

## AN ABSTRACT OF THE THESIS OF

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Abstract approved:

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### Abstract:

Intravenous regional limb perfusion (IVRLP) with tiludronate is a commonly used treatment for distal limb orthopedic disease in horses, but doses and protocols are anecdotal. IVRLP exposes articular cartilage within the perfused area to tiludronate, raising concerns about safety of this treatment, as high tiludronate concentrations ( $\geq 19\text{mg/L}$ ) were harmful for cartilage *in vitro*. This is the first study to evaluate synovial fluid tiludronate concentrations following IVRLP to determine safety for articular cartilage in horses. Synovial fluid cytology variables and tiludronate concentrations were evaluated in the navicular bursa, coffin and fetlock joints following IVRLP of one front limb with low dose (0.5mg, n=6) or high dose (50mg, n=6) tiludronate. The contralateral limb was perfused with saline as a control. Synovial fluid samples were taken 1 week prior and 30 minutes following IVRLP from all structures and 24 hours post-perfusion from coffin and fetlock joints. Synovial fluid tiludronate concentrations were lower in

limbs perfused with 0.5mg in all synovial structures (metacarpophalangeal joint =  $3.7 \pm 1.5$  mg/L, distal interphalangeal joint =  $16.3 \pm 1.9$  mg/L, navicular bursa =  $6.0 \pm 1.9$  mg/L) than in limbs perfused with 50 mg (metacarpophalangeal joint =  $0.04 \pm 0.02$  mg/L, distal interphalangeal joint =  $0.12 \pm 0.06$  mg/L, navicular bursa =  $0.08 \pm 0.03$  mg/L) at tourniquet release. Only limbs perfused with 50mg had tiludronate detectable in synovial fluid 24 hours post-perfusion. There were no significant differences in synovial fluid cytology variables between samples from limbs perfused with saline vs. tiludronate. Thus, perfusion with tiludronate at doses used did not cause synovial inflammation in comparison to saline control limbs. Cytology variables did increase over time in both treated and control limbs, suggesting that IVRLP and/or synovial fluid sample acquisition stimulates an inflammatory response within synovial structures. Doses of 0.5mg or 50mg of tiludronate given via IVRLP resulted in synovial fluid concentrations that can be considered safe for articular cartilage based on previous in-vitro data. Tiludronate concentrations in bone should be explored to determine safety for bone and possible treatment efficacy for orthopaedic diseases of the distal equine limb.

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Intravenous Regional Limb Perfusion with Tiludronate in Horses

by

Barbara G. Hunter

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Barbara G. Hunter, Author

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**Intravenous regional limb perfusion with tiludronate in horses**

## 1. Introduction

Tiludronate is a non-nitrogenous bisphosphonate used in people for the treatment of Paget's disease and osteoporosis (Bonjour et al., 1995). In recent years, tiludronate has found a place in equine medicine for treatment of diseases marked by abnormal bone remodeling (Kamm et al., 2008). Remodeling of bone in any mammal is continuous throughout life. The process is characterized by a delicate balance between resorption of bone and formation of new bone to replace the resorbed bone. In diseases such as equine navicular disease, the balance between resorption and formation is disrupted (Ostblom et al. 1982). Resorption is upregulated in comparison to formation (Ostblom et al. 1989) and the resulting bone loss can lead to chronic pain. Previously, treatment of affected animals has been palliative. Navicular disease is a chronically degenerative disease and as it progresses, horses become refractory to pain management medications. Athletic careers are cut short, resulting in financial loss for owners. Eventually, quality of life becomes substandard due to the presence of chronic constant pain, and horse owners are left with humane euthanasia as the only option for alleviation of their horse's suffering.

Bisphosphonates are a category of pharmaceuticals that modulate bone remodeling. They have provided veterinarians with an opportunity to decrease bone resorption. Tiludronate is injected systemically at 1 mg/kg in horses for the treatment of navicular disease (Kamm, 2008), but the cost of treatment is expensive. A single treatment typically costs circa 1000 USD, and horses are often treated one to two times per year (personal experience). In effort to reduce costs and minimize systemic side effects such as colic, veterinarians are currently administering tiludronate at 0.2 mg/kg by intravenous regional

limb perfusion for treatment of navicular disease. Although this is 1/5 of the cost of systemic treatment, the safety and efficacy of this dose is completely unknown.

## **2. Objectives and Hypothesis**

The objectives of this study were to determine the concentrations of tiludronate achieved in distal limb synovial structures following intravenous regional limb perfusion with a low dose or high dose of tiludronate and to determine the effect of tiludronate on synovial fluid cytology variables in comparison to placebo control limbs. The working hypothesis was that intravenous regional limb perfusion with low dose tiludronate (0.5mg) would result in synovial fluid concentrations that were safe for cartilage ( $\leq 19$  mg/L) and synovial fluid cytology variables would not vary significantly from controls. Intravenous regional limb perfusion with high dose tiludronate (50mg), the lowest of three doses currently used in clinical practice, would result in synovial fluid concentrations that were unsafe for cartilage ( $> 19$  mg/L) and synovial fluid variables in treated limbs would be consistent with inflammation in comparison to control limbs.

### **3. Literature Review**

#### **3.1 Bone Histology and Remodeling**

Bone is a composite material made up of organic matrix surrounded by hydroxyapatite crystals (Samuelson, 2007). Most bones in the horse consist of a hard outer cortex of compact bone surrounding a medulla filled with spiculated trabecular bone (Figure 1). The cortex is surrounded by periosteum, a tough fibrous tissue that contains numerous osteogenic cells for new bone formation in the underlying bone. The medullar bone is lined by endosteum, a thin, loose connective tissue that serves a similar purpose to periosteum in supplying cells for new bone formation.

Remodeling of bone is a constant process that is continuous throughout life. The cell responsible for the growth of new bone is the osteoblast (Samuelson, 2007). This cell produces osteoid, the organic component of bone's extracellular matrix, which consists of type 1 collagen fibers and proteoglycans such as sulfated glycosaminoglycans, chondroitin sulfate and keratan sulfate which combine with hyaluronans to form aggrecan to line inorganic hydroxyapatite crystals. Initially osteoblasts lie on the surface of bone, but as more osteoid is produced they become encased. Once encased, osteoblasts convert to metabolically active osteocytes. Sitting in small pockets within the osteoid called lacunae, osteocytes maintain the extracellular matrix of bone by passing nutrients and messages through small channels extending from each lacuna, called canaliculi, to neighbouring osteocytes. Additionally, osteocytes are responsible for directing bone remodeling in adaptation to mechanical load. When stimulated by parathyroid hormone

during osteocytic osteolysis, osteocytes secrete hydrolases that cause resorption of the extracellular matrix in a limited fashion to allow rapid release of calcium for systemic use or for the formation of new bone. Bone resorption is performed by osteoclasts; multinucleated cells derived from merging monocytes within bone. Osteoclasts resorb bone by secreting acids (eg., lactic and citric) and hydrolytic enzymes such as collagenases within resorption bays (Howship's lacuna). These cells are mobilized into activity when parathyroid hormone, in response to low levels of ionized calcium in the blood, stimulates osteoblasts to secrete osteoclast stimulating factor. Once stimulated, multiple osteoclasts form a cutting cone which resorbs bone (Figure 1) (Cunningham and Klein, 2007). Immediately following the cutting cone are multiple osteoblasts forming new osteoid. This osteoid becomes mineralized by new hydroxyapatite crystals.

### **3.2. Bisphosphonates**

#### *3.2.1. Interaction with bone*

Bisphosphonates (BPs) are analogues of pyrophosphate, the inorganic compound that is released as a by-product of many normal chemical reactions within the body (Drake et al., 2008). Experimentally, pyrophosphate was found to bind to hydroxyapatite crystals inhibiting calcification resulted (Fleisch et al., 1966). The suggestion was made that bone mineralization could be regulated by controlling pyrophosphate concentrations within the body. Similar to pyrophosphate, BPs also have a high affinity for calcium and preferentially bind to hydroxyapatite crystals (Drake et al., 2008). The hydroxyapatite crystals exposed to bisphosphonates during bone remodeling bind to the bisphosphonates



and form a depot of the drug that can be slowly released during future remodeling (Drake et al., 2008). A recent *in vitro* study evaluating the adsorption of the BP tiludronate to nanocrystalline apatites (similar to hydroxyapatite) showed that immature nanocrystals showed the greatest affinity for tiludronate and adsorbed greater quantities in comparison to mature nanocrystals, suggesting that actively mineralizing bone will have greater affinity for BPs than mature bone (Pascaud et al., 2013). Since their original discovery in the 1960's, BPs have been used to manipulate bone metabolism, primarily through reduction of bone resorption (Drake et al., 2008). They are the primary pharmacologic tool against diseases with osteoclast-mediated bone loss such as osteoporosis, Paget's disease, malignant metastatic bone disease, and hypercalcemia of malignancy.

Tiludronate specifically has been shown to increase bone strength and mass in a rat model of induced osteopenia (Ohnishi et al., 1997). In addition to their anti-resorptive action on bone, BPs can also be anti-inflammatory, and they are administered both to prevent bone resorption and reduce pain (Kamm, 2008). Administration in humans can be associated with some complications (Drake et al., 2008). Osteonecrosis of the jaw is the most commonly reported complication; however 94% of these cases occur in patients being treated for cancer. Other complications include hypocalcemia and in rare cases, atrial fibrillation or severe musculoskeletal pain.

In humans, BPs are administered systemically; most commonly either orally or intravenously. Once administered, BPs adsorb quickly to hydroxyapatite crystals within bone, a situation that allows them intimate and prolonged contact with osteoclasts

(Rogers et al., 2011). As osteoclasts acidify the resorption lacuna, exposed bone is demineralized and BPs are taken up into osteoclasts by endocytosis (Coxon et al., 2008).

Once ingested by osteoclasts, BPs induce osteoclast apoptosis (Rogers et al., 2011). The mechanism by which this occurs is determined by the type of bisphosphonate being used for treatment. Bisphosphonates are classified as non-nitrogenous or nitrogenous. The mechanisms of action of each group will be discussed in detail below. Briefly, however, non-nitrogenous BPs mediate osteoclast apoptosis by forming non-hydrolysable ATP analogues that accumulate within osteoclast cytosol and interfere with cellular functions. Tiludronate, the subject of the research in this thesis, is an example of a non-nitrogenous bisphosphonate. Nitrogenous BPs (eg., zoledronate) have a more complex mechanism of action that is mediated through the mevalonate pathway, which is the pathway responsible for cholesterol synthesis. Non-nitrogenous BPs have a basic structure of two phosphate groups attached to a carbon atom, while nitrogenous BPs add an amino group into this basic structure. This amino group substantially increases the potency of the BP to prevent bone resorption (Drake et al., 2008).

In addition to their effect on osteoclasts, BPs also have effects on osteocytes and osteoblasts. Interestingly, in contrast to osteoclasts, the effect on osteocytes and osteoblasts appears to be anti-apoptotic (Plotkin et al., 1999; Plotkin et al., 2006; Bellido and Plotkin, 2011). This effect is mediated via opening of connexin 43 hemichannels which leads to activation of extracellular signal-related kinases (ERKs) that inhibit

apoptosis of osteoblasts/cytes (Plotkin et al., 2006). This anti-apoptotic function is dose-dependent with effects being lost when BPs are administered in higher concentrations.

### *3.2.2. Non-nitrogenous bisphosphonates*

Once internalized by osteoclasts via endocytosis, non-nitrogenous BPs, such as tiludronate, are incorporated into ATP with the help of aminoacyl-tRNA synthetases to form a non-hydrolysable analogue of ATP (Rogers et al., 1994). Nitrogenous BPs cannot form this non-hydrolyzable ATP because their sterically larger shape does not enable them to fit into the active site of the aminoacyl-tRNA synthetase. The non-hydrolysable ATP accumulates intracellularly within affected osteoclasts and promotes apoptosis through inhibition of enzymes important for cellular metabolism. For example, the non-nitrogenous BP clodronate interferes with adenine nucleotide translocase, an important enzyme for maintaining mitochondrial permeability (Lehenkari et al., 2002). Inhibition of this enzyme results in loss of mitochondrial permeability and subsequent activation of the apoptosis promoting enzyme Mst-1 (Reska et al., 1999).

### *3.2.3. Nitrogenous bisphosphonates*

Nitrogenous BPs also precipitate osteoclast apoptosis, but the mechanism through which this is executed is somewhat more complex in comparison to non-nitrogenous BPs. Nitrogenous BPs inhibit enzymes within the mevalonate pathway, which is responsible for cholesterol synthesis (Rogers et al., 2011). Most commonly the enzyme inhibited is farnesyl pyrophosphate synthase, but a few BPs inhibit squalene synthase, an enzyme one step downstream (Figure 2). Inhibition of either of these enzymes ultimately results in an

accumulation of isopentenyl pyrophosphate (IPP). Accumulation of IPP leads to an acute phase response through activation of  $\gamma,\delta$ -T cells and subsequent release of TNF $\alpha$  (Thompson et al., 2004). In addition, IPP can be metabolized to a non-hydrolysable metabolite that causes loss of mitochondrial permeability. This triggers apoptosis in a manner similar to that of non-nitrogenous bisphosphonates (Mönkkönen et al., 2006).

As IPP is accumulating, its downstream products, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), are not being synthesized. These products are vital for post-translational modification, or prenylation, of intracellular signaling proteins (Rogers et al., 2011). Specifically, several small GTPases do not get prenylated and are therefore dysfunctional. Without their function, osteoclasts lose the ability to manipulate their cytoskeleton, thus formation of the ruffled border is impaired. Movement of vesicles within osteoclasts is also impaired, thus secretion of lysosomal enzymes for degradation of mineral matrix cannot occur. Finally, signaling pathways that normally promote cell survival are disrupted when small GTPases are not available for signaling, thus apoptosis occurs. Nitrogenous BPs precipitate osteoclast apoptosis through two separate mechanisms, and they also impede osteoclast function prior to cellular death. Perhaps this multi-faceted mechanism of action is what enables most nitrogenous BPs to be considerably more potent than non-nitrogenous BPs.

#### *3.2.4 Effects of bisphosphonates on non-bone tissues*

While present in circulation, BPs can be endocytosed by monocytes (Roelofs et al., 2009). The resulting effect is determined again by the classification of the BP. Non-

nitrogenous BPs have been shown to inhibit pro-inflammatory cytokine and nitric oxide secretion from monocytes that have been activated in macrophages (Monkkonen et al., 1998). This inhibition is dose-dependent with 1000  $\mu\text{M}$  having a more potent effect than 100  $\mu\text{M}$ . In contrast, the nitrogenous BP ibandronate did not inhibit cytokine or nitric oxide secretion unless present in cytotoxic concentrations. In fact, up to 50 % of patients will develop a transient fever and acute phase response when administered a nitrogenous BP (Monkkonen et al., 1998). This effect occurs because of an accumulation of isopentenyl pyrophosphate; a substrate within the mevalonate pathway that builds up when this pathway is halted (Thompson et al., 2004). Isopentenyl pyrophosphate activates  $\gamma,\delta$ -T cells which results in the release of  $\text{TNF}\alpha$  and activation of an acute phase response.

Of particular relevance to the research presented in this thesis, is the influence of BPs on chondrocytes and articular cartilage. An early study showed that low doses of tiludronate suppressed secretion of collagenase and proteoglycan-degrading proteinases by articular chondrocytes in an *in vitro* model of arthritis (Emonds-Alt et al., 1985). Later studies revealed that treatment with BPs significantly decreased degradation of type II collagen in patients with Paget's disease (Garnero et al., 2001), and BPs ameliorated bovine chondrocyte necrosis and growth suppression associated with dexamethasone treatment *in vitro* (Van Offel et al., 2002). In this same dexamethasone model, therapeutic concentrations of nitrogenous BPs inhibited dexamethasone-induced apoptosis of chondrocytes, while the non-nitrogenous bisphosphonate, clodronate, did not. A recent *in vitro* study using equine cartilage explants, tiludronate demonstrated a

concentration dependent effect on cartilage with low concentrations inhibiting IL-1-induced chondrocyte apoptosis and proteoglycan release from cartilage matrix and high concentrations enhancing chondrocyte apoptosis and proteoglycan release (Duesterdieck-Zellmer et al., 2012). Similar to osteocytes/blasts, the anti-apoptotic effect of tiludronate on chondrocytes may be mediated via connexin 43 hemichannels which have been shown to be present on chondrocytes (Knight et al., 2009). Interestingly, although non-nitrogenous BPs have been shown to inhibit production of inflammatory cytokines by macrophage/monocyte cells (Monkkonen et al., 1998), they do not appear to inhibit production of pro-inflammatory cytokines by arthritic human chondrocytes (Van Offel et al., 2005).

Although BPs are classically equated with treatment of diseases with abnormal bone resorption, several experimental models have shown that they can also be beneficial for treatment of osteoarthritis. This is likely due to beneficial effects on both cartilage and subchondral bone. In a rat model, induction of osteoarthritis with intra-articular monosodium iodoacetate resulted in radiographic loss of epiphyseal bone mineral density and chondrocytes, cartilage thinning, resorption of subchondral bone and calcified cartilage as well as pain (Strassle et al., 2010). Rats treated with subcutaneous injections of the BP zoledronate prior to osteoarthritis induction did not show losses in bone mineral density, subchondral bone, calcified cartilage or articular cartilage in comparison to control rats. Rats that were treated after induction of osteoarthritis showed significant changes to cartilage and subchondral bone in comparison to controls, but less severe changes than rats that did not receive any BP treatment. In dogs with osteoarthritis

induced via transection of the cranial cruciate ligament, serum biomarkers for bone resorption and bone mineral density losses were significantly reduced in dogs treated with zoledronate in comparison to controls (Agnello et al., 2005). In a similar model, dogs treated with tiludronate showed decreased severity of cartilage lesions and lower gene expression for matrix metalloproteases (MMPs) 1, 3, and 13 within cartilage in comparison to controls (Pelletier et al., 2011). These MMPs have been previously correlated with cartilage degradation. An equivalent study in rats treated with the BP alendronate yielded similar results and also reduced the incidence of osteophyte formation (Hayami et al., 2004). Finally, treatment of experimentally induced osteoarthritis in dogs with subcutaneous injections of tiludronate resulted in decreased clinical lameness, pain, joint effusion, and greater subchondral bone surface in comparison to controls (Moreau et al., 2011).

Not all studies evaluating the effect of bisphosphonates on osteoarthritis have shown positive results. In a study evaluating the effect subcutaneous injection of the non-nitrogenous bisphosphonate, NE-10035, on osteoarthritis induced by transection of the cranial cruciate ligament in 5 dogs, NE-10035 treated dogs had decreased turnover of subchondral bone in comparison to controls but there was no significant effect of the BP on osteophyte formation or severity of cartilage damage in comparison to controls (Myers et al., 1999). In another study, the nitrogenous BP alendronate was given subcutaneously to healthy guinea pigs at doses of 10 µg/kg or 50 µg/kg (Ding et al., 2008). Interestingly, both treatment groups showed greater tibial condylar cartilage damage than placebo-treated controls. Alendronate-treated guinea pigs had greater subchondral bone thickening

than controls with subchondral bone thickening increasing in a dose-dependent fashion. Aledronate treated guinea pigs also had greater bone mineral concentrations and lower bone collagen concentrations in comparison to controls. The results of Moreau et al., (2011) lead one to surmise that prevention of subchondral bone lysis secondary to osteoarthritis may decrease cartilage damage and pain associated with cartilage degeneration. By contrast, the results of Ding et al., (2008) lead one to conclude that one should pick cases for treatment with bisphosphonates cautiously as treatment leading to excess thickening of the subchondral bone may actually be detrimental to overlying articular cartilage.

While BPs are associated with chondroprotective effects, the degree of effect and associated pain relief reported is variable. In one study, intra-articular injection of low dose clodronate, a non-nitrogenous BP similar to tiludronate, was associated with decreased knee pain in human subjects in comparison to baseline pain (Cocco et al., 1999). Another study found that people treated with 15 mg/day of oral risedronate for knee osteoarthritis showed decreased pain and better physical function in comparison to placebo controls (Spector et al., 2005). Conversely, a recent meta-analysis review of current literature evaluating use of bisphosphonates for treatment of osteoarthritic (OA) pain in humans determined that the evidence for bisphosphonate efficacy in treating OA pain was limited (Davis et al., 2013).

### *3.2.5 Bisphosphonates in Horses*



The use of only three bisphosphonates has been reported in horses: zoledronate (nitrogenous), pamidronate (nitrogenous) and tiludronate (non-nitrogenous). Tiludronate administered by systemic intravenous route is licensed for treatment of navicular disease and osteoarthritis of the distal hock joints in horses in Europe, but is not FDA approved in the United States. Zoledronate is not currently licensed for use in horses, but a recent study has described the pharmacokinetics and pharmacodynamics of a safe dose administered to healthy horses (Neito et al., 2013). It has also been used with some clinical efficacy for treatment of bone fragility syndrome; a syndrome found in horses from select areas of California and characterized by massive bone resorption of the axial skeleton (Katzman et al., 2012). Pamidronate is also not licensed for use in horses, but it has been used experimentally *in vitro* (Gray et al., 2002) and *in vivo* (McGuigan et al. 2000). When cultured *in vitro* with equine osteoclasts, pamidronate was found to inhibit osteoclast resorption of bone in a dose-dependent fashion with higher doses having a greater effect on bone resorption (Gray et al., 2002). In a small clinical trial, pamidronate was not found to improve lameness in horses with navicular disease in comparison to placebo controls (McGuigan et al., 2000).

Tiludronate has received the most research attention of the 3 BPs reportedly used in horses. It is labeled for use at a dose of 0.1 mg/kg intravenously once a day for 10 days for a total dose of 1 mg/kg (Kamm, 2008). This dose was initially determined to be appropriate using a rat model and tiludronate was still present in bone 6 months following administration (Thibaud et al., 2003). A study in horses found that a single dose of 1 mg/kg was equally as effective as 10 daily doses of 0.1 mg/kg in reducing a serum

biomarker of bone resorption, carboxy-terminal cross-linking telopeptide (CTX-1) (Delguste et al., 2008). Another study found that one intravenous dose at 1 mg/kg was well tolerated in healthy adult horses; with complications being limited to mild tachycardia during administration and transient hypocalcemia 30 minutes post injection (Varela et al., 2002). Administration at 1mg/kg by single, slow intravenous injection is now the more commonly used dose in clinical practice. Because tiludronate, like other BPs, is excreted primarily through the kidneys, evaluation of serum creatinine prior to administration is advisable. If creatinine is elevated, avoiding use of systemic bisphosphonates or pre-treatment with IV fluids would be prudent. In humans, administration of intravenous BPs to patients with renal insufficiency can result in a rapid decline of renal function (Drake et al., 2008). Colic has also been reported to occur intermittently during administration of tiludronate (Kamm, 2008; Delguste et al., 2011). Administration of flunixin meglumine at 1.1 mg/kg intravenously just prior to tiludronate administration is recommended to avoid this complication.

Tiludronate has reportedly been effective in reducing pain associated with navicular disease (Denoix et al., 2003), distal hock osteoarthritis (Gough et al., 2010) and thoracolumbar osteoarthritis (Coudry et al., 2007) in some horses. Horses with bilateral forelimb lameness localized to the heel region with radiographic evidence of navicular disease, were treated with tiludronate at 1 mg/kg intravenously (Denoix et al., 2003). This resulted in soundness in 50% of horses and improved signs of lameness in 66% of horses at 6 months following treatment in comparison to placebo controls when initial lameness had been present for less than 6 months. By contrast, only two out of eight horses with

lameness of > 6 months duration prior to treatment showed improvement with treatment. These findings parallel findings in rat and dog experimental models of osteoarthritis that revealed greater reductions in bony and articular pathology when BPs were administered prophylactically or early in the disease (Strassle et al., 2010; Agnello et al., 2005).

In a double blind, placebo-controlled study in which horses had lameness localized to the distal hock joints, horses treated with tiludronate at 1 mg/kg intravenously were significantly more likely to show a reduction in degree of clinical lameness at 2 and 4 months following treatment than placebo controls (Gough et al., 2010). Another study revealed that horses with clinical back pain and radiographic signs of thoracolumbar osteoarthritis, when treated with 1 mg/kg tiludronate intravenously showed significant improvement in dorsal flexibility 2 and 4 months following treatment in comparison to placebo controls (Coudry et al., 2007). Treatment with tiludronate has also been shown to significantly reduce CTX-1, a serum biomarker of bone resorption, and minimize loss of bone mineral density during long term cast immobilization of distal limbs in horses (Delguste et al., 2007). Inflammation may inhibit the action of non-nitrogenous BPs as inflammation-associated RANKL and TNF $\alpha$  can induce expression of anti-apoptotic proteins (Sutherland et al., 2009). This may help explain why clinical efficacy of tiludronate is reportedly decreased in cases of chronic navicular disease in horses compared to more acute cases (Denoix et al., 2003).

Despite the fact that tiludronate is labeled for systemic intravenous administration in horses, equine practitioners are currently injecting it into joints and giving it via

intravenous regional limb perfusion. Anecdotally a dose of 50 mg (~ 1/10<sup>th</sup> of a systemic dose) per joint, or per intravenous regional limb perfusion is being used, but this or any other dose is completely unproven for both safety and efficacy. Tiludronate concentrations equivalent to this dose have been shown to have negative effects *in vitro* on equine articular cartilage (Duesterdieck-Zellmer et al., 2012), and more recently intra-articular administration of 50 mg tiludronate *in vivo* has been shown to have a temporary catabolic effect on cartilage matrix (Duesterdieck-Zellmer et al., 2013). Administration of tiludronate via intravenous regional limb perfusion has been reported in the form of non-peer reviewed proceedings (Carpenter, 2012), but the safety of this technique for articular cartilage located within the perfused area has not been evaluated.

### **3.3. Navicular Disease**

The term ‘navicular disease’ is somewhat misunderstood as the term is commonly interchanged with ‘caudal heel pain’ or ‘navicular syndrome’; terms which can refer to injury of any soft tissue or bone structure in the caudal heel region of the equine foot, including the navicular bone. In this thesis, the term navicular disease will be used in reference to pain in the palmar aspect of the equine forelimb. Navicular disease is typically bilateral and breeds such as the American Quarter Horse, Thoroughbreds and Warmbloods are predisposed (Dabareiner and Carter, 2003). It is considered the most common cause of forelimb lameness in the horse (Waguespack and Hanson, 2010) with an estimated one third of forelimb lameness thought to originate in the heel (Dabareiner and Carter, 2003). Navicular disease has been reported as early as 1752 (Waguespack and

Hanson, 2010), however, despite long standing awareness of the disease, equine veterinarians are still limited in their understanding of the pathophysiology of the disease, their ability to accurately diagnose precise causes of pain in the heel and administer effective treatment.

Somewhat complicating is the fact that navicular disease can be a result of injury to more than the navicular bone. The anatomy of the equine foot is a complex array of bone and soft tissue structures (Figure 3). Pain can result from injury to the navicular suspensory ligament, distal sesamoidean impar ligament, distal deep digital flexor tendon, tearing of the heel lamina, bruising of the heel sole, injury or fracture of the navicular bone or caudal aspects of the distal phalanx, inflammation of the navicular bursa and potentially the distal interphalangeal joint, as well as navicular bone sclerosis, lysis or erosion of its overlying fibrocartilage (Dabareiner and Carter, 2003). As one might surmise, injury to any of these structures results in similar clinical findings on lameness evaluation and sensation to all of these structures can be blocked with local anesthesia of the palmar digital nerves (Schumacher et al., 2003; Easter et al., 2000; Schumacher et al., 2000) making localization of pain to a specific structure difficult.

Effective treatment of caudal heel pain is reliant on delivery of treatment that is appropriate for the affected structure. Treatment for abnormalities of the navicular bone itself and its associated suspensory apparatus is derived from the current understanding of normal anatomy and microanatomy of these structures.

### *3.3.1. Anatomy and microanatomy of the navicular apparatus*

The navicular apparatus consists of the navicular bone and the structures that suspend it. The navicular bone, born from one center of ossification through endochondral ossification (Rijkenuizen et al., 1989a), is suspended from the palmar aspect of the middle phalanx by the navicular suspensory ligament (also called the collateral sesamoidean ligament) on its proximal border and is attached to the palmar surface of the distal phalanx by the distal sesamoidean impar ligament on its distal border (Figure 3) (Waguespack and Hanson, 2010). Dorsally and distally the navicular bone is covered by articular cartilage where it articulates with the middle and distal phalanges, while the palmar surface that interfaces with the deep digital flexor tendon is covered by fibrocartilage (Waguespack and Hanson, 2010).

The gross morphology of the navicular bone is influenced by breed and exercise in normal horses with small athletic halfbreed horses having smaller navicular bones than small sedentary halfbreeds, Thoroughbreds or larger halfbreed horses (Gabriel et al., 1998). A hereditary influence on navicular bone shapes that predispose to navicular disease has also been supported in Warmbloods (Dik and van den Broek, 1995; Diesterbeck et al., 2007). The navicular bone is composed of trabecular bone within a medulla surrounded by a rim of cortical bone (Wright et al., 1998) (Figure 4). Adhering to the principle of Wolf's law, the dorsal to palmar arrangement of the trabeculae suggests that the primary force acting on the navicular bone is a compressive force applied by the deep digital flexor tendon (Wright and Douglas, 1993).

Vascular source for the navicular bone is supplied by branches of the palmar digital arteries (Rijkenhuizen et al., 1989a). These branches enter the navicular bone from proximal, distal, medial and lateral directions avoiding surrounding synovial structures with the greatest concentration of blood vessels occurring distal to the navicular bone near its nutrient foramen. The ramus navicularis distalis is an arteriole that runs transversely, distal and palmar to the navicular bone, connecting the medial and lateral palmar digital arteries. In addition to supplying multiple arteriole branches to the distal navicular bone, it also supplies branches to the distal sesamoidean impar ligament, distal phalanx and deep digital flexor tendon.

Innervation to the navicular region is supplied by branches of the palmar digital nerves. Silver impregnation and immunocytochemistry of the area has shown an abundance of sensory nerve fibers present within and around the collateral sesamoidean and distal sesamoidean impar ligaments (Bowker et al., 1994). The role that disruption to these nerves may play in navicular disease is not clearly understood. It is possible that injury to the ligaments of the navicular apparatus may result in aberrant innervation and subsequent pain in the navicular bone.

### *3.3.2. Potential etiologies of navicular disease*

Several etiologies for navicular disease have been proposed, yet understanding of the pathophysiology of the disease remains incomplete. Early studies suggested that arteriole obstruction and secondary arteriosclerosis (Rijkenhuizen et al., 1989b) or thrombosis (Colles and Hickman, 1977) resulted in painful ischemic necrosis of the

navicular bone. Arteriograms in Warmbloods with clinical or radiographic signs of navicular disease showed diminished vascularity distal to the navicular bone with compensatory augmentation of the proximal arteriole supply with histologic signs of increased bone remodeling (Rijkenhuizen et al., 1989b). While more recent histologic studies support the finding of increased bone remodeling, evidence of microthrombi were lacking and investigators suggested that bone remodeling was more consistent with degenerative bone diseases such as osteoarthritis (Wright et al., 1998; Blunden et al., 2006). In further support of navicular disease as a degenerative orthopedic disease, an early study evaluating navicular bones from eight horses with clinical signs of navicular disease found histologic evidence of accelerated bone remodeling in all cases (Ostblom et al., 1982). Two of these horses were injected twice with tetracycline prior to euthanasia which acted as an *in vivo* bone marker. A later study showed that the ratio of resorption to formation of bone in horses with navicular disease was significantly increased over that of normal horses (Ostblom et al., 1989). The proposed mechanism driving the increase in bone remodeling was increased pressure applied by the deep digital flexor tendon to the navicular bone secondary to hoof conformation abnormalities such as long toes with low heels or contracted heels (Ostblom et al., 1982). Evaluation of the force applied to the navicular bone by the deep digital flexor tendon has revealed that while peak pressures are equivalent between normal horses and those with navicular disease, horses with navicular disease display approximately double the stress on the navicular bone in the early phase of the stance in comparison to normal horses (Wilson et al., 2001). This lends support to the theory that prolonged pressure, supplied by the deep digital flexor tendon



(DDFT), may stimulate increased remodeling in the navicular bone (Ostblom et al., 1989). This theory is supported by findings that showed histologic evidence of decreased bone resorption of the navicular bone in horses with navicular disease that were treated with corrective trimming and egg bar shoes in comparison to affected horses left untreated (Ostblom et al., 1989).

Normal remodeling of bone is a balanced process where bone is resorbed from areas under minimal mechanical load at the same rate that it is formed in areas under increased mechanical load (Samuelson, 2007). In the horse, the mechanical load applied by the DDFT to the navicular bone should increase with the activity of the horse. As one might expect, athletic horses have increased cortical bone and decreased medullary trabecular bone within their navicular bones compared to sedentary horses (Gabriel et al., 1998). Several histologic studies of the navicular bone have shown excessive resorption of medullary bone in addition to resorption of bone on the flexor surface of the navicular bone secondary to erosions of the palmar fibrocartilage (Wright et al., 1998; Blunden et al., 2006). The driving mechanism behind this bone resorption is not well understood, but one study suggests that this may be a failed adaptive response of bone to cyclical loading (Bentley et al., 2007). This study found increased microcracks, decreased bone mass, low osteocyte density and connectivity with formation of intracortical cysts and increased remodeling of navicular bones in diseased horses compared to healthy controls. Another study found that medullary pseudocysts and enthesseous new bone formation on the navicular bone were common in horses with navicular disease (Wright et al., 1998). In further support of the theory that the navicular bone of affected horses is loaded

abnormally, an earlier study showed increased intraosseous pressures *in vivo* in horses with clinical navicular disease in comparison to age matched controls (Pleasant et al., 1993).

In addition to bone changes, navicular disease also routinely has concurrent damage to the fibrocartilage covering the palmar aspect of the bone (Wright et al. 1998; Blunden et al., 2006). Fibrocartilage lesions most commonly occur on the sagittal ridge of the distal half of the bone and lesions can range from thinning and fibrillation to full thickness defects with subchondral bone necrosis and secondary fibrosis (Blunden et al., 2006). Inflammation of the overlying navicular bursa is common (Blunden et al., 2006; Wright et al., 1998), and up to 80% of deep digital flexor tendons that oppose diseased navicular bones are reported to have fibrillation on the dorsal surface (Wright et al. 1998), while 25% of deep digital flexor tendons form adhesions to diseased navicular bones (Blunden et al., 2006). Whether damage to the deep digital flexor tendon occurs secondary to navicular bone and fibrocartilage damage or in conjunction with it is unknown. Although the fibrocartilage on the palmar surface of the navicular bone is typically more severely affected than the dorsal articular cartilage, biochemical characterization of navicular bone articular cartilage in horses with navicular disease has shown increased concentrations of matrix metalloprotease-2, which is a gelatinase associated increased turnover in cartilage (Viitanen et al., 2003).

### *3.3.3. Diagnosis of navicular disease*

Navicular disease is diagnosed using a combination of patient history, clinical signs, local anesthesia and diagnostic imaging. Affected horses typically have a history of being ‘stiff’ on their forelimbs prior to presence of noticeable lameness. Owners may describe their horse as ‘walking on eggshells’ or sore after working on hard ground. Horses may be deteriorating in their ability to perform athletic endeavors that require high impact forces on the front feet. In some disciplines, the presenting complaint may simply be decreased extension of the forelimbs during performance. Onset is often insidious over several months and severity of lameness tends to increase with increasing duration of lameness (Wright, 1993a).

Lameness due to navicular disease is commonly bilateral and more pronounced when trotting on a hard surface. Trotting in a circle will exacerbate lameness on the forelimb closer to the center of the circle. Horses may show a pain response to application of pressure with hoof testers to the middle third of the sole (Waguespack and Hanson, 2010). Distal limb flexion is reported to intensify lameness in 64% of horses while toe extension accentuates lameness in 41% of cases (Wright, 1993a).

Local anesthesia to the distal forelimbs can be applied by a variety of approaches; however no approach specifically localizes pain specifically to the navicular bone, bursa or suspensory apparatus. Injection of 2% mepivacaine directly into the navicular bursa is reported to decrease clinical pain attributed to navicular disease within 5 minutes in 92% of horses (Wright, 1993a). However, bursal injections of mepivacaine have also been

shown to improve lameness associated with experimentally induced synovitis in the distal interphalangeal joint within 10 to 30 minutes (Schumacher et al., 2003). Intra-articular injection of the distal interphalangeal joint with local anesthetic has been shown to rapidly reduce lameness associated with navicular bursal pain (Pleasant et al., 1997) and injection of that joint is less technically challenging than injection of the navicular bursa (Moyer et al., 2011). However, injection of the distal interphalangeal joint with mepivacaine has also been shown to alleviate sole pain in addition to pain associated with the distal interphalangeal joint (Schumacher et al., 2000).

An alternative to intra-synovial anesthesia is perineural anesthesia of the medial and lateral palmar digital nerves just proximal to the palpable collateral cartilages. Nerve blocks performed in this location with mepivacaine volumes of 2 ml or less are purported to desensitize only the palmar third of the foot, but research has shown that this also blocks experimentally induced toe sole pain (Schumacher et al., 2000) and distal interphalangeal joint pain (Easter et al., 2000). The majority of horses with navicular disease will show improvement in lameness with local anesthesia of the palmar digital nerves, often switching lameness to the contralateral foot once one limb is blocked, but some horses require local anesthesia of the digital nerves at the base of the proximal sesamoid bones to resolve lameness (Dyson and Kidd, 1993).

Once lameness has been localized to the foot, medical imaging is essential to determine which structure is the source of pain. In navicular disease, often multiple abnormal structures contribute to pain (Dyson and Murray, 2007a). Radiographs are

commonly used to evaluate changes in the navicular bone. The diagnostic value of radiographs for navicular disease is a source of some controversy in equine practice. Several radiographic changes have been correlated with navicular disease, but severity of radiographic changes are poorly correlated with clinical lameness (Ackerman et al., 1977; Colles, 1979; Ostblom et al., 1982; Pool et al., 1989). Additionally, variability in interpretation exists between evaluators, and even within the same evaluator when radiographs are repeatedly reviewed (Groth et al., 2009). Radiographic changes correlated with the presence of navicular disease include increased numbers ( $>7$ ) of enlarged synovial invaginations on the distal border of the navicular bone, flexor cortex defects, proximal or distal border enthesophytes, remodeling of the lateral margin of the navicular bone, mineralization of the deep digital flexor tendon, fragmentation of the distal border, thinning of the flexor cortex, poor corticomedullary distinction on the flexor surface, cyst-like lesions within the medullary cavity, and medullary sclerosis of the navicular bone (Wright, 1993b; Groth et al., 2009; Dyson, 2011). The clinical relevance of each type of change varies. For example, increased numbers of enlarged synovial invaginations, fragmentation of the distal border, proximal border enthesophytes, and thinning of the flexor cortex were not found to be significantly associated with degree of lameness, but remodeling of the lateral margin, flexor cortex defects, loss of corticomedullary distinction, medullary sclerosis and medullary trabecular cyst-like lesions were correlated with increasing severity of lameness in one study (Wright, 1993b). Others have suggested that proximal border enthesophytes are consistent with insertional desmopathies of the collateral sesamoidean ligament, a

potentially painful condition (Dyson, 2011). Distal border fragments may be an incidental finding if adjacent bone is normal but if surrounded by radiolucent bone, fractures are more likely to be associated with lameness (Dyson, 2011).

Computed tomography (CT) has been used to improve evaluation of the navicular bone. An x-ray based imaging modality; CT has proven itself superior for evaluation of bone pathology (Vallance et al., 2012). The accuracy of determining distal border synovial invagination depth and number is improved with CT in comparison to radiography (Claerhoudt et al., 2012), and evaluators display a greater degree of certainty in their navicular bone findings on CT in comparison to radiographic findings of the same area (Groth et al., 2009).

Soft tissue lesions in addition to bone abnormalities are common in navicular disease (Dyson and Murray, 2007a). While use of contrast bursography can help diagnose full thickness cartilage erosions of the flexor surface of the navicular bone and adhesions of the deep digital flexor tendon to exposed subchondral navicular bone (Dabareiner and Carter, 2003); the use of imaging modalities other than radiography is often necessary to achieve a complete assessment of abnormalities. Computed tomography (CT), contrast enhanced computed tomography (CECT) and magnetic resonance imaging (MRI) have been used in effort to improve diagnostic accuracy in navicular disease.

When using CT, the addition of intra-arterial contrast (ie: CECT) improves identification of soft tissue lesions in the navicular region, and the combination of CT and CECT supplies a more complete picture of distal limb abnormalities than either modality

alone (Vallance et al., 2012). Recently, a comparison of CECT to histopathology findings in horses with DDFT pathology revealed a 93% sensitivity of CECT for accurate identification of DDFT lesions (van Hamel, 2013). While neither CT nor CECT images can be used to view cartilage, contrast bursography or arthrography with CT can be used to identify full thickness cartilage defects (Puchalski, 2012). In cases of full-thickness cartilage loss, contrast material can be seen in contact with subchondral bone, highlighting the cartilage defect.

MRI has proven to be invaluable for diagnosing soft tissue lesions associated with navicular disease, particularly in horses that have no radiographic abnormalities. MRI images can be achieved with a high field magnet (1-3 Tesla) or a low field magnet that is 0.15 - 0.5 Tesla (Winter, 2012). High field images require general anesthesia for acquisition, while low field images can be obtained in standing sedated horses. Increasing usage of MRI, in particular high field MRI, in the diagnosis of foot lameness has allowed clinicians to realize the diversity of lesions that can result in caudal heel pain, with DDFT injuries being the most common soft tissue abnormality reported (Dyson and Murray, 2007a; Bell et al., 2009; Gutierrez-Nibeyro et al., 2010). Interestingly, while the DDFT may be the primary source of pain, multiple structures are commonly affected according to other reports (Sampson et al., 2009; Dyson and Murray, 2007b; Gutierrez-Nibeyro et al., 2012; Vallance et al., 2012). In one report of 199 horses with foot pain that underwent high field MRI, DDFT injuries were present in 59% of horses (Dyson et al., 2005). In another study of 72 horses, navicular bone pathology was present in 33% of horses and DDFT lesions were the primary abnormalities in 18% (Sampson et al., 2009). Another

study of 46 horses revealed DDFT lesions to be the most severe MRI abnormality in 43% of cases (Dyson et al., 2003). In a study of 264 horses undergoing high field MRI for foot pain, 82.6% of horses had DDFT lesions present with other lesions which were most commonly occurring at the level of the collateral sesamoidean ligament and the navicular bone (Dyson and Murray, 2007b). When DDFT histopathology findings were compared to MRI images of the same area in 77 limbs from lame horses, lesions seen on histopathology were present on MRI studies for all limbs, but the sequences in which lesions were visible varied with chronicity of lameness (Blunden et al., 2009). In that study, histopathology also suggested that DDFT lesions were degenerative in origin rather than inflammatory.

Low field MRI has also proven useful for improving diagnostic and prognostic evaluation of horses with navicular disease. In a comparison of low field MRI to CT and CECT in horses with foot lameness, low field MRI proved superior for identification of soft tissue abnormalities of the distal sesamoidean impar ligament and the DDFT from the level of the proximal aspect of the navicular bone to its insertion on the third phalanx (Vallance et al., 2012). Low field MRI is advantageous in comparison to CT, CECT and high field MRI in that it does not require general anesthesia. While the images obtained are of lower quality in comparison to high field MRI due to motion artifact (Vallance et al., 2012), decreased resolution, and sequence limitations (Winter, 2012); the lack of general anesthesia is appealing to many clients. Retrospective studies have shown lesion distributions similar to those seen on high field MRI studies. One study found that 51% of navicular bones had enlarged synovial invaginations while 63% of imaged feet had



adhesions between the navicular bone and the DDFT (Bell et al., 2009). In another study of 56 horses, low field MRI revealed lesions in multiple structures in 89.2% of horses with the navicular bone and DDFT being most commonly affected (Gutierrez-Nibeyro et al., 2010). A third study evaluating 77 jumper and 21 dressage horses in training revealed similar findings with 77% of horses showing navicular bone abnormalities and 64% showing DDFT lesions (Mitchell et al., 2006). A fourth study evaluating 79 horses showed similar trends with 94% of horses having greater than 1 structure affected and navicular bone and DDFT lesions occurring in 78% and 54% of cases, respectively (Gutierrez-Nibeyro et al., 2012). As low field MRI is a non-invasive diagnostic that does not entail the risks associated with general anesthesia, some clinicians will opt to use follow up MRI's to monitor lesion healing. Milner et al. (2012) showed that low field MRI, repeated at 3month intervals, is a useful tool for monitoring reduction in size of dorsal border lesions of the DDFT. Low field MRI can also be used to identify full thickness defects in the flexor surface fibrocartilage of the navicular bone (Sherlock et al., 2008), but high field MRI using ultrafast T1 gradient echo sequences are necessary to identify partial thickness cartilage defects (Pease, 2012).

Direct visualization of the fibrocartilage flexor surface of the navicular bone and the dorsal surface of the DDFT can be achieved with navicular bursoscopy. Multiple surgical approaches have been described for navicular bursoscopy (Cruz et al., 2001; Haupt and Caron, 2010) and Smith et al. (2007) found that navicular bursoscopy more accurately identified cartilage damage than CT or MRI.

#### *3.3.4. Treatment of navicular disease*

Treatment of navicular disease can be as challenging as diagnosing its cause. Often multiple treatments are combined. Clinical experience suggests that response to therapy varies as widely as the costs associated with therapy. Regardless of the therapy used, responses tend to be time limited. As the chronicity of lameness increases, response to therapy tends to decrease and ultimately, horses are euthanized when owners run out of options to control pain. The treatment options available to many horses are dictated by the financial state of their owners. In some cases, horses may be treated with every treatment option currently available in effort to allow the horse to continue performing, while other horses are simply retired and allowed to live on pasture until their lameness begins to hamper their quality of life.

While multiple treatment options are available, no singular treatment type has shown itself to be exceptional. Treatments can be grouped into two general categories: medical and surgical. In general, medical therapies should precede surgical.

The simplest therapy is rest with controlled exercise in conjunction with therapeutic trimming and shoeing. Three to four weeks of small paddock or stall confinement should be sufficient to allow dissipation of soft tissue inflammation and adaptation to trimming and shoeing changes (Dabareiner and Carter, 2003). If a decrease in lameness is not seen after this time, then treatment with pharmaceuticals is likely to be necessary to improve comfort.

Specific trimming recommendations for navicular syndrome horses are focused on balancing the hoof in a mediolateral plane, ensuring an appropriate hoof pastern angle (Figure 5) and managing conformational problems such as under run heels and long toes in order to reduce the biomechanical forces on the navicular bone (Dabareiner and Carter, 2003). Correction of hoof angles improves breakover, the point in each stride where the heel loses contact with the ground followed by the toe. It is at this point that the greatest forces are applied to the tissues of the caudal heel. During breakover, the dorsal hoof wall, or tip of the toe acts as a fulcrum, and decreasing toe length while increasing heel height decreases the length of this fulcrum thereby reducing the force on the navicular suspensory apparatus and DDFT. The breakover point on a horse's foot is ideally located using radiographic guidance. A line is drawn on the dorsal surface of the third phalanx on a lateral to medial radiographic projection, and the point at which this line crosses the sole is the breakover point that the dorsal hoof wall should be trimmed to (Dabareiner and Carter, 2003). In addition, to improve breakover and enlarge the functional weight-bearing surface of the foot, heels should be trimmed back to the widest part of the frog (Dabareiner and Carter, 2003).

Therapeutic farriery is a mainstay in the treatment of navicular syndrome. Up to 73% of horses with navicular syndrome are reported to improve one grade in lameness within six weeks of corrective shoeing (Turner, 1986). Therapeutic shoeing is often necessary in addition to correct trimming to achieve maximal improvements in lameness. Horses should be shod with shoes that fit full, meaning that the metal of the shoe should be about an eighth of an inch wider than the heels palmar to the last shoe nail (Dabareiner

and Carter, 2003). This allows the heels to expand when the foot is weight-bearing. Additionally, the toe of the shoe can be rockered or dubbed to improve breakover. Heel wedges can be added to shoes to realign the hoof pastern axis in horses with a broken-back conformation and they decrease biomechanical forces on the navicular apparatus and DDFT. Although wedge height depends on each individual horse's conformation, a wedge of 2-3° is recommended (Dabareiner and Carter, 2003) and wedges of 2° have been reported to decrease forces on the DDFT in the navicular region by 24% (Willemen et al., 1999). The wedge can be built directly in the shoe, or can be achieved with heel wedge pads, rim pads or full pads. Specialty shoes that supply added heel support include egg bar shoes, straight bar shoes, and heart bar shoes. Individual horses vary in their therapeutic shoeing needs, thus evaluation by an experienced professional and development of an appropriate shoeing plan is necessary to achieve optimal results.

As navicular disease progresses, treatment with pharmaceuticals often becomes necessary to improve comfort. The majority of pharmaceuticals used in the treatment of navicular disease are palliative. The goal of their use is to decrease inflammation within the foot, thereby supplying comfort. Medications may be given systemically or locally. The most common systemically administered medications are non-steroidal anti-inflammatory drugs (NSAIDs). These drugs inhibit the enzyme cyclooxygenase preventing the production of prostaglandins from arachidonic acid (Dabareiner and Carter, 2003). Prostaglandins play a key role in the development of inflammation and decreasing prostaglandin synthesis can decrease pain. NSAIDs licensed for use in horses include phenylbutazone, flunixin meglumine, and firocoxib.

Other systemic medications for navicular disease include osteoarthritis disease modifying drugs such as hyaluronan, polysulfated glycosaminoglycans (PSGAGs) or pentosan polysulfate sodium (PPS). Hyaluronan is a natural component of synovial fluid and articular cartilage with anti-inflammatory properties and the recommended dosing regimen is 40 mg intravenously once a week for 3 weeks, then once a month thereafter (Dabareiner and Carter, 2003). PSGAGs are a synthetic form of glycosaminoglycans which occur naturally in articular cartilage while PPS is an antiarthritic agent derived from the hemicellulose of beechwood trees. Both are administered intramuscularly. One study has shown that 500 mg of PSGAGs given intramuscularly every 4 days for 8 treatments improves lameness in horses with navicular disease (Crisman et al., 1993). While studies specifically evaluating the effect of PPS on navicular disease are not available, intramuscular PPS has been shown to decrease cartilage fibrillation and increase chondroitin sulfate 846 epitope, a biomarker for aggrecan synthesis, in horses with experimentally induced arthritis (McIlwraith et al., 2012).

Local administration of medications occurs traditionally in the form of intra-synovial injections. For treatment of navicular syndrome, injections are made into the distal interphalangeal joint, the navicular bursa, the digital flexor tendon sheath or a combination of these three structures (Guitierrez-Nibeyro et al., 2010). The medications most commonly used are corticosteroids such as triamcinolone acetonide or methylprednisolone acetate. These may be used alone or in conjunction with hyaluronan. Corticosteroids are potent anti-inflammatories that prevent the production of both

prostaglandins and leukotrienes by inhibiting phospholipase A2 and preventing the release of arachidonic acid from phospholipids (Dabareiner and Carter, 2003).

Injection of the navicular bursa with corticosteroids has been shown to result in temporary return to athletic function in horses with navicular syndrome. In one report, 23 horses with clinical signs of navicular disease that blocked to palmar digital nerve blocks and showed injury to the navicular apparatus and/or DDFT on low field MRI received navicular bursal injections with triamcinolone (Bell et al., 2009). Seventy four percent of these horses returned to their intended use and remained sound for a mean of 7.3 months. Inclusion of hyaluronate in the injection did not significantly affect outcome, but administration of <10 mg of triamcinolone was correlated with a decreased response to therapy. Another study of 56 horses diagnosed with navicular disease on clinical evaluation and injury to the navicular apparatus and/or DDFT on low field MRI reported that only 32.3% of horses were able to return to light riding following injection of sodium hyaluronan and 6 mg of triamcinolone into the distal interphalangeal joint and/or digital flexor tendon sheath and 3 mg of triamcinolone into the navicular bursa (Guitierrez-Nibeyro et al., 2010). A more recent study by Marsh et al. (2012) reported that 35% of 101 horses treated with navicular bursa injections for MRI diagnosed navicular bone or DDFT lesions were sound and 75% of treated horses were able to return to their intended use for a mean of 9.7 months. This study also reported pressurizing bursae with 3 – 5 mls of sodium hyaluronate while the horse was anesthetized to break down adhesion in horses with adhesions of the navicular bursa to the collateral sesamoidean ligament on MRI.

Practitioners can also inject the distal interphalangeal joint with corticosteroid in effort to treat navicular disease. Triamcinolone injected into the distal interphalangeal joint has been shown to diffuse into the navicular bursa regardless of whether it was injected alone or in combination with sodium hyaluronan (Boyce et al., 2010). A comparison of return to soundness following injection of the distal interphalangeal joint versus injection of the navicular bursa has not been reported.

A more recent medical therapy advocated for the treatment of navicular disease is the non-nitrogenous bisphosphonate tiludronate. A double blinded, placebo controlled study evaluating the efficacy of tiludronate for treatment of navicular disease showed that 50% of horses with lameness of less than 6 months duration resolved lameness 6.5 months following treatment with 1 mg/kg tiludronate administered systemically, and 66.7% of horses showed clinical improvement in lameness (Denoix et al., 2003). In another non-peer reviewed study, 80% of horses diagnosed with foot pain resolved lameness and returned to function following corrective shoeing and treatment with systemic tiludronate at 1 mg/kg (Mitchell et al., 2006).

Surgical options for horses with navicular disease can be used in conjunction with medical therapy, or can be turned to when medical therapy fails. Three surgical procedures have proven useful for treatment of navicular disease: navicular bursoscopy, transection of the collateral sesamoidean ligament and palmar digital neurectomy (Smith et al., 2007; Wright, 1993c; Jackman et al., 1993). Two reports evaluated return to function following navicular bursoscopy for debridement of DDFT and navicular

cartilage defects. In one study, 11 of 15 horses (73%) were sound 6 months following bursoscopic debridement (Smith et al., 2007). In the second study, 61% of 84 horses were sound a mean of 17 months following bursoscopic debridement (Smith and Wright, 2012).

Transection of the collateral sesamoidean ligament (CSL) has been recommended for treatment of navicular disease as release of this ligament was thought to decrease the biomechanical forces on the navicular bone, thereby decreasing pain (Wright, 1993c). A retrospective evaluation of 118 cases of navicular disease treated with collateral sesamoidean desmotomy revealed that while 76% of horses were sound 6 months following surgery, only 43% of horses were sound 36 months following surgery suggesting that as the ligament heals, pain returns (Wright, 1993c). Monthly MRI evaluation of transected CSL in an experimental model on normal horses showed that tissue was present on MRI between the transected ends of the CSL 6 months following desmotomy (Sampson et al., 2010). Twelve months following transection, experimental limbs were not significantly different from control limbs on histologic evaluation.

Palmar digital neurectomy can be used for management of navicular disease in horses that become sound when the palmar digital nerves are anesthetized with local anesthetic. This procedure requires life-long post-operative care and is not without complications. As such, it is generally recommended as a palliative procedure rather than a first line of defense. The most common complication associated with palmar digital neurectomy is reinnervation of the transected nerves (Jackman et al., 1993). Employment



of a two incisions pull-through technique allows removal of a longer segment of nerve and has been associated with an increased duration of soundness post-operatively (Black, 1992; Maher et al., 2007). Other complications include incomplete desensitization of the heel and formation of painful neuromas. Post-operative care that minimizes inflammation at the surgical site, such as stall rest and pressure bandages, is thought to be crucial for prevention of reinnervation and formation of painful neuromas (Dabareiner and Carter, 2003). Unusual but catastrophic complications such as sepsis of the third phalanx, DDFT rupture, navicular bone fracture, hoof capsule sloughing and DDFT rupture have also been reported (Maher et al., 2007).

### **3.4. Regional Limb Perfusion**

Regional limb perfusion with antibiotics is used commonly in horses for the treatment of distal limb orthopedic infections as the technique results in considerably higher tissue and synovial fluid concentrations of antibiotics than can be achieved with systemic administration (Rubio-Martinez and Cruz, 2006). Regional limb perfusion can be administered via two routes in horses; IVRLP or intraosseous regional limb perfusion (IORLP). For both techniques, a tourniquet is applied to either the proximal metacarpus/metatarsus, for perfusion of structures below the carpus/tarsus, or to the mid-radius/tibia, for perfusion of the carpal/tarsal area. Following occlusion of venous circulation by the tourniquet, regional limb perfusion is then performed by infusing diluted antibiotics into a bone (IORLP: Figure 6) or vein (IVRLP: Figure 7) below the tourniquet. More specifically, for IORLP a hole is drilled into a bone below the

tourniquet. In foals, this can be achieved with an intramedullary needle (Golenz et al., 1993), while adults require drilling of a unicortical hole. A 4.0 mm hole can be drilled with the horse standing under heavy sedation (Mattson et al., 2004) or under general anesthesia (Butt et al., 2001). Following drilling of the hole a 5.5mm cannulated screw can be inserted, which can attach to the luer tip of an intravenous extension set (Mattson et al., 2004), or the luer tip of an intravenous extension set can be wedged directly into the hole (Butt et al., 2001, Rubio-Martinez, 2006). For IVRLP, a catheter is inserted into a superficial vein distal to the tourniquet. Exsanguination of the limb during tourniquet placement is not necessary and not recommended as it makes insertion of the intravenous catheter more difficult (Rubio-Martinez, 2006). Perfusates are infused under pressure with considerably greater pressure being achieved with IORLP (450 lb/inch<sup>2</sup>) than with IVRLP (<15 lb/inch<sup>2</sup>) (Whitehair et al., 1992a). After administration of the perfusate, the tourniquet is left in place for 30 minutes to allow adequate time for perfusates to diffuse into all tissues below the tourniquet. After 30 minutes, the tourniquet is removed and normal circulation is restored. The depot of antibiotics within the perfused area moves down the concentration gradient into systemic circulation. High concentrations of antibiotics can typically be maintained with the perfused tissues for 24 hours or more (Mattson et al., 2004; Werner et al., 2003) with bone maintaining high concentrations longer than soft tissues (Parra-Sanchez et al., 2006).

Regional limb perfusion is particularly useful when treating distal limbs infected with resistant bacteria (Rubio-Martinez and Cruz, 2006). Antibiotic efficacy is dependent on adequate concentrations of appropriate antibiotics reaching the site of infection, but

fibrin accumulation and ischemia associated with infection can impede penetration of systemically administered antibiotics (Scheuch et al., 2002; Knottenbelt, 1997). Regional limb perfusion overcomes these penetration impediments because antibiotics are able to diffuse into tissues within the perfused region following both pressure and concentration gradients after pressurization of the venous system with an antibiotic (Finsterbush and Weinberg, 1972). The achievement of antibiotic concentrations within tissue or synovial fluid that are greater than 10 times bacterial minimum inhibitory concentrations (MIC) increases bacteria killing and reduces the risk for development of bacterial resistance (Parra-Sanchez et al., 2006; Goldberg and Owens, 2002). These concentrations can be achieved with regional limb perfusion without exposing horses to risks associated with systemic antibiotics such as antibiotic induced colitis (Barr et al., 2012). A recent study has shown that outcomes are improved when regional limb perfusion is included in the treatment regimen (Findley et al., 2013). Outcomes of horses with solar foot penetrations in which synovial structures were penetrated were evaluated and failure to perform an intravenous regional limb perfusion (IVRLP) with antibiotics at the time of surgical debridement was associated with a poorer outcome than horses that received IVRLP.

Maximum concentration achieved with regional limb perfusion varies by tissue (Mattson et al., 2004; Parra-Sanchez et al., 2006). Early work by Whitehair et al (1992b) showed that following IORLP into the metacarpus with gentamicin in normal horses, gentamicin concentrations in carpal synovial fluid were 55 times the MIC of common equine pathogens and 100 times the concentration that could be achieved with systemic administration of gentamicin. Infusion of radiopaque dye with perfusates in these horses

showed that perfusates were following venous circulation from the metacarpus into the synovium, synovial fluid, small carpal bones and radius distal to the tourniquet with maximum concentrations being achieved after the tourniquet had been left in place for 30 minutes, and greater concentrations present in carpal synovial fluid than bone. Repetition of this study in horses with experimentally induced septic arthritis of the carpus revealed that high concentrations were still achieved in carpal synovial fluid, but concentrations were not as high as those achieved in normal joints (Whitehair et al., 1992a). This led to the suggestion that sepsis-associated thrombi may impede synovial vascular circulation, making it more difficult for antibiotics to permeate into synovial fluid. The gentamicin concentrations achieved in synovial fluid following IORLP were still over 30 times the concentrations that could be achieved with systemic administration, however. In contrast to the study by Whitehair et al. (1992a), radiocarpal synovitis experimentally induced with intra-articular lipopolysaccharide revealed that significantly greater concentrations of amikacin were achieved in radiocarpal synovial fluid of joints with synovitis in comparison to control joints following IVRLP (Beccar-Varela et al., 2011).

As the use of regional limb perfusion with antibiotics has become more common clinically, the merits of the two different modes of administration, IORLP and IVRLP, can be compared. Vascular access for IVRLP can be difficult to achieve in swollen, inflamed limbs, however IORLP requires creation of an intraosseous portal; a technically more challenging procedure requiring specialized equipment. IORLP with antibiotics has been effective in resolving cases of chronic osteomyelitis (Whitehair et al., 1992c), septic physitis (Kettner et al., 2003) and experimentally-induced septic arthritis (Whitehair et

al., 1992a). Likewise, IVRLP with antibiotics has been used to resolve septic physitis and arthritis (Kelmer and Hayes, 2009) and is associated with improved survival when used as an adjunct therapy in the treatment of distal limb orthopedic infections (Findley et al., 2013; Cimetti et al., 2004). The two techniques have been compared experimentally and in two studies, IVRLP was found to achieve greater synovial fluid antibiotic concentrations than IORLP (Butt et al., 2001; Scheuch et al., 2002). A third study found that antibiotic concentrations in medullary blood aspirated from the first phalanx were similar following IORLP and IVRLP (Rubio-Martinez et al., 2005a), and a fourth study determined that distribution of technetium Tc 99m pertechnetate to the phalanges was equivalent with IVRLP and IORLP (Mattson et al., 2005).

Despite apparently similar clinical efficacies, IVRLP has become the preferred clinical technique over IORLP as it is technically easier to perform, particularly in an ambulatory setting, and is associated with fewer complications (Rubio-Martinez et al., 2012). The most commonly reported complication associated with IORLP is leakage of perfusate from the bone portal either during or following infusion leading to inflammation of the overlying soft tissues (Butt et al., 2001; Scheuch et al., 2002; Mattson et al., 2004). Screw related complications, such as screw loosening or breakage and discharge around the screw, were reported in 33% of IORLPs in a recent retrospective study evaluating clinical use of IORLP (Rubio-Martinez et al., 2012). More significantly, osteomyelitis and osteonecrosis with a secondary pathologic fracture of the first phalanx has been reported following IORLP with 3 grams of gentamicin (Parker et al., 2010). While considering this complication, it is important to keep in mind that this is

an unusually high dose of gentamicin for IORLP (Watts, 2011). High intraosseous pressure, as can occur with IORLP, has also been cited as a cause of lameness in one horse (Morisset et al., 1999).

Complications associated with IVRLP are reportedly minor (Watts, 2011, Rubio-Martinez et al., 2012). A recent retrospective study evaluating use of clinical IVRLP in 155 horses found a complication incidence of 12.26% (Rubio-Martinez et al., 2012). Complications were limited to hematoma, phlebitis and thrombus formation at the site of the vein used for perfusion. All complications were considered minor. Another study showed that performance of two IVRLPs 24 hours apart resulted in subcutaneous inflammation at injection sites, but this inflammation was reduced with application of topical 1% diclofenac cream following IVRLP (Levine et al., 2009). IVRLPs with 20 – 22 gauge catheters that are removed following each perfusion can result in vascular and subcutaneous inflammation that make repeated access to vessels challenging. As a result, use of in-dwelling catheters has been recommended (Watts, 2011; Kelmer et al., 2012). Twenty gauge, 4 cm long, polytetrafluoroethylene catheters and 16 gauge, 15 cm long polyurethane over-the-wire catheters have been used with clinical success in cephalic and saphenous veins for 3 to 21 days of daily IVRLP (Kelmer et al., 2012). If in-dwelling catheters for repeated IVRLPs are not feasible, use of 26 gauge butterfly catheters is recommended as the smaller gauge results in less endothelial damage on venipuncture (Watts, 2011; Rubio-Martinez, 2006).

Cephalic or saphenous veins are currently routinely used for IVRLP, but previously there has been some concern that perfusates given via an upper limb vein may not penetrate distal limb structures as well as perfusates administered through a distal limb vein (Levine et al., 2010). This concern arises from the fact that substantially more soft tissues must be compressed to achieve venous occlusion when a tourniquet is applied to the proximal limb in comparison to the distal limb. Previous work has shown that tourniquet pressure must be increased to achieve venous occlusion as the diameter of the limb increases (Crenshaw et al., 1988), thus achievement of effective tourniquet pressure for the proximal limb may be more challenging than for the distal limb. Comparison of two studies that measured amikacin in metacarpophalangeal synovial fluid following IVRLP with 1 gram of amikacin diluted to 50 - 60 mls in standing horses showed that greater concentrations were achieved when the tourniquet was placed on the proximal metacarpus (Errico et al., 2008) versus the distal radius (Levine et al., 2010). Comparison between these two studies is not as straight forward. Distal limbs were anesthetized with perineural blocks in the study that used the proximal metacarpal tourniquet and horses stood quietly throughout perfusions, while perineural blocks were not used in the study which used the distal radial tourniquet and authors noted that substantially lower synovial fluid concentrations of amikacin were achieved in horses that moved during perfusions. Movement during IVRLP has been suggested to decrease IVRLP efficacy (Watts, 2011, Levine et al., 2010) and this difference in experimental method makes comparison of the two studies difficult.

Direct comparison of metacarpo/metatarsophalangeal synovial fluid antibiotic concentrations following IVRLP using a palmar/plantar digital, cephalic or saphenous vein has been done with erythromycin (Kelmer et al., 2013a) and amikacin (Kelmer et al., 2013b). Both studies showed dramatically higher antibiotic concentrations in synovial fluid of the metacarpo/metatarsophalangeal joint following IVRLP using the cephalic or saphenous vein in comparison to the palmar/plantar digital vein. In fact, erythromycin was not detectable at all in metacarpo/metatarsophalangeal synovial fluid in 2/6 horses following IVRLP via the palmar/plantar digital vein (Kelmer et al., 2013a). When considering these findings, it is worth noting that tourniquets spanned 10 cm for cephalic/saphenous perfusions while a 5 cm tourniquet was used for palmar/plantar digital vein perfusions. The importance of tourniquet width in IVRLP will be discussed in more detail below, but narrow tourniquets have been associated with decreased tourniquet efficacy and reduced synovial fluid antimicrobial concentrations in the perfused region (Levine et al., 2010). Regardless of the confounding factor of differences in tourniquet width, these two studies demonstrate that antimicrobial concentrations well over 10 times MIC of target pathogens can be achieved with IVRLP via the cephalic or saphenous vein. Other studies demonstrate that antimicrobial concentrations well over 10 times MIC of common equine pathogens can also be achieved in synovial fluid of the distal interphalangeal joint (Butt et al., 2001) and metacarpo/metatarsophalangeal joint following IVRLP via a palmar/plantar digital vein (Errico et al., 2008; Rubio-Martinez et al., 2005b; Butt et al., 2001; Werner et al., 2003; Murphey et al., 1999). Thus upper limb veins and digital veins are viable options for performing IVRLP and both have been



proven clinically effective in resolving distal limb orthopedic infections (Kelmer et al., 2012; Santschi et al., 1998; Palmer and Hogan, 1999).

As previously mentioned, the length of the limb that the tourniquet spans has been associated with tourniquet efficacy as increasing width is correlated with more effective vascular occlusion (Crenshaw et al., 1988). For maximum tourniquet efficacy, tourniquet width should be 20% wider than the diameter of the limb (Grice et al., 1986).

Comparison of a 12.5 cm wide rubber tourniquet to a 1 cm wide rubber tourniquet and 10.5 cm wide pneumatic tourniquet for IVRLP via the cephalic vein with amikacin in horses showed that significantly greater amikacin concentrations were achieved in synovial fluid with the wide rubber and pneumatic tourniquets in comparison to the narrow rubber tourniquet. The use of pneumatic tourniquets yielded greater concentrations of amikacin in synovial fluid in comparison to wide rubber tourniquets (Levine et al., 2010). However, another study compared amikacin concentrations in synovial fluid following IVRLP via a palmar digital vein with a wide rubber tourniquet or a pneumatic tourniquet applied at the level of the proximal metacarpus (Alkabes et al., 2011). Results showed that synovial fluid amikacin concentrations were greater with use of the wide rubber tourniquet. The combination of these two studies indicates that rubber tourniquets may be more effective for IVRLP with tourniquets on the proximal metacarpus and pneumatic tourniquets may be more effective when tourniquets are applied to the distal radius or tibia. Regardless of tourniquet type, however, placing wide enough tourniquets is essential for maximal tourniquet efficacy.

Tourniquet efficacy is reflected by the absence of leakage of perfusate under the tourniquet. Leakage occurs when maximum venous pressure, the maximum intravascular pressure achieved during infusion of a perfusate, is greater than effective tourniquet pressure (Grice et al., 1986). Tourniquet width is the most important factor in maintaining effective tourniquet pressure above maximum venous pressure, but other factors such as rate of injection, volume of injection and tourniquet pressure are also important (Grice et al., 1986). In effort not to overpower effective tourniquet pressures, perfusates are generally injected slowly. In the horse, injection over 3-5 minutes is common (Levine et al., 2010; Werner et al., 2003; Parra-Sanchez et al., 2006; Kelmer et al., 2013a), and more recently, injection over 1-2 minutes has been reported to result in synovial fluid antibiotic concentrations >10 time common bacterial MICs (Alkabes et al., 2011; Beccar-Varela et al., 2011; Mahne et al., 2013). Pressure settings for pneumatic tourniquets are recommended to be between 300 and 500 mmHg (Watts, 2011) and rubber tourniquets are reported to reach tissue pressures of > 1000 mm Hg with pressure being positively correlated with number of wraps around the limb (Abraham and Amirouche, 2000).

Ideal volumes for IVRLP perfusates have not been clearly defined and a wide range of volumes have been recommended and reported for both the distal and proximal limb in horses. In theory, the goal is to pressurize the vascular holding volume of the area of limb distal to the tourniquet without exceeding the effective tourniquet pressure. Vascular holding volume will vary by horse size, individual anatomy and systolic blood pressure. Thus, the perfusate volume needed to pressurize the vascular holding volume is difficult to determine for each individual case. Reported perfusate volumes used in the

cephalic or saphenous veins range from 50 to 100 mL (Levine et al., 2010; Kelmer et al., 2012; Kelmer et al., 2013a, b) with 60 mL being most common (Levine et al., 2009; Beccar-Varela et al., 2011; Parra-Sanchez et al., 2006; Sheuch et al., 2002) and 30 mL to 60 mL for the distal limb in an average 500 kg horse (Werner et al., 2003; Murphey et al., 1999) with 60 mL being most commonly reported (Rubio-Martinez et al., 2005b; Errico et al., 2008; Butt et al., 2001). More recently, use of 15 – 35 mL for total perfusate volume has been recommended for IVRLP (Burba, 2013), supported by a recent study in which synovial fluid gentamicin concentrations were compared following IVRLP with 10 mL, 30 mL and 60 mL perfusate volumes (Hyde et al., 2013). Results showed a trend for concentrations of gentamicin in synovial fluid to increase as perfusate volume decreased, suggesting that diffusion of antibiotics down a concentration gradient may be more important than diffusion down a pressure gradient during IVRLP. Like many studies, this study suffered from a low sample size and high standard deviations in data. Repetition of the study with a larger sample size would be valuable to prove whether the apparent trend can become statistically valid.

Any movement during IVRLP has previously been suggested to substantially decrease efficacy of regional limb perfusion with respect to synovial fluid antimicrobial concentrations (Levine et al., 2010; Watts, 2011). However, the effect of movement on the efficacy of an IVRLP may not be as dramatic as previously thought. A recent study compared synovial fluid amikacin concentrations following IVRLP under general anesthesia, standing sedation, standing sedation with local perineural anesthesia, and standing sedation with local intravenous anesthesia (Mahne et al., 2013). The study

showed that horses undergoing IVRLP with standing sedation alone or with intravenous anesthesia moved significantly more and yielded higher pain visual analogue scores, presumably secondary to tourniquet pain, than horses under general anesthesia or standing sedation with local perineural anesthesia. Despite this, there was no significant difference in amikacin synovial fluid concentrations between groups. Although an association between increased movement and decreased antimicrobial synovial fluid concentrations has been reported (Levine et al., 2010), mild movement during IVRLP may not be as detrimental to IVRLP efficacy as previously thought. Efforts to minimize movement are still recommended during IVRLP in standing horses, particularly when one considers that efforts to decrease movement also increase patient comfort. Clinical experience has shown that substantial movements can result in unplanned tourniquet release or shifting of the tourniquet.

A wide variety of antibiotics have been reported for use in IVRLP in horses. Regardless of the type of antibiotic used, antibiotic concentrations in the perfused area vary by tissue. Concentrations achieved in bone are consistently and significantly lower than concentrations achieved in synovial fluid (Parra-Sanchez et al., 2006; Werner et al., 2003) but disappearance times are slower for bone than synovial fluid (Parra-Sanchez et al., 2006). Subcutaneous tissue shows a similar antimicrobial concentration and disappearance rate to synovial fluid (Parra-Sanchez et al., 2006), and synovial fluid concentrations commonly vary between joints within the same perfused area (Butt et al., 2001; Murphey et al., 1999; Rubio-Martinez et al., 2005b).

While antibiotics are the most common pharmaceuticals to be used in IVRLP, in recent years use of some non-antibiotic substances has been reported. Of particular prominence is the use of mesenchymal stem cells via IVRLP or intra-arterial regional limb perfusion (IARLP). The philosophy behind use of regional limb perfusion for delivery of stem cells to lesions in the foot is derived from studies in rodents that indicate that stem cells administered systemically can home to ischaemic tissues and stimulate regeneration of these tissues (Barbash et al., 2003; Capoccia et al., 2009). For tendon lesions within the hoof capsule that are not reachable for intra-lesional injection, IVRLP and IARLP offer an alternative method of delivery of stem cells to these areas in theory. Evaluation of stem cell distribution following distal limb IARLP and IVRLP showed that IARLP more reliably distributes stem cells to the foot when IVRLP is done via the cephalic vein; however IARLP with a tourniquet routinely results in arterial thrombus formation which can lead to lameness and skin sloughing (Sole et al., 2012; Sole et al., 2013) and is not recommended. In contrast, intraarterial injection without a tourniquet resulted in similar stem cell distributions to those achieved with a tourniquet, but did not result in thrombus formation of the median artery through which the stem cells were delivered (Trela et al., 2013). Uptake of stem cells into experimentally created superficial digital flexor core lesions was lower with IARLP and IVRLP than uptake with intra-lesional injection, but persistence of stem cells, once present, was similar between the two techniques (Sole et al., 2013). IARLP resulted in better lesion uptake than IVRLP and 10 day old lesions had better uptake than 3 day old lesions. IVRLP via the palmar digital vein provides better distribution to the foot than IVRLP via the cephalic vein, but

distribution is lateralized to the side on which venipuncture for the IVRLP was done (Trela et al., 2013).

Morphine has been recently reported to be safe for use in IVRLP (Hunter et al., 2012; Valverde et al., 2013). Initial experiments were done in healthy horses. Although synovial fluid concentrations following IVRLP have been determined, the clinical efficacy of the concentrations in controlling pain has yet to be clearly defined. Similar to synovial fluid concentrations of antibiotics following IVRLP concentrations of morphine in synovial fluid following IVRLP can be highly variable, thus any clinically apparent analgesic effect may also be variable.

Recently, tiludronate has been reported to be administered via IVRLP. Specifically, a non-peer reviewed proceedings article described administration of 50 mg of tiludronate, diluted to a total volume of 60 mL with saline, via the cephalic vein every other week for 3 treatments (Carpenter, 2012). This protocol was used in combination with a course of extracorporeal shock wave therapy for treatment of dorsal metacarpal disease (bucked shins) in 5 horses, and was reportedly successful in resolving lameness in all 5 horses. However, literature evaluating the concentrations of tiludronate achieved in synovial fluid, cartilage or bone following IVRLP and the safety or clinical efficacy of IVRLP with tiludronate via the palmar digital veins for treatment of navicular disease is lacking.

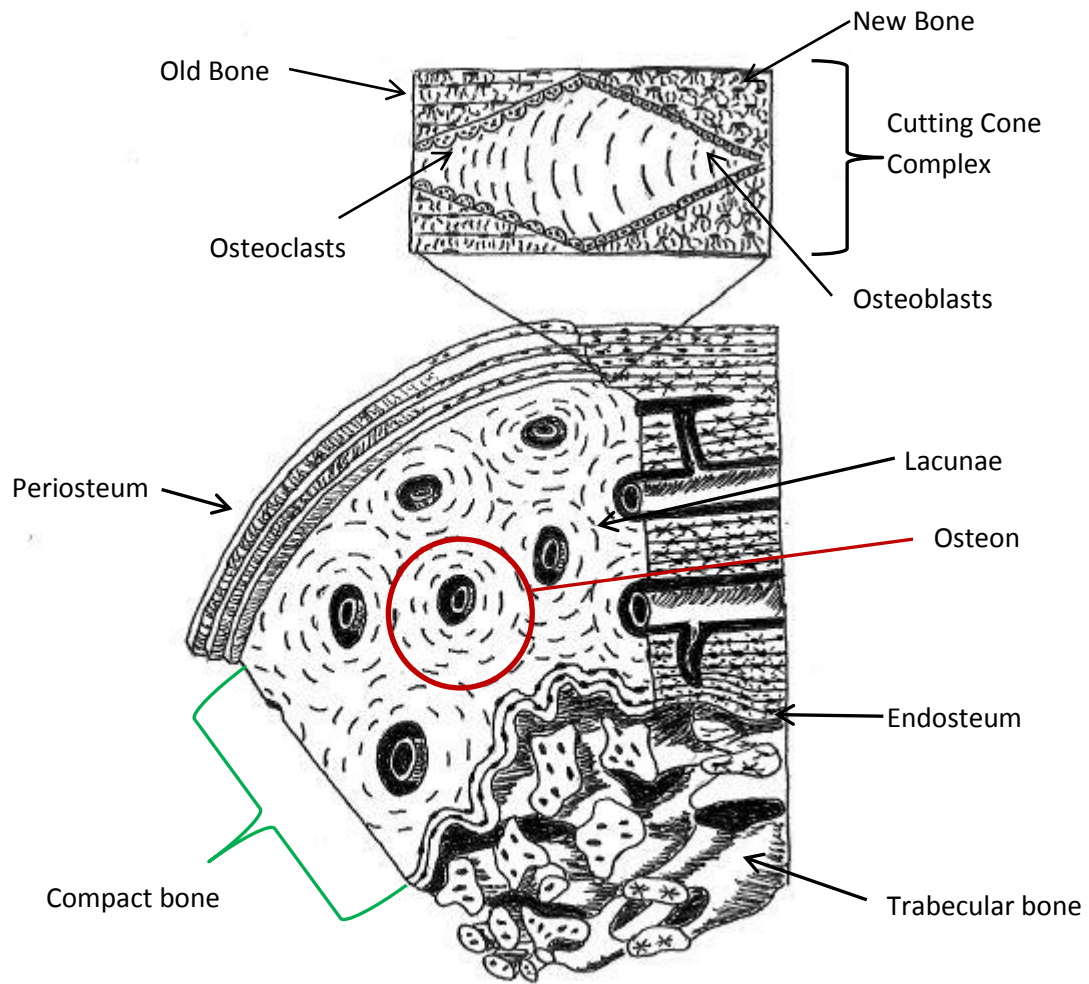


Figure 1: Diagram of normal bone histology (adapted from: Samuelson, 2007 by Shannon Casserly).

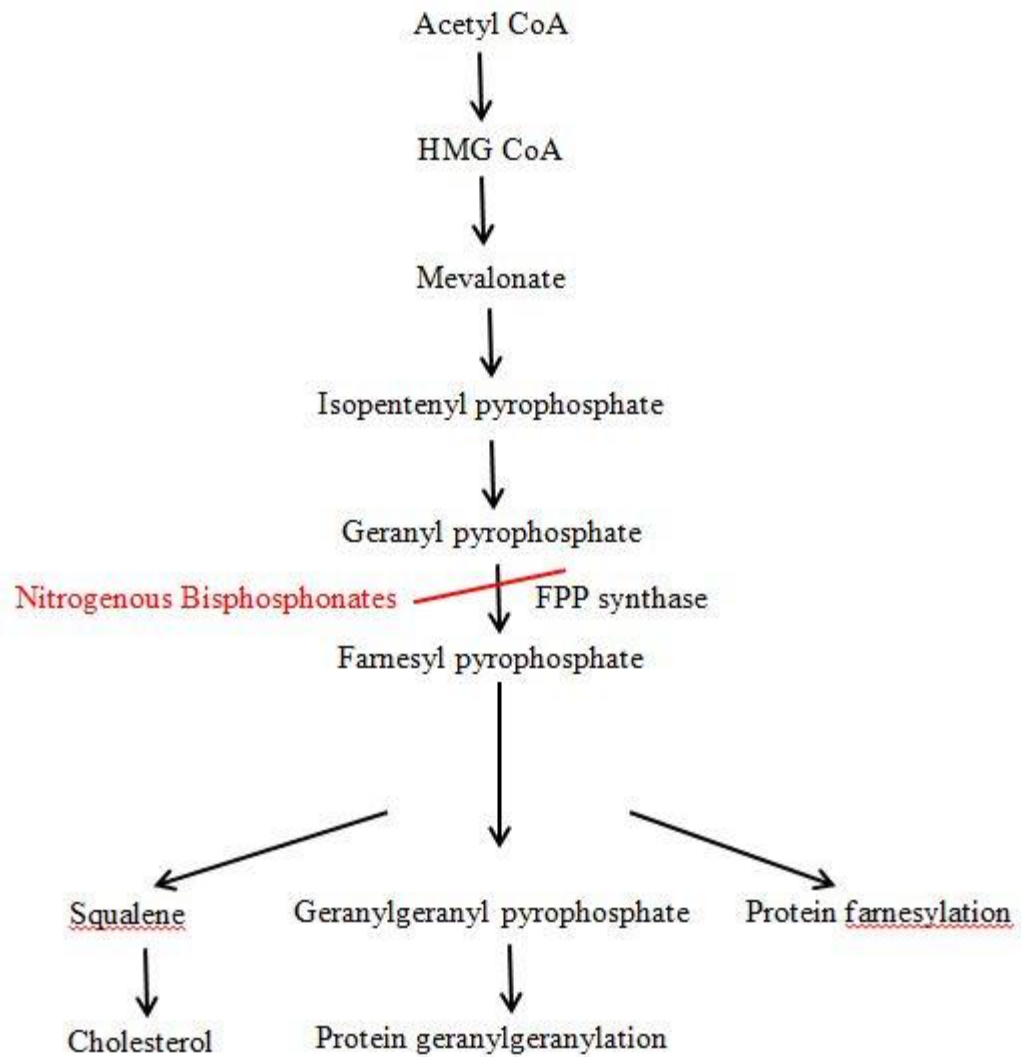


Figure 2: The mevalonate pathway showing point at which nitrogenous bisphosphonates inhibit pathway.



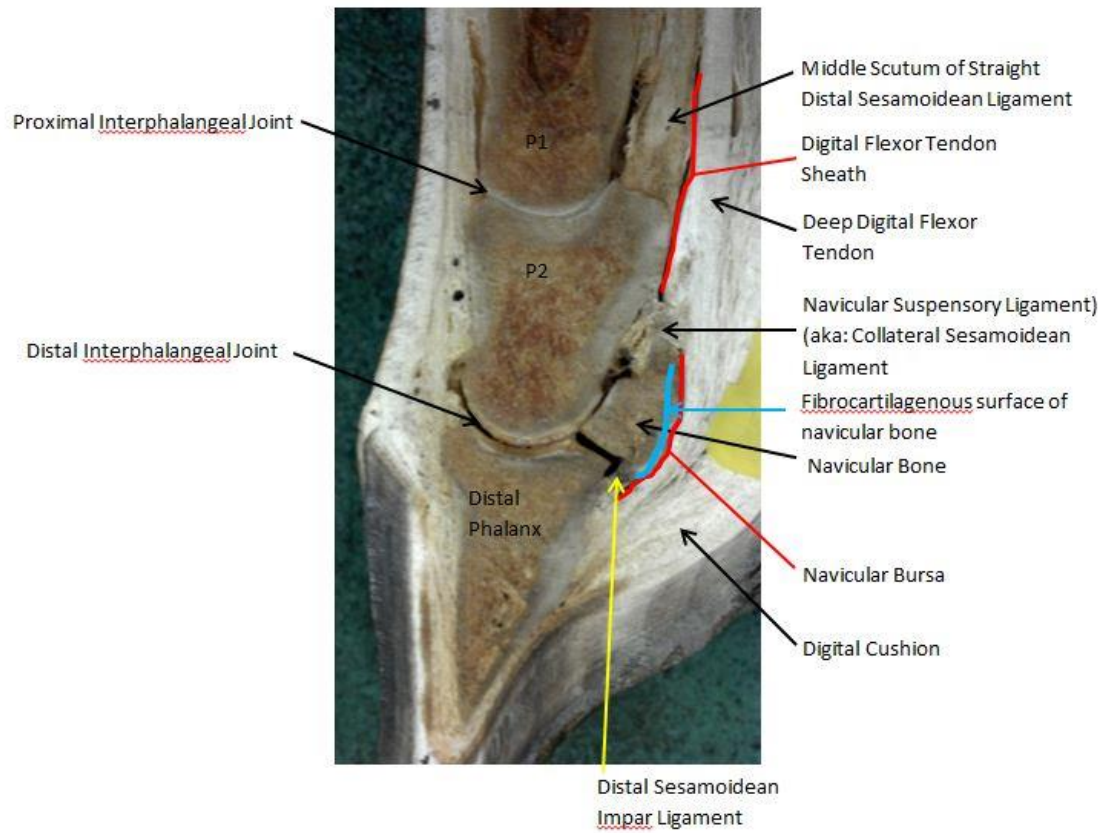


Figure 3: Anatomy of the equine foot: Sagittal view

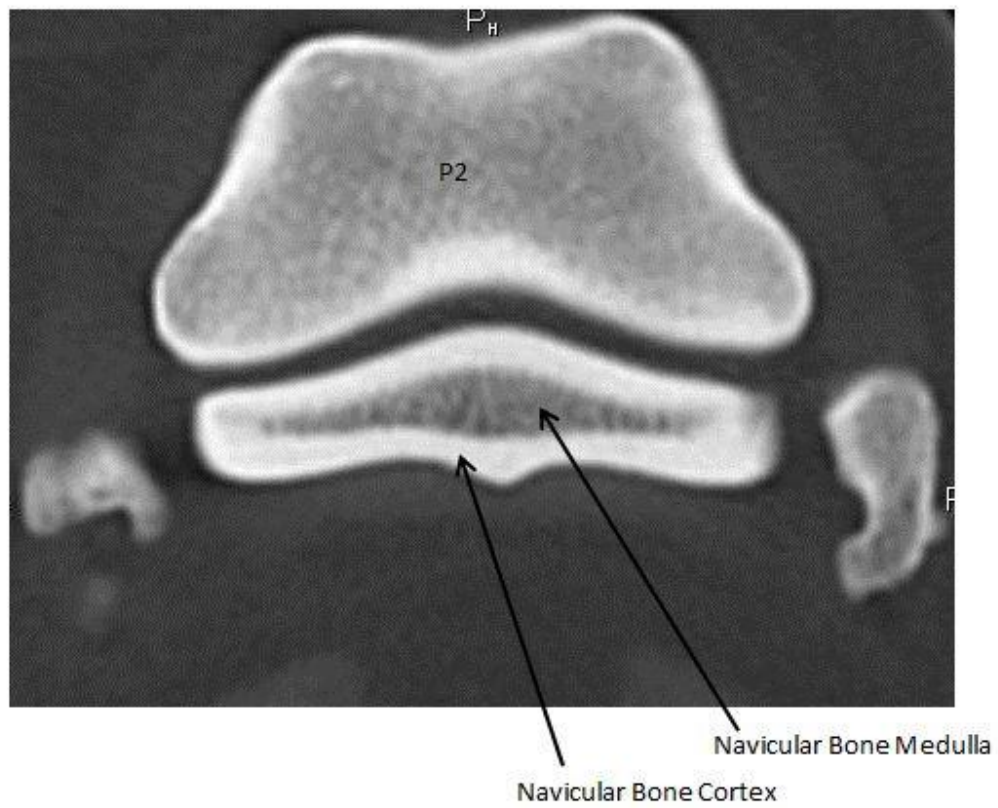


Figure 4: Computed tomography transverse image of navicular bone demonstrating trabecular medullary bone surrounded by a cortical rim.

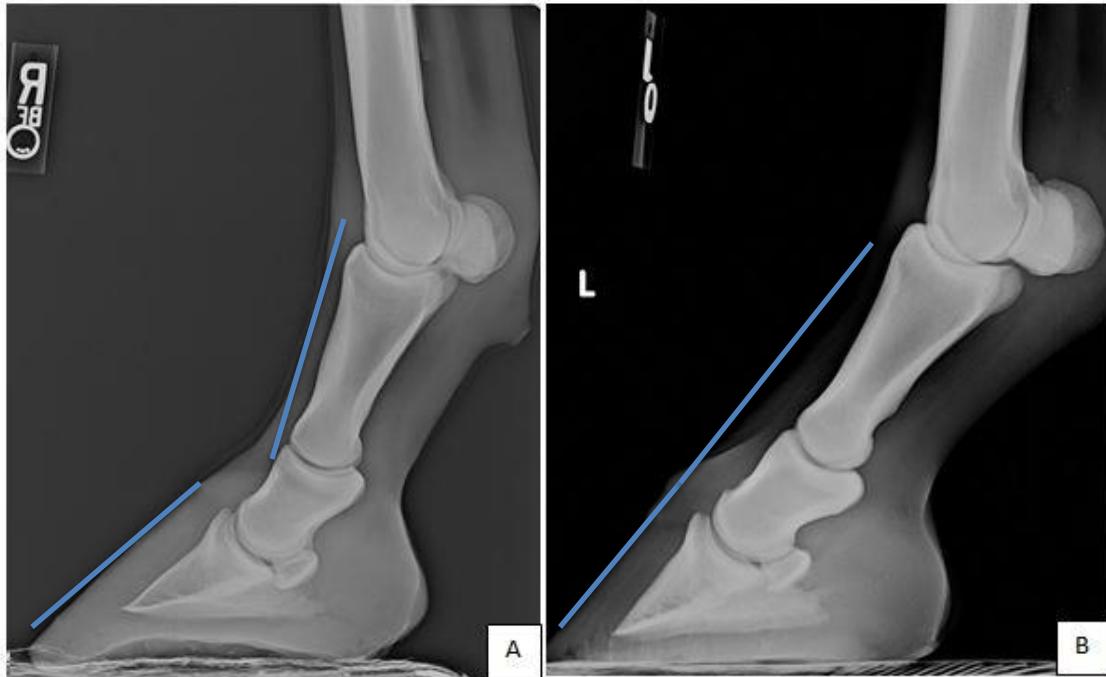


Figure 5: A = Broken back hoof pastern angle. B = Normal hoof pastern angle.



Figure 6: Intraosseous regional limb perfusion.

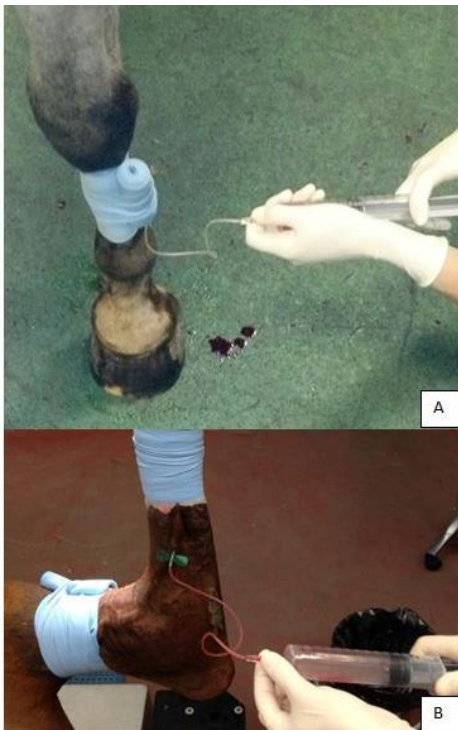


Figure 7: Intravenous regional limb perfusion via the palmar digital vein (A) and the medial saphenous vein (B).

## 4. Materials and Methods

### 4.1. Horses

This experiment was done as two independent trials; a low dose trial and a high dose trial. All experimental procedures were performed with the approval of the Institutional Animal Care and Use Committee. Six adult horses (mean weight, 600 kg; range, 514 to 636 kg; mean age, 10.6 years; range 4 to 17 years) from a university teaching herd were used for the low dose trial. Horses included 1 Thoroughbred, 3 Quarter Horses, 1 Warmblood and 1 Quarter Horse cross. All horses were healthy on clinical examination. Horses were sound at the walk on a paved surface. On subjective lameness evaluation horses were graded from 1-5 according to the AAEP lameness grading scale (Anonymous, 1999) (Grade 1 = lameness difficult to perceive at a trot and only intermittently present, Grade 2 = lameness clearly and consistently visible at the trot under one circumstance [eg., circle to the left on hard ground] but not other circumstances, Grade 3 = lameness clearly visible at the trot under all circumstances, Grade 4 = lameness visible at the walk, Grade 5 = horse unwilling to bear weight on the affected limb when standing still). Baseline lameness grades at the trot are outlined in Table 1. One horse was positive to distal limb flexion of that limb. All other distal limb flexions were within normal limits. Horses were housed in boxstalls or small paddocks (15 x 15 m) for the duration of the experimental trial and given free access to grass hay and water. Following all data collection, horses were returned to pasture with their herdmates.

For the high dose trial, an additional 6 adult horses (mean weight, 480 kg, range, 414 – 545 kg; mean age, 12.5 years; range, 12-19 years) were used. These horses were horses that owners were planning to euthanize for reasons unrelated to this study (eg., chronic hindlimb lameness, hyperkalemic periodic paralysis). When offered the opportunity to participate in this

study, owners donated their horses with the hope that the results of this study can be used to help horses with navicular disease in the future. Horses were euthanized following data collection both for sample collection and in accordance with owner wishes. Horses included 1 Rocky Mountain Horse, 1 Appaloosa, 1 Morgan, 1 Quarter Horse, 1 American Paint Horse and 1 Arabian cross. All horses were healthy on clinical examination and were housed in boxstalls or small paddocks for the duration of the experimental trial. Horses were sound at the walk on a paved surface. Distal limb flexion tests were within normal limits in all horses. Baseline lameness grades at the trot are outlined in Table 1.

## **4.2 Experimental Protocol**

Horses were sedated with detomidine<sup>a</sup> (0.01-0.015 mg/kg IV) and were given additional doses of detomidine<sup>a</sup> or butorphanol<sup>b</sup> (0.01 mg/kg) as needed. Distal forelimbs were clipped circumferentially from mid-metacarpus to coronary band and following aseptic preparation, high four-point blocks were placed bilaterally with bupivacaine<sup>c</sup>. Following aseptic preparation, baseline synovial fluid samples were collected bilaterally from both forelimb distal interphalangeal joints, metacarpophalangeal joints and navicular bursae. Synovial fluid samples were collected from the distal interphalangeal joints and metacarpophalangeal joints with the horse weight bearing by inserting a 20-gauge 3.81-cm hypodermic needle 1 cm lateral to the common digital extensor and 1 cm dorsal to the coronary band or metacarpophalangeal joint margin. Needles were directed in a distal axial direction until synovial fluid filled the needle hub, then 0.5 – 1.5 mL of synovial fluid was aspirated using a 3 mL syringe. Samples were collected from the navicular bursae by inserting an 18 gauge 3.5 inch spinal needle on palmar midline between the heel bulbs 1-2 cm proximal to the coronary band and inserting the needle in a horizontal plane until bone was contacted. A lateral radiographic projection was taken to verify

that the needle was positioned on the caudoproximal surface of the navicular bone (Figure 8). Once the needle was appropriately positioned, the needle stylet was removed. Synovial fluid was aspirated with a 3 mL syringe while the limb was weight bearing. If fluid did not flow freely in this position, the limb was lifted off the ground, the digital joints were slightly flexed, and aspiration was re-attempted. Typically 0.5 – 0.8 ml of synovial fluid could be aspirated from each navicular bursa. Distal limbs were bandaged for 24 hours with light, aseptically applied bandages following synovial centesis.

Synovial fluid samples were partitioned into microcentrifuge tubes for later tiludronate analysis and EDTA containing vials (Monoject)<sup>d</sup> for cytologic analysis. Horses were given a seven day rest period following collection of baseline samples, and then were re-sedated with detomidine<sup>a</sup> (0.01-0.015 mg/kg IV) for IVRLP. Following aseptic preparation, high four point blocks were placed with bupivacaine<sup>c</sup>. Limbs were aseptically prepared bilaterally for all synovial centeses and skin over the medial and lateral palmar digital veins was aseptically prepared bilaterally for IVRLP. A 10.2-cm wide rubber tourniquet<sup>e</sup> was placed on each forelimb covering 15- 18 cm of the proximal half of the metacarpus and 10.2-cm long gauze pads rolled to 1 cm thickness were placed on either side of the flexor tendons on the metacarpus prior to application of the rubber tourniquets. Tourniquets were applied as tightly as possible by the same investigator (BH) each time. Following tourniquet application, IVRLP was performed via a palmar digital vein on each forelimb by inserting a 21-gauge 1.9-cm butterfly catheter and infusing 50 mls of perfusate over 3-5 minutes. Catheters were removed immediately following infusions and temporary bandages of gauze and elastic wrap<sup>f</sup> were tightly applied. Bandages and tourniquets were left in place for 30 minutes.

Each horse had one forelimb randomly assigned using [www.random.org/lists](http://www.random.org/lists) to be treated with tiludronate. A list consisting of 12 times the letter R and 12 times the letter L was entered into the query field and a computer generated list of these letters in random order was created. The first 6 letters in the randomized list were designated as treated limbs. The opposite forelimb was used as a control and was perfused with 50 mls of saline. For the low dose experimental phase, 0.5 mg of tiludronate<sup>g</sup> dissolved in 50 mls of saline was infused into each treated limb. For the high dose experimental phase, 50 mg of tiludronate dissolved in 50 mls of saline was infused into each treated limb. Infusions were all done by the same investigator (BH) who was blinded to treatment allocations.

Tourniquets and bandages were removed 30 minutes after infusions. Just prior to tourniquet removal, a 10 ml jugular blood sample was taken and placed in a sterile vial. Immediately following tourniquet removal, synovial fluid samples were obtained from distal interphalangeal joints, metacarpophalangeal joints and navicular bursae bilaterally using the technique described above. Bandages were aseptically applied following synovial centeses. Twenty-four hours following IVRLP, horses were evaluated for lameness at a walk and trot.

Following lameness evaluation, horses treated with a low dose of tiludronate were sedated with detomidine<sup>a</sup> (0.01-0.015 mg/kg IV) and synovial fluid samples were collected from the distal interphalangeal (DIP) joints and metacarpophalangeal (MCP) joints of both forelimbs. Bandages were placed following synovial centeses. Horses remained on boxstall or small paddock rest for another 24 hours, and then were returned to their herd. Lameness evaluations were performed once a day for 7 days following IVRLP.

Following lameness evaluation on horses treated with the high dose of tiludronate, horses were euthanized with an intravenous injection of pentobarbital<sup>h</sup> (87 mg/kg IV). Synovial fluid



was aspirated from the DIP joints and MCP joints of both forelimbs following euthanasia. Bone and cartilage samples were collected from all structures of the distal forelimbs and banked for future analysis.

### **4.3. Synovial fluid cytology**

Following each sample collection, 200-300  $\mu$ L of synovial fluid was placed in a 2 ml vial containing 7.5% EDTA liquid (Monoject)<sup>d</sup> for analysis of cytology variables. Total solids and total nucleated cells counts were measured within two hours of sample collection. Total solids were measured as total solids using a refractometer<sup>i</sup>. Total nucleated cell counts were determined manually using the BMP Leukocheck<sup>j</sup>. Wright Giemsa stained cytopsin<sup>k</sup> slides were used for manual differential cell counts. Samples were grossly evaluated for presence of grossly visible blood contamination, and blood was noted as present or absent.

### **4.4. Tiludronate concentration determination**

Jugular blood samples were allowed to sit at room temperature for 30 minutes to allow time for coagulation, then were centrifuged at 3500xg for 5 minutes. Serum was separated into microcentrifuge tubes and frozen at -80°C until tiludronate analysis. Synovial fluid was centrifuged at 10,000xg for 30 minutes at 4°C. The synovial fluid supernatant was separated from cellular material and frozen in microcentrifuge tubes at -80°C until tiludronate analysis.

Tiludronate analysis was done using high performance liquid chromatography<sup>l</sup> followed by mass spectrometry<sup>m</sup>. The complete protocol is outlined in Appendix 1.

Briefly, tiludronate in all samples was methylated with 0.2 M trimethylsilyldiazomethane in acetone. Concentrations of methylated tiludronate were determined against a standard curve of tiludronate (0.5 – 64 ng/mL) made in equine synovial fluid from untreated horses euthanized for reasons unrelated to this study. For the serum samples, the standard curve was generated in equine serum from untreated horses. All unknown and standard curve samples were spiked with a known amount of deuterated tiludronate<sup>n</sup> as an internal control. Further, positive and negative control samples were run along each batch of samples. The lower limit of detection for the assay was 0.1 mg/L.

#### **4.5. Statistical analysis**

Results are reported as mean  $\pm$  standard error. Cytology variables and tiludronate for each joint were compared over time and between treated and control limbs for the low dose trial and the high dose trial separately using two-way repeated measures ANOVA. Limbs were nested within horse and horses were treated as an independent variable. Post-hoc analysis was done using a sedilax test. Statistical significance was set at  $p \leq 0.05$  and analyses were done using Graphpad Prism<sup>o</sup> and R Studio<sup>P</sup>.

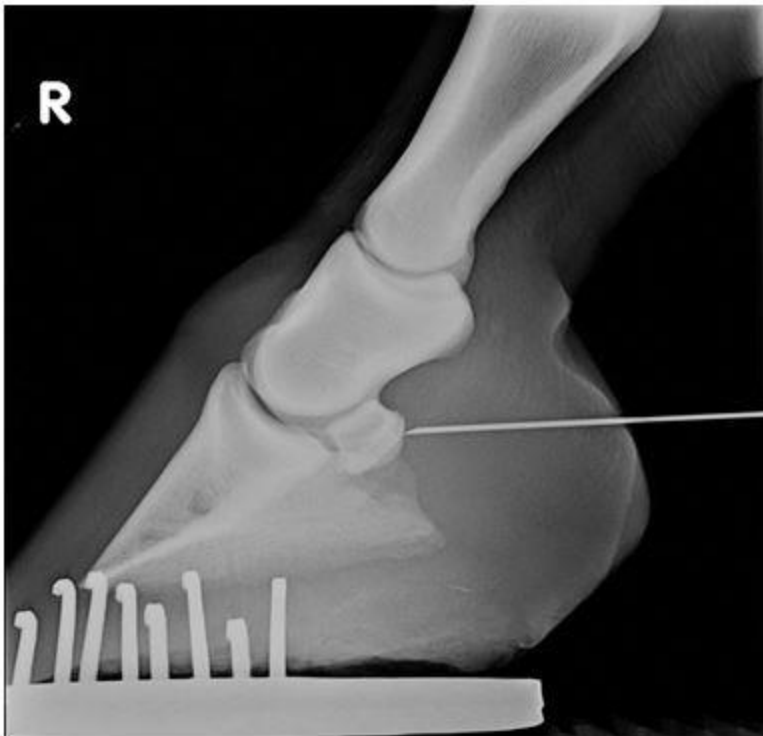


Figure 8: Properly positioned needle for navicular bursa centesis.

Table 1: AAEP Lameness grades of horses on baseline lameness evaluation prior to IVRLP

<b>Horse # for IVRLP with 0.5 mg tiludronate trial</b>	<b>AAEP lameness grade pre-perfusion</b>
1	3 (Left front)
2	1 (Right front)
3	0
4	0
5	0
6	3 (Right front)
<b>Horse # for IVRLP with 50 mg tiludronate trial</b>	
1	0
2	2 (Left front)
3	1 (Left front)
4	0
5	0
6	0

## 5. Results

Clinical evaluation of lameness 24 hours, 48 hours and 7 days following IVRLP in horses treated with 0.5 mg of tiludronate or lameness evaluation at 24 hours post-perfusion in horses treated with 50 mg of tiludronate is outlined in Table 2. One horse was positive to distal limb flexion in one limb prior to IVRLP with 0.5 mg tiludronate continued to be positive to distal limb flexion following IVRLP. Distal limb flexion of the contralateral limb and of the limbs of remaining horses in the low dose trial was within normal limits. No horse receiving 50 mg of tiludronate via IRVLP was positive to distal limb flexion prior to or 24 hours following IVRLP.

The number of structures from which adequate samples ( $> 0.5$  mls) of synovial fluid was obtained in the low dose and high dose trials are outlined in Table 3. Analysis of cytology data in horses perfused with 0.5 mg of tiludronate showed that TNCCs, total solids and percent neutrophils of pre-perfusion samples, 30 minute post-perfusion samples and 24 hour post-perfusion samples were not different between treated and control limbs for any joint (Table 4). Mean  $\pm$  SEM values for cytology variables are depicted in Figure 9.

When analyzing cytology data over time in horses perfused with 0.5 mg of tiludronate, MCP TNCCs ( $P = 0.007$ ) and percent neutrophils ( $P = 0.03$ ) increased significantly with time while total solids ( $P = 0.38$ ) did not change with time. No significant differences were seen in DIP synovial fluid cytology variables over time in this same group of horses (TNCC:  $P = 0.13$ ; % Neuts:  $P = 0.20$ ; TS:  $P = 0.83$ ). Synovial

fluid from the navicular bursa showed an increase in TNCCs ( $P = 0.05$ ) and TS ( $P = 0.03$ ) over time, but percent neutrophils were not different ( $P = 0.88$ ).

In horses perfused with 50 mg of tiludronate, there were no significant differences between treated and control limbs in TNCCs and percent neutrophils or in synovial total solid concentrations prior to perfusion, 30 minutes following perfusion or 24 hours following perfusion (Table 4). Mean  $\pm$  SEM values for cytology variables are depicted in Figure 9. Analysis of cytology data over time in horses perfused with 50 mg of tiludronate showed that TNCCs increased significantly over time in MCPs ( $P < 0.001$ ), DIPs ( $P = 0.003$ ) and navicular bursae ( $P = 0.05$ ) while no significant differences were seen in percent neutrophils ( $P \geq 0.07$ ) or TS ( $P \geq 0.29$ ) in any joint.

Tiludronate was not present in any samples prior to IVRLP. Following perfusion with 0.5 mg, tiludronate was not found in serum, or synovial fluid of saline perfused limbs. Tiludronate was present in synovial fluid of the tiludronate perfused limb at time of tourniquet release, but was no longer detectable by 24 hours post-perfusion (Table 5).

Following perfusion with 50 mg, the greatest concentrations of tiludronate in synovial fluid were present at time of tourniquet release ( $P \leq 0.02$ ) (Table 5). Comparison of synovial fluid concentrations of tiludronate in treated limbs versus controls revealed significantly greater concentrations of tiludronate in treated limbs ( $P \leq 0.02$ ). Tiludronate was present in low concentrations in serum at the time of tourniquet release (Table 5). Concentrations of tiludronate in the synovial fluid of treated limbs were significantly greater than serum concentrations at time of tourniquet release ( $P < 0.001$ ).

Table 2: AAEP Lameness grades of horses on baseline lameness evaluation prior to IVRLP and lameness grades at 24 hours, 48 hours, and 7 days post-perfusion. (N/A = not available)

<b>Horse # for IVRLP with 0.5 mg tiludronate trial</b>	<b>AAEP lameness grade pre-perfusion</b>	<b>AAEP lameness grade 24 hours post-perfusion</b>	<b>AAEP lameness grade 48 hours post-perfusion</b>	<b>AAEP lameness grade 7 days post-perfusion</b>	<b>Limb Treated with tiludronate</b>
1	3 (Left front)	3 (Left front)	3 (Left front)	3 (Left front)	LF
2	1 (Right front)	3 (Left front)	0	0	RF
3	0	1 (Left front)	3 (Left front)	0	LF
4	0	1 (Left front)	0	0	RF
5	0	0	0	0	LF
6	3 (Right front)	1 (Right front)	3 (Right front)	3 (Right front)	RF
<b>Horse # for IVRLP with 50 mg tiludronate trial</b>					
1	0	3 (Right front)	N/A	N/A	RF
2	2 (Left front)	2 (Left front)	N/A	N/A	LF
3	1 (Left front)	1 (Left front)	N/A	N/A	LF
4	0	0	N/A	N/A	RF
5	0	2 (Right front)	N/A	N/A	RF
6	0	0	N/A	N/A	LF

Table 3: Number of synovial structures from which > 0.5 ml of synovial fluid was obtained versus number of structures that synovial centesis was attempted. (N/A = not available)

<b>Phase 1: IVRLP with low dose tiludronate</b>			
	<i>Navicular Bursa</i>	<i>Coffin Joint</i>	<i>Fetlock Joint</i>
Baseline	10/12	10/12	12/12
30 minutes	12/12	12/12	12/12
24 Hours	N/A	12/12	12/12
<b>Phase 2: IVRLP with high dose tiludronate</b>			
Baseline	10/12	11/12	12/12
30 minutes	9/12	11/12	12/12
24 Hours	N/A	12/12	12/12



Table 4: P values associated with comparison of cytology variables between saline controls and tiludronate treated limbs over time where  $P \leq 0.05$  is significant. (MCP = metacarpophalangeal joint, DIP = distal interphalangeal joint, NB = navicular bursa).

<b>IVRLP with low dose (0.5 mg) tiludronate</b>	<b>MCP</b>	<b>DIP</b>	<b>NB</b>
% Neutrophils	0.95	0.27	0.14
TNCC (cells/ $\mu$ L)	0.42	0.51	0.30
Total solids (g/dL)	0.36	0.6	0.66
<b>IVRLP with high dose (50 mg) tiludronate</b>			
% Neutrophils	<b>0.03</b>	0.20	0.88
TNCC (cells/ $\mu$ L)	<b>0.007</b>	0.13	<b>0.05</b>
Total solids (g/dL)	0.38	0.83	<b>0.03</b>

Table 5: Mean  $\pm$  SEM concentrations of tiludronate in synovial fluid or serum following IVRLP with 0.5 mg or 50 mg of tiludronate. Lower limit of assay detection = 0.01 mg/L. (N/A = not available)

<b>IVRLP with low dose (0.5 mg) tiludronate</b>	Baseline <i>mg/L</i>	Tourniquet Release <i>mg/L</i>	24 Hours <i>mg/L</i>
Navicular Bursa			
<i>Treated</i>	0	0.08 $\pm$ 0.03	N/A
<i>Saline</i>	0	0	N/A
Distal Interphalangeal Joint			
<i>Treated</i>	0	0.12 $\pm$ 0.06	0
<i>Saline</i>	0	0	0
Metacarpophalangeal Joint			
<i>Treated</i>	0	0.04 $\pm$ 0.02	0
<i>Saline</i>	0	0	0
Serum	N/A	0	N/A
<b>IVRLP with high dose (50 mg) tiludronate</b>			
Navicular Bursa			
<i>Treated</i>	0	6.05 $\pm$ 1.93	N/A
<i>Saline</i>	0	0.09 $\pm$ 0.03	N/A
Distal Interphalangeal Joint			
<i>Treated</i>	0	16.3 $\pm$ 5.46	0.02 $\pm$ 0.01
<i>Saline</i>	0	0.08 $\pm$ 0.02	0
Metacarpophalangeal Joint			
<i>Treated</i>	0	3.75 $\pm$ 1.54	0.07 $\pm$ 0.01
<i>Saline</i>	0	0.02 $\pm$ 0.01	0
Serum	N/A	0.11 $\pm$ 0.05	N/A

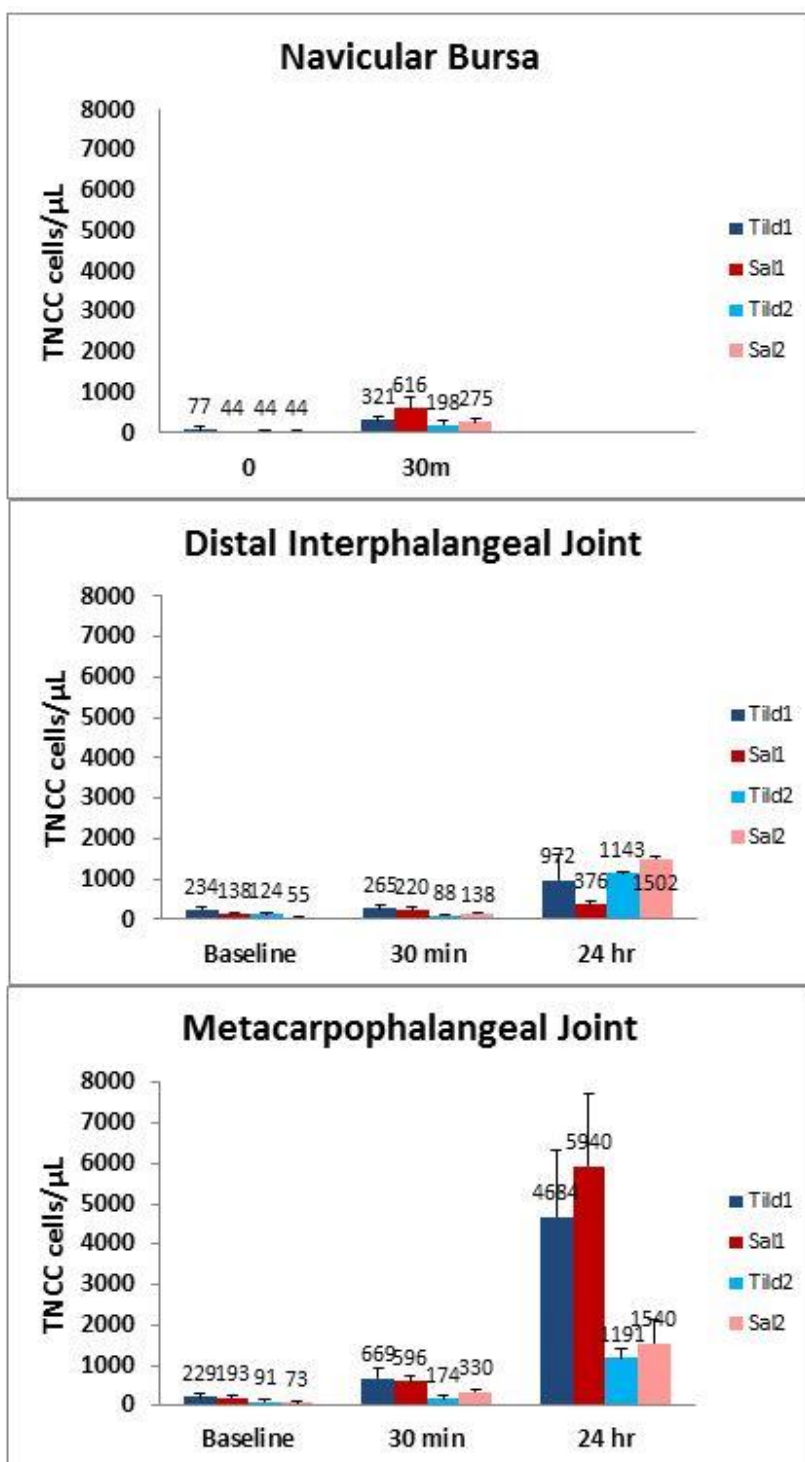


Figure 9a: Mean  $\pm$  SEM total nucleated cell counts (TNCC) in synovial fluid following IVRLP with low dose tiludronate (0.5 mg) or high dose tiludronate (50 mg). Tild1 = IVRLP with 0.5 mg tiludronate, Sal1 = saline control for Tild1, T2 = IVRLP with 50 mg tiludronate, Sal2 = saline control for T2

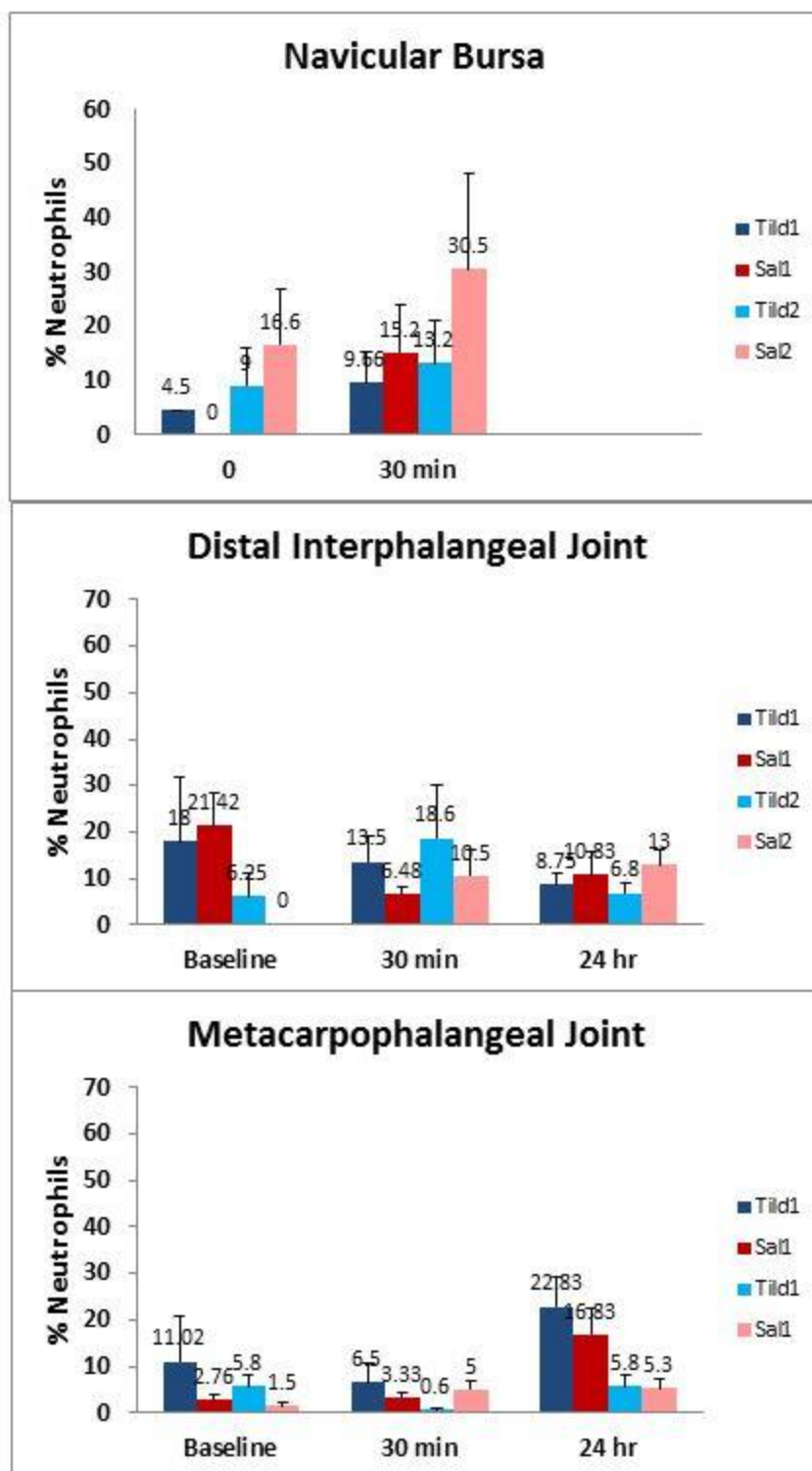


Figure 9b: Mean  $\pm$  SEM % neutrophils in synovial fluid following IVRLP with low dose tiludronate (0.5 mg) or high dose tiludronate (50 mg).

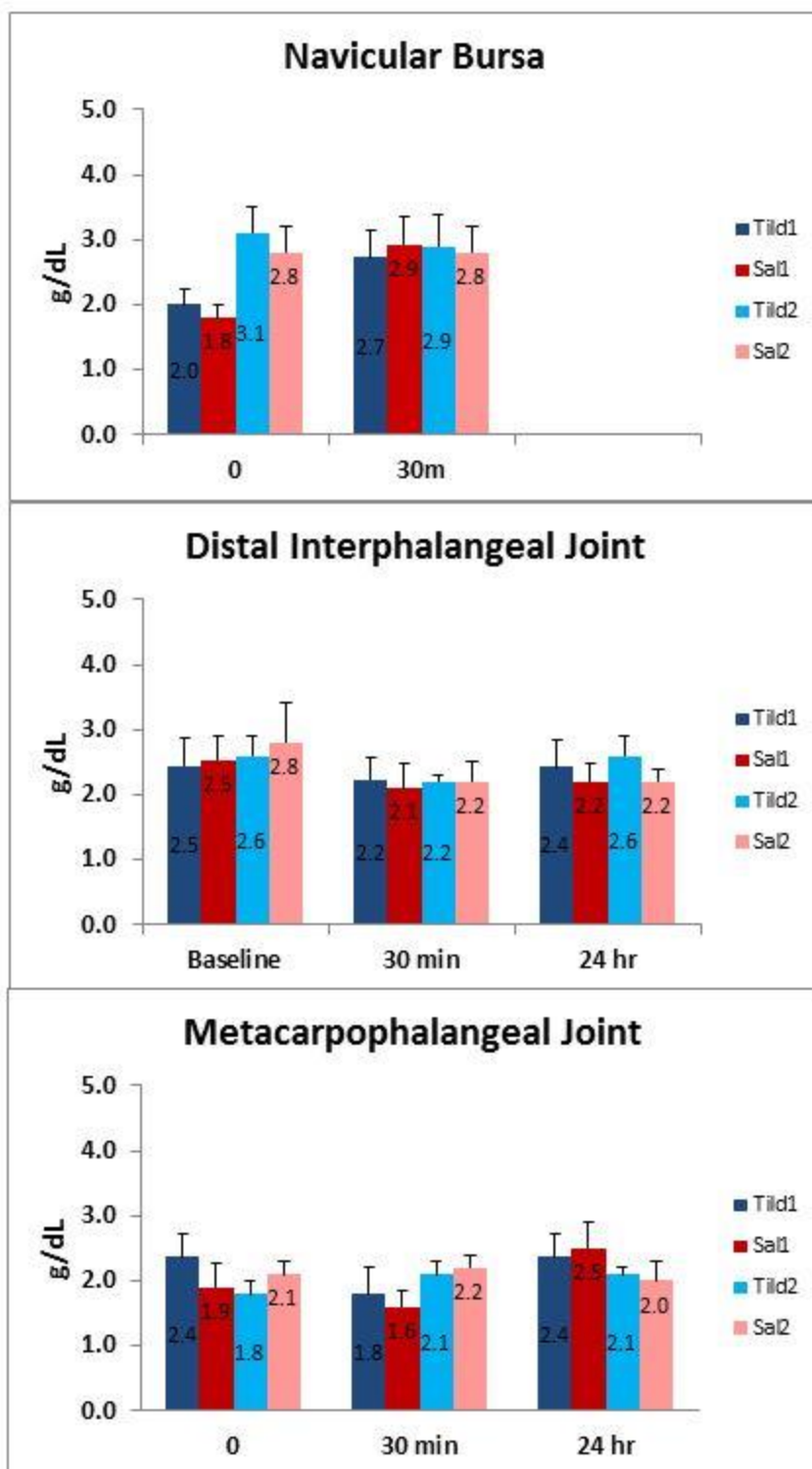


Figure 9c: Mean  $\pm$  SEM total solids concentration in synovial fluid following IVRLP with low dose tiludronate (0.5 mg) or high dose tiludronate (50 mg).

## 6. Discussion

For IVRLP with tiludronate to be considered safe in horses, the concentrations achieved in synovial fluid must be safe for articular cartilage and bone concentrations must not induce osteoblast or osteocyte apoptosis. This study is the first study to determine concentrations of tiludronate in synovial fluid following IVRLP. As expected, synovial fluid concentrations increase in a dose dependent fashion. Maximum concentrations were present immediately following tourniquet removal and minimal to no tiludronate was present in synovial fluid 24 hours following IVRLP. Tiludronate could be drawn by its affinity to calcium and dissipate into articular cartilage and subchondral bone, or it could diffuse down its concentration gradient into systemic circulation and be eliminated via the kidneys. Regardless of how it is eliminated from the joint, previous *in vitro* work suggests that for tiludronate in synovial fluid to be safe for articular cartilage, concentrations should be maintained below 19 mg/L (Duesterdieck-Zellmer et al., 2012). Thus, IVRLP with 0.5 mg and 50 mg for tiludronate in 50 mL of saline resulted in maximum synovial fluid concentrations that can be considered safe for both healthy and diseased articular cartilage.

This study also assessed the potential interaction of tiludronate with synovium by evaluating cytology variables following IVRLP and determined that tiludronate does not significantly change synovial fluid cytology variables in comparison to saline treated controls. The lack of significant difference between treatment and control groups for cytology variables suggests that tiludronate did not cause more synovial inflammation at

the doses used for IVRLP in this study than saline controls. Clinicopathologic elevations were present in both treatment and control groups, however, suggesting that an inflammatory stimulus was present in both groups. The degree of inflammation indicated by each parameter varied by joint and with time of collection. When considering total solids in each synovial structure, the degree in elevation from normal depends on the definition of normal. In general, total solids values of  $< 2.0$  g/dL are considered normal (McIlwraith, 1980), however a range of  $<0.8$  g/dL to  $<2.5$  g/dL has been published as potentially normal (Frisbie, 2012). If  $< 2.0$  g/dL is used as a normal cutoff for total solids, total solids was mildly to moderately elevated in all groups at all time points in the distal interphalangeal joint and in most groups at each time point in the navicular bursa and metacarpophalangeal joint (Figure 9). While these elevations are consistent with synovial inflammation, it is important to remember that these were low volume samples placed in EDTA containing vials prior to analysis and EDTA contamination can falsely elevate total solids values (MacWilliams and Friedrichs, 2003). Because treated and control samples were collected in identical fashion, we can surmise that tiludronate did not elevate total solids in comparison to saline controls. As EDTA can falsely elevate total solids values, the mild degree of synovial inflammation reflected in the total solids concentration cannot be assessed in this study.

Total nucleated cell counts in synovial fluid can be a valuable indicator of inflammation and cell counts  $>1000$  cells/ $\mu$ L are considered elevated (Steel, 2008). TNCCs were within normal limits in all synovial structures in baseline samples and in samples taken at time of tourniquet release regardless of treatment group. Elevations

were seen in all groups in almost all structures at 24 hours post-perfusion. These elevations could be secondary to serial synoviocenteses. Repeated joint sampling every 48 hours has been shown to significantly increase TNCCs in comparison to baseline and result in TNCCs above 1000 cells/ $\mu$ L (Sanchez et al., 2012). Repeated arthrocentesis has also been previously shown to increase inflammatory cytokines in equine healthy joints for up to 14 days (van den Boom et al., 2004; van den Boom et al., 2005). The elevation in TNCCs could also be due to the presence of saline within synovial fluid following IVRLP. Presence of both saline and lactated Ringer's solution have been shown to cause acute stress to synoviocytes and chondrocytes after joint lavage, with the effect of saline being greater than that of lactated Ringer's solution (Straehly, 1985). Repeated intrasynovial injection of lactated Ringer's solution every 12 hours for 24 hours significantly increased TNCCs in comparison to baseline and can result in TNCCs of over 20,000 cells/ $\mu$ L in some horses (Dykgraaf et al., 2007). In this study, saline would be present in synovial fluid in variable quantities as it was carrying tiludronate into the synovial structures. With this in mind, inflammation associated with the combination of intrasynovial saline and repeated arthrocentesis in our horses is a reasonable explanation for the TNCCs elevations seen. This is also a reasonable explanation for the elevations seen in percent neutrophils. Alternatively, neutrophil counts >10% have been reported to be normal in fluid samples with very low cell counts (Mahaffey, 2002.)

Isotonic saline was chosen for the diluent in this study as tiludronate should not be diluted with calcium containing polyionic solutions. As tiludronate has a high affinity for calcium, administering it with a calcium containing solution would be contraindicated as



the drug would be bound to the calcium within the delivery agent before it ever reached the patient. Physiologic saline is an isotonic electrolyte solution that does not contain calcium routinely used to dilute antibiotics to a specific volume for IVRLP in equine practice, thus it was chosen to dilute the tiludronate. A dilution volume of 50 mls was chosen as this is a commonly reported volume for IVRLP (Levine et al., 2010; Kelmer et al., 2013a, b).

Potential benefits of IVRLP with tiludronate via a digital vein in clinical patients with navicular disease has yet to be determined, but there are several factors to consider before pursuing a study to evaluate clinical efficacy in resolving lameness associated with navicular disease. The first of these factors is that bone concentrations of tiludronate following IVRLP with 50 mg, a dose that is already used anecdotally in clinical practice, are unknown. The concentration of tiludronate in bone is important as the effect of bisphosphonates on bone cells is concentration dependent (Plotkin et al., 2006). Low concentrations have an anti-apoptotic effect on osteoblasts and osteocytes while this effect is lost at high concentrations (Plotkin et al., 2006). Conversely, adequate bisphosphonate concentrations must be achieved in order to inhibit osteoclast resorption of bone (Grey et al., 2002). In theory, bone concentrations of tiludronate are positively and linearly correlated with dose administered, but that has not yet been examined to this author's knowledge. The ideal bone concentration of tiludronate to achieve maximum therapeutic effect has not been defined in horses, but the concentrations of tiludronate in bone following systemic administration at a 1 mg/kg dose have been found to range from 3.5 – 12.9 mg/kg (Delguste et al., 2011). Systemic administration of tiludronate at 1

mg/kg has been shown to have therapeutic effect in horses with osteoarthritis or navicular disease (Coudry et al., 2007; Gough et al., 2010; Denoix et al., 2003). Thus, one can surmise that if the bone concentrations of tiludronate following IVRLP with 50 mg are equivalent to bone concentrations following systemic administration of tiludronate at 1 mg/kg, then IVRLP with 50 mg of tiludronate should have a therapeutic effect.

Although bone concentrations of tiludronate were not measured as part of this study, bone concentrations of antibiotics following IVRLP have been measured. Bone concentrations of gentamicin, amikacin and enrofloxacin have all been shown to be significantly lower than concentrations in synovial fluid drawn from adjacent joints (Werner et al., 2003; Parra-Sanchez et al., 2006). Maximum tiludronate concentrations in synovial fluid following IVRLP with 50 mg were highly variable, with the highest mean concentrations being achieved in the DIP joint (Table 5). If similar concentrations are achieved in bone in a mg/kg equivalent, then IVRLP with 50 mg of tiludronate is likely safe for bone and may be efficacious in the treatment of navicular disease. If tiludronate follows a similar trend to antibiotics, then bone concentrations following IVRLP will likely be lower than the range correlated with clinical efficacy for the treatment of navicular disease and IVRLP with 50 mg may not be sufficient to produce a therapeutic effect. As tiludronate should have a much higher affinity for bone than antibiotics, however, it is difficult to predict whether it would follow the same diffusion trends. IVRLP of tiludronate could in fact result in considerably higher bone concentrations due to the high affinity of tiludronate for calcium, thus it is important to evaluate bone concentrations directly. In the absence of tiludronate bone concentrations, future studies

correlating levels of serum biomarkers for bone resorption, such as CTX-1, with clinical efficacy of 50 mg of tiludronate given via IVRLP may be of value. If IVRLP with 50 mg tiludronate does not result in a therapeutic effect, additional studies evaluating the safety and efficacy of higher doses may be warranted. Anecdotally, this author is familiar with doses of 100 and 150 mg being used in practice, thus these are likely appropriate doses to assess.

Another important factor to consider prior to using IVRLP with tiludronate for treatment of navicular disease is the selection of appropriate cases. While tiludronate does have an effect on cartilage (Duesterdieck-Zellmer et al., 2012), and bisphosphonates in general can exert an anti-inflammatory effect (Drake et al., 2008), the primary effect of tiludronate thought to provide clinical benefit is on bone. Use of tiludronate to treat injuries that are primarily soft tissue in origin is unlikely to result in clinical success. As recent studies have shown that injuries to the DDFT, collateral sesamoidean ligament and distal sesamoidean impar ligament are present in a large number of horses with caudal heel pain with or without concurrent navicular bone injury (Dyson et al., 2005; Sampson et al., 2009), accurate diagnosis of the source of caudal heel pain prior to treating with tiludronate is important. While tiludronate may be of benefit to cases with certain navicular bone abnormalities, it is unlikely to be of significant benefit to horses with fibrocartilage erosions on their navicular bones with or without kissing lesions on the DDFT. As with any therapeutic tool, tiludronate has the greatest chance of producing successful results when used to treat specific problems rather than being used as a panacea treatment for caudal heel pain.

Equal in importance to an accurate diagnosis is the idea that treatment with tiludronate should not be done in isolation from other treatments. For example, improvements in navicular bone sclerosis secondary to tiludronate treatment are likely to be short lived if horses are left with long toes and low heels. Treatment with tiludronate should always be combined with therapeutic trimming and shoeing as reduction of DDFT pressure on the navicular bone is a key principle in the treatment of navicular disease. Additionally, high levels of inflammation may inhibit the action of non-nitrogenous bisphosphonates (Sutherland et al., 2009). Inflammatory cytokines were shown *in vitro* to induce expression of anti-apoptotic proteins, thus osteoclasts' apoptosis secondary to tiludronate treatment may be diminished in the face of severe inflammation. With this in mind, one may consider injecting the navicular bursa or distal interphalangeal joint with corticosteroids a few weeks prior to treatment with tiludronate in effort to reduce inflammation within the foot.

This study did have a few limitations that are important to address. The first is the small number of horses used in each trial. Given the high variability in data obtained, ideally more horses would be added to this study. This study was designed to prove the principle that the synovial fluid concentrations of tiludronate following IVRLP would be equal to or lower than a level that can be considered safe for articular cartilage. Although statistical significance should be interpreted with caution, the basic goal of the study is sufficiently demonstrated with the data obtained. The high variability in data is a weakness that is inherent to research studies employing IVRLP and the degree of variability in this study is consistent with that seen in other studies (Levine et al., 2010;

Butt et al., 2001; Scheuch et al., 2002; Beccar-Varela et al., 2011). A final weakness of this study was the use of horses that had variable degrees of baseline lameness. Attempts to compensate for this weakness were made by evaluating horses for changes in lameness from baseline. These were subject evaluations, however, and subjective lameness evaluation has been reported to be less accurate than objective evaluation (Keegan et al., 2013). Ideally horses that were sound on baseline lameness evaluation would have been used so that the evaluator could have noted presence or absence of lameness following IVRLP rather than increase or decrease in lameness. As horses admitted to the study were donated, however, ideal candidates were not always achievable.

## **7. Conclusions**

The findings of this study support the first part of our hypothesis. IVRLP with 0.5 mg of tiludronate does not cause synovial inflammation in comparison to placebo controls and synovial fluid concentrations of tiludronate are within the range that should be considered safe for cartilage. IVRLP with 50 mg of tiludronate also did not cause synovial inflammation in comparison to placebo controls and synovial fluid concentrations of tiludronate were within the range that should be considered safe for cartilage, disproving the second part of our hypothesis.

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## Appendix 1

### *HPLC-MS : Extraction and Derivatization of Tiludronate from Equine Synovial Fluid*

#### *Authentic Material Preparation:*

- 1) Reconstitute 50mg vial of Tildren with 10mL UltraPure Water (MilliQ) using a needle and syringe (5mL syringe, 20 x 1 ½ needle) (→ 5 mg/mL Stock)
- 2) Aliquot reconstituted Tildren into 1.5ml microcentrifuge tubes (500ul in each)
- 3) Place aliquots in -80C freezer (“Fluffy”, top shelf)

#### *Preparation of Tiludronic Acid-d4 (Internal Control):*

To prepare a spiking stock of Tiludronic Acid-d5 Disodium salt (4,000ng/ml):

- 1) Weigh out 1mg and place in 1.5ml epp tube, then add 1ml ultrapure water and vortex for 3min→this gives a 1mg/ml stock
- 2) In a 15ml conical tube add 2988ul ultrapure water to tube and add 12ul reconstituted tiludronate-d5 (1mg/ml)→this gives a 4,000ng/ml spiking stock (3x 1ml aliquots into 1.5ml epp tubes→-80C freezer)
- 3) Check:  $C_1V_1=C_2V_2 \rightarrow (4,000\text{ng/ml})(5\text{ul})=C_2(200\text{ul}) \rightarrow C_2= 100\text{ng/ml}$

#### *Reagent Preparation:*

- 1) Extraction Solvent: 420ml Diethylether + 180ml 2-Propanol → 600ml
- 2) 0.2 M Trimethylsilyldiazomethane: Dilute 2M TMS-DAM (5ml) in 45ml Acetone (1:10)→50ml 0.2M TMS-DAM
- 3) Methanol:Water: 10ml Methanol + 10ml UltraPure Water (MilliQ)→ 20ml
- 4) Disodium Clodronate (Dichloromethylenediphosphonic acid disodium salt): 20mg Disodium Clodronate + 100ml Ultrapure Water in glass flask with magnetic stir bar until dissolved. Filter using a 0.2um bottle-top filter into 250ml glass bottle→ 100ml Disodium Clodronate (0.2mg/ml)
- 5) Oxalic Acid: 1g Oxalic Acid + 20ml UltraPure Water in 50ml centrifuge tube, vortex until dissolved→ 20ml Oxalic Acid (50mg/ml)
- 6) 3M HCL: 12.4ml HCL (12.1M) + 37.6ml UltraPure Water → 50ml HCL (3M)

7) Extraction Mixture: 100ml UltraPure Water + 10 ml Oxalic Acid (50mg/ml in water) + 4ml Disodium Clodronate(0.2mg/ml in water) + 10ml 3M HCL → 124ml Extraction Mixture

*Prepare standards from original 5mg/ml aliquot of Tildren:*

\*First, dilute Stock Soln (5mg/ml) to generate Working Stock (1mg/ml)

Stock (5mg/ml) → Working Stock (1mg/ml):

$$(5\text{mg/ml})V1 = (1\text{mg/ml})(2\text{ml})$$

$$V1 = 400\text{ul}$$

**400ul Stock (5mg/ml) + 1.6ml UltraPure Water**

→ 2ml Working Stock (1mg/ml)

\*Then, begin preparing 10X Standards starting with the most concentrated 10X Standard (10X Standard 9 (5,120ng/ml)):

Working Stock (1mg/ml) → 10X Standard 9 (5,120ng/ml):

$$(1\text{mg/ml}) V1 = (5,120\text{ng/ml}) (5\text{ml})$$

$$V1 = 25.6\text{ul}$$

**25.6ul Working Stock (1mg/ml) + 4974.4ul UltraPure Water**

→ 5ml 10X Standard 9 (5,120ng/ml)

\*Begin serially diluting the 10X standards 1:2 with water to generate the remaining 10X standard solutions:

10X STD 9 (5,120ng/ml) → 10X STD8 (2,560ng/ml)

$$(5,120\text{ng/ml})V1 = (2,560\text{ng/ml})(2\text{ml})$$

$$V1 = 1\text{ml}$$

**1ml 10X STD9 (5,120ng/ml) + 1ml UltraPure Water**

→ 2ml 10X STD8 (2,560ng/ml)

\*Repeat 1:2 dilutions for 10X standards 7 through 2 (Standard 1 is the blank)



***HPLC-MS: Method for the Extraction and Derivatization of Tiludronate from Equine Synovial Fluid***

Extraction:

**HPLC/Mass Spec is very sensitive. Avoid Cross-contamination! Use a separate filtered tip for each sample and solution. Clean cannulas on N Evaporators between samples.**

1. Turn on Nitrogen Evaporator heat block/bath\*. Check N tanks.
2. In a 15ml Corning Centristar (430791) centrifuge tube add, in the following order:
  - a. 200ul sample (this could be comprised of one of the following three options: 20ul Diluted sample plus 180ul Blank Synovial Fluid, 20ul 10X STD plus 180ul Blank Synovial Fluid, or 200ul Undiluted sample)
  - b. 5ul Internal Standard (Tiludronate-d4 4,000ng/ml)
  - c. 195ul nuclease free (nf) water
  - d. 570ul Extraction Mixture (Oxalic acid, Disodium Clodronate, HCL)
  - e. The combined components (totaling 970ul) are then vortexed for 15sec
3. Add exactly 3ml (use FinnPipette) Extraction Solvent (Diethylether:2-propanol) to the tube then place on a rocking platform for 10min. Make sure lids are seated correctly and tight.
4. Centrifuge tube at 11,000rpm for 5min
5. Transfer **2.55ml** (3 x 850ul aliquots, using a new tip each aliquot) of the supernatant to 13 x 100mm polypropylene tube.
6. Evaporate under nitrogen (check for max psi) in a 60C water bath\* or heat block to dryness (~45min).

\*Water bath must be turned off during Ether evap. To compensate for quick cooling of water, set bath at 70C initially and turn off while transferring supernatant (should be at 60C for start of evap). In order to keep evap temp close to 60C, set the first 9 tubes in the heat block for 45 min, prepare the supernatant layers of the remaining tubes, place them in the water bath evap for 35 min, and transfer them to the heat block for last 10 min. NOTE: the cannulas of both evaporators must be cleaned between samples. Use acetone and Kim wipes.

Could be stored in -80 at this point.

**Derivatization (Methylation of phosphoric acid):**

1. Turn Mata's heat block to 70C. Turn water bath to 60C.

2. Add 200ul 0.2M Trimethylsilyldiazomethane in acetone (TMSD) to 13 x 100mm tube containing evaporated extract and vortex for 3min. NOTE: TMSD in acetone only good for 2 weeks. Store away from light.
3. Incubate tubes in 70C heat block for 30min (Dr. Mata's heat block can hold up to 20 samples )
4. Allow tubes to cool to room temp, wrap lid with Parafilm, then spin at 2000 rpm for 1 min to bring all tube contents to the bottom of the tube
5. Add exactly 200ul Formic Acid and 400ul Hexane then vortex for 3min. Wrap lids with parafilm.
6. Centrifuge tubes at 11,000rpm for 7min
7. Transfer **300ul** of the **SUBNATANT** into a new 13 x 100mm polypropylene tube
8. Evaporate in heat block or water bath evaporator at 60C for approx. 30 min. Note: In this evaporation step the water bath can remain on.
9. Wrap lid in Parafilm, label, and store in -80C until ready to submit to Mass Spec. This evaporated product is stable at -80 C (tested in HPLC 6 weeks later).

#### **Preparation for HPLC/Mass Spec:**

**Note: Once in MeOH, the sample will become hydrolyzed over time. Send to Mass Spec within 36 hrs.**

1. Prepare a 50:50 mixture of Methanol: nf H<sub>2</sub>O then add 150ul to each tube, vortex for 15sec
2. Transfer the 150ul of reconstituted sample to a 1.5ml microcentrifuge tube
3. Spin all 1.5ml microcentrifuge tubes at max speed for 30sec to remove any debris (Note: some bloody samples may require a more thorough spin in order to pellet debris)
4. Transfer 100ul sample to polypropylene vials (provided by HPLC staff)
5. Place the remaining sample (i.e. remaining in the 1.5ml microcentrifuge tube) in -80C freezer for (possible) future analysis
6. Bring vials to ALS basement
7. Analyze samples in vials using the ABI 4000 Mass Spectrometer

## Appendix 2

### *Footnotes*

- a. Dormosedan<sup>®</sup>, Pfizer Animal Health, New York, NY
- b. Torbugesic<sup>®</sup>, Zoetis Animal Health, Auckland, New Zealand
- c. Marcaine<sup>®</sup>, Hospira Inc, Lake Forest, IL
- d. Tyco Healthcare Group LP, Mansfield, MA
- e. Esmark Bandage; Cardinal Health, McGraw Park, IL
- f. Coflex<sup>®</sup>, Cavendish Scott Ltd, England
- g. Tildren<sup>®</sup>, CEVA, France
- h. Beuthanasia-D Special<sup>®</sup>, Schering Plough Animal Health
- i. , Kenilworth, NJ
- j. E-line Veterinary, Bellingham+Stanley
- k. BMP Biomedical Polymers, Gardner, MA
- l. CytoSpin\* 4 Cytocentrifuge, Thermo Scientific, Waltham, MA
- m. XBridge phenyl column, Waters
- n. API 4000, Applied Biosystems
- o. Toronto Research Chemicals
- p. Graph Pad Prism, Graph Pad Software, San Diego, CA
- q. R Studio, <https://www.rstudio.com/>