ABSTRACT

HILL, TRACY LYNN. A Canine Gastric Mucosa Injury Model. (Under the direction of Anthony Blikslager).

Gastric ulceration and gastric stress related mucosal disease result in significant human morbidity and mortality. In canine medicine, there are not overall reports of prevalence but mortality can be significant in patients diagnosed with ulceration. Current models of alterations in gastric barrier function include cell culture models, rodent models using various injury techniques, and in vivo studies in humans and canines. All of these existing models have deficiencies that limit clinical and physiologic relevance. We developed an ex vivo Ussing chamber model of gastric mucosal acid injury to allow for further study of changes in barrier function in the dog. Due to similarities between canines and humans, dogs also serve as a useful model for human disease.

In the first study, acid was used to injure tissue and the protective effect of a zinc l-carnosine containing compound was examined. This product did not successfully protect against increases in gastric permeability or increases in numbers of apoptotic cells that occurred with acid injury. This is in accordance with previous studies using the same compound in the dog in an in vivo model.

We next developed a model of acid injury that would allow for the examination of changes in barrier function both during injury and a subsequent recovery period. We describe tissue responses to this injury, including cyclooxygenase (COX) expression, histopathologic changes, and gastroprotective prostanoid concentrations. This injury and recovery model is used in our subsequent work.
Tramadol, a pain relieving medication often giving in conjunction with non-steroidal anti-inflammatory drugs, has reported potential adverse interactions with COX inhibitors. We treated injured tissue with both tramadol and indomethacin, a non-selective COX inhibitor. Neither tramadol nor indomethacin had adverse effects on gastric barrier function, suggesting that any potential interaction is either due to an unexamined metabolite of tramadol or a mechanism that was not investigated.

To further delineate the role of prostanoids in the importance of recovery in this injury model, we examined the effect of selective and non-selective COX inhibitors. Though these drugs did suppress prostanoid production, there was no overall effect on gastric barrier function. This implies that recovery from acid injury could depend on non-prostanoid dependent mechanisms. When misoprostol, a synthetic prostanoid, was applied, however, there was an improvement in recovery from injury. This data suggests that misoprostol may be advantageous in treatment of mucosal acid injury.

In the final examination, another gastroprotectant drug was examined. Sucralfate is a compound with multiple protective mechanisms. In our work, sucralfate protected against changes in barrier function when administered concurrently with acid injury. Additionally, it improved recovery after injury when applied at the beginning of the recovery period.

Overall, this work demonstrates the utility of this canine ex vivo model to examine various physiologic mechanisms important in injury and repair of gastric mucosa. It also provides an avenue to study various drugs with potential adverse effects on mucosal integrity as well as gastroprotectants.
A Canine Gastric Mucosa Injury Model

by

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BIOGRAPHY

I was born and raised in a Minneapolis suburb, Edina. Even though we always had multiple pets, the veterinary vocation did not enter my early ideas of careers. I was a science nerd who loved watching Mr. Wizard and attended Science Camp in the summers. Fantastic social science teachers in high school brought out a love for the social sciences. I entered undergraduate school at the University of Pennsylvania with the intention of majoring in political science. After a year or so, I rediscovered my love of biology in an introductory biology class with Dr. Michael Angilletta. I went on to major in Biology and found my calling in veterinary medicine. I returned to Minnesota to attend veterinary school at the University of Minnesota. Even at that point, I could not have foretold where my path would take me. I had anticipated becoming a mixed animal practitioner of equine and small animal practice. I quickly discovered a preference for small animal practice and decided to move on towards a small animal internship. Rotating on clinics with Dr. Jane Armstrong at Minnesota brought out a passion for the challenging cases of small animal internal medicine. On her recommendation, I was matched for my internship at North Carolina State University. I then entered a dual small animal medicine residency and PhD program at NCSU in 2006. In 2009, I was boarded in Small Animal Internal Medicine. That same summer, I began a PhD with Dr. Anthony Blikslager. Following my PhD, I will be taking a faculty position at the University of Edinburgh in Scotland.
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CHAPTER 1 CANINE GASTRODUODENAL ULCERATION

LITERATURE REVIEW
Introduction

Gastroduodenal ulceration and perforation is an increasingly recognized cause of morbidity and mortality in dogs, although reports of prevalence of canine ulcer disease overall, or prevalence in specific canine subpopulations (e.g. those dogs taking non-steroidal anti-inflammatory drugs [NSAIDs]) are lacking. With escalating use of NSAIDs for pain management, it is possible that the frequency of ulcer disease is increasing in dogs. Gastric perforation is a severe, potentially fatal, sequela of gastric ulcers that may affect up to 27% of dogs (1). Of dogs that have had perforation of gastric ulcers, between 40% and 70% die or are euthanized because of their disease (1-3). Reports of gastroduodenal ulceration in cats are sparse and survival rates highly variable, ranging from 15 to 100% (2,4,5).

Pathophysiology

The pH of a normal dog stomach is low, typically near pH 1.0 in the fed dog. Gastric pH is less than 3 for nearly 90% of the day in a normally fed dog (6). Given this prolonged exposure to high levels of acid, the gastric and duodenal mucosae have several mechanisms to prevent acid injury and to rapidly repair. Awareness of these protective mechanisms helps in understanding the pathophysiology of injury. Most of the protective mechanisms have not been specifically investigated in the dog and cat but are considered to be similar across mammalian species. The first protective mechanism is the gastric mucosal barrier, composed of primarily mucus and bicarbonate, both of which are secreted by surface epithelial cells to form a viscous layer superficial to the epithelium (Figure 1.1). Bicarbonate, secreted by surface epithelial cells, lies within the mucus layer and serves as a buffering mechanism. This
helps maintain a neutral pH near the mucosal surface, neutralizing the direct effects of acid on the mucosal surface. Phospholipids, both within the mucus layer and lining the surface epithelium, add another layer of protection. Their hydrophobicity repels acid- and water-soluble toxic agents. If the mucus layer is penetrated, tight junctions between epithelial cells prevent back diffusion of acid and other injurious agents. If epithelial cells are damaged, the resulting mucosal defect is quickly covered, usually in minutes, by epithelial restitution and later cell proliferation. When there is penetration of acid or other damaging agents deeper than the mucosal epithelium, local mucosal blood flow quickly increases clearing and buffering injurious agents (7). Duodenal surface epithelial cells also secrete bicarbonate and mucus as a protective barrier against acid (Figure 1.2). In addition, it does have epithelial cell tight junctions and can quickly increase blood flow in response to injury. In addition, bicarbonate secreted in biliary and pancreatic secretions act to buffer acidic ingesta.

Many of these mechanisms, including mucus and bicarbonate secretion, mucosal blood flow, reduction of histamine-mediated cAMP to reduce proton pump activity, and epithelial restitution, are prostaglandin dependent. Prostaglandin synthesis is dependent upon cyclooxygenase (COX) enzymes that produce prostaglandins as an end product of the arachidonic acid pathway. COX enzymes are present in two main forms that are important for gastrointestinal health. Both forms of this enzyme convert arachidonic acid to PGH₂, which is then converted to several different prostaglandins by specific prostaglandin synthases (Figure 1.3). Traditionally, COX-1 has been considered the constitutive form, expressed in gastric and duodenal mucosa as well as other tissues, that acts to maintain gastric blood flow and perform other “housekeeping” activities, whereas COX-2 has been
considered to be solely inducible, responding to inflammatory stimuli such as lipopolysaccharides, IL-1, and TNF-alpha. In many tissues and organs in the body, COX-2 is considered to be the main source of prostaglandins involved in pain and inflammation (8). In the gastrointestinal (GI) tract, COX-2 expression is increased at the edges of ulcerated gastric epithelium, presumably in response to pro-inflammatory stimuli, and is important for ulcer healing by inducing synthesis of PGE$_2$. In the acute phase of injury (within the first 2 hours), PGE$_2$ is the primary prostaglandin synthesized at higher levels due to increased COX-2 expression. At 24 to 48 hours of COX-2 upregulation, another PGH$_2$ metabolite, PGD$_2$, is increased, which corresponds to the resolution of inflammation and clearance of polymononuclear cells in a carageenan rat injury model (9). In the early stages, NSAID treatment may inhibit healing by its inhibition of PGE$_2$ but in later stages it may have a pro-inflammatory effect through decreasing PGD$_2$ levels, which would also effect gastric ulcer healing. In addition to the induced expression at times of injury, COX-2 also is constitutively expressed at low levels in normal gastric and duodenal mucosa (10-12). This background level of activity may help the GI mucosa respond to potentially injurious events. Increased COX-2 expression exerts its protective and reparative effects by increasing PGE$_2$ (11,13). This important protective prostaglandin accelerates mucosal recovery and repair.

Gastroduodenal ulceration may occur when any of these protective mechanisms are disrupted. This may be due to one of several mechanisms, including altered mucosal blood flow, direct disruption of the mucosal epithelial barrier, increased acid secretion, and prostaglandin inhibition. Several retrospective studies in dogs and cats have identified many potential causes of spontaneous gastroduodenal ulceration and perforation.
Pathogenesis of gastroduodenal ulcers in dogs

The most commonly identified predisposing factors for gastroduodenal ulceration in dogs include NSAID therapy, corticosteroid therapy, liver disease, shock or sepsis, and underlying neoplasia. Other less commonly reported associations include intervertebral disk disease, inflammatory bowel disease, and extreme exercise/stress in racing sled dogs (1-3,14-17). In most retrospective studies, many of the dogs (up to 40%) showed evidence of more than one predisposing cause, supporting a multifactorial cause of ulceration in many patients (2).

The mechanism of injury is variable depending on the associated cause. NSAIDs cause ulceration though inhibition of prostaglandin production. Likewise, corticosteroids inhibit the production of prostaglandins by inhibiting phospholipase A2 (Figure 1.3). Phospholipase A2 releases arachidonic acid from phospholipids in the cell membrane, which are transformed by cyclooxygenases and prostaglandin synthases into various prostaglandins. Ulcerogenic doses of corticosteroids range from anti-inflammatory doses to higher shock doses (2). The mechanisms involved with ulceration secondary to liver disease are poorly understood, but may include altered submucosal blood flow and decreased gastrin clearance (18). Similarly, shock and sepsis are likely associated with mucosal ulceration due to altered hemodynamics of the submucosal capillary bed (1). Neoplasia (such as lymphosarcoma and adenocarcinoma) may lead to ulceration by directly disrupting the mucosal epithelial layer. Additionally, neoplasias that alter acid secretion, such as gastrinomas and mast cell disease, may also lead to ulceration (1,2). In dogs undergoing surgery for intervertebral disc disease (IVDD), about 75% have gastric mucosal lesions. Many of these had been previously treated
with corticosteroids or NSAIDs as a potential contributing cause of ulceration, but in one report, approximately one third of dogs had received neither (17). Potential mechanisms for ulceration associated with IVDD include alterations in parasympathetic/sympathetic tone causing increased acid secretion and ischemic damage to the mucosa. Up to 50% of racing sled dogs may be affected with gastric erosions after strenuous activity; acute hemorrhage from ulceration is a significant cause of race-related mortality (16,19,20). It is hypothesized that high fat diets fed to these dogs may lead to delayed gastric emptying and hyperacidity. Potential contributing factors may include exercise-induced visceral ischemia, bacterial pathogens, stress, and physical trauma (foreign bodies) (16).

Helicobacter pylori is a significant cause of gastric ulceration in humans; its role in gastric ulceration in dogs has been examined. Although Helicobacter pylori has been reported to be associated with gastric ulcers in certain geographical regions (e.g. India), there has not been a confirmed report of gastric ulceration due to Helicobacter species in the United States and up to 100% of unaffected healthy dogs may be positive for various Helicobacter species (21-23). As opposed to Helicobacter infection in humans, natural infection with Helicobacter in dogs does not significantly increase gastrin levels (24). Although Helicobacter has been associated with gastritis and duodenitis, it has not yet shown a significant association with ulcer disease (23,25).

The most common locations for gastroduodenal ulcerations, regardless of cause, are the antrum, pylorus, and proximal duodenum. In normal dogs, PGE₂ levels are highest in the pylorus and proximal duodenum (10,12,26). These locations may be the most predisposed to ulceration because they may be exposed to high levels of acid and ingesta that may damage
epithelium, either through chemical or mechanical trauma. In addition, bile salts and bile acids may contribute to injury in the pyloric antrum, via biliary reflux into the stomach. Biliary contents can damage mucosa by dissolution of mucosal lipids as well as mucosal absorption of toxic bile acids (27,28).

**NSAIDs and gastroduodenal ulcers**

In humans, chronic NSAID therapy is a common cause of gastric and duodenal lesions, affecting up to 9% of patients within the first two weeks of treatment with NSAID or aspirin therapy. Importantly, up to 40% of these patients can be asymptomatic (29). There are no similar studies in dogs and cats receiving chronic NSAID therapy, but if veterinary patients are at all similar to their human counterparts in the prevalence of subclinical disease then NSAID-induced gastroduodenal irritation is likely to be an under-recognized disease in patients receiving NSAID therapy. It is, however, the most well examined, and most commonly reported, cause of gastric ulcers in dogs. NSAID-associated ulcers are reported with both acute (one to two doses) and chronic (months to years) therapy using these drugs (2). Ulcers have been associated with most NSAIDs, including non-selective COX inhibitors such as aspirin, phenylbutazone, ketoprofen, flunixin, piroxicam, ibuprofen, and naproxen, as well as with preferential COX-2 inhibitors carprofen, and meloxicam, and selective COX-2 inhibitors (coxibs) (3,30-36). Administration at higher than approved doses or close association temporally of two different NSAIDs or an NSAID and a corticosteroid increases the risk of ulceration though there are reports of ulceration in dogs treated with NSAIDs at prescribed doses (3,15).
NSAIDs can induce gastric injury leading to ulcers via a variety of mechanisms. Firstly, aspirin in particular is a weak acid that becomes non-ionized in contact with gastric acid. In its non-ionized form, aspirin more readily diffuses across membranes into epithelial cells. Within the neutral pH of the epithelial cell, aspirin then becomes ionized, releasing hydrogen ions that disrupt cell function. This mechanism does not seem to be a significant cause of injury in other clinically utilized NSAIDs.

For most NSAIDs, the primary mechanism of injury leading to gastroduodenal ulceration is thought to be via cyclooxygenase inhibition, depressing prostaglandin synthesis. Due to the presumed role of COX-1 as the constitutively expressed COX enzyme in the gastrointestinal tract, COX-2 selective inhibitors were developed in an attempt to decrease adverse gastrointestinal events, such as ulceration and perforation. Though many of these COX-2 selective inhibitors (meloxicam, carprofen, deracoxib, firocoxib) do show COX-2 selectivity in whole blood assays, evidence of the selectivity of these drugs at the gastrointestinal mucosa is conflicting; selectivity of NSAIDs in whole blood may be lost at the tissue level (37,38). Several studies do show maintenance of normal prostaglandin levels in mucosal tissue with COX-2 selective NSAIDs, supportive of COX-2 selectivity, while others do show some suppression of baseline prostaglandin levels (26,37-42). When gastric mucosa is examined endoscopically after administration of various NSAIDs, reports are somewhat contradictory as to which NSAIDs produce the most severe lesions, although the non-selective COX inhibitors do tend to induce lesions more reliably than COX-2 selective drugs (26,27). As stated previously, COX-2 is upregulated with gastrointestinal injury associated with the reparative process. The administration of a COX-2 inhibitor concurrent
with another cause of gastrointestinal injury, such as liver disease, sepsis or SIRS, or other causes of ischemic or hypovolemic injury may exacerbate injury, although this theory is unproven in dogs. In people, the presence of concurrent diseases, such as sepsis and liver cirrhosis, has been associated with a poorer outcome in patients with peptic ulcers (44,45).

Another mechanism by which many NSAIDs can induce epithelial injury is via increased leukotriene synthesis and neutrophil-mediated damage. Suppression of cyclooxygenase enzymes may increase leukotriene synthesis by shuttling arachidonic acid conversion to leukotrienes, such as LTB\(_4\), via lipooxygenases. However, whether this occurs in tissues in response to NSAIDs has been debated. Nonetheless, increased LTB\(_4\) would lead to neutrophil recruitment to the gastrointestinal mucosa, causing further mucosal damage (46). Dual cyclooxygenase/lipooxygenase inhibitors (tepoxalin) have been developed in an attempt to reduce gastric injury with this potential mechanism as a consideration. When compared to a highly selective COX-2 inhibitor (firocoxib), the dual COX/LOX inhibitor tepoxalin did inhibit healing after endoscopic biopsy of duodenal mucosa less than the COX-2 selective inhibitor firocoxib (47).

Much is yet to be understood regarding the role of various NSAIDs in gastrointestinal ulceration. It is important to recognize that any NSAID, regardless of selectivity, may cause gastroduodenal ulceration and perforation, especially in dogs with a secondary predisposing cause. There is, however, support that more COX-2 selective inhibitors, such as firocoxib and deracoxib, reduce gastrointestinal side effects in research animals (26,47). Caution should be taken any time an NSAID, even a COX-2 selective inhibitor, is administered and owners should be warned to watch for and report signs suggestive of potential ulceration.
Additionally, switching therapy between NSAIDs or between a corticosteroid and an NSAID should only be undertaken after a sufficient washout period. The appropriate length of this washout period is unknown, but is likely anywhere from a few days up to one week or more, depending on the duration of therapy and drug. It must be noted that this recommendation is the authors’ recommendation, and this is not supported (or refuted) by current evidence.

*Pathogenesis of gastroduodenal ulceration in cats*

Gastroduodenal ulceration and perforation is less frequently recognized in cats. Most commonly, ulceration is described secondary to neoplasia, including systemic mastocytosis, gastric lymphosarcoma and adenocarcinoma, and gastrinoma (5). Additional diseases that have been described and associated with ulceration include inflammatory bowel disease, hypovolemia, renal disease, toxic ingestion, and infectious causes (endoparasites, bacterial granuloma) (2,5). There are reports of cats developing gastroduodenal ulceration secondary to anti-inflammatory therapy with carprofen, meloxicam, and prednisolone (4,50). It is unclear whether cats are more resistant to adverse gastrointestinal side effects of NSAID therapy or if the paucity of reports on NSAID associated ulceration in cats is because these drugs are less commonly prescribed for use in cats than in dogs. This may be due to the fact that there are fewer NSAIDs approved for use in cats (either perioperatively or for long term use) than dogs (51).

*Clinical presentation*

Dogs presenting for spontaneous gastroduodenal ulceration or perforation are most often large breed dogs (average 35kg) that are middle aged (5-7 years). Most common breeds
reported include common larger breed dogs, such as Labrador Retrievers, Doberman Pinschers, German Shepherd Dogs, and Rottweilers (1-3,33). In one report, affected Rottweilers were younger at age of presentation (3.5 years on average) (2). It is not known if there is something inherent in these dogs that predispose them to gastroduodenal ulceration, or if other factors (such as these are the breeds most likely to be administered NSAIDs) skew reports of ulceration. Reported duration of clinical signs prior to presentation ranges from less than 24 hours to several months (1-3,15,33). Most commonly, owners report non-specific signs such as lethargy, vomiting, inappetance, and weakness (Table 1.1). Fewer dogs present with more obvious signs such as hematemesis or melena, although melena is more common than hematemesis. Described physical examination findings in dogs with spontaneous gastroduodenal perforation include dehydration, compensated or decompensated shock, and abdominal pain and abdominal distension (1-3,15,33).

Although reports of gastroduodenal ulceration in cats are less common, affected cats also have non-specific presenting signs including lethargy, weight loss, vomiting, and inappetence (2,4,5). Hematemesis and melena seem to be even less common in cats with gastroduodenal ulceration. Cats are older on presentation, on average 10 years, and were mostly domestic shorthair and domestic longhair (2). In cats with ulceration-associated tumors, duration of clinical signs is longer than in cats with non-neoplastic disease (4-5 months versus about 1 month for non-neoplastic disease) (5). Cats with gastroduodenal perforation present similarly to dogs, with dehydration, shock, and abdominal pain and distension being the most common findings (2,4,5).
Diagnosis of gastroduodenal ulceration

The most common hematologic abnormalities in dogs include a leukocytosis characterized by neutrophilia with a left shift and lymphopenia, and less consistently anemia. Anemia is commonly normocytic normochromic, but may be microcytic hypochromic depending on duration of ulceration and significance of gastrointestinal hemorrhage. Clinical pathologic analyses of ulcerated dogs most commonly show hypoproteinemia and hypoalbuminemia. Other frequent findings include hyponatremia and hypocalcemia. If a concurrent disease is present, supportive abnormalities of that condition may also be seen, such as elevation in liver enzymes (1,2,15,33).

In cats, neutrophilia with a left shift, lymphopenia, and anemia are also most commonly detected. Cats likewise demonstrate hypoproteinemia and hypoalbuminemia as well as hyponatremia and hypocalcemia. In addition, clinical pathologic biochemical abnormalities in cats may include azotemia and elevation in creatine kinase (2,5). It is not clear whether these biochemical changes (azotemia, elevated creatine kinase) are directly related to gastrointestinal ulceration in cats or whether it is due to concurrent diseases.

Orthogonal abdominal radiographs often show evidence of gastroduodenal perforation more consistently than in patients without perforation (Figures 1.4 and 1.5). Dogs and cats with perforation have decreased abdominal detail in about 75% of cases (2,15). Cats with gastroduodenal perforation frequently have pneumoperitoneum (2,4,5) whereas fewer canine cases present with pneumoperitoneum (approximately 50%). Unfortunately, the diagnosis of gastroduodenal ulceration without perforation is often more difficult as plain abdominal radiographs are often normal, although they may demonstrate a mass effect if
there is underlying gastrointestinal neoplasia (33,52). Contrast radiography has a higher sensitivity for gastroduodenal ulceration than plain radiography, although only approximately 25% of gastroduodenal ulcers are detected, and this technique has a higher complication rate than either plain radiography or ultrasound (1). Contrast radiography may show a filling defect representative of the ulcer and/or show delayed gastric emptying (2,36). In patients where perforation is known or suspected, contrast radiography should not be performed due to the risk of barium peritonitis. Abdominal ultrasound is generally considered a better modality with which to diagnose perforation and often ulceration as well. With perforation, dogs and cats commonly (80-100%) have free peritoneal effusion. Additional findings include ‘bright’ (highly echogenic) mesenteric fat, gastric or duodenal mucosal thickening, and pneumoperitoneum (2,15,53). Ultrasonographic changes with ulceration include thickening of the gastric or duodenal wall, loss of normal layering, presence of a crater with accumulation of microbubbles (a collection of small dense echoes associated with strong acoustic shadowing), and decreased gastric motility (Figures 1.6 and 1.7). If there is an underlying gastrointestinal neoplasia, it is often also visualized by ultrasonography (5,54). This modality is also useful for detecting non-gastrointestinal neoplasias that can cause ulcers, such as gastrinomas and mast cell disease. Abdominal cytology may be performed if free fluid is present. About 50% of dogs and cats with perforation of the gastric or duodenal mucosa secondary to ulceration have septic suppurative inflammation; non-septic suppurative inflammation is seen in the remainder of cases. Likewise, culture of abdominal fluid is positive for growth in about half of dogs and cats, though the likelihood of a positive
culture was not associated with presence of bacteria, cytologic neutrophil count or morphology (2,15).

Endoscopy is commonly cited as the most reliable test for gastroduodenal ulceration, detecting up to 100% of ulcers (1,33). Endoscopy offers the advantage that in addition to visualization of the ulcer and assessment for gastrointestinal masses not detected with other modalities, it may allow for biopsy of surrounding mucosal tissue for diagnostic purposes. Though it may be impossible to determine the depth of injury and therefore whether the defect is truly an ulcer or superficial erosion, the severity of lesions can be judged subjectively which can help make the determination whether surgical intervention is required or if medical management is appropriate (Figure 1.8). Hemorrhage from the ulcer or gastric contents may obscure visualization and make diagnosis difficult (2). If a perforation is present or the ulcer is very deep, insufflation of the stomach and duodenum with air may potentially worsen the defect or cause perforation. Endoscopy, though a valuable tool for detection of ulceration, should not be performed without preparation to move to exploratory surgery should a perforation be observed or hemorrhage is severe.

Exploratory celiotomy, along with necropsy, are the gold standard for gastroduodenal ulceration. Examination of the gastric and duodenal mucosa allows both for the assessment of the depth and severity of ulceration, and provides a means of treatment via resection of the ulceration or perforation. The surgeon should ensure all surfaces of the stomach and duodenum are inspected, including the difficult-to-access dorsal surface of the stomach and the cardia area.
Treatment of gastroduodenal ulceration

Effective treatment begins by addressing the underlying causes of ulceration when applicable. NSAID therapy should be discontinued immediately upon suspicion of ulceration. Any underlying hemodynamic abnormalities and dehydration should be corrected. If possible, concurrent diseases should be treated as appropriate (liver disease, renal disease, neoplasia). Minimizing the cause of gastroduodenal ulceration is the first step in treatment. In addition, two important arms of treatment specific for ulceration and perforation are medical management and surgical therapy. Less severely affected animals may often be successfully managed with medical therapy alone. There are many available drug therapies aimed at decreasing or neutralizing acid secretion or directed at cytoprotection. Usually, a combination of acid suppressing treatments and cytoprotective agents are used. Commonly prescribed medications include H₂-receptor antagonists, proton pump inhibitors, prostaglandin analogs, sucralfate, and antacids (Table 1.2).

Acid suppression therapy

Though the minimum level or time of total acid suppression that is required in dogs and cats to best facilitate ulcer healing is unknown, examination of human data suggests that for optimal healing, gastric pH should be maintained above 3 for at least 75% of the day (55). If active hemorrhage is present, a pH above 6 is required to allow platelet aggregation and hemostasis (55). Effective acid suppression may be attempted with one of two main classes of drugs: H₂-receptor antagonists and proton pump inhibitors.

H₂-receptor antagonists exert their activity by reversibly binding the histamine receptor on the gastric parietal cell. Histamine normally acts on the parietal cell by binding to
a G protein-coupled receptor, thereby increasing intracellular cAMP, which increases gastric acid secretion; antagonism of this receptor causes acid suppression. In addition, by decreasing intracellular levels of cAMP, H₂-receptor antagonists reduce reactivity to acetylcholine and gastrin. Of the most commonly used H₂-receptor antagonists, famotidine, cimetidine and ranitidine, famotidine achieves the most effective acid suppression, reducing maximal acid output by 50-70% (6,56). Ranitidine, traditionally considered the next closest in potency to famotidine, did not suppress acid secretion significantly more than saline when administered at recommended doses (2mg/kg IV) (6). Famotidine also prevents NSAID-induced decrease in mucosal blood flow by an unknown mechanism (57). In this way, famotidine may serve in prevention of gastric ulcers, as well as for treatment. Famotidine, administered at 1 mg/kg/day, trended to decrease gastric lesion score following an exercise trial in Alaskan racing sled dogs (58).

Proton pump inhibitors (PPIs), by binding irreversibly to H⁺-K⁺ ATPase, can achieve more effective acid suppression than H₂-receptor antagonists. PPIs are weak bases that diffuse into cells in a non-protonated form. For activation, PPIs need to be exposed to the acidity of the canaliculi within the parietal cell, where they become protonated and therefore activated to bind to the proton pump. In dogs where maximal acid output is stimulated with intravenous histamine and then administered omeprazole, acid suppression was seen as early as one hour following administration (59). Clinically, however, acid output is not generally stimulated as quickly as with intravenous histamine therapy, so time to effective acid suppression is longer (2-6 days) because complete recruitment of proton pumps to the canaliculi is less complete. By day 2 in normal dogs, omeprazole, given at 1 mg/kg once
daily, maintained gastric pH at about 3.8 versus 2.8 in famotidine treated dogs. By day 6, omeprazole (1 mg/kg once daily) maintained gastric pH ≥ 3 for 70% of the time and pH was ≥ 4 for 50% of the time (6). In the same study, omeprazole-bicarbonate suspension (2mg omeprazole/mL of 8.4% sodium bicarbonate) was administered at 1 mg/kg twice daily; this increased the time of pH ≥ 3 and 4 to 91% and 78%, respectively, suggesting that twice daily treatment with omeprazole suspension may be the most effective acid suppressant allowing for maximal ulcer healing (6). In a second study, omeprazole was also superior to famotidine in acid suppression, given at 1.5-2.6mg/kg once a day as a tablet or 1.5-2.6mg/kg once a day as a suspension (Gastrogard® diluted in sesame oil at 40mg/ml) (60). Proton pump inhibitors may have additional beneficial effects besides acid suppression. In human neutrophils, omeprazole treatment in vitro decreased production of free radicals produced with NSAID induced injury (61). Omeprazole may exert this activity by inhibiting proton pumps in the lysosome, increasing lysosomal pH and decreasing oxidative burst. For patients that are unable to tolerate oral medication due to vomiting, PPIs are available in intravenous injectable form, such as pantoprazole, that also effectively increases gastric pH in dogs (62). When administered intravenously (1mg/kg once daily), pantoprazole suppressed acid secretion similarly to, though slightly less than, oral omeprazole at 1mg/kg PO once daily (6). The time to onset of acid suppression was also similar between the injected and oral PPI. Because this class of drugs is dependent on acid for activation, it is generally not recommended that they be co-administered with H₂-receptor antagonists that may inhibit activation of the proton pump and thereby decrease PPI activity. In humans, it is recommended that PPIs be administered approximately 15 minutes prior to feeding, although
reports are somewhat contradictory as to whether administration of omeprazole with or prior to a meal affects bioavailability significantly (63,64).

*Cytoprotection*

Misoprostol is a PGE₁ analog that acts similarly to endogenous prostaglandins in increasing gastric mucosal blood flow, stimulating bicarbonate and mucus secretion, and increasing epithelial turnover (65,66). It has been shown to decrease gastric erosions associated with aspirin treatment in dogs, suggesting its utility in prevention of NSAID induced ulcers (67). The use of misoprostol for treatment of preexisting ulcers, however, has not been examined in dogs but its ability to promote and support protective mechanisms implies that it may serve a role in gastric ulcer treatment as well as prevention. Additional studies examining the efficacy of different dosing intervals of misoprostol (every 8, 12, or 24 hours) showed that misoprostol twice daily prevented the development of gastric lesions associated with aspirin treatment just as well as three times a day treatment (68). Misoprostol, as a prostaglandin analogue, is probably most effectively used in the prevention of NSAID and corticosteroid induced ulcers.

Sucralfate is a mucosal cytoprotective drug that dissociates in the acid contents of the stomach to aluminum hydroxide and sucrose octosulfate. It then electrostatically binds to positively charged proteins that are exposed at the base of the ulcer, forming a barrier against further back diffusion of acid into the mucosa (69). As such, sucralfate is only useful as an ulcer treatment and not as protective therapy against ulceration. Binding to ulcerated gastric mucosa is not adversely affected by concurrent administration with acid suppressants or antacids (69). In addition, sucralfate stimulates PGE₂ production, thereby promoting
prostaglandin-mediated cytoprotective mechanisms (increased mucosal blood flow, decreased acid secretion, increased mucus and bicarbonate secretion) as well as increasing expression of epidermal growth factor (70,71).

Antacids, such as magnesium hydroxide and aluminum hydroxide, act in cytoprotection of the gastric mucosa by neutralizing acid and inactivating pepsin. In addition, they can also induce local PGE$_2$ synthesis (72). The disadvantage of these treatments is that to maintain efficacy they must be given very frequently, up to every 2-3 hours, to prevent rebound hyperacidity between dosing. Given at these high frequencies and therefore relatively high total dose, acid-base derangements, phosphate depletion, and diarrhea can occur which limits their clinical utility in most patients.

Adjunctive treatments

In addition to the above listed targeted therapies for ulceration, additional therapy may be warranted based on individual assessment of the patient. This may include pain management with opioids such as buprenorphine to manage acute pain associated with ulceration. Buprenorphine is a partial mu-agonist that is effective for visceral pain and has less effect on gastrointestinal motility than a more potent mu-agonist such as morphine or hydromorphone but can still be administered intravenously. Vomiting is a common sign in patients with gastroduodenal ulceration that could be due to either locally mediated effects (stimulation of vagal afferents due to ulceration) or centrally acting effects with concurrent diseases (stimulation of the chemoreceptor trigger zone i.e. liver disease, renal disease, neoplasia). If vomiting is present, anti-emetics should be prescribed as well as therapy previously mentioned. Many highly effective intravenous anti-emetics are available and
relatively inexpensive, including serotonin receptor antagonists (ondansetron and dolasetron), NK-1 receptor antagonists (apretitant and maropitant), and more conventional anti-emetics such as metoclopramide and chlorpromazine. Ideally, the selection of an anti-emetic would consider both its locally mediated and centrally acting (in the CRTZ and vomiting center) effects. Serotonin receptor antagonists (ondansetron, dolasetron) are effective peripherally acting anti-emetics and are therefore appropriate for treatment of ulcerogenic vomiting. NK-1 receptor antagonists (apretitant, maropitant) act both locally and centrally to protect against emetogens. Metoclopramide, as a dopaminergic antagonist, works primarily in the CRTZ so may be less effective in treating visceral afferent mediated vomiting as is often present with ulceration. Chlorpromazine, because of its potential hemodynamic and anti-cholinergic effects, is not recommended for most patients with ulceration. Many of the recommended anti-emetic therapies have relatively wide effective therapeutic dosing ranges so dosing may be tailored to each individual patient based on frequency and severity of emesis.

Surgical treatment

For patients with gastroduodenal perforation, severe ulceration with hemorrhage or failure to respond to medical therapy, surgery is typically warranted. In critically ill patients, appropriate stabilization measures should be taken prior to surgery and may include fluid resuscitation and/or blood transfusions if anemia is severe. At surgery, the affected ulcerated mucosa is resected. Occasionally, Billroth procedures are required for more severely affected animals or in patients with gastrointestinal tumors where the lesions are located in the pylorus near the pylorus itself (5). Often, dogs and cats with gastroduodenal perforations will have local or generalized peritonitis that will need to be managed appropriately with
lavage and placement of drains as necessary (2,4). If ulcerations or perforations are difficult to identify, intraoperative endoscopy or ultrasonography may aid in localization of disease. Subsequent to surgery, appropriate medical therapy should also be employed, including antibiotic therapy, acid suppression therapy, and nutritional management.

**Outcome**

There are few reports in the literature describing short and long term survival of dogs and cats with gastroduodenal ulceration, though reported survival rates following medical or surgical management are quite variable. In dogs with perforations, survival-to-discharge rates have ranged from 30-60% (2,3,15). In cases where perforation is not present and treatment consists of aggressive medical management, survival is reportedly as good as 100% (33). Survival rates in cats following spontaneous perforation appears to be higher following surgical management, often reaching 100%. In the few reports of cats with ulceration without perforation, survival rates range from 14% to 100% (4,5). Early recognition of potential ulceration and prompt treatment is the optimal approach to achieve the best outcome. In addition, as effective acid suppression drugs such as PPIs become more common, successful treatment with medical management of ulceration in dogs and cats will likely improve.

**Summary**

Gastric ulceration is a potentially fatal disease affecting an unknown, but likely substantial, number of dogs and cats each year. Though there are multiple mechanisms by which the gastric and duodenal mucosa is able to protect and repair itself, sometimes these are overwhelmed, leading to ulceration. Early signs of ulceration may be very mild, including
vomiting, inappetence and lethargy. In many patients, recognition of ulceration does not occur until perforation has occurred and clinical signs are more obvious. Ulcers have been increasingly associated with NSAID treatment in dogs and occasionally cats, likely due to the increased use of these drugs in animals that may have other risk factors for the development of ulceration. Whenever drugs from this class are prescribed, owners should be cautioned on the need for following instructions on administration, and advised to observe for early signs of ulceration. If this occurs, NSAID treatment should be promptly discontinued and appropriate medical management for ulceration should be initiated. Appropriate therapy includes acid suppression, most effectively achieved with omeprazole administered twice daily, along with sucralfate therapy for cytoprotection. If the ulcer is NSAID- or corticosteroid-induced, misoprostol may accelerate healing, but additional studies are needed to provide evidence for this. If a perforation is suspected, gastrointestinal hemorrhage is severe, or clinical signs are refractory to medical therapy, surgery along with medical therapy should be considered for the best possible outcome.
Figure 0.1 Protective mechanisms of the gastric mucosal epithelium

Surface epithelial cells secrete bicarbonate and mucus, while the submucosal capillary bed carries protective growth factors and carries away injurious agents. Tight junctions form a barrier against back diffusion of acid.
Surface epithelial cells secrete bicarbonate and mucus, while the submucosal capillary bed carries protective growth factors and carries away injurious agents. Tight junctions form a barrier against back diffusion of acid.
Figure 0.3 Prostanoid pathway and the role of cyclooxygenase-1 and 2

The site of inhibition of non-steroidal anti-inflammatory drugs, lipoxygenase inhibitors and glucocorticoids is noted.
Figure 0.4 Lateral abdominal radiographs of a dog

There is peritoneal fluid and pneumoperitoneum, consistent with intestinal perforation. The pylorus appears thickened, consistent with a gastric ulcer.
Figure 0.5 Ventrodorsal abdominal radiographs of a dog

There is peritoneal fluid and pneumoperitoneum, consistent with intestinal perforation. The pylorus appears thickened, consistent with a gastric ulcer.
Figure 0.6 Ultrasonographic image of gastric ulcer

Ultrasonographic image of gastric ulcer, showing gastric wall thickening.
Figure 0.7 Ultrasonographic image of duodenal ulcer

Ultrasonographic image of duodenal ulcer, showing duodenal wall thickening and bright surrounding mesentery.
Figure 0.8 Endoscopic image of gastric ulcer

Endoscopic image of gastric ulcer showing evidence of necrosis and active hemorrhage.
### Table 1.1 Clinical signs of gastroduodenal ulceration

The most common clinical signs with gastroduodenal ulceration and/or perforation in dogs and cats. The three most common signs for each species are highlighted in bold.

<table>
<thead>
<tr>
<th>Clinical sign</th>
<th>Frequency in dogs</th>
<th>Frequency in cats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lethargy</td>
<td>95%</td>
<td>100%</td>
</tr>
<tr>
<td>Vomiting</td>
<td>81%</td>
<td>87%</td>
</tr>
<tr>
<td>Inappetance/anorexia</td>
<td>52%</td>
<td>73%</td>
</tr>
<tr>
<td>Melena</td>
<td>40%</td>
<td>20%</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>29%</td>
<td>50%</td>
</tr>
<tr>
<td>Hematemesis</td>
<td>29%</td>
<td>23%</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>22%</td>
<td>31%</td>
</tr>
<tr>
<td>Weight loss</td>
<td>11%</td>
<td>80%</td>
</tr>
<tr>
<td>Weakness</td>
<td>6%</td>
<td>0%</td>
</tr>
<tr>
<td>Polyuria/polydipsia</td>
<td>5%</td>
<td>33%</td>
</tr>
</tbody>
</table>

(2,5,15,33)
### Table 1.2 Treatment for gastroduodenal ulceration

Frequently prescribed medications for management of gastroduodenal ulceration.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Use</th>
<th>Dose</th>
<th>Route</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Famotidine</td>
<td>Acid suppressant</td>
<td>0.5-1mg/kg</td>
<td>IV, SQ, PO</td>
<td>Q12-24h</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>Acid suppressant</td>
<td>0.5-1mg/kg</td>
<td>PO</td>
<td>Q12-24h</td>
</tr>
<tr>
<td>Pantoprazole</td>
<td>Acid suppressant</td>
<td>0.5-1mg/kg</td>
<td>IV</td>
<td>Q12h on day 1, then Q24h</td>
</tr>
<tr>
<td>Misoprostol</td>
<td>Prostaglandin analog</td>
<td>Dog: 2-5mcg/kg Cat: No known dose</td>
<td>PO</td>
<td>Q12h</td>
</tr>
<tr>
<td>Sucralfate</td>
<td>Cytoprotectant</td>
<td>Dog: 0.5-1g/dog Cat: 0.25g/cat</td>
<td>PO as slurry in water</td>
<td>Q8h-q12h</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>Pain management</td>
<td>0.01-0.02mg/kg</td>
<td>IV, IM, PO in cats</td>
<td>Q6-12h</td>
</tr>
<tr>
<td>Ondansetron</td>
<td>Anti-emetic</td>
<td>0.25-1mg/kg</td>
<td>IV, PO</td>
<td>Q6-12h</td>
</tr>
<tr>
<td>Dolasetron</td>
<td>Anti-emetic</td>
<td>0.5-1mg/kg</td>
<td>IV, PO</td>
<td>Q24h</td>
</tr>
<tr>
<td>Apretitant</td>
<td>Anti-emetic</td>
<td>1-2mg/kg</td>
<td>PO</td>
<td>Q24h</td>
</tr>
<tr>
<td>Maropitant</td>
<td>Anti-emetic</td>
<td>SQ: 1mg/kg PO: 2mg/kg</td>
<td></td>
<td>Q24h for 5 days</td>
</tr>
<tr>
<td>Metoclopramide</td>
<td>Anti-emetic, promotility</td>
<td>0.2-0.5mg/kg 1-2mg/kg/day</td>
<td>IV, IM, PO IV</td>
<td>Q6-8h CRI</td>
</tr>
<tr>
<td>Magnesium hydroxide</td>
<td>Antacid</td>
<td>5-10ml/animal</td>
<td></td>
<td>Q4-6h</td>
</tr>
</tbody>
</table>

(6,60,72)
References


CHAPTER 2 GASTROPROTECTION AND PROSTAGLANDIN E$_2$

LITERATURE REVIEW
Introduction

The pH of a normal dog stomach is quite low, regularly near pH 1.0 in the fed dog. Gastric pH is less than 3 for nearly 90% of the day in a normally feeding dog (1). Given this prolonged exposure to high levels of acid, the gastric mucosa has several mechanisms to prevent acid injury and effect repair. The first protective mechanism is the gastric mucosal barrier, composed of primarily mucus and bicarbonate, both of which are secreted by surface epithelial cells to form a viscous layer superficial to the epithelium. Bicarbonate, secreted by surface epithelial cells, lies within the mucus layer and serves as a buffering mechanism. This helps maintain a neutral pH near the mucosal surface, neutralizing the direct effects of acid on the mucosal surface. Phospholipids, both within the mucus layer and lining the surface epithelium, add another layer of protection. Their hydrophobicity repels acid- and water-soluble toxic agents. Should the mucus layer be penetrated, tight junctions between epithelial cells prevent back diffusion of acid and other injurious agents. If epithelial cells are damaged, the resulting mucosal defect is quickly covered, usually in minutes, by epithelial restitution and later cell proliferation. When there is penetration of acid or other damaging agents past the mucosal epithelium, local mucosal blood flow quickly increases clearing and buffering injurious agents (2).

The molecular control of these protective mechanisms is complex. Many of these mechanisms, including mucus and bicarbonate secretion, mucosal blood flow, and reduction of proton pump activity are prostaglandin dependent. Prostaglandin synthesis is dependent upon cyclooxygenase (COX) enzymes that produce prostaglandins as an end product of the
arachidonic acid pathway. COX enzymes are present in two main forms that are important for gastrointestinal health. Both forms of this enzyme convert arachidonic acid to prostaglandin H\(_2\) (PGH\(_2\)), which is then converted to several different prostaglandins by specific prostaglandin synthases. Traditionally, COX-1 has been considered the constitutive form, expressed in gastric and duodenal mucosa as well as other tissues, that acts to maintain gastric blood flow and perform other “housekeeping” activities, whereas COX-2 is inducible in inflammatory cells like fibroblasts and macrophages, responding to inflammatory stimuli such as lipopolysaccharides, IL-1, and TNF-alpha (3-6). In many areas in the body, COX-2 is considered to be the main source of prostaglandins involved in pain and inflammation (7). In the GI tract, COX-2 expression is increased at the edges of ulcerated gastric epithelium, presumably in response to pro-inflammatory stimuli. It is important for ulcer healing by inducing synthesis of PGE\(_2\). In addition to the induced expression at times of injury, COX-2, is constitutively expressed at low levels in normal gastric and duodenal mucosa (8-10). This background level of activity may help the GI mucosa respond to potentially injurious events. Increased COX-2 expression exerts its protective and reparative effects by increasing PGE\(_2\) (9,11). This important protective prostaglandin accelerates mucosal recovery and repair.

COX-1 and COX-2 are responsible for the synthesis of PGH\(_2\) from arachidonic acid. It must then be converted to various prostaglandins by specific prostanoid synthases. It is the specificity of multiple prostanoid synthases that can exert different cellular effects.

Prostagland E\(_2\) synthase (PGE\(_2\) synthase) is present in several forms. Cytosolic PGE synthase (cPGES) is constitutively expressed and is likely responsible for the maintenance baseline activity of prostaglandin E\(_2\) (PGE\(_2\)) (12). This form of the enzyme is coupled, both
functionally and spatially, to COX-1 enzyme in the cell, near the endoplasmic reticulum. The level of cPGES is not increased after stimulation by inflammatory cytokines. This is in contrast to another form of PGE synthase in a membrane-bound form (mPGES). This form of the enzymes is present at low levels in normal gastric tissue but becomes highly upregulated in response to inflammatory stimuli such as LPS (13). When mPGES is active, there is very efficient conversion of arachidonic acid to PGE2 in the presence of COX-2, but not in the presence of COX-1 (14,15). In addition, mPGES is found at high levels in active ulcers in humans and at lower levels in scarred or healing ulcers. There was none detected in normal gastric mucosa (16). This suggests that mPGES is coupled to COX-2, as cPGES is coupled to COX-1 and that mPGES is important in conjunction with COX-2 for ulcer healing.

In this discussion, the specific pathways by which PGE2 upregulates protective mechanisms in gastric epithelial will be examined, including mucus and bicarbonate secretion, proton pump activity in parietal cells, and vasodilation in the submucosal capillary bed.

**EP receptors**

There are at least 4 subtypes of PGE2-responsive receptors, EP1, EP2, EP3, and EP4. Each of these may work through a different pathway upon PGE2 binding to exert different cell responses. Upon PGE2 stimulation, EP1 receptors increase intracellular Ca via Gq protein stimulated opening of Ca channels, EP2 and EP4 receptors increase intracellular cAMP via the Gs protein pathway. The EP3 receptor has 4 splice variants with different cellular
pathways: EP3A receptor is linked to G\textsubscript{i} protein which is inhibitory to adenylate cyclase and decreases intracellular cAMP, EP3B and EP3C are linked to G\textsubscript{s} protein, which is stimulatory to adenylate cyclase and increases intracellular cAMP, EP3D is linked to G\textsubscript{q} protein and increases intracellular calcium by phosphatidyl inositol turnover (17). This plasticity of EP receptor subtypes and their variants allows one ligand, PGE\textsubscript{2}, to exert many different cellular effects, including increased mucus secretion, increased bicarbonate secretion, and vasodilation or angiogenesis.

The localization of different EP receptors throughout the gastrointestinal tract is somewhat controversial. In one report, EP1 receptors were detected in smooth muscle cells in the muscularis mucosa, implying they are important for modulation of gastrointestinal motility (18). EP3 receptors were detected in gastric fundic gland epithelial cells and myenteric ganglia, whereas EP4 receptors were detected in surface epithelial cells. In contrast, a second report detected all four EP receptor subtypes in rat gastric epithelial cells (19). An additional report found both EP3 and EP4 cells in rabbit gastric epithelial cells (20). It may be that the distribution of different EP receptor subtypes varies between species or that the distribution appears different depending on the method of detection.

**PGE stimulation of mucus**

Mucus, a water insoluble gel that lines the mucosal surface, acts as the first barrier to acid and other cytotoxic agents in the gastric lumen. It is produced both by surface epithelial cells and by mucous neck cells in the gastric pits in response to cholinergic stimulation, cholecystokinin, secretin, and prostaglandin PGE\textsubscript{2} (21,22). Of the four, PGE\textsubscript{2} is the most
effective in enhancing mucus secretion. PGE$_2$ affects the mucus layer rapidly, increasing the thickness of the mucus layer by as much as 70% within 5 minutes (23). The mucus layer acts to prevent mechanical trauma to underlying epithelium. It also traps secreted bicarbonate, which creates a pH gradient from the gastric lumen to the surface epithelial cells. This pH gradient maintains a near neutral pH immediately superficial to the epithelial cell in spite of a strongly acidic gastric lumen.


After binding of PGE$_2$ to the EP4 receptor, mucus production is stimulated by several second messenger systems, including increased intracellular Ca$^{2+}$, protein kinase C, and cAMP though not all of these are important mediators in PGE$_2$-responsive mucus production (21). Additionally, these mediators, when combined in rat gastric epithelial cells, produce a synergistic increase in mucus production. In order to determine which pathway is relevant to PGE$_2$-mediated mucus upregulation, several pathways have been examined further. cAMP does increase mucus secretion (24). Forskolin, an adenylate cyclase activator, was most effective in increasing cAMP levels in a rat gastric cell line. PGE$_2$ also significantly increased cAMP levels and stimulated more cell mucus production than forskolin (21, 26). PGE$_2$ did not increase intracellular Ca$^{2+}$ levels, demonstrating that cAMP is a more important second messenger than Ca$^{2+}$ in mucus production stimulated by PGE$_2$. To examine whether
the target of cAMP is protein kinase A (PKA) or protein kinase C (PKC), cells were stimulated with PGE₂ concurrently with either a PKA or PKC inhibitor. Only the PKA inhibitor prevented increased PGE₂-mediated mucus secretion (21, 24). Interestingly, though intracellular Ca²⁺ was not increased with PGE₂, an intracellular calcium chelator did partially inhibit PGE₂-mediated mucus secretion, suggesting that intracellular calcium may play some role as a second messenger in this pathway (21).

**PGE stimulation of HCO₃ release**

Bicarbonate (HCO₃) is an important factor in the gastric mucosal barrier. It is secreted by lumenal epithelial cells and acts to buffer any acid that penetrates the mucus layer. Bicarbonate may be either produced within the gastric epithelial cell by conversion of CO₂ using carbonic anhydrase or may be transported across the cell from the submucosal capillary bed. Protons that result from carbonic anydrase-mediated bicarbonate production are transported across the basolateral membrane by a sodium-hydrogen antiporter (26). Once bicarbonate is generated or transported into the cell, it is secreted into the gastric lumen via a chloride-bicarbonate antiporter (27).

There are multiple mechanisms that stimulate bicarbonate release, including cholinergic stimulation and PGE₂. Bicarbonate secretion increases in response to injury induced by hydrochloric acid or ethanol exposure (19). PGE₂ has been shown to increase bicarbonate secretion in normal animals in a dose-dependent manner (27,28). Specifically, it is the SLC26A9 chloride-bicarbonate antiporter ion channel that seems to be important for prostaglandin-mediated bicarbonate secretion (27). In mice that are pretreated with an anion
exchange inhibitor or that are knockout mice for this specific ion channel, PGE$_2$ does not stimulate a surface epithelial increase in pH, as it does in wild type and untreated mice.

The pathway by which PGE$_2$ stimulates increased bicarbonate secretion is by binding to the EP1 receptor (28). Treatment of mice and rats with EP1 receptor agonists stimulate bicarbonate secretion; co-treatment with an EP1 receptor antagonist attenuates this effect (28). Additionally, treatment with an EP1 receptor agonist provides cytoprotection in rat gastric mucosa against acid injury by increasing bicarbonate secretion (22). In transgenic knockout mice that lack the EP1 receptor, they are unable to increase bicarbonate secretion after treatment with PGE$_2$ or with acid injury. Secretion of bicarbonate in response to either treatment in EP3 receptor knockout mice was not significantly different than in the wild type (19). Upon binding of PGE$_2$ to the EP1 receptor, the secondary pathway that exerts the cellular effect of increased bicarbonate secretion is via a G$_q$ protein linked calcium ion channel. Treatment of rats with a phosphodiesterase inhibitor did not affect EP1-mediated bicarbonate secretion, but verapamil, a calcium-channel blocker, inhibited bicarbonate secretion by 55% (28).

**PGE$_2$ and acid secretion**

The gastric mucosa has another mechanism by which it may alter gastric pH in response to injury—the modification of gastric acid secretion. In response to injury, acid secretion decreases (29). Treatment of mice with indomethacin, a non-COX selective inhibitor, or a COX-1 selective inhibitor, attenuated the decrease in acid secretion induced by taurocholate, a bile acid, in a mouse gastric injury model (29). This implies that the attenuation of acid
secretion is prostaglandin mediated. When acid secretion is stimulated with either histamine or pentagastrin, treatment with PGE$_2$ decreases acid secretion significantly. In fact, PGE$_2$ decreases basal secretion of acid, even without treatment (30).

EP3 is the receptor that is largely responsible for the control of gastric acid secretion by PGE$_2$. Treatment of rat gastric mucosa with an EP1/3 receptor agonist decreased pentagastrin-stimulated and histamine-stimulated acid secretion. To determine whether EP1 or EP3 was the active receptor, rats were then treated with an EP1 receptor antagonist. This had no effect on the attenuation of acid secretion provided by the EP1/3 receptor agonist, demonstrating that EP3 was the important receptor (30). In these models, EP2 and 4 receptor agonists had no significant effect on acid secretion. In an EP3 knockout mouse, acid secretion is not significantly decreased by injury with taurocholate, in contrast to wild-type mice that had a 60% decrease in acid secretion after injury (29). EP1 knockout mice did not have significantly different acid secretion than wild-type mice. Similarly, treatment with an EP1 receptor antagonist prevented the decrease in acid secretion after injury, whereas treatment with an EP3 receptor antagonist had no effect (29). In addition to PGE$_2$’s effect, through the EP3 receptor, on the parietal cell to decrease acid secretion, this pathway also decreases histamine secretion. Both PGE$_2$ treatment and EP1/2 receptor agonist treatment decreases pentagastrin-stimulated histamine release from enterochromaffin-like cells (ECL) in the gastric mucosa (30). This inhibitory effect of the EP3 receptor in both the parietal cell and the ECL cell is mediated via Gi protein pathway and subsequent decrease in intracellular cAMP. In the parietal cell, cAMP acts to increase proton pump activity. In the ECL cell, cAMP increases histamine release, which stimulates parietal cell function.
PGE stimulation of vasodilation

One of the other mechanisms of gastric mucosal protection is by upregulation of mucosal blood flow. In times of gastric injury, even with a mild irritant, gastric mucosal blood flow (GMBF) is increased (31). This increased flow can then protect against further injury. Though gastric mucosal blood flow may increase in response to neural stimulation, nitric oxide, or prostacyclin, PGE$_2$ is an important mediator of protective increases in gastric mucosal blood flow. PGE$_2$ increases gastric mucosal blood flow up to 1.5 times normal when administered intravenously and this effect is inhibited by treatment with indomethacin (22,32). PGE$_2$ also maintains gastrointestinal hyperemic response after injury, as with taurocholate (33). Some types of injury, such as with ethanol, cause venoconstriction in the collecting venules of the submucosal capillary bed, leading to mucosal venous congestion and exacerbating injury (34). PGE$_2$ protects against this vasoconstriction. In addition, PGE$_2$ causes vasodilation of arterioles in the submucosal capillary bed. This acts to protect against further injury and provides additional oxygen and nutrients at times of cellular stress.

The receptors responsible for this PGE$_2$ response are not as clearly delineated. There is some support that EP1 receptors play an important role in increasing gastric mucosal blood flow. EP1 knockout mice, though they had similar levels of PGE$_2$ upregulation, had significantly decreased hyperemic responses compared to wild-type after injury with taurocholate, whereas an EP3 knockout was unaffected (32,35). Treatment with an EP1 receptor antagonist also blocked the increase in GMBF after taurocholate injury (32). This data all seems to support a primary role for EP1 in mediating GMBF. Additional studies, however, have shown that treatment with EP 1/3, EP2, EP3, and EP3/4 receptor agonists all
increase GMBF, whereas an EP1 receptor agonist had no significant effect (22). When specifically examining the effect on the venous and arteriolar sides of the capillary bed, EP2 and 4 receptor agonists prevented ethanol-induced venule vasoconstriction (34). Only the EP2 agonist effectively dilated arterioles; an EP4 receptor provided some dilation at higher doses. EP3 and EP1 receptor agonists had no effect on arteriolar vasodilation. It is difficult to explain these apparently contradictory findings. It has been hypothesized be that EP1 receptors may, through mediating afferent neuron function, increase mucosal blood flow in some cases by increasing smooth muscle relaxation instead of direct effects on the submucosal capillary bed (32). Additional studies will hopefully continue to explore the specific mechanisms by which PGE₂ increases GMBF.

Conclusion

The gastric mucosa has an impressive degree of mechanisms to protect it against the high acidity of gastric contents. When any of these are disrupted, quick upregulation of PGE₂ levels by cyclooxygenase enzymes prompt rapid recovery. It is not until these protective mechanisms are overwhelmed, or when the function of cyclooxygenases is inhibited, as by non-steroidal anti-inflammatory drugs, along with disruption of a protective mechanism that gastric ulceration occurs. PGE₂ is the prostanoid produced via the cyclooxygenase pathway that is integral to the upregulation of these protective mechanisms: increased mucus secretion, increased bicarbonate secretion, decreased acid secretion, and increased gastric mucosal blood flow.
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CHAPTER 3 A ZINC L-CARNOSINE CONTAINING PRODUCT DOES NOT AMELIORATE ACID INJURY IN CANINE GASTRIC MUCOSA
Introduction

Gastric ulcer disease is an increasingly recognized cause of morbidity and mortality in dogs, although reports of prevalence of ulcer disease are lacking. The causes of gastric and pyloric ulcers are regarded as multifactorial, including liver disease, non-steroidal anti-inflammatory drug (NSAID) treatment, and corticosteroid treatment. Gastritis, inflammatory bowel disease, renal failure, shock, recent surgery, intervertebral disk disease, stress, mast cell disease, gastric neoplasia, *Helicobacter* spp. infection and gastrinoma have also been associated with ulcers (1-4). Gastric perforation is a severe, potentially fatal, sequela of gastric ulcers that may affect up to 27% of dogs with gastric ulcers (4). Of dogs that have had perforation of gastric ulcers, between 37% and 69% were euthanized or died because of their disease (1,4,5).

Several drugs have been investigated in dogs for treatment and prevention of gastric ulcers, including histamine (H₂-receptor) antagonists, proton pump inhibitors (PPI) and prostaglandin analogs. The most commonly used H₂-receptor antagonists are famotidine, cimetidine and ranitidine, of which famotidine achieves the most effective acid suppression, reducing maximal acid output by 50-70% (6,7). Gastric ulceration and perforation is a significant cause of morbidity in Alaskan racing sled dogs after a race (8); famotidine is clinically effective for the prevention of gastric erosions among these dogs when given for 7 days prior to an exercise trial (9). PPIs may be more efficacious for gastric acid suppression. For example, omeprazole is more effective at achieving acid suppression than famotidine (6). Misoprostol is a PGE₁ analog that acts similarly to endogenous prostaglandins with gastric
protective actions such as increasing gastric mucosal blood flow, stimulating bicarbonate and mucus secretion, and increasing epithelial turnover (10,11). Misoprostol has been shown to decrease gastric erosions associated with aspirin treatment in dogs, suggesting its utility in the prevention of NSAID-induced ulcers (12). The use of misoprostol for treatment of preexisting ulcers, however, has not been examined in dogs. Nonetheless, its ability to stimulate protective mechanisms implies that it may serve a role in gastric ulcer treatment as well as prevention.

Gastricalm® is a supplement which purportedly achieves optimal gastric health by adhering to the stomach lining, reinforcing natural defenses and providing enhanced antioxidant activity, thereby providing protection from gastric ulcer disease (13). Gastricalm® is composed of a zinc-carnosine complex with the addition of vitamin E. There are multiple reports on the efficacy of zinc-carnosine complex in rats and humans. In rat epithelial cell culture, zinc-carnosine prevented indomethacin, H\textsubscript{2}O\textsubscript{2} or ethanol-induced cytolysis (14,15). In an \textit{in vivo} rat model, zinc-carnosine complex prevented ulcer formation induced by indomethacin and aspirin (16-18). Administration of zinc-carnosine was also associated with more rapid healing of ulcers induced in rat models of arthritis, diabetes mellitus, and portal hypertension (19,20). Humans treated with zinc-carnosine had reduced signs of ulcer disease and improved endoscopic scores (21). In a separate study, zinc-carnosine prevented a change in gastrointestinal permeability induced by indomethacin (16). Zinc-carnosine complex, a component of Gastricalm®, has been marketed for gastric health and ulcer prevention in dogs.
To date, there is only one report of the clinical efficacy of Gastricalm® in dogs as a protective agent against aspirin-induced gastric injury in 18 healthy mixed-breed dogs; Gastricalm® did not prevent gastric lesions in this model (22). The effect of Gastricalm® on prevention of increased mucosal permeability due to aspirin was not examined. We sought to investigate the efficacy of Gastricalm® in an *ex vivo* canine gastric mucosal model of acid-induced injury.

**Materials and Methods**

*Tissue acquisition*—Tissue samples were obtained from dogs that were previously scheduled to be euthanized at a local animal shelter. The age of the dogs was unknown, but ranged from approximately 10 months to 7 years-of-age. The dogs were typically mixed breed, ranging in size from approximately 10 kg to 30 kg. All dogs were euthanized with an overdose of sodium pentothal. Immediately following euthanasia, the entire antral section of the stomach was excised. This tissue was incised along the greater curvature and placed mucosa side down in oxygenated (95% O₂, 5% CO₂) Ringer’s solution (in Mm: 114.0 NaCl, 5.0 KCl, 1.25 CaCl₂, 1.10 MgCl₂, 25.0 NaHCO₃, 0.3 NaH₂PO₄, 1.65 Na₂HPO₄) at room temperature. After transport to the laboratory, the tissue was transferred to oxygenated Ringer’s solution at room temperature and the seromuscular layer was removed via blunt dissection. The remaining antral mucosa tissue was mounted on Ussing chambers (1.14 cm² diameter), with one tissue sample per treatment group.

*Ussing chambers*—Mucosa was bathed on both mucosal and serosal sides of the chambers with 10 ml of oxygenated Ringer’s solution maintained at 37°C by water-jacketed reservoirs.
Ten mmol/L glucose was added to the serosal bathing solution which was balanced with the addition of 10 mmol/L mannitol in the mucosal bathing solution. The spontaneous PD was measured with Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through silver-silver chloride electrodes with a voltage clamp that corrected for fluid resistance. Resistance (Ω·cm²) was calculated from the spontaneous PD and Isc. If the spontaneous PD was between $-1 \text{ mV}$ and $1 \text{ mV}$, tissues were current clamped at ± 100 µA for 5 seconds and the PD was recorded. The Isc and PD were recorded every 15-minutes for 2-hours. Data were entered into spreadsheets that calculated transepithelial electrical resistance (TER) from Isc and PD using Ohm’s law. After 30-minutes of incubation, one of four treatments was added to the mucosal surface, signified as time 0. Each dog had all three treatments applied to tissue mounted in separate chambers. Dogs in the first treatment group had neutral Ringer’s solution replaced with acid Ringer’s solution, pH 1.45-1.55. In the second treatment group, tissues remained in Ringer’s solution at a pH of 7.4, with the addition of 70mg of pulverized Gastricalm® in a stock 100mg/ml solution (7mg/ml in chamber solution). This dose approximates the canine gastric concentration in vivo based on gastric volume (using 10ml/kg for gastric volume) for a 15 kg dog when given the recommended dose of half of a 70mg tablet and extrapolated to the estimated dose for 10 milliliters of gastric juice. For the third treatment, tissues were subjected to both acid Ringer’s solution and Gastricalm® treatment. A fourth chamber was kept as a control with neutral Ringer’s solution. Tissues were maintained on the Ussing chambers with applied treatments for an additional 150-minutes. pH was checked every 30-minutes and HCl was added as needed to maintain pH between 1.45-1.55 for acid treatment groups. After 180-
minutes total (including the initial equilibration period and 150-minute treatment period), the tissues were removed.

*Histological examination*—Gastric mucosal samples were taken for each dog prior to mounting on Ussing chambers. After 180-minutes, the tissues were collected from each of the four treatment groups in 10% neutral buffered formalin. All 5 samples from each dog were sectioned at 5µm, stained with hematoxylin and eosin, and viewed with a light microscope.

*Immunofluorescence*—Tissue sections were obtained for each treatment group (neutral Ringer’s ± Gastricalm®, acid Ringer’s ± Gastricalm®) and imbedded in OCT media. Sections were thawed, fixed in cold acetone, and blocked with 10% normal goat serum. Following blocking with normal goat serum, sections were incubated with rabbit anti-active caspase-3 in 2% normal goat serum overnight at 4°C. After several washes with PBS and 0.1% Tween in PBS, sections were incubated with goat anti-rabbit FITC diluted in 2% normal goat serum for 60-minutes. Slides were washed with PBS and 0.1% Tween in PBS and hard mounted with Vectashield mounting medium with DAPI stain for DNA. Sections were examined using a Meiji fluorescent microscope and captured using Infinity Analyze software.

*Data analysis*—A 2-way repeated measures ANOVA was used to compare transepithelial resistance data among the four treatment groups (control, acid Ringer’s, Gastricalm®, and acid Ringer’s with Gastricalm®) over the time period the tissues were in the Ussing chambers. The Holm-Sidak post-hoc test was used to detect differences among treatments
and time when significance was detected during the initial ANOVA. Significance was set at 
p<0.05.

**Results**

For the first 30-minutes of incubation time prior to application of treatments, there were no significant changes in TER for any of the treatment groups. There was no significant change in TER over the entire treatment period in either control group (neutral Ringer’s, neutral Ringer’s + Gastricalm®). Treatment with acid Ringer’s produced a significant drop in the TER beginning 75-minutes after acid application in both acid Ringer’s treatment groups (Figure 3.1). There was no effect of Gastricalm® on TER with acid treatment.

*Histological examination*—As compared to baseline gastric mucosa, tissue that had been maintained in neutral Ringer’s solution for 180-minutes (control) appeared normal with no remarkable crypt destruction in studied samples (Figure 3.2). Alternatively, tissues exposed to acid Ringer’s had moderate to marked crypt destruction. These lesions were also observed to the same degree in tissue that had been treated with acid Ringer’s solution and Gastricalm®. The tissue that was treated with Gastricalm® in neutral Ringer’s solution did not appear affected and was similar to control.

*Immunofluorescence*—Neither control nor control tissues treated with Gastricalm® demonstrated evidence of FITC staining for active caspase-3, indicative of apoptosis. In acid injured tissue, active caspase-3 staining was notably increased (Figure 3.3). Similarly, there was active caspase-3 observed in the acid injured tissue co-treated with Gastricalm®,
indicating that Gastricalm® did not protect against the development of apoptosis induced by acid.

**Discussion**

In this model of canine gastric ulcer injury, Gastricalm® was not protective against mucosal damage or apoptosis induced by acid Ringer’s solution. There are several mechanisms by which zinc-carnosine have been proposed to prevent gastric ulceration. For instance, it has been shown to inhibit lipid peroxidation, decrease apoptosis, inhibit neutrophil activity, and suppress NF-κB and IL-8. In addition, Gastricalm® reduced TNF-α, induced IGF-1 and heat shock protein 72-kDa expression (15,17,19,20,23-25). However, these effects may be dependent on factors that are not applicable in Ussing chamber models, such as mucosal blood flow. In support of the current findings, Baan et al found that Gastricalm® was not protective against aspirin induced ulcers in live dogs (22).

For all dogs, sections of gastric mucosa were taken exclusively from the antral and pyloric regions of the stomach. Although this region, along with proximal duodenum, are the most likely sites for ulcer disease in dogs (4), it may be that the effects of Gastricalm® vary depending on the region of the stomach examined. Future studies examining additional regions of gastric mucosa may therefore be warranted.

In canine gastric mucosa, acid Ringer’s solution at a pH of 1.5 produced a reliable and repeatable decrease in TER. This pH is within the normal range for a fasted dog (26,27). In the live dog, there are multiple mechanisms that protect against epithelial injury in the presence of acidic pH in this range. The gastric mucosa is coated by an unstirred and a stirred
layer of mucus and bicarbonate, both of which are secreted by surface epithelial cells. Mucin units form a gel that lines the apical surface of the gastric epithelium, trapping acid within the gel as well as providing a space for bicarbonate and other factors important for protection (28). Bicarbonate, secreted by surface epithelial cells, lies within the mucus layer and serves as a buffering mechanism for any penetrating acid. Phospholipids, both within the mucus layer and lining the surface epithelium, are secreted by epithelial cells and repel acid- and water-soluble agents that could cause cell damage or necrosis. Ussing chamber-mounted tissue likely loses the most superficial most layer of mucus because of the circulation of Ringer’s within the chamber, but maintains mucus and bicarbonate adjacent to the epithelium. In addition, mounted tissue would lose the ability for changes in mucosal blood flow that allows for rapid clearance of penetrating acid as would be expected to occur in the live dog. In addition, cell mediators important for stimulation of restitution and repair, such as epidermal growth factor, transforming growth factor-α, IGF-1 and tumor necrosis factor-α, may be lacking (28). The loss of these mechanisms in mounted mucosal tissue may explain the significant decrease in resistance and histologically evident loss of normal mucosal epithelium after acid application.

Regardless of the limitations of the methodology, because acid Ringer’s induced a repeatable decline in TER and evidence of mucosal epithelial damage, this model will prove useful for further studies of acid induced injury and repair. Based on the current model, however, Gastricalm® did not prevent against acid-induced ulcer injury. Nonetheless, further studies may be helpful to examine the proposed mechanisms of Gastricalm® in the prevention of gastric injury.
Figure 0.9 TER with Gastricalm® treatment

TER decreased significantly (p<0.05) in acid treated tissue beginning at 135-minutes (105 minutes after application of acid). Control and Gastricalm® control tissues did not significantly change over the treatment period. Gastricalm® treatment with acid was not different than acid treatment without Gastricalm®.
Figure 0.10 Histology of tissue after 180 minutes of treatment

Hematoxylin and eosin stain. A) Control tissue showed no evidence of morphologic changes. B) Gastricalm® treatment with neutral Ringer’s did not result in any change in morphology compared to control tissues. C) Acid injured tissue exhibited moderate to severe disruption of normal epithelium. D) Acid injury with Gastricalm® treatment produced a similar level of injury as compared to acid injured tissue alone. Bar = 100mm.
Evaluation of active caspase-3 using a FITC labeled anti-active caspase-3 antibody. DAPI stain shows nuclear material within epithelial cells for reference. Both control and control Gastricalm® groups show little to no staining for active caspase-3, indicating low levels of apoptosis. Acid injured tissue, both with and without Gastricalm® treatment, showed moderate apoptosis of gastric epithelium.
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CHAPTER 4 ACID-INDUCED BARRIER DYSFUNCTION IN CANINE GASTRIC MUCOSA
Introduction

Over the last 40 years, gastroduodenal ulceration and gastrointestinal perforation have been increasingly recognized in dogs (1-5). This may be due in part to improved diagnostic capabilities but may also be related to increased non-steroidal anti-inflammatory drug (NSAID) use for management of acute and chronic pain. NSAIDs are amongst the most implicated causes of gastric ulceration and can be seen with both acute and chronic NSAID therapy (4,5). Multiple other causes of gastric injury and ulceration have been reported in dogs, including liver disease, sepsis/systemic inflammatory response syndrome, renal disease, and cancer (1-5).

Most existing studies of gastric ulcer injury in the dog focus on the role of NSAIDs in ulceration. In these models, NSAIDs are administered and gastric lesions are traditionally scored via endoscopy or lesions are induced with endoscopic biopsy forceps and healing time is noted (10-12). Additional studies have examined prostanoid concentration in gastroduodenal mucosa after NSAID administration (13-15). Some in vivo models have examined ulcer treatment using a non-selective NSAID, aspirin, to induce ulcers (16-18).

There are few reports of gastric injury models in dogs where the injury is not induced by or in association with NSAID therapy. This deficiency leads to a lack of understanding about the injury mediated by NSAIDs on previously injured tissue. Additionally, the lack of other injury models precludes the study of ulcer therapies that are not specifically directed at treatment of NSAID-induced ulceration.
We sought to develop an *ex vivo* model of acid injury that would as closely as possible mimic natural peptic ulcer injury. In this model, hydrochloric acid was applied to the mucosal side of canine gastric tissue mounted on Ussing chambers. Gastric injury and recovery were evaluated using two measurements of gastric barrier function and with histologic examination. Prostanoid production and COX-1 and -2 levels were quantified. After optimizing the model, we developed a reliable and reproducible acid-induced injury model that produced a consistent increase in gastric permeability with subsequent recovery. Acid-injured tissue produced higher levels of gastroprotective prostanoids and increased levels of mucus. This model of *ex vivo* acid injury is useful for further examination of the effects of NSAIDs on pre-existing gastric ulceration and mucosal injury as well as the study of gastroprotective drugs used in treatment of mucosal injury.

**Materials and Methods**

*Tissue acquisition*—Tissue samples were obtained from dogs that were euthanized at a local animal shelter. The precise age of the dogs was unknown in most cases, but ranged from approximately 8 months to 10 years-of-age. The dogs were typically mixed breed, ranging in size from approximately 10 kg to 30 kg. All dogs were euthanized with an overdose of sodium pentothal. Immediately following euthanasia, the entire antral section of the stomach was excised. This tissue was incised along the greater curvature and placed mucosa side down in oxygenated (95% O₂, 5% CO₂) Ringer’s solution (in Mm: 114.0 NaCl, 5.0 KCl, 1.25 CaCl₂, 1.10 MgCl₂, 25.0 NaHCO₃, 0.3 NaH₂PO₄, 1.65 Na₂HPO₄) at room temperature. After transport to the laboratory, the tissue was transferred to oxygenated Ringer’s solution at room
temperature and the seromuscular layer was removed via blunt dissection. The remaining
antral mucosa tissue was mounted on Ussing chambers (1.14 cm$^2$ diameter), with one tissue
sample per treatment group.

_Ussing chambers_—Mucosa was bathed on both mucosal and serosal sides of the chambers
with 10 ml of oxygenated Ringer’s solution maintained at 37ºC by water-jacketed reservoirs.
Ten mmol/L glucose was added to the serosal bathing solution which was balanced with the
addition of 10 mmol/L mannitol in the mucosal bathing solution. After 30-minutes of
incubation, treatments were applied to each dog’s tissue, mounted in separate chambers.

**Stage 1: Model development** Ringer’s solution was applied to the mucosal side of the gastric
mucosa that had been titrated to one of three pH levels: 1.1, 1.2, 1.3 for either 30- or 45-
minutes, giving a total of 6 possible models of acid injury. Each of these treatments was
applied to the gastric mucosa of 4 dogs.

**Stage 2: Hydrochloric acid injury model** After selection of the optimal injury model (pH 1.2
for 45 minutes) based on electrical resistance changes, tissue from an additional 25 dogs was
used in studies to further evaluate this model. Tissue from each dog was mounted on Ussing
chambers as described and injured with acid. Additional tissue from each dog was mounted
without acid injury as control. Outcome measures were transepithelial resistance, $^3$H-
mannitol flux, prostanoid concentrations (thromboxane B$_2$, TXB$_2$, stable metabolite of
thromboxane A$_2$, and prostaglandin E$_2$, PGE$_2$), cyclooxygenase expression and histological
evaluation. This treatment was applied to 38 dogs.

**Stage 3: Sulfuric acid injury** As part of the evaluation of the hydrochloric acid injury model
we tested barrier function in response to injury with sulfuric acid (H$_2$S). Because TER largely
measures flux of sodium and chloride ions and because hydrochloric acid was used to titrate Ringer’s solution to pH 1.2, evidence was sought to confirm that changes in TER were due to changes in barrier function and not reflective of movement of chloride ions across a concentration gradient (mucosal to serosal). Ringer’s solution was titrated to pH 1.2 with sulfuric acid and applied to the mucosa after at 30-minute equilibration for 45 minutes. Acid Ringer’s was then replaced with neutral Ringer’s. This treatment was applied to 5 dogs. Transepithelial resistance and $^3$H-mannitol flux were measured as described.

*Transepithelial resistance* – The spontaneous PD (potential difference) was measured with Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through silver-silver chloride electrodes with a voltage clamp that corrected for fluid resistance. Resistance ($\Omega \cdot \text{cm}^2$) was calculated from the spontaneous PD and Isc (short circuit current). If the spontaneous PD was between –1 mV and 1 mV, tissues were current clamped at $\pm 100$ $\mu$A for 5 seconds and the PD was recorded. The Isc and PD were recorded every 15-minutes for 210-minutes. Data were entered into spreadsheets that calculated transepithelial electrical resistance (TER) from Isc and PD using Ohm’s law.

$^3$H-mannitol flux—As a second indicator of gastric permeability, flux of $^3$H labeled mannitol across the mucosa was measured. 200 uM of $^3$H radiolabeled mannitol was added to the mucosal reservoir. Samples were taken of both serosal and mucosal reservoirs after 3 minutes to establish baseline radioactivity. Two one-hour mucosal to serosal fluxes were performed by sampling serosal bathing solutions at one hour and two hours after addition of radiolabeled mannitol.
Prostanoid quantification—Samples of the serosal bathing solutions were collected at 30 and 210 minutes of tissue incubation after which they were snap frozen in liquid nitrogen and stored at -80°C until analysis. The amount of thromboxane B₂ (the stable metabolite of TXA₂) and prostaglandin E₂ were measured using commercially available ELISA kits.

Cyclooxygenase expression—Western blot analyses for COX-1 and -2 were completed using gastric mucosal tissue obtained at 0 and after 210 minutes followed by semi-quantification using densitometry. β-actin expression was used as a loading control.

Histological examination—Gastric mucosal samples were taken for each dog prior to mounting on Ussing chambers. After 210-minutes, the tissues were collected from each treatment group in Carnoy’s fixative for 24 hours and transferred to 70% ethanol. Samples were sectioned at 5µm, stained with hematoxylin and eosin, and viewed with a light microscope. Additional sections from each treatment was stained with Alcian blue and PAS stain to stain the gastric mucus layer and viewed with a light microscope.

Data analysis—A 2-way repeated measures ANOVA was used to compare transepithelial resistance data. A Kruskal-Wallis ANOVA on ranks was used to analyze prostanoid, western blot, and flux data. The Tukey post-hoc test was used to detect differences among treatments and time when significance was detected during the initial ANOVA. Significance was set at p<0.05.

Results

For model development in stage 1, Ringer’s solution was applied to the mucosal side of the gastric mucosa that had been titrated to one of three pH levels: 1.1, 1.2, 1.3 for either 30- or
45-minutes, giving a total of 6 possible models of acid injury. All treatments induced a significant decrease in TER during the period of injury (Figures 4.1 and 4.2). Once acid Ringer’s was replaced with neutral Ringer’s, TER partially recovered in all treatment groups. 45-minutes of injury was selected based on the apparent more gradual recovery of TER. pH 1.2 was selected because it produced reliable change in barrier function in all dogs without causing electrical overload, as was the case in 1 dog with pH 1.1 for 45 minutes. The acid injury model used for the remainder of the experiments, therefore, was acid Ringer’s pH 1.2 applied for 45-minutes.

Stage 2 was then performed using this selected injury model with 38 dogs. This acid injury produced a reliable decrease in barrier function, measured both by TER and by flux of radiolabeled mannitol. After 45-minutes of acid injury, TER in acid-injured tissue was 34.2±2.4% of control TER. Following 45-minute acid injury period, barrier function partially recovered but remained significantly decreased compared to control; TER at 210-minutes was 71.7±4.6% of control at 210-minutes (Figure 4.3, p<0.001). Flux of radiolabeled mannitol in injured tissue was 183.4±38.4% that of control (Figure 4.4, p=0.006).

In stage 3, 5 dogs were treated with acid Ringer’s solution that was titrated to pH 1.2 with sulfuric acid (Figure 4.5). This injury produced a significant decrease in barrier function slightly greater than with hydrochloric acid injury, without significant recovery (sulfuric acid injured tissue at 210-minutes: 41.1±4.12% of control, p<0.001).

Tissue response to acid injury was then examined by quantifying the concentration of two gastroprotective prostanoids, prostaglandin E₂ (PGE₂) and thromboxane B₂ (TXB₂) (Figure 4.6). Both of these were significantly elevated in acid-injured tissue compared to
control. PGE$_2$ concentration in acid-injured tissue was 1882.79±776.9% of control (p<0.001); TXB$_2$ concentration of injured tissue was 805.4±211.4% of control (p<0.001). As evidenced by standard errors, there was a moderate degree of inter-dog variability in concentrations of both prostanoids with acid injury.

To determine whether increased prostanoid concentrations were a result of increased COX expression or activity, COX-1 and COX-2 were semi-quantified (Figure 4.7). There was no significant difference in COX-1 or -2 expression in control versus acid-injured tissues. There was also no difference in COX expression from baseline (before tissues were mounted) and control (at 210-minutes).

Grossly, gastric mucosa appeared to have a thickened mucus layer in acid injured tissue as compared to control tissue (Figure 4.8). Hematoxylin and eosin stained tissues showed little effect of Ussing chamber mounting on tissue morphology (baseline versus control, Figure 4.9). Acid-injured tissue had moderate to marked sloughing of gastric epithelial cells. Periodic acid-Schiff (PAS)/Alcian blue stains were performed to isolate the gastric mucus layer (Figure 4.10). Uninjured control tissue demonstrated a thin layer of gastric mucus. Acid-injured tissue had a thicker mucus layer; trapped within the mucus were sloughed gastric epithelial cells.

Discussion

This study describes the development of a canine ex vivo model of gastric acid injury using Ringer’s solution titrated to pH 1.2 with hydrochloric acid. Hydrochloric acid was used to replicate as much as possible the pathophysiology of acid injury to gastric antral mucosa in a
Because only the mucosal aspect of the chamber was treated with hydrochloric acid, this brought an imbalance of chloride ions across the tissue. Transepithelial resistance largely measures movement of sodium and chloride ions. To confirm that the change in barrier function was due to a true change in permeability and not simply movement of chloride ions down a concentration gradient, acid injury was induced with sulfuric acid. This acid, also titrated to pH 1.2, produced a similar degree of barrier dysfunction than hydrochloric acid, validating the model. Using two independent measurements of barrier function, TER and $^3$H-mannitol flux, we were able to demonstrate an immediate and persistent change in barrier function due to hydrochloric acid injury.

A pH of 1.2 is within a normal range for canine gastric mucosa (19). It may be unexpected that pH 1.2 would lead to altered gastric barrier function when the normal canine gastric mucosa is exposed to this pH normally without undue effects. The Ussing chamber model has the advantage that it isolates a mucosal epithelium and allows for study of mucosal and serosal aspects independently, it does isolate tissue from its blood supply, removing an important gastroprotective mechanism and allowing for tissue to be more easily injured than in a live dog. In addition, the mucus layer may be disturbed by mounting on the Ussing chamber. The authors feel, however, than the studied acid injury model is still relevant and similar to acid injury effects in vivo.

Several different acid injury models were initially attempted. There were no significant differences among any of the injuries applied. A 45-minute injury was selected because of the longer time of injury to allow for later study of factors affecting degree of barrier dysfunction during injury. When individual dog data was examined (data not shown),
it appeared that pH 1.2 was most reliable in inducing significant injury without causing electrical overload. While a different pH-time combination may also serve as a useful acid injury model, the authors feel that the selected model serves as a relevant tool to study acid injury and recovery.

Prostanoids are considered integral to recovery from many different types of mucosal injury. Gastroprotective prostanoids increase bicarbonate and mucus secretion, increase gastric mucosal blood flow, decrease acid secretion, and modulate gastric motility. Grossly, it was observed that the injured tissue had a thicker mucus layer which might be a result of increased prostanoid production. PAS/Alcian blue stains of acid-injured tissue showed a thick mucus layer superficial to the gastric epithelium. Increased prostanoid concentrations may have contributed to this increased mucus layer leading to partial recovery of barrier function. Other gastroprotective mechanisms induced by prostanoids (bicarbonate secretion, increased gastric mucosal blood flow, decreased acid production) were not examined in this study but could be examined using this model in future work. Additional mechanisms of gastroprotection and repair, such as heat shock proteins, could be also examined using this acid injury model.

This work describes the effect of acid injury on ex vivo canine gastric antral mucosa and demonstrates the utility of this model for further study of gastric injury and recovery. Control tissues maintained relatively stable barrier function and histologically appeared undamaged, demonstrating that canine tissue is viable on Ussing chambers for at least 210-minutes. Additionally, acid produced a significant and partially reversible change in barrier function. Gastric mucosa responded to this injury, at least in part, by dramatically increasing
production of gastroprotective prostanoids. There are several potential avenues for further investigation using this model, including treatment with cyclooxygenase inhibitors, various gastroprotective agents, and any other drug that may have an effect on preventing or worsening gastric acid injury.
Figure 0.12 Acid injury titration-time experiment, 30-minute injury

Transepithelial electrical resistance (TER) was measured as an index of gastric barrier function. pH 1.1, 1.2, and 1.3 were applied to the mucosal side of the tissue for 30 minutes. At all pH values, acid injury caused a significant decrease in TER at 60-minutes (p<0.05). TER of tissue treated with pH 1.1 for 30 minutes was also significantly lower than control. After replacement with neutral Ringer’s solution, TER returned to control values within 15-30 minutes. Values represent means ± SE. n=4.
Transepithelial electrical resistance (TER) was measured as an index of gastric barrier function. pH 1.1, 1.2, and 1.3 were applied to the mucosal side of the tissue for 45 minutes. TER of tissue treated with pH 1.1, 1.2, and 1.3 were all significantly lower than control at 45 minutes. After replacement with neutral Ringer’s solution, TER returned to control values within 15-30 minutes. Acid injury of pH 1.2 for 45 minutes was selected for further investigation. Values represent means ± SE. n=4.
Figure 0.14 Acid injury model

For selected injury, acid Ringer’s solution pH 1.2 was applied to mucosal side for 45 minutes then replace with neutral Ringer’s solution. TER is significantly lower in acid injured tissue during injury and recovery periods (p<0.001, n=38).
Flux of $^3$H labeled mannitol with acid injury is increased, indicating a change in paracellular permeability ($p=0.006, n=29$).
Figure 0.16 TER with sulfuric acid injury

Acid injury with alternate acid (sulfuric acid) to validate model of injury. TER with sulfuric acid injured tissue is significantly lower than control at all time points upon injury (p<0.001) and lower than hydrochloric acid injured tissue from 150-minutes onward (p<0.05). n=5.
Figure 0.17 Effect of acid injury on prostanoid concentration

Acid injury induces a significant increase in both prostaglandin E₂ and thromboxane B₂ (the stable metabolite of thromboxane A2)\((p < 0.001)\). Prostanoid concentration is expressed as change in prostanoid from baseline (time 210-time 30). \(n=29\).
Figure 0.18 COX expression with acid injury

COX-1 and COX-2 are expressed in uninjured and injured tissue. There is no change in expression of either enzyme after Ussing chamber mounting (control versus baseline) or with acid injury (control versus acid injury). n=6.
Figure 0.19 Acid injury induces mucus grossly

Grossly, acid injured tissue (right) had a subjectively thicker mucus layer than control tissue (left).
Control tissue was not significantly altered by placement on Ussing chambers as it appeared similar to tissue processed at the time of euthanasia. Acid injured tissue showed moderate to marked sloughing of gastric epithelial cells superficially.
Uninjured control tissue had a thin layer of mucus. Acid-injured tissue had a thick layer of mucus with trapped sloughed epithelial cells within the mucus.

Figure 0.21 PAS/Alcian blue stain with acid injury
References


CHAPTER 5 TRAMADOL DOES NOT DECREASE RECOVERY OF GASTRIC BARRIER FUNCTION
**Introduction**

As recognition of acute and chronic pain in dogs has increased, so too has the desire to optimize pain therapy. This is frequently achieved by use of non-steroidal anti-inflammatory drugs (NSAIDs) but in some cases multimodal therapy with other centrally acting analgesics, such as tramadol, is prescribed. NSAIDs are a common cause of ulceration in people and canines. In the US, gastric ulceration in the people results in up to 300,000 hospitalizations annually with a mortality of approximately 4% and estimated annual cost of $3.3 billion (1,2). In dogs, mortality with ulceration can be as high as 70% (3-5). Ulceration induced by NSAIDs is primarily due to inhibition of gastro protective prostanoids elaborated by the COX enzymes. There are two principal COX isoforms: COX-1, which is constitutively expressed in most tissues including the gastrointestinal tract and COX-2, which is typically induced at sites of injury and inflammation. Selective COX-2 inhibitors hypothesized to decrease the risk of gastric ulceration by targeting the COX-2 isoform, leaving constitutively expressed COX-1 functional to ensure adequate production of baseline levels of protective prostanoids in the gastric mucosa. However, gastric ulcers have been noted in patients treated with selective COX-2 inhibitors in both people and dogs, albeit at a lower prevalence than non-selective COX inhibitors (6,7). Additionally, co-therapy with NSAIDs and tramadol increases risk of peptic ulcer perforation in people over NSAIDs alone (8). There are anecdotal reports that dogs concurrently receiving an NSAID and tramadol have a higher prevalence of gastric and duodenal perforations as compared with dogs treated with NSAIDs alone (9). In rats, the combination of rofecoxib, a COX-2 selective inhibitor, and tramadol
produced at least twice as many gastric ulcers than either drug administered separately (10).
The mechanisms for this interaction are unknown.

Tramadol has over 30 metabolites in the dog (11, 12). The possible gastrointestinal side effects of tramadol and its metabolites are poorly described. Using an ex vivo model of acid injury, we sought to investigate the effect of the parent compound tramadol on gastric barrier function as well as its potential interaction with a non-selective COX inhibitor indomethacin. We hypothesized tramadol would have an additive or synergistic effect with a non-selective COX-inhibitor indomethacin in decreasing recovery of barrier function after injury.

Materials and methods

Tissue collection—Tissue samples were obtained from 15 dogs that were previously scheduled for euthanasia by shelter veterinarians. The investigators had no influence on dog selection and there was no incentive on the part of investigators for euthanasia. Dogs were included if they are approximately 10 months to 7 years of age, 10-35 kg, and appear normal on physical examination. Dogs were euthanized according to AVMA approved guidelines selected by shelter veterinarians. Immediately following euthanasia, a midline celiotomy was performed and the stomach was exteriorized. The gastric antrum was excised along the greater curvature from the pyloric sphincter to the incisura angularis and placed mucosal side down into oxygenated (95% O₂, 5% CO₂) Ringer’s solution at room temperature. Approximately 20-30 minutes later, the tissue was transferred to dissection pans in the laboratory and bathed in fresh oxygenated Ringer’s solution.
**Ussing chamber**—The antral mucosa was dissected from the seromuscular layer and mounted in Ussing chambers (1.14 cm² surface area). One mucosal sample was used from each dog for each treatment. Canine Ringer’s solution contained (in mM) 112.0 NaCl, 4.0 KCl, 2.4 CaCl₂, 0.8 MgCl₂, 25.0 NaHCO₃, 0.23 NaH₂PO₄, and 1.58 Na₂HPO₄. Ten mmol/L glucose was added to the serosal bathing solution to maintain viability of the tissue and balanced with 10 mmol/L mannitol in the mucosal bathing solution. Tissue was maintained at 37°C in chambers bathed with oxygenated Ringer’s in water-jacketed reservoirs. After a 30-minute equilibration period, tissue was injured by application of Ringer’s solution titrated to a pH of 1.2 with HCl to the mucosal side of the tissue for 45 minutes. The spontaneous potential difference (PD) was measured with Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through silver-silver chloride electrodes with a voltage clamp that corrected for fluid resistance to calculate short-circuit current (Isc). If the spontaneous PD was between –1mV and 1mV, tissues were current clamped at ± 100µA for 5 seconds and the PD was recorded. The Isc and PD were recorded every 15 minutes for 210 minutes. Data was entered into spreadsheets that calculated transepithelial electrical resistance (TER) from Isc and PD using Ohm’s law. One chamber was maintained with neutral pH Ringer’s solution as control. After 45 minutes of acid injury, acidified Ringer’s was replaced with neutral Ringer’s solution. Immediately following acid injury, drug treatments were applied. Drug treatments included: indomethacin 10-5 M, tramadol 10-5 M, and indomethacin + tramadol at 10-5 M. Drugs were applied to mucosal and serosal bathing reservoirs to mimic both topical and systemic effects of each drug. Doses were selected that were two- to ten-fold higher than normal maximum therapeutic serum concentrations for
each drug to overcome implicit barriers to drug penetration in Ussing chamber models (13,14). Controls included uninjured tissue with no drug treatment and acid injured tissue without drug treatment.

Prostanoid levels—Samples of the serosal bathing solutions were collected at 30 and 210 minutes of tissue incubation after which they were snap frozen in liquid nitrogen and stored at -80°C until analysis. The amount of thromboxane B$_2$ (TXB$_2$, the stable metabolite of TXA$_2$) and prostaglandin E$_2$ (PGE$_2$) were measured using commercially available ELISA kits.

Western blot—Western blot analyses for COX-1 and -2 were completed using gastric mucosal tissue obtained at 0 and after 210 minutes followed by semi-quantization using densitometry. β-actin expression was used as a loading control.

Histological examination—Gastric mucosal samples were taken for each dog prior to mounting on Ussing chambers. After 180-minutes, the tissues were collected from each of the five treatment groups in 10% neutral buffered formalin. All 6 samples (baseline plus 5 treatment groups) from each dog were sectioned at 5µm, stained with hematoxylin and eosin, and viewed with a light microscope.

Data analysis/interpretation—A 2-way repeated measures ANOVA was used to compare transepithelial resistance data among the five treatment groups (control, acid Ringer’s, tramadol, indomethacin, and tramadol + indomethacin) over the time period the tissues were in the Ussing chambers. A Kruskal-Wallis one-way ANOVA on ranks was used to analyze western blot results and prostanoid concentrations. The Tukey post-hoc test was used to
detect differences among treatments and time when significance was detected during the initial ANOVA. Significance was set at p<0.05.

Results

Acid injury induced a significant and partially reversible decrease in barrier function (Figure 5.1). At 210-minutes, TER of acid injured tissue was 83.9±9.7% of control tissue. There was no significant effect of any drug application (indomethacin, tramadol, or both) administered with acid injury on TER as compared to acid injured tissue without drug application. Flux of $^3$H-mannitol after acid injury, with or without drug administration, was not significantly different than control (Figure 5.1).

There was a significant effect of treatment on the change in concentrations of both PGE2 and TXB2 (PGE2: p<0.001, TXB2: p<0.001). Acid injury increased synthesis of PGE$_2$ (absolute change; control PGE$_2$: 65.7±26.8pg/ml, acid injured PGE$_2$: 509.3±158.3pg/ml, Figure 5.2); this increase was attenuated when acid-injured tissue was treated with indomethacin or indomethacin + tramadol (absolute change, indomethacin + acid injury PGE$_2$: 182.9±93.8pg/ml, indomethacin + tramadol + acid injury PGE$_2$: 99.7±31.6pg/ml). There was no significant effect of tramadol on PGE$_2$ concentration.

TXB$_2$ also increased with acid injury (absolute change; control TXB$_2$: 7.1±7.8pg/ml, acid injured TXB$_2$: 233.2±90.7pg/ml, Figure 5.2). Similar to PGE$_2$, this increase was also attenuated with indomethacin was administered with acid injury, with or without tramadol co-administration (absolute change, indomethacin + acid injury TXB$_2$: 37.9±16.8pg/ml,
indomethacin + tramadol + acid injury TXB$_2$: 47.5±12.7pg/ml). Likewise, there was no individual or additive effect of tramadol on TXB$_2$ concentration.

COX-1 and COX-2 were both present at baseline in canine gastric mucosa (Figure 5.3). There was no effect of the 210-minute ex vivo experiment on either COX-1 or -2 expression (tissue levels at time of collection were not different than at the end of the experiment). Acid injury, with or without drug administration, did not alter expression of either COX-1 or COX-2 enzyme.

Acid injury induces diffuse moderate to marked sloughing of luminal gastric epithelial cells (Figure 5.4). There is no apparent effect of either indomethacin or tramadol on tissue morphology with acid injury.

**Discussion**

It was surprising that neither indomethacin nor tramadol had a significant effect on barrier function. Dose response studies in 5 dogs prior to this work did not show a significant effect of treatment at this and two lower doses ($10^{-6}$ and $10^{-7}$M for indomethacin, $10^{-7}$ and $10^{-8}$M for tramadol, data not shown) though the highest dose of indomethacin did trend to decrease TER recovery in this earlier pilot study. These dose ranges for indomethacin and tramadol are two- to ten-fold higher than reported maximum serum concentrations for these drugs (13, 15). It is possible, however, than a super-therapeutic dose might have elucidated an effect of drug on barrier recovery. Additionally, only the parent tramadol compound was applied to the tissue. In the dog, there are over 20 metabolites of tramadol with unknown activity (16). It is possible that while the parent compound alone does not affect gastric barrier function or
prostanoid synthesis, one of its metabolites might. The metabolites were not also examined due to lack of commercial availability and cost.

Another unexpected finding was the apparent lack of effect on barrier function of indomethacin, especially considering that indomethacin treatment significantly inhibited the increase in PGE$_2$ and TXB$_2$ induced by acid-injury. Previous work by these investigators investigating indomethacin in _ex vivo_ models in other species have consistently shown a detrimental effect of this COX inhibitor on barrier function at 10- to 100-fold lower concentrations than used in the current study (17,18). It is possible that canine gastric mucosa is more resistant to adverse effects of COX-inhibition than other species previously examined (porcine, murine, equine). It also suggests a non-COX dependent mechanism for recovery after acid injury in this canine model.

COX-expression itself was unchanged by any treatment, including mounting of the tissue on Ussing chambers for 210-minutes. It is possible that 210-minutes is not enough time to appreciate a significant change in COX expression. Interestingly, both COX-1 and COX-2 were present at baseline and expression was not significantly different between the two isozymes. This finding is in accordance with previous canine studies demonstrating the presence of both form of the enzyme (19). The paradigm of COX-1 as “constitutive” and COX-2 as “inducible” seems to be fading as evidence mounts that there is a overlap between the two and both are important in times of health and injury.

There is no evidence, using this canine _ex vivo_ model of acid injury, that tramadol alone has a detrimental effect to gastric barrier function. Likewise, there was no apparent barrier dysfunction when tramadol and indomethacin were co-administered. Concentrations
of both prostanoids measured, PGE$_2$ and TXB$_2$, were unchanged in acid-injured, tramadol-treated tissue versus acid injury control. Similarly, COX-1 and -2 expression was unaffected by tramadol administration. This implies that tramadol has no COX-inhibiting activity ex vivo and no effect on transcription or translation of the COX enzymes. Additionally, tramadol apparently did not induce a tissue reaction that increased production of gastroprotective prostanoids. The parent compound, tramadol, does not adversely affect gastric barrier function ex vivo in the dog, either alone or in conjunction with a non-selective COX inhibitor indomethacin.
Acid injury induces a significant decrease in TER followed by partial recovery. There was no overall significant effect of indomethacin, tramadol or the combination of the two on recovery of TER after acid injury. Likewise, there were no significant differences in $^3$H-mannitol flux between treatments.

**Figure 0.22 TER with acid injury with drug administration**

Acid injury induces a significant decrease in TER followed by partial recovery. There was no overall significant effect of indomethacin, tramadol or the combination of the two on recovery of TER after acid injury. Likewise, there were no significant differences in $^3$H-mannitol flux between treatments.
There is a significant effect of treatment on both PGE$_2$ and TXB$_2$ concentrations (p<0.001). Acid injury induces a significant increase in PGE$_2$ and TXB$_2$. Indomethacin attenuates this increase when administered alone or concurrently with tramadol. Tramadol had no effect on PGE$_2$ or TXB$_2$ concentrations induced by acid injury when administered alone or concurrently with indomethacin.

**Figure 0.23 Prostanoid concentrations with drug administration**

There is a significant effect of treatment on both PGE$_2$ and TXB$_2$ concentrations (p<0.001). Acid injury induces a significant increase in PGE$_2$ and TXB$_2$. Indomethacin attenuates this increase when administered alone or concurrently with tramadol. Tramadol had no effect on PGE$_2$ or TXB$_2$ concentrations induced by acid injury when administered alone or concurrently with indomethacin.
Figure 0.24 Effect of drug administration on COX expression

There was no change in COX-1 and -2 expression from baseline to the end of the experimental period (baseline vs. control). There was also no significant effect of any treatment (acid injury, indomethacin, tramadol) on COX-1 or -2 expression. Both isozymes expressed at baseline (before injury or mounting on the chambers).
Figure 0.25 Hematoxylin and eosin stain, indomethacin and tramadol

Control tissue morphology remains unchanged compared to baseline (before mounting). Acid injury induces moderate to marked gastric epithelial sloughing. These changes are not significantly affected by co-administration with either indomethacin or tramadol.
References


CHAPTER 6 THE EFFECT OF PROSTANOIDS ON RECOVERY AFTER ACID INJURY IN GASTRIC MUCOSA
Introduction

Gastric ulceration in the US human population results in up to 300,000 hospitalizations annually with a mortality of approximately 4%. The estimated annual cost of this disease syndrome is $3.3 billion (1,2). Non-steroidal anti-inflammatory drugs (NSAIDs) are amongst the most implicated causes of gastric ulceration in humans and dogs (3,4). There is strong evidence that NSAID-induced ulceration is primarily due to inhibition of gastroprotective prostanoids elaborated by the cyclooxygenase (COX) enzymes (5-8). Prostanoids (prostaglandin E2, thromboxane A2, and prostacyclin) have multiple gastroprotective properties in the gut, including increasing bicarbonate and mucus secretion, regulating gastric blood flow, increasing epithelial cell turnover, and restitution of damaged epithelium. COX-1 is constitutively expressed and is presumed to be responsible for the physiological functions attributed to prostaglandins, including gastroprotection, in healthy states. COX-2 is considered to be an inducible enzyme that is expressed in injured tissues, including injured gastric mucosa. Its expression and activity has been linked to pain and inflammation (5,7,9-11). Selective COX-2 inhibitors were developed to minimize adverse gastrointestinal events by targeting the pro-inflammatory COX-2 isoform without inhibiting COX-1. In theory, this would allow for continued production of baseline levels of protective prostanoids in the gastric mucosa. Although the prevalence of gastric lesions is lower with more COX-2 selective NSAIDs in humans and dogs, gastric lesions have been reported in clinical studies and experimental models that have evaluated selective COX-2 inhibitors (12,13). Selective COX-2 inhibitors do, however, produce less severe gastric lesions.
compared to treatment with non-selective inhibitors (15). COX-2 has been detected in both normal pyloric tissue and along the margins of gastric ulcers. Additionally, inhibition of COX-2 decreases gastric mucosal lesion healing (16-18). In COX-1 deficient mice, ulcer healing is undisturbed unless mice are also treated with COX-2 inhibitors, implying a role for COX-2 in ulcer repair (19). These findings may explain why gastric ulceration can still be seen with selective COX-2 inhibitors.

Dogs may serve as a useful model for gastric ulcer injury due to similarities in physiology. Several NSAIDs with varying COX-2 selectivity have been approved for use in dogs with similar selectivity profiles as NSAIDs in humans. Overall, studies in dogs suggest that COX-2 selective inhibitors result in decreased gastric lesion formation as compared to a non-selective COX-inhibitor, aspirin (20). The findings of these studies are difficult to reconcile with observations that selective COX-2 inhibitors can induce gastric ulcers in clinical patients. However, the experimental models have been largely performed on healthy laboratory dogs with no gastric injury, limiting their clinical relevance (13, 22). There is one in vivo study using gastric mucosal injury to study repair in the presence of COX-2 selective NSAIDs. In this study, endoscopic biopsy-induced lesions healed more slowly with firocoxib, a selective COX-2 inhibitor, compared to placebo or the COX-1 selective drug tepoxalin (12).

Because COX-1 and COX-2 both appear to play an important role in mucosal repair, it is important to examine the effect of selective COX inhibitors in additional models of gastric injury. The use of Ussing chambers to study barrier function of canine gastric mucosa has been established by previous investigators. In a canine ex vivo model of injury using
Ussing chambers, aspirin, in the presence of acid, significantly reduced transepithelial resistance (23). Using a similar *ex vivo* model that we have independently established, we will examine the effect of three COX-inhibitors with different selectivity on recovery after acid injury. The first, indomethacin, is a non-selective COX-inhibitor. The second, NS-398, is a COX-2 selective inhibitor while the third, SC-560, is a COX-1 selective inhibitor.

To further delineate the role of prostanoids in recovery of barrier function after injury in the dog, we also investigated whether the administration of exogenous prostanoids could accelerate recovery from acid injury. Traditionally, exogenous prostanoid therapy is only administered to patients with NSAID mediated injury but not with other forms of gastric injury, such as acid-mediated injury. Misoprostol is a synthetic prostanoid of the PGE series. Similar to the endogenous product, misoprostol suppresses acid secretion and is cytoprotective by increasing mucus and bicarbonate secretion. Ulceration due to NSAIDs, both COX-2 selective and non-selective, can be minimized with co-administration of misoprostol (9, 15). There is no information on whether or not misoprostol enhances recovery following acid-induced ulceration. We expect that misoprostol will accelerate return of barrier function after acid injury using our *ex vivo* model of injury using canine gastric mucosa, implying that it has a clinically relevant role in treatment of gastric injury that is not NSAID-mediated.

**Materials and Methods**

*Tissue acquisition*—Tissue samples were obtained from dogs that were euthanized at a local animal shelter. The precise age of the dogs was unknown in most cases, but ranged from
approximately 8 months to 10 years-of-age. The dogs were typically mixed breed, ranging in size from approximately 10 kg to 30 kg. All dogs were euthanized with an overdose of sodium pentothal. Immediately following euthanasia, the entire antral section of the stomach was excised. This tissue was incised along the greater curvature and placed mucosa side down in oxygenated (95% O₂, 5% CO₂) Ringer’s solution (in Mm: 114.0 NaCl, 5.0 KCl, 1.25 CaCl₂, 1.10 MgCl₂, 25.0 NaHCO₃, 0.3 NaH₂PO₄, 1.65 Na₂HPO₄) at room temperature. After transport to the laboratory, the tissue was transferred to oxygenated Ringer’s solution at room temperature and the seromuscular layer was removed via blunt dissection. The remaining antral mucosa tissue was mounted on Ussing chambers (1.14 cm² diameter), with one tissue sample per treatment group.

Ussing chambers—Mucosa was bathed on both mucosal and serosal sides of the chambers with 10 ml of oxygenated Ringer’s solution maintained at 37ºC by water-jacketed reservoirs. Ten mmol/L glucose was added to the serosal bathing solution which was balanced with the addition of 10 mmol/L mannitol in the mucosal bathing solution. After 30-minutes of incubation, treatments were applied to each dog’s tissue, mounted in separate chambers.

Stage 1: COX inhibition After the initial equilibration period, Ringer’s solution was applied to the mucosal side of the gastric mucosa that had been titrated to pH 1.2 with hydrochloric acid. The acid Ringer’s solution circulated through the reservoirs for 45-minutes after which it was removed and replaced with neutral Ringer’s solution. Immediately after the acid injury period, selected COX inhibitors were added to give a final concentration of 10⁻⁵ M in both serosal and mucosal bathing reservoirs. Indomethacin was used as a non-selective COX inhibitor. NS-398 was used as a COX-2 selective inhibitor. SC-560 was used as a COX-1
selective inhibitor. Indomethacin was applied to the gastric mucosa of 27 dogs; 13 dogs each were treated with NS-398 and SC-560. Additional tissues were maintained in neutral Ringer’s as uninjured control and acid injury control without drug treatment. Outcome measures were transepithelial resistance, \(^3\)H-mannitol flux, and thromboxane B\(_2\) (stable metabolite of thromboxane A\(_2\)) and prostaglandin E\(_2\) concentrations (TXB\(_2\), PGE\(_2\)).

**Stage 2: Prostaglandin administration** Tissue from an additional 15 dogs was treated with misoprostol to determine the protective effect of this synthetic prostaglandin E\(_1\) analog. Tissue from each dog was mounted on Ussing chambers as previously described for equilibration and acid injury. At the same time that acidified Ringer’s was applied, misoprostol 10\(^{-6}\)M was added to both serosal and mucosal bathing reservoirs and maintained at that concentration for the duration of the experiment. This dose approximates maximal serum therapeutic levels. Additional controls included uninjured mounted tissue and tissue injured with acid without drug treatment. Outcome measures were transepithelial resistance, \(^3\)H-mannitol flux, and histological evaluation.

**Transepithelial resistance** – The spontaneous PD (potential difference) was measured with Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through silver-silver chloride electrodes with a voltage clamp that corrected for fluid resistance. Resistance (\(\Omega \cdot \text{cm}^2\)) was calculated from the spontaneous PD and Isc (short circuit current). If the spontaneous PD was between −1 mV and 1 mV, tissues were current clamped at ± 100 \(\mu\)A for 5 seconds and the PD was recorded. The Isc and PD were recorded every 15-minutes for 210-minutes. Data were entered into spreadsheets that calculated transepithelial electrical resistance (TER) from Isc and PD using Ohm’s law.
$^3$H-mannitol flux—As a second indicator of gastric permeability, flux of $^3$H labeled mannitol across the mucosa was measured. 200 uM of 3H radiolabeled mannitol was added to the mucosal reservoir. Samples were taken of both serosal and mucosal reservoirs after 3 minutes to establish baseline radioactivity. Two one-hour mucosal to serosal fluxes were performed by sampling serosal bathing solutions at one hour and two hours after addition of radiolabeled mannitol.

Prostanoid quantification—Samples of the serosal bathing solutions were collected at 30 and 210 minutes of tissue incubation after which they were snap frozen in liquid nitrogen and stored at -80°C until analysis. The amount of thromboxane B$_2$ and prostaglandin E$_2$ were measured using commercially available ELISA kits.

Histological examination—Gastric mucosal samples were taken for each dog prior to mounting on Ussing chambers. After 210-minutes, the tissues were collected from each treatment group in Carnoy’s fixative for 24 hours and transferred to 70% ethanol. Samples were sectioned at 5µm, stained with hematoxylin and eosin, and viewed with a light microscope.

Data analysis—A 2-way repeated measures ANOVA was used to compare transepithelial resistance data. A Kruskal-Wallis ANOVA on ranks was used to analyze prostanoid, western blot, and flux data. The Tukey post-hoc test was used to detect differences among treatments and time when significance was detected during the initial ANOVA. Significance was set at p<0.05. Data is represented as means±SE.
Results

Stage 1 COX Inhibition

Hydrochloric acid Ringer’s (pH 1.2) produced a reproducible decrease in barrier function (Figure 6.1, p<0.001). At the point of maximal injury (75-minutes), transepithelial resistance (TER) in acid-injured tissue was 37.3±2.8% of control (Figure 6.1). By the end of the recovery period, TER in this tissue remained significantly lower than control, at 73.7±5.0% of control. TER in acid-injured tissue that was then treated with indomethacin at the initiation of the recovery period (75-minutes) was not significantly different than acid injury control. Likewise, inhibition of COX-2 with NS-398 or COX-1 with SC-560 did not alter the effect of acid injury on TER (Figures 6.2 and 6.3). When both selective COX-inhibitors were administered together, TER was also not significantly effected as compared to acid injury control (Figure 6.4). There was no significant effect of any treatment on the second measure of gastric mucosal permeability, ³H-mannitol flux (Figure 6.5).

Stage 2 Prostaglandin admininstration

To ensure that this lack of effect on gastric permeability on COX-inhibitors was not due to ineffective prostanoid suppression, the concentrations of two gastroprotective prostanoids, PGE₂ and TXB₂ (stable metabolite of TXA₂) were quantified (Figure 6.6). In this case, there was a significant effect of treatment. Acid injured control tissues had 9- to 11-fold higher concentrations of both prostanoids than control. Indomethacin, the non-selective COX inhibitor, attenuated this effect in acid injured tissue and was approximately equal to uninjured control tissue for both prostanoids. Selective COX inhibition variably attenuated increases in prostanoids produced by acid injury. The COX-2 inhibitor NS-398 resulted in
TXB$_2$ levels 5-fold higher than control and PGE$_2$ levels 3-fold higher than control; COX-2 inhibition had an apparently stronger attenuating effect on PGE$_2$ versus TXB$_2$. COX-1 inhibition with SC-560 provided a more equivalent attenuating response, a 5-fold increase in TXB$_2$ and a 6.5-fold increase in PGE$_2$ compared to controls (compared to a 9-fold and 11-fold increase in acid injured controls). Dual inhibition with both NS-398 and SC-560 resulted in 6-fold increase in TXB$_2$ concentrations and 3-fold increase in PGE$_2$ concentrations compared to uninjured control.

After establishing that inhibition of prostanoids did not have an overall significant effect on recovery of barrier function, we investigated whether addition of exogenous prostanoids would accelerate recovery. In the next stage of the experiment, misoprostol was administered at the end of the injury period, at 75-minutes. Misoprostol, applied at the beginning of the recovery period, did have a significant effect on TER when administered concurrently with acid injury (Figure 6.7). TER of misoprostol-treated, acid-injured tissue was significantly higher than TER in acid-injured control tissues (p=0.026) though it did remain significantly lower than uninjured, control TER (p=0.017). There was not a significant effect of treatment on permeability to mannitol (Figure 6.7).

To determine if this protective effect was reflected in tissue morphology, these tissues were stained with hematoxylin and eosin and examined. Control, mounted tissue maintained relatively normal morphology compared to control (Figure 6.8) with minimal apoptotic cells. Acid-injured tissue demonstrated moderate to marked superficial epithelial cell sloughing. Treatment of acid injured tissue with misoprostol protected tissue morphology; these tissues appeared more similar to control tissues.
Discussion

In this study, we determined the effect of COX-inhibition and exogenous prostanoid administration on gastric barrier function after acid injury in an *ex vivo* model. Surprisingly, none of the COX-inhibitors had a significant effect on TER. Initially, we were concerned that tissue prostanoids were not sufficiently inhibited but when PGE$_2$ and TXB$_2$ were quantified, it was clear that the inhibitors were having the expected effect of attenuating prostanoid production induced by acid injury. The reasons behind this lack of effect on barrier function are unclear. It is possible that acid injury already induces significant changes in TER that cannot be worsened by further COX-inhibition. It is also possible that the COX-inhibited tissue can depend upon alternate mechanisms of recovery, such as with heat shock proteins. Further investigation would be needed to determine possible alternate mechanisms of recovery.

Though inhibition of prostanoids did not significant effect TER, administration of exogenous prostanoids did accelerate recovery of TER after acid injury. Traditionally, misoprostol is used clinically only for treatment of gastric injury mediated by COX-inhibition, such as occurs with NSAID overdoses. This work suggests that misoprostol may have utility in treatment or prevention of non-NSAID mediated injury. Misoprostol, as a synthetic prostanoid of the PGE class, has several gastroprotective effects including increased bicarbonate secretion and mucus production. Though these specific effects were not examined in the current study, it was apparent that misoprostol did have a protective effect not only on TER but also against tissue sloughing induced by acid injury.
In neither stage of the study was permeability to mannitol significantly affected by treatment, even when TER was affected. TER is a measure of electrical resistance and is largely dependent on the movement of sodium and chloride ions. As such, it is quite sensitive to acute and small changes in permeability. Mannitol is a larger molecule, the size of glucose, so is less sensitive to small changes in permeability. A thickened mucus layer produced with acid injury may prevent passage of mannitol without altering movement of ions. It is also possible that this peracute injury and recovery is not sufficiently long to measure changes in permeability to this larger molecule.

An ex vivo study such as this has inherent advantages and disadvantages over in vivo models or cell culture models of injury. Because the tissue is only viable for a limited period of time after collection, it limits time of the experiment to approximately 3 ½ hours, preventing examination of longer-term injury and recovery. In these experiments, we cannot induce a true ulcer that can be macroscopically observed but instead an acute injury with relatively brief period of recovery. Additionally, by using tissue ex vivo, we are unable to examine the role of submucosal blood flow or inflammation on tissue recovery or the effect of metabolism of any drug administered. Because these are shelter animals, there is a fair degree of variation between dogs that would not be present in a cell culture model using a single cell line. There are, however, several advantages to this model. It is more clinically relevant than cell models as we are using whole gastric mucosa instead of a monolayer of gastric epithelium. In addition, these experiments used tissue from dogs that are similar to those that may be seen clinically (mixed ages, breeds, diet, and environments). Using this ex vivo model is in accordance with institutional animal use policies as we used gastric mucosa
from shelter animals that were already being euthanized, preventing the need for additional laboratory animals.

This study shows the need for further investigation into the effect of prostanoids on recovery after injury in canine gastric mucosa. Alternative modes of non-COX dependent recovery should be examined. Also, the potential use of misoprostol for non-NSAID mediated injury could be further investigated. The authors feel that this model of injury could serve in these subsequent examinations, in concert with in vivo and cell culture models of injury.
Figure 0.26 TER with acid injury and indomethacin

TER was significantly lower with acid injury with partial recovery though TER of acid injured tissue remained significantly lower than control at all time points from 45-minutes onward (n=27, p<0.001). There were no differences between acid injury and acid injured tissue treated with indomethacin.
Figure 0.27 TER with acid injury and COX-2 inhibitor

TER was significantly lower with acid injury with partial recovery though TER of acid injured tissue remained significantly lower than control at all time points from 45-minutes onward (n=13, p<0.001). There were no differences between acid injury and acid injured tissue treated with the COX-2 inhibitor NS-398.
Figure 0.28 TER with acid injury and COX-1 inhibitor

TER was significantly lower with acid injury with partial recovery though TER of acid injured tissue remained significantly lower than control at all time points from 45-minutes onward (n=13, p<0.001). There were no differences between acid injury and acid injured tissue treated with the COX-1 inhibitor SC-560.
Figure 0.29 TER with acid injury and COX-1 and 2 inhibitors

TER was significantly lower with acid injury with partial recovery though TER of acid injured tissue remained significantly lower than control at all time points from 45-minutes onward (n=13, p<0.001). There were no differences between acid injury and acid injured tissue treated with both COX-1 inhibitor SC-560 and COX-2 inhibitor NS-398.
Figure 0.30 Permeability to mannitol with acid injury and COX inhibition

There is no significant effect of treatment to permeability of mannitol (p=0.24).
Figure 0.31 Prostanoid concentrations with acid injury and COX inhibition

COX inhibition significantly attenuates production of both measured prostanoids, PGE₂ and TXB₂ (PGE₂: p=0.017, TXB₂: p=0.028). Indomethacin attenuates increased prostanoid productions most effectively. Selective COX-1 and 2 inhibition, as well as combined COX-1 and COX-2 inhibition, have intermediate attenuating effects.
Acid injury significantly decreases epithelial resistance, an index of barrier function (p<0.001). Misoprostol, given concurrently with acid, attenuates this change (#p=0.026) though misoprostol-treated, acid-injured tissue remains significantly more permeable than control, uninjured tissue (*p=0.017).
Figure 0.33 Permeability to mannitol with acid injury and misoprostol

Permeability to mannitol is not significantly altered with treatment (p=0.179).
Figure 0.34 Hematoxylin and eosin stain, acid injury and misoprostol

Control, mounted tissues maintain nearly normal tissue morphology compared to baseline tissues collected at euthanasia. Acid injured tissue demonstrates moderate to marked superficial epithelial sloughing. Acid injured tissue that has been treated with misoprostol has less severe epithelial cell sloughing.
References


CHAPTER 7 SUCRALFATE PRESERVES GASTRIC MUCOSAL BARRIER FUNCTION WITH ACID INJURY
Introduction

Stress related mucosal disease (SRMD) affects 60-100% of critically ill human patients and is a significant cause of morbidity and mortality (1). There are no reports of prevalence of stress related mucosal disease in dogs, but it is likely that critically ill canine patients suffer from alterations in gastric barrier function similarly to humans. SRMD encompasses a range of severity of alterations in gastric mucosal integrity, ranging from diffuse, superficial mucosal erosions without overt hemorrhage (stress related injury) to stress ulcers that may hemorrhage and necessitate transfusion. SRMD occurs when typical gastric acidity damages the mucosal epithelium due to deficiencies in normal gastroprotective mechanisms. Gastric mucosal blood flow may decrease with hypotension or splanchnic hypoperfusion, prostaglandin release may be inhibited by non-steroidal or corticosteroid therapy, or proinflammatory cytokines may lead to increased oxidative damage to the mucosa.

The mainstay of treatment for SRMD is traditionally acid suppressant medications including histamine receptor agonists (H₂RAs) and proton pump inhibitors (PPIs). These drugs exert their protective effect by increasing gastric pH. As a consequence, bacteria may proliferate in the gastric lumen, including Gram-negative bacilli. In critically ill patients that are recumbent and susceptible to reflux and aspiration or that are on mechanical ventilation, this may lead to tracheobronchial colonization and pneumonia (2). Sucralfate is a does not alter gastric pH and therefore does not increase risk of these complications. In a recent meta-analysis, sucralfate was as effective as H₂RAs for prevention of overt-bleeding events in critically ill patients and had significantly less incidence of ventilator-associated pneumonia.
and lower rates of gastric colonization (3). Though PPIs appear to be more effective than
H2RAs in the prevention of clinically significant bleeding in critically ill patients with
SRMD, they have similar rates of nosocomial pneumonia (4). In addition, because PPIs alter
pH and gastrointestinal flora, human patients treated with PPIs are at 65% increased risk for
Clostridium difficile-associated diarrhea (5). Complication rates between sucralfate and PPIs
have not been compared in large-scale studies, so it is unclear whether PPIs have increased
efficacy or increased risk over sucralfate.

Sucralfate is a complex of sucrose and aluminum salts. In the presence of acid,
sucralfate crosslinks into viscous layer that binds to negatively charged particles such as with
damaged gastric mucosa. It also acts as a cytoprotectant, increasing protective mechanisms
and accelerating migration and repair of damaged tissues (6). Sucralfate stimulates mucus
and bicarbonate secretion, in part by stimulation of the gastroprotective prostanoid
prostaglandin E2 (PGE2) (7). It has the advantage over PPIs that it does not affect overall
gastric pH therefore likely has a lower likelihood of complications such as nosocomial
pneumonia and Clostridium difficile-associated diarrhea. In this study, we examined the
protective effect of sucralfate on acid injury using an ex vivo model in canine gastric mucosa.
Sucralfate was administered concurrent with injury and subsequent to injury to determine its
potency both as a protective and reparative drug.

Materials and Methods

Tissue acquisition—Tissue samples were obtained from dogs that were euthanized at a local
animal shelter. The precise age of the dogs was unknown in most cases, but ranged from
approximately 8 months to 10 years-of-age. The dogs were typically mixed breed, ranging in size from approximately 10 kg to 30 kg. All dogs were euthanized with an overdose of sodium pentothal. Immediately following euthanasia, the entire antral section of the stomach was excised. This tissue was incised along the greater curvature and placed mucosa side down in oxygenated (95% O₂, 5% CO₂) Ringer’s solution (in Mm: 114.0 NaCl, 5.0 KCl, 1.25 CaCl₂, 1.10 MgCl₂, 25.0 NaHCO₃, 0.3 NaH₂PO₄, 1.65 Na₂HPO₄) at room temperature. After transport to the laboratory, the tissue was transferred to oxygenated Ringer’s solution at room temperature and the seromuscular layer was removed via blunt dissection. The remaining antral mucosa tissue was mounted on Ussing chambers (1.14 cm² diameter), with one tissue sample per treatment group.

_Ussing chambers_—Mucosa was bathed on both mucosal and serosal sides of the chambers with 10 ml of oxygenated Ringer’s solution maintained at 37ºC by water-jacketed reservoirs. Ten mmol/L glucose was added to the serosal bathing solution which was balanced with the addition of 10 mmol/L mannitol in the mucosal bathing solution. After 30-minutes of incubation, treatments were applied to each dog’s tissue, mounted in separate chambers.

Stage 1: Sucralfate + acid injury concurrently To examine the protective ability of sucralfate against acid injury, sucralfate and acidified Ringer’s were applied concurrently to the mucosal aspect of the Ussing chamber. Ringer’s solution was titrated to pH 1.2 with hydrochloric acid and applied to the mucosal aspect of the tissue for 45-minutes. After this injury period, acidic Ringer’s was removed and replaced with neutral Ringer’s. For acid injury + sucralfate, acidic Ringer’s was applied as described with the addition of 100mg/ml sucralfate (sucrose octasulfate). When acidic Ringer’s was removed, sucralfate was added
with the neutral Ringer’s solution to maintain 100mg/ml throughout this experiment. Dose was based on estimated gastric volume and average dose for a 20kg dog. Sucralfate was dissolved in DMSO at 1g/ml. One chamber was maintained in neutral Ringer’s as control. Each of these treatments was applied to the gastric mucosa of 9 dogs. Outcome measures were transepithelial resistance, $^3$H-mannitol flux, prostaglandin E$_2$ (PGE$_2$) concentration, and histological evaluation. These treatments were applied to 9 dogs.

Stage 2: Sucralfate following acid injury In order to evaluate sucralfate as a treatment for pre-existing alterations in barrier function, sucralfate was applied after the initial acid injury. The acid injury was applied as described previously. Subsequent to acid injury, sucralfate was applied to the mucosal bathing reservoir to give a concentration of 100mg/ml. Controls included uninjured control, acid injury control, uninjured control + sucralfate, and injured control + vehicle (DMSO). Outcome measures were transepithelial resistance, $^3$H-mannitol flux, PGE$_2$ concentration, and histological evaluation. These treatments were applied to 8 dogs.

Transepithelial resistance – The spontaneous PD (potential difference) was measured with Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through silver-silver chloride electrodes with a voltage clamp that corrected for fluid resistance. Resistance (Ω·cm$^2$) was calculated from the spontaneous PD and Isc (short circuit current). If the spontaneous PD was between –1 mV and 1 mV, tissues were current clamped at ± 100 µA for 5 seconds and the PD was recorded. The Isc and PD were recorded every 15-minutes for 210-minutes. Data were entered into spreadsheets that calculated transepithelial electrical resistance (TER) from Isc and PD using Ohm’s law.
$^3$H-mannitol flux—As a second indicator of gastric permeability, flux of $^3$H-mannitol across the mucosa was measured. 200 uM of $^3$H radiolabeled mannitol was added to the mucosal reservoir. Samples were taken of both serosal and mucosal reservoirs after 3 minutes to establish baseline radioactivity. Two one-hour mucosal to serosal fluxes were performed by sampling serosal bathing solutions at one hour and two hours after addition of radiolabeled mannitol.

Prostanoid quantification—Samples of the serosal bathing solutions were collected at 30 and 210 minutes of tissue incubation after which they were snap frozen in liquid nitrogen and stored at -80ºC until analysis. The amount of prostaglandin E$_2$ was measured using commercially available ELISA kits.

Histological examination—Gastric mucosal samples were taken for each dog prior to mounting on Ussing chambers. After 210-minutes, the tissues were collected from each treatment group in Carnoy’s fixative for 24 hours and transferred to 70% ethanol. Samples were sectioned at 5µm, stained with hematoxylin and eosin (H&E), and viewed with a light microscope.

Data analysis—A 2-way repeated measures ANOVA was used to compare transepithelial resistance data. A one-way ANOVA was used to analyze prostanoid and flux data; a Kruskal-Wallis ANOVA on ranks was used to analyze non-parametric prostanoid and flux data. The Tukey post-hoc test was used to detect differences among treatments and time when significance was detected during the initial ANOVA. Significance was set at p<0.05. Data is represented as mean±SE.
Results

Acidic Ringer’s solution applied to gastric mucosa decreased TER to 43.6±7.8% of control at the completion of the injury period (75-minutes, Figure 7.1). After replacement of acidic Ringer’s with neutral Ringer’s solution, TER of acid injured tissue recovered to 72.8±8.1% of control.

Sucralfate was applied at two different time points in two sets of dogs. For the first set, sucralfate was administered concurrently with acid injury (30-minutes). When sucralfate treatment was simultaneous with acid injury, sucralfate attenuated the decrease in TER induced by acid (Figure 7.1). At 75-minutes, TER of acid injured tissues were 42.5±11.1% of control; TER of sucralfate-treated, acid-injured tissue at 75-minutes was 118.0±15.2% of control. By the end of the recovery period at 210-minutes, acid-injured tissue was 71.4±11.6% of control; sucralfate treatment increased this recovery to 119.1±12.0% of control.

Sucralfate also attenuated the increase in gastric barrier function measured by $^3$H-mannitol flux. Permeability to this molecule was increased in acid injured tissue (Figure 7.2; control flux: 0.17±0.02 µmol/cm².h, acid injury flux: 0.29±0.07 µmol/cm².h). When sucralfate was applied with acid Ringer’s solution, flux of $^3$H-mannitol significantly decreased (sucralfate + acid injury flux: 0.09±0.03 µmol/cm².h, p=0.008).

This protective effect on barrier function was then examined histologically. Control, mounted tissues maintained largely unchanged morphology compared to baseline tissues taken at the time of tissue collection with only occasional apoptotic cells (Figure 7.3). Acid
injury induced moderate to marked sloughing of the superficial gastric epithelium. When sucralfate was administered concurrently with acid, normal tissue morphology remained, with little to no tissue sloughing or apoptosis.

In the second experiment, sucralfate was applied to gastric mucosa immediately after acid injury (at 75-minutes) to examine the effect on previously injured tissue. In this subset of dogs, sucralfate increased TER during recovery (Figure 7.4; acid injury: 75.1±4.8% of control at 210-minutes, sucralfate + acid injury: 111.0±15.5% of control at 210-minutes). TER of sucralfate treated, acid-injured tissue was significantly higher than acid injured control tissue from 150-minutes until the end of the experiment. In this group of 8 dogs, there was not an overall significant effect of treatment on mucosal permeability to radiolabeled mannitol (Figure 7.5; control flux: 0.13±0.01 µmol/cm².h, acid injury flux: 0.15±0.02 µmol/cm².h, sucralfate + acid injury flux: 0.12±0.01 µmol/cm².h).

Sucralfate administered after injury had a similar protective effect on tissue morphology (Figure 7.6). Acid-injured tissue, as in the previous experiment, demonstrated moderate to marked degrees of epithelial sloughing. These changes were not seen in acid-injured tissue that was then treated with sucralfate.

There was no effect of sucralfate or acid injury on concentrations of PGE₂ (Figure 7.7, n=4).

Because sucralfate was administered dissolved in DMSO, an additional vehicle control group was added to 4 dogs. Addition of vehicle control after acid injury did not produce significant changes in barrier function compared to acid injury control (Figure 7.8, n=4).
The effect of sucralfate on uninjured tissue was also examined. TER was not significantly different between control tissue and uninjured control with sucralfate added at 75-minutes (Figure 7.9).

**Discussion**

In this study, the protective effect of sucralfate on acid-injured canine gastric mucosa was examined. In the first group of dogs, sucralfate was administered concurrently with acid injury. Sucralfate had a significant protective effect on both barrier function and tissue morphology. The authors were surprised at the similarity in tissue morphology between control tissue and injured, sucralfate-treated tissue, suggesting that sucralfate is a powerful gastroprotectant against acid injury. Though many patients already have some degree of gastric mucosal injury at the time of presentation, this data suggests the utility of sucralfate as a preventative measure in critically ill patients that are susceptible to stress related mucosal disease. Further work is needed to examine the efficacy of sucralfate as a prophylactic for gastric ulceration through in vivo studies and with clinical patients.

In the second group of dogs, sucralfate’s effect on barrier function subsequent to acid injury was examined. This model of injury more closely mimics treatment of sucralfate in patients with pre-existing damage to the gastric mucosa, either stress related mucosal disease, stress ulceration, or ulceration of other etiologies. In this setting, sucralfate also significantly increased epithelial resistance (a measurement of barrier function) after acid injury and preserved tissue morphology, implying that sucralfate could be used to treat pre-existing gastric erosions and ulceration to recover and maintain barrier function.
This Ussing chamber \textit{ex vivo} model has advantages and disadvantages over \textit{in vivo} injury models. First, the isolation of tissue on the chamber allows for an isolated examination of barrier function, which is very difficult \textit{in vivo}. Second, the acute effects of a drug can be examined with measurements of barrier function being examined every 15 minutes. Transepithelial resistance is a more sensitive indicator of gastric barrier function, as it measures the passage of ions, than available \textit{in vivo} measurements of gastric barrier function. Radiolabeled mannitol is a larger molecule and less sensitive to acute changes in barrier function, which may explain why significant changes were seen in TER but not with permeability to mannitol.

Sucralfate did not significantly affect production of PGE$_2$, though this has been a reported mechanism of action of sucralfate. It is possible that there was an increase in tissue production of PGE$_2$ but it was not significantly secreted into the bathing reservoirs. Additionally, prostanoids were only sampled in four of the dogs with large standard errors and low power. Further work would be required to determine the effect of sucralfate on canine production of PGE$_2$.

There are, however, several disadvantages to the use of this technique to study gastric injury in dogs. First, it allows only for the study of peracute injury. It is not a macroscopic erosion or ulcer that is occurring secondary to acid injury; the changes here are seen microscopically, so it does not exactly mimic a true erosion or ulcer in a dog. Also, because the mucosa is \textit{ex vivo}, the gastric mucosal blood supply is removed. The effect of sucralfate on alterations of submucosal blood flow or influx of inflammatory cells, therefore, cannot be examined. The soluble mucus layer is also disrupted during the mounting process. In spite of
the loss of the protective mechanisms, however, sucralfate still had protective effects on
barrier function.

Sucralfate has been a mainstay of treatment for gastric ulceration in dogs though there
has been little empirical data supporting its use in this species. This *ex vivo* study showed
that sucralfate protects barrier function when gastric mucosa is injured with acid and
improved recovery of barrier function after acid injury. This work suggests that sucralfate has
a protective effect of sucralfate, both as a preventative against acid injury and as a treatment
for pre-existing alterations in barrier function though further work is needed to examine the
effects of sucralfate *in vivo*, including with clinical patients with stress-related mucosal
disease.
Acid Ringer’s solution induced a significant decrease in TER as compared to uninjured control (p=0.003). Sucralfate treatment concurrent with acid injury significantly attenuated the decrease in barrier function induced by acid Ringer’s solution (p<0.001, n=9).

Figure 0.35 TER with acid injury and sucralfate administered concurrently
Figure 0.36 Permeability to mannitol with acid injury and sucralfate co-administered

There was a significant effect of treatment on flux of radiolabeled mannitol across canine gastric mucosa (*p=0.008). Sucralfate administered concurrently with acid injury significantly decreased $^3$H-mannitol flux compared to acid injury control (n=9).
Figure 0.37 H&E stain, acid injury and sucralfate co-administered

Control tissues mounted on Ussing chambers maintained similar tissue morphology to baseline tissues (taken at the time of tissue collection) with occasional apoptotic epithelial cell noted. Acid injury induced moderate to marked epithelial cell sloughing. Treatment with sucralfate concurrent with injury protected against morphologic change induced by acid.
Acid injury induced a significant decrease in TER compared to uninjured control (p=0.002). When Sucralfate was administered after acid injury, TER recovered to a higher level than untreated, injured tissue. Acid injured tissue treated with Sucralfate had a significantly higher TER from 150-minutes until the end of the experiment (p<0.035, n=8).

Figure 0.38 TER with acid injury followed by sucralfate administration
There is not a significant effect of injury or sucralfate treatment on $^3$H-mannitol flux (p=0.214, n=8).
Figure 0.40 H&E stain, acid injury followed by sucralfate

Sucralfate administered at the completion of the acid injury period preserved tissue morphology compared to untreated, acid-injured tissue.
Figure 0.41 Prostaglandin E$_2$ concentration with sucralfate

There was no significant effect of acid injury or sucralfate administration on concentration of PGE$_2$ (n=4).
Sucralfate was administered in DMSO (vehicle). Treatment with DMSO alone (vehicle control) did not produce an overall significant change in barrier function as compared to acid injury alone, whereas sucralfate in DMSO increased TER after acid injury. There was no overall effect of acid injury with sucralfate or acid injury with vehicle control in these 4 dogs (inset).

Figure 0.42 TER and permeability to mannitol with acid injury and vehicle control
Figure 0.43 TER and permeability to mannitol, sucralfate + neutral Ringer’s control

Sucralfate administered without acid injury has no significant effect on TER or ^3^H-mannitol flux (n=4).
References


