Effects of Prednisone or Prednisone with Ultralow-Dose Aspirin on the Gastroduodenal Mucosa of Healthy Dogs

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Abstract:

This study tested the hypothesis that administration of immunosuppressive doses of prednisone in conjunction with ultralow-dose aspirin (0.5 mg/kg/day) would result in gastroduodenal lesion scores similar to those found in dogs administered only immunosuppressive doses of prednisone, but that the gastroduodenal scores from both of these treatment groups would be significantly higher than placebo when administered to healthy dogs for 27 days. Eighteen healthy adult purpose-bred dogs were divided randomly into three groups. Group I received placebo capsules and placebo suspension, Group II received prednisone capsules (mean 2.3 mg/kg, range 2.0-2.4) and placebo suspension, and Group III received prednisone capsules (mean 2.3 mg/kg, range 2.3-2.5) and aspirin suspension (0.5 mg/kg) by mouth once daily for 27 days. Gastroduodenoscopy was performed on days -7 (baseline), 5, 14, and 27 of treatment. Four regions of the stomach (angularis incisura, body, pylorus, and cardia) and the proximal descending duodenum were systematically scored on a scale of 1 (normal) to 11 (perforating ulcer) by an experienced observer who was blinded to the treatment groups

and clinical signs of each subject. Dogs were observed every 8 hours for vomiting, diarrhea, and inappetence. Feces were scored on a scale of 1-5 with diarrhea defined as a fecal score <4.

Lesion scores for each group, at each location, and total scores, at each time period were evaluated for the effects of time and treatment using a Kruskal-Wallis test. Total dog days of vomiting and dog days of diarrhea in each group were compared using a Wilcoxon rank sums test. Significance was determined at $p<0.05$.

There were no significant differences in median total gastric lesion scores between any of the groups at any time during the study. There was no location effect on regional gastroduodenal lesion scores and there was no significant change in gastroduodenal lesion scores over time in any of the groups during treatment. Significantly more dog-days of diarrhea occurred within the prednisone and aspirin group during the experimental period (Period 2) in comparison to Period 1. However, no significant differences were found between any of the groups for dog-days of vomiting, diarrhea or inappetence at any time in the study.

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DECLARATION OF WORK PERFORMED

I declare that I, Heather Graham, performed all work detailed in the materials and methods of this manuscript with the exception of the following procedures. The clinical pathology laboratory at Virginia-Maryland's Regional College of Veterinary Medicine performed complete blood counts and serum biochemical profiles. The parasitology laboratory at Virginia-Maryland's Regional College of Veterinary Medicine performed all zinc sulfate fecal flotations. Statistical analysis was performed by Dr. Stephen Werre.

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ABBREVIATIONS

AA: arachidonic acid
BID: every 12 hours
BMBT: buccal mucosal bleeding time
CBT: cuticle bleeding time
CCK: cholecystikinin
CNS: central nervous system
COX: cyclo-oxygenase
DIC: disseminated intravascular coagulation
dL: deciliter
ECL: enterochromaffin-like cells
ERPF: effective renal plasma flow
FDP: fibrin degradation products
FOBT: fecal occult blood test
G cells: gastrin-secreting cells
GF: growth factor
GFR: glomerular filtration rate
GGT: gamma-glutamyl-transferase
GI: gastrointestinal
HAC: hyperadrenocorticism
IL: interleukin
IMHA: immune-mediated hemolytic anemia
IV: intravenous
IVDD: intervertebral disc disease
kg: kilogram
KP: ketoprofen plus prednisolone
LO: lipoxygenase
LT: leukotriene
mg: milligram
ml: milliliter
MP: meloxicam plus prednisolone
NAG: n-acetyl-beta-glucosaminidase
NC: control group[1]
NN: placebo group
NSAID: non-steroidal anti-inflammatory drug
PA: prednisone and ultralow-dose aspirin
PN: prednisone and placebo
PG: prostaglandin
PO: per os (by mouth)
PPI: proton pump inhibitor
PT: prothrombin time
PTT: partial thromoplastin time
q (e.g. Q12 hours): every
SC: subcutaneous
TID: every 8 hours

TNF-α: tumor necrosis factor- alpha
TNF-ß1: tumor necosis factor beta-one
TxA_2: thromboxane A- two
UPC: urine protein to creatinine ratio
vWF: von Willebrand's factor

INTRODUCTION:

A variety of immunosuppressive drugs have been implemented in the treatment of autoimmune disorders, yet standardized, substantiated treatment protocols are lacking for some of the most devastating disease processes, including immune-mediated hemolytic anemia (IMHA). The mainstay of treatment for IMHA continues to employ immunosuppressive doses of corticosteroids with or without the use of additional immunosuppressive medications, such as azathioprine, cyclosporine, mycofenolate mofetil, cyclosphosphamide, leflunomide, and others. Aside from the morbidity and mortality suffered as a result of severe acute hemolytic anemia, thromboembolic events also occur in 30-100% of these patients.[2-5] Thromboembolic sequelae contribute significantly to the morbidity and mortality of IMHA patients, and various medical treatments are being used to decrease these thromboembolic events.

Aspirin modulates thrombotic events by irreversibly acetylating platelet cyclooxygenase (COX) leading to decreased thromboxane A_2 synthesis, thereby impairing platelet aggregation in a dose-dependent manner.[6-8] In humans, low dose aspirin therapy has been proven to reduce thromboembolic disease in patients suffering from a spectrum of disease processes, including cardiovascular and renal disease.[7,8] However, because aspirin is a non-selective COX inhibitor, it can cause gastrointestinal hemorrhage and ulceration at recommended doses of 25-35 mg/kg every 8-12 hours in dogs.[4,9-13] Ultralow-dose aspirin, at a dose of 0.5 mg/kg/day, may provide the benefits of modulating platelet aggregation while minimizing the detrimental effects of COX-1 suppression on gastric mucosal integrity and renal perfusion.[4,7,8,14] Aspirin at a dose of 0.5 mg/kg/day may reduce platelet aggregation in dogs and therefore may provide an

alternative to standard antithrombotic therapy with heparin.[15,16] In one retrospective study evaluating prognostic factors, survival rates, and treatment protocols for 151 canine IMHA patients, dogs that were treated with prednisone, azathioprine, and ultralow-dose aspirin (with or without mixed-molecular-weight heparin) had a significant survival advantage over those treated with prednisone and azathioprine with or without mixed-molecular-weight heparin.[4] Clinical evidence of adverse effects of ultralow-dose aspirin when administered in conjunction with immunosuppressive doses of glucocorticoids has not been recognized in dogs, but the use of ultralow-dose aspirin as antithrombotic therapy in IMHA patients is a relatively new application.

A significant consideration that must be made when evaluating a treatment protocol involving concurrent use of prednisone and ultralow-dose aspirin in IMHA patients is the fact that distinguishing the effects of medications (prednisone and ultralow-dose aspirin) on gastroduodenal integrity from the effects of sequelae of IMHA (DIC and/or gastroduodenal ischemia secondary to hypoxia and/or thrombosis) may be difficult to achieve in clinically affected patients. Alternately, gastroduodenal mucosal integrity may be subclinically disrupted in these patients secondary to the concurrent administration of a glucocorticoid and a non-selective NSAID, but these lesions go unnoticed because the patients develop no overt clinical signs of gastroduodenal hemorrhage. Our purpose is to determine if there is any appreciable difference in gross endoscopic gastroduodenal scores between healthy dogs receiving immunosuppressive prednisone alone and healthy dogs receiving immunosuppressive prednisone in conjunction with ultralow-dose aspirin. Our hypothesis is that there will be no statistically significant difference in the gastroduodenal endoscopic scores between dogs

treated with prednisone alone and those treated with prednisone and ultralow-dose aspirin, however, both treatment groups will have significantly higher gastroduodenal scores than placebo-treated dogs. The objectives of this study were to: 1) document, score and compare the endoscopically detectable gastroduodenal lesions caused by the administration of prednisone (2.2 mg/kg/day), prednisone (2.2 mg/kg/day) and aspirin (0.5 mg/kg/day) or placebo, and 2) document and compare the occurrence of GI side effects (vomiting, diarrhea, inappetence) associated with the administration of these drugs. By evaluating these differences in healthy dogs, we eliminate any confounding effects that IMHA and its sequelae might have on the gastroduodenal mucosa and determine if the use of ultralow-dose aspirin with immunosuppressive doses of prednisone presents a relatively safe treatment option for canine IMHA patients.

CHAPTER I: LITERATURE REVIEW

A. Gastroduodenal anatomy and physiology

i. Function:

The primary functions of the stomach include serving as a reservoir for ingesta and mechanical and biochemical breakdown of ingesta. This allows controlled emptying of gastric contents into the duodenum, which facilitates optimal digestion and absorption of nutrients. The fundus and body of the stomach accommodate the majority of the gastric contents, dilating with minimal resulting increase in intragastric pressure.[17,18] The capacity of the canine stomach varies depending on body size and age of the patient, however the stomach capacity is approximately 90-110 mL per kilogram.[19]

The pyloric antrum grinds food into smaller particles (physical digestion or triturition) while mixing it with gastric juice (chemical digestion), and the pylorus limits the size of particles that may enter the duodenum.[17,18,20] Gastric emptying is tightly controlled by the autonomic nervous system, and gastric contents empty into the duodenum at a rate that is optimal for further digestion and absorption of nutrients. The food material that reaches the duodenum undergoes further chemical digestion which is mediated by a variety of nutrient-specific hydrolytic enzymes.[18,20,21] Water, electrolytes, peptides, simple sugars, and lipids are absorbed by enterocytes, entering the intestinal capillary network or lymphatics which ultimately enter into the hepatic portal vasculature or the thoracic duct, respectively.[20,21]

ii. Gastroduodenal microanatomy:

The stomach is lined by a continuous layer of surface mucous cells which are columnar epithelial cells that secrete large amounts of viscid mucus to form a protective gel layer. The stomach mucosa also has two subtypes of tubular glands: oxyntic (or gastric) glands and pyloric glands.[18,20-22] Oxyntic glands, found in the fundus and body, have parietal cells that secrete hydrochloric acid and intrinsic factor, chief cells that secrete pepsinogens, and mucous neck cells that secrete mucus.[18,20-22] Pyloric glands, located in the pyloric antrum, contain primarily mucous cells that secrete a thin mucus and specialized gastrin-secreting cells (G cells), with few pepsinogen-secreting chief cells.[18,20] Enterochromaffin-like (ECL) cells are histamine-secreting cells that are found in the oxyntic glands in close association with parietal cells.[18,20]

The duodenal mucosa has a large surface area which is created by finger-like projections of enterocytes called villi which are covered on their apical surface by a brush border composed of microvilli.[20,22] The glycocalyx, a viscous glycoprotein layer, encases the microvilli and its associated digestive enzymes. Interspersed with the enterocytes are goblet cells that secrete mucus which blends with the glycocalyx and assists in protecting the mucosa and entrapping molecules.[21,23] An unstirred water layer, a slow-flowing water layer at the luminal surface, in conjunction with the mucus and glycocalyx, represents a diffusion barrier between the ingesta within the duodenal lumen and the enterocytes.[20,22,23]

iii. Gastrointestinal secretions:

Gastrin, acetylcholine, and histamine stimulate hydrochloric acid secretion by gastric parietal cells located in the body and fundus of the stomach.[21,23] Binding of these substances to the parietal cell basolateral membrane triggers an intracellular biochemical cascade that leads to the activation of a proton pump. The proton pump actively secretes hydrogen ions against a concentration gradient into the gastric lumen, maintaining a low intraluminal pH.[21,23]

Gastric acid secretion results from the synergistic effects of gastric secretalogues (gastrin, acetylcholine, and histamine), and it occurs in three phases: cephalic, gastric, and intestinal.[21-23] The cephalic phase of gastric secretion is a reflex response coordinated in the cerebral cortex. Acetylcholine is released by postganglionic vagal fibers that terminate in the myenteric and submucosal neural plexuses and on G cells, parietal cells, and chief cells in response to the sight, smell or taste of food.[21]

Acetylcholine exerts its effects on acid secretion both directly by binding to muscarinic receptors on parietal cells and indirectly through stimulation of histamine release from the ECL cells and gastrin release from G cells.[23] The gastric phase of secretion occurs when distension of the pyloric antrum (pyloropyloric reflex) and body of the stomach (oxynto-pyloric reflex) with food stimulates G cells to secrete gastrin through both long vagovagal reflexes and short intramural neural pathways. Gastrin stimulates acid secretion by binding to ECL cells and stimulating histamine release.[20] Histamine then binds to H_2 histamine receptors on parietal cells, stimulating acid secretion. Acid secretion is locally stimulated by the presence of amino acids in the ingesta through direct stimulation of parietal cells. Gastrin secretion is ultimately inhibited by a negative feedback mechanism when the antral pH falls below 3.0. Histamine itself plays a major role in acid secretion, with the majority of the histamine pool being stored in enterochromaffin-like cells in the oxyntic glands in the gastric body. Both gastrin and acetylcholine have receptors on these cells and are capable of triggering the local release of histamine which binds to H_2 receptors on gastric parietal cells leading to acid secretion.[20,21,24] Finally, distension of the small intestines and the presence of breakdown products of protein digestion within the small intestinal lumen trigger the production of gastrin which in turn stimulates secretion of small amounts of gastric acid by the parietal cells.[23,24] Gastric acid secretion may be inhibited by the presence of a number of substances, including somatostatin, calcitonin, vasoactive intestinal peptide, epinephrine, secretin, prostaglandins, and nitric oxide.[20,21,24] These substances may act either directly on the parietal cell itself or indirectly through mediation of gastrin secretion. In the dog,

gastric acid secretion occurs only in the presence of food through a combination of neuroendocrine, paracrine, and mechanical stimuli.

The proteolytic enzyme, pepsin, is secreted in the inactive zymogen form (pepsinogen) from the gastric chief cells, located in the gastric body and fundus, during feeding.[18,20,23] While there are a number of different forms of pepsinogen, with differing immunologic and electrophoretic characteristics, in canine gastric secretions, these different forms appear to function similarly.[23] Pepsinogen release is mediated by a variety of agents including secretin, acetylcholine, gastrin, epinephrine, prostaglandins, vasoactive intestinal peptide, and cholecystikinin (CCK).[21,24] The amino terminal peptide of pepsinogen is cleaved under acidic conditions, converting it to pepsin, the active proteolytic form of the enzyme. Although the proteolytic activity of pepsin contributes minimally to protein digestion, the peptides generated by pepsin-mediated protein cleavage stimulate additional secretion of gastrin and CCK, contributing to the regulation of gastric acidity.

Pancreatic secretions accumulate within the duodenal lumen during digestion and are composed primarily of nutrient-specific hydrolytic enzymes, the most important of which are trypsin, amylase, and lipase.[18,20,23] Trypsinogen (zymogen form of trypsin) is activated by enterokinase (an enzyme produced by duodenal enterocytes) to trypsin, which then acts as an autocatalytic agent, activating other trypsinogen and peptidase molecules.[23] Trypsin hydrolyzes proteins and polypeptides in the luminal phase of digestion, producing smaller pepetides.[18,21,23] Amylase is secreted in its active form and hydrolyzes starch molecules to polysaccharides within the intestinal lumen. The resulting peptides and short chain polysaccharides diffuse through the unstirred water layer,

mucus, and glycocalyx to reach the brush border for the final phases of chemical digestion.[18,21,23] Once these nutrients reach the apical surface of the enterocyte, they are hydrolyzed by peptidases and polysaccharide-specific enzymes (e.g. lactase, maltase, sucrase) and are absorbed by the enterocytes through a variety of active and passive transport mechanisms. Lipid digestion requires additional steps due to the hydrophobic nature of lipids. In the duodenum, lipids undergo emulsification by bile acids and are then transported to the jejunum.[18,20,23] In the jejunum, emulsified lipids are hydrolyzed through the combined actions of pancreatic lipase and co-lipase resulting in the formation of free fatty acids which are ultimately complexed with bile salts, forming micelles that are eventually absorbed into the jejunal enterocytes.[18,20,23]

iv. Gastroduodenal mucosal defense

The gastric mucosa is protected from the damaging effects of hydrochloric acid, mechanical irritation, bile acids, pepsin, and other digestive enzymes by a mucosal barrier composed of mucus impregnated with bicarbonate, gastric epithelial cell membranes, and subepithelial vasculature.[25,26] The mucus layer is composed primarily of high molecular weight glycoproteins called mucins and water.[25,26] Mucins are secreted by goblet cells in the crypts, and their secretion is regulated by a number of substances, including acetylcholine, prostaglandin-E_2, secretin, and beta-adrenergic agonists. This layer aids in epithelial surface lubrication, plays a role in the local immune system, and helps to protect the gastric epithelium from toxins, ions, enzymes, drugs, infectious agents, and other noxious substances.[27]

Bicarbonate ion secretion is believed to be mediated by a number of neural and hormonal substances including prostaglandins, CCK, glucagons, and neurotensin.

Bicarbonate diffuses along the concentration gradient from the gastric surface epithelial cells into the mucus layer, forming a pH gradient that protects the gastric mucosa from luminal acid.[28] Other important characteristics of the mucosal barrier include the hydrophobic lipid cell membranes with tight junctional complexes, inhibition of hydrogen ion back-diffusion, rapid neutralization and clearance of back-diffused intracellular hydrogen ions, rapid epithelial cell turnover, protective prostaglandins (PG_I and PG_E), and local mucosal immunity.[25,28,29]

Mucosal blood flow is integral in maintaining the mucosal barrier. Increased blood flow within the submucosal capillary network associated with mucosal injury serves to supply plasma bicarbonate, oxygen, and nutrients to cells to aid in cell repair and removes injurious substances, such as back-diffused acid and inflammatory mediators.[20,27,28,30] Mucosal defects are repaired rapidly by immediate epithelial restitution followed by cellular renewal, thus mitigating further damage extending deeper into submucosa.[31]

Prostaglandins, the products of cyclooxygenase metabolism of arachidonic acid, have been shown to exert a gastroprotective effect, acting in an autocrine or paracrine manner within the mucosa. Secretion of certain prostaglandins (particularly PGI_2, E, E_2, and $F_{2\alpha}$) by the gastroduodenal mucosa exerts cytoprotective effects through a combination of functions.[20,32-35] These cytoprotective mechanisms include stimulation of mucus and bicarbonate secretion, induction of epithelial resistance to cytotoxic injury, stabilization of tissue lysosomes, stimulation of hydrophobic surface active phospholipids, increasing gastric mucosal blood flow, and modulating tissue immunocytes (particularly macrophages and mast cells).[20,36] In particular, PGE_2

suppresses TNF-α production by macrophages and suppresses platelet activating factor, histamine, and TNF-α release from mucosal mast cells, resulting in down-regulation of the local inflammatory response.[20,37,38]

The local mucosal immune system consists of immunocytes, including MHC II positive macrophages that serve as antigen-presenting cells and mast cells, that act as sentinels for foreign antigens.[20,21] A local immune response is coordinated through the release of cytokines and other inflammatory mediators, such as nitric oxide (NO), eicosanoids, neuropeptides, and proteinases, resulting in leukocyte recruitment, vasodilation (hyperemia), and endothelial permeability (edema).[39,40] This inflammatory response is designed to protect the gastrointestinal mucosa, but, in some circumstances, this inflammation can contribute to erosion and ulcer formation. Cell-mediated and humoral immune responses are generated when antigen-presenting cells present antigen to B and T lymphocytes within Peyer's patches and the lamina propria, in some instances contributing to the development of hypersensitivity and an ongoing inflammatory response.

As an inflammatory mediator, nitric oxide typically serves a protective role, primarily by inhibiting neutrophil chemotaxis and mast cell degranulation. In experimental rat models, gastric mucosal injury appears to be potentiated by NO suppression.[40] Accordingly, nitroso derivatives (NO donors) of typically nonselective NSAIDs (e.g. aspirin, indomethacin, phenylbutazone formulated to release NO in vivo) have demonstrated wider safety margins in humans, however no such medications are currently approved for veterinary use.[41,42]

The duodenal mucosa is protected from mucosal injury by a bicarbonate-rich mucus layer produced by glands of Brunner in the upper duodenum and by alkaline pancreatic secretions. Pancreatic secretions contain a large amount of bicarbonate which protects the duodenal mucosa by inactivating pepsinogen and neutralizing hydrochloric acid.[21,23] Gastric acid neutralization is further aided by bicarbonate ions secreted in the duodenal mucus and in bile from the liver. Additional protective mechanisms that are activated when gastric acid is present in the duodenal lumen include: 1) reflexive inhibition of further gastric acid secretion through neural and hormonal feedback and 2) intestinal mucosal liberation of secretin, a molecule that stimulates rapid secretion of alkaline pancreatic juice.[21,23]

B. Gastroduodenal mucosal injury and repair

An erosion is defined as a superficial defect limited to the mucosa, whereas an ulcer is a defect that extends into the lamina muscularis mucosa or deeper. Mucosal erosion results in increased local blood supply and stimulates rapid replication of epithelial cells, and healing typically occurs within 2 days.[31] If injury exceeds the reparative abilities of the mucosa, superficial erosions can progress to ulcers, and ulcers often require several weeks to completely heal, assuming the cause of the injury has been eliminated. The most sensitive and specific method for identifying and differentiating gross lesions of the mucosa is gastroduodenal endoscopy. There are a multitude of underlying causes for mucosal injury including, but not limited to medications (glucocorticoids, NSAIDs), primary gastric mucosal disease (lymphoplasmacytic or eosinophilic gastroenteritis, neoplasia, mechanical irritants), poor perfusion (hypotension,

gastric volvulus, DIC or thrombosis), neurologic disease (intervertebral disc disease), metabolic disorders (renal or hepatic disease, hypoadrenocorticism), and gastric hyperacidity (gastrinoma or mast cell tumor).[22,24,43-46] NSAID administration, neoplasia, hepatic disease, and shock are the most common causes of ulceration in the dog, and the most common clinical sign is vomiting, although other common clinical signs include melena, inappetence, weight loss, and cranial abdominal pain.[31,47]

Ulcer healing proceeds through an immediate phase, followed by a late phase of repair. Mucosal disruption triggers the release of a variety of growth factors (GF) from cells at the ulcer margin and base, including epidermal GF, fibroblast GF, hepatocyte GF, transforming GF (α & ß), insulin-like GF, and gastrin.[31] These growth factors stimulate ulcer repair in both the immediate and late phases of healing. Restitution, characterized by rapid epithelial cell migration over a mucosal defect, characterizes the immediate phase, and helps minimize ongoing damage in the interim between injury and re-epithelialization. Restitution is followed by epithelial proliferation within 1-2 days of injury, and this ultimately leads to re-epithelialization of the ulcer within 10-20 days, with some variation depending on ulcer size and persistence of ulcerogenic factors in the mucosal environment.[24] Finally, reconstruction and regional differentiation of mucosal glands occurs within 20-40 days of ulceration, and results in complete ulcer healing. Ongoing corticosteroid and/or NSAID administration may delay or inhibit ulcer healing, as these drugs inhibit growth factor synthesis, delaying tissue repair and angiogenesis.[31,48-50]

C. Non-steroidal anti-inflammatory drugs, Corticosteroids, and Gastrointestinal injury

i. NSAIDs

a. Pharmacologic effects of NSAIDs

The physiologic and pathologic effects of NSAIDs are related to their modulation of the arachidonic acid (AA) cascade and alteration of resulting eicosanoid production. The profile of eicosanoids produced depends on which of the two major enzymatic pathways is involved in arachidonic acid metabolism: lipoxygenase (5-LO, 12-LO, or 15-LO) or cyclo-oxygenase (COX-1 or COX-2).[36,51] Leukotrienes and lipoxins are produced by lipoxygenase activity, and prostaglandins (PGs) and thromboxane-A_2 (TxA_2) are produced by cyclo-oxygenase activity.[41,52] The subtype of PGs produced depends on the COX isoform that is active.[36,53] Both isoforms are structurally similar, with similar molecular weight and amino acid composition, but are functionally distinct.[53] COX enzymes have two adjacent active sites: the COX site and the peroxidase site. COX enzymes form PGs through two steps: 1) oxidation and cyclization of arachidonic acid to PGG_2 at the COX site; 2) reduction of PGG_2 to PGH_2 at the peroxidase site. Based on x-ray crystallography, it has been determined that while the COX sites of the two isoforms are similar, characterized by a long hydrophobic channel, the active COX site of COX-2 is larger than that of COX-1, a feature which allows the COX-2 enzyme to utilize a wider variety of substrates and likely accounts for differences in NSAID selectivity.[54] NSAIDs inhibit COX activity by blocking substrate access to this hydrophobic channel either by inducing a structural enymatic change or by physically blocking it.[36]

COX-1 is constitutively expressed in all cells (apart from erythrocytes) of the body and plays an integral role in maintenance of homeostasis, with particular

importance in gastroprotection, renal perfusion, platelet function, and reproduction.[36,51,53] PGs produced by COX-1 activity in the gastrointestinal mucosa (particularly PGI_2, PGE, PGE_2, and $PGF_{2\alpha}$), exert their gastroprotective effects by decreasing the volume, acidity and pepsin contents of gastric secretions, increasing mucus and bicarbonate production, stimulating the mucosal cell replication, and maintaining mucosal perfusion.[34,35,53,55]

COX-2 is an inducible enzyme that is formed at sites of inflammation and produces pro-inflammatory PGs from metabolism of arachidonic acid that is released from damaged cell membrane phospholipids through the activity of phospholipase-A_2. COX-2 is expressed in a variety of cell types, including endothelial cells, leiomyocytes, chrondrocytes, fibroblasts, macrophages, monocytes, and synoviocytes in response to pro-inflammatory cytokines and growth factors.[34,35,53] The predominant stimulators of COX-2 expression include interleukin-1α (IL-1α), tumor necrosis factor-α (TNF-α), transforming growth factor-ß (TGF-ß), and bacterial lipopolysaccharides (LPS), although platelet-derived GF, epidermal GF, leukoregulin, and IL-1ß can also induce COX-2 and stimulate PG production.[36,41,53] The resulting prostaglandins (PGE_2, PGI_2, PGD_2) serve a primarily pro-inflammatory role in the acute phase of tissue damage by inducing hyperemia through arteriolar dilation and causing edema and hyperalgesia through synergistic effects with histamine and bradykinin.[41,51] Accordingly, the majority of the therapeutic analgesic, anti-inflammatory, and antipyretic effects of NSAID therapy have been attributed to COX-2 inhibition, and COX-1 inhibition is blamed for adverse events involving damage to the gastroduodenal mucosal barrier, renal ischemia and dysfunction, hepatotoxicity, and impaired platelet aggregation.[41,51-53,56]

In addition to its pro-inflammatory role, COX-2 is also expressed constitutively in the nervous system, reproductive tract, and kidneys, and is believed to play an important role in normal homeostatic functions in these organ systems.[36] Upregulation of COX-2 expression has also been demonstrated in experimental rodent models of gastric erosion and ulceration, indicating that COX-2 likely plays a role in the process of mucosal repair.[32,36,48,57,58] When a gastric ulcer forms, local production of PGE_2 is mediated predominantly by the COX-2 isoform, originating from fibroblasts, macrophages, and granulocytes at the base of the ulcer. These cells produce COX-2 in response to local production of IL-1 and TNF-α in the early phases of healing and TGF-ß1 in the late phases of healing.[36,39] NSAID therapy, in particular the use of COX-2 selective NSAIDs, has been shown to inhibit angiogenesis and epithelial regeneration in experimentally-induced gastric ulcers, delaying gastric mucosal repair and healing.[41]

Recent NSAID research has complicated these premises. Another potential COX isoform, COX-3 (a spliced variant of COX-1) has been identified in the CNS of the dog and may explain some of the central analgesic and anti-inflammatory effects of NSAIDs that were previously unexplained.[41,52,59] This proposed COX-3 isoform appears to be more responsive to paracetamol (acetaminophen) and dipyrone suggesting that these medications may have a relative COX-3 selectivity.[41,60] Additionally, NSAIDs such as tepoxalin that serve as dual inhibitors of COX and 5-LO appear to have a wide gastrointestinal safety margin despite being relatively COX-1 selective, raising the question of what role 5-LO plays in the therapeutic effects of dual inhibitors. It has been proposed that 5-LO inhibition diminishes the production of leukotrienes that may

contribute to gastrointestinal mucosal ischemia and inflammation through their vasoconstrictive and chemoattractant effects.[41,42]

Leukotrienes (LTs) are eicosanoids that are synthesized through lipoxygenase metabolism of AA. They are primarily synthesized by inflammatory cells, have primarily detrimental effects on the gastrointestinal mucosa, and can be subdivided into two subgroups: peptidoleukotrienes and LT-B4.[39] Peptido-leukotrienes (LT-C4, LT-D4, and LT-E4) are synthesized predominantly by mast cells and promote mucosal injury by increasing vascular endothelial permeability, activating mast cells, promoting expression of leukocyte adhesion molecules (P-glycoprotein), and stimulating smooth muscle contraction. LT-B4 is synthesized by neutrophils and functions to activate and recruit more neutrophils, thereby perpetuating inflammation.[61] Inhibition of COX enzymes by NSAIDs leads to upregulation of LT production, contributing to NSAID-mediated gastrointestinal mucosal injury.

Other factors that may contribute to gastric mucosal injury include thromboxane and platelet activating factor. Thromboxane is an eicosanoid that is produced through platelet-associated COX-1 activity. Thromboxane-mediated mucosal ischemia is mediated by stimulation of platelet aggregation in conjunction with potent vasoconstrictive effects.[39] The ulcerogenic properties of platelet activating factor are thought to be due to stimulation of smooth muscle constriction as well as neutrophil activation and eosinophil chemotaxis.[39]

b. Pathophysiology of gastrointestinal toxicity of Aspirin

The adverse gastrointestinal effects of aspirin result from direct and indirect toxicity. When the gastric pH is <3.5, aspirin becomes non-ionized and lipid soluble and is absorbed by gastric mucosal cells where it becomes trapped within the cell as an ionized salt.[12] This alters cellular metabolism and membrane permeability, leading to back-diffusion of hydrogen ions from the gastric lumen into the cell.[12] Impairment of ion transport mechanisms and loss of electroneutrality lead to cellular swelling and death. Histamine is released, mucosal inflammation worsens, and the integrity of tight junctions between mucosal cells is reduced, leading to hemorrhage, erosion, and ulcer formation.[62]

The indirect effects result from the irreversible acetylation of cyclo-oxygenase. Aspirin is a non-selective NSAID (IC_{50} COX-1:COX-2 ratio <0.3), inhibiting the production of protective prostaglandins in the gastric mucosa and renal vasculature and the production of thromboxane in platelets.[52,60,63] In the stomach, production of prostaglandin types E, F, and I is suppressed by aspirin administration, resulting in increased epithelial permeability, alteration of mucus composition and hydrophobicity, suppression of bicarbonate secretion, reduced ulcer healing, and suppression of mucosal immunocyte function.[9,11,12,34,35,64-67] This combination of disrupted mucosal barrier, hyperacidity, and topical irritant effects of aspirin leads to mucosal injury, while the reparative abilities of the mucosa are concurrently compromised. These factors culminate in an ulcerogenic gastroduodenal environment.

Aspirin dosing regimens vary, with therapeutic serum salicylate concentrations (5-30 mg/dL) being achieved with anywhere from 10-35 mg/kg PO q 8-12 hours in dogs.[9,11,12,64-66] At lower dosages, it may take longer to reach therapeutic serum

concentrations, as in one study, dogs receiving either plain or buffered aspirin at 25 mg/kg q 8 hours reached therapeutic serum levels within 2 hours and dogs receiving the lower dose of 10 mg/kg q 12 hours did not attain therapeutic levels until 50 hours (after 5 doses).[66] Additionally, the rate of aspirin absorption was affected by the type of preparation used, dogs receiving enteric-coated aspirin at 25 mg/kg q 8 hours did not achieve therapeutic levels until 4 hours after administration. Finally, duration of effect also appears to be affected by dosage, as dogs receiving the lower dose of buffered or plain aspirin (10 mg/kg q 12 hours) only maintained therapeutic levels for 4 hours after each dose.[66] In this study, gastric mucosal hemorrhages were only identified in the plain aspirin group that received 25 mg/kg q 8 hours, likely reflecting the direct cytotoxicity of this preparation.

c. Aspirin and platelet function

Aspirin irreversibly inhibits cyclo-oxygenase activity, thereby inhibiting the catalytic conversion of arachidonic acid to PGH_2, the precursor to thromboxane-A_2 (TXA_2), PGD_2, PGE_2, PGE_{2a}, and PGI_2.[16] Platelet-associated COX-1 converts PGH_2 to TXA_2, and TXA_2 then stimulates the formation of a platelet plug through induction of platelet aggregation and local vasoconstriction.[16] A counter-regulatory prostaglandin, prostacyclin (PGI_2) is produced by vascular endothelium-associated COX-2. Prostacyclin inhibits platelet aggregation and induces vasodilation, thus impeding primary hemostasis. Due to the relative COX-1 selectivity of aspirin and the ability of vascular endothelial cells to rapidly replace the inhibited COX-2 enzyme, the inhibitory effects on TXA_2 production predominate.[16]

d. Studies demonstrating aspirin-induced gastrointestinal injury

Aspirin has consistently been shown to induce gastroduodenal injury in dogs at anti-inflammatory dosages (10-35 mg/kg every 8 hours), with lesions ranging from mucosal hemorrhages to perforating ulcers at higher doses.[9,11,13,66,68-71] Several studies have demonstrated aspirin-induced lesions throughout the stomach, duodenum, and in one study throughout the small intestines and involving ileocolic junction.[9,11,13,66,68-71] Aspirin injures the gastric mucosa in a dose-dependent manner, however, in humans, a phenomenon known as gastric adaptation occurs with chronic administration.[72] The underlying mechanisms involved in gastric adaptation are poorly understood but is believed to occur in two phases: an initial stabilization phase characterized by restitution (cell migration from underlying layers) and a late stage where mucosal healing occurs despite ongoing aspirin administration. Although gastric adaptation has been proven to occur in humans and in two early studies involving dogs[64,65,67], subsequent canine studies failed to support this finding [69,70].

Cimetidine, a histamine$_2$ receptor blocker, given at doses of 7.5-10 mg/kg every 8 hours failed to prevent GI hemorrhage in dogs in two studies and also failed to have an effect on the healing of mechanically-induced gastric ulcers in aspirin-treated dogs.[9,68] Furthermore, omeprazole, a gastric proton pump inhibitor (PPI), 0.7 mg/kg/day, also failed to improve gastritis or ulcer healing in aspirin-treated dogs.[68] This is in contrast to findings in human patients, where PPIs and H2 blockers have been shown to significantly reduce the risk of gastrointestinal hemorrhage in NSAID users.[73-75] A recent study evaluating the effect of another histamine$_2$ receptor blocker, famotidine, on canine gastric blood flow, measured by laser Doppler flowmetry, found that 0.5 mg/kg of famotidine

administered concurrently with the non-selective NSAID, diclofenac (1 mg/kg per rectum), resulted in significantly better preservation of gastric mucosal blood flow when compared with placebo.[76] In this same study, mucosal prostaglandin E_2 concentrations were not significantly different from placebo treated dogs, indicating that the difference in blood flow was mediated by factors other than prostaglandin synthesis. The exact mechanism is poorly understood, however, there is some evidence, in a rat model, that diclofenac only decreases gastric mucosal blood flow in the presence of gastric acid.[77]

In contrast, a number of studies have demonstrated that the administration of the synthetic prostaglandin misoprostol, at a dose of 2-7.5 mcg/kg every 8-12 hours, significantly decreased the incidence and severity of gastritis in dogs receiving 25-35 mg/kg of aspirin every 8 hours.[11,13,78,79] Dogs treated with misoprostol and aspirin had fewer and less severe gross mucosal lesions and experienced significantly fewer episodes of vomiting than those treated with aspirin alone.[11,13,78,79] These findings further support the role of prostaglandin inhibition in aspirin-induced gastritis and ulceration.

ii. Corticosteroids

a. Immunosuppression

The immunosuppressive effects of corticosteroids are utilized in the clinical management of a variety of immune-mediated and autoimmune diseases. Corticosteroids influence cell-mediated immunity by altering signal transduction, cytokine production, and lymphocyte effector mechanisms. They also inhibit neutrophil emigration into inflamed tissues, suppress neutrophil, monocyte, and eosinophil chemotaxis, and suppress the cytotoxic and phagocytic abilities of neutrophils.[80] Additionally,

corticosteroids are believed to suppress cytokine production by macrophages and T cells, suppress lymphocyte proliferation, and induce thymocyte apoptosis.[80] In the acute phase of inflammation, synthetic corticosteroids can be used to suppress hyperemia and edema. They inhibit lysosomal enzyme release from and antigen processing by macrophages, and they suppress the effects of tissue phospholipases, thus inhibiting eicosanoid production.[80] Through these mechanisms, the immune response is impaired and the adrenal axis is suppressed. The recommended immunosuppressive dose of prednisone for dogs is 2.2-6.6 mg/kg/day.[80] This dosage is typically maintained for weeks then gradually tapered over weeks to months if the desired affect is achieved. Dosage reduction allows the pituitary-adrenal axis to rebound from iatrogenic suppression.

b. Pathophysiology of corticosteroid-mediated gastrointestinal toxicity

Corticosteroids are believed to cause gastrointestinal ulceration through indirect effects on the gastrointestinal mucosa, similar to aspirin-induced injury. Corticosteroids inhibit prostaglandin synthesis by strongly blocking mRNA transcription for COX-2 (with minimal COX-1 blockade) and inhibiting phospholipase-A activity, thereby interrupting the synthesis of arachidonic acid from phospholipid precursors.[1,36,81] As a result, the composition of gastric mucus is altered and the quantity of mucus production, the rate of mucosal cell turnover and bicarbonate production is decreased, mucosal bloodflow is compromised, and restitution is inhibited.[49,50,81,82] Glucocorticoids also inhibit capillary and fibroblast proliferation and enhance collagen breakdown in the late stages of inflammation, resulting in delayed tissue healing.[1,49,50,80,81] Glucocorticoids delay mucosal healing, therefore, when the rate of mucosal damage exceeds the

regenerative ability of the gastrointestinal mucosa, mucosal hemorrhages, erosions, and/or ulcers may form.[49,50,83-86] These effects are exacerbated when glucocorticoids and NSAIDs are used concurrently at therapeutic doses.[1,87-89]

c. Studies demonstrating corticosteroid-induced gastrointestinal injury

A number of studies have documented the adverse effects of NSAIDs and corticosteroids on the gastric, duodenal, and, less commonly, colonic mucosa, when they are used alone, or in combination. In 1983, Sorjonen et al compared the effects of hypotension and dexamethasone administration on gastric mucosa of clinically normal dogs that underwent hemilaminectomy and found that the severity of gastric hemorrhage in dogs treated with dexamethasone (2.2 mg/kg SC q 12 hrs for 8 days) with or without hypotension exceeded that of dogs that had surgical hypotension alone.[86] In another study, methylprednisolone sodium succinate, when given at high doses (30 mg/kg IV once, followed by 15 mg/kg at 2 and 6 hours, then every 6 hours until 48 hours), induced gastric hemorrhages in all 10 healthy dogs, and the hemorrhages were classified as severe in 9 of these dogs.[84] The same group of investigators evaluated the ability of misoprostol (4-6 ug/kg PO TID) to prevent hemorrhagic gastritis and did not demonstrate a protective effect.[85] In 2000, Neiger et. al. found that 19/25 dogs with intervertebral disc disease who were treated with a single dose of dexamethasone (2 mg/kg IV) had endoscopically detectable gastric mucosal lesions (Day 0). These dogs continued to receive prednisone (2 mg/kg on Day 1, 1mg/kg on Day 2, and 0.5 mg/kg for the remainder of hospitalization) and were divided into three groups, one control, one treated with omeprazole (0.7 mg/kg q24 hours), and one treated with misoprostol (2 ug/kg PO TID).

At the time of endoscopic re-evaluation 5-6 days later, no significant differences were found between treatment groups, indicating that neither misoprostol nor omeprazole provided a benefit at these doses. It was unclear from this study whether dexamethasone or prednisone had the more deleterious effect on the gastric mucosa.[83] Finally, perforation of the proximal descending colon on the antimesenteric border resulting in a universally fatal septic peritonitis was reported in 13 dogs that were treated with corticosteroids, with (4 dogs) or without (9 dogs) concurrent NSAIDs, perioperatively for a variety of neurologic (11 dogs) and non-neurologic conditions (2 dogs). The most common corticosteroid administered to these dogs was dexamethasone at a mean cumulative dose of 6.4 mg/kg over an average of 5.1 days following surgery. Clinical signs of vomiting, anorexia, and depression developed an average of 22 hours before death in each of these dogs.[90] In these dogs, lesions were confined to focal necrosis and perforation of the antimesenteric border of the colon near the left colic flexure in all but one dog; that dog that had mucosal hemorrhages diffusely affecting the entire gastrointestinal tract on necropsy.[90]

d. Studies demonstrating gastrointestinal toxicity associated with combination therapy with corticosteroid and NSAIDs

The deleterious effects of corticosteroids combined with NSAIDs have been demonstrated with different NSAIDs that have a variety of COX selectivity profiles. In a study by Dow et al in 1990, the gastrointestinal effects of flunixin meglumine, a COX-1 selective NSAID with IC50 COX-1:COX-2 ratio of 0.7, administered at two different doses (1.1 or 2.2 mg/kg IM q 12h) was compared to flunixin and prednisolone (flunixin

1.1 mg/kg IM q 12h and prednisone 0.55 mg/kg PO q 12h) over a 10 day period.[88] Endoscopic evaluation was performed before the experimental phase and every other day throughout the study period. There were five dogs per group: 1) lower dose flunixin; 2) higher dose flunixin; 3) flunixin + prednisone; 4) placebo control. A more rapid onset of more severe gastric lesions was noted in Group 3, with numerous hemorrhages and erosions in the antrum and pylorus of all dogs by Day 2, progressing to shallow ulcers by Day 4. All dogs in Group 2 developed visible gastric lesions by Day 4 of the study, whereas dogs in the Group 1 developed lesions by Day 6. Additionally, fecal occult blood, diarrhea, and gastrointestinal signs (inappetence, vomiting, diarrhea, melena) developed more rapidly and were more severe in dogs from Group 3. Finally, on post-mortem histologic evaluation of the gastrointestinal mucosa, lesions (ulcers, erosions, and hemorrhages) were most severe in Group 3, followed by Group 2 and then Group 1, indicating that flunixin meglumine causes gastrointestinal injury in a dose-dependent manner and these adverse effects are potentiated by concurrent prednisone administration.[88] Despite these observations, statistical differences between the groups were not found, likely due to a combination of small group sizes and the implementation of a limited 4-point endoscopic scoring system[55].

Boston et al examined the gastrointestinal effects of short term administration of both meloxicam, a COX-2 preferential NSAID (IC_{50} COX-1:COX-2 ratio of 3), and dexamethasone individually and in combination in 2003.[87] Four groups of 5 healthy dogs were used: 1) Saline-saline, 2) Dexamethasone (0.25 mg/kg SC q 12h)- saline, 3) Saline-meloxicam (0.1 mg/kg SC q 24h), 4) Dexamethasone (0.25 mg/kg SC q 12h)-meloxicam (0.1 mg/kg SC q 24 hrs). The groups were administered either saline or dexamethasone

on Days 1, 2, and 3 and either saline or meloxicam on Days 2, 3, and 4, and a sham electrostimulation operation was performed on Day 2, to simulate a case of IVDD that would be treated with dexamethasone on admission, followed by surgery and post-operative analgesia with meloxicam. Gastroduodenoscopy was performed 7 days before the study and on Day 5, utilizing the same 4-point scoring system previously described.[55] No differences were noted between the groups regarding the presence of fecal occult blood. Adverse gastrointestinal signs (vomiting, inappetence, diarrhea, melena, abdominal pain) were not seen in any of the groups at any time. The dogs in the dexamethasone-saline and dexamethasone-meloxicam groups had significantly more severe gastroduodenal lesions than the saline-saline and saline-meloxicam groups, and the dexamethasone-meloxicam group had more severe gastroduodenal lesions than the dexamethasone-saline group, suggesting a synergistic effect.[87] There were no statistical differences in the endoscopic scores between the saline-saline and saline-meloxicam group, indicating that short term administration of meloxicam alone was safe for the gastrointestinal tract. A recent study comparing endoscopic scores (gross mucosal lesions), fecal occult blood tests, and gastrointestinal signs in dogs administered ketoprofen plus prednisolone (KP) and meloxicam plus prednisolone (MP) documented a significant difference between the KP group when compared with the MP and control group (NC).[1] Six healthy dogs were allocated to each group: 1) KP receiving 0.25 mg/kg ketoprofen PO q 24 hours and prednisolone 0.5 mg/kg PO q 24 hours for 30 days; 2) MP receiving 0.1 mg/kg meloxicam PO q 24 hours and prednisolone 0.5 mg/kg PO q 24 hours for 30 days; 3) Control group receiving 2 placebo capsules q 24 hours for 30 days. Endoscopic evaluation was performed weekly throughout the study, and fecal occult

blood tests were performed on the first and last days of the study. All dogs in the KP group had moderate to severe gastric mucosal lesions by day 28. Four of 6 dogs in the KP groups had ulcers in the gastric body or antrum, and the other two dogs had invasive erosions, defined as mucosal epithelial defects with depth and breadth greater than a pinhead-sized mucosal discontinuation (punctuate erosion) but not as wide or deep as an ulcer, and extensive gastric mucosal hemorrhage particularly in the pyloric antrum. No significant differences between endoscopic scores were found between the MP and NC group, however, the gastric lesions in the KP groups, particularly in the body-pyloric antrum regions, were significantly more severe than both the MP and NC groups. Additionally, positive fecal occult blood test (FOBT) results were significantly more common in the KP group (all 6 dogs tested 2 or higher, with 4/6 scoring 3-4 on a scale of 4) than the both the MP (3 dogs had a grade 1 positive FOBT) and NC (1 dog had a grade 1 positive FOBT) groups.[1] All dogs in the KP group had intermittent inappetence or vomiting and 4 had small bowel diarrhea or melena during the study. Based on these findings, the authors concluded that these medications at these dosages should not be used in dogs.

Finally, in a retrospective study evaluating dogs with gastrointestinal perforation that received deracoxib, a COX-2 selective NSAID of the coxib class, 16/29 received deracoxib at a higher than recommended dosage, and 26/29 were given another NSAID or corticosteroid within 24 hours of deracoxib administration.[89] In this report, 3/29 (10%) of the dogs receiving deracoxib developed perforating ulcers at the recommended dose. This occurred without concurrent NSAID or corticosteroid use or a predisposing medical cause for gastrointestinal injury.

D. Coagulation and Thromboembolic Events: Effects of Aspirin and Prednisone on Platelet Function and Coagulation

i. Thromboembolic disease and aspirin therapy

Aspirin modulates thrombotic events by irreversibly acetylating serine residues on platelet COX-1 leading to decreased thromboxane-A2 synthesis, thereby impairing platelet function in a dose-dependent manner.[4,22] In humans, low dose aspirin therapy has been proven to reduce thromboembolic disease in patients suffering from a spectrum of disease processes, including cardiovascular and renal disease.[7,8,91,92] However, because aspirin is a non-selective COX inhibitor, it may cause gastrointestinal hemorrhage and ulceration at doses of 10-35 mg/kg q 8 hours.[9,11,13,60,69,70,93] Ultralow-dose aspirin, at a dose of 0.5 mg/kg/day, may provide the benefits of modulating platelet aggregation while minimizing the detrimental effects of COX-1 suppression on gastric mucosal integrity.[4,7,8,14]

A number of diseases have been associated with prothrombotic tendencies in dogs, leading to the prophylactic use of antithrombotic therapies. These recommendations are based on limited evidence in dogs, and based on recommendations extrapolated from humans. Aspirin, at dosages ranging from 0.5-5 mg/kg q 12-24 hours, has been recommended to reduce platelet aggregation and thromboembolism in dogs, providing an alternative to other antithrombotic therapies that may be more expensive and/or require more intensive monitoring (e.g. heparin, low-molecular weight heparin, and warfarin).[4,16,22] However, despite these recommendations, only one study exists comparing the effect of three different aspirin dosing regimens on canine platelet

aggregation. This study indicated that once daily ultralow-dose aspirin was ineffective at inhibiting in vitro platelet aggregation, but twice daily dosing resulted in a statistically significant decrease in platelet aggregation.[94] However, it is possible that ultralow-dose aspirin has other in vivo effects on hemostasis. Further investigation into the in vivo effects of ultralow-dose aspirin are warranted to determine the ideal dosing regimen for thromboprophylaxis, by evaluation of thromboxane-A_2 and prostacyclin levels and effects on thromboelastography.

One disease associated with a relatively high incidence of thromboembolism is immune-mediated hemolytic anemia (IMHA). In addition to complications resulting from severe acute anemia due hemolysis, at least 30% of IMHA patients have clinically relevant thromboembolic events, involving primarily the lungs.[2,3,95] Two retrospective analyses evaluating prognostic factors for dogs with IMHA found that thromboembolic disease was far more prevalent, identifying histologic evidence of thromboembolic disease in 80-100% of necropsied IMHA patients, with emboli localized primarily in the spleen and the lungs but also in the heart, liver, kidneys, lymph nodes and pituitary gland.[2,4] The presumed mechanisms of hypercoagulability in IMHA patients are numerous, including release of procoagulatory cytokines from activated leukocytes and endothelial cells, upregulation of platelet surface expression of P-selectin, exposure to lysed RBC membranes, the circulation of free hemoglobin in conjunction with endotoxin from gastrointestinal ischemia and gastric mucosal disruption, neutralization of anticoagulant nitric oxide by circulating free hemoglobin, and the possible presence of antiphospholipid antibodies in circulation.[2,4,96-98] Thrombocytopenia has been reported to occur in 29-70% of dogs with IMHA and has been associated with the development of

thrombosis, thromboembolic events, and patient mortality.[2,99] Numerous hemostatic and hematologic abnormalities that are linked to this prothrombotic state have been noted in dogs with IMHA. Up to 60% of IMHA dogs are reported to be in DIC at the time of diagnosis with a spectrum of hemostatic abnormalities including prolonged PT in 10-46%, prolonged PTT in 45-67%, increased fibrinogen in 34-85%, increased FDPs in 57-60%, increased D-Dimer in 80%, and thrombocytopenia in 70-80% of the dogs.[2,100,101] Additional factors that are significantly associated with an increased risk for thrombosis or thromboembolism include presence of an indwelling intravenous catheter and severe thrombocytopenia (defined as <50,000/μL), hyperbilirubinemia (>5 mg/dL), hypoalbuminemia, and increased alkaline phosphatase activity at the time of diagnosis.[2,3,99] Thromboembolic disease contributes greatly to the morbidity and mortality of dogs with IMHA, and various medical treatments have been used to decrease the incidence and severity of thromboembolic events.[2,4,16,98,101]

In one retrospective study evaluating prognostic factors, survival rates, and treatment protocols for 151 dogs with IMHA, those treated with prednisone, azathioprine, and ultralow-dose aspirin (with or without mixed-molecular-weight heparin) had a significantly higher survival rate than those treated with prednisone and azathioprine with or without mixed-molecular-weight heparin. As a result of this retrospective analysis, aspirin therapy, at a dose of 0.5 mg/kg/day, is currently being recommended in dogs with IMHA.[4,16] Clinical evidence of adverse effects of ultralow-dose aspirin when administered in conjunction with immunosuppressive doses of glucocorticoids has not been recognized[4], however, this treatment protocol is relatively new and epidemiologic

data evaluating adverse events in a large population of dogs treated in this manner is not yet available.

ii. Hypercoagulability associated with hypercortisolemia

Immunosuppressive (2.2-6.6 mg/kg/day) dosages of prednisone are usually employed as first line therapy in dogs with IMHA patients, and is often continued for several months. Common side effects associated with prednisone administration (polyuria, polydipsia, polyphagia, muscle wasting, fat redistribution, pot belly, etc.) affect morbidity. It is likely that hemostatic derangements, similar to those that have been documented in spontaneous canine hyperadrenocorticism (HAC), also exist in the iatrogenic condition. Levels of procoagulant factors II, VII, IX, X, XII, fibrinogen, and platelet-derived factors V and vWF are increased, antithrombin III levels decreased, and thrombin-antithrombin complexes increased (indicative of subclinical thrombosis) in dogs with naturally occurring hyperadrenocorticism.[38,102,103] Human patients with hyperadrenocorticism have been shown to have a four-fold increased risk of thromboembolic events and deep vein thrombosis. The hemostatic derangements documented in dogs with hyperadrenocorticism are similar to those found in humans.

More than 50% of dogs with naturally-occurring HAC are hypertensive, a factor that has been shown to contribute to thromboembolic events in humans with HAC.[102,103] The pathophysiology of HAC-associated hypertension is believed to be multifactorial, involving activation of the renin-angiotensin system, excessive renin secretion, reduced production of vasodilatory prostaglandins, increased vascular sensitivity to catecholamines, increased mineralocorticoid secretion, and secondary renal disease.[102,103]

Glomerular disease is a relatively common sequela to untreated hyperadrenocorticism, with proteinuria (UPC (urine protein:creatinine ratio) >1) occurring in 44-75% of dogs with HAC. While the underlying mechanism is unclear, glomerular disease may develop as a result of altered immune complex solubility (due to relative antigen excess), increased incidence of chronic infections due to immunosuppression, and decreased clearance of immune complexes by the reticuloendothelial system.[103,104] Additionally, systemic hypertension associated with HAC can contribute to secondary glomerular damage, including glomerulosclerosis which may result in altered glomerular pore size and permselectivity.[22,102,103] Renal sodium retention, activation of the renin-angiotensin-aldosterone system, increased production of endothelin, and nitric oxide deficiency in dogs with glomerular disease further exacerbate systemic hypertension, creating a viscious feedback loop of hypertensive stimuli. The propensity for thromboembolic events is a serious complication in dogs with glomerular disease, occurring in at least 13% of dogs suffering from protein-losing nephropathies.[22,104,105] This hypercoagulable state results from loss of antithrombin III, increased thromboxane production, hypercholesterolemia-induced platelet hypersensitivity, thrombocytosis, hyperfibrinogenemia, and multiple other poorly-defined factors, including the production of procoagulant cytokines and coagulation factors V, VII, VIII, and X.[22,104-107] However, this hypercoagulable state is most commonly associated with severe proteinuria. The glomerular disease associated with HAC is typically more mild, in the UPC range of 1-5, and its contribution, if any, to thromboembolic sequelae is uncertain.[22,103]

Concern exists regarding the potential prothrombotic state that long term administration of immunosuppressive glucocorticoid dosages may induce. This concern

is even greater in dogs with prothrombotic diseases, such as IMHA. An improved prognosis has recently been documented with the use of ultralow-dose aspirin in conjunction with standard immunosuppressive therapy (prednisone and azathioprine) in patients with IMHA.[4] The presumption in this study was that improved survival was the result of effective thromboprophylaxis, but platelet function tests were not performed. Furthermore, in this study necropsies were performed on only 9 of 37 patients that died or were euthanized in the hospital, and all of those dogs had evidence of thromboembolic disease involving the spleen, lungs, heart, kidney, and/or lymph nodes. Unfortunately, there was no mention of the specific treatment given to any of these 9 dogs.

CHAPTER II: Effects of Prednisone Alone or Prednisone with Ultralow-Dose Aspirin on the Gastroduodenal Mucosa of Healthy Dogs

A. Material and Methods:

Eighteen young adult random source dogs (9 male, 9 female) with a median age of 14 months (range 12-24 months) and a mean weight of 15.6 kg (range 12.2-17.9 kg) were acclimated to research housing conditions for 2 weeks. The dogs were entered into the study based on a normal physical examination, CBC, serum biochemical profile, and urinalysis. Ivermectin (200 ug/kg SC once) and fenbendazole (50 mg/kg PO q 24h for 3 consecutive days, repeated in 2 weeks) was administered to all dogs except those with a herding-type appearance (2 dogs, both from the placebo group, Group NN), which received fenbendazole treatment only.[108] All dogs had a negative zinc sulfate fecal flotation prior to the first gastroduodenoscopy. This study was approved by the

Institutional Animal Care and Use Committee (IACUC Approval #07-024-CVM), Virginia Polytechnic Institute and State University.

The study period followed a two week acclimation period. The study period was subdivided into two periods: Period 1 and Period 2. Period 1 commenced 10 days (Day -10 through Day -1) prior to the initiation of oral medications (starting on Day 1). Period 2 consisted of 27 days (Day 1-Day 27) during which all dogs received oral medications or placebos according to their respective treatment groups. Dogs were observed every 8 hours, starting 10 days before initiation of the treatment period and throughout treatment. The number of bowel movements was recorded and the presence of melena, hematochezia, or mucus was noted. Feces were graded from 1 to 5 using a previously described scale[70], with scores <4 considered to be diarrhea. Vomiting episodes and the presence of hematemesis were recorded. A dog-day of diarrhea or vomiting represented any day in which one or more episodes of diarrhea or vomiting was observed in a dog. Dogs were fed a commercial maintenance cereal based dog food[i] twice daily according to manufacturer's recommendations. Each dog's appetite was scored from 1 to 3 each day; 1 was assigned when all food was consumed, 2 for decreased appetite, and 3 for anorexia.

Dogs were anesthetized for gastroduodenoscopy, photography, and lesion scoring 7 days before drug administration, and 5, 14, and 27 days after initiation of drug administration. Atropine sulfate (0.05 mg/kg IM) and acepromazine (0.1 mg/kg IM) were used as premedications, an intravenous catheter was placed, and anesthesia was induced with thiopental (10-15 mg/kg IV to effect). Dogs were intubated and general anesthesia was maintained with isoflurane in oxygen, and 0.9% NaCl solution was

delivered intravenously at 10 mL/kg/hour. The dogs were placed in left lateral recumbency and gastroduodenoscopy was performed, as previously described, in a manner as not to create iatrogenic lesions prior to endoscopic scoring.[11,13,69,70]

Upon entry into the stomach, the endoscope was advanced into the gastric body, and air insufflated to distend rugal folds and allow lesion scoring of the gastric body. The gastric body was evaluated as the endoscope was advanced along the greater curvature to the level of the pyloric antrum. After the antrum and pylorus were visualized, the endoscope tip was partially retroflexed to further evaluate the angularis incisura and the lesser curvature. The endoscope was then fully retroflexed to evaluate the gastric cardia. The tip of the endoscope was straightened and advanced through the pyloric sphincter and into the proximal descending duodenum, which was also scored. The endoscope was then retracted into the body of the stomach where brush cytology samples were obtained to identify the presence or absence of spiral bacteria (on day -7 only).

During visual evaluation, each region of the stomach (gastric body, pyloric antrum, angularis incisura, and cardia) and the duodenum was photographed using a still video recorder[ii] and gastric lesions were scored by an experienced observer who was unaware of the treatment groups [109]. Scores were assigned (1-11; Table 1) to each region based on a previously described scale (Table 1).[11,13,69,70] Mucosal hemorrhages were defined as small, reddened hemorrhagic lesions with intact mucosa (Figure 1). An erosion was defined as a defect in the mucosal epithelium (Figure 2). An ulcer was defined by a wide mucosal defect with depth in the center and raised margins (Figure 3).

Each region was scored based on the most severe lesions present. Scores for each region were summed, and a total score was given to each dog for each endoscopic examination.

Gastric mucosal brushing samples obtained at baseline (on Day -7) were applied to glass slides, stained using Dip Quick stain[iii], and viewed under 100X magnification for presence of spiral bacteria consistent with *Helicobacter* spp.[110] Since spiral bacteria were observed in all 18 dogs, stratification of groups based on *Helicobacter* spp. status was not necessary.

On day 0, 18 dogs were randomly divided into three groups of 6. Each dog in the placebo group (Group NN) was administered two placebo capsules (containing sucrose powder) and a placebo solution (0.05 mL/kg PO q 24 hr of an almond oil and sucrose powder mixture, identical in appearance to the aspirin-containing solution administered to Group PA). The prednisone group (Group PN) was administered a mean dosage 2.3 mg/kg (range 2.0-2.4) of prednisone (two 17.5 mg compounded capsules orally q 24h and 0.05 ml/kg of the same placebo solution as Group NN. The prednisone and ultralow-dose aspirin group (Group PA) was administered prednisone (two 17.5 mg compounded capsules, mean dosage 2.3 mg/kg [range 2.2-2.5]) orally q 24h and aspirin (0.5 mg/kg of aspirin in the form of an almond oil-based aspirin solution [10 mg/mL], PO q 24h). Medications were given for 27 days. Dogs were weighed weekly and changes in aspirin dosing were made as needed. All dogs receiving prednisone during the treatment period (Groups PN and PA) were administered 5 mg of prednisone per day (mean dose = 0.329 mg/kg, range 0.284-0.379) for 4 days after the last endoscopic evaluation then 5 mg every other day for 4 more doses prior to discontinuing administration entirely. Aspirin

administration was halted immediately in the prednisone and aspirin group (PA) after the final endoscopic evaluation on Day 27.

B. Summary of Statistical Methods:

Both endoscopy scores and clinical signs were summarized as medians surrounded by a range.

Clinical Signs: For each combination of the clinical sign (Vomiting, Diarrhea, or Inappetence) and periods (period 1[Day -10 through Day -1] or period 2 [Day 1- Day 27]), median scores were compared between the 3 treatment groups using the Kruskal-Wallis test. Furthermore, for each combination of clinical sign and treatment group (eg, dog days of vomiting in placebo group), the median scores were compared between period 1 and period 2 using the Friedman's Chi-square test while controlling for dog as a blocking factor (because the length of period 1 was 10 days, and the length of period 2 was 27 days, all scores in period 1 were multiplied by 2.7 before making this comparison).

Endoscopy scores: For each combination of region (body, angularis, pylorus, cardia, duodenum, or total score) and day of endoscopy (-7, 5, 14, or 27), median scores were compared between the 3 treatment groups using the Kruskal-Wallis test. Furthermore, for each combination of region and treatment, the median scores were compared between days of endoscopy using the Friedman's Chi-square test while controlling for dog as a blocking factor. Statistical significance was set to alpha<0.05. All analyses were performed using SAS version 9.1.3[iv].

C. Results:

There were no significant differences in regional or total endoscopic scores between groups at any time point (Appendix 1, Table 2, Figure 10). Additionally, no significant change in regional or total endoscopic scores was noted over time (i.e. between Day -7, 5, 14, 27) within any of the groups (Appendix 1, Table 4, Figure 10). Median total endoscopic scores on Day -7 were 5 for all three treatment groups, $p = 1$. Median total endoscopic scores on Day 5 were 5, 6, and 5 for groups NN, PN, and PA respectively, $p = 0.1629$. Median total endoscopic scores on Day 14 were 5, 5.5, and 5 for groups NN, PN, and PA respectively, $p = 0.8857$. On Day 27, median total endoscopic scores were 5 for all groups, $p = 0.3007$.

Median regional scores did not differ between groups at any time point, nor was there any significant change in median regional scores within any of the groups over time (Appendix 1, Tables 2-4). The median score for each region in each treatment group at each time point was 1 (Appendix 1).

Foreign bodies were identified in two dogs during endoscopic evaluation. An accumulation of approximately 5-8 small (<2 cm) pieces of green plastic bedding (later identified as material that had been chewed from the underside of the elevated dog bed in the subject's run) were identified at Day -7 in the pylorus of one dog (dog 651) in the aspirin and prednisone group (Figure 4). No gastroduodenal lesions were noted at that time, and the authors believed that this material would pass spontaneously, so the foreign matter was not endoscopically removed at that time. However, on endoscopic evaluation of the same dog on day 5, the foreign material was still present and hemorrhages and erosions were noted throughout the angularis, pyloric antrum, cardia, and duodenum, and

the total endoscopic score was 23. Sixteen pieces of hard green plastic bedding ranging from 1-4 cm in size were removed from the stomach of dog 651 using a rat-toothed forceps (Figure 5). Nine days later, on Day 14, all but one erosion in the cardia had resolved, resulting in a total endoscopic score of 9 (Figure 6). On Day 27, one wire spring from a ballpoint pen was found in the stomach of dog 816 from the prednisone group (PN), and a single linear erosion in the cardia of this dog was attributed to superficial trauma caused by the spring (Figures 7 & 8). Follow-up evaluation of this erosion was not performed, as it was detected on the last day of the study.

On the second day of observation (Day -16, 6 days before the start of Period 1), two of the study dogs (Dog 761 from Group PA, Dog 166 from Group PN) got out of their runs and fought, leaving Dog 761 with a wound on her left forelimb that required cleaning, bandaging, and antimicrobial therapy (amoxicillin 15 mg/kg PO BID X 5 days). The wound healed without complications.

During Period 1, Dog 829 from Group PA accounted for 5 of the 6 dog days of diarrhea noted. A second zinc sulfate flotation, a fecal direct saline smear, and *Clostridium perfringens* enterotoxin assay were performed, but an etiology was not identified.

Two dogs vomited (both from group PN) during Period 1 and one dog (from group NN) vomited during Period 2. There were no differences between any of the groups regarding the number of dog days of vomiting throughout the study period ($p=0.3679$), and all vomiting events were self-limiting. Additionally, there were no differences between the number of dog days of vomiting within each group when comparing Period 1 and Period 2 (Appendix 2, Table 6).

There were no significant differences in dog days of diarrhea between any of the groups at any point during the treatment period (Period 2), $p = 0.1917$ (Table 5, Appendix 2). There was a significant increase in the number of dog days of diarrhea within the prednisone and aspirin group (Group PA) between period 1 (median = 0, range 0-5) and period 2 (median = 2.5, range 0-18) with $p = 0.0253$ (Table 6, Figure 11, Appendix 2). There appeared to be a 1 week delay during Period 2 before the increase in dog days of diarrhea was noted in the prednisone and aspirin group, with only 1 dog day of diarrhea during the first week of Period 2, 13 dog days during the second week, 12 dog days during the 3rd, and 13 dog days during the 4th. In the prednisone and aspirin group, one dog (dog 611) had 18 of 39 days of diarrhea and a second dog had (dog 829) 15 of 39 days of diarrhea during the treatment period (Period 2). Dog 829 was the same dog that had 5 of the 6 dog days of diarrhea during Period 1. The median grade of diarrhea for both of these dogs was grade 3, and tenesmus, mucus, hemotochezia, or melena was not noted. At no point during the study period did any dog show signs of dehydration, depression, lethargy, or inappetance. Diarrhea resolved within 5 days of decreasing the prednisone dose to 5 mg/day and discontinuing aspirin administration.

Neither inappetence nor anorexia was noted in any of the dogs at any time point in the study (Appendix 2). Additionally, weight fluctuations were minimal throughout the course of the study (Appendix 3), leading to relatively minor adjustments in aspirin dosing to achieve the target daily dose of 0.5 mg/kg/day.

D. Discussion:

Neither gastroduodenal lesion scores nor clinical signs differed significantly between the prednisone and aspirin group (PA) and the prednisone alone group (PN) at any time point, and neither group differed significantly from the placebo (NN) at any time during the study. Based on these results, prednisone at a dose of 2.2 mg/kg/day with or without ultralow-dose aspirin (0.5 mg/kg/day) may be safely used for a duration of 27 days in healthy adult dogs. Despite this finding, it is important to note that individual dogs from each group developed gastric and/or duodenal mucosal lesions of varying severity at one point or another during the Period 2 (Table 3). Lesions developed in 2 dogs from the placebo group, 3 dogs from the prednisone and aspirin group (one dog had lesions on Days 5, 14, and 27), all 6 dogs from the prednisone alone group. In the placebo group, both dogs had >5 submucosal hemorrhages (region score = 4) in the pyloric antrum on day 14 only. In the prednisone only group, 3 of the 6 dogs developed a regional lesion score of 6 (2-5 erosions) at one endoscopic evaluation, and the remaining 3 dogs developed only submucosal hemorrhages with region scores of 2 (1 hemorrhage) or 3 (2-5 hemorrhages). All lesions in the prednisone only group were confined to the gastric mucosa (no duodenal lesions). While fewer dogs in the prednisone and aspirin group developed lesions, the most severe lesion, a small focal ulcer (region score = 8) in the gastric cardia, was found on Day 14 in Dog 231 from this group (PA). Of the other two dogs in group PA that developed gastric or duodenal lesions, one had erosions in the gastric and duodenal mucosa receiving scores ranging between 5-7 (1 to >5 erosions) on days 5, 14, and 27, and the other dog had >5 submucosal hemorrhages in gastric body receiving a regional score of 4 on day 27. Among these lesions, no predilection site was

noted, which is consistent with some previous reports of NSAID and corticosteroid-induced gastrointestinal injury[9,11,13,89,111], but conflicts with the findings of other reports in which the pyloric antrum was most affected.[55,83,87,112,113] According to these observations, there appears to be a variable response to the administration of either prednisone alone or prednisone and aspirin, and, although no statistical significance was detected when comparing the median endoscopic scores between groups, certain individuals in both the prednisone alone and prednisone with aspirin groups developed more severe gastroduodenal lesions than those observed in any individual in the placebo group. While there is no definitive explanation for this discrepancy between individual response to prednisone with or without aspirin, it is possible that certain individuals that appeared healthy had subclinical gastrointestinal pathology that predisposed them to developing gastrointestinal lesions. Alternately, variations in pharmacokinetics, including drug absorption, metabolism, or tissue distribution may exist between individual dogs, influencing the gastrointestinal effects of these medications.

The combined or individual effects of prednisone and ultralow-dose aspirin on mucosal healing may explain our findings in dog 651 who had foreign material but no mucosal lesions in his stomach on Day -7. In hopes that this material would pass spontaneously, it was left in place and during the second endoscopic examination performed five days after initiation of prednisone and aspirin, numerous pinpoint hemorrhages and multiple erosions were noted in the stomach in the presence of this foreign material. It is feasible that the mucosal irritation induced by the foreign material was initially compensated for by the rapid rate of normal gastric mucosal healing, however, in the presence of prednisone and aspirin administration, the rate of mucosal

damage may have exceeded the compromised regenerative ability of the gastrointestinal mucosa, leading to hemorrhage and erosion formation.[49,50] However, the possibility also exists that the foreign material was either initially ingested immediately before the first endoscopic procedure on day -7 and insufficient time had elapsed to accumulate the mucosal lesions that were evident on day 5 or the dog ingested enough extra bedding in the interim between the first and second evaluation to induce gastric injury that otherwise may not have occurred in the presence of the initial amount of foreign material. Statistical evaluation of endoscopic scores was performed with these lesion scores included, and it was determined that the lesions caused by this foreign material had no significant statistical impact on the median group scores (neither total nor regional).

There was no difference in the incidence of vomiting, diarrhea, or inappetence between treatment groups at any time during the study, but there were significantly more dog days of diarrhea in the prednisone and aspirin group (PA) during Period 2 when compared to Period 1. The diarrhea was small bowel in origin and was mild with a median consistency grade of 3, and diarrhea events were infrequent overall. The median number of diarrhea events per dog day of diarrhea was 1, with one diarrhea event noted on 27 of the 39 dog days of diarrhea during Period 2, two diarrhea events per day on 7 of the 39 days, and three diarrhea events on 5 of the 39 days. None of the dogs became dehydrated, depressed, or inappetent at any time during the study, and the diarrhea resolved in all subjects within 5 days of discontinuing aspirin therapy and decreasing the prednisone dose (mean dose = 0.329 mg/kg/d X 4 days then every other day for 4 doses). This rapid response supports a causal role of the prednisone and aspirin combination in the pathogenesis of the diarrhea noted in group PA. The clinical implications of these

findings are that, while an increased incidence of diarrhea may develop in patients receiving this combination of medications, the diarrhea is likely to be mild, infrequent, not associated with significant patient morbidity, and should resolve rapidly when therapy is ceased.

While there were no grossly detectable differences in gastroduodenal mucosal integrity between our study groups, it is possible that there was mucosal damage involving more distal regions of the gastrointestinal tract beyond the reach of the endoscope. This may explain the significant increase in dog days of diarrhea noted in group PA during period 2 in the absence of significant differences in gastroduodenal score. Lesions have been identified in the large and small intestines in both dogs and humans treated with NSAIDs or corticosteroids,[51,67,90,114,115] and although no gross evidence of intestinal hemorrhage was noted, increased mucosal permeability and/or microscopic intestinal hemorrhage may have been developed in these dogs. The possibility also exists that the study groups may have had significantly different histologic changes that were not detected grossly. Although gross endoscopic appearance has been well-correlated with the presence of histologic disease in canine and feline patients with clinical gastrointestinal disease[116], histologic changes associated with aspirin-induced gastrointestinal disease in dogs have demonstrated less consistent changes relative to gross appearance[64]. Furthermore, dogs involved in this study may have had subclinically increased gastrointestinal permeability or decreased mucosal absorptive capacity in the absence of grossly evident mucosal lesions on endoscopy. Noninvasive evaluation of gastrointestinal permeability and mucosal absorptive capacity employing the oral administration of sugar probes that are differentially absorbed at

different levels of the GI tract has the potential to detect site-specific mucosal damage in a noninvasive manner, however, this was not performed in this study.[109,117-120] Finally, dogs in the present study may have had gastrointestinal hemorrhage that was not evident on gross inspection of feces, as 100-200 mL of digested blood is required for gross evidence of melena.[121] While fecal occult blood testing is a more sensitive method for detecting gastrointestinal hemorrhage, requiring 20-50 times less blood than is necessary to produce melena, the standard guaiac paper fecal occult blood test may yield false positives as a result of dietary protein intake.[121,122] Fecal occult blood testing was not performed in this study.

There have been no previous studies evaluating the effects of chronic administration of immunosuppressive doses of prednisone on canine gastroduodenal mucosa, however, several studies have demonstrated adverse gastrointestinal sequela following the short term administration of high doses of methylprednisolone and dexamethasone alone or in combination with NSAIDs.[83-89] Corticosteroids are believed to cause gastrointestinal ulceration through upstream inhibition of prostaglandin synthesis by phospholipase A blockade.[1,36,81] Corticosteroids have also been shown to inhibit healing of existing ulcers by altering the composition of gastric mucus, decreasing the rate of mucosal cell turnover, inhibiting capillary and fibroblast proliferation, and enhancing collagen breakdown in the late stages of inflammation.[1,49,50,80-82] Aspirin has been shown to have adverse effects on the gastric mucosa through a combination of direct cytotoxicity and COX-1 inhibition, resulting in decreased prostaglandin synthesis, compromised mucosal barrier, hyperacidity, ulceration and delayed mucosal healing.[9,11,13,66-70] There have been no studies evaluating the gastroduodenal effects of

ultralow-doses of aspirin in dogs, so it is unclear whether or not these effects occur at a dose of 0.5 mg/kg/day. Based on the results of this study, in which dogs given aspirin and prednisone were not different from dogs given only a placebo, it seems unlikely that such low doses of aspirin would have any adverse gastrointestinal effects independently. However, it is well-established in the human literature that patients receiving low dose aspirin (75-81 mg/day) are at an increased risk of gastrointestinal ulceration, particularly when there is concurrent use of corticosteroids or other NSAIDs or in the presence of comorbid conditions.[123,124]

This study indicates that the concurrent use of immunosuppressive doses of prednisone and ultralow-dose aspirin in young healthy dogs for 27 days is safe. However, it is noteworthy that individual dogs developed gastric lesions, ranging from submucosal hemorrhages to a single small ulcer, and some experienced an increased frequency of small bowel diarrhea during therapy. These complications may pose a greater risk in clinically ill patients whose underlying disease process may increase the relative incidence and severity of these adverse events and who may require treatment in excess of 27 days. These patients should be monitored accordingly for signs of gastrointestinal pathology, including vomiting, diarrhea, melena, hematemesis, depression, and anorexia. This treatment protocol is currently being recommended in the treatment of IMHA[4,16], and the concurrent use of these medications may also be indicated in other less common disease processes, such as systemic lupus erythematosus and certain types of protein-losing nephropathies.[22,105,125] In these disorders, immunosuppressive dose of corticosteroids are necessary for treatment of the underlying condition but the dog is also prone to thromboembolic events requiring low-dose aspirin

therapy. Clinical studies evaluating these dogs for adverse events associated with this combination therapy are needed.

CHAPTER III: Conclusions:

This study demonstrated that chronic administration of immunosuppressive doses of prednisone with or without concurrent administration of ultralow-dose aspirin to healthy young adult dogs for 27 days resulted in equivalent gastroduodenal endoscopic scores as those dogs receiving placebo. While there was a significant increase in the number of dog days of diarrhea within the prednisone and aspirin group during treatment with these medications when compared with the observation period before treatment began, diarrhea rapidly (within 5 days) resolved with weaning of medications. Within the confines of this study, these findings indicate that the concurrent use of prednisone and ultralow-dose aspirin affords no greater risk than the use of prednisone alone, in healthy dogs. Based on these findings, there is no apparent contraindication for the concurrent use of prednisone and aspirin at these doses. While certain individuals may suffer from increased incidence of diarrhea, in this study, the diarrhea was mild, self-limiting, and not associated with significant morbidity. However, caution must be exercised in extrapolating these findings to older clinically ill patients that require therapy for greater than 27 days, as they may be more susceptible to adverse gastrointestinal events. Clinical studies evaluating the incidence of adverse gastrointestinal effects should be collected from clinically ill dogs treated with this protocol to determine the safety of this therapeutic regimen.

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TABLES:

Table 1: Gastroduodenal lesion scale [a]

Score	Description
1	Normal
2	1 Submucosal hemorrhage
3	2-5 Submucosal hemorrhages
4	>5 Submucosal hemorrhages
5	1 Erosion
6	2-5 Erosions
7	>5 Erosions
8	1 Ulcer
9	2 Ulcers
10	≥ 2 Ulcers
11	Perforating ulcer

[a] Criteria previously published by Ward et al[13]

Table 2. P-values for regional and total endoscopic scores (comparison between groups)

Day	Body	Angularis	Pylorus	Cardia	Duodenum	Total
-7	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
5	1.000	0.5879	0.3637	0.3444	0.3679	0.1629
14	0.1203	0.3679	0.1194	0.2909	1.0000	0.8857
27	0.1194	0.5879	0.3679	0.5861	1.0000	0.3007

Table 3: Ranges for regional and total endoscopic scores for each Group

Group NN						
Day	Body	Angularis	Pylorus	Cardia	Duodenum	Total
-7	1	1	1	1	1	5
5	1	1	1	1	1	5
14	1	1	1-4	1	1	5-8
27	1	1	1	1	1	5
Group PN						
Day	Body	Angularis	Pylorus	Cardia	Duodenum	Total
-7	1	1	1	1	1	5
5	1	1-4	1-4	1-6	1	5-16
14	1-6	1-4	1	1-4	1	5-16
27	1	1-4	1-4	1-6	1	5-11
Group PA						
Day	Body	Angularis	Pylorus	Cardia	Duodenum	Total
-7	1	1	1	1	1	5
5	1	1-4	1-7	1-6	1-5	5-23
14	1	1	1	1-8	1	5-12
27	1-4	1-4	1	1-6	1	5-16

Table 4. P-values for period effect on endoscopic scores (comparison within groups)

Group	Body	Angularis	Pylorus	Cardia	Duodenum	Total
NN	1.0000	1.0000	0.1116	1.0000	1.0000	0.1116
PN	0.1116	0.7325	0.194	0.2004	1.0000	0.1427
PA	0.1116	0.3916	0.3916	0.5319	0.3916	0.4836

Table 5: P-values for clinical signs (comparison between groups)

Period	Diarrhea	Vomiting	Appetite
1*	0.5748	0.1194	1.0000
2**	0.1917	0.3679	1.0000

*Period 1: Observation only period (10 days) prior to initiating medications
**Period 2: Treatment period (27 days)

Table 6: P-values for period effect on clinical signs (within groups)

Group	Diarrhea	Vomit	Appetite
NN	0.6547	0.3173	1.0000
PN	0.6547	0.1573	1.0000
PA	**0.0253**	1.0000	1.0000

FOOTNOTES:

[i] Science Diet Maintenance, Hills Pet Nutrition Inc. Topeka, KS
[ii] Sony Promavica Still Video Recorder , Model MVR5300, Sony Corporation, Japan
[iii] Dip Quick Stain Solution, Jorgenson Laboratories, Loveland, CO
[iv] The SAS system, Version 9.12, SAS Institute Inc. Cary, NC 27513

FIGURES:

Figure 1: Mucosal hemorrhages
Small pinpoint hemorrhages (arrows) in the pyloric antrum of a dog receiving prednisone (Day 5). This area received a gastric lesion score of 4. The white areas are small amounts of retained food.

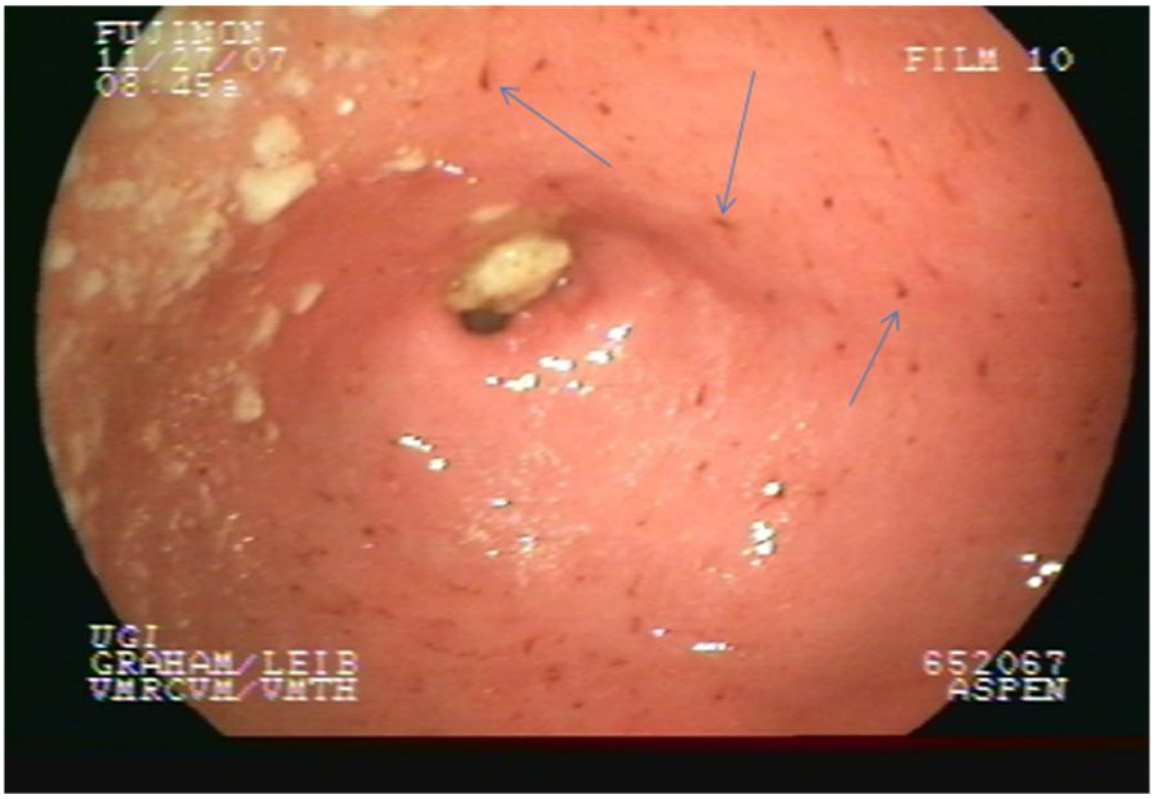


Figure 2: Multiple erosions
Endoscopic appearance of multiple erosions (arrows) in the pyloric antrum, defined as superficial defects in the mucosal epithelium. This area received a gastric lesion score of seven. The other lesions are mucosal hemorrhages. These lesions were identified on Day 5 in a dog receiving prednisone and aspirin.

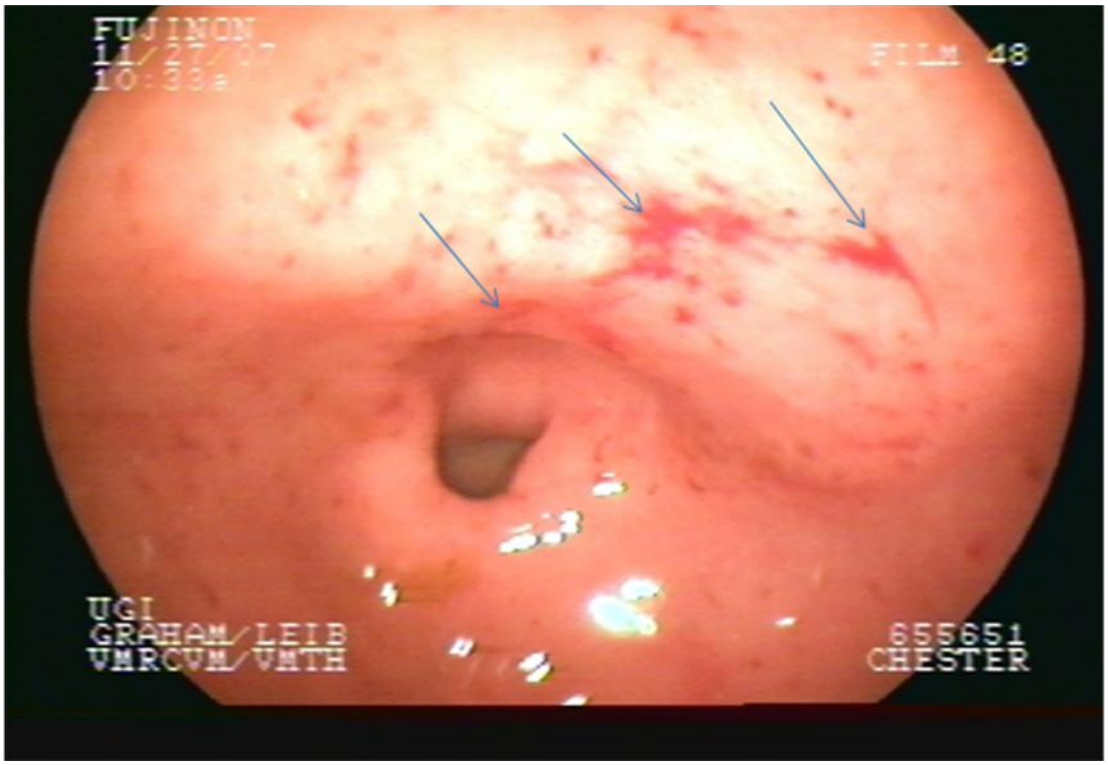


Figure 3: Ulcer
Endoscopic appearance of an ulcer (arrow) in a dog receiving aspirin (a subject enrolled in a previous study[51]). The defect in the mucosa has an observable depth, width and a raised margin. This area received a score 9.

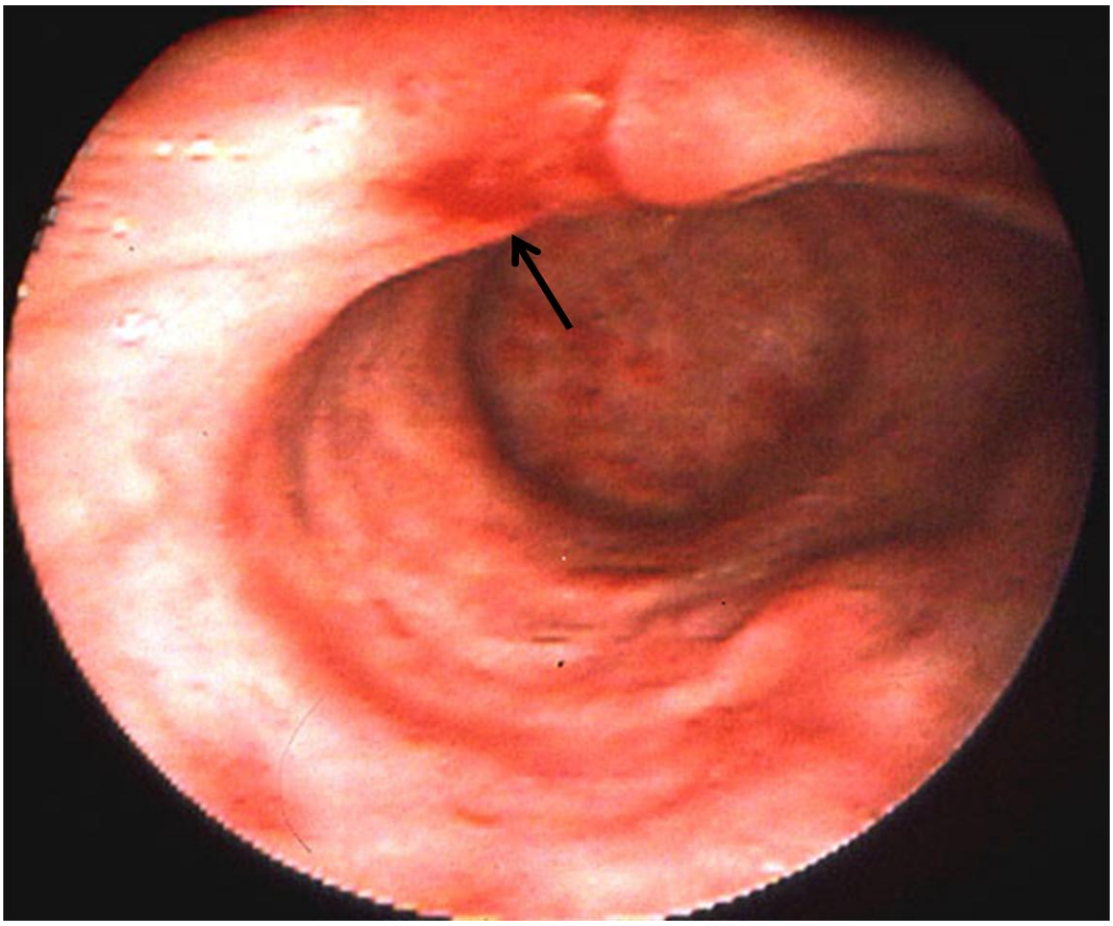


Figure 4: A retroflexed view of lesser curvature, angularis incisura, and pyloric antrum of Dog 651, from the prednisone and aspirin group, on Day -7. Foreign material (plastic bedding) is present but the gastroduodenal mucosa is normal (score for pyloric antrum = 1, total endoscopic score = 5).

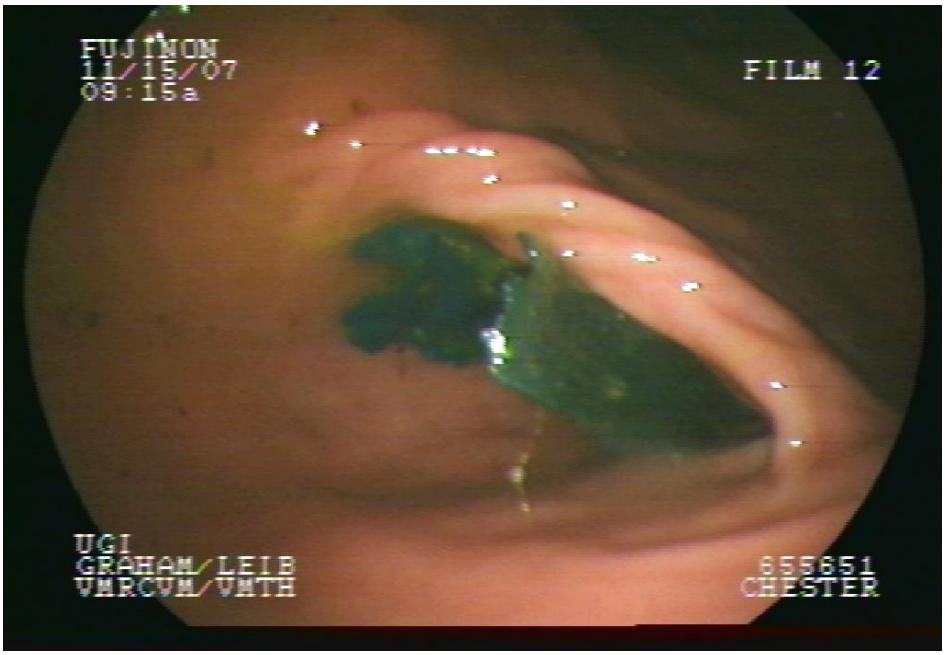


Figure 5: Dog 651 (same dog as Figure 4) on Day 5. Foreign material persists in pyloric antrum (thick arrow) with mucosal hemorrhages (thin arrows).

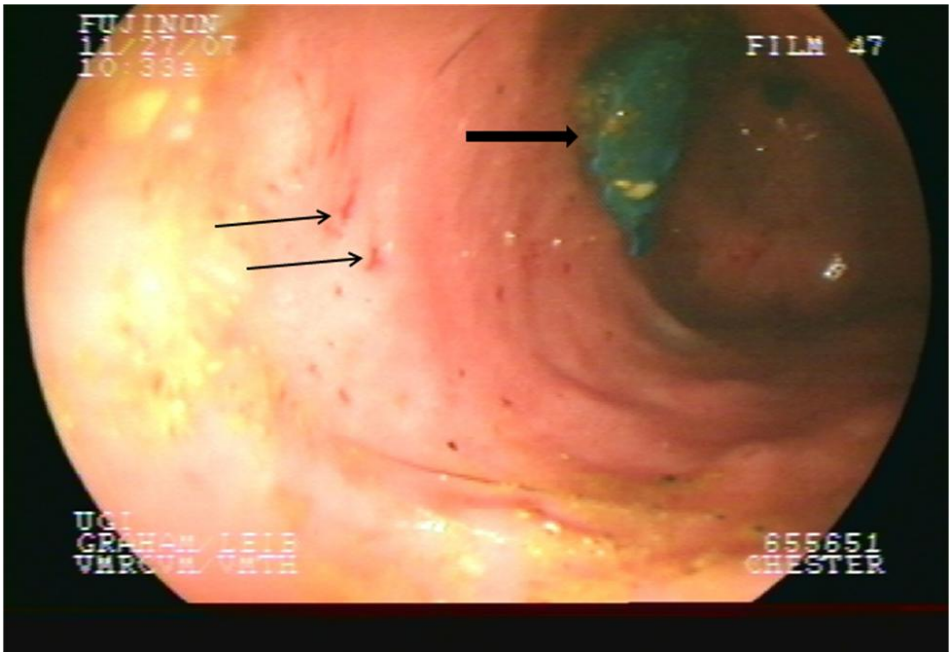


Figure 6: Dog 651 (same dog as in Figure 4 & 5) on Day 14 (1 week after removal of foreign material). A single linear erosion is visible in the cardia of the stomach (thin arrow) on this retroflexed view. The shaft of the endoscope is visible entering the cardia (thick arrow).

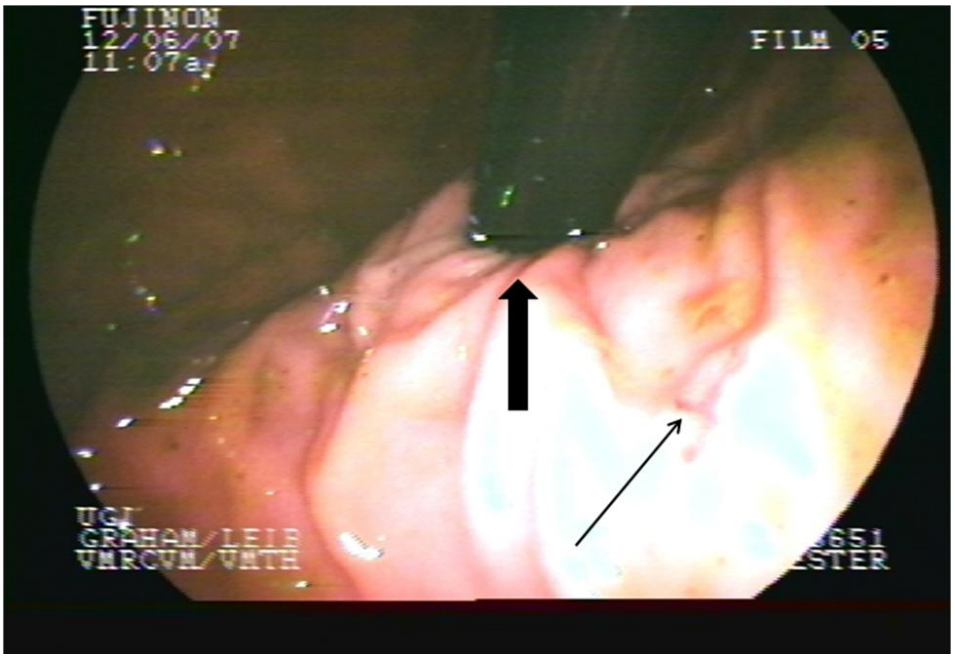


Figure 7: Spring foreign body in a dog from the prednisone group (Dog 816) on Day 27. The brown fluid adhered to the mucosa is a mixture of feces and gastric secretion.

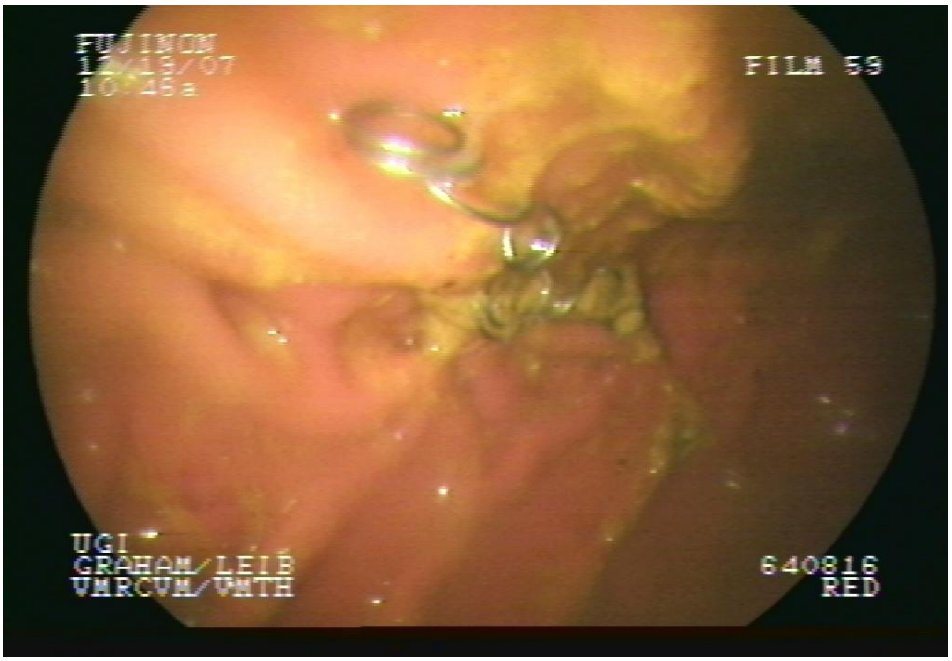


Figure 8: Linear erosions (thin arrows) in cardia of dog 816 on Day 27, apparently caused by spring foreign body. The shaft of the endoscope entering the cardia of the stomach can be seen (thick arrow) on this retroflexed view.

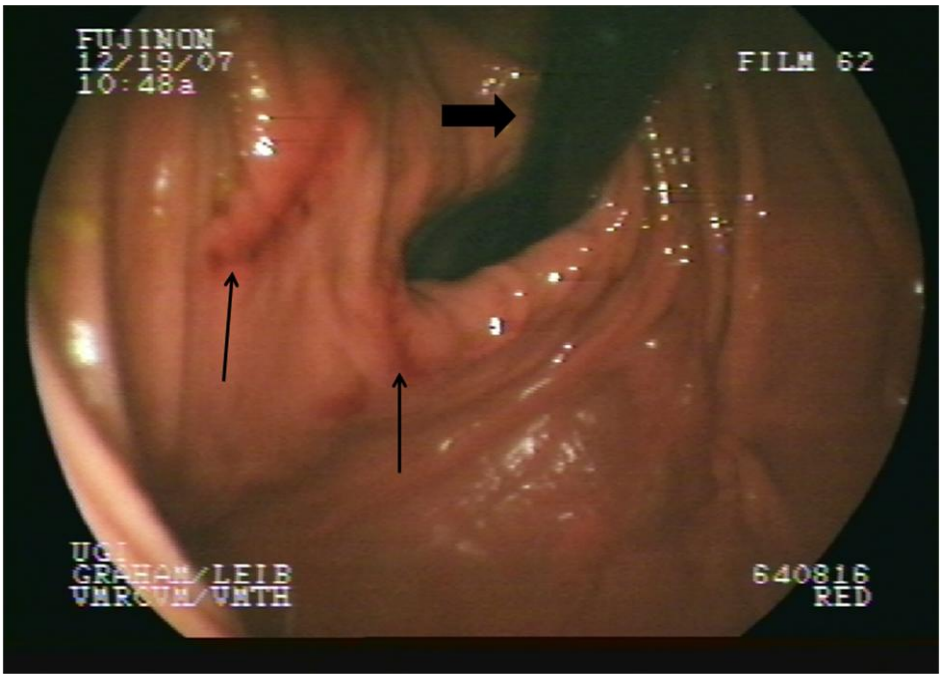


Figure 9: Small ulcer (arrow) in the gastric cardia of dog 231 (from group PA) on Day 14. This area received a score of 8.

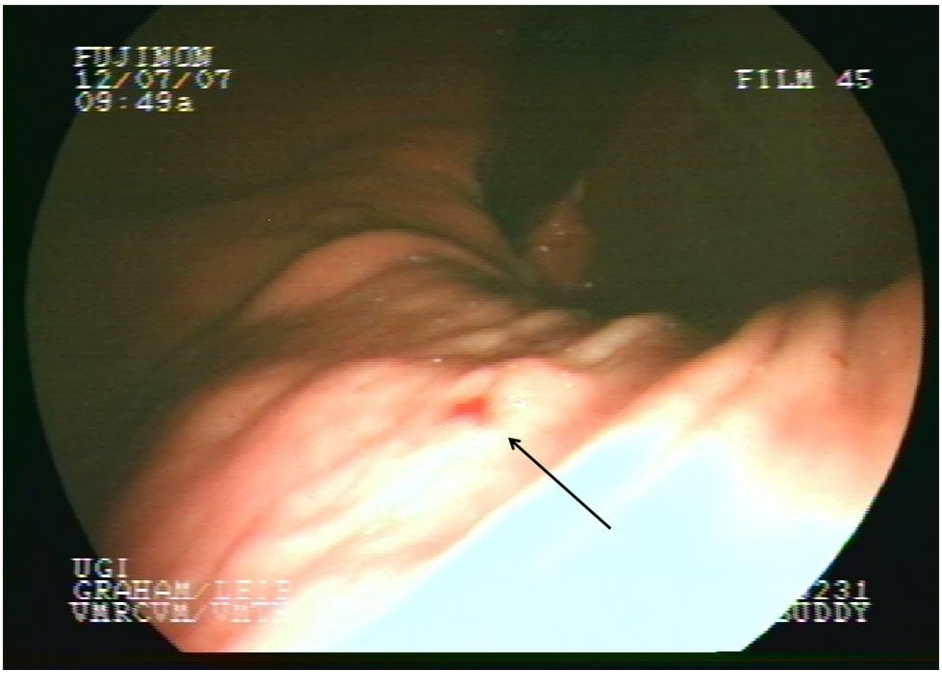
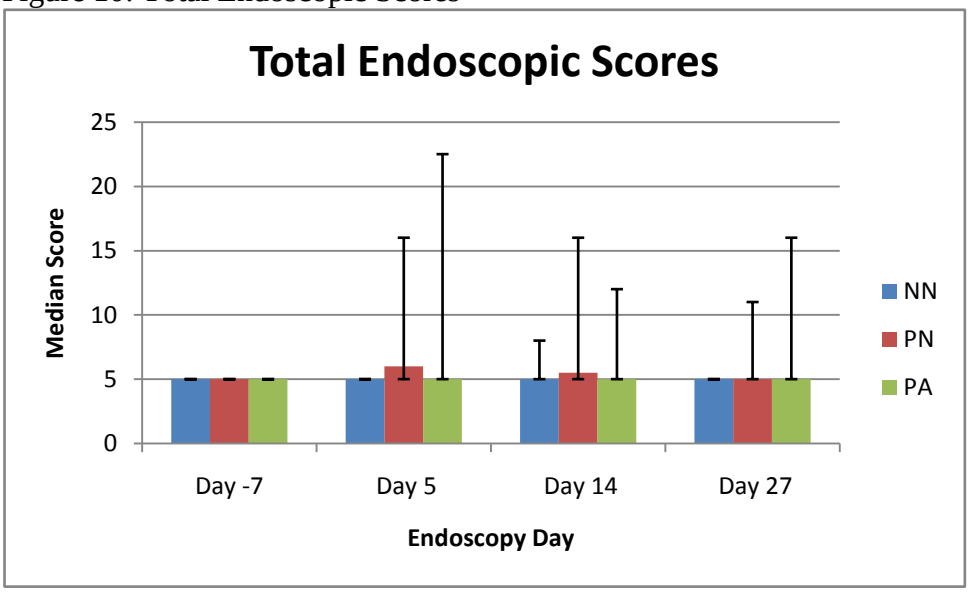
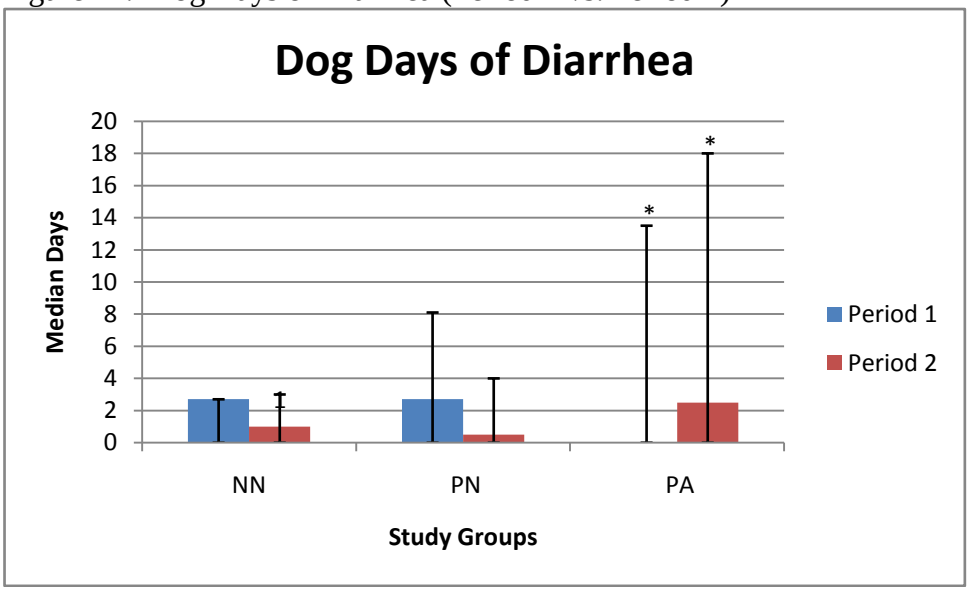


Figure 10: Total Endoscopic Scores



NN: Placebo group
PN: Prednisone group
PA: Prednisone and aspirin group
Error bars represent the range of total endoscopic scores.

Figure 11: Dog Days of Diarrhea (Period 1 vs. Period 2)



NN: Placebo group
PN: Prednisone group
PA: Prednisone and aspirin group
Error bars represent the range of dog days of diarrhea.

APPENDICES:

Appendix 1: Regional and Total Gastroduodenal scores for each dog

A. Day -7

Treatment-Dog Number	Body	Angularis	Pylorus	Cardia	Duodenum	Total
Placebo						
291	1	1	1	1	1	5
309	1	1	1	1	1	5
129	1	1	1	1	1	5
532	1	1	1	1	1	5
069	1	1	1	1	1	5
451	1	1	1	1	1	5
Prednisone						
067	1	1	1	1	1	5
750	1	1	1	1	1	5
873	1	1	1	1	1	5
166	1	1	1	1	1	5
816	1	1	1	1	1	5
566	1	1	1	1	1	5
Prednisone/Aspirin						
611	1	1	1	1	1	5
651	1	1	1	1	1	5
231	1	1	1	1	1	5
829	1	1	1	1	1	5
719	1	1	1	1	1	5
761	1	1	1	1	1	5

B. Day 5

Treatment-Dog Number	Body	Angularis	Pylorus	Cardia	Duodenum	Total
Placebo						
291	1	1	1	1	1	5
309	1	1	1	1	1	5
129	1	1	1	1	1	5
532	1	1	1	1	1	5
069	1	1	1	1	1	5
451	1	1	1	1	1	5
Prednisone						
067	1	4	4	6	1	16
750	1	1	1	1	1	5
873	1	1	3	1	1	7
166	1	1	1	1	1	5
816	1	1	1	1	1	5
566	1	1	1	3	1	7

Prednisone/Aspirin						
611	1	1	1	1	1	5
651	1	4	7	6	5	23
231	1	1	1	1	1	5
829	1	1	1	1	1	5
719	1	1	1	1	1	5
761	1	1	1	1	1	5

C. Day 14

Treatment-Dog Number	Body	Angularis	Pylorus	Cardia	Duodenum	Total
Placebo						
291	1	1	4	1	1	8
309	1	1	1	1	1	5
129	1	1	1	1	1	5
532	1	1	1	1	1	5
069	1	1	4	1	1	8
451	1	1	1	1	1	5
Prednisone						
067	1	1	1	1	1	5
750	2	1	1	1	1	6
873	1	1	1	1	1	5
166	1	1	1	2	1	6
816	1	1	1	1	1	5
566	6	4	1	4	1	16
Prednisone/Aspirin						
611	1	1	1	1	1	5
651	1	1	1	5	1	9
231	1	1	1	8	1	12
829	1	1	1	1	1	5
719	1	1	1	1	1	5
761	1	1	1	1	1	5

D. Day 27

Treatment-Dog Number	Body	Angularis	Pylorus	Cardia	Duodenum	Total
Placebo						
291	1	1	1	1	1	5
309	1	1	1	1	1	5
129	1	1	1	1	1	5
532	1	1	1	1	1	5
069	1	1	1	1	1	5
451	1	1	1	1	1	5
Prednisone						
067	1	4	4	1	1	11
750	1	1	1	1	1	5
873	1	1	1	1	1	5

166	1	1	1	1	1	5
816	1	1	1	6	1	10
566	1	1	1	2	1	6
Prednisone/Aspirin						
611	1	1	1	1	1	5
651	4	4	1	6	1	16
231	1	1	1	1	1	5
829	1	1	1	1	1	5
719	4	1	1	1	1	8
761	1	1	1	1	1	5

Appendix 2: Dog Days of Clinical Signs: Vomiting, Diarrhea, and Inappetence

A. Period 1 (10 days)

Treatment-Dog Number	Vomiting	Diarrhea	Inappetence
Placebo			
291	0	0	0
309	0	1	0
129	0	0	0
532	0	1	0
069	0	1	0
451	0	1	0
Prednisone			
067	1	1	0
750	0	1	0
873	0	0	0
166	0	3	0
816	1	0	0
566	0	2	0
Prednisone/Aspirin			
611	0	5	0
651	0	0	0
231	0	0	0
829	0	1	0
719	0	0	0
761	0	0	0

B. Period 2 (treatment period): (27 days)

Treatment-Dog Number	Vomiting	Diarrhea	Inappetence
Placebo			
291	0	0	0
309	0	1	0
129	0	1	0
532	1	1	0

069	0	3	0
451	0	0	0
Prednisone			
067	0	0	0
750	0	4	0
873	0	0	0
166	0	0	0
816	0	1	0
566	0	1	0
Prednisone/Aspirin			
611	0	15	0
651	0	3	0
231	0	0	0
829	0	18	0
719	0	2	0
761	0	1	0

Distribution of dog days of diarrhea within Period 2 for the prednisone and aspirin group:

Week 1: 1
Week 2: 13
Week 3: 12
Week 4: 13

APPENDIX C: Dog weights (in kg) throughout study

Treatment-Dog Number	Day -7	Day -3	Day 5	Day 14	Day 21	Day 27
Placebo						
291	17.5	18.1	17.7	17.5	17.1	16.8
309	16.5	16.6	17.2	16.8	16	16.8
129	17.4	17.5	17.2	16.1	17.4	17.8
532	16.3	16.4	16.9	16.1	16.3	16.1
069	18.4	18.3	17.6	17.4	18	17.9
451	16.2	16.8	16.1	15.8	16.1	16.5
Prednisone						
067	16.1	16.1	15.8	14.5	14.8	14.9
750	14.8	14.6	14	13.2	14.5	15
873	13.2	14.9	14.5	13.2	14.1	14.1
166	15.9	15.7	15.3	14.6	15.1	14.8
816	17.6	17.5	16.8	15.8	15.8	15.5
566	14.5	14.6	14.2	13.7	14	13.7
Prednisone/Aspirin						
611	16.4	16.3	16	16.1	17.3	17.1
651	15.7	15.7	15.1	13.9	14.6	15.2

231	13.9	13.2	13.3	12.8	11.8	13.4
829	15.2	16	14.4	15.2	16.4	17.4
719	15.3	15.2	14.5	14.2	14.5	14.8
761	13.5	13.4	13	12.2	12.2	12.1