Intra-articular Buprenorphine in Horses

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INTRA-ARTICULAR BUPRENORPHINE IN HORSES

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
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by
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Abstract

Opioid drugs have the potential of provide local analgesia in inflamed joints. To date, morphine is the only opioid that has been tested for intra-articular (IA) administration in horses. Having an alternative drug, other than morphine, could widen the therapeutic options, particularly in cases of drug shortages or inaccessibility to specific drugs. The work presented in this dissertation reports the cytotoxic effects of buprenorphine on cultured equine chondrocytes, and the pharmacokinetic, pharmacodynamic and analgesic effects, of IA administered buprenorphine in horses with experimentally induced synovitis.

To evaluate the potential cytotoxic effects on equine cartilage, chondrocytes were obtained from normal equine joints and cultured. Chondrocytes were exposed to two concentrations of buprenorphine (0.05 mg mL⁻¹ and 0.12 mg mL⁻¹) for 0 and 2 hours. Chondrocyte viability was evaluated after the exposure using four different techniques.

For the pharmacokinetic, pharmacodynamic and analgesic assessment of IA buprenorphine, seven healthy horses were used in a randomized cross-over design. Temporary lameness resulting from acute synovitis was induced following IA administration of lipopolysaccharide. Afterwards, horses received three different treatments: 1) IA buprenorphine (5 µg kg⁻¹) plus intravenous saline, 2) IA saline plus intravenous buprenorphine (5 µg kg⁻¹), and 3) IA saline plus intravenous saline. Each horse received each treatment with a washout period between treatments. Physiologic variables, lameness and pain scores were evaluated at stablished intervals over 48 hours, and synovial fluid and plasma for drug concentrations were determined at selected time-points over 24 hours.

Results from these studies show that a low concentration of buprenorphine does not have appreciable cytotoxic effects on equine chondrocytes in vitro. Buprenorphine was detectable in the
synovial fluid for at least 24 hours post IA administration, with no significant systemic absorption. Intra-articular administration of buprenorphine however, did not significantly improve lameness or pain scores when compared with the saline control. Pharmacodynamically, gastrointestinal sounds were decreased only for 4 hours in the IA and intravenous treatments when compared with saline.

Buprenorphine exhibits a concentration dependent cytotoxic effect in vitro. There was no significant analgesic effect of buprenorphine after IA administration, despite having detectable IA concentrations for at least 24 hours post-administration.
Chapter One.
Introduction

Lameness caused by orthopedic disease is one of the major causes of decreased performance and morbidity of sport horse all around the world (Oliver et al., 1997; Bailey et al., 1999; Perkins et al., 2005). A significant economic impact has been identified in the racehorse industry from training failure and wastage of horses with orthopedic disorders (Lindner & Dingerkus, 1993; Bailey et al., 1997; Olivier et al. 1997; Wilsher et al., 2006; Dyson et al., 2008). Various types of sport horses from multiple disciplines can be affected, including racing, cutting, and jumping horses. The reported incidence of lameness among these horses ranged from 42% to 57% (Jeffcott et al., 1982; Lindner & Dingerkus, 1993; Swor et al., 2019). Causes of lameness in these horses include ligament injuries, desmopathies, tendonitis, fractures, and intra-articular (IA) disorders such as synovitis, arthritis, osteoarthritis, and different types of IA fractures among others (Jeffcott et al., 1982; Penell et al., 2005). Intra-articular disorders causing morbidity in these horses have been overrepresented and have accounted for 37% to 75% of the causes of lameness (Penell et al., 2005; Preston et al., 2008; Swor et al., 2019).

In performance horses, repeated hyperextension while running imposes significant tension upon the synovial lining, which translates in a constant stretching, impingement, and concussion at this lining. This can result in cellular membrane injury leading to inflammatory mediators being released inciting joint inflammation and pain, resulting into the development of primary synovitis (Palmer & Bertone, 1994). Intra-articular fractures also occur leading to synovitis and joint inflammation. Whether the IA disorder requires surgical or medical management, treatment of pain is a cornerstone to regain joint functionality, speed up recovery, prevent the development of
chronic pain states, and subsequently, resume appropriate quality of life and athletic performance (Di Salvo et al., 2021).

Intra-articular pain has been treated with the administration of systemic non-steroidal anti-inflammatory drugs (NSAIDs) (Owens et al., 1995; Hu et al., 2005; Foreman & Ruemmler, 2011; Walliser et al., 2015). In some instances, systemic opioids are also used but are commonly reserved to the perioperative period in those cases where surgery is needed as a therapeutic intervention for the treatment of joint disease (Mircica et al., 2003; Clark et al., 2008). The reluctance to use systemic opioids more regularly is based on their potential to decrease gastrointestinal motility that may lead to ileus, constipation, and colic (KuKanich & Wiese, 2015), or due to their potential central nervous system and locomotor excitatory effects when given without sedatives (Combie et al., 1979; Combie et al., 1981; Kalpravidh et al., 1984; Sojka et al., 1988).

To prevent potential side effects of systemically administered drugs and maximize drug delivery to the affected tissue, IA injection of analgesics can be performed to treat pain from synovitis or osteoarthritis in horses. Perioperative administration of local anesthetics such as lidocaine, mepivacaine and bupivacaine have been used in horses with success. However, their concentration dependent and agent specific cytotoxic effect on chondrocytes both in vivo and in vitro makes them less than ideal for IA administration (Chu et al., 2006; Karpie & Chu, 2007; Lo et al., 2009; Park et al., 2011). Conversely, peripheral opioid receptors are expressed in peripheral tissues and evidence demonstrates that they are upregulated during local inflammation on the equine synovial membrane (Hassan et al., 1993; Stein et al., 1993; Sheehy et al., 2001; van Loon et al., 2013), making opioid drugs a feasible alternative to treat joint pain.

Morphine, a full µ-opioid receptor agonist, has been administered IA to decrease pain in humans, dogs, and horses with consistent and reliable results (Day et al., 1995; Likar et al., 1997;
Lindegaard et al., 2010a). Morphine has the advantage of maintaining adequate concentrations within the joint and producing analgesia lasting for over 24 hours in horses without significant systemic absorption (Lindegaard et al., 2010b). Furthermore, morphine has demonstrated minimal cytotoxic effects on both equine and human chondrocytes *in vitro* independent of the concentration and duration of exposure (Stueber et al., 2014; Ickert et al., 2015; Rubio-Martinez et al., 2017).

With the presence of opioid receptors within the synovia and the evidence of morphine’s efficacy when used IA (Santos et al., 2009; Lindegaard et al., 2010a), other opioid drugs, such as buprenorphine should be assessed. Buprenorphine is a partial µ-opioid receptor agonist with a higher potency and higher receptor affinity than morphine and could possibly be administered IA to manage pain in horses. Buprenorphine has been used systemically for the treatment of perioperative and experimentally induced pain with efficacy in producing analgesia in horses (Carregaro et al., 2007; Love et al., 2012; Love et al., 2013). In humans, buprenorphine has been used for the management of IA pain after knee and shoulder arthroscopy and for the treatment of temporomandibular joint pain showing superior analgesia than morphine (Fellahi et al., 1995; Varrasi et al., 1999; Präger et al., 2007; Sugandarajappa et al., 2016; Das & Samal, 2019).

The option of having an alternative opioid analgesic that could have a similar or better profile than morphine justifies the evaluation of the cytotoxicity, pharmacokinetics, and analgesic efficacy of the IA administration of buprenorphine in horses.
2.1. Nociception

2.1.1. Physiologic pain

As a protective mechanism, noxious stimulus (which can be mechanical, thermal, chemical, or electrical) activates a variety of high threshold uni- and poly-modal nociceptors located throughout the body leading to pain. This type of pain is known as physiologic or nociceptive pain, and its perception by the brain is perpetuated if the noxious stimulus is maintained, thereby serving as a warning to protect the body against damage. Physiologic pain is well localized, transient, and plays an important role as it elicits reflex withdrawal, avoidance responses, as well as neuroendocrine, autonomic nervous, and immune system responses that usually vary in severity in proportion to the intensity of the noxious stimulus (Muir & Woolf, 2001). If the noxious stimulus is intense enough to cause tissue injury and a subsequent inflammatory response, pain persists, and the sensory nervous system undergoes adaptive or maladaptive changes (Muir, 2010).

2.1.2. Pain pathway

2.1.2.1. Transduction

Pain begins when specialized receptors, also called nociceptors, located in the sensory neurons of the peripheral nervous system are activated. Four different types of nociceptors have been described. First, the transient receptor potential (TRP) superfamily of channels, which comprises a diverse group of cation channels that are highly implicated in the immediate detection of noxious stimuli (White et al., 2010). These channels respond to multiple types of stimuli...
including changes in pH and increases in temperature (Caterina et al., 1997; Tominaga et al., 1998). Second, the adenosine and adenosine triphosphate (ATP)-sensitive receptors, also known as purinergic receptors (P2X₃) which are responsible for the transduction of chemical stimuli being activated by ATP released from damaged cells (Ding et al., 2000). Third, the degenerin-epithelial sodium channels (mDEG), which is a large family of voltage gated ion channels that peripherally are responsible for transducing mechanical stimuli (García-Añoveros et al., 2001). Last, voltage-gated sodium channels (VGSCs or NaV channels) which are also expressed by peripheral sensory neurons. These channels are key determinants for the control of excitability and generation of action potentials within terminals and initiating their propagation to the central nervous system (CNS) (Baker & Wood, 2001). Their main function is to convert a chemical, mechanical, or thermal stimulus into an electrical signal that can be propagated into the central nervous system by specific nerve fibers.

### 2.1.2.2. Transmission

Once the nociceptor has been depolarized, an action potential is created and propagated to the CNS. This propagation is carried by specialized sensory nerve fibers of two types: 1) lightly myelinated Aδ- and 2) unmyelinated C-fibers. The Aδ- fibers transmit electrical nerve impulses at a speed of 2-20 m/s and are responsible for the prompt painful sensation that triggers withdrawal from the stimulus. C-fibers conduct at a speed of 0.5-2 m/s and its activation results in a long lasting delayed painful sensation that in humans is associated with a dull throbbing or burning sensation (Koga et al., 2005; Muir, 2010). Both, Aδ- and C-fibers are first-order neurons or afferent fibers that synapse on second-order neurons in the superficial (laminae I and II) and deep (laminae V and VI) layers of the dorsal horn of the spinal cord (Andrew & Craig, 2002; Dinakar et al.,
Aδ-fibers transmit the signal by releasing the neurotransmitter glutamate that binds to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainite, and N-methyl-D-aspartate (NMDA) ligand-gated sodium and calcium channels. C-fibers release substance P, which interacts with the tachykinin receptor family. All these receptors are located in the second-order neurons.

2.1.2.3. Modulation

After the impulse (action potential) from the first-order neuron enters the dorsal horn of the spinal cord and synapses with the second-order neuron it may be sent directly to the brain (by projection tracts) or may be modulated (amplified or inhibited) by interneurons or descending projections (Grubb, 2018). The descending inhibition is a process controlled by the periaqueductal gray (PAG) region which appears to be responsible for the coordination of the endogenous analgesic system. The descending projection neurons from the PAG will synapse in the gap between the first- and second-order neurons, releasing neurotransmitters such as endorphins, enkephalins, dynorphins, norepinephrine, serotonin, gamma-aminobutyric acid (GABA), and glycine. This decreases the propagation of the pain impulse at the synapse by the release of the inhibitory neurotransmitters that will bind to pre- and post-synaptic sites (Millan, 2002; Almeida et al., 2004; Dinakar et al., 2016; Grubb, 2018).

2.1.2.4. Projection

Once the second-order neuron is activated, the signal travels to the contralateral side of the spinal cord and then it is transmitted by ascending nociceptive pathways called tracts. These tracts include the spinothalamic, spinocervicothalamic, spinoreticular and spinomesencephalic tracts.
The ascending spinothalamic is of most importance in mammals, projecting fibers into the reticular formation of the brainstem, the periaqueductal gray of the midbrain, and the diencephalon, to end in the ventral posterolateral nucleus of the thalamus, hypothalamus, and structures of the midbrain (Grubb, 2018). Then in these locations the second-order neuron synapses with a third-order neuron that projects to higher centers (Kuner, 2010; Dinakar et al., 2016).

2.1.2.5. Perception

The impulses from the third-order neurons are transmitted to the somatosensory cortex, PAG region, reticular formation, and the limbic system. The fact that the impulses reach all these regions implies a myriad of responses which include perception of pain (somatosensory cortex), activation of descending facilitatory and inhibitory pathways, activation of autonomic and motor responses (reticular formation), and behavioral reactions like withdrawal from the source of pain, aggression, amongst others (somatosensory cortex) (Grubb, 2018).

2.1.3. Pathologic pain: peripheral and central sensitization

After tissue damage, cells within the tissue release an assortment of neuroactive substances, including prostaglandins, histamine, serotonin, bradykinin, cytokines, proteases, norepinephrine, and nerve growth factor that will bind to receptors in the nerve endings (Beck & Handwerker, 1974; Koda & Mizumura, 2002; Couteaux et al., 2005). These substances activate a variety of intracellular signaling cascades, that conclude in shifting high-threshold nociceptors into low-threshold nociceptors and activating silent nociceptors. The result of these events is the development of primary hyperalgesia (originates from within the damaged tissue) and secondary
hyperalgesia (originates from the surrounding healthy tissue) (Mizumura, 1997; Muir & Woolf, 2001; Couteaux et al., 2005). This phenomenon is called peripheral sensitization.

Intense or prolonged (chronic) noxious stimulation may cause alterations in patterns of gene expression. This will alter the phenotype of each neuron, providing the nervous system with a substantial degree of neuroplasticity that can result in a phenomenon termed central sensitization (Ma & Woolf, 1996; Neumann et al., 1996). Central sensitization is characterized by allodynia (pain from a normally not painful stimulus), and secondary hyperalgesia (Woolf & Thompson, 1991; Schiable & Richter, 2004), and is manifested by an increase in spinal neuron excitability. These hyperexcitable spinal cord neurons are more susceptible to peripheral inputs and respond strongly to stimulation (Woolf & Wall, 1986; Schiable & Richter, 2004). The development and maintenance of central sensitization involves activity of excitatory and inhibitory interneurons, activation of NMDA receptors, and descending influences from the brainstem (D’Mello & Dickensopn, 2008).

The comprehensive understanding of these processes of the pain pathway are critical to treat pain successfully and efficiently with different drugs and techniques to provide outstanding analgesia and improve animal welfare.

2.2. Assessment of orthopedic pain in horses: pain scales

Adequate diagnosis and treatment of painful conditions is dependent on accurate recognition of pain in animals. Pain is a subjective experience that cannot be verbally communicated by animals. Horses, as prey animals tend to mask pain, making its diagnosis even more difficult in clinical practice. Unfortunately, no ‘gold standard’ is available to quantify and qualify pain in horses. Additionally, inter-individual variation in pain expression may be a factor
complicating the accurate assessment of pain (Wagner, 2010). Clinical studies have tended to focus on differences in physiological, endocrine (hormonal/mediator concentrations) and/or behavioral parameters over time and with analgesic treatment in horses with a range of painful conditions, including laminitis, synovitis, and colic. These studies have led to the realization that one pain assessment tool or system may not perform equally well for different types of pain (e.g., visceral vs. somatic pain, acute vs. chronic pain, nociceptive vs. inflammatory vs. neuropathic pain) (de Grauw & van Loon, 2016).

Since pain is a complex physiologic process that involves changes in several neuroendocrine parameters and behaviors, there is no one single measure that reliably assesses pain (de Grauw & van Loon, 2016). For example, physiologic parameters such as heart rate and respiratory rate are non-specific for the presence and severity of pain, since they may be influenced by other factors, including ambient temperature, dehydration, excitement and cardiovascular and/or respiratory disease. Endocrine measures such as circulating endogenous cortisol, β-endorphins and catecholamines may be used to assess pain. However, changes in these parameters may reflect stress responses that may not be pain-induced (de Grauw & van Loon, 2016). On the other hand, unpleasant sensory and emotional experiences that constitute pain give rise to subtle or overt changes in behavior that may offer the strongest indication of the presence, localization, and severity of the pain. Aspects of behavior that may be altered by pain include elements of demeanor, posture, and gait, as well as interactive behavior. Nevertheless, a horse's behavior is influenced by factors other than pain, including breed, temperament, sex, age, and familiarity with environment (Wagner, 2010). Pain expression is also dependent on the type and origin of pain. For instance, somatic and visceral pain are different phenomena that manifest differently and need to be treated accordingly (van Loon & Van Dierendonck, 2019)
Systematic assessment of pain using defined and validated pain scoring systems or scales help to improve recognition and treatment of painful conditions (de Grauw & van Loon, 2016). For a pain scoring system to reliably work in practice, it should be easy to use, with relevant well-defined parameters that can be assessed repeatedly and quickly by different observers with consistent results (Wagner, 2010). The tools that have been investigated and employed for semi-objective assessment of pain in horses are the visual analogue scale (VAS), simple descriptive scale (SDS), numerical rating scale (NRS), composite measure pain scales (CMPS), and scales based on facial expressions of pain (Grimace scales) (Wagner, 2010).

2.3. Orthopedic pain management in horses

Most drugs used for the treatment of pain in horses fall into one of four broad categories: steroidal, NSAIDs, opioids, and local anesthetics (Muir, 2010). Equine orthopedic pain management has become a growing area of interest and is a vital part of equine orthopedic surgery. Its importance has been emphasized in many studies, proving that appropriate pain management results in decreased postoperative complications (chronic pain, support-limb laminitis, gastric ulceration, and gastrointestinal disease), can decrease recovery time, decrease physiologic stress on the animal, and provide maximum comfort during the postoperative period (Baller & Hendrickson, 2002; Goodrich, 2008). Proper pain treatment for limb diseases will also decrease the incidence and risk of support-limb laminitis, which is a sequela of poor orthopedic pain management on the contralateral limb.
2.3.1. Local anesthetics

Local anesthetics (LA) block the propagation of action potentials along nerve fibers because of the inactivation of voltage-gated sodium channels. LAs cross the cell membranes as free bases (unionized). Inside the cells, they become ionized and bind to specific amino acids within the sodium channel thus blocking the pore (Mazoit, 2012). The local anesthetics have a long history of use in horses and are administered by many routes (e.g., intraarticular, perineural, epidural) to facilitate lameness evaluation and surgical procedures (Mama & Hector, 2019).

2.3.2. Nonsteroidal anti-inflammatory drugs

The most common drug class used for the treatment of pain in horses are the NSAIDs (Baller & Hendrickson, 2002; Sanchez & Robertson, 2014; Walliser et al., 2015). Their use in horses is common due to ease of obtaining them, relatively low cost and limited need for record keeping (Mama & Hector, 2019). NSAIDs have potent anti-inflammatory, analgesic, and antipyretic activity (Bacchi et al., 2012). They minimize the effect of inflammatory mediators in the periphery and reduce noxious input to the central nervous system (Mama & Hector, 2019).

The mechanism of action of the NSAIDs is the inhibition of cyclo-oxygenase (COX), the enzyme that metabolizes arachidonic acid for synthesizing the prostaglandins and thromboxane (Lees & Higgins, 1985; Bacchi et al., 2012). The two enzymes that make up this pathway are COX-1 and COX-2. COX-1 is expressed constitutively in most tissues, it is responsible of homeostatic functions such as renoprotection and gastroprotection. COX-2 is also constitutive in the CNS, kidney, eye, pyloric and duodenal mucosa, endothelial cells and reproductive organs but becomes markedly upregulated during inflammation in the affected tissues (Muir, 2010; Lees,
2018). COX-2 is inducible in injured tissues, which results in prostaglandin production and inflammation (Goodrich, 2008). Prostaglandins and leukotrienes are key factors in the production of peripheral sensitization and likely are important in augmenting central sensitization. NSAIDs are effective analgesics for the treatment of mild to moderate inflammatory pain, emphasizing the importance of prostaglandin production in the initiation and maintenance of pain (Muir, 2010). Phenylbutazone, flunixin meglumine, and firocoxib are among the most used (Doucet et al., 2008; Foreman et al., 2012; Soma et al., 2012).

2.3.3. Opioids

2.3.3.1. Opioid receptors

Four types of opioid receptors have been identified thus far: µ, κ, δ, and nociception/orphanin FQ receptor (NOP-R) (KuKanich & Papich, 2018). These receptors belong to the large superfamily of G protein-coupled receptors (GPCRs) and are activated by endogenous opioid peptides (β-endorphin activates predominantly µ-opioid receptors, leucine- and methionine-enkephalin activate δ-opioid receptors, and dynorphin A is the endogenous ligand of the κ-opioid receptors) and exogenous opioid compounds (morphine, buprenorphine, butorphanol, among others) (KuKanich & Papich, 2018). Opioid receptors have a wide distribution in the body tissues including the CNS, where they can be located on the lamina I and II of dorsal horn, periaqueductal grey matter, medial thalamic nuclei, intralaminar thalamic nuclei, nuclei tractus solitarius and peripheral nervous systems (KuKanich & Wiese, 2015). In the peripheral nervous system, the opioid receptors are located on sensory nerve endings. About a third of C fibers carry µ or δ receptors which contribute to the peripheral analgesic effect of opioids (Stein, 1995). Additionally, opioid receptors are present in the gastrointestinal system, kidneys, lungs, adrenal
glands, and reproductive system. They participate in different physiological and pathological processes such as pain modulation, immunomodulation, cardiovascular regulation, sexual activity, food intake and emotional responses (Atweh & Kuhar, 1983; Wittert et al., 1996; Bu et al., 2020).

Depending on the interaction with the receptor and effect elicited upon binding to the receptor, opioid drugs may be classified as full µ agonists, partial agonists, antagonists, or a combination of µ antagonist and κ agonist. Full µ agonists elicit a dose dependent effect and eventually plateaus (maximum effect), a partial µ agonist activates the receptor but plateaus at a sub-maximum response, and an antagonist binds to the receptor but does not activate it and may displace agonists from it (Kukanich & Wiese, 2015; Kukanich & Papich, 2018).

2.3.3.2. Full µ-agonists

2.3.3.2.1. Morphine

Its analgesic effects in horses at clinically relevant doses (0.1-0.2 mg kg⁻¹) are still controversial (Bennett & Steffy, 2002; Clark et al., 2005; Clutton et al., 2010). An experimental study evaluating the analgesic effect of intravenous (IV) morphine at 0.1 and 0.2 mg kg⁻¹, levomethadone at 0.1 and 0.2 mg kg⁻¹, and butorphanol at 0.1 and 0.2 mg kg⁻¹ using thermal threshold testing (contact incremental heat applied to the skin at the withers), found that both doses of butorphanol, both doses of levomethadone, but only the high dose of morphine induced a significant change in thermal thresholds (Dönselmann Im Sande et al., 2017). Interestingly, studies evaluating systemic administered morphine for the treatment of orthopedic pain are inexistent.

Two major side effects have been of concern after the systemic administration of morphine. One is the CNS stimulatory effect and the second is the decrease in GI motility (Combie et al.,
The administration of systemic doses above 0.5 mg kg\(^{-1}\) of morphine have been associated with increased locomotion making its use potentially dangerous to the animal and personnel (Combie et al., 1981). But also, with a dose of 0.05-0.1 mg kg\(^{-1}\) increased locomotor activity can be observed. The effect at this dose range lasted only for 5 minutes after administration. With an increase in dose a prolongation of duration of increased locomotor activity can be seen. A dose of 0.2 mg kg\(^{-1}\) dose can increase locomotion up to 5 hours, and with a dose of 0.5 mg kg\(^{-1}\) such an effect can be seen up to 5 hours after administration (Hamamoto-Hardman et al., 2018). The second concern is that morphine at 0.05, 0.1, 0.2, and 0.5 mg kg\(^{-1}\) IV, was associated with a decrease in gastrointestinal borborygmi with the most prolonged decrease noted in the 0.5 mg kg\(^{-1}\) dose group. Fecal output was also decreased relative to baseline in the 0.5 mg kg\(^{-1}\) dose group. It was concluded that the highest incidence and duration of the effects (increased locomotion and decreased gastrointestinal motility) were seen with the highest dose given (Hamamoto-Hardman et al., 2018).

### 2.3.3.3. K-agonists μ-antagonists

#### 2.3.3.3.1. Butorphanol

K-agonists μ-antagonists’ opioids are drugs that have high affinity for κ-opioid and μ-opioid receptors, with efficacy to activate κ-receptors but no efficacy to activate μ-receptors, thus it acts as a competitive antagonist for μ (Helm et al., 2008). The analgesic properties of the κ-agonist μ-antagonist butorphanol have been investigated over the years. Kalpravidh et al. (1984) compared the effects of butorphanol and pentazocine (another κ-agonist μ-antagonist rarely used in veterinary medicine) administered IV to horses at 0.05, 0.1, and 0.4 mg kg\(^{-1}\) (for butorphanol) and 2.2 mg kg\(^{-1}\) (for pentazocine). They concluded that the highest dose of butorphanol (0.4 mg
kg\(^{-1}\)) provided the most intense and longest analgesia (90 minutes) when compared with pentazocine (30 minutes). In another study, the analgesic effects of IV butorphanol at a dosage of 0.025 mg kg\(^{-1}\) was assessed in detomidine sedated horses. Butorphanol was found to increase the nociceptive threshold up to 75 minutes in a model of pain using pneumatic pressure over the canon bone and an electrical current stimulus over the coronary band (Schatzman et al., 2001). Butorphanol demonstrated to be less efficacious than buprenorphine for the perioperative orthopedic pain management (Taylor et al., 2016).

2.3.3.4. Partial \(\mu\)-agonists

2.3.3.4.1. Buprenorphine

Buprenorphine is a partial \(\mu\)-agonist opioid that has been used in horses for the treatment of pain (Rigotti et al., 2014; Walker, 2007; Taylor et al., 2016). Its thermal antinociceptive effects have been demonstrated experimentally. Compared to butorphanol, buprenorphine elicits a greater analgesic effect (Love et al., 2012). Carregaro et al. (2007) studied the antinociceptive effects and the incidence of spontaneous locomotor activity of increasing doses of buprenorphine (5-10 \(\mu\)g kg\(^{-1}\)). The IV injection of buprenorphine caused a dose-dependent increase in spontaneous locomotor activity, but only the dose of 10 \(\mu\)g kg\(^{-1}\) induced analgesia based on results for the experimental method used (hoof-withdrawal reflex latency and skin-twitching reflex latency after painful stimulation with a heat lamp).

In a multi-center study, the perioperative analgesic effects of IV buprenorphine at 5-10 \(\mu\)g kg\(^{-1}\) and butorphanol at 0.02-0.1 mg kg\(^{-1}\) were compared in horses undergoing soft tissue or orthopedic surgery. Postoperative pain scores were significantly lower in the buprenorphine group (Taylor et al., 2016). In ponies undergoing field castration IV buprenorphine at 5 \(\mu\)g kg\(^{-1}\) provided
a better maintenance of the anesthetic plane and decreased the requirement for rescue analgesia postoperatively, when compared to IV butorphanol at 0.025 mg kg\(^{-1}\) (Rigotti et al., 2014). A further study in ponies showed that the IV administration of 10 µg kg\(^{-1}\) of buprenorphine compared to placebo (5% glucose) prior to surgical castration provided effective postoperative analgesia measured with a dynamic interactive visual analogue scale and a simple descriptive scale (Love et al., 2013).

### 2.3.3.4.2. Pharmacokinetics of systemic buprenorphine in horses

A noncompartmental model analysis fitted the data obtained in the three pharmacokinetic studies where IV buprenorphine was given to adult horses. In each study, a different dose was used (5, 6, and 10 µg kg\(^{-1}\)). The two lower doses (5 and 6 µg kg\(^{-1}\)) showed half-lives of 3.5 and 5.8 hrs., respectively, clearance of 7.9- and 6.1-mL kg\(^{-1}\) min\(^{-1}\), respectively, and a similar volume of distribution (3 and 3.1 L kg\(^{-1}\) min\(^{-1}\), respectively). With the higher dose (10 µg kg\(^{-1}\)) the volume of distribution was like the other doses (2.7 L kg\(^{-1}\) min\(^{-1}\)), but the clearance was remarkably higher (19.6 mL kg\(^{-1}\) min\(^{-1}\)). The different clearance between the high and the low doses may indicate that it is nonlinear, and it increases with dose. A hypothesis to explain this statement could be that with higher doses there might be an increase in hepatic blood flow mediated by an increase in sympathetic stimulation and thus cardiac output leading to greater clearance (Carregaro et al., 2006). The large volume of distribution seen in all the investigations may be reflective of buprenorphine’s high lipophilicity (Messenger et al., 2011; Davis et al., 2012; Love et al., 2015). Furthermore, in these studies the plasma concentrations detected were at levels that are considered to be analgesic in other species (~1 ng mL\(^{-1}\)) (Watson et al., 1982; Nolan et al., 1987; Ko et al., 2011).
Following IM administration of a dose of 5 µg kg\(^{-1}\), the bioavailability of buprenorphine was variable (51-88%) which makes this route of administration less desirable as the concentrations might not be consistent to produce analgesia (Davis et al., 2012).

2.3.4. Intra-articular analgesia

Intra-articular use of analgesic agents has been a successful method of controlling pain in humans, dogs, and horses over the years (Sammarco et al., 1996; Schumacher et al., 2000; Dutton et al., 2014; Richards et al., 2016). It offers pain control directly at the location being manipulated (Baller & Hendrickson, 2002). Therefore, the aim of IA administered analgesics is to decrease the nociceptive input from the afferent articular nerves.

Several drug classes have been used to provide IA analgesia. The analgesic efficacy of local anesthetics, opioids, NSAIDs, α₂-adrenergic receptor agonists, corticosteroids, and tramadol have been evaluated in humans and different animal species (Raekallio et al., 1996; Sammarco et al., 1996; Stein et al., 1999; Alagol et al., 2004; Ozyuvaci et al., 2004; Santos et al., 2009; Lindegaard et al., 2010a; van Loon et al., 2010; Gomes et al., 2021). Of importance is, that any drug administered IA is not chondrotoxic, which in the long term could be more deleterious than the benefit of producing immediate pain relief. Therefore, in the following section a review on the drugs used IA, their potential chondrotoxic effects and clinical efficacy is presented.

2.3.4.1. Local anesthetics for intra-articular analgesia

Single-dose IA injections of local anesthetics are often used for diagnostic tests and to relieve pain after injury, surgery, or in degenerative joint diseases with inflammation (Park et al., 2011; Kreuz et al., 2018). In humans, IA local anesthetics have been shown to reduce postoperative
pain following total knee arthroplasty (Fang et al., 2015). In the past bupivacaine, lidocaine, and mepivacaine have been used in humans, dogs, and horses to provide IA analgesia.

Bupivacaine is an amide type local anesthetic that is commonly used in human and veterinary medicine to provide locoregional anesthesia due to its long-lasting effect. In humans, several reports of its IA use have demonstrated that it decreases pain scores or reduces supplementary analgesia requirements after arthroscopy (Raja et al., 1992; Allen et al., 1993; Boden et al., 1994; Alagöl et al., 2005). In a study in dogs, bupivacaine injected in the stifle joint produced analgesia for up to 24 hours after ruptured cruciate ligament surgery (Sammarco et al., 1996). In another investigation it was seen that IA bupivacaine in dogs undergoing elbow arthroscopy decreased pain scores at 4- and 24-hours post injection compared to IA morphine and no treatment (Gurney et al., 2012).

Even though its use has resulted in analgesia, there are reports about its chondrotoxicity. Park et al. (2011) tested the chondrotoxicity of bupivacaine, lidocaine and mepivacaine in cultured equine chondrocytes. Bupivacaine was the most toxic one of the three local anesthetics assessed, followed by lidocaine and mepivacaine. Adler et al., (2021) tested the chondrotoxic effects of different concentrations of bupivacaine, lidocaine, mepivacaine, and ropivacaine on cultured equine chondrocytes and fibroblast-like synoviocytes. Bupivacaine was the most toxic among the local anesthetics tested to the cultured chondrocytes. However, in vitro results may not necessarily reflect in vivo drug dynamics due to drug removal from the joint over time and dilution of drug with SF, the damage to the chondrocytes may be limited and less severe as seen with in vitro studies (Park et al., 2011). The mechanism for the chondrotoxic effect of local anesthetic has not been completely clarified. It has been hypothesized that by influencing sodium, calcium, and
potassium channels, local anesthetics may lead to mitochondrial dysfunction decreased energy production and subsequent cell necrosis or apoptosis (Kreuz et al., 2018).

Although there is sufficient evidence stating that bupivacaine has immediate chondrotoxic effects, a study in rabbits showed no permanent impairment of cartilage function after three months of exposure to a 48-hours IA infusion of 0.25% bupivacaine. In this study, cartilage metabolism was increased, possibly beyond that of normal chondrocyte physiology, as a reparative response to the damage (Gomoll et al., 2009). The single IA administration of bupivacaine in dogs with and without osteoarthritis showed a rapid clearance of bupivacaine from SF in normal and osteoarthritic joints. This leads to the conclusion, that a single injection of IA bupivacaine is unlikely to damage chondrocytes due to its rapid clearance from the joint (Barry et al., 2015).

2.3.4.2. Opioids for intra-articular analgesia

Opioid receptors can be found in the synovium of humans, dogs, rats, and horses (Keates et al., 1999; Sheely et al., 2001; Hayashi et al., 2002; Mousa et al., 2007). μ-opioid receptors have been detected in the synovium of rats and humans with osteoarthritis, rheumatoid arthritis, and joint trauma. κ- and δ- receptors have been demonstrated in human’s healthy fibroblast-like synoviocytes and they are downregulated in rheumatoid arthritis and osteoarthritis (Shen et al., 2005). The density of μ-opioid receptors is significantly increased in inflamed joints compared to healthy joints which could imply that these receptors also play a role in inflammation (Keates et al., 1999; van Loon et al., 2013). Results of an immunohistochemical analysis and radioligand binding of tissue homogenates suggested the presence of opioid receptors in synovial membranes of horses (Sheely et al., 2006). This finding supports the practice of IA administration of opioids to relieve joint pain in horses.
2.3.4.2.1. Morphine for intra-articular analgesia

A systematic literature review on the effects of IA morphine compared to placebo in humans, summarized that morphine produced a definite reduction in postoperative pain intensity compared with placebo (Gupta et al., 2001).

The cytotoxic effects of morphine and morphine-6-glucuronide (M6G), which is one of the metabolites of morphine have been evaluated in cultured human chondrocytes. No negative effects on cell viability were detectable after exposure to 0.05, 0.025, 0.0125 % morphine or equal concentrations of M6G (Ickert et al., 2015). Another study assessed the effects of 0.285% of morphine in equine synoviocytes and cartilage explants after 2 hours of exposure, showing no cytotoxic effects (Rubio-Martinez et al., 2017). Furthermore, in cultured dog chondrocytes morphine did not demonstrate evidence of chondrotoxicity after 24 and 48 hours of exposure (Anz et al., 2009).

The analgesic and anti-inflammatory effects of IA morphine have been studied in horses with lipopolysaccharide (LPS)-induced synovitis (Santos et al., 2009; Lindegaard et al., 2010a; Lindegaard et al., 2010c). Santos et al. (2010) compared the analgesic effects of IA morphine, ropivacaine, and the combination of both in horses with LPS induced synovitis. Analgesia was measured subjectively using a numerical rating scale, a simple descriptive scale, pain upon maximal flexion of the carpus and by the range of motion exhibited by the affected joint. Ropivacaine had a fast onset of analgesic effect, but of short duration (2.5-3.5 hours approximately) whereas morphine had a slower onset of action and a longer duration of analgesic effect. The combination of both drugs produced a strong analgesic effect with a total duration of 24 hours (Santos et al., 2009).
Lindegaard et al. (2010a) compared the analgesic effects of IA morphine with IV morphine in horses with LPS-induced synovitis. In this paper, pain was evaluated by use of a visual analogue scale of pain intensity (VAS) and a composite measure pain scale (CMPS) and lameness was scored using an eight (0-7) grade scale. An analgesic effect of IA morphine was demonstrated by significantly less lameness than treatment with the same dose of morphine IV.

Opioid receptors are upregulated in inflamed joints of horses (Sheely et al., 2006). Lindegaard et al. (2010b) investigated the anti-inflammatory effects of IA morphine in horses with LPS-induced synovitis. The IA administration of morphine resulted in significantly less joint swelling and lower SF total protein and SF amyloid A concentrations.

Based on the concern about the effects of morphine in horses after systemic administration (decreased gastrointestinal motility and CNS stimulation) the pharmacokinetic profile of IA morphine has been investigated. Raekallio et al. (1996) measured morphine and its metabolites concentrations in plasma of ponies that had been IA injected with morphine. Overall, the plasma concentrations of morphine and its metabolites after IA administration were too low to cause any noticeable systemic effects. After injection of IA morphine in horses with LPS-induced synovitis the IA and plasma concentrations of morphine and its metabolites (morphine-3-glucuronide (M3G) and M6G) were measured repeatedly over a period of 28 hours. Morphine could be detected for at least 24 hours in the SF (above 10.2 ng mL\(^{-1}\)) and low plasma concentrations, less than 5 ng mL\(^{-1}\) were retrieved. M3G and M6G concentrations peaked at 2 hours in SF and plasma and were detected at low concentrations thereafter (Lindegaard et al., 2010c).
2.3.4.2.2. Buprenorphine for intra-articular analgesia

Buprenorphine also has local analgesic effects and has been used to treat IA pain in humans (Fellahi et al., 1995; Varrassi et al., 1999; Präger et al., 2007; Sugandarajappa et al., 2016; Das & Samal, 2019). The injection of 0.3 mg of buprenorphine compared 0.9% sodium chloride (placebo) into the shoulder joint resulted in significant lower pain scores one hour after surgery and longer intervals to the time of rescue analgesia. (Fellahi et al., 1995).

Varrassi et al. (1999) conducted a double-blinded trial in people undergoing knee arthroscopy comparing the analgesic effects IA buprenorphine (0.1 mg) plus IM saline, IA bupivacaine (50 mg) plus IM saline, and IA saline plus IM buprenorphine (0.1 mg). In this study, pain was evaluated using a visual analogue scale (VAS). Patients receiving IM buprenorphine or placebo had higher VAS scores than the ones receiving IA buprenorphine or IA bupivacaine. Intra-articular buprenorphine and IA bupivacaine had similar analgesia but were superior to systemic buprenorphine. (Varrassi et al., 1999).

In a prospective double-blinded clinical trial the analgesic effects of buprenorphine injected in the temporomandibular joint of human patients suffering from anterior disc displacement with or without reduction, were compared to saline. Pain was assessed by means of a VAS. In the buprenorphine group VAS scores and joint mobility were improved compared to saline, concluding that IA buprenorphine provided pain relief in these patients (Präger et al., 2007).

Analgesia from IA buprenorphine has been compared against IA morphine in people (Sugandarajappa et al., 2016; Das & Samal, 2019). In a prospective, randomized, placebo-controlled double-blinded comparative study, 0.1 mg of IA buprenorphine was compared to 3 mg of IA morphine and placebo in patients following arthroscopic knee surgery. Pain was assessed with VAS and hemodynamic data (heart rate and blood pressure) over a period of 8 hours after
surgery. After one hour of administration, buprenorphine and morphine had comparable analgesic efficacy. After 4 hours until 8 hours post treatment buprenorphine had lower VAS scores and reduced supplementary analgesia requirements (Sugandarajappa et al., 2016).

2.3.4.3. Other drugs used for intra-articular analgesia: tramadol, non-steroidal anti-inflammatory drugs, and alpha-2 adrenergic agonists.

2.3.4.3.1. Tramadol for intra-articular analgesia

Tramadol is a weak inhibitor of μ-opioid receptors and an inhibitor of serotonin and noradrenalin reuptake (Di Salvo et al., 2018). In human medicine tramadol is a well-established systemic analgesic for acute and chronic pain. In humans, tramadol is metabolized by cytochrome P450 enzymes. The most important metabolite is O-desmethyltramadol (M1), which has more affinity to the μ-opioid receptors than the parent compound, thus most of the analgesic effect of tramadol has been attributed to its metabolite (Raffa et al., 1992). Several studies have investigated the pharmacokinetics of tramadol in horses after oral, IV and IM administration (Shilo et al., 2008; Stewart et al., 2011; Knych et al., 2013a; Knych et al., 2013b). Bioavailability is poor after oral administration regardless of the dose used (3-10 mg kg\(^{-1}\)), and the plasma concentrations of the M1 metabolite were only transiently maintained (Stewart et al., 2011; Knych et al., 2013a). Following IV and IM administration the plasma concentrations of tramadol and M1 achieved levels that produce analgesia in humans (Shilo et al., 2008; Knych et al., 2013b). However, the analgesic effects of tramadol or M1 have not yet been described in horses.

Tramadol has also been used for IA administration in people providing good pain relief by demonstrating similar analgesia to IA morphine (Akinci et al., 2005). The chondrotoxicity of different concentrations of tramadol were evaluated in cultured equine chondrocytes and
concentrations below 0.4 mg mL$^{-1}$ have low toxic effects on chondrocytes (Di Salvo et al., 2018). In the same study, the analgesic effects of IA tramadol were evaluated. Pain scores obtained in tramadol-treated horses were lower between 1- and 6-hours post-administration than those obtained in the control group. However, the differences were not statistically significant.

2.3.4.3.2. Nonsteroidal anti-inflammatory drugs for intra-articular analgesia

Some NSAIDs such as tenoxicam and ketorolac have been investigated for their potential analgesic effect when injected IA in humans and rats (Cook et al., 1997; Riggin et al., 2014). Tenoxicam has demonstrated confounding results. In one study in people, the IA administration of tenoxicam after knee arthroscopy reduced oral analgesic requirements during the first day after surgery but did not affect the perception of pain measured by VAS (Cook et al., 1997). A potential detrimental effect of IA tenoxicam was described after IA injection in rats, where after 24- and 48-hours of injection joints demonstrated tissue loss and edema (Ozyuvaci et al., 2004). Ketorolac was assessed in rat joints aiming to determine the safety of IA administration. It was found that the parameters measured for joint mobility and histologic evaluation of the articular cartilage after the treatment were not different when compared to a control, demonstrating the potential safety of its IA use (Riggin et al., 2014).

2.3.4.3.3. Alpha-2 adrenergic receptor agonists for intra-articular analgesia

Alpha-2 adrenergic receptor agonist drugs such as dexmedetomidine and clonidine have also been used for the management of IA pain in people and rats (Gentili et al., 1996; Al-Metwalli et al., 2008; Paul et al., 2010; Alipour et al., 2014; Panigrahi et al., 2015; Gomes et al., 2021). Intra-articular administration of dexmedetomidine improved pain outcomes in the early
postoperative period in humans after arthroscopic knee surgery (Peng et al., 2018). The chondrotoxic effects of xylazine and dexmedetomidine were evaluated in cultured equine chondrocytes, showing that there is a dose-dependent decrease in cell viability with both drugs (Mancini et al., 2017).
Chapter Three.
Toxicity assessment of buprenorphine on equine articular chondrocytes in vitro

3.1. Introduction

Successful pain relief after diagnostic or therapeutic procedures has been demonstrated with IA administration of various pain medications in horses (Santos et al., 2009; Lindegaard et al., 2010a). Drugs used IA should have local analgesic effects while having no detrimental effects on cartilage. Local anesthetics appear to lack this attribute, as chondrotoxic effects have been reported in different species, including dogs, cattle, and horses (Anz et al., 2009; Lo et al., 2009, Park et al., 2011).

Opioids present as an alternative to local anesthetics for IA pain management. Morphine has been used for more than twenty years to treat joint pain in humans and horses, exhibiting anti-inflammatory and analgesic effects (Kalso et al., 1997; Lindegaard et al., 2010b). The presence of opioid receptors in the equine joints has been demonstrated (Sheehy et al., 2001), further supporting the IA use of opioids for pain management. Its analgesic efficacy has been linked to the upregulation of μ-opioid receptors in inflamed joints (van Loon et al., 2013).

Buprenorphine is a partial μ-opioid receptor agonist, approximately 25 times more potent than morphine and with a higher receptor affinity (KuKanich & Wiese, 2015). Buprenorphine has been used systemically for the treatment of pre- and post-operative pain in several species such as humans, dogs, cats, and horses (Roughan & Flecknell, 2002; Steagall et al., 2009; Love et al., 2013; Rigotti et al., 2014; Jonan et al., 2018). In humans, IA buprenorphine after arthrocentesis of the temporomandibular joint, provided pain relief and increased joint mobility when compared with saline (Präger et al., 2007). Other studies in humans report that IA buprenorphine provided
superior analgesia when compared with IA morphine after arthroscopic knee surgery (Varrassi et al., 1999; Sugandarajappa et al., 2016).

Before recommending the IA use of buprenorphine in horses it is important to assess its toxicity to cartilage, in particular the chondrocytes. The chondrotoxic effects of buprenorphine have not been investigated before in any species. The aim of this study was to evaluate the chondrotoxic effects of buprenorphine on equine articular chondrocytes in vitro. We hypothesized that buprenorphine would not cause any toxic effects on cultured equine chondrocytes independent of the concentration and duration of exposure.

3.2. Materials and Methods

3.2.1 Chondrocyte culture

Equine articular chondrocytes used in this study were isolated and propagated as previously reported by Nixon et al. (1992). Briefly, equine articular cartilage was aseptically collected from both stifles, left shoulder, and left metacarpophalangeal joints of three horses euthanized for reasons unrelated to this study. After collection, the cartilage was placed in Hank’s Balanced Salt Solution (HBSS) containing penicillin and streptomycin (P/S) (Gibco, MT, USA). Then, the cartilage was minced under sterile conditions and rinsed with Phosphate Buffer Saline (PBS) (GE Healthcare Life Sciences, UT, USA). Afterwards, minced cartilage was enzymatically digested in collagenase type II. The cartilage was incubated at 37°C for 12 hours in the digestion media, after which the cell suspension was filtered through a sterile cell strainer (100 µM). The filtered fluid was then centrifuged at 260xg for 10 minutes at 20°C to pellet the cells, and the supernatant was removed. The cell pellet was resuspended in Dulbecco’s Modified Eagles’ Medium (DMEM)-high glucose/F12 (Gibco, NY, USA) to wash by centrifuging again at 260xg for 10 minutes at
20°C. The supernatant was then removed, and the cell pellet resuspended in DMEM-F12 medium. The cells were counted and plated at a density of 3-4 x 10^5 cells/cm^2 and were incubated at 5% CO_2 and 37°C in a humidified atmosphere until the cells formed adequate monolayers for propagation. Cells from passage 1 through 3 were used for the experiments. Once chondrocytes were confluent, they were plated into wells for the experiment.

### 3.2.2. Treatment groups

Chondrocytes were exposed to the following treatments: 1) media only (MED) (DMEM/F12; Gibco, NY, USA) as a negative control, 2) bupivacaine (BUPI) (Preservative-free Bupivacaine HCl; Hospira, Inc., IL, USA) diluted in DMEM/F12 to achieve a concentration of 2.2 mg mL^{-1} as a positive control, 3) high dose buprenorphine (HIGH BUPRE) (Buprenorphine HCl; Par Pharmaceutical, NY, USA) diluted in DMEM/F12 to achieve a concentration of 0.12 mg mL^{-1}, 4) low dose buprenorphine (LOW BUPRE) (Buprenorphine HCl; Par Pharmaceutical, NY, USA) diluted in DMEM/F12 to get a concentration of 0.05 mg mL^{-1}, and 5) morphine (MOR) (Preservative-free Morphine Sulfate; Hospira, Inc., IL, USA) diluted in DMEM/F12 to achieve a concentration of 2.85 mg mL^{-1}. Chondrocytes were exposed to each of the treatments for 0 hours (T0), and 2 hours (T2). Chondrocyte viability was evaluated using the following techniques: live-dead fluorescent staining, Water-Soluble Tetrazolium salts-8 (WST-8) assay to detect mitochondrial activity, and measurement of lactate dehydrogenase (LDH) concentrations.
3.2.3. Cell viability tests

3.2.3.1. Live-Dead Fluorescence staining of chondrocytes

Equine chondrocytes were cultured in 24-well plates. After confluency was achieved, DMEM/F-12 media was removed from each well and treatments were added in triplicate. Plates were incubated for 0 and 2 hours, respectively. After each treatment time was completed, chondrocytes were stained with Hoechst 33342 (all cells) and propidium iodide (PI; dead) stains. Treatments were not removed prior to the addition of the stains. Hoechst 33342 was diluted to 1:1000 in phosphate buffered saline and 500 ul of Hoechst 33342 was added to each well. PI stain was diluted per manufacturer instructions at 1:500 with phosphate buffered saline and 400 ul of PI stain was added to each well. Following, the addition of both Hoechst 33342 and PI the 24 well plates were incubated at room temperature in the dark for 10 minutes. Equine chondrocytes from each treatment group were observed using Zoe. Cells were observed under phase contrast first, followed fluorescence using the blue (350/380nm) and red (530/617nm) spectrums at 10X magnification. Images were captured using the microscope’s camera.

3.2.3.2. Mitochondrial activity determined by Water-Soluble Tetrazolium salts assay (WST-8)

Chondrocytes were seeded at 3 x 10^4 cells/cm^2 in 96-well plates and incubated at 37°C until confluency was achieved. For the experiment, DMEM/F12 media was removed, and each treatment as described above was added to its respective well and incubated for the designated times. All treatments were evaluated in quadruplicate. A no-cell well (media/treatment only) was included each group as a background control for the WST assay. The WST assay (Dojindo Molecular Technologies, Inc., Rockville, MD) was performed per manufacturer instructions. For
T0, immediately after treatments were added, 10 µL of the WST-8 reagent was also added to each well and incubated at 37° C for 2 hours. After the 2-hour incubation, the optical density for each well was determined at 450 nm using a spectrophotometer (BioTek Synergy, Agilent Technologies, Inc., Santa Clara, CA). For the T2 time-point, treatments were added as described above and the cells were incubated for 2 hours at 37° C. Following that incubation, 10 µL of the WST-8 reagent was added to each well and the plate was incubated for 2 more hours at 37° C. As previously, the plate was read at 450 nm using the spectrophotometer to obtain optical density for each well. No-cell background well readings were subtracted from the optical densities for each well for the respective treatments to eliminate variation in readings due to treatment dilutions.

### 3.2.3.3. Measurement of lactate dehydrogenase (LDH) concentrations

Equine chondrocytes were cultured to confluency in 24 well plates. DMEM/F12 media was removed from each well and the respective treatments added in duplicate. Controls for the LDH assay were also included: no-cell stimulation (media±treatment) as a negative control and killed cells (2 µL of 10% trition-X per 100 µL of media) as a positive control. For the T0 samples, 5 µL of supernatant was removed from each well immediately after the application of the treatments and added to 95 µL of LDH storage buffer. For the T2 samples, 5 µL supernatant was removed from each well after 2 hour incubation at 37° and added to 95 µL of LDH storage buffer. After collection supernatant was immediately frozen at -80°C until further analysis.

LDH analysis was performed using the LDH-Glo Cytotox Assay (Promega, Madison, WS) per manufacture instructions. Briefly, 50 µL of supernatant in LDH storage buffer from each sample was added to their respective wells on a 96 well plate followed by the addition of 50 µL the LDH detection reagent. Serial dilutions of the LDH standard was also included on the plate.
All samples and standards were tested in duplicate. The plate was incubated at room temperature (21°C) for 60 minutes. Following incubation, luminescence was detected for each sample using a plate reader (BioTek Synergy, Agilent Technologies, Inc., Santa Clara, CA) and concentrations determined from the standard curve.

3.3. Statistical analysis

Data analyses were performed using JMP Pro 16.1.0 (SAS Institute Inc.). All continuous variables (LDH, WST) were evaluated with a mixed ANOVA with treatment, time and their interactions as the fixed effects and each animal as the random effect. Assumptions of all ANOVA models (linearity, normality of residuals and homoscedasticity of residuals) and influential data points were assessed by examining standardized residual and quantile plots. When a fixed effect was detected, Tukey post-hoc comparisons were performed with least square means for the effect. Significance was set at $P < 0.05$.

3.4. Results

3.4.1. Live/Dead Fluorescence Staining

Live/Dead staining was subjectively evaluated for each treatment group under phase and fluorescence microscopy. Phase images from the BUPI and HIGH BUPRE treatment groups began to demonstrate cellular changes exhibited by higher numbers of rounded cells at T0 (Figure 3.1). Rounding of the cells continued at T2 for the BUPI and HIGH BUPRE groups with increased distance noted between cells (Figure 3.2). Chondrocytes death was subjectively noted to be increased in the BUPI and HIGH BUPRE groups as greater staining for both Hoechst 33342 (blue) and PI (red) was present in both the T0 and T2 samples (Figures 3.1 and 3.2). While not as
pronounced as the BUPI and HIGH BUPRE group mild subjective increase in dead cells were noted in the MOR and LOW BUPRE groups when compared with MED controls at T2 (Figure 3.2).

Figure 3.1. Live-dead determination at T0: Imaging obtained after equine chondrocytes were stimulated with the following: media only (MED), bupivacaine (BUPI), morphine (MOR), high buprenorphine (HIGH BUPRE), and low buprenorphine (LOW BUPRE). Live and dead staining performed using Hoescht and propidium iodine followed by observation under a fluorescence microscope at T0. The top images display chondrocyte following stimulation under phase at 10X magnification. The bottom images display chondrocytes following simulation with the addition of Hoescht and PI stain. The cells were viewed at 10X magnification via fluorescence from the blue and red spectrums and merged to create the above images. Hoescht dye passes through cell membranes of all cells independent if they are dead or alive and stains them with a blue color. And the propidium iodine dye penetrates only dead cells giving them a red color. Therefore, the merged image demonstrates dead cells as purple.

Figure 3.2. Live-dead determination at T0: Imaging obtained after equine chondrocytes were stimulated with the following: media only (MED), bupivacaine (BUPI), morphine (MOR), high buprenorphine (HIGH BUPRE), and low buprenorphine (LOW BUPRE). Live and dead staining performed using Hoescht and propidium iodine followed by observation under a fluorescence microscope at T2. As above, the top images display chondrocyte following stimulation under phase at 10X magnification. The bottom images display chondrocytes following simulation with the addition of Hoescht and PI stain and viewed at 10X magnification via fluorescence from the blue and red
spectrums. Note the increased cell death in BUPI and HIGH BUPRE groups evident by the increased number of purple/red cells in the merged image shown above.

3.4.2. WST-8 assay

At T0, mitochondrial activity was lowest in BUPI treatment demonstrated by a mean optical density (OD) of 0.051 ± 0.035 (Figure 3.3). Conversely, mitochondrial activity was the highest in the MED, MOR and LOW BUPRE treatments with an OD of 0.235 ± 0.035, 0.283 ± 0.035 and 0.356 ± 0.035, respectively (Figure 3.3). These values were significantly different (P < 0.0001) when compared to the BUPI (0.051 ± 0.035) and HIGH BUPRE (0.141 ± 0.035) treatments. LOW BUPRE OD (0.356 ± 0.035) was significantly higher than MED (0.235 ± 0.035) (P < 0.0001), and BUPI was significantly lower (0.051 ± 0.035) (P < 0.0001) than MED (0.235 ± 0.035) (Figure 3.3).
Figure 3.3. Viability of cultured equine chondrocytes after being exposed for 0 hours (T0) to media (MED), 2.2 mg mL⁻¹ of bupivacaine (BUPI), 0.12mg mL⁻¹ of buprenorphine (HIGH BUPRE), 0.05 mg mL⁻¹ of buprenorphine (LOW BUPRE) and 2.85 mg mL⁻¹ of morphine (MOR) determined with a WST-8 assay. Bars represent the least squares mean of the mean optical density (OD). Error bars represent the standard error. *Significantly higher viability than BUPI and HIGH BUPRE. †Significantly higher viability than BUPI. ‡Significantly higher viability than MED. P < 0.05.

At T2, mitochondrial activity was the highest in the chondrocytes subjected to MED, MOR, or LOW BUPRE with OD means of 0.250 ± 0.035, 0.220 ± 0.035 and 0.236 ± 0.035, respectively. Chondrocytes exposed to BUPI had the least mitochondrial activity with an OD mean of 0.029 ± 0.035 followed by chondrocytes exposed to HIGH BUPRE with and OD mean of 0.057 ± 0.035 (Figure 3.4). MED, MOR, and LOW BUPRE had significantly greater mitochondrial activity (P<0.0001) when compared with chondrocytes exposed to BUPI and HIGH BUPRE (Figure 3.4). There was no difference in mitochondrial activity among chondrocytes exposed to MED, MOR, or LOW BUPRE, nor between those chondrocytes exposed to HIGH BUPRE or BUPI. The only significant difference between time points within treatments was a significant decrease in mitochondrial activity (P = 0.015) noted in the LOW BUPRE between T0 (0.356 ± 0.035) and T2 (0.236 ± 0.035).
Figure 3.4. Viability of cultured equine chondrocytes after being exposed for 2 hours to media (MED), 2.2 mg mL$^{-1}$ of bupivacaine (BUPI), 0.12 mg mL$^{-1}$ of buprenorphine (HIGH BUPRE), 0.05 mg mL$^{-1}$ of buprenorphine (LOW BUPRE) and 2.85 mg mL$^{-1}$ of morphine (MOR) determined with a WST-8 assay. Bars represent the least squares mean of the optical density (OD). Error bars represent the standard error. *Significantly higher viability than BUPI and HIGH BUPRE. P < 0.05.

3.4.3. LDH assay

At T0, supernatant from equine chondrocytes exposed to BUPI (7.71 ± 1.94 mU mL$^{-1}$) and HIGH BUPRE (7.79 ± 1.94 mU mL$^{-1}$) had the highest concentrations of LDH. While supernatant from chondrocytes exposed to MED (2.59 ± 1.94 mU mL$^{-1}$), MOR (2.78 ± 1.94 mU mL$^{-1}$) and LOW BUPRE (3.46 ± 1.94 mU mL$^{-1}$) had lower concentrations of LDH.

At T2, supernatant from equine chondrocytes exposed to MED (0.33 ± 1.94 mU mL$^{-1}$), MOR (2.37 ± 1.94 mU mL$^{-1}$) and LOW BUPRE (3.65 ± 1.94 mU mL$^{-1}$) had the lowest concentrations of LDH. Whereas chondrocytes exposed to BUPI (9.16 ± 1.94 mU mL$^{-1}$) and HIGH BUPRE (12.58 ± 1.94 mU mL$^{-1}$) had higher concentrations of LDH.
Independent of the time of exposure to HIGH BUPRE or BUPI, chondrocyte viability was significantly decreased (P < 0.016) compared to MORP and MED (Figure 3.5). Furthermore, independent of exposure time, chondrocyte viability in HIGH BUPRE was significantly lower (P = 0.0045) than LOW BUPRE (Figure 3.5). There were no significant differences within treatments between timepoints.

![Figure 3.5](image)

Figure 3.5. Viability of cultured equine chondrocytes after being exposed for 0 and 2 hours to media (MED), 2.2 mg mL⁻¹ of bupivacaine (BUPI), 0.12 mg mL⁻¹ of buprenorphine (HIGH BUPRE), 0.05 mg mL⁻¹ of buprenorphine (LOW BUPRE) and 2.85 mg mL⁻¹ of morphine (MOR) determined with a lactate dehydrogenase (LDH) assay. Bars represent the least squares mean of the LDH concentrations. Error bars represent the standard error. *Significantly lower viability than MED and MOR. †Significantly lower viability than LOW BUPRE. P < 0.05.

### 3.5. Discussion

The findings of the present study indicate a concentration-dependent cytotoxic effect of buprenorphine on equine articular chondrocytes *in vitro*. Cultured equine chondrocytes were exposed to two concentrations of buprenorphine. The high concentration of buprenorphine (0.12
mg mL\(^{-1}\)) equals the low end (5 µg kg\(^{-1}\)) of the systemic analgesic dose reported in equids which ranges from 5 – 10 µg kg\(^{-1}\) (Taylor et al., 2016). The low concentration of buprenorphine equals a systemic subclinical dose of 2 µg kg\(^{-1}\). Two studies evaluated the pharmacokinetics and analgesic efficacy of IA morphine in horses with LPS-induced synovitis, using a systemic subclinical dose of morphine of 0.05 mg kg\(^{-1}\) which remained within the joint for at least 24 hours and decreased lameness scores (Lindegaard et al. 2010a, b). Furthermore, studies in humans reported doses of IA buprenorphine as low as 100 µg joint\(^{-1}\), which equals 1.4 µg kg\(^{-1}\) in an average 70 kg person. Systemic analgesic dosages of buprenorphine in humans are in the range of 3 – 8.5 µg kg\(^{-1}\) (Dahan et al., 2006). Even though there is no information on the analgesic efficacy of IA buprenorphine in horses, a subclinical dose of 2 µg kg\(^{-1}\) was chosen since analgesia from IA buprenorphine in humans and IA morphine in horses can be accomplished using lower doses than those used systemically.

Equine chondrocytes exposed to high concentrations of buprenorphine, at 0.12 mg mL\(^{-1}\), had subjectively increased chondrocyte death demonstrated by an increased number of dead cells seen via PI staining 2 hours after administration. Additionally, chondrocytes exposed to high concentrations of buprenorphine demonstrated 4 times less mitochondrial activity than chondrocytes exposed to media, morphine or to low concentration of buprenorphine. Only bupivacaine, which has also been reported to have concentration-dependent cytotoxic effect on canine and human chondrocytes (Brue et al., 2013; Rengert et al., 2021), was seen to have a similar effect in PI staining and mitochondrial activity in this study. Cytotoxicity of bupivacaine to equine chondrocytes \textit{in vitro} has been reported previously at the dose used in this study and therefore was used as positive control (Park et al., 2011; Rubio-Martinez et al, 2017). Furthermore, supernatant from chondrocytes treated with the high concentration of buprenorphine demonstrated the highest
concentration of LDH of all treatments at both timepoints, revealing that cell membrane integrity was disrupted and cell viability decreased.

Morphine, a μ-opioid receptor agonist, when used alone, was previously reported to have no significant cytotoxic effects on equine chondrocytes (Rubio-Martinez et al., 2017) with similar findings demonstrated in this study. As a partial μ-opioid receptor agonist, it was expected that buprenorphine would have a similar response to morphine. However, the current study found a cytotoxic effect of buprenorphine on equine chondrocytes dependent on the concentration tested. Concentration-dependent cytotoxicity has been demonstrated in chondrocytes exposed to select opioids and local anesthetics (Abrams et al., 2017; Adler et al., 2021). Concentration-dependent chondrotoxicity has been reported for the μ-opioid receptor agonist meperidine where the lowest concentration of 0.5% was found to be significantly less chondrotoxic compared with 1% and 1.5% meperidine (Abrams et al., 2017). As mentioned previously, bupivacaine concentrations of 2.5 mg mL\(^{-1}\) and 5 mg mL\(^{-1}\) resulted in decreased canine chondrocyte viability based on significantly less MTT activity when compared to media control and low dose bupivacaine at 0.6 mg mL\(^{-1}\) (Renger et al., 2021). Buprenorphine can act as a local anesthetic by inducing a concentration-dependent blockade of sodium channels when injected perineurally and neuraxially (Helgesen et al., 1990; Jaffe & Rowe, 1996, Leffler et al., 2012). A comparable effect has also been reported with meperidine, which is also μ-opioid receptor agonist and sodium channel blocker (Helgesen et al., 1990; Jaffe & Rowe, 1996). Cytotoxicity from local anesthetics is thought to be related to a concentration-dependent increase in ion influx that leads to changes in oxidative metabolism within the cell leading to an increase in reactive oxygen species (ROS) production, mitochondrial dysfunction followed by disruption in transmembrane potential, ultimately ending in apoptosis or necrosis (Irwin et al., 2002; Unami et al., 2003; Grishko et al.,
Cellular morphological changes after exposure to local anesthetics have also been reported. Chondrocytes exposed to lidocaine showed concentration-dependent spherical protrusions in the cell surface (phenomenon known as membrane blebbing), followed by cell shrinkage and death (Maeda et al., 2016). This effect of local anesthetics on cell morphology and organization of membrane-associated cytoskeletal structures has also been reported to occur in fibroblasts and tenofibroblasts (Nicholson et al., 1976; Sung et al., 2014). The fact that buprenorphine and meperidine share a common mechanism of action with the local anesthetics, such as lidocaine and bupivacaine, and all three exhibit a concentration-dependent chondrotoxic effect, may provide an explanation for the decreased viability observed in chondrocytes exposed to high concentrations, but not low concentrations of buprenorphine. This effect could be mediated by the same cellular processes reported for the local anesthetics and meperidine. Further studies are needed to confirm this hypothesis.

Other possible causes for the cytotoxicity seen in this study could be the pH of the solution and/or crystalline development. An acidic pH has been speculated as a potential source for decrease in chondrotoxicity. However, pH below pH 7.4 was not associated with decreased chondrocyte viability unless extremely acidic (pH 2.4) (Karpie & Chu, 2007; Bogatch et al., 2010). Karpie & Chun (2007) exposed cultured bovine chondrocytes to normal saline solutions of pH 7.4, 7.0, or 5.0 for 15-, 30- and 60-minutes. After exposure, the chondrocytes were re-incubated in chondrocyte growth media and viability was assessed 1-, 24- hours and 1 week later. Viability did not change significantly among the different pH. Bogatch et al. (2010) exposed cultured bovine chondrocytes to phosphate buffer solution of pH 7.1, 4.5, 3.8, 3.2 and 2.4 for 60 minutes. Only the pH of 2.4 was associated with a significant decrease in cell viability. While buprenorphine HCl has a pH of 4–5 (Hedges et al., 2013), the pH of any of the treatments was not controlled or
measured in this study, however, an effect from the pH seems unlikely as previous studies have rule out pH in this range as a cause of cell death.

Mixing local anesthetics with culture media at different pH can cause crystal formation, and crystallization has been suggested to potentially result in micro trauma to the cells and subsequently cell death (Adler et al., 2021). For instance, the local anesthetic ropivacaine forms crystals at physiologic pH (7.37) (Fulling et al., 2000), whereas bupivacaine and lidocaine form crystals at a pH of 7.7 and 12.9, respectively (Hwang et al., 2016). In the present study, no gross crystallization or cloudiness were observed when the treatments were combined with the culture media and were not suspected to be a cause of cell death in this study.

The main limitation of this research is that a two-dimensional (2D) monolayer culture was used which does not accurately recreate the natural microenvironment to which chondrocytes are normally exposed in vivo. In this regard, three-dimensional (3D) cell cultures have been demonstrated to be superior in terms of extrapolating results towards in vivo environments (Jaroch et al., 2018). In contrast to monolayer cultures, 3D cultures better develop in vivo characteristics including cellular morphologies, structural organization, intercellular interactions, and gene expression patterns (Rosser et al., 2019). Despite the superiority of 3D cultures, its use is still limited due to difficulties associated with culturing (Jaroch et al., 2018). Therefore, the results reported here and its extrapolation to an in vivo setting should be interpreted carefully.

In conclusion, the present study revealed that buprenorphine at a concentration of 0.12 mg mL⁻¹ (5 µg kg⁻¹) significantly decreases chondrocyte viability in vitro after two hours of exposure. However, a concentration of 0.05 mg mL⁻¹ (2 µg kg⁻¹) appears to have a negligible effect on chondrocyte viability in vitro. Further studies are necessary to determine the chondrotoxic
mechanism associated with higher concentrations of buprenorphine and to determine if the lower concentration used in this study is clinically relevant for IA administration in the horse.
Chapter Four.
Pharmacokinetics of intra-articular buprenorphine in horses with lipopolysaccharide-induced synovitis.

4.1. Introduction

Intra-articular administration of analgesic drugs, such as opioids has been reported as a modality to provide analgesia in human and veterinary medicine with the potential of causing less systemic side effects (Cook et al., 1997; Kalso et al., 1997; Soto et al., 2014; Ickert et al., 2015). In horses, the use of systemic opioids is limited due to its potential risk of decreasing gastrointestinal motility leading to signs of colic, central nervous system excitation and urinary retention (Roberts & Argenzio, 1986; Sellon et al., 2001; Boscan et al., 2006; Figueiredo et al., 2012). Additionally, it has been shown that µ-opioid receptors are upregulated in inflamed joints (Sheehy et al., 2001; Stain, 2006; Santos et al., 2009; van Loon et al., 2013; Selon et al., 2001). The analgesic efficacy of morphine injected IA in horses with lipopolysaccharide (LPS)-induced synovitis has been demonstrated (Santos et al., 2009; Lindegaard et al., 2010a; van Loon et al., 2010) with minimal systemic uptake and therefore minimal systemic side effects. Up to date, morphine has been the only opioid investigated in equine for IA administration.

Buprenorphine is a partial µ-opioid receptor agonist that has a higher receptor affinity and is 25 times more potent than morphine (KuKanich & Wiese, 2015). In horses, buprenorphine has been studied for the treatment of perioperative pain, showing better analgesic effects than butorphanol for field castration (Rigotti et al., 2014). In human medicine buprenorphine has been used IA for the treatment of joint pain after knee arthroscopy (Varrasi et al., 1999; Sugandarajappa et al., 2016; Das & Samal, 2019), shoulder surgery (Fellahi et al., 1995) and temporomandibular joint arthrocentesis (Präger et al., 2007).
Before recommending the IA use of buprenorphine as an alternative, information on its disposition, pharmacokinetic profile and analgesic efficacy is essential. Up to date there is no pharmacokinetic information available about IA buprenorphine in horses. The aim of this study was to describe the pharmacokinetics of IA buprenorphine. It was hypothesized that IA buprenorphine would not allow for detectable plasma concentrations of buprenorphine, and IA buprenorphine would allow for detectable concentrations within the synovium for 24 hours.

4.2. Materials and methods

4.2.1. Animals

The study was approved by the Louisiana State University Institutional Animal Care and Use Committee (19-113). Six healthy adult horses (4 Thoroughbreds and 2 Quarter Horses), with a median age of 6 years (3–16 years) and a mean weight of 485.2 ± 25.8 kg from the research herd at Louisiana State University were used for this study. All horses were considered healthy and free from any orthopedic disease based on a thorough physical examination and lameness examination done by an experienced equine surgeon (BL) prior to start of the experiment. Horses were housed indoors in separated stalls and fed twice daily their usual ration of pellets (Purina® Strategy®, CA) and hay throughout the study. Horses were fasted for 12 hours prior to drug administration and 1 hour after drug administration. Water was always available ad libitum.

4.2.2. Study design

The study was carried out as a randomized, blinded cross-over study. All horses received two treatments with a minimum washout period of three weeks between treatments.
4.2.3. Treatments

Horses were weighed the day prior to the experiment. The skin over the right or left jugular vein was clipped followed by aseptic placement of a 14-gauge 100 mm IV catheter (Millpledge Veterinary, CA, USA) for blood sampling.

Four hours prior to treatment administration (T-4) one radiocarpal joint was randomly chosen by coin toss (randomized during the first round, the opposite radiocarpal joint was used in the second round). The skin over the joint was clipped and aseptically prepared for arthrocentesis and injection of LPS. Synovial fluid was sampled with a 20-gauge 40 mm hypodermic needle (Covidien Monoject, MA, USA) and analyzed to deem joints healthy by measuring the protein and leukocyte concentrations. Synovitis was induced by the IA injection of 0.5 ng LPS per joint derived from *Escherichia coli* strain 055:B5 (Sigma-Aldrich, MO, USA) performing a standard aseptic technique with a 20-gauge 40-mm needle.

Four hours after LPS injection (T0), SF was sampled from the injected joint to confirm presence of inflammation by an increase in SF protein concentration and leukocyte count. Treatments were randomized (randomizer.org) and the assigned treatments were administered. For the IA treatment administration, a standard aseptic arthrocentesis technique using a 20-gauge 40-mm hypodermic needle (Covidien Monoject, MA, USA) was used. For the IV treatment administration, a 21-gauge winged infusion set (BD Vacutainer, NJ, USA) was used to inject the treatment into the contralateral jugular vein that was not catheterized. Intravenous treatment was administered over a period of 3 minutes. Each horse received two treatments with a minimum washout period of three weeks in between: 1) IAB [IA buprenorphine (Buprenorphine HCl; Par Pharmaceutical, NY, USA) at 5 µg kg⁻¹ and an equivalent volume of IV saline at 0.017 mL kg⁻¹
(sodium chloride 0.9%; Hospira, Inc., IL, USA), 2) IVB [IV buprenorphine 5 \( \mu \text{g kg}^{-1} \) and an equivalent volume of IA saline at 0.017 mL kg\(^{-1}\)].

4.2.4. Sampling

During each one of the two study periods, blood and SF samples were taken at 0 (T0), 0.5 (T0.5), 2 (T2), 6 (T6), 12 (T12), and 24 (T24) hours after treatment administration. Prior to sampling, 12 mL of blood was collected from the jugular catheter and wasted. Following 12 mL of blood was sampled and transferred into heparinized tubes (BD Vacutainer; BD, NJ, USA). Blood was centrifuged within 30 minutes of sampling at 3000 rpm for 10 minutes and the serum was passed into cryogenic vials and stored at -80°C before pharmacokinetic analysis. Synovial fluid was collected (1-3 mL) by repeated arthrocentesis using the above-described technique. Half of the sample was transferred into an EDTA-stabilized tube and stored at -4°C within 15 minutes before the leukocyte count was performed. The remainder was placed into two separate cryogenic tubes (approximately 0.5 mL tube\(^{-1}\)) and stored at -80°C before pharmacokinetic analysis.

4.2.5. Plasma and synovial fluid buprenorphine concentration determination

Buprenorphine plasma calibrators were prepared by dilution of the working standard solutions (Cerilliant, Round Rock, TX) with drug free equine plasma and SF to concentrations ranging from 0.05 to 70 ng mL\(^{-1}\). Calibration curves and negative control samples were prepared fresh for each quantitative assay. In addition, quality control samples (drug free samples fortified with analyte at three concentrations within the standard curve) were included with each sample set as an additional check of accuracy.
Prior to analysis, 0.5 mL plasma was diluted with 2 mL 0.1M pH 6 phosphate buffer and 0.1 mL water containing the d4-buprenorphine internal standard (Cerilliant, Round Rock, TX; 40 ng mL\(^{-1}\)). All samples were vortexed gently to mix and subjected to solid phase extraction using UCT Clean Screen Columns (Bristol, PA). In brief, the columns were conditioned with 2.5 mL of methanol and 3 mL of water and the samples loaded. The columns were then rinsed with 2 mL 50% methanol in water, prior to eluting with 2.5 mL methanol. Samples were dried under nitrogen, dissolved in 120 \(\mu\)L of 10% acetonitrile in water with 0.2% formic acid and 40 \(\mu\)L injected into the liquid chromatography tandem mass spectrometry (LC-MS/MS) system.

The concentration of buprenorphine was measured in plasma by LC-MS/MS using positive electrospray ionization (ESI(+)). Quantitative analysis was performed on a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA) coupled with a LC-10ADvp liquid chromatography system (Shimadzu, Kyoto, Japan). The spray voltage was 3500V and the sheath and auxiliary gas were 45 and 30 respectively (arbitrary units). Product masses and collision energies of each analyte were optimized by infusing the analytes into the mass spectrometer. Chromatography employed an ACE 3 C18 10cm x 2.1mm 3 \(\mu\)m column (Mac-Mod Analytical, Chadds Ford, PA) and a linear gradient of acetonitrile in water with a constant 0.2% formic acid at a flow rate of 0.35 ml min\(^{-1}\). The initial acetonitrile concentration was held at 10% for 0.50 minutes, ramped to 95% over 4.17 minutes and held at that concentration for 0.37 minutes, before re-equilibrating for 3.83 minutes at initial conditions.

Detection and quantification were conducted using selective reaction monitoring (SRM) of initial precursor ion for buprenorphine (mass to charge ratio \((m/z)\) 468.3) and the internal standard d4-buprenorphine \((m/z) 472.3\). The response for the product ions for buprenorphine \((m/z) 101.0, 186.9\) and the internal standard \((m/z) 100.9, 186.9\) were plotted and peaks at the proper retention
time integrated using Quanbrowser software (Thermo Scientific). Quanbrowser software was used to generate calibration curves and quantitate analytes in all samples by linear regression analysis. A weighting factor of 1/X was used for all calibration curves. Extraction and analysis of SF samples was the same as plasma. The response for buprenorphine was linear and gave correlation coefficients of 0.99 or better. Intra- and inter-day accuracy and precision was within ± 10% for all quality control samples at all concentrations. The technique was optimized to provide a limit of quantitation of 0.05 ng mL\(^{-1}\) and a limit of detection of approximately 0.025 ng mL\(^{-1}\) for buprenorphine in both matrices.

### 4.2.6. Pharmacokinetic Analysis

Non-compartmental analysis was performed on buprenorphine concentrations using a commercially available software (Phoenix WinNonlin Version 8.1, Certara, Princeton, NJ, USA). The slope of the terminal portion of the curve, lambda z (\(\lambda_z\)) was used to calculate half-life (HL \(\lambda_z\)) using the equation \(0.693/\lambda\). The area under curve (AUC) from time 0 to infinity (AUC\(_{0\rightarrow\infty}\)) was obtained by using the linear up log down trapezoidal rule, then dividing the last plasma concentration by the terminal slope extrapolated to infinity. Clearance (Cl) and the apparent volume of distribution at steady state (V\(_{ss}\)) were determined by the pharmacokinetic software using the following formulas:

\[
Cl = \frac{Dose}{AUC_{0\rightarrow\infty}}
\]

\[
V_{ss} = MRT_{\text{inf}} \times Cl
\]

where MRT is the mean residence time.
4.3. Results

4.3.1. Buprenorphine in synovial fluid

After IV administration buprenorphine was detected in SF at T0.5 in all horses (0.91 ± 0.43 ng mL\(^{-1}\)). The mean concentration of buprenorphine in SF decreased over time and was detected in all horses up to T6 with a mean concentration of 0.25 ± 0.29 ng mL\(^{-1}\) at that timepoint. In one horse buprenorphine was detected at T12 at a concentration of 0.71 ng mL\(^{-1}\) and in 2/6 horses at T24 with a mean concentration of 0.48 ± 0.45 ng mL\(^{-1}\). Because of not enough time points with measurable concentrations in all horses at every timepoint it was not possible to run a PK analysis of buprenorphine in SF after IV administration in 4/6 horses. Values for the remaining two horses where analysis was possible are summarized in Table 1.

<table>
<thead>
<tr>
<th>% AUC(_{\text{Extrap}}) (%)</th>
<th>AUC (h ng mL(^{-1}))</th>
<th>AUC(_{0-\infty}) (h ng mL(^{-1}))</th>
<th>C(_{\text{max}}) (ng mL(^{-1}))</th>
<th>T(_{1/2\lambda_z}) (h)</th>
<th>(\lambda_z) (h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse 1</td>
<td>76.17</td>
<td>19.89</td>
<td>83.48</td>
<td>1.53</td>
<td>54.92</td>
</tr>
<tr>
<td>Horse 2</td>
<td>63.88</td>
<td>4.2</td>
<td>11.63</td>
<td>0.44</td>
<td>31.94</td>
</tr>
</tbody>
</table>

After IA treatment the mean concentration of buprenorphine in SF at T0.5 was 27358.35 ± 7268.48 ng mL\(^{-1}\), and the drug was detected in all horses at T24 in the SF with a mean concentration of 8.48 ± 10.88 ng mL\(^{-1}\) at that timepoint. Pharmacokinetic data for IA buprenorphine in SF is summarized in Table 2.
Table 2. Pharmacokinetic parameters in synovia after the IA administration of 5 µg kg⁻¹ of buprenorphine in horses with LPS-induced synovitis. Reported as minimum/maximum and geometric mean in parenthesis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Value</th>
<th>Value</th>
<th>Value</th>
<th>Value</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUCₜ₋∞_%Extrap (%)</td>
<td>0.027 / 0.16</td>
<td>25901.1 / 110537.9</td>
<td>25915.4 / 110567.4</td>
<td>0.045 / 0.193</td>
<td>2.41 / 4 (3.06)</td>
<td>0.173 / 0.287</td>
</tr>
<tr>
<td>AUCₜ₋∞ (h ng mL⁻¹)</td>
<td>2.14 / 3.31</td>
<td>2.34 / 3.58</td>
<td>0.93 / 1.68</td>
<td>1395.3 / 2137.6</td>
<td>1.51 / 4.92</td>
<td>0.14 / 0.46</td>
</tr>
<tr>
<td>Cl (mL h⁻¹ kg⁻¹)</td>
<td>2.36 / 16.3</td>
<td>3.58 / 2.76</td>
<td>1.31</td>
<td>1811.8</td>
<td>0.27</td>
<td>5980.8</td>
</tr>
</tbody>
</table>

4.3.2. Buprenorphine in plasma

After IV administration of buprenorphine the mean plasma concentration peaked at T0.5 and was 0.95 (± 0.18) ng mL⁻¹ and decreased in a nonlinear fashion being detectable in all horses up to T6. Pharmacokinetic data in plasma after IV administration of buprenorphine is summarized in Table 3.

Table 3. Pharmacokinetic parameters in plasma after the IV administration of 5 µg kg⁻¹ of buprenorphine in horses with LPS-induced synovitis reported as minimum/maximum and geometric mean.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUCₜ₋∞_%Extrap (%)</td>
<td>6.36 / 16.3</td>
<td>2.14 / 3.31</td>
</tr>
<tr>
<td>AUCₜ₋∞ (h ng mL⁻¹)</td>
<td>2.34 / 3.58</td>
<td>0.93 / 1.68</td>
</tr>
<tr>
<td>Cl (mL h⁻¹ kg⁻¹)</td>
<td>1395.3 / 2137.6</td>
<td>1.51 / 4.92</td>
</tr>
</tbody>
</table>

After the IA administration of buprenorphine, the mean plasma concentration at T0.5 was 0.91 (± 0.43) ng mL⁻¹ and was detectable in all horses at 6 hours, in 2/6 horses at 12 hours and in none of the horses at 24 hours. Because of lack of time points with detectable concentrations pharmacokinetic analysis was not possible in 4/6 horses after IA Buprenorphine. Pharmacokinetic
values for the 2 horses where plasmatic concentrations analysis was possible after IA administration are summarized in Table 4.

Table 4. Pharmacokinetic parameters in plasma after IA administration of 5 µg kg\(^{-1}\) buprenorphine in two horses with LPS-induced synovitis

<table>
<thead>
<tr>
<th></th>
<th>% AUC(_{\text{Extrap}}) (%)</th>
<th>AUC ((\text{h mg mL}^{-1}))</th>
<th>AUC(_{0-\infty}) ((\text{h mg mL}^{-1}))</th>
<th>C(_{\text{max}}) ((\text{ng mL}^{-1}))</th>
<th>T(_{1/2}) ((\text{h}))</th>
<th>λ(_{z}) ((\text{h}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse 1</td>
<td>11.2</td>
<td>2.58</td>
<td>2.9</td>
<td>0.56</td>
<td>3.56</td>
<td>0.195</td>
</tr>
<tr>
<td>Horse 2</td>
<td>7.6</td>
<td>2.53</td>
<td>2.74</td>
<td>0.62</td>
<td>3.18</td>
<td>0.218</td>
</tr>
</tbody>
</table>

4.4. Discussion

The present study demonstrated that buprenorphine at a dose of 5 µg kg\(^{-1}\) administered IA in horses with acute LPS-induced synovitis, remained at detectable concentrations within the radiocarpal joint for a period of up to 24 hours in 6 horses.

Pharmacokinetic information of buprenorphine in horses is available after subcutaneous, sublingual, intramuscular and IV administration (Messenger et al., 2011; Davis et al., 2012; Love et al., 2015; Grubb et al., 2019; Flynn et al., 2021). In the present study, PK data differed from previously reported values. After IV administration the volume of distribution of 5.9 L kg\(^{-1}\) was larger when compared to previous reported values of 2.79 L kg\(^{-1}\) (Love et al., 2015), 3.0 L kg\(^{-1}\) (Davis et al., 2012), and 3.1 L kg\(^{-1}\) (Messenger et al., 2011). Also, the half-life was shorter with 2.37 hours compared to 3.58 hours (Davis et al., 2012), and 5.79 hours (Messenger et al., 2011) and the clearance rate of 30.19 mL kg\(^{-1}\) min\(^{-1}\), exceeded previously reported values of 6.13 mL kg\(^{-1}\) min\(^{-1}\) (Messenger et al., 2011), 7.97 mL kg\(^{-1}\) min\(^{-1}\) (Davis et al., 2012), and 19.61 mL kg\(^{-1}\) min\(^{-1}\) (Love et al., 2015). Inter-individual variation among the different study populations may explain the difference in values. Buprenorphine is metabolized by the P450 system, which contains a large variety of heme-containing enzymes. Polymorphism for these enzymes in the horse has been
demonstrated for the enzyme CYP2D6, which is responsible for the metabolism of tramadol (Corado et al., 2016). This polymorphism may lead to differences in drug availability and plasma levels among individuals. In humans, buprenorphine is metabolized by CYP3A4 enzyme. In the horse seven possible CYP3A genes have been identified (Schmitz et al., 2010) and genetic variants may contribute to inter-individual variability in drug metabolism and pharmacokinetics.

The pharmacokinetic profile of drugs may be altered by inflammation. The clearance of morphine is faster in rabbits after LPS induced mild inflammatory state (Szkutnik-Fiedler et al., 2016). In rats with endotoxemia induced with 10 µg kg$^{-1}$ of intraperitoneal administered LPS the clearance of ketamine is decreased (Veilleux-Lemieux et al., 2012). Palmer & Bertone (1994) reported that a dose of 0.5 ng joint$^{-1}$ of LPS in healthy horses produced systemic signs of endotoxemia, such as fever, depression, and inappetence. In the present trial, none of the animals showed any clinical signs of systemic inflammation, however, systemic white blood cell (WBC) counts were not performed to rule it out. Although unlikely systemic inflammation cannot be excluded as the cause for altered pharmacokinetics of buprenorphine.

Intravenous buprenorphine doses from 5 to 10 µg kg$^{-1}$ were demonstrated to be antinociceptive for surgical pain and experimental models of pain in horses (Carregaro et al., 2007; Love et al., 2012; Love et al., 2013; Rigotti et al., 2014). Love et al. (2015) studied the pharmacokinetic-pharmacodynamic interactions of 10 µg kg$^{-1}$ of buprenorphine in horses following a single IV administration and demonstrated that an increase in the mechanical threshold (pin driven against dorsal aspect of metacarpal bone) was associated with plasma concentrations in a wide range of 0.34–2.45 ng mL$^{-1}$. In the present investigation, after the IV injection of buprenorphine, the highest plasma concentration detected was at 30 minutes post administration with a mean concentration of 0.95 ng mL$^{-1}$, which is a concentration within the range that has been
reported to decrease mechanical thresholds in horses (Love et al., 2015). Buprenorphine after IV administration was detected in the SF, even though the concentrations were low. It is not known if joint inflammation with its consequent increase in blood flow and capillary permeability in the synovium might have augmented the amount of buprenorphine delivered to the joint. Unfortunately, no information is available regarding the synovial concentrations of buprenorphine in healthy or inflamed joints after systemic administration, to allow for comparison. Therefore, the presence of buprenorphine within the synovium after IV administration and its therapeutic implications remain to be elucidated.

The justification of IA administration of buprenorphine is to achieve a high drug concentration within the joint that would produce analgesia and reduce the systemic exposure and potential side effects of the drug. Intra-articular buprenorphine was detected in the SF of every treated joint for at least 24 hours in all horses. Joint inflammation increases blood flow and capillary fenestration, leading to an increase in synovial permeability, which in turn might increase the elimination of drugs injected into the joints (Campbell, 1968; Christensen et al., 1982). In the present study, despite the local inflammation caused with the IA inoculation of LPS, IA buprenorphine was slowly eliminated from the joint. Plasma buprenorphine concentrations of 0.91 ng mL\(^{-1}\) after IA administration were detected from T0.5 and decreased thereafter being detectable in all horses up to T6. It cannot be excluded that after IA buprenorphine administration, systemic analgesic plasma concentrations could be achieved. Whether these plasma concentrations are analgesic or not for horses with LPS-induced synovitis remains to be elucidated as the antinociceptive activity may vary depending on the type and intensity of pain being evaluated.

In conclusion, the IA administration of buprenorphine in horses with LPS-induced synovitis led to a high concentration of the drug within the joint that was detected up to 24 hours.
Intra-articular administered buprenorphine granted low plasma concentrations of the drug that were within the analgesic range previously reported in horses. Pharmacokinetics of the IV administration of buprenorphine was characterized by a high volume of distribution and clearance, and a short half-life. Findings that could potentially suggest that this drug might have long lasting local effects within the synovium. Analgesic evaluation of this dose is warranted before recommending its use.
5.1. Introduction

Lameness is an important reason of decreased performance in horses. It is one of the major causes of wastage and training failure in racehorses, ending in a significant detrimental economic impact in the racing industry (Lindner & Dingerkus, 1993; Bailey et al., 1999; Olivier et al., 1997; Wilsher et al., 2006; Dyson et al., 2008). The incidence of lameness was reported to be 53% (Jeffcott et al., 1982) and 57% (Lindner & Dingerkus, 1993) in horses in race training. From all the possible causes of lameness, joint pain is among the most common. In one prospective study, around 73% of the lameness were attributed to joint injury in Thoroughbred yearlings (Preston et al., 2008). Common conditions affecting the joints include synovitis, capsulitis, cartilage wear and erosion, and stress-related bone injury.

Treatment of IA pain in horses has been addressed by systemic administration of NSAIDs (Owens et al., 1995; Hu et al., 2005; Foreman & Ruemmler, 2011; Walliser et al., 2015). However, the renal and gastrointestinal side effects associated with NSAID administration limit their long-term use for treatment of joint disease. In some instances, systemic administration of opioids is also used for the treatment of IA pain, especially during the perioperative period (Mircica et al., 2003; Clark et al., 2008). Nevertheless, their potential adverse effects on gastrointestinal motility (Roberts & Argenzio, 1986; Boscana et al., 2006; Sano et al., 2011; Figueiredo et al., 2012), in conjunction with their central nervous system and locomotor excitatory effects (Combie et al., 1979; Kalpravidh et al., 1984; Sojka et al., 1988; Mama et al., 1993; Nolan et al., 1994), when given without other sedatives (Combie et al., 1981), has led some practitioners to avoid its use in
Intra-articular drug administration has become part of the analgesic management in humans and animals with joint pain (Soto et al., 2014; Ickert et al., 2015; Das & Samal, 2019). The presence and upregulation of μ-opioid receptors in the synovium during inflammation (Sheehy et al., 2000; Bergström et al., 2005; van Loon et al., 2013) promoted the use of opioids, specifically morphine for this purpose in humans, dogs, and horses (Stein et al., 1991, Keates et al., 1999; Sammarco et al., 1996, Valverde & Gunkel, 2005; Santos et al., 2009; Lindegaard et al., 2010; Van Loon et al., 2010).

Other opioids, such as buprenorphine, might be considered for IA administration to treat joint pain. Buprenorphine is a partial μ-opioid receptor agonist that has 25 times the potency of morphine and shows a higher receptor affinity (KuKanich & Wiese, 2015). Its systemic antinociceptive effects have been demonstrated in horses (Carregaro et al., 2007; Love et al., 2012; Love et al., 2013). A dose range of 5 to 10 µg kg⁻¹ has been used systemically with success for producing analgesia in ponies and horses (Love et al., 2012; Love et al., 2013; Rigotti et al., 2014; Taylor et al., 2016). The IA administration of buprenorphine is an attractive option for providing local analgesic effects in horses and to prevent the systemic side effects of this type of drugs. In humans, IA buprenorphine has been used in different joints proving evidence of analgesic effects for diverse IA conditions (Fellahi et al., 1995; Varrasi et al., 1999; Präger et al., 2007; Sugandarajappa et al., 2016; Das & Samal, 2019).

Up to date, there is no knowledge available on the pharmacodynamic and analgesic effects of IA buprenorphine administered to horses. The objective of the present study was to evaluate the analgesic properties and pharmacodynamic effects of IA buprenorphine in horses with lipopolysaccharide (LPS) induced synovitis. We hypothesized that the IA administration of
buprenorphine would produce analgesia and will not cause systemic side effects in horses with LPS induced synovitis.

5.2. Materials and Methods

5.2.1. Study design

The study was approved by the Louisiana State University Animal Care and Use Committee (19-113). The study was carried out as a randomized, blinded cross-over study. All horses received three treatments with a minimum washout period of three weeks between treatments.

5.2.3. Animals

Nine healthy adult horses (8 geldings and 1 mare) of two different breeds (6 Thoroughbreds and 3 Quarter Horses) with a median age of 7 years (3–17 years) and a mean weight of 495 ± 46.4 kg from the research herd at Louisiana State University were included in this trial. All horses were deemed healthy and free from any orthopedic disease by a thorough physical and lameness examination that was performed by an experienced equine surgeon (BL). The horses were housed in separated stalls during the study period, fed twice a day with a commercially available diet (Purina® Strategy®, CA, USA) and had access to hay and water ad libitum during the trial.

5.2.4. Induction of synovitis and treatment administration

At the first treatment the left or right radio-carpal joint was randomly chosen by coin, and then alternated for the following two treatments. Prior to induction of synovitis (T-4), body temperature, heart and respiratory rate were recorded, and SF was sampled to rule out pre-existing
joint inflammation. Horses were sedated with xylazine hydrochloride (XylaMed, Bimeda, Inc, Cambridge, ON, USA) at 0.1-0.2 mg kg\(^{-1}\) IV, and the assigned radio-carpal joint was aseptically prepared. Arthrocentesis was performed using a 20-gauge 40 mm needle (Covidien Monoject, MA, USA), SF was collected to deem the joint free of inflammation, and 0.5 ng lipopolysaccharide (LPS) derived from *Escherichia coli* strain O55:B5 (Sigma–Aldrich, MO, USA) was inoculated to induce synovitis on the first day of each treatment. This time was set as T-4. Four hours later (T0) a lameness exam was done and the assigned treatment was administered: 1) IAB (IA buprenorphine (Buprenorphine HCl; Par Pharmaceutical, NY, USA) at 5 \(\mu\)g kg\(^{-1}\)) and IV saline (sodium chloride 0.9%; Hospira, Inc., IL, USA), 2) IVB (IV buprenorphine 5 \(\mu\)g kg\(^{-1}\) and IA saline at 0.017 mL kg\(^{-1}\)), 3) and 3) SAL (IA saline 0.017 ml kg\(^{-1}\) and IV saline 0.017 ml kg\(^{-1}\)). A single investigator (BL), blinded to treatments, performed the arthrocentesis for LPS injection and IA treatment administration.

A 22-gauge butterfly winged infusion set (BD Vacutainer, NJ, USA) was used to inject the IV treatment into one of the jugular veins. Intravenous treatments were given over 3 minutes by the same investigator (JC) not blinded to the treatment.

**5.2.5. Monitoring**

A complete physical examination (heart rate, respiratory rate, mucous membrane color, capillary refill time, temperature and abdominal auscultation), fecal pile count, measurement of joint circumference, joint angle at full flexion, joint range of motion, joint angle, pain on palpation, and pain on flexion of the joint were done before injection of LPS (T-4) and at 0 (T0), 0.5 (T0.5), 2 (T2), 4 (T4), 6 (T6), 8 (T8), 12 (T12), 24 (T24) hours after treatment administration. Synovial
fluid samples were collected at T0, T0.5, T2, T6, T12, and T24 to confirm presence of synovitis by measuring the total protein (TP) and mononucleated cell counts.

Lameness was assessed at T-4, T0, T0.5, T2, T6, T12, T24. Horses were instrumented with a lameness locator (Equinosis Lameness Locator®, Columbia MO, USA) and trotted back and forth over 45 meters in a straight line on a firm pavement surface. Additionally, horses were videotaped (Samsung S10e, South Korea) while trotting. Videos were stored in a cloud system, and later assessed by two investigators (BL and LF) blinded to the treatments who graded the lameness using a previously published scoring system (Lindegaard et al., 2010a).

A single investigator (GC), blinded to treatments, assessed pain at T-4, T0, T0.5, T2, T6, T12, T24. Pain was scored using a composite measure pain scale (CMPS) previously published (Pritchett et al., 2003; Lindegaard et al., 2010a). The CMPS consisted of six categories and for each category a score was defined. At each time point, scores for all behavioral categories were summed to yield a final pain score ranging from 0 to 23.

5.3. Statistical analysis

Data analyses were performed using JMP Pro 16.1.0 (SAS Institute Inc.). Non-continuous variables (lameness, pain score, pain on palpation, GI sound, and range of motion) were evaluated via Kruskal-Wallis tests with post hoc Dunn methods to compare with the baseline. Total protein, fecal piles, and vital signs were evaluated with a mixed ANOVA with batch, treatment, time and their interactions as the fixed effects and each animal as the random effect. Assumptions of all ANOVA models (linearity, normality of residuals and homoscedasticity of residuals) and influential data points were assessed by examining standardized residual and quantile plots. When
a fixed effect was detected, Tukey post-hoc comparisons were performed with least square means for the effect. Significance was set at $P < 0.05$

### 5.4. Results

Nine horses were initially enrolled in the study. Two horses were excluded, one horse developed hoof related lameness and one horse was not handleable for arthrocentesis. Data from those two horses was excluded from analysis. In total seven horses finished the study (5 Thoroughbreds, 2 Quarter Horses). Due to the SARS-CoV-2 pandemic the experimental phase of the study had to be interrupted for three months, and two different batches of LPS were used. The distribution of animals within treatments between the two LPS batches (B1 and B2) was unequal: B1 IAB 2 horses, B1 IVB 5 horses, B1 SAL 2 horses, and B2 IAB 5 horses, B2 IVB 2 horses, and B2 SAL 5 horses. The IA administration of LPS produced an inconsistent synovitis. The degree of lameness and pain was different between the two batches of LPS used. Lameness score was significantly increased ($P < 0.0001$) at T0, ($P < 0.0001$), T2 ($P < 0.0001$), T6 ($P = 0.001$) and T12 ($P = 0.015$) in B2 in all treatment groups when compared to baseline (Figure 4.1). There was no difference in lameness score among groups at any timepoint within each batch. When horses received LPS from B1 the lameness score at T0 was significantly different from baseline ($P = 0.02$) but was not different at any other time point within or among treatments. For all treatments receiving B2, the pain on palpation was significantly increased at T0, T2, and T4 ($P < 0.0001$, $P = 0.0003$, $P = 0.007$, respectively), range of motion was significantly decreased at T0 and T2 ($P = 0.0003$, 0.002, respectively) and the overall pain score was significantly increased at T0 and T2 ($P < 0.0001$), when compared to T-4 (Figure 4.2). The TP increased in the SF significantly independent of the LPS used and stayed elevated up to 24 hours ($P < 0.0001$).
A batch effect was also seen for GI sounds. In B1, GI sounds were not different over time, and no significant difference among treatments was detected. With the use of B2, GI sounds in IAB at T2 were significantly decreased compared to baseline (P = 0.003) (Figure 4.3). The number
of fecal piles decreased significantly within each treatment from T2 to T8 compared to T-4 (P < 0.0001). Fecal piles in IAB and IVB were significantly decreased compared to SAL (P = 0.0071 and P = 0.021, respectively).

![Figure 4.3](image)

Figure 4.3. Median (interquartile range) gastrointestinal (GI) scores over time in seven horses with synovitis induced with two different batches of lipopolysaccharide (LPS), receiving intra-articular buprenorphine (IAB), intravenous buprenorphine (IVB) or saline (SAL). *Statistically different from T-4 (P < 0.05). #Statistically different from SAL (P < 0.05).

Within B1 the respiratory rate (RR) was not different among or within treatments. In B2 a significant increased RR from T-4 was seen at T0 (P = 0.01), T0.5 (P = 0.005), T6 (P < 0.0001), and T12 (P = 0.019) for all treatments. No difference among treatments could be detected (Figure 4.4). No significant differences in HR within or among treatments were detected in B1. With the administration of B2 the HR in IVB was significantly higher than SAL (P = 0.003), whereas no difference was detected for IAB when compared to SAL (Figure 4.5).

Rectal temperature showed no significant differences among or within treatments in each batch at all time points.
Figure 4.4. Least square mean ± SE of respiratory rate (RR) (breaths minute⁻¹) over time in seven horses with synovitis induced with two different batches of lipopolysaccharide (LPS), receiving intra-articular buprenorphine (IAB), intravenous buprenorphine (IVB) or saline (SAL). *Statistically different from T-4 (P < 0.05).

Figure 4.5. Least square mean ± SE of heart rate (HR) (beats minute⁻¹) over time in seven horses with synovitis induced with two different batches of lipopolysaccharide (LPS), receiving intra-articular buprenorphine (IAB), intravenous buprenorphine (IVB) or saline (SAL). *Statistically different from SAL (P < 0.05).
5.5. Discussion

This study failed to demonstrate that IA buprenorphine at 5 \( \mu g \ kg^{-1} \) has an analgesic effect in horses with LPS-induced synovitis. Overall, minimal or no effects on the cardiovascular, respiratory, and gastrointestinal system were observed.

Due to the SARS-CoV-2 pandemic the experimental phase of this study had to be interrupted for a period of three months during the mandatory shut down. This led to the use of two different batches of LPS. A significant difference in the physiologic response to the LPS was found between the two batches. With the first batch the expected degree of lameness was not observed, despite the presence of inflammation which was confirmed by measuring the TP and mononucleated cell counts in the SF prior to LPS injection and at several time points thereafter. Handling and preparation of the LPS using the appropriate technique to achieve a homogeneous solution is paramount to guarantee the desired effect (Van de Water et al., 2021). In the present study the LPS solution preparation as well as the injection were always done by the same experienced investigator (BL). It is therefore unlikely that handling and preparation caused the difference in the responses between the two batches. Biological activity or potency of one preparation of LPS can be very different to another preparation coming from the same mass of endotoxin, indicating that different doses of LPS might be needed to elicit a specific biological response (Dawson, 1997). The dose of LPS for aseptic inflammatory and endotoxemia models has been calculated using either endotoxin units (EU) or mass concentration (ng mL\(^{-1}\)). Endotoxin units, in contrast to mass concentration, considers the specific activity of the endotoxin. Therefore, if EU is used instead of mass concentration, a possible difference in potency between batches would be counteracted (Dawson, 1997). There is the possibility that having calculated the dose
using the EU system, instead of mass concentration, could have yielded a more consistent response to the LPS in the present study.

Reported doses of LPS to induce synovitis range from 0.125 ng joint\(^{-1}\) to 50000 ng joint\(^{-1}\) (Palmer & Bertone, 1994; Khumsap et al., 2003; Lindegaard et al., 2010; Lucia et al., 2013). In a previous study, doses above 0.5 ng joint\(^{-1}\) produced systemic signs of endotoxemia, including fever, depression, inappetence, and non-weightbearing lameness (Palmer & Bertone, 1994). Conversely, Lindegaard et al. (2011a) used 5000 ng per joint to induce synovitis in horses and no systemic side effects were reported with that dose. In the present study a dose of 0.5 ng per joint was chosen, as this has been successfully used to produce consistent synovitis in similar studies that evaluated the analgesic effects of IA morphine and ropivacaine in horses (Santos et al., 2009; van Loon et al., 2010). Nevertheless, the fact that with a higher dose of LPS a more consistent effect might have been seen independent of the batch, cannot be excluded.

There are some reports that suggest that there might be a breed variability in the response of the synovium to different doses of LPS. Van de Water et al. (2017) conducted a study in Standardbred horses using 0.5 ng of LPS per joint. The resulting lameness was too mild to be detectable, either visually or by pressure plate evaluation, despite the presence of inflammation. In another study by Pearson et al. (2012) only three out of eight Standardbred horses had a grade 1 lameness score, using the American Association of Equine Practitioners lameness (AAEP) scale, at 12 hours post induction of synovitis using LPS at a dose of 0.3 ng joint\(^{-1}\), and none of them were lame by 24 hours post injection. The horses used in the present study were either Thoroughbreds or Quarter Horses, which are breeds that have been used for other studies that worked with the same LPS model and had reliably respond to it (Lindegaard et al., 2010a; Lucia et al., 2013). Furthermore, all horses in the present study reacted to the second batch of LPS with
a higher degree of lameness, making any animal or breed variability regarding the response to the first LPS less likely.

The IA or IV administration of buprenorphine in horses did not improve lameness and pain scores in the present study. The dose chosen in the present study was 5 µg kg⁻¹ based on previous reports investigating the analgesic efficacy of buprenorphine. Love et al. (2012) studied the thermal antinociceptive effects of IV buprenorphine at 5-, 7.5-, and 10 µg kg⁻¹, and found that all doses significantly increased the thermal threshold in horses. Rigotti et al. (2014) demonstrated that buprenorphine at 5 µg kg IV before castration provided better intraoperative analgesia compared to butorphanol in ponies. In contrast, Carregaro et al. (2007) investigated the thermal antinociceptive effects of IV buprenorphine using the hoof-withdrawal reflex latency and skin-twitching reflex latency, and only a dose of 10 µg kg⁻¹ resulted in consistent antinociception. Based on these previously reported different doses and antinociceptive responses it is possible that 5 µg kg⁻¹ of buprenorphine, independent of route of administration is not sufficient to provide analgesia for joint pain in healthy horses.

Intra-articular administration of buprenorphine did not cause changes in HR over time. Following the IV administration, only in batch 2 an increase in HR was noted over time compared to the control group, however that treatment had only two horses. Love et al. (2012) found higher heart rates in horses receiving buprenorphine at 10 µg kg⁻¹ IV compared to 5 µg kg⁻¹ IV. Carregaro et al. (2006) reported an increase in heart rate, mean arterial blood pressure and cardiac index after a 10 µg kg IV dose. Furthermore, Love et al. (2015) observed an increase in heart rate in healthy horses administered 10 µg kg⁻¹ IV. The increased sympathetic outflow caused by the central nervous system stimulant effect of buprenorphine may explain these effects.
The foundation for IA administration of opioids in horses is to limit the extent of gastrointestinal side effects that may be seen with their systemic administration. Interestingly we observed with the use of batch 2 LPS a significant decrease in gastrointestinal sounds for up to two hours after IA administration of buprenorphine. Also, the number of fecal piles were decreased up to 8 hours after buprenorphine administration. Other studies in horses have reported a decrease in gastrointestinal motility after IV administration of buprenorphine (Carregaro et al., 2006; Cruz et al., 2011; Love et al., 2015). This is explained by the inhibitory effects of opioids on the intestinal propulsive activity (Roger et al., 1994), leading to a decrease in motility and potentially constipation. Interestingly, IV buprenorphine was not associated with a consistent decrease in GI motility in the present study as the GI sounds in B1 were not different among groups or across baseline. Yet, in B2 there was a decrease in GI sounds, still, not significantly different from the control group, which could be due to the decreased number of horses in the IVB group in that batch. IAB in B2 caused a decrease in GI sounds up to T2 that was not seen in B1. This could be explained by the difference in pain seen between the batches, as horses in B2 exhibited marked signs of pain which leads to sympathetic activation and inhibition of GI motility (Garcia-Pereira 2015), whereas horses in B1 did not show the same degree of pain. Another reason could be the systemic uptake of IA buprenorphine, though, this seems to be unlikely as other opioids given IA, such as morphine, have minimal systemic absorption (Lindegaard et al. 2010b). Nonetheless, no studies exist evaluating the pharmacokinetics of IA buprenorphine. Even though there was GI hypomotility in the horses from this study, no signs of abdominal discomfort were observed in the animals during the experimental period.

This study had some limitations. First, the use of two LPS batches resulted in a different lameness response in the horses, which could have confounded the results of this study. Second,
the use of an experimental model of synovitis with a low dose of LPS does not accurately imitate joint pain due to naturally occurring conditions, such as traumatic arthritis and osteoarthritis. Third, only one dose of buprenorphine was investigated. It cannot be excluded that administering a higher dose of buprenorphine could have provided analgesic effect. However, the volume of the radiocarpal joint in horses is estimated to be 12.6 mL on average (Ekman et al., 1981). It is expected that synovitis will cause some degree of joint effusion (van Loon et al., 2010), leading to a higher-than-normal volume. Based on the volume limitation, a higher dose than 5 μg kg⁻¹ dose was excluded as any additional volume would have led to further distention of the joint capsule causing an additional source of pain.

In conclusion, the IA administration of buprenorphine at 5 μg kg⁻¹ did not improve lameness and pain scale scores in horses with LPS-induced synovitis. Independent of route of administration, no colic signs or behavioral changes were observed. A variation in potency should be considered when using different batches of LPS within the same study as this can alter the final outcome. Further research using other doses of buprenorphine, or different models of synovitis may help evaluate its possible analgesic effect in the treatment of joint pain.
Chapter Six.
Conclusions

Treatment of IA pain in horses with joint disease is paramount to improve recovery, resume joint functionality and provide animal welfare. In human pain management, the IA injection of analgesic drugs is an effective way to provide perioperative analgesia for joint surgery, as it offers pain relief with minimal systemic side effects (Di Salvo et al., 2021). Opioids have been used into the joint to provide analgesia. Morphine has been the most popular IA injected opioid for the past three decades in humans (Gupta et al., 2001). It has the benefit of being safe and unharmsful to chondrocytes, providing effective and consistent IA analgesia. Its pharmacokinetic profile is characterized by low systemic absorption and dose dependent effects lasting up to 24 hours (Raj et al., 2004; Ickert et al., 2015). In horses, morphine has shown a comparable profile to what is seen in humans (Lindegaard et al., 2010a; Lindegaard et al., 2010b; Rubio-Martinez et al., 2017). An alternative opioid, buprenorphine, which has greater potency than morphine and bigger receptor affinity has been used clinically in human medicine to treat post-arthroscopic pain of different joints with evidence of effective analgesia. The possibility of having an alternative drug to treat IA pain expands the therapeutic options to provide analgesia in horses.

Based on the above mentioned facts and the absence of studies assessing buprenorphine’s suitability for management of joint pain in horses, the aim of the present dissertation was to assess if buprenorphine was a feasible alternative opioid for IA administration to treat joint pain in horses, as follows: 1) assessing the cytotoxic effects of buprenorphine on equine chondrocytes in vitro, 2) describing the pharmacokinetics of IA administered buprenorphine in horses with experimentally-induced synovitis, and 3) evaluating the pharmacodynamic and analgesic efficacy of IA
administered buprenorphine in horses with experimentally-induced synovitis. The three studies were successfully completed.

The cytotoxicity assessment of two different concentrations of buprenorphine (0.05 mg mL\(^{-1}\) and 0.12 mg mL\(^{-1}\)) on cultured equine chondrocytes showed that independent of the time of exposure (immediate or after 2 hours) a low concentration (0.05 mg mL\(^{-1}\)) of the drug does not have cytotoxic effects. Unexpectedly, a high concentration (0.12 mg mL\(^{-1}\)) was markedly toxic to the cells especially after 2 hours of exposure. While the in vitro nature of this research limits the generalizability of the results because the course of events happening in vivo might not be accurately recreated in vitro, this investigation provides new insight in the concentration dependent chondrotoxicity of buprenorphine. Furthermore, it raises the question on the mechanism for causing cell death and its physiological implication on a live animal.

The pharmacokinetic, pharmacodynamic and analgesic efficacy of buprenorphine in horses was evaluated for the first time with this research. For the pharmacokinetic analysis, samples of SF and plasma were collected throughout 24 hours after the IA injection of buprenorphine to measure its concentrations over time. Synovial fluid concentrations of buprenorphine after IAB were detectable for at least 24 hours which could imply that the effects could be long-lasting. Plasma concentrations of the drug after IAB were only detected up to 12 hours in 2 horses, which could indicate that systemic absorption from the joint might not be very significant. To recreate a painful condition within the joint, an experimental model of synovitis using LPS was implemented. Two different batches of LPS had to be used during the investigation which yielded different responses in the horses of the study. Horses that received the first batch had mild synovitis and almost unnoticeable lameness. The animals that were injected with the second batch had a marked reaction with severe lameness. The lameness and pain scores in the
IAB group were not different from the control group, which implies that IA administered buprenorphine at 5µg kg\(^{-1}\) did not elicit a noticeable analgesic effect. The only pharmacodynamic effect that was altered and different from the control after IAB was the gastrointestinal motility, which was decreased for two hours. Despite this, none of the horses displayed any signs of abdominal discomfort or colic throughout the study.

Based on these findings, the IA administration of buprenorphine at 5µg kg\(^{-1}\) stayed within the joint for at least 24 hours, nevertheless, this dose appeared to have an insignificant effect to alleviate pain caused from experimentally induced local inflammation. Furthermore, researchers using LPS models of synovitis should be aware of the potential variance in potency between different batches that may confound the results. To better understand the implications of these results, further studies with a different model of synovitis, and different doses should be assessed, contemplating the fact that high concentrations might have a deleterious effect on chondrocytes. Finally, different opioids such as hydromorphone and methadone could also be tested and compared.
APPENDIX. Description of the lameness and pain scores

Description of the numerical scoring system employed to evaluate lameness in horses with LPS-induced synovitis (Lindegaard et al., 2010a).

<table>
<thead>
<tr>
<th>Lameness score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No lameness observed</td>
</tr>
<tr>
<td>1</td>
<td>Lameness is difficult to observe. The lameness is inconsistent, and might only be observed while the horse is turned</td>
</tr>
<tr>
<td>2</td>
<td>Lameness difficult to detect at the walk or at the trot on a straight line. Constant lameness when the horse is turned or under similar stress</td>
</tr>
<tr>
<td>3</td>
<td>Lameness difficult to detect at the walk but consistent at the trot regardless of the circumstances</td>
</tr>
<tr>
<td>4</td>
<td>Lameness consistent at the walk and at trot regardless of the circumstances</td>
</tr>
<tr>
<td>5</td>
<td>Obvious lameness with marked head-nod at the trot</td>
</tr>
<tr>
<td>6</td>
<td>Marked head-nod at the walk, and has problems with trotting</td>
</tr>
<tr>
<td>7</td>
<td>Lameness is marked with minimal weight bearing. The horse is reluctant to move (the horse carries the leg)</td>
</tr>
</tbody>
</table>
Description of the CMPS employed to evaluate lameness in horses with LPS-induced synovitis (Lindegaard et al., 2010a)

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Pain score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gross pain behavior</strong>*</td>
<td>None</td>
</tr>
<tr>
<td>No weight bearing</td>
<td>Occasional</td>
</tr>
<tr>
<td>Normal weight bearing or walking</td>
<td>Continuous</td>
</tr>
<tr>
<td>Foot intermittent off the ground/resting more than the other thoracic limb</td>
<td>No weight bearing: foot totally off the ground or toe just touching the ground</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Weight bearing</th>
<th>Foot intermittent off the ground/resting more than the other thoracic limb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal weight bearing or walking</td>
<td>-</td>
</tr>
<tr>
<td>Normal weight bearing or walking</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Head position</th>
<th>Level of withers</th>
<th>Bellow withers</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Above withers or eating</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level of withers</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Above withers or eating</td>
<td>No apparent pain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bellow withers</td>
<td>Mild discomfort</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bellow withers</td>
<td>Slight pain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bellow withers</td>
<td>Moderate pain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bellow withers</td>
<td>Severe orthopedic pain</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Location in stall</th>
<th>Standing in the middle, facing door</th>
<th>Standing in the middle, facing sides</th>
<th>Standing in the middle, facing back or standing in the back</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>At door watching environment</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standing in the middle, facing door</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standing in the middle, facing sides</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standing in the middle, facing back or standing in the back</td>
<td>No apparent pain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standing in the middle, facing back or standing in the back</td>
<td>Mild discomfort</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standing in the middle, facing back or standing in the back</td>
<td>Slight pain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standing in the middle, facing back or standing in the back</td>
<td>Moderate pain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standing in the middle, facing back or standing in the back</td>
<td>Severe orthopedic pain</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Response to open door</th>
<th>Moves to door</th>
<th>Looks at door</th>
<th>-</th>
<th>No response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moves to observer, ears forward</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Looks at observer, ears forward</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Looks at observer, ears forward</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moves away from observer</td>
<td>No apparent pain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moves away from observer</td>
<td>Mild discomfort</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moves away from observer</td>
<td>Slight pain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moves away from observer</td>
<td>Moderate pain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moves away from observer</td>
<td>Severe orthopedic pain</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Gross pain behavior is defined as tooth-grinding, lip-curl, pawing, sweating**
References


Vita

Gabriel Castro-Cuellar was born and raised in Bogotá, Colombia. He received his DVM degree from La Salle University in Bogotá, Colombia, in April 2015. Before finishing veterinary school he earned a scholarship to travel abroad to Argentina, where he did five months of clinical rotations at the Veterinary Teaching Hospital of the Buenos Aires University. After graduation he spent 2 years in small animal practice. Following, he worked as an associate anesthetist and clinical instructor and lecturer in veterinary anesthesia at La Salle University and the Agrarian University of Colombia in Bogotá. In 2019 he started a three-year residency in Veterinary Anesthesia and Analgesia, and a Master of Biomedical and Veterinary Sciences at Louisiana State University School of Veterinary Medicine. He anticipates Master’s graduation in May 2022 and to finish his residency in July 2022.