Pharmacokinetics and Safety of Acetaminophen in Horses

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Academic Abstract

Due to the detrimental side effects of NSAID administration, such as gastrointestinal ulceration and renal papillary necrosis, there is a profound need for clinical pain relief in horses with long term orthopedic disease whereby gastrointestinal side effects are obviated. Acetaminophen is one of the most commonly used analgesic drugs in humans, and is readily available as an inexpensive generic over-the-counter preparation. Acetaminophen has a number of mechanisms of action that differ from NSAIDs, including actions on the serotonergic, opioid, endocannabinoid and lipoxygenase pathways. These alternate pathways may provide greater efficacy against chronic or neuropathic pain in equine patients. Acetaminophen was preferred by physicians over COX-2 and nonselective NSAIDs, even when those drugs were coupled with proton-pump inhibitors to reduce gastrointestinal side effects; due to cost considerations and the occurrence of adverse side effects from those drugs. In horses, acetaminophen has been reported to be efficacious as an adjunct treatment for laminitis in one pony, and was an effective analgesic agent when combined with NSAIDs in a model of inducible foot pain. However, no studies have been performed to validate a dose-response curve in horses. A study recently completed by our group demonstrated rapid absorption following oral administration of acetaminophen. Reported human therapeutic plasma concentrations were achieved within 30 minutes of administration, with no clinical or clinicopathologic evidence of adverse side effects after two weeks of repeated dosing. Dose simulation trials indicate that a change in dosage schedule may be required in order to provide adequate plasma concentrations.

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Public Abstract

The use of non-steroidal anti-inflammatory drugs (NSAIDs) such as phenylbutazone in horses is widespread, and can be associated with detrimental side effects such as gastrointestinal ulceration and kidney damage. The clinical need for pain relief in horses with long-term lameness that minimizes gastrointestinal side effects has led to the development of cyclooxygenase-2 (COX-2) selective NSAIDs, such as firocoxib, but the expense of this therapy is often a major consideration limiting its use and few alternatives are available. Acetaminophen is one of the most commonly used analgesic drugs in humans, and is readily available as an inexpensive generic over-the-counter preparation. Despite the lower efficacy of acetaminophen in trials of human patients with chronic osteoarthritis, acetaminophen remains the preferred analgesic in humans due to its increased tolerance and improved cost-benefit analysis when compared to nonselective and COX2 selective NSAIDs. Acetaminophen has a number of mechanisms of action that differ from the current mainstays of equine analgesic therapy, which may provide greater efficacy against chronic or neuropathic pain in equine patients. A recent study of acetaminophen in horses has shown rapid absorption and achievement of levels reported to be therapeutic in humans, with no adverse side effects after two weeks of repeated dosing. In horses, acetaminophen has demonstrated efficacy as an adjunct treatment for laminitis in one pony, and was an effective analgesic agent when combined with NSAIDs in a model of inducible foot pain.

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Attributions

The research and writing associated with this manuscript (Chapter 3) would not have been possible without aid from colleagues. Contributions are defined below.

Harold McKenzie, DVM, MS, PG Dipl (Vet Ed), DACVIM (LAIM) is an Associate Professor of Large Animal Medicine at the Virginia-Maryland College of Veterinary Medicine. Dr. McKenzie was integral to the idea, design, and logistics of the manuscript, supervised data collection and interpretation, and contributed immensely to manuscript preparation.

Jennifer Davis, DVM, PhD, DACVIM, DACVCP is an Associate Professor of Clinical Pharmacology at the Virginia-Maryland College of Veterinary Medicine. Dr. Davis helped design and perform the study, supervised data analysis and interpretation, was a blinded grader of gastroscopy scores, wrote the pharmacokinetic calculations section of the manuscript, and contributed to manuscript preparation.

Katie Wilson DVM, MS, DACVIM is Clinical Assistant Professor of Large Animal Medicine at the Virginia-Maryland College of Veterinary Medicine. Dr. Wilson contributed to study design, was a blinded grader of gastroscopy scores, and contributed to manuscript preparation.

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Bridgett McIntosh PhD is a Professional Lecturer at the Middleburg Agricultural Research and Extension Center, Virginia Polytechnic University. Dr. McIntosh contributed to study design, organization of staffing, data collection, and facilities and horses utilized in this study.

Thomas E. Cecere, DVM, PhD, Diplomate ACVP is an Assistant Professor of Anatomic Pathology at the Virginia-Maryland College of Veterinary Medicine. Dr. Cecere contributed to the blinded analysis and histopathologic grading of the liver biopsy scores.

Stephen R. Werre, PhD a Research Assistant Professor of Veterinary Medicine Experimental Statistics at the Virginia-Maryland College of Veterinary Medicine. Dr. Werre helped performed statistical analysis on all study data and was critical to data interpretation.

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Chapter 1: Thesis Organization

This thesis is compiled and formatted to discuss the current understanding of knowledge of nociceptive system anatomy and physiology, and the mechanisms of action and side effects of common analgesics utilized in equine veterinary medicine. The following literature review is therefore focused to the understanding of analgesic medications available in equine veterinary medicine, followed by a review of acetaminophen in human and veterinary literature. One manuscript is attached, which is currently in progress for submission to publication. The manuscript is assessment of the pharmacokinetics and safety of repeated oral dosing of acetaminophen in adult horses.

Chapter 2: Literature Review

Defining Pain

The treatment and management of pain is a cornerstone of equine practice, and pain is often the first or only clinical sign displayed to signify underlying disease or dysfunction. Pain has been described in animals as "an aversive sensory experience that elicits protective motor actions, results in learned avoidance, and may modify species-specific traits of behavior, including social behavior."[1] Pain, or nociception, encompasses the action of transduction, transmission, modulation, and central processing of potential, or actual, noxious or painful stimuli.[2] There are, however, many different types of pain states, and it is critical to be able to differentiate these pain states in order to examine potential therapeutic modalities. The most acute form of pain is known as nociceptive, or physiologic pain. This type of pain is evoked by high-intensity noxious stimuli and serves to act as an adaptive and localized pain response to signal to the body the presence of potential tissue damage.[2] Inflammatory pain, however, is evoked by a variety of stimuli that are secondary to tissue injury. Inflammatory pain serves to promote healing and protect the body during the healing process by producing a hypersensitivity state that parallels the duration of active inflammation.[3] Neuropathic pain is also evoked by a variety of stimuli, as well as peripheral and central nervous system neuropathies. Frequently this type of pain is called maladaptive pain, as it results from central sensory amplification that is persistent and maintained independent of a lesion or disease.[3] Finally, dysfunctional pain occurs from a variety of stimuli, and is defined as being of unknown cause.[2]

Anatomy and Physiology of the Nociceptive System

Nociception is characterized by the process in which noxious stimuli is detected by peripheral nerve fibers, called nociceptors. Anatomically, the cell bodies of nociceptors reside in the dorsal root ganglia or the trigeminal ganglion for the body and the face, respectively. The cell body then has a peripheral branch that innervates the target organ, and central branch, which innervates the spinal cord. Nociceptors, then convert the noxious stimuli to electrical impulses in a process called transduction. These electrical impulses can then be transmitted by primary sensory nerve fibers which are either unmyelinated (C fibers) or covered by a thin myelin sheath (Aδ).[4] The difference between these nerve fiber subsets occurs in the rapidity of their transmission, with Aδ nerve fibers faster than C fibers.[5] Aδ nerve fibers therefore transmit quick pain impulses, producing the sharp, acute pain that stimulates rapid withdrawal from a stimulus.[5] It is of note that, in horses, visceral pain action potentials are transmitted by C fibers within the autonomic nervous system.[6]

Aδ receptors can be even further subdivided into type I high threshold heat and mechanical nociceptors, and type II low threshold heat, high mechanical threshold nociceptors. Aδ Type I nociceptors become sensitive to stimuli (either heat or mechanical) in the presence of tissue injury, and are typically associated with the first pain provoked by intense mechanical stimulus.[4] Type II Aδ nociceptors, however, are typically associated with the first pain provoked by an intense heat stimulus.

C fibers are characterized by a heterogenous population of heat and mechanically sensitive nociceptors.[7] In addition to traditional heat and mechanical C-fibers, there is also a class of heat-responsive C fibers that lack mechanical sensitivity.[8,9] These fibers are known as “silent” nociceptors that only develop mechanical sensitivity with tissue injury, and appear to be

more responsive to the chemical pro-inflammatory mediators released with tissue injury.[4] This class of nerve fibers are not only associated with nociception, but appear to also have responses to cooling, itch-inducing molecules and pleasant touch.

The afferent nerve fibers of nociceptors are pseudo-unipolar, which allows for central and peripheral nerve terminals to originate from a shared axonal stalk.[4] This function is significantly different from the typical neuron, and allows for the nociceptor to send and receive stimuli from either end. This phenomenon can better explain the concept of neurogenic inflammation, as the peripheral nerve terminal can release neuropeptides such as substance P or calcitonin gene-related peptide (CGRP), leading to tissue inflammation and vasodilation.[10,11] While both the central and peripheral terminals can respond to endogenous modulators, only the peripheral nerve terminal is able to respond to environmental stimuli.

The afferent nerve fibers of the nociceptors project to the dorsal horn of the spinal cord, and within the dorsal horn there are several different zones with distinct characteristics. Spinal cord neurons that reside in lamina I are typically Aδ and C fibers, which are responsive to noxious stimuli. C fibers also project into lamina II.[9,12] C fibers are typically peptidergic (eg releasing substance P, neurokinin A, CGRP, and tyrosine kinase A)[13] in the dorsal aspect of lamina II, nonpeptidergic affects typically terminate in the mid region of lamina II, and excitatory interneurons that express protein kinase C reside in the ventral region of lamina II.[9,12] Neurons that project into laminae III and IV are responsive to stimulation that is not provoked by tissue damage with Aβ input.[9,12] Finally, neurons in lamina V of the spinal cord receive noxious and undamaging input from A fibers, and indirect C fibers.[9,12] The convergence of noxious and innocuous input in lamina V by indirect polysynaptic C fibers is characterized by wide-dynamic range neurons converging somatic and visceral pain. [4]

The major output from the dorsal horn of the spinal cord to the brain is carried by neurons that travel ascending pathways to the brain in laminae I and V.[4,14] The spinothalamic tract carries messages to the thalamus and allows discernment of the intensity and location of the pain source.[15] The spinoreticulothalamic tract, however, carries messages to the brainstem that are more associated with poor localization of pain.[4,16] Finally, these signals are transmitted to cortical structures such as the somatosensory cortex and anterior cingulate gyrus, or insular cortex, that aid in the processing of sensory or emotive experiences or pain, respectively.[17,18]

Reception and Conduction of Pain

In nociceptive and inflammatory pain states, the presence of a noxious stimulus (thermal, mechanical, chemical, etc) leads to the activation of nociceptive neurons that release action potentials at high-threshold nociceptors, which then convert (transduce) the stimuli to electrical impulses that can then be transmitted by thinly myelinated (Aδ) and unmyelinated (C) primary sensory nerve fibers.[4] A variety of different candidate receptors are implicated in activation of nociceptors.

Heat sensation is most commonly detected by the vanilloid receptor TRPV1, which is part of the transient receptor potential ion channel family.[4] TRPV1 receptors are contributory to acute heat sensation, as evidenced by impairment of response of TRPV1 knockout mice to heat or capsaicin sensitivity.[19] This receptor channel also appears to be partially responsible for heat hypersensitivity seen in tissue injury and inflammation, as the TRPV1 responses are enhanced by pro-inflammatory agents.[20] Cold sensation, however, is commonly detected by TRPA1 or TRPM8 receptors.[4] Mechanical sensation is typically perceived by a combination of high threshold mechanoreceptors (C fibers, Aδ fibers), and low threshold mechanoreceptors (Aδ D-

hair fibers and Aβ nerve fibers). Low threshold mechanoreceptors are typically responsive to light touch, hair vibration, or texture, but not the noxious stimuli to which high threshold mechanoreceptors are typically responsive. [4] Finally, chemical nociception are vital in generating protective withdrawal responses from environmental irritants, and are typically governed by TRP channels, such as TRPA1 (thiols), TRPV1 (capsaicin), and TRPM8 (menthol). [4]

Once nociception takes place by the afferent nerve terminal, the receptor potential proceeds to activate a number of voltage-gated ion channels that transmit action potentials to the dorsal horn of the spinal cord. Voltage gated sodium and potassium channels are essential in generating an action potential, while voltage gated calcium channels release neurotransmitters from nociceptor terminals.[4] These voltage gated ion channels are subsequently emerging as potential therapeutic targets for mitigation of pain sensitization and neurogenic pain.[21,22]

Pain sensitization is caused by the production and distribution of inflammatory tissue byproducts, such as prostaglandins, peptides (bradykinin, substance P, CGRP), cytokines (IL1β, IL6), eicosanoids, endocannabinoids, leukotrienes and ions (hydrogen and potassium). [4,5] These byproducts are then released into the injured area and cause repetitive stimulation of the peripheral sensory nerves which can lead to the pain state of central sensitization via summation and production of prolonged action potentials in dorsal horn sensory neurons of the spinal cord.[5] Central sensitization is what allows low-intensity stimuli to produce pain, contributing to changes in sensory processing in the spinal cord that lead to the release of inhibitory neurotransmitters from descending pathways of the spinal cord, such as 5-hydroxytryptamine, norepinephrine, and endogenous opioids. [5] In instances of acute pain, glutamate is released from the cell bodies of nerves residing in the dorsal horn of the spinal cord. While glutamate typically binds preferentially to NMDA receptors within the spinal cord, NMDA receptor transmission is

blocked by magnesium sitting in the receptor pore site.[23] Therefore, glutamate release begins to generate excitatory post-synaptic currents (EPSCs) from low-affinity AMPA receptors.[4] When enough EPSCs summate to form an action potential, the action potential is then propagated to more central targets. When tissues are significantly injured, there is an increased release of neurotransmitters (substance P, CGRP, glutamate) that remove the magnesium blockade on NMDA receptors, which is the key step for developing central sensitization.[13] Removal of the magnesium blockade on NMDA receptors allows for an increase in entry of cytosolic calcium in the post-synaptic neuron, ultimately resulting in exacerbation of responses to noxious stimuli and hyperalgesia that presents as "wind-up" pain.[4,13,23] Substance P is also involved in central sensitization by causing long-term depolarization within the spinothalamic, spinoparabrachial, and spino-PAG neurons via binding to neurokinin-1 GPCRs.[13] Central sensitization is further exacerbated in nerve injury by activation of the microglia and astrocytes that function to release cytokines (such as TNF-α, IL1β, and IL6), which serve to further increase central sensitivity to pain.[4] Peripheral nerve injury also leads to a loss of inhibitory post-synaptic signaling in the GABAergic or glycinergic inhibitory interneurons that reside primarily in the dorsal horn.[4] The disinhibition of tonic inhibitory control exerted by GABAergic or glycinergic interneurons leads to an increase in excitatory responses generated by nociceptor neurons. Therefore, the peripheral sensitization, central sensitization, and disinhibition ultimately comprise the progression of pain states. [5] Furthermore, peripheral nerve injury caused by trauma or conditions leading to neuropathic pain (i.e. laminitis) can cause a central wind-up phenomenon that leads to increased animal suffering.[24-26]

Neuropathic pain is typically characterized by the presence to two types of hypersensitivity: 1) allodynia, and 2) hyperalgesia. Allodynia is considered to be "pain that is in

response to a non-nociceptive stimulus."[2] Some examples of allodynia include exaggerated pain responses from movement of hair or stroking of skin. Hyperalgesia, however, is an increased pain state in response to a nociceptive insult, with some examples being mechanical pressure on the skin, or small pinpricks.[2,27] Inflammation stimulates activation and migration of macrophages into the dorsal root ganglion of injured nerve, leading to release of proinflammatory cytokines such as TNF α.[28] Nerve injury also leads to upregulation of receptor proteins which are physiologically activated by heat (TRPV1) or cold (TRPM8) states, leading to the abnormal pain response to mild to moderate warm or cold stimulation.[28] In neuropathic pain states, the normally high thresholds of Aδ and C fibers are dramatically lowered, with spontaneous activity seen in both injured and uninjured nociceptive fibers.[28] This spontaneous activity is facilitated by increased expression of sodium channels in affected and unaffected nerve fibers, serving to lower action potential thresholds and leading to increased nerve activity.[21,29] In fact, in mice lacking voltage gated sodium channels (Nav1.7), responses to inflammatory pain, as well as low/high threshold mechano- and thermoreceptors are significantly attenuated.[30] However, in order for allodynia and hyperalgesia to be seen in areas adjacent to injured nerves, CNS involvement is required.[27] The continued activation of afferent nerve fibers releases excitatory amino acids and neuropeptides in the dorsal horn of the spinal cord, which leads to changes such as phosphorylation of NMDA or AMPA receptors, or increased expression of sodium channels.[28]

Neurotransmitters

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine which interacts with 5-HT receptors that primarily reside in the cerebral cortex, hippocampus, and cerebellum.[31] Other ancillary sites of 5-HT receptors include the amygdala, caudate nucleus, hypothalamus,

thalamus, substantia nigra, and the spinal cord.[32] While serotonin is commonly known for its interactions with the central nervous system, the majority of serotonin resides in the gastrointestinal tract, where it is synthesized in intestinal enterochromaffin cells from L-tryptophan.[33,34] The activities of 5-HT within the gastrointestinal tract are incompletely known, but potentially include roles in maintaining normal gastrointestinal tract homeostasis. Serotonin may also play a role in immune cell function and angiogenesis in inflammatory bowel disorders.[34] Peripheral nociceptive activity of 5-HT is typically involved with other proinflammatory mediators at the site of tissue injury leading to excitation of primary Aδ and C fibers.[35] There are a variety of different 5-HT receptors, of which one of the most important in terms of anti-nociception appears to be the 5-HT_{5A} receptor, which has been shown to reduce pain processing in the spinal cord of rats affected by spinal nerve ligation.[32] 5-HT_{5A} is expressed in the dorsal root ganglia, which is compatible with serotonin receptors being present at the site of spinal processing of pain.[32,36] Furthermore, serotonin and 5-carboxamidotryptamine (5-CT) administered intrathecally have also been shown to reduce expression of neuropathic pain in rats with spinal nerve ligation through activation of 5-HT_{5A} and 5-$HT_{1A/1B/1D}$ receptors.[32] Activation of 5-HT_{1A} receptors has also been demonstrated to modulate the nociceptive response through inhibition of adenylyl cyclase, resulting in closure of calcium channels in the post-synaptic neuron, inhibiting conduction of excitatory action potentials in the dorsal horn of the spinal cord.[37] In particular, humans with higher 5-HT_{1A} receptor density have been noted to have an increased capacity for central suppression of pain.[38] Activation of 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_6, and 5-HT_7 receptors, however, have demonstrated pro-nociceptive effects in rat models. [32,35]

The endocannabinoid (EC) system is present throughout the peripheral nerves and spinal cord, but primarily within central nervous system nociceptive regions.[39-43] The EC system is

comprised of endocannabinoids, two G-protein coupled receptors known as cannabinoid type 1 (CB_1) and cannabinoid type 2 (CB_2).[40] CB_1 is expressed centrally within the neocortex, cerebellum, and limbic systems, where activation can lead to initiation of descending inhibition to spinal cord nociceptive mechanisms, as well as modulation of the emotional aspects of pain in humans.[44] CB_1 is typically expressed peripherally on nerve cells, and predominately on pre-synaptic axonal terminals, where it functions to reduction of pre-synaptic neurotransmitter release through inhibition of calcium channels and activation of potassium channels.[39,40] This inhibition of calcium channels leads to inhibition of secretion of neurotransmitters, which can alternatively inhibit or excite the neuron depending on that neuron's function. CB_2 is found mostly on immune system cells (eg mast cells, B cells, macrophages, microglial cells, natural killer cells) and typically has limited neuronal expression.[40] Endocannabinoids are lipid signaling molecules that are produced via enzymatic cleavage of membrane phospholipids to form the arachidonic acid derivatives AEA, 2-AG, NADA, and virodhamine, with AEA and 2-AG being the best characterized and studied.[4] These transmitters can be produced on demand, and therefore allow for plasticity of the neuronal response to synaptic transmission. While 2-AG is a full agonist of both CB_1 and CB_2 receptors, AEA appears to have a selectivity for CB_1 and TRPV1 receptors.[40] Activation of cannabinoid receptors at the peripheral level has been shown to attenuate central sensitization of pain responses due to the location of cannabinoid receptors in the dorsal root ganglion cells of the spinal cord.[40] Activation of these receptors at spinal sites has demonstrated the ability to attenuate pain responses in models of inflammatory and neuropathic pain through suppression of C-fiber responses of dorsal horn neurons.[40] Finally, activation of CB_1 at central sites has been demonstrated to decrease GABA or glutamate neurotransmitter release, particularly within the dorsal horn and ventralposterior lateral nucleus of the thalamus.[42]

Administration of exogenous endocannabinoids has demonstrated anti-nociception in tissue injury models of acute and persistent inflammatory pain, as well as neuropathic pain in animal models.[40,41] However, these studies are limited by the administration of exogenous endocannabinoids, which have a longer half-life than endogenous endocannabinoids. [43] Therefore, the true biologic activity of endogenous endocannabinoids remains unknown.

COX Enzymes and Prostanoids

Prostanoids are considered to be some of the principal mediators of inflammatory pain. Prostanoids are biosynthesized cyclooxygenase mediated metabolites of arachidonic acid that act with paracrine or autocrine function in a variety of health and disease states.[45-47] Phospholipase A_2 (PLA_2) is involved in the inflammatory response of tissues, which releases arachidonic acid from membrane phospholipids, allowing it to be metabolized by lipoxygenases, epoxygenases and cyclooxygenases (COX) enzymes to form prostanoids or leukotrienes.[48] As COX enzymes are a key target of therapeutic interventions in inflammatory pain states, this review will primarily focus on the interactions of COX enzymes and prostanoids with inflammatory pain states.

Introduction to Cyclooxygenases

COX enzymes are membrane bound proteins that are produced by the endoplasmic reticulum and catalyze bis-oxygenase and peroxidase reactions in the arachidonic acid cascade.[49] The bis-oxygenase reaction of COX enzymes functions to convert arachidonic acid to PGG_2, while the peroxidase reaction coverts PGG_2 to PGH_2. PGH_2 then undergoes isomerization,

oxidation, or reduction by an assortment of prostanoid synthases to generate PGE_2, PGI_2, PGD_2, $PGF_{2\alpha}$, and Thromboxane A_2 (see fig. 1).

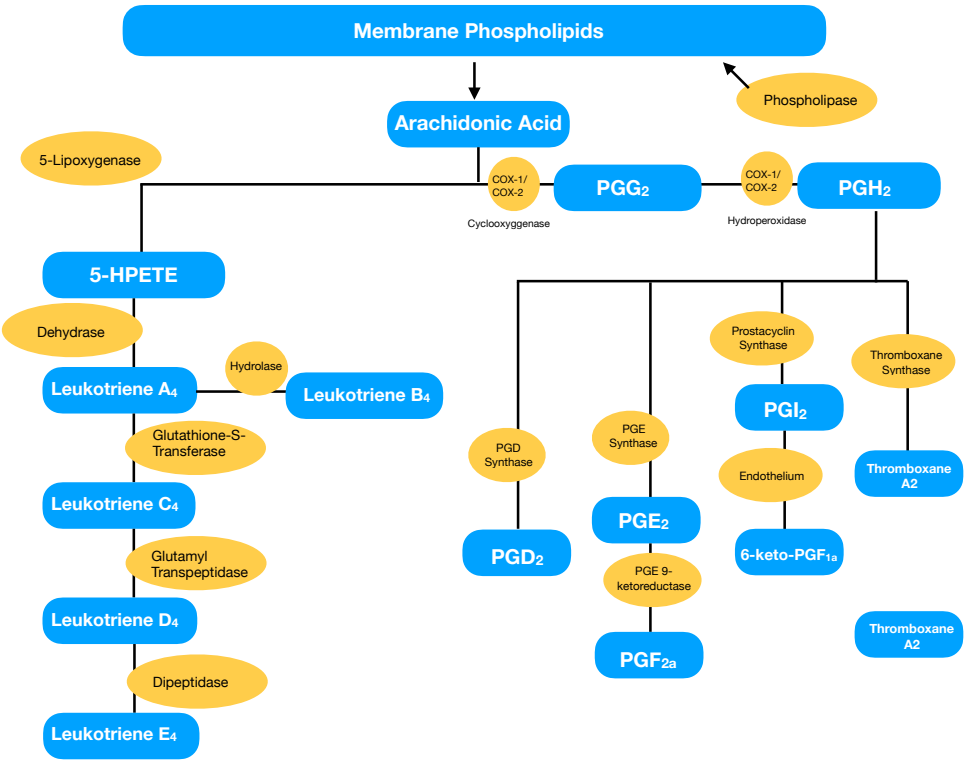

Fig 2.1: Simplified arachidonic acid cascade. Blue boxes signify metabolites of arachidonic acid, yellow ovals represent enzymes that catalyze reactions to produce the metabolites.

The COX enzymes present in mammalian species include COX-1, a variant of COX-1 known as COX-3, and COX-2. COX-1 is expressed in most tissues, and is considered to be the constitutive isoform, maintaining a variety of housekeeping functions including renal blood flow, gastrointestinal mucosal integrity through production of PGE2, and platelet aggregation through production of thromboxane A_2.[50] COX-1 appears to primarily interact with thromboxane synthase, prostaglandin F synthase, and the cytosolic prostaglandin E synthase (PGES) isozyme in the formation of prostanoids; though the physiologic ramifications of this heterogeneity and preferential interaction remain unknown.[47,51] While COX-2 has been historically described as an enzyme that is induced in proinflammatory states in most tissues, this statement has been refuted

in recent years with the discovery of COX-2's various activities in maintaining healthy gastrointestinal, renal, and cardiovascular homeostasis.[48] Similar to COX-1, COX-2 also displays a heterogenic interaction with downstream enzymes in the arachidonic cascade, including prostaglandin I synthase (PGIS) and the microsomal (m) PGES isozymes.[52-54] However, unlike COX-1 preferential interactions, the induction of PGIS and mPGES by COX-2 has been correlated with its activities against induction of inflammatory cytokine release and tumor promotors.[47] The interaction of each of these enzymes in tissues becomes more complex, as the preferential induction of COX-1 or COX-2 depends on the level of available arachidonic acid, with a COX-2 response predominating in low arachidonic acid states, and a COX-1 response prevailing when arachidonic acid in tissues is high.[55] The oversimplification of these enzymes' complex function has led to the introduction of several COX-2 specific inhibitors to the human and veterinary market in order to attempt to improve upon the safety profile of nonselective COX inhibitors. However, these selective COX-2 inhibitors were noted to have several detrimental cardiovascular effects in the humans, resulting in near complete withdrawal from the human market, though many veterinary products still exist.[48] Therefore, it is prudent to further explore the activities of COX-1 and COX-2, particularly in their relation to their differential expression and inflammatory pain.

Cyclooxygenase Functions in Tissues and Consequences of their Inhibition

As stated above, COX-1 is expressed in most tissues, where it mainly functions to maintain a degree of basal prostanoid synthesis. COX-1 activity in the gastrointestinal tract is cytoprotective, where it is primarily responsible for the production of PGE2 and PGI2.[56] Both of these prostanoids serve their cytoprotective function by promoting secretion of bicarbonate

and mucous and decreasing gastric acid secretion in the GI tract to aid in neutralization of acidic GI contents, and as well as by promoting mucosal blood flow.[56,57] Therefore, it was long postulated that the increase in gastric ulceration seen with non-selective COX-inhibitor administration was due to the effects of COX-1 inhibition, as administration of COX-2 inhibitors failed to produce gastric ulcers.[58] However, COX-1 knockout mice have been found to be resistant to gastric ulceration following indomethacin challenge compared to wild type, despite having dramatically lower PGE2 concentrations.[59] The discrepancies between the clinical reports of increasing gastric ulceration with nonselective COX inhibitors vs. COX-2 selective inhibitors and the basic science research may be due to a variety of unknown mechanisms, including potential compensatory upregulation of COX-2 or other enzymes when COX-1 is inhibited.[60] While classically COX-2 inhibition was purported to have less of a risk of gastrointestinal ulceration, recent studies have begun to refute that claim. In a study comparing COX-1 and COX-2 knockout mice, Sigthorsson et al. found that long term COX-2 inhibition or deficiency lead to losses in small intestinal mucosal integrity and increases in inflammation similar to indomethacin challenge, despite normal levels of PGE2 in the small intestine, suggesting a complex relationship between COX-2 and mucosal integrity.[60] As discussed above with COX-2 inhibitors, COX-1 appears to have preferential localization within the gastrointestinal tract, with adverse effects observed with COX-1 inhibition in the stomach, and minimal effect of COX-1 inhibition in the small intestine.[60] However, these theories may be limited in cross-species extrapolation, as differential expression of both COX-1 and COX-2 have been found between monkeys, humans, rats, and dogs.[61,62] In horses, COX-1 has been found to be predominant isoform expressed in squamous mucosa of the healthy stomach, while COX-2 expression was

significantly increased compared to COX-1 expression in ulcerated mucosa, suggestive of COX-2's role in healing gastric ulceration.[63]

As COX-2 inhibition appeared to be helpful in minimizing the risk of gastric ulceration in the human population, a wide variety of COX-2 selective inhibitors were developed for the human market. Unfortunately, the majority of these COX-2 selective drugs have been withdrawn due to the increased risk of myocardial infarction and stroke seen with their use.[64] The underlying physiology of their cardiovascular effects depends on the opposing functions of COX-1 and COX-2 on vascular tone and platelet function. COX-1 in the cardiovascular system is essential for normal platelet function, as only COX-1 is expressed on platelets and the thromboxane A2 (TXA2) subsequently produced promotes vasoconstriction, platelet aggregation and formation of a hemostatic plug.[65] COX-2 is constitutively expressed in the heart. In the cardiovascular system, COX-2 leads to the production of PGI2, which acts as a vasodilator and directly counteracts the effects of TXA on platelet aggregation.[64] Therefore, when COX-2 selective inhibition occurs, the balance shifts toward a pro-thrombotic and vasoconstrictor state, leading to increased risk of myocardial infarction and stroke in susceptible populations. However, the risk of increased blood pressure and development of congestive heart failure also exists with the administration of non-selective COX-inhibitors in susceptible human populations.[66]

In the kidney, COX-1 is predominant in glomerular mesangial cells, collecting duct cells, and arteriolar endothelial cells of the cow, rabbit, guinea pig, rat, and mouse, while in humans it is additionally expressed in the vasa recta.[67] COX-2, however, is expressed—with some species variation—in the glomerulus, macula densa, thick ascending limb, and medullary interstitial cells.[67] There have been dramatic differences noted in the renal function of COX-1 and COX-2

knockout mice, where COX-1 knockout mice have apparently normal renal function, while COX-2 knockouts have severe nephropathy with wide variations in glomerular maturity, suggestive that COX-2 has a greater housekeeping function in the kidney.[59,68,69] The adverse effects of NSAIDs on the kidneys are largely through the perturbations in the production of PGE2, which functions to regulate vasopressin and its effect on osmotic body water balance by promoting diuresis.[67] COX-2 also serves to facilitate the production of PGE2 and PGI2 in the renal cortex, leading to local vasodilation and therefore increases glomerular filtration rate and renal blood flow in the normal kidney.[47] COX inhibitors, both non-selective and COX-2 selective, have been associated with increased risk of acute renal injury in humans[70] and horses.[71] This effect is largely observed under ischemic conditions, where renin-angiotensin and adrenergic system activation leads to further renal vasoconstriction, and under the influence of COX-inhibition, synthesis of vasodilatory prostaglandins such as PGE2 and PGI2 is halted, preventing compensatory vasodilation and normalization of GFR.[67] The unchecked renal vasoconstriction then causes medullary ischemia due to redistribution of blood flow to the renal cortex, producing characteristic renal papillary necrosis in NSAID toxicity of horses.[71] Furthermore, as PGI2 synthesis is primarily COX-2 mediated, the previous paradigm of improved renal safety of COX-2 selective inhibitors in patients with ischemic or volume depletion conditions, is likely erroneous.[72,73]

COX-3 is a splice variant of COX-1, which was first discovered in the CNS of dogs[74], and has been further elucidated in rats[75] and humans.[76] In the central nervous system, COX-3 has been noted to be expressed in the hypothalamus, pituitary and choroid plexus.[75] This enzyme initially appeared to be one of the central nervous system targets of analgesic/anti-pyretic drugs such as acetaminophen, dipyrone, and phenacetin which lack anti-inflammatory activity,[77] but its

involvement in pyresis has been more recently disputed, which is discussed further in conjunction with acetaminophen.

COX-1 and Inflammation

Recent research performed in COX-1 deficient mice has begun to elucidate the complex interactions between COX-1 and inflammatory states. COX-1 deficient mice have been shown to have reduced inflammation in ear edema and subdermal air pouch models when compared to wild type and COX-2 deficient controls.[59,78] Furthermore, while COX-2 is the predominant enzyme in the induction of a prostaglandin response to LPS, increased COX-1 expression was also found in the circulating monocytes of mice treated with LPS, demonstrating induction of COX-1 expression as a direct result of a profound inflammatory insult.[79] COX-1 induction has also been implicated in modulation of neuropathic and post-operative pain, where intrathecal administration of a nonselective COX inhibitor resulted in a delay in onset of hypersensitivity following peripheral nerve injury in rats, and intrathecal administration of a COX-1 selective inhibitor resulted in complete blockade of the development of hypersensitivity.[80] Furthermore, additional studies of peripheral nerve injury in rats demonstrated a time- and location-dependent change in expression of COX-1 in the spinal cord in response to injury, suggesting that COX-1 plays a role in modulating central sensitization and processing of pain. [81]

COX-2 and Inflammation

Induction of the COX-2 enzyme in inflammatory states is much better recognized. COX-2 induction primarily begins at the local tissue level with inflammation, where high levels of COX-2 have been found in injured cells, as well as macrophages, neutrophils, and mast cells which have infiltrated the site of tissue injury in murine models.[48,82] COX-2 also has central activity with pain states, where induction of expression of COX-2 in the dorsal root ganglion has

been found with periganglionic or peripheral nerve inflammation, where it was not present prior to inflammatory stimulus.[48,83] The central activity of COX-2 may also be mediated through its interaction with the endocannabinoid system. COX enzymes have been shown to metabolize AEA and 2-AG, which contributes to the short half-life of these compounds, particularly in inflammatory states.[84] Therefore, it has been postulated that COX inhibition could promote a longer half-life of endocannabinoids centrally by decreasing endocannabinoid breakdown.[85] Following the induction of inflammatory knee pain in rats, intrathecal selective COX-1, selective COX-2 and non-selective COX inhibitors were all found to attenuate spinal hyperalgesia before or during inflammation via reduced release of spinal PGE_2.[85] However, only selective COX-2 inhibitors reversed spinal hyperalgesia once established, and only COX-2 inhibitors prevented the breakdown of 2-AG.[85] Intrathecal injection of a COX-2 selective inhibitor (nimesulide) in rats demonstrated a decreased response to mechanical stimuli in rat dorsal horn neurons, and these effects were eliminated by administration of a CB1 receptor antagonist, however this study failed to demonstrate concomitant elevations in 2-AG and AEA.[86] Therefore, while induction of COX-2 in inflammatory pain states has been well established, the underlying mechanisms activated following this induction have not been fully elucidated.

Prostanoids

The most well studied and principal pro-inflammatory prostanoid generated from the arachidonic acid cascade is PGE_2, which interacts with both peripheral and central nociceptive pathways.[48] In peripheral sites, PGE_2 interacts with protein kinase A or C to lead to sensitization of the TRPV1 receptor, as well as tetrodotoxin resistant sodium channels (TTX-R Na), calcium channels, and voltage gated potassium channels.[48] Sensitization of the TRPV1 receptor by PGE_2 leads to lower activation thresholds and increase in thermal nociception.[87] Other local activities

of PGE_2 include increasing sensitivity to bradykinin, or capsaicin on peripheral nerves, resulting in lasting hyperalgesia to mechanical and chemical stimuli.[48,88] Centrally, PGE_2 has been found to increase dramatically within the spinal cord following inflammation or nociception. PGE_2 has been found to facilitate release of glutamate, substance P, and CGRP via inward calcium currents at the pre-synaptic level.[89,90] Post-synaptically, PGE_2 enhances nociceptive transmission through potentiating AMPA and NMDA currents via activation of PKA or direct mechanisms.[91,92]

Other prostanoids implicated in inflammatory pain include PGI_2 and PGD_2. Prostaglandin I_2 (PGI_2) production has also been found to rapidly increase at the site of tissue injury following inflammatory stimuli, which is chiefly modulated through the PGI_2-IP pathway.[48] PGD_2, however, has demonstrated both pro- and anti-inflammatory properties in tissues.[48] PGD_2 has been implicated in promoting PGE_2 mediated pain states, as PGDS-knockout mice did not exhibit allodynia associated with intrathecal PGE_2 administration in mice, while the mice continued to exhibit thermal sensitivity.[93]

Equine Pain

Methods of determining pain in equids include both physiologic and behavioral methods. Among veterinarians in the UK, behavioral indicators of pain severity (as graded by a supplied pain severity scoring rubric) and heart rate were determined to be the two most common factors in determining the dose and frequency of analgesic administration.[94] Physiologic measures of pain in horses have classically included variations in heart rate, respiratory rate, and non-invasive blood pressure. Yet these measures of pain may be complicated by concurrent disorders, such as endotoxemia, cardiovascular compromise, or dehydration. Changes in heart rate were not found to be significant between treatment and control groups in wound sensitivity, perioperative analgesia, or post-operative arthroscopy patients in horses.[95-97] However, heart rate and plasma

cortisol concentrations have been found to be linked to post-operative pain following exploratory celiotomy in horses.[98] Heart rate variability (HRV) has become an increasing field of research in determining the effect of autonomic nervous system activation from pain or psychosomatic states on heart rate and variability of beat to beat intervals. This can be determined from evaluating the frequency content of the peaks in HRV, with increased low-frequency peaks associated with sympathetic activity, and high frequency peaks due to vagal activity.[99] Heart rate and heart rate variability have also been found to be correlated with pain and anxiety states due to autonomic system activation, suggested by an increase in heart rate and a decrease in the R-R interval.[100] In horses with laminitis prior to NSAID administration, there was a positive correlation toward an increase in low-frequency peaks and decrease in high frequency peaks in horses with higher Obel grade and orthopedic laminitis index, suggestive of greater sympathetic nervous system activation in more severe pain states.[99] Additionally, horses with ischemic gastrointestinal disease have been found to have reduced heart rate variability, which has been correlated to non-survival in these patients.[101] Therefore, heart rate variability may be able to improve the identification of pain on physiologic assessment of horses. Measures of systemic stress, such as serum cortisol and serum beta-endorphins, have also been used as part of a physiologic assessment of pain in horses. However, the use of these measures as a sole indicator of pain has been unrewarding, and elevations have been seen both in acute pain, chronic lameness, and stress of travel.[95,102,103]

Behavioral methods of measuring pain in equids have been described in the literature in order to detect the presence of pain, but have historically been challenging in their utility to assess the severity of pain.[103] Considerable variation exists between the outward expression of pain amongst equids, with donkeys, mules, and feral horses being significantly less likely to

demonstrate pain compared to domesticated horses when faced with similar insults.[104-106] Common postures and behaviors associated with generalized pain in equids include restlessness, agitation, anxiety, lowered head carriage, reluctance to move, and aggression.[103,104,107-109] An equine pain face has been recently described using a combination of mechanical (tourniquet) and chemical (topical capsaicin) pain, which was consistent and statistically significant when compared to control.[110] This face was demonstrated as a combination of asymmetric/lowered ears, angled eye, tension of the facial mimic muscles, withdrawn/tense state, dilation of the nostrils, and tension of the muzzle.[110] Behavioral associations with pain in equids have been most consistently associated with abdominal and orthopedic pain. Research associated with behavioral expressions of abdominal pain in horses has been linked to significant increases in odds ratios of non-survival, with horses expressing the highest pain scores being 10 times more likely to die in a retrospective evaluation of horses with colic.[111] Behavioral signs of abdominal pain in horses that have been regularly associated with colic include rolling, kicking at the abdomen, flank watching, and stretching.[5,103,112,113]

In contrast, horses faced with orthopedic pain typically demonstrate signs of altered weight distribution, seen as weight shifting, lifting the affected limb, and attempting to off-load painful areas. Laminitis is an incapacitating disease of the distal limb in the horse and is widely considered a global problem in equine welfare, with epidemiologic estimates of prevalence at 7-14%.[24,114] Laminitis results in pathologic changes to the distal limb which result in derangements of foot placement and loading due to severe discomfort, and the inability to pharmacologically and therapeutically mediate the severe, unremitting pain is the most common cause of euthanasia.[24] Behavioral changes in horses with laminitis include leaning backward, pointing, rotating limbs, weight shifting, and reluctance to move. The severity of weight shifting and the

time spent facing the back of the stall in horses with laminitis has been shown to correlate not only with severity of pain, but also stage of disease and number of limbs affected.[26,115]

The challenge of treating pain in the laminitic horse arises from the complex, and incompletely understood pathobiology of the disease, and the multiple pain pathways that laminitis affects, and thus makes it an excellent discussion point for chronic pain states in equids. While subacute and acute laminitic pain largely arises from inflammatory and nociceptive mediators, prolonged activation of the inflammatory cascade leads to peripheral and central sensitization that is refractory to traditional non-steroidal anti-inflammatory therapy.[25,26] The neuropathic changes seen in chronic laminitis cases mirror those seen in laboratory models of neuropathic pain, such as chronic constriction injury, diabetic neuropathy, and crush injury.[26] Sensory innervation to the foot is through a combination of thick myelinated A_β-fibers that are responsible for the transmission of low threshold mechanical stimuli, C fibers, and A_δ fibers that are encased in a thin myelin sheath that transmit high-threshold nociceptive information through expression of peptides, and sympathetic nerve fibers.[25] Damage to A and C-fibers in the hoof capsule seen in chronic laminitis appears to be critical in the development of hyperalgesia and allodynia in laminitic horses, which can ultimately lead to central sensitization of pain.[26] This damage has been postulated to be due to a combination of sensitization of the nerve fibers due to chronic soft tissue deformation, and stretching of the nerve fibers due to distal phalanx displacement.[24] Furthermore, chronic inflammatory states expose the nerve fibers to reactive mediators (eg prostaglandins, bradykinins, cytokines) that serve to further lower the excitation threshold.[24] This ultimately leads to a decrease in myelinated and unmyelinated nerve fibers per unit area and an increase in solitary unmyelinated fibers, which may represent an increase in neuropathic pain states due to a loss of spinal inhibitory control.[26] Finally, maladaptive pain

develops from a combination of inflammatory pain secondary to the underlying disease pathology, and neuropathic pain due to lowering of nervous tissue excitation thresholds and continued activation, which leads to functional pain due to abnormal nervous system function.[24,28]

In states of hyperalgesia and allodynia, expression of calcium channel subunit α2δ is increased, aiding in signal transmission between first and second order sensory neurons in the dorsal horn of the spinal cord.[24] This protein has also been found to be expressed in the dorsal root ganglia of refractory exacerbated laminitic horses.[24] Therefore, it has been postulated that the addition of calcium channel α2δ-ligands (gabapentin, pregabalin) may be of benefit in horses exhibiting neuropathic pain. These drugs, however, have poor oral bioavailability in the horse (~16%), and intravenous administration is limited in its clinical utility.[25] Other drugs that have been used for neuropathic pain modulation, local anesthetics and NMDA receptor antagonists such as ketamine, are impractical for long-term repeated dose administration in the horse. Therefore, the need for adjunctive analgesics in horses with chronic laminitic pain that is not responsive to NSAID administration is a priority for minimizing equine suffering and improving equine welfare and treatment outcomes.

Pharmacologic Treatment of Pain in Horses

Non-Selective COX Inhibitors

The most commonly used drugs in equine pain management are non-selective COX-inhibitors, such as phenylbutazone and flunixin meglumine, largely due to their combination of relative low-cost and clinical efficacy. While the underlying mechanism of pharmacologic

action is similar between phenylbutazone, flunixin meglumine, and the less-commonly used ketoprofen, the clinical impression of these drugs has prevailed that phenylbutazone has improved efficacy for orthopedic pain, while flunixin meglumine appears to be more efficacious at the treatment of visceral pain in horses.[5,116] This difference in clinical effect may be that tissue distribution of COX isoforms can vary between tissues, or there may even be heterogeneity within tissues, leading to the variable effects of different non-selective COX inhibitors, but conclusive proof of this clinical suspicion has not been found.[50] Yet these impressions may be confounded by evidence of a relative equivalency of these drugs in mediation of induced foot pain[117,118], and improvement of gastrointestinal motility following phenylbutazone administration when compared to flunixin meglumine in post-operative cases of colic.[119] What is conclusive in the difference between the nonselective COX-inhibitors in horses is their effects in endotoxemia.[120,121] Flunixin meglumine has been found to be more efficacious at attenuating the cardiovascular changes[119,122] , clinical signs[123,124], and production of vasoactive agents[121,125,126] when compared to placebo, and similar effects have not been consistently demonstrated with phenylbutazone administration.[50,71,121,127]

When considering the pharmacokinetics of the non-selective NSAIDs, it is important to note that plasma drug concentrations are not a direct correlator of prostaglandin inhibition or analgesic duration, and this is largely due to accumulation of NSAIDs directly at sites of inflammation.[116] Phenylbutazone has demonstrated variably high oral bioavailability (30-90%)[128] with a half-life of 3-10 hours, and undergoes primary hepatic biotransformation into active metabolites which are excreted via the urinary system.[129] Flunixin meglumine has a similarly variably high oral bioavailability (70-85%)[130,131] with a short half-life of 1.2h and rapid urinary excretion.[131] Ketoprofen, a non-selective COX inhibitor, is not available in an oral formulation,

and has a short half-life of 0.88-1.6h.[132,133] Ketoprofen remains in its ionized form under urinary excretion in horses, minimizing the risks of nephrotoxicity due to tubular reabsorption.[132,133]

Given the concerns of adverse side effects of NSAID administration, COX-2 inhibitors were developed and introduced into the veterinary market. The most commonly used and only labeled COX-2 selective inhibitor in horses is firocoxib, but meloxicam[134] and numerous others have been examined including etodolac,[135] deracoxib,[136] nimesulide[137] and cimicoxib.[138] While some of the risks of impaired mucosal healing and ulceration may be mitigated with COX-2 selective inhibitors their efficacy as analgesics when compared to non-selective COX inhibitors remains questionable. Some studies have supported equivalent analgesia to non-selective COX inhibitors in post-operative colics,[139,140] and osteoarthritis.[141-143] However, another reports relative inefficacy of COX-2 selective inhibitors in an induced lameness model when compared to phenylbutazone.[144] In investigating the safety of COX-2 selective inhibitors, oral ulceration, hypoproteinemia, and nephropathy were noted in the higher dose groups.[145]

Side Effects of COX Inhibitors in Horses

The major side effects of COX inhibition in horses, as alluded to above, include gastric and right dorsal colon ulceration, delayed mucosal repair, and nephrotoxicity. Mechanisms of nephrotoxicity in relation to selective and non-selective COX inhibition has been discussed above. Studies have demonstrated the nephrotoxicity of non-selective COX inhibitors, with particular emphasis on phenylbutazone and flunixin meglumine.[146,147]

Gastric ulceration is one of the key side effects of NSAID administration in people, and the paradigm has continued into veterinary medicine. Conclusive evidence of NSAID induced gastric ulcers in horses on a population level, however, is currently lacking.[148] In a population study of thoroughbred racehorses, arguably the most at risk population for gastric ulceration,

NSAID use was not a significant variable in the development of gastric ulcers.[149] Further conflicting evidence has been found in a prospective study of Arabian horses, where therapeutic phenylbutazone administration was noted to significantly worsen both squamous and glandular gastric ulcer scores,[150] and an additional study noted that supratherapeutic administration of phenylbutazone was noted to cause glandular and squamous gastric ulcers.[151] A separate study, however, found that repeated therapeutic dosing of phenylbutazone in horses did not lead to the development of gastric ulcers.[152] Therefore, while evidence is present on an individual level that NSAID use is a correlator to gastric ulceration in horses, broad-based population data has yet to demonstrate a similar correlation.

Right dorsal colitis (RDC), however, is a well-documented and potentially fatal complication of long term or high dose NSAID administration, with particular emphasis on non-selective COX inhibitors.[71,146,153,154] While the underlying mechanism of NSAID-induced RDC remains incompletely elucidated, prolonged or high dose NSAID administration has been linked to changes in colonic blood flow, and volatile fatty acid production.[155] Furthermore, the right dorsal colon has been found to be more susceptible to ischemic injury than other segments of the gastrointestinal tract,[156] and horses with RDC have been demonstrated to have alterations in lipoxygenase metabolism in the right dorsal colon, which may contribute to disease.[157] The first clinical manifestation of RDC in horses is a protein-losing enteropathy characterized by hypoalbuminemia, which progresses to diarrhea, colic, fever, edema, and weight loss.[155,157] While historically the mortality of RDC in horses neared 100%, due to improved therapeutic monitoring measures and early recognition of disease modern mortality rates are <20%.[157]

The effect of NSAIDs on equine gastrointestinal mucosal repair is controversial and incompletely understood. As stated above, NSAIDs impair prostaglandin synthesis, particularly

PGE2. Prostaglandins within the GI tract enable mucosal repair through a combination of mechanisms: 1) villous contraction[71] and 2) direct repair through closure of intraepithelial tight junctions via the ClC-2 chloride channel.[158,159] When comparing the different classes of COX-inhibitors in horses, flunixin meglumine has been demonstrated to impair mucosal healing mechanisms, allowing permeability to lipopolysaccharide (LPS) to continue up to 18 hours post-insult.[160] This effect has not been demonstrated to the same extent with COX-2 selective inhibitors, such as firocoxib and meloxicam, leading to the postulation of improved safety against endotoxemia in compromised GI tract.[139,161,162] However, the claims against flunixin meglumine's impairment of mucosal healing have remained contested by the work of in vitro studies, and much remains to be known about the specific effects of COX-inhibition on mucosal repair.[163] Nevertheless, given the side effects of NSAID administration, there has been increasing interest in alternative approaches to analgesic control in horses.

Opioids

Opioids are common analgesics used in equids, particularly for refractory pain associated with colic or orthopedic disorders, predominantly in the form of butorphanol.[164] Ultimately, the analgesic effects of opioids are dependent on the stimulated opioid receptor, and there are two major opioid receptors present throughout the body, in addition to multiple minor receptors. The mu opioid receptor is located in the peripheral neurons, spinal cord, brainstem, midbrain, and cortex, while the kappa opioid receptors are located in the peripheral nerves, spinal cord, and brain.[165] In terms of their utility in treatment of pain in horses, mu-receptor agonists such as morphine and fentanyl are more potent suppressors of orthopedic pain, while kappa-receptor agonists such as butorphanol are more effective against visceral pain.[164]

Tramadol is an opioid that is receiving increasing attention in the equine marketplace as a potential adjunctive treatment for laminitic pain. Tramadol is a weak mu-receptor agonist that also interacts with the 5-HT and adrenergic systems.[165] Its utility in the human for reduction of post-operative pain, plus its potential to be combined with NSAIDs to further improve analgesia,[166] make it an appealing target for the equine veterinary market. While the pharmacokinetics of parenteral administration were appealing, the pharmacokinetics of oral administration of tramadol have been found to be extremely variable in horses, with a bioavailability ranging 3-64% depending on the dose and formulation and variability in detection of the active M1 metabolite, making it an impractical option for home administration.[167-169] Additionally, studies in horses have demonstrated its analgesic efficacy as a single agent in orthopedic pain to be unrewarding, but there is potential for its use as a combination agent.[170,171] Side effects of opioid administration in horses include transient hyperexcitability, gastrointestinal ileus, respiratory depression, and miosis.[164,165] While opioids are potent analgesics, they are controlled substance, which limits their utility in the equine market.

NMDA Receptor Antagonists

Similar to opioids, NMDA receptor antagonists such as ketamine are also controlled substances, limiting their use as analgesics in equids. The mechanism of action of NMDA receptor antagonists is to prevent the release of the excitatory neurotransmitter glutamate, thus reducing the potential for central sensitization as discussed in more detail above. While ketamine has been commonly used as an anesthetic drug in the equine market, subanesthetic doses may be efficacious in treating neuropathic as well as nociceptive pain in horses.[164] While limited studies have been performed in horses regarding ketamine's utility as an analgesic, there is evidence of

its efficacy in combination with tramadol for relief of orthopedic pain[170], and its use epidurally for management of wound-associated pain.[96]

Gabapentin

Gabapentin is an anticonvulsant drug that has been gaining popularity for its utility in the treatment of neuropathic pain states. Gabapentin's mechanism of action is through inhibition of calcium influx into presynaptic nerve terminals of the dorsal root ganglion or dorsal horn of the spinal cord through binding to the $\alpha 2\delta$ subunit of voltage dependent calcium channels, and thus preventing excitatory aspartate or glutamate release.[172,173] Interestingly, gabapentin's analgesic effects are only noted in hyperactive sensorimotor states, indicating that a degree of spinal hypersensitivity must be present for its effect to take place.[172,174] Gabapentin has been successfully used in the treatment of neuropathic pain associated with nerve injury,[175] laminitis,[25] and headshaking disorders,[176] though its bioavailability in oral forms is questionable.[177]

Acetaminophen

Drug Properties and Mechanisms of Action

Acetaminophen (chemical name: N-acetyl-para-aminophenol or APAP, INN: paracetamol) is one of the most commonly used analgesic and antipyretic drugs in the human population.[178] A small, moderately lipid soluble weak organic acid, acetaminophen's high pKa causes it to be completely unionized at all physiologic pH values, which allows it to penetrate into the CNS.[178,179] Its relatively high margin of safety and increased tolerance has made it a mainstay of clinical pain management in humans. Acetaminophen has been combined with NSAIDs, particularly COX-2 inhibitors to provide greater analgesic and anti-inflammatory effects than NSAIDs alone in humans. [178] Despite the lower efficacy of acetaminophen in trials

of human patients with chronic osteoarthritis, acetaminophen remains the preferred analgesic due to its increased tolerance and improved cost-benefit analysis when compared to nonselective and COX2 selective NSAIDs in humans. [180,181] Acetaminophen was found to be preferred by physicians over COX-2 and nonselective NSAIDs even when those drugs were coupled with a proton-pump inhibitor to reduce gastrointestinal side effects, due to the increased cost of treatment of the adverse side effects of NSAIDs.[180,181] Furthermore, while APAP has a similar pharmacologic profile to the non-selective NSAIDs, particularly the selective COX-2 inhibitors, it is considered a weak prostaglandin inhibitor.[182]

While the exact pharmacologic mechanism of action of APAP remains incompletely elucidated, it is known that APAP has effects on endogenous opioid and cannabinoid receptors, as well as involvement with the 5-hydroxytryptamine (serotonin) system and central inhibition of COX enzymes.[178] APAP's analgesic and anti-pyretic effects are largely centrally mediated, where inhibition of the formation of prostaglandin E_2, substance P, and 5-HT-3 receptor antagonists occurs. [178,183,184] The conduction and maintenance of fever is largely mediated by inflammatory cytokines and pyrogens inducing the synthesis of prostanoids, particularly PGE2, in the pre-optic nucleus of the hypothalamus.[185] APAP is a potent anti-pyretic, and while previous research had postulated that the mechanism of action was through COX-3 inhibition,[74,186] more recent studies have demonstrated that APAP's antipyretic action may instead be mediated through central COX-2 inhibition, limiting PGE2 production.[187] Yet if APAP is a COX-2 inhibitor, the question remains why APAP does not demonstrate the same level of peripheral anti-inflammatory activity or similar side effects and toxicities as other NSAIDs.

Ultimately, the variation in prostaglandin synthesis inhibition by APAP is due to cellular health, selectivity and the level of arachidonic acid and peroxides available.[182] While traditional NSAIDs appear to inhibit COX enzymes through competition for active binding sites with arachidonic acid, APAP appears to inhibit COX enzymes through acting as an oxidizable substrate in the peroxidase reactions of COX-1 and COX-2, thereby inhibiting the production of PGG2.[178,188] Similar to the selectivity of induction of COX enzymes discussed above, the efficacy of APAP as a COX inhibitor is dependent on local concentrations of arachidonic acid and peroxides. Studies on broken[189] and intact cell[190] preparations have indicated that APAP does not function in a high peroxide or arachidonic acid environment, leading to the appearance of COX-2 selectivity.[191] However, APAP is unlike traditional COX-2 selective drugs, such as the coxib class, which are not peroxide dependent, and may elicit a COX-1 response in the presence of low peroxide concentrations.[178] This elicited COX-1 response in times of low peroxide tone may be why there was an apparent link of APAP to COX-3 inhibition in the CNS, where there are low peroxide concentrations.[178,182] The variation in the anti-inflammatory activity between APAP and COX-inhibitors relies on these mechanisms. APAP has historically been deemed to not have an anti-inflammatory action due to its lack of demonstrable effects on rheumatoid arthritis in humans,[192,193] but newer studies have demonstrated anti-inflammatory activity in osteoarthritis in humans[194] and dogs[195], human dental extractions[196], and laboratory inflammation models.[197,198]

The analgesic effects of APAP have been consistently linked to involvement with the serotonergic (5-HT) system. Its analgesic effects have been demonstrably attenuated by the administration of 5-HT3 receptor antagonists in humans[199,200] and laboratory animal species.[201,202] APAP's effects on the serotonergic system may also be linked to interactions in the

opioid system, where administration of the opioid receptor antagonist, naloxone, prevented the anti-nociceptive effects of APAP administered to rats prior to noxious stimuli, and was demonstrated to inhibit the central increase of 5-HT associated with APAP administration.[203] Another system that appears critical in APAP's analgesic effects is the endocannabinoid system. Mechanisms postulated for these effects include inhibition of COX-2 mediated metabolism of endocannabinoids[204], and central diversion from a prostaglandin synthesis pathway to an endocannabinoid synthesis pathway via COX inhibition.[205] While not much is known specifically in APAP's connection to the endocannabinoid system, a study has demonstrated that an active metabolite of APAP conjugated with arachidonic acid via a fatty acid amide hydrolase (FAAH) to form AM404, a compound which is structurally similar to AEA and 2-AG and may interact centrally with the endocannabinoid system.[179,206-208] Furthermore, CB1 knockout mice and administration of CB1 receptor antagonists each mitigated the analgesic effects of APAP to noxious stimuli.[209] However, the interaction between APAP and AM404 remains controversial, with other studies demonstrating no effect following administration of a FAAH antagonist[210]and that administration of a 5HT receptor antagonist blocked the action of APAP, but not AM404.[211] APAP also appears to block pain associated with central release of excitatory neurotransmitters glutamate and substance P following intrathecal injections of these substances in mice.[184] Therefore, despite many years of administration, the mechanism of APAP still remains largely unknown, and while it appears to interact with many of the same therapeutic targets as traditional NSAIDs, its mechanism of action is distinct enough to deny it classification within this group.[178,182]

Acetaminophen Metabolism and Comparative Pharmacokinetics

In all species, the area under the plasma concentration–time curve (AUC) following oral or intragastric administration is dependent on the rate of gastric emptying, as APAP is absorbed transmucosally from the proximal small intestine.[212-215] Furthermore, in humans, the time to maximal drug plasma concentration (T_{max}) is directly proportional to the rate of gastric emptying, suggesting that once APAP contacts the small intestinal mucosa, drug absorption is extremely rapid.[216] Consequently, APAP has been used as a marker of gastric emptying in man[215,217] and horses.[218,219]

Absorption of acetaminophen is rapid, reaching a maximal plasma concentration (C_{max}) of 21.8 μg/mL in 23 minutes in humans after a 20 mg/kg dose.[215] In a study of veterinary species, acetaminophen reached a C_{max} of 8.61μg/mL in 10 minutes in chickens, 3.08 μg/mL in 15 minutes in dogs, 4.41 μg/mL in 23 minutes in pigs and 14.44μg/mL in 36 minutes in horses following a 10 mg/kg dose.[214] Acetaminophen has a short half-life in plasma ($t_{1/2}$), with a range of 1.5-4 hours in adult humans after standard oral dosing[220,221] and 0.45h in chickens, 0.38h in dogs, 1.41h in pigs, and 2.4-3.97h in horses after 10mg/kg oral dosing.[213,214]

Following absorption in humans, around 25% of administered APAP undergoes first-pass metabolism in the liver, where the majority of it undergoes glucuronidation or sulfonation.[215,222] This first pass metabolism effect is demonstrated by the mid-range bioavailability seen following oral dosing in humans (60-89%).[215,223] A very minor proportion undergoes oxidative metabolism where it is reduced by cytochrome P450 to the reactive intermediate N-acetyl-p-benzoquinoneimine (NAPQI).[179] Under normal situations, NAPQI is then combined with a reduced form of glutathione where it is converted to non-toxic cysteine or mercapturic conjugate forms that are readily eliminated through the biliary or urinary systems.[224,225] Within all age

categories, urinary excretion of acetaminophen in all of its metabolized forms, is complete within 30h of administration in humans.[225]

In veterinary species, the limited data on acetaminophen suggests variability in the primary metabolic pathways of the drug. Lower bioavailability (39-44%) has been reported in dogs, chickens, and turkeys in comparison to humans, which is likely due to higher first-pass effects.[214] This assumption was confirmed when oral bioavailability significantly increased following β-glucuronidase/sulfatase treatment of acetaminophen prior to administration in these species.[214] Horses had demonstrably higher bioavailability compared to other species at 91%, which was attributed to the significantly increased plasma protein binding of acetaminophen minimizing the drug's exposure to first pass effects by hepatic enzymes.[214] Yet because of the higher plasma protein binding of acetaminophen in horses, a lower clearance rate was noted compared to other species.[214]

Acetaminophen Toxicity

Humans

When APAP dosing exceeds therapeutic thresholds, the primary hepatic conjugation pathways become saturated and CYP450 oxidative metabolism predominates to produce excessive levels of NAPQI as reduced glutathione stores become depleted.[179] NAPQI can then bind to hepatocyte mitochondrial proteins to generate reactive oxygen species and inhibit mitochondrial respiration leading to mitochondrial dysfunction and cell necrosis.[226] The hallmark of APAP toxicity on liver biopsy is diffuse centrilobular necrosis with hepatocyte vacuolar degeneration.[227] Clinical signs of APAP hepatotoxicity in humans are accompanied by dramatic elevations of aminotransferases, and acute onset of liver failure including jaundice,

hyperammonemia, coagulopathies, stupor, lactic acidosis, and cerebral edema within 48 hours of acute overdose.[179,228]

Although APAP has a reputation for hepatotoxicity, acetaminophen-induced hepatotoxicity was not reported in a prospective clinical trial of over 30,000 human patients at therapeutic doses. [229] The safety margin of acetaminophen has been found to be exceedingly wide, with therapeutic plasma concentrations found to be between 10-20 μg/mL at a dose of 50 mg/kg/day and hepatotoxicity threshold at plasma concentration of ≥150 μg /mL at a dose of 150 mg/kg/day in human adults.[178,184,230] Despite this wide safety margin, APAP has been found to be the leading cause of acute liver failure in multiple epidemiologic surveys from the United States and the United Kingdom.[231-235] Acetaminophen overdose in humans has been reported to be due to several different risk factors, including willful overdose (suicide), unrecognized self-medication of multiple APAP containing products, under recognition of APAP's toxic potential, excessive consumption of opioid/APAP combination products due to opioid addiction, and concomitant use of recreational drugs or alcohol.[236] While previous studies have described an overwhelming proportion (86%) of APAP overdoses in the United States to be attributable to suicide,[237] the recent trends in excessive opiate prescribing and addiction in the United States has shifted the balance of APAP toxicity toward unintentional overdose.[236] APAP poisoning appears to be on the decline since 2009, however, with fewer emergency department visits and hospitalization, yet the proportion of visits of APAP induced liver failure doubled from 6% in 1998 to 13% in 2011.[234] As there was a 38% increase in prescribing of acetaminophen and opioid combination products between 2001 and 2005, an increase in hepatotoxicity of APAP is closely linked to the opioid epidemic in the United States,[238] with acetaminophen/hydrocodone combination products being the most prescribed medication in the United States in 2003.[239] Up

to 50% of reported APAP overdoses are now due to acetaminophen and opioid combination products, as larger doses of acetaminophen are prescribed in order to attempt to achieve better pain relief.[240] Unfortunately, unintentional overdoses carry a poorer prognosis than willful overdoses as the chronic nature of these overdoses causes patients to typically present to hospital later in the course of disease when therapeutic intervention and antidote (N-acetylcysteine) administration is less likely to be successful.[236,241]

Given that APAP has demonstrated COX enzyme inhibition similar concerns exist over its safety profile in the gastrointestinal tract as other NSAIDs. APAP does not have any reported gastrointestinal side effects at recommended therapeutic dosing ranges in humans[242,243] and the relative risk of acetaminophen causing upper gastrointestinal tract complications was only 1.3.[244] Furthermore, due to its reported safety, APAP has been recommended for use in humans with peptic ulcers as an analgesic.[245] Other studies have refuted these claims, with findings of increased risk of gastrointestinal bleeding with APAP administration. [246-248] However, it has been suggested that these studies may be subject to selection bias due to the increased usage of acetaminophen in populations at risk for gastrointestinal disease.[242]

APAP has not been associated with any of the cardiovascular or renal side effects that are present with other COX-2 inhibitors.[249] While the underlying mechanism of why APAP is relatively non-toxic to kidneys in contrast to other inhibitors of COX enzymes is unknown, controversy over these findings remains. Studies in volume and sodium depleted humans[250] and dogs[251] both demonstrated significant reductions in GFR in NSAID treated groups when compared to APAP. Other literature, however, has suggested that chronic administration of APAP is linked with chronic renal failure in humans.[252] A recent epidemiologic investigation

concluded that "there is currently insufficient evidence to conclude that the habitual use of [acetaminophen] is associated with an increased risk of chronic renal disease."[253]

Toxicity in Veterinary Species

Toxicity of acetaminophen is well recognized in small animal medicine, with over 1,000 cases reported in a 2 year period to the National Animal Poison Control Center.[254] While APAP has therapeutic efficacy as an analgesic in dogs when dosed at 10 mg/kg Q12h, there is no known safe dosage in cats.[254] Clinical signs of APAP toxicity in small animals include methemoglobinemia, hemolysis (which predominate in cats), and hepatotoxicity in dogs, which differs from humans who rarely exhibit blood dyscrasias.[178,254-257]

In cats, the historical paradigm for acetaminophen toxicity has been that cats exhibit a deficiency in glucuronidation, leading to rapid saturation of sulfonation pathways, thus producing excessive NAPQI.[258-260] The NAPQI and production of reactive oxygen species were thought to be particularly toxic to cats due to the increased numbers of exposed sulfhydryl groups on cat hemoglobin, leading to oxidative stress and Heinz body formation.[261] However, a recent study has suggested that para-aminophenol (PAP) is the underlying metabolite linked to feline methemoglobinemia in APAP toxicity.[262] When exposed to cat and dog erythrocytes in vivo, PAP produced methemoglobin, while NAPQI and APAP did not.[262] The mechanism underlying prolonged PAP exposure to erythrocytes may be due to a defect in N-acetyltransferase, leading to decreased clearance of PAP, prolonged redox cycling and methemoglobin production.[262]

While dogs are not immune to the development of blood dyscrasias associated with APAP administration, methemoglobinemia has only been noted in cases of profound APAP toxicity (dose > 200mg/kg).[254,255] Hepatotoxicity is much more common in dogs, with the toxic

threshold dose at 100 mg/kg.[254,258] The pathologic changes and mechanism for hepatotoxicity in dogs is similar to humans, with the development of hepatic centrilobular necrosis at toxic doses.[258]

Acetaminophen Usage in Veterinary Species

APAP, as discussed above, has the reputation of lethal toxicity in small animal medicine, and for many years has been neglected as an analgesic in dogs despite purported therapeutic utility. In dogs, APAP has been demonstrated to be effective in the management of post-surgical pain,[195] osteoarthritis,[263] as an antiarrhythmic,[264] and protective against myocardial infarction.[265]

Acetaminophen in Horses

Acetaminophen has previously been used in the horses as a marker of gastric emptying, given its poor gastric absorption and rapid small intestinal absorption.[218] In recent reports acetaminophen has demonstrated efficacy as an adjunct treatment for laminitis in one pony, and as an effective analgesic agent when combined with NSAIDs in a model of inducible foot pain.[266,267] These reports show promise for the utility of acetaminophen as a standalone or adjunctive NSAID in horses with laminitis pain. These reports, however, utilized a 20 mg/kg dose, which is twice that reported in the limited equine pharmacology studies, and that data was only for a short-term period, or for a single dose, respectively. A recent study examined the analgesic efficacy of acetaminophen (6g/hr), tramadol (3mg/kg/hr), and acetaminophen+tramadol constant rate infusion on mechanical nociception in horses.[171] In that study, acetaminophen was not found to be an effective analgesic as a single agent, but was effective when combined with tramadol.[171] However, the study had a wide range of individual dose variation (13.95-18.75 mg/kg/hour), as horses ranged between 320-430kg, and the acetaminophen+tramadol group used a higher (unspecified) dose rate.[171] It is also important to note that acetaminophen (paracetamol)

administration to horses is considered off-label, as there is not a labeled product for equine use, and its use is currently banned by the FEI (International Federation for Equestrian Sports) and is subject to NSAID restrictions by the United States Equestrian Federation. The aim of the following study was to determine the pharmacokinetics and safety of acetaminophen given for 14 days of repeated oral dosing at 20 mg/kg every 12 hours.

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Chapter 3: Pharmacokinetics and Safety of Repeated Oral Dosing of Acetaminophen in Adult Horses

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Abstract

The safety and pharmacokinetics of acetaminophen (APAP, INN: paracetamol) were determined using 8 healthy adult Thoroughbred geldings. Commercially available APAP (20 mg/kg; 500 mg tablets) was administered orally as a single dose followed by multiple doses every 12 hours for 14 days. Blood samples were collected for determination of plasma APAP concentrations using HPLC-UV. Serum biochemistries and gastroscopy were performed prior to initiation of dosing and following the final dose. Following a single dose, mean maximum concentration (Cmax) was 16.61 ug/mL at 1.35 hours (Tmax). APAP remained above presumed therapeutic concentrations (10 ug/mL) for two hours post-administration and below the lower limit of detection by 12 hours. Elimination half-life (T½) was 2.78h. No significant accumulation was noted following multiple doses. Average Cmax of APAP following repeated oral dosing was 15.85 ug/mL, with a Tmax of 0.99 hours and T1/2 of 4 hours. SDH was significantly decreased (pre: 13.84 U/L, post: 10.52 U/L, p = 0.013) and total bilirubin was significantly increased (pre: 1.99 mg/dl, post: 3.47 mg/dl, p = 0.004) following the last dose. No statistically significant changes were noted in gastroscopy scores (pre: 9.67 mean, post: 10.08 mean, p = 0.75). Dose simulations suggested higher doses and/or shorter dosing intervals may be indicated.

This study demonstrated the safety of acetaminophen with repeated oral dosing over 14 days. This study also confirms that the 20 mg/kg dose used in previous clinical reports reaches proposed therapeutic concentrations after oral administration to fed horses, however a shorter 6-8 hour dosage interval may be required for improved efficacy.

Introduction

Traditional non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly used analgesic agents by equine practitioners, largely because the most commonly recognized causes of pain in horses are mediated by inflammatory processes.[1] The NSAIDs most commonly used in equine practice are the non-selective COX inhibitors, which exert their anti-inflammatory effects via inhibition of COX 1 and COX 2 enzymes.[2,3] COX-1, the constitutive isoform, has a variety of functions that include maintaining renal blood flow, gastrointestinal mucosal integrity, and platelet aggregation. [4] COX-2 expression is induced by inflammatory signals, such as bacterial lipopolysaccharides and pro-inflammatory cytokine release. The suppression of constitutive COX-1 activity is thought to be a major factor in a number of side effects associated with prolonged or high dose non-selective NSAID administration. These include blood dyscrasias, renal papillary necrosis, gastroduodenal ulceration, and right dorsal colitis.[2,3]

Due to the detrimental side effects of COX-1 inhibition, the use of COX-2 selective inhibitors has increased in the equine market. COX-2 selective inhibitors, however, have been found to be less effective in reducing experimentally induced lameness when compared to non-selective inhibitors.[5] The safety of COX-2 selective inhibitors has also been questioned, as COX-2 selective inhibitors have been found to increase gastric mucosal damage, exacerbate inflammatory bowel disease, and worsen colonic inflammation in a rat model.[6,7] Furthermore,

the presence of COX-2 constitutive expression in renal and colonic tissues has not lead to a reduction in risk of renal injury or gastrointestinal side effects with COX-2 selective inhibitor administration. [8-10]

Acetaminophen (chemical name: N-acetyl-para-aminophenol or APAP, INN: paracetamol) is one of the most commonly used analgesic and antipyretic drugs in human medicine, where its relatively high margin of safety and tolerance for prolonged administration has made it a mainstay of clinical pain management.[11,12] Acetaminophen has a similar pharmacology and toxicology profile to the selective COX-2 inhibitors, and is not reported in humans to have the detrimental gastrointestinal side effects seen with non-selective NSAIDs.[13] Despite its reputation for hepatotoxicity, acetaminophen-induced hepatotoxicity was not reported in a prospective clinical trial of over 30,000 human patients at therapeutic doses.[14] The safety margin of acetaminophen in humans has been found to be exceedingly wide, with therapeutic plasma concentrations between 10-20 μg/mL at a dose of 50 mg/kg/day and hepatotoxicity threshold at a plasma concentration of 150 μg/mL at a dose of 150 mg/kg/day in adults.[11]

The safety of acetaminophen in other companion animal species is variable, and has been reported to be compatible with human toxicity thresholds in dogs (100 mg/kg), or unsafe at as little as 10 mg/kg in cats due to deficiency in glucuronyl transferase leading to deficiency in glutathione conjugation of acetaminophen metabolites.[15,16] Despite a long history of use, the mechanism of action of acetaminophen is still not fully understood. It is considered a weak prostaglandin inhibitor, because its effects on prostaglandins are limited to the peroxidase functions of COX-1 and COX-2 and therefore it has limited effects in models of severe inflammation where there is a high peroxide tone.[11] Evidence demonstrating acetaminophen's effects on endogenous opioid and cannabinoid receptors and interactions with the 5-

hydroxytryptamine system suggest mechanisms of action different than other NSAIDs.[11] Acetaminophen's analgesic and anti-pyretic effects are largely centrally mediated, where inhibition of prostaglandin E_2, substance P, and 5-HT-3 receptor antagonists occurs.[11] Due to its wide margin of safety and lack of peripheral effects, acetaminophen has been combined with NSAIDs, particularly COX-2 inhibitors, to provide greater analgesic and anti-inflammatory effects than NSAIDs alone in humans.[11] Despite the lower efficacy of acetaminophen in trials of human patients with chronic osteoarthritis, acetaminophen remains the preferred analgesic due to its superior tolerance and improved cost-benefit analysis when compared to nonselective and COX-2 selective NSAIDs.[17] Acetaminophen was preferred by physicians over COX-2 and nonselective NSAIDs, even when those drugs were coupled with a proton-pump inhibitor to reduce gastrointestinal side effects, due to the increased cost of treatment associated with the adverse side effects of nonselective COX inhibitors.[17,18]

Acetaminophen has previously been used in horses as a marker of gastric emptying, given its poor gastric and rapid small intestinal absorption.[19,20] In recent clinical reports acetaminophen has demonstrated efficacy as an adjunct treatment for laminitis in one pony, and as an effective analgesic agent when combined with NSAIDs in a model of inducible foot pain.[21,22] These clinical reports show promise for the utility of acetaminophen as a primary or adjunctive NSAID in horses with laminitis pain. However, these clinical reports utilized a dose (20 mg/kg) twice that reported in limited equine pharmacology studies for a short-term period, or for a single dose.[5,21,23,24] Previous pharmacokinetics studies in horses demonstrated that acetaminophen was rapidly absorbed, with a high bioavailability of 91%, lower clearance, and longer elimination half-life (2.4h) than other species; potentially due to high levels of plasma protein binding. [23,24] There have been no published research studies on the pharmacokinetics of

acetaminophen at the dosage utilized clinically, nor has the safety of repeated dosing in the horse been investigated. The objectives of this study were to examine the pharmacokinetics of acetaminophen in horses when administered at 20 mg/kg per os as a single dose and when administered at 20 mg/kg per os twice daily for 14 days and to assess safety of acetaminophen through evaluation of bloodwork, gastroscopy, and hepatic ultrasound and biopsy.

Materials and Methods

The study protocol was approved by the Institutional Animal Care and Use Committee at Virginia Polytechnic Institute and State University (15-238).

Animals

Eight healthy adult Thoroughbred geldings aged 5 to 11 years and with body weights ranging between 450 to 571 kg were utilized. Horses were current on vaccinations and anthelmintic treatments, with no history of liver disease or dysfunction. Horses were determined to be healthy by physical exam, complete blood count, serum biochemistry profile, and liver panel (sorbitol dehydrogenase (SDH), serum ammonia, and serum bile acids). Horses were housed in individual stalls during the study, to which they were acclimated 12 hours prior to study protocol initiation. They were fed grass hay and water ad libitum during the sample collection period other than the 12 hours prior to gastroscopic examination, when roughage was withheld. Horses were maintained on grass pasture for the duration of the repeated dose trial.

Instrumentation and drug administration

Each horse was hand restrained, and a 14g x 5.25 inch polyurethane over-the-needle catheter (MILA International, Inc. Erlanger KY USA), and a 22cm extension set with 3-way

stopcock (Clearlink, Baxter International, Deerfield IL) was aseptically placed and secured in place using suture. The total volume of the catheter and extension set was 5.2 mL. Catheters were flushed with 10 mL heparinized saline (10 U/mL heparin in 250 mL 0.9% sodium chloride solution) every 6 hours and/or following administration of medications. Acetaminophen (500 mg tablets, Extra-Strength Tylenol®, Johnson and Johnson, Indianapolis IN) was ground finely using an electric coffee grinder exclusively used for this study, mixed with water and dark corn syrup (Karo, ACH Food Companies, Inc. Cordova, TN) to a total volume of 60 mL, and administered orally at 20 mg/kg via 60 mL catheter tip syringe.

Single Dose Pharmacokinetics Sample Collection

Horses were left free in a stall for the entire sampling period and offered free choice hay and water. Blood samples were collected for plasma acetaminophen analysis immediately prior to drug administration (time 0), and at 10, 20, 30, 45, 60, 90 min, 2, 3, 4, 6, 8, 12, 16, 24, 36, and 48 hours after oral administration. Blood was collected from catheters by first aspirating approximately 12 mL of blood which was discarded, and then 6 mL of blood which was retained for analysis. Catheters were removed 24 hours post-medication administration, and remaining samples (6mL) were taken by percutaneous venipuncture of the left jugular vein at 36 and 48 hours after administration. Blood samples were immediately placed into glass tubes containing sodium heparin and stored on ice for a maximum of 1hr until centrifugation for collection of plasma. Blood samples were centrifuged at 5,000 rpm for 10 min, plasma was collected and frozen at -30°C until analysis.

Repeated Dose Pharmacokinetics Sample Collection

A seven-day washout period followed the single dose pharmacokinetic study. Horses were evaluated again immediately prior by physical exam, complete blood count, plasma biochemistry profile, and liver panel (sorbitol dehydrogenase (SDH), serum ammonia, and serum bile acids), and found to be healthy. Horses were administered 20 mg/kg acetaminophen per os as described above every 12 hours for 14 days (28 doses). Horses were examined twice daily for signs of colic, inappetence, jaundice, or discomfort. Complete blood count, plasma biochemistry profile, and liver panel were repeated on days 7 and 14. Blood was collected for evaluation of acetaminophen plasma concentrations as described above following the final dose.

Analysis of Plasma Concentrations of acetyl-para-aminophenol

Plasma concentrations of acetaminophen were measured using high performance liquid chromatography with ultraviolet detection (HPLC-UV). Calibration curves were prepared by fortifying blank equine plasma with stock solutions of acetaminophen dissolved in methanol. Standards and samples were prepared for analysis in an identical manner by the following method. One gram of plasma was weighed and vortexed with 100 µL of 10 µg/mL 2-acetamidophenol solution in acetonitrile (internal standard), and 4 mL of 50:50 acetonitrile:ethyl acetate solution for 5 minutes. The samples were then centrifuged for 10 minutes at 5,000 RPM, the supernatant transferred to 15 mL polypropylene centrifuge tubes containing 0.4 g of sodium chloride, and vortexed for an additional 5 minutes. The samples were centrifuged again for 5 minutes at 5,000 RPM, and the organic layer pipetted into a 4 mL clear glass vial. The organic layer was dried with nitrogen gas, reconstituted in 250 µL of methanol, and centrifuged at 13,200

RPM for 15 minutes. The samples were aliquoted into 2mL vials with polypropylene low volume inserts.

HPLC analysis was performed on an Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA, USA). Separation was carried out with a Luna C18 column (150 x 4.60mm, 5 µm; Phenomenex, Torrance, CA, USA). Ultraviolet detection was carried out at 254nm for acetaminophen and 280nm for the internal standard. A mobile phase comprised of 0.1 % formic acid in water (solvent system A) and 0.1% formic acid in acetonitrile (solvent system B) was employed with a gradient elution of 98% B at 0 to 3.5 min, 70% B at 3.5 to 6 min, 0 % B at 6 to 8 min, and returning to 98% B at 8-11 min. Flow rate was 1.5 mL/min and injection volume was 5 µL. Column and samples were maintained at room temperature.

Using this method, acetaminophen calibration curves were linear ($R^2 > 0.99$) over a concentration range of 0.1-100 µg/mL. The lower limit of quantification (LLOQ) was determined to be 0.05 µg/mL and the lower limit of detection (LLOD) was 0.025 µg/mL plasma. Method validation was performed using data from five consecutive standard curves. Intraday accuracy was within 2.97 ± 3.22% of the true value, and precision was within 3.08 ± 3.09% of the mean for concentrations of 0.1, 10 and 100 µg/mL.

Gastroscopy

Gastroscopic evaluation was performed immediately prior to the repeated dosing phase of the study, as well as 2 days following the final dose in the repeated dosing study. Feed was withheld for 12 hours and water withheld for 3 hours prior to gastroscopic exam. Horses were sedated with detomidine (Dormosedan®, Zoetis US, Parsippany, New Jersey, USA) at 0.01-0.02 mg/kg intravenously and examined for the presence of gastric ulceration using a 3 m flexible video endoscope (Olympus). Standardized images were captured of the margo plicatus at the

greater curvature (MPGC), the margo plicatus at the right site (MPRT), the lesser curvature (LC), the dorsal fundus (FUND) and the pylorus (PYL). Still images of each region were digitally stored and all image files were coded for blinding. The images were graded by 3 independent, blinded observers (DRH, JLD, KEW) using the Equine Gastric Ulcer Council scoring system.[25] Scores were assigned for each region from 0 to 4, corresponding to the following findings: 0 (intact epithelium), 1 (intact mucosa, evidence of hyperkeratosis or hyperemia), 2 (small, single, or multifocal lesions), 3 (large, single or multifocal lesions or extensive superficial lesions) and 4 (extensive lesions with areas of apparent deep ulceration). An individual score was given to each segment of the squamous (MPGC, MPRT, LC, FUND) and glandular regions (PYL, GLAND) of the stomach. The segmental scores were averaged to obtain a squamous score (mean of the 4 squamous segments), a glandular score (mean of the 2 glandular segments), and a composite score (mean of all 6 segments).

Hepatic Ultrasound and Biopsy

Hepatic ultrasound and biopsy were performed two days following termination of repeated dosing. A 5 MHz curvilinear ultrasound probe was utilized to identify hepatic parenchyma located on the right side, roughly between intercostal spaces 12-16. The liver was examined for any evidence of hepatopathy (ie rounded liver margins, increased size, presence of vacuolation, biliary hyperplasia, or abscessation). Liver biopsy specimens were collected in a technique modified from Rendle 2010.[26] Briefly, horses were sedated with detomidine hydrochloride at a dose of 0.01-0.02 mg/kg intravenously. A roughly 4cm x 4cm area of skin over the biopsy site was clipped and surgically prepared. The subcutaneous tissue and intercostal muscles at the site of biopsy were desensitized with 2 mL of mepivacaine (Carbocaine-V®,

Zoetis US, Parsippany, New Jersey, USA). A 5MHz ultrasound probe was placed in a sterile rectal sleeve with ultrasound gel to facilitate ultrasound-guided biopsy. A stab incision was made in the skin with a #15 scalpel blade. The biopsy needle (14 g Tru-Cut® Biopsy Needle, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) was advanced percutaneously until observed to be placed in the hepatic parenchyma by ultrasound guidance, and a single sample was collected. Samples were placed in 10% formalin solution. Slides were stained with Wright-Giemsa and Prussian blue, and examined by a board certified veterinary anatomic pathologist (TC). Biopsies were graded in a standardized format as previously reported.[27] Horses were monitored for 24 hours following biopsy by physical exam every 6 hours for any evidence of internal hemorrhage or discomfort.

Pharmacokinetic Calculations

Drug concentrations were analyzed using commercially available software (Phoenix® WinNonlin® version 6.3, Certara USA, Inc., Princeton, NJ, USA) to determine noncompartmental pharmacokinetic parameters for each horse. The area under the plasma concentration versus time curve extrapolated to infinity ($AUC_{0-\infty}$) was calculated using the linear trapezoidal rule. Additional parameters reported include the terminal phase rate constant (λ_z), half-life of the terminal phase ($t_{½\lambda}$), area under the first moment curve (AUMC), and mean residence time. The true bioavailability (F%) of acetaminophen could not be calculated as an intravenous dose was not administered. Because of this, the volume of distribution and clearance are reported as Vd/F and Cl/F, respectively. The time to maximum plasma concentration (T_{max}) and the maximum plasma concentration (C_{max}) are reported directly from the data. Using parameters derived from the initial pharmacokinetic analysis, nonparametric superposition was

used to determine a dose and dosing interval that would result in plasma concentrations above the therapeutic concentration reported for humans (10 μg/mL).

Statistical Analyses

All analyses were performed using commercially available software (SAS version 9.4 Cary, NC, USA). Data was assessed for normality, and all variables were normally distributed except for amylase, serum bile acids, sorbitol dehydrogenase and basophils, which followed a non-normal distribution. Normally distributed variables were summarized as means ±standard deviation while non-normally distributed variables were summarized as median (range). For normally distributed variables, the 3 time points (baseline, before treatment and after treatment) were compared using mixed-model analysis of variance followed by Tukey's procedure for multiple comparisons. The linear model specified time point as a fixed effect with Kenward-Roger approximation as the denominator degrees of freedom. Horse identification was specified as the random effect. Because gastroscopy scores were assessed on only two occasions, scores for the two time points were compared using a paired t-test. Non-normally distributed data points were compared nonparametrically between time points using Friedman's chi-square with horse identification as a blocking factor. P-values for the two-way comparisons were adjusted for multiple comparisons using Bonferroni's procedure. To assess inter-rater variability for gastroscopy scores, coefficients of variation were estimated. Statistical significance was set to $p<0.05$.

Results

Single Dose Pharmacokinetics:

Mean ± SD plasma concentrations of APAP following a single oral dose of 20 mg/kg are demonstrated in Fig. 1. Acetaminophen was found to be rapidly absorbed, and plasma concentrations reached a mean C_{max} of 16.61 ± 7.48 ng/mL with a mean T_{max} of 1.35 ± 1.69 hours of administration (Fig. 1). Plasma APAP concentrations remained above presumed therapeutic concentrations (10 ug/mL) for at least two hours post-administration, and below the LLD of the analyzer by 24 hours post- administration. Mean elimination half-life was 2.78 ± 0.60 hours. Other relevant pharmacokinetic parameters are presented in Table 1.

Repeated Dose Pharmacokinetics:

Mean ± SD plasma concentrations of acetaminophen following 14 days oral dosing of 20 mg/kg every 12 hours are demonstrated in Fig. 2. Pharmacokinetic parameters for APAP were calculated and are presented in Table 1. The mean C_{max} achieved following repeated oral dosing was 15.85 ng/mL ± 6.64 with a mean T_{max} of 0.99 ± 0.86 hours and a mean half-life of 3.99 ± 0.69 hours. Troughs following repeated dosing were below the LLD of the analyzer at 36 hours post-administration. Significant accumulation was not noted after multiple doses.

Results of dose simulations indicate that to achieve plasma concentrations above the target therapeutic concentration in humans, a dose regimen of 100 mg/kg q6h would be required. Simulated dosing regimens of 30 mg/kg every 6 hours or 40 mg/kg every 8 hours predict that the target therapeutic concentration could be reached within 30 minutes post-administration and remain above proposed therapeutic dose ranges for approximately 50% of the dosing interval.

Repeated Dose Safety:

Statistically significant reductions in platelet count and total protein were noted over the course of the study, but did not range outside of laboratory reference intervals (Table 2, Table 3). Statistically significant increases in albumin, alkaline phosphatase, calcium, creatine kinase, and potassium were noted over the course of the study, but did not range outside of laboratory reference intervals (Table 3). Changes in SDH and total bilirubin were significant and ranged outside of laboratory reference intervals, with a reduction in SDH and increase in total bilirubin over the course of the study (Table 2). There were no statistically significant changes in gastroscopy scores over the course of the study (Table 4). Liver biopsy scores were not suitable for statistical analysis as these were only obtained at a single point in time, but evidence of inflammation was noted in all horses, and irreversible changes were noted in 1 horse (Table 5).

Parameter	**Single dose Mean ± SD**	**Multiple dose Mean ± SD**
T_{max} (hr)	1.35 ± 1.69	0.99 ± 0.86
C_{max} (ng/mL)	16.61 ± 7.48	15.85 ± 6.64
λz (hr^{-1})	0.26 ± 0.06	0.18 ± 0.03
$T_{1/2}$ (hr)	2.78 ± 0.60	3.99 ± 0.69
$AUC_{0-\infty}$ (hr*ng/mL)	63.01 ± 15.58	45.69 ± 11.74
Vd/F (L/kg)	1.34 ± 0.48	2.72 ± 1.12
Cl/F (mL/kg/min)	335.35 ± 86.06	474.14 ± 170.81
$AUMC_{0-\infty}$ (hr*hr*ng/mL)	273.08 ± 102.34	171.71 ± 84.70
MRT (hr)	4.26 ± 1.24	3.59 ± 1.29

Table 3.1. Noncompartmental pharmacokinetic parameters for acetaminophen (20 mg/kg) given orally as a single dose or multiple doses to healthy adult horses (n=8). T_{max} = time to maximum concentration; C_{max} = maximum concentration; λz = slope of the terminal phase; T ½ λz = half-life of terminal phase; $AUC_{0-\infty}$ = area under the concentration-time curve extrapolated to infinity; Vd/F = apparent volume of distribution dependent on bioavailability; Cl/F = clearance dependent on bioavailability; $AUMC_{0-\infty}$ = area under the first moment curve extrapolated to infinity; MRT = mean residence time

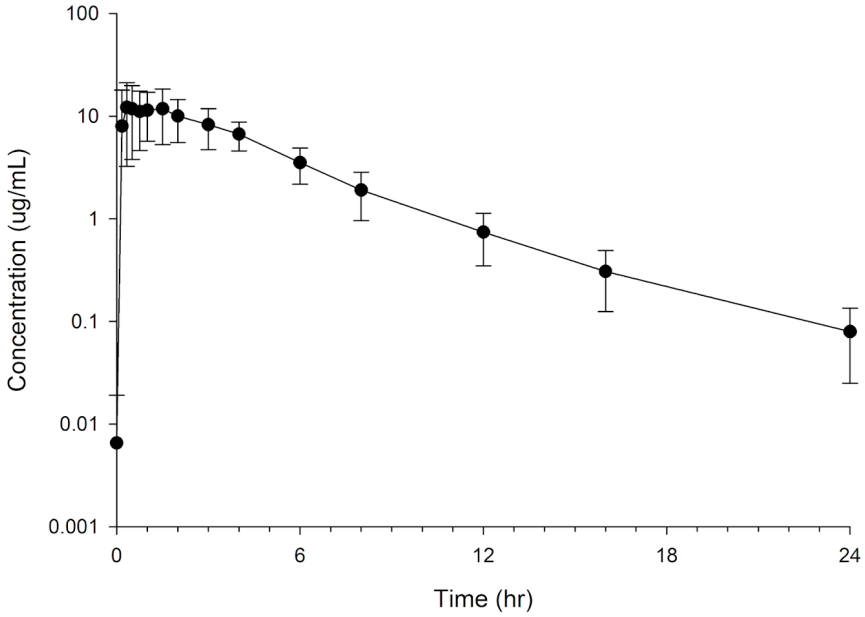

Figure 3.1: Single dose acetaminophen (20 mg/kg) plasma concentration vs. time curve presented as a mean ± standard deviation for 8 horses

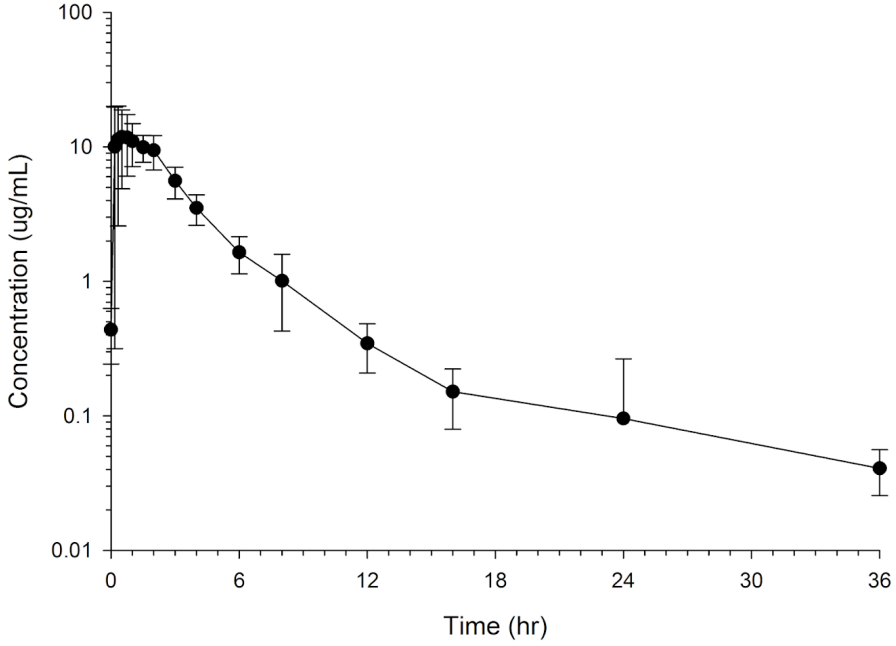

Figure 3.2: Multiple dose acetaminophen (20 mg/kg) plasma concentration vs. time curve presented as a mean ± standard deviation for 8 horses

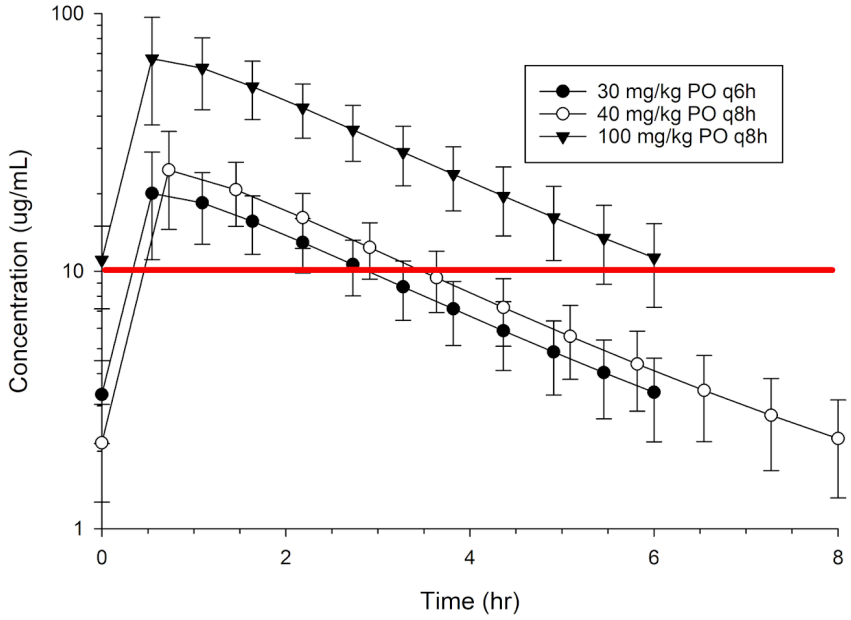

Figure 3.3: Nonparametric superposition modeling of repeated dosing of acetaminophen to achieve concentrations > 10 µg/mL. Graph depicts concentrations at steady state.

Table 3.2: Hepatic Enzymes Following Multiple Dose APAP Administration

Enzyme	**Laboratory Reference**	**Mean (SD) Day 1**	**Mean (SD) Day 23**	**Standard Error**	**P-Value**
GGT (U/L)	15-27	24.12 (2.10)	30.12 (14.58)	2.09	0.34
SDH*	1.2-8.5	13.84 (1.18)	10.52 (2.01)	8.61	0.013
Bile Acids (umol/L)	1.2-8.5	5.95 (1.57)	4.42 (1.20)	0.55	0.24
Total Bilirubin (mg/dL) *	0.6-2.2	1.99 (0.34)	3.47 (1.45)	0.38	0.004
Direct Bilirubin (mg/dL)	0.1-0.2	0.07 (0.07)	0.1 (0.05)	0.03	0.67
AST (U/L)	240-587	366.5 (67.6)	393.87 (54.6)	34.68	0.71
Albumin (g/dL)*	2.8-3.6	2.84 (0.13)	3.25 (0.28)	0.79	0.0003
Total Protein (g/dL)*	5.0-7.7	5.80 (0.26)	6.80 (0.64)	0.21	0.0003
LDH (U/L)	267-512	311.2 (36.5)	317.5 (27.9)	10.85	0.83
ALP (U/L)*	67-268	145.0 (12.3)	208.6 (32.6)	9.08	0.00005

GGT=Gamma Glutamyl-Transferase, SDH= Sorbitol Dehydrogenase, AST= Aspartate Transferase, LDH= Lactate Dehydrogenase

Table 3.3: Statistically Significant Variations in Clinicopathologic Variables Following Multiple Dose APAP Administration

Value	Laboratory Reference	Mean (SD) Day 1	Mean (SD) Day 23	Standard Error	P-Value
Platelets ($x10^3$ cells/μL)	100-350	179.5 (67.76)	125.4 (25.98)	17.9	0.02
Calcium (mg/dL)	11.4-13.4	10.2 (0.21)	11.9 (0.94)	0.3	0.0005
Creatine Kinase (U/L)	183-542	159.0 (31.25)	318.0 (76.06)	19.5	0.00003
Potassium (mEq/L)	3.0-4.5	3.3 (0.70)	4.3 (0.59)	0.26	0.006

Table 3.4: Gastroscopy Data Values Reported as Difference Between Initial and Post- Multiple Dose Trial As Analyzed by Paired T-Test

Location	Mean Day 1	Mean Day 14	Mean Change	SD	P Value
Squamous Mucosa	7.70	8.04	0.33	1.32	0.81
Glandular Mucosa	1.70	1.87	0.17	1.58	0.77
Total	9.67	10.08	0.41	3.52	0.75

Table 3.5: Liver Biopsy Data utilizing liver biopsy scoring system[27]

Category	Horse 10	Horse 11	Horse 12	Horse 13	Horse 14	Horse 15	Horse 17	Mean Total Score
Fibrosis	0	0	0	0	0	0	0	0
Irreversible Cytopathology	1	0	0	0	0	0	0	0.14
Reversible Cytopathology	2	2	0	0	0	1	0	0.71
Inflammation	2	2	1	1	1	1	1	1.28
Hemosiderosis	2	0	1	1	2	1	1	0.88
Biliary Hyperplasia	1	1	0	0	0	0	0	0.28

Table 3.6: Equine Gastric Ulcer Council Gastric Ulcer Grading Scale[25]

Grade	Mucosal Appearance
0	Intact Epithelium
1	Intact mucosa, evidence of hyperkeratosis or hyperemia
2	Small, single, or multifocal lesions
3	Large, single or multifocal lesions or extensive superficial lesions
4	Extensive lesions with areas of apparent deep ulceration

Discussion

The oral absorption of acetaminophen in this study was variable, as determined by the large standard deviations seen in both C_{max} and T_{max} of both the single and repeated dose pharmacokinetics, which was similar to previous reports.[23,24] APAP is absorbed in the proximal small intestine via passive diffusion, and therefore the rate-limiting step in the absorptive process is gastric emptying.[212] Several factors have been noted to influence this process in humans, such as diet, drug formulation, concomitant drug administration, gastric ulceration, surgery, or pregnancy.[28] This variability in oral absorption was likely due to variances in gastric emptying and small intestinal absorption rates between horses at different states of gastric distention with feeding.[19,20] Horses were fed a diet consisting of free choice grass hay during this study to mimic the clinical setting. In humans, administration of APAP in the fed state has been demonstrated to decrease the C_{max} and increase the T_{max}.[29]

Acetaminophen was rapidly absorbed in this study, with APAP concentrations reaching a peak average concentration of 12.19 µg/mL at 30 minutes post-administration, and plasma APAP concentrations reaching a mean maximum concentration of 16.61 ug/mL within an average of

1.35 hours of administration. Elimination half-life was found to be similar to previous reports at 4 hours.[23] Plasma APAP concentrations remained above proposed minimum therapeutic levels (10 μg/mL) in humans for two hours post-administration. The effective concentration that elicits 50% of the maximum drug response (EC_{50}) in humans has been estimated between 15.2 μg/mL and 16.55 μg/mL for acetaminophen, with minimum therapeutic concentration for analgesia set at 10 μg/mL, however therapeutic concentrations have yet to be determined in horses.[30-33] The Rumack-Matthews normogram was developed in the 1970s to predict hepatotoxicity and death secondary to APAP overdose in humans, with a cut-point of measured plasma concentrations of APAP at 200 μg/mL at 4 hours after ingestion and 25 μg/mL at 16 hours.[34] While extrapolation of cut points across species must be approached with caution, at no point in this study did plasma concentrations of APAP in horses even remotely approach these cut-points for hepatoxicity with repeated dosing. The pharmacokinetics following single and multiple doses did not demonstrate significant drug accumulation with 12 hour dosing based on similar C_{max} concentrations following one or 14 doses.

Dose-simulation pharmacokinetics were performed on the data in order to suggest an optimum dosage schedule that would maximize time above the target therapeutic concentrations in humans while minimizing dose frequency and maintaining total daily APAP administration below thresholds for human toxicity (150 mg/kg/day). The results suggest that, in order to maintain concentrations > 10 μg/mL for the entire dosing interval, potentially toxic doses would be reached. Other dosing regimens would reach target concentrations for at least part of the dosing interval, however frequent administration would be required. A dosing regimen of 30 mg/kg per os every 6 hours or 40 mg/kg PO q8h may yield improved pharmacokinetic variables, yet neither repeated dose safety trials nor pharmacodynamic studies have been performed to

determine if this proposed dosage schedule is safe or efficacious. In humans, twice daily administration of sustained-release APAP formulations has been found to have a significantly longer time above a targeted plasma concentration of 4 μg/mL, greater T_{max}, a longer half-life, and lower C_{min} when compared to extended release and immediate release formulations administered at 3 times and 4 times daily dosing, respectively.[35,36] This study utilized the immediate release formulation of acetaminophen due to its ready availability and ease of administration in horses, as these tablets can be crushed prior to administrations. Further studies investigating the pharmacokinetics of a sustained release formulation designed for oral administration in horses may be desirable to provide a less frequent dosage interval, which may aid owner compliance.

No changes in gastric ulceration scores were observed following two weeks of repeated dosing in this study. While APAP has been historically noted to have minimal gastrointestinal effects, recent studies in humans have suggested that there is a dose-responsive increased risk of gastric ulceration and bleeding at the upper end of the dosage range, or when APAP is combined with other NSAIDs that was independent the duration of treatment of either drug.[37-39] APAP has been found to have weak non-selective COX-inhibition in vitro, in addition to prostaglandin inhibition.[11,40,41] The association between high dose APAP and upper gastrointestinal tract ulceration in humans may be a combination of dose-dependent effects of APAP on COX inhibition, in addition to selection bias of APAP administration to patients with increased risk of gastrointestinal ulceration.[37,42] However, low dose APAP has been found to have a decreased risk of gastrointestinal complications in humans when compared to non-selective COX inhibitors.[43] The addition of a proton pump inhibitor at higher doses of acetaminophen has been found to be helpful in mitigating the deleterious effects of prostaglandin inhibition on the gastric

mucosa in humans.[42] Therefore, caution should be exercised when combining APAP with other non-selective COX inhibitors in horses due to potential increased risk for gastrointestinal ulceration, and the addition of a proton pump inhibitor may be advised when combining APAP with other NSAIDs. Clinically significant gastric ulceration scores (≥2)[51] were present at the initiation of this study, which may have been due to the recent variation in daily routine as the horses were introduced to hot-walker low impact exercise, or due to social hierarchy within a pastured herd. Previous studies have found a prevalence of gastric ulceration (EGUS score ≥2) in mature pastured horses of 43-70%, suggesting that pasture management is not protective against gastric ulceration in horses.[51-53] While high gastric ulcer scores were present at the initiation of this study, administration of acetaminophen for two weeks did not demonstrate any significant worsening of squamous or glandular gastric ulcer scores in horses.

The significant increases in total protein and albumin over the course of the repeated dosing study did not vary outside of reference intervals and trended toward normalization rather than increasing deviation from normal. The significant increase of calcium over the course of the study may have been due to improvement in protein binding from rising albumin concentrations, or to increased dietary intake with supplemental feeding provided during the study period. The significant increases in potassium, ALP, and CK were deemed clinically insignificant as they did not vary outside of normal laboratory reference ranges. The significant decrease in platelet concentration could be due to iatrogenic causes (such as platelet clumping), transient platelet sequestration, or from drug administration of acetaminophen. While the platelet count did not range outside of normal laboratory reference intervals, the interaction with acetaminophen and platelets could not be excluded. Thrombocytopenia has been reported in 3.4% of humans presenting with acute acetaminophen toxicity in a retrospective evaluation of 174 patients, with

the mechanism suspected to be a direct toxic effect on platelets.[44] However, the presence of thrombocytopenia in these patients was strongly correlated with hepatic failure and increased AST levels, while none of the horses in this study developed elevations in hepatocellular enzymes.[44]

In most species, around 25% of administered APAP undergoes first-pass metabolism in the liver primarily via conjugation with glucuronic acid or sulphate.[45] When APAP dosing exceeds therapeutic thresholds, the primary hepatic conjugation pathways become saturated and CYP450 oxidative metabolism predominates to produce excessive levels of NAPQI as reduced glutathione stores become depleted.[46] NAPQI can then bind to hepatocyte mitochondrial proteins to generate reactive oxygen species and inhibit mitochondrial respiration leading to mitochondrial dysfunction and cell necrosis.[45] Bioavailability of acetaminophen in horses is 91%, which is higher than dogs, turkeys, and pigs.[23] This suggests that horses rely less upon first pass extraction of the drug by beta-glucuronidase/sulfatase than other species.[23] Clinical signs of APAP toxicity in humans are accompanied by dramatic elevations of aminotransferases, and acute onset of liver failure including jaundice, hyperammonemia, coagulopathies, stupor, lactic acidosis, and cerebral edema within 48 hours of acute overdose.[46,47] None of the horses in this study demonstrated any clinical or clinicopathologic signs of hepatic dysfunction throughout the repeated dose trial.

The hallmark of APAP toxicity on liver biopsy is diffuse centrilobular necrosis with hepatocyte vacuolar degeneration.[48] In the scant reports of equine APAP administration, clinical evidence of hepatotoxicity has not been reported, but no studies have examined liver biopsy sections to determine the presence of any underlying hepatic injury.[19-24] A grading system was utilized in this study that was previously developed for the evaluation of liver biopsy specimens in

horses with clinical signs of liver disease in regards to prognostication for survival.[27] While results of the hepatic biopsy samples taken following two weeks of repeated dosing of acetaminophen in this study indicate that there was evidence of inflammation in all horses and some degree of cytopathology, none of the horses in this study developed clinically significant elevation in hepatobiliary enzymes, nor did any horse demonstrate clinical signs of hepatic dysfunction. The mean total scores using this scoring system were all well below 2, and scores less than 2 were associated with 96% survival rates for horses exhibiting clinical evidence of hepatic disease in the original report.[49,50] A major limitation of this study, however, is that no biopsy specimens were taken prior to repeated acetaminophen administration, due to concerns that hepatic biopsy may incite an inflammatory reaction that would preclude adequate assessment of acetaminophen induced hepatic change. The horses utilized in this study grazed on local grass pasture, which has since been found to contain saponin containing plants (fall panicum), and pre-existing hepatic pathology could not be excluded as a cause of increased SDH observed prior to study initiation or the changes seen on hepatic biopsy. Panicum hepatotoxicity in horses is manifested by elevations in AST, SDH, GGT and ALP on clinicopathologic evaluation, with liver biopsy specimens demonstrating mild lymphocytic and histiocytic inflammation, mild vacuolar change, and individual hepatocyte necrosis.[51] While all the horses in this study demonstrated inflammatory changes, with one horse demonstrated irreversible cytopathology, none of the horses were noted to have clinical evidence of hepatic dysfunction prior to or during the study. The gradual decline in SDH over the course of the study suggests that their previous management may have played a role in the elevations observed. The increase in total bilirubin observed over the course of the study was likely related to fasting prior to gastroscopy on the final day of the repeated dosing study,

which was manifested by an increase in total and indirect bilirubin, with no change in direct bilirubin. Therefore, acetaminophen's effects on hepatic pathology requires further study.

This study has demonstrated the safety of acetaminophen administered at 20 mg/kg via repeated oral dosing over a 14-day period. Results reveal that acetaminophen achieves plasma concentrations within presumed therapeutic ranges within 30 minutes of administration, and remains above 10 μg/mL for two hours post-administration at a dose of 20mg/kg. This preliminary study suggests a shorter 6-8 hour dosage interval than previously used in clinical reports, and supports that the 20 mg/kg dose used in previous clinical reports of analgesia reaches proposed therapeutic concentrations for 2 hours after administration. Further evaluation of the pharmacokinetics of an increased dose and/or increased frequency of dosing, as well as the pharmacodynamics of acetaminophen are necessary to determine the therapeutic efficacy of this drug in horses.

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Chapter 4: Final Comments

This thesis investigated the pharmacokinetics and safety of repeated oral dosing of acetaminophen in adult horses. This revealed that acetaminophen achieves plasma concentrations within the reported human therapeutic range within 30 minutes of oral administration and remains above 10 ug/mL for two hours post-administration when given at a dose of 20 mg/kg per os. This preliminary study supports that the 20 mg/kg dose used in previous clinical reports of analgesia reaches therapeutic concentrations after administration but suggests a shorter 6-8 hour dosage interval than previously used in clinical reports. While there have been reports of efficacy of acetaminophen as an effective analgesic when combined with other NSAIDs, there have been no published studies on the safety or multiple dose pharmacokinetics of acetaminophen in the horse, and therefore this thesis serves to improve that knowledge base. Future directions of this research include investigation of the pharmacodynamics of acetaminophen in adult horses, and its application as an antipyretic.