Abstract

TOMLINSON, JULIA ELIZABETH. Cyclooxygenase inhibitors affect recovery of ischemic-injured jejunum. (Under the direction of Anthony Blikslager)

The cyclooxygenase (COX) enzyme produces prostaglandins. There are 3 isoforms of COX (including COX-1 and COX-2). As prostaglandins are involved in intestinal repair, the objective of this study was to examine the effects of COX inhibitors on healing of jejunal mucosa following ischemia, with the following specific aims.

Aim 1: Assess effects of *in vivo* administration of flunixin (non-selective inhibitor) and etodolac (COX-2 preferential inhibitor) on barrier function of ischemic-injured equine jejunum after 18hrs recovery.

Aim 2: Assess effects of *in vitro* treatment with a selective COX-2 inhibitor, deracoxib on the permeability of ischemic equine jejunum.

Aim 3: Assess effects of ischemia and flunixin on absorption of lipopolysaccharide (LPS) *in vitro*.

Aim 4: Examine the role of COX-2 in jejunal recovery using COX-2 knockout mice.

Ussing chambers were used to assess the barrier function of ischemic and normal jejunal mucosa when exposed to the different inhibitors mentioned above. Transepithelial electrical resistance (TER), a sensitive index of barrier function, was calculated and the permeability of the mucosa assessed using mannitol. The amount of epithelial denudation following ischemia was determined and compared between treatments. Levels of expression of the COX-1 and –2 enzymes before and after ischemia were assessed by Western blot. For aim 3, permeability to lipopolysaccharide (LPS) was measured and the passage of fluorescent-labeled LPS into mucosa assessed with histology. Statistical significance level for all tests was chosen at $p < 0.05$.

Both flunixin and etodolac treatment retarded *in vivo* recovery of jejunal barrier function after ischemia. These drugs act on the paracellular space as treatments did not alter epithelial denudation or restitution. Normal equine jejunal mucosa expressed both COX-1 and COX-2 and ischemia upregulated both isoforms. The selective COX-2 inhibitor deracoxib also adversely affected mucosal recovery *in vitro* but to a lesser extent; deracoxib did not significantly increase permeability to mannitol. The increased

permeability of mucosa to mannitol after flunixin treatment did not reflect increased LPS absorption despite a trend toward this finding. Ischemia alone increased the absorption of LPS. Although the precise route of LPS across the mucosa was not determined, it was postulated to be mostly through the defects from epithelial cell loss. Attempts to clarify the role of COX-2 in mucosal recovery from ischemia using COX-2 knockout mice produced conflicting results. COX-1 inhibition increased permeability to mannitol in both COX-2 knockout and wild-type mice. There was a trend towards increased permeability due to COX-2 inhibition in wild-type mice. For knockout mice, the COX-2 inhibitor also unexpectedly increased permeability. COX-1 protein was present in all mice and COX-2 was present in normal mucosa of wild-type mice. Both isoforms were not upregulated by ischemia.

Untreated horses recovered baseline levels of TER and permeability by 18hrs after ischemia. Both flunixin and etodolac did not allow sufficient prostaglandin production for recovery. *In vitro* treatment with the selective COX-2 inhibitor deracoxib also adversely affected recovery but to a lesser extent than the other drugs tested. The increased permeability due to flunixin did not exacerbate LPS absorption in this model. The clinical significance of the effects of COX inhibitors on permeability will require further *in vivo* testing. COX-1 and –2 are both present in normal jejunal mucosa from both horses and mice, thus it is possible that the action of both enzymes is required for complete recovery from ischemia.

CYCLOOXYGENASE INHIBITORS AFFECT RECOVERY OF ISCHEMIC-INJURED JEJUNUM

by

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For my mother

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Chapter 1:

Introduction and Literature review

Part I: The role of non-steroidal anti-inflammatory drugs in gastrointestinal injury and repair

Part II: The interaction of lipopolysaccharide with intestinal epithelium

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Part I: The role of non-steroidal anti-inflammatory drugs in gastrointestinal injury and repair

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used in clinical practice for a variety of conditions including arthritis and colic in horses. However, it has been documented that NSAIDs cause direct injury to the gastrointestinal tract.[1-3] This group of drugs has also been shown to inhibit healing of injured tissue in the gastrointestinal tract,[4,5] suggesting caution when treating animals with gastrointestinal disease. Nonsteroidal anti-inflammatory drugs cause injury to gastrointestinal mucosa in 2 ways: direct topical injury,[6] and inhibition of prostaglandin synthesis.[7] Although NSAIDs are thought to interfere with mucosal protective mechanisms such as optimal mucosal blood flow and mucus and bicarbonate secretion,[3] the exact pathways of NSAID-induced injury have not been fully elucidated.

Nonsteroidal anti-inflammatory drugs inhibit prostaglandin production by acting on the cyclooxygenase (COX) enzyme. This enzyme converts arachidonic acid to prostaglandins. The enzyme has several isoforms, including the 'housekeeping'COX-1 and the inducible enzyme COX-2. The latter is upregulated by a variety of inflammatory stimuli, including bacterial lipopolysaccharide, and is constitutively expressed in select tissues such as the kidneys.[8] Nonsteroidal anti-inflammatory drugs that inhibit both COX isoforms inhibit the production of housekeeping prostaglandins and so may render the gastrointestinal tract more susceptible to injury. Thus, the development of drugs that selectively inhibit inducible COX-2 has been hailed as the answer to preventing NSAID-

induced gastrointestinal injury while inhibiting inflammation and providing analgesia. However, recent research has revealed a more complex picture.

Mechanisms of Gastrointestinal Injury induced by NSAIDs

Topical injury (Table 1)

Acute topical injury occurs within an hour of ingestion of 600mg of aspirin in humans, and is manifested as petechial hemorrhages and superficial erosions in the mucosa of the stomach. Aspirin is a weak acid (along with many other NSAIDs, such as ibuprofen, indomethacin, diclofenac, and ketoprofen), and remains in a nonionized form in the acidic stomach contents. A nonionized molecule can readily diffuse through the lipid membranes of epithelial cells. Once within the neutral pH environment of the epithelial cell, the molecule becomes ionized, resulting in release and intracellular trapping of hydrogen ions, which disrupt cell function.[9] However, continued aspirin administration results in mucosal adaptation and reduced pathologic changes, although the exact mechanism of this adaptation is unknown.[6]

Breaking of the mucosal barrier was one of the earliest recognized toxic mechanisms of NSAIDs. These drugs can diminish the hydrophobicity of mucus, destroying the protective barrier against acid and bacteria in the gastrointestinal tract.[10] Part of this change occurs as a result of a direct effect on mucus-producing cells of the stomach via uncoupling oxidative phosphorylation in the mitochondria.[11] Another direct drug effect on cell metabolism has been noted in colonocytes exposed to ibuprofen, which resulted in inhibition of fatty acid metabolism and effects on intracellular enzyme activity.[12]

Small intestinal mucosal injury is a particular problem with NSAIDs that undergo enterohepatic circulation, as the small intestinal mucosa is exposed to active metabolites secreted within bile.[2] However, the presence of an NSAID in the small intestinal lumen is not necessarily sufficient to induce damage. For example, an *in vitro* study determined that indomethacin required the presence of bile to be toxic to intestinal epithelial cells. The active component of bile has not been elucidated, but likely involves the detergent effect of bile on mucus and luminal cell membranes.[13]

Prostaglandin inhibition (Table 2).

Topical injury does not appear to be the principal mechanism of NSAID-induced gastrointestinal injury. For instance, prodrugs (which require activation) and alternative routes of administration other than the oral route decrease the potential for topical injury, but mucosal injury is still noted in response to NSAIDs.[14] Thus, inhibition of prostaglandin production is thought to account for the major component of NSAID-induced mucosal injury. This may result from the fact that prostaglandins have important protective effects on the gastrointestinal mucosa,[15] including stimulation of epithelial mucus and bicarbonate secretion, regulation of mucosal blood flow, and induction of epithelial proliferation.[16,17]

Because prostaglandins have so many effects, it has been difficult to discern which component is most important to the health of the gastrointestinal mucosa once their production has been inhibited by NSAIDs. However, there are studies suggesting that the

regulation of mucosal blood flow by PGE and PGI_2 is critical to the protective effects of prostaglandins. For example, administration of the solely vasoactive substance endothelin in a rat model caused a direct reduction in gastric mucosal blood flow and resulted in gastric damage coupled with decreased mucosal protective responses similar to those noted with NSAIDs.[18] Decreased mucosal blood flow is found in acute NSAID injury, but it is not known whether this is a precursor to injury or a result of injury. This NSAID-induced decrease in blood flow returns to normal following short-term NSAID use, possibly because of upregulation of other vasoactive substances such as nitric oxide.[19,20] However, long-term NSAID use can result in continued decreases in blood flow.[21] For example, studies in rats revealed diminished small intestinal villous perfusion 7 days after subcutaneous administration of indomethacin.[22]

Inhibition of prostaglandin synthesis results in a decrease in the concentration of sodium and mucus in the gastric secretions and an increase in hydrogen ion concentration.[23] Prostaglandins inhibit histamine-mediated cyclic AMP production, thereby decreasing the stimulus on the H+, K+ ATPase in the parietal cell to produce acid. This effect may be mediated by an inhibitory G protein linked to specific prostaglandin E receptors.[24]

The role of neutrophils

Data from animal experiments strongly suggest that neutrophils have an important role in the initiation of NSAID damage. There is increased synthesis of tumor necrosis factor α and leukotriene B_4 and upregulation of leukocyte adhesion molecules during NSAID-

induced damage.[25,26] The mechanisms responsible for these effects have not been fully determined, but inhibition of prostaglandin synthesis appears to result in activation of granulocytes and their subsequent targeting to the gastrointestinal tract.[27] For instance, administration of indomethacin to mice resulted in increased numbers of mucosal granulocytes in all portions of the gastrointestinal tract, particularly in the colon.[27] This may be the result of neutrophils inappropriately targeting the normal bacterial flora in the colon, but neutrophil infiltration of the jejunal mucosa also occurred within 6 hours of treating mice with indomethacin.[28] Once neutrophils infiltrate the mucosa, they become activated and release reactive oxygen metabolites and proteases that may damage mucosal epithelium.[29,30] Mice deficient in fucosyltransferase-VII, required for extravasation of leukocytes, were protected against NSAID induced gastrointestinal damage.[31] However, mice deficient in mature B and T lymphocytes were not protected from damage, indicating a specific role for neutrophils rather than other leukocytes.[31]

The response of rat jejunum to indomethacin-induced injury prior to neutrophil infiltration was characterized by focal deposition of fibrin at the villous tips in the first few hours. This was accompanied by villous contraction.[28] These changes may indicate that the early stage of NSAID mucosal injury is neutrophil independent, possibly stemming from endothelial cell injury and microvascular changes attributed to the inhibition of PGE_2 and PGI_2.[28] These prostaglandins are important for the normal maintenance of the vascular endothelium. Blood vessels may subsequently become obstructed as NSAIDs induce upregulation of cell surface adhesion molecules that increase neutrophil adherence to endothelium.[26] It has also been postulated that NSAID

inhibition of prostaglandin synthesis results in the arachidonic acid surplus being metabolized to leukotrienes. Leukotrienes B_4 and E_4 are involved in the induction of leukocyte adhesion in post-capillary venules of rat mesentery after indomethacin administration.[32]

Other injurious effects of NSAIDs

The ability of NSAIDs to impair hemostasis by inhibiting the action of platelet thromboxane A_2 production may contribute to hemorrhage noted with severe ulcers. However, although this has been noted in human studies, it has not been documented in animal studies.[33,34] Nonsteroidal anti-inflammatory drugs have also been shown to increase permeability of the large intestine in healthy human patients.[35] The use of NSAIDs may be associated with an increased risk of emergency admission to hospitals because of colitis attributed to inflammatory bowel disease, particularly among patients with no history of the disease.[36] Nonsteroidal anti-inflammatory drugs have also been documented to cause colonic ulcers in humans who don't have inflammatory bowel disease.[37]

Effect of NSAIDs on gastrointestinal repair

One of the vital components of intestinal barrier function is the ability of the mucosa to repair rapidly following a variety of injurious events, including ischemia and gastrointestinal infectious disease. Prostaglandins appear to have an important reparative role in the gastrointestinal tract. For instance, it has been demonstrated that the NSAID indomethacin retards recovery of ischemic-injured ileum in swine, and mucosal repair

can be restored by addition of PGE_2 and PGI_2.[38] The initial components of mucosal repair include migration of residual epithelial cells to cover mucosal defects; a mechanism termed restitution.[39] Factors regulating this process include trefoil peptides, polyamines, and growth factors.[40] Although prostaglandins do not directly stimulate epithelial restitution, they appear to be required for growth factor-stimulated restitution.[4] In addition, PGE_2 also stimulates villous contraction in small intestinal mucosa, which may facilitate epithelial restitution by reducing the denuded surface area.[41] During these early repair processes, prostaglandins I_2 and E_2 act synergistically to reseal interepithelial tight junctions via their second messengers Ca^{2+} and cAMP, restoring intestinal barrier function following ischemic injury.[42,43] Although the precise mechanisms for the effect of prostaglandins on interepithelial tight junctions have not been fully characterized, this process appears to be clinically relevant. For example, in swine with ischemic-injured ileum treated with indomethacin or indomethacin and prostaglandins I_2 and E_2, morphologic differences in the appearance of tight junctions correlated with substantial differences in mucosal permeability to bacterial lipopolysaccharide.[42] Therefore, while providing necessary analgesia, NSAIDs may delay the healing process, thereby increasing morbidity rate and duration of hospitalization.

Nonsteroidal anti-inflammatory drugs may also affect subacute reparative events such as epithelial proliferation.[5] For example, NSAIDs decreased the number of proliferative cells in the glandular mucosa of rat stomach; an effect reversed by the prostaglandin analogue misoprostol, [5] NSAIDs decrease cell replication, increase necrosis, and increase apoptosis (programmed cell death). Studies in cultured guinea pig gastric mucosal cells

suggest that the mechanisms for NSAID-reduced epithelial proliferation are necrosis and apoptosis.[44] The increase in epithelial necrosis appears to be an effect of short-term treatment. However, in the long term, there was an increase in apoptosis.

An additional factor that appears to delay healing of NSAID-induced mucosal ulcers in the stomach is the acid environment. In particular, ulcer healing is facilitated by reduction of gastric acidity following treatment with histamine-2 receptor antagonists (eg, ranitidine) or proton pump inhibitors (eg, omeprazole) despite continued NSAID use. However, continued NSAID therapy delays gastric ulcer healing.[45,46] Administration of the exogenous PGE_1 analog misoprostol during NSAID therapy aids in healing of ulcers.[47,4]

The contribution of COX-1 and COX-2 to injury and repair

The search for NSAIDs with reduced toxic effects has been aided by the discovery of 2 isoforms of cyclooxygenase: COX-1 and COX-2.[49-51] Since COX-1 is constitutively expressed in most tissues, it has been postulated that this enzyme plays a housekeeping role, whereas COX-2, which is an inducible enzyme in most tissues, has been regarded as proinflammatory.[3] However, it should be remembered that both enzymes produce the same prostanoids from arachidonic acid. Cyclooxygenase catalyzes a 2-step conversion of arachidonic acid to PGG_2 (peroxidation) and PGH_2 (reduction). The final fate of PGH_2 is determined by the presence of local prostanoid synthases, and this likely plays an important role in which prostanoids are elaborated following initial COX activity. The best example of this is co-localization of COX-1 and thromboxane synthase in platelets,

such that platelet TXB_2 production can be used as an indicator of COX-1 activity. [52] Similarly, COX-2 and PGE synthase are co-localized in LPS-stimulated macrophages.[49]

Using the simplified concept that COX-1 is responsible for normal physiologic function whereas COX-2 is proinflammatory, so-called 'GI-safe' NSAIDs have been developed that selectively inhibit COX-2. Available drugs in human medicine include rofecoxib and celecoxib, which have been among the fastest-selling prescription medications in recent history. However, all available drugs inhibit COX isoforms to some extent, which may be measured by a variety of COX selectivity assays.[52] Therefore, there is a range of NSAIDs available with different selectivity for the COX isoforms. For example, etodolac and nabumetone preferentially inhibit COX-2 at low doses, but this effect is diminished at higher doses.[3] Selectivity is also greatly dependent on the type of assay performed, and the target species,[3] making blanket assertions as to the COX selectivity of NSAIDs tenuous. In addition to COX selectivity, other factors may contribute to the reported increase in safety of certain 'GI-safe' COX inhibitors. For instance, etodolac has a low level of enterohepatic circulation and a short half-life in people, and nabumetone has no enterohepatic circulation.[53]

Recent findings have complicated the postulated roles for COX-1 and COX-2. In particular, COX-2 plays an important role in repair of mucosal injury in certain circumstances. For example, COX-2 is upregulated after gastric injury, and this upregulation has been correlated with an increase in gastric epithelial cell proliferation that is inhibited by NSAIDs.[54] It must also be remembered that because COX-2 is

expressed constitutively in tissues such as the kidney, the use of selective COX-2 inhibitors may induce renal damage.[8] Furthermore, selective COX-2 inhibitors delayed healing of acute-stage ulcers in mice[55] and induced colitis in rats.[56] In addition, prostaglandins produced at a site of mucosal injury by COX-2 may modulate inflammation. For instance, concentrations of COX-2 derived prostaglandin D_2 was increased in a model of colitis, and inhibition of COX-2 inhibited PGD_2 production and resulted in a doubling of granulocyte infiltration.[57] Cyclooxygense-2 is also an important source of vascular prostacyclin, the inhibition of which may retard ulcer healing.[2] It may also contribute to maintenance of gastrointestinal barrier function. A recent study determined that there were similar concentrations of COX-1 and COX-2 mRNA in the normal stomach of young rats. This implies a physiologic role for COX-2 in the rat stomach. However, it was not determined how much of the COX protein was transcribed from this mRNA.[58] Interestingly, inhibition of both COX-1 and COX-2 was required for the development of gastric erosions after NSAID administration in rats.[59]

The roles of COX isoforms in inflammation are equally as complex as the roles of these enzymes in gastrointestinal barrier function and repair. An investigation into the anti-inflammatory effects of the specific COX-2 inhibitors nimesulide, celecoxib, NS-398, and DuP697 in carrageenan-induced inflammation of rat paw revealed that substantial anti-inflammatory effects were only observed at doses high enough to inhibit both COX isoforms. This resulted in inhibition of gastric prostaglandin synthesis and resulted in mucosal erosions.[60] Another study determined that COX-2 inhibition by celecoxib did not substantially affect wound collagen deposition in the carrageenan-induced inflammation

model, implying a role for COX-1 in inflammation induced fibrosis.[61] A comparison of indomethacin and nimesulide, a drug that is 40 times more selective for COX-2, revealed that at doses required for substantial prostanoid reduction, nimesulide caused a substantial increase in intestinal permeability.[62]

Gene knockout models have been used in an attempt to clarify the roles of the COX isoforms, but these studies do not always provide clear-cut information, partially because animals adapt to the loss of one of the COX genes.[63] For example, mice in which the COX-1 gene was knocked out did not develop spontaneous gastric ulceration,[64] possibly because up-regulation of COX-2 takes over important housekeeping functions. Alternatively, COX-2 knockout mice have developmental renal abnormalities, highlighting the importance of constitutive COX-2 expression in the kidneys. However, these COX-2 knockout mice do not have an altered inflammatory response in most standard tests.[65] Therefore, in these mice, COX-1 may take over the inflammatory role.

To summarize on the roles of COX-1 and COX-2 in physiologic and pathologic events, one can conclude that at least partial inhibition of both COX-1 and COX-2 is needed in most inflammatory conditions to achieve meaningful anti-inflammatory effects. However, some remaining COX-1 activity may be required for maintenance and protection of the gastrointestinal mucosa, whereas some COX-2 activity may be needed to optimize repair of gastrointestinal injury. Nonetheless, it will be difficult to achieve this fine balance. Drugs that are preferential rather than selective for COX–2, such as

etodolac and nimesulide, may ultimately fit this role at specific dosages, although the current trend is the generation of increasingly selective COX-2 inhibitors.

Future Alternatives

Nitric oxide (NO) synthesis can also protect the gastrointestinal mucosa using some of the same mechanisms as prostaglandins[66] and may compensate when prostaglandins are absent.[67,68] Newer developments include NO-NSAIDs. In animal studies, these drugs induce less damage than their NSAID counterparts and do not retard ulcer healing.[69] Recently, there has also been the development of zwitteronic NSAIDs, which are complexed with phospholipids to reduce topical damage.[70] These newer drugs may provide a more viable alternative to conventional NSAIDs, particularly if the selective COX-2 inhibitors ultimately fail to achieve a balance between anti-inflammatory and gastrointestinal protective effects.

Part II: The Interaction of Lipopolysaccharide with Intestinal Epithelium

Introduction

Intestinal ischemia often results in endotoxemia.[71] For example, in clinical studies of horses with colic, endotoxemia was detected in 25% of cases.[72,73] Endotoxemia significantly contributes to morbidity and mortality in clinical patients recovering from ischemic intestinal disease and subsequent surgery.[74] Although ischemic intestine is resected when possible at surgery, remaining intestine may be injured as a result of distention,[75] or the surgeon's inability to detect or adequately resect all damaged intestine. In fact, a recent study found evidence of serosal injury and neutrophil infiltration in the proximal resection margins of ischemic intestine indicating that not all damaged intestine had been removed.[76]

Endotoxemia is attributable to absorption of lipopolysaccharide (LPS) from intestinal gram-negative bacteria across a compromised intestinal mucosal barrier. Gram-negative bacteria release LPS during rapid proliferation and cell death.[77] Once absorbed, LPS triggers pathophysiological effects in mammals ranging from mild fever to fatal septic shock.[78] LPS stimulates host cells to produce and release endogenous mediators of inflammation. The principal cells that interact with LPS are monocytes/macrophages. Although intestinal epithelial cells were once regarded as merely a barrier to LPS, it has recently been determined that these cells interact specifically with the LPS molecule.

Intestinal epithelial cells are exposed to high levels of intact bacteria and bacterial products and are the first line of the innate immune response to intestinal microbial invasion.[79] For instance, cecal contents of healthy horses contain up to 80 μg LPS/ ml.[80] The intestinal epithelial cell must recognize and react to pathologic bacteria whilst avoiding responses to normal gastrointestinal flora. Inappropriate responses to resident flora likely play a role in development of inflammatory bowel disease.[81]

The intestinal epithelial cell is regularly exposed to LPS, even with a functioning intestinal barrier, and small amounts of LPS may be found in portal blood of healthy individuals.[82] Kupffer's cells in the liver remove LPS before it can reach the systemic circulation.[83] However, in the case of clinical endotoxemia, the amount of LPS in the portal circulation overwhelms the liver's mechanisms for LPS removal, resulting in the appearance of LPS in the systemic circulation. Circulating LPS interacts with host cells via specific cell surface receptors, and the intercalation of these molecules is considered to be an important step in the activation cascade.[84,85]

The Structure of LPS

Lipopolysaccharide functions as a permeability factor associated with the outer membrane of the gram-negative bacterial cell wall. It is released when the bacteria undergoes autolysis, or lysis by the invaded host. LPS is attached to the outer membrane and projects outward from the cell. Bacterial LPS consists of lipid A and a

polysaccharide side chain (Figure 1.). LPS undergoes antigenic variation whereby length and carbohydrate content are varied.[86]

Lipid A is the most highly conserved portion; it appears to be the principal component that is responsible for LPS activity.[86] Slight variation in the number and configuration of fatty acids in lipid A confers the degree of its toxicity.[87] The toxicity of lipid A is primarily associated with its ability to activate host defenses and to stimulate the release of host cytokines. Lipid A has been chemically synthesized. Lipid A alone has the same biologic activity as LPS. Changes in the chemical composition of lipid A result in changes in biologic activity. [88]

Lipopolysaccharides are amphiphilic molecules and therefore form aggregates in aqueous environments if the molecules are present in large enough concentrations. This results in complex phase behavior and structural polymorphism depending on the conditions (pH, temperature etc.) and may have some importance in biological action.[89] It has been shown that it is the monomeric form of LPS and not the aggregated form that is the biologically active unit.[90]

Molecular events in endotoxic shock

Most of the deleterious effects of LPS are due to an overproduction of host inflammatory mediators [87] resulting in endotoxic shock. Once LPS enters the bloodstream, a cascade of events occurs resulting from recognition of the LPS molecule by the innate immune system. Production of prostaglandins, leukotrienes, cytokines and complement

components leads to dramatic pathophysiological reactions such as fever, leukopenia, tachycardia, tachypnea, hypotension, disseminated intravascular coagulation and multi-organ failure. [86]

Monocytes and macrophages are the principal host cells that interact with LPS. This interaction results in activation of the enzyme phospholipase A_2 and production of prostaglandins and leukotrienes from arachidonic acid. Both prostaglandins and leukotrienes have hemodynamic effects resulting in hypotension and inadequate tissue perfusion. These molecules also act as chemoattractants for host immune cells. [77]

Bacterial LPS molecules are potent stimulators of interleukin production by monocytes. Interleukin –1 (IL-1) induces fever, an increase in the number of circulating neutrophils and increased prostaglandin production. [77] Among the other interleukins produced in response to LPS stimulation are IL-6, and IL-8, which both act as chemoattractants and encourage leukocyte differentiation. [77]

Molecules that bind LPS resulting in activation of cellular and humoral defenses include cluster differentiation antigen 14 (CD14). CD14 is the main LPS receptor that activates monocytes. LPS binding and IL-1 cytokine production in human mononuclear cells was blocked by anti-CD14 monoclonal antibodies. [91] CD14 may be present on cell membranes, attached by a lipid tail, or can be secreted into the body fluids, which may provide a source for non-myeloid cells to use this signaling molecule (see later). CD14 is present on the membranes of myeloid cells and in low numbers on B-lymphocytes,

mammary cells and fibroblasts. Epithelial cells do not produce endogenous CD14. [92] CD14 has also been identified in the liver, lungs and kidneys of LPS or interleukin-1β (IL1β)-stimulated mice. [93] LPS stimulation can upregulate CD14 expression. [92] The soluble form of CD14 is always present in the serum at a low concentration [94] and is elevated in septic patients. [95] CD14-LPS complexes are able to stimulate cells not bearing the membrane bound CD14.[96]

Although CD14 can bind LPS, the presence of LPS binding protein (LBP) enhances the interaction 100-1000 times. [97] Lipopolysaccharide binding protein is produced by the host and forms a complex with LPS by binding lipid A. LBP is present in normal human serum and the concentration increases markedly in response to inflammation.[98,99] LBP was identified in bovine serum by Western blot [100] indicating that elevated levels may be a useful clinical indicator of endotoxemia.

LBP binds to and delivers LPS to its final destination and is unaltered in the process. [87] The biological activity of lipoprotein-bound LPS is markedly decreased; therefore soluble CD14 and LBP play a role in the elimination and neutralization of LPS in serum and tissue fluids. [92] High concentrations of LBP have been shown to protect mice against septic shock.[101] It is unknown whether the increased amount of LPS-LBP complex downregulates receptor responses to LPS. However LBP can also lower the threshold for generation of inflammatory cytokines and so enhance cellular response to LPS. The effect may be dependent on the concentration of LBP present.[102]

Although CD14 recognizes the LPS-LBP complex, membrane-bound CD14 does not span the cell membrane and is thought to function solely as a ligand binding protein.[103] In order to transmit a signal into the cell, at least one additional molecule is required, in conjunction with CD14, to form a functional LPS receptor.[104] This molecule is the Toll-like receptor.

Toll-like receptors are so named because of their homology to the Toll receptor involved in the innate immune response of the fruit fly, Drosophila. It is the Drosophila Toll receptors that recognize bacterial products and initiate a cascade resulting in production of antibacterial and antifungal peptides.[105] Both Toll-like receptors and CD14 are termed pattern recognition receptors, since they recognize a predetermined molecular pattern and are essential in differentiating between self and non-self in the initial rapid response to infection. [106]

At least nine different Toll-like receptors have been identified in mammals.[106] The Toll-like receptors are members of the IL-1 family, these receptors share a common intracellular domain, the last 15 amino acids of which are required for signal transduction. This domain interacts with a similar domain of the myeloid differentiation protein, MyD88. MyD88 is an adaptor molecule and associates with the IL-1 receptor associated kinase (IRAK), an enzyme which activates kinase enzymes resulting in increased transcription of inflammatory genes (e.g. IL-1β, IL6 IL8, IL-12) by stimulating the transcription factor nuclear factor κB (NFκB). [92] (Figure 2).

There has been much debate as to which TLR is responsible for recognizing the LPS molecule and initiating the signaling cascade that results in inflammatory cytokine production. A constitutively active mutant of TLR-4 activated inflammatory genes such as IL-1.[107] Positional cloning was used to determine that TLR-4 is the major receptor mediating the LPS signaling pathway. Mice with mutations in the gene coding for TLR-4 had defective LPS signaling.[108,109] Other co-receptors are involved in regulating the TLR response. Subsequent studies identified a secreted protein, MD2 that was shown to physically associate with TLR-4 and is necessary for its responsiveness to LPS (Figure 2). [106,110] In fact, MD2 enhances LPS-induced cytokine production in cells expressing TLR-4 by activating alternate pathways to increase the transcription factor NFκB. [111]

Lipopolysaccharide-epithelial interactions (Figure. 2)

Intestinal epithelial cells are the first line of the innate immune response to intestinal microbial invasion.[79] These cells secrete and respond to a wide variety of chemokines and cytokines.[112] Human intestinal epithelial cells have been shown to produce and release LBP, indicating that they are active in the intestinal immune response.[98] Epithelial cells also have Toll-like receptors (TLR) that bind to and recognize LPS at concentrations less than 1nM.[104] Normal intestinal epithelial cells express low levels of TLR-4 and MD2. [113] Expression of TLR-4 is increased in inflamed intestine.[114] Immunohistochemistry using antibodies against TLR-4 showed that the receptor was expressed on the apical (luminal) surface of the epithelial cells suggesting that these cells interact directly with LPS from the intestinal lumen.[115]

Soluble CD14-LPS complexes are able to stimulate cells not bearing membrane bound CD14.[96] Epithelial cells do not produce endogenous CD14, it is provided by the serum and becomes membrane bound.[116] As previously stated; it is the monomeric form of LPS that is the biologically active unit. Complexes of LPS with soluble CD14 consist of 1-2 LPS molecules bound to a single CD14 molecule. LBP catalyzes LPS binding to soluble CD14.[102] This soluble CD14-LPS-LBP complex binds to surface receptors on the intestinal epithelial cell, resulting in cellular activation.

The normal intestinal epithelial cell does not respond to physiologic bacterial loads. The mechanism of this adaptation has not been fully elucidated. Intestinal epithelial cells normally express low levels of TLR-4 and MD2 and are unresponsive to LPS. [113] In order for the intestinal epithelial cell to respond to LPS from pathogenic bacteria, the levels of TLR-4 and MD2 must increase. The inflammatory mediators interferon (IFN)-γ and tumor necrosis factor (TNF) –α increased intestinal cell responsiveness to LPS through expression of TLR-4.[113,117] Enteroinvasive (pathogenic) bacteria stimulate production of these inflammatory cytokines. Toll-like receptor 4 has been seen intracellularly in the Golgi apparatus of murine intestinal epithelial cells in the absence of surface expression, and the receptors were colocalized with LPS.[118] Infected epithelial cells show the release of LPS into vesicular structures such as the Golgi apparatus.[119] This uptake was stimulated in intestinal epithelial cells by IFN-γ. This may be a mechanism of recognizing infection. [117] Dysregulated production of cytokines in the presence of commensal bacteria has been implicated in the pathogenesis of inflammatory bowel disease. [113]

Transepithelial movement of LPS

There are conflicting reports about the mechanism of LPS passage across the intestinal epithelium. In human studies, endotoxemia has been shown to result from translocation of whole bacteria across a compromised intestinal barrier causing bacteremia. The bacteria then release the LPS when they are killed by cells of the immune system.[78] In horses; bacteremia has never been described in the literature following intestinal ischemia. Only LPS has been directly isolated from the blood.[72,73] *In vitro* studies in rats showed that fluorescent-labeled *E. Coli* C_{25} LPS did not cross normal rat ileal mucosa nor did it cross after damage from hemorrhagic shock, but whole bacterial translocation did occur in this study.[120] Lipopolysaccharide can increase the permeability of normal intestine to other molecules. *In vitro* exposure of the serosal side of rat small intestine to LPS increased the permeability to bovine serum albumin but mucosal exposure to LPS did not.[121]

There are two possible ways that the LPS molecule can be absorbed across the epithelium into the systemic circulation: across the epithelial cell membrane (transcellular route) or between cells, across the intercellular tight junctions (paracellular route). The tight junctions act as gateways to the space between cells and are regulated by active cellular processes.

Studies using a T84 cell monolayer (derived from human colonic carcinoma cells) showed that LPS placed on the apical side of the intestinal epithelial cell was transcytosed in the presence of human serum (which provides CD14) and found on the

basolateral side of the cells. This was not associated with any cell damage. When cells of different maturities were examined, it was found that only the mature, differentiated intestinal cells were capable of this transcytosis.[122] This provides more evidence for the active involvement of the intestinal epithelial cell in the response to LPS as immature intestinal cells are unable to transcytose macromolecules.

Ischemia may affect uptake of LPS by damaging the transepithelial barrier and opening the gateway provided by the tight junctions.[123] An *in vitro* measure of epithelial barrier function is transepithelial electrical resistance, which is measured by passing a current across the epithelium and measuring the resistance with Ohms law.[42] This measurement reflects the function of the tight junctions in the absence of cell loss, and so reflects the paracellular pathway. Ischemia increased the permeability of a human intestinal cell monolayer (Caco2) to fluorescent-labeled LPS despite a lack of change in transepithelial resistance. This implies a transcellular route as there was no change in the paracellular function.[124] However, it appears that LPS can cross ischemic tissue by both transcellular and paracellular pathways. For example, transcellular and increased paracellular uptake of fluorescent-labeled LPS was seen on fluorescence microscopy in rat ischemic jejunum, whereas the uptake in the non-ischemic jejunum was only transcellular.[124]

Distention of rat ileum, simulating intestinal obstruction, resulted in an increase in passage of intraluminally placed LPS into the mesenteric artery. The mechanisms of intestinal LPS translocation following obstruction were found to be mediated by serotonin. This effect was inhibited by a serotonin-3 ($5HT\text{-}_3$) receptor antagonist. This

antagonist may present a therapeutic option in the prevention of LPS absorption from obstructed intestine. [125]

LPS and epithelial turnover

It has been shown that LPS can affect normal intestinal epithelial cell turnover. LPS inhibited growth of a human non-transformed immature epithelial crypt cell line (HIEC) but it stimulated growth in a mature intestinal epithelial cell line (IEC-6). Tumor necrosis factor (TNF-α), which is produced in response to LPS, also had the same effect. The LPS effect was abolished by anti- TNF-α neutralizing antibody. In intestinal ischemia, loss of mature epithelial cells occurs, and LPS may retard growth of the replacement immature cells.[126]

Clinical Relevance

Current therapy to prevent or ameliorate endotoxic shock includes drugs aimed at limiting the overproduction of inflammatory mediators and supporting the cardiovascular system. Glucocorticoids are used in many species as they are potent inhibitors of prostaglandin and leukotriene production from arachidonic acid. They also inhibit macrophage synthesis of IL-1, but this effect only occurs if the steroid is given before the onset of endotoxemia. [77] In horses, where glucocorticoids have been associated with serious side effects such as laminitis, the non-steroidal anti-inflammatory drug flunixin meglumine is frequently used and is effective at inhibiting prostaglandin synthesis. A low dose (0.25mg/kg) of flunixin is ideal as it inhibits prostaglandin production without masking the physical manifestations of endotoxemia required for clinical monitoring of

the equine patient. [126] Pentoxyfilline is a phosphodiesterase inhibitor used in people to increase regional blood flow. In horses, it has also been shown to inhibit LPS-induced cytokine production. [87] However, the use of an inhibitor of one aspect of the inflammatory cascade is unlikely to improve overall survival. [77]

The mechanisms by which LPS crosses from the intestinal lumen to the systemic circulation are not fully understood. Passage appears to be partially dependent on an active, transcellular process. Ischemia opens up a second, paracellular route. By understanding these mechanisms, it may become possible to block the passage of LPS across the intestinal epithelium and so decrease the effects of endotoxic shock secondary to intestinal disease. However, LPS does not have to reach the systemic circulation to effectively trigger pro-inflammatory events. These events are stimulated by LPS binding to intestinal epithelial cell receptors.

As we begin to understand the interaction of the intestinal epithelial cell with LPS, more therapeutic options to ameliorate or abrogate endotoxic shock may become available.

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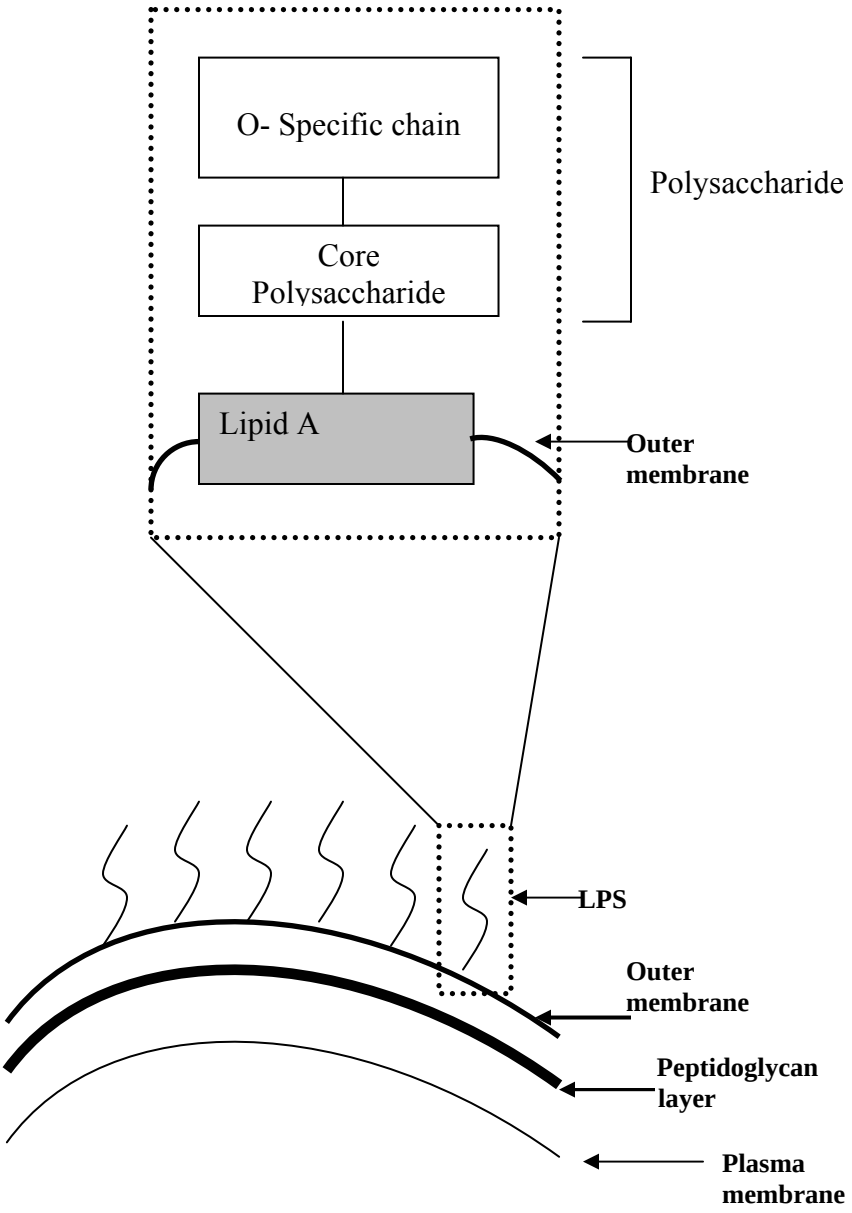
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Table 1: Topical injury caused by NSAIDs

CAUSE	***MECHANISM OF INJURY***	***EFFECT***
Intrinsic properties of acidic drug	Ionization in epithelial cell of stomach, release H^+	Disruption of cell function
	Decrease in fatty acid metabolism in colon	
	General inhibition of goblet cell enzymes	Breakdown of mucosal barrier
	Inhibition of oxidative phosphorylation	
	Enterohepatic circulation	Returns drug to stomach (bile may exacerbate NSAID injury)

Table 2: NSAID injury due to inhibition of prostaglandin production

CAUSE	*MECHANISM*	*EFFECT*
Cyclooxygenase-1 inhibition	Decreased levels of PGI2 and PGE2	Decreased mucosal blood flow
	Decreased mucosal blood flow	Decreased bicarbonate secretion
	Loss of prostaglandin stimulus	Decreased mucus production
	Neutrophil infiltration, activation and release of free radicals and proteases	Tissue injury
	Neutrophil plugging of microvasculature	Microvascular injury
Cyclooxygenase-2 inhibition	Decreased mucosal capacity for repair	Delayed healing
	Decreased PGD_2 in colon	Increased granulocyte infiltration



Gram-negative bacterial cell wall

Figure 1: Structure of lipopolysaccharide.
Bacterial lipopolysaccharide is part of the outer membrane of the Gram negative bacterial cell wall. LPS consists of lipid A and a polysaccharide chain, which is characteristic of the bacterial strain. Lipid A is anchored in the outer membrane; it appears to be the principal component that is responsible for LPS activity

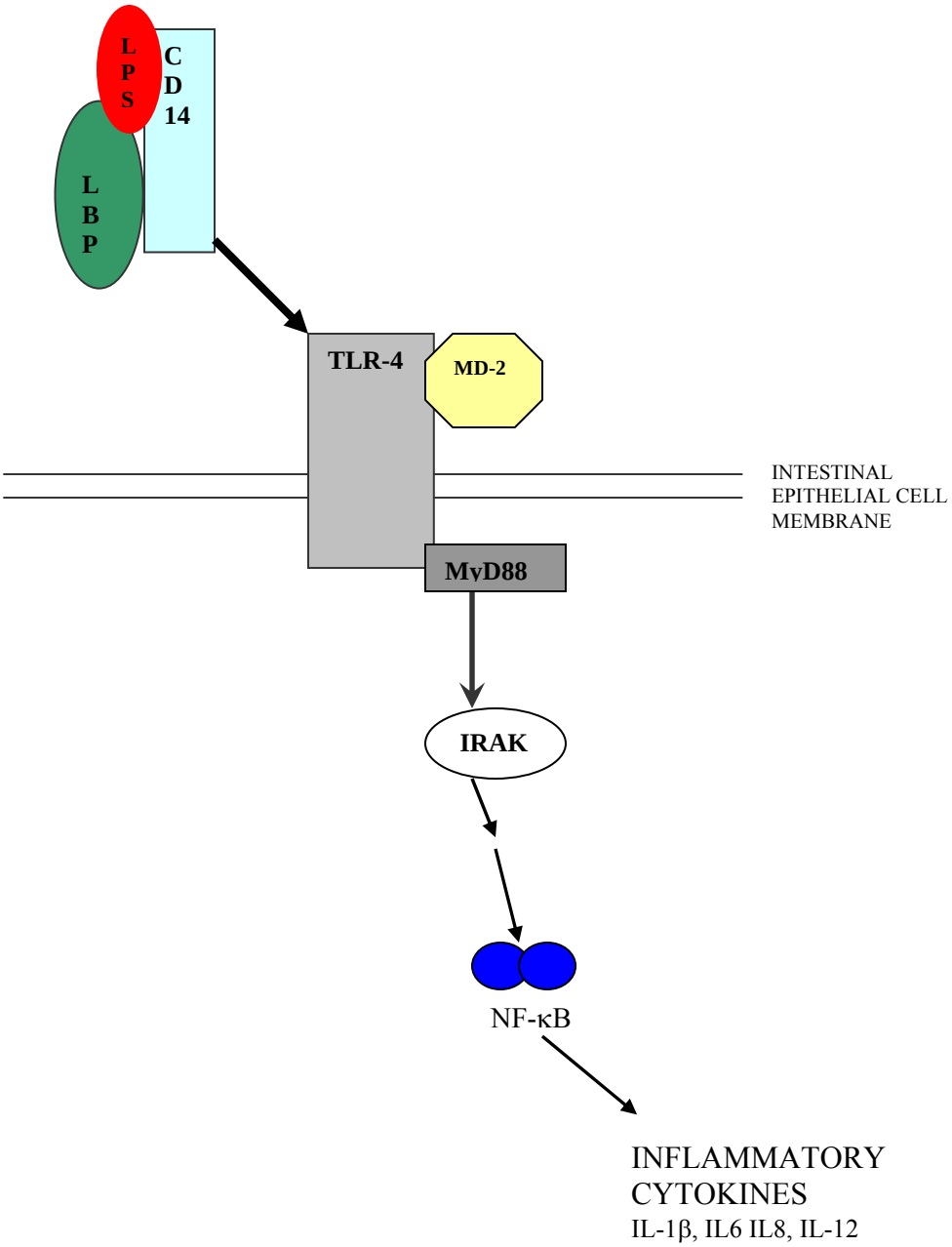


Figure 2: Interaction of lipopolysaccharide with the intestinal epithelial cell.
Epithelial cells have Toll-like receptor 4 (TLR-4) that binds to and recognizes LPS with the aid of the soluble CD14 molecule. In order to recognize LPS, the molecule must first be bound to lipopolysaccharide binding protein (LBP). The LPS-LBP-CD14 complex binds to TLR-4. MD-2, present on the cell membrane, aids in production of a transmembrane signal. The adaptor molecule, MyD88 associates with the intracellular portion of TLR-4 and with the IL-1 receptor associated kinase (IRAK), an enzyme which activates kinase enzymes resulting in increased transcription of inflammatory genes (e.g. IL-1β, IL6 IL8, IL-12) by stimulating the transcription factor nuclear factor κB (NFκB).

Chapter 2:

Systemic administration of the non-steroidal anti-inflammatory drugs flunixin meglumine or etodolac inhibits mucosal recovery of equine post-ischemic jejunum

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Abstract

Objective: To determine whether the cyclooxygenase inhibitors flunixin meglumine and etodolac inhibit healing of the jejunal mucosal barrier in a model of strangulating obstruction followed by 18h recovery.

Animals: 24 horses

Procedure: Horses underwent 2h ischemia of 30cm jejunum under anesthesia. Animals received intravenous saline, flunixin meglumine (1.1mg/kg) or etodolac (23mg/kg) q 12h. Horses were euthanized after 18h recovery. Jejunal mucosa was mounted in Ussing chambers. Two sensitive methods, transepithelial electrical resistance and transepithelial flux of ^{3}H-mannitol measured mucosal permeability. Denuded villous surface area and mean epithelial neutrophil count per mm^2 were calculated on tissues taken at surgery and after 18h recovery. Western blot for COX -1 and 2 was performed. Pharmacokinetics of flunixin and etodolac, as well as eicosanoid levels were assessed on plasma samples.

Results: Both flunixin and etodolac-treated ischemic-injured tissue had a significantly lower transepithelial resistance and significantly increased permeability to mannitol when compared with saline-treated horses. Epithelial denudation after ischemia and 18h recovery was not significantly different between treatments. Western blot showed expression of COX-1 and COX-2 in both ischemic-injured and non-ischemic jejunum. Ischemia significantly upregulated both COX isoforms. Eicosanoid levels were significantly decreased in ischemic tissue from flunixin and etodolac treated groups.

Conclusions: Flunixin and etodolac retarded recovery of intestinal barrier function in 18h post-ischemic jejunum, whereas saline-treated horses recovered baseline levels of transepithelial resistance and permeability to mannitol by 18h.

Clinical relevance: Systemic treatment with flunixin or etodolac retards intestinal recovery following ischemia and 18h reperfusion.

Introduction

Intestinal disease is the leading known cause of death in horses.[1-3] The estimated annual cost of intestinal disease in the United States is $115,300,000.[2] Mortality is largely due to strangulating intestinal disease resulting in loss of intestinal barrier function, hypovolemia and endotoxic shock.[4,5] Although ischemic intestine is resected when possible at surgery, remaining intestine may be injured as a result of distention, [6] or the surgeon's inability to detect or adequately resect all damaged intestine. In fact, a recent study found evidence of serosal injury and neutrophil infiltration in the proximal resection margins of ischemic intestine indicating that not all damaged intestine had been removed. [7]

Non-steroidal anti-inflammatory drugs (NSAIDs), particularly flunixin meglumine, are frequently administered to horses with colic in order to provide analgesia and to ameliorate the signs of endotoxemia. [8] Unfortunately, NSAIDs can have adverse effects. They have been shown to cause gastrointestinal ulcers in horses, rats and humans; colonic ulceration in horses [9-13] and also to retard healing of injured intestinal tissue. [14]

Non-steroidal anti-inflammatory drugs inhibit the prostaglandin producing enzyme cyclooxygenase (COX). This enzyme produces prostaglandin H_2 in a two-step reaction from arachidonic acid. Prostaglandins are local inflammatory hormones and are involved in leukocyte chemotaxis, nociception, vasodilatation and other aspects of the inflammatory cascade.[15] However, prostaglandins also have a physiologic function in the maintenance of gastrointestinal mucosa.[16,17] There are 3 principal COX isoforms.[18,19]

Cyclooxygenase-1 is constitutively expressed in most tissues including the gastrointestinal tract and is thought to be responsible for basal prostaglandin production under physiologic conditions. [11] Alternatively, COX-2 is not detectable in most tissues under normal conditions but is upregulated in response to inflammation.[11] However, there is some constitutive expression of COX-2 in the gastrointestinal tract in humans [20] and the kidney. [21] Cyclooxygenase -3 is a constitutive enzyme and a variant of the COX-1 gene; [22] it is principally found in the cerebral cortex and heart. Its function remains to be fully determined. [19] Non-selective COX inhibitors such as flunixin meglumine block the action of all known COX isoforms and hence markedly decrease prostaglandin levels.

Using the concept that COX-1 is responsible for normal physiologic function whereas COX-2 is pro-inflammatory, so-called 'GI-safe' NSAIDs have been developed that selectively inhibit COX-2. However, all available drugs inhibit both COX isoforms to some extent, which may be measured by a variety of COX selectivity assays. [23] Therefore, there are a range of NSAIDs available with different selectivity for the COX isoforms. For example, etodolac and nabumetone preferentially inhibit COX-2 at low doses, but this effect is diminished at higher doses. [11] Selectivity is also greatly dependent on the type of assay performed, and the target species. [11,23-25] There are no reported studies of etodolac selectivity in horses. In humans, the IC_{50} values of etodolac for COX-1 and COX-2 have been reported to be 15 μM and 1.4 μM respectively. This shows a COX-1 /COX-2 ratio of 10.7. The window of selectivity for etodolac is only 10 fold in humans; once this dose range is exceeded, COX-1 is inhibited. [25] More recent reports show a variation in etodolac selectivity ratios from 2 to 100, depending on the assay

performed. [24,26] Flunixin appears to show a slight preferential inhibition of COX-1 in equine blood with an IC_{50} (mean $\pm$SE) for COX-1 of 0.06 $\pm$ 0.02 μM and for COX-2 of 0.18 $\pm$ 0.03 μM, which gives a COX-1 /COX-2 ratio of 0.3. [27] We have recently shown that *in vitro* treatment of ischemic-injured equine jejunum with flunixin meglumine retarded recovery of intestinal barrier function, whereas etodolac permitted mucosal recovery. [28] Etodolac was chosen because of its availability on the veterinary market although it is not yet licensed for use in horses. Horses that undergo surgery for ischemic small intestinal disease have the severely ischemic tissue removed and it is the remaining reperfused tissue that must undergo recovery. The hypothesis of this study was that flunixin would retard recovery of barrier function in 18h post-ischemic jejunum whereas etodolac would permit enough prostaglandin production to allow adequate recovery from ischemia *in vivo*.

Materials and Methods

The North Carolina State University Institutional Animal Care and Use Committee approved the experimental protocol.

Four groups of six healthy horses 2 - 15 years of age weighing 350-620kg were included in the study. The animals underwent a two-week quarantine period involving vaccination, deworming and observation and were housed at the university research facility for 2-4 months prior to use in the study.

Anesthetic and Surgical Procedures

Prophylactic antibiotic (ceftiofur sodium, 2.2mg/kg IV) was administered 1 hour prior to the onset of the surgical procedure. Each horse was premedicated with intravenous xylazine (1.1mg/kg), followed by placement of an intravenous catheter in the left jugular vein. Anesthesia was induced with intravenous diazepam (0.1mg/kg) and ketamine (2.2mg/kg). Horses were orotracheally intubated and a surgical plane of anesthesia maintained with halothane vaporized in oxygen. Venous blood was sampled prior to the onset of and during the procedure. A midline celiotomy was performed using aseptic technique and the terminal jejunum located. Two 30-cm sections of jejunum were selected 90-cm apart; the first segment was 60-cm oral to the antimesenteric band of the ileum. The local jejunal blood supply was occluded in one of the segments with Kelly hemostats placed over a Penrose drain to avoid damage to the blood vessels. The segment was cross-clamped using Doyen forceps to ensure that no blood flow was received from adjacent non-experimental bowel. Blood supply was occluded for 2 hours. Following the period of ischemia, the clamps were released. At this time, the animals received 12ml intravenous saline (6 horses), flunixin meglumine (6 horses) at 1.1mg/kg q12h or etodolac (6 horses) at 23mg/kg q12h. The dose of etodolac was based on a therapeutic range extrapolated from previously published pharmacokinetic studies in dogs and humans [29-31] followed by an institutional study (unpublished) on 6 unrelated horses. [a] Full thickness wedge biopsies (1cm) were taken from the ischemic-injured intestinal segment and from non-ischemic (control) jejunum 90-cm oral to the ischemic segment, which had previously been unhandled. The defect was sutured using 2-0 polyglactin 910 [b] in a Lembert pattern. The abdomen was then closed in three layers using a simple continuous

suture of #3 polyglactin 910 in the linea alba, a simple continuous suture of 2-0 polyglactin 910 in the subcutaneous tissues and staples in the skin. The fourth group of six horses underwent the surgically induced ischemia but received no treatment and were not recovered, these horses were solely used to provide tissues for western blotting (see later) and then were used as part of a separate study.

The animals were recovered in a padded equine recovery room. Once moved to a stall, each animal was monitored for pain behavior and a pain score was determined using a previously established behavioral scoring system [32-34] (Table 1). Following pain scoring, animals in all treatment groups received butorphanol for the first 8 hrs post operatively (0.05mg/kg intramuscularly q 4h) to alleviate pain. The experimental groups continued to receive flunixin or etodolac every 12h until euthanasia.

Horses were pain scored at 18h following the cessation of ischemia (10h after the last butorphanol administration) and then euthanized with an overdose of sodium pentobarbital (100mg/kg). Following euthanasia, the jejunum was harvested for *in vitro* studies.

Ussing chamber studies

Intestinal loops were incised along the antimesenteric surface. The mucosa was then stripped from the seromuscular layer in oxygenated (95% O_2/5% CO_2) equine Ringer's solution, as prepared in previous studies. [35] The tissue was then mounted in 3.14cm^2 aperture Ussing chambers as described in previous studies. [36] Tissues were bathed on the serosal and mucosal sides with 10ml equine Ringers solution. The serosal solution

contained 10mmol/l glucose and was osmotically balanced on the mucosal side with 10mmol/l mannitol. Bathing solutions were oxygenated (95% O_2/5% CO_2) and circulated in water-jacketed reservoirs at 37^{o}C. After a 15 min equilibration period, the spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes [c] using a voltage clamp that corrected for fluid resistance. Resistance ($\Omega.cm^2$) was calculated from the spontaneous PD and short-circuit current (I_{sc}). If the spontaneous PD was between –1.0 and 1.0mV, tissues were current clamped at $\pm$ 100μA for 5 s and the PD recorded. Short-circuit current and PD were recorded every 15 min for 2 h. The data were entered into spreadsheets that calculate the transepithelial electrical resistance (TER) from I_{sc} and PD using Ohm's law.

Mannitol flux

Mucosal-to-serosal fluxes were performed by adding ^{3}H-mannitol (10μCi/ml) to the mucosal bathing solutions once tissues were mounted in the Ussing chambers. Time zero samples were taken from the reservoirs after a 15-min equilibration period. Samples were collected at 30, 60 and 90 min in scintillation vials and assessed for β emission (counts/minute).

Histology

Mucosal samples of ischemic-injured and non-ischemic (control) jejunum were taken immediately after the 2h period of ischemia (surgical biopsies) and at euthanasia (after 18h recovery). Five-micrometer cross-sections taken at 300μm intervals were stained

with hematoxylin and eosin. For each tissue, 2 investigators independently evaluated 3 sections. Three well-orientated villi and crypts were identified in each section. The length of the crypt and villus and the width at the midpoint of the villus were obtained using a micrometer in the eyepiece of a light microscope. In addition, the height of the epithelial covered portion of each villus was measured. Data from the 2 evaluators was pooled prior to any data manipulations. The surface area of the villus was calculated using a modified formula for surface area of a cylinder. Villus surface area = $(2\pi \cdot \frac{1}{2} [(4/\pi)d]h)$, where π = 3.14, d = villus diameter at midpoint and h = villus height. [36] The denuded villous surface area was calculated from the total surface area of the villus and surface area of the villus covered by epithelium. Epithelial neutrophils were counted in a $10\mu m^2$ grid on 3 different villous tips and then the mean count per mm^2 calculated.

Prostanoid and pharmacokinetic measurements

Plasma was taken under anesthesia, immediately before the onset of ischemia (0h) and at 6h after the end of ischemia, frozen at -70^0C and subsequently evaluated for the cyclooxygenase products PGE_2, 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) and thromboxane B_2 (a stable metabolite of TBA_2). Assays were performed using ELISA kits.[d] Plasma was also taken at 2 and 4h after the end of ischemia however these were lost due to problems with sample storage. For this same reason, only 2 animals in the saline-treated group had plasma samples evaluated at 0h and 6h. Flunixin and etodolac pharmacokinetics were evaluated by a reverse-phase HPLC method with UV detection on plasma samples taken 0, 2 and 4 h after first administration of the drug (from the opposite jugular) to confirm therapeutic levels.

Gel electrophoresis and Western blotting

Mucosal samples of ischemic-injured and non-ischemic jejunum, from the group of 6 horses that were not recovered, were taken immediately after the 2h period of ischemia. Mucosal samples from ischemic-injured and non-ischemic (control) jejunum were taken at euthanasia (after 18h recovery) from the remaining three treatment groups. Tissue was snap frozen in liquid nitrogen and stored at -70^0C. Protein was extracted for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE): One gram of each tissue thawed to 4^oC was added to 2ml of chilled RIPA buffer (0.15 M NaCl50 mM sodium Tris [pH 7.2], 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS), including protease inhibitors (PMSF and Aprotinin). The mixture was homogenized on ice, centrifuged at 4^oC (10,000G for 10 min), and the supernatant saved. Protein analysis of extract aliquots was performed. [e] Extracted protein (100μg) was mixed with an equal volume of 2X SDS-PAGE sample buffer and boiled for 4 min at 100^oC. The resulting lysates were loaded on a 10% SDS-polyacrylamide gel, and electrophoresis carried out according to standard protocols. Proteins were transferred to a nitrocellulose membrane [f] using an electroblotting mini-transfer apparatus. Membranes were blocked overnight at 4^oC in Tris buffered saline (TBS) and 5% dry powdered milk. Membranes were washed twice with TBS containing 0.05% Tween (TBS-T) and incubated for 2 hours in a 1:5000 solution of primary antibody (COX-1 or COX-2). The antibody used was polyclonal anti-human, shown to recognize mouse, rat and porcine COX proteins. [g] Recognition of equine COX was not assessed by the manufacturer. The membranes were incubated for one hour with horseradish peroxidase conjugated secondary antibody, and developed for

visualization of protein by addition of enhanced chemiluminescence agent. [h] Densitometry was performed on scanned images using specialized software. [i]

Statistical analysis

The measurements of transepithelial resistance and ^{3}H mannitol flux data were analyzed using a two-way analysis of variance (ANOVA) for the effects of treatment and ischemia over time. Where a significant time/treatment interaction was detected, one-way ANOVA was used to identify the source of this interaction. A post hoc Tukey pairwise multiple comparison test was then performed. The interaction of transepithelial resistance and treatment was tested after 15 min equilibration on the Ussing chamber. Histological measurements (denuded villus surface area, epithelial neutrophil count per mm^2) were analyzed with a one-way ANOVA for effect of treatment on histological indices of repair. Denuded villus surface area was also analyzed within each treatment group by one-way repeated measures ANOVA for the effect of ischemia. The repeated eicosanoid measurements were calculated as a percentage of each individual's baseline value, due to a wide variation in baseline levels. A mean and standard error was calculated for each treatment group. One-way ANOVA tested for the effects of treatment between the flunixin and etodolac groups, the saline group was omitted as results were available from only 2 individuals. Flunixin and etodolac pharmacokinetics were reported as a mean and standard error for each treatment group and time period. Western blot densitometry measurements (COX-1 and –2 protein levels) were analyzed in each treatment group with a one-way repeated measures ANOVA for the effect of ischemia. Pain scores in each

treatment group were calculated as median and range. Significance level for all tests was chosen at $p < 0.05$.

Results

Transepithelial electrical resistance (Figure 1): There was a significant time/treatment interaction on the two-way ANOVA. One-way ANOVA for the effect of treatment over the whole time period did not detect a significant difference between groups. As the experimental design aimed to examine the effects of *in vivo* flunixin/etodolac treatment on intestinal recovery from ischemia, the transepithelial resistance data from the first 45 min was analyzed in order to minimize the potential effects of *in vitro* recovery of mucosa exposed to ideal conditions of oxygenation and glucose levels; and in the absence of the cyclooxygenase inhibitors given *in vivo* (this recovery can be seen after 45 min in figure 1). One-way ANOVA over this period showed that ischemic-injured tissue from horses treated with flunixin or etodolac had a significantly lower transepithelial electrical resistance than ischemic-injured tissue from saline-treated horses ($p=0.006$, $p=0.04$ respectively). However, there was no statistical difference between the flunixin and etodolac treatment groups. There was no significant difference between treatments for non-ischemic (control) tissue.

Mannitol flux (Figure 2): There was a significant time/treatment interaction on the two-way ANOVA. Analysis of the 30 min flux using a one-way ANOVA showed that ischemic-injured tissue from both flunixin and etodolac-treated horses had significantly increased permeability to mannitol when compared with tissue from saline-treated horses

(p=0.024, p=0.014 respectively). Ischemic-injured tissue from saline-treated horses was significantly less permeable to mannitol than control tissue (p=0.02). The 60 min flux only showed a significant increase in permeability in the etodolac-treated group (p=0.038). The flunixin group showed wide variation between individuals at 60 min (see error bar, figure 2). By the 90 min flux, there was no significant difference in mucosal permeability between treatment groups. In the non-ischemic (control) tissue, there was no significant difference between treatment groups at any time point.

Histology (Figure 3):

- ***Ischemic-injured jejunum***: Ischemia resulted in epithelial denudation in all treatment groups. Mean denudation of ischemic tissue in all treatment groups immediately after 2h ischemia was 49%. Evidence of restitution was seen after 18h recovery, with a decrease in denudation to between 10.2-18.8% in all treatment groups (see table 2 for each treatment and standard error). There was no significant difference between treatment groups in amount of denudation immediately following ischemia, and there was no significant effect of treatment on the amount of denudation after 18 h recovery. However, analysis within each treatment group of the amount of denudation remaining after recovery (18h), showed that residual denudation was significantly different to non-ischemic tissue in the flunixin-treated group but not in the saline and etodolac-treated groups.
- ***Non-ischemic (control) jejunum:*** Control tissue from all treatment groups showed no evidence of denudation at surgical biopsy. After 18h recovery, the control jejunum showed a small amount of epithelial denudation (mean 4.8%) but

this was not significantly different between treatments. It was also not significantly different from the 0% denudation at biopsy within any of the treatment groups. The cause of epithelial denudation in all control tissues was unknown.

Neutrophil numbers (Table 3): There was a significant elevation in the number of infiltrating neutrophils in ischemic-injured tissue at 18h compared to the time of biopsy ($p=0.001$). There was no significant effect of treatment on ischemic-injured tissue neutrophil numbers at the time of biopsy or after 18h recovery. There was no significant difference in neutrophil numbers for each treatment in the non-ischemic (control) tissue at the time of biopsy. However after 18h recovery, neutrophil numbers were significantly higher in non-ischemic (control) tissue from etodolac-treated horses than in the flunixin and saline-treated groups ($p=0.02$).

Prostanoid Measurements: Baseline plasma levels of each eicosanoid varied widely between individuals (e.g. thromboxane B_2 varied from 9 – 423 pg/ml). Therefore results are reported as percentage of baseline for each individual.

Prostaglandin E_2 levels increased 6h after ischemia to a mean $\pm$ SE of 275 $\pm$ 3.72% baseline in the saline-treated group. Levels remained at 89 $\pm$ 5.9% of baseline for the flunixin-treated group and at 98 $\pm$ 14.9% baseline for the etodolac-treated group. Thromboxane B_2 levels increased 6h after ischemia to a mean $\pm$ SE of 3000 $\pm$ 7.6% of baseline levels at 6h in the saline-treated group. In the flunixin-treated group, the 6h levels were decreased to 65 $\pm$ 6% of baseline. The etodolac group results were similar,

with a reduction to 63 ± 3.2% baseline at 6h. 6-keto-$PGF_{1\alpha}$ levels increased at 6h to 3205 ± 19% baseline in the saline-treated group. In the flunixin-treated group, levels were decreased at 6h to 65 ± 6.4% of baseline. The etodolac-treated group showed a decrease to 47.9 ± 13.2% of baseline at 6h after ischemia. However, there was a wide variation in values. Statistical analysis did not include the saline-treated group due to the low numbers (n=2). There was no significant difference in prostanoid levels between the flunixin and etodolac-treated groups.

Pharmacokinetics: Mean ± SE flunixin level at 2h post administration was 5.63 ± 0.37 μg/ml which agrees with previous studies.[37] Mean ± SE etodolac plasma concentration at 2h after administration was 20.2 ± 2.16 μg /ml (range in 6 normal horses from a separate unpublished pharmacokinetic study = 9.73-19.18 μg/ml Mean = 15.97 μg/ml. [a] See Table 4 for mean plasma levels at 0, 2 and 4h after first administration.

Western blot analysis for COX-1 and –2 (Figure 4):

- ***Jejunal mucosa taken immediately after 2h ischemia (untreated group):*** In the non-ischemic (control) tissue, both COX-1 and COX-2 were expressed. In ischemic-injured jejunum, densitometric analysis showed that the amount of both COX isoforms was significantly greater when compared with non-ischemic (control) tissue ($p = 0.035$ for COX-1 and $p= 0.046$ for COX-2). Optical density values (OD) are shown below each lane in figure 4a.
- ***Jejunal mucosa taken after 18h recovery:*** COX-1 and COX-2 were also expressed in the 18h non-ischemic sample. There was no significant difference in

levels between treatments. The levels of COX-1 and COX-2 in the ischemic jejunum after 18h recovery were not significantly higher than non-ischemic tissue except for COX-2 expression in the etodolac-treated horses ($p<0.001$). Optical density values (OD) are shown below each lane in figure 4b.

Pain (Table 5):

Pain scores for the flunixin and etodolac-treated horses appeared similar and differed from the saline-treated horses at 2h and 18h. Median and range are shown in table 5.

Discussion

Non-steroidal anti-inflammatory drugs are frequently used in equine patients with ischemic intestinal disease as they are effective analgesics and ameliorate signs of endotoxemia.[8] However, it is important to elucidate whether clinical use of these drugs exacerbates intestinal injury and/or retards recovery. Previous studies have shown that *in vitro* treatment of ischemic-injured equine jejunum with the COX-1 and -2 inhibitor flunixin meglumine retards recovery of barrier function.[28] The *in vitro* exposure of ischemic–injured jejunum to NSAIDs may not apply to the clinical setting, where an ischemic episode with subsequent reperfusion of the intestine occurs and NSAIDs are often administered systemically for days in order to ameliorate pain and endotoxemia. NSAIDs could also add to ischemic injury during reperfusion by decreasing local blood flow due to decreased PG production.[38] The present study investigated whether the *in vitro* effect of NSAID treatment was prolonged after 18h *in vivo* recovery of jejunum from ischemia. As flunixin meglumine is 3 times more selective for COX-1 in the

horse,[27] the study also examined whether a drug that reportedly preferentially inhibits COX-2 (etodolac) [26] would allow enough prostaglandin production for adequate recovery. The data shows that both flunixin and etodolac treatment significantly retard recovery of intestinal barrier function in equine jejunum that had undergone a period of ischemia followed by 18h reperfusion; whereas saline-treated horses recovered baseline levels of transepithelial resistance and permeability to mannitol.

As the experimental design aimed to examine the effects of *in vivo* flunixin/etodolac treatment on intestinal recovery from ischemia, the transepithelial resistance data from the first 45 min was analyzed in order to minimize possible *in vitro* recovery of mucosa exposed to ideal conditions of oxygenation and glucose levels within Ussing chambers, and in the absence of the cyclooxygenase inhibitors given *in vivo.* In a previous study, which examined the effects of NSAIDs on the *in vitro* recovery of equine jejunum subjected to 1h ischemia, the initial TER (before NSAID treatment) of the ischemic-injured jejunum was about 40 $\Omega.cm^2$. [28] In the present study, jejunum was subjected to 2h ischemia, therefore the level of injury should be more severe. Thus, in an 18h *in vivo* recovery period, the TER of ischemic-injured jejunum recovered from a probable value of less than or equal to 40 $\Omega.cm^2$, to 65 $\Omega.cm^2$ in the saline-treated group and only to 45-50 $\Omega.cm^2$ in the etodolac and flunixin-treated groups. After the *in vitro* (no longer exposed to NSAIDs) period of 2h on the Ussing chamber in the present study, the TER in all treatment groups had recovered to between 60 and 70 $\Omega.cm^2$, indicating that there was an *in vitro* recovery response superimposed on the 18h *in vivo* recovery response.

Hence, it was concluded that the data taken early in the *in vitro* period was a more valid representation of the effects of *in vivo* NSAID treatment.

It appears that although etodolac preferentially inhibits COX-2 in some species,[26] this drug does not allow sufficient prostaglandin production in horses for adequate recovery from ischemia at 18 hours. There are several reasons that could account for this, including a lack of selectivity of etodolac for COX-2 in the horse (thereby potentially serving as a non-selective COX inhibitor) at the dosage administered. An alternative explanation is that COX-2-produced prostanoids are important for mucosal recovery, as has been suggested in some other studies. For example, COX-2 is upregulated after gastric injury, and this upregulation has been correlated with an increase in gastric epithelial cell proliferation that is inhibited by NSAIDs.[39] Furthermore, selective COX-2 inhibitors delayed healing of acute-stage ulcers in mice.[40]

Blood samples were taken in order to assess COX activity by measuring prostanoid levels. Although there was a dramatic reduction in the level of 6-keto-$PGF_{1\alpha}$ (the stable metabolite of PGI_2), TXB_2 (the stable metabolite of TXA_2), and PGE_2 in response to treatment with etodolac or flunixin as compared to saline, there was no significant difference in the levels of these prostanoids between NSAID treatment groups. Whole blood TXB_2 has been used as an index of COX-1 activity because of the co-localization of COX-1 with thromboxane synthase in platelets.[41] This indicates that the action of the etodolac at the dose we used is not COX-2 selective. As previously stated, etodolac selectivity for COX-2 in humans is only approximately 10- fold. [25] In the present study,

the level of etodolac conferring COX-2 selectivity in the horse may have been exceeded at 23mg/kg. This is in contrast to previous *in vitro* studies performed in our laboratory,[28] in which etodolac was administered to tissues at a level of $2.7x10^{-5}$M, and did not inhibit TXB_2, whereas it did partially inhibit PGE_2 and 6-keto-$PGF_{1\alpha}$ levels, suggesting a COX-2 selective profile. However, it can be argued that the prostanoid analyses used in this study are not specific for select COX isoforms, since both COX-1 and COX-2 serve to produce the same intermediary prostanoid (PGH_2), and local synthases produce the specific prostanoids measured in this study. Hence the prostanoids produced depend on which synthase enzyme is associated with which COX isoenzymes, a fact which may be tissue dependent.

It appears from the Western blot analyses that in equine jejunal tissue, both COX-1 and COX-2 are constitutively expressed, despite the fact the COX-2 is typically regarded as an inducible enzyme in gastrointestinal mucosa. Studies in rats have identified mRNA from both COX-1 and COX-2 in normal stomach. [42] An alternative interpretation in this case is that the apparent constitutive expression of COX-2 may reflect induction due to ischemia in adjacent tissue. Tissue sampled immediately after ischemia was from the untreated group of horses that were not recovered. In these horses no biopsies were taken, and non-ischemic jejunum was harvested several feet oral to the ischemic jejunum where the intestine was deep in the abdomen during the period of ischemia, not adjacent to the ischemic section. Therefore the authors refute this theory for the samples taken at surgery. However, in the 18h samples there probably is an effect from the adjacent ischemic-injured intestinal inflammation.

In the present study, both COX isoforms were upregulated in response to ischemia despite the fact that COX-1 is typically regarded as constitutive in gastrointestinal mucosa. COX-1 upregulation has been recently reported in microglial cells in the brain in response to injury.[43] These findings challenge the paradigm that COX-2 is the sole inflammatory isoform of the enzyme that is upregulated in response to injury. At 18h, densitometry results only showed a significant increase in COX-2 expression in etodolac-treated horses. It may be that the expression is upregulated in the other treatment groups but we were unable to detect a difference, as the power of the ANOVA test used was only 0.2 (below the desired power of 0.8) and probably results from insufficient numbers of animals to discern smaller differences in other treatment groups. Another reason for no detectable increase in the COX enzymes in ischemic tissue at 18h recovery could be that COX levels are no longer increased because the tissue has partially recovered.

There was no significant difference detected in the extent of villus denudation between treatment groups immediately after ischemia and after 18h recovery. Restitution occurred during the recovery period, but after 18h recovery, epithelial restitution was not complete (denudation = 10.2-18.8%). The amount of residual denudation in post-ischemic mucosa when compared to non-ischemic (control) tissue within each treatment group was still significantly increased in the flunixin-treated group but not in the etodolac and saline-treated groups. This indicates that there was still significant histologic damage in the flunixin-treated group, which may have contributed to the increased TER and permeability to ^{3}H mannitol. However it should be noted that etodolac treatment still significantly decreased TER and increased mannitol permeability despite no significant

residual epithelial denudation; and that the effects of flunixin and etodolac treatment on TER were not significantly different to each other. Therefore, the changes resulting in increased permeability of the jejunal tissue in the etodolac group appear to be solely due to changes in the paracellular pathway; a pathway that is regulated by inter-epithelial tight junctions.[44] It is also highly probable that changes in the flunixin-treated group are mainly due to the effect on tight junctions, despite significant (18.8%) epithelial denudation. A recent study on bile-injured porcine ileum showed that it was mainly prostaglandin mediated regulation of paracellular permeability (tight junctions) that was responsible for the recovery of transepithelial resistance, and not restitution. The paracellular pathway contributes a large surface area that is potentially permeable to small molecules. Even when the epithelium was 42% denuded in that study, it was still the paracellular pathway that provided the bulk of the surface area for these molecules to cross. After stimulation of restitution from 49-100% using growth factors, the TER of porcine ileum did not recover to baseline values without endogenous prostaglandin-mediated closure of tight junctions. [45] Re-sealing of tight junctions during recovery from ischemic injury appears to be largely regulated by prostaglandins based on recent porcine studies in our laboratory.[44]

In the present study, post-ischemic tissues were able to fully recover normal indices of transepithelial resistance and mannitol permeability within 18h in horses treated with saline despite neutrophil infiltration and despite residual denudation, suggesting compensation by adjacent restituted epithelium, possibly as a result of heightened apposition of tight junctions. The lack of complete restitution in all post-ischemic tissues

may have been due to inflammation, considering the significant elevation in mucosal neutrophil numbers in post-ischemic tissues at 18h. It has recently been shown in porcine studies that mucosal repair events can be inhibited by neutrophil infiltration 6-18 h following injury. The neutrophils migrate between restituting epithelial cells [46] and release reactive oxygen metabolites, which damage the epithelium.[47]

Flunixin and etodolac may have enhanced ischemic injury in addition to inhibiting recovery. Drugs were administered immediately after 2h ischemia to reflect a clinically relevant scenario, but the peak drug concentrations occurring at the time of reperfusion could have resulted in greater inhibition of prostaglandin synthesis, thus resulting in decreased blood flow. It is not possible to discern the exact contribution of enhanced damage during reperfusion versus retardation of intestinal recovery. However, the extent of histologic damage at 18h in NSAID treated groups was not significantly different from the saline-treated group.

There was some evidence of epithelial denudation in the 18h non-ischemic (control) tissues (~5%). The amount of denudation in these 18h control tissues was not significantly different from non-ischemic tissues at the time of surgical biopsy. The denudation may have been due to handling at surgery, as it was necessary to take biopsies, or due to inflammation in the adjacent, ischemic-injured jejunum. However, neutrophil numbers were not significantly different in non-ischemic (control) tissue at 18h when compared with surgical biopsies.

The pain scores in the NSAID-treated groups were less than in the saline-treated group, as expected. The time points when pain scores were taken were prior to or at least ten hours following butorphanol administration, indicating that the pain scores were likely an accurate reflection of the analgesic properties of the NSAIDs alone. There may have been residual butorphanol levels in the animals at 18h, but butorphanol was given at a consistent level in all treatment groups. Based on the conditions of this study, it appears that both flunixin and etodolac are equally effective analgesics. It should be mentioned that butorphanol is a partial μ opioid agonist. Stimulation of μ receptors has been shown to result in decreased intestinal permeability, particularly during inflammation.[48,49] This may have affected our experiments, but as previously stated, butorphanol dosing was consistent between treatment groups.

Although we can confirm from this study that non-selective inhibition of prostaglandin production (by flunixin meglumine) retards mucosal recovery, we cannot be certain of the precise role of COX-1 and COX-2 because of the potential lack of selectivity of etodolac. Thus, it is possible that etodolac is inhibiting mucosal prostaglandin production to the same extent as flunixin, resulting in inhibition of mucosal recovery. The systemic prostanoid levels would tend to support this premise. Alternatively, it is conceivable that etodolac is preferentially inhibiting COX-2, as has been suggested by our previous studies, [28] but that COX-2-elaborated prostanoids are required for full mucosal recovery. The latter is suggested by studies in other species, in which selective inhibition of COX-2 has reduced gastrointestinal epithelial repair. [40,42] In order to answer these questions conclusively, a highly selective COX-2 inhibitor in the horse will be required, based on appropriate selectivity assays.

Footnotes

[a] N.B. Campbell, M.G. Papich, K. Eager, A.T. Blikslager, unpublished observations

[b] Vicryl, Ethicon Inc. Somerville NJ 08876

[c] Fisher Scientific, Fair Lawn NJ 07410

[d] R &D Systems, Minneapolis MN, USA

[e] D_c protein assay, Bio-Rad, Hercules CA. USA

[f] Hybond ECL, Amersham Life Science, Birmingham, UK

[g] Affinity purified goat polyclonal antibodies. Santa Cruz Biotechnology, Inc., Santa Cruz, CA. USA

[h] Amersham, Princeton NJ. USA

[i] Zero Dscan. Quantitative Gel and Blot Analysis for Windows. Scanlytics Inc. Fairfax, VA, USA

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Table 1: Behavioral pain scoring system.
Each behavior category is scored 0-3 using the descriptions of behavior in the relevant column. Scores are added to give a total subjective pain score.

Behavior Category	***Behavioral score to be assigned for each category***			
	0	**1**	**2**	**3**
Gross pain	None		Occasional	Continuous
Head position	Above withers		At withers	Below withers
Ear position	Forward, frequent movement		Slightly back, little movement	
Location	At door watching environment	Standing in middle, facing front of stall	Standing in middle, facing sides of stall	Standing in middle, facing back of stall
Spontaneous Locomotion	Moves freely	Occasional steps		No movement
Response to horse	Ears forward, head up, moves to door	Ears forward, head up, no movement to door	Ear flick, no movement to door	No response
Response to open door	Moves to door	Looks at door		No response
Response to approach	Moves to observer, ears forward	Looks at observer, ears forward	Moves away	Does not move, ears back
Lifting feet	Freely when asked	After mild Encouragement		Unwilling

Table 2: Percentage epithelial denudation of mucosa in non-ischemic (control) and ischemic tissue immediately after ischemia (0h) and after 18h recovery.
Results are shown as mean for each treatment group and standard error (SE). There was no significant difference between treatment groups. Analysis within each treatment group showed significant denudation as a result of ischemia, this denudation was no longer significant at 18h in the saline and etodolac-treated groups but was still significant in the flunixin group. * denotes a significant difference.

Treatment	**Tissue type**	**Mean ± SE at 0h**	**Mean ± SE at 18h**
None	Control	0	4.7 ± 3
Flunixin	Control	0	7.5 ± 3.7
Etodolac	Control	0	2.4 ± 2.4
None	Ischemic	44.5* ± 4.4	15.5 ± 5.2
Flunixin	Ischemic	49.2* ± 7.8	18.8* ± 4.1
Etodolac	Ischemic	54.4* ± 4.2	10.2 ± 6.3

Table 3: Quantitative count of neutrophils in the epithelium.
A mean number is shown for each treatment group immediately after ischemia (0h) and after 18h recovery. Neutrophil numbers in ischemic tissue were significantly higher at 18h than at 0h. There was no significant difference between treatments. Non-ischemic (control) tissue in the etodolac group at 18h had significantly more neutrophils than other treatments. * denotes a significant difference.

Treatment	Tissue type	Mean ± SE at 0h	Mean ± SE at 18h
None	Control	33 ± 17.7	33.2 ± 14.9
Flunixin	Control	28.3 ± 11.3	40.3 ± 8
Etodolac	Control	72 ± 20.5	88.7* ± 11
None	Ischemic	77.5 ± 14.7	330.5* ± 61.5
Flunixin	Ischemic	47 ± 11.7	355.5* ± 58
Etodolac	Ischemic	97 ± 19.5	385.8* ± 39.1

Table 4a: Plasma levels (mean and standard error) of flunixin at 0,2 and 4h after initial administration
Intravenous flunixin was administered at a dose of 1.1mg/kg; plasma samples were taken at 0, 2 and 4h after first administration. Results are shown as mean values for the 6 horses at each time period with standard error.

Sample time (h)	Mean plasma level (μg/ml)	Standard Error
0	11.9	1.7
2	5.63	0.37
4	2.67	0.22

Table 4b: Plasma levels (mean and standard error) of etodolac at 0,2 and 4h after initial administration
Intravenous etodolac was administered at a dose of 23mg/kg; plasma samples were taken at 0, 2 and 4h after first administration. Results are shown as mean values for the 6 horses at each time period with standard error.

Sample time (h)	Mean plasma level (μg/ml)	Standard Error
0	89	11.4
2	20.2	2.16
4	4.7	0.43

Table 5: Median pain scores for each treatment group 2h after recovery from anesthesia and at 18h. See Table 1 for reference. The pain scores in the flunixin and etodolac-treated groups had the same median score 2h after recovery and at 18h. Range is shown in parentheses.

Treatment	Median (range) 2h pain score	Median (range) 18h pain score
Saline	17.5 (16-20)	12 (11-16)
Flunixin	12 (11-14)	9 (9-11)
Etodolac	12 (10-14)	9 (9-10)

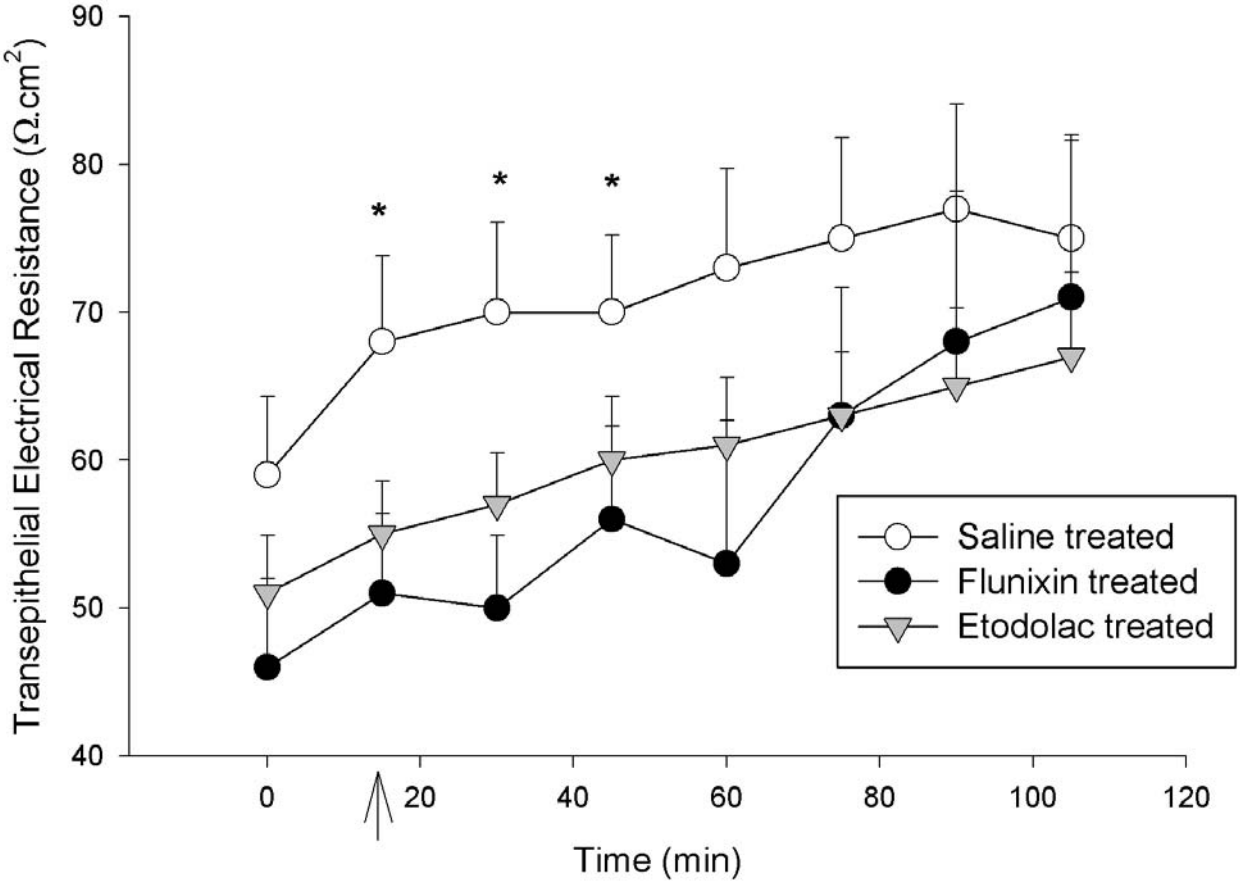


Figure 1: Transepithelial electrical resistance (TER) of ischemic-injured jejunal mucosa after 18h recovery. Mean values are shown with error bars.
The arrow on the x-axis denotes the end of the 15 min *in vitro* equilibration period. Ischemic tissue from flunixin and etodolac-treated groups had significantly lower TER (marked with *) than saline-treated for the first 45 min on the Ussing chamber. There was no significant difference in TER for treatment groups in non-ischemic (control) tissue (data not shown).

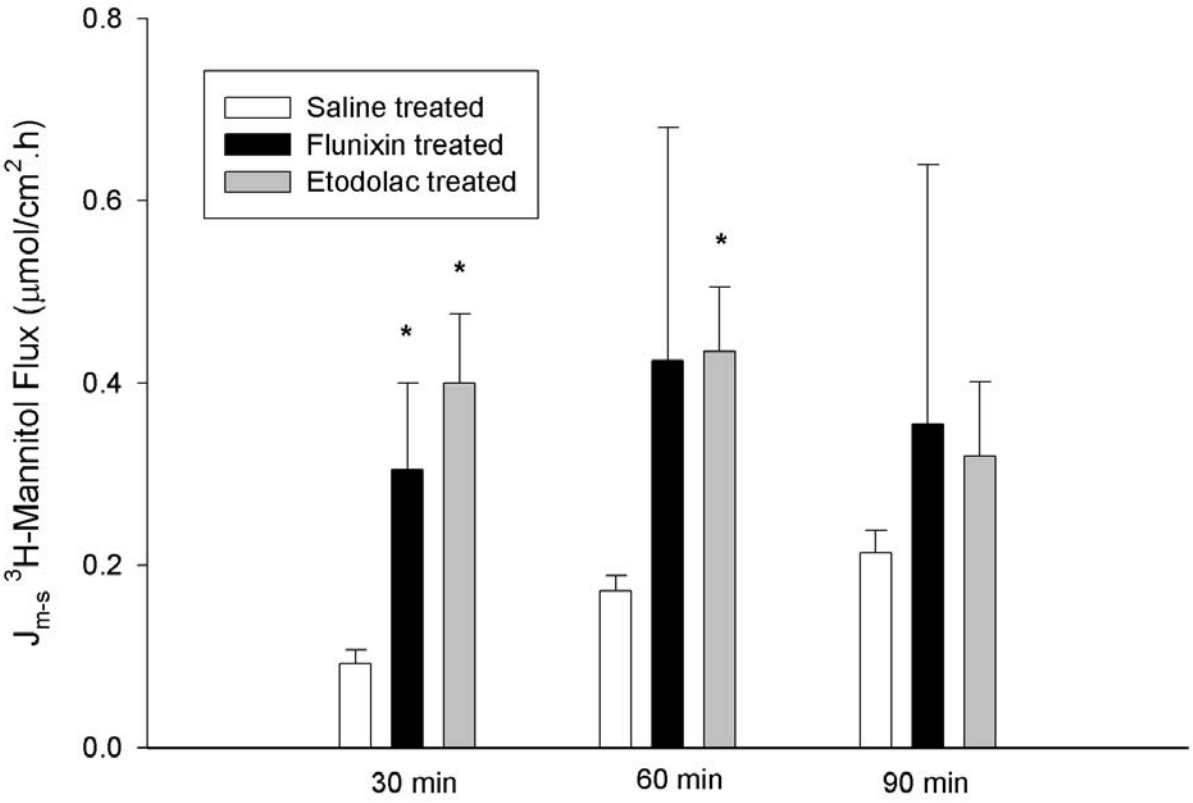


Figure 2: Permeability of ischemic-injured jejunal mucosa to ^{3}H mannitol after 18h recovery. 30, 60 and 90 minute mucosal to serosal ^{3}H mannitol fluxes (J_{m-s}). Mean values are shown with error bars. Jejunal mucosa in the flunixin and etodolac-treated groups was significantly more permeable to ^{3}H mannitol at 30 min than the saline-treated group. At 60 min only the etodolac group was significantly more permeable (significant data is marked with *). There was no significant difference in permeability between treatment groups in non-ischemic (control) tissue (data not shown).

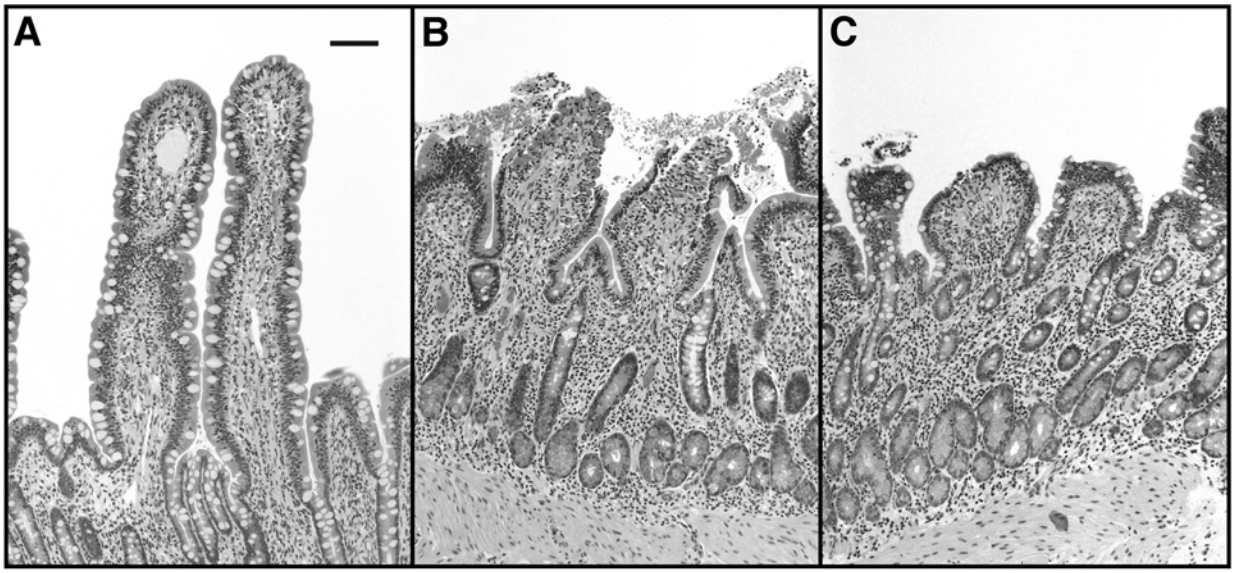


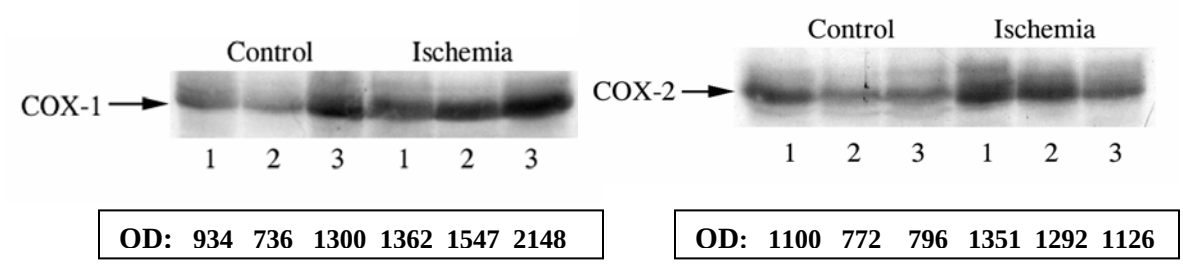
Figure 3: Histological appearance of jejunal mucosa

A: Non-ischemic (control) mucosa

B: Ischemic-injured mucosa immediately after 2h ischemia, note the loss of epithelium at the villous tip

C: Ischemic-injured mucosa after 18h recovery, the villi have contracted down and epithelium now covers most of the villus.

4 a)



OD: 934 736 1300 1362 1547 2148

OD: 1100 772 796 1351 1292 1126

4 b)



OD: 595 538 844 712 944 1206

OD: 665 529 746 798 943 1169

Figure 4: Western blot analysis of non-ischemic (control) and ischemic jejunal mucosa for the cyclooxygenase (COX)-1 and –2 enzymes.

4a) Tissue immediately after 2h ischemia: Individual animals are labeled 1-3. Both COX-1 and COX-2 appeared to be expressed constitutively as evidenced by their presence in non-ischemic (control) tissue. Densitometry showed that COX-1 and –2 were significantly upregulated by ischemia. Optical density values (OD) are shown below each lane.

4b) Tissue after 18h recovery: Individual animals are labeled 1-3. These individuals were from the saline-treated group; densitometry showed no significant difference in appearance between treatments for non-ischemic tissue. The levels of COX-1 and COX-2 in the ischemic jejunum were not significantly higher than non-ischemic tissue except for COX-2 expression in the etodolac-treated horses. Optical density values (OD) are shown below each lane.

Chapter 3:

Effects of the cyclooxygenase inhibitors flunixin meglumine and deracoxib on permeability of ischemic-injured equine jejunum

Grant Support: Morris Animal Foundation, DO2EQ-07

Submitted as a manuscript to Equine Veterinary Journal (Authors: JE Tomlinson and AT Blikslager)

Abstract

Background: Flunixin meglumine is frequently used in post-surgical colic cases. Our recent *in vivo* study, showed flunixin prevented recovery of equine jejunum at 18 hours post-ischemia. The use of a purported cyclooxygenase (COX)-2 preferential inhibitor, etodolac also prevented recovery.

Objective: To examine the effects of the highly selective COX-2 inhibitor deracoxib on *in vitro* recovery of equine jejunum from ischemia when compared with the non-selective COX inhibitor flunixin.

Methods: Six healthy horses underwent 2 hours jejunal ischemia under anesthesia. Following euthanasia, mucosa was mounted in Ussing chambers and recovered for 240 minutes, during which time transepithelial electrical resistance (TER) and mucosal-to-serosal fluxes of ^{3}H-mannitol were monitored as indices of barrier function in the presence of flunixin and deracoxib.

Results: The TER was significantly lower in ischemic-injured tissue immediately following the ischemic period, and recovered significantly over 240 minutes in the presence of no treatment, but not in the presence of flunixin and deracoxib. In addition, flunixin-treated ischemic jejunum was significantly more permeable to mannitol when compared with untreated tissue by the end of the recovery period, whereas deracoxib treatment did not increase permeability. Addition of the prostaglandin E_1 analogue, misoprostol to flunixin-treated tissue restored recovery of TER.

Potential relevance: Treatment of horses with ischemic jejunal disease with flunixin may result in a prolonged permeability defect in recovering mucosa. Addition of

misoprostol or replacement of flunixin with deracoxib may ameliorate effects of COX inhibitors on recovering mucosa.

Introduction

Colic is the leading cause of death in horses behind old age.[1,2] Post surgical deaths are attributable to complications such as endotoxemia, which results from increased permeability of injured intestine to endotoxin.[3,4] In a survival study of colic cases, 69% of deaths occurred prior to discharge from the hospital.[5] During this critical time period, much of the pain and signs of endotoxemia are attributable to prostaglandins. Therefore, flunixin, an inhibitor of prostaglandin production, is frequently administered to post-surgical colic cases. However, we have shown that prostaglandins are critical for recovery of ischemic-injured intestine.[6-8] Non-steroidal anti-inflammatory drugs (NSAIDs) such as flunixin inhibit prostaglandin production by blocking the action of the cyclooxygenase (COX) enzyme, which converts arachidonic acid to prostaglandins. There are two principal isoforms of COX: the 'housekeeping' COX-1, responsible for maintenance of gastrointestinal barrier function, and inducible COX-2, which is upregulated by a variety of inflammatory stimuli (COX-3 has been recently discovered in the brain and spinal cord). NSAIDs that block both COX-1 and -2 isoforms inhibit production of housekeeping prostaglandins, and retard the recovery of injured intestine.[9] These findings have important implications for the use of NSAIDs in patients that have suffered intestinal damage during an episode of colic, since delayed recovery of intestinal barrier function may result in increased permeability to small molecules and endotoxin. We have recently shown that the *in vivo* use of flunixin at a dose of 1.1mg/kg in horses prevents recovery of ischemic-injured equine jejunum at 18 hours post-ischemia (measured *in vitro* as transepithelial electrical resistance) when compared with untreated tissue. The use of a reportedly more COX-2 selective inhibitor, etodolac also prevented

intestinal recovery. Blood prostanoid levels from that study indicated that the dose of etodolac used was not COX-2 selective.[10] Therefore the use of a COX-2 inhibitor, deracoxib, which is highly selective in the dog[11] is examined in the present study.

We hypothesized that the selective COX-2 inhibitor deracoxib would allow sufficient prostaglandin production for *in vitro* recovery of ischemic-injured equine jejunum when compared with the negative effect on barrier function produced by the non-selective cyclooxygenase inhibitor flunixin. We also hypothesized that the prostaglandin E_1 analogue, misoprostol would counteract the effect of flunixin on ischemic-injured tissues.

Materials and Methods

The experimental protocol was approved by the North Carolina State University Animal Care and Use Committee.

Six healthy horses 5- 15 years of age weighing 400-600kg were included in the study. The animals had previously undergone a 4-week quarantine period involving vaccination, deworming and observation. Each horse was premedicated with intravenous xylazine (1.1mg/kg), an intravenous catheter placed in the left jugular vein, and induction achieved with intravenous diazepam (0.1mg/kg) and ketamine (2.2mg/kg). Horses were orotracheally intubated and a surgical plane of anesthesia maintained with isoflurane vaporized in 100% O_2.

A midline celiotomy was performed under aseptic technique and the jejunum located. Two 30cm sections of jejunum were selected 60cm apart; the first segment was 60cm oral to the antimesenteric band of the ileum. The local jejunal blood supply was occluded in one of the segments with silk ligatures. The segment was cross-clamped using Doyen

forceps to ensure that no blood flow was received from adjacent non-experimental bowel. Blood supply was occluded for 2-hours prior to euthanasia using an overdose of sodium pentobarbital (100mg/kg IV). Following euthanasia, the ischemic and non-ischemic jejunum was harvested for *in vitro* studies.

Ussing chamber studies

Intestinal loops were incised along the antimesenteric surface. The mucosa was then stripped from the seromuscular layer in oxygenated (95% O_2/5% CO_2) equine Ringer's solution, as prepared in previous studies.[12] The mucosa was mounted in 3.14cm^2 aperture Ussing chambers as described in previous studies.[12] Tissues were bathed on the serosal and mucosal sides with 10ml equine Ringers solution. The serosal solution contained 10mmol/l glucose and was osmotically balanced on the mucosal side with 10mmol/l mannitol. Chambers contained either no treatment, 2.7 x 10^{-5} mol/l flunixin[a] (equated with tissue levels following systemic administration of 1.1mg/kg dose)[8] an equimolar concentration of deracoxib[b] or flunixin with 1 x 10^{-6} mol/l misoprostol[c,13] added at 45 minutes. The concentration of misoprostol was used in a previous study and was shown to aid intestinal recovery in porcine ileum.[13] Bathing solutions were oxygenated (95% O_2/5% CO_2) and circulated in water-jacketed reservoirs at 37^{o}C. After a 15-minute equilibration period, the spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes using a voltage clamp that corrected for fluid resistance. Transepithelial electrical resistance (TER) (Ω.cm^2) was calculated from the spontaneous PD and short-circuit current (I_{sc}). If the spontaneous PD was between –1.0 and 1.0mV,

tissues were current clamped at ± 100μA for 5-seconds and the PD recorded. Short-circuit current and PD were recorded every 15 minutes for 240 minutes. The data were entered into spreadsheets that calculate TER from I_{sc} and PD using Ohms law.

Mannitol flux

After the tissue was incubated with the treatments for 60 minutes on the Ussing chambers, three 60-minute mucosal-to-serosal mannitol fluxes were performed by adding ^{3}H-mannitol[a] (10μCi/ml) to the mucosal bathing solutions, and monitoring its appearance in the serosal bathing solutions. Samples were collected in scintillation vials at 0, 60, 120 and 180 minutes after addition of ^{3}H-mannitol to the Ussing chambers and assessed for β emission (counts/minute). Mucosal fluxes of mannitol were calculated for each time period (120 minutes, 180 minutes and 240 minutes after the onset of the experiment) as an assessment of mucosal permeability to mannitol.

Histology

The amount of epithelial denudation was measured in mucosal samples taken from ischemic and control jejunum following euthanasia and at the end of the experimental period. Five-micrometer cross-sections taken at 300μm intervals were stained with hematoxylin and eosin. For each tissue, 2 investigators independently evaluated 3 sections. Three well oriented villi and crypts were identified in each section. The length of the crypt and villus and the width at the midpoint of the villus were obtained using a micrometer in the eyepiece of a light microscope. In addition, the height of the epithelial covered portion of each villus was measured. Data from the 2 evaluators was pooled prior

to any data manipulations. The surface area of the villus was calculated using the formula for surface area of a cylinder. The formula was modified by subtracting the area of the base of the villus and multiplying by a factor accounting for the variable position at which each villus was cross-sectioned.[12] The denuded villous surface area was calculated from the total surface area of the villus and surface area of the villus covered by epithelium.

Prostanoid levels

Samples of serosal bathing solutions were taken from each Ussing chamber after the 45-minute incubation period and at the end of the experiment (240 minutes). Samples were snap frozen in liquid nitrogen, stored at -70^0C and analyzed for prostaglandin E_2, 6-keto-prostaglandin $F_{1\alpha}$ (a stable metabolite of PGI_2) and thromboxane B_2 (a stable metabolite of TBA_2). Assays were performed using ELISA kits.[d]

Statistical analysis

The transepithelial electrical resistance values were evaluated using a two-way repeated measures analysis of variance (RM-ANOVA) for the effects of treatment and time. Flux data was analyzed by one-way ANOVA for the effect of treatment within each flux period. Histological data (denuded villus surface area) was analyzed with a 1-way ANOVA for effect of treatment on denudation. Chamber prostanoid levels were analyzed for each time period (45 minutes, 240 minutes) using a one-way ANOVA for the effect of treatment. Post hoc analyses were performed with Tukey's tests for parametric data.

When data was non-parametric, ANOVA on ranks was performed with a post hoc Dunn's multiple comparison test. For all tests, a significance level of $p < 0.05$ was chosen.

Results

Transepithelial electrical resistance (Figure 1): The TER of ischemic jejunum was significantly lower for both flunixin ($p<0.001$) and deracoxib-treated tissue ($p<0.001$) than for untreated ischemic jejunum over the recovery period. There was no significant difference between treatments for control tissue. Flunixin and deracoxib-treated ischemic tissue failed to recover during the 240-minute time period on the Ussing chamber. Untreated ischemic jejunum recovered by 240 minutes to a mean TER of 49 $\Omega.cm^2$ but did not reach the mean untreated control value of 59 $\Omega.cm^2$. Treatment with misoprostol (figure 2) ameliorated the effect of flunixin and significantly increased the TER above that of flunixin-treated ischemic tissue alone ($p < 0.001$).

Mannitol flux (figure 3): Deracoxib-treated ischemic tissue was not significantly more permeable than untreated ischemic tissue in any of the flux periods. Ischemia alone and ischemia with deracoxib treatment did not significantly increase permeability to mannitol when compared with control tissue during all fluxes. Ischemic jejunum treated with flunixin was significantly more permeable to ^3H mannitol when compared with untreated ischemic tissue ($p = 0.046$) and control tissue ($p = 0.017$) by the third flux (240 minutes after onset of experiment).

Histopathology (figure 4, table 1): There was significant epithelial denudation immediately following ischemia ($p < 0.001$). Mean ± SE denudation due to ischemia was 55.6 $\pm$ 1.9%. (Table 1). After 240 minutes on the chambers, partial restitution had occurred. The amount of denudation had reduced to 22.1 ± 6.6% and was significantly less than immediately following ischemia ($p = 0.016$). Denudation of ischemic tissue after the experimental recovery period (240 minutes) was not significantly different from the 240-minute control tissue, which had become partially denuded during the recovery period. There was no significant effect of treatment on amount of epithelial denudation for both control and ischemic tissues.

Prostanoid levels: Chamber prostanoid levels did not differ significantly at 45 minutes. At 240 minutes, levels of thromboxane B_2 (TXB_2) were significantly higher in ischemic-untreated tissue compared to control tissue ($p = 0.025$). Flunixin treatment significantly decreased TXB_2 levels in ischemic and control tissue when compared with untreated tissue ($p = 0.004$). Deracoxib treatment did not significantly alter TXB_2 levels (Table 2). Prostaglandin E_2 and 6-keto prostaglandin $F_{1\alpha}$ levels were not significantly elevated due to ischemia. Both flunixin and deracoxib significantly decreased 6-keto $PGF_{1\alpha}$ levels in ischemic tissue when compared to untreated samples ($p < 0.001$) (Table 3). Flunixin ($p < 0.001$) and deracoxib ($p = 0.014$) also significantly decreased PGE_2 levels in ischemic tissue (Table 4) when compared to untreated samples.

Discussion

The non-selective COX-inhibitor, flunixin and the COX-2 selective inhibitor deracoxib (marketed as highly selective in dogs) both had a detrimental effect on recovery of intestinal mucosal TER after ischemia. However, only flunixin-treatment increased mucosal permeability to mannitol. TER is a sensitive index of mucosal barrier function calculated principally from the electrical movement of Na and Cl ions and hence is a measure of intestinal permeability to these ions.[6] Ischemia results in loss of intestinal barrier function and this produces reduced measurements of TER. Recovery of TER is dependent on prostaglandin-induced closure of the paracellular space.[6,8] Flux measurements were used in this study to assess mucosal permeability to mannitol, which is much larger than a single Na or Cl ion. It is interesting that permeability to mannitol is not increased by 2h ischemia alone or affected by deracoxib treatment, despite the latter preventing recovery of TER. The effects of flunixin on TER were not significantly different to deracoxib but flunixin also significantly increased permeability of ischemic mucosa to mannitol. It seems that although TER is a sensitive index of barrier function, measurements do not directly relate to the permeability of intestinal mucosa to small molecules. Mannitol flux measurements seem to be the most direct indicator of increased intestinal permeability. The clinical relevance of increased intestinal permeability after ischemia may be the indiscriminate absorption of small molecules. Increased intestinal permeability could exacerbate endotoxin absorption and contribute to ileus. A retrospective study of colic cases showed that 69% of all deaths occurred prior to hospital discharge. There is a sharp drop in survival during the first 24-48 hours after surgery.[5] These early time periods are critical for survival, and any pharmaceutically induced delay

in the restoration of intestinal barrier function could contribute to high levels of morbidity.

A previous *in vivo* study conducted in our laboratory showed increased permeability of jejunal mucosa from horses treated with flunixin at 18 hours post-ischemia. The permeability of untreated post-ischemic mucosa was not different to control tissue; it was presumed that the untreated jejunal mucosa had recovered in 18 hours and so was less permeable. It now appears that increased permeability to mannitol is not a normal intestinal response to 2 hours of ischemia. Rather than only retarding prostaglandin-induced recovery from ischemia, flunixin appears to cause an abnormal increase in mucosal permeability. We do not know whether this effect is due to inhibition of prostaglandin production. We do know that there was no significant effect of treatment on epithelial denudation and that restitution was not inhibited in any treatment group. Hence the increased permeability in flunixin-treated mucosa is not due to epithelial cell loss or an inhibition of restitution. In fact, mannitol crosses the intestinal mucosa via the paracellular pathway, which is regulated by prostaglandins.[6] These findings agree with previous studies,[7] that the mechanism of intestinal recovery of barrier function is not due to restitution but to the closure of interepithelial tight junctions.

Prostanoid levels from the intestinal tissue fluid were significantly reduced by flunixin. Deracoxib reduced levels of PGE_2 and 6-keto$PGF_{1\alpha}$ but did not affect TXB_2 levels. Levels of TXB_2 were significantly increased after ischemia unlike the other prostanoids measured, therefore TXB_2 may play a role in recovery of intestinal barrier function. If the

mechanism of increased permeability in flunixin-treated mucosa is entirely due to prostaglandin inhibition, deracoxib may allow enough prostanoid production to avoid this effect. We do not know if deracoxib is selectively inhibiting COX-2 in equine intestinal tissue as we did not perform COX selectivity assays. However, given the highly selective nature of coxibs such as deracoxib in a variety of species, it is probable that deracoxib is COX-2 selective, and it appears that this may be advantageous compared to flunixin during mucosal recovery from ischemia.

In vitro results cannot always be extrapolated to *in vivo* effects. We had previously shown that after 1 hour of ischemia, the *in vitro* use of an alternate COX-2 selective inhibitor etodolac did not retard recovery of TER on the Ussing chamber, but permeability to mannitol was not assessed in this study.[8] Following this, an *in vivo* study with a 2-hour ischemic insult followed by 18 hours of recovery resulted in etodolac retarding recovery of TER and increasing permeability to mannitol.[10] Tissue prostanoids from the *in vitro* study showed that etodolac partially decreased 6-keto$PGF_{1\alpha}$ and did not affect TXB_2 production whereas flunixin significantly inhibited all prostanoids measured.[8] Blood prostanoid levels from the *in vivo* study showed that etodolac inhibited prostanoid production to the same extent as flunixin. As whole blood TXB_2 has been used as an index of COX-1 activity because of the co-localization of COX-1 with thromboxane synthase in platelets,[14] this could indicate that etodolac was not acting in an entirely COX-2 selective manner *in vivo*. However selectivity assays were not performed. In the present study, we decided to use a different COX-2 selective inhibitor, deracoxib that is

reported to be highly selective in dogs.[11] The next step will be to use deracoxib in an *in vivo* study.

In the present study, we determined that the prostaglandin E_1 analogue, misoprostol reversed the effect of flunixin on TER. We plan to test the effect of misoprostol on the permeability of flunixin-treated ischemic intestine *in vivo*. This drug could be administered to ameliorate the adverse gastrointestinal effects of NSAIDs; side effects such as abdominal discomfort may preclude the use of this drug.[c] Like PGE_2, misoprostol elevates cellular cyclic AMP levels to increase intestinal barrier function.[15] An alternative mechanism to increase cellular cyclic AMP is the phosphodiesterase inhibitor, pentoxyfilline. Pentoxyfilline has been reported to prevent NSAID-induced enteropathy in diclofenac treated rats and presents another therapeutic option.[16]

Based on the collective results of our studies, we have major concerns about the use of the non-selective COX inhibitor flunixin in post-operative colic cases. Addition of misoprostol may ameliorate the effects of non-selective COX inhibitors on recovering mucosa. Alternatively, the use of a selective COX-2 inhibitor such as deracoxib could be more appropriate.

Footnotes

[a] Sigma Pharmaceuticals, St Louis, Missouri ,USA

[b] Novartis Animal Health, Greensboro, North Carolina, USA

[c] Cayman Chemicals Ann Arbor, Michigan, USA

[d] R &D Systems, Minneapolis, Minnesota, USA

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Table 1: Percentage epithelial denudation of control and ischemic tissue at the (0 minutes) and at the end of the experiment (240 minutes) in the various treatment groups. Ischemia resulted in significant epithelial denudation.[a] By the end of the experiment, partial restitution had occurred, and the amount of denudation was significantly different to the 0-minute ischemic tissue.[b] There was no significant effect of treatment on amount of denudation for ischemic or control tissues.

Treatment	Tissue type	Time (min)	Mean ± SE
None	Control	0	0 ± 0
None	Ischemic	0	55.6[a] ± 1.9
None	Control	240	9.4 ± 7.2
None	Ischemic	240	22.1[b] ± 6.6
Flunixin	Control	240	23.1± 8.1
Flunixin	Ischemic	240	33.8 ± 6.7
Deracoxib	Control	240	25.1 ± 9.2
Deracoxib	Ischemic	240	36.7 ± 6.5

Table 2: Thromboxane B_2 levels (pg/ml) in control and ischemic tissues mounted in Ussing chambers. There were no significant differences at 45 minutes. At 240 minutes, flunixin had significantly decreased TXB_2 levels in control tissue.[a] By 240 minutes, untreated-ischemic tissue had significantly elevated TXB_2 levels [b] compared to untreated control tissue. Only flunixin significantly reduced TXB_2 levels in ischemic [c] tissues whereas deracoxib treatment had no significant effect.

Treatment	Tissue type	Mean ± SE 45 min	Mean ± SE 240 min
None	Control	0	8.96 ± 1.18
Flunixin	Control	0	2.92[a] ± 1.71
Deracoxib	Control	0.98 ± 0.36	8.4 ± 2.2
None	Ischemic	0	34.4[b] ± 10
Flunixin	Ischemic	0.15± 0.06	3.35[c] ± 1.5
Deracoxib	Ischemic	1.3 ± 0.59	19.7 ± 5.8

Table 3: 6-keto prostaglandin $F_{1\alpha}$ levels (pg/ml) in control and ischemic tissues. There were no significant differences at 45 minutes. By 240 minutes, although untreated-ischemic tissue had no significant elevation of this prostanoid when compared to control tissue, flunixin and deracoxib both significantly decreased levels in ischemic tissue.[a]

Treatment	Tissue type	Mean ± SE 45 min	Mean ± SE 240 min
None	Control	1354 ± 747	13575 ± 2281
Flunixin	Control	759 ± 166	516 ± 98
Deracoxib	Control	1446 ± 55	1637 ± 338
None	Ischemic	2691 ± 1360	11695 ± 2986
Flunixin	Ischemic	317 ± 144.6	1462[a] ± 485
Deracoxib	Ischemic	447 ± 96.7	7184[a] ± 634.4

Table 4: Prostaglandin E_2 levels (pg/ml) in control and ischemic tissues. There were no significant differences at 45 minutes. By 240 minutes, although untreated ischemic-injured tissues had no significant increase in PGE_2 levels, both flunixin and deracoxib significantly decreased PGE_2 levels.[a]

Treatment	Tissue type	Mean ± SE 45 min	Mean ± SE 240 min
None	Control	1757 ± 873	1889 ± 462
Flunixin	Control	76.8 ± 33	44 ± 26.9
Deracoxib	Control	72 ± 20.5	114 ± 44.6
None	Ischemic	1180 ± 759	2111 ± 407
Flunixin	Ischemic	187 ± 128.3	89.5[a] ± 67.6
Deracoxib	Ischemic	157 ± 56.8	485[a] ± 220.7

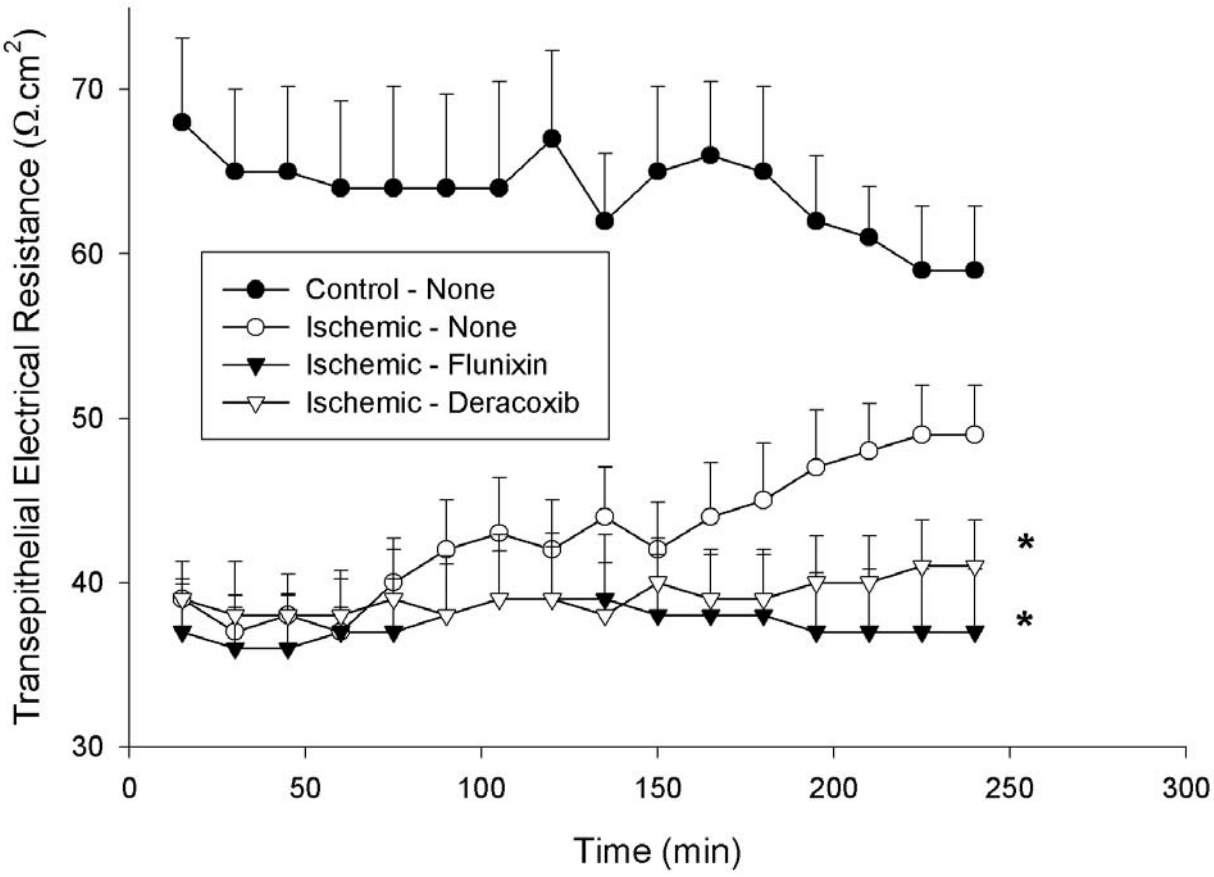


Figure 1: Transepithelial electrical resistance (TER) of control and ischemic jejunum during the 240 minutes on the Ussing chamber. Mean values are shown with error bars. Both flunixin and deracoxib-treated ischemic tissue had significantly lower TER values when compared with untreated ischemic tissue allowing for effects of time (* denotes a significant difference). Untreated ischemic tissue showed a recovery response (increase in TER) by 240 minutes. There was no significant effect of treatment on TER for control tissue. The control values shown are from untreated tissue.

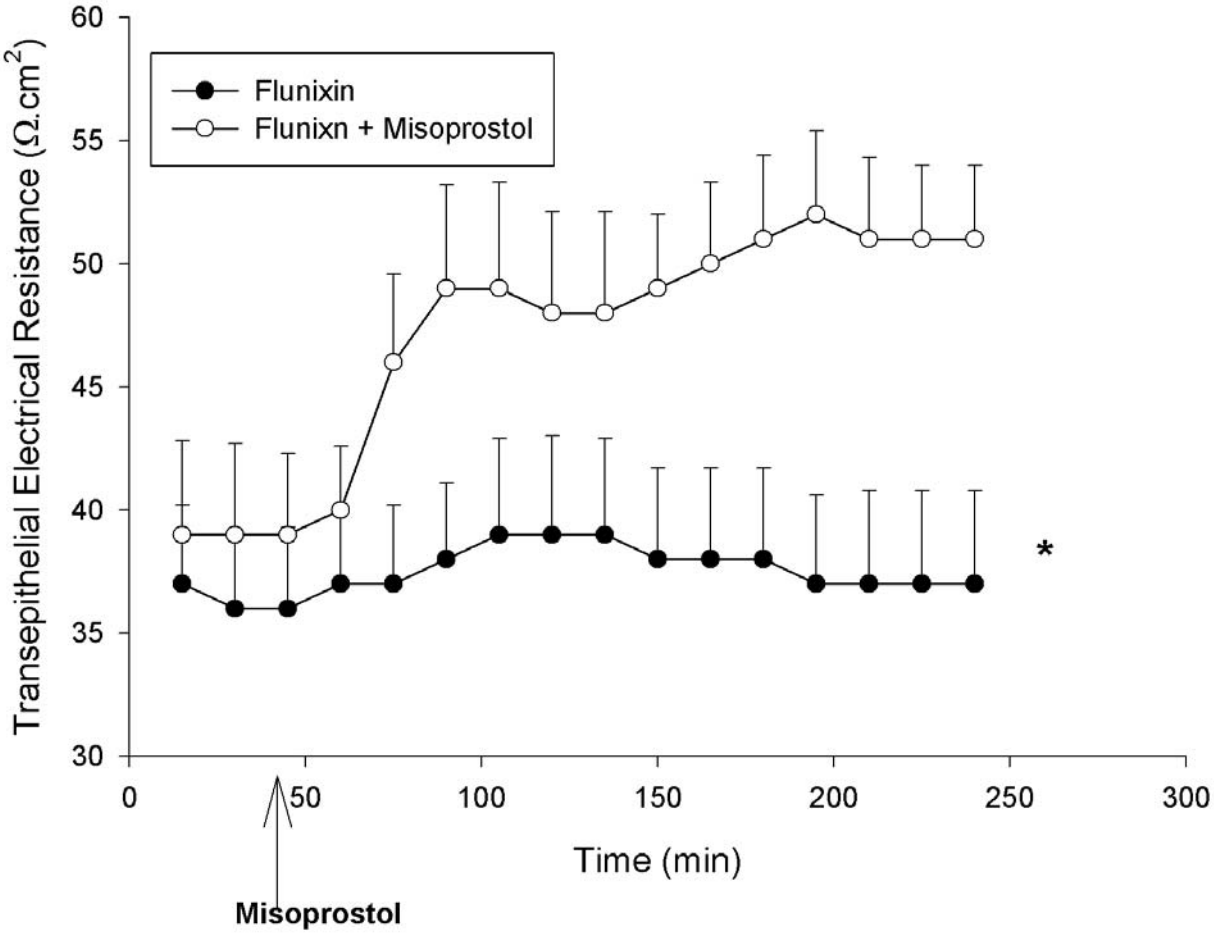


Figure 2: Transepithelial electrical resistance (TER) of flunixin-treated ischemic jejunum during the 240 minutes on the Ussing chamber compared with flunixin-treated ischemic tissue with the addition of misoprostol at 45 minutes (arrow). Mean values are shown with error bars. The TER of flunixin-treated ischemic tissue without misoprostol was significantly lower (*) than tissue with misoprostol added.

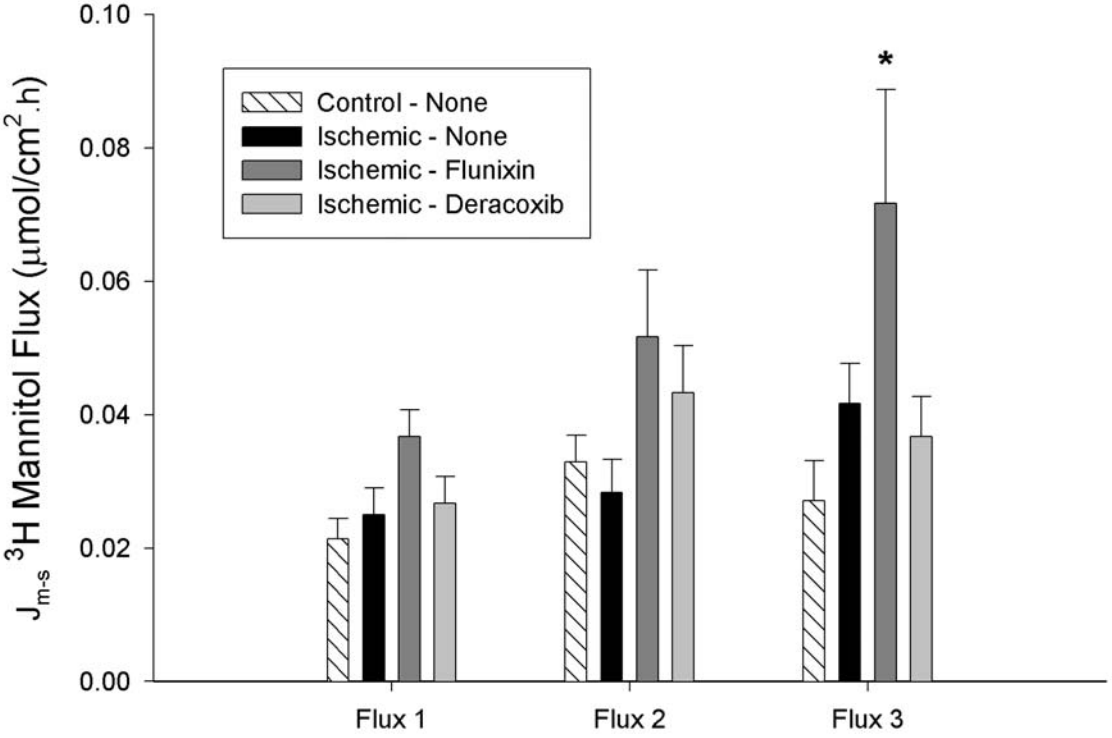


Figure 3: Mucosal permeability of ischemic-injured and control jejunum to ^{3}H mannitol (mannitol flux). Mean values are shown with error bars. During the first and second flux periods (120 and 180 minutes after the onset of the experiment) there was no significant effect of ischemia or treatment on permeability to mannitol. By the third flux period (240 minutes), flunixin-treated ischemic tissue was significantly more permeable to mannitol than untreated ischemic tissue.

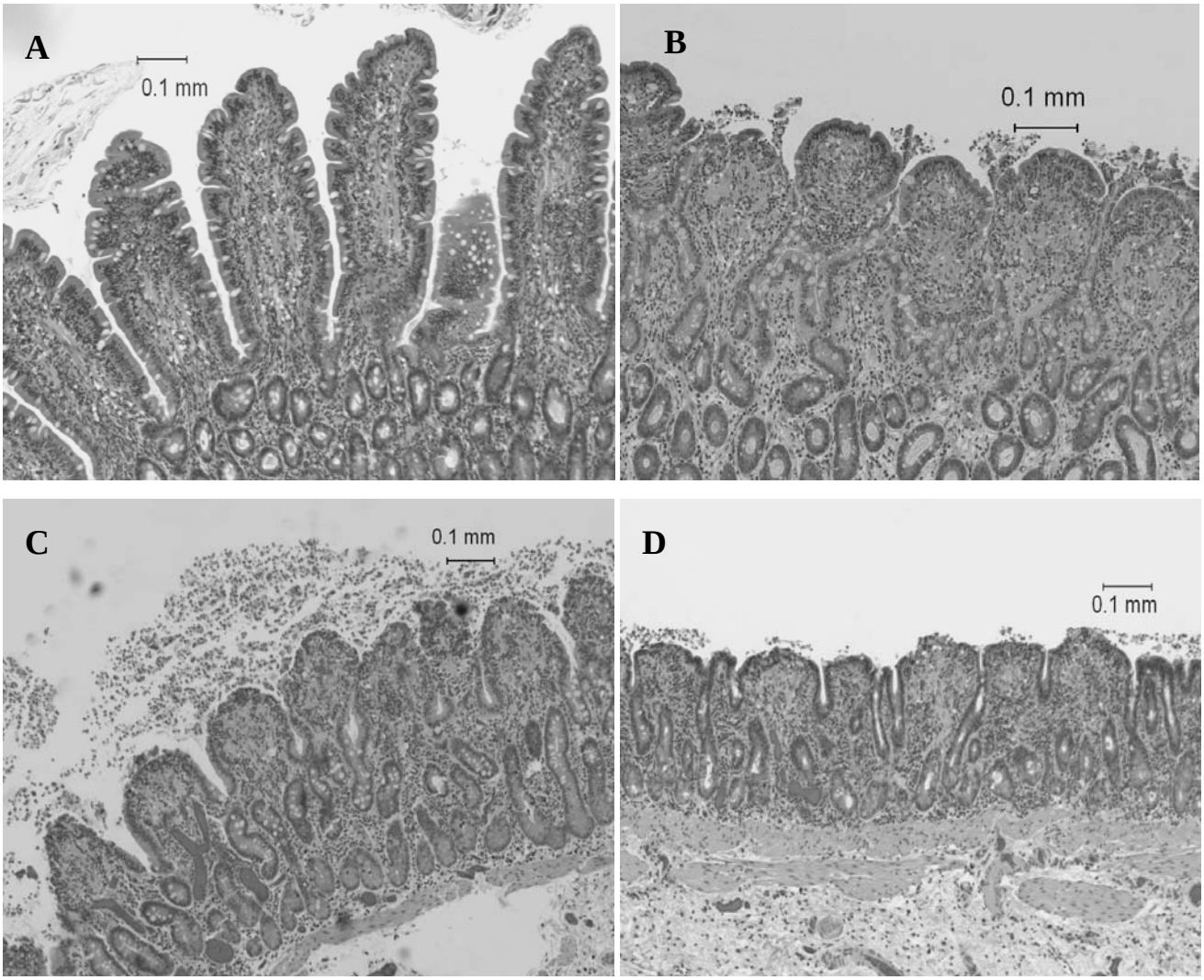


Figure 4: Sections of jejunal mucosa were used to determine the extent of epithelial denudation. There was no significant effect of treatment on epithelial denudation.
A: Control mucosa immediately before the onset of the experiment. No denudation is seen on this slide
B: Control mucosa after 240 minutes on the Ussing chamber. Minimal denudation can be seen at the villous tips.
C: Ischemic mucosa immediately before the onset of the experiment. Mean denudation was 55.6%.
D: Ischemic mucosa after 240 minutes on the Ussing chamber. There was no significant effect of treatment on the amount of denudation, untreated tissue is shown here. By 240 minutes, restitution had partially occurred and mean denudation (untreated) was 23.1%.

Chapter 4:

Effects of ischemia and the cyclooxygenase inhibitor flunixin meglumine on in vitro passage of lipopolysaccharide across ischemic-injured equine jejunum

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Abstract

Objective: Although flunixin adversely affects jejunal barrier function after ischemia, it is not known whether flunixin alters lipopolysaccharide (LPS) absorption. The purpose of this study was to determine whether ischemia and flunixin affect LPS absorption in equine jejunum.

Animals: Twelve horses.

Procedure: Animals were subjected to 120 minutes jejunal ischemia under anesthesia then euthanized. Mucosa was mounted on Ussing chambers treated with or without flunixin. 6 horses were used for assessment of permeability to radiolabeled LPS. Mucosa from the remaining 6 was incubated with fluorescent labeled LPS (FITC-LPS) and examined histologically. Production of tumor necrosis factor-α (TNFα) and of LPS binding protein (LBP) was assessed as an indicator of mucosal response to LPS.

Results: Ischemia significantly increased LPS permeability, but by 180 minutes, mucosa had recovered and was not more permeable than control tissue. Flunixin adversely affected intestinal barrier function throughout the experiment, however this did not result in increased permeability to LPS. LBP production was increased by ischemia, and reduced by exposure to LPS. FITC-LPS entered the lamina propria of ischemic tissue but TNFα was produced on the mucosal side only, indicating little response to the absorbed LPS.

Clinical relevance: Ischemia increased LPS passage across equine jejunal mucosa. Although flunixin delayed mucosal recovery, it did not exacerbate LPS absorption in this model. The clinical significance of flunixin-induced delayed mucosal recovery will require further *in vivo* testing.

Introduction

Colic is a major cause of death in horses.[1,2] Post surgical deaths from colic are attributable to complications such as endotoxemia, which results from increased permeability of injured intestine to lipopolysaccharide (LPS).[3-5] In clinical studies of horses with colic, endotoxemia was detected in up to 25% of cases.[6,7] Endotoxemia significantly contributes to morbidity and mortality in clinical patients recovering from ischemic intestinal disease and subsequent surgery.[8] Although ischemic intestine is resected when possible at surgery, injured intestine may remain as a result of distention,[9] or the surgeon's inability to detect or adequately resect all damaged intestine. In fact, a recent study found evidence of serosal injury and neutrophil infiltration in the proximal resection margins of ischemic intestine indicating that not all damaged intestine had been removed.[10]

Endotoxemia is attributable to absorption of LPS from intestinal gram-negative bacteria across a compromised intestinal mucosal barrier. Gram-negative bacteria release LPS during rapid proliferation and cell death.[11] Once absorbed, LPS triggers pathophysiological effects in mammals ranging from mild fever to fatal septic shock.[12] LPS stimulates host cells to produce and release endogenous mediators of inflammation, for example tumor necrosis factor-α (TNFα), which can be measured to evaluate response to LPS.[5,12] The principal cells that interact with LPS are monocytes/macrophages. However, although intestinal epithelial cells have been regarded as serving solely as a barrier to LPS, it has recently been determined that these

cells interact specifically with the LPS molecule. Intestinal epithelial cells are exposed to high levels of intact bacteria and bacterial products (cecal contents of healthy horses contain up to 80 μg LPS/ ml[13]) and are the first line of the innate immune response to intestinal microbial invasion.[13] The interaction between LPS and intestinal epithelium is in part mediated by epithelial secreted lipopolysaccharide binding protein (LBP) which forms a complex with LPS. LBP is present in normal human serum and increases markedly in response to inflammation.[15,16] The biological activity of LBP-bound LPS is markedly decreased; therefore LBP plays a role in the elimination and neutralization of LPS in serum and tissue fluids.[17,18,19] LBP was identified in bovine serum by Western blot[20] indicating that elevated levels may be a useful clinical indicator of endotoxemia. We aimed to investigate whether ischemia and LPS increased LBP levels in equine jejunal mucosa.

Since much of the pain and signs of endotoxemia are attributable to COX-elaborated prostaglandins, flunixin, a COX inhibitor, is frequently administered in cases of endotoxemia. We have recently shown that the use of flunixin at a dose of 1.1mg/kg in horses prevents recovery of ischemic-injured equine jejunum (measured *in vitro* as transmucosal electrical resistance) when compared with untreated tissue.[21,22] Prostaglandins are critical for recovery of ischemic-injured intestine,[21,23] leading us to postulate that the use of flunixin will paradoxically exacerbate endotoxemia by impairing intestinal barrier function. By retarding recovery of intestinal barrier function after ischemia, the use of NSAIDs in patients that have suffered intestinal damage during an episode of colic has important implications. While NSAIDs provide analgesia and reverse

the systemic effects of endotoxin, these drugs may delay or prevent the healing process, thereby potentially increasing morbidity and mortality. Although the failure of ischemic jejunum to recover normal levels of transmucosal electrical resistance indicates a residual permeability defect in horses treated with flunixin, we did not know if this was sufficient to exacerbate LPS absorption.

Absorption of LPS was increased in ischemic-injured rat intestine via the paracellular route across tight junctions,[24] and we have previously shown that prostaglandins regulate repair of the paracellular space during intestinal recovery.[21,25] However, the basic mechanisms of intestinal absorption of LPS are not fully understood. Nonetheless, it has been shown clinically that horses with extensive small intestinal injury become endotoxemic.[6] The present study further explores the mechanisms of LPS absorption in ischemic-injured equine small intestine. The hypotheses of this study were that absorption of LPS across the intestinal mucosal barrier would occur in the horse; that absorption would be increased by ischemia and that flunixin would exacerbate LPS absorption across ischemic mucosa due to increased paracellular permeability. We also hypothesized that the levels of lipopolysaccharide binding protein would increase in intestinal epithelial cells in direct correlation with increased LPS absorption and therefore could be used as a quantitative indicator of LPS absorption.

Materials and Methods

The North Carolina State University Institutional Animal Care and Use Committee approved the experimental protocol. Two groups of six healthy horses 5 - 15 years of age,

weighing 400-600 kg were included in the study. The animals underwent a four-week quarantine period involving vaccination, deworming and observation prior to being housed at the research facility. Each horse was premedicated with intravenous xylazine (1.1mg/kg), an intravenous catheter was placed in the left jugular vein, and induction achieved with intravenous diazepam (0.1mg/kg) and ketamine (2.2mg/kg). Horses were orotracheally intubated and a surgical plane of anesthesia maintained with isoflurane vaporized in 100% oxygen. A midline celiotomy was performed and the jejunum located. One 30-cm section of jejunum was selected, 60 cm oral to the antimesenteric band of the ileum. The local jejunal blood supply was occluded with silk ligatures. The segment was cross-clamped using Doyen forceps to ensure that no blood flow was received from adjacent non-experimental bowel. Blood supply was occluded for 120 minutes prior to euthanasia using an overdose of sodium pentobarbital (100mg/kg). Following euthanasia, the jejunum was harvested for in vitro studies.

Ussing chamber studies

Intestinal loops were incised along the antimesenteric surface. The mucosa was then stripped from the seromuscular layer in oxygenated (95% O_2/5% CO_2) equine Ringer's solution, as prepared in previous studies [21,26,27] The tissue was then mounted in 3.14 cm^2 aperture Ussing chambers. [26,27] Tissues were bathed on the serosal and mucosal sides with 10 ml equine Ringers solution. The serosal solution contained 10 mmol/l glucose and was osmotically balanced on the mucosal side with 10 mmol/l mannitol. Chambers contained either no treatment or 2.7 x 10^{-5} mol/l flunixin.[a, 21] 500 ng/ml of cold or radiolabeled lipopolysaccharide from *E.coli K12 strain LCD25*[b] was added at 60 minutes

to some of the chambers. Bathing solutions were oxygenated (95% O_2/5% CO_2) and circulated in water-jacketed reservoirs at 37 ^{0}C. After a 15 minutes equilibration period, the spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes using a voltage clamp that corrected for fluid resistance. Resistance ($\Omega.cm^2$) was calculated from the spontaneous PD and short-circuit current (I_{sc}). If the spontaneous PD was between –1.0 and 1.0mV, tissues were current clamped at $\pm$ 100 μA for 5 s and the PD recorded. Short-circuit current and PD were recorded every 15 minutes for 180 minutes. The data were entered into spreadsheets that calculate transepithelial electrical resistance (TER) from I_{sc} and PD using Ohms law.

Lipopolysaccharide flux

This was performed on the first group of 6 horses. After 60 minutes (to allow incubation with flunixin) two 60-minute mucosal-to-serosal endotoxin fluxes were performed by adding radiolabeled LPS to the mucosal bathing solutions and monitoring its appearance in the serosal solutions. ^{3}H-labeled LPS from *E. Coli K12 strain LCD25*[b] (2 μCi/8.8 x 10^6 DPM) was mixed with non-radioactive *E. Coli* LPS of the same strain[b] to a final concentration of 500 ng/ml before being added. Samples were collected in scintillation vials at 0, 60 and 120 minutes after addition of radiolabeled LPS to the Ussing chambers and assessed for β emission (counts/minute). Mucosal fluxes of ^{3}H were calculated as an assessment of mucosal permeability to LPS.

FITC-Lipopolysaccharide studies

Jejunal tissue from the second group of 6 horses was used to determine the location of any lipopolysaccharide that had entered the intestinal mucosa. Control and ischemic jejunum, with or without flunixin, was incubated on the Ussing chambers with fluorescein-isothiocyonate labeled lipopolysaccharide (FITC-LPS) from *E. Coli* serotype 0111:B4 at a dose of 25 μg/ml.[a] [28] The incubation period was 120 minutes and was initiated after 60 minutes incubation with or without flunixin. Following incubation, tissue was removed and snap frozen in liquid nitrogen then stored at -70^0C. Frozen sections were cut for examination under a light microscope equipped with epifluorescence to determine the position of the FITC-LPS. Concurrent sections were stained with hematoxylin and eosin to aid in tissue orientation.

TNF α Measurements

In order to further examine the effects of ischemia and flunixin on LPS-exposed gastrointestinal mucosa; 1 ml of the serosal and mucosal bathing fluids from 3 horses was taken after 120 minutes exposure to FITC-LPS[a] Samples were snap frozen in liquid nitrogen and stored at -70^0C prior to measurement of tumor necrosis factor alpha (TNFα) by ELISA.[c]

Histopathology

Tissues were taken immediately at euthanasia and from the Ussing chamber after the 180 minutes experiments. Five-micrometer cross-sections taken at 300 μm intervals were stained with hematoxylin and eosin. For each tissue, 2 investigators independently

evaluated 3 sections. Three well-oriented villi and crypts were identified in each section. The length of the crypt and villus and the width at the midpoint of the villus were obtained using a micrometer in the eyepiece of a light microscope. In addition, the height of the epithelial covered portion of each villus was measured. The surface area of the villus was calculated using a modified formula for surface area of a cylinder. Villus surface area = $(2\pi \cdot \frac{1}{2} [(4/\pi)d]h)$, where $\pi = 3.14$, d = villus diameter at midpoint and h = villus height.[27] The denuded villous surface area was calculated from the total surface area of the villus and surface area of the villus covered by epithelium.

Gel Electrophoresis and Western Blotting for LBP

Mucosal tissue from control and ischemic jejunum with cold LPS $\pm$ flunixin was treated to extract the epithelial cells with citrate phosphate buffer and PBS with EDTA.[29] Samples were stored at -70^0C prior to preparation for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Tissue was thawed to 4^0C and each sample was added to 1.5 ml of chilled RIPA buffer (0.15 M NaCl, 50 mM sodium Tris [pH 7.2], 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS), including protease inhibitors (PMSF and Apoprotinin). The mixture was homogenized on ice, centrifuged at 4^oC (10,000 G for 10 minutes), and the supernatant saved. Protein analysis of extract aliquots was performed.[d] Protein extracts (50 μg) were mixed with an equal volume of 2X SDS-PAGE sample buffer and boiled for 4 minutes at 100^oC. Lysates were loaded on a 10% SDS-polyacrylamide gel and electrophoresis carried out according to standard protocols. Proteins were transferred to a nitrocellulose membrane[e] using an electroblotting mini-transfer apparatus according to the manufacturers' protocol.

Membranes were blocked overnight at 4°C in Tris buffered saline (TBS) and 5% dry powdered milk. Membranes were washed twice with TBS containing 0.05% Tween (TBS-T) and incubated for 2 hours in primary antibody (rabbit anti-human polyclonal vs. LBP).[f] After washing three times for 5 minutes each with TBS-T, the membranes were incubated for one hour with horseradish peroxidase conjugated secondary antibody. After washing twice for 5 minutes each with TBS-T, and once with TBS for 15 minutes, the membranes were developed for visualization of protein by addition of enhanced chemiluminescence agent.[g] Densitometry was performed on scanned images using specialized software.[h]

Statistical Analysis

The transepithelial electrical resistance data was tested using a two-way repeated measures analysis of variance (RM-ANOVA) for the effects of treatment and time. The interaction of transepithelial resistance and treatment from the 60 minute time point onward was chosen to allow adequate tissue equilibration and exposure to flunixin. LPS flux data was tested using a one-way ANOVA for the effect of treatment at each flux period. TNFα measurements were analyzed using a 1-way ANOVA and are reported as mean + standard error of the mean (SE). Histomorphometric data (epithelial denudation) was analyzed with a 1-way ANOVA. Western blot densitometry measurements were analyzed with a one-way ANOVA for the effect of treatment. The post hoc test used in all cases was a Tukey test as the data was parametric. A significance level of $p < 0.05$ was chosen for all tests.

Results

Transepithelial electrical resistance: Flunixin-treated ischemic mucosa exposed to LPS had a significantly lower transepithelial resistance when compared with untreated ischemic mucosa exposed to LPS ($p = 0.004$) (Figure 1). By 180 minutes, the untreated ischemic mucosa had recovered to a resistance level that was not significantly different to controls. There was no significant effect of treatment on control tissue.

Lipopolysaccharide flux: The first flux (60 minutes after addition of LPS) revealed that permeability to LPS in ischemic mucosa was significantly greater than controls ($p=0.042$, Figure 2). By the second flux period (120 minutes after addition of LPS, 180 minutes after the onset of the experiment), the ischemic tissue was not significantly more permeable to LPS than controls. There was no significant difference in permeability to LPS between flunixin-treated and untreated groups in either time period.

TNFα Measurements: TNFα was detected only in the mucosal, and not the serosal tissue fluid. TNFα levels were significantly higher in the untreated ischemic samples (Mean ±SE: 1.35 ± 0.17 pg/ml) when compared with controls (0.11 ± 0.1 pg/ml) ($p < 0.001$). There was no significant difference in levels of TNFα between untreated ischemic samples and ischemic samples treated with flunixin (Mean ±SE: 1.4 ± 0.09 pg/ml). There was no effect of treatment on control tissue.

Histopathology: Ischemia resulted in significant epithelial denudation when compared with control tissue ($p < 0.001$, Table 1). There was no significant effect of treatment on

the amount of denudation for control or ischemic tissues. There was no evidence of restitution as epithelial denudation at 180 minutes was not significantly less than immediately after ischemia. The small amount of denudation present in the control tissue at 180 minutes was not significantly different to the onset of the experiment (Figure 3).

FITC-Lipopolysaccharide studies: In the control tissue, a small amount of epifluorescence was detected, mostly in the villi. The ischemic samples were more fluorescent and FITC-LPS had entered the lamina propria (Figure 4). There did not appear to be a difference in the amount of fluorescence between the ischemic tissue and ischemic tissue treated with flunixin.

Western blot analysis for Lipopolysaccharide binding protein: The presence of LPS significantly decreased the amount of LBP detected in the blots when compared to untreated tissue ($p < 0.001$, Figure 5). This was also true within control ($p = 0.029$) and ischemic tissues ($p = 0.02$). Ischemia significantly upregulated LBP when compared with controls in the none LPS-treated group ($p = 0.029$). This was not detected within the LPS-treated group. Flunixin treatment did not significantly affect tissue LBP levels.

Discussion

Flunixin-treated ischemic mucosa had a significantly lower TER than untreated-ischemic mucosa throughout the *in vitro* recovery period. As shown in previous studies, flunixin prevents recovery of intestinal mucosal barrier function after ischemia.[21,22] Transepithelial electrical resistance is an *in vitro* measure of epithelial barrier function.

This measurement predominantly reflects the permeability of the paracellular pathway.[30] A lowered TER is often accompanied with increased paracellular permeability to small molecules such as mannitol.[22,23,25,30] Ischemia increased the permeability of equine jejunal mucosa to LPS. However, despite flunixin having previously been shown to increase the permeability of ischemic mucosa to mannitol,[22] this did not appear to translate to increased LPS absorption. Thus, the combined effect of ischemia and flunixin treatment on barrier function did not produce a detectable increase in mucosal permeability to lipopolysaccharide over and above that of ischemia alone.

There are conflicting reports about the mechanism of LPS passage across the intestinal epithelium and whether it does cross in some species.[12,28] In horses LPS has been directly isolated from the blood. [6,7] There are two possible ways that the LPS molecule can be absorbed through the intestinal epithelium: across the epithelial cell membrane (transcellular route) or between cells, across the intercellular tight junctions (paracellular route). Ischemia may affect uptake of LPS by damaging the transepithelial barrier and by opening the gateway provided by the tight junctions (paracellular route).[31] Ischemia has been reported to increase the permeability of a human intestinal cell monolayer to FITC-LPS despite a lack of change in transepithelial resistance. This implies a transcellular route as there was no change in the paracellular function.[24] However, it has also been shown that LPS can cross ischemic tissue *in vivo* by both transcellular and paracellular pathways, whereas uptake in non-ischemic jejunum was only transcellular.[24] *In vitro* studies in rats showed that fluorescent-labeled *E. Coli* C_{25} LPS did not cross normal rat ileal mucosa nor did it cross after damage from hemorrhagic shock.[28] From the results of

our present study, we cannot definitely state whether the increased permeability to LPS after ischemia is via the paracellular or transcellular route. It seems likely that the artificially increased 'paracellular' space resulting from epithelial cell loss was the principal route of access. There was no difference in the extent of epithelial denudation between treatment groups and this is a finding in agreement with previous studies.[22,23] Therefore flunixin does not increase epithelial denudation or retard restitution. Flunixin affects the permeability of the tight junctions between epithelial cells [21,22,30] and this additional increase in permeability may have been undetectable in the presence of the large defect created by epithelial cell loss. This would explain why flunixin does not appear to exacerbate LPS absorption despite reducing recovery of TER. There was a trend towards increased LPS absorption in flunixin-treated mucosa but the standard error in this group was large (figure 2). It may be that once restitution has occurred, the increased paracellular permeability due to flunixin would contribute more to LPS absorption. Flunixin has previously been shown to increase the permeability of ischemic intestinal mucosa to mannitol, but this was at a stage when epithelial restitution was near-complete (18-hours following the initial ischemic insult).[23] Mannitol crosses the epithelium exclusively via the paracellular pathway.[21,25,30] LPS is a much larger molecule than mannitol (182.2Da vs. 3000Da) but smaller than inulin (mw. 11,000Da) which can cross the tight junction.[21] However, lipopolysaccharides are large amphiphilic molecules that form aggregates in aqueous environments.[32] Aggregates of LPS may have been too large to cross the tight junction even in compromised intestinal epithelium.

We showed that FITC-LPS enters the lamina propria of equine jejunal mucosa in ischemic tissue. However, this does not appear to have been enough to result in an inflammatory reaction. Ischemia did increase TNFα production but we only detected TNFα in the mucosal chamber fluid and not on the serosal (lamina propria) side. LPS does not have to reach the circulation to mediate its effects, it can activate cells in the lamina propria especially macrophages. These cells produce inflammatory cytokines, including TNFα. [14,19,33] In addition, intestinal epithelial cells themselves produce TNFα in response to LPS. [34] The receptor that responds to LPS is toll-like receptor-4 (TLR-4). This receptor is expressed apically in the epithelial cell and responds to LPS present in the intestinal lumen.[35] In fact, response to normal non-pathogenic bacteria can induce TNFα secretion in intestinal epithelial cell culture.[36] It appears that the intestinal epithelial cells in our study only responded to the LPS placed on the apical side (the equivalent of the lumen). There seems to have been little response to the LPS that crossed into the lamina propria. Previous studies have found increased blood levels of TNFα in response to LPS exposure [12,19,37] hence we expected TNFα production on the serosal (lamina propria) side. It may have been that only cells exposed mucosally to the large amount of FITC-LPS in the chamber fluid reacted and that insufficient LPS molecules crossed into the lamina propria to result in serosal TNFα production. Immune reactions to LPS and bacteria are known to be downregulated in normal intestine [38] so this could explain why only ischemic and not control mucosa reacted to the LPS placed on the mucosal side.

Lipopolysaccharide binding protein was identified in the intestinal epithelial cells. LBP binds to LPS monomers to prevent aggregation,[15,16] and it may be in this form that LPS crosses the intestinal epithelium. Based on evidence of previous studies, we had postulated that ischemia would increase levels of LBP and that exposure to LPS would further increase these levels.[15,16] Ischemia did increase levels of LBP and levels were not further altered by flunixin. However, exposure to LPS significantly decreased LBP levels isolated by Western blot when compared to unexposed tissue making differences difficult to detect. As previously stated, LBP forms complexes with LPS, which is a large, lipid soluble molecule. Since these complexes are predominantly composed of lipid, they would likely be removed by the protein extraction process in preparation for Western blotting, explaining lower LBP levels in tissues exposed to LPS.

We conclude that despite an adverse effect on mucosal barrier function, flunixin did not increase the permeability of ischemic-injured intestinal mucosa to LPS. However, during the experiment, insufficient LPS crossed the mucosa to stimulate production of the inflammatory cytokine TNFα; this may indicate that the amount of LPS crossing the mucosa in the experiment was clinically insignificant. Thus any differences in the permeability of flunixin-treated ischemic mucosa may not have been detected. Flunixin has been shown to increase paracellular permeability of ischemic intestine [21] but LPS may not cross the intestinal epithelium via this route. Alternatively, it may be that once restitution has occurred after ischemia, the remaining increase in paracellular permeability due to flunixin treatment would contribute more significantly to LPS

absorption. Further detailed studies into the mechanism of LPS passage across equine intestinal epithelium are warranted.

Footnotes:

[a] Sigma Aldrich Pharmaceuticals, St Louis Missouri. USA

[b] List Biological, Campbell CA. USA

[c] R & D systems, Minneapolis MN. USA

[d] Dc protein assay, Bio-Rad, Hercules CA. USA

[e.] Hybond ECL, Amersham Life Science, Birmingham, UK

[f] Cell Sciences Inc. Norwood MA. USA

[g.] Amersham, Princeton NJ. USA

[h.] Zero Dscan. Scanlytics Inc. Fairfax, VA. USA

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Table 1: Percentage of epithelial denudation in control and ischemic jejunal mucosa at the onset (0 min) and after the experimental period on the Ussing chamber (180 min). Ischemia resulted in significant epithelial denudation when compared with control tissues (* denotes significance). After a 180-minute recovery period on the Ussing chamber, the amount of denudation was not significantly less than at 0 minutes. There was no effect of treatment (flunixin or LPS) on the amount of denudation.

Treatment	Tissue type	Time (min)	Mean $\pm$ SE
None	Control	0	2.4 $\pm$ 2.4
None	Ischemic	0	38.1* $\pm$ 3.3
None	Control	180	5.2 $\pm$ 5.2
None	Ischemic	180	34 $\pm$ 5.3
LPS	Control	180	5.4 $\pm$ 4.8
LPS	Ischemic	180	26.8 $\pm$ 5.3
Flunixin	Control	180	10.2 $\pm$ 7
Flunixin	Ischemic	180	32.5 $\pm$ 7

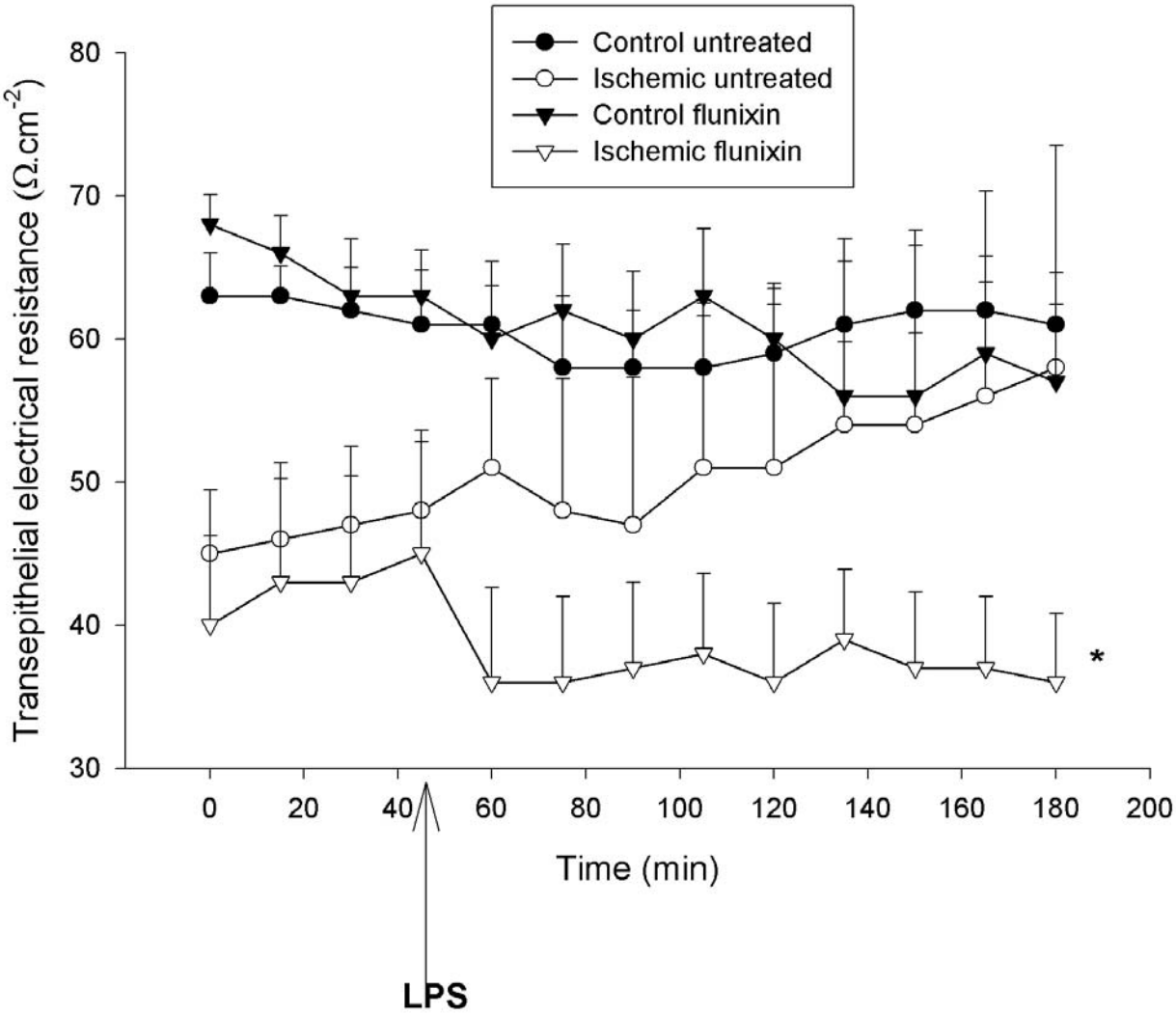


Figure 1: Transepithelial electrical resistance (TER) of control and ischemic jejunal mucosa exposed to lipopolysaccharide (LPS – arrow) with or without flunixin treatment. Mean values are shown with error bars. The TER of flunixin-treated ischemic tissue from 60 minutes to the end of the experiment (180 minutes) was significantly lower than untreated ischemic tissue (*). By 180 minutes the TER of untreated ischemic tissue was not significantly different to control values.

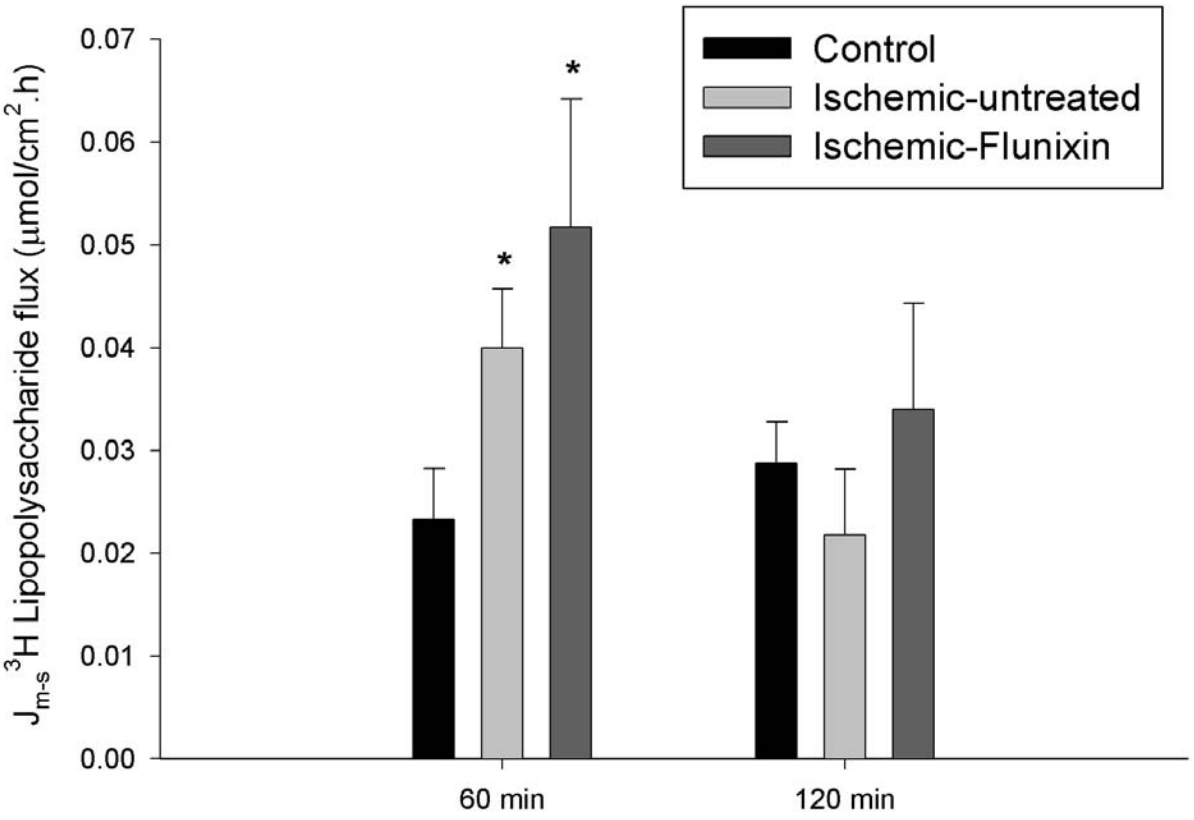


Figure 2: Permeability of control and ischemic jejunum to radiolabeled (^{3}H) lipopolysaccharide (LPS) calculated as mucosal to serosal flux (J_{m-s}). Mean values are shown with error bars. At the 60-minute flux, the permeability to LPS was significantly greater in ischemic tissues when compared with controls (*). Flunixin-treated ischemic tissue was not significantly more permeable than ischemic tissue alone. There was no effect of treatment on permeability of control tissue. Untreated control tissue is shown here.

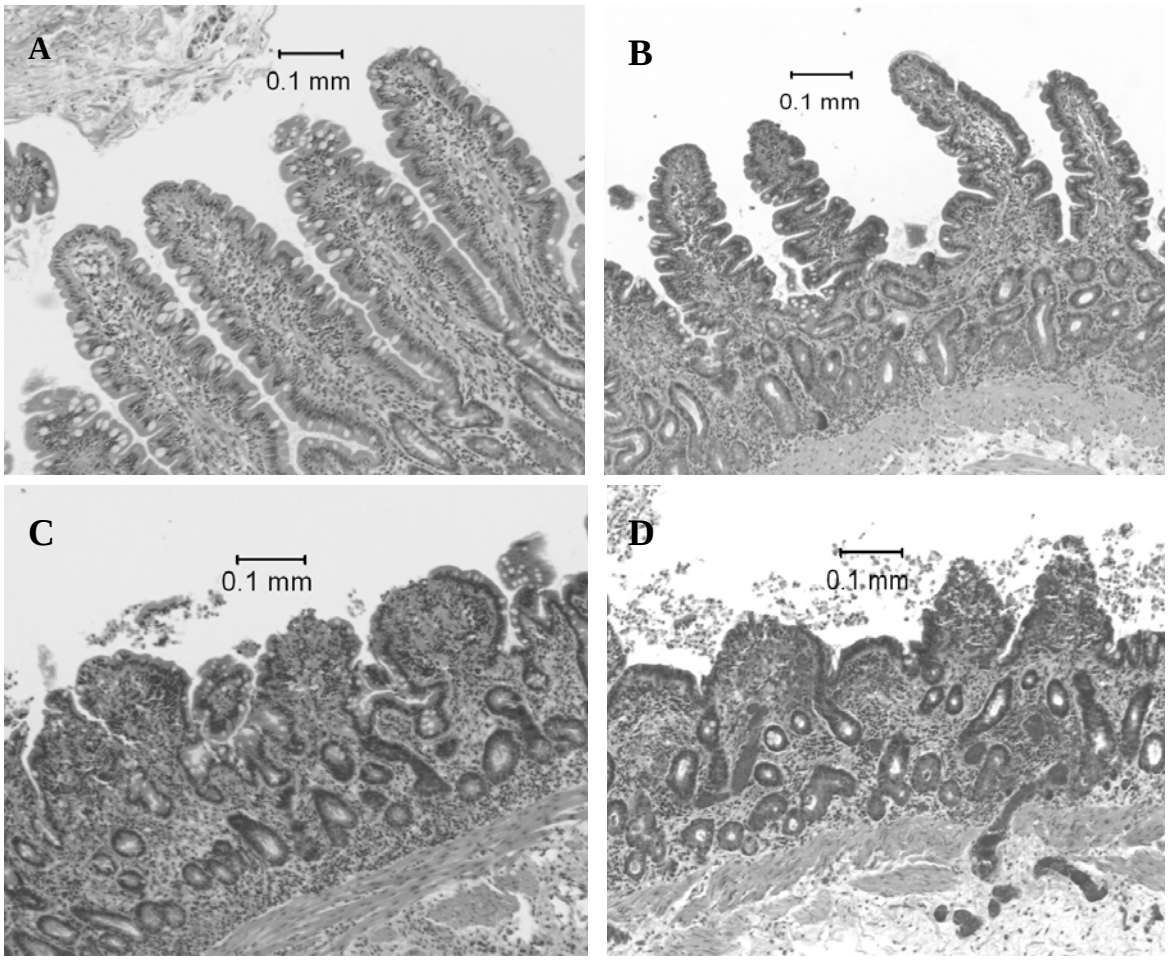
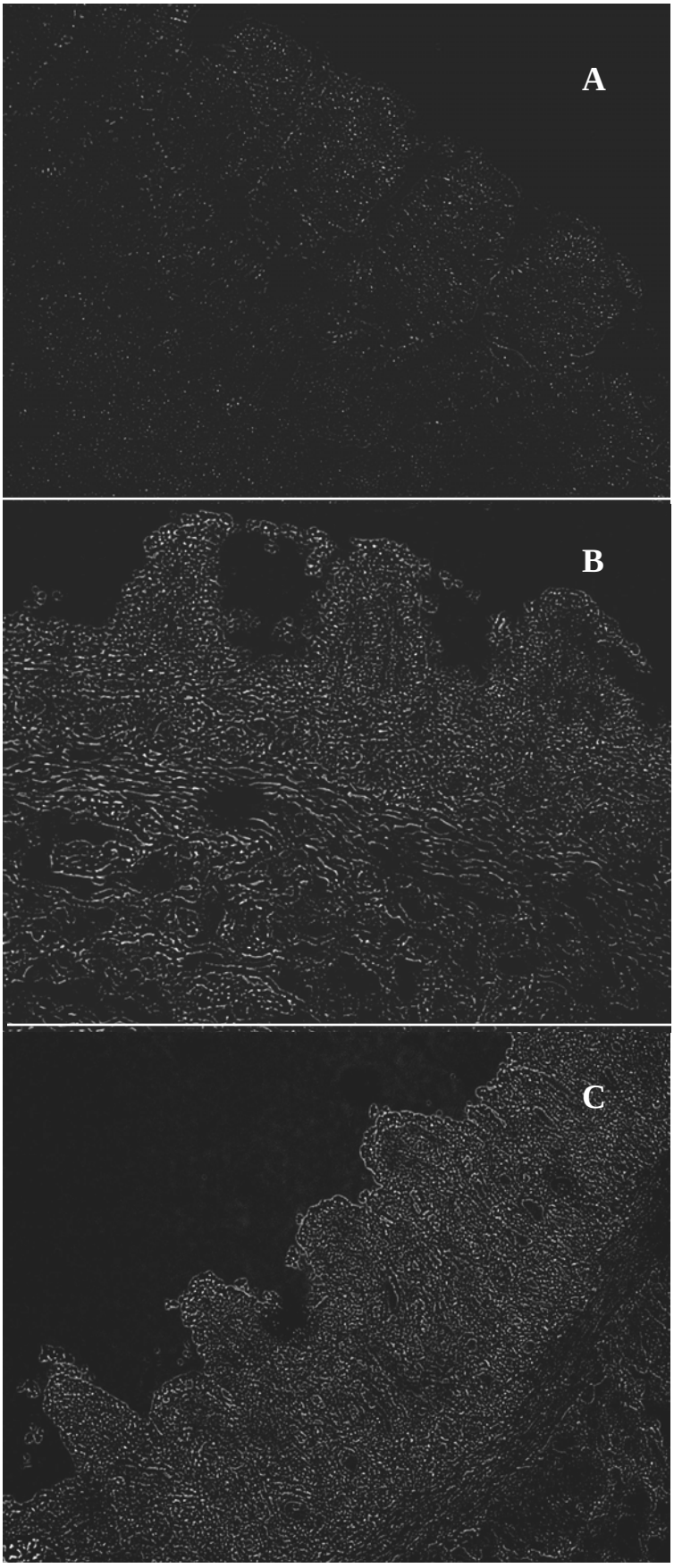
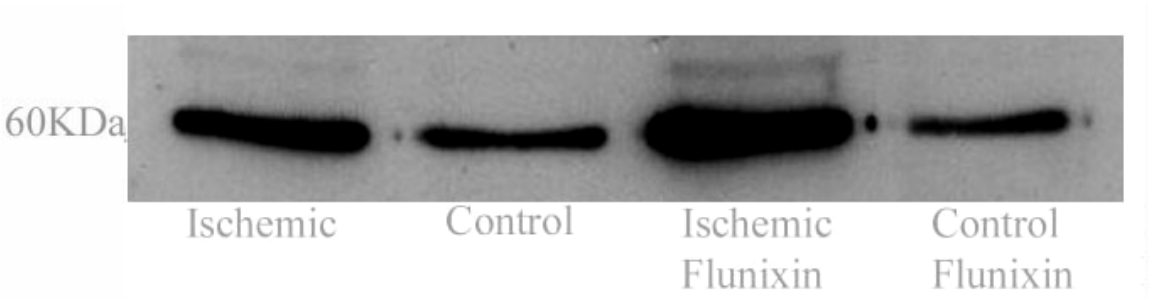


Figure 3: Histological sections of jejunal mucosa.
A: Normal (control) jejunum before placement on the Ussing chamber.
B: Control jejunum after 180 minutes on the Ussing chamber, there was no evidence of epithelial denudation.
C: Ischemic jejunum immediately after 120 minutes of ischemia. Significant epithelial denudation occurred.
D: Ischemic jejunum after 180 minutes on the Ussing chamber, the epithelial denudation remained and was not significantly different to immediately after ischemia.

Figure 4: Histological sections of jejunal mucosa exposed to fluorescent-labeled lipopolysaccharide for 120 minutes on the Ussing chamber.
A: Control jejunum. The epifluorescence is mostly in the villi, near the tips.
B: Ischemic jejunum. There is more epifluorescence than in control tissue. The epifluorescence appears in the lamina propria as well as the villi.
C: Ischemic jejunum pre-treated with flunixin for 45 minutes prior to exposure. There is more epifluorescence than in control tissue. The epifluorescence appears in the lamina propria as well as the villi.


A
B
C

5a:



5b:

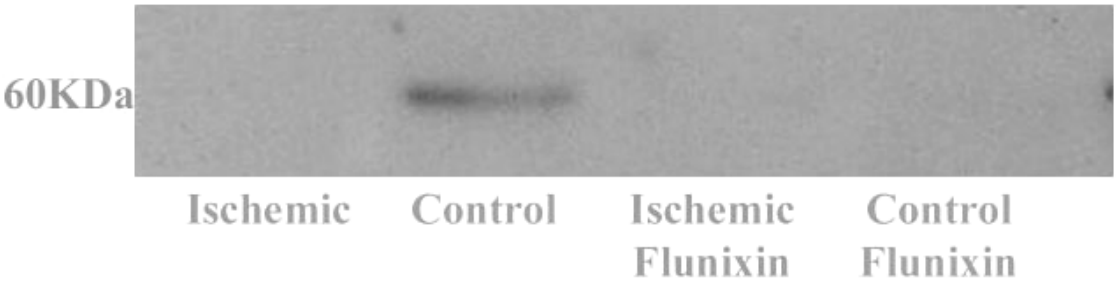


Figure 5: Ischemic and control mucosal samples examined by Western blot analysis for lipopolysaccharide binding protein (LBP).
5a: Tissues not exposed to LPS: The 60 kDa protein was detected in both control and ischemic mucosa that was not exposed to LPS. Densitometry showed significant upregulation of LBP in response to ischemia. There was no effect of flunixin treatment on the amount of LBP.
5b: Tissues exposed to LPS: Minimal LBP was detected, this was probably due to the binding of LBP to the lipid soluble LPS and subsequently being removed during protein extraction.

Chapter 5:

Effects of ischemia and the selective cyclooxygenase inhibitors NS-398 and SC-560 on recovery of jejunum in COX-2$^{-/-}$ and wild-type mice

Grant support: USDA-NRI 0302369 (Blikslager)

Abstract

Procedure: In order to further investigate the role of COX-2 in intestinal recovery, COX-$2^{-/-}$ and wild-type (B6) mice were subjected to 45 minutes of jejunal ischemia, after which transepithelial electrical resistance and mucosal-to-serosal fluxes of mannitol were monitored *in vitro* to measure recovery of barrier function. Control and ischemic jejunal tissues were bathed in Ringers containing COX-selective inhibitors, and prostanoid levels measured in serosal solutions after 120 minutes recovery. Denuded villous surface area was calculated after ischemia and 120 minutes recovery as an index of restitution. Western blot analyses were performed for COX isoforms.

Results: There was no significant effect of treatment on transepithelial resistance. After 120 minutes of recovery, ischemic-injured tissue was significantly less permeable to mannitol than control tissue in wild-type mice. Treatment with the selective COX-1 inhibitor SC-560 significantly increased permeability to mannitol in recovering tissues compared to control tissues, associated with reductions in prostanoid levels, whereas mannitol permeability was not significantly different from control tissues following treatment with the selective COX-2 inhibitor NS-398. There was no significant effect of any of the treatments on epithelial restitution, suggesting changes in mannitol permeability were paracellular in nature. In COX-$2^{-/-}$ mice, mannitol permeability in ischemic-injured tissue recovered to control levels by 120 minutes, but the significant reduction in mannitol permeability noted in wild-type tissues was not seen. Ischemic tissue from COX-$2^{-/-}$ mice was significantly more permeable than that from wild-type. Similar to wild-type tissues, SC-560 significantly increased permeability to mannitol, associated with significant reductions in prostanoid levels, but no change in epithelial

restitution. Paradoxically, NS-398 also caused increased permeability in ischemic tissue from COX-2$^{-/-}$ mice whilst not affecting prostanoid levels.

COX-1 protein was present in wild-type and COX-2$^{-/-}$ mice, and was not significantly upregulated by ischemia. COX-2 was also detected in control and ischemic samples from wild-type mice, but was absent in COX-2$^{-/-}$ mice.

Conclusions: Permeability of ischemic-injured jejunum recovered within 120-minutes in wild-type and COX-2$^{-/-}$ mice, but an overshoot reduction in mannitol permeability was only seen in wild-type mice. The latter was also blocked by NS-398 in wild-type tissues, suggesting this was a COX-2-driven process. Alternatively, inhibition of COX-1 prevented recovery of barrier function in both sets of mice, suggesting a more important role in acute recovery of ischemic-injured jejunum for COX-1.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are frequently used in clinical practice as analgesics and for their anti-inflammatory effects. However, it has been documented that NSAIDs cause direct injury to the gastrointestinal tract.[1-3] This group of drugs has also been shown to inhibit healing of injured tissue in the gastrointestinal tract,[4,5] suggesting caution when treating animals with gastrointestinal disease. NSAIDs inhibit prostaglandin production by acting on the cyclooxygenase (COX) enzyme, which converts arachidonic acid to prostaglandins. The COX enzyme has several isoforms, including the constitutive COX-1 and the inducible enzyme COX-2 (COX-3 is a variant of the COX-1 gene and is found in the brain, spinal cord and heart).[6-8] COX-2 is upregulated by a variety of inflammatory stimuli.[6] NSAIDs have been developed that selectively inhibit COX-2 with a view to decreasing adverse gastrointestinal effects. However, recent findings have complicated the postulated roles for COX-1 and COX-2 in the gastrointestinal tract. For example, COX-2 is upregulated after gastric injury, and this upregulation has been correlated with an increase in gastric epithelial cell proliferation.[9] Furthermore, selective COX-2 inhibitors delayed healing of acute-stage ulcers in mice[10] and induced colitis in rats.[11] In addition, prostaglandins produced by COX-2 at a site of mucosal injury may modulate inflammation. For instance, concentrations of COX-2 derived prostaglandin D_2 were increased in a model of colitis, and inhibition of COX-2 inhibited PGD_2 production and resulted in a doubling of granulocyte infiltration.[12] Cyclooxygense-2 may also contribute to maintenance of gastrointestinal barrier function. A recent study determined that there were similar concentrations of COX-1 and COX-2

mRNA in the normal stomach of young rats. This implies a physiologic role for COX-2 in the rat stomach.[13] Interestingly, inhibition of both COX-1 and COX-2 was required for the development of gastric erosions after NSAID administration in rats.[14]

Gene knockout models have been used in an attempt to clarify the roles of the COX isoforms, but these studies may not always provide accurate information because animals adapt to the loss of one of the COX genes.[15] For example, mice in which the COX-1 gene was knocked out did not develop spontaneous gastric ulceration,[16] possibly because up-regulation of COX-2 takes over important housekeeping functions. Alternatively, COX-2 knockout mice have developmental renal abnormalities, highlighting the importance of constitutive COX-2 expression in the kidneys. However, these COX-2 knockout mice do not have an altered inflammatory response in most standard tests,[17] suggesting they can be used in experimental models of inflammation.

We have shown that prostaglandins are critical for recovery of ischemic-injured intestine.[18-21] Studies have defined a role for both COX-1 and-2 in intestinal recovery from injury [9-11,22] leading us to postulate that the selective COX-2 inhibitors will retard recovery of ischemic jejunum by impairing intestinal barrier function. The aim of this study was to further elucidate the role of the COX-1 and –2 enzymes in intestinal recovery from ischemia. COX-$2^{-/-}$ and wild-type (B6) mice were subjected to jejunal ischemia and the effects of the selective COX inhibitors SC-560 (selective for COX-1) and NS-398 (selective for COX-2) on *in vitro* recovery from ischemia were examined.

Materials and methods

Six female COX-$2^{-/-}$ mice[a] and 6 corresponding wild-type mice were used in the study. The North Carolina State University Animal Care and Use committee approved the experimental protocol. Each group of mice was further subdivided into controls (3 mice) and those undergoing 45 minutes of jejunal ischemia (3 mice).

Anesthesia was achieved using an induction chamber. The inhalant anesthetic used was isoflurane vaporized in 100% oxygen. Following induction, the control mice were then euthanized and the small intestine removed; the mice undergoing ischemia were transferred onto a coaxial Bain circuit with mask to continue anesthesia. A midline celiotomy was performed and the jejunum located. The small intestinal blood supply was occluded by ligating the cranial mesenteric artery. Blood supply was occluded for 45 minutes prior to euthanasia by cervical dislocation. Following euthanasia, the jejunum was harvested for *in vitro* studies.

Transepithelial electrical resistance

Intestinal loops were incised along the mesenteric surface in oxygenated (95% O_2/5% CO_2) murine Ringer's solution, as prepared in previous studies.[23] The tissue was then mounted in 0.13cm^2 aperture Ussing chambers as described in previous studies.[19-22] Tissues were bathed on the serosal and mucosal sides with 8 ml murine Ringers solution. The serosal solution contained 10 mmol/l glucose and was osmotically balanced on the mucosal side with 10 mmol/l mannitol. Control and ischemic jejunal tissues were bathed in Ringers on the mucosal and serosal sides, which contained 5 x 10^{-6}M NS-398; SC-560;

NS-398 & SC-560 or no treatment. Bathing solutions were oxygenated (95% O_2/5% CO_2) and circulated in water-jacketed reservoirs at 37^{o}C. After a 15-minute equilibration period, the spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes using a voltage clamp that corrected for fluid resistance. Resistance ($\Omega.cm^2$) was calculated from the spontaneous PD and short-circuit current (I_{sc}). If the spontaneous PD was between –1.0 and 1.0mV, tissues were current clamped at $\pm$ 25μA for 5 seconds and the PD recorded. Short-circuit current and PD were recorded every 15 minutes for 60 minutes. The data was entered into spreadsheets that calculate transepithelial electrical resistance (TER) from I_{sc} and PD using Ohms law. In order to evaluate recovery in each of the treatment groups, data was calculated as percentage change from baseline (the value recorded after a 15-minute equilibration on the Ussing chamber) before undergoing statistical analysis.

Mannitol flux

Following the TER measurements, mucosal-to-serosal mannitol flux was performed by adding ^{3}H-mannitol (10 μCi/ml) to the mucosal bathing solutions and monitoring its appearance in the serosal bathing solutions. Samples were collected in scintillation vials at 0 and 60 minutes after addition of radiolabeled mannitol and assessed for β emission (counts/minute). Mucosal fluxes of ^{3}H were calculated as an assessment of mucosal permeability to mannitol.

Histopathology

Tissues were taken at euthanasia and after the Ussing chamber experiments. Five-micrometer cross-sections taken at 300μm intervals were stained with hematoxylin and eosin. For each tissue, 2 investigators independently evaluated 3 sections. Three well oriented villi and crypts were identified in each section. The length of the crypt and villus and the width at the midpoint of the villus were obtained using a micrometer in the eyepiece of a light microscope. In addition, the height of the epithelial covered portion of each villus was measured. Data from the 2 evaluators was pooled prior to any data manipulations. The surface area of the villus was calculated using the formula for surface area of a cylinder. The formula was modified by subtracting the area of the base of the villus and multiplying by a factor accounting for the variable position at which each villus was cross-sectioned. [21] The denuded villous surface area was calculated from the total surface area of the villus and surface area of the villus covered by epithelium.

Gel electrophoresis and western blotting

Mucosal tissue from control and ischemic jejunum was snap frozen in liquid nitrogen. Samples were stored at -70^0C prior to preparation for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Tissue was thawed to 4^oC and each sample was added to 1 ml of chilled RIPA buffer (0.15 M NaCl, 50 mM sodium Tris [pH 7.2], 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS), including protease inhibitors (PMSF and Apoprotinin). The mixture was homogenized on ice, centrifuged at 4^oC (10,000 G for 10 minutes), and the supernatant saved. Protein analysis of extract aliquots was performed.[b] Protein extracts (amounts equalized by protein concentration) were

mixed with an equal volume of 2X SDS-PAGE sample buffer and boiled for 4 minutes at 100^{o}C. Lysates were loaded on a 10% SDS-polyacrylamide gel and electrophoresis carried out according to standard protocols. Proteins were transferred to a nitrocellulose membrane[c] using an electroblotting mini-transfer apparatus according to the manufacturers protocol. Membranes were blocked overnight at 4^{o}C in Tris buffered saline (TBS) and 5% dry powdered milk. Membranes were washed twice with TBS containing 0.05% Tween (TBS-T) and incubated for 2 hours in primary antibody (affinity purified goat polyclonal antibodies for COX-1 or COX-2).[d] After washing three times for 5 minutes each with TBS-T, the membranes were incubated for one hour with horseradish peroxidase conjugated secondary antibody. After washing twice for 5 minutes each with TBS-T, and once with TBS for 15 minutes, the membranes were developed for visualization of protein by addition of enhanced chemiluminescence agent.[e] Densitometry was performed on scanned images using specialized software.[f]

Prostanoid levels

Samples of serosal bathing solutions were taken from each chamber at the end of the experiment (120 minutes). Samples were snap frozen in liquid nitrogen, stored at –70^{0}C and analyzed using ELISA kits [g] for prostaglandin E_2 (PGE_2), 6-keto prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) (a stable metabolite of PGI_2) and thromboxane B_2 (TXB_2) (a stable metabolite of TBA_2).

Statistical analysis

The transepithelial resistance data (% change from baseline) was analyzed using a two-way analysis of variance (ANOVA) for the effects of treatment and time. Mannitol flux values were tested using a 1-way ANOVA for the effects of treatment. Histomorphometric data was analyzed with a 1-way ANOVA for effect of treatment on histological indices of repair. Prostanoid and western blot densitometry measurements were analyzed in each group with a one-way ANOVA for the effect of treatment. Post hoc tests used were Tukey's test for parametric data, and Dunn's test when data was non-parametric following ANOVA on ranks. For all tests a significance level of $p < 0.05$ was chosen.

Results

Transepithelial electrical resistance:

- ***Wild-type mice (figure 1):*** The TER was significantly lower in ischemic mucosa when compared to control tissue ($p = 0.001$). SC-560, NS-398 and NS-398 combined with SC-560 (NS-398&SC-560) did not significantly alter the transepithelial resistance from that of untreated ischemic tissue over the period it was measured. There was no significant difference in transepithelial resistance between treatments for control tissue.
- ***COX-2$^{-/-}$ mice (figure 2):*** Ischemic mucosa had a significantly lower TER than control tissue ($p < 0.001$). There was no significant difference in TER between treatments for ischemic tissue over the period measured. There was also no significant difference between treatments for control tissue.

Mannitol flux:

- ***Wild-type mice (figure 3):*** Ischemic untreated tissue was significantly less permeable to mannitol than control tissue (p=0.009). SC-560 and NS-398&SC-560 significantly increased permeability to mannitol when compared to untreated ischemic tissue (p = 0.014 and p = 0.007 respectively). NS-398 alone did not significantly increase permeability to mannitol; the permeability of NS-398-treated ischemic tissue was not significantly different from untreated tissue or from the other treatments.
- ***COX-2$^{-/-}$ mice (figure 4):*** Unlike wild-type mice, untreated ischemic tissue was not significantly more or less permeable to mannitol than control tissue. It was significantly more permeable than in wild-type mice (p=0.027). SC-560 (p = 0.03), NS-398 (p = 0.032) and NS-398&SC-560 (p <0.001) significantly elevated permeability to mannitol when compared to untreated ischemic tissue.

Histopathology: (table 1)

- ***Wild-type mice (figure 5):*** There was significant epithelial denudation after ischemia (p = 0.007). By 120 minutes, partial restitution had occurred but denudation was not significantly different to the 0-minute ischemic tissue or to control tissue. There was no significant effect of treatment on epithelial denudation. The control tissue showed a small amount of denudation after the period on the Ussing chambers but this was not significant.

- ***COX-2$^{-/-}$ mice (figure 6):*** There was significant epithelial denudation after ischemia ($p < 0.001$). By 120 minutes, restitution had occurred so that the extent of denudation was significantly less than ischemic tissue at 0-minutes ($p = 0.03$) and was not significantly different to control tissue. The control tissue showed no evidence of denudation by the end of the experiment. There was no significant effect of treatment on epithelial denudation.

Prostanoid levels:

- ***Wild-type Mice:*** Levels of PGE_2, TXB_2 and 6-keto-$PGF_{1\alpha}$ were not significantly elevated in tissue fluid following ischemia. In ischemic tissue, only treatment with NS-398&SC-560 significantly decreased PGE_2 levels ($p = 0.04$). However in control tissue, all treatments significantly decreased PGE_2 levels (table 2). TXB_2 levels (table 3) were significantly decreased in ischemic tissue by NS-398&SC-560 ($p<0.001$) but SC-560 and NS-398 alone did not significantly decrease levels. In fact, treatment with SC-560 alone significantly elevated TXB_2 levels when compared with untreated tissue ($p<0.001$). Chamber treatments did not significantly alter TXB_2 levels in control tissue. 6-keto-$PGF_{1\alpha}$ levels were significantly decreased by all treatments in control tissue ($p < 0.001$) but not in ischemic tissue (table 4).

- ***COX-2$^{-/-}$ mice:*** As with wild-type mice, levels of PGE_2, TXB_2 and 6-keto-$PGF_{1\alpha}$ were not significantly elevated in chamber fluid following ischemia. PGE_2 levels in ischemic tissue were significantly decreased by NS-398&SC-560 ($p < 0.001$) and by SC-560 ($p = 0.048$) but NS-398 had no effect (Table 2). There was no

effect of treatment on PGE_2 levels in control tissue. TXB_2 levels were not significantly decreased by SC-560 alone or by NS-398&SC-560 (Table 3). Treatment with NS-398 paradoxically resulted in a significant elevation of TXB_2 levels in ischemic tissue when compared to untreated tissue ($p = 0.006$). 6-keto-$PGF_{1\alpha}$ levels in ischemic tissue were significantly decreased by SC-560 alone ($p = 0.034$) and NS-398&SC-560 ($p = 0.03$) (Table 4) whereas NS-398 alone had no effect. Treatments did not significantly alter control tissue levels of 6-keto-$PGF_{1\alpha}$.

TXB_2 levels were significantly lower in COX-$2^{-/-}$ mice than in wild-type mice ($p<0.001$) except for NS-398 treated ischemic tissue. 6-keto-$PGF_{1\alpha}$ and PGE_2 levels were not significantly different in COX-$2^{-/-}$ mice when compared with wild-type mice.

Western blot analysis:

COX-1 was present in wild-type and COX-$2^{-/-}$ mice and was not significantly elevated by ischemia (figure 7). COX-2 was present in control and ischemic samples from wild-type mice. There was no detectable difference in the levels of COX-2 protein between ischemic and control tissue on densitometry. There was no COX-2 protein detected in the COX-$2^{-/-}$ mice (figure 8).

Discussion

In both wild-type and COX-$2^{-/-}$ mice, no treatments significantly altered transepithelial electrical resistance when compared with untreated ischemic tissue. TER is a sensitive index of mucosal barrier function calculated principally from the electrical movement of

Na and Cl ions.[19] As in studies of other species,[18-22] ischemia resulted in low values of measured TER indicating a loss of intestinal barrier function. Recovery of TER is dependent on prostaglandin-induced closure of the paracellular space.[18-20] It appears that the inhibition of prostaglandin production by the selective COX-1 inhibitor SC-560, the selective COX-2 inhibitor NS-398 or a combination of the two was not enough to affect TER during the 60 minutes it was measured.

Flux measurements were taken in the second 60-minute time period to assess mucosal permeability to mannitol, which is much larger than a single Na or Cl ion. Mannitol flux measurements seem to be the most direct indicator of increased intestinal permeability to small molecules. The clinical relevance of increased intestinal permeability may be indiscriminate absorption of molecules such as endotoxin. By 120 minutes, permeability to mannitol was decreased in ischemic intestine from wild-type mice when compared to controls. We have seen this overshoot reduction in mannitol permeability before, in post-ischemic equine jejunum and this may be a protective mechanism.[24,25] The phenomenon was not seen in COX-$2^{-/-}$ mice or in NS-398 treated wild-type mice. In fact, ischemic tissue from COX-$2^{-/-}$ mice was significantly more permeable to mannitol than that from wild-type mice, therefore it appears that the COX-2 enzyme plays a role in recovery of barrier function after ischemia.

Both SC-560 alone and in combination with NS-398 significantly increased the permeability of ischemic mucosa in wild-type mice. This may indicate that the increase in permeability was due principally to COX-1 inhibition by SC-560. However there was a

trend towards increased permeability in NS-398 treated tissues, but the standard error was large in this group. Therefore we cannot rule out a contribution for both COX-1 and COX-2 produced prostanoids in restoring barrier function of ischemic intestine. In COX-$2^{-/-}$ mice, all treatments increased the mucosal permeability of ischemic intestine. This includes the COX-2 inhibitor NS-398. As these mice lack the COX-2 enzyme, NS-398 could be acting on another COX isoform or could have an effect through a different mechanism of action.

After the 120 minute period on the Ussing chambers, there was histologic evidence of partial restitution. There was no significant difference in the extent of denudation between treatment groups. Hence changes in mannitol permeability were likely due to changes in paracellular function. As NS-398 increased permeability in COX-$2^{-/-}$ mice, we hypothesized that this could be due to non-specific action on COX isoforms or due to a direct toxic effect. The former explanation may be ruled out as NS-398 had no significant effect on prostanoid levels. The adverse effects of non-steroidal anti-inflammatory drugs can also be due to direct topical mucosal damage.[26] There was no detectable histologic damage in the NS-398 treated group, but topical damage remains a plausible explanation for the increased permeability.

Prostanoid levels were not significantly elevated by ischemia but were inhibited to various extents by the different treatments. We are principally concerned with the effects of the treatments on prostanoid levels in the ischemic mucosa. In wild-type mice, both NS-398 and SC-560 alone appeared to decrease PGE_2 levels but this was only significant

for NS-398 and SC-560 combined due to large standard errors in the other groups. NS-398 appeared to decrease TXB_2 when compared with untreated ischemic tissue, but again this decrease was only significant for NS-398 and SC-560 combined. The levels of 6-keto $PGF_{1\alpha}$ (a stable metabolite of PGI_2) appeared to be decreased by all treatments in wild-type mice but these changes were not significant. In COX-$2^{-/-}$ mice, SC-560 significantly decreased PGE_2 and 6-keto $PGF_{1\alpha}$ but did not significantly decrease TXB_2 production despite a trend towards this finding. As PGE_2 and PGI_2 have been shown to be required for recovery of intestinal barrier function after ischemia,[21] a reduction in levels of these prostanoids should result in increased intestinal permeability. Although the prostanoid levels measured in this study were not always significantly decreased by the treatments that increased intestinal permeability to mannitol, there appears to be a correlation.

Western blot showed that COX-2 is present in normal mucosa from wild-type mice, this indicates that this enzyme is constitutively expressed in the jejunum of these mice and may contribute to the maintenance of the intestinal mucosal barrier. COX-2 was also found in normal jejunal mucosa from the horse [24] and as stated earlier COX-2 mRNA was found in normal rat stomach.[13] It appears that in these species, COX-2 has a physiologic role. The present study attempted to clarify the roles of COX-1 and COX-2 in restoration of barrier function and therefore recovery after ischemic insult. This study cannot rule out the requirement of both COX-1 and –2 elaborated prostanoids for intestinal recovery from ischemia. The protective overshoot response of decreased mucosal permeability seen in untreated wild-type mice after ischemia did not occur in

NS-398 treated wild-type mice or in COX-$2^{-/-}$ mice. However, it appears that inhibition of COX-2 alone is at least less damaging than inhibition of COX-1 because COX-2 inhibition did not significantly increase permeability when compared to controls. Therefore the authors continue to recommend the clinical use of a COX-2 selective inhibitor in post-ischemic patients over non-selective and selective COX-1 inhibition.

Footnotes:

[a] Jackson Laboratories, Bar Harbor ME. USA

[b] D_c protein assay, Bio-Rad, Hercules CA. USA

[c] Hybond ECL, Amersham Life Science, Birmingham, UK

[d] Santa Cruz Biotechnology, Inc., Santa Cruz, CA. USA

[e] Amersham, Princeton NJ. USA

[f] Zero Dscan. Scanlytics Inc. Fairfax, VA, USA

[g] R & D Systems Minneapolis MN. USA

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Table 1: Percentage epithelial denudation (Mean ± SE) in control and ischemic mucosa from wild-type (WT) and COX-2$^{-/-}$ mice before (0 min) and after (120 min) placement on the Ussing chambers. Ischemia caused significant epithelial denudation in both wild-type [a] and COX-2$^{-/-}$ [b] mice. After 120 minutes, partial restitution had occurred but denudation was only significantly less than 0 min ischemic tissue for COX-2$^{-/-}$ mice [c]. There was no significant effect of treatment on epithelial denudation.

Treatment	Tissue type	Time (min)	Mean ± SE Denudation WT	Mean ± SE Denudation COX-2$^{-/-}$
None	Control	0	0 ± 0	0 ± 0
None	Control	120	5.8 ± 4	0 ± 0
NS-398	Control	120	1.1 ± 0.6	0 ± 0
SC-560	Control	120	1.3 ± 1.3	2.1 ± 2.1
NS-398&SC-560	Control	120	5.9 ± 5.9	2.0 ± 1.2
None	Ischemic	0	25[a] ± 3.8	32.4[b] ± 7.1
None	Ischemic	120	10.8 ± 5.4	10.3[c] ± 4.5
NS-398	Ischemic	120	7.8 ± 0.2	10.9 ± 5.2
SC-560	Ischemic	120	5.7 ± 5.7	8.4 ± 7.0
NS-398&SC-560	Ischemic	120	4.7 ± 4.7	11 ± 1.7

Table 2: PGE_2 levels (pg/ml) in serosal tissue fluid at 120 minutes. Levels were not significantly elevated by ischemia. For wild-type mice (WT), only NS-398 and SC-560 combined (NS-398&SC-560) significantly decreased PGE_2 in ischemic tissue [a]. All treatments significantly decreased levels in control tissue [b]. In COX-$2^{-/-}$ mice, both SC-560 and NS-398&SC-560 significantly decreased PGE_2 levels in ischemic tissue [c] but no treatments affected control tissue.

Treatment	Tissue type	Mean ± SE WT	Mean ± SE COX-$2^{-/-}$
None	Control	1471 ± 176	959 ± 312
NS-398	Control	509[b] ± 61	565 ± 199.7
SC-560	Control	510[b] ± 87	301 ± 169
NS-398&SC-560	Control	443[b] ± 132	319.5 ± 173
None	Ischemic	845 ± 216	636 ± 55
NS-398	Ischemic	347 ± 87	654 ± 144
SC-560	Ischemic	286 ± 39.6	423[c] ± 81
NS-398&SC-560	Ischemic	159[a] ± 68	347[c] ± 137

Table 3: TXB_2 levels (pg/ml) in serosal tissue fluid at 120 minutes. Levels were not significantly elevated by ischemia. For wild-type mice (WT) both SC-560 and NS-398&SC-560 significantly decreased TXB_2 levels [a] in ischemic tissue fluid. In COX-$2^{-/-}$ mice, TXB_2 levels were not significantly decreased by any treatment but were increased significantly by NS-398 [b] in ischemic tissue. There was no effect of treatment on control tissue in either group.

Treatment	Tissue type	Mean ± SE WT	Mean ± SE COX-$2^{-/-}$
None	Control	48 ± 22	1.2 ± 0.19
NS-398	Control	14.7 ± 4.5	7.1 ± 0.04
SC-560	Control	69 ± 14.7	0 ± 0.02
NS-398&SC-560	Control	5.3 ± 1.09	2 ± 0.21
None	Ischemic	19 ± 4.6	6.3 ± 1.96
NS-398	Ischemic	8 ± 0.47	33.7[b] ± 5.93
SC-560	Ischemic	60.7[a] ± 3.8	3 ± 0.82
NS-398&SC-560	Ischemic	5[a] ± 2.1	5 ± 2.05

Table 4: 6-keto $PGF_{2\alpha}$ levels (pg/ml) in serosal tissue fluid at 120 minutes. Levels were not significantly elevated by ischemia. All treatments significantly decreased 6-keto $PGF_{2\alpha}$ levels in control tissue fluid of wild-type (WT) mice [a]. Treatments did not affect levels in ischemic tissue. For COX-$2^{-/-}$ mice, both SC-560 and NS-398&SC-560 significantly decreased 6-keto $PGF_{2\alpha}$ levels [b] in ischemic tissue but not in control tissue.

Treatment	Tissue type	Mean ± SE WT	Mean ± SE COX-$2^{-/-}$
None	Control	5404± 798	3097 ± 1240
NS-398	Control	1008[a] ± 127	1533 ± 149
SC-560	Control	570[a] ± 217	199 ± 73.9
NS-398&SC-560	Control	390[a] ± 137	805 ± 439
None	Ischemic	2458 ± 858	1515 ± 409
NS-398	Ischemic	778 ± 248	1045 ± 149
SC-560	Ischemic	248 ± 94	260[b] ± 83
NS-398&SC-560	Ischemic	114 ± 53	161[b] ± 70

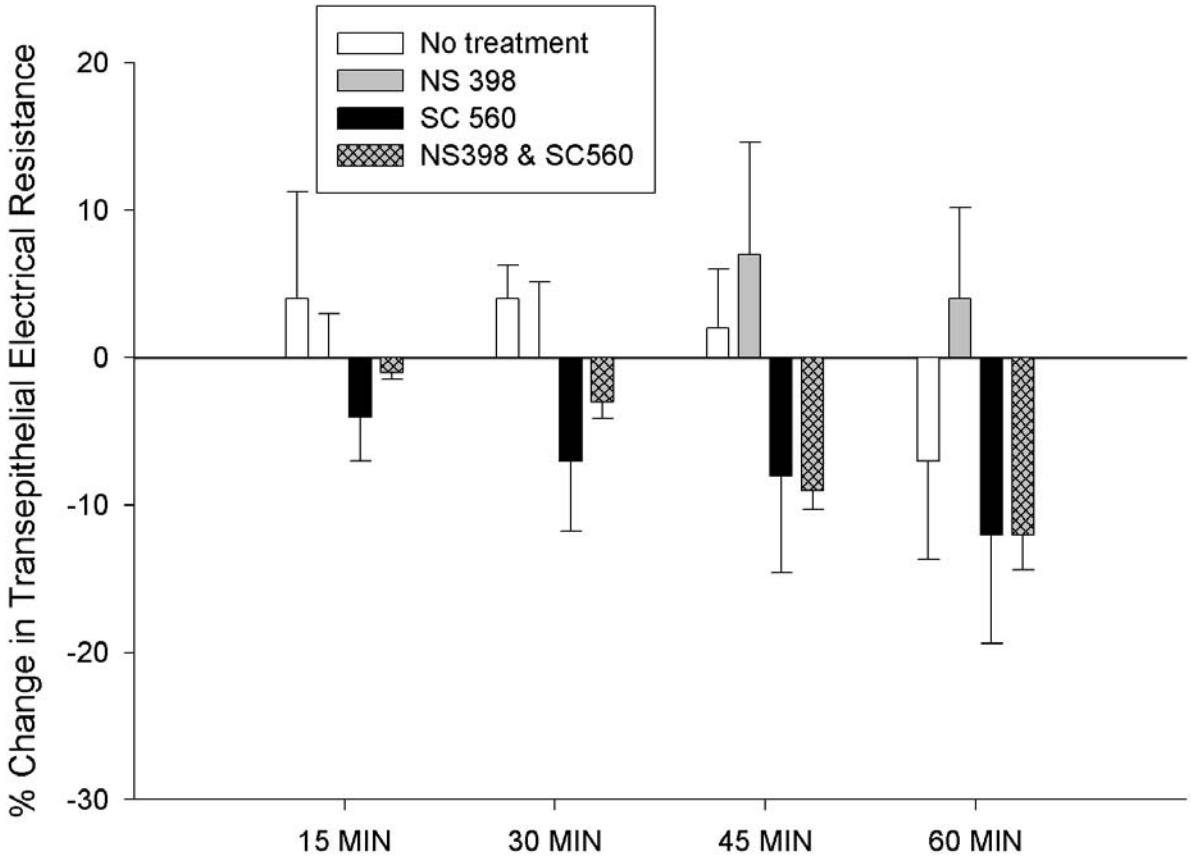


Figure 1: Percentage change in transepithelial electrical resistance of ischemic jejunal mucosa from wild-type mice. Mean values are shown with error bars. Mucosa was exposed to no treatment, the selective COX-1 inhibitor SC-560, the selective COX-2 inhibitor NS-398 or a combination of NS-398 & SC-560. There was no effect of any treatment on change in TER.

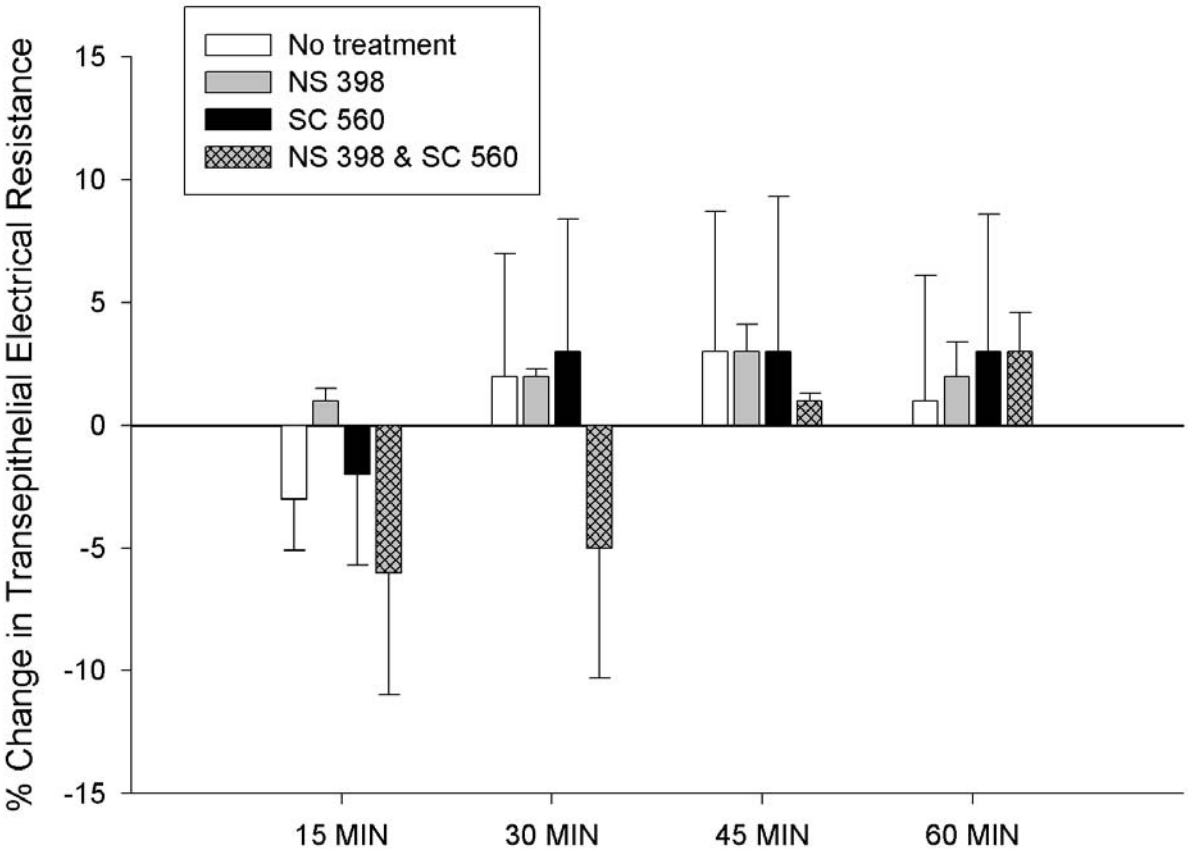


Figure 2: Percentage change in transepithelial electrical resistance of ischemic jejunal mucosa from COX-$2^{-/-}$ mice. Mean values are shown with error bars. Mucosa was exposed to no treatment, the selective COX-1 inhibitor SC-560, the selective COX-2 inhibitor NS-398 or a combination of NS-398 & SC-560. There was no effect of any treatment on change in TER.

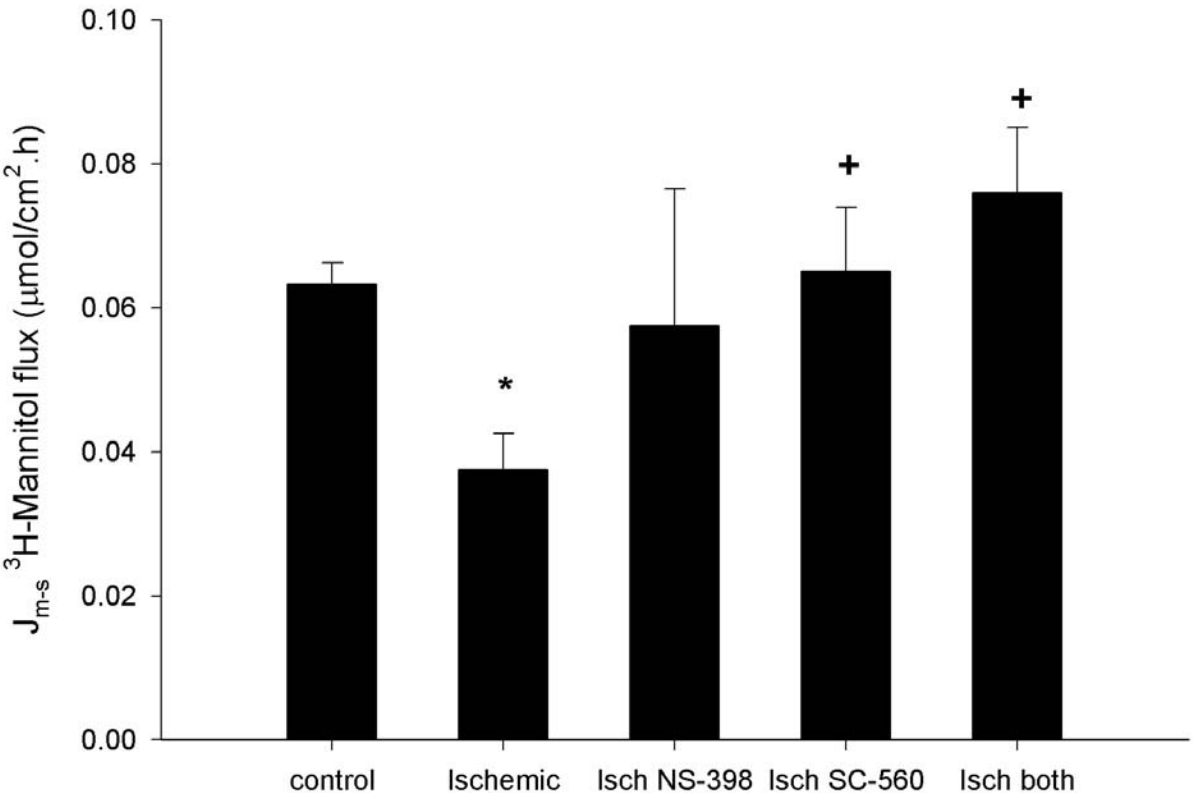


Figure 3: Mucosal to serosal ^{3}H-mannitol flux (J_{m-s}) across control and ischemic (Isch) jejunal mucosa from wild-type mice. Mean values are shown with error bars. Ischemic untreated tissue was significantly less permeable to mannitol than control tissue (*). SC-560 and NS-398&SC-560 (Isch both) significantly increased permeability of ischemic tissue to mannitol (+).

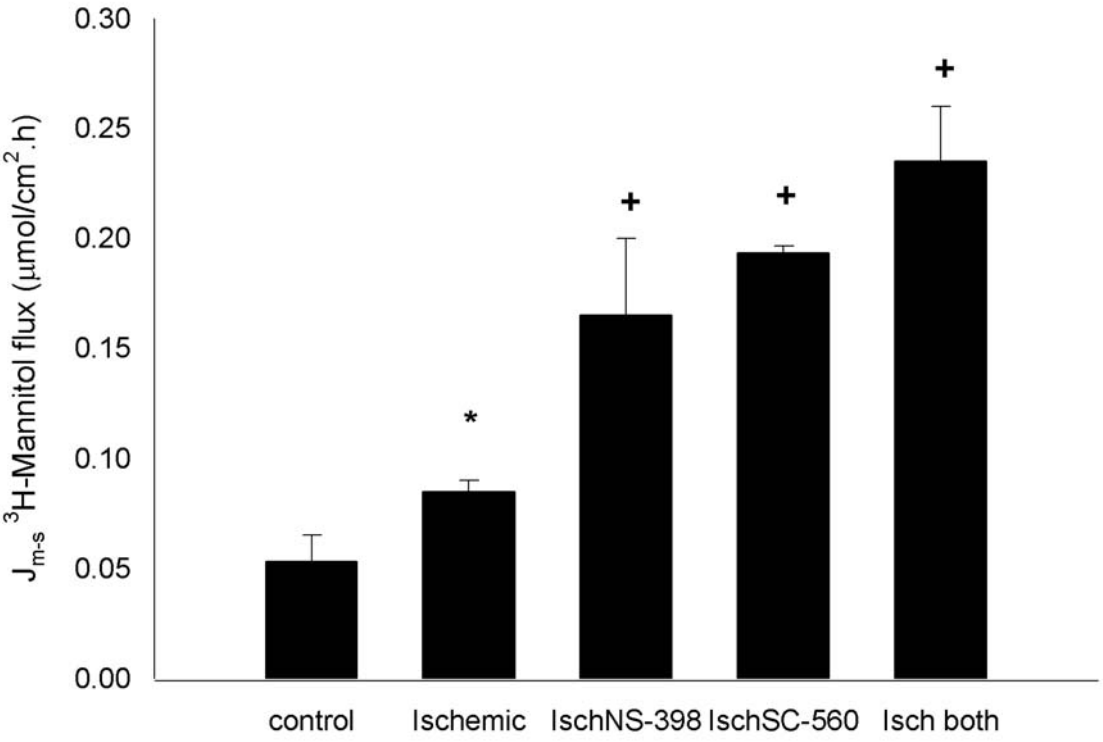


Figure 4: Mucosal to serosal ^{3}H-mannitol flux (J_{m-s}) across control and ischemic (Isch) jejunal mucosa from COX-2$^{-/-}$ mice. Mean values are shown with error bars. Permeability of untreated ischemic tissue was not significantly different to control tissue. Permeability of ischemic tissue was significantly increased compared to wild-type mice All treatments significantly increased permeability of ischemic tissue to mannitol (*).

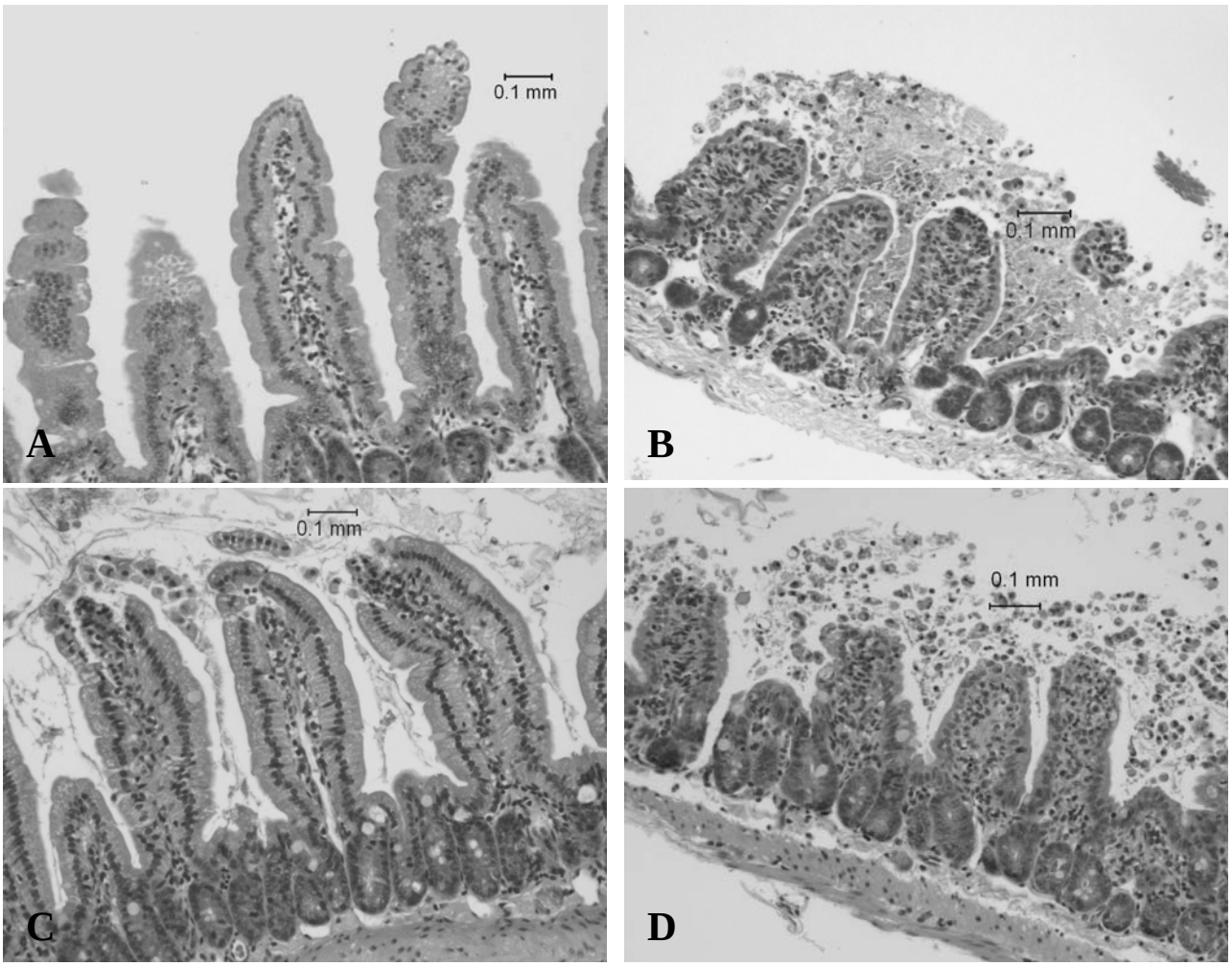


Figure 5: Histological sections of control and ischemic jejunal mucosa from wild-type mice.
A: Control mucosa before placement on the Ussing chamber. There was no evidence of epithelial denudation.
B: Control mucosa after 120 minutes on the Ussing chamber. There was a small amount of epithelial denudation but the amount was not significantly different to mucosa before placement on the Ussing chamber.
C: Ischemic mucosa immediately before placement on the Ussing chamber. There was significant epithelial denudation at the villous tips.
D: Ischemic mucosa after 120 minutes on the Ussing chamber. Partial restitution had occurred.

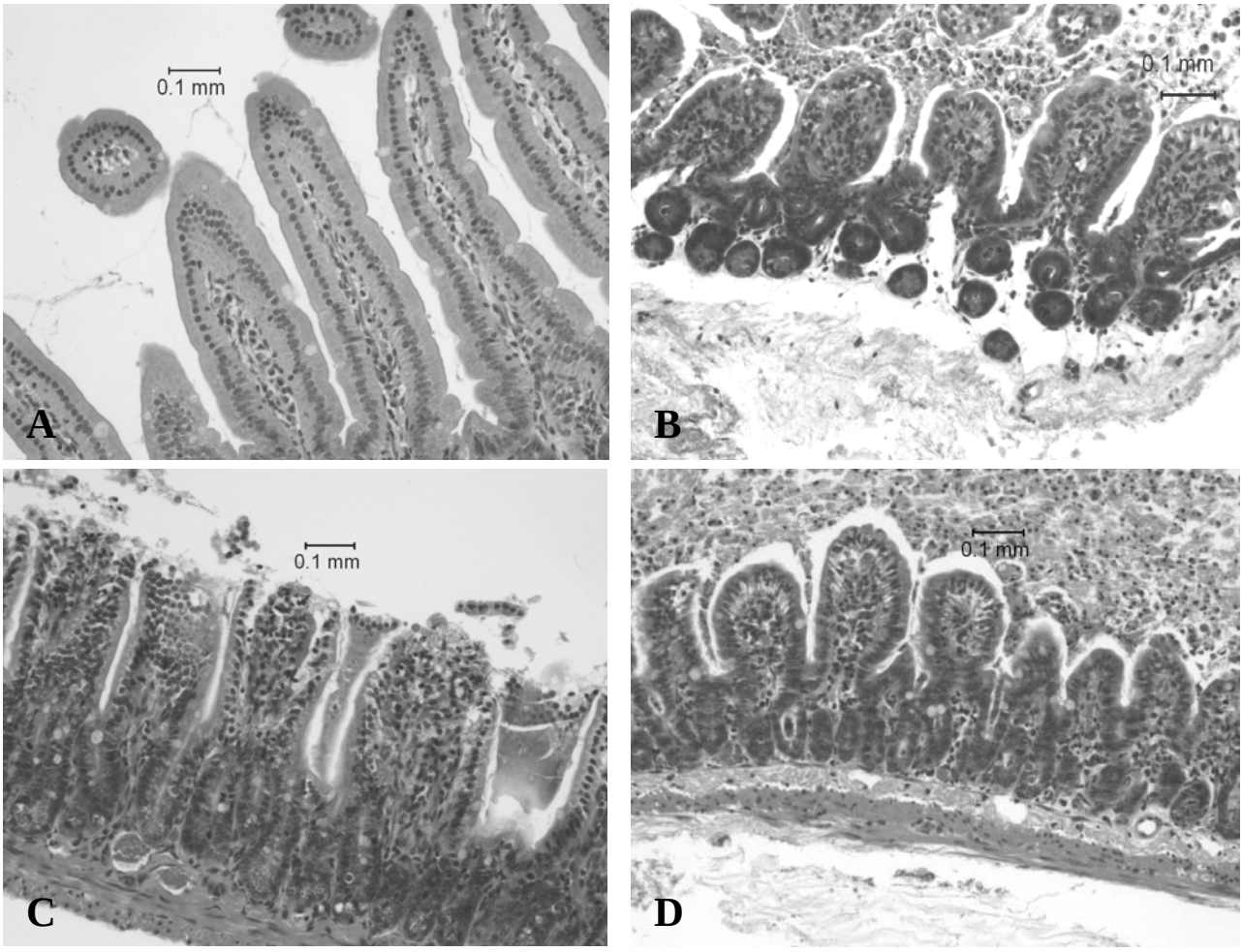


Figure 6: Histological sections of control and ischemic jejunal mucosa from COX-2$^{-/-}$ mice.
A: Control mucosa before placement on the Ussing chamber. There was no evidence of epithelial denudation.
B: Control mucosa after 120 minutes on the Ussing chamber. There was no evidence of epithelial denudation.
C: Ischemic mucosa immediately before placement on the Ussing chamber. There was significant epithelial denudation at the villous tips.
D: Ischemic mucosa after 120 minutes on the Ussing chamber. Significant restitution had occurred.

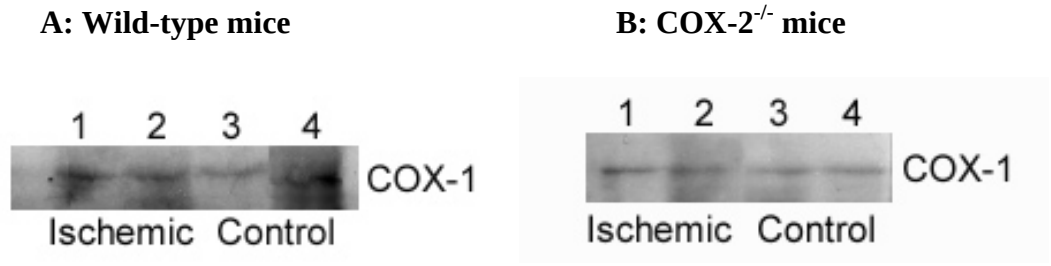


Figure 7: Western blot for the COX-1 protein in 2 control and 2 ischemic jejunal mucosal samples from wild-type (A) and COX-$2^{-/-}$ (B) mice. Individuals were numbered 1-4 in each group. COX-1 was present in control and ischemic tissue; densitometry showed that levels were not significantly elevated by ischemia.

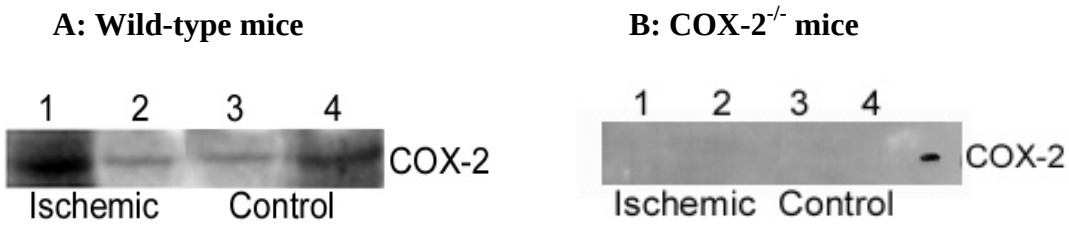


Figure 8: Western blot for the COX-2 protein in 2 control and 2 ischemic jejunal mucosal samples from wild-type (A) and COX-$2^{-/-}$ (B) mice. Individuals were numbered 1-4 in each group. COX-2 was present in control and ischemic tissue from wild-type mice; densitometry showed that levels were not significantly elevated by ischemia. As expected, COX-2 was not present in COX-$2^{-/-}$ mice.

FINAL CONCLUSIONS AND FUTURE STUDIES PLANNED

Final conclusions

These studies have indicated that prostanoid production from both COX-1 and COX-2 is required for adequate recovery of jejunal barrier function from ischemia in the horse and mouse. It appears that inhibition of both COX isoforms has the most damaging effect on mucosal barrier function, evidenced by increased permeability to the small molecule mannitol. The mouse studies have shown that selective inhibition of COX-1 also has this effect, though this has not been proven in the horse. Therefore, despite affecting some aspects of mucosal recovery from ischemia, it appears that selective inhibition of COX-2 is least damaging to intestinal barrier function as it has not been definitively shown to increase mucosal permeability to mannitol.

It was postulated that increased mucosal permeability of post-ischemic intestine exposed to non-selective COX inhibitors would result in increased absorption of lipopolysaccharide and so exacerbate endotoxemia. This was not proven in this study; however there was a strong trend towards increased LPS absorption in flunixin-treated equine jejunum post-ischemia.

The most significant conclusion that can be drawn from this study is that the use of selective COX-2 inhibitors for the treatment of pain and endotoxemia in post-ischemic equine patients is preferable to the use of non-selective (and COX-1 selective) inhibitors until safer therapeutic options are identified.

Future directions

The selective COX-2 inhibitor, deracoxib must be evaluated in the horse using an 18h recovery model as described in chapter 2, as *in vitro* results do not always mirror *in vivo* findings. The analgesic properties of deracoxib must also be evaluated.

Mucosal permeability to LPS after ischemia and COX inhibition should be evaluated in an 18h recovery model following adequate epithelial restitution in order to fully assess the effects of flunixin. Then it can be definitively shown whether flunixin exacerbates paracellular absorption of LPS.

The use of mice lacking the COX-2 gene should be continued. Increasing animal numbers should reduce standard error levels and confirm findings.