ABSTRACT

CAMPBELL, NIGEL BRENT. THE ROLE OF THE ENZYME CYCLOOXYGENASE AND BILE IN THE DAMAGE AND REPAIR OF INTESTINAL EPITHELIUM. (Under the direction of Anthony Thomas Blikslager)

Colic describes poorly localized abdominal pain in horses and accounts for the majority of emergency calls received by equine veterinarians. The main drugs used to treat colic are the nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit the enzyme cyclooxygenase (COX), and prevent endotoxin-induced elaboration of prostaglandins. There are 2 isoforms of cyclooxygenase: COX-1, which constitutively produces prostaglandins and COX-2, which is induced by inflammation. **Study 1:** We hypothesized that the non-specific cyclooxygenase inhibitor flunixin would retard repair of ischemic intestinal injury by preventing production of reparative prostaglandins whereas the selective COX-2 inhibitor, etodolac, would permit repair as a result of continued COX-1 prostaglandin production. Equine jejunum was subjected to ischemia for 1 hour, and recovered for 4 hours in Ussing chambers. In ischemic tissue treated with flunixin, production of prostaglandins was inhibited, and there was no evidence of recovery based on measurements of transepithelial resistance (TER). Conversely, untreated ischemic tissues or tissues treated with etodolac had significant elevations in prostaglandins, and significant recovery of TER. These studies suggest that specific COX-2 inhibitors may provide an advantageous alternative to non-specific cyclooxygenase inhibitors in horses with colic. **Study 2:** A potential adverse effect of NSAIDS in horses is colitis. It was hypothesized that the non-selective COX inhibitor flunixin would retard repair of bile-injured colon by preventing production of reparative prostaglandins, whereas the selective COX-2 inhibitor, etodolac would not inhibit repair

as a result of continued COX-1 activity. Equine colon was exposed to 1.5mM deoxycholate for 30-minutes, after which they were recovered for 4 hours in Ussing chambers. Contrary to the proposed hypothesis, recovery of bile-injured colonic mucosa was not affected by flunixin or etodolac, despite significantly depressed prostanoid production. However, treatment of control tissue with flunixin led to increases in mucosal permeability, whereas treatment with etodolac had no significant effect. Therefore, although recovery from bile-induced colonic injury maybe independent of COX-elaborated prostanoids, treatment of control tissues with non-selective COX inhibitors may lead to marked increases in permeability. Alternatively, selective inhibition of COX-2 may reduce the incidence of adverse effects in horses requiring NSAID therapy. **Study 3:** It has been shown that rapid *in vitro* recovery of barrier function in porcine ischemic-injured ileal mucosa, is attributable principally to reductions in paracellular permeability. However, these experiments did not take into account the effects of normal luminal contents, such as bile salts, which, according to our preliminary studies, reach concentrations as high as 10^{-5}M in the porcine ileum. The objective of this study was to evaluate the role of deoxycholic acid in recovery of mucosal barrier function. Porcine ileum was subjected to 45-minutes of ischemia, after which mucosa was mounted in Ussing chambers, and exposed to varying concentrations of deoxycholic acid. The ischemic episode resulted in significant reductions in TER, which recovered control levels of TER within 2-hours, associated with significant reductions in mucosal-to-serosal ^{3}H-mannitol flux. However, treatment of ischemic-injured tissues with 10^{-5}M deoxycholic acid fully inhibited recovery of TER with significant increases in mucosal-to-serosal ^{3}H-mannitol flux, whereas 10^{-6}M deoxycholic acid had no effect. Histologic

evaluation at 2 hours revealed complete restitution regardless of treatment, indicating the breakdown in barrier function was due to changes in paracellular permeability. Similar effects were noted with application of 10^{-5}M taurodeoxycholic acid, and the effects of deoxycholic acid were reversed with application of the Ca^{2+} mobilizing agent thapsigargin. Deoxycholic acid at physiologic concentrations significantly impairs recovery of epithelial barrier function by an effect on paracellular pathways, and these effects appear to be Ca^{2+}-dependent.

THE ROLE OF THE ENZYME CYCLOOXYGENASE AND BILE IN THE DAMAGE AND REPAIR OF INTESTINAL EPITHELIUM

by
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BIOGRAPHY

For as long as I can remember, my life has always involved interaction with animals. From the earliest days of my childhood, my family has had dogs, usually between 4-6 in number and of different breeds, as well as keeping rabbits, guinea pigs, and hamsters at varying times.

I started work on a mixed dairy/pig farm when I was twelve, which continued every weekend, and during vacation until the age of 16 while still attending school. After this introduction to farming, I then began work on a larger dairy farm, which required getting up every morning at 4am to milk the cows. During this time in my life I became more involved in the health and welfare aspects of agriculture. I was responsible for medicating sick animals, helping with difficult calvings, and occasionally working at a local veterinary hospital. Also, I helped my mother with her herd of 30 Angora goats and sometimes assisted my sister with her horses. These activities instilled in me a strong desire to work with animals and I decided to pursue a career in veterinary medicine

My veterinary education began at the Royal Veterinary College (RVC) in London, England, and was one of the best experiences of my life. I initially had a deep interest in bovine medicine, but during the clinical training developed a stronger interest in equine medicine, specifically colic. As a result of this, I completed a clinical study measuring electrolyte levels in colic cases and examined the incidence of post-operative ileus. It was in my fifth and final year of undergraduate veterinary education that I decided to specialize in horses.

I graduated in July 1994 from the RVC and began a career in a mixed animal practice carrying out 50% equine and 50% food animal duties. My first job was a great

experience with a multitude of cases and good mentoring, however I desired additional experience in equine medicine. I then completed an internship in equine medicine and surgery at the Animal Health Trust in Newmarket, England, under the supervision of Dr. Sue Dyson. During the internship, I decided to pursue an equine medicine residency in the USA and was accepted into an equine internal medicine residency at North Carolina State University in July 1997.

ACKNOWLEDGEMENTS

When I started my residency in equine internal medicine, I had no intention of earning a PhD or staying in the USA. This all changed once I met Dr. Anthony Blikslager and conducted a research study in his laboratory during the summer of 1998. His seemingly limitless energy, enthusiasm and support were an inspiration to me. From this first project, my appetite for research was awakened and I decided to pursue a PhD in physiology and became Dr. Blikslager's first graduate student.

I would also like to acknowledge the support from the other members of my committee: Dr. Robert Argenzio, Dr. Mark Papich and Dr. Sarah Gardner, who all helped me develop into a better scientist and researcher.

My deepest gratitude and thanks go to Karen Young for her help and infinite patience, which enabled me to learn the techniques necessary for my research. Without her assistance I believe my research would never have been completed.

My enthusiasm for clinical veterinary medicine would not have been possible if it had not been for the mentoring of Mr. Colin Sitford and Ms. Vanessa Bulmer during my first job. They were available at anytime of the day and night to answer questions or to come and help me without one word of complaint, enabling me to learn and become a better veterinarian.

Finally, none of this would have been possible without the love and support of my mother, Audrey; my father, Alan, and my sister, Jo. Despite the 5 hour time difference and thousands of miles between us, they have always been there for me. The death of my father during my PhD was the worst time of my life. However, his words of support and encouragement will forever be with me. I will always miss him.

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INTRODUCTION

Colic is a general term for poorly localized abdominal pain in horses and accounts for the vast majority of out-of-hours emergency calls received by veterinarians in equine practice. The most commonly used drugs to treat colic in the horse are the nonsteroidal anti-inflammatory drugs (NSAIDs).

These drugs act by inhibiting the enzyme cyclooxygenase, which produces pro-inflammatory mediators, including prostaglandins (PGs), from arachidonic acid. As well as being involved in inflammation, PGs are also involved in the pain response to inflammation. One of the initial stimuli for the production of PGs by COX is absorption of endotoxin into the bloodstream of the horse caused by damage to the intestinal epithelial barrier. This damage to the epithelial wall can occur for a number of reasons, the most common ones being ischemic injury to or excessive gas distension of small or large intestine. In approximately 95% of these horses initial treatment with NSAIDs at the barn will be sufficient. However, 5% of horses will require more intensive therapy and possibly even surgical intervention, usually in a hospital environment.

As well as their inflammatory effects, PGs are also involved in normal functions of the body. For example, they are involved in maintenance of the intestinal epithelial barrier and functions of the renal system. It is now known that PGs are produced by at least two isoforms of COX. The basic hypothesis is that COX-1 is constitutively expressed and is involved in homeostatic functions, whereas COX-2 is induced by inflammatory mediators and is involved in inflammation. The role of a recently cloned third isoform, COX-3, remains to be determined. However, its expression principally in the central nervous system suggests it may be involved in homeostatic functions such as

thermoregulation.

As well as being excellent anti-inflammatory and analgesic compounds, NSAIDs can also have adverse effects, including gastrointestinal mucosal injury and renal disease in both humans and horses. This is thought to be due to inhibition of COX-1, preventing production of PGs involved in homeostasis. This has led to the introduction of selective inhibitors of COX-2, which has resulted in a dramatic decrease in cases of gastric ulceration in human medicine but with continued good anti-inflammatory and analgesic properties.

The purpose of this study was to evaluate the role of the cyclooxygenase isoforms on the healing of ischemic-injured small intestine and bile-injured colon in the horse. As an extension of these studies, the role of bile, a normal constituent of intestinal contents, in recovery of ischemic-injured porcine ileum was also assessed.

CHAPTER ONE

COLIC, NONSTEROIDAL ANTI-INFLAMMATORY DRUGS, THE ENZYME CYCLOOXYGENASE AND BILE

INTRODUCTION

Colic is a general term for poorly localized abdominal pain in horses. In human medicine, the term is frequently used to describe nonspecific abdominal discomfort shown by infants. Therefore, colic describes a syndrome including obstructive, inflammatory, and ischemic disease. According to a recent national survey conducted by the USDA[1], colic is the leading cause of death in the horse behind old age. Colic is also the number one cause for equine insurance claims in nationwide and accounts for the majority of emergency calls taken by veterinarians in equine practice.[2] The principal reason for death in horses, with colic is shock associated with the absorption of bacterial endotoxin from damaged intestine [3,4] which induces production of prostaglandins (PGs) via the enzyme cyclooxygenase (COX) from the substrate arachidonic acid, that exacerbate circulatory shock and abdominal pain. Therefore, equine clinicians administer inhibitors of cyclooxygenase (e.g. NSAIDs) such as flunixin meglumine to combat the effects of endotoxin.

Abdominal Pain

Abdominal pain in the horse can be demonstrated in a number of ways, many of which can be quite dramatic.[2] Horses react to pain by pawing the ground and looking back at their sides. They may curl their upper lip. They may get up and down frequently, as if trying to find a position that is comfortable for them. Often horses will sweat a lot. When the pain is more severe the horse may violently roll around or thrash on the ground. However, some horses with conditions known to be extremely painful (in most horses) may just be slightly depressed or altered in their demeanor. This shows that horses that experience abdominal pain exhibit a variety of signs, and although horses

exhibiting signs associated with severe pain tend to have conditions that require surgical intervention, this is not always the case and vice-versa. In the horse, unlike other species, the pain produced by various abdominal conditions can be caused or exacerbated by endotoxemia.

Abdominal pain can result from one or a combination of the following:[5]

1. Mechanical Obstruction
2. Gas or fluid distention
3. Increased tension on the mesentery
4. Ischemia
5. Inflammation of the bowel wall or peritoneum

The most common cause of mechanical obstruction is an impaction. Pain is produced as the smooth muscle in the wall of the affected intestine contract against the unyielding impaction. Distention of the intestine with fluid or gas can also be a cause of abdominal pain. This distention can be primary (e.g. gastric dilation and cecal tympany), or secondary in conditions that produce obstruction of the lumen of the intestine, strangulation or impaction of the bowel or abnormalities associated with intestinal inflammation (e.g. anterior enteritis). The increased pressure within the lumen of the intestine stretches the intestinal wall where it directly stimulates pain receptors. Excessive tension on the mesentery is a major cause of severe abdominal pain. Usually more apparent clinical signs (e.g. violent rolling) are seen, as this pain is thought to be far more intense, and normally occurs secondary to conditions causing pain by mechanical obstruction or fluid/gas distention, potentiating the pain response. Ischemia and inflammation also cause abdominal pain and are usually closely associated with each

other. In both cases a primary intestinal abnormality results in the local production of cytokines and proinflammatory substances that reduce the threshold to painful stimuli (e.g. mild intestinal distension) that under normal conditions would not produce a painful response or the degree of pain associated with such stimuli is enhanced.

Perception of Pain

To understand how this pain is perceived, knowledge of the mechanisms of pain perception is needed. In man and probably in most domestic species the great majority of the peripheral pathways from the viscera, travel in sympathetic nerves. The exceptions to this rule are: (1) the pelvic viscera whose pain pathways are via pelvic and pudendal nerves and then through the dorsal roots of sacral spinal nerves and (2) the respiratory tract and esophagus, which send their signals via the vagus nerve.[6] For abdominal pain the pathways begin with nociceptors in the intestine. When the intestine distends with fluid and/or gas, for example, the stretch of the bowel wall causes the nociceptors to begin the propagation of action potentials. These potentials travel via visceral afferent nerves, typically C-type fibers, to the dorsal horn of the spinal cord.[5] Further transmission to secondary afferent fibers in the dorsal horn is by substance-P mediated synapses. The signal then travels to the thalamus via spinal tracts.

In man the central pathways of these pain sensations from the viscera are of basically two types. Some impulses travel through the spinothalamic tract essentially via three main neurons, which probably enable the cerebral cortex to localize approximately the source of the pain.[3] The axons are arranged somatotropically alongside the axon carrying somatic pain. Other impulses reach the cerebral cortex via spinoreticular fibers. These probably carry a sensation of more severe pain from generalized visceral areas. In

the domestic animals the central pathways of pain remain obscure, but it is thought that the spinothalamic tract is the principal pain pathway that propagates impulses to the somatosensory cortex where the pain is perceived.[5,6] Descending inhibitory pathways, which affect the transmission of impulses in the dorsal horn of the spinal cord, can modulate the general pain pathway.

During inflammation numerous events occur resulting in nociception of peripheral stimuli. Production of PGs results in change of high threshold mechanoceptors into nociceptors. Changes also occur in the central nervous system especially the dorsal horn of the spinal cord where reflex and metabolic activity of neuronal cells is increased.[7] Together, this results in hyperalgesia. PGs alone do not produce pain, but rather sensitize afferent nociceptors to the effects of other pain producing substances such as bradykinin and histamine.[8] The hyperalgesic effects of bradykinnin are not only potentiated by PGs but may also involve induction of PGs. The major PGs involved in hyperalgesia are PGE_2 and PGI_2. The resultant hyperalgesia produced by these two mediators are different. PGE_2 results in a delayed onset but long lasting hyperalgesic state whereas PGI_2 effects are more rapid and quickly subside.[8,9] PGI_2 is a more potent hyperalgesic agent than PGE_2.[10]

In addition to direct hyperalgesic action of PGs they are also involved in the processing of nociceptive signals induced by other agents. Activation of excitatory amino acid receptors (EAA) such as N-methyl-D-aspartate receptors and metabotropic EAA receptors results in an influx of extracellular calcium (Ca^{2+}) and intracellular mobilization of Ca^{2+} respectively. This stimulates the release of arachidonic acid by phospholipase A_2.[11] Metabotropic EAA receptor activation also stimulates phospholipase C that acts on

cell membranes and results in an increase in arachidonic acid. This increase in arachidonic acid is preceded by transient increases in diacylglycerol and arachidonoyl-monoacylglycerol. This arachidonic acid is then metabolized by cyclooxygenase into PGs, which then act on nociceptors to further potentiate perception of painful stimuli.[12] The function of intestinal nociceptors may also be up regulated by several inflammatory mediators including tumor necrosis factor (TNF), interlukin-1β (IL-1β) and interlukin-8 (IL-8). PGs are also released in response to these mediators, for example IL-1β stimulates the production of proinflammatory PGs by COX.[4] Initially these mediators are produced by peripheral nerves and inflammatory cells e.g. macrophages and neutrophils.

Endotoxemia

Endotoxin or lipopolysaccharide (LPS) is a component of outer bacterial cell walls of gram-negative bacteria. LPS is liberated from gram-negative bacteria following rapid multiplication or bacteriolysis. Gastrointestinal disorders, particularly those causing mucosal damage to the gastrointestinal tract, e.g. strangulating obstruction, are the main cause of endotoxemia in the horse. Concentrations as high as 80μg of endotoxin per milliliter of cecal contents have been measured in clinically normal horses.[13] The normally functioning mucosal barrier allows only minute quantities of endotoxin into the portal circulation or peritoneal cavity. Binding of endotoxin by bile salts within the lumen of the bowel serves as an additional defense to systemic absorption of endotoxin.[14] Severe mucosal inflammation, enterocyte necrosis, and loss of epithelial barrier function may occur secondary to various gastrointestinal insults. Invasive enteric pathogens, including *Salmonella* and *Clostridial* species, are capable of causing direct mucosal damage. Strangulating and non-strangulating obstruction may result in intestinal ischemia,

reperfusion injury and mucosal damage.[15] Toxicosis, e.g. oak, can also lead to severe mucosal inflammation and subsequent endotoxin absorption. Non-steroidal anti-inflammatory drugs (NSAIDs) can cause damage to the integrity of the mucosal barrier at toxic doses, thought to be due to decreasing production of PGs, especially PGE_2 and PGI_2 which are important for the maintenance of mucosal blood flow, epithelialization and the mucus-bicarbonate barrier.[16] Toxicity associated with NSAIDs has resulted in clinical signs of endotoxemia secondary to severe ulcerative lesions throughout the gastrointestinal tract.[17-19]

Normal resident gastrointestinal microbes are important contributors to the luminal environment. Commensal organisms reduce the risk of virulent bacterial overgrowth by competing for limited substrates, binding sites and through release of metabolic by-products that inhibit the growth of competing bacteria. A number of clinical situations pose a threat to the normal gut flora. Rapid dietary changes to rations containing easily fermentable carbohydrate with minimal roughage can alter the luminal environment allowing pathogenic organisms to flourish. Experimentally induced carbohydrate overload caused decreased cecal pH and elevated endotoxin levels.[13] Enteral and parenteral anti-microbials, e.g. trimethoprim-sulfa, have been associated with enterocolitis and subsequent endotoxemia due to loss of mucosal integrity. This process may be initiated by destruction of commensal organisms, with subsequent proliferation of pathogenic organisms.

In the horse, intact epithelium such as that in the gastrointestinal tract provides the first line of defense against invading microorganisms. Host phagocytic cells, lymphocytes and humoral factors including immunoglobulins, complement and acute phase proteins

constitute the secondary host defense mechanisms. Functional deficiencies of these protective barriers, such as occurs during colic, facilitate absorption of LPS into the circulation. LPS is a potent inflammatory stimulant capable of both direct and indirect induction of multiple host inflammatory and immunologic processes. Endotoxemia is associated with LPS-induced activation of host cellular populations and the liberation of prostanoids, inflammatory peptides, complement, kinins and reactive oxygen radicals. Once the inflammatory cascades are initiated, alterations in homeostatic mechanisms governing endothelial integrity, hemodynamics, hemostasis and metabolism may lead to tissue ischemia and organ failure.[20] When endotoxin contacts blood, most of it binds to LPS-binding protein (LBP), a normal plasma constituent. The endotoxin-LBP complex then binds to CD14 on the membranes of intravascular macrophages and monocytes. Endotoxin is shuttled from CD14 to toll-like receptor molecules, which aggregate with other membrane proteins to initiate intracellular responses. Early intracellular events include the activation of NFκB, which induces transcription of a number of genes including the pro-inflammatory cytokines TNFα and IL-1β. At the same time, there is also induction of important pro-inflammatory enzymes, primarily in macrophages and endothelial cells, including COX-2 and inducible NO synthase (iNOS).[20]

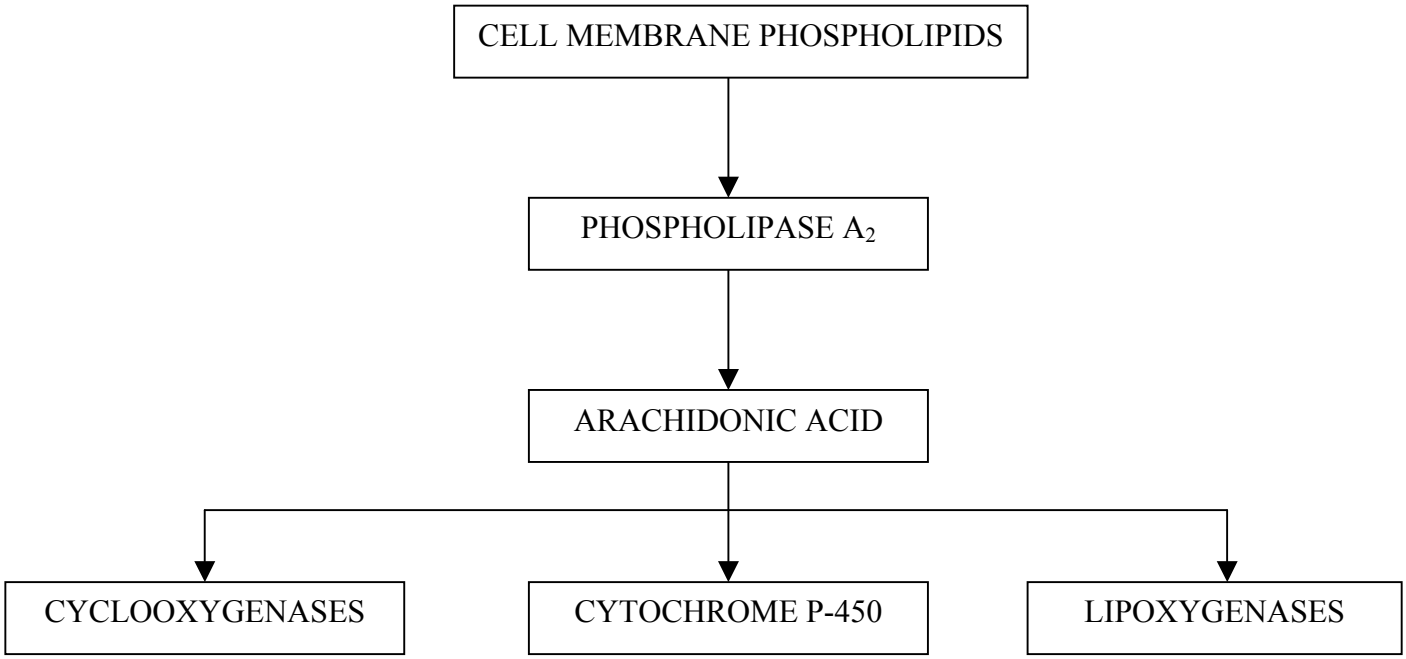
In the horse with colic, endotoxemia can occur in conditions that may or may not require surgery. The most common cause of endotoxemia in colic is a lesion involving compromise of the blood supply to the intestine, causing ischemia and tissue death. These lesions all require surgery. While the compromised bowel is present in the abdomen, large quantities of endotoxin are being absorbed into the systemic circulation, causing numerous effects via an upregulated inflammatory response, many of which are

deleterious to the patient. Even after surgical removal of the ischemic intestine, the effects of endotoxin are still seen, and continued treatment is required because of activation of host defenses and pro-inflammatory responses.

Arachidonic Acid Metabolism

Arachidonic acid is the most abundant polyunsaturated fatty acid in the phospholipid component of cell membranes. Arachidonic acid is released from the cell membrane by the enzyme phospholipase A_2. This enzyme is activated by a variety of stimuli including physical trauma, hormones, growth factors, immunoglobulins, phagocytosis, lectins, thrombins, endotoxin, tumor necrosis factor and hypoxia.[21] The release of arachidonic acid serves as a precursor for the synthesis of the different eicosanoids, mediated through the COX isoforms, and also the lipoxygenase and cytochrome P-450 pathways (CYP450), as is shown in Fig. 1.

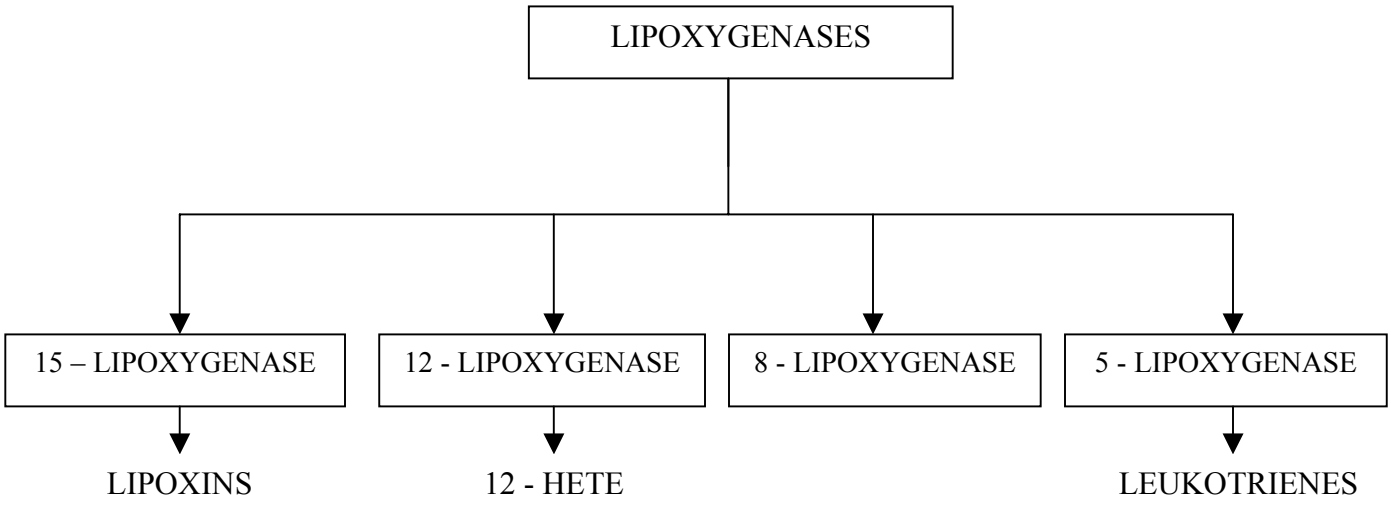
Fig. 1: The arachidonic acid cascade



Arachidonic acid can be metabolized by CYP450 enzymes to 5, 6-, 8, 9-, 11, 12- and 14, 15-epoxyeicosatrienoic acids (EETs) and their corresponding dihydroxyeicosatrienoic acids and 20-hydroxyeicosatetraenoic acid. These metabolites of arachidonic acid are involved in the regulation of renal epithelial transport and vascular function.[22]

The metabolism of arachidonic acid can also be catalyzed by the lipoxygenases. The family of lipoxygenases is divided into four subtypes according to tissue distribution: 5-, 8-, 12- and 15-lipoxygenase,[23] as is shown in Fig. 2.

Fig. 2: The lipoxygenase family



5-lipoxygenase (5-LOX) converts arachidonic acid to leukotrienes (LTs) and is principally found in cells of myeloid origin, such as polymorphonuclear leukocytes, macrophages, eosinophils, mast cells, monocytes, basophils, and B lymphocytes that are involved in inflammatory and immune reactions. Leukotriene C_4, LTD_4 and LTE_4 are potent bronchoconstrictors, whereas LTB_4 is chemotatic for leukocytes. These mediators are involved in a range of allergic and inflammatory conditions, such as asthma,

rheumatoid arthritis and ulcerative colitis.[24] 8-lipoxygenase has been found in skin and may be involved in skin tumor development.[25] 12-lipoxygenase has been found in platelets, erythroleukemia cells and umbilical endothelial cells. Metabolites of 12-LOX, such as 12-hydroxyeicosatetraenoic acid (12-HETE) have been found to play a central role in the various stages of the metastatic process in tumors.[23] 15-Lipoxygenase produces 15- hydroxyeicosatetraenoic acid (15-HETE) from arachidonic acid, which is subsequently converted to lipoxins, which have been shown to have anti-inflammatory properties.[26] There is evidence supporting cytoprotective and proresolution roles for lipoxin in intestinal inflammation.[27,28] Lipoxins also inhibit neutrophil chemotaxis, adhesion to epithelium and epithelial cell chemokine release.[29,30]

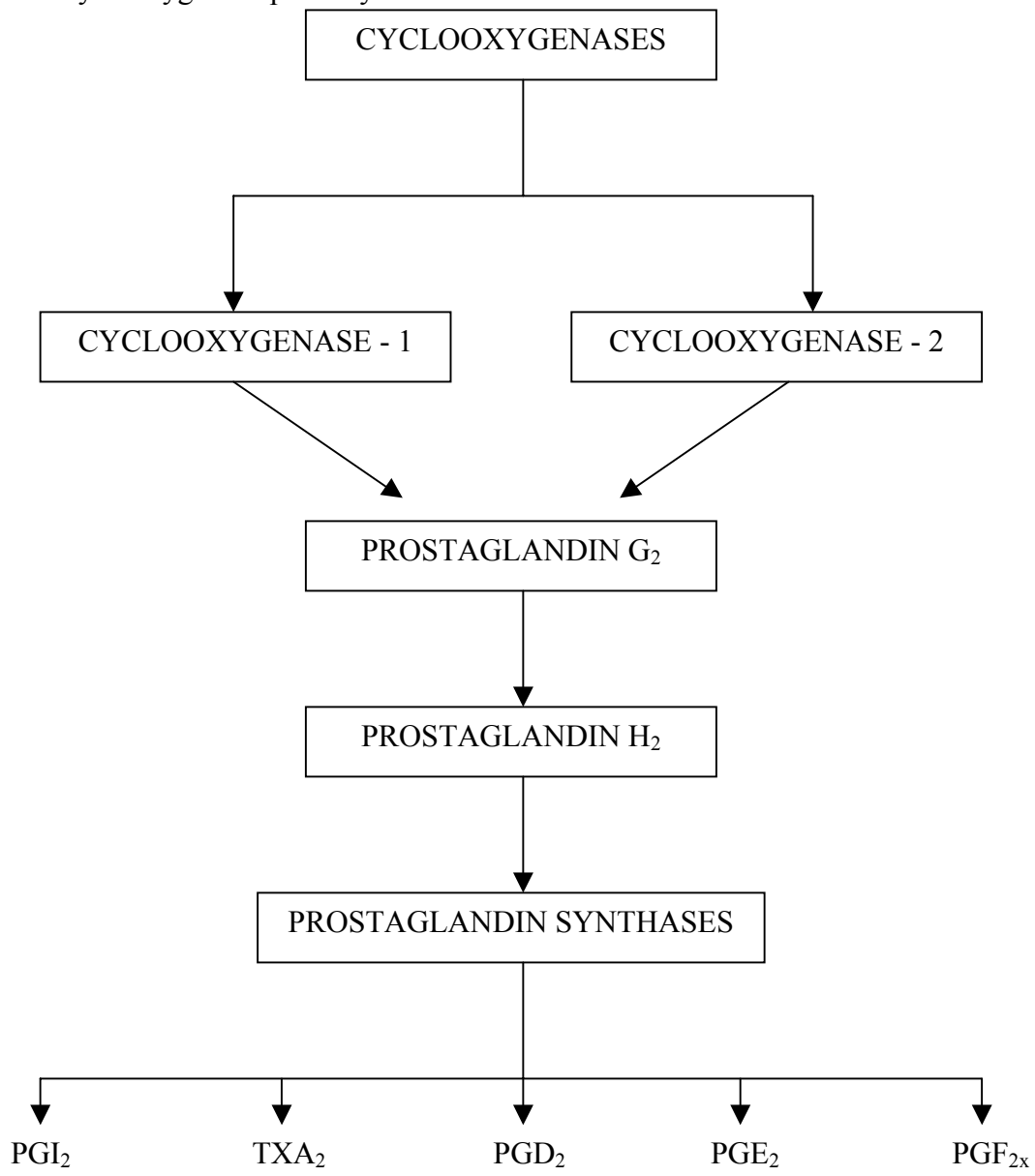
At a molecular level the mechanism of action of NSAIDs was first discovered in 1971, when it was shown that NSAIDs inhibit the enzyme prostaglandin endoperoxide synthase or cyclooxygenase (COX).[31] Cyclooxygenase catalyzes a 2-step conversion of arachidonic acid to PGG_2 (peroxidation) and PGH_2 (reduction). It is now known that PGs are produced by two distinct cyclooxygenases: COX-1 and COX-2. COX-1 is a constitutively expressed gene that is not up regulated in response to inflammatory stimuli. Alternatively, COX-2 is not expressed under normal conditions but is induced by a variety of agents including inflammatory mediators, particularly LPS[32], and is predominantly expressed at sites of inflammation, including inflamed gastrointestinal mucosa. Because of their pattern of expression, it has been suggested that COX-1 is responsible for "house-keeping functions" such as maintenance of the intestinal epithelial barrier, whereas COX-2 is likely responsible for producing prostaglandins that amplify

shock, inflammation and pain[33]. However, it is now known that COX-2 is also constitutively expressed in the brain, kidneys and uterus.

Cyclooxygenase-1 and -2 are structurally distinct proteins, the amino acid sequence of their complementary DNA showing only 60% homology. Cyclooxygenase-1 contains 576 amino acids and COX-2 contains 587 amino acids. They have similar active sites for their natural substrate arachidonic acid and for blockade by NSAIDs. Both consist of a long narrow channel with a hairpin bend at the end. They are membrane associated, so arachidonic acid released from membrane sites adjacent to the opening of the enzyme channel, which is largely hydrophobic, is sucked in, twisted around the hairpin bend, after which two oxygens are inserted and a free radical extracted, resulting in a five carbon ring that characterizes PGs. NSAIDs inhibit COX activity by excluding access for arachidonic acid into the channel. X-ray crystallography has suggested that this blocking occurs by hydrogen bonding to the polar arginine at position 120. At position 523 there is an isoleucine molecule in COX-1 and a valine (smaller by a single methyl group) in COX-2. The smaller valine molecule in COX-2 leaves a gap in the wall of the channel, allowing access to a side-pocket, which is thought to be the binding site of many COX-2 selective drugs. The bulkier isoleucine in COX-1 is large enough to block access to the side pocket. The difference at position 523 is compounded by the substitution of isoleucine 434 in COX-1 with a valine molecule in COX-2. This is within the second shell of amino acids surrounding the COX active site, further increasing the effective size of the active site channel by enhancing the mobility of side chains within the side pocket. The larger main channel combined with the extra nook increases the volume of the COX-2 NSAID binding site by about 20% over that of COX-1.[21]

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, as is shown in Fig. 3.

Fig. 3: The cyclooxygenase pathway



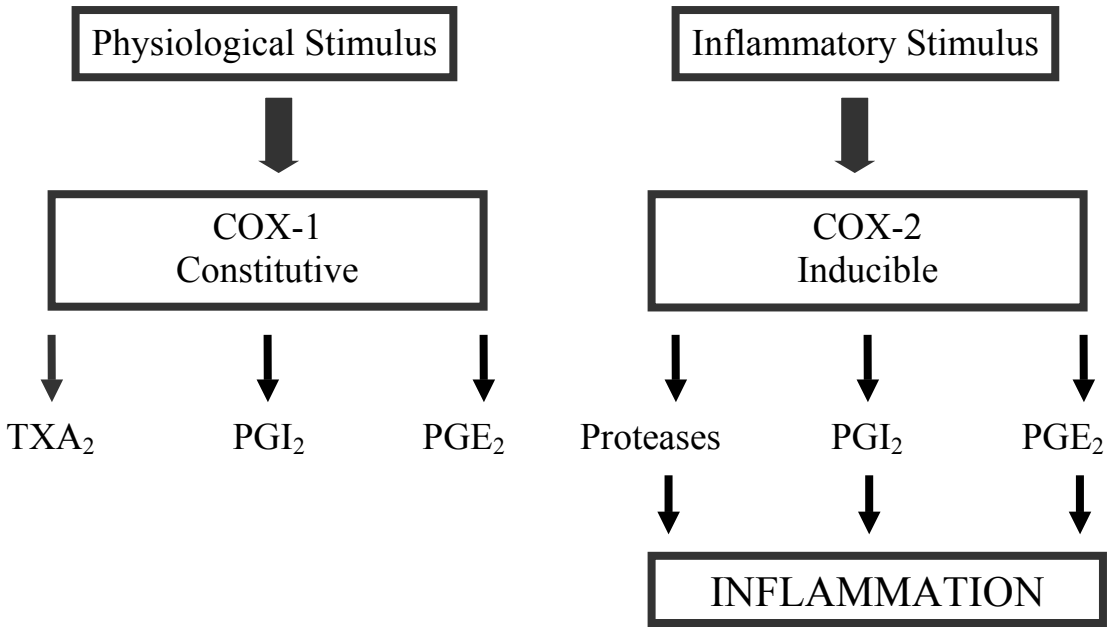
The best example of this is co-localization of COX-1 and thromboxane synthase in platelets, such that platelet TXB_2 production can be used as an indicator of COX-1 activity. [34] Similarly, COX-2 and PGE synthase are co-localized in LPS-stimulated

macrophages.[35] Therefore, selective COX-2 inhibitors may inhibit the production of PGs responsible for amplifying abdominal pain and circulatory shock, while allowing continued COX-1 production of PGs responsible for repair of the epithelial barrier. This shows that arachidonic acid can be metabolized by a number of enzymes. However, only two of these enzymes lead to the production of pro-inflammatory mediators, COX and 5-LOX.

Cyclooxygenase-1 and -2

The initial hypothesis for the functions of COX-1 and COX-2 is shown in Fig 4. Early studies looked at anti-inflammatory and ulcerogenic effects of specific COX-2 inhibitors and concluded they were beneficial, being anti-inflammatory with no or minimal gastrointestinal effects[36,37]. A model using chemically induced subcutaneous

Fig. 4: Initial hypothesis for the functions of COX-1 and COX-2



inflammation in rats showed that a specific COX-2 inhibitor, inhibited the inflammatory response but had no effect on PG production compared to controls.[36] Whereas a NSAID,

that inhibits both COX-1 and COX-2, was also anti-inflammatory but caused gastric lesions due to decreased production of PGs. However another study showed that COX-2 inhibitors were only beneficial at dosages that also inhibited COX-1[38]. This study suggests COX-1 also plays a role in inflammation, meaning there would be no advantage using specific COX-2 inhibitors. But the authors did note that this may be dependent on the model of inflammation used, as this study used a rat inflammation model initially and then a mouse model to try to explain the results of the rat experiment. Studies also looked at inflammation in rodent models where the rodents already had underlying gastrointestinal (GI) disease. In mice with pre-existing gastric lesions COX-2 inhibition impaired mucosal healing[39], despite the fact that COX-1 is most frequently cited as producing cytoprotective and reparative PGs. Also in rats with experimentally induced colitis, treatment with a specific COX-2 inhibitor resulted in increased mortality as a result of colonic perforation compared to untreated controls. The rats were also more likely to die if treated with specific a COX-2 inhibitor compared to a non-specific COX-1/COX-2 inhibitor[40], indicating COX-2 may serve to produce reparative PGs during acute GI mucosal injury. Also, 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.[41]

In addition, prostaglandins produced at a site of mucosal injury by COX-2 may modulate inflammation or even be classed as anti-inflammatory. One study showed that concentrations of COX-2 derived PGD_2 were increased in a model of colitis, and inhibition of COX-2 abolished this increase in PGD_2 production and resulted in a worsening of the condition, seen as a doubling of granulocyte infiltration .[42] Another

study looked at carrageenin-induced pleurisy in rats[43]. In this model, COX-2 protein expression peaked initially at 2 hours, associated with maximal PGE_2 production (inflammation). However, at 48 hours there was a second increase in COX-2 expression, 350% greater than at 2 hours, and this coincided with inflammatory resolution. The use of a selective COX-2 inhibitor inhibited inflammation at 2 hours, but exacerbated the inflammatory response at 48 hours, along with decreases in PGD_2. Cyclooxygense-2 is also an important source of vascular prostacyclin, the inhibition of which may retard ulcer healing.[44] 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.[45] Interestingly, inhibition of both COX-1 and COX-2 was required for the development of gastric erosions after NSAID administration in rats.[38]

This data shows that the initial hypothesis of COX-1 as a beneficial homoeostatic isoform and COX-2 as a pro-inflammatory isoform is an over-simplification, and that both enzymes appear to have overlapping functions. Hopefully, as more research is carried out, a clearer role for these enzymes will be found. It must also be remembered that COX-2 is expressed constitutively in tissues such as the kidney where it is involved in such functions as regulation of sodium secretion, so the use of selective COX-2 inhibitors may induce renal damage[46] and may also be involved in the development of hypertension.[47,48] Also, in COX-2 knockout neonatal mice, the kidneys fail to develop and the mice die.[49,50] Inhibition of COX-2 in neonatal rats can impair glomerulonephrogenesis and produce renal cortical damage.[51] Therefore COX-2 is also

involved in the development as well as some constitutive functions of the kidney. There are also concerns that selective COX-2 inhibitors may be prothrombotic and increase the risk of myocardial infarction. This concern has arisen because of the unexpected finding of a higher rate of myocardial infarction in patients receiving the selective COX-2 inhibitor rofecoxib, compared with patients receiving a much less selective COX-2 inhibitor naproxen in a study of gastrointestinal toxicity.[52] Cyclooxygenase-2 may also be involved in mechanisms to protect the myocardium from ischemic damage and use of selective COX-2 inhibitors may predispose some individuals to myocardial infarction.[53] It has also been shown that COX-2 inhibition may affect fertilization, implantation, and maintenance of pregnancy as well as ovulation.[54]

As previously mentioned, the mechanism of most NSAIDs is inhibition of COX in the peripheral tissues, preventing the production of PGs involved in the inflammatory response. Because of its potent analgesic and anti-pyretic actions, acetaminophen is generally regarded as an NSAID. However, it lacks the other typical actions of NSAIDs, such as anti-platelet activity and gastrotoxicity.[55] Acetaminophen is only a weak inhibitor of PG synthesis *in vitro* and appears to have very little anti-inflammatory activity, although some reduction in tissue swelling after dental surgery has been reported.[56,57]. The mechanism of action of acetaminophen has yet to be fully elucidated. There has been some evidence that acetaminophen has a central analgesic action, rather than acting peripherally as other NSAIDs do.[58] A very recent study has begun to elucidate the mechanism of action of acetaminophen. A variant of the COX-1 enzyme was cloned from the cerebral cortex in dogs and humans. This enzyme has been named COX-3.[59] It is a splice variant of the COX-1 gene but, retaining intron 1 in its mRNA. It was also shown

that acetaminophen is a selective inhibitor of COX-3. This explains the central action of acetaminophen and also why it is a weak inhibitor of peripheral PG synthesis via COX. However, further work needs to be done to fully elucidate the role of COX-3. It has also been hypothesized that there may be more COX variants that have yet to be cloned.

NSAIDs and the Gastrointestinal mucosa:

In human medicine, NSAIDs are mainly used for the control of pain associated with osteoarthritis. The use of NSAIDs, especially non-specific COX inhibitors, can lead to the development of gastric ulceration.[60]

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.[61] Thus, inhibition of prostaglandin production is thought to account for the major component of NSAID-induced mucosal injury. However, acute topical injury does occur 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.[62] 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.[63]

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.[64] 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.[65] 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.[66]

Prostaglandins have so many effects in the gastrointestinal tract, it has been difficult to ascertain 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.[67] 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 up-regulation of other vasoactive substances such as nitric oxide.[68,69] However, long-term NSAID use can result in continued decreases in blood flow.[70] For example, studies in rats revealed diminished small intestinal villous perfusion 7 days after subcutaneous administration of indomethacin.[71] 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.[72] Prostaglandins inhibit histamine-mediated cAMP 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.[73]

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 up-regulation of leukocyte adhesion molecules during NSAID-induced damage.[74,75] 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.[76] 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.[76] 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.[77] Once neutrophils infiltrate the mucosa, they become activated and release reactive oxygen metabolites and proteases that may damage mucosal epithelium.[78,79] Mice deficient in fucosyltransferase-VII, required for extravasation of leukocytes, were protected against NSAID-induced gastrointestinal damage.[80] However, mice deficient in mature B and T lymphocytes were not protected from damage, indicating a specific role for neutrophils rather than other leukocytes.[80]

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.[77] 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.[77] 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.[75]

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.[81,82] Nonsteroidal anti-inflammatory drugs have also been shown to increase permeability of the large intestine in healthy human patients.[83] The use of NSAIDs may be associated with an increased risk of colitis attributable to inflammatory bowel disease, particularly among patients with no history of the disease.[84] Nonsteroidal anti-inflammatory drugs have also been documented to cause colonic ulcers in humans who don't have inflammatory bowel disease.[85]

One of the vital components of intestinal barrier function is the ability of the mucosa to repair rapidly following, for example, 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.[86] The initial components of mucosal repair include migration of residual epithelial cells to cover mucosal defects; a mechanism termed restitution.[87] Factors regulating this process include trefoil peptides, polyamines, and growth factors.[88]Although prostaglandins do not directly stimulate epithelial restitution, they

appear to be required for growth factor-stimulated restitution.[89] In addition, PGE_2 also stimulates villous contraction in small intestinal mucosa, which may facilitate epithelial restitution by reducing the denuded surface area.[90] During these early repair processes, PGI_2 and PGE_2 act synergistically to close interepithelial tight junctions via their second messengers Ca^{2+} and cAMP, restoring intestinal barrier function following ischemic injury.[91,92] 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, with increased permeability occurring in the tissue treated with indomethacin alone.[91] Therefore, while providing necessary analgesia, NSAIDs may delay the healing process, thereby increasing morbidity rate and duration of hospitalization.

NSAIDs may also affect subacute reparative events such as epithelial proliferation.[93] For example, NSAIDs decreased the number of proliferative cells in the glandular mucosa of rat stomach; an effect reversed by the PG analogue misoprostol, [93] 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.[94] 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.

In equine medicine NSAIDs are used to control orthopedic pain (usually due to degenerative joint disease) and abdominal pain (colic) by inhibiting the enzyme cyclooxygenase (COX), which prevents the production of inflammatory prostaglandins. Adverse effects in horses include gastrointestinal ulceration[95,96], inflammation of the right dorsal colon[97] (right dorsal colitis) and renal papillary necrosis.[98] Gastric ulceration may be caused by a decrease in PGE_2 in the stomach, reducing the cytoprotective role of PGs. Of the NSAIDs routinely used in equine practice, phenylbutazone is associated with the greatest risk of toxicity.[99] Both phenylbutazone and flunixin meglumine have been associated with the development of right dorsal colitis (RDC).[100] The pathogenesis of RDC is unknown. It is thought that there are both systemic and local factors contributing to the development of the condition.[101,102] For example, it has been hypothesized that NSAID inhibition of PGE_2 production decreases mucosal blood flow resulting in hypoxic or ischemic mucosal damage. The importance of blood flow may explain why toxicity is most frequently noted in hypovolemic patients.[97] However, toxic effects of the NSAID phenylbutazone in the colon have also been noted in normovolemic horses given therapeutic dosages,[103] suggesting as yet unidentified non-vascular mechanisms may also be involved in the pathogenesis of RDC. Reduced levels of PGs may decrease renal perfusion potentially leading to renal damage[98]. Therefore, equine practitioners are becoming increasingly concerned about NSAID toxicity, especially in horses that are hemoconcentrated and hypovolemic, e.g. patients with gastrointestinal disease requiring surgery.

Bile and the Gastrointestinal Tract

Bile is a normal constituent of the luminal contents of the mammalian small intestine, the main function of which is to aid fat digestion and absorption. The primary bile acids, cholic acid and chenodeoxycholic acid are synthesized in the liver from cholesterol and are conjugated with taurine or glycine to form taurocholic and glycocholic acids. They can undergo further metabolism, by enteric bacteria for example, to form secondary bile acids, such as deoxycholic acid, lithocholic acid, and taurodeoxycholic acid.[104]

The effect of bile salts on the intestinal epithelium has been studied by a number of investigators. For example, taurodeoxycholic acid caused an increase in the permeability of the gastric mucosa to varying sizes of polyethylene glycol molecules.[105] In the rabbit small intestine, chenodeoxycholic acid and ursodeoxycholic acid (a tertiary bile acid, derived form a secondary bile acid) increased the rate of transmural flux of lactulose in the jejunum and ileum[106], while the salt of deoxycholic acid, deoxycholate, increased the mucosal-to-serosal flux of mannitol in the rat and the rabbit.[107] Electron microscopy revealed wider intercellular spaces with loss of contact points between the cellular membranes. Deoxycholate also caused increases in mucosal permeability in porcine colon[108,109] and porcine ileum.[110] Damage to the colonic mucosa ranged from slight (with loss of single cells) with 1.5mM deoxycholate to extensive (with a barren mucosal surface and sloughed epithelial sheets) with 3 to 21mM deoxycholate. Removal of the bile salts was followed by rapid recovery of mucosal barrier function in both the ileal and colonic studies.

Although this work has provided important insight into the mechanisms of injury

and repair in intestinal mucosa subjected to bile salts, all of these studies used bile salt concentrations that are much higher (0.1mM to 21mM) than physiological concentrations. Furthermore, most of these studies assessed the effects of bile salts on normal mucosa. However, one study evaluated the effects of bile on rat jejunum subjected to 30-minutes of ischemia, and showed that 1mM taurodeoxycholic acid and taurochenodeoxycholic acid did not exacerbate gut permeability to sodium fluorescein.[111] In another study, low dose deoxycholate (50μM) added prior to the addition of 250μM deoxycholate attenuated the injury produced by 250μM deoxycholate alone on gastric mucosa.[112] The mechanism of attenuation triggered by low dose deoxycholate may have involved an effect on intracellular calcium. In addition, increasing calcium levels to 4mM inhibited the damaging effect of 5mM deoxycholic acid on colonic epithelium.[113]

The Biliary System and NSAIDs

There have been a number of studies that have looked at the toxicity of NSAIDs and bile, especially in relation to the amount of drug that is excreted via the biliary system and enterohepatic circulation. In one study, the gastric and intestinal damage noted when the NSAIDs aspirin, diclofenac, diflunisal, ibuprofen and indomethacin were administered orally or intravenously to fasted or fed rats was assessed.[114] It was concluded that, in the rat, acute gastric and intestinal toxicity of NSAIDs was due to different mechanisms. Whereas gastric toxicity is strongly influenced by the amount of drug dissolved under the pH conditions of the stomach, intestinal toxicity appears to depend on biliary excretion and enterohepatic circulation of a drug as well as on its potency as an inhibitor of PG synthesis.

Another study looked at the mechanisms of acute and chronic inflammation induced by indomethacin. It showed that enterohepatic circulation of indomethacin is important in promoting the acute phases of injury and inflammation.[115] The NSAID diclofenac (which does undergo enterohepatic circulation) caused a progressive increase in intestinal permeability, marked increases in enteric gram-negative bacterial numbers and frank intestinal ulceration when administered to rats. In the same study, nitrofenac, aspirin, and nabumetone (which all undergo no enterohepatic circulation) did cause some increase in intestinal permeability, which was not progressive and did not cause any changes in enteric bacterial number or intestinal ulceration. It was concluded that in the case of diclofenac, enterohepatic recirculation was of paramount importance in the pathogenesis of the enteropathy seen.[116]

Indomethacin has been shown to significantly increase bile acid-induced small intestinal mucosal injury whereas pretreatment with topical 16,16-dimethyl PGE_2 significantly reduced the bile acid-induced small intestinal mucosal injury, showing that there is a role for PGs in this rat model.[117] A similar finding was found in a model of bile salt-induced damage of porcine colon. Indomethacin administration accelerated the degenerative changes whereas the effects of indomethacin were abolished by PGE_2 administration.[118] In a human study it was concluded that indomethacin increased intestinal permeability whereas nabumetone did not and that it was the biliary excretion of indomethacin that was the cause of this difference.[119] Further evidence for the adverse effects of the biliary excretion of indomethacin were found in a study where bile from rats given indomethacin was added to a cell culture of rat intestinal cells and was found to be cytotoxic.[120]

The administration of indomethacin and diclofenac to rats was found to significantly decrease the surface hydrophobicity of mucus in the stomach and duodenum associated with a reduction in phosphatidylcholine.[121] A reduction in hydrophobicity impairs the physicochemical barrier against luminal acidity and renders the mucosa susceptible to injury. The same study carried out in rats that had first undergone bile duct ligation revealed no decrease in the surface hydrophobicity of mucus. This shows that excretion of NSAIDs in bile appears to play a key role in this effect. Phosphatidylcholine has been shown to play a physiologically important role in reducing the cytotoxic activities of bile salts, most likely by forming mixed micelles.[122,123] Secretion of NSAIDs (e.g. indomethacin) into the bile has been shown to abrogate this protective property due to the affinity of NSAIDs to chemically interact with phosphatidylcholine, transforming mixed micelles back into cytotoxic bile salt micelles.[124,125] These studies show that for those NSAIDS that undergo biliary excretion and enterohepatic circulation, these processes are very important for the development of some of their adverse effects.

The actions of COX-1 and COX-2 can no longer be considered as completely separate entities, with an overlapping of functions, especially with respect to maintenance of the gastrointestinal barrier, inflammation and renal function. With the recent discovery of COX-3, it is going to become even more confusing. Further research is required to further define their functions.

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CHAPTER TWO

FUTURE TRENDS IN NONSTEROIDAL ANTI-INFLAMMATORY DRUG THERAPY

INTRODUCTION

While the development of selective COX-2 inhibitors has led to significant reductions in the incidence of adverse effects including gastrointestinal ulceration, while still providing good analgesia,[1] the situation is still not as good as it could be. Use of selective COX-2 inhibitors may still cause some adverse effects, e.g. in patients with underlying disease such as gastric ulceration or colitis. Selective COX-2 inhibitors have also been associated with the development of hypertension and renal damage and may predispose some patients to myocardial infarction. Therefore, other therapeutic modalities are being looked at.

Co-administration of Gastroprotective Drugs

This involves administration of an NSAID concurrently with a drug that is considered to be gastroprotective to potentially reduce the gastrointestinal side effects of NSAIDs alone.

Misoprostol

This is an analog of PGE_2 and there is endoscopic evidence that shows misoprostol protects against gastroduodenal damage from NSAIDs in humans.[2] The clinical relevance of these findings is supported by a human study which showed a 40% reduction in hospital admissions for ulcer complications when misoprostol was given concurrently with NSAIDs.[3] Misoprostol has also been shown to prevent aspirin-induced gastric ulceration in dogs.[4] One study has also shown that misoprostol is an effective inhibitor of the basal gastric acid secretion in the horse.[5] Misoprostol has been used with some success to help treat phenylbutazone toxicosis in horses.[6] Therefore, misoprostol may be of benefit in the reduction and/or treatment of the adverse effects of NSAIDs in

the horse.

Proton pump inhibitors

Proton pump inhibitors (PPIs) inhibit the proton pump found in the stomach, essentially stopping acid secretion within the stomach. In human medicine, endoscopic studies in patients receiving PPIs have shown they are very effective in enhancing healing of NSAID-associated gastric and duodenal ulcers and in preventing relapse.[7-9] When used in patients receiving NSAIDs who also had bleeding ulcers, PPIs reduced ulcer bleeding by 4-6 fold compared with *Helicobacter pylori* eradication alone.[10] In horses, the PPI omeprazole has been shown to be very effective in inhibiting both basal and pentagastrin stimulated acid secretion.[11] Omeprazole has also been shown to be very effective in the treatment of gastric ulcers and their re-occurrence in the horse.[12,13] Therefore, omeprazole may be effective in reducing the incidence of gastric ulceration in horses receiving NSAIDs.

New Drug Formulations

NO-NSAIDs

One of the modalities being investigated for reducing NSAID-induced gastrointestinal injury is the use of nitric oxide releasing nonsteroidal anti-inflammatory drugs (NO-NSAIDs). They are synthesized by the ester linkage of an NO releasing moiety to conventional NSAIDs, such as aspirin, flurbiprofen, naproxen, diclofenac and ibuprofen.

There have been numerous studies that have shown fewer gastrointestinal side effects with NO-NSAIDs compared to the parent NSAID. In one study a single dose of naproxen to rats produced gastric lesions within 24 hours. In contrast, an equimolar dose

of NO-naproxen showed no ulcerogenic activity.[14] Long-term administration of flurbiprofen to rats resulted in weight loss, reduction in hematocrit and significant GI bleeding. Equimolar doses of NO-flurbiprofen had no such deleterious effects.[15] Similar results were seen when comparing naproxen (gastric damage) to NO-naproxen (no gastric damage).[16] These studies show the beneficial effects of NO-NSAIDs in both acute and chronic administration compared to the parent NSAID. NO-NSAIDs also exert a positive effect on the gastric damage caused by noxious stimuli. For example, NO-aspirin dose dependently decreased the severity of hydrochloric acid/ethanol-induced stomach lesions in rats, whereas aspirin had no effect.[17] Also, NO-NSAIDs may be of value for the healing of pre-existing lesions. Aspirin pre-treatment delayed the healing of thermal cauterization-induced stomach ulcers in rats, whilst an equimolar dose of NO-aspirin actually accelerated the healing process.[18]

The precise mechanism(s) behind the beneficial effects of NO-NSAIDs have yet to be fully elucidated. However, a number of mechanisms have been proposed. NSAID administration leads to a decrease in gastric mucosal blood flow due to decreases in PGE_2 and PGI_2. A study looking at the mesenteric post-capillary venules in rats showed flurbiprofen constricted them by 16.6%, whereas NO-flurbiprofen dilated them by 6.7%.[15] This led to the proposal that NO-NSAIDs cause vasodilation of the gastric vasculature and this minimizes their ulcerogenic potential. As NSAID-induced gastric damage can be potentiated by leukocyte adherence, this may be a way for NO-NSAIDs to reduce their toxicity. Administration of flurbiprofen but not NO-flurbiprofen significantly increased leukocyte adherence to mesenteric post capillary venules in rats.[15] Reduced leukocyte infiltration has also been shown by looking at myeloperoxidase activity which

was significantly reduced by NO-aspirin, NO-flurbiprofen and NO-naproxen compared to their parent NSAID.[19]

Caspases are a family of enzymes involved in apoptosis. Activation of caspase-3 is involved in cytokine-induced apoptosis.[20] Low levels of NO from NO donors inhibit cell apoptosis by post-translational inactivation of caspases 1 and 3.[21] These results suggest NO-NSAIDs might inhibit caspase activity in the gastrointestinal tract. Pre-treatment of rats with aspirin, but not NO-aspirin increased activity of both caspase 1 and 3[19] and NO-flurbiprofen inhibited caspase-3 activity and apoptosis compared to flurbiprofen in gastric mucosa.[22] Overall, the inactivation of caspase(s) appears to be an important factor in the gastrointestinal tolerability of NO-NSAIDs.[23]

There is a considerable body of evidence that supports the view that NO-NSAIDs are less damaging to the gastrointestinal tract than the parent NSAID. This beneficial response is likely due to a combination of mechanisms rather than a single process. As well as having less adverse effects than their parent NSAID, NO-NSAIDs appear to provide just as good or sometimes better anti-inflammatory and analgesic effects. This has been shown in a number of studies. For example, similar suppression of carrageenan-induced rat hind paw edema by NO-naproxen and NO-indomethacin compared to naproxen and indomethacin.[24]

What accounts for this these effects? We know the NSAID component of the NO-NSAID will inhibit COX and decrease the production of pro-inflammatory PGs, but what about other possible mechanisms? The inhibition of caspases by NO-NSAIDS reduces the formation of pro-inflammatory interleukin-1β which may contribute to the anti-inflammatory effects.[19] Studies have shown that NO-NSAIDs do not have any greater

inhibitory action on COX than the parent NSAIDs.[15,25] However, it has been shown that NO-NSAIDs inhibit induction of COX-2. NO-aspirin inhibited induction of COX-2 in LPS pre-treated cultured J774 macrophages.[26] NF-κB contributes to cytokine-driven generation of leukocyte adhesion molecules[27] leading to the migration of neutrophils to a site of inflammation. NO may modulate NF-κB activity[28,29] and evidence for an effect on NF-κB by NO-NSAIDs has recently been shown.[30] NO-NSAIDs also appear to have enhanced anti-nociceptive activity.[23] It has been suggested that NO or one of its redox species may interact with and reduce transmission via NMDA receptors in the spinal cord[31] reducing or even inhibiting hyperalgesia.

Therefore, NO-NSAIDs appear to have numerous advantages of their parent NSAIDs. They are currently undergoing clinical trails in human patients and hopefully will soon be available for clinical use.

NSAIDs and zwitterionic phospholipids

The association of NSAIDs with a zwitterionic phospholipid (a phospholipid having both a positive and negative charge) is another approach to improving tolerability and safety of NSAIDs. The rationale for this is that since the gastric mucosa possesses hydrophobic properties that are protective against luminal acid, the association of a NSAID with a zwitterionic phospholipid should result in a decrease in, or the prevention of , the interaction between the NSAID and the mucosa, an interaction that could decrease the protective ability of the mucosa.[32] When dipalmitoylphosphatidylcholine (DPPC) was complexed to the NSAIDs indomethacin, diclofenac and naproxen, they were better tolerated and had a reduction in GI injury compared to the parent NSAID, with comparable anti-inflammatory and analgesic effects.[33] These results have only been

obtained in rodent models. These beneficial effects have yet to be demonstrated in other species.

Intestinal Trefoil Factors

Intestinal trefoil factors comprise a family of proteins expressed almost exclusively in the gastrointestinal tract. They are stable at a wide range of pH values, resistant to protease degradation, and induction and localization during gastric insult suggest that these peptides function in gastroprotection and restitution.[34,35] Knockout mice lacking intestinal trefoil factor have an impaired mucosal healing response[36] and transgenic mice that overexpress a trefoil peptide have increased resistance to NSAID-induced damage.[37] Another study showed that oral administration of trefoil peptides to rats protected against indomethacin-induced gastric injury.[38,39](162,163) Intravenously infused hTFF2 and pTFF2 (both intestinal trefoil factors) protected the gastric mucosa from ethanol-induced damage in anesthetized rats.[40] In addition, topical application of these trefoil factors also was effective at protecting the gastric mucosa from injury.[40] Another study has shown that hTFF2 enhances the rate of colonic epithelial repair, and reduces local inflammation in a rat model of colitis, and suggests that luminal application of trefoil peptides may have therapeutic potential in the treatment of inflammatory bowel disease.[41] Intestinal trefoil peptide also promotes protection of epithelial cells from complement activation (caused by mucosal damage) via up-regulation of decay-accelerating factor expression, contributing to a robust mucosal defense.[42] The exact mechanism of these effects is not fully understood, but may be independent of PGs.[38] Further studies are required before instituting clinical use.

Dual inhibitors of COX/5-LOX

There have been several studies that postulate cyclooxygenase inhibition by NSAIDs, besides causing a reduction in the synthesis of vasodilatory and gastroprotective PGs, diverts arachidonic acid to the 5-LOX pathway, thus increasing the formation of LTs.[43,44] This causes vasoconstriction of the gastric mucosa and increases the formation of reactive oxygen metabolites from the perioxidative cleavage of hydroxyeicosatetraenoic acids[45] (products of 5-LOX), with further mucosal injury. Indeed, gastric and intestinal mucosal lesions by NSAIDs are prevented by the concurrent administration of 5-LOX inhibitors.[43,44] Also, inhibition of 5-LOX is considered the ideal treatment for allergic disorders and asthma.[45]

However, it has already been shown that some COX inhibitors induce adverse reactions in patients with asthma either because of shunting of arachidonic acid to the 5-LOX pathway[46-48] or because of a reduction in vasodilatory PGs, PGE_2 and PGI_2, that have been shown to protect the airways.[49-51] To avoid these adverse reactions of NSAIDs an alternative method of treatment needs to be sought. This could be in the form of drugs that inhibit both COX and 5-LOX, which could lead to compounds with improved efficacy and reduced side effects when compared to selective COX inhibitors.[52] These dual inhibitors may also exhibit anti-inflammatory activity with a wider spectrum than classical NSAIDs by inhibiting 5-LOX product-mediated inflammatory reactions, against which NSAIDs are ineffective.[53-55]

There have been several research compounds that have been developed. S-2474 displayed dual inhibition with a good selectivity towards COX-2. In rats it produced excellent anti-inflammatory activity without ulcerogenic effects.[55] ER-34122 is an orally

active dual inhibitor and in mice had an enhanced anti-inflammatory effect when compared to indomethacin (non-selective COX inhibitor), which was linked to the inhibition of 5-LOX LT synthesis.[54] Tepoxalin significantly inhibited gastric LTB_4 synthesis in rats and markedly suppressed PG synthesis at a site of peripheral inflammation[56] and inhibited IL-2, IL-6 and TNF-α production.[57] Orally administered tepoxalin also exhibited anti-inflammatory activity in rats with adjuvant-induced arthritis and showed good analgesia.[58] Another promising compound is ML3000. Given orally, ML3000 and indomethacin were compared in a study of carrageen-induced rat paw edema. While the ED_{50} for ML3000 was significantly higher than indomethacin, both drugs were anti-inflammatory with ML3000 producing significantly less gastric injury.[59] Thus, while more clinical data is needed, the data so far suggests that dual COX/5-LOX inhibitors may represent a valid therapeutic alternative to standard NSAIDs and selective COX-2 inhibitors.

The clinical use of NSAIDs will continue despite their potential adverse effects, as they are the most effective drugs available for the treatment of colic in the horse and osteoarthritis in the horse and in humans. However, it is becoming clear that the future of NSAID therapy is going to rely on the discovery of new compounds with less adverse effects. The use of NO-NSAIDs and dual COX/5-LOX inhibitors may soon become common place in both human and veterinary medicine.

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CHAPTER THREE

THE ROLE OF CYCLOOXYGENASE INHIBITORS IN REPAIR OF ISCHEMIC-INJURED JEJUNAL MUCOSA IN THE HORSE

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SUMMARY

Cyclooxygenase inhibitors are administered to horses to prevent endotoxin-induced elaboration of prostaglandins. However, PGE_2 and PGI_2 stimulate repair of injured intestine. There are 2 isoforms of cyclooxygenase: COX-1, which constitutively produces prostaglandins and COX-2, which is induced by inflammation. We hypothesized that the non-specific cyclooxygenase inhibitor flunixin meglumine would retard repair of ischemic intestinal injury by preventing production of reparative prostaglandins whereas the selective COX-2 inhibitor, etodolac, would permit repair as a result of continued COX-1 prostaglandin production. Segments of equine jejunum were subjected to ischemia for 1 hour, and recovered for 4 hours in Ussing chambers. In ischemic tissue treated with the non-specific cyclooxygenase inhibitor flunixin meglumine (2.7×10^{-5}M), production of PGE_2 and PGI_2 was inhibited, and there was no evidence of recovery based on measurements of transepithelial resistance. Conversely, untreated ischemic tissues or tissues treated with the specific COX-2 inhibitor etodolac (2.7×10^{-5}M) had significant elevations in PGE_2 and PGI_2, and significant recovery of transepithelial resistance. These studies suggest that specific COX-2 inhibitors may provide an advantageous alternative to non-specific cyclooxygenase inhibitors in horses with colic.

INTRODUCTION

Colic is the second most common cause of death in the horse in the United States behind old age.[1] The principal cause of colic-associated mortality is strangulating obstruction.[2] The primary reason for death is shock associated with the absorption of bacterial endotoxin from damaged intestine.[3,4] Bacterial endotoxin induces production of prostaglandins that exacerbate circulatory shock and abdominal pain. Therefore, equine clinicians administer cyclooxygenase inhibitors such as flunixin meglumine to combat the effects of endotoxin. However, not all prostaglandins amplify the shock-associated effects of endotoxin, particularly constitutively produced prostaglandins of the E series.[5]

Recent studies suggest that Prostaglandin E_2 (PGE_2) maintains and repairs the intestinal epithelial barrier.[6,7,8] For example, PGE_2 stimulates restitution, an epithelial repair mechanism that re-surfaces denuded intestinal villi.[9] Furthermore, PGE_2 triggers villous contraction, which aids in the process of restitution by reducing villous surface area.[10,11] It has also been shown that PGE_2 and PGI_2 have a synergistic role in restoration of intestinal barrier function by increasing intracellular cAMP and Ca^{2+}, respectively, which in turn signal cytoskeletal-mediated tight junction closure.[12]

It has recently been determined that prostaglandins are produced by two distinct cyclooxygenases: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). Cyclooxygenase-1 is a constitutively expressed gene that is not up-regulated in response to inflammatory stimuli. Alternatively, COX-2 is not expressed under normal conditions but is induced by a variety of agents including inflammatory cytokines and is predominantly expressed at sites of inflammation, including inflamed gastrointestinal mucosa.[13,14] Because of their pattern of expression, it has been suggested that COX-1 is

responsible for "house-keeping functions" such as maintenance of the intestinal epithelial barrier, whereas COX-2 may be responsible for producing prostaglandins (PGs) that amplify inflammation.[15] It has recently been demonstrated that the non-specific COX inhibitor, indomethacin retards recovery of porcine ischemic injured ileum.[16] With the advent of specific COX-2 inhibitors,[17,18] it may be possible to prevent the production of prostaglandins responsible for amplifying abdominal pain and circulatory shock (exacerbated by COX-2), while allowing the continued production of prostaglandins responsible for repair of the epithelial barrier (produced by COX-1).

We hypothesized that the non-specific COX inhibitor flunixin meglumine would retard repair of ischemic intestinal injury by preventing production of reparative PGs whereas the selective COX-2 inhibitor, etodolac[19] would permit repair as a result of continued COX-1 PG production.

MATERIALS AND METHODS

Experimental horses and surgery

Eight 2 year-old intact male ponies were quarantined for 2 weeks prior to the start of the study. They were equine infectious anaemia negative, vaccinated against tetanus, eastern and western equine encephalitis, and rabies and dewormed with ivermectin paste. All ponies had physical exam parameters within normal limits. The ponies were sedated with xylazine (1.1mg/kg IV), anesthetized with diazepam (0.1mg/kg IV) and ketamine (2.2mg/kg IV), intubated and maintained on halothane vaporized in 100% oxygen. Anesthetic monitoring included intra-arterial systemic blood pressure, electrocardiography, and arterial blood gas analysis. Maintenance intravenous fluids

(lactated Ringers solution at 15ml/kg/hr) were administered via a jugular catheter. The abdomen was approached via a 20cm ventral midline incision. Two 1-meter jejunal loops were created approximately 3 meters proximal to the ileocecal fold by ligating the intestinal lumen with 0.5cm umbilical tape. Loops were randomly identified as control or ischemic. Ischemia was induced by ligating the mesenteric vessels using 3.0 metric silk. After one hour of ischemia, ischemic and control loops were resected and the ponies were euthanatized with an overdose of pentobarbital.

Ussing chamber studies

Intestinal loops were incised along the anti-mesenteric surface. The mucosa was then stripped from the seromuscular layer in oxygenated (95% O_2/5% CO_2) equine Ringer's solution containing either no treatment, $2.7x10^{-5}$ M etodolac or $2.7x10^{-5}$ M flunixin meglumine and mounted in 3.14cm^2 aperture Ussing chambers, as described in previous studies.[20] The concentration of flunixin meglumine was extrapolated from the serum level of flunixin reached at a dose of 1.1mg/kg IV according to previous studies.[21] The dose of etodolac was matched to that of flunixin meglumine. Tissues were bathed on the serosal and mucosal sides with 10ml equine Ringer's solution. The serosal bathing solution contained 10mM glucose, and was osmotically balanced on the mucosal side with 10mM mannitol. Bathing solutions were oxygenated (95% O_2/5% CO_2) and circulated in water-jacketed reservoirs at 37^oC. 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 1.0 mV, tissues

were voltage clamped at ±100μA for 5 sec and the PD recorded. Short-circuit current and PD were recorded every 15 min for 4 hours. The data was entered into spreadsheets that calculate resistance (R) from I_{sc} and PD using Ohm's Law. The data was analyzed for the effect of time, treatment and time-treatment interaction with 2-way ANOVA on repeated measures. For significant time-treatment interactions, select time points were analyzed with 1-way ANOVA for the effect of treatment. Differences between treatments were analyzed with a post-hoc Tukey's test.

Histologic studies

Tissues were taken immediately following the surgical procedures, and at the end of Ussing chamber experiments for routine histologic evaluation (5-μm cross-sections taken at 300μm intervals and stained with hematoxylin and eosin). For each tissue, two investigators blinded to treatment groups independently evaluated three sections. Four 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 two evaluators was pooled prior to any data manipulations. The surface area of the villus was calculated using the formula for the 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.[20] The denuded villous surface area was calculated from the total surface area of the villus and the surface area of the villus covered by epithelium. Statistical analysis involved 1-way ANOVA for effect of treatment on histologic indices of repair.

Eicosanoid measurements

Samples of the serosal bathing solutions (0.5ml) were taken at 1 hour from the start and at the end of the experiments (4 hours). These samples were snap-frozen in liquid nitrogen, stored at –70^{o}C and analyzed for PGE_2, 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) and Thromboxane B_2 (a stable metabolite of Thromboxane A_2). Assays were performed by the Center for Gastrointestinal Biology and Disease, Chapel Hill, NC, using commercial ELISA kits (R & D Systems, Minneapolis, MN, USA). Statistical analysis was done using 1-way ANOVA for effect of treatment on eicosanoid levels.

RESULTS

Ussing chamber studies

Tissues subjected to ischemia for 1 hour had transepithelial resistance (TER) measurements, approximately half that of control tissues, at the beginning of the experiment (figure 1). Control tissue showed a gradual decline in TER over the 4 hour period of the experiment. The TER of untreated ischemic tissue showed a gradual recovery response over the recovery period and reached levels not significantly different from control after 4 hours. Similarly, tissues subjected to ischemia and treated with etodolac also showed a recovery response, whereas the TER of tissues exposed to ischemia and treated with flunixin meglumine showed no recovery response. There was a significant interaction between time and treatment (2-way ANOVA on repeated measures, $P< 0.05$). At 4 hours, TER of flunixin-treated tissues was significantly below that of untreated and etodolac-treated tissues (1-way ANOVA, $P< 0.05$).

Histologic studies

Histopathologic examination of mucosa immediately after 1 hour of ischemia showed epithelial sloughing from the tips of the villi (figure 2), which resulted in substantial denuded villous surface area (figure 3). After 4 hours in the Ussing chambers, evidence of restitution and marked reductions in villous height were noted (figure 2). Ischemic tissues showed a significant reduction in denuded surface area (figure 3) and villous height (figure 4). Tissues subjected to ischemia and treated with etodolac or flunixin meglumine showed similar histologic evidence of villous atrophy and restitution compared to ischemic tissue alone (figure 2). There was no significant difference between untreated, flunixin-treated or etodolac-treated tissues in denuded surface area (figure 3) or villous height (figure 4) at the end of the recovery period. Control tissue showed some epithelial sloughing from the tips of villi over time (figure 2) and a small decrease in villous height (figure 4). Measurements of crypt death showed minimal changes over time (figure 5).

Eicosanoid measurements

There were significant increases in PGE_2, 6-keto-$PGF_{1\alpha}$ and TXB_2 concentrations in the serosal bathing solution in control tissues and untreated and etodolac-treated ischemic tissues at hour 4 compared to hour 1 of the experiment ($P<0.05$, figs 6-8). However, tissues treated with flunixin had minimal levels of eicosanoids at hour 1 and hour 4 and significantly less than any other treatment group ($P<0.05$, figs 6-8). Ischemic tissues treated with etodolac had TXB_2 levels similar to those of control and untreated ischemic tissues (fig 6), but significantly less PGE_2 and 6-keto-$PGF_{1\alpha}$ by hour 4 ($P<0.05$,

figs 7,8). There was no difference in the levels of eicosanoids in control or ischemic tissues (figs 6-8).

DISCUSSION

Transepithelial resistance (TER) is a highly sensitive measure of the permeability of the intestinal mucosa. For example, recent studies have shown close correlations between recovery of TER and reductions in mucosal permeability to inulin and mannitol in ischemic-injured tissues treated with PGE_2 and PGI_2.[12] The lack of recovery of TER in ischemic-injured equine tissues treated with flunixin meglumine suggests that there were sustained increases in mucosal permeability compared to untreated or etodolac-treated ischemic tissues. This lack of recovery was most likely attributable to inhibition of eicosanoid production by flunixin. Clinically, such increases in permeability may result in increased absorption of bacterial toxins, including bacterial endotoxin. Thus, it is conceivable that treatment of horses with intestinal injury with non-specific COX inhibitors may contribute to increased absorption of endotoxin.

The significant recovery of TER in ischemic and etodolac-treated tissues compared to flunixin-treated tissues was not accompanied by any appreciable differences in the histologic indices between the three groups. However, previous studies have associated PG-induced recovery of TER with closure of dilated paracellular spaces and tight junctions; ultrastructural changes that cannot be detected with light microscopy.[22] The specific PGs implicated in mucosal repair via this mechanism are PGE_2 and PGI_2.[12] In the present studies, control, ischemic, and etodolac-treated tissues produced significantly greater levels of PGE_2 and 6-keto-$PGF_{1\alpha}$ (the stable metabolite of PGI_2)

compared to flunixin-treated tissues, which likely explains the poor recovery of TER in flunixin-treated tissues. In previous studies, the inhibitory effect of the non-specific COX inhibitor indomethacin on repair of ischemic-injured porcine ileum was reversed following the addition of PGE_2 and carbacyclin, an analogue of PGI_2.[12]

Etodolac is a specific COX-2 inhibitor,[19,23] whereas flunixin meglumine is a non-specific inhibitor of COX-1 and COX-2. In equine ischemic tissue, treatment with etodolac allowed continued production of PGs compared to flunixin (figs 6-8). However, less PGs were produced in etodolac-treated tissues compared to untreated tissues (figs 6-8). This was probably due to inhibition of COX-2 by etodolac, while allowing PG production by COX-1. This is further supported by the observation that etodolac treatment had no effect on TXB_2 levels when compared to control and untreated ischemic tissues. Thromboxane B_2 is exclusively produced by COX-1 in platelets, and has been used as a marker of COX-1 activity.[23,24] However etodolac did partially inhibit PGE_2 and 6-keto-$PGF_{1\alpha}$ production, suggesting that some reparative PGs are produced by COX-2. Despite the lower levels of PGs in etodolac-treated tissues compared to untreated tissues, recovery of TER was similar. This suggests that PGs produced by COX-1 are sufficient to stimulate repair of injured intestinal mucosa in this equine model. However, other studies have shown an inhibitory action of specific COX-2 inhibitors on repair of gastric ulcers in rats,[25] suggesting that in some instances COX-2 PG production is also required for mucosal repair.

In the present studies, we have shown that COX-2 inhibition via etodolac may be beneficial in the repair of ischemic-injured intestine compared to tissues treated with non-specific COX inhibitors. However, we do not know if a COX-2 inhibitor will be as good

an analgesic as, for example, flunixin meglumine in horses with colic. Etodolac has been shown to be as good an analgesic as non-specific COX inhibitors in a model of arthritis in rats,[26] and similar effects have been demonstrated in humans[27] and dogs.[28] However, in order to assess definitively the effectiveness of selective COX-2 inhibitors in horses with colic, clinical trials will ultimately be required.

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FIGURE LEGENDS

Figure 1: Transepithelial resistance (TER) recorded from equine jejunal mucosa in Ussing chambers. Tissues subjected to 1 hour of ischemia have TER values markedly below those of control tissues at the beginning of the recovery period. Within 4 hours, untreated ischemic tissues and tissues treated with the selective cyclooxygenase-2 inhibitor etodolac (2.7×10^{-5} M) have recovered to values not significantly different from control, whereas tissues treated with flunixin meglumine (2.7×10^{-5} M) showed no evidence of recovery. *$P<0.05$ vs. untreated and etodolac-treated ischemic tissues, 1-way ANOVA, n=8.

Figure 2: Photomicrographs of equine jejunal mucosa. A. Control tissue immediately following resection from the pony. B. Control tissue after 4 hours within an Ussing chamber. Note reduction in villous height, and abnormal appearance to the superficial epithelium at the tip of the villous associated with declining transepithelial resistance documented in figure 1. C. Histologic appearance of tissue immediately following 1-hour of ischemia. Note sloughing of epithelium from the upper third of the villus, exposing sub-epithelial tissues. D. Histologic appearance of untreated ischemic tissue after 4 hours in an Ussing chamber. Note evidence of villous contraction, and flattening of epithelium towards the tip of the villus (epithelial restitution). E. Appearance of flunixin meglumine-treated ischemic mucosa and F. etodolac-treated tissue after 4 hours in an Ussing chamber. There is no apparent difference between treated ischemic tissues and untreated ischemic tissues (D). 1cm bar = 200μm.

Figure 3: Denuded villous surface area in equine jejunal mucosal tissues, as determined by morphometric analysis. Control tissues have essentially no denuding of villi at the beginning of the Ussing chamber experiment (0hr), but develop significant increases in denuded surface area by the end of the Ussing chamber experiment (4hr). Ischemic-injured tissues have markedly elevated denuded surface area at the beginning of the Ussing chamber experiments, but show evidence of significant decreases in denuded surface area by the end of the experiments regardless of treatment. $^{\#}P<0.05$ vs. 0hr control tissues, $*P<0.05$ vs. 0hr ischemic tissues, 1-way ANOVA, n=8.

Figure 4: Villous height in equine jejunal mucosa. Villous height is significantly reduced in both control tissues and ischemic tissues at the end of the Ussing chamber experiment (4hr) compared to tissues at the beginning of the experiment (0hr). $^{\#}P<0.05$ vs. 0hr control tissues, $*P<0.05$ vs. 0hr ischemic tissues, 1-way ANOVA, n=8.

Figure 5: Crypt depth in equine jejunal mucosa. There were significant reductions in the depth of crypts in control tissues during the 4hr recovery period in Ussing chambers, whereas there were no detectable differences in crypt depth in ischemic tissues. $^{\#}P<0.05$ vs. 0hr control tissues, 1-way ANOVA, n=8.

Figure 6: Thromboxane B_2 (the stable metabolite of TXA_2) levels in the serosal bathing solution of equine jejunal mucosal tissues mounted in Ussing chambers. All treatment groups developed significant increases in TXB_2 between hour 1 and hour 4 of the Ussing chamber experiments except flunixin-treated tissues, which had minimal TXB_2 levels

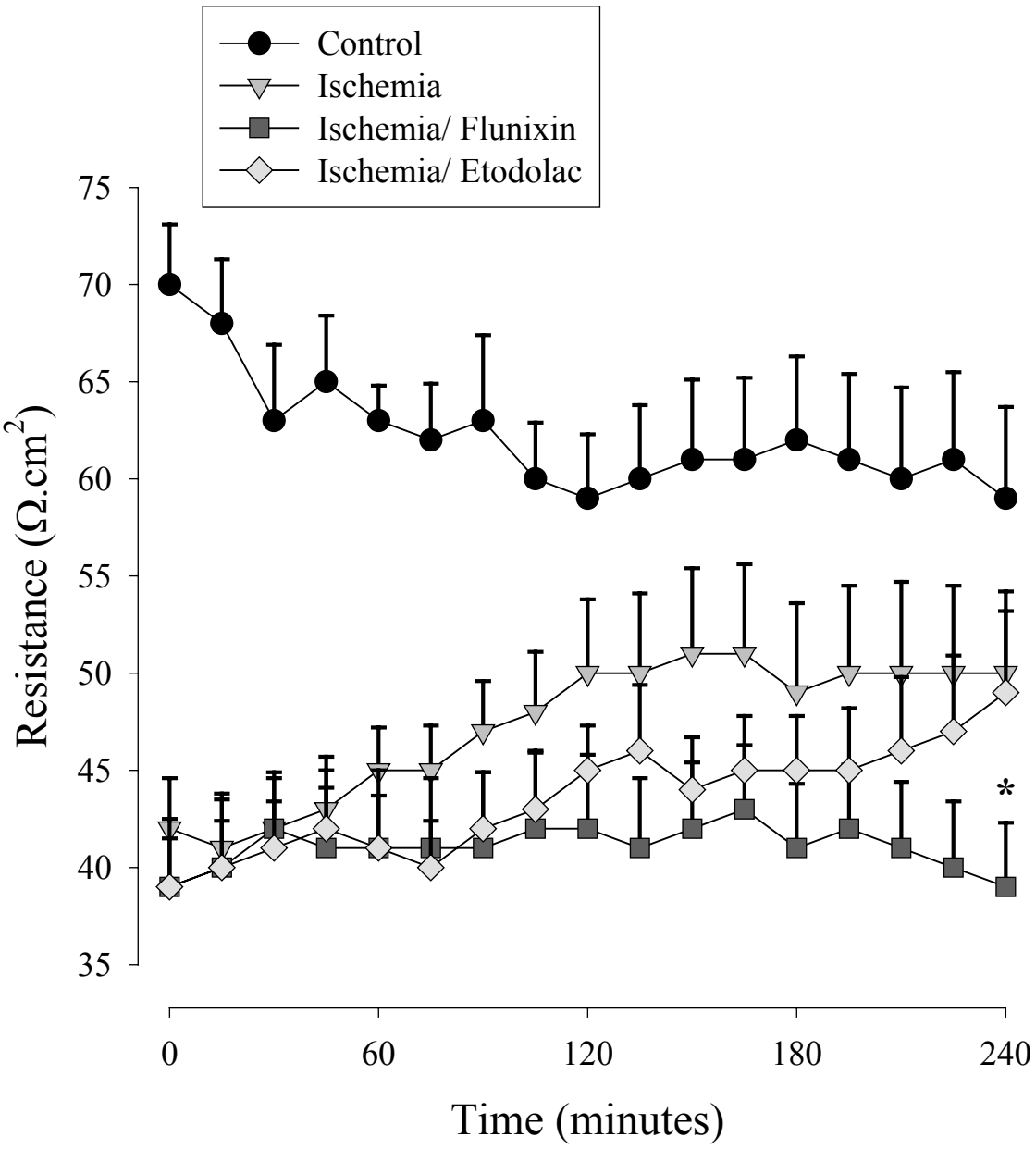
throughout the experiment. There were no significant differences between control, untreated-ischemic, and etodolac-treated ischemic TXB_2 levels at either the hour 1 or hour 4 time points. *P<0.05 vs. hour 1 levels from the same treatment group, #P<0.05 vs. the other treatment groups at the respective time period, 1-way ANOVA, n=8.

Figure 7: Prostaglandin E_2 levels in the serosal bathing solution of equine jejunal mucosal tissues mounted in Ussing chambers. All treatment groups developed significant increases in PGE_2 levels between hour 1 and hour 4 of the Ussing chamber experiments except flunixin-treated tissues, which had minimal PGE_2 levels throughout the experiment. Although etodolac-treated tissues had significant increases in PGE2 levels by hour 4 of the experiments, these levels were significantly below those of control and untreated ischemic tissues at the hour 4 time point. *P<0.05 vs. hour 1 levels from the same treatment group, #P<0.05 vs. the other treatment groups at the respective time period, 1-way ANOVA, n=8.

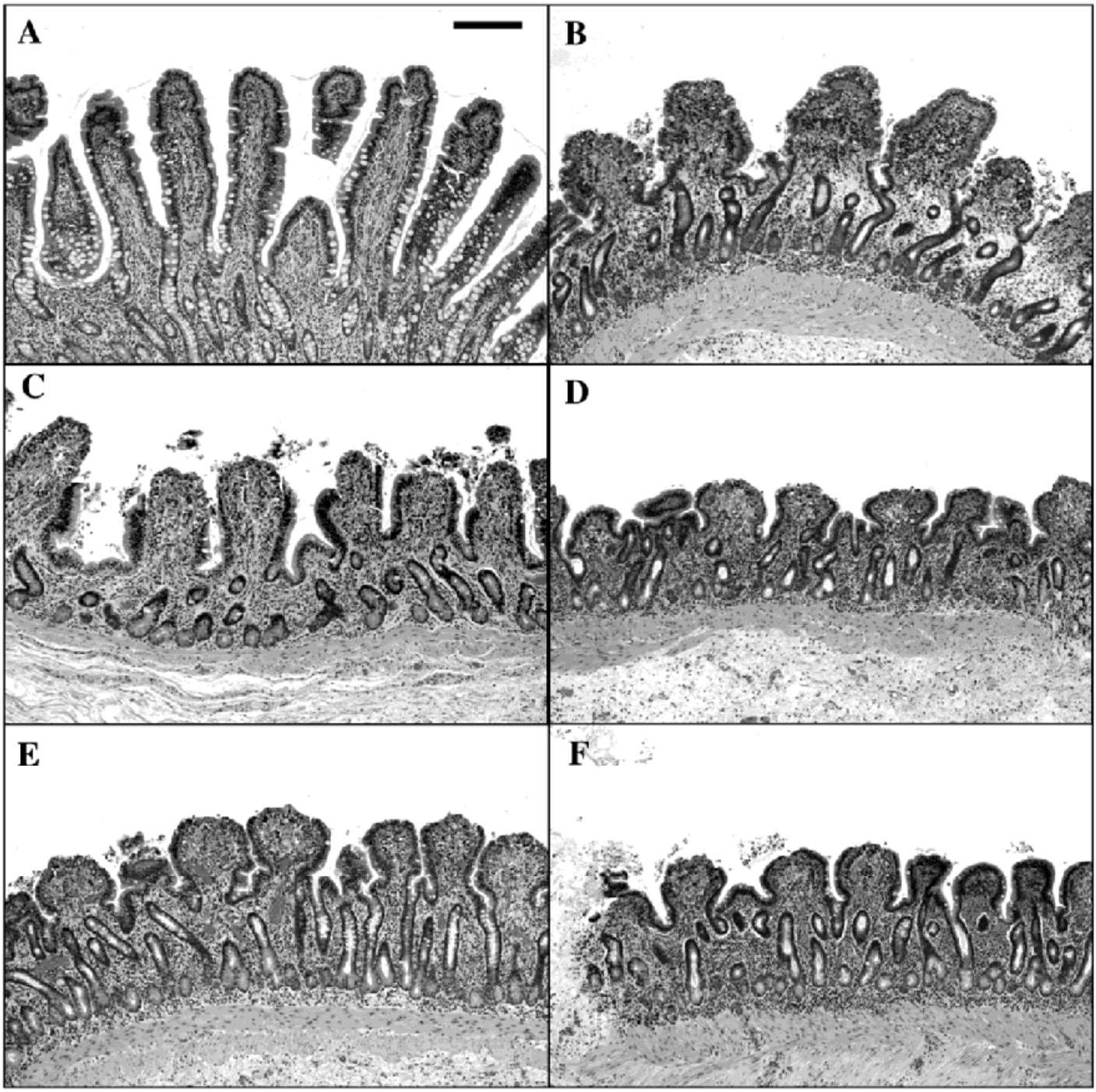
Figure 8: 6-keto $PGF_{1\alpha}$ (the stable metabolite of PGI_2) levels in the serosal bathing solution of equine jejunal mucosal tissues mounted in Ussing chambers. All treatment groups developed significant increases in $PGF_{1\alpha}$ levels between hour 1 and hour 4 of the Ussing chamber experiments except flunixin-treated tissues, which had minimal $PGF_{1\alpha}$ levels throughout the experiment. Although etodolac-treated tissues had significant increases in $PGF_{1\alpha}$ levels by hour 4 of the experiments, these levels were significantly below those of control and untreated ischemic tissues at the hour 4 time point. *P<0.05

vs. hour 1 levels from the same treatment group, $^{\#}P<0.05$ vs. the other treatment groups at the respective time period, 1-way ANOVA, n=8.

Campbell et al. Figure 1



Campbell et al. Figure 2



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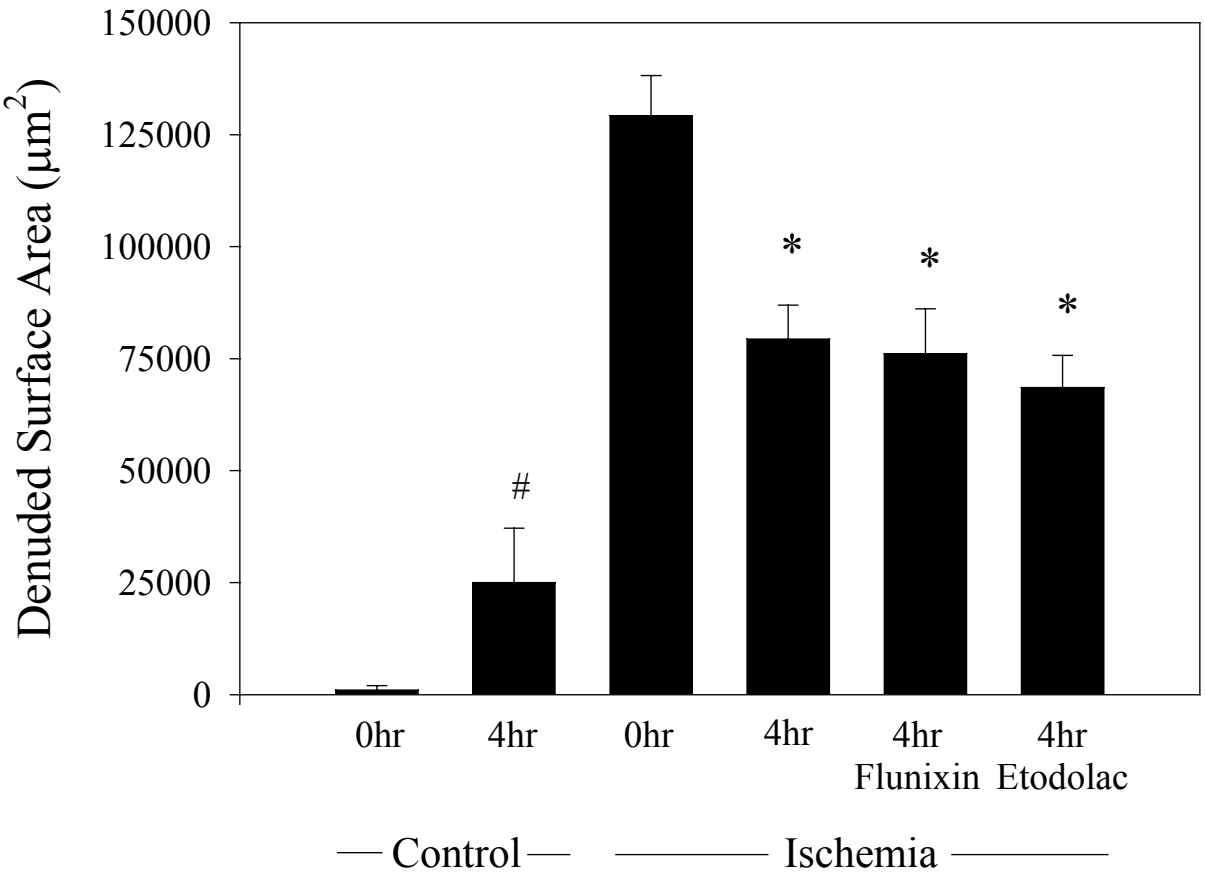

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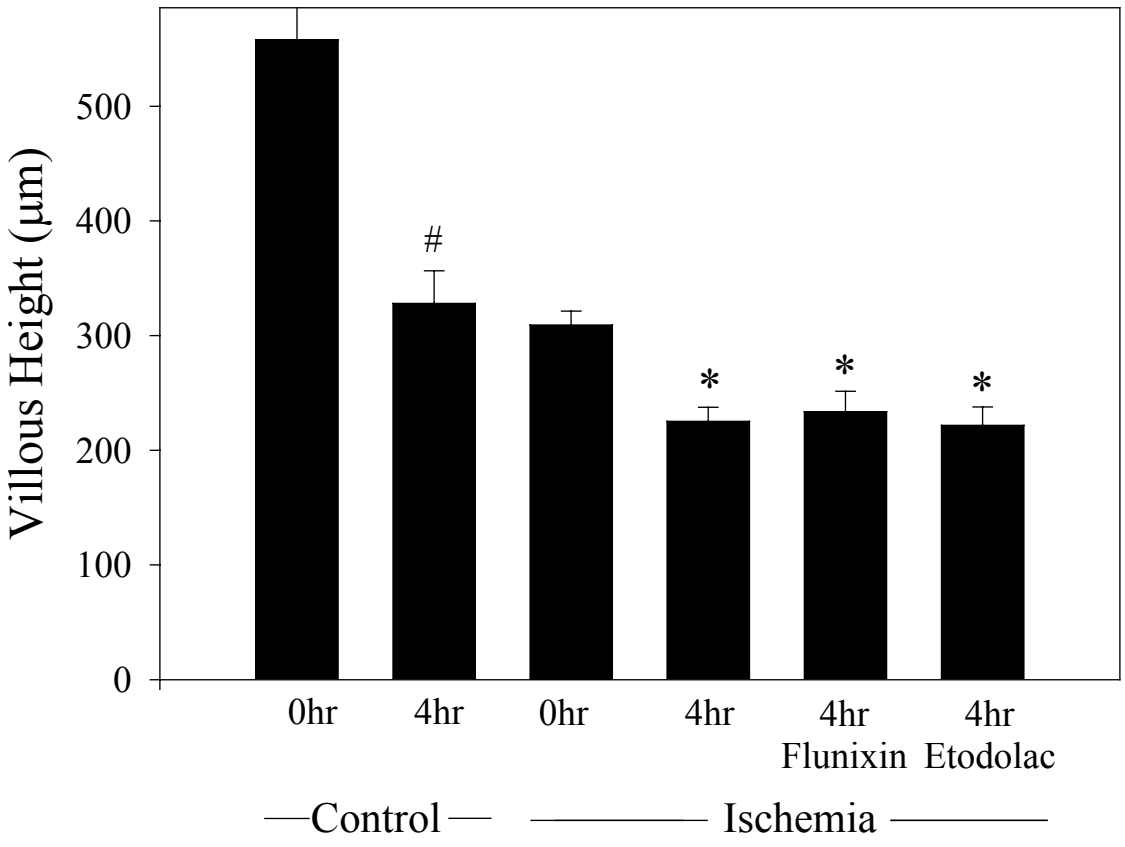

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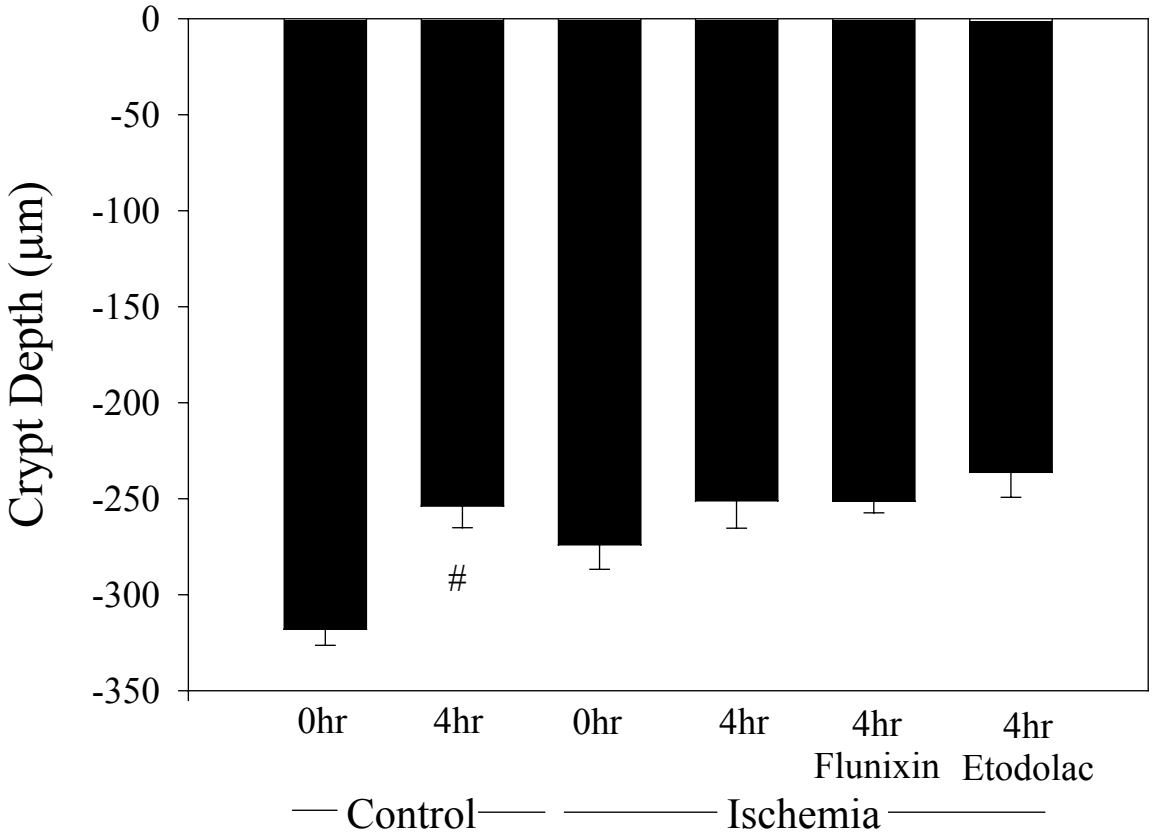

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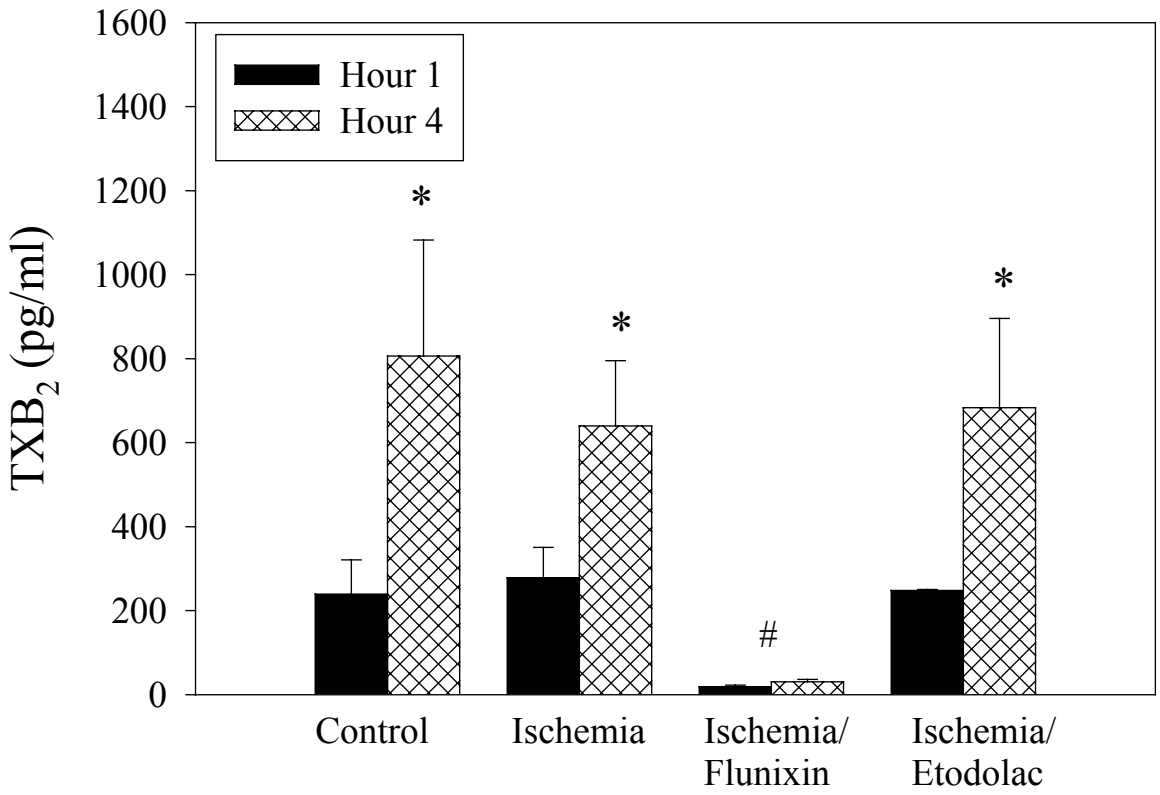

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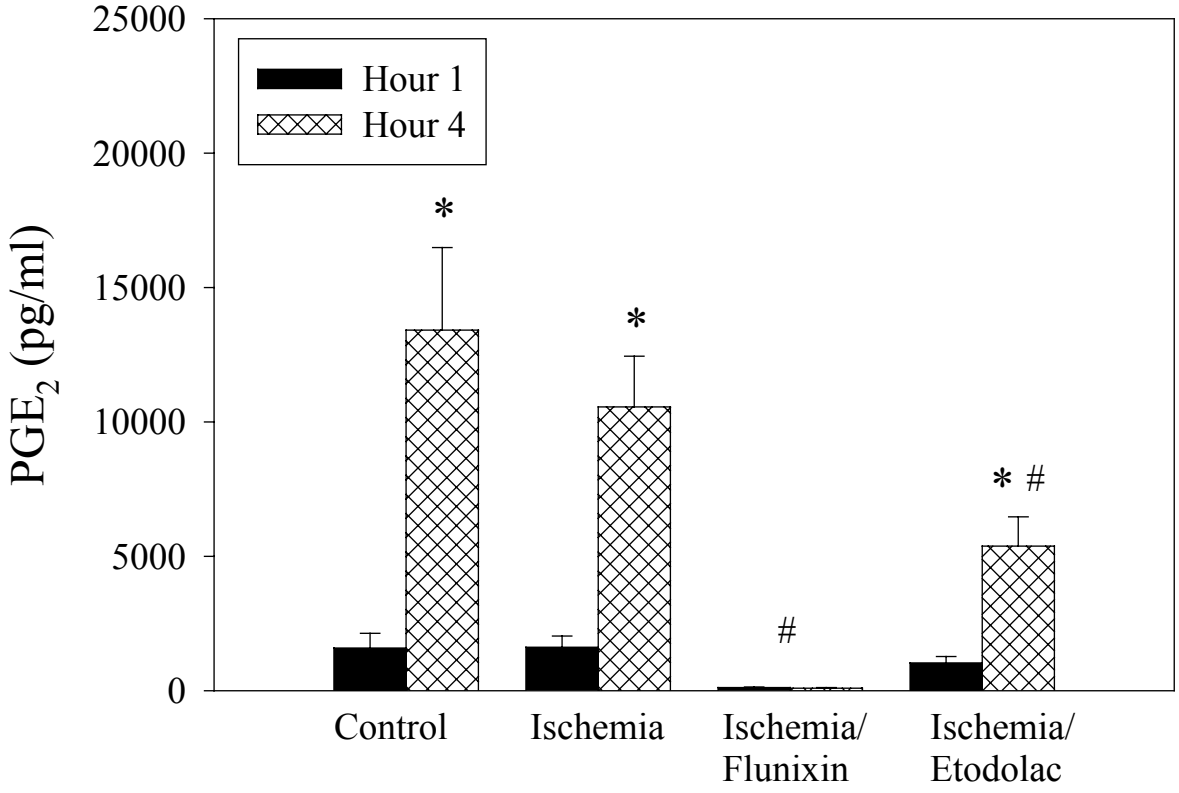
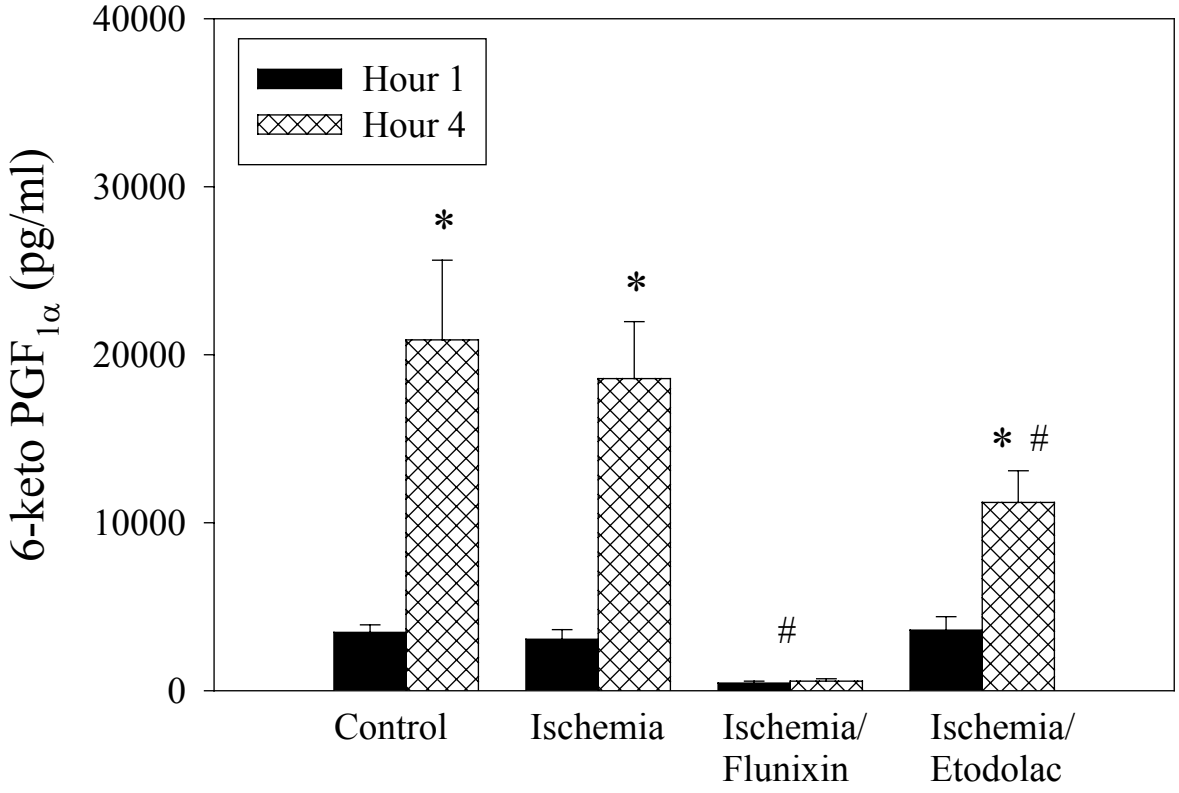


Campbell et al. Figure 8



CHAPTER FOUR

THE EFFECTS OF CYCLOOXYGENASE INHIBITORS ON BILE-INJURED AND NORMAL EQUINE COLON.

by
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SUMMARY

A potential adverse effect of cyclooxygenase (COX) inhibitors (non-steroidal anti-inflammatory drugs [NSAIDs]) in horses is colitis. In addition, we have previously shown an important role for COX-produced prostanoids in recovery of ischemic-injured equine jejunum. It was hypothesized that the non-selective COX inhibitor flunixin would retard repair of bile-injured colon by preventing production of reparative prostaglandins, whereas the selective COX-2 inhibitor, etodolac would not inhibit repair as a result of continued COX-1 activity. Segments of the pelvic flexure were exposed to 1.5mM deoxycholate for 30-minutes, after which they were recovered for 4 hours in Ussing chambers. Contrary to the proposed hypothesis, recovery of bile-injured colonic mucosa was not affected by flunixin or etodolac, despite significantly depressed prostanoid production. However, treatment of control tissue with flunixin led to increases in mucosal permeability, whereas treatment with etodolac had no significant effect. Therefore, although recovery from bile-induced colonic injury maybe independent of COX-elaborated prostanoids, treatment of control tissues with non-selective COX inhibitors may lead to marked increases in permeability. Alternatively, selective inhibition of COX-2 may reduce the incidence of adverse effects in horses requiring NSAID therapy.

INTRODUCTION

In equine medicine, non-steroidal anti-inflammatory drugs (NSAIDs) are used to control orthopaedic pain (usually due to degenerative joint disease) and abdominal pain (colic) by inhibiting the enzyme cyclooxygenase (COX) and preventing the production of prostaglandins (PGs). However, a number of adverse effects of NSAIDs have been noted in horses, including gastrointestinal ulceration[1], right dorsal colitis[2] and renal papillary necrosis.[3] Mucosal blood flow to the right dorsal colon can be increased or decreased by PGs. It is hypothesized that a decrease in blood flow attributable to administration of NSAIDs may be involved in triggering colitis. This may explain why toxicity is most frequently noted in hypovolemic patients.[2] However, toxic effects of NSAIDs (phenylbutazone) in the colon have also been noted in normovolemic horses given therapeutic dosages.[4] There may also be non-vascular mechanisms that contribute to NSAID toxicity that have yet to be identified.

Colitis is frequently fatal in the horse. The primary reason for death is shock associated with the absorption of bacterial lipopolysaccharide (LPS) from damaged intestine. Lipolysaccharide induces production of PGs that exacerbate circulatory shock and abdominal pain.[5] However, prostaglandins also have beneficial effects in the gastrointestinal tract. For example, PGE_2 stimulates restitution; an epithelial repair mechanism that re-surfaces denuded mucosa.[6] Prostaglandins have also been shown to restore barrier function of ischemic-injured intestinal mucosa, possibly via an action on interepithelial tight junctions.[7] Thus, the complete inhibition of prostanoids may prevent intestinal recovery in horses with colitis.

Prostaglandins are produced by two distinct cyclooxygenases: COX-1 and COX-

2.[8] Cyclooxygenase-1 is constitutively expressed in most tissues, including the gut. Cyclooxygenase-2 is not expressed under normal conditions in the gastrointestinal tract, but is induced by a variety of stimuli, particularly LPS[9], and is predominantly expressed at sites of inflammation. Based on their pattern of expression, it has been suggested that COX-1 is responsible for "house-keeping" functions such as maintenance of the intestinal epithelial barrier, whereas COX-2 is responsible for producing prostaglandins that amplify shock, inflammation and pain.[10] Therefore, selective COX-2 inhibitors may inhibit the production of PGs responsible for amplifying pain and shock, while allowing continued COX-1 production of PGs responsible for repair of the epithelial barrier. Recent studies in our laboratory support this by showing that ischemic-injured equine jejunal mucosa fails to recover during *in vitro* testing in the presence of flunixin, while recovery was not inhibited by the COX-2 inhibitor etodolac.[11] However, other studies have shown an important role for COX-2-elaborated prostanoids in recovery of ulcerated gastric mucosa,[12] suggesting that local upregulation of this enzyme may provide important reparative prostanoids. Cycloxygenase-2 may also have anti-inflammatory properties, especially in chronic inflammatory conditions.[13]

In the present study, a model of colonic injury extrapolated from previous porcine studies was used[14] in which equine colon was treated with bile acid, and recovery was monitored *in vitro* within Ussing chambers. It was hypothesized that the non-specific COX inhibitor flunixin[15] would retard repair of bile-injured large colon by preventing production of reparative PGs whereas the selective COX-2 inhibitor, etodolac[16] would permit repair as a result of continued COX-1 PG production.

MATERIALS AND METHODS

Experimental horses and surgery

All procedures were approved by the North Carolina State University Institutional Animal Care and Use Committee. Seven horses (ages 5-15 and weight 450 kg-550 kg) were quarantined for 2 weeks prior to the start of the study and had physical examination parameters within normal limits. The horses were sedated with xylazine (1.1mg/kg IV), anesthetized with diazepam (0.1mg/kg IV) and ketamine (2.2mg/kg IV), intubated and maintained on halothane vaporized in 100% oxygen. Anesthetic monitoring included arterial blood pressure, electrocardiography, and arterial blood gas analysis. Maintenance intravenous fluids [Lactated Ringer's solution (LRS) at 15ml/kg/hr] were administered via a jugular catheter. The large colon was exteriorized via a ventral midline incision and the pelvic flexure identified. Two 30cm loops were created in the pelvic flexure without compromising the blood supply and randomly identified as control or bile- injured. A 5-liter bag of LRS containing deoxycholic acid at a final concentration of 1.5 mM was connected to an IV fluid pump via IV pump tubing. The tubing was placed into the lumen of one end of the colon loop and secured with a pursestring suture. A separate piece of IV pump tubing was placed into the lumen of the colon at the other end of the loop, sutured into place, and re-attached to the bag of deoxycholic acid solution. This created a re-circulating pump system to perfuse the colonic lumen with 1.5mM deoxycholic acid. A similar system was created in the control loop, except that LRS containing no additional treatment was used. After 30 minutes, bile treated and control loops were resected and the horses were humanely destroyed with an overdose of pentobarbital.

Ussing chamber studies

The colon loops were incised along the anti-mesenteric surface. The mucosa was stripped from the seromuscular layer in oxygenated (95% O_2/5% CO_2) equine Ringer's solution[11] containing either no treatment, $2.7x10^{-5}$ M etodolac[a] or $2.7x10^{-5}$ M flunixin meglumine[c] and mounted in Ussing chambers, as described in previous studies.[17] The concentration of flunixin was extrapolated from a peak serum level of 8μg/ml of flunixin reached at a dose of 1.1mg/kg IV according to previous studies.[18] The dose of etodolac was matched to that of flunixin and both drugs were diluted in dimethyl sulphoxide to form stable solutions. Tissues were exposed to treatments from the start of the *in vitro* portion of the experiment. Tissues were bathed on the serosal and mucosal sides with 10ml equine Ringer's solution. The serosal bathing solution contained 10mM glucose, and was osmotically balanced on the mucosal side with 10mM mannitol. Bathing solutions were oxygenated (95% O_2/5% CO_2) and circulated in water-jacketed reservoirs at 37°C. 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 1.0 mV, tissues were current clamped at ±100μA for 5 sec and the PD recorded. Short-circuit current and PD were recorded every 15 min for 4 hours. The data were entered into spreadsheets that calculate resistance (R) from I_{sc} and PD using Ohm's Law.

Histologic studies

Tissues were taken immediately following the surgical procedures, and at the end of Ussing chamber experiments for routine histologic evaluation (5μm cross-sections taken at 300μm intervals and stained with hematoxylin and eosin). A grade scale (0, no damage to 4, extensive damage) was used to assess the amount of damage according to previous descriptions.[19]

Eicosanoid measurements

Samples of the serosal bathing solutions (0.5ml) were taken at 1 hour from the start and at the end of the experiments (4 hours). These samples were snap-frozen in liquid nitrogen, stored at -70^{o}C and analyzed for 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) and Thromboxane B_2 (a stable metabolite of Thromboxane A_2). Assays were performed by the Center for Gastrointestinal Biology and Disease, Chapel Hill, NC, using commercial ELISA kits.[d]

Flux measurements

To assess tissue permeability to LPS, mucosal-to-serosal fluxes of ^{14}C-labeled Salmonella typhimurium LPS[e] were initiated by adding 0.2μCi/ml to the mucosal solution of tissues. Following a 15-minute equilibration period, standards were taken from the bathing reservoirs. Subsequently, three 1-hour fluxes were performed by taking samples from the serosal bathing reservoirs. Samples were assessed for β emission (counts/minutes). Mucosal-to-serosal fluxes of ^{14}C-LPS (J_{ms}) were determined using standard equations.[20]

Statistical analysis

Since all data were normally distributed, parametric tests were used for all analyses. For experiments with multiple values recorded over a 240-minute time period, 2-way ANOVA on repeated measures was performed for the effects of time and treatment, and time-treatment interactions. A post-hoc Tukey's test was used to test for statistical differences between treatments. For analyses with a significant time-treatment interaction, 1-way ANOVA was performed at select time points to distinguish differences between treatments. For all other data, a 1-way ANOVA was performed to discern significant differences between treatments. For all statistical analyses a P value of <0.05 was considered significant. Each data point represents a single animal used throughout the study.

RESULTS

Ussing chamber studies

Tissues exposed to 1.5 mM deoxycholic acid for 30 minutes had transepithelial resistance (TER) measurements approximately one third that of control tissues at the beginning of the experiment (Fig. 1). Control tissue showed a relatively stable TER over the 4-hour period of the experiment. The TER of untreated bile-injured tissue showed a gradual recovery response with a 2-fold increase in TER over 4 hours. However, there were no significant differences between the treatment groups, although there was a tendency for flunixin and etodolac to reduce recovery of TER (Fig. 1).

Treatment effects were far more noticeable in control tissues. Over the last 2-hour period of the experiment, there was a significant decrease in TER between control tissue

and control tissue treated with etodolac or flunixin (Fig. 2). In addition, there was also a significant decrease in TER between control tissue treated with etodolac compared with control tissue treated with flunixin.

Histologic studies

Histopathologic examination of mucosa immediately after 30 minutes of exposure to 1.5 mM deoxycholic acid regardless of treatment, showed a median damage grade of 3.5 (range 1-4, loss of single cells to barren surface with sloughed epithelial sheets). After 4 hours in the Ussing chambers, evidence of epithelial restitution was noted, resulting in continuous epithelial coverage regardless of treatment group (Fig. 3). In control tissues, there was no histologic evidence of mucosal damage at the beginning of the Ussing chamber experiments or at the end of the 240-minute recovery period regardless of treatment.

Eicosanoid measurements

There were significant ($P < 0.05$) increases in 6-keto-$PGF_{1\alpha}$ and TXB_2 concentrations in the serosal bathing solution in control tissues and bile-injured tissue that received no treatment at hour 4 compared to hour 1 of the experiment (Figs. 4-7). There was also a significant difference between 6-keto-$PGF_{1\alpha}$ and TXB_2 concentrations in bile-injured tissue that received no treatment compared to bile-injured tissue treated with etodolac and flunixin. However, there was no significant difference in eicosanoid concentrations in bile-injured tissue treated with etodolac or flunixin at hour 4 when compared to hour 1 of the study. There was no significant difference in TXB_2 concentration in control tissue treated with etodolac or flunixin. However, there was a

significant difference in the 6-keto-$PGF_{1\alpha}$ concentration in control tissue treated with flunixin when compared to control tissue treated with etodolac ($P<0.05$).

Flux measurements

Because of the dramatic changes in TER in control tissues treated with flunixin, we wanted to assess the clinical relevance of these findings by determining tissue permeability to LPS. Accordingly, there was a significant increase in the flux of ^{14}C-labeled LPS when comparing tissues treated with flunixin to either untreated control tissue or etodolac-treated control tissues (Fig. 8). However, there was no significant difference in flux of ^{14}C-labeled LPS between untreated control tissue and control tissue treated with etodolac.

DISCUSSION

Transepithelial resistance (TER) is a highly sensitive measure of the ionic permeability of intestinal mucosa. Recent studies have shown close correlations between recovery of TER and reductions in mucosal permeability in ischemic-injured tissues treated with prostaglandins.[7] From the TER data in the present studies, it can be seen that exposure to 1.5 mM deoxycholic acid caused significant damage to the mucosa of the colon ($35\pm1.8\ \Omega.cm^2$ compared to $104\pm14.7\ \Omega.cm^2$ in control tissue). The TER of the bile-injured tissue increased to $92\pm11.7\ \Omega.cm^2$ after 4 hours, indicating the ability of the tissue to initiate a recovery response in this ex vivo preparation. The significant recovery of TER in bile-injured tissues occurred in conjunction with an improvement in the histologic indices measured, suggesting that recovery of TER was attributable to epithelial restitution. In contrast to previous studies on ischemic-injured jejunum, there

was no significant inhibitory effect of flunixin on tissue recovery.

Some studies indicate that etodolac is less selective for COX-2 than other inhibitors,[21] suggesting that the lack of difference between the effects of flunixin and etodolac in bile-injured tissue may relate to their ability to inhibit both COX isoforms. The selectivity, which is usually calculated using COX-1:COX-2 IC_{50} ratio, is dependent on the type of COX *in vitro* selectivity assay used. Thus, a pharmacokinetic/ pharmacodynamic approach assessing whole animal measurements of potency and selectivity should be used to support or refute the results of *in vitro* assays.[22]

The reasons that flunixin inhibited recovery of equine ischemic-injured tissue, but not equine bile-injured tissue, may relate to distinct mechanisms of repair in the two models. In ischemic-injured jejunum, there was no observable histologic difference between tissues treated with or without flunixin, showing that changes in paracellular resistance, regulated by inter-epithelial tight junctions, may be responsible for differences in treatment groups.[11] This supposition is supported by a number of studies performed in ischemic-injured porcine ileum, which have shown that prostaglandins play a critical role in recovery of mucosal paracellular resistance.[7,23] In contrast, the repair mechanism that appears to dominate recovery of bile-injured colon would appear to be epithelial restitution. Porcine studies utilizing the same model support this premise.[14,19]

Treatment of control tissue with etodolac or flunixin had more dramatic effects than treatment of bile-injured tissues. In particular, there were significant decreases in TER over the last two hours of the experiment under the influence of either etodolac or flunixin, but flunixin was significantly more damaging than etodolac. These data are suggestive of an important role of prostanoids in the maintenance of mucosal barrier

function, as has been shown in numerous studies in other species.[24,25] Although COX inhibitors may have direct topical irritant effects,[26] eicosanoid analyses in the present study suggest that the effects of flunixin and etodolac on control tissue are attributable to reductions in prostaglandin and thromboxane levels. Although both drugs prevented elevations in TXB_2 over the 4-hour recovery period in control tissues, etodolac inhibited 6-keto-$PGF_{1\alpha}$ to a lesser extent than flunixin. This suggests that the residual concentration of this prostanoid following etodolac treatment is produced by COX-1, and helps ameliorate NSAID damage in colonic tissue. Clinically, use of NSAIDs such as flunixin may increase colonic permeability, which may in turn result in increased absorption of bacterial toxins such as LPS, and protein loss. This supposition is supported by the significant increase in LPS flux noted in equine colonic tissues treated with flunixin.

The data on TXB_2 levels in the present study were somewhat unexpected, because this prostanoid has been used as a marker of COX-1 activity in some tissues,[27,28] including equine jejunum.[11] However etodolac, a COX-2 inhibitor, inhibited production of TXB_2 to the same extent as the non-selective COX inhibitor flunixin. These data can be interpreted in two ways: either etodolac is not selective enough for COX-2 to allow discrimination between COX isoforms, or TXB_2 is also produced by COX-2 in bile-injured colon. The latter may be true, since studies have clearly shown elevations in TXB_2 in horses given intravenous LPS,[29] a potent stimulus for COX-2 up-regulation. However, this would also mean that control tissues in the present study had COX-2 activity, which may have been induced by surgical manipulation or stripping in preparation for mounting in Ussing chambers, and which may account for the significant

increase in TXB_2 noted at the end of the study.

The decrease in TER in control tissues treated with etodolac or flunixin was not accompanied by any significant change in the histologic indices, suggesting changes in paracellular resistance are responsible for decreases in TER. The paracellular space is regulated by inter-epithelial tight junctions, which form a continuous gate at the apical aspect of adjoining cells. The relative permeability of tight junctions is tissue specific. In particular, the small intestine has relatively 'leaky' tight junctions, whereas those in the colon are more tightly apposed.[30] This results in a higher baseline TER in colon compared to jejunum.[11] Thus, fairly minor damage to tight junctions, which may have occurred in the present studies in tissues treated with COX inhibitors, could result in relatively large changes in TER. However, the significant increase in the flux of ^{14}C-labeled LPS across the mucosa in flunixin-treated control tissue suggests that these junctional changes are of clinical significance, which could lead to LPS absorption or protein loss.

In the present studies, we have shown that exposure of colonic mucosa to 1.5 mM deoxycholic acid for 30-minutes leads to injury of mucosa and the recovery from that injury is for the most part independent of COX-elaborated prostanoids. In addition, we have shown that non-selective COX inhibition in normal colonic mucosa, with flunixin, leads to a marked increase in permeability. Cyclooxygenase-2 inhibition, with etodolac, also triggered increases in permeability, but to a lesser extent than non-selective COX inhibition. This suggests etodolac may have reduced side effects in horses requiring NSAID therapy.

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FIGURE LEGENDS

Figure 1: Transepithelial electrical resistance (TER) of tissues subjected to luminal instillation of deoxycholic acid (1.5mM) for 30-minutes. Resistance values progressively increase over the 240-minute in vitro experiment, indicating recovery of mucosal barrier function. There were no significant differences between treatment groups, although there was a trend toward inhibition of recovery of TER in tissues treated with the non-selective COX inhibitor flunixin, or the selective COX inhibitor etodolac. (Mean ± S.E.M. from seven animals)

Figure 2: Transepithelial electrical resistance (TER) of normal colonic mucosa. Untreated tissues had relatively stable TER values during 240-minute *in vitro* studies, whereas as those treated with either flunixin or etodolac had progressive reductions in TER. There was a significant reduction in TER when comparing flunixin-treated tissues to control tissues and etodolac-treated tissues ($P<0.05$, 2-way ANOVA on repeated measures). (Mean ± S.E.M. from seven animals)

Figure 3: Histologic appearance of tissues following exposure to 1.5mM deoxycholic acid for 30-minutes and subsequent treatment with COX inhibitors. A. Appearance of normal colonic mucosa prior to placement in Ussing chambers. B. Exposure of colonic mucosa to 1.5 mM deoxycholic acid for 30-minutes resulted in sloughing of superficial surface epithelium, although crypt epithelium remained in tact. C. Deoxycholic acid-injured tissues repair while maintained for 240-minutes *in vitro.* Epithelium adjacent to previously denuded regions have undergone restitution, resulting in a flattened

appearance to surface epithelial cells. D. Normal colonic mucosa following 240-minute *in vitro* experiments looks similar to tissues prior to mounting in Ussing chambers. E. Despite reductions in TER in tissues treated with flunixin, continuous epithelial coverage is maintained over the 240-minute recovery period. 1cm bar = 200μm.

Figure 4: TXB_2 (the stable metabolite of TXA_2) levels in deoxycholic acid-treated colonic tissues during 240-minute *in vitro* experiments. There was a significant increase in TXB_2 levels in the bathing solution of injured colonic tissues when comparing 240-minute samples with 60-minute samples, and these levels were significantly increased compared to tissues treated with either etodolac or flunixin (#P<0.05, 1-way ANOVA). (Mean ± S.E.M. from seven animals)

Figure 5: 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2) levels in deoxycholic acid-treated colonic tissues during 240-minute *in vitro* experiments. Similar to TXB_2 measurements, there was a significant increase in 6-keto-$PGF_{1\alpha}$ levels in the bathing solution of colonic tissues when comparing 240-minute samples with 60-minute samples, and these levels were significantly increased compared to tissues treated with either etodolac or flunixin (#P<0.05, 1-way ANOVA). (Mean ± S.E.M. from seven animals)

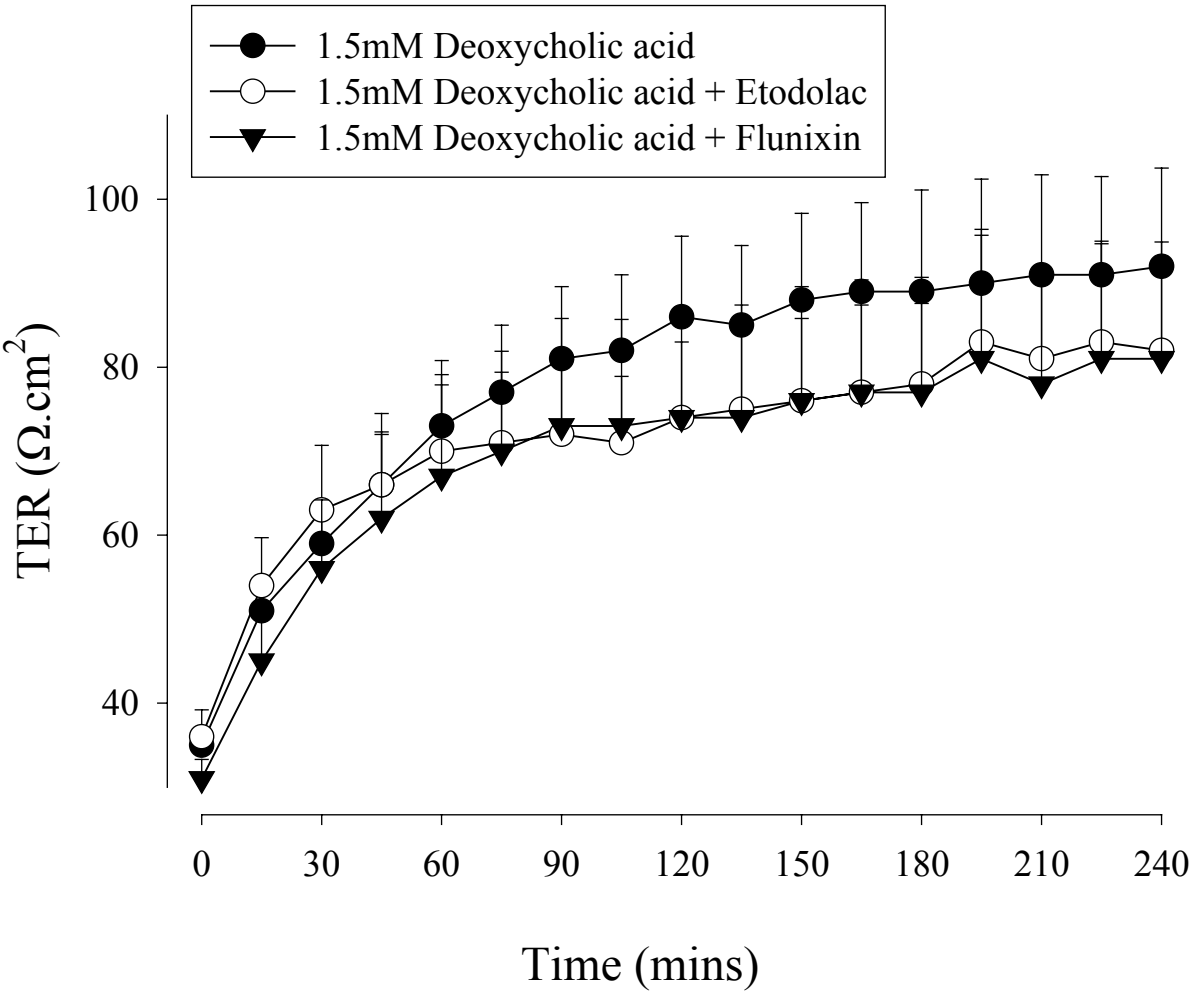
Figure 6: TXB_2 (the stable metabolite of TXA_2) levels in normal colonic tissues during 240-minute *in vitro* experiments. There was a significant increase in TXB_2 levels in the bathing solution of colonic tissues when comparing 240-minute samples with 60-minute samples, and these levels were significantly increased compared to tissues treated with

either etodolac or flunixin (#P<0.05, 1-way ANOVA). (Mean ± S.E.M. from seven animals)

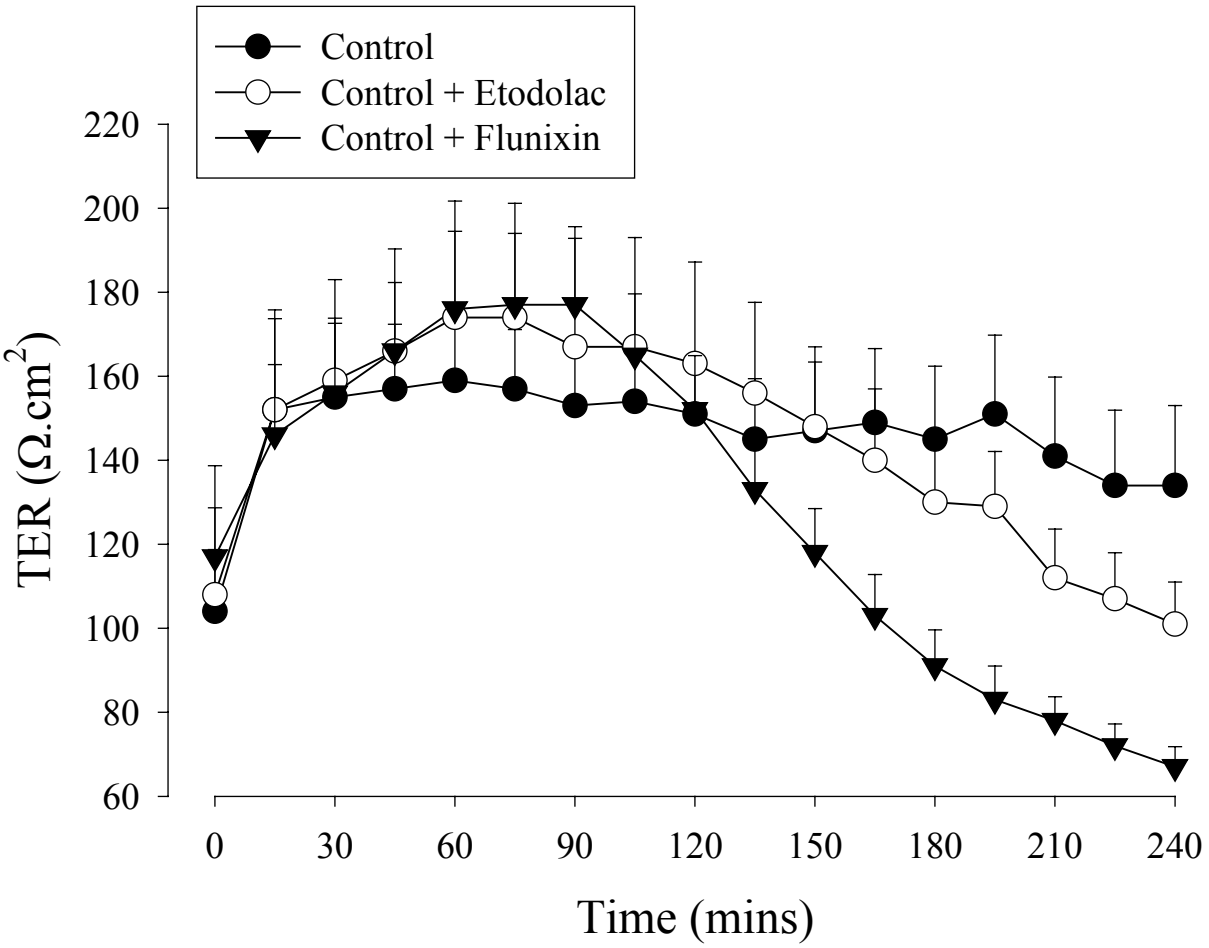
Figure 7: 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2) levels in normal colonic tissues during 240-minute *in vitro* experiments. There was a significant increase in 6-keto-$PGF_{1\alpha}$ levels in the bathing solution of injured colonic tissues when comparing 240-minute samples with 60-minute samples, and these levels were significantly increased compared to tissues treated with either etodolac or flunixin (#P<0.05, 1-way ANOVA). There was also a significant difference between tissues treated with etodolac and tissues treated with flunixin at 240-minutes *P<0.05, 1-way ANOVA). (Mean ± S.E.M. from seven animals)

Figure 8: Mucosal-to-serosal fluxes of ^{14}C-labeled lipopolysaccharide (LPS) in normal colonic tissues. As further evidence of diminished colonic barrier function in tissues treated with flunixin, there was a significant increase in flux of LPS when compared to tissues that remained untreated, or tissues treated with etodolac for 240-minutes (#P<0.05 vs., control or etodolac-treated tissues, 1-way ANOVA). (Mean ± S.E.M. from seven animals)

Campbell et al. Figure 1



Campbell et al. Figure 2



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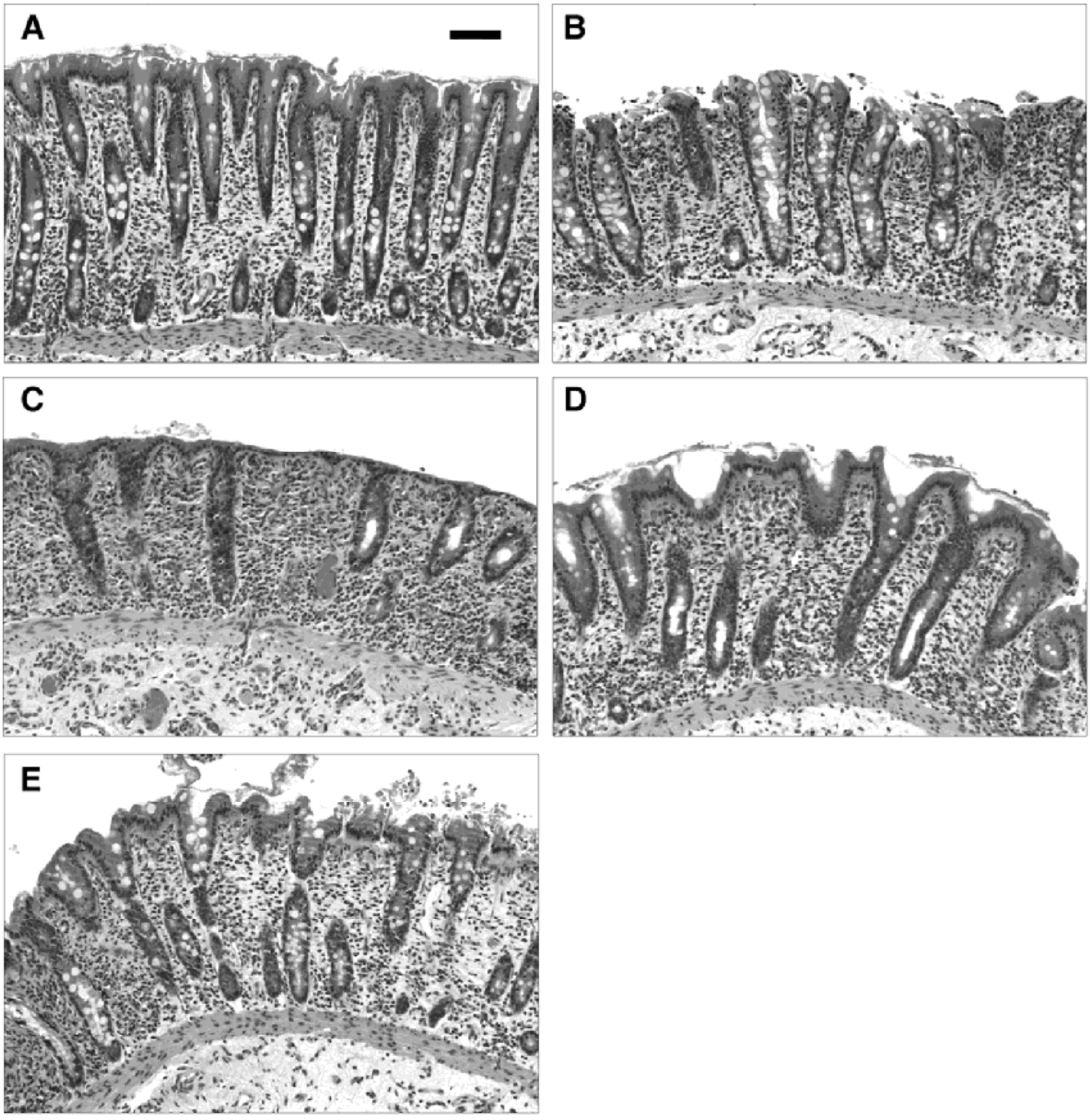

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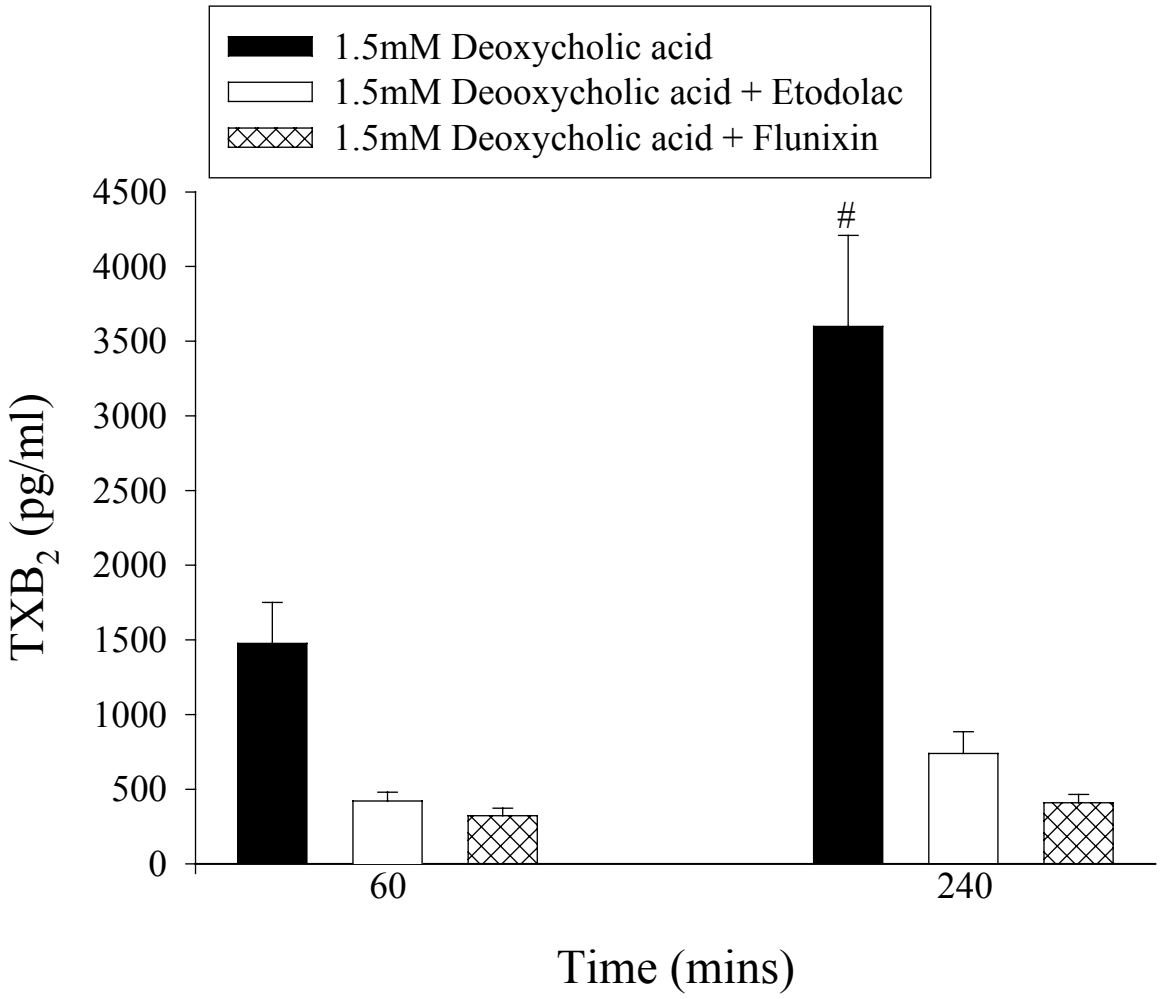

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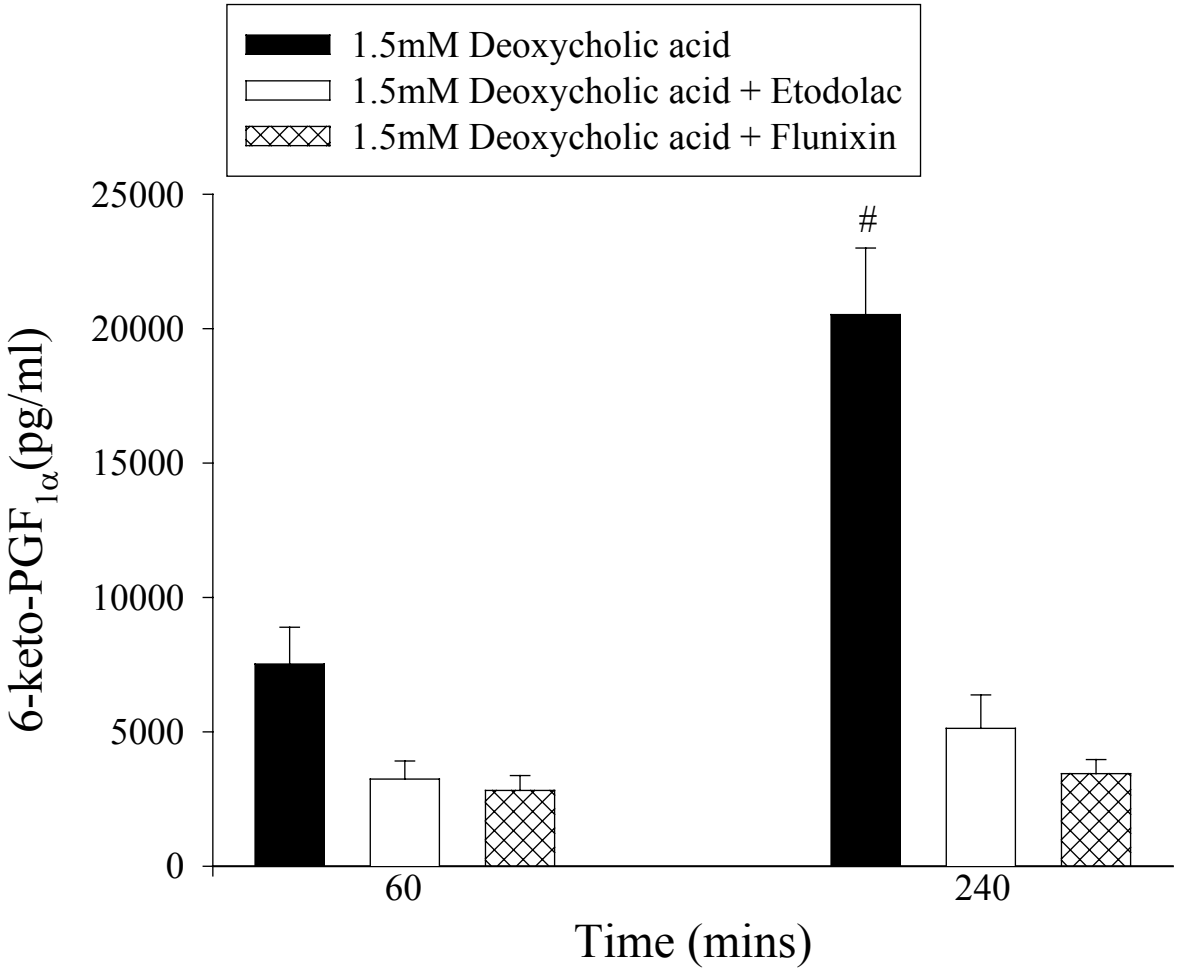

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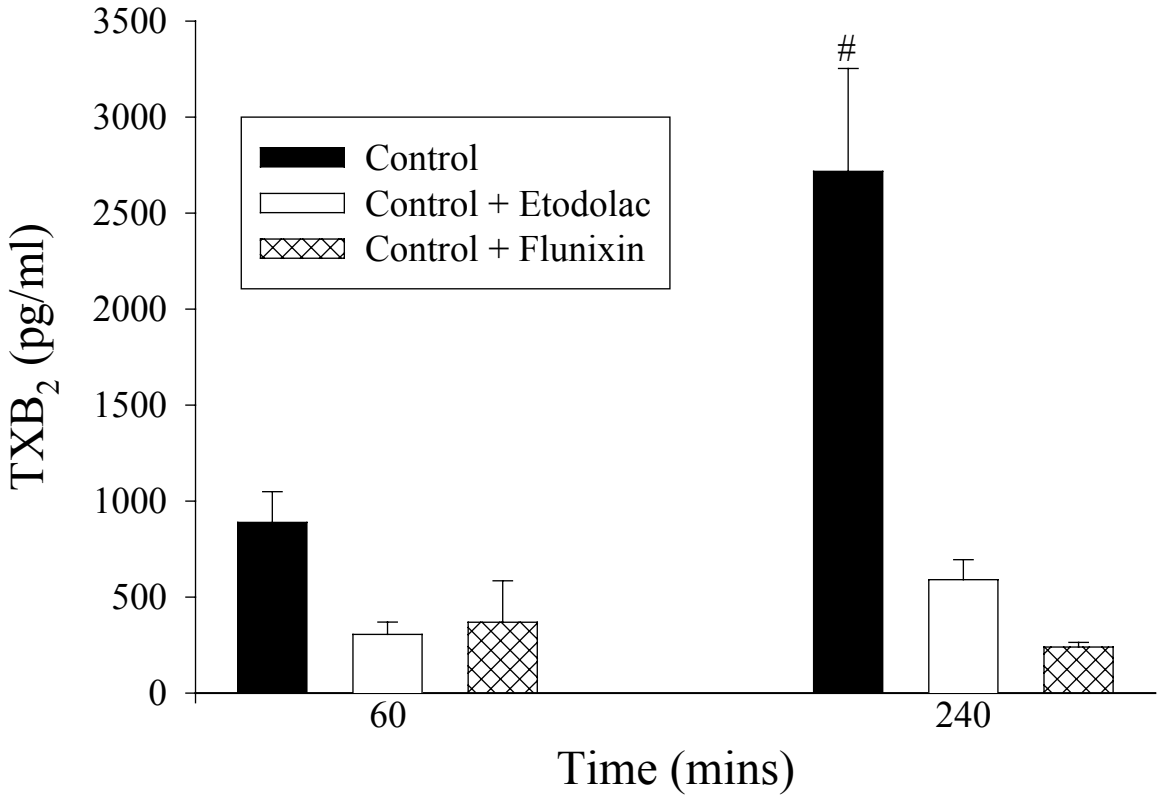

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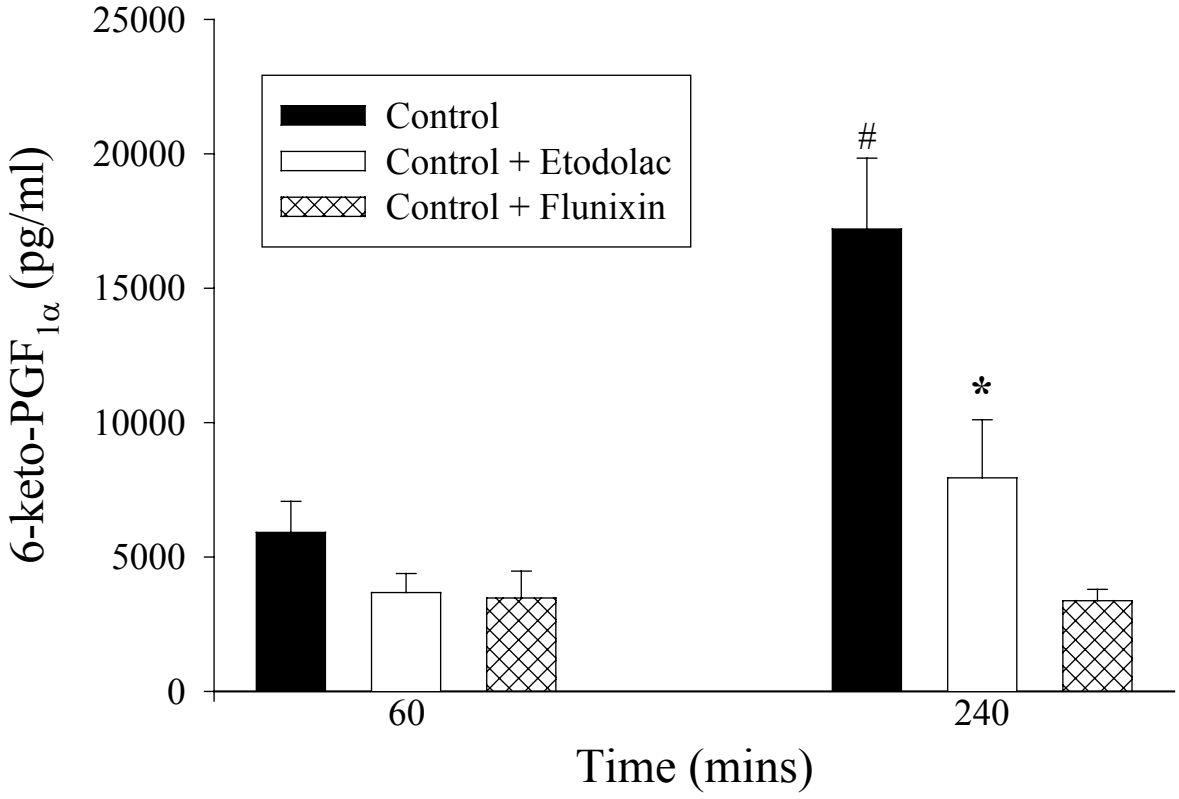

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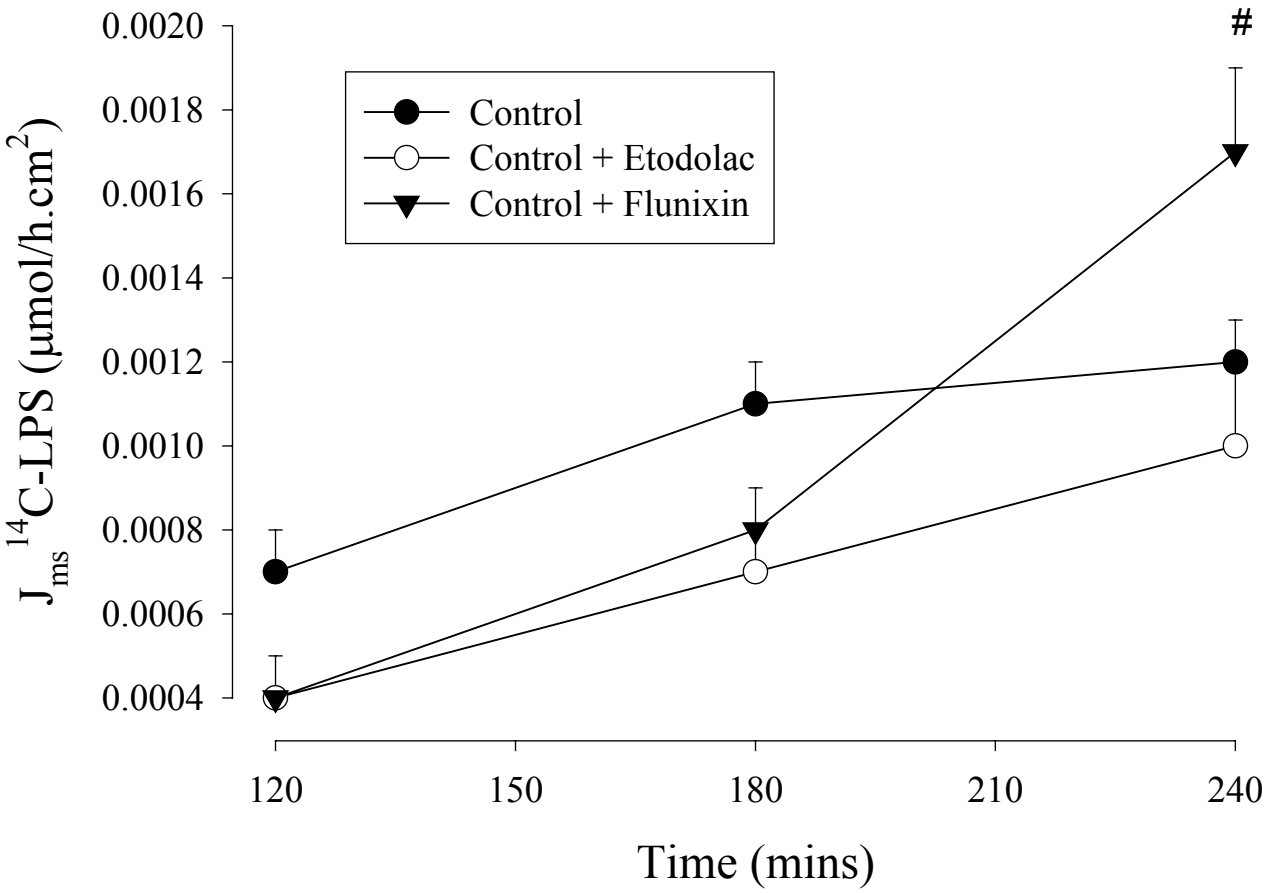

CHAPTER FIVE

PHYSIOLOGIC CONCENTRATIONS OF BILE SALTS INHIBIT RECOVERY OF ISCHEMIC-INJURED PORCINE ILEUM

by
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Abbreviations used in this paper: Isc, short circuit current; TER, transmucosal electrical resistance

SUMMARY

Physiologic concentrations of bile salts inhibit recovery of ischemic-injured porcine ileum – We have previously shown rapid *in vitro* recovery of barrier function in porcine ischemic-injured ileal mucosa, attributable principally to reductions in paracellular permeability. However, these experiments did not take into account the effects of normal luminal contents, such as bile salts, which, according to our preliminary studies, reach concentrations as high as 10^{-5}M in the porcine ileum. Therefore, the objective of this study was to evaluate the role of deoxycholic acid in recovery of mucosal barrier function. Porcine ileum was subjected to 45-minutes of ischemia, after which mucosa was mounted in Ussing chambers, and exposed to varying concentrations of deoxycholic acid. The ischemic episode resulted in significant reductions in transepithelial electrical resistance (TER), which recovered control levels of TER within 2-hours, associated with significant reductions in mucosal-to-serosal ^{3}H-mannitol flux. However, treatment of ischemic-injured tissues with 10^{-5}M deoxycholic acid fully inhibited recovery of TER with significant increases in mucosal-to-serosal ^{3}H-mannitol flux, whereas 10^{-6}M deoxycholic acid had no effect. Histologic evaluation at 2 hours revealed complete restitution regardless of treatment, indicating the breakdown in barrier function was due to changes in paracellular permeability. Similar effects were noted with application of 10^{-5}M taurodeoxycholic acid, and the effects of deoxycholic acid were reversed with application of the Ca^{2+} mobilizing agent thapsigargin. Deoxycholic acid at physiologic concentrations significantly impairs recovery of epithelial barrier function by an effect on paracellular pathways, and these effects appear to be Ca^{2+}-dependent.

INTRODUCTION

Bile is a normal constituent of the luminal contents of the mammalian small intestine, the main function of which is to aid fat digestion and absorption. The primary bile acids, cholic acid and chenodeoxycholic acid are synthesized in the liver from cholesterol and are conjugated with taurine or glycine to form taurocholic and glycocholic acids. They can undergo further metabolism, by enteric bacteria for example, to form secondary bile acids, such as deoxycholic acid, lithocholic acid, and taurodeoxycholic acid.[1]

The effect of bile salts on the intestinal epithelium has been studied by a number of investigators. For example, taurodeoxycholic acid caused an increase in the permeability of the gastric mucosa to varying sizes of polyethylene glycol molecules.[2] In the rabbit small intestine, chenodeoxycholic acid and ursodeoxycholic acid (a tertiary bile acid, derived form a secondary bile acid) increased the rate of transmural flux of lactulose in the jejunum and ileum,[3] while the salt of deoxycholic acid, deoxycholate, increased the mucosal-to-serosal flux of mannitol in the rat and the rabbit.[4] Electron microscopy revealed wider intercellular spaces with loss of contact points between the cellular membranes. Deoxycholate also caused increases in mucosal permeability in porcine colon[5,6] and porcine ileum.[7] Damage to the colonic mucosa ranged from slight (with loss of single cells) with 1.5mM deoxycholate to extensive (with a barren mucosal surface and sloughed epithelial sheets) with 3 to 21mM deoxycholate. Removal of the bile salts was followed by rapid recovery of mucosal barrier function in both the ileal and colonic studies.

Although this work has provided important insight into the mechanisms of injury

and repair in intestinal mucosa subjected to bile salts, all of these studies used bile salt concentrations that are much higher (0.1mM to 21mM) than physiological concentrations of bile salts documented in the intestinal lumen ($6\pm3x10^{-6}$M from our preliminary studies of porcine ileal contents). Furthermore, most of these studies assessed the effects of bile salts on normal mucosa. However, one study evaluated the effects of bile on rat jejunum subjected to 30-minutes of ischemia, and showed that 1mM taurodeoxycholic acid and taurochenodeoxycholic acid did not exacerbate gut permeability to sodium fluorescein.[8] In another study, low dose deoxycholate (50μM) added prior to the addition of 250μM deoxycholate attenuated the injury produced by 250μM deoxycholate alone on gastric mucosa.[9] The mechanism of attenuation triggered by low dose deoxycholate may have involved an effect on intracellular calcium. In addition, increasing calcium levels to 4mM inhibited the damaging effect of 5mM deoxycholic acid on colonic epithelium.[10]

Previous studies of mucosal healing in porcine ischemic-injured ileum have shown that recovery of barrier function following ischemic injury occurs rapidly in studies performed *in vitro*,[11] but these experiments did not take into account the effects of luminal contents. Since bile salts cause dose-dependent injury to intestinal epithelium, and relatively high concentrations of bile may be present in mammalian small intestine, the objective of the present study was to evaluate the role of physiologic doses of deoxycholic acid in recovery of mucosal barrier function in porcine ischemic-injured ileum.

MATERIALS AND METHODS

Experimental animal surgeries

All studies were approved by the North Carolina State University Institutional Animal Care and Use Committee. Six to eight-week-old Yorkshire crossbred pigs of either sex were housed singularly, and maintained on a commercial pelleted feed. Pigs were held off feed for 24 hours prior to experimental surgery. General anesthesia was induced with xylazine (1.5 mg/kg, IM), ketamine (11 mg/kg, IM) and pentobarbital (15mg/kg, IV) and was maintained with intermittent infusion of pentobarbital (6-8mg/kg/hr). Pigs were placed on a heating pad and ventilated with 100% O_2 via a tracheotomy using a time-cycled ventilator. The jugular vein was cannulated, and blood gas analysis was performed to confirm normal pH, and partial pressures of CO_2 and O_2. Lactated Ringers solution was administered intravenously at a maintenance rate of 15ml/kg/hr. Blood pressure was continuously monitored via a transducer connected to the carotid artery. The ileum was approached via a ventral midline incision. Samples of ileal luminal contents were removed and ileal segments delineated by ligating the intestinal lumen at 10-cm intervals, and then subjected to ischemia by ligating the local mesenteric blood supply for 45 minutes.

Ussing chamber studies

Following the ischemic period, the mucosa was stripped from the seromuscular layer in oxygenated (95% O_2/ 5% CO_2) Ringer's solution, and mounted in 3.14 cm^2 aperture Ussing chambers. Tissues were bathed on the serosal and mucosal sides with 10ml Ringer's solution. The serosal bathing solution contained 10mM glucose, and was osmotically balanced on the mucosal side with 10mM mannitol. Bathing solutions were

oxygenated (95% O_2/5% CO_2) and circulated in water-jacketed reservoirs. Bile acids (deoxycholic acid or taurodeoxycholic acid) were added to the mucosal side of the Ussing chamber to achieve a final concentration of 10^{-3}M, 10^{-4}M, 10^{-5}M and 10^{-6}M. 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 ($\Omega \cdot cm^2$) was calculated from the spontaneous PD and short-circuit current (Isc). If the spontaneous PD was between -1.0 and 1.0 mV, tissues were current clamped at ±100 μA for 5 seconds and the PD recorded. Short-circuit current and PD were recorded every 15 minutes for 4 hours.

Bile acid assays

Ileal luminal samples were centrifuged in a microcentrifuge at 3000 RPM for 15-minutes. Supernatants were collected, and the 3α-hydroxy bile acid content was determined by a standardized enzymatic colorimetric assay (Enzabile®, Nycomed Pharma AS, Oslo, Norway).Concentrations of conjugated and unconjugated bile acids in porcine bile were measured as described previously, with some modifications as described below.[12,13] Five microliters of porcine bile was diluted 1:200 with sterile filtered phosphate buffered saline, before addition of 50nmol internal standard. After liquid-solid extraction of bile acids from serum and separation of unconjugated bile acids by lipophilic anion exchange chromatography, the unconjugated bile acid fraction was converted to the methyl ester form by reaction with 2,2-dimethoxypropane in acidic methanol. The conjugated bile acids were recovered by elution from the lipophilic anion exchange column using 0.3M acetic acid in 72% ethanol (pH adjusted to 9.6 with

ammonium chloride). Following elution, the conjugated bile acids were deconjugated by enzymatic hydrolysis and sulfolysis as previously described.[12] The resultant unconjugated bile acid species were dissolved in water, extracted and derivatized as for the unconjugated bile acids. Detection and quantification of the unconjugated bile acids was achieved using gas chromatography-mass spectrometry with selected-ion monitoring. Gas chromatography-mass spectrometry was performed in a GC8000 gas chromatograph coupled to a Voyager mass spectrometer (Thermoquest Corporation, Schaumburg, IL). The mass-spectrometer was operated in selected-ion monitoring, electron-impact mode. Ions monitored were mass/charge (M/Z) 368, 370 and 372, representing relatively strong, specific ions generated by monohydroxy- (cholic acid), dihydroxy- (deoxycholic, chenodeoxycholic and ursodeoxycholic acid) and trihydroxycholanoates (lithocholic acid) respectively.[14] The internal standard, nordeoxycholic acid, was monitored at M/Z 521. Quantification of the bile acids in samples was achieved by calculation of the ratio of the peak area for each bile acid to the peak area of the internal standard, followed by interpolation on calibration curves generated with mixtures of pure standards. The total bile acid concentration reported is the sum of the five individual bile acids in their conjugated and unconjugated forms.

Mucosal-to-serosal fluxes of ^{3}H-mannitol and ^{14}C-LPS

To assess paracellular permeability following experimental treatments, 0.2μci/ml ^{3}H-mannitol or ^{14}C -LPS was placed on the mucosal side of Ussing chamber mounted tissues. After a 15-minute equilibration period, standards were taken from the mucosal side of each chamber and a 30-minute flux period was established by taking 0.5ml samples from the serosal compartment. The presence of ^{3}H and ^{14}C was established by

measuring β-emission in a liquid scintillation counter. Unidirectional ^{3}H-mannitol or ^{14}C-LPS fluxes from mucosa to serosa were determined using standard equations.

Morphometric measurements

Tissues were taken at 0 and 240 minutes for routine histologic evaluation. Tissues were sectioned (5 μm) and stained with hematoxylin and eosin. For each tissue, 3 sections were evaluated. Four well-oriented villi were identified in each section. The length of the villus, and the width at the midpoint of the villus were obtained using a micrometer in the eye piece 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 the formula for the 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. The percentage of the villous surface area that remained denuded was calculated from the total surface area of the villus and the surface area of the villus covered by epithelium. The percent-denuded villous surface area was used as an index of epithelial restitution.

Electronmicroscopy

Tissues were removed from Ussing chambers after 240 minutes during 3 separate experiments (n=3 for each treatment). Tissues were placed in Trump's 4F:1G fixative, and prepared for transmission electron microscopy using standard techniques. For each tissue evaluated, 5 well-oriented interepithelial junctions were evaluated.

Data analysis

Data were reported as mean ± SE. All data were analyzed using an ANOVA for repeated measures except where the peak response was analyzed using a standard one

way ANOVA or paired t-test (Sigmastat, Jandel Scientific, San Rafael, CA). A Tukey's test was used to determine differences between treatments following ANOVA, and $P < 0.05$ was considered significant.

RESULTS

Bile acid assay

Ileal luminal samples from 8 pigs were assayed for total bile acid concentration, using a commercial assay. The assay showed that the mean concentration of total 3α-hydroxy bile acid in the porcine ileum was $6\pm3x10^{-6}$M. The specific bile acids present in the pig ileum were also elucidated in samples from the same 8 pigs by mass spectrometry (Table 1). In general, the concentrations of unconjugated bile acids were in the same range as that determined using the commercial assay kit, with deoxycholic acid having the highest concentration. However, conjugated bile acid concentrations reached levels in the millimolar range, with conjugated lithocholic acid having the highest concentration.

Deoxycholic acid inhibits recovery of ischemic-injured porcine ileum

One-hour of ischemia resulted in significant reductions of TER ($34\pm2.7\Omega.cm^2$) compared to controls ($51\pm4.8\Omega.cm^2$; $P<0.05$), but ischemic-injured tissues recovered control levels of TER within 2 hours (Fig. 1). Mucosal treatment of ischemic-injured tissues with 10^{-6}M deoxycholic acid had no effect, whereas treatment with 10^{-5}M deoxycholic acid markedly inhibited recovery of TER. Higher doses of deoxycholic acid above the physiologic concentrations (10^{-3} M, 10^{-4}M) completely inhibited recovery (data not shown). Histologic evaluation of tissues revealed denuding of villous tips immediately following the ischemic period (Fig 2A), but denuded villi were fully

restituted with flattened epithelium within 60-minutes (Fig 2B). Histologic evaluation of control and ischemic-injured tissues treated with 10^{-5}M or 10^{-6}M deoxycholic acid revealed no effect on epithelial morphology (Fig 2C, D), whereas higher doses of deoxycholic acid (10^{-3}M, 10^{-4}M) caused disruption of epithelial restitution (data not shown). Morphometric analysis revealed significant reductions in villous height in control tissues treated with 10^{-5}M and 10^{-6}M deoxycholic acid (Fig. 3). The villous height of ischemic-injured tissues was significantly lower than control tissues. Furthermore, addition of 10^{-5}M deoxycholic acid to ischemic-injured tissues resulted in further significant reductions in villous height (Fig. 3). There was no effect of either concentration of deoxycholic acid on normal tissues (data not shown).

Deoxycholic acid increases paracellular permeability

Because 10^{-5}M deoxycholic acid inhibited recovery of TER in ischemic-injured tissues without any histologic changes in epithelial restitution, we considered the possibility that the deoxycholic acid inhibited recovery of TER via an action on the paracellular space. This paracellular premise was also supported by previous work in which we have shown that *in vitro* recovery of ischemic-injured porcine ileal mucosa is associated with progressive reductions in paracellular permeability.[11,15,16] However, the finding that 10^{-5}M deoxycholic acid stimulated significant villous contraction did not agree with this premise, since this action would tend to increase measurements of TER by reducing the surface area of the villous.[17] Therefore, we chose to perform mucosal-to-serosal fluxes of the paracellular probe mannitol. Accordingly, these fluxes were significantly elevated in tissues treated with 10^{-5}M deoxycholic acid compared to tissues treated with 10^{-6}M deoxycholic acid, or tissues that had no treatment (Fig. 4). We also

performed mucosal-to-serosal fluxes of ^{14}C -labeled lipopolysaccharide (LPS) from *Salmonella typhimurium*, a bacterial toxin that has been shown to traverse epithelium via a transcytotic pathway,[18] but which may gain access to the serosal surface of intestinal mucosa via the paracellular route following ischemia.[19] The close correlation of ^{14}C-LPS fluxes and ^{3}H-mannitol fluxes suggested the possibility of paracellular flux of LPS in ischemic-injured porcine mucosa that was enhanced in the presence of 10^{-5}M deoxycholic acid (Fig. 5).

In further experiments, we sought to determine the effects of deoxycholic acid washout on recovery of TER, since the effects of treatments that have an action on paracellular structures are typically reversible.[20-23] Paired tissues were treated with 10^{-5}M deoxycholic acid at the beginning of the recovery period, and deoxycholic acid was washed out after 60-minutes in one of each pair of tissues. This experiment revealed that the effect of 10^{-5}M deoxycholic acid was fully reversible following washout (Fig. 6).

Electronmicroscopic evidence of paracellular changes in the presence of deoxycholic acid.

As an additional method of assessing the role of deoxycholic acid in altering paracellular permeability, we performed electronmicroscopic analysis of ischemic-injured tissues after a 240-minute recovery period (Fig. 7). Untreated ischemic-injured tissues had evidence of closely apposed tight junctions in 3 separate experiments, whereas ischemic-injured tissues exposed to deoxycholic acid (10^{-5}M) for 240-minutes had evidence of widened tight junctions and dilated paracellular spaces in tissues from the same experimental animals.

Taurodeoxycholic acid has similar effects as deoxycholic acid

Since deoxycholic acid is only one of a number of bile salts that may be present within the gastrointestinal lumen, we also chose to evaluate the effect of taurine conjugation of deoxycholic acid on mucosal recovery. The bile acid taurodeoxycholic acid was added to the mucosal surface of ischemic-injured tissue, and had a similar effect as that of deoxycholic acid at 10^{-5}M (Fig. 8).

Deoxycholic acid appears to mediate its effects via Ca^{2+}

Because of the importance of Ca^{2+} in regulation and re-assembly of the tight junction,[24] and in view of studies implicating Ca^{2+} in bile salt-induced injury[9,10] we wanted to investigate the effects of chelating Ca^{2+} and increasing intracellular Ca^{2+} in our ischemia model. Accordingly, EGTA (1mM) was added to the mucosal and serosal sides of ischemic-injured tissue, and significantly impaired recovery of TER (Fig. 9). Peak TER of tissue treated with EGTA was almost identical to that of ischemic-injured tissue treated with 10^{-5}M deoxycholic acid. In addition, we attempted to reverse the effects of10^{-5}M deoxycholic acid with thapsigargin, which has been shown to elevate intracellular calcium from intracellular and extracellular calcium sources without stimulating other second messenger signaling mechanisms.[25] Application of thapsigargin (10^{-6}M) to the mucosal and serosal sides of ischemic-injured tissue resulted in recovery of TER to levels similar to those of untreated ischemic-injured tissue (Fig. 10).

DISCUSSION

Transepithelial resistance (TER) is a highly sensitive measure of the ionic permeability of intestinal mucosa. Recent studies have shown close correlations between

recovery of TER and reductions in mucosal permeability to inulin and mannitol in ischemic-injured tissues treated with prostaglandins.[11] During the 240-minute period of the present experiments, the TER of ischemic-injured tissue receiving 10^{-6}M deoxycholic acid started at 34±2.7Ω.cm^2 and increased to 52±3.6Ω.cm^2 by the end of the experiment, which was very similar to control tissues (51±4.4Ω.cm^2). However, the TER of ischemic-injured tissues treated with 10^{-5}M deoxycholic acid was significantly less than that of control tissue at the end of the recovery period. Similar results were seen with application of 10^{-5}M taurodeoxycholic acid. The reduced recovery of TER in ischemic-injured tissues treated with 10^{-5}M deoxycholic acid correlated with a significant increase in the mucosal-to-serosal flux of ^{3}H-manitol and ^{14}C-lipopolysaccharide, indicating these tissues failed to recover mucosal barrier function. Despite the increases in mucosal permeability in tissues treated with 10^{-5}M deoxycholic acid, there was no appreciable difference in the histologic appearance of ischemic-injured tissues treated with 10^{-5}M deoxycholic acid. This suggests that changes in the paracellular space may account for the decrease in TER and increase in mucosal permeability. ^{3}H-mannitol has been shown to traverse leaky epithelia via the paracellular space[26,27] and ^{14}C-LPS may gain access to the serosal surface of intestinal mucosa via the paracellular route following ischemia,[19] further supporting the possibility of disruption of paracellular pathways. In previous studies, taurochenodeoxycholic acid (4mM) has been shown to alter the integrity of tight-junctional complexes between the epithelial cells of rabbit colon.[28] Furthermore, electron microscopy revealed wider intercellular spaces with loss of tight junctional integrity in rabbit small intestine treated with chenodeoxycholic acid (1mM).[3] Similarly, in the present studies, the tight junctions and paracellular spaces appeared widened in repairing

tissues in the presence of 10^{-5}M deoxycholic acid compared to tissues in the absence of bile acid. One additional property of the tissue response to deoxycholic acid in the present study that is consistent with changes in paracellular permeability is the rapidity with which the effect of deoxycholic acid was reversed upon washout. This reversal of the effects of bile salts agrees with previous studies in rabbit small intestine[3] in which the effects of bile salts were confined to the paracellular space.

Previous studies have indicated that dihydroxy secondary bile salts (taurodeoxycholic acid) have a more pronounced effect on gastric mucosal permeability than trihydroxy primary bile acids (taurocholic acid).[2] However, in another study of rat small intestine after 30 minutes of ischemia, neither taurodeoxycholic acid nor taurochenodeoxycholic acid increased mucosal permeability.[8] In the present study, both deoxycholic acid and taurodeoxycholic acid had similar effects on TER of ischemic-injured tissue at the doses tested. This may be due to the increased amount of ischemia used in the present study compared to others.[8] In addition, distinct segments of the gastrointestinal tract may respond differently to various bile acids.

The role of calcium in the regulation of tight junctions has previously been studied. For example, increases in intracellular calcium have been implicated in cytoskeletal-mediated tight junction closure in recovering ischemic mucosa.[11,29] In the present study, a decrease in available calcium in ischemic-injured tissues by the use of the calcium binding agent EGTA led to a decrease in TER, similar to that seen in ischemic-injured tissues treated with 10^{-5}M deoxycholic acid. Alternatively, addition of thapsigargin to ischemic-injured tissues treated with 10^{-5}M deoxycholic acid reversed the

effects of deoxycholic acid, suggesting that the mechanism by which deoxycholic acid increases paracellular permeability involves intracellular Ca^{2+}.

In summary, this study shows that bile acids at physiologic concentrations significantly impair mucosal recovery of epithelial barrier function, and these effects appear to be due to changes in paracellular permeability. Thus, bile acids result in increased permeability in tissues to the paracellular probe mannitol. Furthermore, increased permeability to lipopolysaccharide was also shown, heightening the clinical significance of these findings, considering the importance of LPS in sepsis.[30] Although the precise mechanism whereby bile acids disrupt recovery of ischemic-injured mucosa, there appears to be a role for intracellular Ca^{2+}. However, further studies will be required to fully understand these pathways

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Table 1. Bile acid concentrations (mean±SE) obtained from n=8 porcine ileal ingesta sample using mass spectrometry

Bile Acid	Unconjugated (μMol/L)	Conjugated (μMol/L)
Cholic	2.2±0.4	15.6±10.5
Deoxycholic	5.5±2.0	130.3±124.4
Chenodeoxycholic	3.0±1.2	138.4±119.4
Lithocholic	3.8±1.7	2066.3±1290.1
Ursodeoxycholic	1.7±0.7	8.1±5.6

FIGURE LEGENDS

Figure 1: Transepithelial resistance (TER) recorded from porcine ileal mucosa in Ussing chambers exposed to varying concentrations of deoxycholate. One-hour of ischemia resulted in significant reductions of TER ($34\pm2.7\Omega.cm^2$) compared to controls ($51\pm4.8\Omega.cm^2$; $P<0.05$), but ischemic-injured tissues recovered control levels of TER within 2 hours. Mucosal treatment of ischemic-injured tissues with 10^{-6}M deoxycholic acid had no effect, whereas treatment with 10^{-5}M deoxycholic acid markedly inhibited recovery of TER ($P<0.05$).

Figure 2: Photomicrographs of porcine ileal post-ischemic mucosa. **A.** Histologic appearance of tissue immediately following 1-hour of ischemia. Note sloughing of epithelium from the upper third of the villus, exposing sub-epithelial tissues. **B.** Ischemic-injured tissue after 1 hour of recovery in Ussing chambers. Villous contraction and epithelial restitution are evident, leading to complete epithelial coverage of the villi. Ischemic-injured tissues treated with deoxycholic acid had similar evidence of restitution at 60-minutes (not shown). Furthermore, ischemic-injured tissues treated for 240-minutes with 10^{-6}M deoxycholic acid (**C**) or 10^{-5}M deoxycholic acid (**D**) had continued evidence of complete restitution. Bar = 100μm.

Figure 3: Morphometric analysis of normal and ischemic-injured porcine ileal mucosa. Following the 240-minutes in vitro incubation period, significant reductions in villous height in control tissues treated with 10^{-5}M deoxycholic acid were noted ($^aP<0.05$ vs. untreated control tissues). Ischemic-injured tissues had significantly reduced villous

height compared to control tissues ($^{b}P<0.05$ vs. control tissues). Furthermore, 10^{-5}M deoxycholic acid treatment resulted in further significant reductions in villous height in ischemic-injured tissue ($^{c}P<0.05$ vs. control and the other ischemic-injured treatment groups).

Figure 4: Mucosal-to-serosal fluxes of the paracellular probe mannitol. Treatment of ischemic-injured tissue with 10^{-5}M deoxycholic acid caused a significant increase in the flux of mannitol by the end of the recovery period compared to tissues treated with 10^{-6}M deoxycholic acid, untreated ischemic-injured tissue and control tissues (*$P<0.05$).

Figure 5: Mucosal-to-serosal fluxes of ^{14}C -labeled lipopolysaccharide (LPS) from *Salmonella typhimurium*. Treatment of ischemic-injured tissue with 10^{-5}M deoxycholic acid caused a significant increase in the flux of LPS compared to tissues treated with 10^{-6}M deoxycholic acid, ischemic-injured tissue that had no treatment or control tissues by the end of the recovery period (*$P<0.05$). The close correlation of ^{14}C-LPS fluxes and ^{3}H-mannitol fluxes suggested the possibility of paracellular flux of LPS in ischemic-injured porcine mucosa that was enhanced in the presence of 10^{-5}M deoxycholic acid.

Figure 6: Effects of deoxycholic acid washout on recovery of TER. Paired tissues were treated with 10^{-5}M deoxycholic acid at the beginning of the recovery period, after which deoxycholic acid was washed out at 60-minutes in one of each pair of tissues. This experiment revealed that the effect of 10^{-5}M deoxycholic acid was fully reversible following washout.

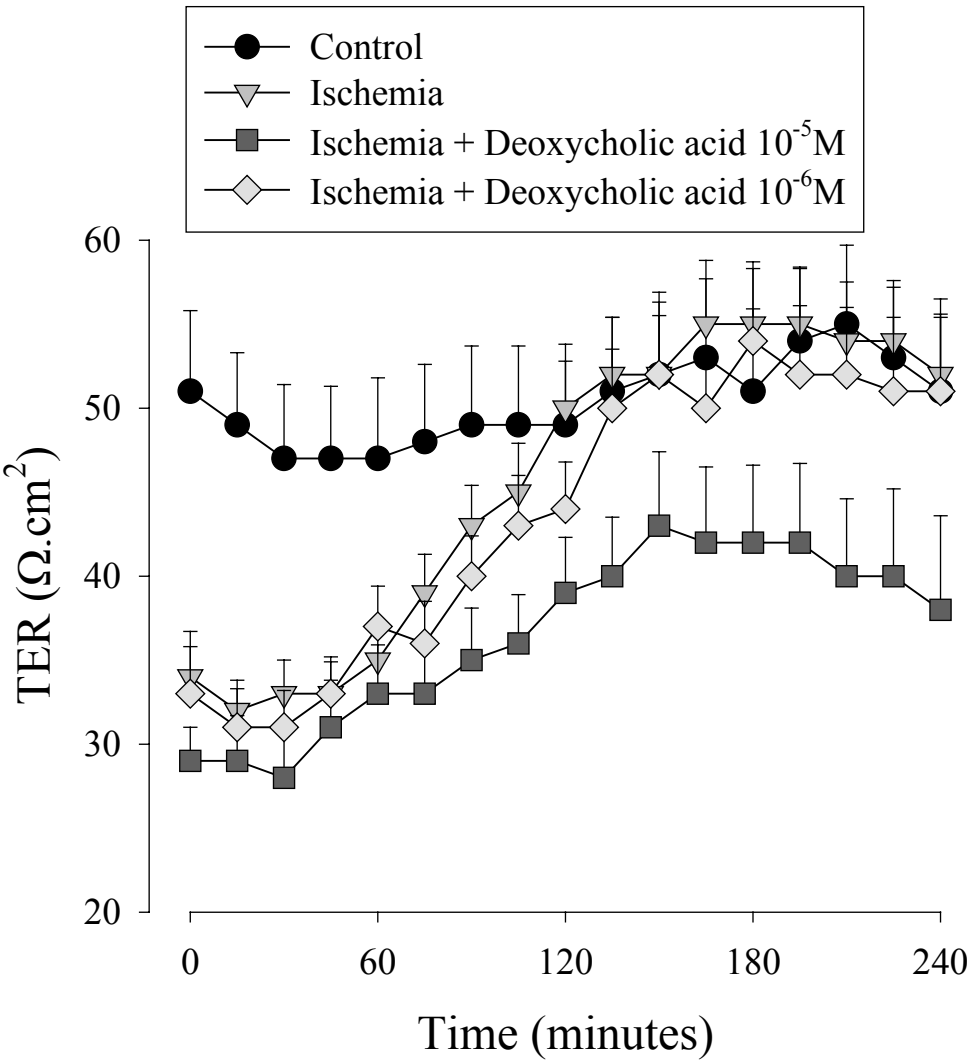
Figure 7: Electronmicroscopic evaluation of ischemic-injured mucosa. A. Ischemic-injured tissues at the end of the 240-minute recovery period appeared to have closely apposed tight junctions (arrows). B. Ischemic-injured tissues treated with mucosal deoxycholic acid (10^{-5}M) had evidence of dilated tight junctions (arrows) and dilated paracellular spaces. Bar = 300nm.

Figure 8: Effects of the bile acid taurodeoxycholic acid on ischemic-injured mucosa. Mucosal treatment of ischemic-injured tissues with 10^{-5}M taurodeoxycholic acid markedly inhibited recovery of TER ($P<0.05$) associated with no observable disruption of restituting epithelium (not shown). This was a similar effect as that of deoxycholic acid at 10^{-5}M (Fig. 1).

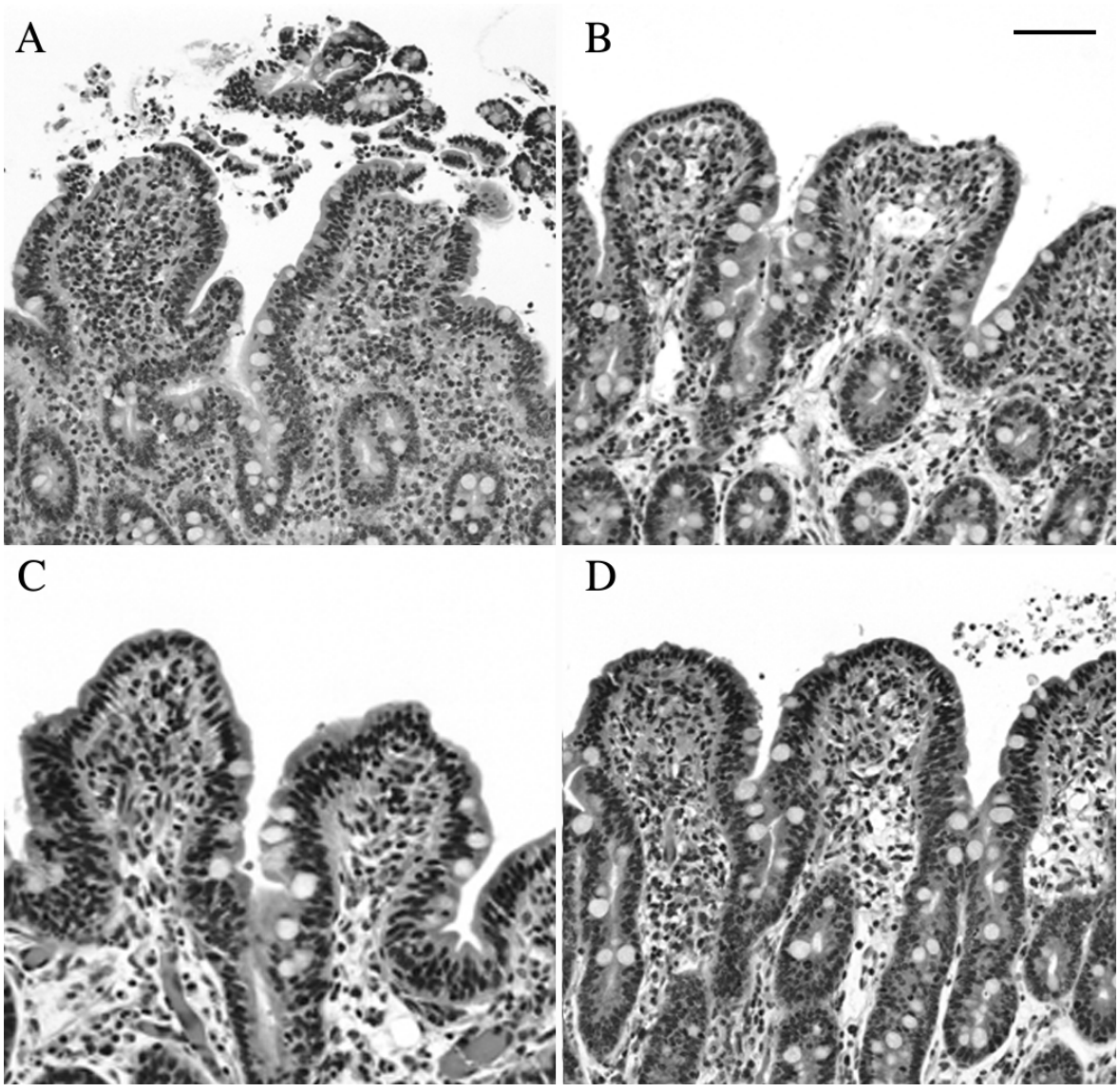
Figure 9: Effect of the calcium chelating agent EGTA on ischemic-injured mucosa. EGTA (1mM) was added to the mucosal and serosal sides of ischemic-injured tissue, and significantly impaired recovery of TER ($P<0.05$). Peak TER of tissue treated with EGTA was almost identical to that of ischemic-injured tissue treated with 10^{-5}M deoxycholic acid (Fig. 1).

Figure 10: Effect of the calcium mobilizing agent thapsigargin. Application of thapsigargin (10^{-6}M) to mucosal and serosal sides of ischemic-injured tissue treated with 10^{-5}M deoxycholic acid resulted in recovery of TER to levels similar to those of untreated ischemic-injured tissue (Fig.1) ($53\pm6.7\Omega.cm^2$ compared to $55\pm2.4\Omega.cm^2$), whereas tissues treated with deoxycholic acid alone failed to recover.

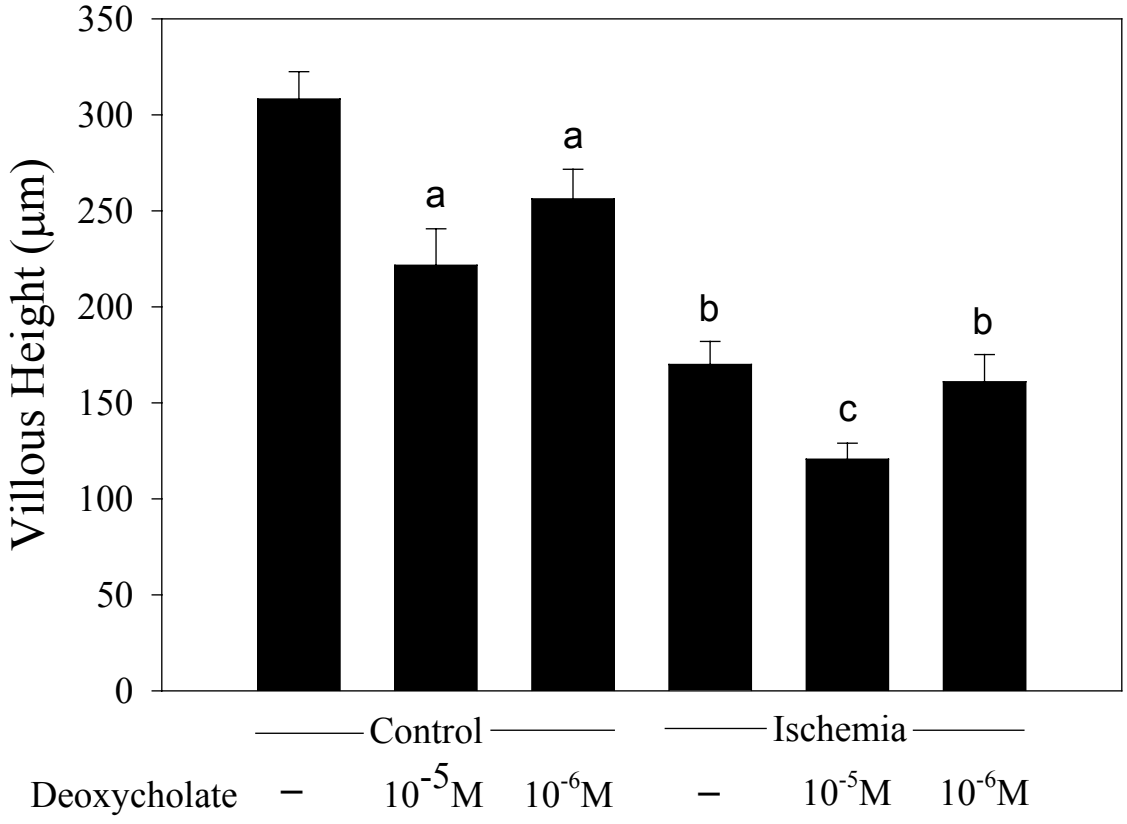
Campbell et al. Figure 1



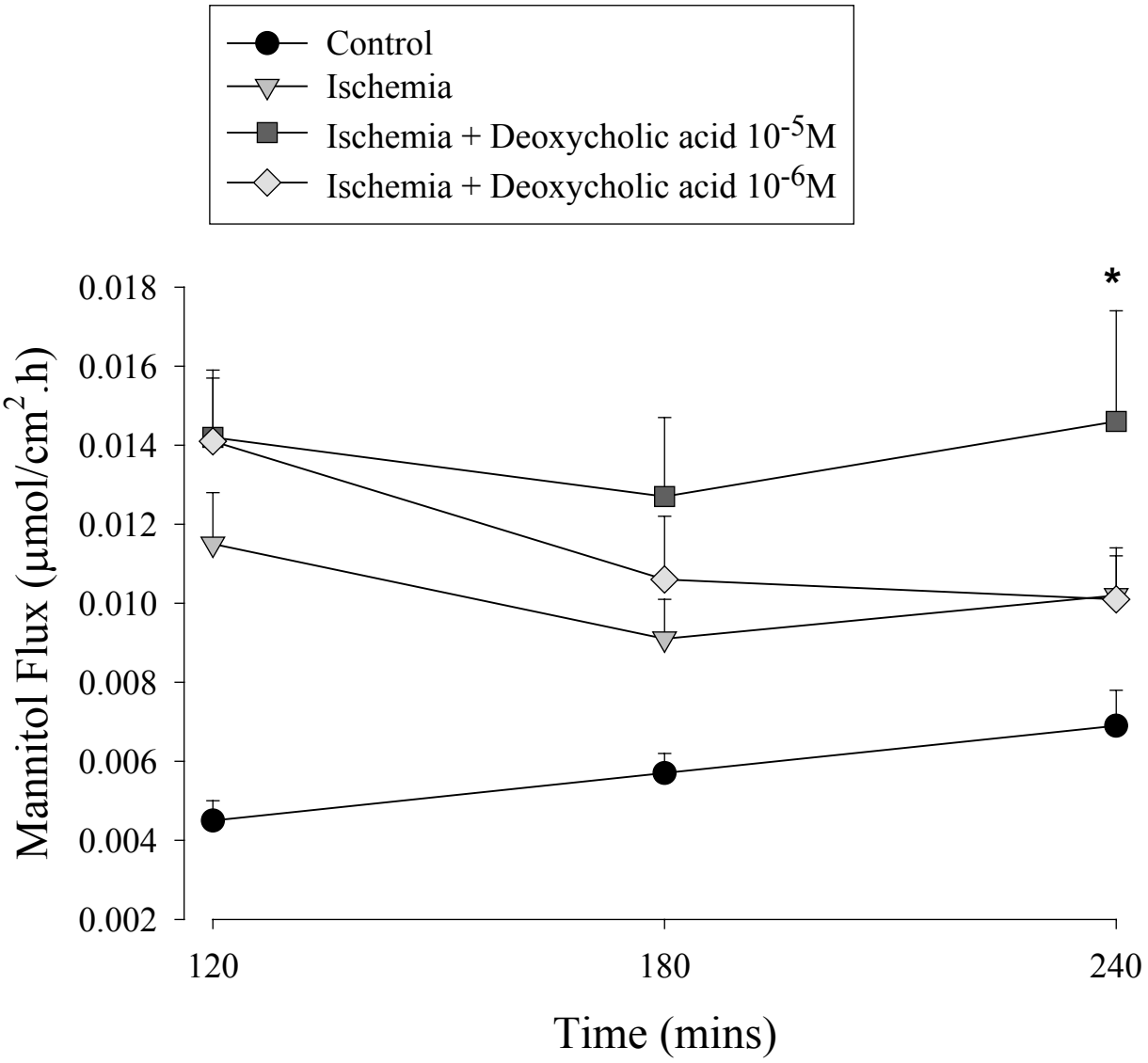
Campbell et al. Figure 2



Campbell, et al. Figure 3



Campbell et al. Figure 4



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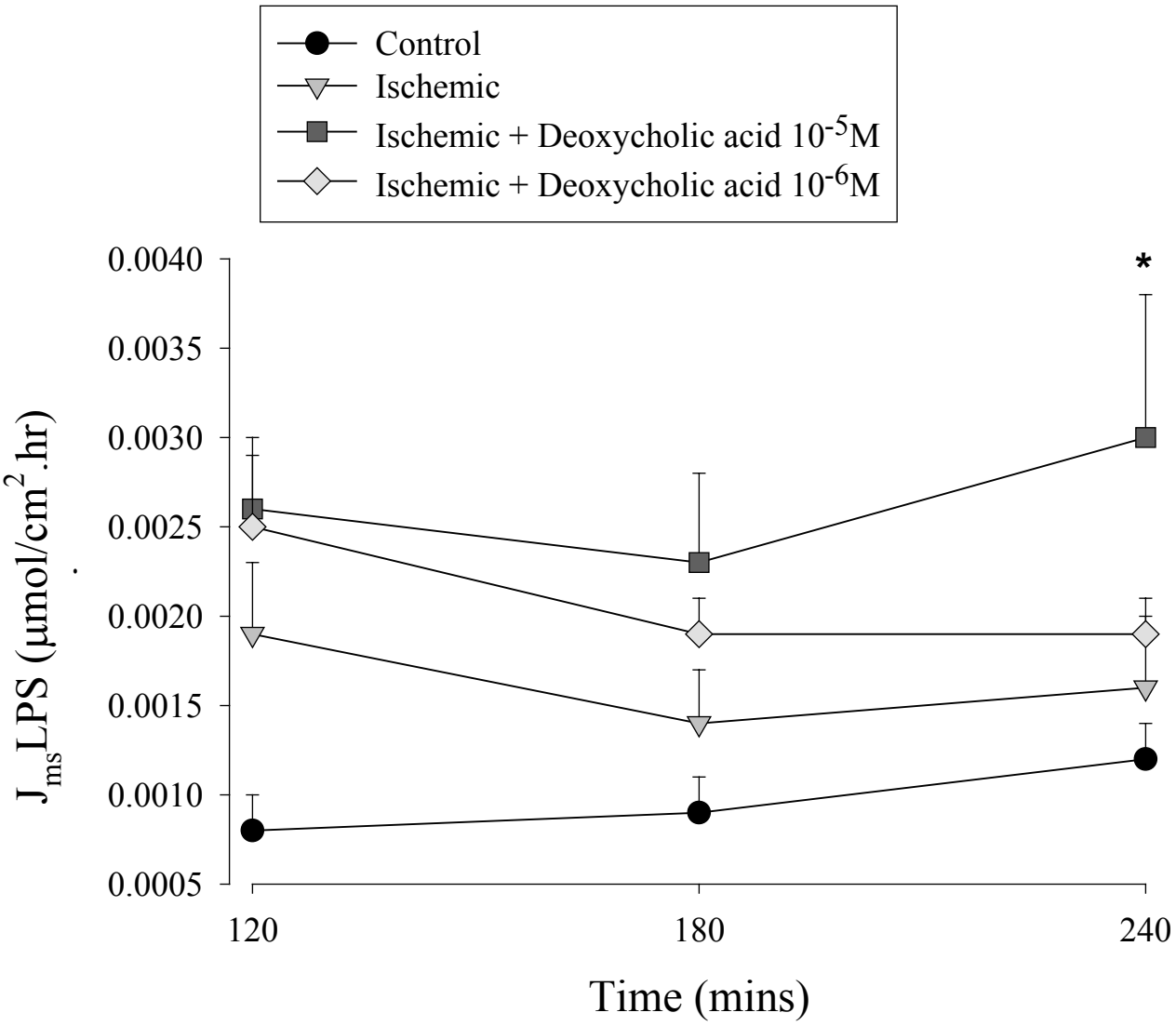

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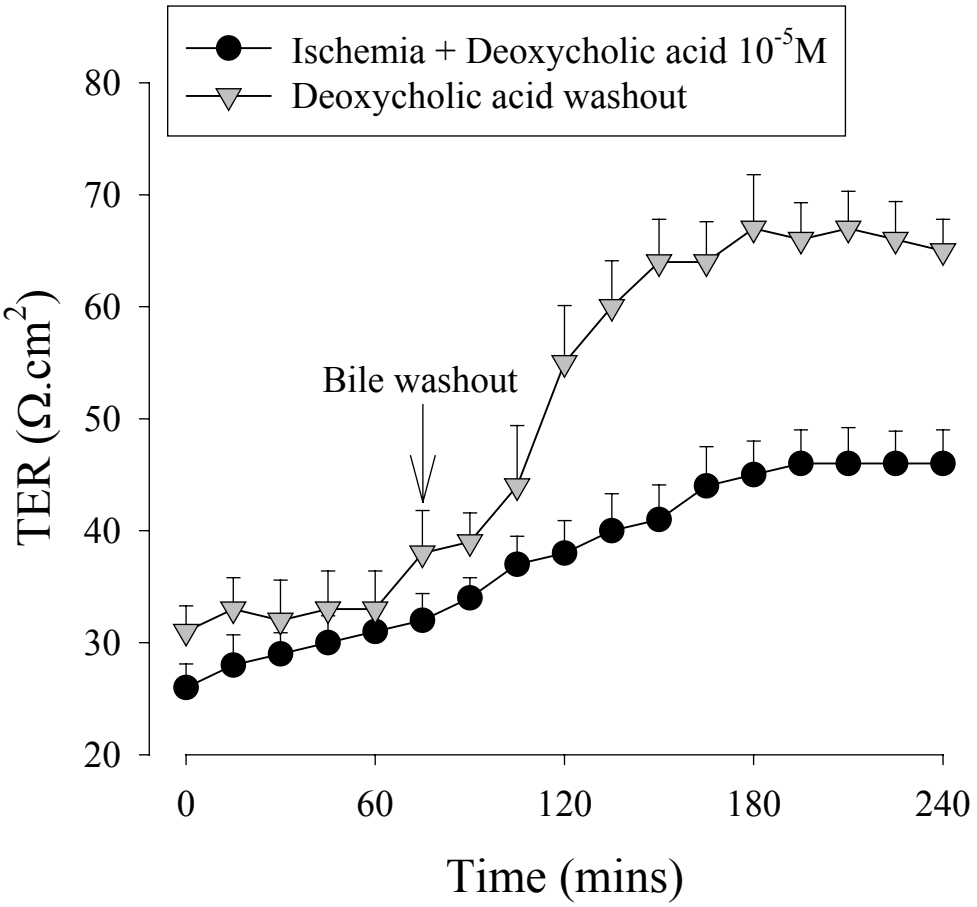

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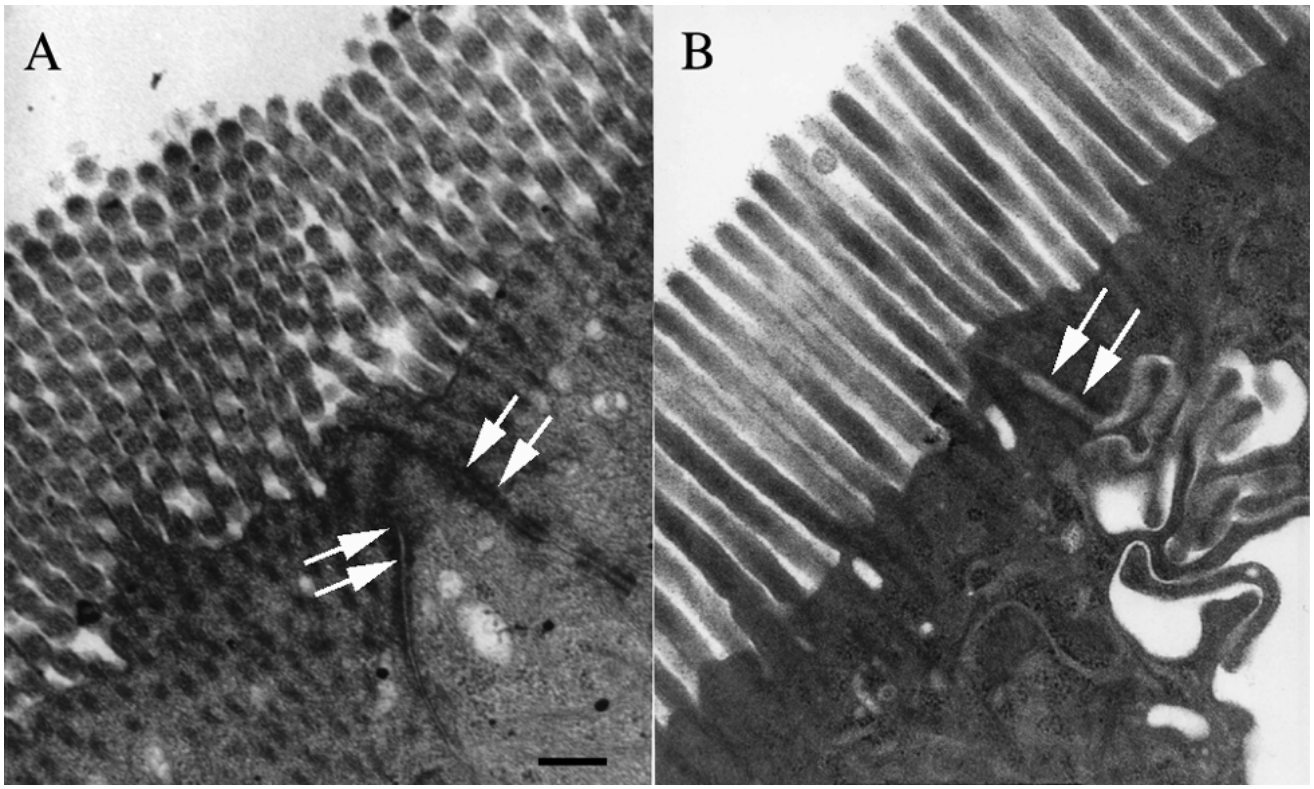
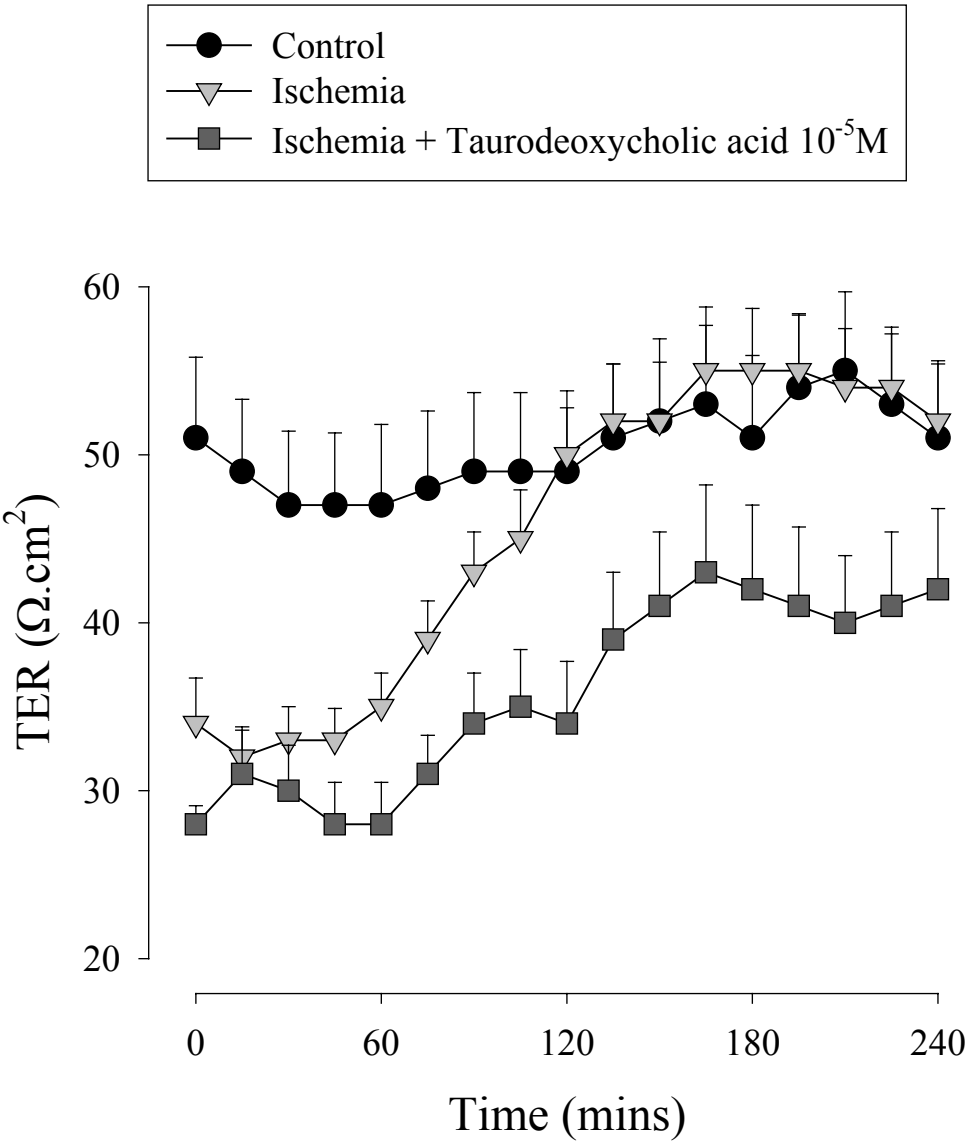


Campbell et al. Figure 8



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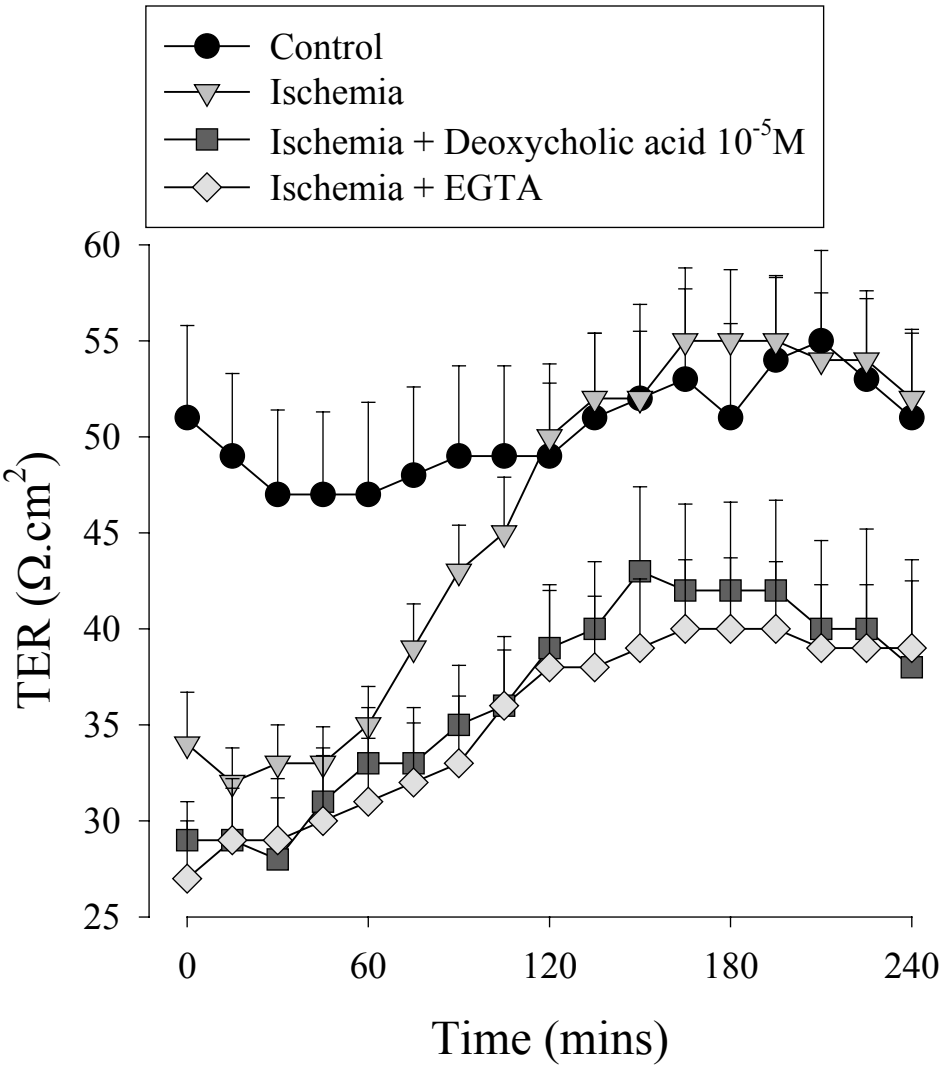

Campbell, et al. Figure 10