ABSTRACT

MARSHALL, JOHN FRASER. The Effect of Novel Anti-inflammatory Drugs on the Cyclooxygenase Enzymes and Recovery of Mucosal Barrier Function. (Under the direction of Anthony T Blikslager).

The non-steroidal anti-inflammatory drugs (NSAIDs) are a large group of drugs that are commonly used for the treatment of pain and inflammation in the veterinary species. The NSAIDs inhibit the action of the cyclooxygenase (COX) enzymes, COX-1 and COX-2, to reduce the production of prostaglandins. However, their use is associated with adverse effects, particularly in the gastrointestinal tract which may be related to the inhibition of COX-1. To reduce the incidence of these effects, NSAIDs have been designed to selectively inhibit COX-2 while allowing physiologic prostaglandin production by COX-1.

The first experiments of this thesis aimed to determine the effect of three NSAIDs in the horse. Flunixin meglumine is commonly used to treat pain and endotoxemia associated with colic in the horse. Deracoxib and firocoxib have been shown to be COX-2 selective in the dog. This study used *in vitro* whole blood assays to determine the effect of flunixin meglumine, deracoxib, and firocoxib on the COX enzymes. Using this model, flunixin meglumine was shown to non-selectively inhibit COX-1 and COX-2 in the horse. In contrast, deracoxib and firocoxib selectively inhibited COX-2 in the horse.

Using an *in vitro* equine whole blood assay, the next project determined that the novel NSAID robenacoxib is COX-2 selective in the horse. The effect of robenacoxib and

flunixin meglumine on the recovery of ischemic-injured equine jejunum was then compared using an equine *ex vivo* model. While flunixin meglumine significantly inhibited the production of prostaglandin $E_2$ ($PGE_2$) and the recovery of barrier function, robenacoxib allowed barrier function to recovery and production of $PGE_2$.

The mechanism of action of the novel anti-inflammatory compound AHI-805 is currently unknown. Using an *in vitro* equine whole blood model, the effect of AHI-805 on COX-1 and COX-2 was determined. While AHI-805 did not inhibit COX-1, it did significantly inhibit the action of COX-2. The effect of AHI-805 on the recovery of mucosal barrier function in ischemic injured equine jejunum was determined using an equine *ex vivo* model. Treatment of ischemic injured equine jejunum with AHI-805 significantly inhibited the recovery of mucosal barrier function. Furthermore, in this *ex vivo* model AHI-805 significantly inhibited the action of both COX-1 and COX-2.

# The Effect of Novel Anti-inflammatory Drugs on the Cyclooxygenase Enzymes and Recovery of Mucosal Barrier Function

by
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DEDICATION

For Zamantha, Avery and Dually.

# BIOGRAPHY

I grew up on the south west coast of Scotland in the town of Ayr, where I attended Kyle Academy. The influences of my father's work for the Scottish Agricultural College and a family involved in dairy farming and farriery led me to study veterinary medicine at the University of Glasgow. I graduated with my BVMS degree in 2003 which gave me an excellent foundation for my further studies. My first position following graduation was an internship in equine medicine and surgery at North Carolina State University (NCSU). It was during this time that I was fortunate to meet Anthony Blikslager who has been a constant source of advice and support throughout my postgraduate studies. In 2004, I moved to Stillwater Oklahoma to begin an equine surgery residency at Oklahoma State University (OSU). Following the residency I returned to Raleigh NC in 2007 to undertake a PhD in Physiology under the direction of Anthony Blikslager. In March of 2008, I passed the examination to become a Diplomate of the American College of Veterinary Surgeons (DACVS). Following completion of the PhD in May 2010, I am returning to the University of Glasgow to undertake the position of Lecturer in Equine Surgery.

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It was in a pub by a canal during a visit to Birmingham, England that I discussed the possibility of returning to NCSU with Anthony Blikslager. I am very grateful to Anthony for encouraging me to enter the PhD program after my residency and supporting me throughout the three years. My research would not have been possible without Anthony's willingness to discuss new ideas and results. In addition to the mentoring I have received academically from Anthony, I must also acknowledge his enthusiasm for enjoying life outside of the lab. With the help of Anthony and Becky Blikslager, the annual Burns Supper has become the highlight of the social calendar.

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# CHAPTER 1

# The Cyclooxygenase Selectivity of NSAIDs – Biochemical Basis, Pharmacology, and Clinical Relevance

**John F Marshall BVMS**

## Introduction

In both veterinary and human medicine, non-steroidal anti-inflammatory drugs (NSAIDs) are among the most frequently administered medications. Despite the long history associated with the medicinal use of NSAIDs, it was not until relatively recently that their mechanism of action was elucidated. In the 1970s, a reduction in prostaglandin production due to the inhibition of cyclooxygenase by aspirin and indomethacin was described.[1] However, at that time knowledge of the cyclooxygenase enzymes was limited to a single enzyme, and a further 20 years would pass before a second, inducible form of cyclooxygenase would be identified.[2, 3] As the roles of the constitutively expressed isoform of cyclooxygenase (COX-1), and the inducible isoform (COX-2) were investigated the deleterious effects of NSAID use were attributed to the inhibition of COX-1. Efforts to identify NSAIDs which would inhibit the COX-2 enzyme specifically, while allowing continued production of prostaglandins by COX-1, have been made in an attempt to maximize the beneficial effects of NSAIDs while reducing side-effects.[4] Recently, COX-2 selective NSAIDs, or coxibs, have been introduced in veterinary medicine for the treatment of pain and inflammation in a range of companion animal species including the dog, cat, and horse.[5-7] The aim of this review is to describe the pharmacology of the NSAIDs with particular emphasis on the design of COX-2 selective drugs, and relate this information to their use in the veterinary clinic.

## Cyclooxygenase Enzyme Structure

The cyclooxygenase (COX) enzymes catalyze the reaction whereby arachidonic acid is converted to prostaglandin $H_2$ ($PGH_2$) via prostaglandin $G_2$ ($PGG_2$). They exist as homodimers and are located mainly in the endoplasmic reticulum and nuclear envelope of the cell.[8] Each monomer of the active cyclooxygenase enzyme possess three major domains including the active catalytic site, an epidermal growth factor (EGF)-like domain, and a membrane binding domain. Currently, three COX enzymes have been described and designated COX-1, COX-2, and COX-3. The COX-3 enzyme is a product of the COX-1 gene, but includes intron 1 and is therefore referred to as a spice variant.[9] It will not be covered further in this review. The human COX-1 gene produces a 2.8kB transcript while the COX-2 gene produces a longer, 4.8kB transcript.[10, 10] While the mRNA transcripts are significantly different, the COX-1 and COX-2 proteins are similar in amino acid sequence (60% homology).[11] However, they have several structural differences which are important when considering the pharmacology of NSAIDs. A critical difference between the COX isoforms when considering the action of COX-2 selective NSAIDs, is the substitution of isoleucine in COX-1, for a valine in COX-2 at positions 434 and 523. These changes result in a 25% increase in the size of the channel and active site in COX-2 compared to COX-1. This size increase is the result of access to a side pocket and a conformational change in phenylalanine 518.[12]

**Cyclooxygenase Enzyme Function**

COX-1 is often referred to as the constitutive isoform, and is expressed in the majority of tissues during normal physiological conditions. In contrast, COX-2 is an inducible form of cyclooxygenase whose expression is increased following stimulation by a range of mediators including lipopolysaccharide (LPS). This is reflected in the arrangement of the COX genes, whereby the COX-2 gene possesses a TATA box 31 base pairs upstream of the transcriptional start site while the COX-1 gene does not.[10] The COX-2 gene also contains several putative response elements including NF-Kβ, C/EBP, CRE, and NF-IL-6.[10] During inflammation and injury, these elements allow COX-2 to respond to a wide variety of stimuli through upregulation of protein expression. In contrast, the COX-1 gene lacks these elements and possesses the characteristics of a 'housekeeping' gene.[13]

Although the genes, mRNA transcripts, and protein structures of the COX enzymes differ, the COX enzymes both use the same substrate to produce an identical product.[11] The first reaction catalyzed by the COX enzymes is the oxidation and cyclization of arachdonic acid (AA) to prostaglandin $G_2$ ($PGG_2$) at the COX site. This is followed by the reduction of $PGG_2$ to prostaglandin $H_2$ ($PGH_2$) at the peroxidase site. The final product of the COX enzymes, $PGH_2$ is subsequently metabolized further by enzymes which produce a variety of products with a range of biological activities.[11] Thromboxane synthase in platelets converts $PGH_2$ into thromboxane $A_2$ ($TXA_{2)}$) which is essential for

platelet aggregation. Prostaglandin $I_2$ ($PGI_2$) or prostacyclin is produced by prostacyclin synthase.[11]

The COX-1 enzyme is expressed in many tissues throughout the body including the stomach, kidney, reproductive tract, and blood platelets.[14] Its varied functions include maintenance of gastric mucosal integrity, regulation of renal perfusion, and platelet aggregation. Although it is inducible, the COX-2 enzymes has also been found to be constitutively expressed in tissues including the kidney, and brain where it plays a role in renal homeostasis and the central pain response.[14] During inflammation, the expression of COX-2 is upregulated in a variety of cell types. Endothelial cells, chondrocytes, fibroblasts, monocytes, macrophages, and synovial cells may all express COX-2 during inflammation. At sites of injury, the upregulation of COX-2 leads to increased $PGE_2$ production which mediates pain and inflammation. Inhibition of COX-2 in models of inflammation results in a decreased inflammatory response.[14]

**Non-Steroidal Anti-inflammatory Drugs (NSAIDs)**

**Determination of Cyclooxygenase Selectivity**

It has been established that the NSAIDs can be compared by examining their relative effects on COX-1 and COX-2.[15] Using this system, NSAIDs are described as belonging

to one of four categories. Members of the first group of drugs selectively inhibit the COX-1 enzyme. This effect is desirable in certain circumstances, such as the inhibition of platelet COX-1 to reduce aggregation or the use of SC-560 in the experimental inhibition of COX-1.[15] The second group of non-selective NSAIDs is equally effective at inhibiting both COX-1 and COX-2. This group includes ibuprofen, ketoprofen, and flunixin meglumine.[16] Alternatively, an NSAID may inhibit COX-2 to a greater extent than COX-1 and is described as COX-2 preferential. The COX-2 preferential group includes meloxicam and carprofen.[16] Those NSAIDs which significantly inhibit COX-2, but do not significantly inhibit COX-1 at clinically relevant concentrations, are known as COX-2 selective NSAIDs.[15] As this group reduces pain and inflammation through their potent inhibition of COX-2, while allowing the normal production of prostaglandins by COX-1, they may offer significant advantages over non-selective COX inhibitors.[4] COX-2 selective NSAIDs have recently become available for use in veterinary medicine and include firocoxib and robenacoxib.[16]

When evaluating an NSAID it is important to evaluate several important factors which determine the cyclooxygenase selectivity.[17] Two crucial factors include the species in which the NSAID is being administered, and the method used to determine the cyclooxygenase selectivity. It is not possible to extrapolate the cyclooxygenase selectivity of an NSAID from one species to another as significant differences in selectivity may exist between species. Whereas data obtained in the dog suggests carprofen to be COX-2 preferential with selectivity ratios at $IC_{50}$ and $IC_{80}$ of 16.6 and

101.2 respectively,[18] experimental evidence has found carprofen to be non-selective in the horse with selectivity ratios at $IC_{50}$ and $IC_{80}$ of 1.9 and 1.7 respectively.[19] Phenylbutazone is an NSAID frequently used in equine medicine which is associated with side-effects such as renal medullary necrosis and right dorsal colitis.[20, 21] These side effects can be attributed to the inhibition of COX-1 by this non-selective NSAID, with cyclooxygenase selectivity ratios of 0.3 and 0.7 at $IC_{50}$ and $IC_{80}$ respectively in equine models.[19] This is in contrast to canine experimental models which have shown that phenylbutazone is both a less potent cyclooxygenase inhibitor in the dog, and has a COX-2 preferential action with selectivity ratios of 9.7 and 21 at $IC_{50}$ and $IC_{80}$ respectively.[18]

When interpreting the data available on cyclooxygenase selectivity, a second major factor is the influence of experimental method. A variety of experimental methods have been described for determining the cyclooxygenase selectivity of NSAIDs including recombinant enzymes, cell culture, and both *in vitro* and *ex vivo* whole blood methods.[22] The method used may affect concentration required to inhibit the cyclooxygenase enzymes, the resulting selectivity ratio, and the ability for results to be applied to *in vivo* and clinical situations. In the veterinary literature, recombinant canine cyclooxygenase enzymes produced using a canine kidney cDNA library and a mammalian cell expression system have been used to determine the selectivity of several NSAIDs including deracoxib and carprofen.[23] This *in vitro* uses purified recombinant enzymes to which arachadonic acid is added before the resulting prostaglandin $E_2$ ($PGE_2$) is measured. It should be noted that this system differs from the *in vivo* situation in several significant

aspects. Importantly, the protein concentration of the experimental conditions is lower than the concentration found *in vivo*.[17] This is important in determining the potency of NSAIDs, which are generally highly protein bound (>95%) *in vivo.* The addition of arachidonic acid to the *in vitro* reaction is different from the naturally occurring reaction which relies upon endogenously liberated arachidonic acid. Furthermore, the incubation time of 10 minutes used in these experiments is short when compared with other experimental methods and the clinical situation. The combination of these factors results in significant differences in cyclooxygenase selectivity ratios for NSAIDs when compared to other methods. For example, using purified recombinant canine enzymes deracoxib was found to have a highly COX-2 selective cyclooxygenase selectivity ratio of 1275.[23] However, another group determined the cyclooxygenase selectivity ratio of deracoxib to be 12 using a canine whole blood assay, a difference of over 100-fold.[24]

An alternative to purified recombinant enzymes is the use of cultured mammalian cells which express the cyclooxygenase enzymes. Kay-Mugford *et al* described an *in vitro* system for determining the cyclooxygenase selectivity of NSAIDs in the dog using a macrophage-monocyte cell line derived from a dog with malignant histiocytosis.[25] Similar to the use of recombinant enzymes, this system also relies upon the addition of exogenous arachidonic acid. However, in contrast to recombinant enzymes, COX-2 expression is induced in this model. A concern with this model is the use of unstimulated cells as a model of COX-1 inhibition. In this study, COX-1 and COX-2 mRNA could not be detected in unstimulated cells by Northern blot analysis and the very low basal levels

of $PGE_2$ were therefore attributed to low levels of COX-1. Stimulation with lipopolysaccharide (LPS) resulted in increases in both COX-2 mRNA and $PGE_2$ which was used as a model for COX-2 inhibition.[25]

In order to create more physiologic experimental conditions, whole blood assays have been developed and used in several species including the dog, cat and horse.[6, 18, 19] There are two main methods of performing whole blood assays. The first uses measurement of coagulation induced thromboxane $B_2$($TXB_2$), a stable metabolite of the thromboxane $A_2$($TXA_2$) produced during blood clotting, as a measure of COX-1 activity.[22] Thromboxane synthase is colocalized with COX-1 in blood platelets and therefore the $TXB_2$ produced during coagulation is attributed to the action of COX-1. The inhibition of COX-1 by an NSAID results in a decrease in the production on $TXB_2$. As an indicator of COX-2 activity, this method relies on the measurement of induced-prostaglandin $E_2$ ($PGE_2$).[22] The expression of COX-2 in blood leucocytes is induced, for example with lipopolysaccharide (LPS), and the resulting $PGE_2$ is measured. The inhibition of COX-2 by an NSAID results in a decrease in the production of $PGE_2$. A potential criticism of this method is the different incubation periods for the COX-1 (1 hour) and COX-2 assays (24 hours) which may affect the selectivity of time-dependant COX-2 selective NSAIDs.[22] In an effort to standardize incubation times, a method of measuring $TXB_2$ in heparinized blood as a measure of both COX-1 and COX-2 activity has been described.[6] To measure COX-2 activity, aspirin is used to inhibit the activity of COX-1, and the expression of COX-2 is stimulated with LPS by for measurement of $TXB_2$ after the incubation period. [6]

Both of these assay systems offer several distinct advantages over recombinant enzymes and cell culture systems.[22] The first is the use of physiologically relevant cell types for each assay. The COX-1 assay relies on the activity of COX-1 in platelets which is important in the inhibition of coagulation by NSAIDs. The COX-2 assay is based on the inhibition of COX-2 in leucocytes, an important target of NSAIDs in the relief of pain and inflammation. As NSAIDs are very highly protein bound (>95%), the second important feature of these assays is the physiologic protein concentration at which these reactions take place. Unlike recombinant enzyme systems or transfected cell lines, blood assays require the induction of COX-2 which is physiologically relevant. Also, the blood assays possess endogenous sources of arachidonic acid and do not require the addition of arachidonic acid to the experimental conditions. Therefore, the use of *in vitro* whole blood assays to determine the potency and COX selectivity of NSAIDs is currently the accepted standard.[22, 26]

However, other systems aimed at improving the validity and clinical relevance of these assays have been described. These include the use of gastric cells, chondrocytes, and synoviocytes as clinically relevant models of COX inhibition.[22, 26] This approach has been has been applied to the dog and horse. Studies using canine whole blood, gastric mucosa, duodenal mucosa and synovial fluid have compared the *ex vivo* effects of a range of NSAIDs including aspirin, meloxicam and firocoxib in the dog.[27] A study by Landoni *et al* measured $PGE_2$ produced by LPS stimulated equine synoviocytes to

compare the effect of flunixin meglumine, tolfenamic acid, and ketoprofen on cyclooxygenase in the horse.[26]

## Cyclooxygenase Selectivity in Veterinary Medicine

### Effect of NSAIDs in the Dog

A variety of NSAIDs are available for use in the dog for the treatment of pain and inflammation. NSAID use in the dog has been associated with a range of adverse effects including gastrointestinal ulceration and perforation, renal failure, liver disease, and coagulopathy. Several NSAIDs have been shown to non-selectively inhibit COX in the dog including aspirin, ketoprofen, ibuprofen, etodolac, and piroxicam.[18] Meloxicam and carprofen appear to preferentially inhibit the activity of COX-2 *in vitro*.[18, 28] The COX selectivity ratio of carprofen varies wildly between reports. This may be due to the existence of two enantiomers, S-carprofen and R-carprofen.[16] When these were examined individually, S-carprofen had a selectivity ratio of 17.6, while R-carprofen had a ratio of 5.8.[29] Three COX-2 selective NSAIDs have been described for use in the dog including deracoxib, firocoxib, and robenacoxib. In two separate whole blood assay experiments, the COX selectivity ratio of deracoxib was determined to be 12 and 49,[24] while a recombinant enzyme assay found the ratio to be 1275.[23] While deracoxib does selectively inhibit COX-2, administration of doses above the recommended level has been associated

with gastric perforation in dogs.[30] Robenacoxib is a recently described NSAID with a COX selectivity ratio of 129 in the dog.(King Vet Sci 2010) However, due to its recent introduction, studies examining its clinical use and adverse effects are currently unavailable. Unlike robenacoxib, firocoxib has been approved for use in the US by the FDA and current studies have not shown an increased incidence of adverse effects with its clinical use.(Ryan WG Vet Ther 2006) Firocoxib currently has the highest COX selectivity ratio described in a canine whole blood model of 384.[24]

**Effect of NSAIDs in the Cat**

Studies examining the cyclooxygenase selectivity of NSAIDs in the cat are limited. Meloxicam and carprofen have been demonstrated to preferentially inhibit COX-2 in feline whole blood models.[31] The recently described coxib, robenacoxib, appears to be highly selective for COX-2 with selectivity ratios of 502 and 477 at $IC_{50}$ and $IC_{80}$ respectively.[6]

**Effect of NSAIDs in the Horse**

Although the use of NSAIDs in the horse is extremely common, there is a limited range of products available. Phenylbutazone is routinely administered for the treatment of musculoskeletal pain and is effective in reducing lameness. However, while

phenylbutazone is an effective analgesic and anti-inflammatory in the horse, is has been shown to cause gastric ulceration, kidney failure and right dorsal colitis.[20, 21, 32, 33] These adverse effects on the renal and gastrointestinal systems are due to non-selective inhibition of both COX-1 and COX-2. While phenylbutazone is used for the treatment of musculoskeletal disorders, flunixin meglumine is routinely used for the treatment of colic in the horse. Although flunixin meglumine is effective in treating pain and endotoxemia associated with gastrointestinal disease, it has also been shown to cause adverse effects including gastric ulceration.[33] Furthermore, flunixin meglumine has been shown to inhibit the recovery of ischemic injured equine jejunum and increase permeability to LPS.[34, 35] The recovery of the injured intestinal mucosa depends on the action of prostaglandin induced chloride secretion and the closure of the paracellular spaces in the epithelium.[36] As flunixin is a non-selective COX inhibitor, it significantly inhibits the production of prostaglandins following injury and retard the recovery of the mucosal barrier. The increased mucosal permeability to LPS caused by flunixin is clinically relevant as endotoxemia is a significant cause of morbidity and mortality in the colic patient. Although unavailable for use in the US, the COX selectivity of carprofen and meloxicam has been investigated in the horse.[19] However, while they have both been shown to be COX-2 preferential in the dog,[18] both of these NSAIDs are non-selective COX inhibitors in the horse.[19] While an *in vitro* blood model did not demonstrate a COX-2 selective effect in the horse, meloxicam did allow the recovery of mucosal barrier function in an equine model of ischemic intestinal injury.[37] Ketoprofen was shown to produce fewer

adverse effects on the gastrointestinal tract than flunixin meglumine in an experimental model.[33] However, an earlier study comparing their *ex vivo* effects on $TXB_2$ and $PGE_2$ found no significant difference between the effects of these drugs.[38] Recently, firocoxib, a member of the COX-2 selective coxibs, was introduced for the treatment of musculoskeletal conditions in the horses.[39] While, firocoxib has been shown to be comparable to phenylbutazone in reducing lameness at a clinically relevant dose,[40] there is currently no published study describing the effect of firocoxib on the COX enzymes in the horse. However, firocoxib did not inhibit the recovery of mucosal barrier function in a model of equine ischemic injury.[41] Furthermore, when compared to flunixin meglumine, firocoxib did not inhibit the production of $TXB_2$ in horses following experimental abdominal surgery suggesting it may not significantly inhibit COX-1.[41]

## Conclusions

There are currently a variety of NSAIDs available for use in the veterinary species. The popularity of NSAIDs stems from their effective treatment of pain and inflammation across a range of conditions. However, the use of NSAIDs is not without risk, and adverse effects as a result of NSAID administration have been reported. It is therefore important for the veterinarian to understand the mechanism of action of NSAIDs, and the physiologic basis of their beneficial and adverse effects. It is then possible to select an

appropriate NSAID for the individual patient to maximize the control of pain and inflammation while minimizing the risk of gastrointestinal and renal toxic effects.

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# CHAPTER 2

## The Effect of NSAIDs on the Gastrointestinal Tract of the Horse

**John F Marshall BVMS**

## Introduction

The gastrointestinal tract of the horse is anatomically and physiologically complex. Due to certain anatomical features, the horse is susceptible to a variety of gastrointestinal lesions including volvulus and entrapment of the small and large intestines.[1, 2] The equine gastrointestinal tract requires endogenous prostanoids not only for the maintenance of mucosal blood flow and integrity, but for control of hind gut fermentation and volatile fatty acid production.[3] In the event of gastrointestinal injury, prostaglandins have been shown to be necessary for the restoration of mucosal barrier function.[4] As non-steroidal anti-inflammatory drugs (NSAIDs) are routinely administered to horses suffering from gastrointestinal disease, the effect of NSAIDs on the recovery of barrier function has been investigated.[5-8] Using models of mucosal injury including ischemia,[5] bile acids,[9] and reactive oxygen metabolites,[10] studies have examined the effect of NSAIDs in both the small and large intestine. These studies have highlighted several differences in the response of the large and small intestines to injury and recovery, and to NSAID treatment. In order to understand the potential adverse effects of NSAIDs in the horse, knowledge of these differences is essential.

**Relevance of barrier function recovery in the horse**

Colic represents a major equine welfare and economic issue with an estimated 4.2 colic episodes per 100 horses each year, and an approximate annual cost of $115 million dollars.[11] Despite advances in medical and surgical care of horses, the National Animal Health Monitoring System estimates that colic has a fatality rate of 11%.[11] During severe colic caused by strangulating obstruction, reduced blood supply causes ischemic injury necessitating surgical resection of the affected intestine. However, complete removal of damaged intestine is not always possible and restoration of blood flow may result in further damage from reperfusion injury.[12] The high level of morbidity and mortality associated with colic in the horse is linked to the absorption of lipopolysaccharide (LPS) from Gram negative bacteria across damaged intestine.[13] After gaining access to the systemic circulation from the compromised intestine, LPS-induced endotoxemia is responsible for several major disease syndromes including post-operative ileus and laminitis.[13] These post-operative complications frequently result in extended hospitalization, increased cost, and the death of the horse. It is therefore important that barrier function is rapidly restored following injury.

**The role of prostaglandins on the recovery of mucosal barrier function**

Following mucosal injury, the recovery of mucosal barrier function occurs as the result of three major processes.[14] The villi of the small intestinal epithelium are capable of contracting in response to injury. This villus contraction is a major part of the mucosal response to injury and reduces the surface area of exposed basement membrane to be covered by epithelium. The first phase of contraction by the villus myofibroblasts is mediated by the enteric nervous system. This is followed by a second phase of contraction which is requires endogenous prostaglandin synthesis.[15, 16] The second major process in the restoration of mucosal integrity is the closure of the paracellular spaces.[14] In order for the paracellular spaces to close and repair barrier integrity, the tight junctions located apically between the epithelial cells must be assembled. This process involves the recruitment of tight junction proteins to the junctions and is dependent on the action of endogenous prostaglandins.[14] While the mechanisms by which prostaglandins stimulate the assembly to tight junctions are currently unclear, experimental studies suggest they are mediated by their effects on ion channels and transporters. The effect of prostaglandins on recovery of mucosal barrier function is related to the initial stimulation of chloride secretion, mainly via ClC-2 chloride channels,[17] and inhibition of NHE2 electroneutral sodium exchangers.[18] Whether this effect is directly related to this secretory effect of prostaglandins, or tight junction signaling by ClC-2 and NHE2 is currently unknown. The third process in the repair of the mucosal epithelium and recovery of barrier function is epithelial restitution.[14] Following injury, the epithelial cells

surrounding the denuded basement membrane produce plasma membrane extensions and begin to migrate into the defect. In contrast to villus contraction and assembly of the tight junctions, epithelial restitution is not dependent on the production of endogenous prostaglandins.[19]

**Effect of NSAIDS on recovery of barrier function**

The roles of two cyclooxygenase enzyme isoforms have been described in the normal and healing intestine. Cyclooxygenase-1 (COX-1) is constitutively expressed in most tissues, and is believed to be responsible for PG production during normal physiological processes while cyclooxygenase-2 (COX-2) is expressed at only low levels in normal tissue, but is up regulated in response to injury.[20] In the equine gastrointestinal tract, constitutive expression of both COX-1 and COX-2 has been identified in the jejunum and the pelvic flexure of the large colon.[5, 21] Nonsteroidal anti-inflammatory drugs (NSAIDs), particularly flunixin meglumine, are frequently used to treat colic in the horse, both to provide analgesia, and to ameliorate signs of endotoxemia.[22] NSAIDs inhibit the COX enzyme, which is critical in the conversion of arachidonic acid to prostaglandin (PG) $H_2$. Prostaglandin $H_2$ is then converted to variety of prostanoids by a range of specific synthases.[20] Studies involving the porcine ileum have shown that prostaglandins $I_2$ and $E_2$ are required to restore mucosal barrier function following ischemic injury.[23]

## The Equine Small Intestine

Studies of the effects of NSAIDs on the small intestine of the horse have focused on ischemic injury of the jejunum. This injury is encountered clinically in cases of strangulating lesions of the jejunum such as small intestinal volvulus or strangulation by a pedunculated lipoma. It has been demonstrated that flunixin meglumine retards recovery of the mucosal barrier in ischemic-injured equine jejunum as determined by the *ex vivo* measurement of transepithelial electrical resistance (TER) and the mucosal-to-serosal flux of mannitol.[5] A clinically relevant measurement of mucosal barrier function is the mucosal-to-serosal paracellular flux of LPS. Importantly, treatment with flunixin meglumine results in an increased mucosal permeability to LPS following ischemic injury and an 18 hour recovery period.[6, 24] Flunixin meglumine inhibits the increase in thromboxane $B_2$ ($TXB_2$) and prostaglandin $E_2$ ($PGE_2$) observed during recovery from ischemic injury both *in vitro* and *ex vivo*.[5, 24] When ischemic injured equine jejunum is treated with a combination of flunixin meglumine and the prostaglandin $E_1$ ($PGE_1$) analogue misoprostol, the inhibitory effect on the recovery of barrier function is prevented.[5] Therefore, the inhibition of barrier function in ischemic injured equine jejunum by flunixin meglumine is likely due to the inhibition of cyclooxygenase in the intestine.

Following ischemic injury, chemoattractants are released resulting in neutrophil migration and infiltration of injured tissues. This neutrophil infiltration and degranulation

causes further tissue damage through physical opening of paracellular spaces, and the release of proteases e.g. elastase.[14] It has been shown in the pig that mucosal neutrophil infiltration following ischemic injury is detrimental to mucosal barrier function.[25] Treatment with flunixin meglumine results in an increase in mucosal neutrophil infiltration in ischemic injured equine jejunum over an 18 hour recovery period.[26] The reason for this increase neutrophil infiltration and its significance in the inhibition of mucosal barrier function recovery in the horse is unclear at this time.

Following the identification of the detrimental effect of flunixin meglumine on mucosal barrier function recovery, several studies have examined alternative medications. Etodolac was studied in an *ex vivo* model of equine ischemic intestinal injury, however there was no significant difference between the effect of etodolac and flunixin meglumine on the recovery of barrier function.[7] A later study revealed that etodolac is not a COX-2 selective inhibitor in the horse which explains the inhibition of barrier function recovery by etodolac.[27] Another NSAID that has been shown to preferentially inhibit COX-2 in the dog is meloxicam.[28] When meloxicam was examined in a model of ischemic injury and recovery, it was found that it did not inhibit the recovery of mucosal barrier function as measured by TER and mucosal-to-serosal passage of inulin.[29] This study did not find a significant effect of either flunixin meglumine or meloxicam on the passage of LPS through ischemic injured tissue.[29] Both meloxicam and flunixin meglumine were shown to increase the mucosal neutrophil infiltration.[29] However, as meloxicam was shown to have an equal or greater effect than flunixin meglumine on postoperative pain, and it did

not inhibit the recovery of mucosal barrier function, it appears to be a suitable alternative to flunixin meglumine for the treatment of colic.[29]

In an effort to reduce adverse gastrointestinal effects in human medicine, COX-2 selective drugs have been developed. These drugs are often referred to as coxibs, and are designed to be potent inhibitors of COX-2 while allowing normal production of prostaglandins by COX-1 at clinically relevant concentrations.[30] Deracoxib is a member of the coxibs that is available for use in veterinary medicine and has been shown to selectively inhibit COX-2 in the dog.[31] A study of the *in vitro* recovery of ischemic injured equine jejunum showed that deracoxib was similar to flunixin meglumine and inhibited the recovery of mucosal barrier function as meaured by TER.[5] However, unlike flunixin meglumine, deracoxib treatment did not result in a significant increase in the mucosal-to-serosal passage of mannitol.[5] Furthermore, deracoxib inhibited the increase in $PGE_2$ and $PGF_1$ without inhibiting an increase in $TXB_2$.[5] As this was an *in vitro* study the effect of deracoxib on neutrophil infiltration was not determined. Therefore, deracoxib may offer an alternative to flunixin meglumine in cases of small intestinal ischemic injury.

Although meloxicam and deracoxib appear to offer advantages over flunixin meglumine for the treatment of small intestinal ischemic injury,[5, 29] in the US neither is currently available for use in the horse. However, a member of the coxibs has been recently approved for the treatment of musculoskeletal pain in the horse. This NSAID, firocoxib,

has been shown to be a highly selective inhibitor of COX-2 and its use has not been associated with an increased risk of adverse gastrointestinal effects in the dog.[32] In an *ex vivo* study of ischemic injury and recovery, treatment with firocoxib did not inhibit mucosal barrier function as measured by TER.[24] In this study, flunixin meglumine, but not firocoxib, was again shown to increase the permeability of the mucosa to LPS.[24] In horse treated with flunixin meglumine or firoxoxib there was no significant increase in the plasma $PGE_2$ during the recovery period suggesting both NSAIDs inhibit the action of COX-2.[24] Only flunixin meglumine inhibited the increase in plasma $TXB_2$ suggesting that firocoxib did not significantly inhibit the action of COX-1.[24] The effect of firocoxib on mucosal neutrophil infiltration is currently unknown.

The role played by mucosal neutrophils in the inhibition of mucosal barrier function recovery in the horse is currently unclear. Several studies have shown that there is an increase in mucosal neutrophil infiltration of injured jejunum in horses treated with flunixin meglumine which is associated with reduced barrier function.[7, 24] In contrast, treatment with meloxicam resulted in increased neutrophil infiltration without inhibition of barrier function recovery.[29] However, a recent study examining the effect of lidocaine on ischemic injury and recovery of the equine jejunum did suggest a role of mucosal neutrophil infiltration in barrier function.[26] In this study, while treatment with flunixin meglumine inhibited the recovery of mucosal barrier function, a combination of flunixin meglumine and lidocaine did not.[33] Interestingly, treatment with lidocaine significantly inhibited the mucosal infiltration of neutrophils associated with flunixin meglumine

treatment.[26] However, an *in vitro* study revealed that lidocaine does not inhibit the migration and adhesion of equine neutrophils.[34] It therefore appears that lidocaine prevents the inhibition of barrier function recovery associated with flunixin meglumine by an anti-inflammatory mechanism that is unknown.

**The Equine Large Intestine**

The large intestine of the horse is divided into several anatomically and physiologically distinct regions. Following mixing in the cecum, ingesta enters the ventral colon every 3 – 4 minutes. The right and left ventral regions of the large colon are succulated and contain a large volume of mixed ingesta for fermentation.[2] While the ventral colon does secrete a significant volume of fluid, in the normal situation there is a net absorption of water and sodium chloride.[35] However, when the right ventral colon is examined *in vitro,* production of endogenous prostanoids causes inhibition of sodium absorption and stimulation of chloride secretion.[36] The *in vitro* treatment of normal right ventral colon with flunixin meglumine inhibits this increased chloride secretion.[37] Exposure of right ventral colon to reactive oxygen intermediates resulted in an increase in chloride secretion which was abolished by treatment with flunixin meglumine.[10] However, as this model did not result in tissue injury or changes in mucosal barrier function the effect of flunixin meglumine on the recovery of the right ventral colon is unknown.[10] Since the recovery of barrier function has been associated with the stimulation of chloride

secretion[4] and flunixin meglumine inhibits secretion by the right ventral colon,[37] it may inhibit recovery from ischemic injury. However, at this time ischemic injury of the right ventral colon has not yet been investigated.

Following the large, sacculated ventral colon the large colon of the horse loses its sacculations and narrows at the pelvic flexure.[2] Although studies of the right ventral colon suggest that flunixin meglumine would inhibit recovery of the colon from ischemic injury,[37] a study of the pelvic flexure found that treatment with flunixin meglumine did not adversely affect the recovery of mucosal barrier function.[8] However, several differences between the response of the pelvic flexure and jejunum to ischemic injury were discovered. In contrast to the results of previous studies in the jejunum,[7, 24, 29] the transepithelial electrical resistance of colonic mucosa following injury and recovery was significantly lower than uninjured mucosa.[8] Also, treatment with flunixin meglumine did not increase mucosal permeability to mannitol.[8] While injury and recovery resulted in neutrophil infiltration of the lamina propria, treatment with flunixin meglumine did not increase accumulation.[8] The effect of ischemic injury or flunixin meglumine on the transmucosal passage of LPS is unknown. Therefore, would it appear that there are significant differences in the responses of the equine jejunum and pelvic flexure to ischemic injury and flunixin meglumine.

The right dorsal colon is the shortest and widest section of the equine ascending colon.[2] Like the ventral colon, it is sacculated and is a major site of fluid secretion and

absorption.[35] In contrast to the ventral colon, a net secretion of fluid occurs in the dorsal colon.[35] Also, unlike the ventral colon the right dorsal colon is well attached to the abdominal roof, cecal base and root of the mesentery. It is therefore not susceptible to volvulus and the resultant ischemic injury.[2] However, the administration of phenylbutazone, a non-selective NSAID, has been shown to cause right dorsal colitis which may result in death.[38, 39] Experimental studies have shown that prolonged (21 days) administration of phenylbutazone decreases colonic volatile fatty acid production and mucosal blood flow.[38] While no study has examined the effect of prolonged phenylbutazone treatment on mucosal barrier function, the *in vitro* treatment of oxidant-injured right dorsal colon with phenylbutazone did not affect the recovery of barrier function.[40]

While studies of the effect of NSAIDs on the recovery of the colon from injury are limited, they do suggest that there may be significant differences in the response of the jejunal and colonic mucosal barrier to injury. As the colon is a physiologically complex organ, with varying functions throughout its length, further studies are required to determine the effect of injury location on the response to NSAID treatment. However, this may be difficult to assess *in vitro* as significant differences in the absorptive function have been identified when compared to the *in vivo* situation.

## Conclusions

The equine gastrointestinal tract may be affected by a variety of conditions resulting in injury to the mucosal barrier. Restoration of barrier function is essential to prevent the passage of luminal contents, including LPS, into the systemic circulation resulting in endotoxemia.[41] The routine use of NSAIDs in the treatment of pain and endotoxemia in the horse has led to investigation of their effects on barrier function recovery. In the jejunum, the non-selective COX inhibitor flunixin meglumine has been shown to inhibit the recovery of mucosal barrier function, and increase neutrophil infiltration and permeability to LPS.[6, 7] The COX-2 selective NSAIDs deracoxib[5, 24] and firocoxib,[24] do not inhibit the recovery of barrier function in the jejunum. The inhibition of chloride secretion, an essential element in recovery of barrier function after ischemic injury, has been demonstrated in the right ventral colon by treatment with flunixin meglumine.[37] Investigation of the effects of ischemic injury in the large colon has focused on the pelvic flexure.[8] In contrast to the jejunum, flunixin meglumine did not inhibit recovery of mucosal barrier function or increase neutrophil infiltration.[8] Therefore, the regions of the equine gastrointestinal tract appear to vary in their response to ischemic injury and recovery. Furthermore, the response to treatment with NSAIDs depends on which region of the gastrointestinal tract is injured.

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# CHAPTER 3

## The cyclooxygenase selectivity of flunixin meglumine, deracoxib, and firocoxib in the horse

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and Anthony T Blikslager

**Abstract**

**Objective –** To determine the cyclooxygenase (COX) selectivity of flunixin meglumine, deracoxib, and firocoxib in the horse.

**Animals –** 6 horses.

**Procedure –** Inhibition of COX-1 and COX-2 by each NSAID was determined by measuring coagulation-induced thromboxane $B_2$ ($TXB_2$) and lipopolysaccharide (LPS) stimulated prostaglandin $E_2$ concentrations respectively. The concentration at which each NSAID inhibited COX-1 and COX-2 by 50%, 80%, and 95% ($IC_{50}$, $IC_{80}$, and $IC_{95}$) was calculated using a four-parameter logistic equation. COX selectivity at $IC_{50}$, $IC_{80}$, and $IC_{95}$ was determined by dividing the COX-1 value by the COX-2 value. Results were analyzed using a 2-way ANOVA for the effect of treatment and concentration, with a statistical significance of $p<0.05$.

**Results –** Coagulation and LPS significantly increase serum $TXB_2$ and plasma $PGE_2$ respectively. Flunixin meglumine non-selectively inhibited both COX-1 and COX-2 with selectivity ratios at $IC_{50}$ and $IC_{80}$ of 3.27 and 3.32 respectively. Deracoxib was COX-2 selective at lower concentration ($IC_{50}$ ratio 46.92), but COX-2 selectivity decreased at higher concentrations ($IC_{95}$ ratio 7.89). Firocoxib was highly COX-2 selective at both low and high concentrations with COX selectivity ratios of 63.27 and 139.72 at $IC_{50}$ and $IC_{80}$ respectively.

**Conclusions and Clinical Relevance –** Deracoxib and firocoxib selectively inhibit COX-2 in equine whole blood. The COX selectivity of deracoxib appears to be

concentration dependant in the horse. Flunixin meglumine is a non-selective COX inhibitor in the horse.

## Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in the horse for the treatment of pain and inflammation. These drugs act by inhibiting the production of prostaglandins by the cyclooxygenase (COX) enzymes. The COX enzymes exist in two major isoforms designated COX-1 and COX-2 which vary in their tissue distribution and physiologic roles. COX-1 is generally considered to be the constitutively expressed isoform, present in many tissues and important for normal physiologic processes. In contrast, COX-2 is an inducible isoform important for the production of prostaglandins during injury and disease.[1] Traditional NSAIDs, including flunixin meglumine, generally inhibit both isoforms with equal potency and may be described as non-selective COX inhibitors.[2] The adverse effects observed during treatment with NSAIDs including renal failure and colitis are often attributed to non-selective inhibition of COX-1.[3, 4] In order to treat the symptoms of pain and inflammation while avoiding the adverse effects of COX-1 inhibition, NSAIDs have been designed to selectively inhibit COX-2.[5] These include deracoxib, which is available in the US for use in the dog, and firocoxib which is available in the US for use in both the dog and horse. In the horse, firocoxib was shown to alleviate lameness to a similar extent as phenylbutazone.[6] However, the effect of firocoxib on the cyclooxygenase enzymes in the horse has not been described.

The potency of an NSAID is established by determining the concentration at which each cyclooxygenase enzyme is inhibited by a defined percentage, most commonly 50%

($IC_{50}$).[7] Several experimental models have been used to determine the potency of NSAIDs including recombinant enzymes, cell culture, tissue, and whole blood assays.[8] Whole blood assays offer several advantages including a physiologic protein concentration, an endogenous arachdonic acid source, and the induction of COX-2. Using the potency of an NSAID for COX-1 and COX-2, the selectivity of the NSAID can be determined by calculating the ratio of the COX-1 $IC_{50}$ to the COX-2 $IC_{50}$ to describe the relative effect on each enzyme.[8]

As the selectivity of an NSAID may vary between species,[7] we aimed to investigate whether deracoxib and firocoxib are COX-2 selective in the horse. Furthermore, we aimed to describe the effect of concentration on selectivity for flunixin meglumine, deracoxib, and firocoxib by establishing COX selectivity ratios for each at $IC_{50}$, $IC_{80}$, and $IC_{95}$.

## Materials and Methods

### Animals

All procedures were approved by the Institutional Animal Care and Use Committee of North Carolina State University. A maximum of sixty milliliters of whole blood was collected from 6 healthy female horses three times with a minimum of 21 days between collections.

### *In vitro* COX-1 inhibition assay

The effect of flunixin meglumine, deracoxib, and firocoxib on COX-1 enzyme activity was determined using an *in vitro* whole blood coagulation assay as previously described.[9, 10] Briefly, anti-coagulant-free vacuum tubes were prepared by adding 10µl of vehicle control (DMSO), flunixin meglumine, deracoxib or firocoxib. Blood (2.5ml) was collected in each tube resulting in a final treatment concentration of 0.01µM - 1000µM. A total of twenty four 2.5 ml tubes of blood were collected for each experiment. A 2.5ml sample of heparinized (uncoagulated) blood was collected to serve as a baseline and negative control. The blood was allowed to clot for 1 hour at 37°C before being centrifuged at 8000rpm for 10 minutes. Following centrifugation, 100µl of serum was added to 400µl of methanol and centrifuged at 8000rpm for 10 minutes. A 50µl aliquot of the supernatant was collected and diluted in 150µl of thromboxane $B_2$ ($TXB_2$) enzyme immunoassay (EIA) kit[a] buffer resulting in a final 20-fold dilution. The amount of $TXB_2$

in each sample was determined using a commercially available $TXB_2$ EIA kit.[a] The baseline and coagulation induced $TXB_2$ in equine serum were determined and expressed as a mean ± SE. The assay coefficient of variance (CV) was determined using the vehicle control treated serum samples. The intra-animal CV was determined using the three vehicle control serum samples per animal with a minimum of 21 days between sample collections.

***In vitro* COX-2 inhibition assay**

The effect of flunixin meglumine, deracoxib, and firocoxib on COX-2 activity was determined using an *in vitro* lipopolysaccharide (LPS) stimulated equine plasma prostaglandin $E_2$ ($PGE_2$) assay as previously described.[9, 10] Briefly, blood (12ml) was collected into heparinized vacuum tubes and incubated with 100µg/ml of LPS in 0.1% bovine serum albumin in phosphate buffered saline (PBS) for 5 minutes. A sample of blood which was not treated with LPS was prepared as a negative control. After 5 minutes stimulation with LPS, 500µl aliquots of blood were treated with vehicle control (DMSO), flunixin meglumine, deracoxib, or firocoxib at a final concentration of 0.01 - 1000µM and incubated at 37°C for 24 hours. Following incubation, the blood was centrifuged at 8000rpm for 10 minutes to separate the plasma. Following centrifugation, 100µl of serum was added to 400µl of methanol and centrifuged at 8000rpm for 10 minutes. A 50µl aliquot of the supernatant was collected and diluted in 150µl of prostaglandin $E_2$ ($PGE_2$) enzyme immunoassay (EIA) kit[b] buffer resulting in a final 20-

fold dilution. The amount of $PGE_2$ in each sample was determined using a commercially available $PGE_2$ EIA kit.[b] The baseline and coagulation induced $PGE_2$ in equine serum were determined and expressed as a mean ± SE. The assay coefficient of variance (CV) was determined using the vehicle control treated serum samples. The intra-animal CV was determined using the three vehicle control serum samples per animal with a minimum of 21 days between sample collections.

**Analysis of cyclooxygenase inhibition data**

The effect of coagulation and LPS stimulation on $TXB_2$ and $PGE_2$ respectively was determined using a Student's t-test. The effect of treatment with each NSAID on COX-1 and COX-2 was analyzed using a two-way ANOVA with a Holm-Sidak *post hoc* test. Significance was set at $p<0.05$. The results of each cyclooxygenase assay were expressed as a percentage of the negative control sample to determine the percentage inhibition. Using the percentage inhibition of each concentration, a four-parameter logistic curve model of COX-1 and COX-2 inhibition was created for each treatment using commercially available software.[c] The concentrations of each treatment resulting in 50, 80 and 95% inhibition ($IC_{50}$, $IC_{80}$ and $IC_{95}$ respectively) of COX-1 and COX-2, and the ratio of the COX-1 and COX-2 $IC_{50}$, $IC_{80}$ and $IC_{95}$ values were calculated to determine the COX selectivity of flunixin meglumine, deracoxib, and firocoxib in the horse.

## Results

### *In vitro* COX-1 inhibition assay

The baseline $TXB_2$ in equine whole blood was determined to be 23.56±3.49pg/ml. Coagulation for a period of 1 hour significantly increased the serum $TXB_2$ in vehicle control samples to 984.79±193.12pg/ml ($p<0.05$) (Table 1). The assay and intra-animal CV were 47% and 55% respectively (Table 2).

### *In vitro* COX-2 inhibition assay

The baseline $PGE_2$ in equine plasma following 24 hours incubation was determined to be 155.99±13.65pg/ml. Following LPS stimulation, the $PGE_2$ in equine plasma increased significantly to 496.31±49.92pg/ml ($p < 0.05$) (Table 1). The assay and intra-animal CV were 32% and 13% respectively (Table 2).

### Cyclooxygenase inhibition analysis

There was no significant difference in the inhibition of COX-1 and COX-2 by flunixin meglumine at any concentration tested (Fig 1). At a concentration of 1 or 10µM, deracoxib inhibited COX-2 to a significantly greater percentage than COX-1 (Fig 2). Firocoxib inhibited COX-2 significantly more than COX-1 at a concentration of 10, 100, or 1000µM.

Following analysis of the COX inhibition assays, flunixin meglumine was determined to be the most potent inhibitor of COX-1. Deracoxib inhibited COX-1 by 50%, 80% and

95% at a lower concentration than firocoxib (Table 3). The COX-1 and COX-2 inhibition curve for flunixin meglumine revealed a similar curve for both enzymes and parallel slopes (Fig 1). The COX selectivity ratios for flunixin meglumine at $IC_{50}$ and $IC_{80}$ were similar at 3.27 and 3.32 respectively demonstrating non-selective inhibition of the COX enzymes. The slopes of the COX-1 and COX-2 inhibition curves for deracoxib were not parallel (Fig 2). This was reflected in the COX selectivity ratios for deracoxib which decreased from 46.92 at $IC_{50}$ to 7.89 at $IC_{95}$. Therefore, the COX-2 selectivity of deracoxib was concentration dependant and decreased at higher concentrations. The COX-2 inhibition curve for firocoxib had a typical sigmoid dose-response curve. In contrast, although the firocoxib COX-1 inhibition curve showed increasing inhibition with concentration the inhibition of COX-1 did not reach 100% at the concentrations tested in this experiment (Fig 3). The COX selectivity ratios determined for firocoxib revealed selective inhibition of COX-2 at $IC_{50}$ (selectivity ratio 63.27), and $IC_{80}$ (selectivity ratio 139.72).

**Discussion**

This study compared the effect of flunixin meglumine on the activity of the COX enzymes with the effect of two newer NSAIDs, firocoxib and deracoxib. While all three of these drugs reduce pain and inflammation by inhibiting the cyclooxygenase enzymes, firocoxib and deracoxib have been designed to selectively inhibit COX-2.[5] An *in vitro* whole blood assay was used in this experiment as it was believed this best simulated the

*in vivo* effect of NSAIDs.[8] The COX-1 assay is based on the inhibition of COX-1 in platelets which are a clinical target of NSAID therapy. The COX-2 assay involved the induction of COX-2 by leukocytes and subsequent inhibition by the test NSAID. Again, the inhibition of induced COX-2 in leukocytes is clinically relevant in the therapeutic use of NSAIDs. Also, using a whole blood assay allowed a physiologic protein concentration to be used which is important given the very high protein binding by NSAIDs (>95%).[8]

Flunixin meglumine has previously been shown to non-selectively inhibit both COX-1 and COX-2 based upon $IC_{50}$ and $IC_{80}$ ratios.[9, 10] It has been suggested that as a high level of COX inhibition is required to produce a clinically relevant analgesic and anti-inflammatory effect, the ratio of the concentrations of NSAID required to inhibit COX-1 and COX-2 by 95% should be compared ($IC_{95}$).[7] Therefore, in this study we attempted to determine the $IC_{50}$, $IC_{80}$ and $IC_{95}$ for each NSAID. This study confirmed the results of previous studies which demonstrated that flunixin meglumine inhibits both of the cyclooxygenase isoenzymes in a non-selective manner. Non-selective cyclooxygenase inhibition has been associated with the adverse effects of NSAID administration including gastrointestinal and renal complications.[3, 4] Furthermore, flunixin meglumine has been shown to inhibit the recovery of mucosal barrier function in equine ischemic injured jejunum and increase permeability to LPS.[11]

The COX selectivity of deracoxib has not previously been studied in the horse. A study of the effect of deracoxib on the activity of recombinant canine COX enzymes showed

that it was COX-2 selective with a selectivity ratio of 1275.[12] However, when deracoxib was examined using a canine whole blood assay the COX selectivity ratio was considerably lower at 12.[13] It has been recognized that the selectivity of NSAIDs may vary depending on the experimental method used and this may explain the difference in these results.[8] The results of this study show that although deracoxib selectively inhibits COX-2 in the horse at $IC_{50}$, this selectivity decreases at higher concentrations due to the non-parallel slope of the dose-response curve. It was previously shown that while deracoxib inhibited the recovery of mucosal barrier function in ischemic injured equine jejunum as measured by transepithelial resistance (TER), it did not increase permeability to mannitol.[14] However, this previous study used a deracoxib concentration of 27µM and based on the findings of this study we would expect that a clinically effective dose would result in a concentration of between 1 and 10µM.

Firocoxib is a recently introduced NSAID which has been shown to effectively treat equine lameness.[15] Furthermore, it has been shown that firocoxib does not significantly inhibit the recovery of ischemic injured equine jejunum.[16] However, the COX selectivity of firocoxib in the horse has not been described. This study demonstrates that firocoxib is a COX-2 selective NSAID which inhibited COX-2 significantly more than COX-1 at a concentration of 10 - 1000µM. This concentration range is greater than the plasma concentration of firocoxib obtained following repeated oral administration.[17] This may be due the *in vitro* experimental method used and suggest that further studies using an *ex vivo* model are warranted.

This study has shown that while there is no significant difference in the inhibition of COX-1 and COX-2 by flunixin meglumine, both deracoxib and firocoxib are selective inhibitors of COX-2. Importantly, our results highlight the effect of treatment concentration on COX-2 selectivity. Therefore, when determining the dose of NSAID for experimental or clinical purposes this effect should be considered.

**Footnotes:**

a Thromboxane B2 EIA kit, Cayman Chemical, Ann Arbor MI

b Prostaglandin $E_2$ EIA kit (Monoclonal), Cayman Chemical, Ann Arbor MI

c SigmaPlot 10, Systat Software, San Jose CA

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**Table 1:** The baseline and coagulation stimulated $TXB_2$ were measured in equine plasma and serum respectively. The baseline $PGE_2$ was measured in equine plasma obtained from whole blood incubated without LPS for 24 hours. The LPS stimulated $PGE_2$ was measured in equine plasma obtained from whole blood incubated with 100µg/ml of LPS for 24 hours. Results are expressed as the mean ± SE. Coagulation resulted in a significant increase in serum $TXB_2$. LPS stimulation resulted in a significant increase in plasma $PGE_2$.

| | Baseline (pg/ml) | Stimulated (pg/ml) | Percentage increase (%) |
|---|---|---|---|
| Thromboxane $B_2$ | 23.56 ± 3.49 | 984.79 ± 193.12 | 4180 |
| Prostaglandin $E_2$ | 155.99 ± 13.65 | 496.31 ± 49.92 | 318 |

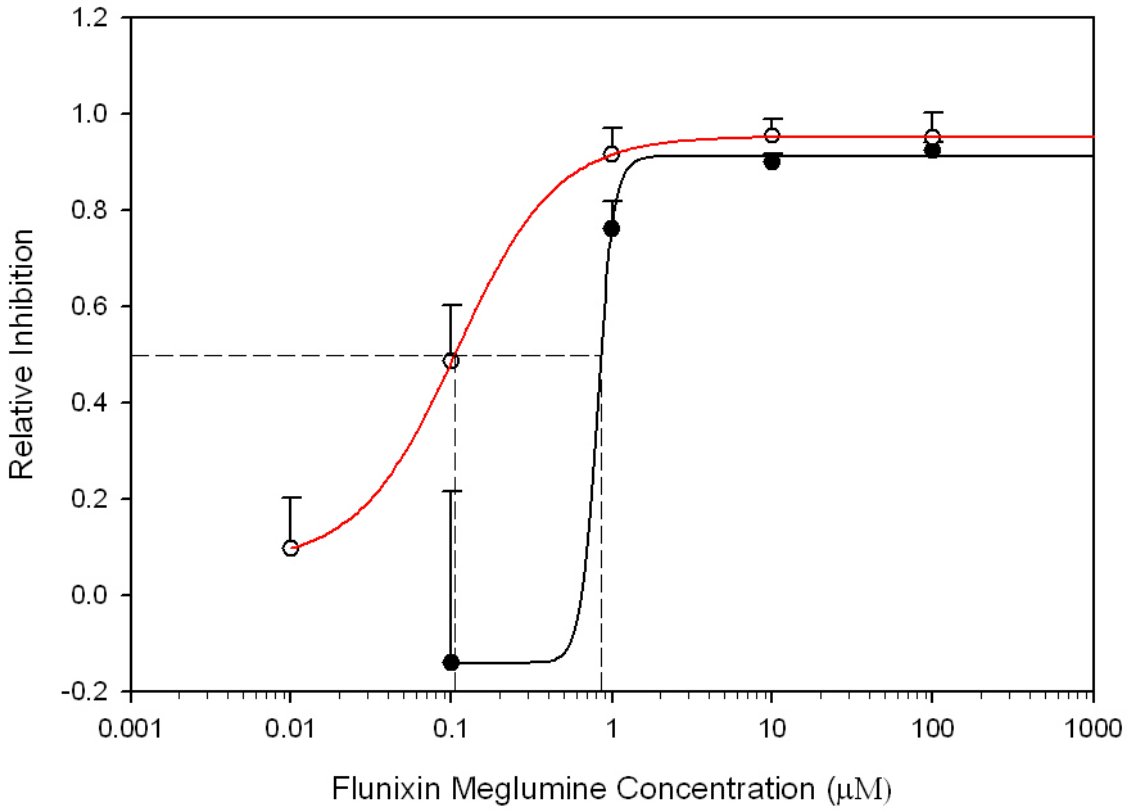


**Figure 1:** The mean ± SE relative inhibition of COX-1 (closed circles) and COX-2 (open circles) by flunixin meglumine. The predicted inhibition of COX-1 and COX-2 by flunixin meglumine is represented by the black and red lines respectively. The expected $IC_{50}$ is represented by dashed lines. There was no significant difference in the inhibition of COX-1 and COX-2 at any concentration tested.

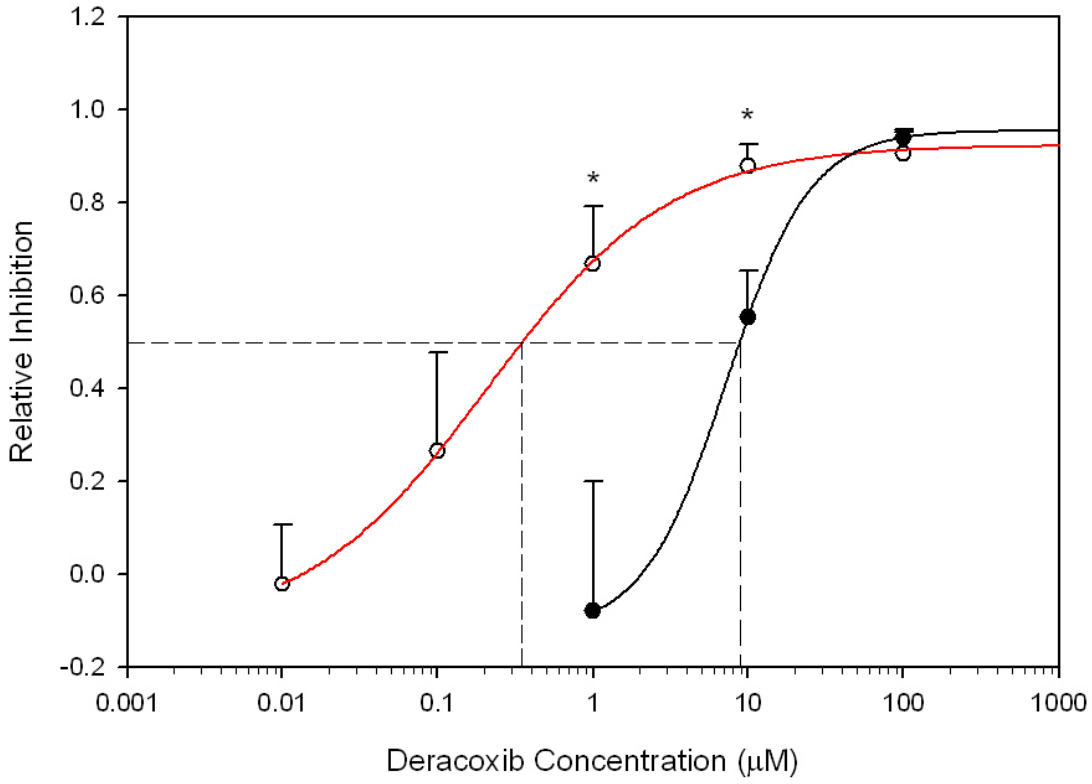


**Figure 2:** The mean ± SE relative inhibition of COX-1 (closed circles) and COX-2 (open circles) by deracoxib. The predicted inhibition of COX-1 and COX-2 by deracoxib is represented by the black and red lines respectively. The expected $IC_{50}$ is represented by dashed lines. The inhibition of COX-2 was significantly greater than COX-1 at a concentration of 1 and 10µM ($p<0.05$).

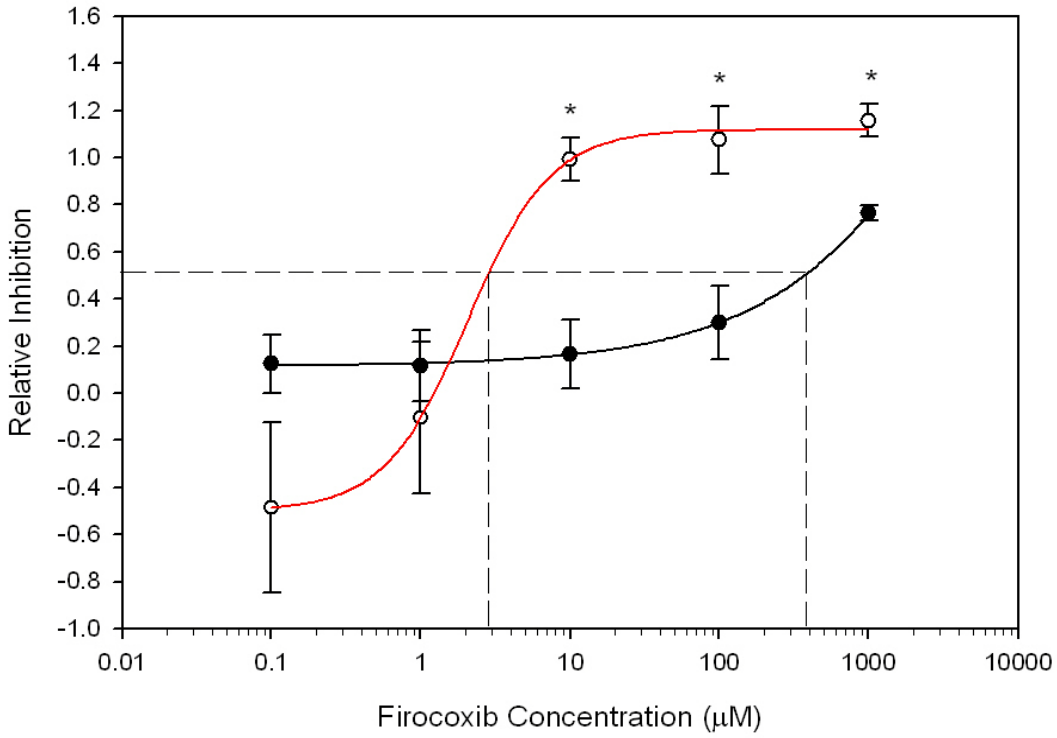


**Figure 3:** The mean ± SE relative inhibition of COX-1 (closed circles) and COX-2 (open circles) by firocoxib. The predicted inhibition of COX-1 and COX-2 by firocoxib is represented by the black and red lines respectively. The expected $IC_{50}$ is represented by dashed lines. The inhibition of COX-2 was significantly greater than COX-1 at a concentration of 10, 100 and 1000µM ($p<0.05$).

**Table 2:** The *in vitro* potency of cyclooxygenase inhibition by flunixin meglumine, deracoxib, and firocoxib. For each NSAID, the $IC_{50}$, $IC_{80}$, and $IC_{95}$ values for COX-1 and COX-2 were calculated.

N/A – This value could not be predicted based on the model.

| | COX-1 | | | COX-2 | | |
|---|---|---|---|---|---|---|
| | $IC_{50}$ (µM) | $IC_{80}$ (µM) | $IC_{95}$ (µM) | $IC_{50}$ (µM) | $IC_{80}$ (µM) | $IC_{95}$ (µM) |
| Flunixin meglumine | 0.36 | 1.26 | N/A | 0.11 | 0.38 | 2.21 |
| Deracoxib | 12.2 | 50.15 | 493.81 | 0.26 | 4.7 | 62.59 |
| Firocoxib | 345.46 | 1081.43 | N/A | 5.46 | 7.74 | 9.86 |

**Table 3:** The COX selectivity of flunixin meglumine, deracoxib, and firocoxib in equine whole blood. The selectivity of each NSAID was determined by calculating the ratio of COX-1/COX-2 at $IC_{50}$, $IC_{80}$, and $IC_{95}$.

N/A – No ratio could be determined.

| | COX-1/COX-2 Ratio | | |
|---|---|---|---|
| | $IC_{50}/IC_{50}$ | $IC_{80}/IC_{80}$ | $IC_{95}/IC_{95}$ |
| Flunixin meglumine | 3.27 | 3.32 | N/A |
| Deracoxib | 46.92 | 10.67 | 7.89 |
| Firocoxib | 63.27 | 139.72 | N/A |

# CHAPTER 4

## The cyclooxygenase selectivity and effect of robenacoxib on the recovery of ischemic-injured equine jejunum *ex vivo*

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**Abstract**

**Objective –** To determine the cyclooxygenase (COX) selectivity of robenacoxib and its effect on mucosal recovery following ischemic injury in the horse.

**Animals –** 12 horses.

**Procedure –** Robenacoxib selectivity was determined by assaying its $IC_{50}$ on COX-1 and COX-2 activity by measuring coagulation-induced thromboxane $B_2$ ($TXB_2$) and lipopolysaccharide-stimulated prostaglandin $E_2$ concentrations respectively. COX selectivity was defined as the $IC_{50}$ for COX-1 divided by the $IC_{50}$ for COX-2. Horses (n=6) were anesthetized and jejunum was subjected to 2 hours ischemia. Control and ischemic-injured mucosa was placed in Ussing chambers and treated with Ringer's solution (control), flunixin meglumine ($2.7\times10^{-5}$M) or robenacoxib ($2.7\times10^{-5}$M). Transepithelial electrical resistance (TER) and $^{3}$H-mannitol flux were measured over a 4-hour recovery period. Bathing solution $TXB_2$ and prostaglandin E metabolites (PGEM) were measured to assess COX-1 and COX-2 function respectively. Histological injury grade and percentage epithelial denudation were determined by microscopy. Results were analyzed using ANOVA, with a statistical significance of $p<0.05$.

**Results –** The $IC_{50}$ of robenacoxib for COX-1 and COX-2 was 11.46±4.46μM and 0.19±0.07μM respectively, resulting in a COX selectivity ratio of 61.01. TER of ischemic-injured jejunum treated with flunixin meglumine was significantly lower than that of control and robenacoxib treatment. There was a significant increase in PGEM and

$TXB_2$ in control and robenacoxib-treated tissues but not flunixin meglumine-treated tissues.

**Conclusions and Clinical Relevance -** Robenacoxib selectively inhibits COX-2 and allows recovery of barrier function in ischemic-injured equine jejunum *ex vivo*.

## Introduction

Colic has a major impact on the equine industry due largely to the high fatality rate, which has been estimated at 11% based on data from the National Animal Health Monitoring System.[1] In particular, horses with colic due to a strangulating lesion, which results in ischemic injury to the intestine, have a dramatically reduced prognosis for survival compared to horses with simple obstruction of the intestine.[2] This reduction in prognosis is principally because of endotoxemic shock, which is the leading cause of early postoperative mortality.[3] Many of the signs of endotoxemia, and much of the pain associated with strangulating obstruction are attributable to prostaglandins (PG) elaborated by the cyclooxygenase (COX) enzymes.

The roles of the COX-1 and COX-2 isoforms have been described in the normal and healing intestine.[4] Cyclooxygenase-1 is constitutively expressed in most tissues, and is believed to be responsible for PG production during normal physiological processes while COX-2 is expressed at only low levels in normal tissue, but is upregulated in response to injury and bacterial toxins such as lipopolysaccharide. Both COX-1 and COX-2 have been shown to be expressed in equine jejunum.[4] Non-steroidal anti-inflammatory drugs (NSAIDs), particularly flunixin meglumine, are frequently used to treat colic in the horse, both to provide analgesia, and to ameliorate signs of endotoxemia.[5,6] NSAIDs inhibit the COX enzyme, which metabolizes arachidonic acid to the intermediary prostanoid $PGH_2$. As we have previously shown, prostaglandins are

critical for the closure of epithelial tight junctions and resultant recovery of barrier function after ischemic injury.[7]

The non-selective NSAIDs, which include flunixin meglumine, bind to and inhibit both COX-1 and COX-2, and therefore decrease PG concentrations in all tissues.[8,9] The coxibs are a group of NSAIDs which selectively inhibit the COX-2 isoform, and include robenacoxib.[10] The relative COX-2 selectivity of the coxibs is due to differences in binding between the drug and COX-1 and COX-2. Binding of coxibs with COX-1 is a weak and rapidly reversible reaction, in contrast to binding with COX-2 which results in a close association that prevents arachidonic acid accessing the enzyme. This selective inhibition of COX-2 is generally believed to be due to the greater size of the COX-2 hydrophobic channel and differences in the amino acid structure of COX-2 which creates a side pocket for the relatively large coxib molecules.[10] A COX-2 selective NSAID would potentially be ideal for the treatment of equine colic as it would allow production of prostaglandins by an uninhibited COX-1 for normal homeostasis and repair while blocking production of the high levels of prostaglandins elaborated by COX-2 responsible for pain and endotoxemia. Robenacoxib is a recently described COX-2 selective NSAID developed for use in veterinary species.[11] The chemical structure of robenacoxib differs significantly from firocoxib, which is currently the only coxib available for use in the horse in the US.[11] Initial *in vitro* and laboratory animal studies of robenacoxib revealed a selective COX-2 inhibition profile and a reduction in adverse

gastrointestinal effects when compared with diclofenac.[11] An *ex vivo* study of the inhibition of COX-1 and COX-2 in feline blood by robenacoxib demonstrated a COX-1/COX-2 selectivity ratio of 502.3.[12] However, the COX-1/COX-2 selectivity of robenacoxib in the horse is currently unknown.

We hypothesized that robenacoxib would be COX-2 selective in the horse, and would allow the recovery of mucosal barrier function in ischemic-injured equine jejunum.

## Materials and Methods

### COX Selectivity Assay

#### Animals

All procedures were approved by the Institutional Animal Care and Use Committee of North Carolina State University. A maximum of sixty milliliters of whole blood was collected from 6 healthy female horses twice with a minimum of 21 days between collections.

#### Effect of robenacoxib on COX-1 *in vitro*

The effect of robenacoxib on COX-1 enzyme activity was determined as previously described.[8,9] Briefly, anti-coagulant-free vacuum tubes were prepared by adding 10µl of vehicle control (DMSO) or robenacoxib. Blood (2.5ml) was collected in each tube

resulting in final robenacoxib concentrations of 0.01μM - 1000μM. A total of twenty four 2.5 ml tubes of blood were collected for each experiment. The blood was allowed to clot for 1 hour at 37°C before being centrifuged at 8000rpm for 10 minutes. Following centrifugation, 100μl of serum was added to 400μl of methanol and centrifuged at 8000rpm for 10 minutes. A 50μl aliquot of the supernatant was collected and diluted in 150μl of thromboxane $B_2$ ($TXB_2$) enzyme immunoassay (EIA) kit[c] buffer resulting in a final 20-fold dilution. The amount of $TXB_2$ in each sample was determined using a commercially available $TXB_2$ EIA kit.[a]

**Effect of robenacoxib on COX-2 *in vitro***

The effect of robenacoxib on COX-2 activity was determined as previously described[8,9]. Briefly, blood (12ml) was collected into heparinized vacuum tubes and incubated with 100μg/ml of LPS in 0.1% bovine serum albumin in phosphate buffered saline (PBS) for 5 minutes. A sample of blood which was not treated with LPS was prepared as a negative control. After 5 minutes stimulation with LPS, 500μl aliquots of blood were treated with vehicle control (DMSO) or robenacoxib at a final concentration of 0.01 - 1000μM and incubated at 37°C for 24 hours. Following incubation, the blood was centrifuged at 8000rpm for 10 minutes to separate the plasma. Following centrifugation, 100μl of serum was added to 400μl of methanol and centrifuged at 8000rpm for 10 minutes. A 50μl aliquot of the supernatant was collected and diluted in 150μl of prostaglandin $E_2$ ($PGE_2$)

enzyme immunoassay (EIA) kit[d] buffer resulting in a final 20-fold dilution. The amount of $PGE_2$ in each sample was determined using a commercially available $PGE_2$ EIA kit.[d]

**Analysis of cyclooxygenase inhibition data**

The results of each cyclooxygenase assay were expressed as a percentage of the negative control sample to determine the percentage inhibition. Using the percentage inhibition of each concentration, a four-parameter logistic curve model was created using commercially available software.[e] The concentrations of robenacoxib resulting in half the maximal inhibition ($IC_{50}$) of COX-1 and COX-2, and the ratio of the COX-1 $IC_{50}$ and COX-2 $IC_{50}$ was calculated to determine the selectivity of robenacoxib in the horse.

**Effect of Robenacoxib on Recovery of Ischemic Injured Jejunum**

**Animals**

Six healthy horses, aged 5 – 20 years and weighing 400 – 600 kg were included in the study. All horses underwent a minimum of 2 weeks quarantine, which included vaccination, de-worming and observation at the Equine Health Center of North Carolina State University prior to entering this study. The horses were moved to a Laboratory Animal Resources facility adjacent to a centralized research surgery suite a minimum of 72 hours prior to surgery where they were housed in individual stalls and fed ad libitum grass hay until 8 hours prior to surgery when hay but not water was withdrawn.

**Anesthesia and surgery**

An intravenous catheter was placed in the left jugular vein and each horse was premedicated with xylazine (1.1 mg/kg IV) before induction of anesthesia using diazepam (0.01 mg/kg IV) and ketamine (2.2 mg/kg IV). The horses were orotracheally intubated and anesthesia was maintained with isoflurane vaporized in 100% oxygen. A midline celiotomy was performed and the jejunum located. Two 30cm sections of jejunum per horse were selected 60cm from the ileum and 60cm apart. One section was randomly designated the control section and the other was designated the ischemic section. The jejunal blood supply to the ischemic segment was occluded by placing vascular clamps on the mesenteric vasculature and cross-clamping the segment of jejunum with Doyen forceps to ensure that no blood flow was received from adjacent non-experimental bowel. After a period of 2 hours the control and ischemic-injured jejunum was harvested. The horses were then euthanatized with an overdose of sodium pentobarbital (100mg/kg IV).

**Ussing chamber studies**

The control and ischemic injured jejunum was incised along the anti-mesenteric border and placed in oxygenated (95% $O_2$/ 5% $CO_2$) equine Ringer's solution formulated to match the electrolyte composition and pH of equine plasma.[13] The mucosa was stripped from the seromuscular layer and mounted in Ussing chambers (3.14 $cm^2$ aperture). The mucosal and serosal sides of the tissue were bathed in equine Ringer's solution

containing mannitol (10mmol/L) or glucose (10 mmol/L) respectively, which was oxygenated and warmed to 37°C by a waterbath. The tissue bathing solutions additionally contained either no treatment, flunixin meglumine[a] ($2.7\times10^{-5}$M) or robenacoxib[b] ($2.7\times10^{-5}$M) and allowed to equilibrate for a period of 15 minutes. The spontaneous potential difference (PD) was measured using agar bridges connected to calomel electrodes, and the PD was short circuited through Ag-AgCl electrodes by use of a voltage clamp that corrected for fluid resistance. If the spontaneous PD was between -1.0 and 1.0 mV the tissue was current clamped at ±100μA for 5 seconds and the PD was recorded. The short-circuit current (Isc) and PD were measured every 5 minutes for 240 minutes. Ohm's law was used to calculate the transepithelial electrical resistance (TER) using the PD and Isc.

**Mannitol flux**

Following 45 minutes of incubation within the Ussing chambers, $^{3}$H-mannitol (10μCi/ml) was added to the mucosal side of the tissues. Samples of the mucosal and serosal bathing solutions were collected 0, 60, 120 and 180 minutes following the addition of $^{3}$H-mannitol and assessed for β emission (counts/min) in a scintillation counter. The mucosal-to-serosal flux of mannitol was calculated for each 60 minute period as an indicator of mucosal permeability.

**Prostanoid levels**

Samples of the serosal bathing solutions were collected after 45 and 240 minutes of tissue incubation in the Ussing chambers. The samples were snap frozen in liquid nitrogen and stored at -80ºC until analysis. The amount of immediate prostaglandin E metabolites and thromboxane $B_2$ were measured as indicators of COX-2 and COX-1 function respectively. The assays were performed using commercially available ELISA kits.[c,f]

**Immunoblot analysis**

Following 240 minutes of incubation within the Ussing chambers, the tissues were removed and mucosal scrapings harvested. Protein was extracted from the mucosal scrapings according to standard protein extraction protocols.

Briefly, each sample was homogenized in chilled radioimmunoprecipitation buffer (0.15M NaCl, 50mM sodium Tris [pH, 7.2], 0.5% deoxycholic acid, 1% Triton X-100, and 0.1% SDS) with protease inhibitors (phenylmethylsulfonyl fluoride, sodium orthovanadate and apoprotinin). The mixture was centrifuged at 10000 × *g* at 4°C for 10 minutes, and the supernatant was collected. Protein analysis of extracted aliquots was performed using the Lowry assay to determine protein concentration of each sample. Following the determination of sample protein concentration, the protein concentration of samples was standardized and protein extracts (50μg) were mixed with SDS-PAGE sample buffer and reducing agent (Biorad superscript) and boiled for 5 minutes at 100°C. Lysates were loaded on a 4 -12 % gradient pre-cast Bis-Tris polyacrylamide gel and

electrophoresis was carried out according to standard protocols. Proteins were transferred to a nitrocellulose membrane by use of an electroblotting transfer apparatus according to the manufacturers' protocol. Membranes were blocked for 2 hours at room temperature in Tris-buffered 150mM NaCl solution (TBS) and 5% dry powdered milk. Membranes were incubated for 12 hours in either a 1:200 dilution of anti-human COX-1, a 1:200 dilution of anti-human COX-2, or a 1:10,000 dilituin of beta-actin. After washing three times for 10 minutes each with TBS- 0.05% Tween solution, the membranes were incubated with horse radish peroxidase conjugated secondary antibody at a dilution of 1:2000 (COX-1, COX-2). After washing three times for 10 minutes each with TBS-0.05% Tween solution, the membranes were developed for visualization of proteins by addition of enhanced chemiluminescence agent. Densitometry was performed on digitized images using specialized software,[g] to quantify COX-1 and COX-2 expression using β-actin as a loading control.

**Histology**

Mucosal samples were obtained prior to and following incubation of tissues in Ussing chambers. All samples obtained were placed in 10% neutral buffered formalin and stored in 70% ethanol prior to embedding in paraffin blocks using standard techniques. For each tissue sample three five-micrometer cross-sections taken at 300μm intervals were stained with hematoxylin and eosin. Three investigators (AB, DS, NM) independently evaluated 3 sections for each tissue. The investigators were blinded to the source of the tissue and

data was pooled before any statistical analyses were performed. The mucosal injury was graded using the grading scheme previously described by Chiu et al.[14] Briefly, the grading scale for mucosal damage involves six grades ranging from a morphologically normal villus (Grade 0) to a villus with complete epithelial loss and necrosis of the lamina propria and crypt cells (Grade 5). The earliest lesion is the separation of the epithelial cells from the basement membrane to form a fluid filled gap termed Gruenhagen's space (Grade 1). This is followed by progressive separation of the epithelial cells from the lamina propria starting at the tip and progressing toward the base of the villus (Grades 2 – 4). Grade 5 mucosal damage is characterized by complete epithelial loss from the villus and necrosis of the lamina propria and crypt epithelium.

For each tissue section, three well oriented crypts and villi were chosen. The length of the crypt and villus, width at midpoint of the villus, and the length of the villus covered by epithelium were measured. The total surface area of the villus and the area of the villus covered by epithelium were calculated using a modified equation for the area of a cylinder as previously described.[15] The percentage of epithelial denudation was then calculated for each villus. The percentage of epithelial denudation was compared between treatment groups and injury.

**Statistical Analysis**

Transepithelial electrical resistance and $^{3}$H-mannitol flux measurements were analyzed using a 2-way repeated measures analysis of variance (RM-ANOVA). Correlation between the results of histological grading and measurement by the three independent observers was tested using the Pearson's Moment Product Correlation Test before data was pooled. Results of histological evaluation were evaluated by 2-way analysis of variance (ANOVA) of ranked data. Using the results of densitometry, COX-1 and COX-2 expression was evaluated by ANOVA for the effect of injury (control or ischemic injury) and treatment (control, flunixin meglumine, and robenacoxib). Where an effect of type of injury or treatment was identified, post hoc analysis was performed using Tukey's test to further evaluate the effect of treatment or injury. Statistical significance was set at $p<0.05$.

## Results

### Effect of robenacoxib on COX-1 and COX-2 *in vitro*

The $IC_{50}$ values of robenacoxib for COX-1 and COX-2 were 11.46 ± 4.46 μM and 0.19 ± 0.07μM respectively. The resulting COX selectivity ratio at $IC_{50}$ of robenacoxib in the horse was 61.01.

### Transepithelial electrical resistance

The transepithelial resistance (TER) of ischemic injured equine jejunum treated with flunixin meglumine was found to be significantly lower than control treatment tissue over the course of the recovery period ($p < 0.05$) (Fig 1). The TER of ischemic jejunum treated with robenacoxib was not significantly different from untreated ischemic tissue (Fig 1). There was no significant difference in TER between treatments in control (uninjured) tissue.

### Mannitol flux

There was no significant effect of ischemic injury on mucosal-to-serosal flux of $^{3}H$-mannitol (Fig 2). Overall, there was no significant difference between the three successive 60 minute flux periods. Treatment with flunixin meglumine, or robenacoxib, had no significant effect on mannitol flux (Fig 2).

**Prostanoid levels**

There was no significant difference in the serosal bathing solution prostanoid levels following 45 minutes of recovery (Figs 3 and 4). Following the 240 minute recovery period, the level of prostanoids in the ischemic tissue bathing solution were significantly higher than those of the control tissue bathing solution ($p=0.001$) (Figs 3 and 4). Treatment with robenacoxib had no significant effect on the concentration of thromboxane $B_2$ (Fig 3) or prostaglandin E metabolites (Fig 4) when compared with untreated ischemic tissue. In contrast, flunixin meglumine significantly inhibited the increase in $TXB_2$ and PGEM observed in the ischemic injury tissue group ($p<0.001$).

**Immunoblot analysis**

Cyclooxygenase-1 and -2 were both found to be constitutively expressed in the uninjured jejunum. Ischemic injury had no significant effect on expression of COX-1 but resulted in significant upregulation of COX-2 expression ($p = 0.04$) (Fig 5). There was no significant effect of treatment on expression of either COX-1 or COX-2.

**Histology**

There was a strong correlation between the results of the independent observers ($r=0.90$). The mean ± SE grade of injury of control uninjured tissue and ischemic injured tissue was 0.0 ± 0.0 and 3.44 ± 0.16. Following the 240 minute recovery period, there was no significant difference in the grade of injury of control tissue (2.93±0.29) and ischemic

injured tissue (3.76 ± 0.12). The mean ± SE percentage epithelial denudation of ischemic injured tissue was 58.39 ± 3.49% prior to recovery, and 53.21 ± 3.64% following recovery. Epithelial denudation was not observed in control uninjured tissue immediately following harvest (mean ± SE, 0±0%) but increased to 52.76 ± 4.37 % following the 240 minute Ussing chamber period. The grade of mucosal injury and the percentage of epithelial denudation was significantly increased by ischemic injury ($p<0.001$ and $p=0.026$ respectively). In the ischemic injured tissue, there was no significant difference in the grade of mucosal injury and percentage epithelial denudation prior to and following the 240 minute recovery period. Treatment with flunixin meglumine, or robenacoxib had no significant effect on the grade of mucosal injury or the percentage of epithelial denudation.

**Discussion**

This study examined the effects of the novel COX inhibitor robenacoxib, on the COX enzymes in both a whole blood and a gastrointestinal model. The results of the *in vitro* COX inhibition assay revealed that robenacoxib is COX-2 selective in the horse with a COX selectivity ratio of 61.01. While this selectivity ratio is less than that reported for robenacoxib in the cat, it is substantially greater than the selectivity ratios reported for flunixin meglumine (0.3), phenylbutazone (0.3 and 1.6) or meloxicam (3.8) in the horse.[8,9]

Ischemic injury resulted in a significant decrease in the TER of equine jejunal mucosa. In this study, treatment of ischemic-injured equine jejunum with robenacoxib did not inhibit the recovery of mucosal barrier function as determined by TER. In contrast, flunixin meglumine significantly inhibited the recovery of TER. This inhibitory effect of flunixin meglumine on TER recovery has been previously described and has been attributed to the inhibition of prostaglandin-mediated closure of the paracellular spaces between repairing cells.[4] While TER measures the movement of ions across the mucosa, the mannitol flux experiment examines the movement of a larger molecule across the paracellular space. The results of this experiment revealed no effect of ischemic injury on mannitol flux and are similar to those of a previous study.[16] However, this previous study showed that treatment with flunixin meglumine caused an increase in permeability to mannitol.[16] In contrast, the current study found no significant effect of treatment with flunixin meglumine or robenacoxib on mannitol permeability. Therefore, only the TER measurements indicated an increased mucosal permeability in response to ischemic injury, and identified differences between the treatment groups. This may be due to the fact that TER is a more sensitive indicator of mucosal permeability and accurately reflects the transmucosal passage of ions.[7]

Previous studies examining the effect of flunixin meglumine on the equine jejunum have reported using a concentration of $2.7\times10^{-5}$M.[16] The maximum plasma concentration of flunixin meglumine achieved after intravenous administration of a clinically relevant

dose (1.1mg/kg) is 9.3±0.76mg/l.[17] This is the equivalent of $1.73\times10^{-5}$ – $2.05\times10^{-5}$M, which is less than the dose of $2.7\times10^{-5}$M used in this study. However, as this experiment was performed using equimolar doses of flunixin meglumine and robenacoxib, it is possible to directly compare the effects of these medications. The effect of each treatment on COX-1 and COX-2 was assessed by performing ELISAs to detect thromboxane $B_2$ ($TXB_2$) and stable prostaglandin $E_2$ metabolites (PGEM) respectively as previously described.[16] These assays revealed that ischemic injury resulted in a significant increase in the production of $TXB_2$ and PGEM during the 4 hour recovery period. Although treatment with robenacoxib decreased the production of PGEM during the recovery period, this difference was not significant. This low level of inhibition was unexpected as our study demonstrated an $EC_{50}$ for COX-2 in the horse of 0.19μM which is significantly lower than the $2.7\times10^{-5}$M used in this study.[12] Based upon our COX-2 inhibition data we expected a concentration of $2.7\times10^{-5}$M to inhibit COX-2 by 97%. This difference may therefore be related to the different models used in this experiment. Treatment with flunixin meglumine resulted in significantly lower production of $TXB_2$ and PGEM demonstrating the ability of this drug to significantly inhibit the action of both COX-1 and COX-2. This non-selective inhibition of both COX isoforms by flunixin meglumine has been demonstrated in previous studies.[16]

Immunoblot analysis of COX-1 protein expression revealed that while the enzyme is constitutively expressed in the equine jejunum, it is not upregulated by ischemic injury. This finding is in contrast to a previous study which suggested that COX-1 expression is

upregulated by ischemic injury in the horse.[4] However, this previous study examined COX expression following an 18 hour *in vivo* recovery period which is not directly comparable to this study. In addition, the findings of this current study are consistent with other models of ischemic injury in which the expression of COX-1 is unaffected by injury.[18,19] The results of immunoblot analysis of COX-2 protein expression revealed constitutive expression in the equine jejunum which is upregulated in response to ischemic injury over the course of a four hour recovery period. The upregulation of COX-2 in ischemic injured equine jejunum has been previously described and is important in the production of prostaglandins necessary for repair of injured tissues.[4]

This study examined a novel analgesic and anti-inflammatory compound to assess its potential suitability for the treatment of equine gastrointestinal disease. Previous studies have shown that non-selective NSAIDs are associated with side-effects including colitis and renal failure.[20,21] Furthermore, non-selective NSAIDs have been shown to inhibit the recovery of mucosal barrier function and increase the transmucosal passage of lipopolysaccharide following ischemic injury.[22] This study has shown that robenacoxib is a more COX-2 selective that those NSAIDs currently in use in the horse.[8,9]. Furthermore, it did not inhibit the recovery of mucosal barrier function in ischemic injured equine jejunum. Robenacoxib may be clinically useful in the colic patient as a treatment for pain and inflammation, while allowing the recovery of mucosal barrier function. Therefore,

robenacoxib warrants further study to determine its analgesic and anti-inflammatory properties in the horse.

**Footnotes:**

a Flunixin meglumine USP, Professional Compounding Centers of America, Houston, TX

b Robenacoxib, Novartis Animal Health, Switzerland

c Thromboxane B2 EIA kit, Cayman Chemical, Ann Arbor MI

d Prostaglandin $E_2$ EIA kit (Monoclonal), Cayman Chemical, Ann Arbor MI

e SigmaPlot 10, Systat Software, San Jose CA

f Prostaglandin E Metabolite EIA kit, Cayman Chemical, Ann Arbor MI

g SigmaScan, Systat Software, San Jose CA

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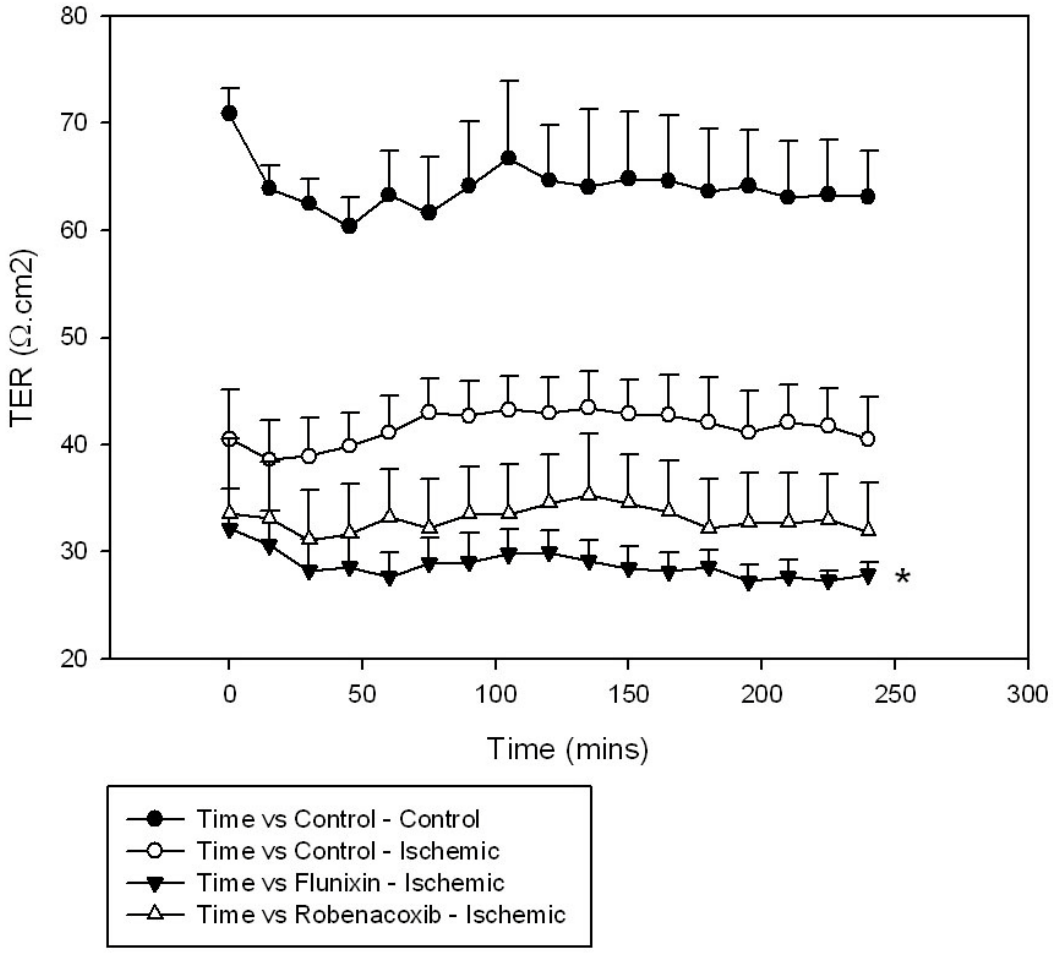


Figure 1: TER of control and ischemic jejunal mucosa was measured every 15 mins during the 240 minute recovery period on the Ussing chamber. Flunixin treated tissue had significantly lower ($p<0.05$) TER values than untreated (control) ischemic tissue (*). ●=Control-control, ○=control-ischemic, ▼=flunixin-ischemic, Δ=Robenacoxib-ischemic

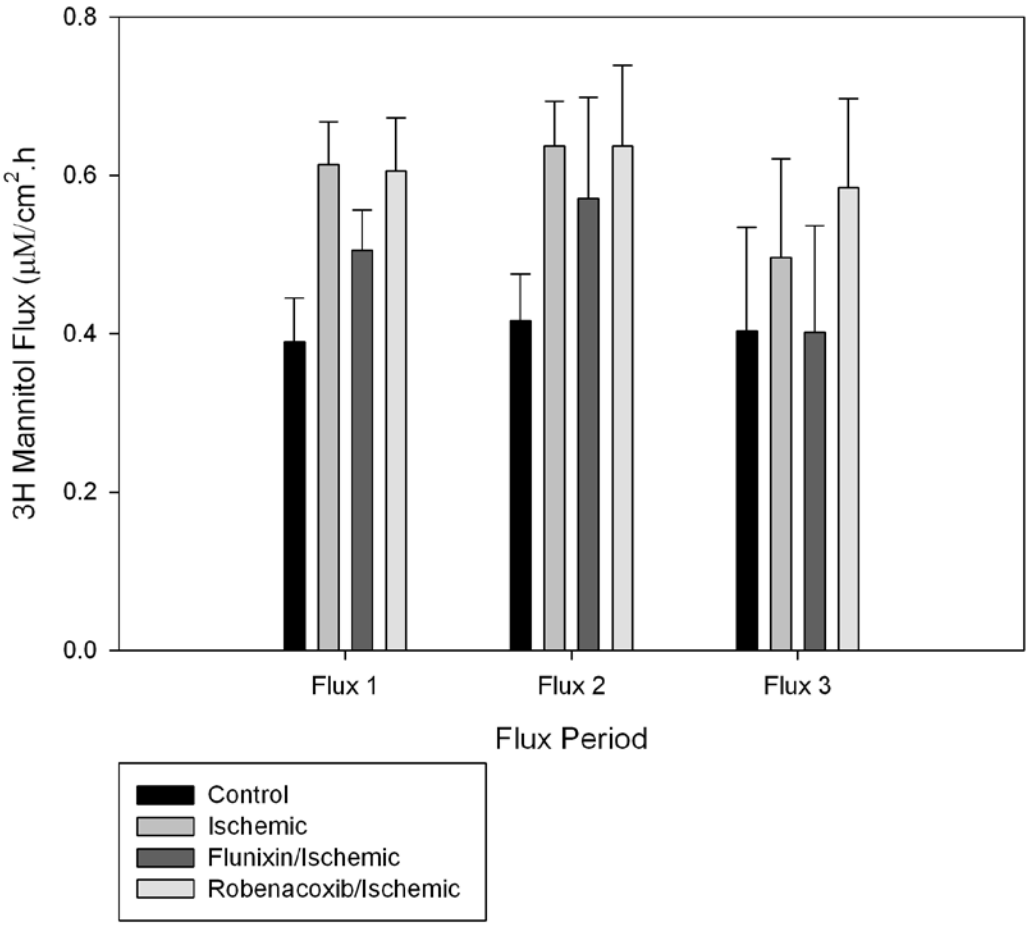


Figure 2: Permeability of control (uninjured) and ischemic-injured equine jejunum was assessed by mucosal-to-serosal flux of $^{3}$H-mannitol over three flux periods. Flux periods were defined as flux 1 (0-60mins), flux 2 (60-180mins) and flux 3 (180-240mins). There was no significant effect of ischemic injury or treatment on mannitol flux.

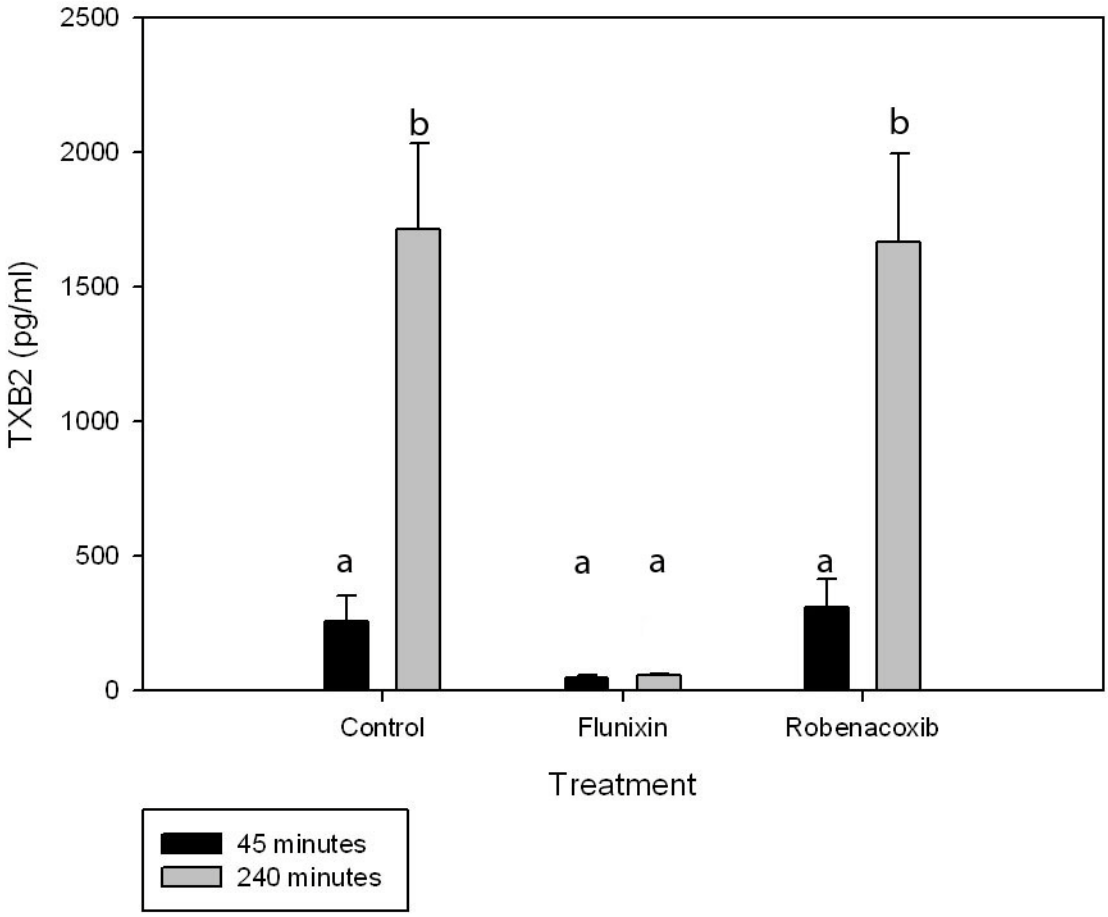


Figure 3: Thromboxane B2 ($TXB_2$) levels were measured in ischemic tissue bathing solution after 45 and 240 minutes of recovery. There was no significant difference in $TXB_2$ after 45mins of recovery in control, flunixin, and robenacoxib treated tissues (a). TXB2was significantly increased after 240mins of recovery in control and robenacoxib treated tissue (b, $p<0.05$).
There was no increase in TXB2 in flunixin meglumine treated tissues at 240mins (a).

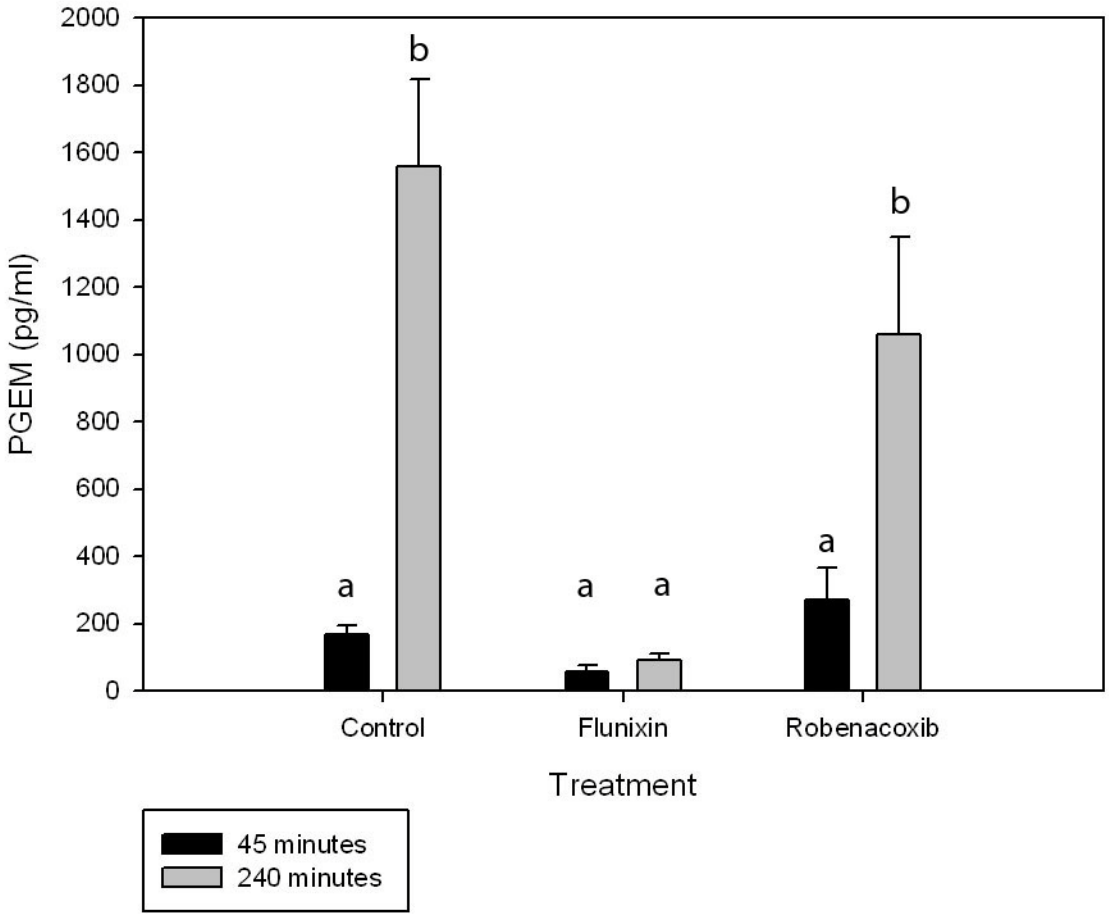


Figure 4: Prostaglandin E Metabolite (PGEM) levels were measured in ischemic tissue bathing solution after 45 and 240 minutes of recovery. There was no significant difference in PGEM after 45mins of recovery in control, flunixin, and robenacoxib treated tissues (a). PGEM was significantly increased after 240mins of recovery in control and robenacoxib treated tissue (b, $p<0.05$).

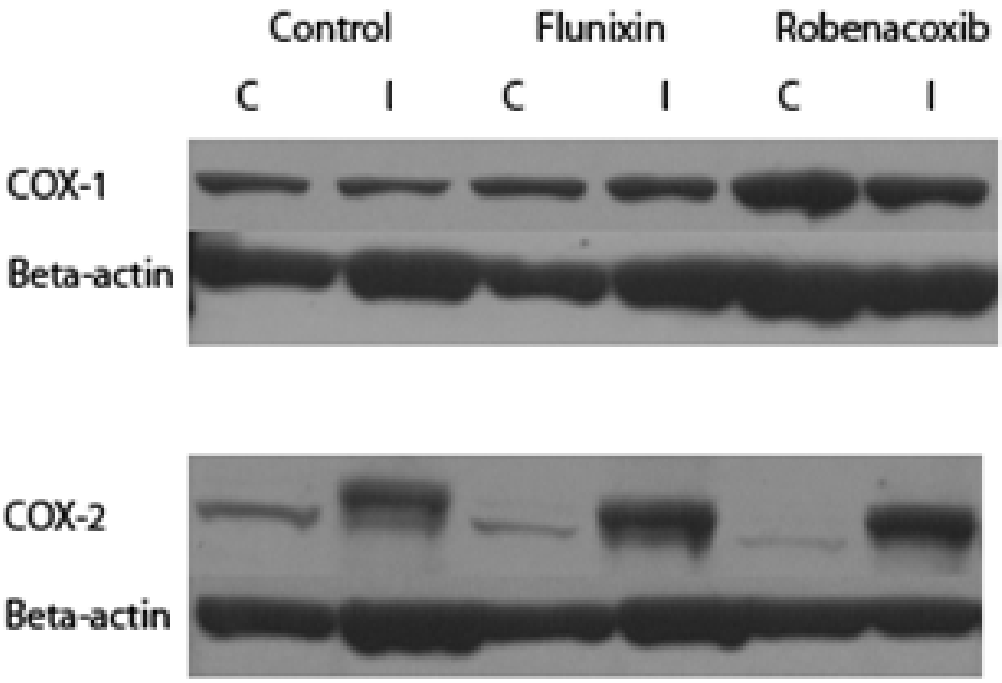


Figure 5: Immunoblot analysis of COX-1 and COX-2 expression in control (C) and ischemic-injured equine jejunum (I). Ischemic injury significantly increased the expression of COX-2 only ($p<0.05$).

# CHAPTER 5

## The effects of the novel anti-inflammatory compound AHI-805 on cyclooxygenase enzymes and the recovery of ischemic-injured equine jejunum *ex vivo*

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**Abstract**

**Objective –** To determine the effect of AHI-805, an aza-thia-benzoazulene derivative, on the cyclooxygenase enzymes, and the recovery of mucosal barrier function following ischemic injury.

**Animals –** 12 horses.

**Procedures –** The effect of AHI-805 on COX-1 and COX-2 activity was determined by measuring coagulation-induced thromboxane $B_2$ ($TXB_2$) and lipopolysaccharide-stimulated prostaglandin $E_2$ concentrations respectively in equine whole blood. Horses (n=6) were anesthetized and jejunum was subjected to ischemia for 2 hours. Control and ischemic-injured mucosa was placed in Ussing chambers and treated with Ringer's solution containing control treatment (DMSO), or flunixin meglumine ($2.7\times10^{-5}$M), or AHI-805 ($2.7\times10^{-5}$M). Trans-epithelial electrical resistance (TER) and mucosal-to-serosal flux of $^{3}$H-mannitol were measured over a 4-hour recovery period to assess mucosal permeability. Bathing solution thromboxane $B_2$ ($TXB_2$) and prostaglandin E metabolites (PGEM) were measured by EIA to assess COX-1 and COX-2 function respectively. Histological grade of injury and percentage of epithelial denudation were determined. Results were analyzed for effect of injury and treatment. Statistical significance was set at $p<0.05$.

**Results -** The baseline and coaglulation induced $TXB_2$ in equine whole blood were 68.8±33.3pg/ml and 1232.1±232.3pg/ml respectively. The baseline and LPS-stimulated $PGE_2$ in equine whole blood were 241.1±70.9pg/ml and 647.4±330.0pg/ml respectively. Treatment with AHI-805 at a concentration of 1µM or greater significantly inhibited the production of LPS-induced $PGE_2$. TER of ischemic-injured jejunum treated with flunixin or AHI-805 was significantly lower than control treated injured tissue over the course of the recovery period. Ischemic injury significantly increased grade of histological damage, and percentage epithelial denudation. However, there was no significant effect of treatment. There was a significant increase in PGEM and $TXB_2$ in control tissues over the 240 minute recovery period, but not in flunixin or AHI-805-treated tissues.

**Conclusions and Clinical Relevance -** This study indicates that flunixin meglumine and AHI-80 5 inhibit recovery of barrier function in ischemic-injured equine jejunum *in vitro* through inhibition of the COX enzymes.

## Introduction

Pain and inflammation in the horse is most frequently treated by the administration of non-steroidal anti-inflammatory drugs (NSAIDs). The NSAIDs act by inhibiting the action of the cyclooxygenase enzymes and thereby reducing the production of prostaglandins.[12] There are two major cyclooxygenase isoforms designated cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). Although these enzymes differ in their pattern of expression and protein structure, they both produce prostaglandin $H_2$ ($PGH_2$). [2]COX-1 is expressed in the majority of tissues under normal physiological conditions.[2] In contrast, the expression of COX-2 is generally low but is up-regulated in response to inflammation.[2] Although the NSAIDs are effective in reducing pain and inflammation, they are also associated with significant side-effects, particularly of the gastrointestinal tract and kidneys. The administration of phenylbutazone has been associated with right dorsal colitis and renal medullary crest necrosis.[3-5] Furthermore, flunixin meglumine, an NSAID commonly administered for the treatment of pain, inflammation, and endotoxemia associated with colic in the horse has been shown to retard the recovery of mucosal barrier function following jejuna ischemic injury.[6] By inhibiting the recovery of barrier function, flunixin meglumine increases the permeability of the mucosa to lipopolysaccharide (LPS).[7] If LPS if allowed access to the systemic circulation, this results in endotoxemia which is associated with significant morbidity and mortality in the horse.[8]

The association of the NSAIDs with significant side effects has led to the investigation of other medications for the treatment of post-operative pain in the colic patient. These include COX-2 selective NSAIDs, opiates, and local anesthetics. The COX-2 selective NSAIDs including firocoxib and deracoxib have been demonstrated to allow uninhibited recovery of ischemic injured jejunum.[9, 10] Of these medications only firocoxib is currently available for use in the use. However, it is available only as an orally administered preparation which may not be suitable for use in the post-operative colic patient. Butorphanol has been shown to reduce post-operative pain in colic patients when administered in combination with flunixin meglumine.[11] In addition to the potential adverse effects of flunixin meglumine, the administration of butorphanol significantly delayed the passage of feces after surgery, a significant concern in the post-operative colic patient.[11] The systemic use of the local anesthetic lidocaine has been investigated but studies have shown that it is not an effective analgesic when administered alone.[12] Therefore, the alternatives to NSAIDs for the treatment of pain and inflammation in the horse are currently limited. The aza-thio-benzoazulene derivatives are a recently described class of medications possessing analgesic and anti-inflammatory properties.[13] Although their mechanism of action is currently unclear, they have not been previously shown to inhibit the COX enzymes. Therefore, the aza-thio-benzoazulene derivative AHI-805 could potentially provide analgesia and anti-inflammatory effects without inhibiting mucosal repair following injury. The aim of this study is to determine if AHI-

805 inhibits the cyclooxygenase enzymes, and whether it inhibits the recovery of ischemic injured equine jejunum following ischemic injury.

## Materials and Methods

### Effect of AHI-805 on the Cyclooxygenase Enzymes

#### Animals

All procedures were approved by the Institutional Animal Care and Use Committee of North Carolina State University. A maximum of sixty milliliters of whole blood was collected from 6 healthy female horses twice with a minimum of 21 days between collections.

### Effect of AHI-805 on COX-1 *in vitro*

The effect of AHI-805 on COX-1 enzyme activity was determined as previously described.[1, 14] Briefly, anti-coagulant-free vacuum tubes were prepared by adding 10µl of vehicle control (DMSO) or AHI-805. Blood (2.5ml) was collected in each tube resulting in final AHI-805 concentrations of 0.01µM - 1000µM. A total of twenty four 2.5 ml tubes of blood were collected for each experiment. The blood was allowed to clot for 1 hour at 37°C before being centrifuged at 8000rpm for 10 minutes. Following centrifugation, 100µl of serum was added to 400µl of methanol and centrifuged at 8000rpm for 10 minutes. A 50µl aliquot of the supernatant was collected and diluted in 150µl of thromboxane $B_2$ ($TXB_2$) enzyme immunoassay (EIA) kit[c] buffer resulting in a final 20-fold dilution. The amount of $TXB_2$ in each sample was determined using a commercially available $TXB_2$ EIA kit.[a]

**Effect of AHI-805 on COX-2 *in vitro***

The effect of AHI-805 on COX-2 activity was determined as previously described.[1, 14] Briefly, blood (12ml) was collected into heparinized vacuum tubes and incubated with 100µg/ml of LPS in 0.1% bovine serum albumin in phosphate buffered saline (PBS) for 5 minutes. A sample of blood which was not treated with LPS was prepared as a negative control. After 5 minutes stimulation with LPS, 500µl aliquots of blood were treated with vehicle control (DMSO) or AHI-805 at a final concentration of 0.01 - 1000µM and incubated at 37°C for 24 hours. Following incubation, the blood was centrifuged at 8000rpm for 10 minutes to separate the plasma. Following centrifugation, 100µl of serum was added to 400µl of methanol and centrifuged at 8000rpm for 10 minutes. A 50µl aliquot of the supernatant was collected and diluted in 150µl of prostaglandin $E_2$ ($PGE_2$) enzyme immunoassay (EIA) kit[d] buffer resulting in a final 20-fold dilution. The amount of $PGE_2$ in each sample was determined using a commercially available $PGE_2$ EIA kit.[d]

**Analysis of cyclooxygenase inhibition data**

**Effect of AHI-805 on COX-1 and COX-2**

The results of each cyclooxygenase assay were expressed as a percentage of the negative control sample to determine the percentage inhibition. To determine the effect of AHI-805 on COX-1, a one-way ANOVA of the $TXB_2$ data was performed to compare the control samples with AHI-805 treatment at each concentration. To determine the effect of

AHI-805 on COX-2, a one-way ANOVA of the $PGE_2$ data was performed to compare the control samples with AHI-805 treatment at each concentration. Post hoc analysis was performed using a Holm-Sidak test.

**Logistic regression modeling of cyclooxygenase inhibition data**

Using the percentage inhibition of each concentration, a four-parameter logistic curve model was created using commercially available software.[e] The concentrations of AHI-805 resulting in 50, 80 and 95% inhibition ($IC_{50}$, $IC_{80}$, and $IC_{95}$) of COX-2 were determined. These values could not be determined for COX-1.

**Effect of AHI-805 on recovery of ischemic injured equine jejunum**

**Animals**

All procedures were approved by the Institutional Animal Care and Use Committee of North Carolina State University. Six healthy horses, aged 5 – 20 years and weighing 400 – 600 kg were included in the study. All horses underwent a minimum of 2 weeks quarantine, which included vaccination, deworming and observation at the Equine Health Center of North Carolina State University prior to entering this study. The horses were moved to a Laboratory Animal Resources facility adjacent to a centralized research surgery suite a minimum of 72 hours prior to surgery where they were housed in

individual stalls and fed ad libitum grass hay until 8 hours prior to surgery when hay but not water was withdrawn.

**Anesthesia and surgery**

An intravenous catheter was placed in the left jugular vein and each horse was premedicated with xylazine (1.1 mg/kg IV) before induction of anesthesia using diazepam (0.01 mg/kg IV) and ketamine (2.2 mg/kg IV). The horses were orotracheally intubated and anesthesia was maintained with isoflurane vaporized in 100% oxygen. A midline celiotomy was performed and the jejunum located. Two 30cm sections of jejunum per horse were selected 60cm from the ileum and 60cm apart. One section was randomly designated the control section and the other was designated the ischemic section. The jejunal blood supply to the ischemic segment was occluded by placing vascular clamps on the mesenteric vasculature and cross-clamping the segment of jejunum with Doyen forceps to ensure that no blood flow was received from adjacent non-experimental bowel. After a period of two hours the control and ischemic injured jejunum was harvested. The horses were then euthanatized with an overdose of sodium pentobarbital (100mg/kg IV).

**Ussing chamber studies**

The control and ischemic injured jejunum was incised along the anti-mesenteric border and placed in oxygenated (95% $O_2$/ 5% $CO_2$) equine Ringer's solution formulated to match the electrolyte composition and pH of equine plasma. The mucosa was stripped from the seromuscular layer and mounted in Ussing chambers (3.14 $cm^2$ aperture). The mucosal and serosal sides of the tissue were bathed in equine Ringer's solution containing mannitol (10mmol/L) or glucose (10 mmol/L) respectively, which was oxygenated and warmed to 37ºC by a waterbath. The tissue bathing solutions additionally contained either vehicle control treatment (DMSO), flunixin meglumine ($2.7\times10^{-5}$M), or AHI-805 ($2.7\times10^{-5}$M) and allowed to equilibrate for a period of 15 minutes. The spontaneous potential difference (PD) was measured using agar bridges connected to calomel electrodes, and the PD was short circuited through Ag-AgCl electrodes by use of a voltage clamp that corrected for fluid resistance. If the spontaneous PD was between -1.0 and 1.0 mV the tissue was current clamped at ±100 μA for 5 seconds and the PD was recorded. The short-circuit current (Isc) and PD were measured every 5 minutes for 240 minutes. Ohm's law was used to calculate the transepithelial resistance (TER) using the PD and Isc.

**Mannitol flux**

Following 45 minutes of incubation within the Ussing chambers, $^{3}$H-mannitol (10μCi/ml) was added to the mucosal side of the tissues. Samples of the mucosal and serosal bathing solutions were collected 0, 60, 120 and 180 minutes following the addition of $^{3}$H-mannitol and assessed for β emission (counts/min) in a scintillation counter. The mucosal-to-serosal flux of mannitol was calculated for each 60 minute period as an indicator of mucosal permeability.

**Prostanoid levels**

Samples of the serosal bathing solutions were collected after 45 and 240 minutes of tissue incubation in the Ussing chambers. The samples were snap frozen in liquid nitrogen and stored at -80ºC until analysis. The amount of immediate thromboxane $B_2$ and prostaglandin E metabolites were measured as indicators of COX-1 and COX-2 function respectively. The assays were performed using commercially available ELISA kits.[a,d]

**Immunoblot analysis**

Following 240 minutes of incubation within the Ussing chambers, the tissues were removed and mucosal scrapings harvested. Protein was extracted from the mucosal

scrapings according to standard protein extraction protocols. Briefly, each sample was homogenized in chilled radioimmunoprecipitation buffer (0.15M NaCl, 50mM sodium Tris [pH, 7.2], 0.5% deoxycholic acid, 1% Triton X-100, and 0.1% SDS) with protease inhibitors (phenylmethylsulfonyl fluoride, sodium orthovanadate and apoprotinin). The mixture was centrifuged at 10000 × *g* at 4°C for 10 minutes, and the supernatant was collected. Protein analysis of extracted aliquots was performed using the Lowry assay to determine protein concentration of each sample. Following the determination of sample protein concentration, the protein concentration of samples was standardized and protein extracts (50μg) were mixed with SDS-PAGE sample buffer and reducing agent (Biorad superscript) and boiled for 5 minutes at 100°C. Lysates were loaded on a 4 -12 % gradient pre-cast Bis-Tris polyacrylamide gel and electrophoresis was carried out according to standard protocols. Proteins were transferred to a nitrocellulose membrane by use of an electroblotting transfer apparatus according to the manufacturers' protocol. Membranes were blocked for 2 hours at room temperature in Tris-buffered 150mM NaCl solution (TBS) and 5% dry powdered milk. Membranes were incubated for 12 hours in either a 1:200 dilution of anti-human COX-1, a 1:200 dilution of anti-human COX-2, or a 1:10,000 dilution of beta-actin. After washing three times for 10 minutes each with TBS-0.05% Tween solution, the membranes were incubated with horse radish peroxidase conjugated secondary antibody at a dilution of 1:2000 (COX-1, COX-2). After washing three times for 10 minutes each with TBS-0.05% Tween solution, the membranes were developed for visualization of proteins by addition of enhanced chemiluminescence

agent. Densitometry was performed on digitized images using specialized software, to quantify COX-1 and COX-2 expression using β-actin as a loading control.[e]

**Histology**

Mucosal samples were obtained prior to and following incubation of tissues in Ussing chambers. All samples obtained were placed in 10% neutral buffered formalin and stored in 70% ethanol prior to embedding in paraffin blocks using standard techniques. For each tissue sample three five-micrometer cross-sections taken at 300μm intervals were stained with hematoxylin and eosin. Three investigators (AB, DS, NM) independently evaluated 3 sections for each tissue. The investigators were blinded to the source of the tissue and data was pooled before any data analysis was performed. The mucosal injury was graded using the grading scheme previously described by Chiu et al.[15] Briefly, the grading scale for mucosal damage involves six grades ranging from a morphologically normal villus (Grade 0) to a villus with complete epithelial loss and necrosis of the lamina propria and crypt cells (Grade 5). The earliest lesion is the separation of the epithelial cells from the basement membrane to form a fluid filled Gruenhagen's space (Grade 1). This is followed by progressive separation of the epithelial cells from the lamina propria starting at the tip and progressing to the base of the villus (Grades 2 – 4). Grade 5 mucosal damage is characterized by complete epithelial loss from the villus tip and necrosis of the lamina propria and crypt cell.

For each tissue section, three well oriented crypts and villi were chosen and the length of the crypt and villus, width at the middle of the villus, and length of the epithelium covered portion of the villus were measured. The total surface area of the villus and the area of the villus covered by epithelium were calculated using a modified equation for the area of a cylinder as previously described.[16] The percentage of epithelial denudation was then calculated for each villus. The percentage of epithelial denudation was compared between treatment groups and injury.

**Statistical Analysis**

Transepithelial electrical resistance and $^{3}$H-mannitol flux measurements were analyzed using a 2-way repeated measures analysis of variance (RM-ANOVA). Correlation between the results of histological grading and measurement by the three independent observers was tested using the Pearson's Moment Product Correlation Test before data was pooled. Results of histological evaluation were evaluated by 2-way analysis of variance (ANOVA) of ranked data. Using the results of densitometry, COX-1 and COX-2 expression was evaluated by ANOVA for the effect of injury (control or ischemic injury) and treatment (control, flunixin meglumine, and AHI-805). Where an effect of type of injury or treatment was identified, post hoc analysis was performed using Tukey's test to further evaluate the effect of treatment or injury. Statistical significance was set at $p<0.05$.

## Results

### Effect of AHI-805 on the Cyclooxygenase Enzymes

### Effect of AHI-805 on $TXB_2$

The baseline $TXB_2$ concentration in equine blood was 68.8±33.3pg/ml which increased to 1232.1±232.3pg/ml following coagulation. Treatment with AHI-805 did not significantly inhibit the production of $TXB_2$ by COX-1 (Fig 1).

### Effect of AHI-805 on PGE2

The $PGE_2$ concentration in un-stimulated equine plasma was 241.1±70.9pg/ml. LPS-stimulated plasma contained 647.4±330.0pg/ml of $PGE_2$. Although there was no effect at lower concentrations, treatment with AHI-805 at a concentration of 1, 10, or 100µM resulted significant inhibition of COX-2 and a significant decrease in the amount of $PGE_2$ in equine plasma ($p<0.05$) (Fig 2). Regression modeling of the $PGE_2$ data using a four-parameter logistic curve revealed the $IC_{50}$, $IC_{80}$, and $IC_{95}$ of AHI-805 to be 2.35, 11.72, and 29.9µM respectively (Fig 3).

## Effect of AHI-805 on recovery of ischemic injured equine jejunum

### Transepithelial electrical resistance

The transepithelial resistance (TER) of ischemic injured equine jejunum treated with flunixin meglumine or AHI-805 was found to be significantly lower than control treatment tissue over the course of the recovery period ($p < 0.05$) (Fig 4). There was no significant difference in TER between treatments in control (uninjured) tissue.

### Mannitol flux

Ischemic injury significantly increased the mucosal-to-serosal flux of $^{3}$H-mannitol during flux period 1 (60-120mins), and flux period 2 (120-180mins) (Fig 5). During the third flux period (180-240mins), there was no significant effect of ischemic injury. Overall, there was no significant difference between the three successive 60 minute flux periods. Treatment with flunixin meglumine or AHI-805 had no significant effect on mannitol flux during any of the flux periods (Fig 5).

### Prostanoid levels

There was no significant difference in the serosal bathing solution prostanoid levels following 45 minutes of recovery (Figs 6 and 7). Following the 240 minute recovery period, the level of prostanoids in the ischemic tissue bathing solution were significantly

higher than those of the control tissue bathing solution ($p<0.05$). Flunixin meglumine and AHI-805 both significantly inhibited the increase in $TXB_2$ and PGEM observed in the ischemic injury tissue group over the 240 minute recovery period ($p<0.001$). The increase in $TXB_2$ and PGEM was significantly greater in AHI-805 treated tissues than flunixin meglumine treated tissues ($p<0.05$).

**Immunoblot analysis**

Cyclooxygenase-1 and -2 were both found to be constitutively expressed in the uninjured jejunum. Ischemic injury had no significant effect on expression of COX-1 but resulted in significant upregulation of COX-2 expression ($p<0.05$) (Fig 8). There was no significant effect of treatment on expression of either COX-1 or COX-2.

**Histology**

There was a strong correlation between the results of the independent observers ($r=0.896$). The mean ± SE grade of injury of control uninjured tissue and ischemic injured tissue was 0.0 ± 0.0 and 3.44 ± 0.16. Following the 240 minute recovery period, the mean ± SE grade of injury of ischemic injured tissue was 3.66 ± 0.12. The mean ± SE percentage epithelial denudation of ischemic injured tissue was 58.39 ± 3.49% prior to recovery, and 57.14 ± 7.51% following recovery. Epithelial denudation was not observed

in control uninjured tissue immediately following harvest (mean ± SE, 0±0%) but increased to 39.57 ± 4.99 % following the 240 minute Ussing chamber period. The grade of mucosal injury and the percentage of epithelial denudation was significantly increased by ischemic injury ($p<0.001$ and $p<0.05$ respectively). In the ischemic injured tissue, there was no significant difference in the grade of mucosal injury and percentage epithelial denudation prior to and following the 240 minute recovery period. Treatment with flunixin meglumine or AHI-805 had no significant effect on the grade of mucosal injury or the percentage of epithelial denudation.

**Discussion**

Although flunixin meglumine is a commonly administered analgesic for the treatment of equine colic, research continues in an attempt to identify a suitable alternative. The aim of this research is to identify a medication possessing analgesic and anti-inflammatory properties, while avoiding the detrimental effects of cyclooxygenase inhibition on the gastrointestinal and renal systems. This study examined the effects of the novel analgesic and anti-inflammatory compound AHI-805 on the cyclooxygenase enzymes. Coagulation induced thromboxane $B_2$ ($TXB_2$) has previously been used to measure COX-1 activity as the production of thromboxane $A_2$ ($TXA_2$) is linked to COX-1 in platelets.[17] In this study, we found no significant effect of AHI-805 on the production of $TXB_2$ and therefore no effect on COX-1. Based on the available information regarding AHI-805, this finding

was not unexpected.[13] However, when LPS-induced prostaglandin $E_2$ ($PGE_2$) was measured as an indicator of COX-2 activity, AHI-805 at a concentration of 1μM or greater was found to significantly reduce $PGE_2$. The $IC_{50}$ value of AHI-805 for COX-2 was determined to be 2.35μM. In comparison, a previous study showed that the $IC_{50}$ value of flunixin meglumine for COX-2 is lower than that of AHI-805, at 0.18μM.[1] However, the $IC_{50}$ value of AHI-805 is lower than that previously reported for phenylbutazone (3.79μM).[1] Therefore, this experiment suggests that AHI-805 can inhibit COX-2 at a concentration comparable to that of commonly used NSAIDs.

Ischemic injury resulted in a significant decrease in the TER of equine jejunal mucosa. In this study, treatment of ischemic injured equine jejunum with both flunixin meglumine and AHI-805 significantly inhibited the recovery of TER. This inhibitory effect of flunixin meglumine on TER recovery has been previously described and has been attributed to the inhibition of prostaglandin-mediated closure of the paracellular spaces between repairing cells.[6] While TER measures the movement of ions across the mucosa, the mannitol flux experiment examines the movement of a larger molecule across the paracellular space. The results of this experiment demonstrated an increase in the paracellular movement of mannitol during the first three hours of the recovery period in ischemic injured tissue. There was no significant difference in mannitol flux between control and ischemic injured tissue during the fourth hour of recovery which is most

likely due to closure of the paracellular space and further demonstrates the recovery of barrier function. In contrast to a previous study, this current study found no significant effect of treatment with flunixin meglumine on mannitol permeability.[10] Therefore, only the TER measurements identified differences between the treatment groups. This may be due to the fact that TER is a more sensitive indicator of mucosal permeability and accurately reflects the transmucosal passage of ions.[18]

In the Ussing chamber model, treatment with either flunixin meglumine or AHI-805 resulted in significantly lower production of PGEM which implies that both of these drugs significantly inhibit the action of COX-2. This inhibition of COX-2 by flunixin meglumine has been demonstrated in previous studies.[10] This result confirms the findings of the LPS-induced $PGE_2$ assay and suggests AHI-805 inhibits the action of COX-2. However, AHI-805 also reduced the production of $TXB_2$ by the equine jejunum. This is in contrast to our finding that AHI-805 did not significantly reduce coagulation induced $TXB_2$ and may reflect differences in the two models. For example, the Ussing chamber model has a lower protein concentration in the bathing solution than the whole blood model.[17] The results of this study suggest that AHI-805 is an inhibitor of both COX-1 and COX-2 and significantly reduces the production of prostanoids by ischemic injured equine jejunum. This may therefore be the mechanism by which AHI-805 inhibits the recovery of mucosal barrier function following ischemic injury.

Immunoblot analysis of COX-1 protein expression revealed constitutive expression in the equine jejunum, without upregulation by ischemic injury. This is consistent with other models of ischemic injury in which the expression of COX-1 is unaffected by injury.[19, 20] The results of immunoblot analysis of COX-2 protein expression demonstrate low level constitutive expression in the equine jejunum which is upregulated in response to ischemic injury over the course of a four hour recovery period. The upregulation of COX-2 in ischemic injured equine jejunum has been previously described.[10] There was no significant effect of flunixin meglumine or AHI-805 on the expression of either COX-1 or COX-2.

This study examined a novel analgesic and anti-inflammatory compound to investigate its mechanism of action. As AHI-805 did not appear to inhibit COX-1 in an in vitro whole blood model, we then assessed its suitability for the treatment of equine gastrointestinal disease using a model of ischemic injury and recovery. The aza-thio-benzoazulene derivative AHI-805 significantly inhibited the recovery of mucosal barrier function and may therefore be contraindicated in the treatment of equine colic. However, future studies to further elucidate the mechanism of the anti-inflammatory action of AHI-805 are warranted.

**Footnotes:**

a Thromboxane B2 EIA kit, Cayman Chemical, Ann Arbor MI

b Prostaglandin $E_2$ EIA kit (Monoclonal), Cayman Chemical, Ann Arbor MI

c SigmaPlot 10, Systat Software, San Jose CA

d Prostaglandin E Metabolite EIA kit, Cayman Chemical, Ann Arbor MI

e SigmaScan, Systat Software, San Jose CA

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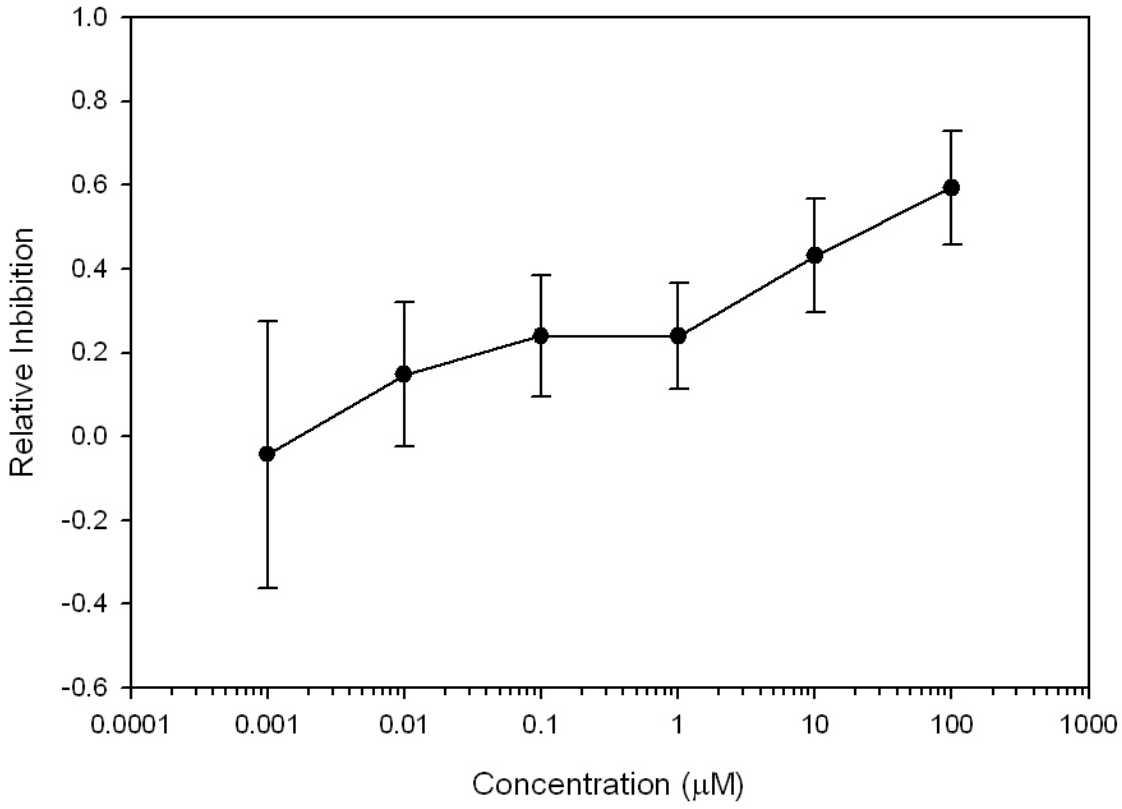


**Figure 1:** The effect of AHI-805 on the inhibition of COX-1 as determined by measurement of coagulation induced $TXB_2$ in an *in vitro* equine whole blood assay. AHI-805 did not significantly inhibit the action of COX-1.

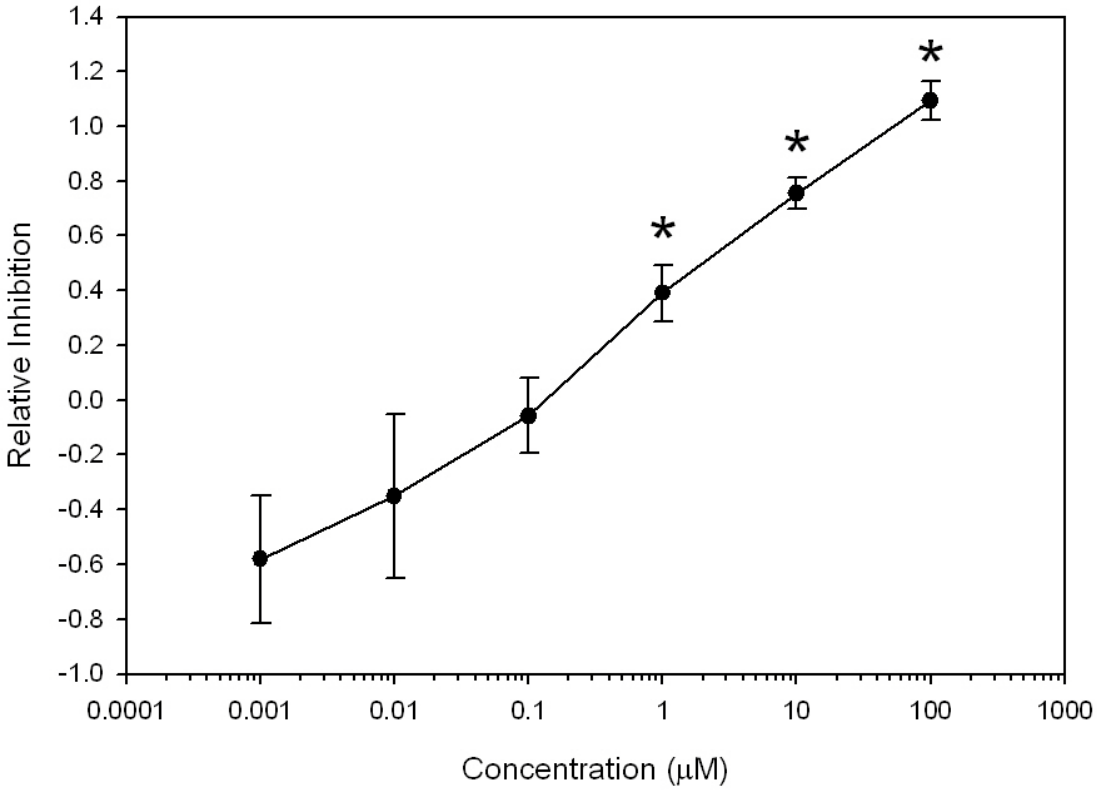


**Figure 2:** The effect of AHI-805 on the inhibition of COX-2 as determined by measurement of LPS- induced $PGE_2$ in an *in vitro* equine whole blood assay. AHI-805 significantly inhibited the action of COX-2 at 1, 10 and 100μM concentrations (* $p<0.05$).

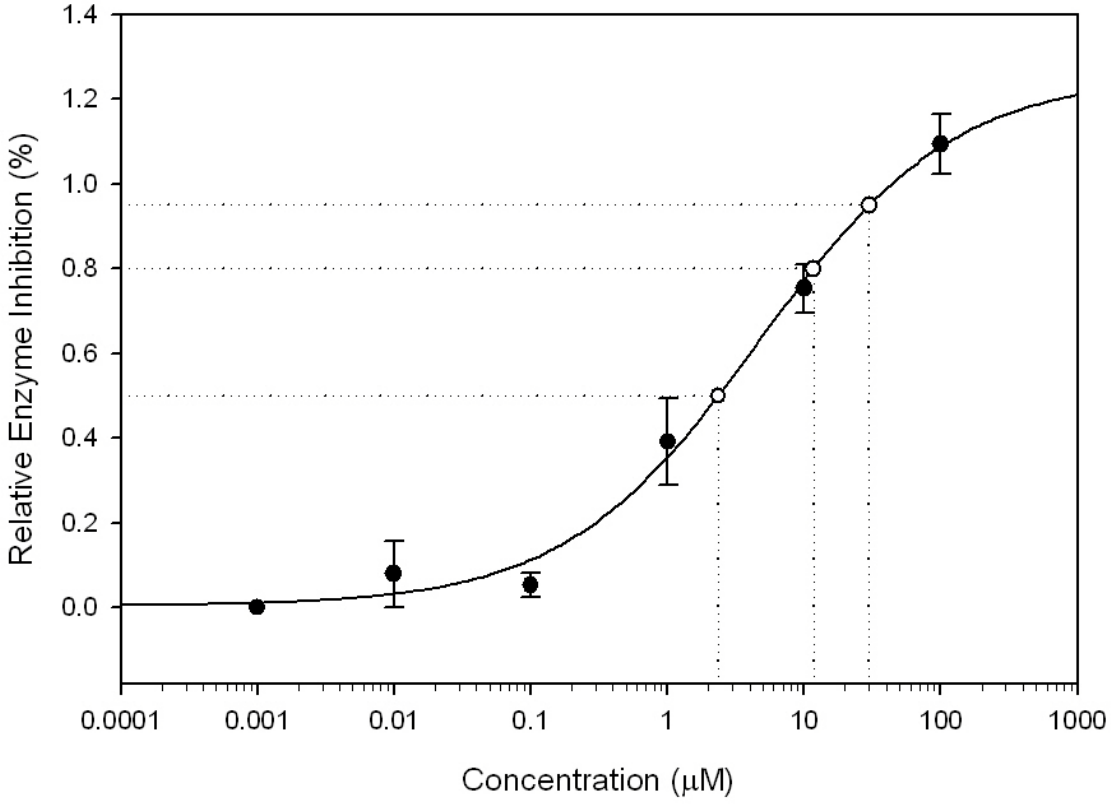


**Figure 3:** A four-parameter logistic equation was used to produce a model of COX-2 inhibition by AHI-805. Closed circles (●) represent the actual COX-2 inhibition, open circles (○) represent predicted COX-2 inhibition at $IC_{50}$, $IC_{80}$ and $IC_{95}$.

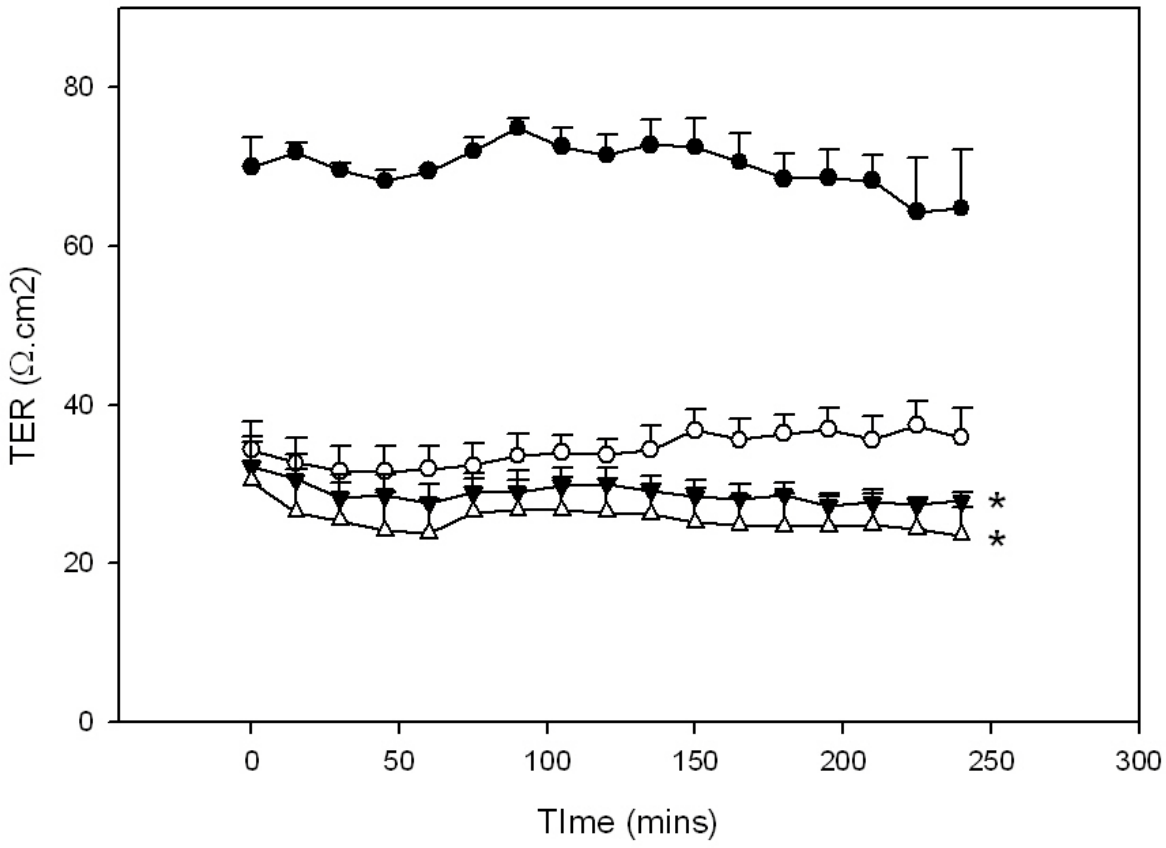


**Figure 4:** The trans-epithelial resistance (TER) of control and ischemic injured equine jejunum was measured every 15 minutes during a 240 minute recovery period on the Ussing chamber. Flunixin and AHI-805 treated tissue had significantly lower ($p<0.05$) TER values than untreated (control) ischemic tissue (*). ●=Control treatment-control tissue, ○=control treatment –ischemic tissue, ▼=flunixin treatment –ischemic tissue, Δ=AHI-805 treatment –ischemic tissue.

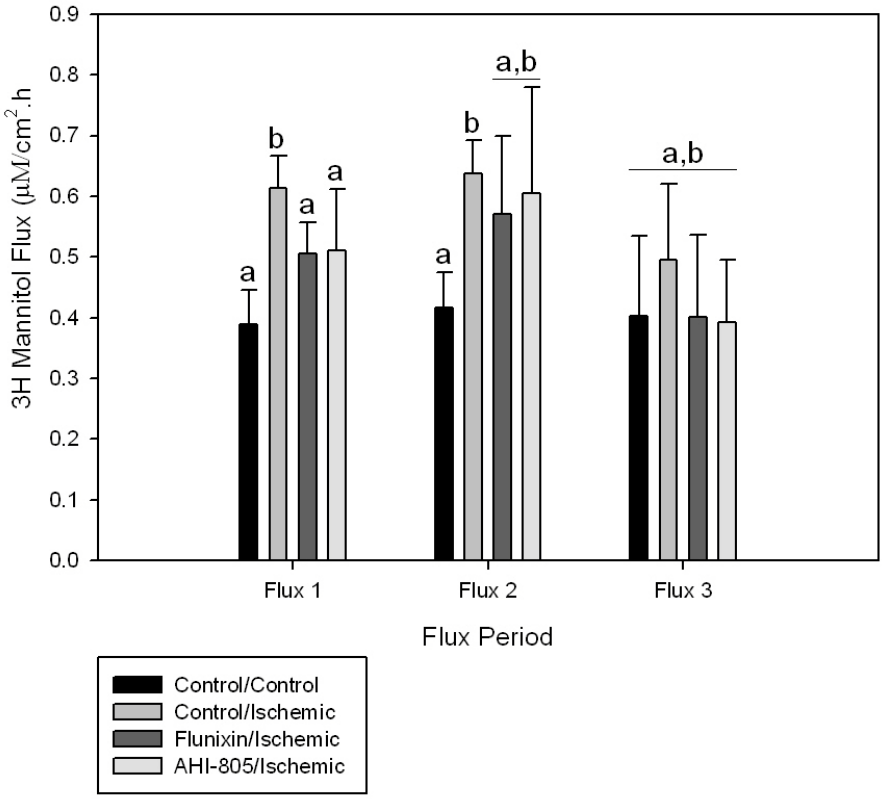


**Figure 5:** The mucosal to serosal flux of $^{3}$H-Mannitol across control and ischemic injured equine jejunum was measured over three flux periods. Tissues were treated with control, flunixin meglumine, or AHI-805. During flux period 1 (60 – 120mins), there was a significantly greater flux across ischemic injured tissue when compared to control tissue ($p<0.05$). During flux period 2 (120 – 180mins), there was a significantly greater flux across ischemic injured tissue when compared to control tissue ($p<0.05$). There was no significant effect of flunixin meglumine or AHI-805 during any flux period.

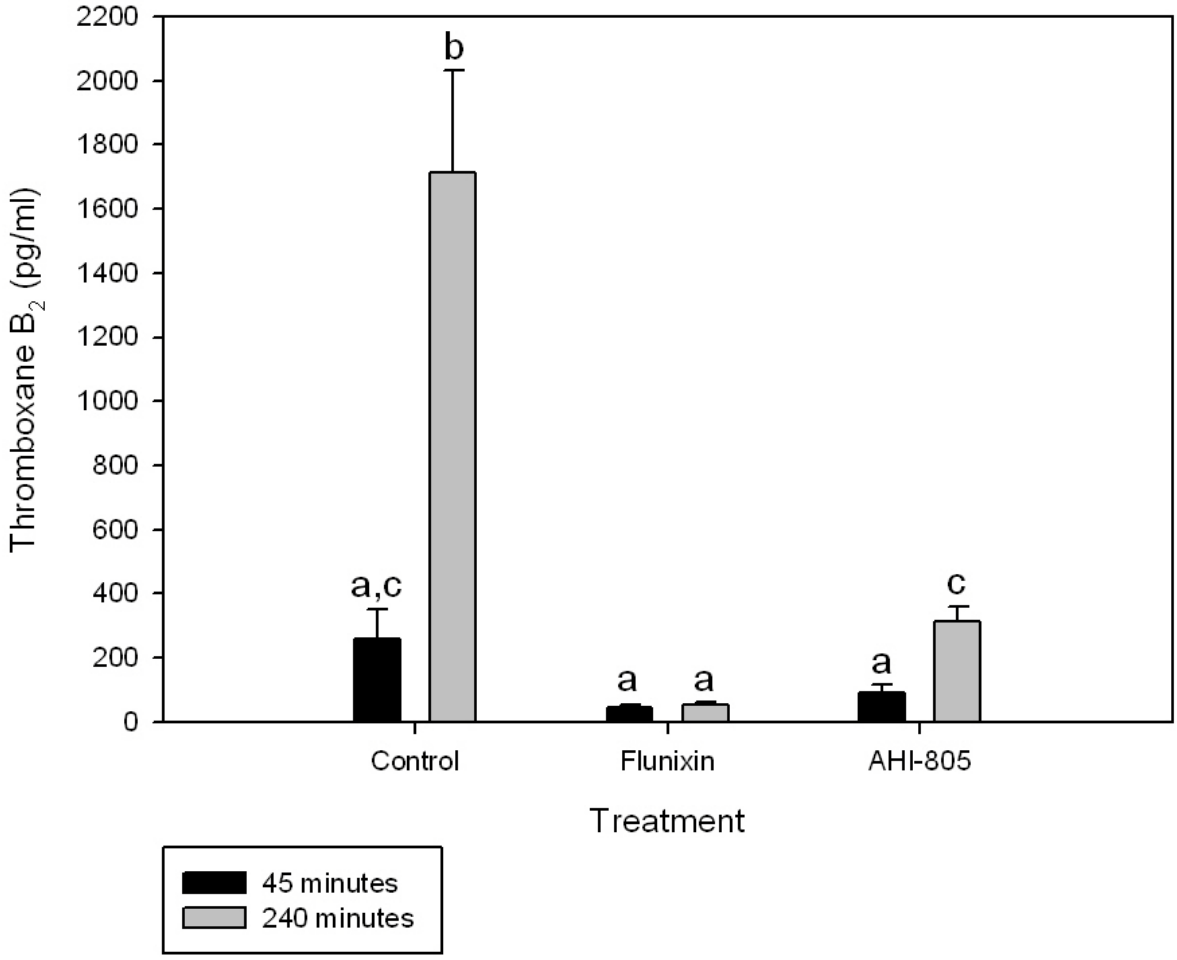


**Figure 6:** Thromboxane B2 (TXB2) levels were measured in ischemic tissue bathing solutions after 45 and 240 minutes of recovery. $TXB_2$ increased significantly over the recovery period in the control and AHI-805 treated tissue ($p<0.05$) but not in flunixin treated tissue. Flunixin meglumine and AHI-805 treated tissue produced significantly less PGEM than control treated tissue at 240 minutes ($p<0.05$).

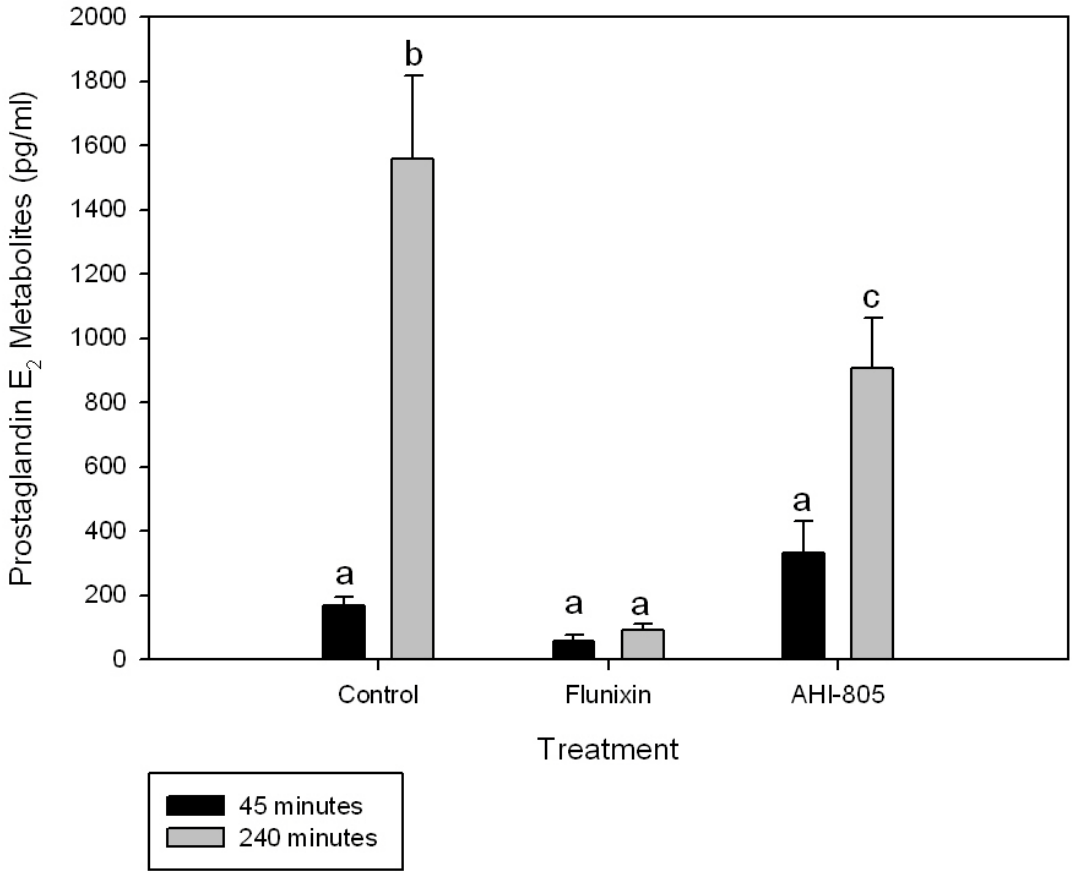


**Figure 7:** Prostaglandin $E_2$ metabolite (PGEM) levels were measured in ischemic tissue bathing solutions after 45 and 240 minutes of recovery. $PGE_2$ increased significantly over the recovery period in the control and AHI-805 treated tissue ($p<0.05$) but not in flunixin treated tissue. Flunixin meglumine and AHI-805 treated tissue produced significantly less PGEM than control treated tissue at 240 minutes ($p<0.05$). Flunixin meglumine treated tissue produced significantly less PGEM than AHI-805 treated tissue ($p<0.05$).

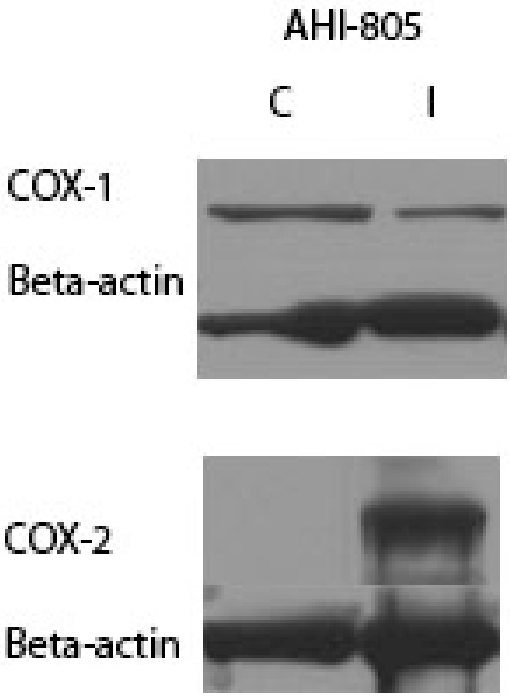


**Figure 8:** Immunoblot analysis of COX-1 and COX-2 expression in control (C) and ischemic-injured equine jejunum (I) treated with AHI-805. Ischemic injury significantly increased the expression of COX-2 only ($p<0.05$). There was no significant effect of treatment on either COX-1 or COX-2 expression.

## Summary

The overall aim of this study was to describe the effect of novel anti-inflammatory drugs on the cyclooxygenase enzymes of the horse. Furthermore, this study aimed to assess the effect these drugs on the recovery of ischemic-injured equine jejunum. Several NSAIDs were studied including flunixin meglumine, deracoxib, firocoxib, robenacoxib and the novel anti-inflammatory compound AHI-805.

The commonly used NSAID flunixin meglumine was shown to non-selectively inhibit both COX-1 and COX-2. It was also demonstrated the flunixin meglumine inhibits the recovery of ischemic injured equine jejunum. Therefore, the use of flunixin meglumine in the treatment of pain and endotoxemia in the post-operative colic patient may be contraindicated.

Deracoxib, firocoxib, and robenacoxib were all shown to selectively inhibit the COX-2 enzyme in the horse and therefore may reduce the risk of adverse gastrointestinal and renal effects. Robenacoxib was examined in a model of intestinal ischemic injury and recovery. Treatment with robenacoxib did not significantly inhibit mucosal barrier function recovery.

AHI-805 is a recently described novel anti-inflammatory compound with an unknown mechanism of action. This study showed that AHI-805 significantly inhibited the action of COX-2. However, an *ex vivo* model of intestinal ischemic injury and recovery revealed

that AHI-805 significantly inhibited the recovery of mucosal barrier function throught the inhibition of both COX-1 and COX-2.

**Future Directions**

This study examined the effect of NSAIDs in the horse using *in vitro* and *ex vivo* models. To determine the clinical significance of these findings, future studies will focus on the effect of NSAIDs administered at recommended dosages in the horse. An *ex vivo* blood model of cyclooxygenase inhibition will determine the inhibition of COX-1 and COX-2 following oral or intravenous NSAID administration.

In addition to the study of individual NSAIDs, future studies will examine the effect of combined NSAID administration using an *in vitro* and *ex vivo* models. While the combined use of NSAIDs does occur, it is associated with adverse gastrointestinal effects including right dorsal colitis. These studies will assess the effect of combined NSAIDs on the cyclooxygenase enzymes.