WOOTEN, JENNA GRAY. The Role of Cyclooxygenase (COX)-2 in the Canine Proximal Gastrointestinal Tract. (Under the direction of Drs. Anthony Blikslager and Duncan Lascelles.)

In veterinary medicine, NSAIDs are the most commonly prescribed analgesic and anti-inflammatory medications; unfortunately, they are also commonly associated with ulceration and perforation in dogs. Recent studies have indicated that the role of COX-2 appears to be more complicated than originally thought and its inhibition may lead to corresponding benefits or risks. Therefore, we investigated NSAIDs with differing degrees of selectivity and examined the role of COX-2 in the pylorus and duodenum.

Each dog received carprofen (4.4 mg/kg, q 24 h), deracoxib (2 mg/kg, q 24 h), aspirin (10 mg/kg, q 12 h), and placebo (1 dog treat, q 24 h) orally for 3 days (4-week interval between treatments). Prostanoid synthesis was greater in pyloric mucosa than it was in duodenal mucosa. Nonselective NSAIDs significantly decreased prostanoid concentrations in these mucosae, compared with the effects of deracoxib.

Following the same model dogs received deracoxib (2mg/kg q24h PO), firocoxib (5mg/kg q24h PO), meloxicam (Day 1=0.2mg/kg q24h PO, Day 2-3=0.1mg/kg q24h PO), or placebo (1 dog treat, q 24 h). There were no significant effects of varying COX-2 selectivity on gastric and duodenal tissue prostanoid concentrations, and no significant relationship between the degree of selectivity and gross or histological appearance of the mucosa, suggesting that there are no differences among the preferential and selective COX-2 inhibitors with regard to adverse effects on the upper GI tract.
Twenty-seven clinically normal dogs were evaluated to determine if gastrointestinal lesions were present, and to determine if COX-1 and COX-2 expression were different in lesioned tissue compared to normal tissue. Findings show the gross appearance of a dog’s stomach will likely not provide definitive evidence of whether or not disease is present. From our results, COX-2 appears to be upregulated at the sites of inflammation and erosion and so in these situations, non-selective NSAIDs and COX-2 inhibitors could both be problematic, if this elevated COX-2 is actually playing a protective role. It is still not known if there is any difference between the selective COX-2 inhibitors and the non-selective NSAIDs in their ability to inhibit this upregulated COX-2 that is functioning in a protective role.
The Role of Cyclooxygenase (COX)-2 in the Canine Proximal Gastrointestinal Tract

by

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DEDICATION

For my Mom & Nana

Terry Barnes & Bertha Davis

For teaching me that an education is priceless
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CHAPTER 1

Nonsteroidal anti-inflammatory drug (NSAID)-induced gastric mucosal injury in the proximal gastrointestinal tract of dogs

Jenna G. Wooten
Introduction

The gastric mucosa is continuously exposed to a number of injurious factors. This is particularly the case in the stomach, where the contents may become highly acidic. Lining the gastric mucosa is a complex barrier of interacting physical and biochemical defense mechanisms that separate luminal contents from the interstitium. The gastric mucosal barrier is designed to sustain structural integrity and function, despite exposure to a harsh environment, making barrier function critical to the overall health of the gastrointestinal (GI) tract. Several conditions can disrupt barrier function, leading to acid back diffusion and mucosal damage.\(^1,2\) Causative factors include bile salts, primary GI disease, and administration of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs).

In veterinary medicine, NSAIDs are the most commonly prescribed analgesic and anti-inflammatory medications; unfortunately, they are also commonly associated with ulceration and perforation in both human beings and dogs.\(^3,4\) Dogs may be more sensitive to some NSAIDs than human beings because of differences in absorption, metabolism, and enterohepatic recirculation.\(^5-8\) Inhibition of cyclooxygenase (COX), a key enzyme in the arachidonic acid pathway, and subsequent inhibition of prostaglandins (PGs) appears to be the primary mechanism of action for NSAIDs, although some exert effects on alternate signaling pathways.\(^9,10\) Prostaglandins mediate a variety of physiological features involved in mucosal protection. Other lipid mediators, such as lipoxins, act similarly to PGs and are involved in protection. Suppression of PG synthesis is a critical event in GI toxicity,\(^8,11,12\)
where erosion, ulceration, and perforation have all been associated with NSAID use in several species.\textsuperscript{5,13-16}

At least two isoforms of the COX enzyme exist (COX-1 and COX-2) and the manner in which NSAIDs interact with these isoforms is likely accountable for the variability in efficacy and toxicity amongst this class of drugs. NSAIDs approved for the veterinary market differ in their degree of COX selectivity. Traditional NSAIDs act as non-selective inhibitors of the COX enzyme, inhibiting both isoforms. Since COX-1-derived PGs are believed to play a crucial role in gastric mucosal defense and cytoprotection,\textsuperscript{17} a coxib class of NSAIDs was developed to selectively inhibit COX-2 (primarily expressed in response to inflammatory stimuli), intending to provide a safer alternative to traditional NSAIDs.\textsuperscript{18} Selectively blocking COX-2 was believed to maintain the integrity of the gastric epithelium, while reducing pain and inflammation.\textsuperscript{18} While this drug development was conceptually appealing, increasing evidence has established that COX-2 is constitutively expressed in GI mucosa and plays a role in gastric mucosal protection.\textsuperscript{19-21} Inhibition of PG production is primarily responsible for both the beneficial and deleterious effects provided by NSAIDs and the amount and type of PG inhibition is contingent upon the drug chosen and administered dose.\textsuperscript{22}

Although COX-1 is the predominant isoform in the gastric mucosa, increasing evidence of both constitutive and inducible COX-2 mRNA and protein have been found in specific areas of the GI tract.\textsuperscript{23,24} Recent studies have indicated that the role of COX-2 appears to be multi-faceted and depending on the physiological process involved, its inhibition will lead to corresponding benefits or risks. The COX-2 isoenzyme seems to be of
lesser importance in the mucosal lining under normal conditions, but in the face of injury or preexisting ulceration, it appears to be of crucial importance.

**Gastric Mucosal Barrier Function**

Under normal conditions, the functioning of the GI tract with its balanced microflora depends on the preservation of distinct chemical and physical defense mechanisms, which are essential in order to maintain mucosal integrity and resist auto-digestion. The mucosa is lined with a monolayer of columnar epithelial cells and interepithelial tight junctions that provide an effective barrier against the invasion of unwanted solutes, luminal antigens, and microorganisms. The luminal environment of the stomach contains nutrients, but is laden with bile acids, proteolytic enzymes, pepsin, and acid. When epithelial continuity is breached, erosion, ulceration, and perforation can occur. Several factors contribute to gastric mucosal barrier function: a superficial mucus-bicarbonate-phospholipid layer, continuous epithelial cell proliferation and restitution, an increased rate of blood flow in the mucosa, and PG production.

The first line of defense is the mucus-bicarbonate-phospholipid “barrier”. This preepithelial barrier between the lumen and epithelium is the most superficial component of the gastric mucosal barrier. A continuous layer of surface epithelial cells that secrete a mucus gel containing membrane phospholipids, line the stomach mucosa preventing unwanted solutes, microorganisms, and luminal antigens from entering the body (Figure 1). The glycoprotein mucus adheres to the mucosal surface and traps bicarbonate in order to sustain a neutral environment. The mucus-bicarbonate-phospholipid layer works
along with gastric epithelial tight junctions to prevent acid and pepsin diffusion (i.e. back diffusion) from the gastric lumen back into the mucosal cells.\textsuperscript{1,40-43} Mucus secretion is initiated by several GI hormones, such as gastrin. It is also stimulated by prostanoids such as PGE\textsubscript{2}.\textsuperscript{1} Secretion of HCO\textsubscript{3}\textsuperscript{-} into the mucus layer creates a nearly neutral pH at the apical cell surface.\textsuperscript{1,29} Within the epithelium are gastric parietal cells, which when stimulated secrete hydrochloric acid (HCl) into the lumen. After forming HCl, the parietal cells release bicarbonate into the plasma, where it is transported up toward the base of the surface epithelium and into the gastric lumen in order to maintain intracellular homeostasis.\textsuperscript{1,29} Due to bicarbonate secretion, the venous blood leaving the gastric mucosa is more alkaline than the arterial blood delivered to it.\textsuperscript{44}

The structural integrity of the gastric mucosa is maintained by mucosal cells that undergo constant renewal. This well-coordinated process completely replaces normal or damaged surface epithelial cells in about 3-7 days, depending on the health of the tissue (inflammation, ulceration, carcinogenesis).\textsuperscript{45} Independent of cell proliferation is a process known as epithelial restitution in which preserved epithelial cells migrate to reseal denuded regions of the mucosa within minutes of superficial injury.\textsuperscript{46,47} Epithelial cells, stimulated in part by PGs,\textsuperscript{32} extend lamellipodia over injured mucosa, reestablishing an intact epithelial barrier, thereby preventing further damage.\textsuperscript{4,46,48} A diverse array of peptides and growth factors stimulate mucosal growth and wound closure.\textsuperscript{49,50} For example, salivary glands produce epidermal growth factor (EGF) which travels with saliva into the gastric lumen, where it is thought to play a crucial role in mucosal integrity and healing.\textsuperscript{36,37,39} Another mucosal integrity peptide, TGF\textalpha\textsuperscript{a} (transforming growth factor \alpha), is produced principally in
intestinal mucosa, and signals through the same receptor as EGF. Peptides produced by mucosal epithelial cells and paneth cells, protect both the mucosal surface and its crypts, respectively. Trefoil peptides found in goblet cells bind to glycoproteins in the brush border and promote migration. At sites of injury and inflammation such peptides have been shown to be rapidly upregulated.

At the level of the muscularis mucosae, gastric arteries branch into capillaries that drain into mucosal venules at the base of surface epithelial cells supplying them with nutrients and oxygen. Lining the microvessels are endothelial cells which generate potent vasodilators, such as nitric oxide (NO) and PGI₂, which oppose the mucosal damaging action of vasoconstrictors (e.g. leukotrienes) and therefore prevent compromise of microcirculation. In the submucosa, vessels are surrounded by primary afferent sensory neurons and nerves creating a dense plexus. Luminal content is ‘sensed’ by nerve fibers from this plexus and if activated, these nerves have a direct effect on submucosal arterioles, altering mucosal blood flow.

Prostaglandins stimulate and/or facilitate most of the gastric mucosal defense mechanisms. Continuous mucosal generation of PGE₂ and PGI₂ is a crucial component of barrier function. Several studies have shown that PGs, PGE₂ in particular, enhance recovery of transmucosal resistance, an index of barrier function, in ischemia-injured intestine. The importance of PGE₂ and PGI₂ is further emphasized by a study that found that immunoneutralizing antibodies to these PGs caused gastric and duodenal ulcers in rabbits and dogs, matching those produced by NSAID PG inhibition. These PGs stimulate mucus, bicarbonate, and phospholipid secretion, enhance blood flow of the mucosa,
stimulate secretion of epithelial cell growth, inhibit mast cell activation along with leukocyte and platelet adherence, and inhibit acid secretion in order to protect the mucosa from ulcerogenic and necrotizing agents.\textsuperscript{4,26,56,57} Inhibition of PGs in gastric secretions decrease sodium and mucus concentrations and increase hydrogen ion concentrations. Biological actions exerted by PGs are mediated via EP receptors. The EP-1 receptor is associated with bicarbonate secretion and blood flow to the mucosa.\textsuperscript{64,65} Also involved in protection are the EP-3 and EP-4 receptors, which affect acid and mucus secretion, respectively.\textsuperscript{66,67}

**NSAID Pharmacology and Mechanism of Action**

*Pharmacokinetics*

The pharmacokinetic properties of all NSAIDs are relatively similar.\textsuperscript{68} Some general pharmacokinetic features of NSAIDs are low volumes of distribution, a high degree of binding to plasma protein, limited urinary excretion of the parent drug, and inter-species differences in clearance and elimination half-life.\textsuperscript{69} These drugs normally have good bioavailability from subcutaneous, intramuscular, and oral administration. The chemical make-up of NSAIDs (i.e. weak acids) allows for efficient absorption from the GI tract, with the exception of aspirin (and possibly diclofenac, tolfenamic acid and fenbufen) which undergoes presystemic hydrolysis to form salicylic acid.\textsuperscript{70} In some cases, concomitant administration of NSAIDs with food or antacids may lead to delayed or reduced absorption.\textsuperscript{70} NSAIDs absorbed in the stomach and small intestine make their way into circulation, where they become highly bound (90-99\%) to plasma proteins (mainly albumin), limiting their body distribution to extracellular spaces.\textsuperscript{69,70} Therefore, volumes of distribution of NSAIDs
are low and usually less than 0.2 L/kg; only 5-10% of the compounds appear in the plasma and exert clinical effects.\textsuperscript{70} The majority of NSAIDs are metabolized largely by hepatic biotransformation and excreted by the kidney.\textsuperscript{71} Total body clearance is low and differences among NSAID clearance account for the variability in half-life among this class of drugs.\textsuperscript{70} Some NSAIDs undergo extensive enterohepatic recirculation. Highly lipophilic drugs, such as celecoxib, have a large first-pass effect because they are highly metabolized. Drugs with a lower lipophilicity are therefore less extensively metabolized, resulting in a lower first-pass effect, creating an increase in time to reach therapeutic levels.\textsuperscript{72}

NSAIDs are involved in a number of drug interactions, by several mechanisms, including protein-binding-displacement interactions, induction or inhibition of hepatic drug metabolism, and competition for active renal tubular secretion with other organic acids.\textsuperscript{69} The potential for interactions in which these drugs displace other highly bound drugs is not surprising, since NSAIDs have such a high degree of protein binding. NSAIDs have also been known to induce or inhibit other drugs with hepatic metabolism. Phenylbutazone and sulfinpyrazone are two prominent drugs associated with this type of interaction, although the exact features of these compounds that account for this are unknown.\textsuperscript{69} Hepatic disease can result in a significant change in NSAID pharmacokinetic properties due to the central role of the liver in the overall elimination of the majority of these compounds.\textsuperscript{70} Another mechanism of NSAID drug interaction is competition for active renal tubular secretion at the proximal tubule.\textsuperscript{69} NSAIDs are organic acids, and will compete for transport with other organic acids. The extent to which they alter transport varies depending on the NSAID administered.\textsuperscript{73-75}
**Mechanism of action**

Cyclooxygenase-1 is constitutively expressed and mediates formation of PGs responsible for homeostasis, renal blood flow, and gastrointestinal protection (PGE$_2$, PGI$_2$, and PGD$_2$).$^{31,48,56,69,76}$ In contrast, COX-2 is expressed at sites of inflammation, producing PGs that enhance inflammation.$^{48,56,77}$ Inhibition of these enzymes and subsequent suppression of PGs, produced from the substrate arachidonic acid, is the principal mechanism of action associated with NSAIDs.$^{9,10}$ The analgesic, antipyretic, anticoagulant, and anti-inflammatory properties of different NSAIDs are directly related to the type and amount of PG inhibition.$^{78}$ Although both isoforms of COX appear to have different physiologic functions, they are similar in structure, comprised of approximately 600 amino acids per isoform.$^{79}$ Each isoform consists of a long hydrophobic channel with a hairpin turn at the end and have approximately 60% amino acid homology.$^{22,79}$

**Role of Lipoxins and Aspirin-Triggered Lipoxins**

Various physical aspects of mucosal defense can be attributed to PG production and when suppressed NO can exert many of the same protective effects as PGs. Recently, another group of lipid mediators, known as lipoxins, have also been found to protect the stomach from injury. Lipoxins represent a unique class of lipid mediators with well characterized anti-inflammatory properties.$^{80,81}$ In the stomach, recent studies have shown potent protective effects from lipoxin A$_4$ and its epimeric counterpart, which is synthesized via aspirin-acetylated COX-2. Identification of the aspirin-acetylated COX-2 derived lipoxin, as an endogenous mediator of mucosal protection, has attributed to an abundant amount of human research, whereas fewer studies have focused specifically on dogs.
Multiple pathways contribute to the synthesis of various members of the lipoxin family. The aspirin-acetylated COX-2 pathway is of particular interest when examining NSAIDs. A conformational change altering the ability of COX enzymes to metabolize arachidonic acid is caused by aspirin acetylation of serine residues in both COX enzymes.\textsuperscript{82-84} The acetylation by aspirin completely blocks the PG production in both COX isoforms. Aspirin-acetylated COX-2 can convert arachidonic acid to 15-R-HETE, where subsequent metabolism occurs by 5-lipoxygenase to 15-epi-lipoxin A\textsubscript{4}.\textsuperscript{82} This lipoxin is called the aspirin-triggered lipoxin (ATL). Its epimeric counterpart, lipoxin A\textsubscript{4} (LXA\textsubscript{4}) is synthesized from arachidonic acid production of 15-lipoxygenase, however, both LXA\textsubscript{4} and ATL bind to the same G protein-coupled receptor and exert virtually identical anti-inflammatory actions.\textsuperscript{85,86} Both lipoxins inhibit recruitment of neutrophils by attenuating their chemotaxis, adhesion, and transmigration across vascular endothelial and epithelial cells.\textsuperscript{82,85} This is important since several studies have provided considerable evidence that neutrophils play a role in the pathogenesis of aspirin-induced gastric damage.\textsuperscript{87-89} These findings suggest that ATL interference with aspirin-stimulated neutrophil adherence to the vascular endothelium will likely result in diminished gastric damage – a biological mechanism for limiting aspirin associated gastric damage.

Several studies have indicated that NSAID-induced gastric damage requires inhibition of both COX isoforms.\textsuperscript{20,21,90} This was further emphasized when a recent study examining co-administration of a COX-2 selective inhibitor and aspirin found a significant increase in GI damage compared to either drug alone.\textsuperscript{80} Cyclooxygenase-2-derived LXA\textsubscript{4} increased gastric resistance to aspirin-induced damage.\textsuperscript{91} This study also found LXA\textsubscript{4} dose-
dependently reduced injury from aspirin, again suggesting a role in mucosal protection. Therefore, it is possible that increased GI damage following administration of aspirin and a selective COX-2 is due to the inhibition of acetylated COX-2’s conversion of arachidonic acid to 15-R-HETE. Another rat model of gastric inflammation, found that administration of aspirin enhanced capacity in the stomach to produce LXA₄, presumably related to increased expression of acetylated COX-2, and a concomitant increased resistance to damage caused by aspirin. Aspirin-triggered lipoxins share several protective effects with the native LXA₄ and it has been shown that they may also exert gastric protection by releasing NO from the vascular endothelium. The reduction in severity of gastric damage is thought to be due to increased mucosal blood flow and reduced adherence of leukocytes. Though lipoxins synthesized following aspirin-administration seem to play a role in gastric protection; their role is still not clear in the absence of aspirin administration.

**NSAID Selectivity in Dogs**

Based on the premise that inhibition of COX-1 would hinder basal physiological PG functions, a coxib class of NSAIDs was developed to selectively inhibit COX-2, with hopes of fewer adverse effects. NSAIDs are classified according to their ability to preferentially inhibit one isoform versus the other. Non-selective NSAID molecules are small and linear and can therefore block either COX isoenzyme. The coxib NSAID molecules were created to be too large to fit in the receptor site of the COX-1 enzyme, sparing COX-2 mediated PGs.
The specificity of a drug is typically expressed as a ratio of concentration at which a specific compound inhibits each isoenzyme. The concentration that inhibits 50% of COX activity (IC$_{50}$) has been the standard in determining selectivity ratios, however it has been suggested that this does not accurately approximate plasma concentrations achieved with therapeutic dosing.$^{94-96}$ One study concluded that IC$_{80}$ values may more closely resemble steady-state plasma concentrations and therefore seems to be a more accurate reflection of concentrations and activity in vivo.$^{96}$ A study comparing the two inhibitory concentrations found most NSAID selectivity ratios remained the same among the drugs studied, with the exception of etodolac, ibuprofen, and piroxicam (all of which became less selective with ratios close to 1).$^{97}$ A COX-2:COX-1 ratio of <1.0 is considered ideal, indicating that a given NSAID preferentially inhibits COX-2. However, it is not uncommon for the ratio to be reported as COX-1:COX-2, in which the ratio should be >1.0 in order to achieve selectivity.$^{98}$ Prostaglandin E$_2$ and TXB$_2$ concentration assays are generally the standard outcome measurements for inhibitory concentrations of COX-2 and COX-1 activity, respectively.$^{97}$ Nonetheless, various protocols have been used to elicit their production. Lipopolysaccharide (LPS) is commonly used to stimulate COX-2, however LPS stimulation has occurred at different time points within studies and incubation periods vary, ranging from 6$^{95}$ to 24$^{99-101}$ hours.

Combined with inconsistent data and variation among studies, drug comparison and interpretation of clinical applicability has been made difficult.$^{102}$ In some studies,$^{99}$ NSAIDs have assayed canine platelets and macrophage cell lines to determine COX-1 and COX-2 activities. Kay-Mugford et al. measured COX activity using a developed assay in a canine
monocyte/macrophage and a kidney cell line. Based upon the previous data, a whole blood assay was developed for use in vitro to compare NSAID selectivity in humans. Since this method does not rely on isolated cell lines, it was thought to more closely simulate physiological activity due to the presence of plasma proteins and other potential cofactors. Reviews of the aforementioned methods have indicated that the same NSAID can exhibit different selectivity ratios by using different cell types or assay techniques.

Most NSAIDs have a high degree of protein binding in circulation, affecting drug distribution and in vivo activity. Since COX selectivity is dependent on drug concentrations in target tissues, the selectivity of a drug could be lost at higher concentrations. Higher drug concentrations could result in toxic effects in tissues that rely upon basal physiologic levels of PGs. For these reasons, in vitro assays should not be used to predict in vivo efficacy and toxicity. With these limitations in mind, a few studies have assessed in vivo effects of NSAIDs on gastric mucosal production of prostanoids.

Sessions et al. stimulated PG production ex vivo on gastric biopsies taken from dogs treated with carprofen, deracoxib or etodolac. Interestingly, no drug altered the target tissue PG concentrations, which was unexpected and contradicts the classic COX-2 preferential and selective classification scheme. However, the protocol used in this study may not be of clinical relevance as it assessed the drug effect on the total possible production of prostanoids by the tissue, and therefore does not reflect the effect of a drug treatment on the actual tissue levels of prostanoids.

Difficulty in comparison is increased due to marked interspecies variation among experimental models, and differences within species between in vivo and in vitro
models. In vitro and ex vivo human studies were performed and both found etodolac to be COX-2 selective, suggesting an increased safety profile of this NSAID. In clinical observations similar safety profiles for etodolac administration in dogs have been seen. However, in vitro results from two different studies in dogs found etodolac to be COX-1 selective, suggesting species variation in the structure or activity of the COX enzyme. These results also reveal the importance of testing agents on target species in order to obtain the correct activity profile.

Several canine studies have accessed NSAID selectivity, most using some form of in vitro methodology. The selectivity of carprofen for COX-2 has varied by almost 100-fold across different studies. Only one study reported carprofen as a highly selective drug. In two in vitro and one in vivo study, etodolac appeared to be COX-1 sparing. However, another study evaluating etodolac found that it inhibited COX-2 more than 5 times as much as COX-1. Yet another study found etodolac to selectively inhibit COX-2, although this same study found carprofen inhibited COX-2 about 19 times as effectively as etodolac. That same study also found deracoxib to inhibit COX-2 approximately 375 times as much as etodolac and 20 times as much as carprofen. Another in vitro study, using a canine whole blood assay, found deracoxib to be 12 fold more selective for COX-2 than carprofen, and carprofen 5-6 fold selective for COX-2. The same study found firocoxib to be 384-fold more selective for COX-2 than COX-1, making it the most selective COX-2 inhibitor tested in vitro in canine blood. Another commonly used NSAID, meloxicam, was evaluated and inhibited COX-2 10-12 fold more than COX-1. The
inconsistency of the \textit{in vitro} data has made interpretation difficult and may inadequately reflect drug actions \textit{in vivo}. The clinical relevance of these findings is yet to be determined.

The efficacy of NSAIDs does not appear to be associated with COX selectivity; rather responses differ depending on individual patients. Studies suggest all veterinary NSAIDs are comparable in regards to anti-inflammatory and analgesic effects.\textsuperscript{114,115} Apart from a few studies that have examined the effect of NSAIDs on gastric mucosal production of prostanoids, COX selectivity has largely been determined using \textit{in vitro} assays, and assumptions made about the GI effects based upon this. Given the variability in results from these assays, and our lack of understanding of the COX physiology in the canine proximal GI tract, assumptions regarding the effects of various NSAIDs based on \textit{in vitro} results may lead to erroneous conclusions. Caution should be implemented when attempting to predict \textit{in vivo} efficacy and toxicity of an NSAID.\textsuperscript{105}

\textbf{NSAID-Induced Mucosal Damage in Dogs}

NSAIDs are the most commonly prescribed veterinary medication, yet studies have shown their administration can cause direct injury to gastric mucosa and even inhibit healing of tissue that has already been damaged.\textsuperscript{116-120} Although the mechanism of NSAID-induced ulceration is not completely understood, it is known that gastric damage related to NSAID administration is twofold: a topical effect upon the gastric mucosa and a systemic effect induced by impairment of gastroprotective PGs.\textsuperscript{56,121-123}

Direct topical injury is initiated by the weakly acidic and lipid-soluble characteristics of NSAIDs.\textsuperscript{77,117} The weak acids, in their nonionized lipophilic form within the lumen,
diffuse across plasma membranes into surface epithelial cells where they eventually dissociate into the ionized form, releasing hydrogen ions into the cell, leading to loss of cellular function.\textsuperscript{117,31,77,123} This acute topical injury can also cause a decrease in mucosal hydrophobicity by the uncoupling of the mitochondrial oxidative phosphorylation in mucus producing cells.\textsuperscript{31} Also, NSAIDs that undergo enterohepatic circulation may expose small intestine mucosa to reactive metabolites secreted within bile, causing mucosal damage.\textsuperscript{116,124} However, an \textit{in vitro} study found that that the presence of NSAIDs in the gastric lumen are not necessarily sufficient to provoke damage, suggesting bile must be present to induce toxic effects.\textsuperscript{124}

While acute topical injury is possible, the principal mechanism of NSAID-induced GI injury is thought to be inhibition PG synthesis.\textsuperscript{31} This mechanism is central in the development of side effects associated with the inhibition of gastroprotective PGs. Cyclooxygenase catalyzes arachidonic acid to PGH\textsubscript{2}. Following COX activity, an assessment of local prostanoids determines which prostanoids PGH\textsubscript{2} should elaborate. Co-localization of COX-1 and thromboxane synthesis in platelets is a good example of this. The platelets produce TXB\textsubscript{2}, which can be used as an indicator for COX-1 activity.\textsuperscript{102} Likewise, in LPS-stimulated macrophages, COX-2 and PGE\textsubscript{2} synthase are colocalized.\textsuperscript{125} However, there is no current technology to determine which isoform is producing PGE\textsubscript{2}, so co-localization with COX-2 is a simplistic statement. Inhibition of certain PGs can lead to decreased mucosal blood flow, epithelial mucus and bicarbonate secretion, and epithelial restitution, which can all attribute to ulceration.\textsuperscript{31,56,76,117,122} Ideally, a COX-2 inhibitor would
tip the balance toward gastric protection by not inhibiting COX-1 associated prostanoid synthases.

The incidence of NSAID-induced gastrointestinal complications in dogs is unknown; however, several canine studies have shown a marked increase of endoscopically visible, but clinically silent erosions in the mucosa in almost all dogs administered aspirin compared with placebo administration.\textsuperscript{123,126-129} A study examining the effects of buffered aspirin, carprofen, and etodolac in healthy dogs found higher lesion scores (hemorrhage, erosions, ulcerations) in dogs administered aspirin compared to placebo.\textsuperscript{123} They did not find a significant difference in median gastrointestinal lesion scores among dogs administered carprofen or etodolac.\textsuperscript{123} Likewise, very few, if any, differences have been seen during endoscopy of the gastric mucosa amongst dogs administered carprofen,\textsuperscript{107,122,123,130} deracoxib,\textsuperscript{107,130-132} etodolac,\textsuperscript{107,123,129} firocoxib,\textsuperscript{132} ketoprofen,\textsuperscript{122} and meloxicam.\textsuperscript{76,122,132} Luna et al. found that the NSAIDs they studied (carprofen, etodolac, meloxicam, ketoprofen) induced only minor damage, which was clinically unimportant, even after long-term use.\textsuperscript{133} In this study, carprofen induced the lowest number of GI adverse effects, followed by meloxicam, etodolac, and ketoprofen.\textsuperscript{133}

\textit{Role of neutrophils in NSAID-induced mucosal damage}

Recently it has been suggested that neutrophils play a role in initiation of mucosal damage. Several studies have found neutrophil adherence and neutrophil-derived factors to initiate injury.\textsuperscript{117,134} The presence of leukocyte adhesion molecules and an increase in TNF\textsubscript{\alpha} and leukotriene B\textsubscript{4} synthesis have been reported.\textsuperscript{89,135-137} At the vascular endothelium, neutrophil adherence can result in reduction of mucosal blood flow by obstructing capillaries,
thereby inducing injury via ischemia.\textsuperscript{56,77,134,138} One study found that NSAID-induced damage was significantly reduced in rats pretreated with antineutrophil serum or methotrexate to produce a neutopenic state.\textsuperscript{56} It is possible that neutrophil adherence occurs as a consequence of endothelial cell injury. These microvascular changes could be attributed to a deficiency of PGs important for normal vascular endothelium maintenance (primarily PGE\textsubscript{2} and PGI\textsubscript{2}).\textsuperscript{139} However, the exact mechanism responsible for these effects is still unclear.\textsuperscript{134,140}

Once neutrophils infiltrate the mucosa and become activated they can release reactive oxygen metabolites and mediate lipid peroxidase\textsuperscript{134,141,142} that may damage the mucosa.\textsuperscript{143,144} In rodents exhibiting ulceration, the degree of lipid peroxidase and the degree of NSAID-induced mucosal damage have been directly linked to the degree of reactive-oxygen species, likely derived by xanthine oxidase.\textsuperscript{134,142,145} Studies have found that vitamin E and/or melatonin can prevent lipid peroxidation, and could possibly be used to protect the gastric mucosa from NSAID-induced injury.\textsuperscript{134,146-148}

It has also been postulated that inhibition of the COX enzymes with traditional NSAIDs may result in a surplus of arachidonic acid, which may then become metabolized via the 5-LOX pathway, into leukotrienes.\textsuperscript{108} Following NSAID administration, overproduction of leukotrienes have been shown in human gastric mucosa.\textsuperscript{108,149} Leukotriene B\textsubscript{4} is a potent stimulus for neutrophil chemotaxis, adhesion and degranulation; it also increases microvascular permeability, all of which could contribute to mucosal damage.\textsuperscript{68,108,150,151}
Tepoxalin is a drug created for dogs with chronic arthritis and it is designed to inhibit COX-1, COX-2, and LOX activity where it theoretically suppresses leukotrienes, thereby assisting in damage prevention.\textsuperscript{108} In studies with laboratory animals, tepoxalin did not display ulcerogenic activity when administered within its therapeutic range, and pretreatment with tepoxalin prevented indomethacin (a non-selective NSAID) induced lesions.\textsuperscript{150-152} Although the aforementioned studies are interesting, the studies have been of short duration. Very little information on LOX inhibitors is available, and no controlled clinical trial has been done to evaluate the relative efficacy and safety of this dual inhibitor against COX inhibiting NSAIDs in dogs.

**Role of Cyclooxygenase-2 in the Gastrointestinal Tract**

*Mucosal Protection and Resolution of Inflammation*

In the GI tract of healthy humans and various animals, COX-2 has been either undetectable or expressed at very low levels.\textsuperscript{19} However, it has been well documented that COX-2 is responsible for increased PG levels at sites of inflammation and disease. When the gastric mucosa is damaged, COX-2 is rapidly expressed and studies suggest it may play a role in facilitating ulcer repair; delayed ulcer healing was noted in rodents administered selective COX-2 inhibitors.\textsuperscript{153-156} Other studies have found COX-2 expression to be upregulated in the margins of healing gastric ulcers.\textsuperscript{48,56,77,138} Administration of a selective COX-1 inhibitor did not affect gastric healing.\textsuperscript{156,157}

The aforementioned observations complicates the postulated roles for both COX isoforms; principally COX-2, suggesting that COX-2 somehow contributes to mucosal
protection. This seems paradoxical given the aforementioned observations of such low levels of COX-2 expression in the stomach. Davies et al., observed that while COX-2 expression was indeed very low in a healthy stomach, upregulation could occur rapidly. Within 1 hour of oral administration of aspirin or indomethacin (non-selective NSAIDs), COX-2 expression levels were markedly increased. Administration of PGs prevented induction of COX-2, therefore this observed increase in expression was probably in response to diminished mucosal PGs, suggesting upregulation occurred as a protective mechanism. Wallace et al., found administration of a COX-1 inhibitor did not disturb mucosal integrity in rats. However, a combination of a selective COX-1 inhibitor and a selective COX-2 inhibitor elicited gastric damage. Likewise, another study found simultaneous blockade of both isoforms to be necessary in order to induce lesions and that selective COX-1 inhibitors alone may not be ulcerogenic.

A study examining paw edema in wild type and COX-2 deficient mice emphasized the role of COX-2 in resolution of inflammation. Carrageenan-injections induced inflammation to a similar extent in both COX-2 deficient and wild type mice. This study found significant leukocyte infiltration in the paws of both mice. In wild type mice, the swelling subsided within 24-48 hours. In COX-2 deficient mice, NSAIDs reduced paw swelling, suggesting COX-1 derived PGs were responsible for the inflammatory response. The paw swelling in the COX-2 deficient mice (not administered an NSAID) was still apparent up to one week after injection. These findings indicate that both COX-1 and COX-2 contribute to PG production at the site of inflammation. This study concluded that PGs derived from COX-2 have a role in the resolution phase, as well as in the early stages of the
inflammatory response.\textsuperscript{161} Another study, found COX-2 to be directly involved in regulating inflammatory resolution in a carrageenan-induced pleurisy model.\textsuperscript{162} This study showed that there were two phases of COX-2 expression. An early peak (at about two hours) was associated with onset of inflammation, leukocyte infiltration, PGE\textsubscript{2} production and COX activity.\textsuperscript{162} Non-selective and COX-2 selective NSAIDs inhibited this early phase of the inflammatory response.\textsuperscript{163} A second and much greater peak in COX-2 expression occurred 48 hours after irritant injection.\textsuperscript{162} At this peak, the number of leukocytes in the pleural cavity decreased to normal levels, resulting in the resolution of the inflammation. No detectable PGE\textsubscript{2} was present, however another PGH\textsubscript{2} metabolite, PGD\textsubscript{2}, accompanied the rise of COX-2 expression.\textsuperscript{162} Administration of COX-2 selective inhibitors from 24 h to 48 h post-injection of irritant (during the resolving phase) abolished PGD\textsubscript{2} production and prolonged the inflammatory response by preventing a decrease in leukocytes. A normal course of resolution was seen with co-administration of PGD\textsubscript{2} and cyclopentenone PGs, indicating products of PGD\textsubscript{2} may be responsible for initiating resolution of inflammation.\textsuperscript{162}

These studies have established that the expression of COX-2 appears to provide a second line of defense for the gastric mucosa and COX-2 may also be a crucial mediator in mucosal repair process. Cyclooxygenase-2 is now recognized as a source of mediators, which can be both beneficial and detrimental. Such information would suggest that COX-2 inhibitors may not be beneficial in cases of previously damaged mucosa.

\textbf{COX-2 expression pathways}

Cyclooxygenase-2 expression is upregulated in both acute and chronic inflammation. During inflammation, two general pathways regulate COX-2 expression. These pathways
respond to extracellular stimuli, such as lipopolysaccharide (LPS), and regulate several cellular activities including mitosis, differentiation, and gene expression. One pathway involves activation of the nuclear factor-κB (NFκB), a nuclear transcription factor, resulting in increased gene transcription and the synthesis of COX-2 mRNA. The second involves several highly conserved intracellular signaling molecules, known collectively as mitogen-activated protein kinases (MAPKs). The three major MAPK-signaling pathways that lead to altered gene expression are the extracellular regulated kinases (ERK1/2), p38 MAPK, and c-Jun NH (2)-terminal kinase (JNK).

Four isoforms of p38 MAPK have been identified, α, β, γ and δ, and the expression of these isoforms varies depending on the cell type. Inflammatory cells, including neutrophils, monocytes, macrophages and CD4⁺ T cells, predominantly express p38α and δ, whereas endothelial cells express all isoforms of p38 MAPK. Studies have indicated involvement of p38 MAPK in the upregulation of COX-2, at the level of transcription. Also, by stabilizing mRNA it is involved with post-transcription and has a direct effect on the rate of translation. Transcription factors, such as NFκB are activated by MAPKs, however, because p38 MAPK can activate the COX-2 gene promoter directly, activation of transcription factors are not required for upregulation of COX-2.

The p38 MAPK pathway can regulate gene expression by one of two mechanisms, activation of transcription and stabilization of mRNA transcripts. The mRNA encoding these genes is unstable due to an AU-rich 3’ untranslated region. These regions are bound by AU-binding proteins, therefore mRNA is unable to be translated in a resting cell. Upon activation of the cell, mRNA levels are increased due to phosphorylation of the AU-binding
proteins by p38 MAPK. These proteins are released, stabilizing mRNA and promoting translation.

Lipopolysaccharide can trigger inflammation and activate multiple intracellular signaling pathways, including the NFκB and the mitogen activated protein kinase (MAPK) pathways.\textsuperscript{177-180} Inflammation is triggered when LPS binds to and activates its receptor complex on cellular membranes, comprised of CD14, toll-like receptor-4 (TLR-4), and MD-2. Recent studies have demonstrated that LPS stimulation of equine leukocytes results in the activation of the p38 MAPK pathway.\textsuperscript{181} It has also been shown that upon LPS stimulation of equine leukocytes, COX-2 expression increases and COX-2-dependent prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) production is upregulated by the p38 MAPK pathway.\textsuperscript{181-183}

The importance of the p38 MAPK pathway in pro-inflammatory gene expression has been demonstrated in several studies.\textsuperscript{184-189} The exact mechanisms involved in COX-2 expression is still to be determined, but evidence indicates that the MAPK pathways, p38 in particular, play a pivotal role in pro-inflammatory gene expression. Further evidence suggests the p38 MAPK pathway may be a suitable target for therapeutics designed for the treatment of endotoxemia and other pro-inflammatory diseases.

Conclusion

Many unanswered questions remain and it is clear that the role of both COX isoforms is more complex than initially anticipated. Several studies indicate that the classical COX hypothesis oversimplifies the role of COX-2, as it appears to do more than just mediate pain and inflammation. We know that the upregulation of COX-2 is commonly associated with
inflammation or mucosal injury, but some evidence of constitutive expression in humans suggests COX-2 may represent a second line of defense in the gastric mucosa. While significant discoveries have been made, the exact role and relevance of COX-2, requires further investigation. Thus, the need for more research to access the role of these enzymes is needed, in particular COX-2.
REFERENCES


CHAPTER 2
Cyclooxygenase expression and prostanoid production in pyloric and duodenal mucosae in dogs after administration of nonsteroidal anti-inflammatory drugs

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Objective—To assess cyclooxygenase (COX) expression and prostanoid concentrations in pyloric and duodenal mucosae of dogs after administration of nonsteroidal anti-inflammatory drugs (NSAIDs).

Animals—8 healthy dogs.

Procedures—Each dog received carprofen (4.4 mg/kg, q 24 h), deracoxib (2 mg/kg, q 24 h), aspirin (10 mg/kg, q 12 h), and placebo (1 dog treat, q 24 h) orally for 3 days (4-week interval between treatments). Before study commencement (baseline) and on day 3 of each treatment, pyloric and duodenal mucosal appearance was assessed endoscopically and biopsy specimens were obtained for histologic examination. Cyclooxygenase-1 and COX-2 protein expressions were assessed via western blotting, and prostanoid concentrations were measured via ELISAs. An ANOVA was used to analyze data.

Results—Treatments had no effect on mucosal appearance and ulceration was not evident histologically. In pyloric and duodenal mucosae, COX-1 expression was unaffected by treatments. Cyclooxygenase-2 expression remained unchanged in pyloric mucosa; in duodenal mucosa, aspirin significantly increased COX-2 expression, compared with effects
of deracoxib and carprofen. At baseline, total prostaglandin and thromboxane B_2 concentrations in pyloric mucosa were significantly greater than those in duodenal mucosa. Aspirin significantly decreased both prostanoid concentrations in both mucosal tissues, compared with other treatments. In pyloric mucosa, carprofen administration significantly decreased total prostaglandin and thromboxane B_2 concentrations, compared with deracoxib administration.

Conclusions and Clinical Relevance—In dogs, prostanoid synthesis was greater in pyloric mucosa than it was in duodenal mucosa. Nonselective NSAIDs significantly decreased prostanoid concentrations in these mucosae, compared with the effects of a selective COX-2 NSAID. (Am J Vet Res 2008;69:457–464)

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ABBREVIATIONS

<table>
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<th>NSAID</th>
<th>COX</th>
<th>PG</th>
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<td>Nonsteroidal anti-inflammatory</td>
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In veterinary medicine, the use of NSAIDs for treatment of pain is increasing. Once administered primarily to dogs with osteoarthritis to reduce joint pain and decrease synovitis, NSAIDs are now frequently used to control postoperative pain and inflammation. However, these drugs can be associated with adverse effects, most notably gastrointestinal tract ulceration. In dogs, NSAID-associated gastrointestinal tract ulceration most commonly develops in the region of the pylorus and proximal portion of the duodenum. The principal mechanism of action of NSAIDs is inhibition of COX. However, results of recent studies have indicated that there are multiple isoforms of COX, and the degree to which each NSAID inhibits these COX isoforms varies. The COX enzymes catalyze the conversion of arachidonic acid to PGH$_2$, which is subsequently metabolized by local prostanoid synthases. Prostanoids, particularly PGE$_2$, play a role in gastrointestinal tract protection and repair. Cyclooxygenase-1, which is constitutively expressed in the gastrointestinal tract, is thought to exert homeostatic properties that are crucial for gastric physiologic function, including mucosal protection. Cyclooxygenase-2 is inducible in most mammalian tissues in response to proinflammatory stimuli and has been linked to inflammation. However, it is now known that COX-2 is constitutively expressed in certain tissues, such as ovaries and kidneys. Because COX-1–derived PGs are thought to play a dominant role in gastric mucosal defense and cytoprotection, drugs that selectively inhibit COX-2 are considered to be potentially less damaging to the gastrointestinal tract than those that also block COX-1. Nevertheless, COX-2 mRNA is present in specific locations within the gastric mucosa.
Little is known about the in vivo effects of NSAIDs on pyloric and duodenal mucosae in dogs. Assumptions have been made regarding those in vivo effects based on the degree to which a given NSAID inhibits the COX isoforms, despite a lack of knowledge of the role of various COX enzymes in different tissues. Although results from in vitro assays of canine whole blood vary from study to study, \(^{18,19}\) comparison of carprofen and deracoxib revealed that the selectivity of these drugs was 5- to 6-fold and 12-fold as high for COX-2 as for COX-1, respectively. \(^{20}\) Aspirin is considered a nonselective NSAID in dogs. \(^{17,21}\) The purpose of the study reported here was to assess COX-1 and COX-2 protein expression and prostanoid concentrations in the pyloric and duodenal mucosae in dogs after administration of selective and nonselective or less-selective COX inhibitors (i.e., NSAIDs). We hypothesized that COX-1 and COX-2 proteins would be expressed in pyloric and duodenal mucosal tissues and that inhibition of COX enzymes by a more selective inhibitor would have less effect on prostanoid synthesis than would a less selective inhibitor.

**Material and Methods**

This study was approved by the Animal Care and Use Committee at North Carolina State University and was conducted in accordance with the National Institutes of Health and the International Association for the Study of Pain policies on the use of clinical subjects.

**Dogs**—Eight adult purpose-bred mixed-breed dogs (4 females and 4 males) that weighed 8 to 13 kg were used in the study. All dogs underwent a physical examination to ensure they were healthy prior to inclusion in the study. In addition, a CBC, serum biochemical analysis,
and urinalysis were performed immediately prior to study commencement. Also, prior to the start of the study, gastroduodenoscopy was performed on each dog to rule out preexisting gastroduodenal disease.

**Experimental protocol**—The study was a randomized, placebo-controlled, crossover design. Each dog randomly received carprofen\(^a\) (4.4 mg/kg, q 24 h), deracoxib\(^b\) (2 mg/kg, q 24 h), aspirin\(^c\) (10 mg/kg, q 12 h), or placebo\(^d\) (1 dog treat, q 24 h) orally for 3 days with a 4-week washout period between treatments. Each dog received each treatment by administration per os. Drug treatments were not given with anything else. Commercially available products were used, and dogs received a drug dose that was within ± 10% of the recommended dose according to package inserts. Dogs were treated for 3 days with each drug to attain a theoretical steady state concentration in the plasma and presumably in the tissue of the gastrointestinal tract.

Prior to initiation of the study (baseline) and on day 3 following administration of each treatment, the pyloric and duodenal mucosae were examined endoscopically and mucosal biopsy specimens were obtained. On day 3, endoscopy took place 2 hours after treatment administration. Food was withheld from the dogs for 24 hours prior to endoscopy. Anesthesia was induced with propofol (10 to 15 mg/kg according to effect) and maintained with isoflurane vaporized in 100% oxygen to effect following orotracheal intubation. Gastroduodenoscopy and biopsies were performed by 1 investigator (SLM) with a flexible videogastroscope,\(^e\) and the procedures were recorded electronically. All endoscopic procedures were performed at the same time each morning (10:00 am) to avoid diurnal and feeding-associated effects. Within a region, biopsy locations were at least 2 cm from one
another. Mucosal biopsy specimens obtained from the pylorus and duodenum were immediately (within 6 to 8 seconds) snap frozen in liquid nitrogen, stored at –80°C, and subsequently used for western blot analysis of COX-1 and COX-2 expression and measurement of total PG and TXB₂ concentrations. Care was taken to ensure that each biopsy sample was treated identically. In addition, other mucosal biopsies were immediately placed in neutral-buffered 10% formalin for histologic evaluation.

**Western blot analysis**—One biopsy sample from the pylorus and 1 biopsy sample from the duodenum of each dog was each added to 200 µL of modified radio-immunoprecipitation buffer including the protease inhibitors aprotinin, phenylmethylsulfonyl fluoride, and sodium orthovanadate. The samples were homogenized on ice, and the supernatants were extracted via centrifugation. Protein analysis of extracted samples was performed, and equal concentrations of protein from each sample were mixed and boiled with sample buffer. The lysates were then loaded into wells of precast gels and protein electrophoresis was performed according to standard protocols.

After the protein was transferred to a polyvinylidene fluoride membrane and blocked in 5% milk with 0.05% Tween-20, washed membranes were incubated overnight (approx 18 hours) in a 1:300 solution of either polyclonal anti-COX-1 or anti-COX-2 primary antibody. The membranes were then incubated in a horseradish-peroxidase–conjugated secondary antibody and developed by addition of enhanced chemiluminescence reagents. β-Actin expression was used as an internal verification that the same amount of protein had been loaded into each well. Recombinant COX protein was used as a positive control sample, and
a molecular weight indicator (protein standard) was used to ensure that the canine COX protein bands corresponded to the appropriate measurement (in kilodaltons) for COX. Negative control samples were occasionally used. This approach is similar to that used in a previous study\(^{22}\) in horses when a specific antibody was not available. For each dog, samples from the pylorus and duodenum for all 4 treatments were processed on a single gel; this allowed comparison of the levels of COX expression following each treatment within each dog. By use of the densitometry values, COX protein concentrations following each treatment were expressed as a percentage of the baseline value for each region (pylorus and duodenum) in each dog. To compare the overall levels of COX protein expression in the duodenum and pylorus, the densitometric values for each dog were expressed as a percentage of the baseline value for the pylorus.

**Prostanoid analysis**—Each biopsy sample was added to 200 µL of Tris buffer (50mM Tris-HCl, 150mM NaCl, and 1mM EDTA; pH, 7.4), including aprotinin, phenylmethylsulfonyl fluoride, and sodium orthovanadate. Samples were homogenized on ice and the supernatants were extracted via centrifugation. Protein analysis of extracted samples was performed. Prostaglandin and TXB\(_2\) concentrations were measured by use of commercially available ELISA assay kits.\(^{jk}\) Results were expressed as picogram of prostanoid per microgram of protein in the tissue.

**Mucosal lesion scoring and histologic analysis**—After completion of the study, the endoscopy videos were all reviewed by 1 investigator (SLM) who was unaware of the treatment protocols. The pyloric and duodenal mucosae were assessed for lesions, and a score of 0 (apparently normal) to 4 (severely affected) was assigned by use of a subjective
scoring system (Appendix). Hematoxylin and eosin-stained slides of pyloric and duodenal biopsy specimens were evaluated for inflammation and ulceration by a board-certified veterinary pathologist (JML) who was not aware of the treatment groups.

**Data analysis**—A 2-way repeated measures ANOVA was used to compare the densitometric data for COX-1 and COX-2 protein concentrations, PG and TXB₂ concentrations, and mucosal scores to detect any differences among treatments. A Tukey test was used to identify specific differences among treatments, and significance was set at a value of \( P < 0.05 \). An ANOVA on ranks was used when the data were not normally distributed.

**Results**

Prior to commencement of the study, no abnormalities were detected via physical examination for any dog, and clinicopathologic values were within the reference ranges used at the North Carolina State University Veterinary Teaching Hospital clinical pathology laboratory. No clinically important adverse effects were observed after any drug administration.

Histologic examination revealed no evidence of ulceration or clinically important inflammation in any biopsy specimen. *Helicobacter* spp were detected in 10 of 32 (31%) histologic samples. These dogs were not omitted from the study on the basis of this finding. No treatment-induced bleeding was observed in the pylorus or duodenum. Some pyloric biopsy samples revealed small lymphoid follicles or mild lymphoplasmacytic enteritis. Mild to moderate abnormalities were evident in 27 of 32 (84%) and 26 of 32 (81%) pyloric antral
and duodenal biopsy specimens, respectively. These findings were not related to a specific drug or dog. The mucosal lesion score data were normally distributed; region \((P = 0.62)\) and treatment \((P = 0.28)\) had no effect on the lesion score.

At baseline, densitometric data indicated that the mean amount of COX-1 in the duodenum was 79\% of the amount in the pylorus and the mean amount of COX-2 was 81\% of the amount in the pylorus, but there was considerable variation from dog to dog. Drug administration had no effect on COX-1 protein expression in the pyloric mucosa or duodenal mucosa and no effect on COX-2 protein expression in the pyloric mucosa. In the duodenal mucosa, aspirin significantly \((P < 0.05)\) increased COX-2 expression, compared with the effects of deracoxib and carprofen (Figures 1 and 2).

**Figure 1: COX-2 Blot**

![COX-2 Blot](image)

Figure 1—Representative western blot of COX-2 protein expression in biopsy specimens of duodenal mucosa obtained endoscopically from 1 dog before (baseline; B) and after each of 4 treatments. At 4-week intervals, the dog received 3-day treatments with a placebo (P; 1 dog treat, q 24 h), carprofen (C; 4.4 mg/kg, q 24 h), deracoxib (D; 2 mg/kg, q 24 h), or aspirin (A; 10 mg/kg, q 12 h). Notice that compared with baseline, aspirin administration caused an increase in mucosal COX-2 protein expression, whereas administration of carprofen or deracoxib had no effect.

At baseline, PG concentration was significantly \((P < 0.05)\) greater in pyloric mucosa, compared with duodenal mucosa (mean ± SEM baseline concentrations, 630 ± 66 pg/µg of protein vs 311 ± 21 pg/µg of protein; **Table 1**). Aspirin significantly \((P < 0.05)\) decreased PG concentrations in pyloric and duodenal mucosae, compared with effects of all other
treatments. In pyloric mucosa, carprofen significantly ($P < 0.05$) reduced the PG concentration, compared with the effect of deracoxib. At baseline, the mean ± SEM concentration of $\text{TXB}_2$ was significantly ($P < 0.05$) greater in pyloric mucosal tissue (600 ± 105 pg/µg of protein), compared with the value in duodenal mucosal tissue (201 ± 50 pg/µg of protein). Aspirin significantly ($P < 0.05$) decreased $\text{TXB}_2$ concentration in pyloric and duodenal mucosae, compared with effects of all other treatments and baseline values. In pyloric mucosa, carprofen significantly ($P < 0.05$) reduced the PG concentration, compared with the effect of deracoxib; this effect was not evident in duodenal mucosa.

**Figure 2: COX-2 protein expression**

![Graph showing COX-2 protein expression](image)

Figure 2—Effects of 3-day oral treatments with a placebo (1 dog treat, q 24 h), carprofen (4.4 mg/kg, q 24 h), deracoxib (2 mg/kg, q 24 h), or aspirin (10 mg/kg, q 12 h) on COX-2 protein expression in biopsy samples of duodenal mucosa obtained from 8 dogs in a crossover study. Values are expressed as mean ± SEM percentage of baseline level (determined prior to any treatment). Data were analyzed by use of an ANOVA, and post hoc analyses were performed with a Tukey test. *Value was significantly ($P < 0.05$) increased, compared with values associated with deracoxib and carprofen treatments.
Table 1: Total PG and TXB₂ concentrations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total PG concentration</th>
<th>TXB₂ concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µg of PG/µg of tissue protein)</td>
<td>(µg of TXB₂/µg of tissue protein)</td>
</tr>
<tr>
<td>Baseline</td>
<td>Pyloric mucosa 630 ± 66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Duodenal mucosa 311 ± 21</td>
</tr>
<tr>
<td>Placebo</td>
<td>640 ± 62</td>
<td>302 ± 37</td>
</tr>
<tr>
<td>Carprofen</td>
<td>410 ± 77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>359 ± 33</td>
</tr>
<tr>
<td>Deracoxib</td>
<td>707 ± 41</td>
<td>284 ± 12</td>
</tr>
<tr>
<td>Aspirin</td>
<td>147 ± 52&lt;sup&gt;c&lt;/sup&gt;</td>
<td>119 ± 22&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Baseline PG concentrations in the pyloric mucosa were significantly higher than findings in the duodenal mucosa. <sup>b</sup>Carprofen administration significantly (<i>P</i> < 0.05) reduced PG concentration, compared with the effect of deracoxib administration and baseline values. <sup>c</sup>Administration of aspirin significantly (<i>P</i> < 0.05) reduced PG and TXB₂ concentrations in pyloric and duodenal mucosa, compared with effects of all other treatments. <sup>d</sup>Baseline TXB₂ concentrations in pyloric mucosa were significantly higher than findings in the duodenal mucosa. <sup>e</sup>Carprofen administration significantly (<i>P</i> < 0.05) reduced TXB₂ concentration, compared with the effect of deracoxib administration.

Table 1—Mean ± SEM concentrations of PG and TXB₂ in pyloric and duodenal tissue specimens collected before (baseline) and after 3-day treatments with placebo (1 dog treat, PO, q 24 h), carprofen (4.4 mg/kg, PO, q 24 h), deracoxib (2 mg/kg, PO, q 24 h), and aspirin (10 mg/kg, PO, q 12 h) in 8 dogs in a crossover study (4-week intervals between treatments). Data were analyzed by use of an ANOVA to compare treatment groups within a region and also to compare baseline values between regions.

Discussion

In small animal veterinary medicine, deracoxib and carprofen are among the most commonly prescribed NSAIDs for treatment of acute and chronic pain. Carprofen has been described as a COX-1–sparing drug because of its profile of COX-1 and COX-2 inhibition; deracoxib has been described as a selective COX-2 inhibitor. So, both drugs spare the COX-1 enzyme, thereby potentially improving the safety profile of NSAIDs while retaining efficacy. Efficacy of these NSAIDs is presumed to be associated with COX-2 inhibition. In several <i>in vitro</i> studies, the selectivity of carprofen for COX-2 has varied extensively (almost a 100-fold difference in values), and in 1 investigation, the COX-2 selectivity of carprofen was high. In another of those <i>in vitro</i> studies, the COX selectivity of carprofen and deracoxib was assessed and results indicated that the selectivity of these drugs was 5- to
6-fold and 12-fold as high for COX-2 as for COX-1, respectively. In an in vivo study to compare the effects of carprofen with those of deracoxib by use of platelet function tests, carprofen decreased clot strength and decreased platelet aggregation, whereas deracoxib did not significantly alter platelet function.26 Synthesis of TXA₂ in platelets is a key step in platelet aggregation and is mediated exclusively by COX-1.27 From review of the findings of the in vitro and in vivo studies, it is clearly difficult to predict what in vivo effects an NSAID will have on the basis of its theoretical mechanism of action. To our knowledge, the present study is the first to evaluate the in vivo effects of NSAIDs on COX-1 and COX-2 protein expression in both the pyloric and duodenal mucosae of dogs.

The present study was designed to assess the in vivo effects of short-term oral administration of NSAIDs on pyloric and duodenal mucosae, which are tissues at risk for NSAID-induced ulceration.2 In our study, no dog developed any clinical signs of gastrointestinal disease, although the duration of each treatment was only 3 days. Ideally, such a study would involve collection of data at several time points over an extended period. One limitation of such an approach is the effect that one endoscopic episode would have on a subsequent endoscopic episode, even several days later. Therefore, as a starting point, the present study was designed to evaluate the effect of initial administration of NSAIDs within a short period. We chose a 3-day treatment period partly because assessments had been made at a 3-day time point in another study,24 and findings indicated that gastric PGE₂ concentration was significantly decreased by all of the drugs evaluated, including deracoxib and carprofen. We wanted to confirm those findings and also to evaluate the response of the
In the pyloric and duodenal mucosal tissue samples collected from the dogs in our study, histologic findings were unremarkable regardless of treatment. Examination of some pyloric biopsy specimens revealed small lymphoid follicles or mild lymphoplasmacytic enteritis, but these findings were not related to a specific drug or dog. Inflammation is often detected in clinically normal dogs, and histologic findings of a previous endoscopic study were similar. In that study, all of the dogs had *Helicobacter* spp present in histologic samples. Among the biopsy specimens collected from the dogs of the present study, there was also some histologic evidence of *Helicobacter* spp. The species of *Helicobacter* was not determined, and the clinical relevance of *Helicobacter* spp in the gastrointestinal tract of dogs is unclear. *Helicobacter* organisms are commonly found in clinically normal dogs, and in a study of 31 healthy laboratory dogs, all had various *Helicobacter* spp without any evidence of gastrointestinal disease. Compared with the latter finding, the number of *Helicobacter*-positive dogs in the present study was low. There was no correlation between the mucosal lesion scores and the histologic findings in the present study.

It was unexpected to find an effect of NSAIDs on COX protein expression because NSAIDs are thought to inhibit COX enzyme action, but not alter the expression of the COX enzymes. However, there is some evidence that suggests NSAIDs may alter (decrease) expression of the COX mRNA or enzyme. Such an effect has also been identified in equine intestinal mucosa by our group. During the present study, drug administration did not affect the expression of COX-1 protein in the pyloric or duodenal mucosal tissue in dogs;
in addition, there was no detectable effect on COX-2 protein expression in the pyloric mucosa. However, in the duodenal mucosa, aspirin significantly increased COX-2 expression, compared with the effects of deracoxib and carprofen. The mechanism and relevance of this finding will require further investigation. One limitation of our study was the fact that tissue concentrations of each NSAID were not measured; thus, we could not relate our findings to tissue concentrations of the drugs.

In the present study, the finding of constitutive expression of COX-2 protein in canine gastroduodenal mucosa was novel. Historically, in most species, COX-2 has been regarded as inducible in the gastrointestinal tract; our findings are in contrast to those of another study in which no COX-2 protein expression was detected in gastrointestinal tissues in dogs that had not received any NSAIDs. In humans and rodents, COX-2 expression was upregulated in inflamed gastrointestinal tissue, consistent with its role as an inducible enzyme under conditions of inflammation. Results of other investigations have indicated that COX-2 expression is upregulated in the margins of healing gastric ulcers. In addition, research has determined that selective inhibition of COX-1 alone may not cause ulcers, but simultaneous blockade of both COX isoforms induces lesions, suggesting a possible housekeeping role of COX-2 in the gastrointestinal tract. The finding in our study of constitutive expression of COX-2 protein in pyloric and duodenal mucosae of dogs both prior to and after being treated with NSAIDs supports the suggestion that COX-2 has some housekeeping role in the proximal portion of the gastrointestinal tract. Nevertheless, its exact role remains to be determined.

Gastric mucosal injury induced by NSAIDs is thought to be attributable to inhibition
of the synthesis of PGs. Synthesis of PGs is a normal function of COX-1 in the stomach, and the PGs provide protection to the gastrointestinal mucosa. The protective effects of PGs include stimulation of epithelial proliferation, regulation of mucosal blood flow, and stimulation of mucus-bicarbonate secretion. It is postulated that PGs generated by COX-2 also participate in gastrointestinal tract protection, but the exact role of the enzyme is not known, and the conditions under which such activity might occur are not understood. In the present study, total PG concentrations were measured. With current techniques, it is not possible to determine which COX isoform induces each prostanoid in tissues other than whole blood. In our study, measurement of total PG synthesis allowed the investigation of synthesis of all PGs by both COX enzymes, and the actual tissue concentrations of PG (and TXB₂) at the time of biopsy were assessed. Other investigators have measured the total amount of prostanoids that can be generated when tissue biopsy specimens are subsequently stimulated ex vivo. This latter approach is probably a good indicator of total COX enzyme activity in tissue but it does not reflect the prostanoid level in that tissue at the time of biopsy specimen collection. We consider the protocol used in our study more clinically relevant because it reflects the effect of a drug treatment on the actual tissue concentrations of prostanoids and not the effect of a drug treatment on the total possible synthesis of prostanoids by a tissue. The 2 approaches have not been directly compared. Moreover, results obtained from a study to evaluate the concentrations of PGE₂ at different regions of the rat gastrointestinal tract by use of these 2 approaches indicate that the 2 techniques may not be comparable. In the dogs of our study, baseline PG concentrations in the pyloric mucosa
were significantly higher than those in the duodenal mucosa, which may be explained by differences in COX expression in those 2 areas of the gastrointestinal tract. Baseline COX-1 expression was higher in the pyloric mucosa, compared with findings in the duodenal mucosa. This finding in our study is similar to the results of a study of 4 dogs by Kargman et al., although no statistical evaluation was performed on their data. In that investigation, PGE₂ synthesis in human duodenal tissues was higher than that in gastric tissues, but the converse was true for rat and rhesus monkey tissues; PGE₂ concentrations in tissues from dogs were not examined. Those investigators concluded that, in general, higher PGE₂ concentrations correlated with greater concentrations of COX-1 protein. In the present study, carprofen and aspirin decreased the total concentration of PGs in gastric mucosa, whereas PG concentrations were not altered by deracoxib. In another study in which an assay method involving stimulation of PG synthesis was used, both carprofen and deracoxib decreased gastric PGE₂ concentration, but not PGE₁ concentration, after 3 days of oral administration. In our study, it appeared that inhibition of PG synthesis was only detected after administration of drugs that were considered to inhibit COX-1 activity. This suggests that the PG concentrations measured in our study were more reflective of COX-1 activity, despite the fact that COX-2 protein was also detected in the mucosa. The decrease in pyloric mucosal PG concentration in association with aspirin administration in dogs has been reported previously. To further assess the effect of drug administration of 3 days’ duration on COX-1 and COX-2, an *ex vivo* investigation of blood samples could have been conducted as part of the present study. However, it is unknown how the results of such an *ex vivo*
investigation of a different tissue type (ie, blood) would relate to NSAID-induced COX inhibition in the gastrointestinal mucosa.

To our knowledge, measurements of TXB\textsubscript{2} concentrations in intestinal mucosal samples obtained from dogs following NSAID treatments have not been previously reported. In the dogs of the present study, TXB\textsubscript{2} concentrations were significantly higher in the pyloric mucosa, compared with findings in the duodenal mucosa, which appeared to reflect higher concentrations of COX protein expression in the region of the pylorus. Thromboxane is indicative of COX-1 activity in the gastrointestinal tract in pigs.\textsuperscript{45} However, it is not known whether TXB\textsubscript{2} can be linked to COX-1 activity in the gastrointestinal tract of dogs. Nevertheless, the drug with the least COX-1–sparing activity (aspirin) significantly reduced TXB\textsubscript{2} concentration in pyloric mucosa, compared with effects of all other treatments. Carprofen also significantly decreased TXB\textsubscript{2} concentrations, compared with the effect of deracoxib; this suggested that carprofen also inhibits COX-1 activity in the gastric mucosa of dogs, whereas deracoxib has no effect on that activity. In the duodenal mucosa, the only significant decrease in TXB\textsubscript{2} concentrations was detected in dogs treated with aspirin. The differences between the effects of carprofen and deracoxib on total PG and TXB\textsubscript{2} concentrations in the pyloric and duodenal mucosae may reflect a degree of activity-dependant inhibition by carprofen. If true, such a phenomenon has not been reported previously. In the pyloric mucosa, which had greater tissue concentrations of PGs and TXB\textsubscript{2} (and presumably greater COX-1 activity) than the duodenal mucosa, treatment with carprofen affected prostanoid synthesis to greater extent than deracoxib. The yet greater
ability of aspirin to inhibit prostanoid synthesis may explain results from several studies in which almost all dogs that were administered aspirin developed gastric lesions. However, the clinical relevance of those lesions is debated.

The findings of the present study suggest that different NSAIDs reduce prostanoid synthesis to a different degree in the pylorus and duodenum of dogs, and this appears to relate to the drugs’ COX selectivity. Apart from a few studies that have examined the effect of NSAIDs on gastric mucosal production of prostanoids, COX selectivity has largely been determined by use of in vitro assays, and assumptions about the gastrointestinal tract effects of NSAIDs have been based on those determinations. Because of the variability in results from such assays and our lack of understanding of the physiologic actions of COX in the proximal portion of the gastrointestinal tract of dogs, making assumptions about the effects of various NSAIDs on the basis of in vitro experimental results may lead to erroneous conclusions. The purpose of our study was to assess the in vivo action of NSAIDs in the region of the gastrointestinal tract that appears to be at greatest risk for ulceration in dogs. This then leads to the question of why drugs that appear to be highly selective for COX-2 have been associated with perforating ulcers in the pylorus and duodenum in dogs in reports received by the FDA. It must be remembered, however, that there is no control on the quality of information received by the FDA, and that information does not take account of how the drugs were used, underlying diseases, other drugs administered at the same time, and other non–drug-related possible causes of gastrointestinal ulceration. Despite this, the possibility of gastrointestinal tract erosion and ulceration is also indicated on the drug labels.
for the selective COX-2 inhibitors deracoxib and firocoxib. To date, 2 short clinical studies have assessed the association between selective (deracoxib) and preferential (meloxicam) COX-2 inhibitors and gastroduodenal perforation. The study of deracoxib revealed that for almost all dogs that were receiving the drug and had ulceration, an inappropriately high dose of deracoxib was administered, other NSAIDs or corticosteroids were administered concurrently, or treatments were rapidly switched from one NSAID to another. In the present study, there was no significant effect of administration of the selective COX-2 inhibitor deracoxib on gastroduodenal prostanoid production.

b. Deramaxx, Novartis Animal Health, Greensboro, NC.
c. Aspirin (low strength, enteric coated), CVS Pharmacy Inc, Woonsocket, RI.
e. Olympus GIF 160 videoscope, Olympus America Inc, Center Valley, Pa.
f. Anti–COX-1 goat polyclonal IgG (SC-1752), Santa Cruz Biotechnology, Santa Cruz, Calif.
g. Anti–COX-2 goat polyclonal IgG (SC-1745), Santa Cruz Biotechnology, Santa Cruz, Calif.
i. COX-1 from sheep (C-0733) and COX-2 (C-0858) from a human, recombinant expressed in Sf21 cells, Sigma-Aldrich Inc, St Louis, Mo.
k. Thromboxane B1 ELISA kit, Cayman Chemical Co, Ann Arbor, Mich.
REFERENCES


### APPENDIX A: Scoring System

<table>
<thead>
<tr>
<th>Score</th>
<th>Pyloric mucosa</th>
<th>Duodenal mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Apparently normal appearance</td>
<td>Apparently normal appearance</td>
</tr>
<tr>
<td>1</td>
<td>Erythema</td>
<td>Increased granularity</td>
</tr>
<tr>
<td>2</td>
<td>5 to 10 punctate hemorrhages or 1 to 4 erosions</td>
<td>Increased friability</td>
</tr>
<tr>
<td>3</td>
<td>11 to 20 punctate hemorrhages</td>
<td>Bleeding</td>
</tr>
<tr>
<td>4</td>
<td>Confluent hemorrhage; 10 to 20 erosions, or any ulcer</td>
<td>Erosions or any ulcer</td>
</tr>
</tbody>
</table>

Scoring system used to subjectively assess pyloric and duodenal mucosal lesions in dogs during review of video recordings of endoscopic procedures.
CHAPTER 3

Effect of non-steroidal anti-inflammatory drugs with varying cyclooxygenase (COX)-2 selectivity on COX protein and prostanoid concentrations in canine pyloric and duodenal mucosa

Jenna G. Wooten, Anthony T. Blikslager, Steve L. Marks,
J. Mac Law, Elizabeth C. Graeber, and B. Duncan X. Lascelles
ABSTRACT

Objective–To assess the in vivo effects of short-term administration of nonsteroidal anti-inflammatory drugs (NSAIDs) with varying cyclooxygenase (COX)-2 selectivity on pyloric and duodenal mucosa.

Animals–8 healthy dogs.

Procedures–Each dog received deracoxib (2mg/kg q24h PO), firocoxib (5mg/kg q24h PO), meloxicam (Day 1=0.2mg/kg q24h PO, Day 2-3=0.1mg/kg q24h PO), or placebo (1 dog treat, q 24 h) orally for 3 days (4-week interval between treatments). Prior to, and on day 3 of drug administration, pyloric and duodenal mucosal appearance was assessed endoscopically and biopsy specimens were obtained for histological examination. Cyclooxygenase (COX) -1 and COX-2 protein expression were assessed by western blotting, and prostanoids were measured with ELISAs. Data were analyzed using ANOVA.

Results–There was no significant effect of drug on endoscopic mucosal scores or histological scores. Drug administration had no significant effect on COX-1 or COX-2 protein expression. COX-1 expression was significantly higher in the pylorus compared to the duodenum (p<0.05). Total prostaglandin (PG) and thromboxane B2 (TXB2) concentrations were significantly greater in pyloric versus duodenal mucosa (p<0.05). Drug administration had no effect on PG or TXB2 concentrations.

Conclusions and Clinical Relevance–There were no significant effects of varying COX-2 selectivity on gastric and duodenal tissue prostanoid concentrations, and no significant relationship between the degree of selectivity and gross or histological appearance of the mucosa. These findings suggest that, under the experimental conditions of this study, there
are no differences among the preferential and selective COX-2 inhibitors with regard to adverse effects on the upper GI tract.

**Funded by:** A Grant from Novartis Animal Health through their competitive research grants program

**Abbreviations:**

NSAID: non-steroidal anti-inflammatory drug; COX: cyclooxygenase; ELISA: Enzyme-linked ImmunoSorbent assay; PG: prostaglandin; TXB_2_: thromboxane B_2_
Introduction

Administration of non-steroidal anti-inflammatory drugs (NSAIDs) in dogs, while efficacious, has been associated with adverse events, most notably gastrointestinal ulceration. NSAID-associated gastrointestinal ulceration is most commonly found in the pylorus and proximal duodenal region of the dog.\textsuperscript{1,2} Many factors likely contribute to GI ulcer formation in dogs. However, the incidence of ulceration in dogs is unknown because of the nonspecific or mild clinical signs associated with erosions and minor gastric ulceration.\textsuperscript{3} Risk factors considered to play a role in the development of NSAID-associated ulcers in humans and dogs include a history of GI bleeding or gastric ulceration, concurrent administration of multiple NSAIDs, concomitant use of corticosteroids or anticoagulants, liver dysfunction, renal dysfunction and higher than approved NSAID dosage.\textsuperscript{4,5} A number of these factors were present in a recent report on gastric perforation in dogs.\textsuperscript{1}

While the mechanisms are not well understood, gastric damage related to NSAID administration is twofold: a topical effect on gastric mucosa and a systemic effect, most likely due to decreased production of gastroprotective prostaglandins (PGs).\textsuperscript{6} Injury to the mucosa is initiated topically by the weakly acidic NSAIDs such as aspirin.\textsuperscript{4} For the majority of NSAIDs, including the recently introduced cyclooxygenase (COX)-2 selective NSAIDs, the more important component of NSAID-induced ulceration is inhibition of COX-mediated PG synthesis. This mechanism is responsible for the desired anti-inflammatory effect of NSAIDs and is also believed to be crucial in the development of side effects associated with the inhibition of gastroprotective PGs.
Since COX-1-derived PGs are believed to play a dominant role in gastric mucosal defense and cytoprotection, drugs that selectively inhibit COX-2 are considered to have a safer gastrointestinal profile than those that also block COX-1. However, studies have found constitutive COX-2 protein expression in the canine central nervous system and kidney. Of greater pertinence to the present study, our laboratory has recently published data showing constitutive expression of COX-2 protein in canine gastroduodenal mucosa. In studies in humans and rats, COX-2 was found to be upregulated in inflamed GI tissue, suggesting a role in GI mucosal defense and cytoprotection. Other studies have demonstrated upregulation of COX-2 expression in the margins of healing gastric ulcers. In addition, selective inhibition of COX-1 does not predictably cause ulceration, indicating that blockade of both isoforms may be necessary to induce lesions. Mucosal damage induced by ischemia/reperfusion has been shown to be markedly aggravated by the inhibition of COX-2. These studies and several others have established that COX-2 expression appears to provide a second line of defense for gastric mucosa as well as a crucial mediator in mucosal repair. Thus, there appear to be two important scenarios under which the effects of selective COX-2 inhibitors should be evaluated – normal GI mucosa, and abnormal or reparative mucosa. The purpose of the present study was to assess the in vivo effects of short-term administration of varying COX-2 selectivity of NSAIDs on normal pyloric and duodenal mucosa. We hypothesized that with greater COX-2 selectivity there would reduced adverse effects on the mucosa associated with increased prostanoid production.
Materials and Methods

This study was approved by the Animal Care and Use Committee at North Carolina State University.

Dogs–Eight adult purpose-bred mongrels (4 females and 4 males) weighing 8 to 13kg were used in the study. All dogs were given a physical examination to ensure they were healthy prior to the beginning of the study. In addition, CBC, serum biochemical analysis and urinalysis were performed immediately prior to study commencement. Gastroduodenoscopy was performed on each dog prior to the start of the study to rule out preexisting gastroduodenal disease.

Experimental protocol–The study was a randomized, placebo controlled, crossover design. Each dog randomly received deracoxib\(^{a}\) (2mg/kg q24h PO), firocoxib\(^{b}\) (5mg/kg q24h PO), meloxicam\(^{c}\) (Day 1=0.2mg/kg q24h PO, Day 2-3=0.1mg/kg q24h PO), or placebo\(^{d}\) for 3 days with a 4-week washout period between drugs. Each dog received each drug treatment. Commercially available product was used, and dogs were dosed within +/- 10% of the recommended dose according to package inserts. Dogs were treated for 3 days to allow each drug to reach a theoretical steady state concentration in the plasma, and presumably the gastrointestinal tract. Prior to initiation of the study (baseline), and on day 3 following administration of each drug, the pyloric and duodenal mucosa were scored and mucosal biopsies were obtained endoscopically. Baseline biopsies were taken 4 weeks prior to any drug administration. On day 3, endoscopy took place 2 hours following drug administration. The dogs were fasted for 24 hours prior to endoscopy. Anesthesia was induced with propofol (10-15mg/mg according to effect) and maintained with isoflurane vaporized in 100% O\(_2\) to
effect following orotracheal intubation. Gastroduodenoscopy and biopsy were performed by one investigator (SLM), using a flexible videoscope (Olympus GIF 160 videogastroscope) and the procedure was recorded onto DVD. All endoscopic procedures were performed the same time each morning (10:00 am) to avoid diurnal and feeding-associated changes in stomach physiology. Biopsies were taken at least 2cm from adjacent biopsy locations. Mucosal biopsies taken from the pylorus and duodenum were snap frozen in liquid nitrogen within 6-8 seconds, stored at -80°C, and subsequently used for western blot analysis of COX-1 and COX-2 expression and measurement of total PG and thromboxane B₂ (TXB₂) concentrations. Careful consideration was taken to make certain each biopsy sample was treated identically. In addition, separate mucosal biopsies were immediately placed in 10% neutral buffered formalin for histological evaluation.

**Western analysis**—One biopsy sample from the pylorus and duodenum of each dog was added to 200μl of modified radioimmunoprecipitation buffer, including the protease inhibitors aprotinin, phenylmethylsulfonyl fluoride and sodium orthovanadate. The samples were homogenized on ice and the supernatants were extracted by centrifugation. Protein analysis of extracted samples was performed and equal concentrations of protein from each sample were mixed and boiled with sample buffer. The lysates were then loaded into wells of Criterion™ XT Precast Gels and protein electrophoresis was performed according to standard protocols. After transferring the protein to a PDVF membrane, and blocking in 5% milk with 0.05% Tween-20, washed membranes were incubated overnight in a 1:300 solution of either polyclonal COX-1 or COX-2 primary antibody. The membranes were then incubated in a horseradish peroxidase conjugated secondary antibody and developed by
addition of enhanced chemiluminescence reagents. Beta-actin\(^h\) expression was used to verify that the same amount of protein had been loaded into each well. Recombinant COX protein\(^i\) was used as a positive control and a molecular weight indicator (protein standard) was used to be certain canine COX protein bands corresponded to the appropriate kD measurement. Negative controls were used as a quality control step in some gels. This approach is similar to previous studies in other species where a specific antibody was not available.\(^{17}\) Each dog had samples from the pylorus and duodenum for all the treatments run on a single gel. This made comparison of the levels of COX expression following each treatment possible within each dog. Using the densitometry values, COX protein levels following each treatment were expressed as a percentage of the baseline value for each dog in each region (pylorus and duodenum). To compare the overall level of COX protein expression in the duodenum and pylorus, the densitometric values within each dog were expressed as a percentage of the baseline level.

**Prostanoid analysis**—Each biopsy sample was added to 200\(\mu\)l of Tris buffer (50mM Tris-HCl, 150mM NaCl, 1mM EDTA at pH 7.4), including aprotinin, phenylmethylsulfonyl fluoride and orthovanadate. The samples were homogenized on ice and the supernatants were extracted by centrifugation. Protein analysis of extracted samples was performed. Prostaglandin concentrations and TXB\(_2\) concentrations were measured using commercially available ELISA assay kits.\(^{1, k}\) Results were expressed as picogram of prostanoid per microgram of protein in the tissue.

**Mucosal scoring and histological analysis**—After completion of the study, the endoscopy videos were all reviewed by a single investigator (SLM) who was blinded to the
treatment protocols. The mucosa was scored for lesions using a subjective scoring system (Appendix). Hematoxylin and eosin stained slides of gastric and duodenal biopsy specimens were evaluated for inflammation and ulceration by a board-certified veterinary pathologist (ML). The pathologist was not aware of the treatment groups.

**Data analysis**—Two way repeated measures ANOVA was used to compare the densitometric data for COX-1 and COX-2 protein levels, and PG and TXB2 levels. A Tukey’s test was used to identify specific differences between treatments and significance was set at P<0.05. An ANOVA on ranks was used when the data were not normally distributed.

**Results**

All dogs had normal physical examination findings, and laboratory values within the reference range for the North Carolina State University Veterinary Teaching Hospital clinical pathology laboratory. No clinically significant adverse effects were observed for any drug administered.

Histopathology showed no evidence of ulceration or significant inflammation in any biopsy. Mild inflammation was seen in 16 of 80 (20.0%) biopsies. There was no significant effect of drug on histological scores (P=0.518 for pylorus, P=0.918 for duodenum). *Helicobacter spp.* were seen in 30 of 80 (37.5%) histological samples. These dogs were not omitted from the study on the basis of this finding. During endoscopic evaluation, no bleeding was observed in the pylorus or duodenum. Endoscopic mucosal scores were
significantly higher in the pylorus compared to the duodenum (P<0.05). Overall, there was no significant effect of drug on mucosal scores.

Drug administration had no effect on COX-1 or COX-2 protein expression in the pylorus or duodenum. COX-1 expression was significantly higher in the pylorus compared to the duodenum (P<0.05).

Overall, total PG concentrations were significantly greater in pyloric versus duodenal mucosa (baseline concentrations [+/- SEM] 1043 +/- 222 pg/mcg protein versus 283 +/- 49 pg/mcg protein) (p<0.05) (Figure 1A and B). Drug administration had no effect on PG concentrations in pyloric and duodenal mucosa. TXB₂ concentrations were significantly greater in pyloric versus duodenal mucosal tissue (baseline concentrations [+/- SEM] 1649 +/- 125 pg/mcg protein versus 187 +/- 50 pg/mcg protein) (p<0.05) (Figure 2A and B). Drug administration had no effect on TXB₂ concentrations in the pylorus or duodenum.

**Figure 1 A and B: Total PG concentrations in the pylorus (A) and duodenum (D)**

<table>
<thead>
<tr>
<th>Drug Administration</th>
<th>Baseline</th>
<th>Placebo</th>
<th>Deracoxib</th>
<th>Firocoxib</th>
<th>Meloxicam</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG/ug protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline Pylorus</td>
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<td>222</td>
<td>283</td>
<td>49</td>
<td>1043</td>
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<tr>
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<td>49</td>
<td>187</td>
<td>50</td>
<td>283</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug Administration</th>
<th>Baseline</th>
<th>Placebo</th>
<th>Deracoxib</th>
<th>Firocoxib</th>
<th>Meloxicam</th>
</tr>
</thead>
<tbody>
<tr>
<td>TXB₂ concentrations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline Pylorus</td>
<td>1649</td>
<td>125</td>
<td>187</td>
<td>50</td>
<td>1649</td>
</tr>
<tr>
<td>Baseline Duodenum</td>
<td>187</td>
<td>50</td>
<td>187</td>
<td>50</td>
<td>187</td>
</tr>
</tbody>
</table>
Figure 1: (A) Mean (+/- SEM) effect of different drug treatments on total PG levels in pyloric mucosa. Levels of PG are expressed as picogram of PG per microgram of protein. Drug administration had no effect on PG concentration in the pylorus. Data were analyzed using ANOVA. (B) Mean (+/- SEM) effect of different drug treatments on total PG levels in duodenal mucosa. Levels of PG are expressed as picogram of PG per microgram of protein. Drug administration had no effect on PG concentration in the duodenum. Overall, total PG concentrations were significantly greater in pyloric versus duodenal mucosa (baseline concentrations [+/- SEM] 1043 +/- 222 pg/mcg protein versus 283 +/- 49 pg/mcg protein) (p<0.05). Data were analyzed using ANOVA.

Figure 2: (A) Mean (+/- SEM) effect of different drug treatments on thromboxane B2 concentrations in pyloric mucosa. Levels of TXB2 are expressed as picogram of TXB2 per microgram of protein. Drug administration had no effect on TXB2 concentration in the pylorus. Data were analyzed using ANOVA. (B) Mean (+/- SEM) effect of different drug treatments on thromboxane B2 concentrations in duodenal mucosa. Levels of TXB2 are expressed as picogram of TXB2 per microgram of protein. Drug administration had no effect on TXB2 concentration in the duodenum. TXB2 concentrations were significantly greater in pyloric versus duodenal mucosal tissue (baseline concentrations [+/- SEM] 1649 +/- 125 pg/mcg protein versus 187 +/- 50 pg/mcg protein) (p<0.05). Data were analyzed using ANOVA.

Discussion

To date there has been no in vivo study comparing the short-term administration of NSAIDs with varying COX-2 selectivity while assessing COX protein and prostanoids in dogs. Meloxicam is considered a ‘preferential’ COX-2 inhibitor in vitro, as it is has been shown to inhibit COX-2 more readily than COX-1.18,19 In vitro studies have shown
meloxicam to inhibit COX-2 activity 10-12 times more effectively than COX-1 activity.\textsuperscript{18,20} Deracoxib and firocoxib are called coxibs and are described as selective COX-2 inhibitors.\textsuperscript{21-23} The selectivity of deracoxib is reported to be 12-fold as high for COX-2 as for COX-1 in canine blood.\textsuperscript{20} The same study found firocoxib to be 384-fold more selective for COX-2 than COX-1, making it the most selective COX-2 inhibitor in dogs to date.\textsuperscript{20} Several \textit{in vitro} studies have evaluated selectivity ratios. The results vary depending which \textit{in vitro} assay is used and how well these results translate to \textit{in vivo} circumstances is not known. Each of the aforementioned drugs is considered to spare the COX-1 enzyme, thereby potentially increasing the safety profile of NSAIDs while retaining efficacy, however this postulated increased safety has not been proven in canine medicine. There is still potential for COX-1 inhibition depending on the tissue concentration of the drug. Two retrospective clinical studies have assessed the association between NSAIDs and gastroduodenal perforation.\textsuperscript{1, 24} In one study, in almost all cases of ulceration in dogs, an inappropriately high dose, or concurrent administration with other NSAIDs or corticosteroids, or rapid switching from one NSAID to another was noted.\textsuperscript{1} In another study, Enberg et al.\textsuperscript{24} suggested that at higher doses of the NSAID meloxicam, its selectivity for COX-2 may be decreased, thereby increasing COX-1 inhibition. They suggested this increase in COX-1 inhibition may explain GI adverse events.\textsuperscript{24}

The present study was designed to assess the \textit{in vivo} effects of NSAIDs on pyloric and duodenal mucosa, which have been shown to be at risk for ulceration.\textsuperscript{1} No dog displayed any clinical signs of gastrointestinal disease in this study, but the duration of treatment was only 3-days. Ideally, such a study would look at a multiple time points over an
extended period of time. However, such an approach has its limitations, including the potential trauma to the stomach with multiple endoscopies. With that in mind, this study was designed to initially assess an early time period, evaluating the effect of initial administration of NSAIDs. The 3-day time period was chosen based on another study\textsuperscript{22} that looked at the 3 day time point, where all the drugs evaluated significantly suppressed of gastric PGE\textsubscript{2}. In previous work, we had found significant differences between aspirin, deracoxib and carprofen at the 3-day time point.\textsuperscript{12} As a follow up to this, we wanted to compare NSAIDs with varying COX-2 selectivities.

In the present study, there was no significant effect of drug administration on histological scores of the mucosa. Few gastric biopsy specimens revealed mild inflammation, but these findings were not related to a specific drug or dog. Histological findings similar to our own were seen in a previous endoscopic study.\textsuperscript{22} In that study, \textit{Helicobacter} spp. was present histologically in all of the dogs. In a previous study, we also found Helicobacter spp. in histological analyses.\textsuperscript{12} In the present study, \textit{Helicobacter} spp. were present in 36% and 20% of the pylorus and duodenum biopsies, respectively. We did not determine the species of \textit{Helicobacter}, and no dogs were omitted based on these findings. The clinical relevance of \textit{Helicobacter} is unclear in canine gastroenterology. However, \textit{Helicobacter} organisms are commonly found in clinically normal dogs\textsuperscript{25} and the number of positive results found in the present study were very low compared to a report that found 100% of healthy laboratory dogs tested positive for \textit{Helicobacter} spp. without any evidence of gastrointestinal disease.\textsuperscript{26} In the present study, mucosal scores were significantly higher in the pylorus compared to the duodenum indicating greater mucosal irritation in the pylorus.
This may be due to irritating effects of ingesta and gastric acid on the pyloric mucosa, with bile acids having a protective effect in the duodenum.

In the present study, drug administration did not affect the expression of COX-1 or COX-2 protein in the pylorus and duodenum over the time period of this study. COX-1 expression was significantly higher in the pylorus compared to the duodenum. The clinical relevance of this is unclear, but it may suggest higher levels of COX-1 aid the protection of this region of the stomach. In a prior study, we noted constitutive expression of COX-2 protein in gastric tissues, and this was also detected in this study. The findings from this study and the previous study contrast with a study, using tissue from a single dog, that found no COX-2 protein expression in gastrointestinal tissues. In the gastrointestinal tract of healthy humans and a number of animals, COX-2 was found to be either undetectable or expressed at very low levels. Consistent with its role as an inducible enzyme under conditions of inflammation, COX-2 was found to be upregulated in inflamed GI tissue in humans and rodents. COX-2 is generally reported to be expressed under inflammatory conditions. However, our study suggests a more complicated role of the COX-2 isoform since we found it constitutively expressed under normal conditions. Cyclooxygenase-2 may play a role in GI mucosal protection and resolution of inflammation. Indeed, evidence from several studies suggests COX-2 plays a role in mucosal protection. This protective role was further emphasized in the same studies when administration of a selective COX-1 inhibitor did not affect gastric healing, which was in contrast to the findings with a selective COX-2 inhibitor. Additionally, studies performed on COX-1 deficient mice suggest that COX-2 derived PGs contribute to mucosal protection and that inhibition of those PGs by
NSAIDs can cause mucosal injury. Other studies have revealed that COX-2 expression can be upregulated rapidly within 1 hour of oral administration of aspirin or indomethacin, suggesting upregulation occurred as protective mechanism. Studies evaluating carrageenan-induced paw edema and carrageenan-induced pleurisy in wild type and COX-2 deficient mice have also emphasized the role of COX-2 in resolution of and regulation of inflammation. This latter study showed that there were two phases of COX 2 expression. An early peak (at about two hours) was associated with onset of inflammation, leukocyte infiltration, PGE2 production and COX activity. Non-selective and COX-2 selective NSAIDs can inhibit this early phase of the inflammatory response. A second and much greater peak in COX-2 expression occurred 48 hours after irritant injection. At this peak, the number of leukocytes in the pleural cavity decreased to normal levels resulting in the resolution of the inflammation. No detectable PGE2 was present, however another PGH2 metabolite, PGD2, accompanied the increase in COX-2 expression. Administration of COX-2 selective inhibitors from 24 h to 48 h post-injection of irritant (i.e., during the resolving phase) abolished PGD2 production and prolonged the inflammatory response by preventing a decrease in leukocytes. A normal course of resolution was seen with co-administration of PGD2 and cyclopentenone PGs, indicating products of PGD2 may be responsible for initiating resolution of inflammation. From the above discussion it appears that COX-2 may play a significant role in mucosal defense and the repair process. Our study found no adverse effects of inhibition of COX-2 in normal canine gastro-duodenal mucosa under the conditions of this study, however the role of COX-2 and the consequences of its inhibition require further study.
In complex tissues such as gastrointestinal mucosa, it is difficult to conclude which isoform of the COX enzyme produces which prostanoid. In this study, total PG production was measured in order to investigate the synthesis of all PGs from both COX enzymes. At the time of biopsy, the tissue concentrations of prostanoids were measured. Other studies have taken biopsies and measured the total amount of prostanoids that can be produced when the biopsies are subsequently stimulated *ex vivo.* However, measurement of tissue prostanoid levels reflects the effect of a drug treatment on the actual tissue levels of prostanoids *in vivo.* This study, as well as previous studies, found COX-1 expression to be higher in the pylorus and stomach compared to the duodenum. This may explain why PG levels were significantly higher in the pylorus than in the duodenum. Kargman et al found higher PGE₂ production from human duodenal tissue compared to gastric tissue, but the reverse was true for rat and rhesus monkey tissue. Dog tissue PG levels were not examined. In the present study, higher PG levels correlated with greater levels of COX-1 protein. A previous study, using the assay method involving stimulation of PG production, found that deracoxib decreased gastric PGE₂, but not PGE₁ after 3 days of oral administration. In our study, none of the drugs appeared to inhibit PGs. Because we were using drugs with reported preferential selectivity for COX-2, it is possible that the PG concentrations measured in this study were more reflective of COX-1 activity, despite the fact that COX-2 protein was also detected in the mucosa.

Thromboxane has been shown to be a likely indicator of COX-1 activity in the gastrointestinal tract of pigs. The previous study from our laboratory suggests TXB₂ can be linked to COX-1 activity in the canine gastrointestinal tract. In both the prior and present
study TXB\textsubscript{2} concentrations were significantly higher in the pylorus versus the duodenum. This phenomenon appeared to reflect higher concentrations of COX-1 protein expression in the pylorus. Drug administration had no effect on TXB\textsubscript{2} concentrations in the pylorus or duodenum, suggesting no clinically relevant inhibition of COX-1 at the dosages chosen over a 3-day period.

The present study showed that various selective and preferential COX-2 inhibitors had no significant effects on prostanoid production in gastro-duodenal mucosa, and that there was no significant relationship between degree of selectivity and gastrointestinal injury or histologic appearance.
Footnotes

a. Deramaxx®, Novartis Animal Health, Greensboro, NC

b. Firocoxib®, Merial Limited, Duluth, GA

c. Metacam®, Boehringer Ingelheim, Burlington, ON, Canada

d. Science Diet® Jerky Plus® Treats, Hill’s Pet Nutrition, Inc., Topeka, KS.

e. Videoscope (Olympus GIF 160), Olympus America Inc., Center Valley, PA.

f. Anti-COX-1 goat polyclonal IgG (SC-1752), Santa Cruz Biotechnology, Santa Cruz, CA.

g. Anti-COX-2 goat polyclonal IgG (SC-1745), Santa Cruz Biotechnology, Santa Cruz, CA.

h. Anti-beta-actin rabbit polyclonal IgG (Ab8227-50), Abcam, Inc., Cambridge, MA.

i. COX-1 from sheep (C-0733) and COX-2 (C-0858) is a human, recombinant expressed in Sf21 cells, Sigma-Aldrich, Inc., St. Louis, MO.

j. Prostaglandin Screening ELISA Kit, Cayman Chemical Co, Ann Arbor, MI.

k. Thromboxane B₂ ELISA Kit, Cayman Chemical Co, Ann Arbor, MI.
REFERENCES


APPENDIX A: Scoring System

<table>
<thead>
<tr>
<th>Score</th>
<th>Pyloric mucosa</th>
<th>Duodenal mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Apparently normal appearance</td>
<td>Apparently normal appearance</td>
</tr>
<tr>
<td>1</td>
<td>Erythema</td>
<td>Increased granularity</td>
</tr>
<tr>
<td>2</td>
<td>5 to 10 punctate hemorrhages or 1 to 4 erosions</td>
<td>Increased friability</td>
</tr>
<tr>
<td>3</td>
<td>11 to 20 punctate hemorrhages</td>
<td>Bleeding</td>
</tr>
<tr>
<td>4</td>
<td>Confluent hemorrhage; 10 to 20 erosions, or any ulcer</td>
<td>Erosions or any ulcer</td>
</tr>
</tbody>
</table>

Scoring system used to subjectively assess pyloric and duodenal mucosal lesions in dogs during review of video recordings of endoscopic procedures.
CHAPTER 4

Evaluation of the relationship between gastrointestinal irritation and cyclooxygenase (COX) expression in clinically normal dogs

Jenna G. Wooten, B. Duncan X. Lascelles,
Vanessa L. Cook, Mac. J. Law, Anthony T. Blikslager
Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used to alleviate both acute and chronic pain. NSAIDs are frequently used to control postoperative pain and inflammation, however, they have been associated with adverse effects, most notably gastrointestinal ulceration. When administering NSAIDs, veterinarians are often not familiar enough with the possibility of underlying disease and the potential for adverse effects. Several studies have indicated that NSAID administration is a risk factor for gastrointestinal tract ulceration and perforation in dogs. Some of these same studies have also found that NSAID-associated ulceration and perforations are most commonly noted in the pylorus and proximal duodenum. With preexisting conditions, such as inflammation and erosion of the gastric mucosa, the adverse effects from NSAID administration could be intensified, leading to grave results. However, little is understood about the physiology of such preexisting conditions or the circumstances under which NSAID administration may be associated with an increased risk of gastrointestinal toxicity.

NSAIDs act on the cyclooxygenase (COX) enzyme to inhibit the production of prostaglandins (PGs). PGs have important protective effects on the gastrointestinal mucosa. Thus, inhibition of PG synthesis is thought to account for most NSAID-induced mucosal injury. The COX enzyme has at least two isoforms, including a constitutive COX-1 and an inducible (in most tissues) COX-2. The COX-1 isoform is associated with homeostatic physiological functions, such as gastric protection. The COX-2 isoform has been regarded as proinflammatory and is well documented as being responsible for the rise in PG levels at site of inflammation. Nonselective NSAIDs inhibit both of these isoforms, thereby blocking
the production of the PGs thought to be responsible for the “housekeeping” functions. This PG-inhibition leaves the GI tract more susceptible to injury. Thus, a drug class, known as the coxibs, which would selectively inhibit COX-2 (reducing inflammation), while preventing NSAID-induced gastrointestinal injury was developed. Recent findings, however, suggest a more complicated role of the COX isoforms, in particular, COX-2. Several studies have discovered some aspects of mucosal protection and repair may depend on COX-2 expression.\textsuperscript{15-17}

We hypothesized that gastrointestinal erosion and ulceration would be found in clinically normal dogs and that this pathology would be associated with increased COX-2 expression. Our aims were firstly to collect post-mortem material from clinically normal dogs and determine if gastrointestinal lesions were present, and secondly to determine if COX-1 and COX-2 expression were altered in tissues with lesions compared to normal tissue.

\textbf{Materials and Methods}

This study was approved by the Animal Care and Use Committee at North Carolina State University.

\textbf{Dogs}—Twenty seven adult dogs (18 females and 9 males) weighing approximately 7-45kg were used in the study. Dogs were obtained either from the College of Veterinary Medicine Laboratory Animal Resources, or from a county animal shelter. Dogs from the College of Veterinary Medicine were considered unadoptable. Dogs from the county shelter were euthanized because their inappropriate social behavior made them unadoptable. No dogs were euthanized solely for the collection of material for this study.
Experimental protocol–Dogs were euthanized according to standard protocol by a member of the staff from each location. Photographs were taken of the pylorus and duodenum prior to obtaining mucosal samples. We then obtained our samples of the pylorus and duodenum. Mucosal samples taken from the pylorus and duodenum were snap frozen in liquid nitrogen within 6-8 seconds, stored at -80°C, and subsequently used for western blot analysis of COX-1 and COX-2 expression. In addition, separate mucosal samples from the same area (close to where the samples for COX analysis were taken from) were immediately placed in 10% neutral buffered formalin for histological evaluation.

Western analysis–Samples from the pylorus and duodenum were used from select dogs and were added to 1ml of modified radioimmunoprecipitation buffer, including the protease inhibitors aprotinin, phenylmethylsulfonyl fluoride and sodium orthovanadate. The samples were homogenized on ice and the supernatants were extracted by centrifugation. Protein analysis of extracted samples was performed and equal concentrations of protein from each sample were mixed and boiled with sample buffer. The lysates were then loaded into wells of Criterion™ XT Precast Gels and protein electrophoresis was performed according to standard protocols. After transferring the protein to a PDVF membrane and blocking in 5% milk with 0.05% Tween-20, washed membranes were incubated overnight in a 1:300 solution of either polyclonal COX-1 or COX-2 primary antibody. The membranes were then incubated in a horseradish peroxidase conjugated secondary antibody and developed by addition of enhanced chemiluminescence reagents. This approach is similar to previous studies in other species where a specific antibody was not available.18
**Histological analysis**—Hematoxylin and eosin stained slides of gastric and duodenal biopsy specimens were evaluated for inflammation and ulceration by a board-certified veterinary pathologist (ML) using a scoring system (Appendix). Following classification the samples were then designated as falling into one of three broad categories: (Figure 1): normal appearance of the pyloric and duodenal mucosa (labeled CD for control dog); evidence of inflammation (I) in either the pylorus (IP), duodenum (ID), or both; evidence of an epithelial erosion (E) in either the pylorus (EP), duodenum (ED), or both.

**Results**

All dogs appeared normal upon a brief physical examination prior to euthanasia. Therefore, we used histopathology of stomach mucosa as the ‘gold standard’ for detection of lesions, and based the remainder of the study on our determination of histopathological lesions.

Five dogs had unremarkable histological results in both the pylorus and duodenum. Inflammation was found in the pylorus of 10 dogs (duodenum was normal for these dogs) and in the duodenum of 5 dogs (pylorus was normal for these dogs). Only 2 dogs had inflammation in both regions. Evidence of epithelial erosion was seen in the pylorus of 1 dog (duodenum was normal for this dog) and in the duodenum of 3 dogs (pylorus was normal for these dogs). Only 1 dog had evidence of erosion on both regions, totaling 27 dogs in all. *(Tables 1 and 2; Figure 1)*
Table 1: Histological categories

<table>
<thead>
<tr>
<th>Histology Category</th>
<th>Pylorus</th>
<th>Duodenum</th>
<th>Pylorus and Duodenum</th>
</tr>
</thead>
<tbody>
<tr>
<td>0=Normal/Unremarkable</td>
<td>-</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>1-2=Inflammation</td>
<td>10</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>3=Erosion</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>11</strong></td>
<td><strong>8</strong></td>
<td><strong>8</strong></td>
</tr>
</tbody>
</table>

Table 1- Of the 27 dogs used in the study, 5 were histologically unremarkable in both regions, thus considered normal. Inflammation was present in the pylorus of 10 dogs and in the duodenum of 5 dogs. Only 2 dogs had inflammation in both regions. Evidence of an epithelial erosion was seen in the pylorus of 1 dog and in the duodenum of 3 dogs. Only 1 dog had evidence of erosion.

Figure 1 A and B: Histopathology categories in the pylorus and duodenum

Figure 1 A: The pylorus (P) and duodenum (D) of a control dog (CD) is unremarkable, thus considered within normal limits. Inflammation (I) is shown in figure A. Multiple follicles were identified in I1-P. Moderate eosinophilic enteritis was identified in the duodenum of I2 and I3 had evidence of inflammation in both regions, small de novo follicles in the pylorus and reactive lymphoid follicles in the duodenum.
Figure 1 B: The pylorus (P) and duodenum (D) of a control dog (CD) is unremarkable, thus considered within normal limits. Epithelial erosions (E) were identified in the 3 examples shown above. The first erosion dog (E1) has a focal erosion of the mucosal surface in the pylorus region. The second dog (E2) showed evidence of mild lymphoplasmic enteritis with central erosion in the duodenum. E3 displays moderate eosinophilic enteritis along with a small central erosion in the pylorus and evidence of a mild central erosion in the duodenum.

Photographs of the gross appearance of the stomach (Figure 2) are labeled and displayed the same as the histology for continuity. Grading of the stomachs was done at the time of removal and photographs were taken to archive the results. The pyloric and duodenal mucosae were assessed (Appendix), and a score of 0 (apparently normal) to 4 (severely affected). No stomachs were graded above a 3. Gross anatomy did not always correlate with histological analysis (pylorus: \( R^2 = 0.5162 \), duodenum: \( R^2 = 0.3781 \)). Gross appearance of the stomachs appeared fairly similar; no erosion was visible on any of the stomachs. The demarcation between the pylorus and duodenum was consistently seen in all dogs. Stomachs CD (Figure 2B) and E1 (Figure 2B) had some bile staining, but this did not seem to effect histology. Histopathological examination found epithelial erosions in E1-E3 displayed a hyperemic appearance beginning in the duodenum of each stomach, but no visible erosion was identified (Table 2).
Figure 2: Photographs revealing gross appearance of the stomach are presented in the same categories as histopathological analyses for the same of continuity. The gross appearance of the stomachs appeared similar regardless of histopathological categorization. A: Control dog (CD) and inflammation (I) dogs (1-3). B: Control dog (CD) and erosion (E) dogs (1-3). CD and E1 had some evidence of bile staining. E1-E3 displayed a hyperemic appearance beginning in the duodenum of each stomach, but no visible erosion was identified.

Table 2 A and B: Gross appearance and histological evaluation scores

<table>
<thead>
<tr>
<th>A</th>
<th>Dogs</th>
<th>Pylorus</th>
<th>Duodenum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gross</td>
<td>Histology</td>
</tr>
<tr>
<td></td>
<td>CD</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>I1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>I2</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>I3</td>
<td>2</td>
<td>2</td>
<td>2</td>
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</table>
Equal amounts of protein were loaded into western blots to evaluate COX-1 and COX-2. Histological evidence was used to categorize the dogs. Cyclooxygenase-1 expression was not upregulated due to inflammation or erosion (Figure 3). Upregulation of COX-2 in sites of inflammation is shown in Figure 3A. The dog with histological evidence of pyloric inflammation (I1) shows upregulation in the pylorus compared to the duodenum. Likewise, inflammation found in the duodenum of I2 shows upregulation of COX-2 in the duodenum compared to the pylorus. The dog with evidence of inflammation in both regions displays evidence of upregulation of COX-2 in both regions. This same phenomenon was seen in the sites of erosion (E1-P, E2-D, and E3-P and D) in Figure 3B.

<table>
<thead>
<tr>
<th>Dogs</th>
<th>Pylorus</th>
<th>Duodenum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gross</td>
<td>Histology</td>
</tr>
<tr>
<td>CD</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>E2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>E3</td>
<td>1</td>
<td>3</td>
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</table>

Table 2- Gross appearance and histological evaluation scores. (A)-Control dog scores and scores for the dogs with inflammation. (B)-Control dog scores and scores for the dogs having evidence of an erosion.
Figure 3: Upregulation of COX-2 in sites of inflammation and erosion.

Discussion

Cyclooxygenase-2 is generally reported to contribute to a proinflammatory response. However, new evidence suggests COX-2 has a role in resolution of inflammation. One study examining paw edema in wild type and COX-2 deficient mice emphasized the role of COX-2 in resolution of inflammation. Carrageenan-injections induced inflammation to a similar extent in both COX-2 deficient and wild type mice. This study found significant leukocyte infiltration in the paws of both mice. In wild type mice, the swelling subsided within 24-48
hours. In COX-2 deficient mice, NSAIDs reduced paw swelling suggesting COX-1 derived PGs were responsible for the inflammatory response.

The paw swelling in the COX-2 deficient mice was still apparent up to one week after injection. These findings indicate that both COX-1 and COX-2 contribute to PG production at the site of inflammation. Also, it can be concluded that PGs derived from COX-2 have a role in the resolution phase, as well as in the early stages of the inflammatory response.

Expression of COX-2 increased in inflamed gastric tissue\(^{20}\) and it appeared to play an important role in repair of mucosal damage. Upregulation of COX-2 after gastric injury has been correlated with an increase in epithelial cell proliferation.\(^{21}\) Also, several studies which administered selective COX-2 inhibitors to rodents with gastric ulcers found delayed ulcer healing.\(^{22-25}\) A potential COX-2 role in the repair process of the gastrointestinal tract was further emphasized when administration of a selective COX-1 inhibitor did not effect gastric healing.\(^{25,26}\) Additionally, a study performed on COX-1 deficient mice suggested that COX-2 derived PGs contribute to mucosal protection\(^ {26}\) and that inhibition of those PGs by NSAIDs caused mucosal injury.\(^ {16}\) The suggestion that COX-2 may contribute to mucosal protection seems paradoxical given the aforementioned observations of low levels of COX-2 expression in the stomach. However, other studies that revealed similar findings of low levels of COX-2 expression also found it could be upregulated rapidly within 1 hour of oral administration of aspirin or indomethacin,\(^ {27}\) suggesting upregulation occurred as protective mechanism.

We made the assumption that accurate detection of gastric mucosal lesions would be difficult using gross appearance of the gastric mucosal surface. Therefore, we used histopathology of stomach mucosa as the ‘gold standard’ for detection of lesions, and based
the remainder of the study on our determination of histopathological lesions. Histopathology revealed three general categories of findings: normal appearance of the pyloric and duodenal mucosa; evidence of inflammation either in the pylorus, duodenum, or both; evidence of an epithelial erosion in either the pylorus, duodenum, or both.

Inflammation is often detected in clinically normal dogs, and histological findings from an endoscopic study are similar to the present study. Histological appearance could not be predicted from the gross appearance of the gastro-duodenal surface. However, a hyperemic appearance in the duodenum was noted in all dogs having evidence of an erosion, but visual evidence of the erosion was not seen in any of the dogs.

In the diagnosis and management of gastrointestinal disease, the characterizations of inflammatory conditions are usually based on the interpretation of mucosal biopsy samples obtained endoscopically. Willard et al., found the clinical outcome of this characterization could be influenced by non-intentional induced error in several stages including; the endoscopic biopsy procedure. We did not perform endoscopic biopsy, therefore eliminating one of the possible induced errors in collection of our samples. Willard et al., states that interpretation of samples has proved to be the most contentious and frustrating step. We had a board certified veterinary pathologist (JML), blinded to any gross interpretation, characterize our samples. The absence of a standard way to define morphological and inflammatory conditions makes it difficult to compare results from different studies. In order to develop set standards for the diagnosis and treatment of GI disease in small companion animals, the World Small Animal Veterinary Association (WSAVA) established a Gastrointestinal Standardization Group. They developed
histological standards for interpreting the nature and severity of morphological changes. Recently, this group presented a monograph of the histopathological changes that occur in the small intestine, due to inflammatory disease.\textsuperscript{30} The hope is that this standard template will be accepted internationally, thus advancing GI disease in small companion animals.\textsuperscript{30} Our evaluation of samples was based on this system although only one pathologist examined the samples.

Several studies consider endoscopy (gross appearance, not histological analysis) to be reliable method of evaluating gastric hemorrhage and ulceration.\textsuperscript{31-33} However, the current study shows the importance of endoscopy and histological evaluation of biopsies to accurately reveal any underlying disease. It is unrealistic to suggest all dogs prescribed NSAIDs should undergo endoscopy. However, it is important to note that assessment of the gross appearance of a dog’s stomach will likely not provide definitive evidence of whether or not disease is present. From our results, COX-2 appears to be upregulated at the sites of inflammation and erosion and so in these situations, non-selective NSAIDs and COX-2 inhibitors could both be problematic if this elevated COX-2 is actually playing a protective role. Additionally, it is not known if there is any difference between the selective COX-2 inhibitors and the non-selective NSAIDs in their ability to inhibit this upregulated COX-2 that is functioning in a protective role.

The products of COX-2 produce many beneficial and detrimental effects. In injured gastric mucosa, COX-2 appears to play a significant role in mucosal defense and the repair process. In intact mucosa, COX-2 expression seems to be involved in GI maintenance. COX-2 has been shown to play a role in both the proinflammatory response and in resolution
of inflammation. The role of COX-2 appears to be multi-faceted and depending on the physiological process involved, its inhibition will lead to corresponding risks or benefits.
REFERENCES


## Appendix A: Histological Grading System

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>unremarkable (no remarkable changes)</td>
</tr>
<tr>
<td>1</td>
<td>Mild/ Mild Inflammation</td>
</tr>
<tr>
<td>2</td>
<td>Moderate</td>
</tr>
<tr>
<td>3</td>
<td>Marked/Evidence of erosion</td>
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## Appendix B: Gross Anatomy Scoring System

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Apparently normal appearance</td>
</tr>
<tr>
<td>1</td>
<td>Mild bile staining or very mild changes</td>
</tr>
<tr>
<td>2</td>
<td>Mild hyperemia</td>
</tr>
<tr>
<td>3</td>
<td>Moderate hyperemia</td>
</tr>
<tr>
<td>4</td>
<td>Any erosion or evidence of ulceration</td>
</tr>
</tbody>
</table>