineral content that are less than 30% (Lepage, 2001). Many studies (Hoekstra et al., 1999; Bell et al., 2001; Nielsen et al. 2002; Hiney et al., 2004) use RBAEs in conjunction with bone markers to determine any loss or gain in bone mineral content that radiographs cannot detect. Developing appropriate bone mineral content and preventing injuries are not the only problems plaguing the racehorse industry. Another major challenge is the prevalence of Exercise-Induced Pulmonary Hemorrhage.

Exercise-Induced Pulmonary Hemorrhage

Exercise-Induced Pulmonary Hemorrhage, EIPH, is a disorder affecting up to 75% of racehorses and 46% of polo ponies (Sullivan and Hinchcliff, 2015), but its prevalence has not been established in other disciplines or breeds. In EIPH, extreme

pressure differences in blood and alveoli associated with intense exercise cause

pulmonary capillaries to burst (Sullivan and Hinchcliff, 2015). This bursting causes blood

to appear in the trachea and, sometimes, results in bleeding from the nose, known as

epistaxis. Epistaxis is the most outwardly visual sign of EIPH but only occurs in 1 to

3.5% of horses diagnosed with pulmonary hemorrhage (Pascoe et al, 1981; Takashi et al,

2001; Sullivan and Hinchcliff, 2015). Before the development of fiber optic endoscopes,

bloody noses and excessive swallowing after exercise were the only signs of what is now

called EIPH (Pascoe et al, 1981). It is possible that EIPH has been in literature for almost

3 centuries, but bleeding from the nose was believed to be a problem in the horse's head

and nose rather than in the lungs (Pascoe et al., 1981). Risk factors for this condition

include speed, race type (flat or hurdles), distance, age, cumulative racing volume, and

possibly ambient temperature (Takashi et al., 2001; Sullivan and Hinchcliff, 2015). EIPH

will almost certainly decrease performance and, though rare, can result sudden death

(Johnson et al., 1994).

If a trainer or owner suspects a horse has EIPH, a veterinarian can use a variety of

tests to confirm. These tests need to be performed shortly after near-maximal exercise

and can include tracheobronchoscopic assessment, bronchoalveolar lavage, or histologic

sampling of the lungs (Hinchcliff et al., 2005; Sullivan and Hinchcliff, 2015). In a

tracheobronchoscopic assessment, the veterinarian will pass an endoscopy tube through

one of the nares and into the trachea. The EIPH is given a grade of 0 through 4,

depending on severity and amount of blood present as shown in Figure 1, A-D

(Hinchcliff et al., 2005). Grade 0 is no blood present in the pharynx upon examination

(not shown). A horse is considered Grade 1 if there is one or more flecks of blood or two or fewer short, narrow streams of blood in the trachea (A). Grade 2 can possess more than two short, narrow streams of blood or one long stream of blood that is greater than half the length of the trachea (B). Grade 3 occurs when there are multiple, distinct streams of blood covering over a third of the tracheal circumference (C). Grade 4 is the most severe form of EIPH and is assigned when there are multiple streams of blood covering more than 90% of the trachea with blood pooling at the thoracic inlet (D) (Hinchcliff et al., 2005).
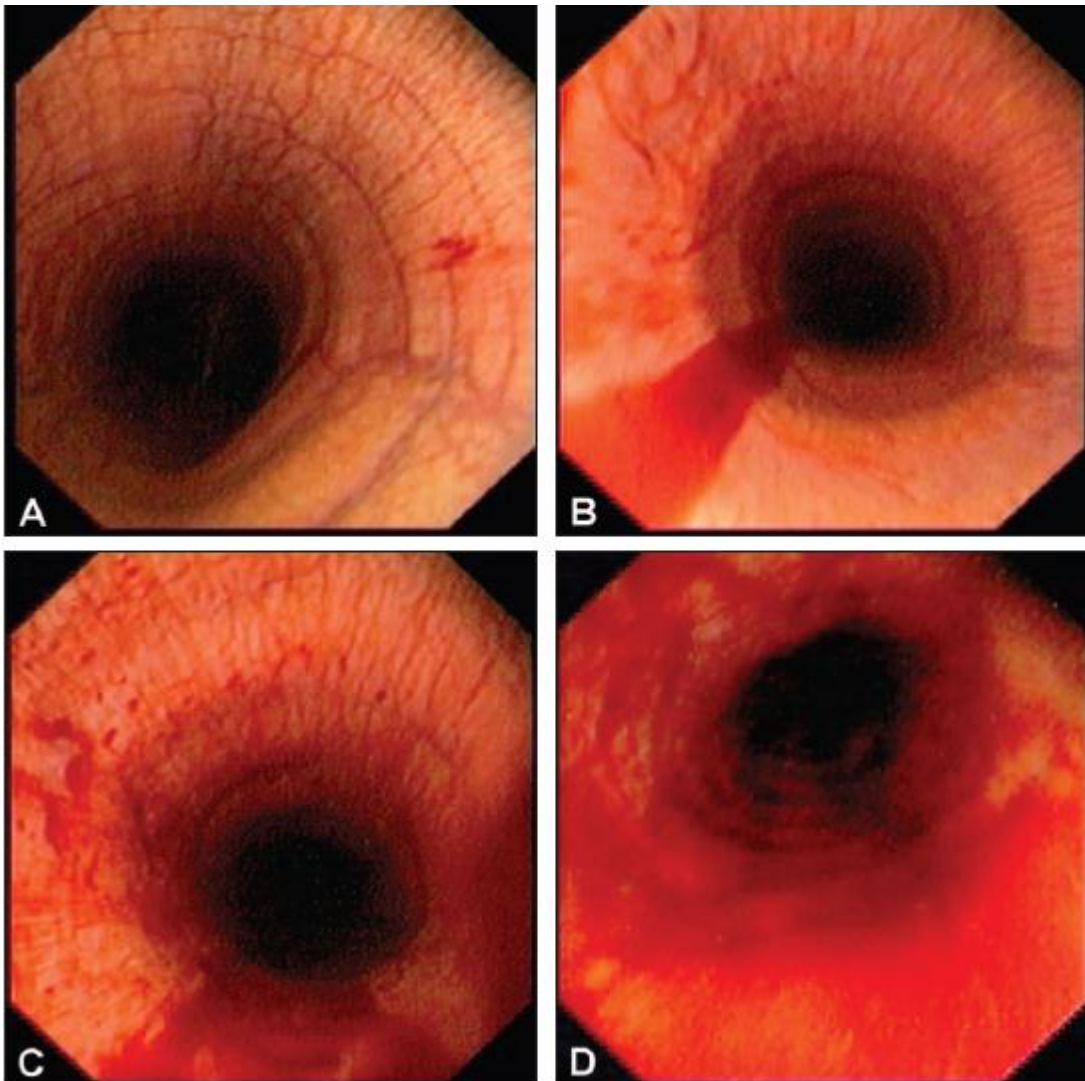
Figure 1. (Hinchcliff et al., 2005) Grades of severity of Exercise-Induced Pulmonary Hemorrhage as seen through tracheobronchoscopic assessment

Exercise induced pulmonary hemorrhage is associated with impaired performance in racehorses (Morley et al., 2014). Typically, Grades 1 to 2 do not markedly affect performance or longevity, but Grades 3 and 4 have been associated with greater distance behind race winners and less earnings (Hinchcliff et al., 2005). Theoretically, Grade 4 EIPH could contribute to greater losses over time because of the damage to the pulmonary capillaries and lungs and the buildup of subsequent scar tissue, and it is believed to be progressive (Sullivan and Hinchcliff, 2015). There is no treatment that will abolish EIPH because the exact mechanism to reduce or prevent the condition is unknown (Sullivan and Hinchcliff, 2015). Many recommendations to heal or lessen EIPH include rest and decreasing irritants that may cause lower airway inflammation (Sullivan and Hinchcliff, 2015). Most horses receive regular doses of furosemide as a prophylactic to lessen the severity of the bleed, via a reduction in circulating blood volume and thus lower pulmonary pressures, but this treatment has received mixed results (Pascoe et al., 1981; Soma and Uboh, 1998; Kindig et al., 2001). However, the racing industry in most of the United States and Canada continues to use furosemide, in spite of the contrary evidence on its usefulness with EIPH and lack of research on the effects of long-term use in the horse.

Furosemide

Furosemide has been used in racehorses for over 30 years in an attempt to manage or prevent EIPH (Sullivan et al., 2015). Many racing jurisdictions in the United States and Canada allow for the use of furosemide on race day with limits on when and who

may administer the dose and how much can be present in the blood after the race (Spencer et al., 2008). Trainers must declare their horses that will run on furosemide and must ensure that the horse receives its dose as part of "the public's need-to-know" on pari-mutuel betting (Soma and Uboh, 1998). Horses may also receive doses of furosemide given during training once they are confirmed to have EIPH, so horses may be getting furosemide frequently over long periods of time spent in training and active racing.

Furosemide is a diuretic that has long been used in humans and other species with signs of heart or renal failure. This drug acts directly on renal tubular function in the loop of Henle, where it binds and inhibits Na^+-K^+-$2Cl^-$ cotransporter activity. With the inhibition of the cotransporter, sodium and chloride increases in concentration in the distal tubule, drawing fluid it, and causes the animal to produce large volumes of urine (Hinchcliff and Muir, 1991). Renal effects include increased renal blood flow through decreased vascular resistance and redistribution of blood flow as well as increased natriuresis, chloruresis, and urinary hydrogen ion excretion (Hinchcliff and Muir, 1991). This elimination of fluid through the kidneys results in overall reduction of fluid in the body and subsequently a decrease in body weight through loss of water. In humans, hemodynamic changes include decreased pulmonary blood volume and reduced left ventricular filling pressure, which is why furosemide is often used in patients with congestive heart failure (Hinchcliff and Muir, 1991).

Furosemide has several immediate adverse effects. In humans, it is known to cause dehydration and hypotension (Spino et al., 1978). It can also contribute to

hypochloremia, hypokalemia, and hyponatremia in about one-third of patients (Spino et al., 1978). The dehydration effect has been used in horses to test for rehydration (Butudom et al., 2004), but few of the other effects have been investigated in horses.

Long-term use in humans and dogs usually leads to furosemide resistance, so the dosage must be adjusted somewhat frequently to combat this (Asare, 2009). Decreases in water weight loss are used to gauge this resistance. In horses, an initial dose of furosemide can cause a horse to lose around 2% of its total body weight, usually through increased urine output (Butudom et al., 2004), but additional doses and resistance have not been researched to date. Because furosemide increases urine excretion and affects electrolyte and calcium balance, current and past use has been associated with higher risk for hip fractures in elderly humans when controlling for other factors (Heidrich et al., 1991). Another study (Rejnmark et al., 2005) showed that long-term treatment with loop diuretics like furosemide increased calcium loss without a difference in bone mineral density. However, patients treated with loop diuretics had higher bodyweights than nonusers, which could explain why no difference was seen as higher weights would typically be associated with greater BMC.

Furosemide can be effective at decreasing pulmonary resistance in ponies with recurrent obstructive pulmonary disease, but the drug had no effect on healthy ponies or on arterial oxygen and carbon dioxide concentrations (Broadstone et al., 1990). Increasing pulmonary compliance is thought to be one of the ways furosemide acts on EIPH, but the exact mechanism is unknown. Some studies speculated that it could be from a reduction in pulmonary pressure (Kindig et al., 2001) while others (Soma and

Uboh, 1998) theorized that furosemide does not reduce pulmonary pressure enough to explain its effects on the severity of EIPH.

Through its natuiresis and chloruresis abilities, furosemide affects mineral excretion through urine. Urine calcium, phosphorus, sodium, and chloride excretions increased over a 24-hour period following administration of furosemide to normal horses (Pagan et al., 2014). Sodium and phosphorus levels returned to normal fairly quickly, but calcium balance remained negatively affected for 72 hours after the dose. This study did not determine when calcium balance returned to normal, but it does suggest that if furosemide is used chronically and frequently, then the negative calcium balance could become detrimental as seen in previous human studies (Heidrich et al., 1991; Rejnmark et al., 2005).

Furosemide use has potential adverse effects in humans, and these effects have not been studied in horses. The racing industry has used furosemide for several decades to mitigate one of its most prevalent problems, but little has been researched on its potential adverse impact on other systems. Researchers have placed much of the focus on furosemide's ability to treat or prevent EIPH with mixed results and paid little attention to other uses or problems that could occur with the drug.

CHAPTER II: INFLUENCE OF LONG-TERM FUROSEMIDE USE ON BONE MINERAL CONTENT, BONE METABOLISM MARKERS, AND WEIGHT LOSS IN HORSES

While fatal injuries in horse racing are uncommon, these injuries have a huge impact on public perception of the sport, and non-fatal musculoskeletal injuries are common among racehorses. Previous literature has suggested bone mineral content is lost during prolonged stalling (Hoekstra et al., 1999; Bell et al., 2001; Nielsen et al., 2002; Hiney et al., 2004) and may take weeks or months to return to baseline (Firth, 2006). Preventing such loss or specifically strengthening bone could prevent certain injuries from occurring (Nunamaker, 2000). Along with musculoskeletal injuries, exercise-induced pulmonary hemorrhage (EIPH) is one of the top welfare and health concerns for racehorses. Use of furosemide to treat this condition has led to various studies questioning its efficacy (Soma and Uboh, 1998; Kindig et al., 2001; Sullivan and Hinchcliff, 2015), but, nonetheless, its use is still permitted and commonplace at U.S. racetracks and usually continues through a horse's career. While the short term effects of furosemide on equine systems have been reported (Broadstone et al., 1990; Hinchcliff and Muir, 1991; Soma and Uboh, 1998; Kindig et al., 2001; Pagan et al., 2014), long term effects in horses have not been studied. One dose of furosemide is enough to affect calcium balance for 72 h (Rejnmark et al., 2005; Pagan et al., 2014), yet repeated doses have been shown to have decreasing diuretic effect (Hinchcliff and Muir, 1991). No research has been done to determine if these repeated doses that may decrease in efficacy also decrease in their effect on calcium balance. Over long periods of time such negative impact, if persistent, could reduce bone mineral content and contribute to the large

number of skeletal injuries in the racing industry. The current study seeks to evaluate the long term effects of furosemide on bone mineral content and biochemical markers for bone metabolism as well as weight loss as a measure of drug efficacy.

Materials and Methods

Eleven healthy horses of mixed breed and age (17±4 yr) were used to test the effects of furosemide on bone mineral content, bone metabolism markers, and weight loss. Horses were obtained from the teaching and research herd of Middle Tennessee State University (MTSU). The study was approved by the MTSU Institutional Animal Care and Use Committee (Protocol #15-009, Appendix).

All horses had dorsal-palmar and medial-lateral radiographs of the left third metacarpal taken on day -28 for determination of bone mineral density using radiographic bone aluminum equivalence. Each cassette was affixed with an aluminum step wedge pentrometer. The X-ray was set to 70 kV with an exposure of 0.16 seconds and focal length of 90 cm.

All eleven horses were then turned out on pasture for 28 d and given access to free choice exercise to serve as a backgrounding period. Horses received a commercial pelleted concentrate (Purina Strategy) daily at a level needed to maintain body condition. On day 0, a second set of radiographs was taken, and each horse was placed in a $9.3m^2$ stall for 28 d. Horses were randomly assigned to either the control (CON, n=5) or treatment (FUR, n=6) group. During this stalling portion of the study, horses were provided with prairiegrass hay and a commercial pelleted concentrate (Purina Strategy)

twice daily to maintain body condition. Horses had *ad libitum* access to water, except on days of furosemide treatment.

Both CON and FUR horses exercised on a mechanical panel exerciser for 60 min, 6 d per week from day 0-28. The exercise protocol involved 17 min walk, 10 min trot, and 3 min canter in both directions, never exceeding 8 m/s. On day 28, a third set of radiographs was taken, and horses were returned to pasture for an additional 28 d. A fourth and final set of radiographs was taken on day 56, and horses were returned to normal use in the MTSU Horse Science Program.

On day 0, 7, 14, 21, 28, 35, 42, and 49, FUR horses were administered IV furosemide (Salix® 5% Injectable, Intervet Inc./Merck Animal Health, Madison, NJ) at 1 mg/kg BW, a "normally accepted" dosage utilized in most racing jurisdictions. On these days, food and water was removed from all horses (both CON and FUR) for 4 hours post-administration to simulate racetrack conditions for horses administered furosemide. Feed and water was immediately returned after weighing horses at 4 h post administration. CON and FUR horses were weighed on an electronic scale before administration and at 2 h, 4 h, 8 h, 24 h, and 48 h after administration to monitor weight loss and weight recovery.

Venous blood samples were collected via jugular venipuncture into heparinized and untreated tubes on treatment days (before furosemide administration) and for two days after furosemide administration. Samples were centrifuged, placed into plasma and serum aliquots, and frozen at -4°C until analysis. Plasma osteocalcin and serum pyridinoline cross-links were analyzed using enzyme-linked immunosorbent assays

(MicroVue™ Serum PYD EIA and MicroVue™ Osteocalcin EIA, Quidel Corporation,
San Diego, CA), and concentrations were generated from absorbance densities using a 4-
Parameter equation (Microplate Manager 6 Software, Bio-Rad Laboratories, Inc.,
Hercules, CA). Change from baseline was determined.

Bone mineral content of the third metacarpal was determined from the
radiographs using radiographic bone aluminum equivalency (RBAE) for all bone cortices
and total BMC, with measurements taken immediately distal to the nutrient foramen.
Radiographs were analyzed using BioRad Quantity One software to form a regression
model using the known thickness of the aluminum stepwedge via the method of
O'Connor-Robison and Nielsen (2013).

Changes in BMC, bone metabolism markers, and weight loss were analyzed for
day, treatment, and time effects (when appropriate) using a mixed model ANOVA with
repeated measures (SAS Inst. Ver 9.2, Inc., Cary, NC). Tukey's adjustment was utilized
when making multiple comparisons. A P-value less than 0.05 was considered significant
and trends were considered when P was less than 0.10.

Results

The BMC in all cortices of both groups were not different at the beginning of the
study (Table 1). Data were placed into periods to reflect change from baseline. Period 1
constituted the time from day -28 to day 0, Period 2 reflected day 0 to day 28, and Period
3 showed day 28 to day 56. No treatment effect was observed ($P = 0.90$), but there was

Table 1. Bone mineral content in mmAl as determined by radiographic bone aluminum equivalency (RBAE) across medial, lateral, dorsal, and palmar cortices of the left third metacarpal bone in control horses and horses administered furosemide over the duration of the study.

		Bone Mineral Content, mmAl				
		Day -28	Day 0	Day 28	Day 56	*P*-Value
Medial	CON	35.24±1.39	35.84±1.39	38.38±1.39	55.58±1.39	0.76
	FUR	36.75±1.27	35.63±1.27	39.35±1.27	56.15±1.27	0.53
	P-Value	0.43	0.91	0.61	0.76	
Lateral	CON	35.05±1.47	35.84±1.47	37.22±1.47	47.52±1.47	0.69
	FUR	36.93±1.34	35.63±1.34	39.22±1.34	52.58±1.34	0.47
	P-Value	0.35	0.92	0.32	0.02	
Dorsal	CON	35.52±0.98	35.34±0.98	36.99±0.98	47.62±0.98	0.9
	FUR	35.27±0.90	35.11±0.90	38.49±0.90	48.60±0.90	0.89
	P-Value	0.86	0.86	0.27	0.47	
Palmar	CON	35.14±1.05	35.34±1.05	36.12±1.05	45.86±1.05	0.89
	FUR	34.93±0.96	34.59±0.96	36.95±0.96	45.34±0.96	0.8
	P-Value	0.88	0.6	0.56	0.72	

a period effect across all cortices ($P < 0.0001$) but not total BMC ($P = 0.15$). Period 1 and Period 2 were not different in any cortex ($P = 0.42$). Total BMC was not different in any period ($P = 0.92$). Period 3 saw an increase in BMC in medial, lateral, dorsal, and palmar cortices of 17.01±1.15 mmAl, 11.83±1.11 mmAl, 10.37±0.87 mmAl, 9.07±0.82 mmAl, $P < 0.0001$, respectively (Figures 2, 3, 4, and 5).

Bone metabolism markers also reflect changes from baseline. OC concentrations represent change from the beginning to the end of a two week period (e.g. from day 0 to day 14). OC levels showed no difference between groups ($P = 0.26$) or days ($P = 0.25$) with a mean concentration change of -1.6±0.92 ng/mL and -0.16±0.84 ng/mL for CON and FUR, respectively (Table 2; Figure 6). PYD concentrations reflect change within a 24h period. PYD showed a trend towards a treatment effect ($P = 0.058$) and a day effect ($P < 0.0001$)(Figure 7). The change in PYD concentrations decreased from day 1 to day 15 ($P < 0.0001$), then increased to day 29 ($P = 0.0042$), and decreased again to day 43 ($P < 0.0001$) (Table 3).

Figure 2. Change in bone mineral content in mmAl as determined by radiographic bone aluminum equivalence (RBAE) in the medial cortex of the left third metacarpal bone over the three periods in the study. Asterisk indicates difference ($P < 0.001$) between periods.
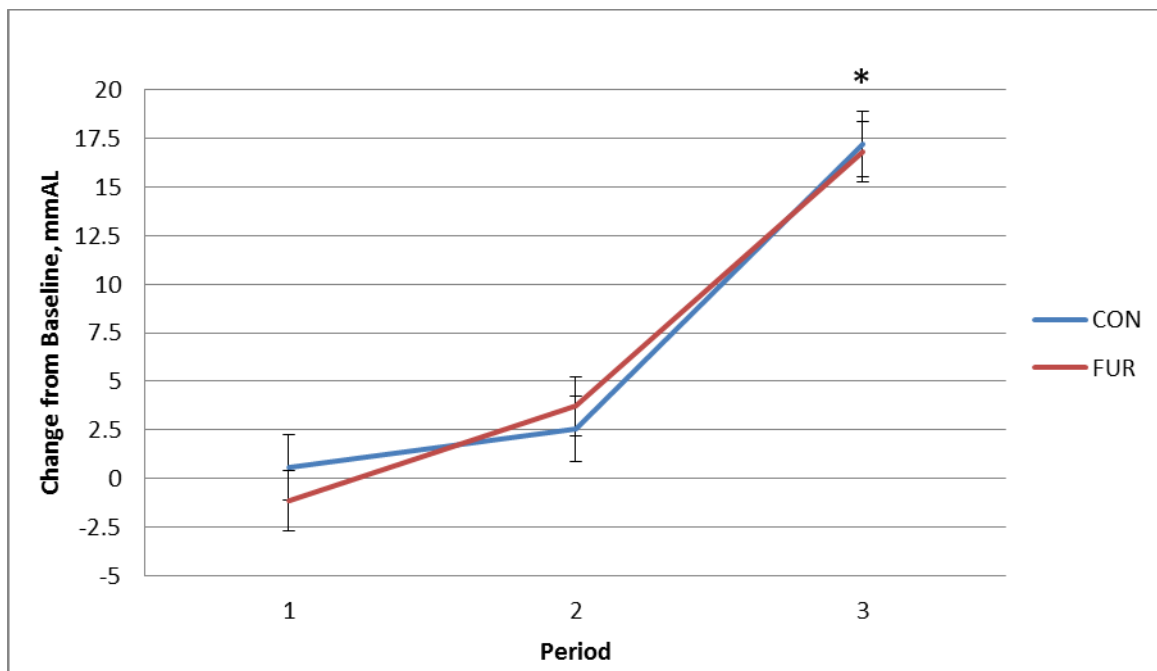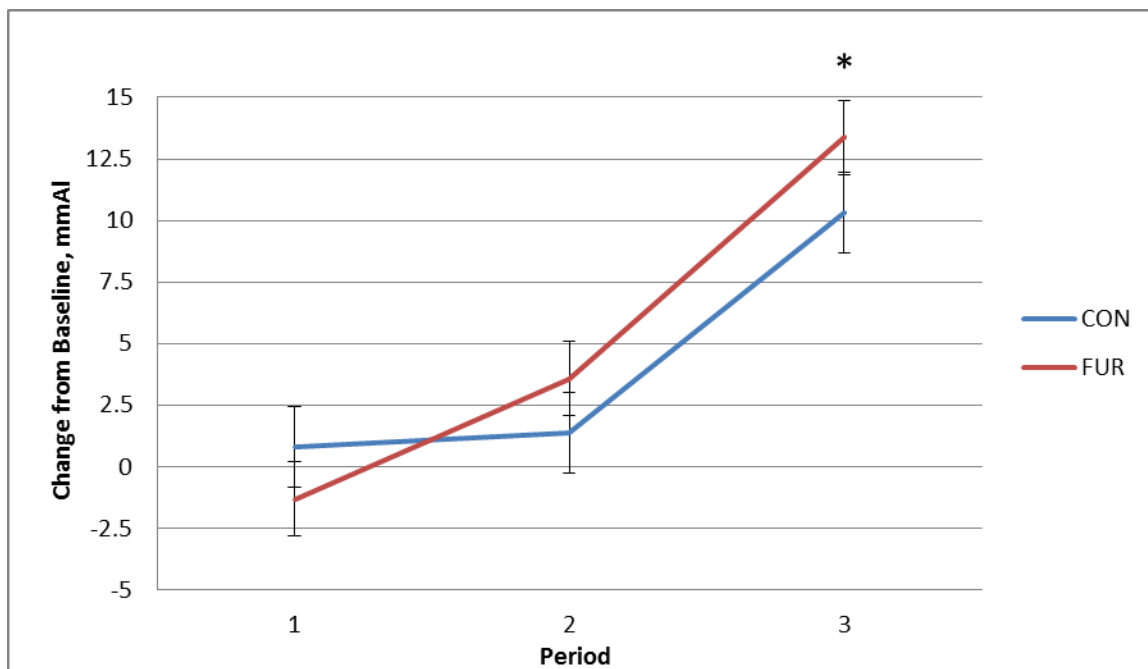
Figure 3. Change in bone mineral content in mmAl as determined by radiographic bone aluminum equivalence (RBAE) in the lateral cortex of the left third metacarpal bone over the three periods in the study. Asterisk indicates difference ($P < 0.001$) between periods.

Figure 4. Change in bone mineral content in mmAl as determined by radiographic bone aluminum equivalence (RBAE) in the dorsal cortex of the third left metacarpal bone over the three periods in the study. Asterisk indicates difference ($P < 0.001$) between periods.
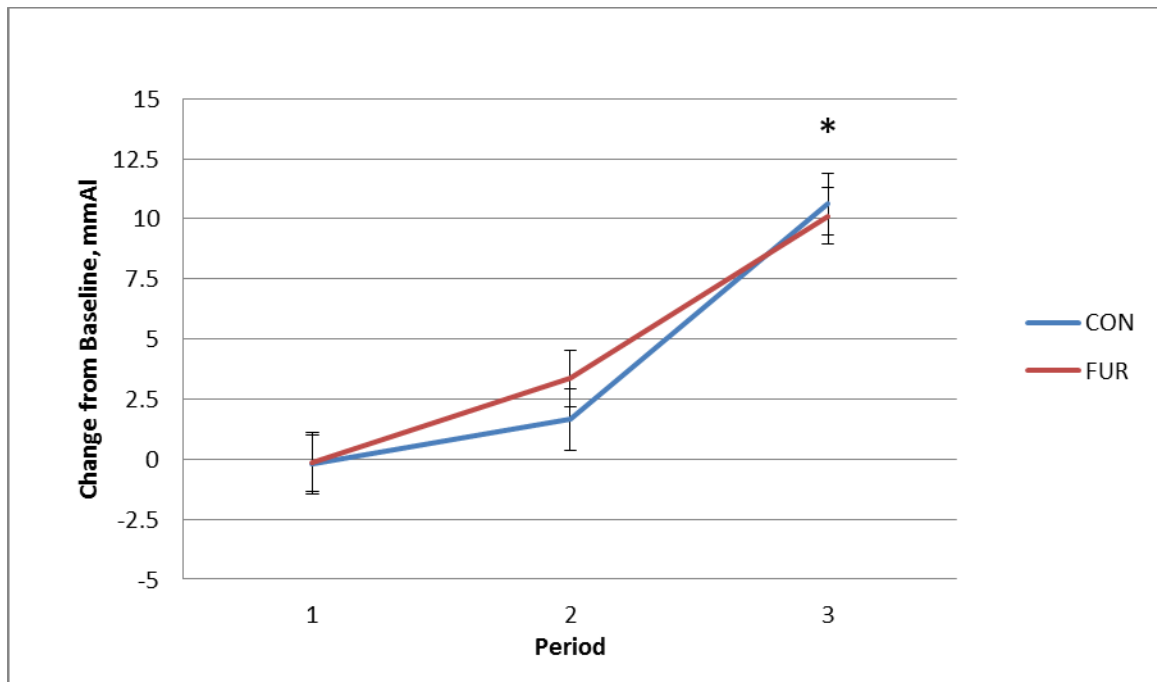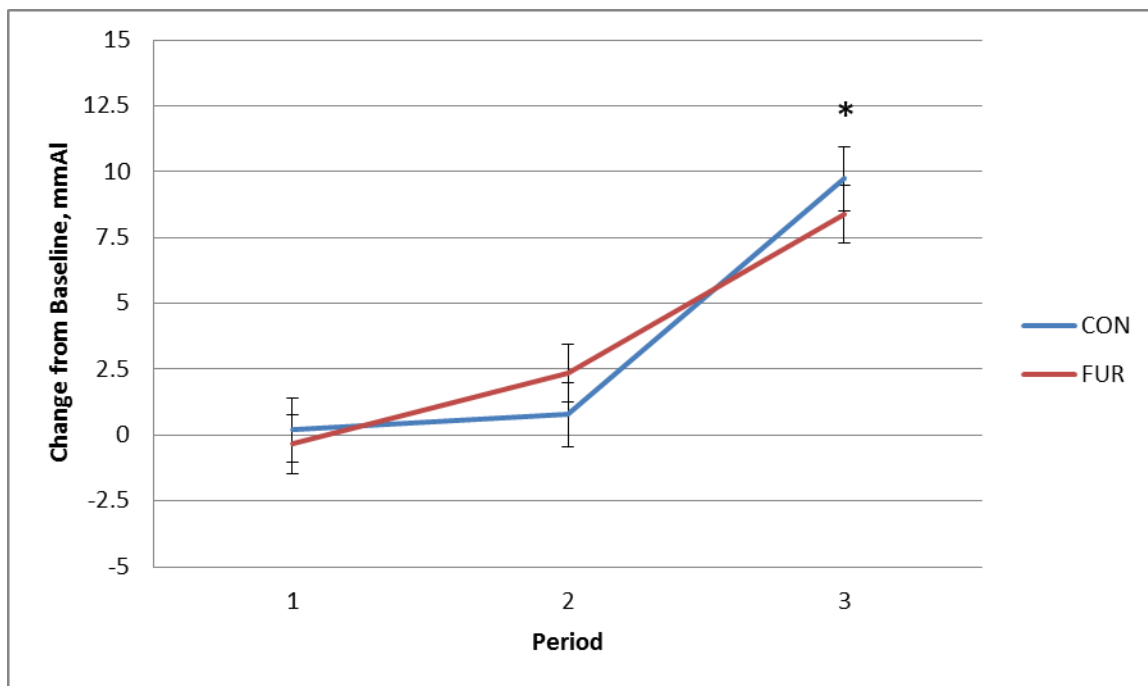
Figure 5. Change in bone mineral content in mmAl as determined by radiographic bone aluminum equivalence (RBAE) in the palmar cortex of the third left metacarpal bone over the three periods in the study. Asterisk indicates difference ($P < 0.001$) between periods.

Table 2. Mean plasma osteocalcin concentrations in control and furosemide horses in two-week intervals showed no differences due to treatment or day ($P < 0.99$).

	Osteocalcin Concentration, ng/mL				
	Day 0	Day 14	Day 28	Day 42	Day 56
CON	17.98±2.9	16.1±2.9	18.42±2.9	13.94±2.9	11.59±2.9
FUR	12.62±2.65	11.32±2.65	17.22±2.65	16.81±2.65	11.98±2.65

Table 3. Mean serum pyridinoline cross-link concentration changes over 24 hours in control and furosemide horses. [abc]Days lacking a common superscript differ ($P < 0.05$).

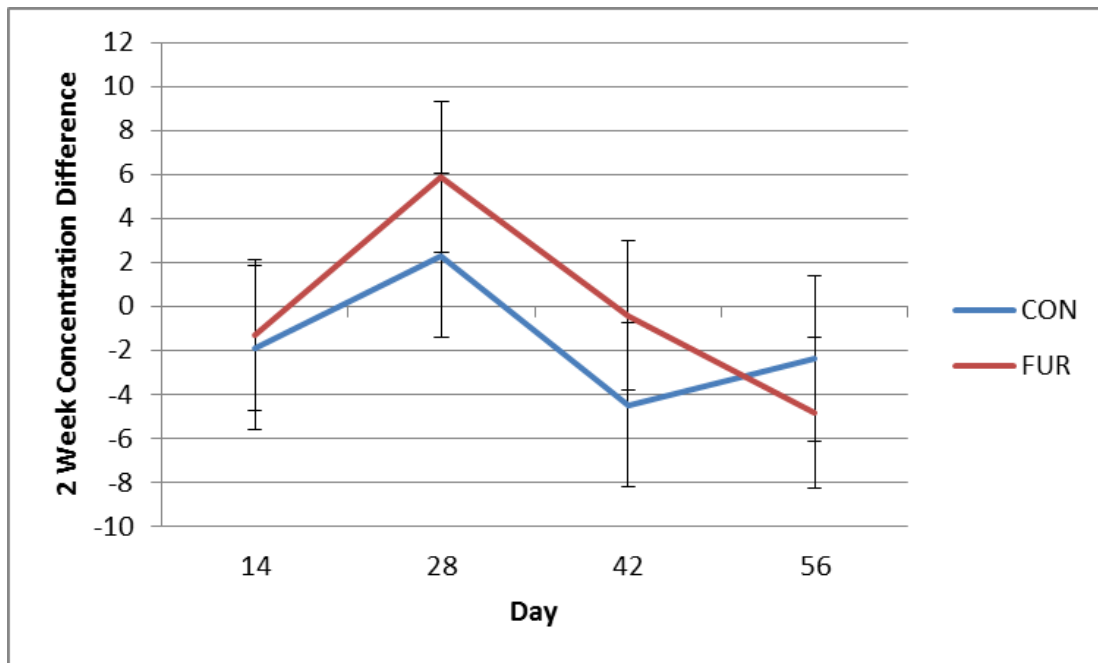|	Pyridinoline Concentration Change, ng/mL			
	Day 1	Day 15	Day 29	Day 43
CON	3.2004±0.65[a]	-2.1428±0.65[b]	-0.3068±0.65[c]	-3.6286±0.65[b]
FUR	4.0227±0.59[a]	-1.5422±0.59[b]	1.1030±0.59[c]	-2.5492±0.59[b]

Figure 6. No difference (P < 0.99) in mean plasma osteocalcin concentration in control and furosemide horses over two-week intervals.
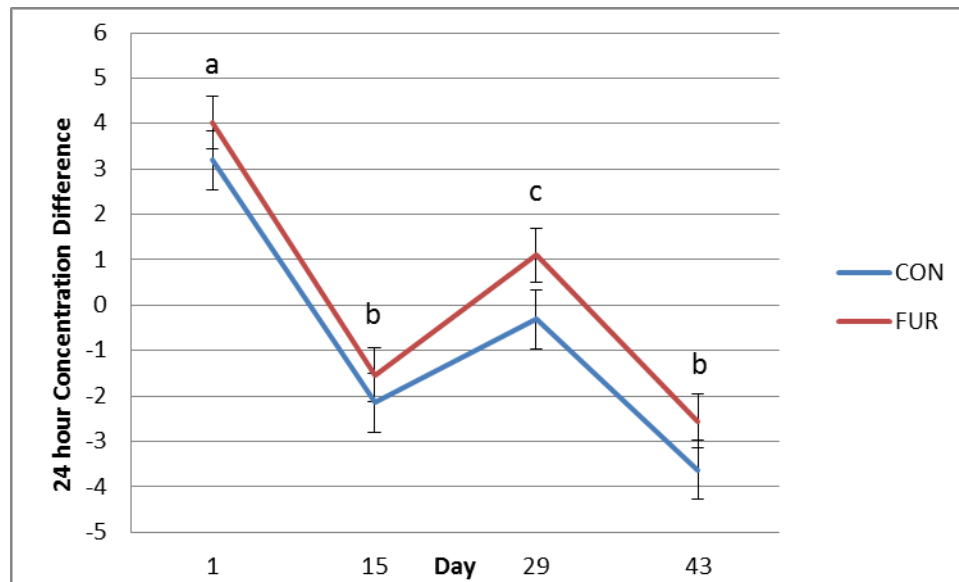
Figure 7. Mean serum pyridinoline cross-links concentration changes over 24 hours in furosemide and control horses. [abc]Days lacking a common superscript differ ($P < 0.05$).

For BW, there was no difference in experiment starting weights between groups (CON = 521.65 kg ± 30.78, FUR = 530.13kg ± 28.16, $P < 0.0001$). Data reflect change from baseline, where baseline is represented here at the starting weight for that date. The differences between FUR and CON are shown in Table 4. There were day by time ($P = 0.0001$), treatment by time ($P < 0.0001$), and day by treatment ($P < 0.0001$) interactions. Percent change in BW was different between groups on day 0 ($P = 0.028$), day 14 ($P < 0.0001$), day 21 ($P < 0.0001$), and day 42 ($P < 0.0001$; Figure 6). Differences occurred between groups at 2h ($P < 0.0001$), 4h ($P < 0.0001$), and 8h ($P = 0.024$; Figure 7). There was no difference in BW loss between day 0 and day 49 in CON ($P = 1.00$), but there was a difference between day 0 and day 49 in FUR ($P = 0.0473$).

Table 4. Percent change in bodyweight in control (CON) and furosemide (FUR) horses up to 48 hours post-administration. [abcde]Days lacking a common superscript differ ($P < 0.05$)

Percent Change in BW									
2h		4h		8h		24h		48h	
CON	FUR	CON	FUR	CON	FUR	CON	FUR	CON	FUR
-0.38 $\pm 0.27^{cd}$	-2.20 $\pm 0.25^{b}$	-1.02 $\pm 0.27^{c}$	-3.29 $\pm 0.25^{a}$	0.04 $\pm 0.27^{d}$	-1.21 $\pm 0.25^{c}$	0.59 $\pm 0.27^{e}$	-0.07 $\pm 0.25^{de}$	0.74 $\pm 0.27^{e}$	0.03 $\pm 0.25^{de}$

Figure 8. Time by treatment interaction in percent change in bodyweight in control (CON) and furosemide (FUR) horses up to 48 hours post-administration. Asterisk indicates difference ($P < 0.05$) between groups.
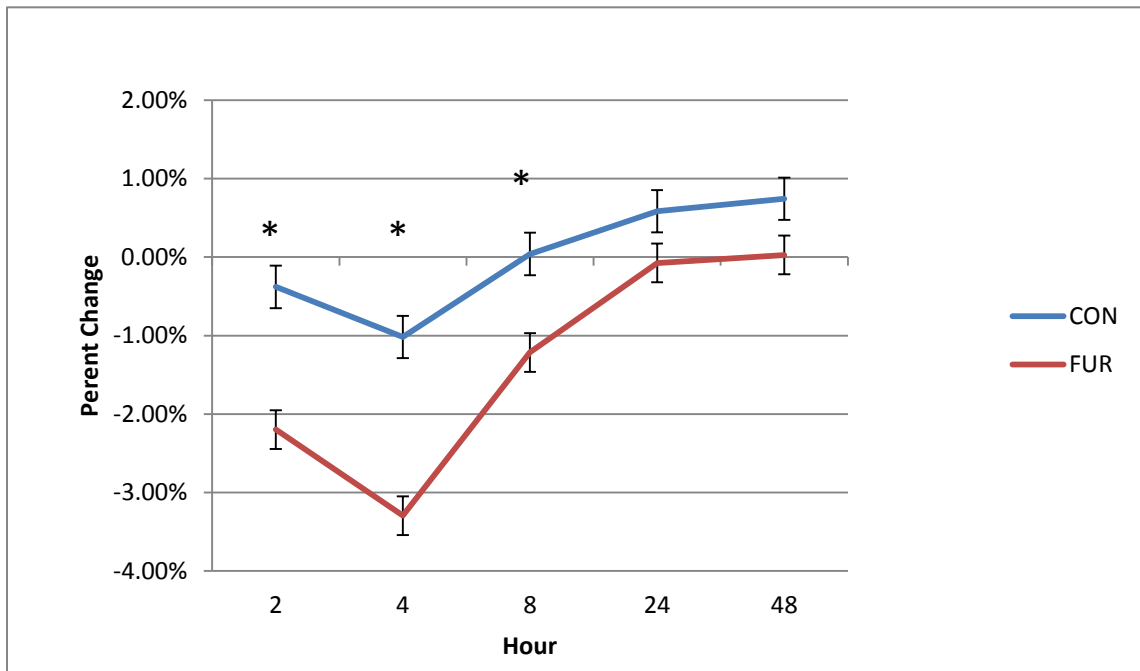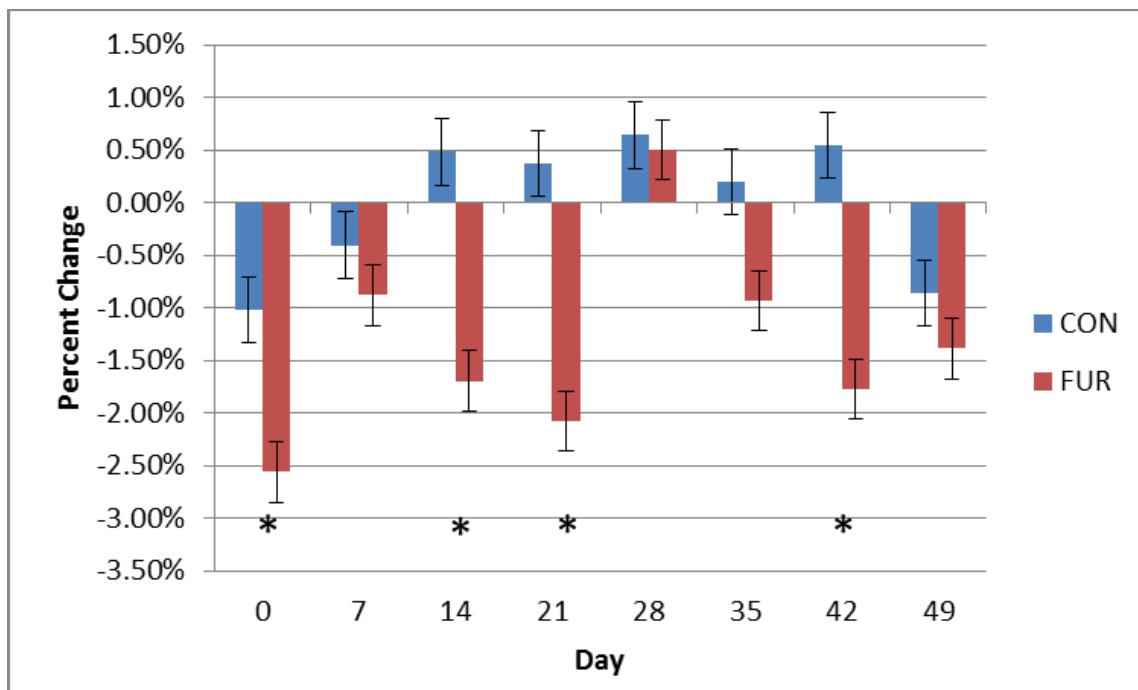
Figure 9. Day by treatment interaction in percent change in bodyweight in control (CON) and furosemide (FUR) horses up to 48 hours post-administration. Asterisk indicates difference ($P < 0.05$) between groups.
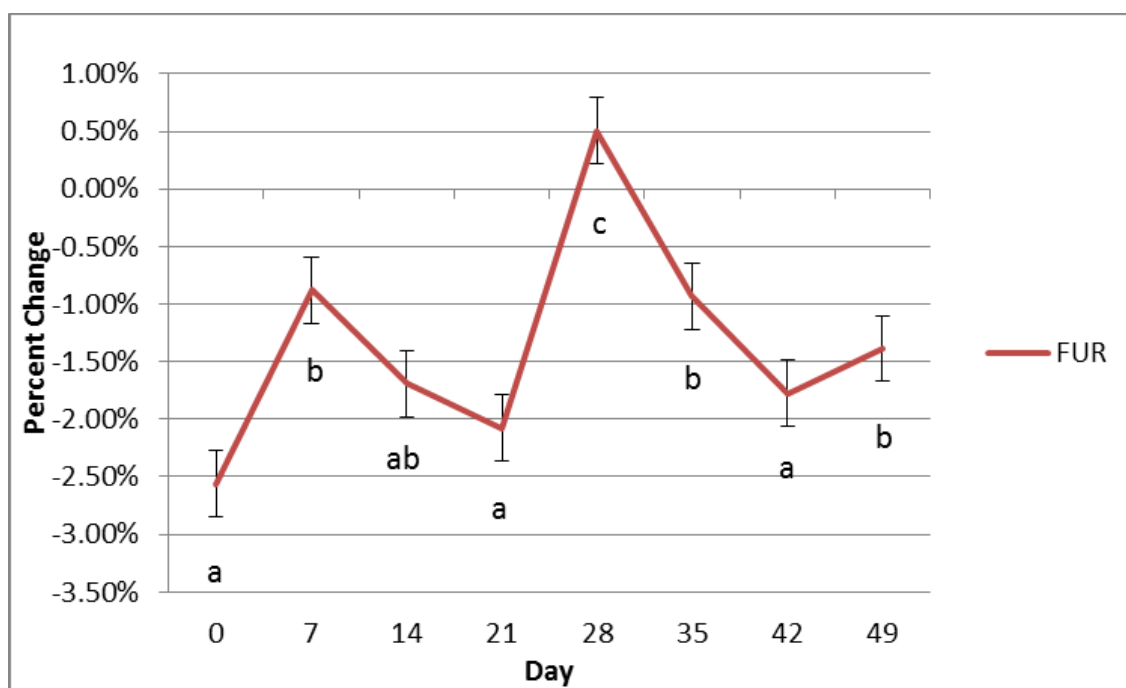
Figure 10. Percent change in bodyweight over time in horses administered furosemide. [abc]Days lacking a common superscript differ ($P < 0.05$).

Discussion

Most studies conclude that stalling lowers BMC in horses (Nielsen et al., 1997; Nielsen et al., 2002), but this study saw no change in BMC between Period 1 and Period 2. This effect could have been due to a lack of activity when the horses were at pasture caused by high temperatures (National Oceanic and Atmospheric Administration). Hiney et al. (2004) found no difference in activity between confined and group housed weanlings, similar to this study. However, researchers in that study may not have seen a difference because their observation window was only 15 minutes on certain days, so extra activity from the group housed horses may not have been captured on video. Even with this short observation window, there is still a possibility that when horses are on pasture with free choice access to exercise that they may choose not to exercise at all. Since horses in this study were older and the area was experiencing early summer heat, these conditions likely led to inactivity and a lack of difference in BMC between the first two periods.

While there was no difference between Period 1 and Period 2 in BMC, the increase in Period 3 observed in all cortices might be expected as the horses went from stalling with forced moderate exercise to pastures with free choice exercise. This finding agrees with previous research suggesting putting horses on pasture and giving them free choice exercise will increase BMC after a period of stalling (Hoekstra et al., 1999; Bell et al., 2001).

No effect of treatment or period was seen in OC concentrations, possibly because of the age of the horses. Previous research (Lepage et al., 1990; Chiappe et al., 1999;

Lepage et al., 2001) indicates that OC concentrations are inversely correlated with age, declining significantly after 3 years of age with less variation between horses. Since the mean age of horses in this study was much higher than 3 years, OC levels remained relatively low and unchanged. Other research (Lepage et al., 1991) has shown that OC has a circadian rhythm. Since all blood samples in this study were obtained in the morning when OC concentrations were near their peak as noted in Lepage et al. (1991), this rhythm does not explain the lack of difference between groups and days. Other studies (Nielsen and Spooner, 2008) that saw differences in OC concentrations used young, growing horses which naturally have higher OC concentrations and are more prone to changes in those concentrations (Lepage et al., 1990). Likely, OC, as a marker of bone formation, remained relatively stable throughout the duration of the study because horses were "mature" and no longer growing.

In contrast to OC and RBAE data from this study, PYD concentrations showed a trend towards a treatment effect and a significant day effect. Because PYD is an indicator of bone degradation (Lepage et al., 2001; Nielsen and Spooner, 2008), any increase in concentrations in 24h should be indicative of an increase in the breakdown of bone, and furosemide appears to contribute to this breakdown. This contribution could be from prolonged negative calcium balance as seen in Pagan et al. (2014) which would force the horse to draw from the calcium stored in the bones in order to meet its requirement. The difference in days may be reflective of a change in activities, whether that was coming in to stalls from pasture on Day 0 to Day 1 or being turned out on pasture from being stalled on Day 28 to 29. While these changes were not seen in BMC, there may not have been

enough time for these changes to accumulate to be seen on radiographs as it may take up to a 30% difference from baseline to be determined by radiograph (Lepage et al., 2001; Firth, 2004). Previous research used a period of at least 62 days from the onset of stalling and training to observe a decrease in BMC (Nielsen et al., 1997) while this study lasted only 56 days from the onset of stalling.

Body weight losses were typical in FUR horses post administration, as determined in previous studies (-4.1 ± 0.3%, Warren et al., 1999; -2.1 ± 0.3%, Butudom et al., 2004). However, previous studies did not determine BW loss within a control group. This study demonstrated furosemide administration lead to significant loss of BW over controls (FUR: -1.35 ± 0.2%, CON:- 0.01 ± 0.2%, P < 0.0001), and dehydration persisted for up to 8h post-administration before the FUR horses' weights returned to baseline. As noted in Butudom et al. (2004), this dehydration is comparable to endurance exercise and presents similar rehydration and possible electrolyte balance problems. Warren et al. (1999) reported greater percent loss in BW, but researchers used intramuscular furosemide injection. Horses in that study also had higher starting BW (lowest BW group: 569.5±16 kg) than horses in the current study, suggesting heavier horses might have more BW to lose, particularly if they had greater body fat.

In day by treatment interactions, a difference was noted between day 0 and day 49 in FUR (P = 0.047) but not in CON (P = 1.00). This difference in FUR but not CON could be attributed to drug resistance. Loop diuretic resistance is well known in intensive care units and usually requires dosage adjustment to maintain urine output (Asare, 2009). Because no long-term studies have been conducted with horses, this difference could be

the first documentation of furosemide resistance in horses. Resistance could mean that furosemide would become less effective as a treatment for EIPH and would need to be adjusted to maintain efficacy.

Based on these results, further research into long-term effects of furosemide is warranted. The treatment trend seen in the PYD changes could present a serious problem over time. More research is needed to determine if this trend is an effect either with more horses or over a longer period of time. The point at which changes in PYD concentrations translate into changes in BMC needs to be established. The majority of horses in this study were also well above the average age for typical racehorses on furosemide for treatment of EIPH, so potential investigations into adverse effects of furosemide should use horses closer to the typical age of most racehorses as two or three year olds. Younger horses also have different mineral requirements than older horses, and if the negative calcium balance as seen in Pagan et al. (2014) is prolonged, it could impact younger horses even more than older horses. Racehorses typically have different workloads than horses used in the current study, so future research should also focus on horses in heavy or very heavy workloads. These workloads involve higher speeds as well as greater overall amounts of work which will stress and load the bone differently than moderate exercise and can influence BMC. Furosemide's negative effect on calcium balance could diminish total BMC in these horses but not the more stressed cortices (medial and dorsal). Finally, drug resistance or acclimatization presents problems when considering efficacy of furosemide as a prophylactic treatment for EIPH. Because our study

demonstrated decreased BW losses over time, the number of doses or amount of time on treatment until no change from baseline occurs needs to be determined.

Summary and Conclusion

While catastrophic bone failure is uncommon in racehorses, it has an enormous negative impact on the public's perception of horse racing. Due to the prevalence of EIPH and the popularity of treating this condition with furosemide, determining furosemide's role in bone health should be a top priority. Though some of furosemide's short-term effects like dehydration and mineral excretion have been investigated, many of its long-term effects in horses remain unknown. This lack of research is troubling since many racehorses with EIPH will remain on frequent doses of furosemide for most of their careers. Even though this study lasted two months, a very short time for a race career, these results raise interesting questions about furosemide use. While BMC and OC was not impacted by treatment in this study, the trend towards an increased amount of PYD, a marker of bone degradation, after furosemide administration could have an impact on bone strength over a longer period of time. This study also found that furosemide increases BW losses over control through dehydration. At 4h post-administration, FUR horses lost more weight than CON horses receiving no treatment and were unable to regain hydration status until after 8h post-administration. Additionally, the difference between day 0 and day 49 in FUR horses but not in CON horses could indicate drug acclimatization or resistance. This resistance should be considered when using furosemide as a long-term treatment for EIPH as doses would need to be adjusted

periodically, a common practice in human medicine but relatively forgotten in treating horses. Adjusting doses to account for drug resistance could impact the severity of EIPH over time. The findings on PYD and BW losses warrant further investigation to establish the safety of using frequent doses of furosemide over long periods of time.

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APPENDIX

APPENDIX A: IACUC Approval

IACUC
INSTITUTIONAL ANIMAL CARE and USE COMMITEE
Office of Research Compliance,
010A Sam Ingram Building,
2269 Middle Tennessee Blvd
Murfreesboro, TN 37129

MIDDLE TENNESSEE STATE UNIVERSITY

PROTOCOL APPROVAL NOTICE

5/18/2015

Investigator(s) Name: John Haffner, Abby Pritchard, Kayleigh Maher, Helen Hardy, Andrea Smith, Andrea Cole, Holly Nobbe and Devin Lintzenich
Investigator(s) Email: holly.spooner@mtsu.edu and john.haffner@mtsu.edu
Investigator(s)' Department: Agribusiness

Protocol Title: "Does chronic furosemide use influence markers of bone metabolism and bone density in the horse"
Protocol ID: 15-009

Dear Investigator(s),

The MTSU Institutional Animal Care and Use Committee has reviewed the animal use proposal identified above and has approved your protocol in accordance with PHS policy. This approval is effective for three (3) years from the date of this notice. Your study expires 5/18/2018. Investigator(s) MUST file a Progress Report annually regarding the status of the study and submit an end-of-project report.

MTSU Policy defines an investigator as someone who has contact with animals for research or teaching purposes. Anyone meeting this definition needs to be listed on your protocol and needs to complete IACUC training through the CITI program. Addition of investigators requires submission of an Addendum Approval to the Office of Research Compliance.

The IACUC must be notified of any proposed protocol changes prior to their implementation. Unanticipated harms to subjects or adverse events must be reported within 48 hours to the Office of Compliance at (615) 494-8918.

Also, all research materials must be retained by the MTSU faculty in charge for at least three (3) years AFTER the study is completed. Be advised that all IACUC approved protocols are subject to audit at any time and all animal facilities are subject to inspections at least biannually. Furthermore, IACUC reserves the right to change, revoke or modify this approval without notice.

Sincerely,

Compliance Office
(On behalf of IACUC)
Middle Tennessee State University
Tel: 615 494 8918
Email: compliance@mtsu.edu

IACUCN001   Version 1.2   Revision Date 04.27.2015