FUROSEMIDE AND ITS EFFECTS ON BONE DEVELOPMENT IN HUMANS & EQUINE MODELS: IMPLICATIONS FOR HUMAN AND ANIMAL USE

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ABSTRACT

Furosemide is a type of loop diuretic whose mechanism of action targets the activity of the Na^+-K^+- $2Cl^-$ cotransporter in the Loop of Henle of the kidney. By its action in the kidney, Furosemide produces an extreme natriuretic effect resulting in profound diuresis. This diuretic effect of Furosemide makes it a common treatment in volume-related diseases such as Congestive Heart Failure (CHF), hypertension and a variety of renal disorders in the human. In horses, particularly racing animals, Furosemide is commonly administered for the treatment of Exercise-Induced Pulmonary Hemorrhage (EIPH), a disorder caused by pulmonary capillary failure due to increased pulmonary vascular pressure at peak speed during exercise. While the diuretic effect of Furosemide is successful in treating volume-related ailments in the human and the horse, this diuresis, when not carefully monitored, causes volume depletion and electrolyte imbalance in both species. Depletion of sodium, calcium, chloride and magnesium has been shown to have detrimental effects in both humans and horses, especially with regard to calcium depletion. A meta-analysis of the literature available suggests that Furosemide may have a detrimental effect on bone development and remodeling in the human and the horse. Further, in horses administered frequent doses of Furosemide, a negative balance of calcium ensues causing bone resorption and a weakening of bone to occur, thus putting horses at increased risk of fracture. Additional research is required to confirm this hypothesis, but if true, this holds important ramifications for the use of Furosemide in human medicine, for populations at increased risk for fracture and in race horses.

BIOGRAPHICAL SKETCH

Jeanne Lee Schnell is a Masters of Professional Studies candidate in the Department of Animal Science. Upon receiving her Associates of Arts in Humanities from the State University of New York College of Agriculture and Technology at Cobleskill, Jeanne transferred to Cornell University where she received her Bachelor of Science in Animal Science with a minor in Communication. After working in the thoroughbred industry for several years, Jeanne decided to return to the classroom to pursue her dream of becoming a veterinarian. A life-long student, she recognizes that an individual can never stop learning.

This research is dedicated to my family (both through blood and through friendship – and a shared love of Biochemistry), my advisor Dr. Jerrie Gavalchin, for her 2AM e-mails and constant reassurance, and to all the horses I have loved before. Without all of you I would never have gotten to where I am in the first place.

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LIST OF ABBREVIATIONS

BMD – Bone Mass Density

CHF – Congestive Heart Failure

CNT – Connecting Tubule

DCT – Distal Convoluted Tubule

FDA – Food and Drug Administration

EIPH – Exercise Induced Pulmonary Hemorrhage

IGF1 – Insulin-Like Growth Factor 1

IM – Intramuscular

IV – Intravenous

MCSF – Macrophage Colony Stimulating Factor

NSAIDs – Non-Steroidal Anti-Inflammatory Drugs

OPG – Osteoprotegrin

PG – Prostaglandins

PGE2 – Prostaglandin E2

PKA – Protein Kinase A

PKC – Protein Kinase C

PTH – Parathyroid Hormone Hormones

TAL – Thick Ascending Limb

TRAP – Tartrate-Resistant Acid Phosphatase

TXB_2 – A stable metabolite of thromboxane B2

PREFACE

Furosemide is a loop-diuretic commonly used in the treatment of fluid retention (edema) and swelling associated with Congestive Heart Failure (CHF), hypertension and renal disease in children and adults. Its fast, almost immediate action in decreasing plasma volume and pressure on the heart and renal system through increased urine output make Furosemide a popular choice amongst physicians for the treatment of volume-based ailments in individuals suffering from heart failure and renal disease. Though it is helpful in decreasing fluid-association complications, adverse side effects include volume depletion and electrolyte imbalance include substantial loss of sodium, calcium, chloride and magnesium ions.

In veterinary medicine, Furosemide is used in the treatment of Exercise-Induced Pulmonary Hemorrhage (EIPH), a condition affecting racing animals including, greyhound dogs, rabbits, camels and thoroughbred horses. Its use in thoroughbreds has been hotly debated as Furosemide's action is not to prevent incidence of the disease, but to mitigate the chance of or grade of EIPH affecting the afflicted equine. Further, Furosemide has been shown to cause a loss of 1-2% of the horse's body weight which has been thought to be a performance-enhancing effect of the drug.

The excretion of calcium in urine may cause a loss in bone mineral density which can lead to thinning of bone or osteoporosis putting the individual at increased risk for fracture. Using humans and the horse as a model, this paper examines present and past literature for a potential causative link between Furosemide use and impaired bone development in association with fracture risk. Also described are inflammatory factors that may increase the risk of Furosemide-associated bone disease as well as the identification of areas requiring further research.

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CHAPTER 1

CHARACTERISTICS OF FUROSEMIDE

History

Sharp and Dohme Incorporated established a program dedicated to renal formulary in 1943 that focused on manipulating actions of renal tubular function through pharmaceuticals. When Sharp and Dohme merged with industry giant Merck in 1952, the renal drug program continued. Initially, the program produced a class of thiazides (one of several types of diuretics), one of which was Chlorothiazide. Marketed in 1958, and considered a landmark development, Chlorothiazide's action increased sodium and chloride excretion in the kidney causing diuresis. Compared to the carbonic anhydrase inhibitors, Sulfanilamide and Acetazolamide, which were introduced in 1937 and 1954 respectively, Chlorothiazide was shown to relieve edema with fewer side effects and had the novel effect of lowering blood pressure (Turner 2018). One year after marketing Chlorothiazide, the renal formulary looking to improve potency and diuretic effect, synthesized a more potent derivative, Furosemide. In 1966, the Food and Drug Administration (FDA) approved its use in humans (Food and Drug Administration 1966) for the treatment of conditions associated with volume overload and edema secondary to Congestive Heart Failure (CHF), liver failure, or renal failure including the nephrotic syndrome (Khan and Siddiqu 2018).

Furosemide has been marketed under several names which include: Aisemide, Apo-Furosemide, Beronald, Desdemin, Discoid, Diural, Diurapid, Dryptal, Durafurid, Edemid, Errolon, Eutensin, Flusapex, Frudix, Frusetic, Frusid, Fulsix, Fuluvamide, Furesis, Furix, Furo-Puren, Furon, Furosedon, Fusid.frusone, Hydro-rapid, Impugan, Katlex, Lasilix, Lasix, Lodix, Lowpston, Macasirool, Mirfat, Nicorol, Odemase, Oedemex, Profemin, Rosemide, Rusyde,

Salix, Seguril, Teva-Furosemide, Trofurit, Uremide, and Urex. Previously, Furosemide was approved by its British Approved Name (BAN), Frusemide, but this derivation has fallen out of favor and Furosemide is the preferred name when referring to the drug in the medical literature (U.S. National Library of Medicine 2018). The terms Lasix and Salix are the common names for Furosemide in the field of veterinary medicine and animal health.

General Pharmacology and Mechanism of Action

There are three classes of diuretic: thiazides, loop diuretics and potassium sparing. Furosemide, due to its action on the Loop of Henle is classified as a loop diuretic [see Figure 1], but its mechanism of action has not yet been fully elucidated because the cellular mechanism of sodium transport by the kidney continues to be poorly understood. Initially, Furosemide was understood to act similarly to thiazides, as a milder inhibitor of carbonic anhydrase but this was disproved. It was found that Furosemide targets sodium transport (Kirkendall and Stein, 1966), and animal model studies by Lipson and Hays showed that this diuretic agent acted to decrease ionic permeability (Lipson and Hays 1966).

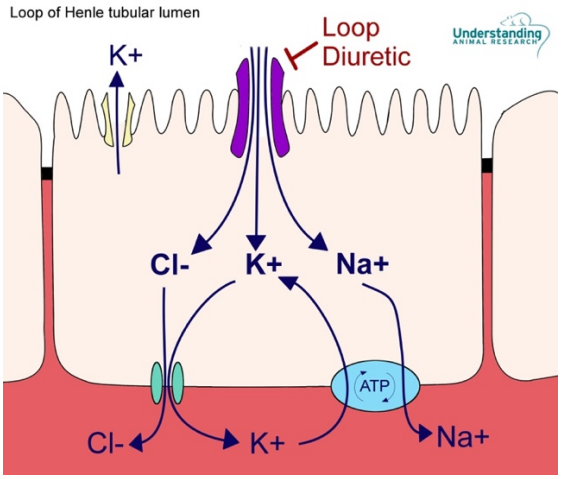


Figure 1: Mechanism of action of loop diuretics such as Furosemide (Animalytix LLC. 2018).

Furosemide is used in all species for its diuresis-producing effects. It does this by first reducing absorption of electrolytes in the thick ascending portion of the Loop of Henle of the

kidney. Through inhibition of reabsorption of sodium and chloride in the ascending portion, Furosemide binds to and inhibits the activity of the Na^{+}-K^{+}- $2Cl^{-}$[1] cotransporter at the luminal membrane (Breyer and Jacobson 1990). The drug competitively binds to the Cl- active site of the luminal Na+- K^{+}- $2Cl^{-}$ cotransporter. Inhibition of cotransporter action leads to increased amounts of sodium and chloride ion to the distal tubule [see Figure 2]. Furosemide also increases the excretion of plasma potassium and calcium in the distal tubule. Because Furosemide blocks the renal system's ability to concentrate and dilute urine appropriately, this increased concentration of sodium and chloride ions produces isotonic urine (Weiner and Mudge 1985) Also excreted, albeit in lesser amounts are chloride, hydrogen, ammonium, and bicarbonate (Plumb 2008). Loop diuretics deliver an increased load of Na+ ions to the distal tubules resulting in a renin-angiotensin-aldosterone-driven increase in K^{+} and H^{+}[2] in exchange for Na+ (Kochevar 2009). This process temporarily increases renin secretion because of action on the nephron while also increasing the glomerular filtration rate. This is due to the drug's chemistry. Being a derivative of anthranilic acid, Furosemide is rapidly absorbed in the gastrointestinal tract and possesses low lipid solubility. The poor lipid solubility and rapid renal excretion minimize the possibility of its accumulation in tissues and organs or crystalluria (Animalytix LLC. 2018) making it desirable for long-term use.

[1] Na^{+} - Sodium ion; K^{+}- Potassium ion; Cl^{-} - Chloride ion.
[2] H^{+} - Hydrogen ion

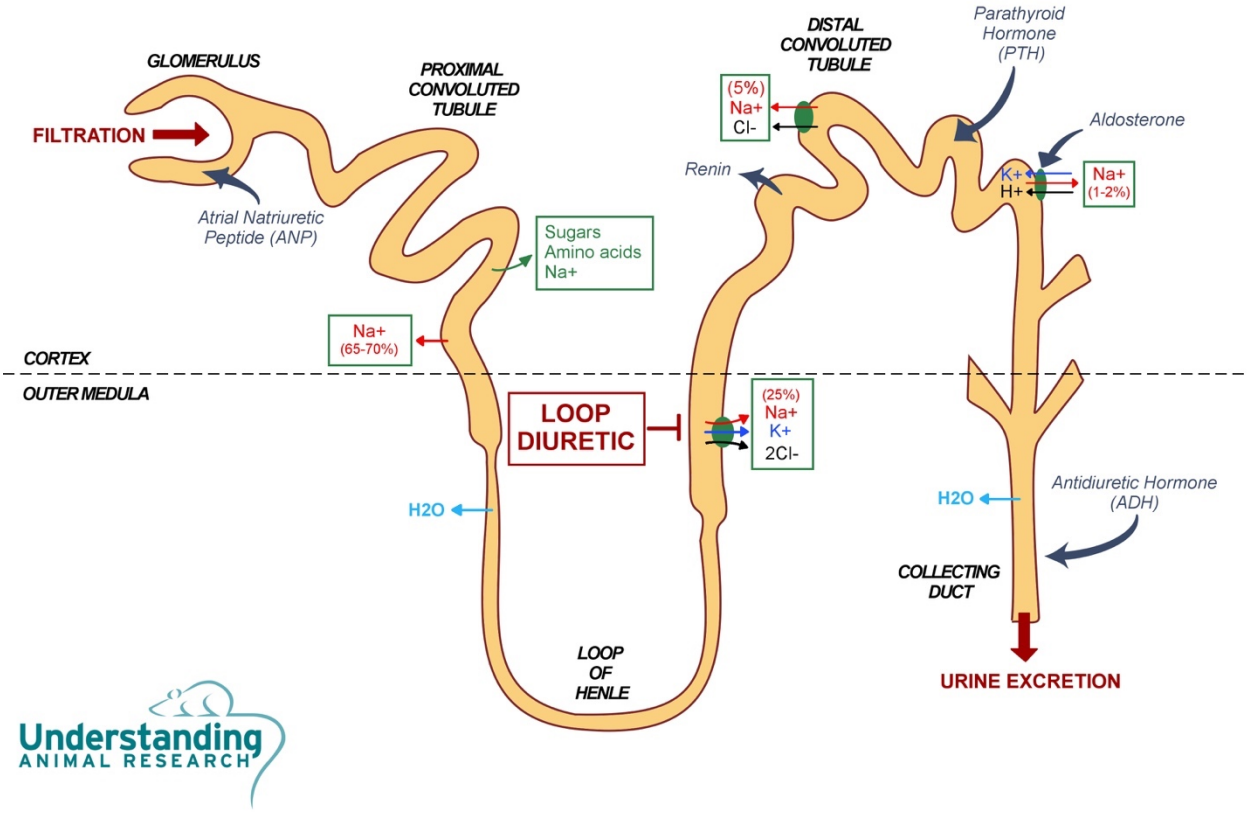


Figure 2: The effect of loop diuretics on sodium, potassium, calcium and chloride ions in the Loop of Henle (Animalytix LLC. 2018).

While more research is required, the pharmacokinetics of Furosemide vary greatly from species to species.. In humans, approximately 60-75% of Furosemide is absorbed when taken orally and the rest of the drug is excreted in the urine unchanged. Its diuretic effect can be observed within 60 minutes of oral administration, with the peak impact of diuresis occurring 120 minutes. Intravenous (IV) administration produces much more radical effects with diuresis being observed within five minutes of administration. In normal individuals and those with CHF or renal disease, the drug is bound to approximately 95% of plasma proteins. Its serum half-life is approximately two hours but can delayed in individuals with CHF, advanced renal disease, and

in neonates (Plumb, 2008). Diuretic effects in humans can last six to eight hours regardless of the route of administration.

Adverse effects of Furosemide are volume imbalance, fluctuation in acid-base homeostasis, glucose intolerance and lipid abnormalities (Greenberg 2000). Short-term side effects include dizziness, lightheadedness, and impaired balance. Other detrimental effects that have been observed with continuous or long-term Furosemide use include ototoxicity, increased risk of fracture and drug-induced osteoporosis.

CHAPTER 2

FUROSEMIDE USE IN HUMANS

Treatment

In 2018, Furosemide was the 15th most prescribed drug in the United States (Fuentes, et al., 2018). The drug is commonly prescribed to individuals of all ages mainly for the treatment of edema across a variety of clinical fields. Furosemide's action on the kidney make it a novel treatment for a variety of ailments. Its fast action make the drug a first line of treatment for disorders that affect the body's ability to maintain fluid balance.

Furosemide can produce extra-renal vascular effects in conjunction with the synthesis of prostaglandins, specifically PGF2α in the cortical and medullary limbs of the Loop of Henle. This increase in PGF2α aids in the release of interstitial fluids causing edema as well vasodilation (The European Agency for the Evaluation of Medicinal Products 2009). In individuals prone to high blood pressure but who are otherwise healthy, Furosemide is prescribed to lower overall osmotic volume in the body so as to decrease the risk of damage to blood vessels and major organs. Other outcomes are a decrease in abdominal girth and weight.

In humans, Furosemide is referred to as a "water pill," that is taken by mouth. Long-term use of Furosemide is associated with oral route, having an onset of 30-60 minutes and a six to eight hour duration (Davis' Drug Guide 2018). Other routes of administration are intramuscular (IM) and intravenous (IV) with intravenous administration leading to the most rapid action of the drug, but the shortest duration.

Evidence for Risk of Fracture

Due to its action on the Na^+-K^+- $2Cl^-$ cotransporter of the nephron, Furosemide can cause marked electrolyte imbalances in humans, especially those with poor renal function. In

individuals who are prescribed Furosemide as an on-going treatment, electrolyte panels must be performed on a regular basis, as well as monitoring of calcium and potassium levels. While uncommon, interruption of calcium and potassium ionic concentrations can cause irregular or impaired cardiac activity especially in older adults.

A study by Greenblatt et al, found that of 2,367 patients who received Furosemide, 10.1% were given potassium and calcium supplements. The study also found that the severity of volume depletion increased when Furosemide was given continuously over a 30-day period and that Furosemide, regardless of the administration route, increased the likelihood of adverse reactions associated with electrolyte imbalance. In several cases, prolonged administration impaired cardiac functions (Greenblatt, et al. 1977).

Furosemide can also increase calcium excretion at the distal tubule of the nephron and has been prescribed in patients with renal failure for the treatment and/or prevention of hypercalcemia. Renal calcium handling accounts for 50% of plasma calcium[3] through the glomerulus; 99% of this portion is reabsorbed in the tubules. This allows only 200 mg of calcium in the healthy individual to be excreted per day (Jeon 2008). Ionized calcium is tightly regulated by endocrine hormones such as Parathyroid Hormone (PTH), 1,25-dihydroxyvitamin D_3 (vitamin D) and levels of calcium[4]. Hormones such as PTH and vitamin D enhance calcium reabsorption in the thick ascending limb (TAL), distal convoluted tubule (DCT) and or connecting tubules (CNT). Another hormone, estrogen, also promotes calcium absorption in the DCT/CNT (Mount and Yu, 2008). Loop diuretics, however, inhibit calcium reabsorption at these sites. In the treatment of hypercalcemia, Furosemide affects $Ca2^+$ ion transport via its action on the Na^+-K^+-$2Cl^-$ cotransporter. It also reduces lumen-positive voltage in the TAL further inhibiting

[3] Plasma calcium composition consists of Ca^{2+} ions, unfilterable fractions and unbound calcium proteins.
[4] Calcium can regulate its own secretion through negative feedback on parathyroid glands which secrete PTH.

reabsorption (Jeon 2008). This action is in response to increased calcium delivery and compensatory adaptation of the downstream renal segments (Lee, et al. 2007). In a 2007 study by Lee, et al., mice dosed with a single treatment of Furosemide experienced a significant increase in urinary excretion of calcium after a four hour period. In this same study, it was observed that mice had a fourfold increase in calcium excretion (Lee, et al. 2007). While this study did not directly observe the effects of calcium excretion on bone density, it can be postulated that Furosemide treatment resulted in excretion of more than the body's allowable limit of calcium due its mechanism of action: 1) inhibition of the Na^{+}-K^{+}- $2Cl^{-}$ cotransporter; , 2) inhibition of Ca^{2+} ions at the TAL in the Loop of Henle and other sites; 3) decreasing sodium reabsorption which interferes with the process of urine concentration in the medulla leading to increased fluid loss.

Only 1% of calcium is found circulating in plasma fluid. The other 99% percent is found circulating in teeth and bones. In the bone, calcium is stored with phosphorus in a matrix of hydroxyapatite crystals that make up both the cortical[5] and cancellous bone. Osteoblast and osteoclast cells function to release calcium ions from the bone matrix in response to factors that are driven by plasma calcium levels. Low plasma calcium causes osteoclasts to release more calcium into plasma. Furosemide's action in the Loop of Henle causes a negative calcium balance. This negative balance has been associated with a decrease in bone mineral density due to the body's homeostatic mechanism necessitating a balance of plasma calcium.

Bone mineral density decreases with increasing need for plasma calcium. Loop diuretics such as Furosemide have been known to increase the incidence of hip and other osteoporotic fractures in several studies (Rejnmark, et al., 2006; Lim, et al., 2008; and Carbone et al., 2009).

[5] The majority of calcium is stored in cortical bone. Osteoclasts cells move over this matrix secreting factors that release Ca^{2+} and P ions from the bone matrix.

Lim, et al performed a cohort study of men of varying ages who were prescribed loop diuretics. Of the 3,269 individuals in the study, 87% took Furosemide. The study found that although vitamin D and calcium intake were similar among all participants, those participants taking Furosemide experienced a greater decline in total hip bone mass density compared to men who were not taking Furosemide. The study also found that men whose usage of loop diuretics was continuous had the greatest average rate of bone loss at the hip, compared to intermittent and non-users. Their rate of bone loss was almost 3-fold greater than non-users and 1.5 greater for intermittent users (Lim, et al. 2008), suggesting long-term effects of Furosemide use. In a similar study but smaller study of 1005 men, 471 who had mild to moderate reductions in glomerular filtration rate (GFR) were shown to be at increased risk for hip and other osteoporotic fracture due to decreased bone mass density (Ishani, et al. 2008).

A 2006 clinic trial of 87 healthy, postmenopausal women given Bumetanide (a loop diuretic whose mechanism of action is also directed to the Loop of Henle) or a placebo for one year found that urinary calcium excretion increased by 17%, and bone mineral density in the hip decreased by 2%, while overall body density decreased by 1.4% in women who received Bumetanide compared to those who had received the placebo. These effects occurred despite supplementation with calcium and vitamin D (Rejnmark, et al. 2006). Furthermore, the study found that there was a 51% increase in fracture risk in the hip, spine and forearm region in the women who had been treated with the loop diuretic.

While these data support a potential role for Furosemide in loss of bone mass density, conflicting evidence does exist. An observational study of 133,855 individuals enrolled in the Women's Health Initiative found no significant correlation between loop diuretic use and changes in bone mass density (BMD) or fractures in the 3,411 women who took loop diuretics.

It is interesting to note however, that one-third of the women were taking calcium supplements of at least 1,000 mg per day which could explain the lack of correlation since calcium supplementation would mitigate both the loss in bone mass density and fracture risk (Carbone, et al., 2009).

While Furosemide use may be more often associated with risk of fracture in elderly or postmenopausal women, Atkinson et al found that infants treated with diuretics, loop or otherwise, had large renal-specific calcium, sodium and chloride losses. Such losses caused volume depletion and electrolyte imbalances (Atkinson, et al. 1988) and put developing bones at increased risk for stress and impaired development. A 2017 retrospective cohort study of 53,725 children (furosemide-adherent, n = 466; furosemide nonadherent, n = 2810; no furosemide, n = 50,449) found that the incident rate of fracture was significantly higher in the furosemide-adherent group compared with both the furosemide nonadherent group and the no furosemide group (Heo, et al. 2018). Osteoporotic fracture and fractures of the radius and ulna accounted for the most common site of fractures, with 26.5 and 24.8 percent respectively. Additionally, as age increased, fracture risk also increased, rising 1.2% per year. Also in this study, the researchers found that children on continuous Furosemide therapy were nearly twice as likely to experience a fracture compared to patients who were not on this therapy. The potential role of diuretics in fracture was further supported by the increased risk of fracture in those patients who had more prescription refills for a diuretic, suggesting a dose-response type effect (Heo, et al. 2018).

Ototoxicity

The incidence of ototoxicity in individuals prescribed Furosemide is well documented in medical literature. Ototoxicity is defined as the tendency of certain therapeutic agents and other chemical substances to cause functional impairment and cellular degeneration of the tissues of

the inner ear, and especially the end-organs and neurons of the cochlear and vestibular divisions of the eight cranial nerve (Hawkins 1976). It is associated with bilateral hearing loss and can be accompanied by tinnitus. The onset of hearing loss associated with Furosemide is unpredictable and can be sudden or progressive with complete or gradual loss occurring even after a single dose of the drug. In the cochlea of the inner ear, loop diuretics such as Furosemide cause edema in the epithelium of the stria vascularis that eventually leads to hearing loss (Dalian, et al. 2016).

In a study by Santos and Nadol, researchers observed pathologic changes in the inner structure of human temporal bone including, cystic changes in the Hensen's cells, collapse of Reisner's membrane and the tectorial membrane and diffuse loss of inner and outer ear hair cells in patients who had received Furosemide as part of their treatment for renal disease (Santos and Nadol 2017). The degree of degeneration and hearing loss correlated to dose-dependent effects. In one case, the study found that longer exposure to Furosemide resulted in extensive damage to stria vascularis. Santos and Nadol posit that ototoxicity may occur without significant clinical signs at first dosing, making it imperative to measure hearing capacity before and after Furosemide administration.

A multivariate analysis of thirty-five neonates diagnosed with sensorineural hearing loss and seizures found Furosemide-associated hearing loss. An examination of treatment records for these infants found that while both Furosemide and Kanamycin had been used to treat seizures in this population, only Furosemide was associated with hearing loss (Brown and Sabo, 1991).

Other studies also support the recommendation that Furosemide may not be appropriate for certain patients. In patients whose renal system function is impaired, high dose or bolus Furosemide infusion should be avoided to prevent ototoxicity (Oh and Han 2015). Studies in animal models have also found that the incidence of ototoxicity increases and care should be

taken when prescribing Furosemide to an individual who is also receiving aminoglycoside[6] therapy, (Lee, et al. 2018).

Conclusions

Furosemide's novel action on the Loop of Henle, targeting the Na^+-K^+- $2Cl^-$ cotransporter and its ability to cause rapid diuresis make it ideal for treating edema and other fluid imbalances. Care must be taken however due to adverse side effects associated with Furosemide use including volume depletion and electrolyte imbalance. Prolonged or continuous-use has shown increased risk of fracture and drug-induced osteoporosis. Short-term use has also shown side effects that may or may not be dose dependent. In patients experiencing vestibular issues, symptoms of lightheadedness or dizziness and ototoxicity (hearing impairment), these side effects can be long-term.

[6] A class of antibiotics used for gram-negative bacterial infections

CHAPTER 3

FUROSEMIDE USE IN HORSES

Use in the Horse

The use of Furosemide in horses, particularly in racing thoroughbreds has been at the heart of heated controversy for several years and has even come under congressional scrutiny. Furosemide is used primarily for the mitigation of Exercise Induced Pulmonary Hemorrhage (EIPH) in horses but can also be used for the treatment of ascites, cellulitis, edema, respiratory distress syndrome in foals and (although rare in the horse) renal failure (Hinchcliff and Muir 1991).

Pharmacokinetics in the Horse

Furosemide's action as a loop diuretic that targets the activity of the Na^{+}-K^{+}- $2Cl^{-}$ cotransporter at the luminal membrane allows for the concentration and/or dilution of tubular fluid and increases the sodium and chloride delivery to the distal tubule. It also increases the excretion of plasma potassium and calcium in the distal tubule. Thus these actions require consideration and close observation when administering this drug to horses.

Furosemide is administered IM or IV to horses due to the poor response when it is orally administered, likely due to the animal not receiving a dose sufficient to produce diuresis[7] (see Figure 3). In horses, elimination of IV Furosemide is slightly less than one hour if the animal possesses normal renal function. Studies of its elimination in horses that had undergone bilateral ureteral ligation found that the time for elimination for the drug increased to 164 minutes, demonstrating that Furosemide elimination in the horse is primarily renal. Additionally, hemodynamic effects prevented by ureteral ligation provided evidence that the effects of the

[7] It can be difficult to dose a horse via oral administration because a horse may refuse to eat if the medication is in feed, or because the animal can refuse to swallow or spit out the medication if given in a bolus form.

drug in the horse are diuresis-dependent (Dyke, et al. 1998). Unlike its use in humans, Furosemide can be administered to horses up to four times per day (Johansson, et al., 2004). With the more frequent administration in horses, further hemodynamic changes were observed transiently and included a decrease in stroke volume index, and increases in left ventricular filling pressure, heart rate, mean arterial pressure, and systemic vascular resistance. These hemodynamic changes were associated with activation of the neurohumoral axis, as evidenced by increases in plasma renin activity, and plasma norepinephrine and arginine vasopressin concentration (Hinchcliff and Muir 1991).

Dosages of Diuretics

Drug	Dosage
Furosemide	4–6 mg/kg IV, IM, or SC, as needed for acute therapy
	Dogs: 2–4 mg/kg, PO, once to three times daily
	Cats:1–2 mg/kg, PO, once or twice daily
	Large animals: 0.5–1 mg/kg/day, IV or IM

Figure 3: Dosage of Furosemide in horses (Dowling 2016).

Frequent IM or IV administration of Furosemide has been shown to lead to complications that include, accidental injection into the carotid artery, local infection, perivascular administration of the drug, and thrombosis (Agne, et al. 2018). Further complications include hypovolemia, electrolyte imbalance (hyperuricemia, hypokalemia, hypocalcemia) that increased in severity with increased dose and frequency, and more rarely, irregular cardiac activity. It should be noted that acute rhabdomyolysis[8] and myoglobinuria due to hypokalemia can also occur.

[8] Also known as "tying up" in the horse. Rhabdomyolysis is a "syndrome of muscle pain and , cramping…associated with overexertion, overtraining, and electrolyte imbalance in horses (Valberg 2018)."

Whole body electrolyte imbalance is a common adverse side effect of Furosemide administration in the race horse, resulting in a synchronous diaphragmatic flutter also known as "thumps." This is due to a loss of large quantities of calcium chloride, magnesium, and potassium, usually through extensive sweating due to intense or prolonged exercise (Crandell 2015). The syndrome can be so severe as to produce a "thumping" noise that can be heard outside of the horse's body, hence the name of the aforementioned syndrome.

In acute short-term therapy with Furosemide, a single larger dose (4-6 mg/kg) is given IV, IM, or SC. In the case of this regimen, the major adverse effect is acute intravascular volume reduction, which worsens cardiac output and hypotension and may precipitate acute renal failure (Dowling 2016).

EIPH

EIPH is an affliction primarily seen in racing animals; greyhounds, camels, and horses. It refers to the presence of blood in the airway of the animal which may be seen in the trachea and lungs upon endoscopic examination or bronchoalveolar lavage. In severe cases, epistaxis[9] is observed from one or both nostrils. In horses, the condition is graded on a scale from zero (no blood observed) to four (excessive blood in the broncheotrachial area and bleeding from one or both nostrils) (see Figure 4).

EIPH is thought to be caused by pulmonary capillary failure due to the increased pulmonary vascular pressure that occurs during exercise at peak speed (galloping) resulting in the scarring or thickening of pulmonary vein walls and, decreased luminal diameter and increased intravascular pressure at the level of the pulmonary capillaries (Rush, 2016).

[9] Bleeding from the nose.

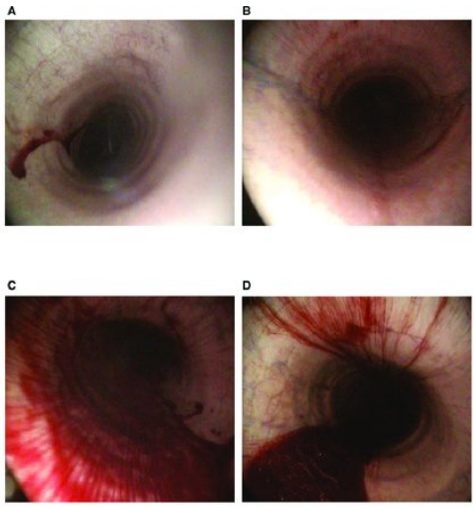


Figure 4: Grading scales for exercise-induced pulmonary hemorrhage (EIPH). A: Grade 1, B: Grade 2, C: Grade 3, D: Grade 4, according to Hinchcliff et al. 20 : Grade 1 = 1 or more flecks of blood or 2 or fewer short, narrow streams of blood in the trachea or mainstem bronchi visible from the tracheal bifurcation; Grade 2 = 1 long stream of blood or more than 2 short streams of blood occupying less than a third of the tracheal circumference; Grade 3 = multiple, distinct streams of blood covering more than a third of the tracheal circumference, with no blood pooling at the thoracic inlet; Grade 4 = multiple, coalescing streams of blood covering more than 90% of the tracheal surface, with blood pooling at the thoracic inlet (Léguillette, et al. 2016).

The incidence of occurrence of EIPH in horses has been debated. Rush, in her 2016 review of EIPH stated that it can be observed 30-90 minutes after exercise in 45%–75% of racehorses via endoscopic examination, with hemorrhage detected by cytologic examination of bronchoalveolar lavage in >90% of racehorses (Rush, 2016). A 2010 study by Birks, et al, however, found that EIPH occurred in 50% of racing horses, and included both thoroughbred and standardbreds. (Birks, et al. 2010). While there is no evidence that EIPH is breed-specific, there are differences in gait and speed of each individual breeds. The standardbred is a pacing/trotting animal with a variation in gait (compared to the standard gait pattern associated with horses) while the thoroughbred is a horse who races at the gallop rather than the trot or pace. Thus the variation in speed, velocity and impact are likely to make a difference in the incidence of EIPH in these animals. The amount an animal races may also impact the incidence of EPH. When

Sullivan and Hinchcliff examined the incidence of EIPH after a single or after multiple races, as diagnosed by tracheobronchoscopy performed within 2 hours of racing, approximately 43% to 75% of thoroughbred racehorses exhibited blood within the trachea after a single examination. With repeated examinations, however, the prevalence of EIPH increased to greater than 85% (Hinchcliff, et al., 2009).

Regulation of Furosemide Use in Racing

The debate over Furosemide's use and efficacy in horse racing has been on-going since it was first allowed in racing jurisdictions in Maryland in 1973 (Lieberman, 2015). As medications such as Furosemide and Butazolidin[10] came into wider use, state officials and jockeys noticed increased breakdowns, reversal of form and longer layoffs (Haskin, 2012). However, many of the early studies in the 1970s and better part of the 1980s that investigated this proved inconclusive, so the medication continues to be used. By 1990, every racing jurisdiction in the United States permitted Furosemide, with the exception of the New York State Racing and Wagering Board[11] which didn't allow its use until 1995. In most jurisdictions, including New York, a horse is eligible to be treated with Furosemide if a practicing veterinarian finds blood in a horse's lungs after a workout or race. Furosemide must be administered through an IV injection given between four and four and one half hours before post time[12]. The New York State Gaming Commission has set the permissible range for Furosemide dosing (Lieberman 2015). This range, as determined by Article 1: The Rules of Racing by the New State Gaming Commission is:

[10] Also known as Phenylbutazone, a pain reliever used in horses.
[11] Now known as the New York State Gaming Commission
[12] Post-time refers to the time that the horse in question will break from the starting gate.

"A single intravenous (IV) injection of no less than 150 milligrams 3cc and no more than 500 milligrams (10cc) on the grounds of a licensed or franchised racing association or corporation during the time period from four to four and one-half hours before the scheduled post time of the race in which the horse is to compete (New York State Gaming Commission 2018)*."*

For short-term action in the horse, the recommended dose is 0.5 – 1.0 mg/kg IM or IV per day (Rush 2016) but for prevention of epistaxis associated with EIPH, the recommended dose is 0.3 – 0.6 mg/kg 60 – 90 minutes prior to race or 250 mg IV 4 hours prior to racing (Plumb 2008). Racetracks around the country regulate Lasix treatment similarly, however dosage regimens are not uniform and may not be at adequate or appropriate levels to produce the diuresis intended to mitigate EIPH or prevent it across racing jurisdictions.

There have been hundreds of research articles published between the 1970s and the present that argue for the efficacy of Furosemide for EIPH. A systematic-review of these studies by Sullivan et al found that there were a total of seventeen studies that effectively demonstrated that Furosemide reduced the incidence and severity of EIPH in thoroughbred and standardbred racehorses (Sullivan, et al. 2014). A well-known and often-quoted 2009 study by Hinchcliff, et al., examined 167 racing thoroughbreds in South Africa placed in fields of nine to sixteen horses. The horses were raced two times, one week apart. The two races consisted of the same field of horses running for the same distance, and the fields either received 500 mg of Furosemide IV or a placebo of saline solution. The severity of EIPH after racing was determined by tracheobronchoscopy and scored on a scale from 0 to 4. Researchers in this study found that prerace administration of Furosemide resulted in a decreased the incidence and severity of EIPH under these conditions (Hinchcliff, et al., 2009).

Another study examined the effects of co-administration of non-steroidal anti-inflammatory drugs (NSAIDs) such as Flunixin with Furosemide and found that when the two drugs interact in the equine body, the pulmonary and renal effects of Furosemide were mitigated due to the inhibitory effect of NSAIDs on renal prostaglandins, which reduced the natriuretic response of Furosemide (Soma and Uboh, 2002). Racehorses are commonly prescribed NSAIDs for pain relief. Phenylbutazone, one such NSAID prescribed to racehorses, was found to abolish exertion-induced increased in plasma TxB_2 in the horse (Soma and Uboh, 2002). Racehorses will often be administered both Furosemide and NSAIDs during training, so that while natriuresis is the intended effect, the loss of "water weight" which enhances performance in the horse, comes at the cost of electrolyte balance.

The mechanism of action by which Furosemide reduces the incidence and severity of EIPH is still unknown. Olsen, et al postulated that Furosemide-induced reductions in body weight suggested reductions in body fluid volume and intravascular fluid volume which would lessen exercised-induced increases in pulmonary arterial blood pressure typically associated with exercise with a consequent reduction in the incidence of alveolar capillary rupture and decreased hemorrhage (Olsen, et al., 1992). In the 2009 Hinchcliff et al study, researchers argued that there was no association between the amount of weight lost and prevention of EIPH but that Furosemide did improve performance. Thus, it is conflicting to say that this reduction in fluid volume does not enhance performance because it is by this action that the severity and possible incidence of EIPH is reduced.

Furosemide causes swift and substantial urine loss, a 2% to 4% loss in body weight, and, only 30 minutes after administration, a 13% reduction in plasma volume (Stack 2015). In a 2014 interview with *The Guardian*, for an article entitled, "Lasix: the drug debate which is bleeding

US horse racing dry," Hinchcliff stated that normal urine output for a healthy horse is about 10 to 15 liters per day but that a horse can pass between 10 to 15 liters of urine in the first hour after Furosemide is administered (Ross, 2014). This profound increase in urination after Furosemide treatment in horses, combined with its ability to cause increased excretion of sodium, calcium, potassium and chloride, as well as evidence in humans that Furosemide treatment may contribute to increased risk of fracture[13] is of great concern with regard to risk of fracture or breakdown in the racing thoroughbred.

Conclusion

As of this writing, there have been no studies investigating the potential role of Furosemide in fracture risk in race horses, nor have studies been undertaken to research the long-term effects of Furosemide in the horse. Furosemide is a diuretic whose natriuretic properties affect the balance of electrolyte and mineral excretion in the urine. A single dose of Furosemide was found to negatively affect the balance of urinary calcium, phosphorus, magnesium, sodium and chloride for 24 hours post administration in animal models. While the sodium imbalance was corrected within the same time-span, Pagan et al found that a single dose of Furosemide caused an imbalance of calcium, chloride and magnesium ions that was still decreased 72 hours after treatment (Pagan, et al., 2014). Thus, further research is required to understand the nature of Furosemide on bone development and remodeling in the horse.

[13] As evidenced by previously mentioned studies by Atkinson, et al; Carbone, et al; Greenblatt, et al; Heo et al; Ishani, et al; Lim, et al and Rejnmark, et al.

CHAPTER 4

BONE DEVELOPMENT AND REMODELING

Introduction

The development and remodeling of bone is a complex system with many factors at play. In the body, osteoblast cells develop an extracellular matrix on which to deposit calcium and phosphorus that will form bone. Calcium and phosphate are deposited onto this matrix in the form of hydroxyapatite crystals that can be broken down and released into the plasma when changes in the body call for more calcium or phosphorus. Over 99 percent of total body calcium is found as calcium hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$) in bones and teeth, where it provides bone tissue with the strength and resilience that is required for the mobility associated with everyday life (Ross, et al. 2011).

Bone Development

In all species, bone development begins in the fetus. Bone formation, or osteogenesis is a process undertaken by osteoblasts that secrete factors which recruit calcium and phosphate ions to the bone matrix. Non-mineralized portions of bone around blood vessels form spongy bone known as cancellous or trabecular bone. Cortical bone, the hard, white outer layer associated with the human skeleton forms 80% of the skeleton with trabecular bone forming the other 20%. Cortical bone protects the body's organs, aids in the mechanics of motion and absorbs most of the shock associated with movement. Trabecular bone is a storehouse of calcium and phosphorus.

Two types of bone development occur: intramembranous ossification and endochondral ossification. Intramembranous ossification is the process by which mesenchymal tissue is transformed into bone. This type of development occurs in the bones of the skull and differentiation of mesenchymal cells into cartilage that will later be transformed into bone. Cartilage intermediate is formed and replaced by bone cells through the process of endochondral ossification (Gilbert, 2000).

Mesenchymal stem cells can develop into cartilage that will be transformed into bone while other mesenchymal stem cells differentiate to become bone precursors known as osteoblasts. Osteoblasts are responsible for creating the osteoid matrix upon which hydroxyapatite crystals are stored. Osteoblasts trapped in the matrix become bone cells known as osteocytes that are important in the resorption of bone and in the process of bone remodeling. Both types of cells are important in the formation of bone.

Bone ossification requires bone morphogenetic proteins and activation of transcription factor CBFA1 (Gilbert, 2000). Bone morphogenetic proteins such as BMP2, BMP4, and BMP7 which activate signal factor CBFA1 cause mesenchymal cells to become bone cells directly (Hall, 1988). These bone proteins also activate genes required for the production of osteopoetin and osteocalcin which are bone-specific extracellular matrix proteins (see Figures 5 and 6).

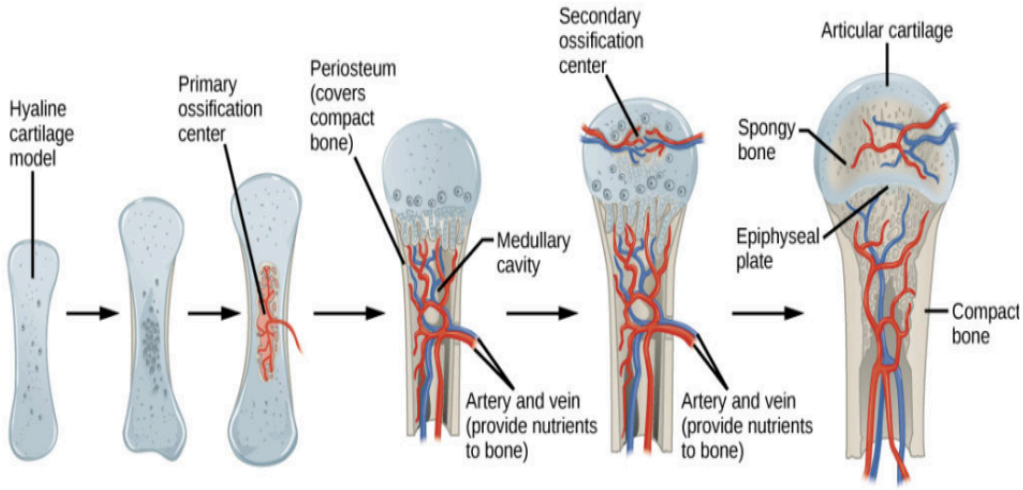


Figure 5: Endochondral ossification is the process of bone development from hyaline cartilage. The periosteum is the connective tissue on the outside if bone that acts as the interface between bone, blood vessels, tendons and ligaments (Rice University, 1999).

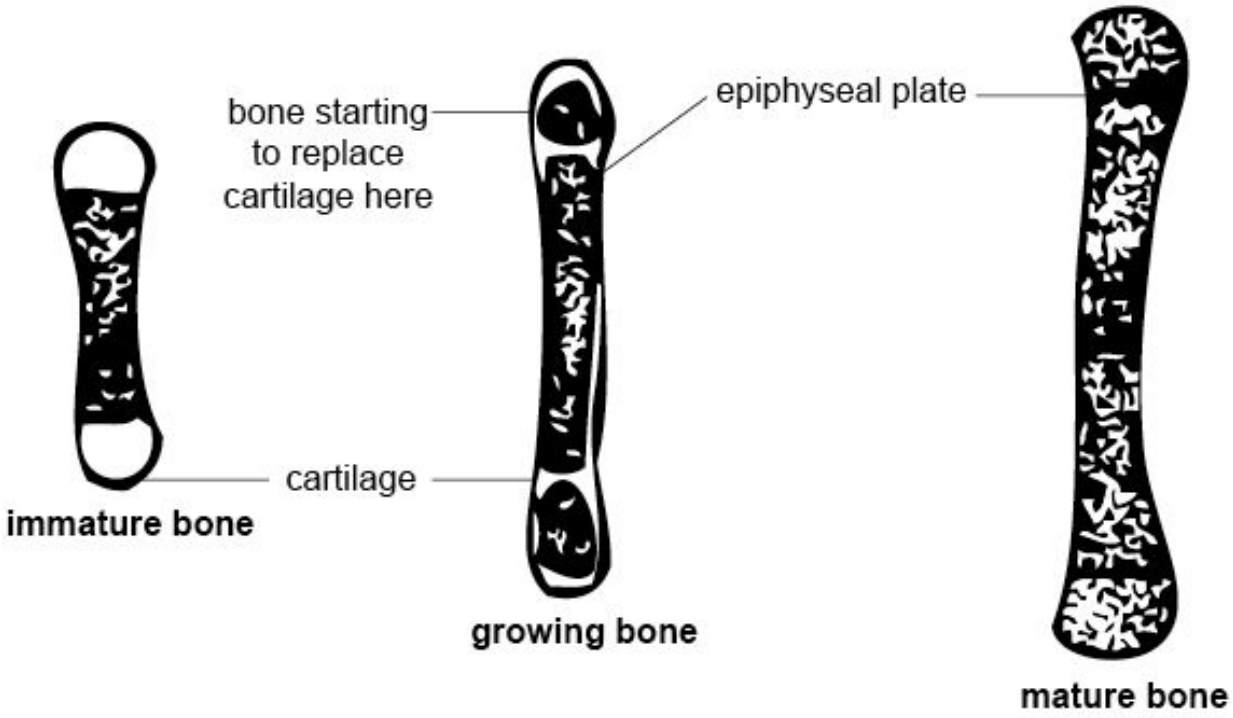


Figure 6: The growth of bone (Rice University,1999)

Five stages are required in order for cartilage tissue to form for the process of endochondral ossification, First, mesenchymal cells are stimulated by paracrine transcription

factors, Pax1 and Scleraxis to commit to cartilage cell lineage. During the second stage, mesenchymal cells differentiate into cartilage cells called chondrocytes. N-CAM, a bone factor and *SOX9,* a gene that encodes a DNA-binding protein, are also expressed in these cells and are important for their maintenance (Oberlender and Tuan,1994). In the third stage, chondrocytes form the model for bone. As they proliferate, chondrocytes secrete extracellular matrix factors that are cartilage specific. In the fourth stage, chondrocyte division ceases and the cells become hypertrophic. This alters osteoid (matrix) through the addition of collage X and fibronectin to support mineralization by calcium carbonate (Gilbert 2000). The fifth and final stage is the proliferation of blood vessels and the generation of osteoblasts. Hypertrophic chondrocytes die by apoptosis and become bone marrow. Cells surrounding this space become osteoblasts which form bone matrix that eventually become bone. The replacement of chondrocytes by osteoblasts is dependent on the mineralization of the extracellular matrix and calcium is especially important (Tuan, 1987). Hypertrophic chondrocytes play a role in the mineralization of the extracellular matrix in that they secrete membrane-bound vesicles (into the extracellular matrix) that contain activated enzymes which generate calcium and phosphate ions that become part of the osteoid during the mineralization process (Gilbert, 2000).

Bone Remodeling

As with bone development, the process of bone remodeling is dependent on a multitude of factors including nutrition, the intensity and frequency of exercise, and disease status. Bone remodeling in the body occurs dynamically through an interplay between bone-forming osteoblasts and bone-resorbing osteoclasts. These cells work in conjunction to alter the inner wall of the bone and endosteum region. In this region, there are osteoblastic niches for hematopoietic stem cells that release factors to drive migration of leukocytes and progenitor cells

from the bone marrow to injured bone and tissue for repair. Bone marrow plays a pivotal role in bone remodeling in that it helps transport mobilized cells and chemotactic signals between blood and bone marrow aid in communication between the body's other organs and the injured bone.

Osteoclasts play a dual role in regulation of bone resorption and homeostatic release or stress-induced mobilization of hematopoietic stem/progenitor cells. It well-known that stress plays an important factor in the response to injury and recruitment of cells in the repair of tissue damage. In bone especially, stem cell recruitment is a key component of the repair mechanism. In multiple studies, it has been shown that stem cell recruitment in humans include BM CD34+ cells which are implicated in cell motility, whereas circulating CD34+ cells secrete MMP-2 or MMP-9, as do G-CSF mobilized progenitors (Janowska-Wieczorek, et al., 1999) to further recruit stem cells. Also playing a role are proteolytic enzymes such as MMP-9 that drive progenitor cells to increase their expression of CXCR4 and SDF-1 which amplify cell recruitment in circulation (Kollet, Dar and Lapidot 2007). Increased SDF-1 expression further recruits stem cells from bone marrow and sends them to the site of injury. Additionally, SDF-1 regulates osteoclast precursors determining their fate, leading to recruitment of leukocytes to the affected tissue (in this case bone). These signals direct stem cells to the site of growth or remodeling.

Bone remodeling is a constant process required to maintain the strength of bones. Dynamic action is required to keep the balance between bone formation and the reabsorption process. T cells are important in producing the activation factor RANKL and macrophage-colony stimulating factor (MCSF). RANKL is vital to the proliferation and stimulation of osteoclasts that aid in bone resorption, while MCSF is important in the formation of both osteoclast precursors and TRAP-positive osteoclast formation. Together, these two factors are critical for

the formation and adhesion of multinucleated cells expressing osteoclasts specific markers such as tartrate-resistant acid phosphatase (TRAP), Cathepsin K, Calcitonin receptor (CTR) and Integrin receptors (Singh, et al., 2012).

Other transcription factors, cytokines, and chemokines are shared by both the immune system and skeletal system, but their interactions and the exact role that they play in each system is an area of ongoing research. Hormones and prostaglandins (PG) also interact with bone cells. Prostaglandin E2 (PGE2) can have stimulatory or inhibitory effects on bone dependent on which signal the bone cells receive. In the presence of MCSF and protein kinase A (PKA), PGE2 stimulates osteoclast formation regardless of the presence or absence of osteoblasts. In the absence of MCSF or PKA, PGE2 inhibits remodeling of bone. It is important to note that PGE2 and Prostaglandin F2α (PGF2α) are critical in bone metabolism by regulating a variety of signaling pathways and PGF2α via activation of protein kinase C (PKC), which stimulates the Na-dependent inorganic phosphate transport system in osteoblasts that is required for their function (Agas, et al., 2013). Administration of Furosemide, especially at high doses, interferes with the levels of these prostaglandins by decreasing the amount of PGE2 and increasing the amount of PGF2α, which may impair the synthesis and function of osteoblasts. This may lead to the disruption of the dynamic interplay between osteoblasts and osteoclasts in the bone remodeling process and further research is needed to identify any long-term effects of such action. The cytokine IL-1 inhibits the synthesis of osteoclasts and is produced in the parathyroid which also monitors plasma calcium levels. Bone resorption is also regulated through the production of IL-1, IL-6 and RANKL (indirectly through the activation of osteoblasts that produce it) to increase the level of calcium in the blood (Singh, et al. 2012). Osteoprotegrin (OPG), which is secreted by osteoblasts, protects the skeleton from excessive bone resorption by

binding to RANKL and preventing it from interacting with RANK. This is an important feature in the mechanism of bone remodeling and calcium homeostasis in the determination of all species for fracture risk (Boyce and Xing, 2007).

CHAPTER 5

OTHER FACTORS IN CONSIDERATION OF FRACTURE RISK

In the human, as in the horse, normal accretion of bone is a dynamic process in healthy individuals. Being metabolically active, bone responds to both innately determined and environmental factors that determine its structure and integrity. Factors considered here are genetics, nutrition and environmental factors such as level and intensity of exercise and surface (racehorses).

Genetics

Bone modeling is the process by which growth occurs, resulting in an increase in the size and length of bone. Modifications to the bone such as change is size, shape and density can occur as a result of genetic factors such as the height of the parents, mutagenetic effects of pharmaceuticals on the fetus that can affect development and other aberrations that result in genetic abnormality or deficiency. A longstanding and continuous examination of scientific literature has established genetic factors as the principle element of bone mass which then determines bone strength (Krall and Dawson-Hughes 1993; Jouanny, et al. 1995; Jones and

Nguyen 2000; Sigurdsson, et al. 2008; Perez-Lopez, Chedraui and Cuadros-Lopez 2010). While genetics may be the chief determinate in establishing bone mass density, influences on genetics such as the environment also play a role in bone development across life stages. The idea of "peak bone mass" that then can be achieved on a lasting basis through dietary manipulation and/or use of supplements has not been completely resolved (Ross, et al.,. 2011).

Calcium homeostasis is required for healthy bone development and remodeling. This homoeostasis is maintained by serum and urinary calcium levels, both of which are heritable traits in humans. Both levels are tightly regulated by levels of parathyroid hormone (PTH) and vitamin D, which are also a result of heritable genetic factors as well as feedback loops (see Figure 7). Although tightly regulated, calcium excretion and reabsorption can also be affected by genetic disorders or by disruption of calcium transporters through the actions of pharmaceutical agents. Similar to sodium homeostasis, calcium balance is regulated by a short-term G-protein-coupled receptor-dependent system[14] and the long-term action of aldosterone, both of which are controlled by genetic transcription and regulation (Bonny and Bochud, 2014).

[14] Controlled by angiotensin 2

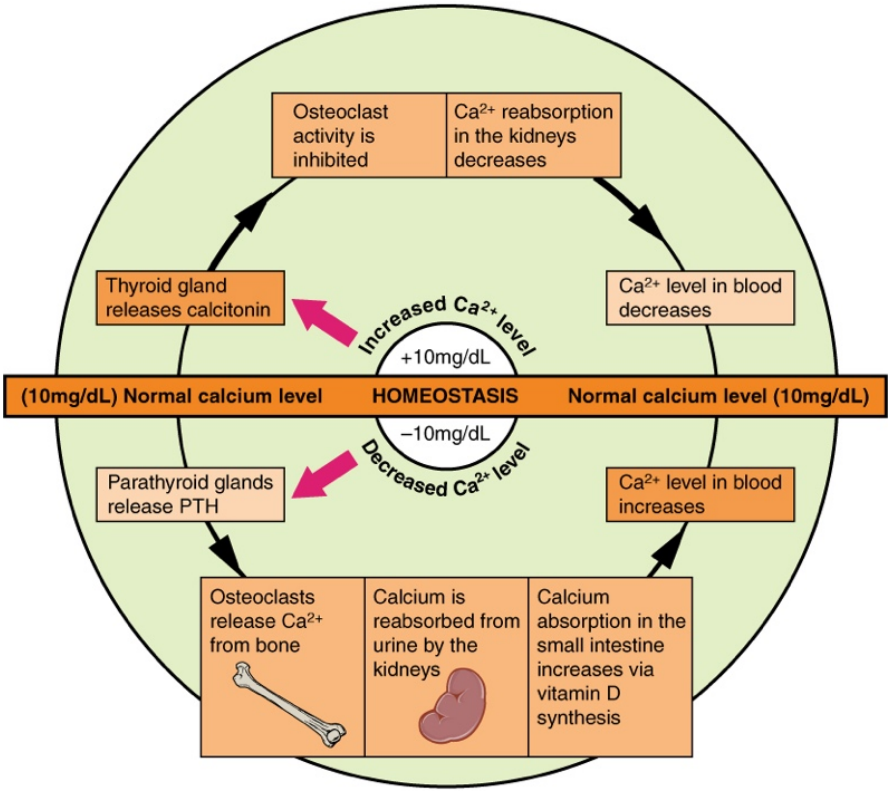


Figure 7: Calcium homeostasis in the body is a delicate balance that is maintained by many different factors including the action of PTH and vitamin D; accretion and bone resorption and the absorption of dietary calcium from the small intestine (Betts, et al., 2019).

Nutrition

Humans

Just before and during puberty, children require 1,300 mg of calcium to ensure healthy bone growth and development. The average human aged 19-50 years requires 1,000 mg of calcium intake daily to maintain calcium homeostasis. After age 50, that requirement increases to 1,200 mg of calcium for both sexes (National Institutes of Health, 2018). Approximately 30% of calcium comes from the diet, typically from foods that are calcium-rich such as dairy, whole

grains and some fruits. Supplementation is generally not needed, however factors that necessitate use of a supplement are: impaired health status, gender, pregnancy status and age. Those with impaired calcium absorption as a result of sickness may require supplement. Females require more calcium both during pregnancy and post-menopause when a decrease in estrogen results in a decrease in the amount of Osteoprotegrin, the factor that helps to regulate resorption of bone. As a person ages, absorption of calcium decreases and intake must increase to maintain calcium homeostasis (National Institutes of Health 1994; Hearney, et al. 1989).

Some foods may contain components such as phytic and oxalic acid that bind to calcium and decrease absorption in the body. In Western diets, protein and sodium are consumed in great quantities, and such consumption can result in hypocalcemia that results in the body increasing resorption of bone to maintain calcium homeostasis. Such dietary practices tap into the alkali reservoir in bone, causing gradual dissolution of bone mineral (Roughead, 2006). Excessive sodium intake in the form of NaCl[15] causes a hypercalciuric effect due to a shared renal absorption pathway with calcium in the proximal tubule and the ascending loop of Henle (Nordin, et al., 1993). This can also cause excessive water intake in individuals with fluid regulation abnormalities such as those with hypertension, CHF, or renal issues can cause edema or ascites. However, an increase in urinary excretion of sodium (such as that that occurs with Furosemide administration), has been shown to be accompanied with an increase in urinary calcium loss (Roughead, 2006).

As new food sources are being discovered and prepared in novel ways, the balance between calcium intake and homeostasis is in flux. As such, dietary requirements of the

[15] Sodium chloride

individual must be taken into consideration so as to ensure a proper balance so that bone mineral reserves remain adequate and the risk for osteoporosis and fracture remain low.

Horses

It should be noted that the mechanism of absorption of dietary calcium is different in the horse than in the human. Unlike humans, who convert vitamin D to its active form 1,25 dihydroxycholecalciferol[16] in order to aid in the absorption of calcium in in the intestine, absorption of calcium in the horse is independent of vitamin D. Horses absorb up to 75% of calcium from their diet compared to other species (Toribio 2011). With regard to excretion, the horse is also unlike other species in that renal excretion of calcium is a major mechanism for excreting excess dietary calcium; a much larger percentage of absorbed dietary calcium is excreted by horses than in other species (Cornell University College of Veterinary Medicine: ECLINPATH 2013).

In all species, the ratio of calcium to phosphorus intake is 2:1 for the average, healthy adult. In young growing horses, however, the ratio required for healthy bone growth and development is 1:1. This is primarily met by feeding quality forage and a balanced concentrate to the horse. Nevertheless, the complex nature of a horse's age, environment (stabled, pastured, or a combination of the two) coupled with its exercise status make ensuring all of the dietary needs required are met a challenge. The typical horse requires 20 grams of calcium and 14 grams of phosphorus per day. This need increases for lactating mares to 85 grams of calcium and 39 grams of phosphorus. Young horses require the most calcium at 36-40 grams of calcium and 20 to 22 grams of phosphorus per day (National Research Council Committee on Nutrient Requirements for Horses 2007). Forage and concentrates vary in composition and while this

[16] Via the kidney that produces an enzyme 1α-hydroxylase that facilitates conversion to the more active form.

paper cannot delve into every aspect of equine nutrition, a brief discussion is merited for the sake of causality in fracture risk. Horses are fed two types of forage: legumes such as alfalfa that are high in calcium or grass which contains calcium, but at a lower amount (see table on page 39). The major type of concentrate fed to horses are cereal type (oats, corn and barley) with the bulk of commercial feed preparations being made from oats. Oats are high in phosphorus and can tip the Ca:P ratio towards phosphorus causing the equine body to call on bone mineral reserves to restore the proper ratio of calcium in the blood.

Calcium deficiency in the horse puts a horse at any age for an increased risk of fracture due to thinning of the bone (osteoporosis) and deficiency causes skeletal deformities in the growing horse (Huntington, 2011). Bone that is demineralized becomes weakened and its integrity unstable, putting the animal for at risk for fracture or complete bone breakdown.

Environmental Factors: Exercise and Activity; Mechanical Loading: Surface (Race Horses)

It is widely accepted that exercise and regular physical activity cause modulations in bone density. The strength and intensity of exercise can lead to increases in in bone mass in areas that act as load-bearing sites [see appendix]. In humans these load bearing sites include the tibia, femur and the patella (Rice University, 1999). Strength training using weights and exercise that exerts steady stress on bone such as jogging or running for short duration can have positive effects on bone. In the horse, load-bearing sites include the third metacarpal or cannon bones, and bones in the equine hoof such as the coffin bone (Rubin, et al., 2013). Bone that has had time to adjust to stress and mechanical loading, responds favorably with increases in mass and density. Physical exercise causes anabolic effects that further strengthen the integrity of bone due to pressure of mechanical restraints applied by an exercise.

Humans

Like any other living tissue, bone responds to pressure exerted on it. Those individuals who exercise regularly achieve maximum bone strength and density compared to more sedentary individuals. Maimoun et al found that bone mineral density was higher ($P < 0.05$) in triathletes at the total proximal femur and lower limbs. They concluded that bone turnover differed in athletes compared with controls, suggesting that bone turnover may be sport-practice dependent (Maimoun, et al. 2004). Exercise helps individuals maintain bone and muscle strength as well as balance and coordination. Such attributes are important especially in the infirmed and elderly who are at an increased risk for fractures due to factors such as coalescence in the infirmed and decreased bone mineral deposition in the infirmed and elderly alike. Other factors which are modified during physical activity include some hormones. Estrogen and insulin-like growth factor 1 (IGF1) have long been known to affect bone growth and strength. It remains unknown what effect exercise has on the major hormones (PTH, vitamin D and calcitonin) that regulate bone growth and development (Maïmoun and Sultan, 2009).

Lack of exercise and increased periods of inactivity weaken bone because it causes the bone to lose tensile strength in the muscle that surrounds it as well as deposition of minerals such as calcium which cause the bone to become thinner and more susceptible to fracture. In older individuals, the rate of bone resorption is greater than the rate and ability to lay down new bone, especially if the individual did not achieve peak bone mass in their youth. A sedentary lifestyle is associated with poor bone health in both young and old individuals alike. A study by Chastin et al, found that the frequency of osteogenic activities are important in counteracting the effect of sedentary behavior on bone health (Chastin, et al., 2014). This study further found that bone accretion was more likely to occur with short, but frequent exercise that emphasized mechanical stress on the bones. This data highlights the importance of exercise in maintaining bone health

and combatting loss of bone mineral deposition in the older adult who may also have an exacerbated risk for loss due to other factors such as poor diet, impaired mobility or prescription drugs.

Horses

In the horse like the human, exercise has been shown to increase bone density. In young, developing horses, osteoblast activity is higher than osteoclast activity, allowing for advanced bone development. By the age of six months, a healthy thoroughbred weanling will have reached 84% of its adult height with 97% of its full height reached at the age of 22 months (Kentucky Equine Research, 2008). Unlike humans whose skeletal growth continues until the early 20's (Farr and Khosla, 2015), the horse's skeleton is fully matured between the age of five and six years old[17] demonstrating that the equine skeleton is dynamic and responds to a variety of factors that play a role in bone maturity.

While racehorses are typically stalled loose in a box-stall that allows them to walk around, this type of activity does not allow for healthy bone development in the horse. For such horses, exercise is achieved by hand-walking or in the course of training either through trotting, cantering or breezing on the track. These activities involve the horse being outside of its stall for approximately 60 to 90 minutes in a 24-hour period[18]. Several studies have showed that keeping a horse stalled for long durations as opposed to allowing them access to pasture for sustained periods will have detrimental effects on bone (Porr, et al. 1998; Hoekstra, et al. 1999; Hiney, Nielsen and Rosenstein. 2004). Studies of prolonged stalling have also shown that it takes weeks or months for bone mass density to return to basal levels after long periods of inactivity (Firth, 2006). The environment of the thoroughbred puts the horse at an increased risk of fracture due to

[17] For larger breeds such as draft horses, the skeleton matures in upwards of eight years old.
[18] This time period includes the time it takes to tack, warm-up, gallop or breeze, cool-down and bathe (if necessary).

the nature of the training program – high intensity exercise, even with proper conditioning cannot decrease fracture risk because the animal may be losing bone mass density due to prolonged stalling. This is especially true of older racehorses whose may have experienced long periods of inactivity between races thereby losing peak condition and vital bone mass density. Strenuous exercise increases the need for calcium and phosphorus in the body, thus bone modeling in the racehorse is continuous and requires proper nutrition and conditioning. (Huntington, 2011).

Young racehorses are brought into training between 18 and 20 months of age (Geor, 2001). Typically, these horses come from a farm setting where they have access to turnout in a paddock while slowly being introduced to saddle. At the track, they are introduced to a demanding exercise program to which the skeleton must adapt in order to meet the increased workload. A study by Day, et al., found that when immature horses are put into race training, they experienced a period of demineralization of the third metacarpal that resulted in lowered bone density at 40-60 days following the onset of track work. This period of demineralization was followed by remineralization and remodeling of the bone in response to specific stress on the bone (Day, et al. 1998).

Due to the economics of the racetrack and demand from owners, this is also the period of time that trainers begin high intensity breezing in order to prepare horses for the demands of racing. This practice also puts the horse at increased risk of fracture, especially considering demineralization occurs in a load-bearing bone site, the third metacarpal.

Previous injury may also predispose racehorses to increased risk of fracture. A task-force formed to investigate a cluster of racing fatalities at Aqueduct racetrack in Ozone Park, N.Y. found a preponderance of horses whose deaths were caused by musculoskeletal failure. Through

necropsy of 129 racehorses who were euthanized due to catastrophic injury in a six-month period[19], it was found that 79% suffered musculoskeletal failure and 50% of horses in this group were affected by failure of the metacarpal bones that make up the equine fetlock (Palmer, et al., 2017). Researchers studied the frequency and duration of workouts prior to the fatal incident and whether or not corticosteroids were administered[20] but were unable to further examine whether there was a correlation between these factors and breakdowns due to limitations of the study[21]. They did find that condylar fractures of the third metacarpal bone, diaphyseal fractures of the third metacarpal bone, proximal sesamoid bone, carpal fractures and humeral fractures were the most common fatal musculoskeletal injury (Palmer, et al., 2017). Upon further examination, Palmer's group also found evidence of progressive subchondral bone sclerosis or necrosis which has been shown to be associated with fatal metacarpal failure underscoring that the presence of subchondral bone sclerosis and necrosis in the third carpal bones, the distal aspect of the third metacarpal bones, and the proximal sesamoid bones likely plays an important role in the pathophysiology of catastrophic injury of these structures (Palmer, et al., 2017).

In addition to factors such as nutrition and the conditioning program on the racetrack, the surface of the track also plays a role in fracture risk. Bone remodeling occurs in response to stimulation by surface-dependent factors initiated by damage or mechanical loading. It includes bone resorption and deposition but does not alter the size or shape of bone (Seeman 2009). In a cohort study using data based on 2,201,152 racing starts in the USA and Canada from January 1st, 2009 to 31st December 2014 and 3,990,000 workout starts made by the 171,523 Thoroughbreds who were declared runners, researchers found that horses had 32% higher chance

[19] The Aqueduct fall-winter and Aqueduct spring meets of 2011-2012.
[20] An anti-inflammatory used for the treatment of injured joints or tendons of racehorses.
[21] These limitations include small sample size, incomplete health records and the length of study period conducted.

of sustaining a fracture when racing on a dirt surface compared to a synthetic surface and a 35% higher chance of fracture if they had sustained a previous injury during racing (Georgopoulos and Parkin, 2017). Data from the Equine Injury Database compiled by *The Jockey Club* registry further supports increased fracture risk associated with dirt surfaces. The frequency of breakdown from 2,277,622 dirt races conducted between 2009-2017 was 1.98, while turf racing from 476,117 races in the same time period was 1.50 (The Jockey Club, 2018). Additionally, the *Jockey Club* identified an increased incidence of breakdown in races whose distances were six furlongs[22] or less suggesting that short distance in conjunction with the high speed and intensity of a race were likely to compound the risk of fracture or breakdown.

There are many factors that come into play when examining the risk of fracture in humans and horses alike. Certain similarities such as the effect of the nature and frequency of exercise on bone development and remodeling is an area of research that may further identify risk factors to both species and provide vital information into the role that pharmaceuticals prescribed to individuals may have in the process. In the horse, Furosemide is administered for the mitigation of EIPH. Its action as a loop diuretic results in sodium and chloride delivery to the distal tubule while also increasing excretion of plasma potassium and calcium in the distal tubule. Excretion of potassium and calcium, key minerals required for bone growth and remodeling put the horse at risk for fracture. Furosemide, coupled with environment in which horses train and race (long hours in the stall and prolonged periods of inactivity) could also contribute to increased risk of fracture and deserves further investigation.

[22] A furlong is equal to 1/8th of a mile. 6 furlongs is 0.75 of a mile.

CHAPTER 6

FURTHER DISCUSSION

Furosemide is a loop diuretic whose action in the nephron of the kidney produces a natriuretic effect and rapid diuresis. Its effects and fast action make it an ideal method for

treating edema and a variety of other volume-related disorders in a number of species, including humans and horses. While it is very effective in treating such disorders, Furosemide can have adverse reactions including volume depletion, electrolyte imbalance, hyponatremia, hypokalemia, hypocalcemia and ototoxicity. Lesser side effects of the drug include lightheadedness and dizziness in humans. Furosemide has also been shown to impair cardiac function in some individuals. Both short-term and long-term use of the drug has been shown to cause excretion of large amounts of $Ca2^{+}$ ions. This adds stress to the already delicate balance of calcium homeostasis. Due to its action on the Na^{+}-K^{+}- $2Cl^{-}$ cotransporter that allows for calcium ($Ca2^{+)}$ ion transport, studies have shown increased incidence of fracture in individuals who have been prescribed Furosemide (Hearney, et al. 1989; Jones and Nguyen, 2000; Rejnmark, et al. 2006; Ishani, et al., 2008; Carbone, et al., 2009, Heo, et al. 2018).

In the human, Furosemide is used to treat Congestive Heart Failure, hypertension and a variety of diseases affecting renal output. Its diuretic effect and fast action make it the 15^{th} most common drug prescribed drug in the United States. Contraindications of Furosemide make its use in the elderly and in growing children a concern due to the potential to lose bone mass density from increased calcium excretion through the diuretic effect of Furosemide. In elderly populations at increased risk of fracture, it should be noted that the drug is known to cause dizziness and lightheadedness, and this can increase the risk of falls in elderly patients. Thus, extreme care must be taken when prescribing the drug long-term in those populations who have been identified as at-risk for incidence of fracture.

The horse is a unique species upon which humans place many demands for transportation and pleasure whether through draft power, riding or racing and one whose body requires a delicate balance between calcium and phosphorus to maintain the integrity of bone. This is due

to the nature of renal excretion of a much larger percentage of dietary calcium in the horse than most species. In the racehorse, Furosemide is mainly used for the treatment of EIPH, an affliction known to cause varying degrees of epistaxis. Used in 95% of racehorses in North America, it has come under scrutiny both by the public and in congressional hearings. While many researchers have found that Furosemide decreases the incidence of and mitigates the effect of EIPH in racehorses, these studies have often had small sample sizes and have not adequately simulated racing conditions. To date, no studies have been undertaken in the horse to determine the effect of long-term use of Furosemide on the incidence of fracture risk. Given the body of work and evidence demonstrating increased fracture risk in humans that is dose-related and or long-term, and the similarity of administration in the racehorse, in that Furosemide is given long-term over the course of the horse's career and at varying doses based on desired effect (performance-enhancing or otherwise), future research should be undertaken to examine the long-term effects of Furosemide on bone development and remodeling in racehorses. Additionally, more research is needed to determine whether EIPH is a static episode, occurring only once after a racing event or whether it occurs at multiple instances over time. Such information could reduce the frequency and amount of Furosemide given, potentially sparing the horse from unnecessary electrolyte excretion and therefore preventing the body's need to tap into vital calcium reserves in the bone.

The process of bone development and remodeling is a complex, dynamic process enhanced by many factors. In order for bone to form, intrinsic factors such mineral deposition of calcium and phosphorus are required in varying amounts depending on the age, gender, pregnancy status and state of health of the individual. Healthy bone undergoes both accretion and resorption on a daily basis and this process is dependent on how much calcium is available to the

individual for bone to form or remodel. Extrinsically, this depends on nutrition; adequate intake of calcium is crucial to maintain the calcium homeostasis required for bone health and secondly, the amount of activity an individual engages in is vital to bone mass density. Studies have long supported moderate but frequent exercise that provides stress and does not overload the bone, but rather strengthens it. For individuals whose bone mineral density may be at risk or compromised, Furosemide may not be the best course of action for ailments requiring diuretic treatment. Since a racehorse's bones are already subjected to intense athletic demand and its environmental conditions do not support bone health, Furosemide use is a factor that can no longer be overlooked.

Ongoing research to establish any definitive link between Furosemide use and fracture risk in racing has been limited due to the many variables that need to be considered. Such variables include age, gender, diet, amount and duration of exercise, surface (for horses only), calcium homeostasis and genetics. This student proposes the study below to test the effects of Furosemide use on the rate of bone loss if any, to determine the risk of fracture using a population of racing thoroughbreds as a model.

Thoroughbred race horse trainers vary in their determination of when to have a veterinarian administer Furosemide. Some trainers give their horses Furosemide before training only, some trainers give their horses Furosemide before racing only and other trainers give Furosemide before racing and training. Having identified these parameters, a population of two-year old thoroughbred race horses who are entering training[23] will be placed in groups according to the aforementioned parameters along with one control group that will receive no Furosemide. The groups will be as follows:

Group 1: Control (No Furosemide)

[23] These horses will come from breeding farms and will not have had any training or racing experience.

Group 2: Furosemide (5 cc dose[24]) before training only
Group 3: Furosemide (5 cc dose) before racing only
Group 4: Furosemide (5 cc dose) before training and (5cc dose) before racing.

Once divided into these groups, the horses will be under the care and regimen of a group of trainers according to their preference for timing of Furosemide administration. Trainers will be provided with hay, concentrates and bedding to normalize for nutritional and environmental conditions. To establish a baseline of bone health and density, each horse will be examined by the study veterinarian and given a thorough health exam, at which time, X-rays of each leg, carpus, ankle, fetlock and hoof will be taken[25]. Over the period of one year, divided into 6 months of training/breeze work and 6 months 6 months in which to race[26]. X-rays will be taken before and after each timed workout and each race. Horses will be x-rayed for visual detection of bone remodeling to determine whether or not Furosemide had any effect on bone loss.

At the end of this trial's one year duration, samples from the carpus and proximal sesamoid bones (as these sites are most often linked to fracture in the racing thoroughbred) will be taken using minimally invasive arthroscopy. Using the Osteoarthritis Research Society International's (OARSI) scoring system, we will determine the histopathological changes in the bone and note the presence or absence of bone lesions and/or microfracture, as these signs indicate a loss of bone density.

The next step would be to establish the dosage at which Furosemide treatment is effective in the mitigation of Exercise-Induced Pulmonary Hemorrhage (EIPH) but does not result in any bone loss or changes in bone density. Groups would remain the same as for the first experiment

[24] A 5 cc dose of Furosemide is the allowable dose given to race horses in most but not all U.S. thoroughbred racing jurisdictions.
[25] According to a body of evidence, these sites are most at-risk for bone loss and increased incidence of fracture
[26] Horses will record 3 timed workouts (breezes) as mandated for any 2-year old prior to starting their first race. These breezes will be 6 weeks apart to correspond with the 6 month timeframe for training. Each horse, deemed fit to race by the examining veterinarian will have 3 races, 6 weeks apart to correspond with the 6 month timeframe for racing.

(Control; Furosemide administered before training; Furosemide administered before racing; and Furosemide administered before racing and training), however horses in each group would be further divided into dosage categories as follows:

- Horses given 1 cc of Furosemide
- Horses given 2 cc of Furosemide
- Horses given 3 cc of Furosemide
- Horses given 4 cc of Furosemide
- Horses given 5 cc of Furosemide

Horses again would go through a period of one-year, however, they would train for four months and race during an eight month period. During this period, they would ,record 2 timed workouts, 4 weeks apart and race 4 times, with eight weeks between each race. X-rays would be taken before the horses receive Furosemide to establish a baseline of bone density and also before and after workouts and before or after racing. Further, horses from each group will be subjected to endoscopy to observe the degree of EIPH as defined by the EIPH scale created by Hinchcliff[27] (2009). Endoscopy examinations will be performed during the cooling out period after training and racing events. From the data collected, the appropriate dose to mitigate the incidence of exercise-induced pulmonary hemorrhage while maintaining bone integrity can be determined and further recommendations to the use of Furosemide in racing thoroughbred can be made.

Other factors that will not be examined here could be involved and include surface, distance of training, distance of racing; gender of the horse; and method of training to train the horses as each race horse trainer has their own, unique training style. Another challenge of this

[27]Grade 1 = 1 or more flecks of blood or 2 or fewer short narrow streams of blood in the trachea or mainstem bronchi; Grade 2 = 1 long stream of blood or more than 2 short streams of blood occupying less than a third of the tracheal circumference; Grade 3 = multiple distinct streams of blood covering more than a third of the tracheal circumference, with no blood pooling at the thoracic inlet. Grade 4 = multiple, coalescing streams of blood covering more than 90% of the trachea with blood pooling at the thoracic inlet.

study is also its duration. Racehorses, especially two-year old's, are not always able to withstand the rigors of training. In this case, horses may be given time off and then brought back as a three-year old year or they may be retired from racing and retrained for less demanding careers. Additionally, not all horses will race during their two-year old year and train until their three-year old year. However, once it has been determined whether Furosemide has any effects on bone loss, further studies could examine the effect of additional variables such as surface composition or race distance on bone loss and whether or not such variables contribute to the rate of bone loss.

Humans and animals have been linked to each other throughout history, especially in the field of medicine. The advent of Furosemide provided physicians with a "magic bullet" to treat a wide variety of ailments from congestive heart failure to hypertension. Its action to cause profound diuresis has indeed helped patients with these conditions lead relatively normal lives. In the racehorse, Furosemide has been hailed as a wonder drug by veterinarians and trainers alike for its ability to decrease the incidence of EIPH while allowing the horse to be competitive. This relief from excess fluid build-up may come at a cost, however. Studies have shown a correlation between Furosemide and increased fracture risk in humans. The horse, whose life depends on its lungs and legs, is at a disadvantage if it requires a drug to breathe that increases its risk for fracture. As stewards of both the human and equine population, the responsibility is ours to find the balance between pharmacology and physiology.

TABLES

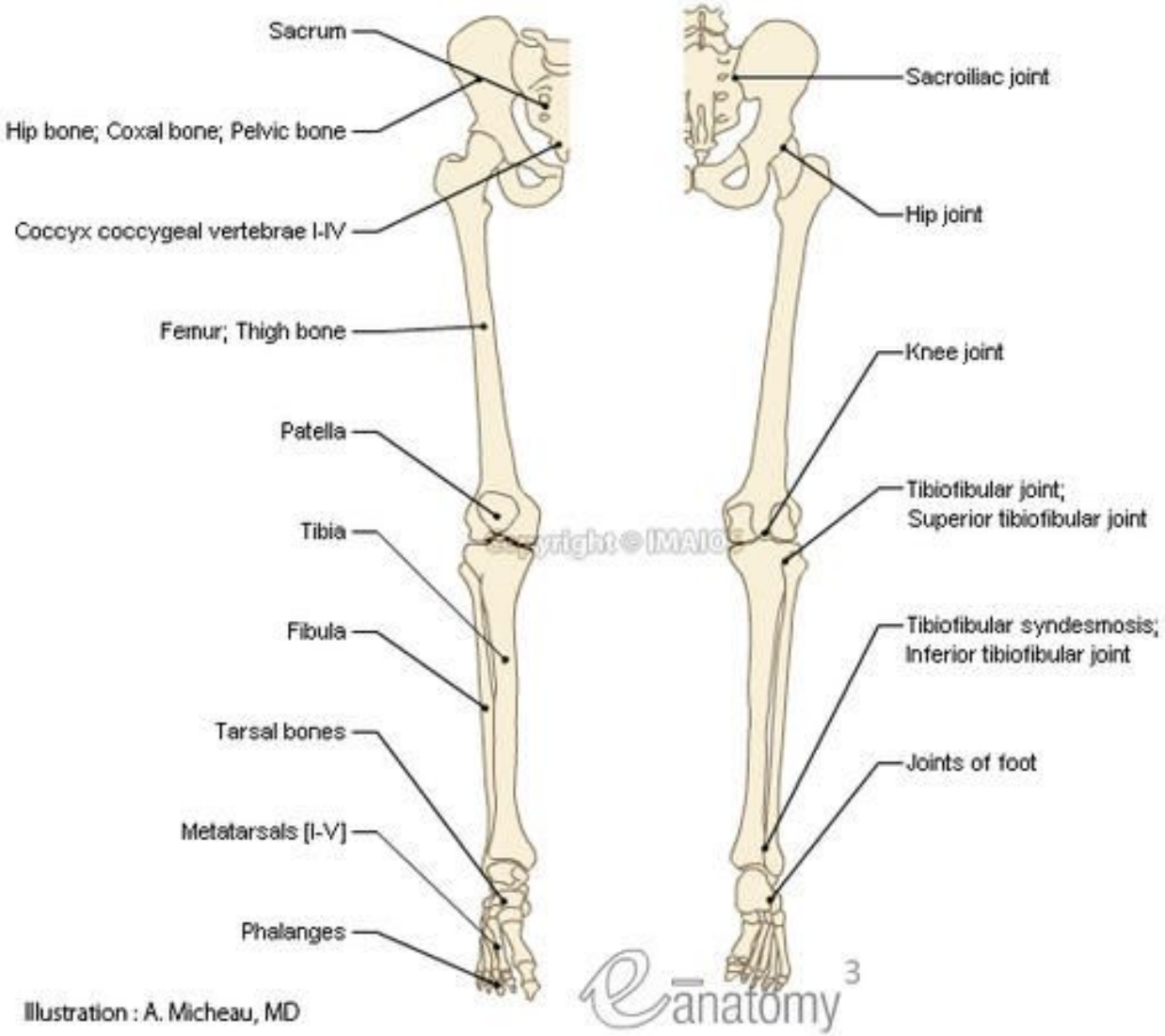
Table 1: General Nutrient Characteristics of Forages Commonly Fed to Horses

Nutrient[1]	Cool Season Grasses	Warm Season Grasses	Legumes
DE (Mcal/kg)	1.7-2.5	1.7-2.3	2-2.5
CP (%)	6-20	7-13	14-20
NDF (%)	55-65	70-80	35-45
ADF (%)	30-40	30-40	30-40
Ca (%)	0.25-.050	0.20-0.40	0.8-1.5
P (%)	0.20-0.40	0.20-0.40	0.2-0.35

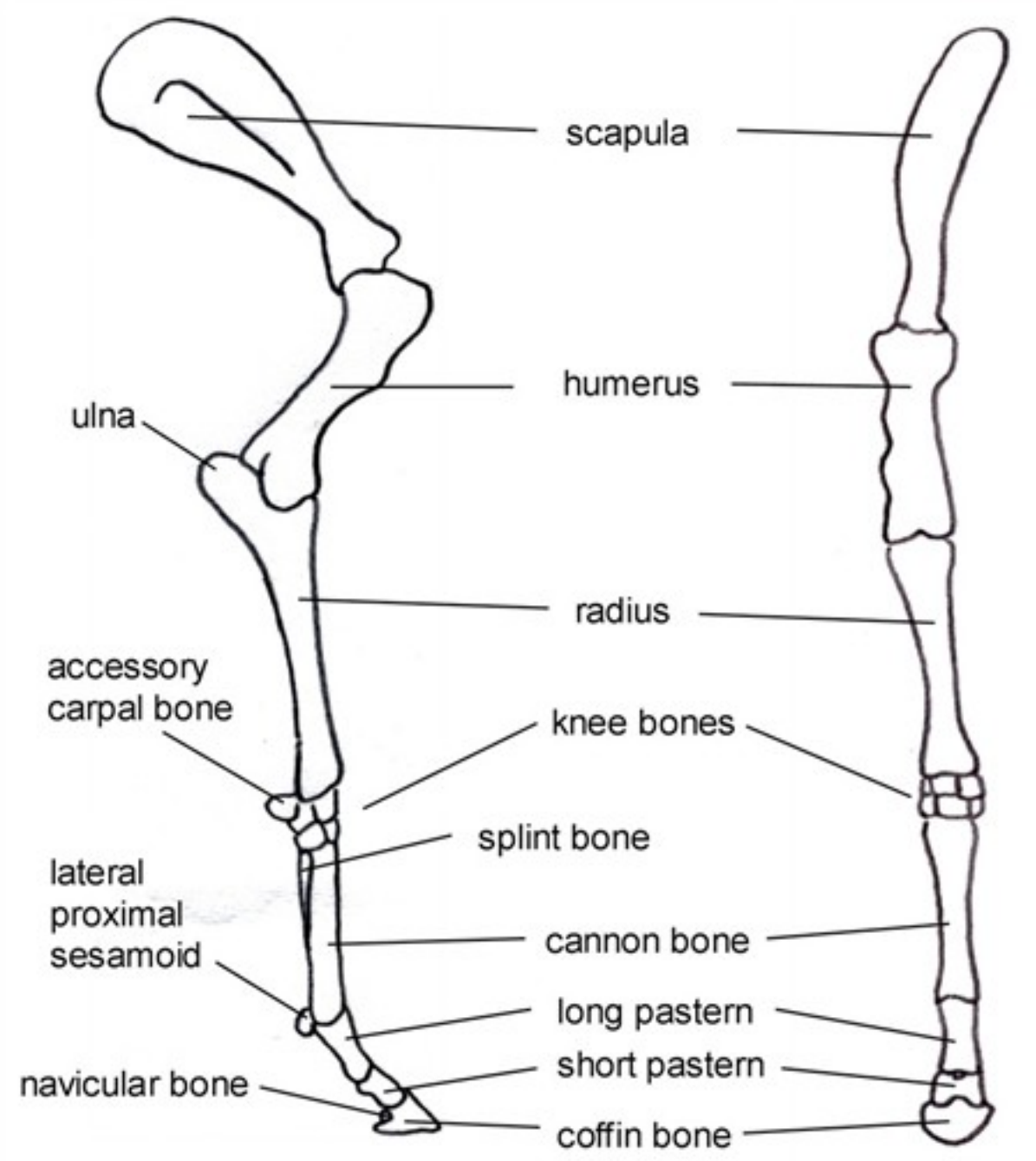
[1]DE, digestible energy; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; Ca, calcium; and P, phosphorus.

Differences in average nutritive values of forages commonly fed to horses are shown in Table 1 (Nutrena and Cargill Nutrition 2012).

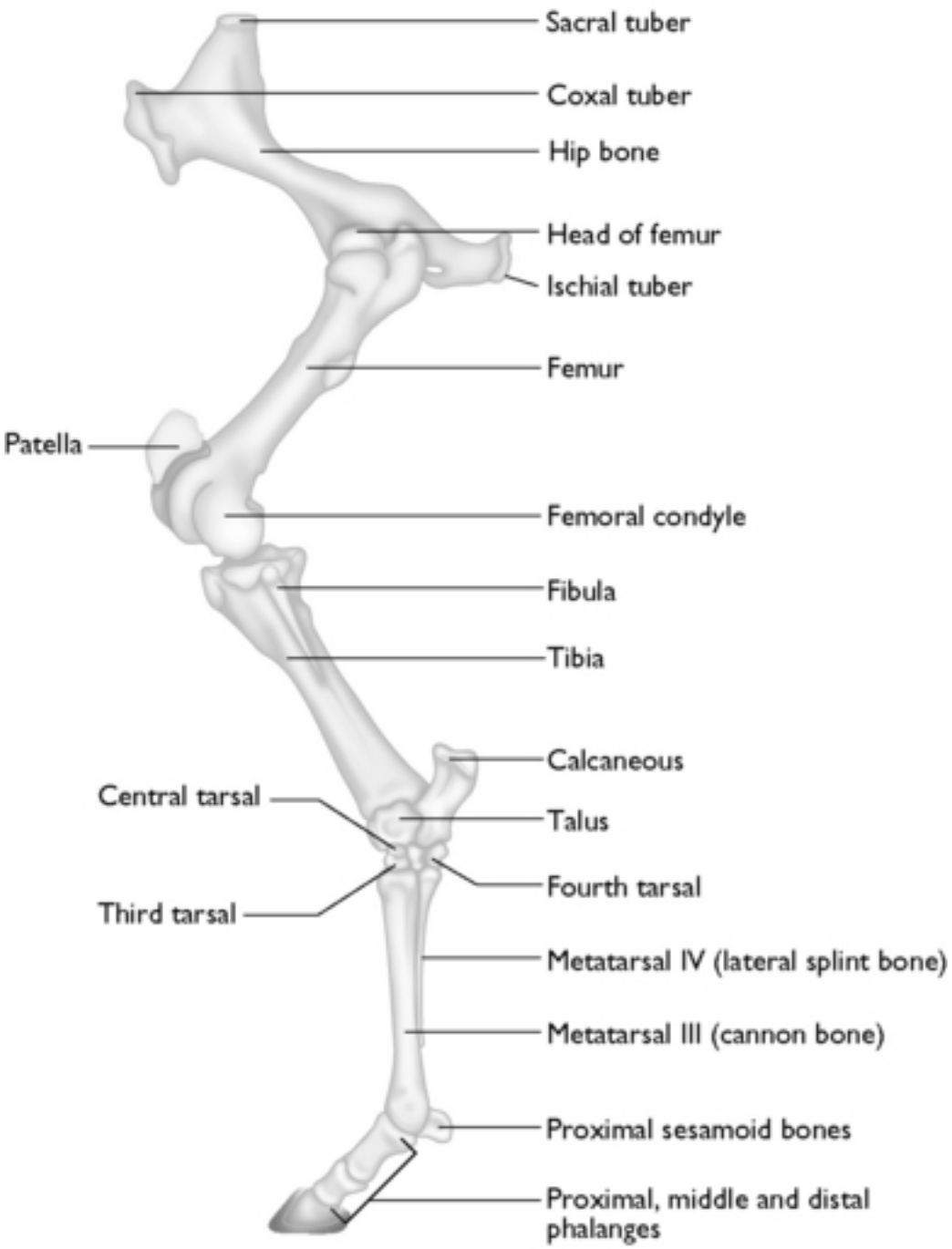
APPENDIX I: ANATOMY OF THE LOWER LIMB – HUMANS & HORSES



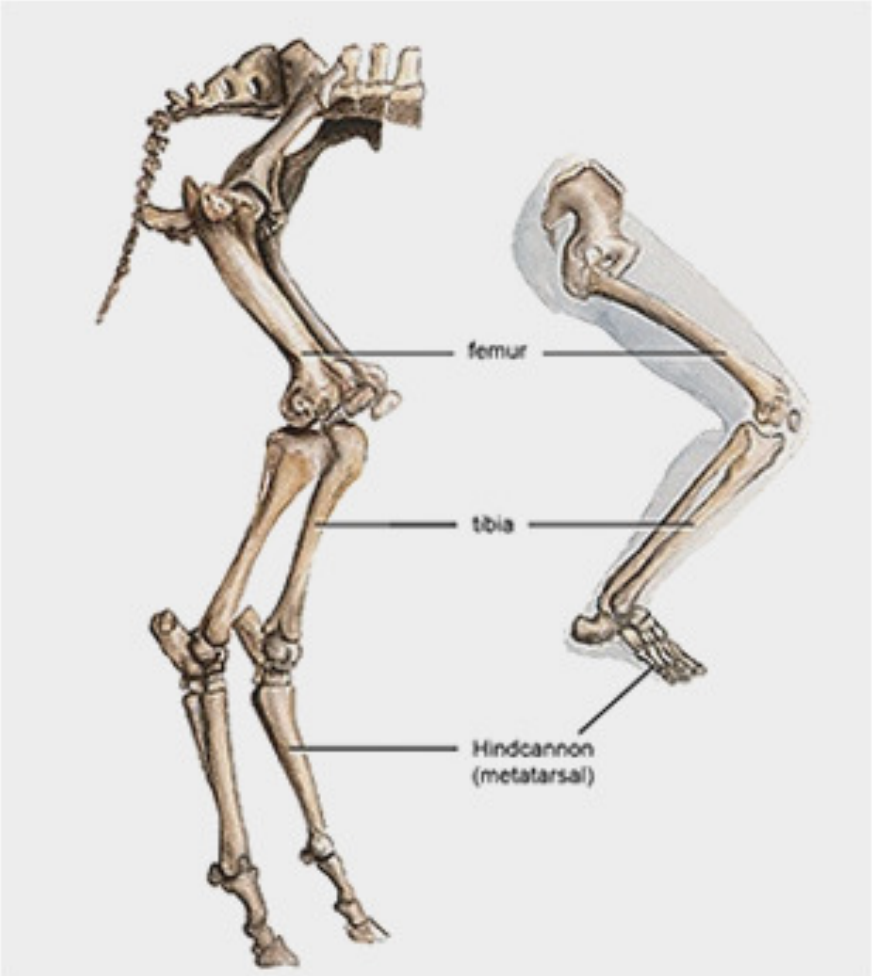
Bones and corresponding joints of the human limb and foot (Imaios 2018)



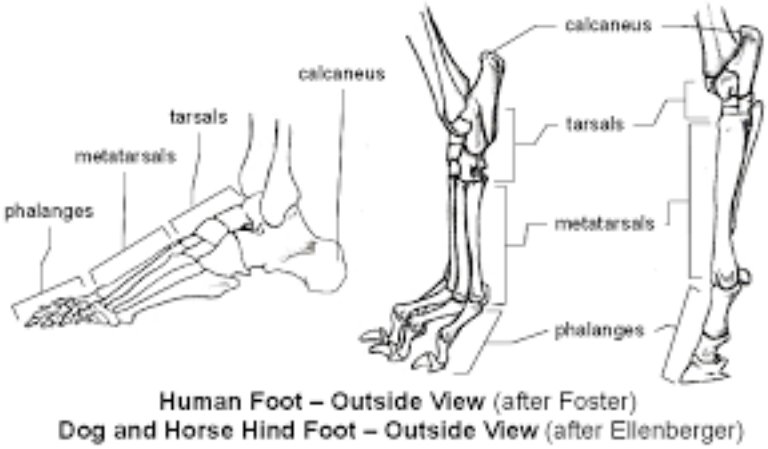
Bones of the Equine Fore Limb (Copper Mare Media 2013)



Bones of the Equine Hind Limb (DuoVital-Health LLC 2019)



Comparison of the bones of the human leg and the equine hind limb (Ferguson 2015)



Comparison of the bones of the human foot and the canine and dog hind foot (Liszewski 2011)

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