

## ABSTRACT

COOK, VANESSA LOUISE. The Role of Novel Anti-Inflammatory Drugs in the Repair of Ischemic-Injured Equine Jejunum. (Under the direction of Anthony Blikslager).

Following colic surgery, ischemic-injured intestine may remain which must recover for the horse to survive. However, the commonly used analgesic, flunixin meglumine, a non selective cyclooxygenase (COX) inhibitor, may retard the repair of ischemic-injured jejunum. Therefore, we investigated alternative anti-inflammatory drugs which may allow recovery of ischemic-injured jejunum whilst providing effective analgesia.

The effect of 0.9% saline 1ml/50kg, flunixin meglumine 1mg/kg IV every 12 hours, lidocaine 1.3mg/kg loading dose and 0.05mg/kg/minute constant rate infusion IV, or the two drugs combined, was evaluated on recovery of mucosal barrier function in equine jejunum following 2 hours of ischemia and 18 hours of recovery (n=6 horses/group). Flunixin meglumine inhibited the recovery of mucosal barrier function as evidenced by a lower transepithelial resistance (TER) and increased LPS flux across ischemic-injured mucosa from horses in that treatment group. When treatment with flunixin meglumine was combined with lidocaine, recovery of mucosal barrier function was not retarded. The mucosal influx of neutrophils seen with flunixin meglumine treatment was ameliorated by treatment with lidocaine. Lidocaine inhibited upregulation of COX-2 in ischemic-injured jejunum.

The same model was used to evaluate the effect of a COX-2 preferential inhibitor, firocoxib at 0.09mg/kg IV. Pain scores did not increase after surgery in horses treated with flunixin meglumine or firocoxib. Unlike flunixin meglumine, firocoxib allowed recovery of

TER and did not increase LPS flux across ischemic-injured jejunum. Analyses of plasma prostanoids suggested that firocoxib is COX-2 selective in horses.

The effect of lidocaine on neutrophils was evaluated by incubating isolated equine neutrophils with 0.1-1000 $\mu$ g/ml of lidocaine *in vitro*. Neutrophil adhesion and migration in response to stimulants was subsequently evaluated. LTB<sub>4</sub> and IL-8 induced adhesion were increased at 1mg/ml of lidocaine. Migration increased with increasing concentration of lidocaine, in response to the same stimulants.

Therefore, the use of firocoxib, or lidocaine in combination with flunixin meglumine, may be advantageous for horses recovering from ischemic intestinal injury, compared to treatment with a non-selective COX inhibitor, such as flunixin meglumine, alone.

The Role of Novel Anti-Inflammatory Drugs in the Repair of  
Ischemic-Injured Equine Jejunum

by  
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DEDICATION

*For my husband and best friend*

*Dr Tony Pease*

*You complete me*

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## **CHAPTER 1**

### **Use of Systemically Administered Lidocaine in Horses with Gastrointestinal Tract Disease**

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## Reference Point

### Use of systemically administered lidocaine in horses with gastrointestinal tract disease

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Lidocaine hydrochloride is widely used in veterinary medicine as a local anesthetic and as an antiarrhythmic for treatment of animals with ventricular tachycardia.<sup>1,2</sup> The use of systemically administered lidocaine has increased dramatically in equine hospitals as a treatment for horses with intestinal ileus.<sup>3-5</sup> Lidocaine has novel anti-inflammatory properties that may also ameliorate the effects of ischemia-reperfusion injury.<sup>6-9</sup> The purpose of the information reported here was to evaluate the current use of systemically administered lidocaine in the treatment of horses with 2 specific gastrointestinal tract problems (ie, ileus and ischemic intestinal injury).

#### Abbreviation

NHE      Na<sup>+</sup>-H<sup>+</sup> exchanger

#### Pharmacologic Properties of Lidocaine

All local anesthetics are composed of 3 subunits: a lipophilic aromatic group, such as a

benzene ring; a hydrophilic amine group; and an intermediate chain that connects those 2 structures.<sup>10</sup> The nature of the intermediate chain subclassifies local anesthetics into esters or amides. Lidocaine has an amide link as the intermediate bond and is therefore classified as an aminoamide local anesthetic. All amide local anesthetics, such as bupivacaine, ropivacaine, and prilocaine, have a prefix that ends with the letter i (ie, bupi-, ropi-, and pri-, respectively), whereas ester anesthetics, such as cocaine, benzocaine, and procaine, do not. The 2 classes of local anesthetics are metabolized differently. Ester anesthetics are hydrolyzed by cholinesterase in the plasma to form the metabolite para-amino benzoic acid, which is a known allergen. Amide anesthetics are primarily metabolized by the liver. Hepatic cytochrome P450 enzymes first dealkylate and then hydrolyze the compound. Lidocaine is dealkylated to the main metabolites monoethylglycinexylidide and glycinexylidide, which retain local anesthetic properties, before being hydrolyzed further.<sup>11</sup> Because lidocaine is metabolized by the liver, serum concentrations can increase in animals with hepatic disease<sup>12</sup> and cause toxicosis at standard administration rates.

Lidocaine acts as a local anesthetic by binding to voltage-gated sodium channels to block the influx of  $\text{Na}^+$  into a cell and hence prevent propagation of the action potential. Nonetheless, the effectiveness of systemically administered lidocaine in animals with gastrointestinal tract disease is probably not achieved through its anesthetic action. A bolus of 2 mg of lidocaine/kg followed by a constant rate infusion at a rate of 50  $\mu\text{g}/\text{kg}$  can provide somatic analgesia, as evidenced by a significant increase in thermal threshold of the skin.<sup>13</sup> However, the same dose had no effect on visceral pain, which was assessed by duodenal and

colorectal distention with a balloon.<sup>13</sup> Therefore, the perceived clinical benefit of systemic administration of lidocaine in horses with gastrointestinal tract disease is likely achieved through novel anti-inflammatory effects on neutrophils, which take place at a concentration that is less than the concentration necessary for blockade of sodium channels.<sup>14</sup>

### **Use of Lidocaine in the Management of Ileus**

In horses, interest in lidocaine has recently focused on its use as an agent that enhances gastrointestinal tract motility in colic patients with postoperative ileus.<sup>3</sup> Its systemic use in humans was first described for treatment of patients with ileus following cholecystectomy, for which it decreased the duration of postoperative ileus.<sup>15</sup> A survey of Diplomates of the American College of Veterinary Surgeons revealed that IV infusion of lidocaine was the most frequently used method to enhance motility (prokinetic) in horses.<sup>16</sup> Intraoperative administration of lidocaine to surgical colic patients may have been associated with a reduction in the incidence of postoperative ileus from 19% to 9% in 1 study.<sup>4</sup>

In a multicenter study,<sup>5</sup> investigators evaluated the effectiveness of a constant rate infusion of lidocaine, compared with that for a constant rate infusion of saline (0.9% NaCl) solution, for 24 hours in horses with nasogastric reflux attributable to postoperative ileus or enteritis. Sixty-five percent of horses treated with lidocaine in that study had a cessation of refluxing within 30 hours, which was significantly better than the proportion of horses treated with saline solution in which only 27% had a cessation of refluxing during the same period. Furthermore, lidocaine administration significantly reduced the hourly volume of reflux

during and after the infusion. Duration of hospitalization was significantly less (mean of 6 days less) in lidocaine-treated horses than in saline solution–treated horses, although short-term survival did not differ significantly.

### **Mechanisms of Action of Lidocaine in Ileus**

Analysis of results from the aforementioned studies suggests that there is a clinical benefit to systemic administration of lidocaine in the management of animals with ileus. However, the mechanism of action by which this effect is achieved is unclear. An in vitro study<sup>17</sup> on isolated strips of intestinal muscle treated topically with lidocaine resulted in contraction of muscle obtained only from the proximal portion of the duodenum and not from the pyloric antrum or midjejunum. Additionally, systemic administration of lidocaine to clinically normal horses did not decrease the duration of the migrating myoelectrical complex, increase the number of phase III events in the migrating myoelectrical complex, or result in more frequent spiking activity, when compared with results after administration of saline solution,<sup>18</sup> which would be expected if it were a prokinetic agent. Although both studies<sup>17,18</sup> were performed in clinically normal horses and results from horses with ileus may differ, the results suggest that lidocaine does not have a direct prokinetic effect. Therefore, it is likely that the clinical effectiveness of lidocaine is attributable to other mechanisms of action.

Experiments in mice have determined the importance of intestinal inflammation as a cause of ileus 12 to 24 hours after surgical manipulation.<sup>19</sup> Macrophages within the muscularis layers of the intestine are activated by surgical manipulation of the intestine,

which causes them to release proinflammatory cytokines and prostaglandins.<sup>20</sup> In turn, these inflammatory mediators cause influx of monocytes and neutrophils into the muscularis layers and further release of inflammatory products, which reduce muscle contractility and ultimately result in ileus.<sup>21,22</sup> In horses, neutrophilic inflammation has been identified in the jejunum 18 hours after experimentally induced ischemia.<sup>23</sup> The temporal relationship between onset of clinical signs of ileus and peak intestinal inflammation suggests that intestinal inflammation may mediate postoperative ileus in horses. Accordingly, the beneficial effects of lidocaine in the management of horses with ileus may be related to its anti-inflammatory properties.

Local anesthetics inhibit many of the immune functions of neutrophils.<sup>14</sup> In vitro assays to evaluate these various functions have revealed that lidocaine inhibits neutrophil adhesion, phagocytosis, and the production of free radicals.<sup>24,25</sup> Inhibition of these functions could reduce the deleterious effect of neutrophils on intestinal contractility, although many of these effects are only evident at plasma concentrations higher than those achieved clinically.<sup>26</sup> In addition, lidocaine can reduce the expression of endothelial adhesion molecules, which is the first step in migration of neutrophils into the intestines.<sup>6</sup> There is also evidence of anti-inflammatory effects of lidocaine on the intestines. In rats with experimentally induced obstructive ileus, IV administration of lidocaine prevents fluid secretion into the lumen of the small intestine and reduces edema formation in the intestinal wall.<sup>27</sup> Additionally, topical administration of lidocaine gel and systemic administration of lidocaine reduce inflammation in humans with ulcerative colitis.<sup>28,29</sup> Therefore, the beneficial effects of systemic

administration of lidocaine in horses with ileus are likely attributable to anti-inflammatory effects, although additional studies are necessary to investigate this hypothesis.

### **Use of Lidocaine in Ischemia-Reperfusion Injury**

Ischemic lesions of the gastrointestinal tract have a poorer prognosis than do simple obstructions.<sup>30</sup> Reperfusion injury is believed to contribute further to deterioration in viability of the intestines after surgery.<sup>31</sup> During reperfusion, transepithelial migration of neutrophils physically disrupts the mucosal barrier and then causes additional damage and enterocyte death via the release of free radicals and toxic substances.<sup>32,33</sup> In horses, neutrophils accumulate in the large colon during ischemia, with further influx during the reperfusion period,<sup>34</sup> which suggests that neutrophils are an important contributor to ischemia-reperfusion injury in horses. Generation of free radicals by xanthine oxidase when blood flow is restored is believed to initiate reperfusion injury in the small intestines by causing lipid peroxidation of cell membranes and generation of chemoattractants, such as leukotriene B<sub>4</sub>.<sup>35</sup> Increases in xanthine oxidase have been detected during ischemia in the equine small intestines,<sup>36</sup> although similar increases have not been detected in the large colon.

Currently, there is little information on the effectiveness of lidocaine in the management of intestinal ischemia and reperfusion in any species. Its use has been investigated in animals used experimentally to evaluate stroke, heart attack, and organ transplantation, all of which are likely to have ischemia-reperfusion injury as a central mechanism. Many studies have focused on the effect of systemic administration of lidocaine in dogs with experimentally induced myocardial ischemia and reperfusion. These studies have revealed that treatment

with lidocaine reduces lipid peroxidation in membranes,<sup>9</sup> reduces the size of infarcts,<sup>9</sup> reduces accumulation of neutrophils,<sup>37</sup> and improves subsequent cardiac contractility.<sup>8</sup> Similar beneficial effects of systemic administration of lidocaine have been reported in dogs and rodents with experimentally induced cerebral ischemia and reperfusion.<sup>38-40</sup> With regard to lung and kidney transplants, addition of lidocaine to the organ storage solution reduces reperfusion injury as evidenced by a decrease in histologic damage, a reduction of neutrophil influx into the graft, and improvements in organ function.<sup>41,42</sup>

Effects of treatment with systemically administered lidocaine in ischemic-injured equine jejunum have been evaluated.<sup>a</sup> In one of those studies,<sup>b</sup> systemic administration of lidocaine 15 minutes prior to reperfusion significantly reduced the mean grade for mucosal damage, compared with results after treatment with saline solution. In the other study,<sup>a,b</sup> treatment with lidocaine ameliorated the negative effects of flunixin meglumine on recovery of the mucosal barrier, as evidenced by a higher transepithelial resistance when the 2 treatments were combined, compared with effects after administration of flunixin meglumine alone.

### **Mechanisms of Action of Lidocaine in Ischemia-Reperfusion Injury**

Mechanisms by which treatment with lidocaine reduces ischemia-reperfusion injury in any organ are not completely understood. As mentioned previously, influx of neutrophils from the circulation into the tissues has been associated with reperfusion injury. Similar to the effects in ileus, inhibition of neutrophil activation by lidocaine is likely to play an important role in amelioration of reperfusion injury. Isolated human neutrophils subjected to oxygen depletion and then reoxygenation have decreased expression of endothelial adhesion

molecules when they are incubated with lidocaine before depletion-reoxygenation.<sup>7</sup> A similar effect of lidocaine is evident when isolated neutrophils from healthy volunteers are incubated with plasma from a human arm that has undergone ischemia and reperfusion by tourniquet application and release.<sup>6</sup> Such adhesion molecules are also necessary for neutrophil migration across the intestinal epithelium.<sup>32</sup> Therefore, a reduction in the expression of neutrophil adhesion molecules by treatment with lidocaine may reduce damage to intestinal epithelial cells by reducing neutrophil influx into the intestines during reperfusion, although this has not yet been investigated.

Integrity of the vascular endothelium is also damaged by neutrophil diapedesis, which results in leakage of plasma proteins (such as albumin) from the capillaries and a subsequent reduction in oncotic pressure and circulating plasma volume.<sup>43</sup> In rats with experimentally induced endotoxemia, treatment with lidocaine before administration of endotoxin reduced adherence of leukocytes to the vascular endothelium and reduced leakage of albumin across venules, compared with results for preendotoxin treatment with saline solution.<sup>44</sup> In that study, lidocaine also prevented an endotoxin-induced decrease in mean arterial pressure. Absorption of endotoxin across intestinal mucosa that has been damaged by ischemia and reperfusion is a cause of early postoperative fatalities in many colic patients.<sup>30</sup> The resulting increase in capillary permeability makes it challenging to maintain circulating plasma volume and organ perfusion.<sup>45</sup> The anti-inflammatory effects of lidocaine on neutrophil adhesion may ameliorate the increase in capillary permeability evident clinically in colic patients after surgery, which ultimately would improve fluid dynamics in these patients.

Another mechanism by which lidocaine may reduce ischemic injury is prevention of ischemic-induced intracellular  $\text{Na}^+$  overload<sup>46,47</sup> (Figure 1).

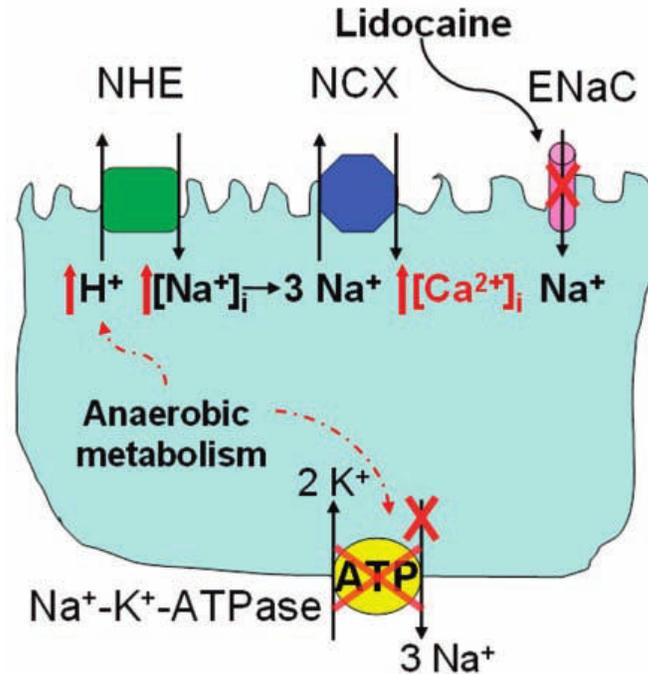


Figure 1—Schematic depicting an ischemic cell and the mechanisms through which anaerobic metabolism results in an increase in the intracellular  $\text{Na}^+$  concentration. Excessive  $\text{H}^+$  from anaerobic metabolism is removed from the cell by the NHE, which results in an increase in the intracellular  $\text{Na}^+$  concentration  $[\text{Na}^+]_i$ . In addition, an increased influx of  $\text{Na}^+$  through the epithelial  $\text{Na}^+$  channel (ENaC) is also important in toxic effects induced by ischemia. Because of the depletion of ATP during ischemia, excess  $\text{Na}^+$  cannot be removed by  $\text{Na}^+-\text{K}^+-\text{ATPase}$  on the basolateral border. Instead, the high  $[\text{Na}^+]_i$  results in reversal of the  $\text{Na}^+-\text{Ca}^{2+}$  exchanger (NCX) to expel excess  $\text{Na}^+$ , which results in an increase in the intracellular  $\text{Ca}^{2+}$  concentration  $[\text{Ca}^{2+}]_i$  and subsequent activation of inflammatory enzymes. Lidocaine may block the ENaC to prevent  $\text{Na}^+$  influx via the ENaC and ameliorate intracellular  $\text{Na}^+$  overload.

The intracellular  $\text{Na}^+$  concentration increases during ischemia because of  $\text{Na}^+$  influx

through the epithelial  $\text{Na}^+$  channel (which is blocked by lidocaine) and the NHE. The NHEs are responsible for electroneutral absorption of  $\text{Na}^+$  in exchange for  $\text{H}^+$  as well as regulation of intracellular pH. During ischemia, there is an increase in the concentration of  $\text{H}^+$  in cells as a result of anaerobic metabolism. Excess  $\text{H}^+$  is exported by the NHEs, which results in an increase in the intracellular  $\text{Na}^+$  concentration.<sup>48</sup> The intracellular  $\text{Na}^+$  concentration is further increased because the principal mechanism for  $\text{Na}^+$  efflux from the cell (ie,  $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ ) is inhibited during ischemia as a result of ATP being depleted.<sup>49</sup> The  $\text{Na}^+ - \text{Ca}^{2+}$  exchanger typically pumps  $\text{Ca}^{2+}$  out of cells in exchange for  $\text{Na}^+$  in a 1:3 ratio. However, during reperfusion, the  $\text{Na}^+ - \text{Ca}^{2+}$  exchanger reverses to expel excess  $\text{Na}^+$  from the cells, which causes  $\text{Ca}^{2+}$  influx and intracellular  $\text{Ca}^{2+}$  overload.<sup>50</sup> An increase in the intracellular  $\text{Ca}^{2+}$  concentration is responsible for activation of many key inflammatory enzymes, including xanthine oxidase and calcium-dependent phospholipase  $\text{A}_2$ ,<sup>51</sup> and this ultimately results in cell death.<sup>52</sup>

Although these mechanisms have been predominantly evaluated for myocardial and cerebral ischemia, intracellular  $\text{Na}^+$  overload is also likely to play a role in intestinal ischemic injury. In porcine ileum subjected to ischemic conditions for 45 minutes, pharmacologic inhibition of the NHE2 isoform improves the recovery of the injured mucosal barrier and reduces mucosal-to-serosal flux of  $\text{Na}^+$ .

Treatment with lidocaine can significantly reduce the increase in intracellular  $\text{Na}^+$

concentration during myocardial ischemia by blocking the Na<sup>+</sup> channel.<sup>47,54,55</sup> Such treatment results in improvements in functional and metabolic recovery in rat hearts.<sup>47,54</sup> The gastrointestinal tract undergoes changes during ischemic injury similar to those in other organs, and the gastrointestinal tract may be even more susceptible to damage.<sup>35</sup>

## **Conclusions**

An understanding of the anti-inflammatory properties of lidocaine has opened up a novel and exciting treatment option for horses with inflammatory conditions of the gastrointestinal tract, including ileus and recovery from ischemic injury. Equine clinicians principally use systemic administration of lidocaine for the management of ileus, with its effectiveness likely attributable to its ability to inhibit neutrophil activation, rather than through its actions as a prokinetic. The benefits of systemic administration of lidocaine in ischemia-reperfusion injury have been determined predominantly in other species and other organs. Its benefit in cardiac and cerebral ischemia-reperfusion injury is achieved by preventing intracellular Na<sup>+</sup> overload and through its anti-inflammatory properties. Although these mechanisms have not been evaluated in ischemic-injured intestines, it is likely that the effect of lidocaine is similar in this tissue. Given the high mortality rate after colic surgery that results from the absorption of endotoxin across injured intestinal mucosa, a drug that reduces intestinal ischemia-reperfusion injury could also reduce the mortality rate in these horses. Therefore, further evaluation of the clinical effectiveness of lidocaine in horses with gastrointestinal tract disease is urgently needed.

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## **CHAPTER 2**

### **The Role of Neutrophils in Equine Ischemic Intestinal Injury**

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## **Introduction**

Ischemic injury to the intestine can occur when the blood supply is reduced below critical levels or completely occluded. In the majority of cases of equine intestinal ischemia, venous drainage is occluded, whilst arterial supply initially continues, resulting in a hemorrhagic strangulation with edema and hemorrhage of the intestine.<sup>1,2</sup> Occlusion of both venous and arterial supply occurs less frequently, and results in an ischemic strangulating obstruction characterized by a lack of edema and hemorrhage.<sup>3</sup> Ischemic injury can also occur subsequent to an arterial thrombus or embolus.<sup>4</sup> In horses, the usual vessel affected is the cranial mesenteric artery.<sup>5</sup> Microvascular thrombosis in local submucosal vessels can also occur as a sequelae to a strangulating obstruction.<sup>1</sup> Non occlusive ischemia can be documented after any situation that causes reduced cardiac output, such as cardiac disease or severe hypovolemia and shock;<sup>6</sup> a situation that is less common in horses than in people.

The reduction in blood supply causes damage to the epithelial cells as a result of oxygen and nutrient deprivation.<sup>7</sup> The mucosal epithelial cells are particularly susceptible to ischemia due to their high metabolic rate,<sup>8</sup> and, in the small intestine, the architecture of the villus blood supply.<sup>9</sup> Thus epithelial cells near the villus tip are injured first, resulting in a characteristic pattern of progressive injury from the villus tip to the crypt.<sup>10</sup>

Regardless of the underlying cause of ischemic injury, emergency surgical intervention and resection of the injured intestine results in the best prognosis.<sup>11</sup> Despite this, strangulating intestinal lesions have a higher post operative mortality rate than simple intestinal obstructions.<sup>12</sup> Unfortunately, determining the viability of affected intestine at surgery is difficult, and ischemic-injured intestine frequently remains.<sup>13,14</sup> Absorption of

endotoxin across remaining injured mucosa can occur and contributes to the high post operative mortality rate.<sup>15,16</sup> Repair of this injured intestine and recovery of the mucosal barrier is essential to prevent multiple organ failure and death.<sup>11,17</sup>

Neutrophils are one of the principal cells which respond to acute intestinal injury, infiltrating the mucosa to control infection.<sup>18</sup> However, neutrophil influx also contributes to tissue damage.<sup>19</sup> Therefore, the purpose of this review is to evaluate the mechanisms by which neutrophils respond to a breach of the mucosal barrier, and how they contribute to both repair and further injury. The effect of different drugs on this process and possible treatments to improve recovery are discussed.

### **Neutrophil Recruitment to Injured Mucosa**

Mucosal injury results in infection, and also inflammation. Infection occurs because damage to the intestinal barrier allows bacteria and toxins to penetrate the underlying lamina propria, whereas injured epithelial cells produce sterile inflammation as a result of necrotic cell death.

The innate immune response provides a rapid response to the presence of foreign substances in the lamina propria, and is the primary defense against invading organisms.<sup>20</sup> Toll-like receptors (TLRs) which are found on cells such as macrophages, dendritic cells and intestinal epithelial cells are an important part of this defense.<sup>21</sup> They recognize conserved microbial products, known as pathogen-associated molecular patterns (PAMPs), which include bacterial peptides, lipopolysaccharide (LPS), flagellin and bacterial DNA.<sup>22</sup> TLRs play a key role in the response of the epithelium to injury.<sup>23</sup> Activation of TLRs trigger 2

intracellular signaling cascades which ultimately results in the activation of NF- $\kappa$ B and p38 MAP kinase resulting in the production of proinflammatory cytokines, which are tailored to the original stimulant (Figure 1).<sup>24</sup>

Cell necrosis due to ischemic injury can also trigger the innate immune response through the activation of TLR2.<sup>25</sup> Initially it was thought that the stimulant was high-mobility group box 1 protein (HMGB1), a substance that is released from cells in response to trauma and inflammation.<sup>26,27</sup> However, it is now known that IL-1 $\alpha$  is released from macrophages in response to cell necrosis, and results in the production of chemokines which attract and activate neutrophils (Figure 1).<sup>28,29</sup> It is currently hypothesized that the macrophage stimulant released from necrotic cells is N-formylmethionyl mitochondrial proteins.<sup>30</sup>

The principal TLR which initiates the response to intestinal injury is TLR4 which is located on the basolateral side of the epithelial cells and on monocytes in the lamina propria and recognizes LPS.<sup>31</sup> Stimulation of TLR4 results in the production of several inflammatory cytokines and chemokines.<sup>32</sup> These include IL-1 $\beta$  and IL-8, with TNF $\alpha$  being the key to the early inflammatory response, all of which are key neutrophil chemoattractants (Figure 1). Neutrophils must migrate across the vascular endothelium into the submucosa and then cross to the apical side of the injured epithelium to control the invading pathogens. Initially, upregulation of L, P and E selectins on the neutrophils and endothelial cells allows tethering of the neutrophils to the vascular endothelium.<sup>33,34</sup> The presence of chemoattractants, such as platelet activating factor (PAF) and IL-8, causes neutrophils to express  $\beta_2$  integrins which then bind to intercellular adhesion molecules (ICAMs) on the endothelium resulting in firm adhesion between the neutrophils and the endothelium (Figure 1).<sup>34</sup> Transendothelial

migration of neutrophils into the tissues occurs through the endothelial tight junctions and is mediated by platelet and endothelial cell adhesion molecule (PECAM-1) and binding to proteins in the extracellular matrix.<sup>33</sup> Once in the submucosa, neutrophils either phagocytose opsonized pathogens, or, if the pathogen is too large to engulf, release destructive enzymes such as proteases and their own ROMs into the extracellular matrix to kill it.<sup>35</sup>

Neutrophils also migrate between the epithelial cells to the luminal side of the mucosa to kill bacteria in the lumen. This entails the neutrophils adhering to the basolateral side of the intestinal epithelium, and migrating through the apical junction complex to the apical side of the epithelium (Figure 1).<sup>36</sup> Although this process is similar to endothelial migration, migration across epithelial cells is mainly stimulated by IL-8, whereas endothelial migration is dependant on IL-8 and PAF.<sup>37</sup> Again, the  $\beta$ 2 integrin CD11b/CD18 on neutrophils allows adhesion to ligands on the basolateral side of epithelial cells.<sup>38</sup> Such ligands have not been completely identified, but may include fucosylated glycoproteins.<sup>39</sup> Subsequently migration continues with binding of neutrophils to junctional adhesion molecules.<sup>40</sup>

Overall, the entire inflammatory response to mucosal damage is very tightly regulated in order to avoid unwanted injury, or an inappropriate response to commensal bacteria. Therefore, a binary system of control is in place whereby the presence of both microbial antigens and host triggers are necessary to allow inflammation to proceed.<sup>41</sup>

The presence of neutrophils can promote the recovery of the mucosal barrier. A previous study documented that the addition of neutrophils to ischemic-injured porcine mucosa enhanced the recovery as shown by an increase in transepithelial electrical resistance

(TER).<sup>42</sup> This beneficial effect was inhibited by treatment with an IL-1 $\beta$  inhibitor or a COX-2 selective inhibitor. As neutrophils release IL-1 $\beta$  which in turn up regulates COX-2, it is likely that the beneficial effect of neutrophils is achieved via up regulation of COX-2, and subsequent release of prostaglandins (PGs) which promote repair (Figure 1).<sup>42</sup>

### **Contribution of Neutrophils to Mucosal Injury**

Despite the importance of neutrophils in the innate immune response, their presence in ischemic-injured intestine results in further mucosal damage in two ways.

First, the proteases and ROMs released from activated neutrophils are indiscriminate and kill neighboring cells as well as the pathogen they are trying to destroy.<sup>19</sup> One particular protease, elastase, has been shown to promote neutrophil extravasation by digesting matrix components.<sup>43</sup> Cellular membranes contain large amounts of polyunsaturated fatty acids which are particularly susceptible to free radicals.<sup>44</sup> ROMs cause lipid peroxidation of cell membranes causing the release of peroxidation products such as malondialdehyde (MDA).<sup>45</sup> An increase in these products has been detected in equine jejunum following 2 hours of ischemia and 2 hours of reperfusion.<sup>46</sup> ROMs can further increase neutrophil adhesion and migration as they modify ligand binding and increase endothelial cell permeability.<sup>47</sup> Antioxidants such as superoxide dismutase protect cells from free radicals, but these are easily overwhelmed by the large volumes of superoxide, hydrogen peroxide and hypochlorous acid released by activated neutrophils.

Second, the physical act of neutrophils migrating between epithelial cells increases mucosal permeability in recovering intestine.<sup>48</sup> This study evaluated the effect of neutrophils on recovery of ischemic-injured porcine intestine. Electron microscopy revealed neutrophils

migrating between epithelial cells and widened intercellular spaces where neutrophils had previously migrated.<sup>48</sup> Migration of neutrophils disrupted epithelial barrier integrity as evidenced by a reduction in TER.<sup>48</sup> Similar results have been found in human epithelial cell culture. Neutrophil migration across T84 monolayers in response to a chemotactic gradient of fMLP resulted in a reduction in TER that was proportional to fMLP concentration, and neutrophil number.<sup>49</sup> The same group subsequently determined that this decrease in TER was due to the physical act of neutrophils separating the epithelial cells and was not due to cell death, or release of proteases or ROMs.<sup>50</sup> It is likely that elastase is important in degrading the transmembrane protein E-cadherin to open the epithelial adherens junction and hence allow neutrophil migration.<sup>51</sup>

The contribution of neutrophils to further damage after reperfusion has been shown by experimentally blocking each step of neutrophil activation, adhesion and migration, and documenting the attenuation in intestinal injury. These studies predominately use antagonists against specific chemoattractants,<sup>52-54</sup> monoclonal antibodies against adhesion molecules,<sup>52,54</sup> and polyclonal anti-neutrophil serum.<sup>55,56</sup>

### **Evidence of Neutrophilic Infiltration in Equine Ischemic Intestine**

Detection of neutrophils in the mucosa can be achieved by direct counting on histologic samples. However, identification of neutrophils on histologic sections stained with H&E can be difficult because of altered neutrophil morphology following migration into the tissues. Therefore, immunohistochemistry against myeloperoxidase<sup>57</sup> or calprotectin,<sup>58</sup> which are found in the neutrophil cytoplasm, can be used to aid identification. Alternatively,

measurement of myeloperoxidase concentration itself, can be performed in tissue or body fluids as an indicator of neutrophil activation.<sup>59,60</sup> However, one equine study found a poor correlation between neutrophil counts and tissue myeloperoxidase activity.<sup>61</sup>

Using calprotectin immunohistochemistry, it was shown that following 2 hours of colonic ischemia, neutrophil counts peaked in the subcolonic venules after 30 minutes of reperfusion.<sup>62</sup> Neutrophils were evident in close proximity to the vessel wall, or seen migrating through it, suggesting that transendothelial migration was occurring.<sup>62</sup> Neutrophil numbers in the mucosa peaked after 18 hours of reperfusion,<sup>62</sup> consistent with a possible role in the post-ischemic response to injury. This peak in neutrophilic infiltration after 18 hours of reperfusion has been previously documented in equine jejunum subjected to 2 hours of ischemic injury.<sup>63,64</sup> A previous study evaluating ischemia and reperfusion in the equine colon also documented an increase in neutrophils during ischemia, with a further increase after 3 hours of reperfusion.<sup>61</sup>

When the proximal resection margin of clinical cases of strangulating small intestinal obstruction were evaluated, it was found that there was a significant increase in neutrophil numbers compared to normal intestine,<sup>65</sup> and this occurred in all intestinal layers.<sup>58</sup> Additionally, when experimental strangulating small intestinal lesions were examined after 18 hours of reperfusion, neutrophil counts in all intestinal layers were further increased, and these cells were calprotectin positive, suggesting that the neutrophils were activated.<sup>58</sup>

Myeloperoxidase concentration in plasma and peritoneal fluid was found to be increased in horses with a strangulating intestinal lesion, compared to those with non strangulating lesions, at the time of admission to a referral hospital.<sup>66</sup> This suggests that

activation of neutrophils in the plasma and peritoneal fluid occurs in naturally occurring disease, and may be linked to the severity of the intestinal lesion. Tissue myeloperoxidase concentration was also found to be elevated in ileal resection margins in horses with distal small intestinal strangulations,<sup>65</sup> most likely because it is impossible to resect all injured intestine in these situations.

These findings suggest that neutrophils are activated in horses with ischemic intestine; with peak tissue infiltration occurring approximately 18 hours after blood flow is restored.

### **Therapeutic Approaches to Reduce Neutrophilic Infiltration**

Due to the association between an increase in mucosal neutrophil counts, and a reduction in mucosal barrier function,<sup>48</sup> treatments that could reduce neutrophil activation, adhesion and migration into the intestine have been sought.

The analgesics most commonly selected for horses with injured intestine are non steroidal anti-inflammatory drugs (NSAIDs), and flunixin meglumine in particular.<sup>67</sup> It would be expected that treatment with flunixin meglumine could potentially reduce neutrophilic infiltration of ischemic-injured mucosa. However, evaluation of neutrophil numbers in horses treated with saline, flunixin meglumine or etodolac found no effect of treatment.<sup>58,64</sup> In fact, treatment with certain NSAIDs, including flunixin meglumine, etodolac and meloxicam, may actually increase neutrophilic infiltration compared to treatment with saline solution, even when treatment is initiated prior to the onset of ischemia.<sup>63,64</sup> The effect of flunixin meglumine and etodolac can be explained by their

inhibition of COX-1 in horses, and hence a reduction in prostaglandins that are required for mucosal repair. Additionally, meloxicam, despite being a COX-2 selective NSAID, has been shown to cause gastric mucosal damage and increase oxygen free radicals.<sup>68,69</sup> Therefore it appears that NSAIDs do not have a direct effect on neutrophils, but may increase mucosal damage and hence neutrophilic infiltration.

During organ transplantation, loss of blood supply and then reperfusion occur in a similar way to that in intestinal reperfusion.<sup>70</sup> Therefore, solutions that are used for organ preservation may reduce injury in intestine that undergoes ischemia and then restoration of blood flow. Carolina rinse is a transplant solution composed of several substances that are each aimed at inhibiting further damage during reperfusion of the organ.<sup>71</sup> Intra-arterial and topical administration of this solution to ischemic equine jejunum resulted in a reduction in serosal neutrophil counts after 60 minutes of reperfusion, compared to treatment with lactated Ringer's solution.<sup>72</sup> This effect is most likely due to the osmotic reflection coefficient being maintained in ischemic sections treated with Carolina rinse,<sup>72</sup> therefore endothelial integrity is maintained and neutrophil diapedesis is reduced.

Local anesthetics are already in widespread veterinary use to provide analgesia by blocking voltage gated sodium channels. However, local anesthetics also modulate the inflammatory response, primarily by inhibiting many of the immune functions of neutrophils via mechanisms unrelated to sodium channel blockade.<sup>73,74</sup> *In vitro* assays to evaluate these different functions have found that lidocaine inhibited neutrophil adhesion, phagocytosis, and the production of free radicals in human neutrophils.<sup>75,76</sup> In addition, lidocaine has been shown to reduce the expression of endothelial adhesion molecules, which is the first step in

migration of neutrophils into the intestine.<sup>77</sup> Systemic lidocaine has also been used in horses, as a promotility agent in colic patients to treat postoperative ileus.<sup>78-80</sup> Although the mechanism of action of lidocaine in reducing ileus has not yet been evaluated, it is probable that its benefit arises from anti-inflammatory effects rather than it acting as a prokinetic. There has been limited investigation into the effectiveness of lidocaine in the management of ischemic-injured intestine. However, in a canine model of cardiac ischemia, treatment with lidocaine was shown to reduce neutrophil infiltration.<sup>81</sup> To date, only two studies have evaluated the effect of lidocaine on ischemic intestine. These studies both investigated the use of epidural lidocaine in rabbits, and documented a beneficial effect of treatment on post ischemic motility and mucosal pH.<sup>82,83</sup> We have recently evaluated the effect of lidocaine on neutrophil counts in equine ischemic-injured intestine after 18 hours of recovery (Chapter 3). In agreement with previous findings,<sup>63</sup> neutrophilic infiltration was greatest in ischemic-injured mucosa from horses treated with flunixin meglumine. Interestingly, when this treatment was combined with lidocaine, mucosal neutrophil counts were reduced, and were not significantly different from control mucosa.

It is also possible to inhibit neutrophil infiltration by specifically blocking each step in the recruitment of neutrophils to ischemic tissue. Blockade of the initial chemotactic signals for neutrophil migration has been performed with the use of 2 different antagonists against LTB<sub>4</sub> receptors. These antagonists were administered after the induction of small intestinal ischemia in rats, and were effective in reducing neutrophil accumulation in the intestine and its mesentery.<sup>84</sup> Alternatively, it is possible to block selectin or integrin dependant interactions of neutrophils. In a porcine model of ischemic intestinal injury,

pretreatment with CD11/CD18 monoclonal antibodies resulted in a reduction in neutrophilic infiltration and an improvement in mucosal barrier function.<sup>48</sup> Intravenous administration of a monoclonal antibody against ICAM-1, which is required for neutrophil diapedesis, was also shown to reduce neutrophil infiltration into ischemic injured small intestine in rats.<sup>85</sup> Although specific monoclonal antibodies are in use to treat various diseases,<sup>86,87</sup> none are currently in use which specifically target neutrophils. Additionally, such treatment could have widespread effects and result in unwanted side effects, and most are prohibitively expensive for therapeutic use in veterinary patients.

An alternative to using treatments that directly affect neutrophils, is to reduce ischemic tissue damage and hence the signals that stimulate neutrophil recruitment. Classically, it is believed that reperfusion injury occurs due to the generation of free radicals by tissue xanthine oxidase when blood supply is restored.<sup>88</sup> However, the importance of this phase and indeed whether it occurs in equine intestine is unclear.<sup>89,90</sup> Despite this, antioxidants and free radical scavengers have been evaluated in models of equine intestinal ischemia. Dimethyl sulfoxide (DMSO) a hydroxyl radical scavenger, may reduce injury after reperfusion when used at a low dose of 20mg/Kg,<sup>91</sup> whereas a higher dose of 1g/Kg may be detrimental.<sup>92</sup> Other antioxidants including ascorbic acid, and acetylcysteine have been evaluated in equine intestine *in vitro*.<sup>93,94</sup> However, none of these studies examined whether treatment reduced neutrophilic infiltration.

Therefore, the only treatment currently identified which could be used in clinical cases to reduce neutrophilic infiltration after intestinal ischemic injury may be systemically administered lidocaine. However, it is important to realize that this does not reduce

neutrophilic influx compared to saline treatment, but does ameliorate the inflammatory effects of flunixin meglumine, which may allow flunixin meglumine to be used without detriment. The mechanism by which lidocaine achieves this effect is unclear. It is possible that it has direct effects on neutrophils to reduce adhesion and migration, or it may reduce the production of inflammatory enzymes, such as COX-2, as we document in Chapter 3. When attempting to reduce neutrophilic inflammation, caution must be exercised as a global reduction in neutrophil function, and hence increased susceptibility to infection, may occur.

### **Conclusions**

Improving the recovery of injured intestine *in situ* is likely to reduce the short term mortality of horses after colic surgery. Neutrophils are recruited to injured intestine in response to a breach of the mucosal barrier, with peak inflammation occurring after 18 hours of reperfusion. However, neutrophils result in further damage by releasing free radicals at the site of injury, and by widening paracellular spaces as they migrate.<sup>95</sup> Therefore, inhibition of neutrophil influx is an important therapeutic target. However, to date, a viable therapeutic option is not available. Systemically administered lidocaine may reduce neutrophilic influx in horses treated with flunixin meglumine, but its precise mechanism of action is unclear.

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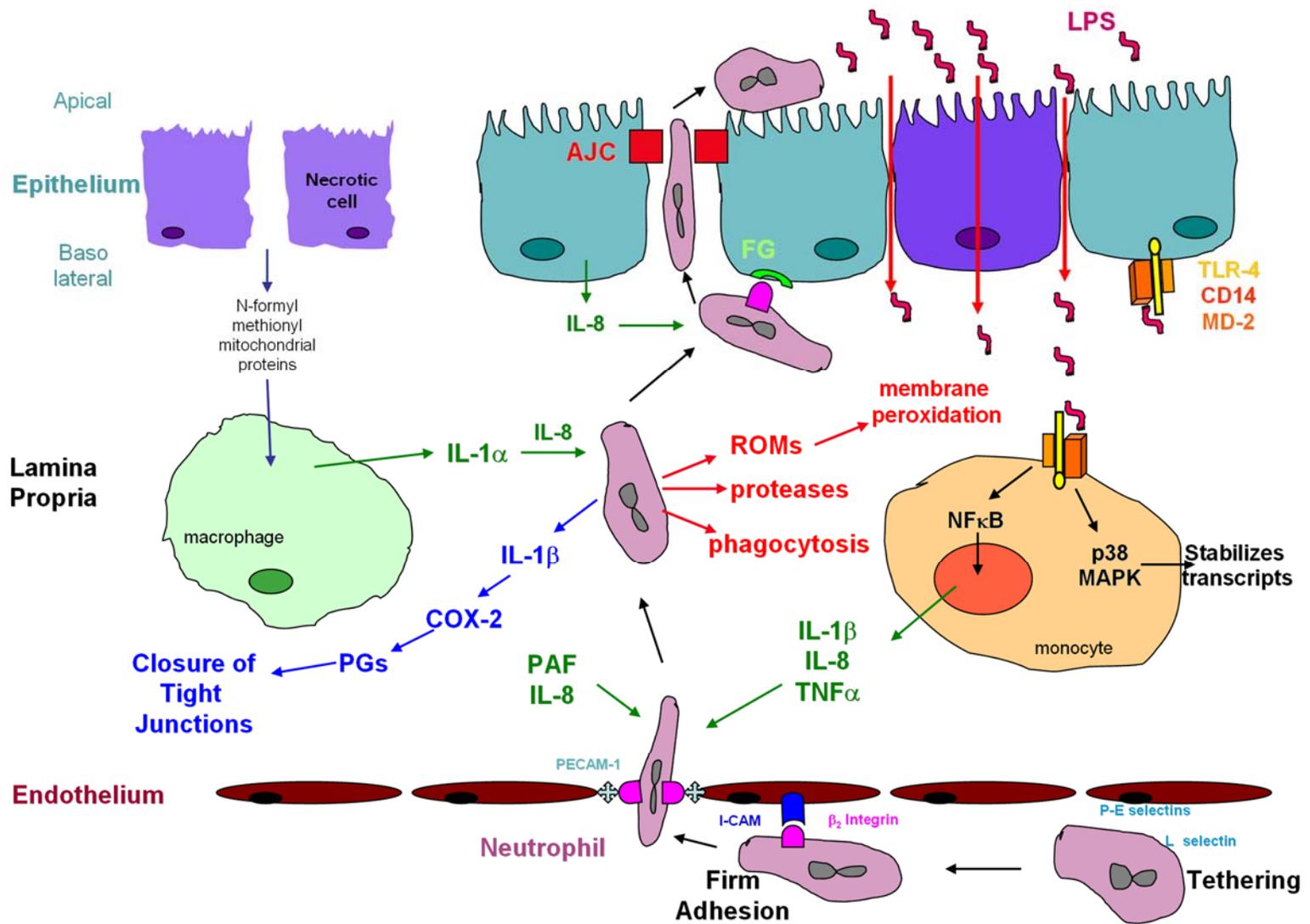
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**Figure 1:** Recruitment of Neutrophils to Injured Mucosa. Injury to the mucosa allows LPS to access the lamina propria where it triggers the production of neutrophil chemoattractants (green) via TLR signaling and the NF $\kappa$ B pathway. Necrotic cells also stimulate neutrophil recruitment via release of N-formylated mitochondrial proteins. Neutrophils cause further damage through the release of ROMs and proteases (red) and migration through tight junctions, but also contribute to mucosal repair via production of PGs (blue).

**Abbreviations:**

AJC	Apical Junction Complex
LPS	Lipopolysaccharide
PGs	Prostaglandins
TLR-4	Toll-like Receptor 4
PAF	Platelet Activating Factor
IL	Interleukin
ROMs	Reactive Oxygen Metabolites
ICAM	Intercellular Adhesion Molecule
PECAM	Platelet and Endothelial Cell Adhesion Molecule
TNF	Tumor Necrosis Factor
FG	Fucosylated glycoproteins



## **CHAPTER 3**

### **Attenuation of Ischaemic Injury in the Equine Jejunum by Administration of Systemic Lidocaine**

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## Attenuation of ischaemic injury in the equine jejunum by administration of systemic lidocaine

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**Keywords:** horse; colic; ischaemia; lidocaine; mucosal repair

### Summary

**Reasons for performing study:** Absorption of endotoxin across ischaemic-injured mucosa is a major cause of mortality after colic surgery. Recent studies have shown that flunixin meglumine retards mucosal repair. Systemic lidocaine has been used to treat post operative ileus, but it also has novel anti-inflammatory effects that could improve mucosal recovery after ischaemic injury.

**Hypothesis:** Systemic lidocaine ameliorates the deleterious negative effects of flunixin meglumine on recovery of mucosal barrier function.

**Methods:** Horses were treated i.v. immediately before anaesthesia with either 0.9% saline 1 ml/50 kg bwt, flunixin meglumine 1 mg/kg bwt every 12 h or lidocaine 1.3 mg/kg bwt loading dose followed by 0.05 mg/kg bwt/min constant rate infusion, or both flunixin meglumine and lidocaine, with 6 horses allocated randomly to each group. Two sections of jejunum were subjected to 2 h of ischaemia by temporary

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occlusion of the local blood supply, via a midline celiotomy. Horses were monitored with a behavioural pain score and were subjected to euthanasia 18 h after reversal of ischaemia. Ischaemic-injured and control jejunum was mounted in Ussing chambers for measurement of transepithelial electrical resistance (TER) and permeability to lipopolysaccharide (LPS).

**Results:** In ischaemic-injured jejunum TER was significantly higher in horses treated with saline, lidocaine or lidocaine and flunixin meglumine combined, compared to horses treated with flunixin meglumine. In ischaemic-injured jejunum LPS permeability was significantly increased in horses treated with flunixin meglumine alone. Behavioural pain scores did not increase significantly after surgery in horses treated with flunixin meglumine.

**Conclusions:** Treatment with systemic lidocaine ameliorated the inhibitory effects of flunixin meglumine on recovery of the mucosal barrier from ischaemic injury, when the 2 treatments were combined. The mechanism of lidocaine in improving mucosal repair has not yet been elucidated.

**Potential relevance:** Recovery of ischaemic-injured jejunum in post operative colic cases may be improved when treatment with flunixin meglumine is combined with lidocaine.

## **Introduction**

Colic has a major impact due largely to the high fatality rate, estimated at 11% based on data from the National Animal Health Monitoring System (Traub-Dargatz *et al.* 2001). In

particular, horses with colic due to a strangulating lesion have a reduced prognosis for survival compared to horses with a simple obstruction (Mair and Smith 2005). The continued presence of ischaemic intestine after surgery has also been shown to reduce short term survival to 33.4% compared to 85.8% in horses in which all ischaemic intestine is resected (Mair and Smith 2005). Injured intestine may remain because it is impossible to resect, is difficult to identify (Freeman *et al.* 1988) and because intraluminal distension alone can cause ischaemic injury (Dabareiner *et al.* 2001). Even just 50 min of ischaemia has been shown to cause sufficient mucosal damage to allow systemic absorption of endotoxin upon reperfusion (Moore *et al.* 1981).

Flunixin meglumine is frequently administered to post operative colic cases to provide analgesia and ameliorate the production of prostaglandins (PGs) associated with endotoxaemia by inhibiting the cyclooxygenase (COX) enzyme (Baskett *et al.* 1997). However, PGs are essential for repair of ischaemic-injured mucosa (Blikslager *et al.* 1997; Campbell and Blikslager 2000) and treatment with nonselective COX inhibitors, such as flunixin meglumine, impairs recovery of barrier function (Tomlinson *et al.* 2004; Tomlinson and Blikslager 2004, 2005; Little *et al.* 2007). Therefore, alternative anti-inflammatory drugs must be evaluated that could replace NSAIDs used currently or be used in combination with them to ameliorate their negative effects on recovery of ischaemic-injured intestine.

Lidocaine is used widely as a local anaesthetic, but has been recently administered systemically in horses to treat post operative ileus (Brianceau *et al.* 2002; Cohen *et al.* 2004; van Hoogmoed *et al.* 2004; Malone *et al.* 2006). The exact mechanism of action of lidocaine in reducing ileus is not known, but it appears to lack a direct prokinetic effect (Nieto *et al.*

2000; Milligan *et al.* 2007), suggesting that other actions probably contribute to its apparent therapeutic effect. It is known that local anaesthetic agents modulate the inflammatory response via mechanisms unrelated to sodium channel blockade (Hollmann and Durieux 2000). Lidocaine has been shown to reduce secretion of inflammatory cytokines (Lahav *et al.* 2002) and inhibit neutrophil function (Lan *et al.* 2004). Additionally, in studies evaluating its effect on ischaemia and reperfusion injury in other organs, lidocaine has been shown to reduce lipid peroxidation attributable to oxidant release (Lantos *et al.* 1996) and inhibit neutrophil adhesion and migration (Schmid *et al.* 1996) Superoxide radicals have been implicated in reoxygenation injury of the equine jejunum (Johnston *et al.* 1991) and neutrophils have been found to accumulate in the equine large colon during low-flow ischaemia and reperfusion (Johnston *et al.* 1991; Moore *et al.* 1994). However, the effect of lidocaine on ischaemic-injured intestine has not yet been evaluated.

Therefore, the objective of this study was to investigate the effects of systemic lidocaine, the non selective COX inhibitor flunixin meglumine and the 2 drugs combined on recovery of mucosal barrier function in ischaemic-injured equine jejunum. It was hypothesised that treatment with systemic lidocaine would ameliorate the deleterious effects of flunixin meglumine on intestinal repair, while providing effective analgesia.

## **Materials and methods**

### *Horses*

The experimental protocol was approved by the North Carolina State University Institutional Animal Care and Use Committee. Twenty-four horses of either sex, age 3–24 years,

weighing 382–605 kg with no history of gastrointestinal problems were used in this study. All horses first underwent a 2 week quarantine period, were vaccinated and received anthelmintic treatment. Horses were housed in individual stables with access to *ad libitum* hay and water. Immediately before surgery, a complete clinical examination was performed and horses evaluated for signs of pain using a previously established equine behavioural pain scoring system (Pritchett *et al.* 2003).

### *Surgical procedures*

Horses were premedicated with xylazine (1.1 mg/kg bwt, i.v.), an i.v. catheter placed in the left jugular vein and ceftiofur sodium (2.2 mg/kg bwt, i.v.) administered. Horses were assigned randomly to one of 4 treatment groups, with 6 horses in each group: 1) 0.9% saline 1 ml/50 kg bwt i.v. every 24 h; 2) flunixin meglumine 1 mg/kg bwt i.v. every 12 h; 3) lidocaine 1.3 mg/kg bwt loading dose administered over 15 min, followed by 0.05 mg/kg bwt/min constant rate infusion; and 4) both flunixin meglumine and lidocaine at the previously described doses.

Anaesthesia was induced using diazepam (0.1 mg/kg bwt, i.v.) and ketamine (3 mg/kg bwt, i.v.). The horses were intubated orotracheally and a surgical plane of anaesthesia maintained with isoflurane vapourised oxygen. Treatment with flunixin meglumine or saline was administered immediately prior to induction of anaesthesia, while the loading dose of lidocaine was administered immediately after the horse was placed on gas anaesthesia. Butorphanol (0.05 mg/kg bwt) was administered immediately i.v. after induction and subsequently i.m. every 6 h, for additional analgesia. Using aseptic technique, a midline

celiotomy was performed and the ileum and distal jejunum located. Two 30 cm sections of jejunum approximately 1 m apart, with the most aboral one located 60 cm orad to the antimesenteric band of the ileum, were isolated with Doyen forceps to prevent blood flow from collateral segments. Kelly haemostats, placed over a section of a Penrose drain to minimise damage to the blood vessels, were used to occlude the local jejunal blood supply to these 2 sections for 2 h. Following 2 h of ischaemia, the clamps were removed and the abdomen closed routinely.

Following recovery from anaesthesia, each horse was moved to a stable and monitored using the equine behavioural pain scoring system, and clinical examinations were recorded at 4, 8 and 16 h after recovery from anaesthesia. Horses were allowed access to water immediately and were offered small handfuls of hay. Horses in *Groups 3* and *4* were given an additional loading dose of lidocaine over 15 min on return to the stall, prior to recommencing the constant rate infusion. Horses in *Groups 2* and *4* received an additional dose of flunixin meglumine 12 h after the initial treatment. All horses were subjected to euthanasia with an overdose of sodium pentobarbital (100 mg/kg bwt, i.v.) 18 h after the end of ischaemia. Sections of both ischaemic-injured and adjacent uninjured control jejunum were collected immediately after euthanasia, cut longitudinally along the antimesenteric border and placed into preoxygenated equine Ringer's solution for use in the *in vitro* part of the study.

#### *Serum lidocaine measurements*

Heparinised venous blood samples were obtained for measurement of plasma lidocaine

concentration immediately after administration of the initial loading dose, at the end of the ischaemic period, after the second loading dose, and at 8 and 16 h after the end of ischaemia. Plasma lidocaine concentration was determined by high pressure liquid chromatography with ultraviolet detection.

#### *Ussing chamber studies*

The mucosa was stripped from the seromuscular layer of ischaemic-injured and control jejunum in oxygenated equine Ringer's solution and mounted in 3.14 cm<sup>2</sup> aperture Ussing chambers within 20 min, as described in previous studies (Argenzio *et al.* 1993; Blikslager *et al.* 1997). Tissues were bathed on the serosal and mucosal sides with 10 ml equine Ringer's solution with 10 mmol/l glucose added to the serosal side, osmotically balanced on the mucosal side with 10 mmol/l mannitol. The Ringer's bathing solution was maintained at 37°C with circulating water-jacketed reservoirs and was oxygenated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes and the PD was short-circuited through silver-silver chloride electrodes using a voltage clamp that corrected for fluid resistance. Following an initial 15 min equilibration period, short-circuit current (I<sub>sc</sub>) and PD were recorded every 15 min for 3 h. The transepithelial electrical resistance (TER) ( $\Omega \cdot \text{cm}^2$ ) was calculated from the I<sub>sc</sub> and PD using Ohm's law. If the spontaneous PD was between -1.0 and 1.0mV, a current clamp of 100 $\mu$ A was applied briefly and the PD recorded, to increase the accuracy of the measurement.

### *Lipopolysaccharide (LPS) flux*

After a 30 min equilibration period, mucosal-to-serosal LPS fluxes were performed by adding 83 µg of lipopolysaccharide from *Escherichia coli* serotype 0111:B4 labelled with fluorescein isothiocyanate<sup>1</sup> (FITC-LPS) to the mucosal bathing solutions of control and ischaemic-injured mucosa, and monitoring its appearance in the serosal bathing solutions. Aliquots of 200 µl were collected in triplicate from the serosal side after 15 min (baseline) and after 1 and 2 h, and assessed for fluorescence. Following the 2 h flux period, mucosa from the Ussing chamber was placed in 10% neutral buffered formalin for 24 h before being transferred to 70% ethanol, while protecting the samples from light. Unstained sections (5 µm thick) were cut for examination under a light microscope with epifluorescence to evaluate the location of the FITC-LPS subjectively.

### *Statistical analysis*

Heart rate and temperature were compared by use of a 2-way repeated-measures ANOVA for the effects of treatment and time from 0–16 h. TER were tested by use of a 2-way repeated-measures ANOVA for the effect of treatment and ischaemia over time. When a significant time and treatment interaction was detected, a one-way ANOVA was used to identify the source of the interaction and a *post hoc* pairwise multiple comparison procedure (Fisher LSD method) was performed. Behavioural pain scores were analysed by an ANOVA on ranks for the effects of treatment and time from 0–16 h. When a significant time and treatment interaction was detected, a one-way ANOVA was used to identify the source of the interaction and a *post hoc* Tukey pairwise multiple comparison procedure was performed.

Flux data were tested by use of a 1-way ANOVA, with a *post hoc* pairwise multiple comparison procedure (Fisher LSD method) to determine the effect of treatment and loop (control vs. ischaemic-injured). For each statistical test, a power analysis was performed and determined to be  $\geq 0.94$  in all analyses. For all tests, significance was set at  $P < 0.05$ .

## **Results**

### *Clinical parameters and behavioural pain scores*

During the course of the study, no horse demonstrated gross signs of pain, nor required additional analgesia. Median behavioural pain scores were 0 in all groups prior to surgery. Horses treated with flunixin meglumine, either alone or combined with lidocaine, had no significant change in behavioural pain score after surgery compared to preoperative scores. However, horses treated with saline or lidocaine alone had a significant increase in behavioural pain scores at 4 and 8 h after ischaemia compared to those before surgery ( $P < 0.05$ ) (Fig 1). Additionally, at no time were scores in horses treated with lidocaine significantly different to those of horses treated with saline. At 4, 8 and 16 h after ischaemia, horses treated with flunixin meglumine and lidocaine had significantly lower behavioural pain scores than horses treated with saline at that time point ( $P < 0.05$ ).

Temperature was not significantly different between groups at any time point. However, temperature was increased significantly in all groups, 8 and 16 h after ischaemia, compared to before surgery, regardless of treatment ( $P < 0.05$ ).

Heart rate was not significantly different between treatment groups prior to surgery, nor at any time point after surgery. Heart rate was significantly increased in all groups at 4, 8 and

16 h after ischaemia compared to before surgery, regardless of treatment.

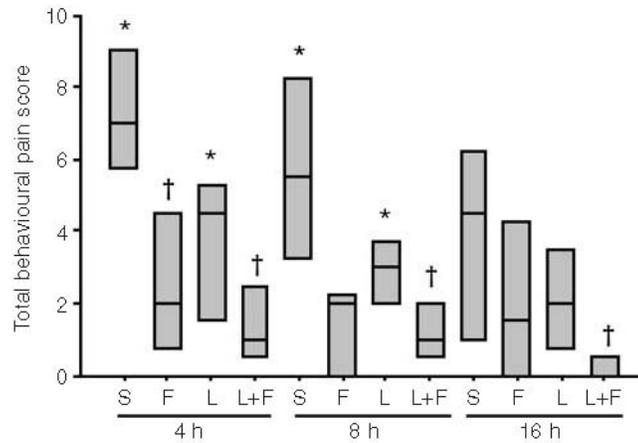


Fig 1: Total behavioural pain score for horses treated with saline 1 ml/ 50 kg bwt (S), flunixin meglumine 1 mg/kg bwt every 12 h (F), lidocaine 1.3 mg/kg bwt loading dose followed by 0.05 mg/kg bwt/min constant rateinfusion (L), or both flunixin meglumine and lidocaine at the previous doses (L+F) at 4, 8 and 16 h after the end of ischaemia. Lines in the boxes represent the median behavioural pain score, while the upper and lower limits of the box represent the 75th and 25th percentile. Median behavioural pain score in all groups prior to surgery was 0. \* Value is significantly increased ( $P<0.05$ ) compared to preoperative score for that treatment group. † Value is significantly lower ( $P<0.05$ ) than the score for saline treated horses at that time point after ischaemia.

### Serum lidocaine concentration

No horse showed clinical signs of lidocaine toxicity. Serum lidocaine concentration in horses treated with lidocaine was above the minimum therapeutic concentration of 0.98  $\mu\text{g/ml}$  at all times. Lidocaine concentration was highest after 2 h of ischaemia (mean  $\pm$  s.e.  $4.11 \pm 0.02$   $\mu\text{g/ml}$ ).

### Assessment of transepithelial electrical resistance (TER) (Fig 2)

There was a significant effect of time and treatment on TER ( $P < 0.001$ ). There was no significant difference in TER of control tissue between treatment groups over the entire period. There was no significant difference in TER of ischaemic-injured tissue between horses treated with saline, lidocaine or lidocaine and flunixin meglumine combined, over the recovery period. However, TER of ischaemic-injured tissue from horses treated with only flunixin meglumine was significantly lower than that of ischaemic-injured tissue from horses treated with saline ( $P = 0.016$ ), lidocaine ( $P = 0.005$ ) or lidocaine and flunixin meglumine combined ( $P = 0.008$ ) over the entire period.

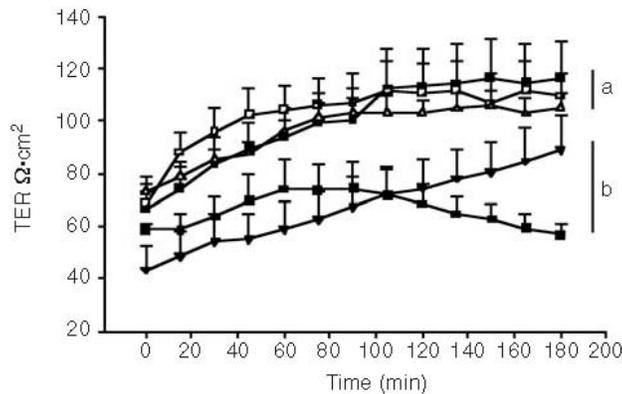


Fig 2: Mean  $\pm$  s.e. transepithelial electrical resistance (TER) of control and ischaemic-injured jejunum from horses treated with saline 1 ml/50 kg bwt, flunixin meglumine 1 mg/kg bwt every 12 h, lidocaine 1.3 mg/kg bwt loading dose followed by 0.05 mg/kg bwt/min constant rate infusion, or both flunixin meglumine and lidocaine at the previous doses, 18 h after the end of ischaemic injury. There was no significant difference in TER of control jejunum between treatment groups, therefore only saline treated control values are shown for clarity. TER of ischaemic-injured jejunum from horses treated with flunixin meglumine was significantly lower than ischaemic-injured jejunum from all other treatment groups over the entire recovery period. ■ = control-saline; ○ = ischaemic-lidocaine; ● = ischaemic lidocaine and flunixin; ▼ = ischaemic-flunixin; Δ = ischaemic-saline.

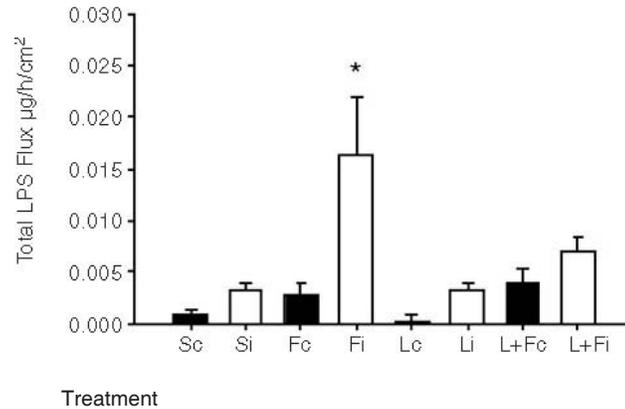


Fig 3: Total  $\mu\text{g}$  of lipopolysaccharide (LPS) flux/h/cm<sup>2</sup> mucosa in horses treated with saline 1 ml/50 kg bwt (S), flunixin meglumine 1 mg/kg bwt every 12 h (F), lidocaine 1.3 mg/kg bwt loading dose followed by 0.05 mg/kg bwt/min constant rate infusion (L), or both flunixin meglumine and lidocaine at the previous doses (L+F) across control (black bars) and ischaemic-injured (white bars) jejunum. \* = LPS flux is significantly increased across ischaemic-injured mucosa from horses treated with flunixin meglumine compared to control mucosa in all treatment groups and compared to ischaemic-injured tissue in horses treated with saline ( $P < 0.001$ ) lidocaine ( $P < 0.001$ ) and lidocaine and flunixin combined ( $P = 0.005$ )

#### Lipopolysaccharide (LPS) flux (Fig 3)

The total flux of FITC-LPS in  $\mu\text{g}/\text{h}/\text{cm}^2$  of mucosa was significantly increased in ischaemic-injured mucosa from horses treated with only flunixin meglumine compared to all control groups and ischaemic-injured mucosa in all other groups. In no other treatment group was there a significant difference in LPS flux between ischaemic-injured and control tissue. With microscopy of mucosa from the Ussing chambers, epifluorescence was predominately visible on the villous tips. Qualitatively, it appeared that FITC-LPS had entered the *lamina propria* of the samples from horses treated with flunixin meglumine.

## Discussion

Intravenous administration of lidocaine resulted in serum concentrations within the therapeutic range at all times except at the end of ischaemia. Previous studies in horses have documented a peak in lidocaine concentration at the end of the loading dose in anaesthetised horses (Feary *et al.* 2005, 2006). However, in the present study, peak lidocaine concentration was documented at the end of the ischaemic period, approximately 2.5 h after the loading dose was given. The same dose has been shown to result in higher serum concentrations of lidocaine in anaesthetised compared to conscious horses, possibly as a result of decreased cardiac output and hepatic blood flow (Feary *et al.* 2005) and may explain the peak in serum concentration that was documented in the present study towards the end of the anaesthetic period. Mean lidocaine concentration at this time point was above the mean toxic concentration reported in horses of 3.24 µg/ml (Meyer *et al.* 2001); however, no signs of toxicity were observed in these horses.

In this study, flunixin meglumine and lidocaine was administered prior to the onset of ischaemia. This early treatment was considered to be the best method to evaluate the effect of a drug in a model situation, as it amplifies the differences in drug treatment. In the clinical situation, intestinal ischaemia probably occurs prior to treatment with flunixin meglumine or lidocaine. Therefore, a clinical trial to evaluate the effect of these drugs in clinical cases is the next logical step.

This model was designed to evaluate the effect of flunixin meglumine and lidocaine on recovery of ischaemic-injured jejunum *in vivo*. To evaluate this process accurately *in vitro*, the time from tissue collection to mounting it in the Ussing chambers was minimised. The

process of harvesting the tissue and stripping the mucosa from the seromuscular layer has been previously shown to stimulate the generation of endogenous prostaglandins which could affect *in vitro* results (Argenzio and Liacos 1990). In mucosa harvested from horses treated with flunixin meglumine, residual tissue levels of the drug could reduce this endogenous release of prostaglandins and influence *in vitro* results. However, *in vitro* measurements are essential to assess recovery of the mucosal barrier after ischaemic injury objectively.

The present study confirmed that administration of flunixin meglumine to horses retards repair of ischaemic-injured jejunum, compared to treatment with saline solution (Tomlinson *et al.* 2004; Little *et al.* 2007). This reduction in barrier function was evidenced by a significant decrease in TER, and increase in LPS permeability in ischaemic-injured mucosa from horses treated with flunixin meglumine alone. However, by combining treatment of flunixin meglumine with systemically administered lidocaine, the effects of flunixin meglumine on recovery of ischaemic injured jejunum were ameliorated. Ischaemic-injured mucosal TER was not significantly different between horses treated with saline, and flunixin meglumine and lidocaine combined. Additionally, LPS flux across ischaemic-injured mucosa in horses treated with both lidocaine and flunixin meglumine was not significantly different from control mucosa.

Flunixin meglumine is a nonselective COX inhibitor and, therefore, prevents the COX-1 dependant production of PGs necessary for mucosal repair in ischaemic-injured intestine (Blikslager *et al.* 1997). The mechanism by which lidocaine overcomes the inhibitory effect of flunixin meglumine on recovery of intestinal barrier function is unknown. It is possible that administration upregulates production of COX-1, allowing sufficient production of PGs

to promote mucosal repair. Alternatively, the novel anti-inflammatory effects of lidocaine, particularly inhibition of neutrophil activation and migration (Schmid *et al.* 1996), could reduce mucosal damage during the 18 h recovery period.

Providing effective analgesia is a critical part of a post operative treatment regime (Sellon *et al.* 2004). Use of our behavioural pain scoring system allowed a more objective method for evaluation of pain and determining the visceral analgesic effects of different treatments. Evidence of pain in all horses was subtle and could be detected only by monitoring changes in the horse's interaction with its environment, people and other horses. Treatment with flunixin meglumine alone or combined with lidocaine prevented a significant increase in pain score after surgery, confirming its effectiveness as a visceral analgesic. However, horses treated with lidocaine, without the inclusion of flunixin meglumine, had a significant increase in behavioural pain scores at 4 and 8 h after surgery, compared to their scores prior to surgery. Median pain scores in horses treated with lidocaine were lower than scores in horses treated with saline, at all time points after surgery, but this difference was not statistically significant. This lack of statistical difference may be due to the small sample size, but these findings are supported by those from a previous study, in which systemic lidocaine was found to be an effective somatic analgesic, but was not an effective visceral analgesic (Robertson *et al.* 2005). The current findings suggest that lidocaine should not be relied upon as the sole method to provide post operative analgesia.

In conclusion, the hypothesis is accepted that systemic lidocaine ameliorated the negative effects of flunixin meglumine on recovery of ischaemic-injured jejunum. When these drugs are used together, the analgesic benefits of flunixin meglumine can be obtained, without it

impairing recovery of the mucosal barrier. This could improve the outcome of colic cases in the immediate post operative period. However, the mechanism by which lidocaine ameliorates the effects of flunixin meglumine remains to be elucidated.

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### **Manufacturer's address**

<sup>1</sup>Sigma-Aldrich, St Louis, Missouri, USA

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## **CHAPTER 4**

### **Anti-Inflammatory Effects of Systemically Administered Lidocaine in Ischemic-Injured Equine Jejunum**

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## **Abstract**

**Objective-** To determine the mechanism by which systemically administered lidocaine ameliorates the deleterious effects of flunixin meglumine on recovery of ischemic-injured equine jejunum.

**Animals-** Jejunal biopsies and plasma from 24 horses participating in a previous study.

**Procedures-** Jejunum was obtained from horses treated IV with 0.9% saline 1ml/50kg, flunixin meglumine 1mg/kg every 12 hours, lidocaine 1.3mg/kg loading dose then 0.05mg/kg/min constant rate infusion, or both flunixin meglumine and lidocaine (n=6/group). Full thickness jejunal biopsies collected from uninjured and ischemic-injured jejunum after 2 hours of ischemia and 18 hours after reversal of ischemia, were analysed for histopathologic grade of injury, villus denudation, and mucosal neutrophil counts.

Concurrent mucosal biopsies were immunoblotted for cyclooxygenase (COX)-1 and 2.

Plasma was collected prior to ischemia and 8 hours after ischemia, and analyzed for thromboxane B<sub>2</sub> (TXB<sub>2</sub>) and prostaglandin E<sub>2</sub> metabolite (PGEM) concentration.

**Results-** No difference was detected between treatment groups in histopathologic grade of injury or epithelial restitution. After 18 hours of recovery, neutrophil counts were significantly elevated in ischemic-injured mucosa from horses treated with flunixin meglumine alone compared to all other treatment groups. After 2 hours of ischemia, COX-2 expression was significantly decreased in horses treated with lidocaine compared to horses treated with saline or flunixin meglumine. Flunixin meglumine and lidocaine, alone or combined, prevented the post-ischemic increase in PGEM found in saline treated horses. Saline or lidocaine alone, allowed a post-ischemic increase in TXB<sub>2</sub>.

**Conclusions and Clinical Relevance-** Systemically administered lidocaine reduced mucosal neutrophil counts and COX-2 expression. When used in combination with flunixin meglumine, the inflammatory effects of flunixin meglumine on mucosal repair are ameliorated.

## Introduction

A survey of diplomates of the American Colleges of Veterinary Internal Medicine and Veterinary Surgeons identified gastrointestinal compromise as a major cause of endotoxemia in horses, and flunixin meglumine as the non steroidal anti-inflammatory drug most frequently administered to these cases.<sup>1</sup> Administration of flunixin meglumine has been documented to alleviate many of the clinical signs of endotoxemia, including elevated heart rate, respiratory rate, fever and colic.<sup>2,3</sup> The effect of flunixin meglumine is primarily achieved through inhibition of inflammatory prostaglandins produced via cyclooxygenase enzymes.<sup>4</sup> However, several previous studies have shown that treatment with flunixin meglumine inhibits the recovery of ischemic-injured jejunum,<sup>5-7</sup> because these prostaglandins are also essential for mucosal repair.<sup>8</sup> Therefore, the ideal treatment for horses with compromised intestinal mucosa would be one that ameliorates the inflammation and pain associated with endotoxemia, whilst still permitting recovery of injured intestine.

We have previously determined that when treatment with flunixin meglumine is combined with systemic administration of lidocaine, the inhibitory effects of flunixin meglumine on recovery of ischemic-injured mucosa are avoided, whilst effective analgesia is provided.<sup>9</sup> Systemically administered lidocaine is primarily used in equine hospitals as a treatment for post-operative ileus.<sup>10</sup> However, in other species, it has been shown to reduce ischemic injury in the heart and brain.<sup>11-13</sup>

The mechanism by which lidocaine exerts its beneficial effects in ischemic injury is not completely understood. *In vitro* studies have shown that incubation of neutrophils with lidocaine reduces neutrophil respiratory burst,<sup>14</sup> adhesion and phagocytosis.<sup>15,16</sup> Neutrophilic

infiltration has been found in equine intestine after ischemic injury<sup>17,18</sup> and is thought to contribute to intestinal injury after ischemia.<sup>19</sup> We have previously shown that treatment with flunixin meglumine increases mucosal inflammation as evidenced by an increase in mucosal neutrophil counts in ischemic-injured jejunum, compared to treatment with saline solution.<sup>6</sup> Therefore, lidocaine could inhibit activation and infiltration of neutrophils into ischemic-injured intestine in horses treated with flunixin meglumine.

Given that the detrimental effects of flunixin meglumine on mucosal recovery are due to inhibition of COX-1, an alternative theory is that lidocaine upregulates this enzyme and allows sufficient production of PGs for mucosal healing. However, a previous study found that local administration of lidocaine prior to tooth extraction resulted in no effect on COX-1 gene expression and production of COX-1 associated TXB<sub>2</sub>.<sup>20</sup> Alternatively, lidocaine may reduce COX-2 expression. This same study did evaluate the effect of lidocaine on COX-2 expression and COX-2 associated PGE<sub>2</sub> production, but due to the impossibility of performing tooth extraction without local anesthetic, a negative control could not be included, so it was not possible to determine if there was an effect of lidocaine on COX-2.<sup>20</sup>

Our objective was therefore to determine the mechanism by which lidocaine ameliorates the inhibitory effect of flunixin meglumine on recovery of ischemic-injured equine jejunum. We hypothesized that treatment with systemic lidocaine would reduce mucosal inflammation after ischemic injury in horses treated with flunixin meglumine. To evaluate this, we analyzed histologic indices of mucosal healing and inflammation, and the expression of COX-1 and -2, and their associated prostanoids.

## **Materials and Methods**

**Horses-** The samples used in this study were obtained from horses participating in a previous study.<sup>9</sup> All procedures were approved by the North Carolina State University Institutional Animal Care and Use Committee. Twenty four horses (either sex, age 3-24 years, weight 382–605 kg) with no history of gastrointestinal problems were used after a two week quarantine period. Physical examinations performed prior to surgery were within normal limits. Horses were housed in individual stalls with access to free choice hay and water.

**Surgical Procedures-** Immediately before surgery each horse was premedicated with xylazine (1.1 mg/kg IV), and an intravenous catheter placed in the left jugular vein. One dose of ceftiofur sodium (2.2 mg/kg IV) was administered prophylactically. Each horse was randomly assigned to one of the following treatment groups (n= 6 horses/ group): 1) 1ml/50kg 0.9% saline IV every 24 hours, 2) 1mg/kg flunixin meglumine IV every 12 hours, 3) 1.3mg/kg loading dose lidocaine administered over 15 minutes, followed by 0.05mg/kg/min constant rate infusion (CRI), 4) both flunixin meglumine and lidocaine at the previously described doses. For horses receiving flunixin meglumine or saline, treatment was administered immediately prior to anesthesia. Anesthesia was induced with diazepam (0.1 mg/kg IV) and ketamine (3 mg/kg IV) followed by intubation and maintenance with isoflurane vaporized in 100% oxygen. For horses receiving lidocaine, the loading dose was administered immediately after the start of gas anesthesia and the CRI started subsequently. All horses received butorphanol (0.05 mg/kg) IV immediately after induction, and subsequently IM every 6 hours, to provide analgesia. Two 30cm ischemic-injured sections of jejunum were created using aseptic technique via a midline celiotomy. Doyen forceps were

used to isolate the segments and prevent collateral blood flow, and Kelly hemostats, placed over a Penrose drain to minimize damage to the blood vessels, were used to occlude the local jejunal blood supply to these two sections for 2 hours. After 2 hours, all the clamps were removed and a full-thickness wedge biopsy taken from the anti-mesenteric border of both an ischemic-injured and an adjacent section of uninjured (control) jejunum. Half of each biopsy was pinned to card, to maintain morphology, and placed in 10% neutral-buffered formalin for 24 hours for subsequent histopathologic evaluation. The mucosa was separated from the other half of the biopsy, snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for subsequent western blot analysis. The biopsy sites were closed using a single layer inverting pattern and then the abdomen was closed routinely.

After recovery from surgery, horses were returned to the stall and a physical examination performed every 4 hours. Free access to water was allowed and small handfuls of hay were offered. Horses receiving lidocaine treatment were given an additional loading dose of lidocaine over 15 minutes on return to the stall, prior to recommencing the CRI. Horses receiving treatment with flunixin meglumine were given an additional IV dose 12 hours after the initial treatment. Eighteen hours after the end of ischemia, all horses were euthanized with sodium pentobarbital (100 mg/kg IV). Immediately following euthanasia, mucosal scrapings from both ischemic-injured and uninjured control jejunum were collected, snap frozen in liquid nitrogen, and held at  $-80^{\circ}\text{C}$  for subsequent western blot analyses. Full thickness sections of both ischemic-injured and uninjured control jejunum were collected, pinned to card to maintain morphology, and placed in 10% neutral-buffered formalin for subsequent histopathologic evaluation.

**Histological examination** – Samples from ischemic-injured and uninjured control jejunum taken at the end of ischemia and after 18 hours of recovery were placed in 10% neutral buffered formalin for 24 hours before being transferred to 70% ethanol to allow processing for immunohistochemistry. After paraffin embedding, 5µm thick sections were taken at 300µm intervals parallel to the crypt villus axis and stained with hematoxylin & eosin. Samples were evaluated by 3 blinded observers. Three well oriented villi from each section were evaluated for histopathologic grade of injury, graded from 0 to 5 using a previously described scale.<sup>21</sup> The height and diameter of the villus at the midpoint were measured using a micrometer in the eyepiece of a light microscope. The height of the epithelium denuded from each villus was also recorded. The surface area of each measured villus was calculated using a modified formula for the surface area of a cylinder:

$$\text{Villus surface area} = (2\pi \cdot 1/2 [(4/\pi)d]h)$$

Where  $\pi = 3.14$ ,  $d =$  villus diameter and  $h =$  villus height. The denuded villus surface area was calculated by subtracting the surface area of the villus covered by epithelium from the total surface area of the villus. This value was then expressed as a percentage of the total villus surface area.

**Immunohistochemistry-** To evaluate neutrophil infiltration myeloperoxidase immunohistochemistry was used as a marker for mature granulocytes to aid with detection of migrated neutrophils.<sup>22</sup> The same paraffin embedded sections were cut as described above and deparaffinized prior to antibody incubation. Evaluation of staining of myeloid cells in the bone marrow was first performed as a positive control to optimize antibody concentration and incubation time. Therefore, experimental sections were incubated with 1:10 rabbit anti-

human myeloperoxidase<sup>a</sup> overnight at 4°C. The numbers of neutrophils infiltrating repairing intestinal villi were counted by two independent blinded investigators (VLC and JFM) in a 10µm<sup>2</sup> grid on 5 separate villi, and the mean count per mm<sup>2</sup> calculated.

**Western blot analyses-** Analyses were performed on samples obtained at the end of the ischemic period (0 hours) and at the time of euthanasia (18 hours) from uninjured control and ischemic-injured jejunum from 3 representative horses in each treatment group. Samples were snap frozen in liquid nitrogen and held at -80°C for bulk analysis. Protein was extracted by adding 1mL of chilled modified radioimmunoprecipitation buffer (0.15M NaCl, 50mM sodium Tris [pH 7.2], 0.5% deoxycholic acid, 1% Triton X-100, 0.1% SDS, and 1% NP-40) to thawed tissue at 4°C with the protease inhibitors phenylmethylsulfonyl fluoride, sodium orthovanadate and aprotinin. The mixture was homogenized on ice and centrifuged twice at 4°C (2,000 rpm for 10 minutes followed by 10,000 rpm for 10 minutes), and the supernatant retained. The protein concentration of extracted samples was determined by the biuret reaction.<sup>b</sup> Equal amounts of protein from each sample were mixed with 4X sample buffer and 20X reducing agent and boiled for 5 minutes at 100°C. Thirty five µg of denatured protein was loaded into each well of an 18 well 4-12% Bis-Tris precast polyacrylamide gel,<sup>c</sup> and electrophoresis performed according to standard protocols. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane<sup>d</sup> activated with methanol, by the use of an electroblotting transfer apparatus. Membranes were boiled in 1X PBS for 5 minutes to increase antigen retrieval and blocked for 2 hours at room temperature in Tris buffered saline tween and 5% powdered milk. Membranes were incubated overnight at 4°C in primary antibody (1:200 dilution of goat polyclonal to COX-1 or COX-2,<sup>e</sup> 1:20,000 dilution of rabbit

polyclonal to  $\beta$  actin<sup>f</sup>). Following 3 washes, the membranes were incubated for 1 hour at room temperature with horseradish peroxidase (HRP) conjugated secondary antibody. After a further 3 washes the membranes were developed for visualization of protein by the addition of enhanced luminol substrate for HRP.<sup>g</sup> Densitometry was performed on scanned images by the use of specialized software.<sup>h</sup>

**Prostanoid concentrations** - Plasma samples were obtained prior to induction of anesthesia (baseline) and 8 hours after ischemia. These were frozen and stored at  $-80^{\circ}\text{C}$  and later evaluated for prostanoid concentrations. Prostaglandin  $\text{E}_2$  concentration was evaluated by converting all unstable metabolites to the stable 13,14-dihydro-15-keto prostaglandin  $\text{A}_2$  for quantification by competitive enzyme immunoassay (EIA).<sup>i</sup> Thromboxane (TX) $\text{A}_2$  concentration was determined by measurement of its stable metabolite TXB<sub>2</sub> by competitive EIA.<sup>j</sup>

**Statistical analyses**- Histopathologic grade of injury were compared by a 1-way ANOVA on Ranks. Histological measurements (denuded villus surface area) were analyzed with a 1-way ANOVA for effect of treatment on histological indices of repair. Denuded villus surface area was also analyzed within each treatment group by a 1-way repeated-measures ANOVA for the effect of ischemia. The measurements for prostanoid concentrations were expressed as a percentage of each horse's baseline value. A one-way ANOVA tested for the effect of treatment between the groups, followed by a post hoc pairwise multiple comparison procedure (Fisher LSD method). Western blot densitometry measurements (COX-1 and COX-2 protein concentrations) were analyzed with 1-way repeated measures ANOVA for

the effect of ischemia. For each statistical test, the power of analysis was determined. A value of  $P < 0.05$  was considered significant.

## **Results**

**Histopathologic examination-** After 2 hours of ischemic injury, median histological grade of biopsies from ischemic sections was 3 in all treatment groups, and 0 in biopsies from uninjured control jejunum in all treatment groups. Following 18 hours of recovery, median grade (25th-75<sup>th</sup> percentile) was 0 (0-0.5), 0.75 (0.5-1.5), 1.25 (0.5-1.5), 1.25 (1-1.5) in horses treated with saline, flunixin meglumine, lidocaine, or flunixin meglumine and lidocaine combined, respectively. There was no significant difference in histopathologic grade between treatment groups.

There was no denudation of villi observed in biopsies obtained from uninjured control jejunum at the end of the ischemic period. Mean denudation  $\pm$  SEM in biopsies from ischemic injured sections after 2 hours of ischemia across all treatment groups was significantly increased at  $38.1 \pm 2.8\%$  with no significant difference in denudation between treatment groups (Fig 1). Restitution was mostly complete 18 hours after the end of ischemia, with villus denudation significantly reduced compared to percentage denudation after 2 hours of ischemic injury. Mean percentage denudation  $\pm$  SEM was  $8.7 \pm 8.3\%$ ,  $9.5 \pm 8.0\%$ ,  $14.8 \pm 15.4\%$ , and  $9.8 \pm 7.3\%$  in ischemic-injured biopsies after 18 hours of recovery from horses treated with saline, flunixin meglumine, lidocaine or flunixin meglumine and lidocaine combined, respectively, with no significant difference between treatment groups.

**Immunohistochemistry-** Mucosal neutrophil counts were lowest in biopsy samples from uninjured (control) jejunum collected at the end of the 2 hour ischemic period, with no

significant difference between treatment groups. Counts were increased in ischemic-injured mucosa after 2 hours of ischemia, but these were not significantly different from control sections, and did not differ significantly between treatment groups (Fig 2a). Mucosal neutrophil counts were highest in all treatment groups in ischemic-injured mucosa after 18 hours of recovery, and were significantly increased compared to counts from both control and ischemic-injured biopsies taken at the time of surgery ( $P < 0.008$ ). The greatest number of neutrophils was observed in ischemic-injured mucosa taken from horses treated with flunixin meglumine alone (Fig 2b). Counts in this group were significantly greater than in ischemic-injured mucosa from all other treatment groups ( $P < 0.001$  compared to saline, and lidocaine and flunixin meglumine combined,  $P = 0.015$  compared to lidocaine treatment). Significantly more neutrophils were observed in ischemic-injured mucosa than in control mucosal biopsies from the same treatment group after 18 hours of recovery in all groups, except for biopsies from horses treated with flunixin meglumine and lidocaine combined.

**Western blot analyses-** Densitometry analysis showed that there was no significant difference in beta actin concentration, confirming equal protein loading across lanes.

Although control and ischemic samples from an individual horse were run on the same gel, samples from 3 horses in all 4 treatment groups were not all on one gel. Therefore, to allow for differences in transfer and developing between blots, the percentage change between control and ischemic samples for each horse was calculated, rather than using absolute densitometry units. A band at approximately 70kDa consistent with COX-1 could be clearly seen in lanes containing protein from control tissues obtained at 0 and 18 hours, confirming constitutive expression of this protein (Fig 3a & 3b). At 0 hours, the expression of COX-1 in

ischemic-injured mucosa was virtually unchanged from that in control mucosa (Fig 4a), with no significant difference between treatment groups (percentage change  $\pm$ SEM for saline - 3.54 $\pm$  6.6, flunixin meglumine 1.14 $\pm$ 1.0, lidocaine -6.39 $\pm$ 3.9, lidocaine and flunixin meglumine -1.04 $\pm$ 1.4). Multiple positive bands of various molecular weights were detected with the COX-2 antibody. The band at approximately 70 kDa was assumed to be COX-2. As with COX-1, COX-2 was also observed in control tissue at 0 and 18 hours, suggesting that this protein is also constitutively expressed. After 2 hours of ischemia, COX-2 expression was decreased in ischemic-injured mucosa compared to control mucosa, in horses treated with lidocaine. This percentage change was significantly different to horses treated with flunixin meglumine or saline (Fig 3a and 4b, P=0.003 compared to flunixin meglumine, P=0.02 compared to saline). There was also a trend toward a decrease in COX-2 expression in ischemic-injured mucosa in horses treated with both flunixin meglumine and lidocaine combined (P= 0.058 compared to flunixin meglumine).

After 18 hours of recovery, the percentage difference in COX-1 expression between control and ischemic-injured tissue was significantly greater than that found at 0 hours in all treatment groups (P $\leq$ 0.01, Fig 4a). COX-2 expression was increased in ischemic-injured mucosa compared to control mucosa in all treatment groups after 18 hours of recovery, with no significant difference between the groups. In horses treated with saline, or lidocaine and flunixin meglumine combined, the percentage change at 18 hours was significantly different to that seen with lidocaine, and lidocaine and flunixin meglumine treated horses at 0 hours (P $\leq$ 0.048, Fig 4b) In horses treated with flunixin meglumine or lidocaine alone, this change was significantly different to that seen at 0 hours in horses treated with lidocaine (P $\leq$ 0.02).

**Prostanoid concentrations-** There was wide variation in plasma prostanoid concentrations between individual horses, as we have previously described.<sup>23</sup> Therefore, to more accurately examine the effect of the different treatments, the data were examined by calculating the percentage change between the preoperative concentration (baseline) and concentration 8 hours after the end of ischemia for each horse.

Prostaglandin E metabolite (PGEM) concentration in saline treated horses was increased by a mean  $\pm$  SEM of  $55.8 \pm 12.5\%$ , 8 hours after the end of ischemia (Fig 5) which was significantly higher than all other treatment groups. Treatment with lidocaine, or flunixin meglumine alone, resulted in a change of  $8.7 \pm 11.0\%$  and  $-2.9 \pm 14.3\%$  from baseline respectively. Treatment with lidocaine and flunixin meglumine combined resulted in a significant decrease in PGEM concentration by  $-29.2 \pm 3.7\%$  compared to treatment with saline or lidocaine alone.

The plasma concentration of TXB<sub>2</sub> in one horse in the flunixin meglumine group was below the limits of detection of the assay at 8 hours after the end of ischemia, and therefore % change in TXB<sub>2</sub> concentration could not be calculated for this horse. Plasma TXB<sub>2</sub> concentration in horses treated with saline or lidocaine alone was increased 8 hours after ischemic injury by  $39.7 \pm 29.6\%$  and  $71.9 \pm 22.7\%$  respectively (Fig 6). Plasma concentration of TXB<sub>2</sub> was decreased by  $-55.3 \pm 13.8\%$  in horses treated with flunixin meglumine alone, and by  $-72.4 \pm 8.4\%$  when treatment was combined with lidocaine, at 8 hours after ischemic injury. These values were significantly different to those in horses treated with saline or lidocaine alone ( $P \leq 0.005$ ).

## Discussion

Mortality after colic surgery is greatest in the first 10 days.<sup>24</sup> Additionally, short term survival is lower in horses with an ischemic lesion, than in those with a simple obstruction.<sup>25</sup> These facts highlight the importance of optimizing care in the immediate post-operative period. Providing adequate analgesia after celiotomy may also reduce post-surgical stress and minimize additional weight loss.<sup>26</sup> However, in our previous studies we have shown that flunixin meglumine, the most commonly used analgesic, inhibits mucosal recovery.<sup>5,6,23</sup> Additionally, flunixin meglumine may contribute to mucosal inflammation, as indicated by an increase in mucosal neutrophil counts compared to counts in ischemic-injured mucosa from horses treated with saline.<sup>6</sup> Although flunixin meglumine is a non-selective COX inhibitor, it does not prevent a post-ischemic increase in mucosal expression of COX-1 or COX-2.<sup>6,23</sup> However, we have previously described that combining treatment with flunixin meglumine with systemically administered lidocaine, recovery of ischemic-injured jejunum can occur.<sup>9</sup> In this study, we document that this effect is associated with a reduction in mucosal inflammation with lidocaine administration.

We had previously documented a low transepithelial resistance (TER) in ischemic-injured mucosa from horses treated with flunixin meglumine.<sup>9</sup> This decrease in TER indicates a loss of mucosal barrier function and could be attributed to either loss of epithelial cells, failure of restitution, or failure of closure of the paracellular space.<sup>27</sup> Here, we found no difference between treatment groups in histologic grade of injury, nor villus denudation. This suggests that the low TER in ischemic-mucosa from horses treated with flunixin meglumine in our previous study is most likely due to failure of closure of paracellular spaces. In

ischemic-injured porcine jejunum, it has been shown that COX-1 derived prostaglandins, acting via the second messengers cAMP and  $\text{Ca}^{2+}$ , stimulate closure of the paracellular space by stimulating  $\text{Cl}^-$  secretion in the crypts, and inhibiting  $\text{Na}^+$  absorption in the villus tips.<sup>28-30</sup> Flunixin meglumine is a non-specific COX inhibitor and would therefore reduce the production of these PGs, thereby inhibiting closure of paracellular spaces.

Neutrophil counts peaked in ischemic-injured mucosa 18 hours after the end of ischemia and were significantly greater than after 2 hours of ischemia, which is in agreement with results from previous studies.<sup>6,23</sup> Infiltration of neutrophils into the mucosa has been shown to disrupt mucosal repair as they traverse the paracellular spaces between restituting epithelial cells.<sup>31</sup> Peak neutrophilic infiltration was seen in ischemic-injured mucosa from horses treated with flunixin meglumine alone. Interestingly, when this treatment was combined with lidocaine, neutrophil counts were not significantly different in ischemic-injured sections compared to control sections at the 18 hour time point. Lidocaine may achieve this effect through a direct effect on the neutrophils to inhibit adhesion and migration, or because it reduces cellular damage and release of neutrophil chemoattractants.

COX-2 is traditionally thought of as an inducible enzyme but may also have some constitutive roles.<sup>32</sup> In particular, COX-2 has been shown to contribute to gastric ulcer healing by promoting epithelial cell proliferation and migration.<sup>33</sup> In this study, we documented the presence of COX-2 in uninjured jejunal mucosa, suggesting it is constitutively expressed, which is in accordance with the findings of a previous study.<sup>23</sup> Its constitutive role in the small intestine is unknown, but COX-2 has been shown to increase neutrophil infiltration and histologic injury, and reduced motility, after small intestinal

ischemia.<sup>34</sup> This same study also found that COX-2 mRNA levels were rapidly increased after just 30 minutes of ischemia.<sup>34</sup> This would coincide with the rapid increase in COX-2 protein levels that we observed after 2 hours of ischemia in horses treated with saline or flunixin meglumine. Interestingly, we found that in horses treated with lidocaine, COX-2 protein levels were decreased in ischemic-injured mucosa after 2 hours of ischemia, suggesting an anti-inflammatory effect of lidocaine. There was also a trend toward this effect when treatment with lidocaine was combined with flunixin meglumine and may explain, in part, how the effects of flunixin meglumine on mucosal healing are overcome when the two drugs are combined.

Plasma prostanoid concentrations are frequently used to assess COX activity.<sup>35</sup> The concentration of TXB<sub>2</sub>, the stable metabolite of TXA<sub>2</sub>, is used as an indicator of COX-1 activity, as COX-1 is the sole source of TXA<sub>2</sub> from platelets.<sup>36</sup> We found that treatment with flunixin meglumine inhibited the post-surgical increase in TXB<sub>2</sub>, which is consistent with its action as a non-selective COX inhibitor. When treatment with flunixin meglumine is combined with lidocaine, TXB<sub>2</sub> production remains inhibited. This suggests that lidocaine has no effect on COX-1. These results are also in agreement with the western analyses of COX-1 at 18 hours, in which there was no difference in the increase in COX-1 protein in ischemic mucosa from horses treated with flunixin meglumine, or lidocaine and flunixin meglumine combined. Few studies have examined the effect of local anesthetics on the COX enzyme. However, when bupivacaine and lidocaine were used to provide local anesthesia for molar extraction, there was, likewise, no effect of either local anesthetic on COX-1 gene expression or TXB<sub>2</sub> concentration in inflammatory transudate.<sup>20</sup>

Plasma PGE<sub>2</sub> metabolite concentration is similarly used to assess COX-2 activity, as the PGE<sub>2</sub> synthase enzyme is preferentially coupled to COX-2.<sup>37</sup> As expected, the non-selective COX inhibitor, flunixin meglumine, prevented the post-surgical increase in plasma PGEM metabolite concentration, indicating COX-2 inhibition. However, treatment with lidocaine alone also significantly reduced the post-operative increase in plasma PGE<sub>2</sub> concentration, and a combination of the two drugs resulted in a decrease in PGE<sub>2</sub> compared to preoperative values. These results are in agreement with the COX-2 western analyses, and an inhibitory effect of lidocaine on COX-2 expression.

In conclusion, we have shown that systemically administered lidocaine exerts anti-inflammatory effects in jejunal mucosa and plasma of horses after ischemic injury. We have determined that this effect is not achieved through a change in histologic injury or epithelial restitution, or via an effect on COX-1. However, treatment with lidocaine does result in a reduction in mucosal neutrophil counts, mucosal COX-2 expression, and plasma PGE<sub>2</sub> concentration. What remains unclear is whether lidocaine inhibits neutrophil activation and hence reduces tissue injury and COX-2 expression, or if reduced tissue COX-2 expression results in a reduced chemotactic stimulus for neutrophil migration. Therefore further studies remain to fully elucidate this effect.

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<sup>a</sup> Anti-Myeloperoxidase, BioGenex, San Ramon, CA

<sup>b</sup> BCA Protein Assay Kit, Pierce, Rockford, IL

<sup>c</sup> Criterion XT Precast Gel, Bio-Rad Laboratories, Hercules, CA

<sup>d</sup> Immobilon P Transfer Membrane, Millipore Corporation, Bedford, MA

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<sup>c</sup> SantaCruz Biotechnology Inc, Santa Cruz, CA

<sup>f</sup> AbCam, Cambridge, MA

<sup>g</sup> ECL Western Blotting Substrate, Pierce, Rockford, IL

<sup>h</sup> SigmaScan Pro5, Systat Software Inc, San Jose, CA

<sup>i</sup> Prostaglandin E Metabolite EIA Kit, Cayman Chemical, Ann Arbor, MI

<sup>j</sup> Thromboxane B<sub>2</sub> EIA Kit, Cayman Chemical, Ann Arbor, MI

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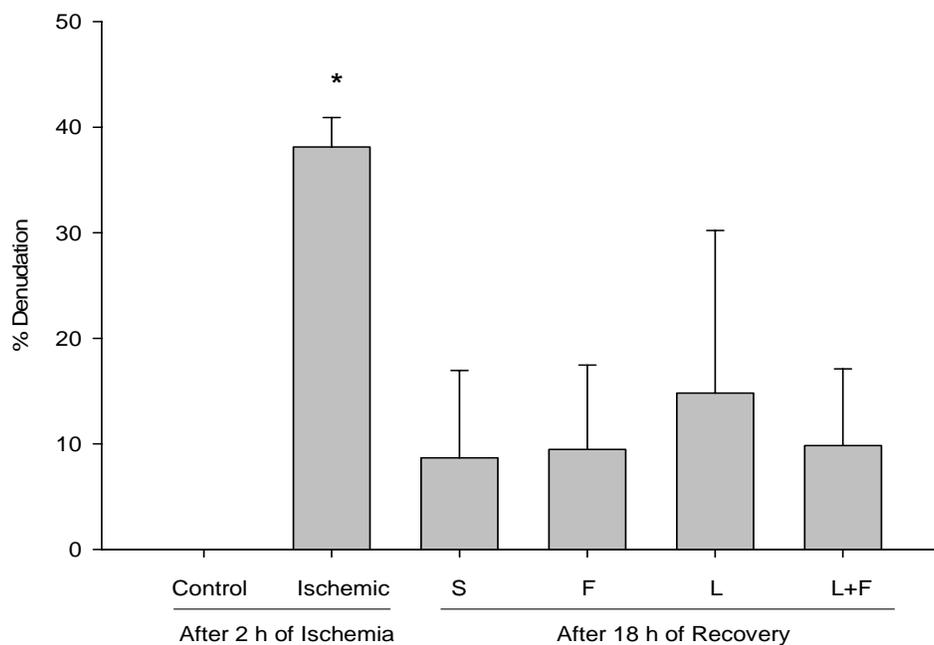
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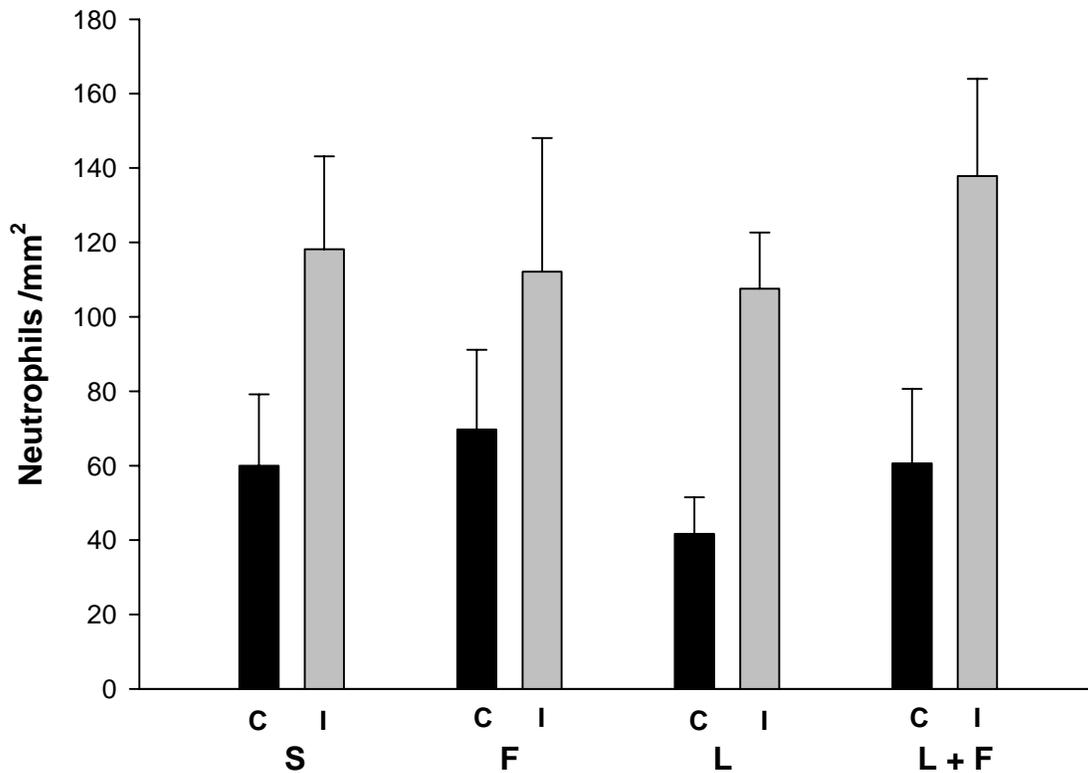
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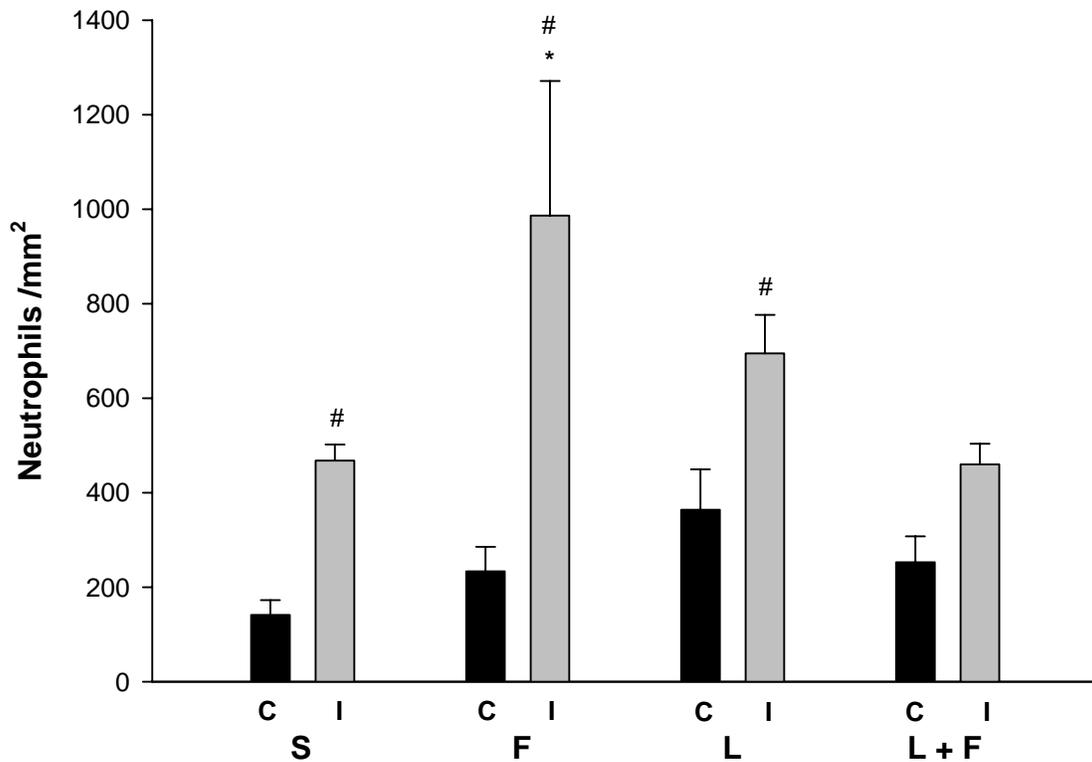
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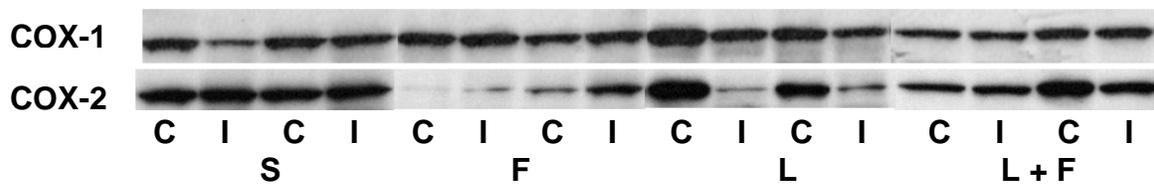
**Figure 1-** Mean  $\pm$ SEM percent villus denudation in biopsies from uninjured (control) or ischemic jejunum after 2 hours of ischemia, and in biopsies from ischemic-injured jejunum 18 hours after the end of ischemia. Horses were treated with saline 1ml/50kg (S), flunixin meglumine 1mg/kg IV every 12 hours (F), lidocaine 1.3mg/kg loading dose followed by 0.05mg/kg/min constant rate infusion (L), or both flunixin meglumine and lidocaine at the previous doses (L+F). There was no significant difference between treatment groups after 2 hours of ischemia so the data were pooled. \* Value is significantly increased compared to all other values ( $P < 0.05$ ).



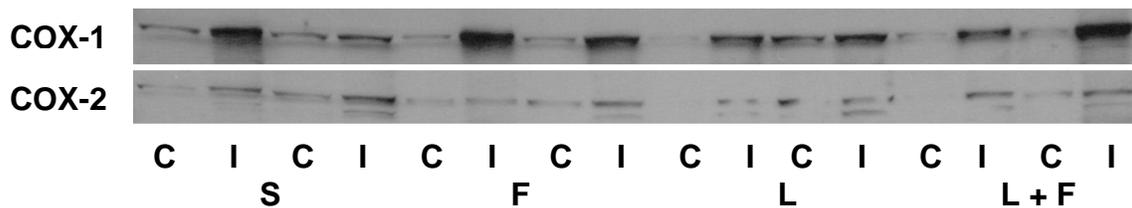
**Figure 2a-** Mucosal neutrophil counts/ mm<sup>2</sup> in biopsies taken from uninjured (control (C)) jejunum and from jejunum after 2 hours of ischemic-injury (I) from horses treated with saline 1ml/50kg (S), flunixin meglumine 1mg/kg IV every 12 hours (F), lidocaine 1.3mg/kg loading dose followed by 0.05mg/kg/min constant rate infusion (L), or both flunixin meglumine and lidocaine at the previous doses (L+F). Although neutrophil counts were increased in ischemic-injured mucosa, there were no significant differences found between control and ischemic-injured jejunum, nor between treatment groups.



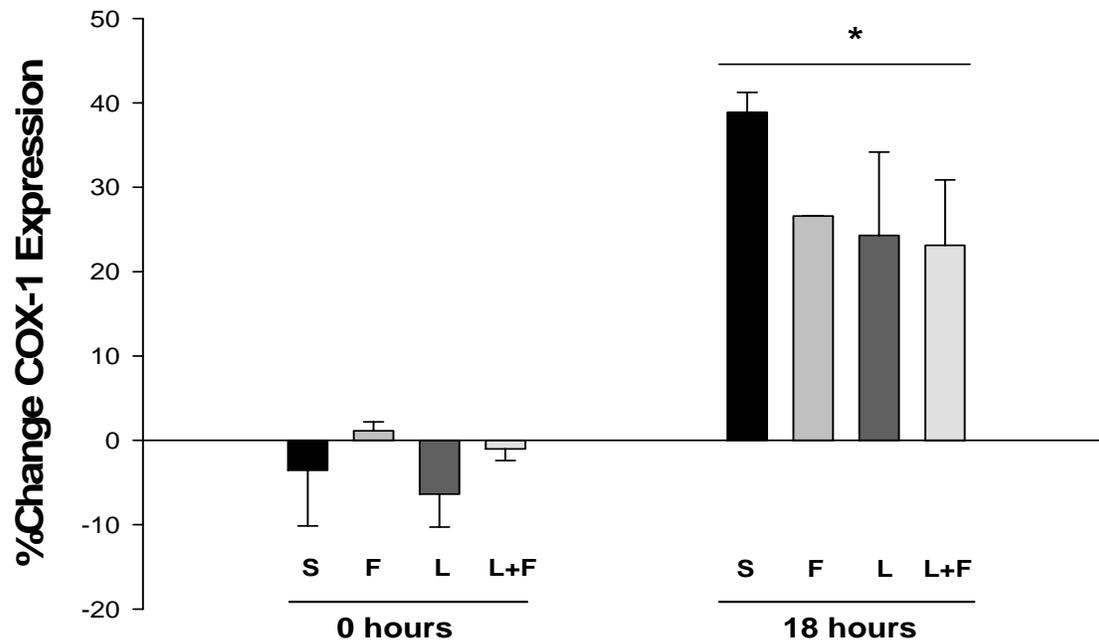
**Figure 2b-** Mucosal neutrophil counts/ mm<sup>2</sup> in biopsies taken from uninjured (control (C)) jejunum and from jejunum after 2 hours of ischemic-injury and 18 hours of recovery (I), from horses treated with saline 1ml/50kg (S), flunixin meglumine 1mg/kg IV every 12 hours (F), lidocaine 1.3mg/kg loading dose followed by 0.05mg/kg/min constant rate infusion (L), or both flunixin meglumine and lidocaine at the previous doses (L+F). \* Value is significantly increased compared to counts in ischemic-injured mucosa in all other treatment groups (P<0.015). # Value is significantly increased compared to counts in control mucosa for that treatment group (saline P=0.007, flunixin meglumine P<0.001, lidocaine P=0.006).



**Figure 3a-** COX-1 and COX-2 expression in uninjured (control (C)) and ischemic-injured (I) mucosa after 2 hours of ischemia (0 hours) from representative horses treated with either saline 1ml/50kg (S), flunixin meglumine 1mg/kg IV every 12 hours (F), lidocaine 1.3mg/kg loading dose followed by 0.05mg/kg/min constant rate infusion (L), or both flunixin meglumine and lidocaine at the previous doses (L+F). COX-1 and COX-2 appear to be constitutively expressed, as evidenced by their presence in uninjured (control) jejunum at this time point.

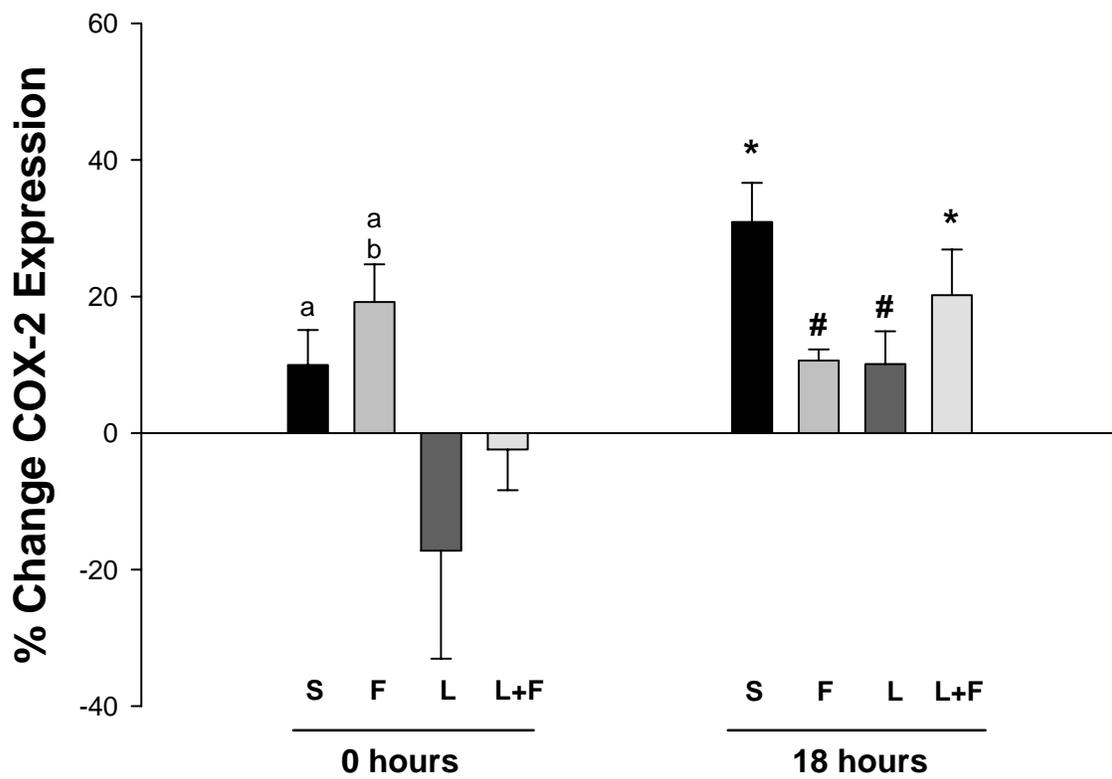


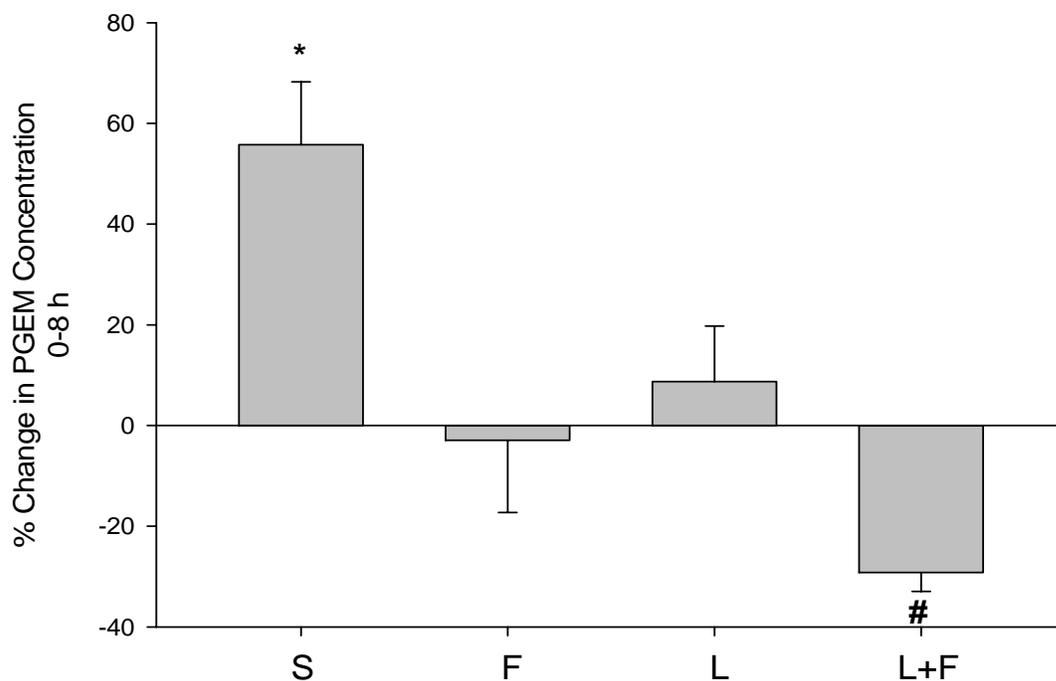
**Figure 3b-** COX-1 and COX-2 expression in uninjured (control (C)) and ischemic-injured (I) mucosa after 2 hours of ischemia and 18 hours of recovery (18 hours) from representative horses treated with either 1mL/kg 0.9% saline IV every 24 hours (S), 1.1mg/kg flunixin meglumine IV every 12 hours (F), lidocaine 1.3mg/kg loading dose followed by 0.05mg/kg/min constant rate infusion (L), or both flunixin meglumine and lidocaine at the previous doses (L+F). Both COX-1 and COX-2 appear to be up regulated in ischemic-injured mucosa.



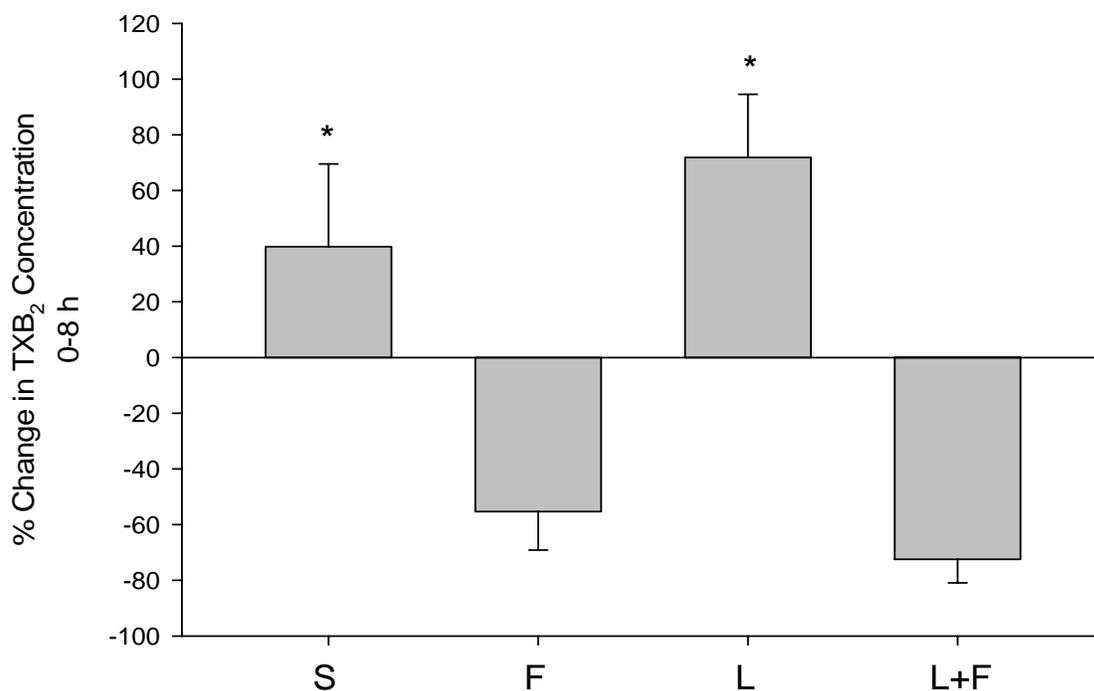
**Figure 4a-** Percentage change in densitometry units of COX-1 western analyses between uninjured control and ischemic-injured jejunal mucosa taken at the end of the ischemic period (0 hours) and after 18 hours of *in vivo* recovery (18 hours). Horses were treated with saline 1ml/50kg (S), flunixin meglumine 1mg/kg IV every 12 hours (F), lidocaine 1.3mg/kg loading dose followed by 0.05mg/kg/min constant rate infusion (L), or both flunixin meglumine and lidocaine at the previous doses (L+F). \* Values are significantly greater than those at 0 hours in all treatment groups ( $P \leq 0.01$ ).

**Figure 4b-** Percentage change in densitometry units of COX-2 western analyses between uninjured control and ischemic-injured jejunal mucosa taken at the end of the ischemic period (0 hours) and after 18 hours of *in vivo* recovery (18 hours). Horses were treated with saline 1ml/50kg (S), flunixin meglumine 1mg/kg IV every 12 hours (F), lidocaine 1.3mg/kg loading dose followed by 0.05mg/kg/min constant rate infusion (L), or both flunixin meglumine and lidocaine at the previous doses (L+F). <sup>a</sup> Value is significantly greater than that in horses treated with lidocaine at 0 hours (saline P=0.02, flunixin meglumine P=0.003). <sup>b</sup> There is a trend for this value to be significantly greater than that in horses treated with L+F at 0 hours (P=0.058) \* Value is significantly greater than that in horses treated with L or L+F at 0 hours (P≤0.048) . # Value is significantly greater than in horses treated with L at 0 hours (P≤0.02).





**Figure 5-** Percentage change in plasma prostaglandin E metabolite (PGEM) concentration between preoperative values and those at 8 hours after the end of ischemia in horses treated with saline 1ml/50kg (S), flunixin meglumine 1mg/kg every 12 hours (F), lidocaine 1.3mg/kg loading dose followed by 0.05mg/kg/min constant rate infusion (L), or both flunixin meglumine and lidocaine at the previous doses (L+F). \* Value is significantly increased compared to all other groups ( $P \leq 0.007$ ). # Value is significantly less than in horses treated with saline ( $P < 0.001$ ) or lidocaine ( $P = 0.026$ ).



**Figure 6-** Percentage change in plasma TXB<sub>2</sub> concentration between preoperative values and those at 8 hours after the end of ischemia, in horses treated with saline 1ml/50kg (S), flunixin meglumine 1mg/kg IV every 12 hours (F), lidocaine 1.3mg/kg loading dose followed by 0.05mg/kg/min constant rate infusion (L), or both flunixin meglumine and lidocaine at the previous doses (L+F). \* Values are significantly different to those in horses treated with flunixin meglumine ( $P \leq 0.005$ ) or flunixin meglumine and lidocaine combined ( $P \leq 0.001$ ).

## **CHAPTER 5**

### **Effect of Firocoxib or Flunixin Meglumine on Recovery of Ischemic-Injured Equine**

#### **Jejunum**

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Anthony T. Bliklager, DVM, PhD

## **Abstract**

**Objective:** Determine if treatment of horses with firocoxib would provide as effective analgesia as flunixin meglumine, while allowing recovery of ischemic-injured jejunum.

**Animals:** 18 horses

**Procedures:** Horses were administered 0.9% NaCl 1mL/50Kg IV, flunixin meglumine 1mg/Kg IV q12 hours or firocoxib 0.09mg/kg IV q24 hours (n=6/group), before two hours of jejunal ischemia. Horses were monitored via behavioral pain scores and received butorphanol for analgesia. After 18 hours, ischemic-injured and control mucosa was placed on Ussing chambers for measurement of transepithelial resistance (TER) and permeability to LPS. Histomorphometry was used to determine denuded villus surface area. Western blots for COX-1 and COX-2 were performed. Plasma thromboxane (TX)<sub>B2</sub> and prostaglandin E<sub>2</sub> metabolite (PGEM) concentrations were determined pre and post operatively. Statistical significance was set at P<0.05.

**Results:** Pain scores did not significantly increase after surgery in horses treated with flunixin meglumine or firocoxib. TER of recovering ischemic-injured jejunum from horses treated with flunixin meglumine was significantly lower than saline or firocoxib treatment groups. LPS permeability across recovering mucosa was significantly increased in horses treated with flunixin meglumine. Treatment had no effect on epithelial restitution. COX-1 was constitutively expressed and COX-2 was upregulated after 2 hours of ischemia. TXB<sub>2</sub> concentration decreased with flunixin meglumine treatment, but increased with firocoxib or saline treatment. Flunixin meglumine and firocoxib prevented an increase in PGEM concentration after surgery.

**Conclusions:** Flunixin meglumine and firocoxib were effective analgesics in horses recovering from jejunal ischemic injury, but flunixin retarded mucosal recovery whereas firocoxib did not.

**Clinical Relevance:** Firocoxib may be advantageous for horses recovering from ischemic intestinal injury compared to flunixin meglumine.

## **Introduction**

Colic is second only to old age as the leading cause of death in horses, with an overall fatality rate of 11%.<sup>1</sup> Death occurs largely as a result of strangulating obstruction of the intestine, which results in disruption of intestinal barrier function, endotoxemia, hypovolemia and shock.<sup>2</sup> Although it is usually possible to resect ischemic small intestine at surgery, the remaining proximal jejunum is commonly damaged by distension,<sup>3</sup> and ongoing microscopic injury occurs as indicated by neutrophilic infiltration during the initial 18 hours after the end of ischemia.<sup>4,5</sup> Many of the signs of endotoxemia, and much of the pain associated with strangulating obstruction are attributable to prostaglandins elaborated by the cyclooxygenase (COX) isoenzymes.

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.<sup>6,7</sup> NSAIDs inhibit COX isoenzymes, which are rate-limiting in the production of prostaglandin (PG) H<sub>2</sub> from arachidonic acid. Local PG synthases then modify PGH<sub>2</sub> to form a wide range of other prostanoids, such as PGI<sub>2</sub>, Thromboxane (TX) A<sub>2</sub>, and PGE<sub>2</sub>.<sup>8</sup> However, administration of NSAIDs is not without adverse side effects, such as gastric ulceration and renal crest necrosis.<sup>9-11</sup> Additionally, they retard the recovery of intestinal barrier function in ischemic-injured equine jejunal mucosa,<sup>5,12</sup> because, prostaglandins are critical for recovery of barrier function after ischemic injury.<sup>13</sup>

Three COX isoforms have been identified.<sup>14</sup> COX-1 is constitutively expressed in most tissues, including the equine jejunum,<sup>5</sup> and is thought to be responsible for PG production during normal physiologic processes. COX-2 is expressed at low levels in most normal

tissue, including equine jejunum,<sup>5</sup> but is upregulated in response to injury. The role, if any, of COX-3, a constitutively expressed isoform, in the gastrointestinal tract has not been determined.

Different NSAIDs have different selectivity for the COX isoforms. For example, flunixin meglumine is a non-selective inhibitor of the COX enzymes, and therefore decreases PG concentrations in tissue. A COX-2 selective NSAID may be advantageous for the treatment of equine colic since it would inhibit the COX-2 isoform which contributes to pain and inflammation, while allowing PG mediated intestinal repair through COX-1 associated PGs.<sup>5,15</sup> Such benefits were observed with the use of the COX-2 selective inhibitor meloxicam, which was shown to provide adequate analgesia whilst permitting recovery of ischemic-injured equine jejunum.<sup>15</sup> However, an equine injectable solution of meloxicam, suitable for use in horses with colic, is not available in the United States.

Firocoxib is a NSAID that shows excellent COX-2 selectivity in canine, feline and equine blood.<sup>16-18</sup> In horses, firocoxib was shown to be 265 times more selective for COX-2 than COX-1 and provided significant analgesia in a lameness model.<sup>16</sup> Additionally, firocoxib was found to be as effective an analgesic as phenylbutazone in horses with lameness due to naturally occurring osteoarthritis, with no adverse effects detected.<sup>19</sup> Due to the COX-2 selectivity, and the musculoskeletal analgesic properties of firocoxib in horses, we believed that investigation into the effect of an injectable form of firocoxib on recovery of ischemic-injured intestine was warranted. Our hypothesis was that treatment with firocoxib would allow optimal recovery of mucosal barrier function in ischemic-injured equine jejunum, compared to treatment with flunixin meglumine, whilst providing effective visceral

analgesia.

## **Materials and Methods**

**Horses-** All procedures were approved by the North Carolina State University Animal Care and Use Committee. Eighteen horses, age 3-20 years and weighing between 378 and 605 kg were used in this study. Horses had no history of colic, and were quarantined for 2 weeks, vaccinated, and received anthelmintic treatment prior to use in the study. Before surgery a complete physical examination was performed and baseline pain scores determined using an established behavioral pain scoring system.<sup>20</sup>

**Surgical procedure-** Horses were randomly assigned to one of three treatment groups, with 6 horses in each group: Group 1 horses received 1mL/kg 0.9% saline IV every 24 hours, Group 2 horses received 1.1mg/kg flunixin meglumine IV every 12 hours, and Group 3 horses received 0.09mg/kg firocoxib<sup>a</sup> IV every 24 hours. Immediately before surgery each horse was sedated with xylazine (0.5-1mg/kg IV) and an intravenous catheter was placed aseptically in the left jugular vein. Prophylactic antibiotics (2.2mg/kg ceftiofur IV once) and the test drug, according to the allocated treatment group, were administered immediately before anesthetic induction. Anesthesia was induced with diazepam (0.1mg/kg IV) and ketamine (3mg/kg IV), and then maintained with isoflurane vaporized in oxygen via an orotracheal tube. To provide additional analgesia, all horses received 0.05mg/kg butorphanol IV immediately after anesthetic induction, and subsequently IM every 6 hours. After routine aseptic preparation, a ventral midline approach to the abdomen was made, and a 30-cm loop of jejunum was isolated and cross-clamped using Doyen forceps to prevent

collateral blood flow. The local mesenteric jejunal blood supply was temporarily occluded for two hours using Kelly clamps, placed over a Penrose drain to minimize trauma to the vessels, to simulate a strangulating obstruction. At the end of the two hour period of ischemia, all clamps were removed, and full thickness biopsies of ischemic-injured and adjacent uninjured control jejunum were obtained via an enterotomy at the antimesenteric border of the jejunum. Biopsy sites were closed with an inverting suture pattern, and then the abdomen was closed routinely. Horses were recovered from anesthesia in a dedicated padded recovery stall and then returned to their stalls, where they had access to water and were offered small amounts of hay. All horses were monitored postoperatively at 4, 8 and 16 hours after the end of ischemia for vital parameters, gastrointestinal borborygmi, appetite, defecation and urination. Additionally at these times, pain was assessed using the behavioral pain scoring system.<sup>20</sup> Group 2 horses received an additional dose of flunixin meglumine 12 hours after the end of ischemia. Eighteen hours after the end of ischemia, each horse was humanely euthanatized with an overdose of sodium pentobarbital (100mg/kg IV). Immediately after euthanasia, ischemic-injured and control non-ischemic jejunal tissues were harvested for *ex-vivo* experiments. The tissues were incised along the anti-mesenteric border, rinsed in equine Ringer's solution to remove intestinal contents, and then placed in pre-oxygenated equine Ringer's solution for immediate transport to the laboratory.

**Plasma firocoxib concentration-** Venous blood samples were collected 5 minutes after IV administration of firocoxib and 8 and 18 hours later. The plasma was separated and stored at -80°C for subsequent analysis of firocoxib concentration using reverse-phase high-pressure liquid chromatography with ultraviolet detection.<sup>21</sup>

**Plasma eicosanoid concentrations-** Venous blood was collected into EDTA tubes prior to surgery and at 8 hours after the end of ischemia. The plasma was separated and stored at  $-80^{\circ}\text{C}$  for bulk analysis.  $\text{PGE}_2$  concentration was estimated by first converting all its unstable metabolites to the stable metabolite 13,14-dihydro-15-keto prostaglandin  $\text{A}_2$  for quantification by competitive enzyme immunoassay (EIA).<sup>b</sup>  $\text{TXA}_2$  concentration was determined by measuring its stable metabolite  $\text{TXB}_2$  by EIA.<sup>c</sup>

**Ussing chamber electrical studies-** The mucosa was stripped from the seromuscular layers of ischemic-injured and non-ischemic control jejunal tissues in oxygenated (95%  $\text{O}_2$  and 5%  $\text{CO}_2$ ) equine Ringer's solution. Ischemic and control mucosal tissues were mounted in  $3.14\text{cm}^2$  aperture Ussing chambers. Tissues were bathed on the mucosal and serosal sides with 10ml oxygenated equine Ringer's solution. The solution on the serosal side additionally contained 10mmol of glucose/L and was osmotically balanced on the mucosal side with 10mmol of mannitol/L. The bathing solutions were circulated and maintained at  $37^{\circ}\text{C}$  by use of water-jacketed reservoirs. After a 15 minute equilibration period, spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes and the short-circuit current ( $I_{\text{sc}}$ ), measured by an automated voltage clamp. Electrical measurements were recorded every 15 minutes for 3 hours. Transepithelial resistance (TER) was calculated from the  $I_{\text{sc}}$  and PD using Ohm's law.

**Permeability studies-** After 30 minutes of equilibration in the Ussing chambers, 83  $\mu\text{g}$  of fluorescein isothiocyanate (FITC)-labeled lipopolysaccharide (LPS) (E.coli O55:B55) was added to the mucosal bathing solutions of control and ischemic tissues. Ussing chambers used for these studies were protected from light. Time zero samples (200  $\mu\text{l}$ ) were

taken from both mucosal and serosal sides in triplicate, with sampling repeated at 60 and 120 minutes after addition of the FITC-LPS. A standard concentration curve was constructed using 0-0.005 µg/ml concentrations of FITC-LPS to determine the concentration of FITC-LPS that crossed to the serosal side of the mucosa after two hours of *in vitro* incubation, for control and ischemic loops of each of the three treatment groups, using a fluorometer.

**Histology-** Light microscopy was performed on three 5 µm sections stained with H&E, well aligned on the villus-crypt axis, from each biopsy obtained at the end of ischemia, and at 18 hours after the end of ischemia. The total height, epithelial covered height, and width of three well-oriented villi in each section were measured using a micrometer in the eyepiece of a light microscope by 3 blinded investigators. The total surface area denuded of epithelial cells was calculated using a modified formula for the surface area of a cylinder.

First, the total villus surface area was calculated using the formula:

Villus surface area =  $2\pi \cdot 1/2 [(4/\pi)d]h$  where d = villus width at its midpoint and h = villus height. The villus surface area remaining denuded of epithelium was then calculated by measuring the height of the epithelial covered portion and subtracting it from the total surface area. This denuded surface area was then expressed as a percentage of the total villus surface area.

**SDS-PAGE and Western analysis-** Mucosal scrapings of control and ischemic-injured jejunum from each horse were obtained immediately following surgery and at the time of euthanasia, snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis. One gram of each tissue sample was thawed to  $4^{\circ}\text{C}$  and added to 2ml of modified radioimmunoprecipitation buffer, including the protease inhibitors aprotinin,

phenylmethylsulfonyl fluoride and sodium orthovanadate. The sample was homogenized on ice and then the supernatant extracted by centrifugation. Protein analyses of aliquots of extracted samples were obtained, and then equal concentrations of protein from each sample were mixed and boiled with sample buffer. The lysates were loaded into wells of a pre-cast 10% SDS-polyacrylamide gel and protein electrophoresis performed according to standard protocols. After transfer to nitrocellulose membranes, and blocking in 5% milk, the membranes were cut horizontally at the approximate 50 KDa molecular weight mark to allow separate incubation with COX primary antibody (upper part of the membrane) or  $\beta$  actin primary antibody (lower part of the membrane). Incubations with the respective primary antibody were performed overnight at 4<sup>o</sup>C in a 1:200 solution of goat polyclonal COX-1 or COX-2 primary antibody<sup>d</sup> or a 1:20,000 solution of rabbit polyclonal  $\beta$  actin primary antibody.<sup>e</sup> The membranes were then incubated in a horseradish peroxidase conjugated secondary antibody and developed by addition of enhanced chemiluminescence reagent.<sup>f</sup> Densitometry of resulting blots was performed by use of specialized software.<sup>g</sup>

**Statistical analysis-** Behavioral pain scores were examined with a one-way analysis of variance (ANOVA) on ranks for the effect of treatment and time from 0-16 hours. When a significant interaction was detected a pairwise multiple comparison procedure (Tukey test) was used to identify the source of the interaction. The percentage change in TXB<sub>2</sub> and PGEM concentration between 0 and 8 hours was analyzed by a 1-way ANOVA followed by a multiple comparison procedure (Holm-Sidak method). A two-way repeated measures ANOVA was used to analyze TER for the effect of treatment and ischemia over time. When a significant interaction was detected, a multiple comparison procedure (Fisher LSD method)

was performed. A one-way ANOVA was performed on the ischemic and control groups for the effect of treatment on LPS flux, followed by a multiple pairwise comparison procedure (Fisher LSD method). Statistical analysis of histological data was performed using a 2-way ANOVA for the effects of time and treatment, followed by a multiple comparison procedure (Fisher LSD method) when a significant interaction was detected. For all tests,  $P < 0.05$  was considered significant.

## **Results**

**Plasma firocoxib concentration-** Plasma concentration peaked 5 minutes after IV administration at mean  $\pm$  SEM of  $0.217 \pm 0.032$  mg/mL. It then declined to a mean  $\pm$  SEM of  $0.046 \pm 0.005$  and  $0.044 \pm 0.005$  mg/mL at 8 and 16 hours after administration respectively.

**Behavioral pain scores-** No horses showed gross signs of pain at any time during the study. Median pain score was zero in all groups prior to surgery. Horses treated with saline had significantly higher pain scores at 4 and 8 hours after surgery than horses in that group prior to surgery ( $P = 0.002$ , Figure 1). However, horses treated with either flunixin meglumine or firocoxib had no significant change in postoperative pain score compared to preoperative values for the respective group. Additionally, at 4 hours after surgery, horses treated with saline had significantly higher pain scores than those treated with flunixin meglumine or firocoxib ( $P < 0.05$ , Figure 1). At 8 hours after surgery, pain scores were significantly higher in horses treated with saline compared to those treated with flunixin meglumine ( $P = 0.033$ ). Pain scores in horses treated with flunixin meglumine were not significantly different from those treated with firocoxib at any time point. Pain scores at 16 hours after surgery were not significantly different between treatment groups, or from preoperative values.

**Plasma eicosanoid concentrations-** Plasma concentrations of both TXB<sub>2</sub> and PGEM varied widely between individual horses. Therefore, the results are expressed as the percentage change in concentration between 0 hour (preoperative) and 8 hour values. Plasma TXB<sub>2</sub> concentration increased significantly by 8 hours after ischemia in horses treated with firocoxib by a mean  $\pm$  SEM of  $110.1 \pm 70.5\%$ , compared to those treated with flunixin meglumine which decreased by a mean of  $-61.5 \pm 12.9\%$  from baseline (Figure 2). Plasma PGEM concentrations were significantly increased at 8 hours after ischemia compared to baseline, in horses treated with saline (mean  $\pm$  SEM  $55.8 \pm 12.5\%$ ) compared to horses treated with flunixin meglumine or firocoxib ( $P=0.006$ , Figure 3). PGEM concentrations remained virtually unchanged after ischemia in horses treated with flunixin meglumine or firocoxib, with a mean  $\pm$  SEM % change of  $-2.9 \pm 14.3\%$  and  $-3.5 \pm 11.1\%$  from baseline respectively.

**Electrical Studies-** There was no significant difference in TER of control jejunum from horses treated with saline, flunixin meglumine or firocoxib during the *in vitro* incubation period. There was also no significant difference in TER of ischemic-injured mucosa from horses treated with flunixin meglumine and control tissues in all treatment groups over the entire *in vitro* incubation period (Figure 4a). TER was significantly increased in ischemic-injured mucosa from horses treated with saline or firocoxib compared to ischemic-injured mucosa from horses treated with flunixin meglumine (vs. saline  $P=0.009$ , vs. firocoxib  $P<0.001$ ) and compared to all control mucosa ( $P<0.008$ ) over the *in vitro* recovery period.

Immediately following stripping of ischemic-injured mucosa from the seromuscular

layer and mounting in Ussing chambers, an increase in  $I_{sc}$ , an indicator of  $Cl^-$  secretion, was detected in tissues from horses treated with saline or firocoxib (Figure 4b). The  $I_{sc}$  remained virtually unchanged in mucosa from horses treated with flunixin meglumine and was significantly lower than that of saline treated horses at 30 minutes ( $P=0.006$ ) and significantly lower than both saline and firocoxib treated horses at 45 and 60 minutes ( $P<0.027$ ). After the initial 60 minute *in vitro* period,  $I_{sc}$  returned to baseline and was not significantly different between treatment groups for the remainder of the experiment.

**Permeability Studies-** Lipopolysaccharide flux was significantly increased across ischemic-injured mucosa from horses treated with flunixin meglumine compared to control mucosa from all treatment groups, and across ischemic-injured mucosa from all other treatment groups ( $P<0.006$ , Figure 5). In no other treatment group was LPS flux across ischemic-injured mucosa significantly different to that across control mucosa.

**Histology-** No denudation of villi was detected in biopsy sections taken from control jejunum at the end of the 2 hour ischemic period. There was no significant difference in the percentage of villus denudation after 2 hours of ischemia between the three treatment groups. Therefore, the data were pooled to give a mean overall villus denudation of  $39.8 \pm 3.8\%$  after 2 hours of ischemia. By 18 hours, restitution was mostly complete, with a reduction in the percentage denudation to a mean of  $8.7 \pm 8.3\%$  in saline treated horses,  $9.5 \pm 8.0\%$  in horses treated with flunixin meglumine and  $0 \pm 0\%$  in horses treated with firocoxib (Figure 6). There was no significant difference in the percentage of villus denudation between the treatment groups after 18 hours of recovery. Some mild epithelial loss ( $<0.5\%$ ) was detected in control jejunum at 18 hours after surgery (data not shown).

**Western analyses-** Samples from 3 representative horses in each treatment group were evaluated from uninjured (control) and ischemic-injured mucosa obtained at the end of the ischemic period (0 hours recovery) and immediately after euthanasia (18 hours recovery). Densitometry on  $\beta$  actin bands showed no significant difference across all lanes, confirming equal protein loading. Blots were first probed for COX-2, then stripped and re-probed for COX-1. Multiple bands of various molecular weights were detected with the COX-2 antibody. The band immediately below the 75KDa molecular weight marker was considered to be COX-2. This was also confirmed by comparing the selected COX-2 band to the position of the single clear band seen on the corresponding stripped COX-1 blots, which were found to be in an almost identical position. Because samples from different horses were run on different gels, data were expressed as the percentage change between control and ischemic-injured mucosa for each horse, rather than as absolute densitometry units, to allow for differences in transfer and developing between the blots.

After 2 hours of ischemia (0 hours), COX-1 was found in control samples from all horses confirming its constitutive expression (Figure 7a). Densitometry showed that there was virtually no change in COX-1 expression in ischemic-injured mucosa relative to control mucosa at 0 hours, with no significant difference between treatment groups (% change  $\pm$  SEM: saline  $-3.54 \pm 6.6\%$ , flunixin meglumine  $1.14 \pm 1.04\%$ , firocoxib  $0.49 \pm 3.53\%$ , Figure 7b). In contrast, only faint COX-2 signal was detected in control mucosa at 0 hours indicating low constitutive expression (Figure 7a). COX-2 signal was detectable in ischemic-injured mucosa after 2 hours of ischemia, with no significant difference between treatment groups (% change between control and ischemic mucosa  $\pm$ SEM: saline  $9.94 \pm 5.14\%$ , flunixin

meglumine  $19.19 \pm 5.50\%$ , firocoxib  $8.38 \pm 6.84\%$ , Figures 7a and 7d).

Eighteen hours after the end of ischemia, COX-1 expression was significantly increased between ischemic-injured mucosa and control mucosa in all treatment groups (mean % increase  $\pm$  SEM: saline  $38.89 \pm 2.35\%$ , flunixin meglumine  $26.60 \pm 0.03\%$ , firocoxib  $23.72 \pm 6.57\%$ ,  $P \leq 0.003$ , Figure 7c). The % increase in COX-1 expression in ischemic tissue was significantly greater in horses treated with saline than in those treated with firocoxib ( $P=0.025$ , Figure 7b). COX-2 expression was increased in ischemic-injured mucosa compared to control mucosa in all treatment groups after 18 hours of recovery, with no significant difference between groups, nor between the change at 0 hours (mean % increase  $\pm$  SEM: saline  $30.90 \pm 5.74\%$ , flunixin meglumine  $10.61 \pm 1.62\%$ , firocoxib  $23.10 \pm 15.56\%$ , Figures 7c and d).

## **Discussion**

Flunixin meglumine is currently the drug most commonly administered after colic surgery for its analgesic effects, and because it reduces clinical signs of endotoxemia.<sup>7</sup> Providing adequate analgesia after colic surgery has been shown to reduce post-surgical weight loss and hospital stay<sup>22</sup> and therefore is a critical part of postoperative management. However, in this study we again document<sup>5,15</sup> that, although flunixin meglumine provides effective visceral analgesia, it retards recovery of ischemic-injured jejunum. Normally, ischemic-injured mucosa has a higher TER than control tissue after 18 hours of recovery.<sup>15</sup> This overshoot phenomenon is likely due to the PGs produced in response to ischemic-injury stimulating closure of the paracellular spaces in the crypts during recovery,<sup>13,23,24</sup> and is

exaggerated due to the high density of tight junctions in crypt epithelium.<sup>25,26</sup> This elevation in TER can be observed in horses treated with saline, but is inhibited by treatment with flunixin meglumine. Of particular concern from a clinical perspective is the elevation in LPS flux across ischemic mucosa from horses treated with flunixin meglumine. Horses are particularly sensitive to endotoxin absorption, with administration of only 0.03 µg/Kg causing tachypnea, tachycardia, fever and reduced gastrointestinal motility.<sup>27</sup> In this study, we showed absorption of 0.0164 µg of LPS per hour per cm<sup>2</sup> of jejunal mucosa from horses treated with flunixin meglumine. The time for complete recovery of barrier function in horses treated with flunixin meglumine is unknown, but given the current data, it is obviously greater than 18 hours. Therefore, even if a small area of injured mucosa remains after surgery, absorption of endotoxin could be significant in horses treated with flunixin meglumine. However, treatment with firocoxib did allow recovery of the mucosal barrier, as indicated by a high TER and low permeability to LPS, which were not significantly different to measurements in horses treated with saline.

Recovery of the epithelial barrier after ischemic injury first requires villus contraction and restitution of the epithelium to cover the denuded basement membrane.<sup>28</sup> We found that restitution was mostly complete after 18 hours of recovery and was not affected by treatment. Therefore, the reduction in TER and increase in LPS flux seen in ischemic-injured jejunum from horses treated with flunixin meglumine is unlikely to be due to inhibition of this phase of healing. The relative lack of importance of restitution in recovery of TER has been previously documented in bile injured porcine ileum.<sup>24</sup> This study, and others,<sup>29</sup> suggest that it is more likely the subsequent phase in recovery of the epithelial barrier, closure of the

paracellular space, that is critical for recovery of barrier function. This phase of repair is mediated by COX derived prostaglandins through stimulation of Cl<sup>-</sup> secretion and inhibition of electroneutral Na<sup>+</sup> absorption.<sup>29-32</sup> Inhibiting the production of COX derived PGs by administration of a non-selective COX inhibitor, such as flunixin meglumine, would retard closure of the paracellular space. This effect of flunixin meglumine can be evaluated by measuring the short circuit current that is generated by Cl<sup>-</sup> secretion in response to endogenous tissue prostaglandins, which are released when the mucosa is stripped from the seromuscular layer. In this study we showed that Cl<sup>-</sup> secretion is increased during the first 60 minutes of *in vitro* incubation of mucosa from horses treated with saline or firocoxib, but that this increase is inhibited in tissue from horses treated with flunixin meglumine. This data suggests that the effect of flunixin meglumine on recovery of TER and paracellular permeability to LPS in ischemic-injured equine jejunum is due to inhibition of COX-1 derived PGs, and ultimately prevention of closure of the paracellular space. Conversely, in tissues from horses treated with firocoxib or saline, short circuit current is elevated after stripping of the mucosa, suggesting that COX-1 derived PGs are produced, and stimulate closure of the paracellular space. This would imply that firocoxib allows sufficient production of COX-1 derived prostaglandins to allow mucosal recovery in horses.

Further evidence of the COX-1 sparing effect of firocoxib is observed in the results of plasma prostanoid concentrations. Thromboxane A<sub>2</sub> is produced in platelets solely by the COX-1 enzyme with no contribution from COX-2.<sup>33</sup> Measurement of its stable metabolite, TXB<sub>2</sub> in whole blood and plasma has been used as a determinant of COX-1 activity.<sup>34,35</sup> We found that treatment with firocoxib allowed an increase in plasma TXB<sub>2</sub> concentration after

surgery, whereas this increase was inhibited by treatment with flunixin meglumine. This suggests that firocoxib has a sparing effect on the generation of COX-1 derived TXA<sub>2</sub>, whereas production of TXA<sub>2</sub> is inhibited by flunixin meglumine. PGE synthase is the enzyme which is responsible for the conversion of COX derived PGH<sub>2</sub> to PGE<sub>2</sub>.<sup>36,37</sup> There are several forms of PGE synthase, but the enzyme is preferentially coupled to COX-2 rather than COX-1,<sup>36,37</sup> and therefore measurement of PGE<sub>2</sub> concentration in blood and plasma is primarily used as an indicator of COX-2 activity.<sup>35</sup> Our results show that treatment with either flunixin meglumine or firocoxib inhibited the post-surgical increase in plasma PGE<sub>2</sub> found in horses treated with saline solution, implying that both drugs inhibit the COX-2 isoform.

Western analyses confirmed the findings of previous studies: COX-1 is constitutively expressed in equine jejunum as evidenced by its presence in control tissue at the time of ischemia and after 18 hours of recovery.<sup>5,15</sup> COX-1 protein was upregulated in ischemic-injured tissue after 18 hours of recovery in all treatment groups, suggesting that it is not purely a constitutive enzyme. An increase in COX-1 has also been documented in microglia after cerebral ischemia,<sup>38</sup> and has been found in equine ischemic jejunum at this time point in a previous study.<sup>15</sup> This increase in COX-1 in ischemic-injured mucosa at 18 hours was partly abolished by treatment with firocoxib, a finding we have previously documented with meloxicam, a COX-2 preferential NSAID.<sup>15</sup> However, the elevated TER and reduced LPS flux found in firocoxib treated horses indicate that firocoxib still permits sufficient production of PGs to allow mucosal recovery, and this reduction in COX-1 may be due to alternative anti-inflammatory mechanisms of NSAIDs.<sup>39</sup>

In contrast to COX-1, COX-2 appears to be rapidly upregulated after ischemic injury with increased amounts in ischemic tissue after just 2 hours of ischemia. This rapid increase in COX-2 protein levels by 2 hours has been also found in rats after intestinal ischemia induced by superior mesenteric artery occlusion.<sup>40</sup> COX-2 derived prostaglandins are known to contribute to intestinal inflammation and injury after small intestinal ischemia/ reperfusion and COX-2 is therefore a potential therapeutic target.<sup>40</sup> Although no differences were detected between treatment groups for COX-2 expression, the power of this test was 0.091 which is below the desired power of 0.8, probably due to the small number of horses analyzed in each treatment group. Therefore it is possible that there is a difference in the effect of the treatments on COX-2 expression, but we were unable to detect it.

The goat polyclonal COX-2 antibody that we used resulted in several strong false positive signals of various molecular weight proteins. This has been previously described as a complication of COX-2 immunoblotting and underscores the importance of using accurate molecular weight markers when identifying the COX-2 signal.<sup>41</sup> The use of real-time quantitative polymerase chain reaction to identify COX-2 mRNA would be an appropriate alternative to immunoblotting for COX-2, and has been previously reported in the determination of COX-2 mRNA levels in equine laminae.<sup>42</sup>

Firocoxib remained at detectable concentrations in the plasma 16 hours after intravenous administration, and appears to have a very long elimination phase. This suggests that the dose used in this study is appropriate for once daily intravenous dosing of this drug in normal horses. However, horses with colic frequently have compromised cardiovascular function such as reduced cardiac output,<sup>43</sup> and hypotension,<sup>44</sup> which may affect elimination

and metabolism of drugs, necessitating a change in dose or frequency of administration.<sup>45</sup>

Therefore, a pharmacokinetic study of firocoxib in the target population would be advisable.

An established behavioral pain scoring system<sup>20</sup> was used in this study as a more objective method to assess the visceral analgesic effects of the different treatments. Although pain scores in saline treated horses were significantly increased at 4 and 8 hours after the end of ischemia compared to preoperative values, no evidence of gross pain was detected at any time. Firocoxib has been shown to be effective as a somatic analgesic in horses, as it was documented to reduce pain associated with osteoarthritis.<sup>19</sup> In this study, postoperative pain scores were not significantly increased compared to preoperative values in horses treated with firocoxib, suggesting that this treatment provides effective visceral analgesia.

Based on the data obtained in this study, this intravenous formulation of firocoxib appears to be COX-2 selective in horses. Administration resulted in effective visceral analgesia whilst permitting recovery of mucosal barrier function in ischemic-injured intestine. Further *in vivo* studies are indicated to determine its suitability for use as an alternative to flunixin meglumine in postoperative colic patients with ischemic-injured intestine.

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<sup>a</sup> 2% firocoxib w/v injectable solution, Merial, Duluth, GA

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<sup>b</sup> Prostaglandin E Metabolite EIA Kit, Cayman Chemical, Ann Arbor, MI

<sup>c</sup> Thromboxane B<sub>2</sub> EIA Kit, Cayman Chemical, Ann Arbor, MI

<sup>d</sup> SantaCruz Biotechnology, Inc., Santa Cruz, CA

<sup>e</sup> AbCam plc, Cambridge, MA

<sup>f</sup> ECL Western Blotting Substrate, Pierce, Rockford, IL

<sup>g</sup> SigmaScan Pro 5, Systat Software Inc., San Jose, CA

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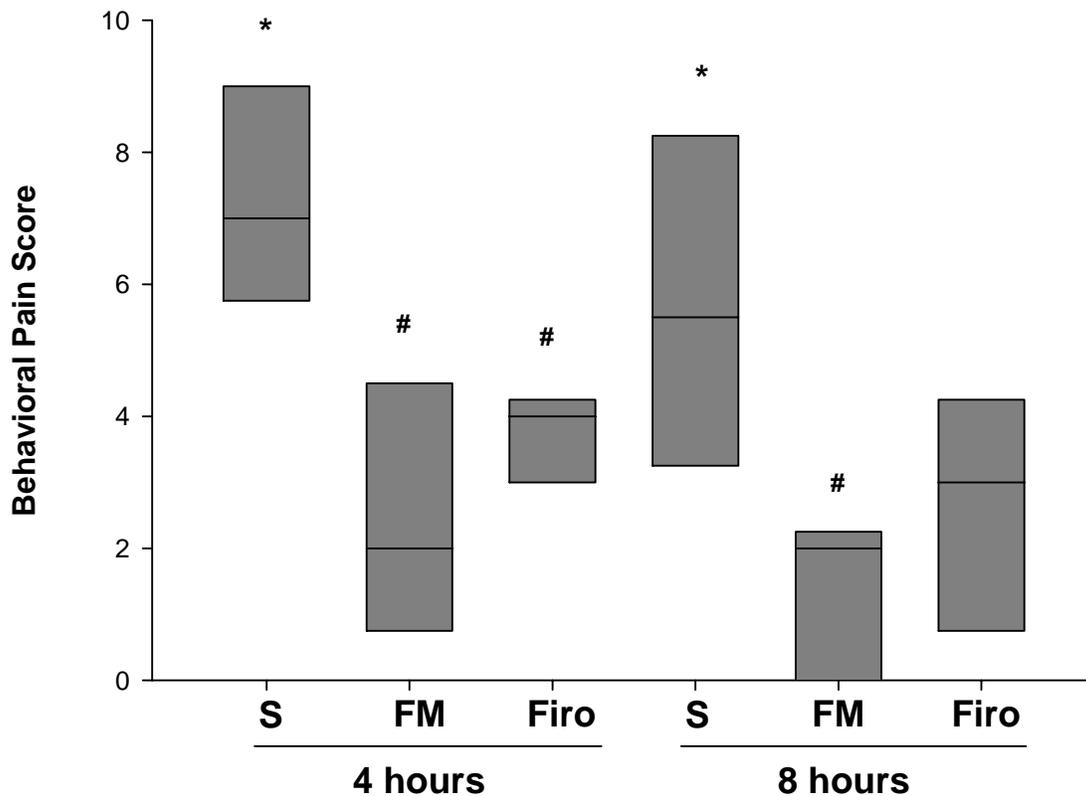
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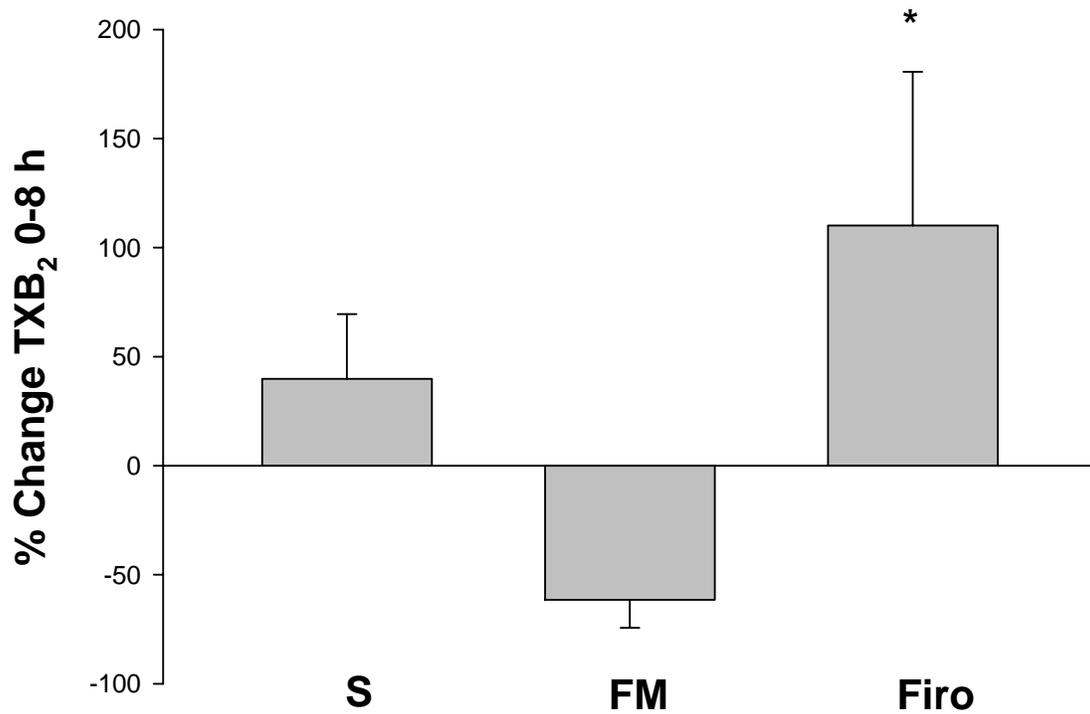
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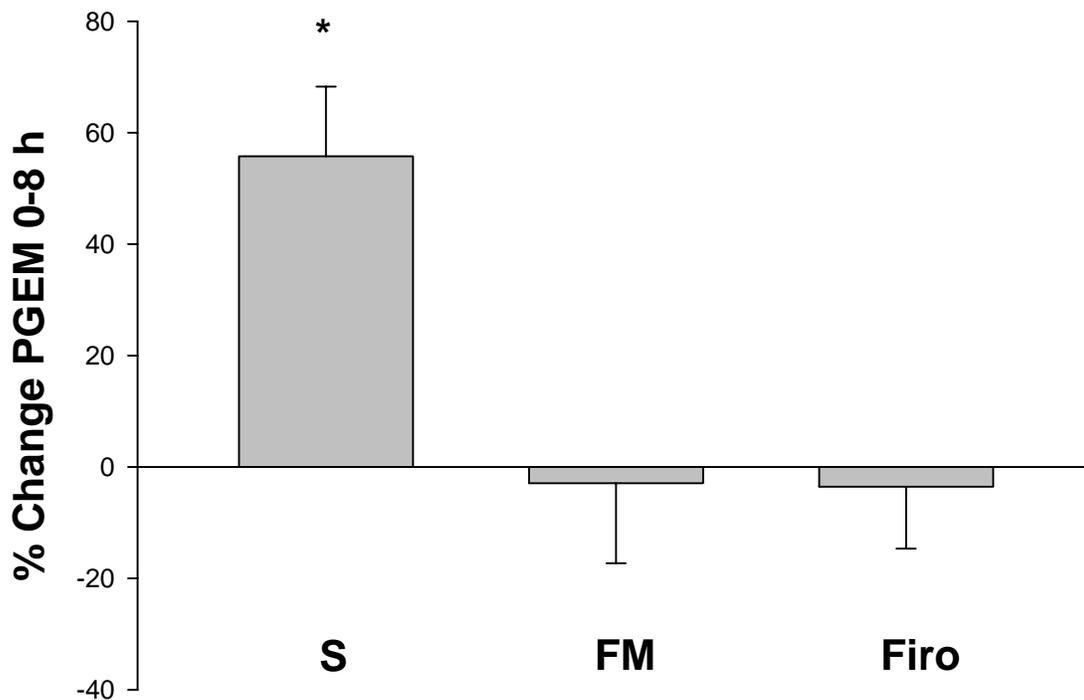
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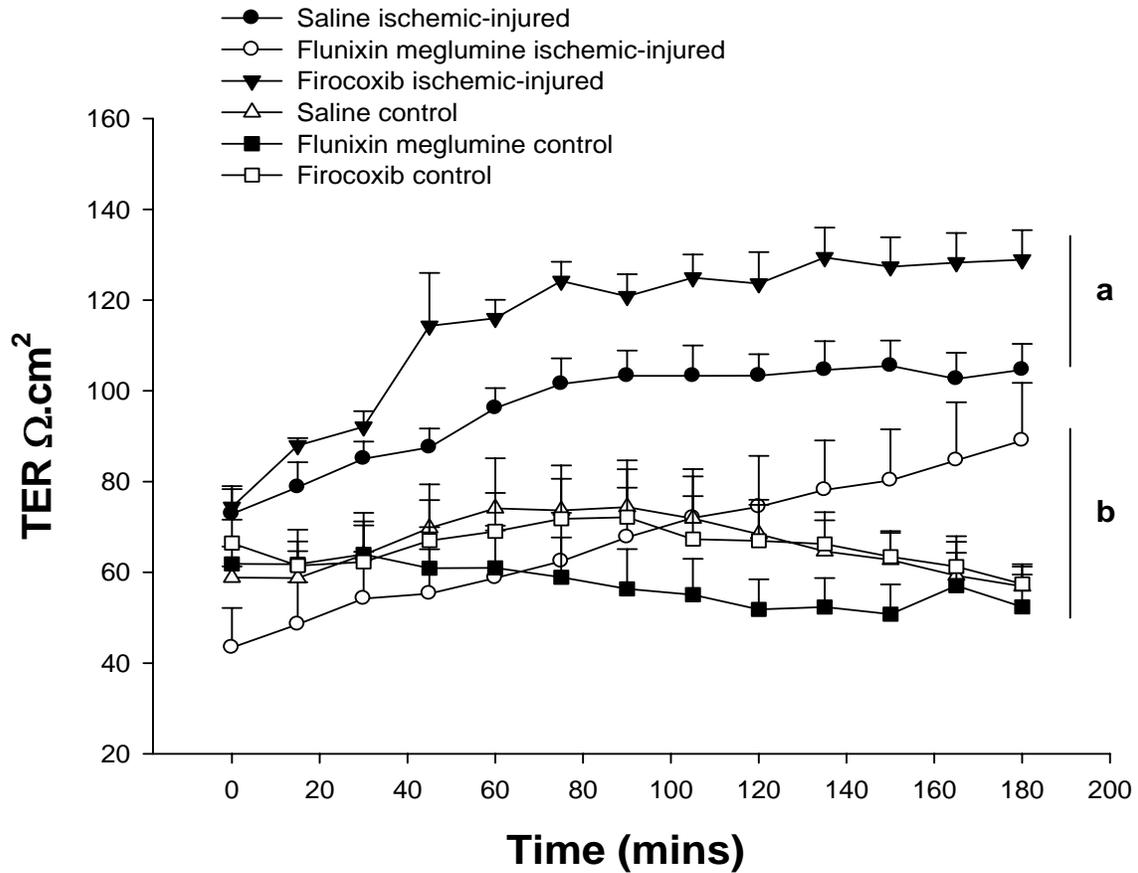
**Figure 1:** Behavioral pain scores for horses treated with 1mL/kg 0.9% saline IV every 24 hours (S), 1.1mg/kg flunixin meglumine IV every 12 hours (FM), or 0.09mg/kg firocoxib IV every 24 hours (Firo) at 4 and 8 hours after the end of 2 hours of jejunal ischemia. Lines in the boxes represent the median pain score, while the upper and lower limits of the box represent the 75<sup>th</sup> and 25<sup>th</sup> percentile. \* Value is significantly greater than the preoperative score for that treatment group (P=0.002). # Value is significantly lower than the score for saline treated horses at that time point (P<0.05).



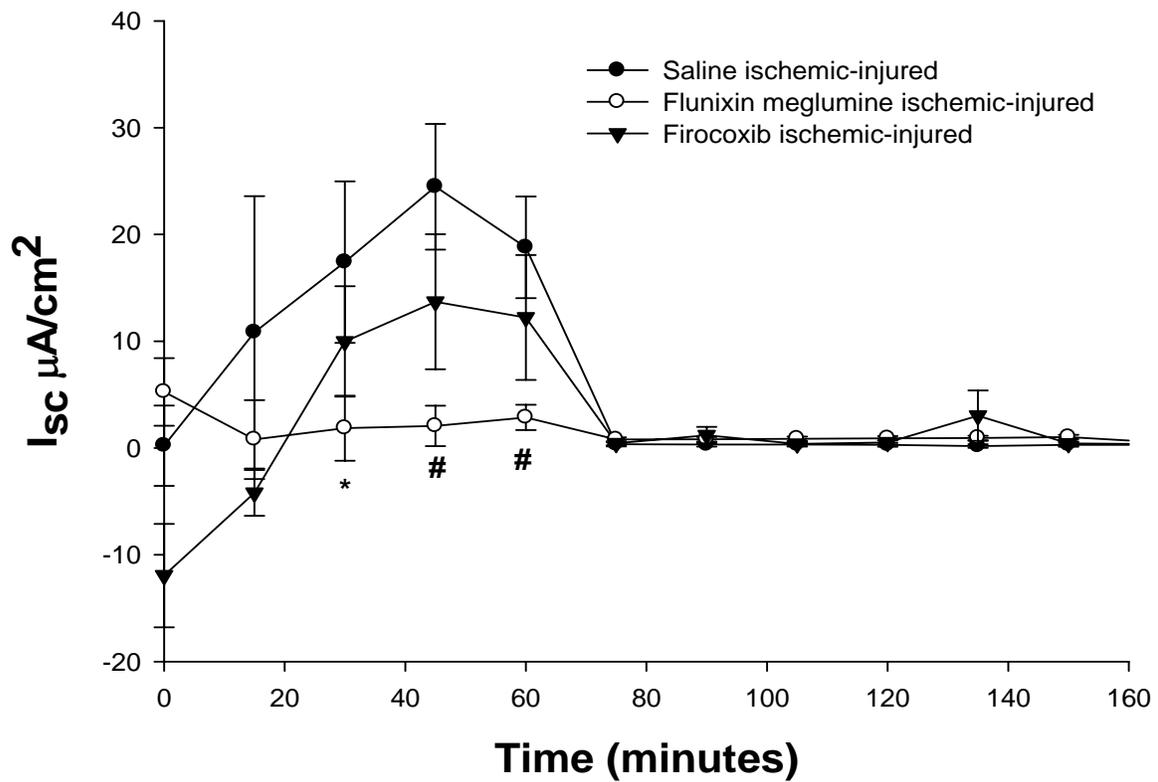
**Figure 2:** Percentage change in plasma thromboxane (TX) B<sub>2</sub> concentration from 0 hours (preoperative) values to 8 hours after jejunal ischemic injury in horses treated with 1mL/kg 0.9% saline IV every 24 hours (S), 1.1mg/kg flunixin meglumine IV every 12 hours (FM), or 0.09mg/kg firocoxib IV every 24 hours (Firo). \* Value is significantly different to horses treated with flunixin meglumine (P=0.049).



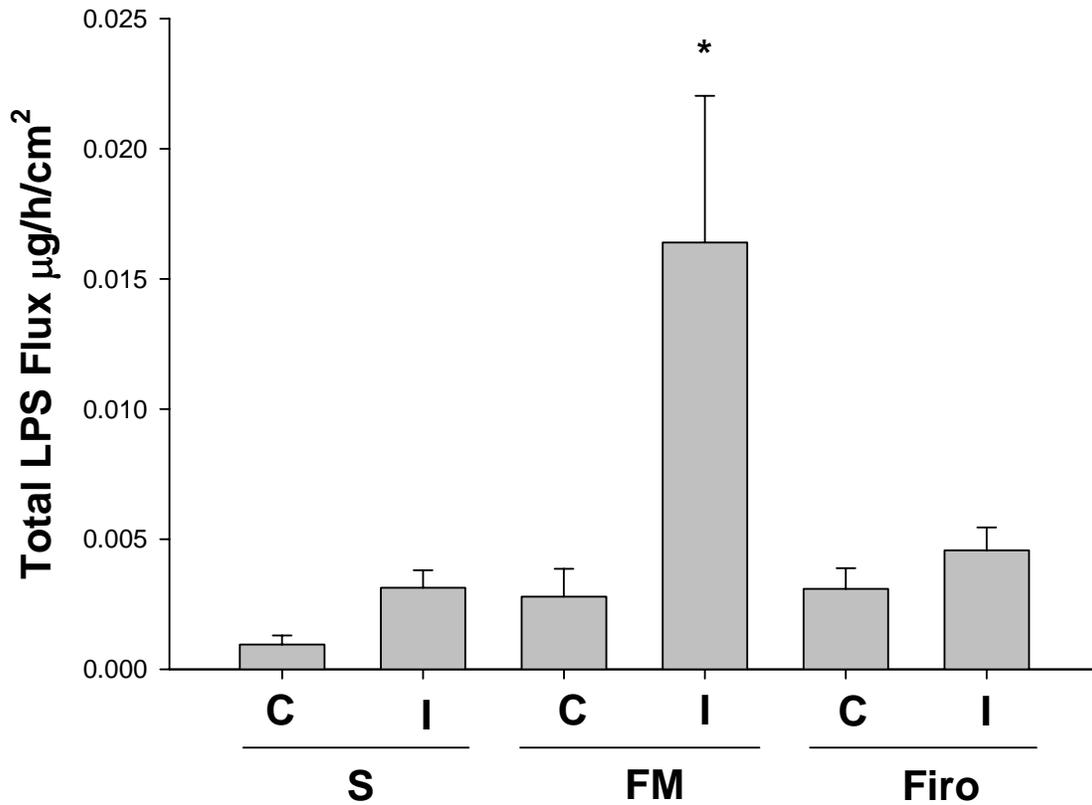
**Figure 3:** Percentage change in plasma prostaglandin E<sub>2</sub> metabolite (PGEM) concentration from 0 hours (preoperative) values to 8 hours after jejunal ischemic injury in horses treated with 1mL/kg 0.9% saline IV every 24 hours (S), 1.1mg/kg flunixin meglumine IV every 12 hours (FM), or 0.09mg/kg firocoxib IV every 24 hours (Firo). \* Value is significantly different to horses treated with flunixin meglumine or firocoxib (P=0.006).



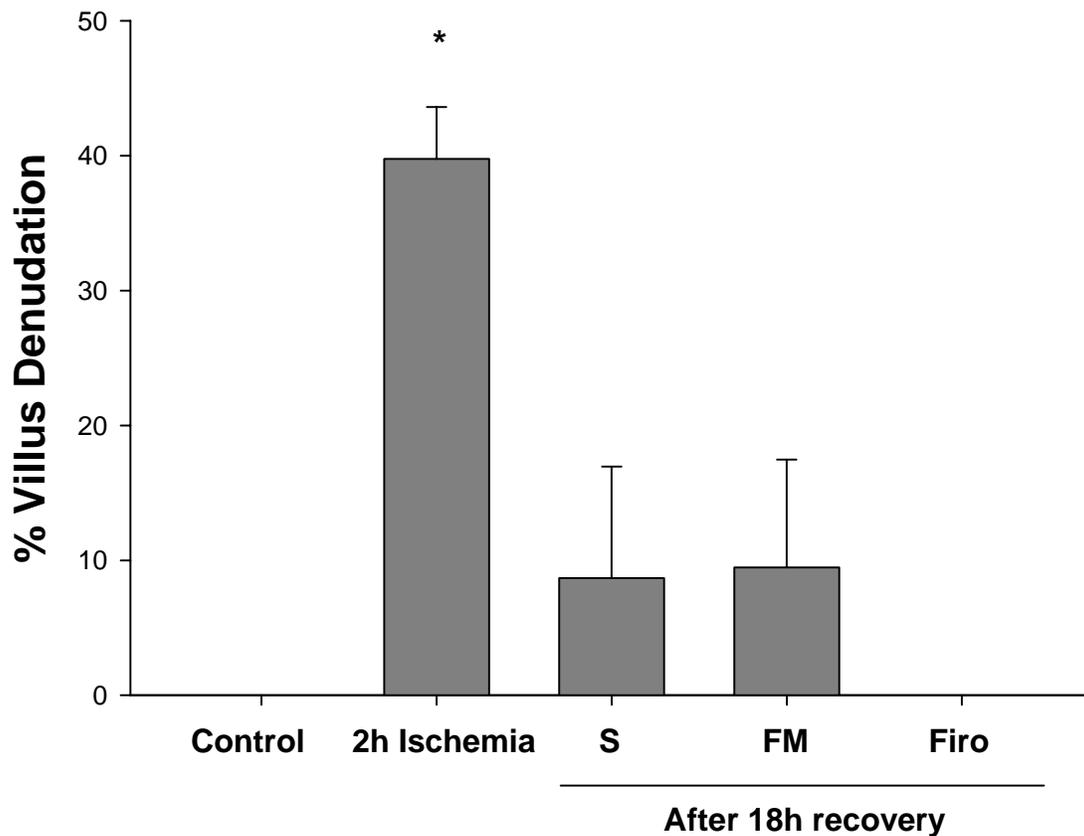
**Figure 4a:** Mean  $\pm$  SEM transepithelial resistance (TER) of control or ischemic-injured jejunal mucosa from horses treated with 1mL/kg 0.9% saline IV every 24 hours, 1.1mg/kg flunixin meglumine IV every 12 hours, or 0.09mg/kg firocoxib IV every 24 hours, at 18 hours after ischemic injury. TER of control mucosa did not vary between treatment groups and was significantly lower than TER of ischemic-injured mucosa from horses treated with saline or firocoxib (b vs. a,  $P < 0.008$ ). TER of ischemic-injured mucosa from horses treated with flunixin meglumine was significantly lower than that of ischemic-injured mucosa from horses treated with saline or firocoxib (b vs. a,  $P < 0.009$ ).



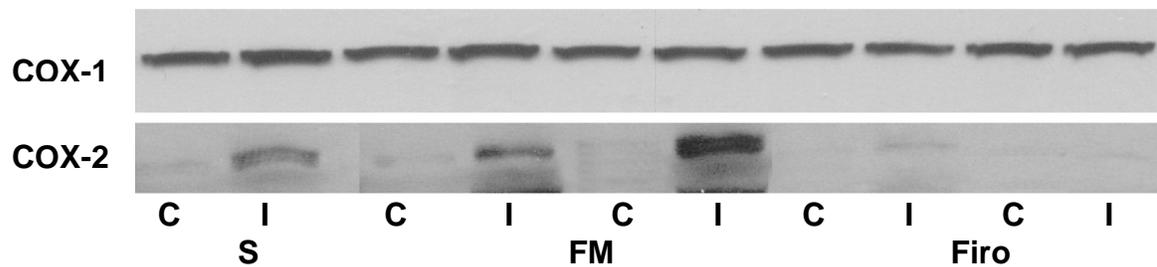
**Figure 4b:** Mean  $\pm$  SEM short circuit current ( $I_{sc}$ ) of ischemic-injured jejunal mucosa from horses treated with 1mL/kg 0.9% saline IV every 24 hours, 1.1mg/kg flunixin meglumine IV every 12 hours, or 0.09mg/kg firocoxib IV every 24 hours. \* Value is significantly lower than in mucosa from horses treated with saline ( $P=0.006$ ). # Value is significantly lower than in mucosa from horses treated with saline or firocoxib ( $P<0.027$ ).



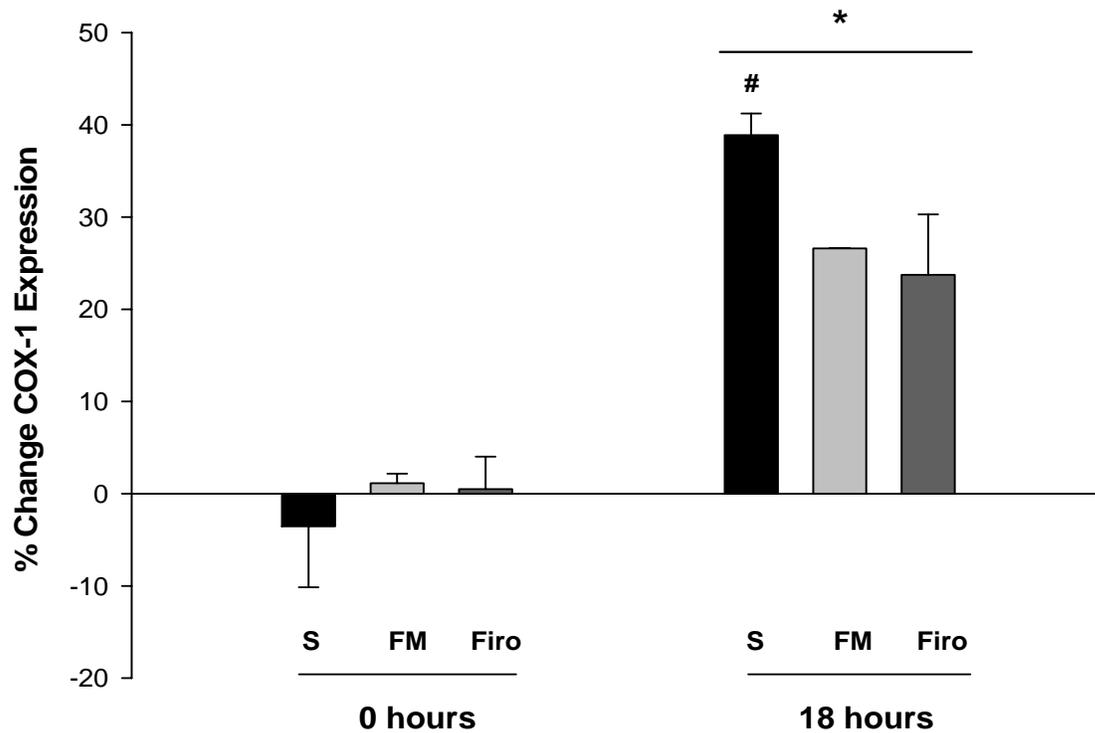
**Figure 5:** Amount of lipopolysaccharide (LPS) in  $\mu\text{g}/\text{hour}/\text{cm}^2$  crossing from the mucosal to serosal side of jejunal mucosa mounted in Ussing chambers. Mucosa was harvested from jejunum that was either undamaged (control (C)) or subjected to 2 hours of ischemia by occlusion of the local blood supply (ischemic-injured (I)) from horses that had been treated with 1mL/kg 0.9% saline IV every 24 hours (S), 1.1mg/kg flunixin meglumine IV every 12 hours (FM), or 0.09mg/kg firocoxib IV every 24 hours (Firo). \* Value is significantly greater than all other groups ( $P < 0.006$ )



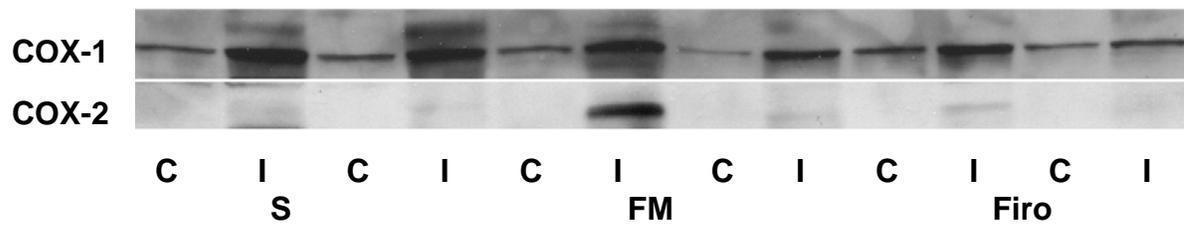
**Figure 6:** The percentage of villus denudation measured in biopsy samples taken from horses treated with 1mL/kg 0.9% saline IV every 24 hours (S), 1.1mg/kg flunixin meglumine IV every 12 hours (FM), or 0.09mg/kg firocoxib IV every 24 hours (Firo). Biopsy samples were taken from ischemic-injured jejunum after 2 hours of ischemia (2h Ischemia) and adjacent uninjured jejunum (Control). No significant difference was detected between treatment groups at this time, so the data were pooled. Biopsy samples taken after 18 hours of recovery showed no significant difference in restitution between treatment groups. \* Value is significantly different to all other groups ( $P < 0.001$ ).



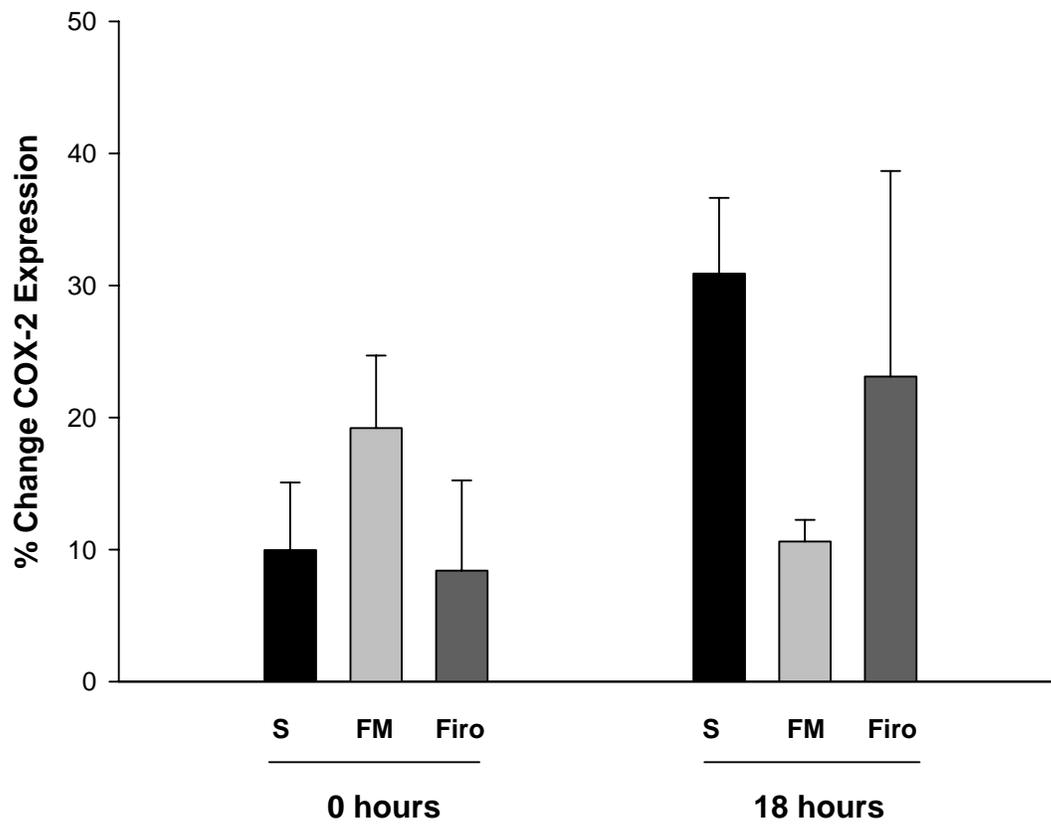
**Figure 7a:** COX-1 and COX-2 expression in uninjured (control (C)) and ischemic-injured (I) mucosa after 2 hours of ischemia (0 hours) from representative horses treated with either 1mL/kg 0.9% saline IV every 24 hours (S), 1.1mg/kg flunixin meglumine IV every 12 hours (FM), or 0.09mg/kg firocoxib IV every 24 hours (Firo). COX-1 is constitutively expressed, as evidenced by its presence in uninjured (control) jejunum at this time point.



**Figure 7b:** Percentage change in COX-1 expression between uninjured (control) and ischemic-injured mucosa based on densitometry of Western analyses on samples taken at the end of 2 hours of ischemia (0 hours) and after 18 hours of recovery (18 hours). Horses were treated with either 1mL/kg 0.9% saline IV every 24 hours (S), 1.1mg/kg flunixin meglumine IV every 12 hours (FM), or 0.09mg/kg firocoxib IV every 24 hours (Firo). \* Values are significantly greater than those at 0 hours in all treatment groups. # Value is significantly different to horses treated with firocoxib (P=0.025) at 18 hours.



**Figure 7c:** COX-1 and COX-2 expression in uninjured (control (C)) and ischemic-injured (I) mucosa after 2 hours of ischemia and 18 hours of recovery (18 hours) from representative horses treated with either 1mL/kg 0.9% saline IV every 24 hours (S), 1.1mg/kg flunixin meglumine IV every 12 hours (FM), or 0.09mg/kg firocoxib IV every 24 hours (Firo). Both COX-1 and COX-2 appear to be up regulated in ischemic-injured mucosa.



**Figure 7d:** Percentage change in COX-2 expression between uninjured (control) and ischemic-injured mucosa based on densitometry of Western analyses on samples taken at the end of 2 hours of ischemia (0 hours) and after 18 hours of recovery (18 hours). Horses were treated with either 1mL/kg 0.9% saline IV every 24 hours (S), 1.1mg/kg flunixin meglumine IV every 12 hours (FM), or 0.09mg/kg firocoxib IV every 24 hours (Firo). No significant differences were detected between 0 and 18 hours, or between treatment groups.

## CHAPTER 6

### **The Effect of Lidocaine on *In Vitro* Adhesion and Migration of Equine Neutrophils**

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## **Abstract**

**Objective:** Determine if *in vitro* migration and adhesion of equine neutrophils is inhibited by lidocaine

**Animals:** Blood from 6 adult horses

**Procedures:** Neutrophils were isolated from equine whole blood using a sediment-gradient centrifugation protocol. Purified neutrophils were incubated with lidocaine at concentrations from 0.1 -1000 $\mu$ g/ml for 30 minutes at 37<sup>0</sup>C, after calcein loading. Adhesion of neutrophils to serum substrates in the presence of HBSS or the stimulants 100nM LTB<sub>4</sub> or PAF, or 100ng/ml IL-8 was assessed by fluorescence and expressed as a percentage of total adhesion. Neutrophil migration in response to HBSS, 100nM LTB<sub>4</sub>, 150nM PAF, or 100ng/ml IL-8 was likewise measured and expressed as a percentage of total migration. Statistical significance was set at P<0.05.

**Results:** Neutrophil adhesion was significantly increased in response to all stimulants, regardless of incubation with lidocaine. IL-8 stimulated adhesion was significantly increased when neutrophils were incubated with 1mg/ml of lidocaine, compared to lower lidocaine concentrations. LTB<sub>4</sub> stimulated adhesion was significantly increased when neutrophils were incubated with 1mg/ml of lidocaine compared to that at 5 $\mu$ g/ml of lidocaine. Migration was significantly increased in response to IL-8 at all lidocaine concentrations, and in response to LTB<sub>4</sub> at lidocaine concentrations  $\geq$ 1 $\mu$ g/ml.

**Conclusions:** Incubation of equine neutrophils with lidocaine at 1mg/ml increased LTB<sub>4</sub> and IL-8 stimulated adhesion. Increasing concentrations of lidocaine resulted in a concurrent increase in LTB<sub>4</sub> and IL-8 stimulated neutrophil migration.

**Clinical Relevance:** Lidocaine did not inhibit neutrophil migration or adhesion *in vitro* at therapeutic concentrations, and increases migration and adhesion at supraphysiologic concentrations.

## Introduction

Neutrophils are a critical component of the immune response, but they have also been shown to retard the recovery of ischemic-injured intestine.<sup>1</sup> This occurs due to their release of reactive oxygen metabolites (ROMs),<sup>2</sup> and physical disruption of epithelial tight junctions as they migrate.<sup>3</sup> Therefore, a treatment that could reduce neutrophil infiltration into recovering injured intestine is desirable and could improve recovery of the mucosal barrier after ischemic injury.

Local anesthetics appear to have novel anti-inflammatory effects that are independent of their anesthetic effect of Na<sup>+</sup> channel blockade.<sup>4</sup> An *in vitro* study on isolated human neutrophils determined that incubation with lidocaine inhibited the production of ROMs by neutrophils.<sup>5</sup> However, this effect was only observed at lidocaine concentrations in the milligram range, which is consistent with the levels obtained with local infiltration of lidocaine. Another study showed that lidocaine suppressed chemotaxis and phagocytosis of isolated human neutrophils at concentrations of 20 µg/ml and higher, whereas this effect was not observed with other local anesthetics.<sup>6</sup> These *in vitro* studies have all been performed on human neutrophils and the effect of lidocaine on immune functions of equine neutrophils has not yet been evaluated.

We have previously documented that systemic administration of lidocaine and flunixin meglumine to an equine model of jejunal ischemic injury resulted in a decrease in mucosal neutrophil counts after 18 hours of recovery, compared to treatment with flunixin meglumine alone (Chapter 4). The plasma lidocaine concentration in these horses was a maximum of  $4.11 \pm 0.02$  µg/ml,<sup>7</sup> which is considerably lower than the concentrations at

which inhibitory effects of lidocaine have been observed in the previously described studies. The mechanism by which lidocaine reduced the influx of neutrophils into ischemic-injured mucosa has not been established. Lidocaine could have a direct inhibitory effect on equine neutrophil adhesion and migration, as has been observed with human neutrophils *in vitro*. Alternatively, lidocaine could reduce tissue damage during ischemic injury and repair, and therefore reduce chemoattractants that would stimulate neutrophil recruitment.

Therefore, we sought to determine if incubation of equine neutrophils with lidocaine would inhibit neutrophil adhesion and migration *in vitro*. By determining the precise affect of lidocaine on neutrophils *in vitro*, we may further understand the mechanisms of action of this drug *in vivo*.

## **Materials and Methods**

**Isolation of Equine Neutrophils-** Neutrophils were isolated from whole equine blood by an established sedimentation-gradient centrifugation protocol.<sup>8,9</sup> Briefly, 20mL of blood was collected from 6 adult healthy horses and transferred to polycarbonate tubes. The blood was allowed to stand at room temperature for 45 minutes to separate the plasma and red cells. The plasma was layered over 5ml of Ficoll-Paque<sup>a</sup> and centrifuged at 1800 rpm for 20 minutes at room temperature. The supernatant was aspirated and the remaining red cells lysed with a hypotonic solution. The pellet was resuspended in Hanks buffered salt solution (HBSS) and washed once. Neutrophil concentration and viability was determined on an aliquot of sample by trypan blue staining and counting using a hemocytometer. The purified neutrophils were then used to determine the effect of lidocaine on *in vitro* adhesion and migration assays.

**Neutrophil Adhesion-** a 96 well Immulon plate<sup>b</sup> was coated with adhesion substrates by adding 100 $\mu$ L of sterile filtered 5% fetal calf serum (FCS) in PBS and incubating at room temperature for 2 hours followed by 3 washes with PBS. Purified neutrophils were resuspended in HBSS at a concentration of  $1 \times 10^7$  cells/mL and loaded with 2 $\mu$ g/mL calcein by incubation for 30 minutes at room temperature. Cells were washed once and then resuspended in HBSS with 1mM Ca<sup>2+</sup> and 1mM Mg<sup>2+</sup> (HBSS<sup>++</sup>) at a concentration of  $2 \times 10^6$  cells/mL. Cells were incubated with lidocaine solution<sup>c</sup> at 0, 0.1, 1, 5, 10, 100 and 1000  $\mu$ g/mL for 30 minutes at 37°C. A 50 $\mu$ L aliquot of each neutrophil solution was added to the appropriate well of the preprepared plate and incubated at 37°C for 10 minutes to allow the neutrophils to settle into the bottom of the wells. Seventeen  $\mu$ L of the adhesion stimulants 100nM leukotriene (LT)B<sub>4</sub>,<sup>d</sup> 100ng/mL interleukin (IL)-8,<sup>e</sup> or 100nM platelet activating factor (PAF),<sup>f</sup> or HBSS<sup>++</sup> as a negative control were then added to the appropriate wells and the plate incubated for 3 minutes at 37°C in a water bath. Calcein fluorescence was determined in a fluorescence plate reader at wavelengths of 485nm excitation and 530nm emission before and after 4 washes with 150 $\mu$ L of PBS. Percent adhesion for each of the 4 wash steps was then calculated by dividing the fluorescence after washing by the fluorescence before washing.

**Neutrophil Migration-** Neutrophils were resuspended and loaded with calcein as described above. Because of the high protein binding of lidocaine, the technique of our previous described migration assay was modified to initially exclude FCS from the incubation.<sup>10</sup> Therefore after washing, cells were resuspended in HBSS<sup>++</sup> to a concentration of  $2 \times 10^6$  cells/mL and incubated with lidocaine as previously described. Cells were then spun at

1200rpm for 10 minutes, and resuspended in HBSS<sup>++</sup> with 2% FCS (chemotaxis buffer) with the appropriate concentration of lidocaine again added, to provide binding substrates for migration. The chemotactic stimulants 100nM LTB<sub>4</sub>, 100ng/mL IL-8 or 150nM PAF, or HBSS<sup>++</sup> with 2% FCS as a negative control were added to the appropriate lower wells of a 2µm ChemoTx plate<sup>s</sup> and 20µL of untreated cells added to 3 untreated lower wells to serve as the 100% migration control. The filter was placed over the lower wells, 20µL of treated cells added to the appropriate top wells, and the plate incubated for 1 hour at 37°C. After incubation, residual cells on top of the filter were removed with a rubber squeegee and washed away with PBS. Five µL of EDTA (0.5M) was added for 5 minutes to the top wells to detach adherent migrated cells and then the plate was centrifuged at 500rpm for 1 minute to dislodge cells from the lower side of the filter. The top of the plate and filter were then removed and the fluorescence in the lower wells measured as before at the same wavelengths. The % migration was determined by dividing the fluorescence of each well by the fluorescence in the 100% migration wells.

The EC<sub>50</sub> for lidocaine for IL-8 and LTB<sub>4</sub> stimulated migration was determined as follows: First, the % excitation was calculated by subtracting the % migration at 0µg/mL of lidocaine from the migration at each concentration of lidocaine for each stimulant. This value was then divided by the % migration at 0µg/mL of lidocaine for the respective stimulant and expressed as a percentage. These values were plotted against the log lidocaine concentration µg/mL and the concentration of lidocaine resulting in a 50% increase in migration calculated by linear regression.

**Statistical Analyses-** Percentage adhesion and migration were evaluated by the use of a 2-way repeated measures ANOVA for the effect of stimulant and lidocaine concentration. When a significant interaction was detected a pairwise multiple comparison procedure (Fisher LSD method) was used to identify the source of the interaction. For each test, the power was determined, and found to be >0.9. Significance was set at  $P < 0.05$ .

## **Results**

**Neutrophil Isolation and Viability-** Isolated neutrophils were determined to be viable if they excluded the trypan blue dye. Using this technique, it was determined that the isolated neutrophils had a viability of >98%.

### **Neutrophil Adhesion-** (Figure 1)

Neutrophil adhesion was significantly increased in response to all stimulants compared to HBSS<sup>++</sup> with no lidocaine (HBSS<sup>++</sup> 0), regardless of incubation with lidocaine ( $P \leq 0.005$  for PAF,  $P \leq 0.004$  for IL-8,  $P \leq 0.008$  for LTB<sub>4</sub>). IL-8 stimulated adhesion was significantly increased when neutrophils were incubated with 1000  $\mu\text{g/ml}$  of lidocaine compared to that seen with IL-8 stimulation at all other lidocaine concentrations ( $P \leq 0.024$ ). LTB<sub>4</sub> stimulated adhesion was significantly increased when neutrophils were incubated with 1000  $\mu\text{g/ml}$  of lidocaine compared to that seen with LTB<sub>4</sub> stimulation of neutrophils incubated with 5  $\mu\text{g/ml}$  of lidocaine ( $P = 0.044$ ).

### **Neutrophil Migration-** (Figure 2)

Migration was significantly increased in response to IL-8 compared to chemotaxis buffer with no lidocaine (chemotaxis buffer 0), regardless of incubation with lidocaine ( $P \leq$

0.034). Migration was significantly increased in response to LTB<sub>4</sub> compared to chemotaxis buffer with no lidocaine (chemotaxis buffer 0) when neutrophils were incubated with lidocaine at concentrations  $\geq 1 \mu\text{g/ml}$  ( $P \leq 0.049$ ). IL-8 stimulated migration was significantly increased when neutrophils were incubated with 1000  $\mu\text{g/ml}$  of lidocaine compared to that seen with IL-8 stimulation at all other lidocaine concentrations ( $P \leq 0.017$ ). LTB<sub>4</sub> stimulated migration was significantly increased when neutrophils were incubated with 1000  $\mu\text{g/ml}$  of lidocaine compared to that seen with LTB<sub>4</sub> stimulation at all other lidocaine concentrations ( $P \leq 0.037$ ).

The log EC<sub>50</sub> for IL-8 was determined to be 2.3752, which equated to a lidocaine concentration of 237  $\mu\text{g/ml}$  (Figure 3a). For LTB<sub>4</sub> the log EC<sub>50</sub> value was 2.1316, which was equivalent to 135  $\mu\text{g/ml}$  of lidocaine (Figure 3b).

## **Discussion**

Previous *in vitro* studies have documented suppression of the immune functions of isolated human neutrophils by lidocaine.<sup>5,6</sup> To our knowledge the effect of local anesthetics on isolated equine neutrophils has not been previously reported. Our study documented an increase in neutrophil adhesion in response to IL-8 and LTB<sub>4</sub> at the highest concentration of lidocaine evaluated. Additionally, IL-8 and LTB<sub>4</sub> stimulated migration was increased with increasing concentrations of lidocaine.

These results are obviously in contrast to similar studies using human neutrophils, and suggest that there may be significant species differences in the response of neutrophils. Although a direct comparison between the responses of human and equine neutrophils has not been performed, differences have been found between other species. A comparison

between isolated human and rabbit neutrophils showed significant species differences in the generation of reactive oxygen intermediates by neutrophils in response to n-formyl-methionyl-leucyl-phenylalanine (fMLP) and zymogen activated plasma.<sup>11</sup> An *in vivo* model of endometritis in cows and mares found that neutrophil migration was 15 times higher in mares, despite using a 4 fold lower concentration of IL-8 as a stimulant.<sup>12</sup> Additionally, when the response of neutrophils from horses and ponies were compared in an inflammatory wound model, chemotaxis was found to be increased in horses compared to ponies, despite a lower local concentration of chemoattractants.<sup>13</sup> These studies suggest that neutrophils of adult horses may be particularly sensitive to local chemoattractants. It is possible, therefore, that the concentration of chemoattractants that we used was relatively high for equine neutrophils, and may have obscured an inhibitory effect of lidocaine.

Our results suggest that high concentrations of lidocaine may actually stimulate neutrophil adhesion and migration, particularly at 1mg/ml. In a previous study, the plasma concentration of lidocaine obtained after systemic administration in horses did not exceed a mean of 4.11 µg/ml.<sup>7</sup> Plasma lidocaine concentrations greater than 3.24µg/ml in horses have been documented to produce toxic side effects.<sup>14</sup> Additionally, the EC<sub>50</sub> determined for lidocaine in this study were over 40 times greater than the reported toxic concentration. Therefore, this documented stimulation of neutrophils by lidocaine is unlikely to be an issue in a clinical situation.

The reason for the observed increase in neutrophil migration and adhesion is unclear. During the study, the solution of neutrophils in HBSS containing lidocaine at 1mg/ml was observed to have a color change compared to the other concentrations of lidocaine. Local

anesthetics are known to be acidic<sup>15</sup> and therefore the pH of the solutions were tested, but were not different from HBSS itself (data not shown). The lidocaine that was used for this study was a 2% solution of lidocaine containing the preservative methyl paraben. Previous *in vitro* studies have used a solution of lidocaine powder in distilled water with no preservatives.<sup>6</sup> However, the 2% solution is the same as that which is used clinically, and therefore, to allow extrapolation from *in vitro* to *in vivo* effects, it is logical to use the same formulation. Methyl p-hydroxybenzoate (methyl paraben) is commonly used in food, drugs and cosmetics as an antimicrobial preservative and is considered to be non toxic.<sup>16</sup> However, a study evaluating its presence in an insulin preparation showed that it reduced neutrophil function.<sup>17</sup> The concentrations of lidocaine for incubation with neutrophils were diluted in PBS and therefore lower concentrations of methyl paraben would also have been present. Therefore it is possible that at 1mg/ml concentration of lidocaine, the correspondingly high concentration of methyl paraben could have affected the isolated neutrophils.

Due to the extremely high protein binding of lidocaine,<sup>15</sup> it was necessary to exclude FCS from the buffer to ensure that free lidocaine was available to interact with the neutrophils during incubation. However, binding proteins present in serum, such as fibrinogen, are essential to allow neutrophil migration.<sup>18</sup> Therefore, following incubation with lidocaine it was necessary to centrifuge and resuspend neutrophils in HBSS with FCS in order for migration to occur during the assay. Excluding FCS from the buffer resulted in migration of <20% in response to the different stimulants (data not shown). This additional wash step could have caused activation of neutrophils and masked an inhibitory effect of lidocaine.

In summary, in contrast to *in vitro* studies of human neutrophils, we documented an increase in Il-8 and LTB<sub>4</sub> stimulated adhesion and migration in response to incubation with supraphysiologic concentrations of lidocaine. It is unlikely that these concentrations would be reached with systemic administration of lidocaine and this is therefore unlikely to be of clinical significance. The cause for the increase in migration and adhesion is unknown. We have previously documented amelioration of flunixin meglumine induced neutrophilic infiltration in ischemic injured equine jejunum by systemically administered lidocaine (Chapter 4). However, the results of this study suggest that this effect is unlikely to be due to a direct effect on neutrophil migration and adhesion by lidocaine. Therefore, it is more likely the documented decrease in mucosal COX-2 expression and plasma PGE<sub>2</sub> concentration, which reduces neutrophilic infiltration.

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<sup>a</sup> Ficoll-Paque PLUS, GE Healthcare, Piscataway, NJ

<sup>b</sup> Immulon Immunoassay Plate, Thermo Fisher Scientific, Waltham, MA

<sup>c</sup> Lidocaine 2% injectable, The Butler Company, Columbus, OH

<sup>d</sup> Leukotriene B<sub>4</sub>, Cayman Chemical, Ann Arbor, MI

<sup>e</sup> Interleukin-8 human, Sigma Aldrich, St Louis, MO

<sup>f</sup> PAF C-16, Cayman Chemical, Ann Arbor, MI

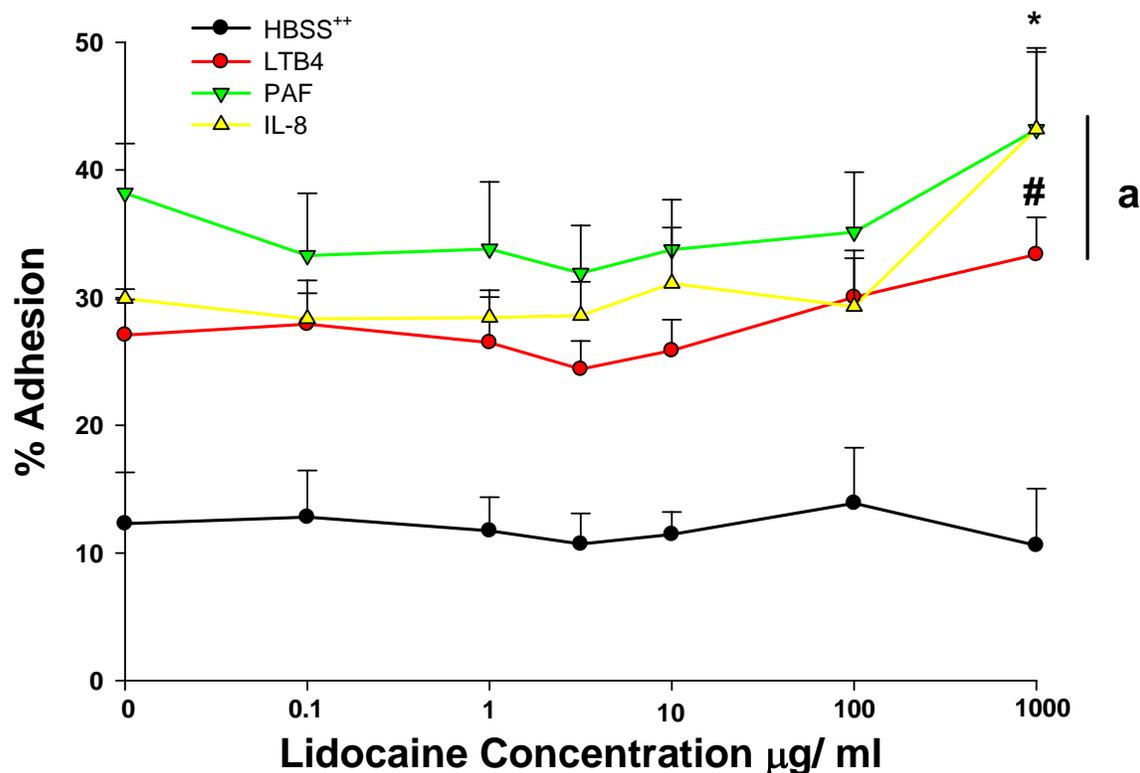
<sup>g</sup> ChemoTx, NeuroProbe Inc, Gaithersburg, MD

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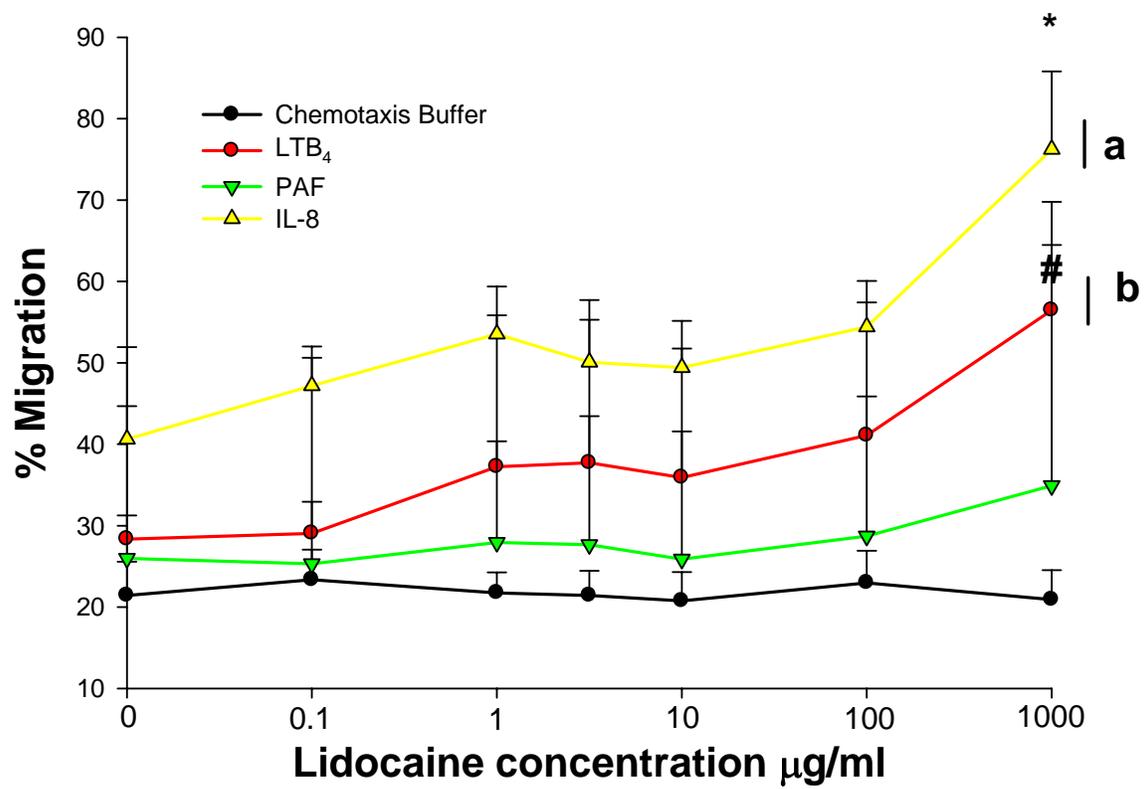
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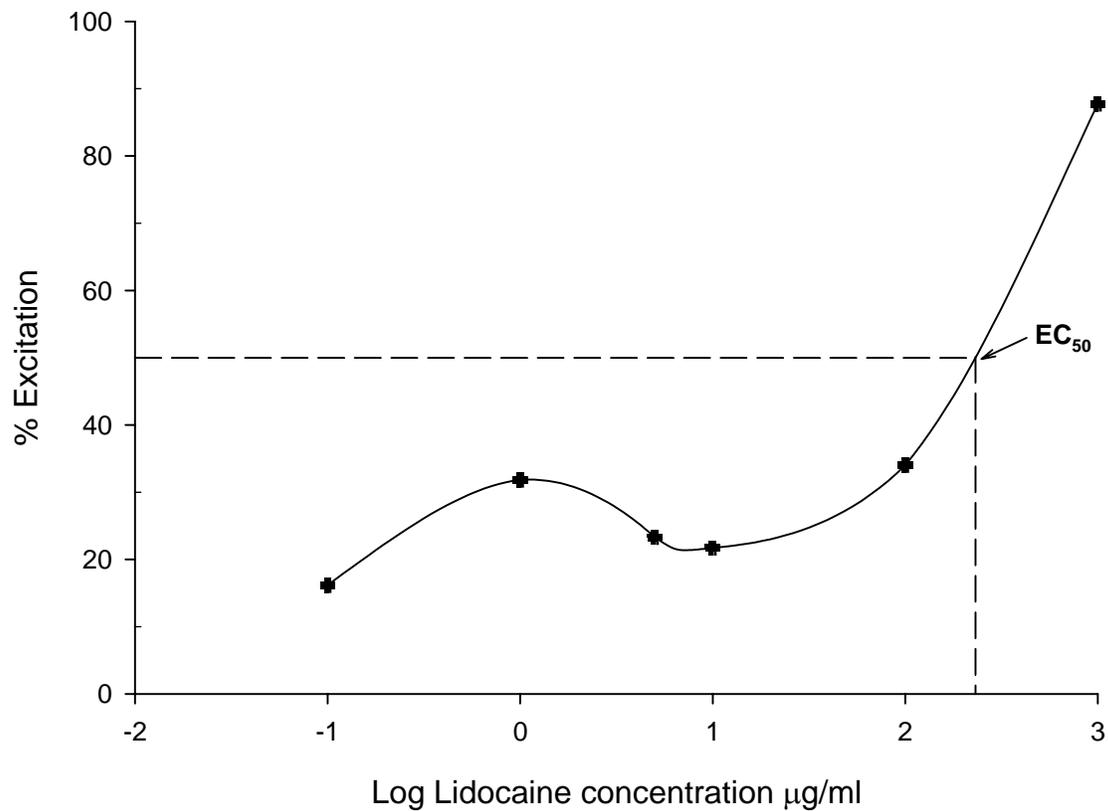
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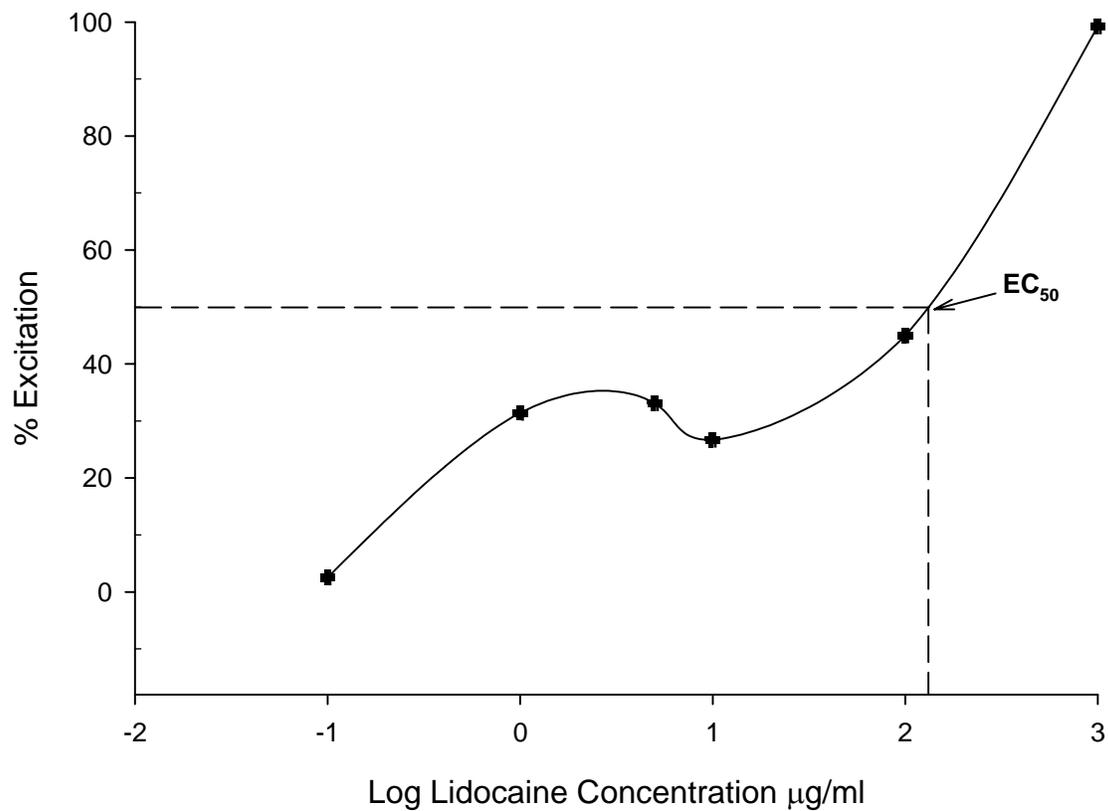
**Figure 1-** Percentage adhesion of equine neutrophils isolated from 6 horses incubated for 30 minutes with varying concentrations of lidocaine in HBSS<sup>++</sup>. Adhesion stimulants were HBSS<sup>++</sup> (negative control), 100nM LTB<sub>4</sub>, 100ng/mL IL-8 or 100nM PAF. Adhesion was significantly increased in response to all stimulants compared to HBSS<sup>++</sup> with no lidocaine (HBSS<sup>++</sup> 0), regardless of incubation with lidocaine (a). \* IL-8 stimulated adhesion was significantly increased when neutrophils were incubated with 1000 µg/ml of lidocaine compared to that seen with IL-8 stimulation at all other lidocaine concentrations. # LTB<sub>4</sub> stimulated adhesion was significantly increased when neutrophils were incubated with 1000 µg/ml of lidocaine compared to that seen with LTB<sub>4</sub> stimulation of neutrophils incubated with 5 µg/ml of lidocaine.

**Figure 2-** Percentage migration of equine neutrophils isolated from 6 horses, incubated for 30 minutes with varying concentrations of lidocaine in HBSS<sup>++</sup>, spun and resuspended in chemotaxis buffer. Migration stimulants were chemotaxis buffer (negative control), 100nM LTB<sub>4</sub>, 100ng/mL IL-8 or 150nM PAF. Migration was significantly increased in response to IL-8 compared to chemotaxis buffer with no lidocaine (chemotaxis buffer 0), regardless of incubation with lidocaine (a). Migration was significantly increased in response to LTB<sub>4</sub> compared to chemotaxis buffer with no lidocaine (chemotaxis buffer 0) when neutrophils were incubated with lidocaine at concentrations  $\geq 1\mu\text{g/ml}$  (b). \* IL-8 stimulated migration was significantly increased when neutrophils were incubated with 1000  $\mu\text{g/ml}$  of lidocaine compared to that seen with IL-8 stimulation at all other lidocaine concentrations. # LTB<sub>4</sub> stimulated migration was significantly increased when neutrophils were incubated with 1000  $\mu\text{g/ml}$  of lidocaine compared to that seen with LTB<sub>4</sub> stimulation at all other lidocaine concentrations.





**Figure 3a-**  $EC_{50}$  for lidocaine in response to IL-8 stimulated migration. The % excitation was calculated by subtracting the % migration at  $0\mu\text{g/ml}$  of lidocaine from the migration at each concentration of lidocaine for IL-8. This value was then divided by the % migration at  $0\mu\text{g/ml}$  of lidocaine for IL-8 and expressed as a percentage. For IL-8 induced migration the  $EC_{50}$  of lidocaine was determined to be  $237\mu\text{g/ml}$ .



**Figure 3b-** EC<sub>50</sub> for lidocaine in response to LTB<sub>4</sub> stimulated migration. The % excitation was calculated by subtracting the % migration at 0µg/ml of lidocaine from the migration at each concentration of lidocaine for LTB<sub>4</sub>. This value was then divided by the % migration at 0µg/ml of lidocaine for LTB<sub>4</sub> and expressed as a percentage. For LTB<sub>4</sub> induced migration the EC<sub>50</sub> of lidocaine was determined to be 135µg/ml.

## **CHAPTER 7**

### **Dissertation Summary and Future Directions**

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## Summary

The goal of this work was to evaluate treatments that could improve the recovery of ischemic-injured intestine in post-operative colic cases. Two different drugs were evaluated, each of which have novel anti-inflammatory effects.

The first drug we investigated was the local anesthetic lidocaine. This drug has been reported to improve recovery after ischemic injury to the myocardium and cerebrum, but its effect on ischemic-injured intestine had not been previously evaluated. Our first study showed that by using systemically administered lidocaine in conjunction with the non-selective COX inhibitor flunixin meglumine, the inhibitory effects of flunixin meglumine on recovery of the mucosal barrier were ameliorated.

We then further sought to determine the mechanism by which this effect was obtained. It was shown that neutrophilic infiltration into ischemic-injured mucosa peaked in horses treated with flunixin meglumine, but this was reduced by combining treatment with lidocaine. Additionally, lidocaine was found to reduce mucosal COX-2 expression after 2 hours of ischemia, and post-operative plasma PGE<sub>2</sub> concentrations. Therefore it remained unclear whether lidocaine reduced mucosal inflammation through a direct inhibitory effect on neutrophils, or if reduced tissue COX-2 expression was responsible for a decreased stimulus for neutrophilic influx.

To evaluate this further, we performed *in vitro* studies to determine the effect of different concentrations of lidocaine on neutrophil adhesion and migration. In contrast to expected results, lidocaine did not inhibit neutrophil adhesion or migration, and actually increased these functions at high concentrations.

Therefore these results suggest that systemically administered lidocaine ameliorates the inhibitory effects of flunixin meglumine on mucosal repair through a reduction mucosal COX-2 expression and hence a reduction in neutrophilic infiltration.

An alternative approach to treatment of post-operative colic patients would be to administer a COX-2 preferential drug which provides effective analgesia but allows repair of ischemic injured intestine through the production of COX-1 associated prostaglandins. Therefore we evaluated the effect of firocoxib, a COX-2 selective inhibitor, on recovery of ischemic injured jejunum. Firocoxib and flunixin meglumine both provided effective visceral analgesia. However, flunixin meglumine inhibited mucosal repair, whereas firocoxib did not.

### **Future Directions**

Both systemically administered lidocaine in combination with flunixin meglumine, and firocoxib provided effective analgesia whilst allowing recovery of the mucosal barrier in our model of equine jejunal ischemic injury. Therefore, a prospective randomized clinical trial to compare the currently used “gold standard”, flunixin meglumine, to these treatment regimens is warranted. Particular attention should be placed on whether effective analgesia is obtained in the clinical case, and the incidence of post operative complications, in addition to short term survival.

Although we determined that lidocaine appears to reduce mucosal COX-2 expression, and COX-2 associated plasma PGEM concentration, the pathway by which this effect is achieved is not known. Further evaluation of lidocaine in a model of ischemic injury would

determine if this is achieved through  $\text{Na}^+$  channel blockade as we postulated in Chapter 1 figure 1, or if different receptors and signaling mechanisms are involved.