PROTECTIVE EFFECTS OF ZINC-L-CARNOSINE /VITAMIN E ON ASPIRIN-INDUCED GASTRODUODENAL INJURY IN DOGS

MASTER’S THESIS

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By

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ABSTRACT

Zinc plays a role in many biochemical functions, including DNA, RNA, and protein synthesis. The dipeptide carnosine forms a stable complex with zinc, which has a protective effect against gastric epithelial injury *in-vitro* and *in-vivo*. This randomized double-blinded placebo-controlled study investigated the protective effects of zinc-L-carnosine in combination with alpha-tocopheryl acetate (vitamin E) on the development of aspirin-induced gastrointestinal (GI) lesions in dogs. Eighteen mixed-breed dogs (mean 20.6 kg) were negative for parasites, and had normal blood work evaluations, and gastroduodenoscopic exams. On days 0 – 35, dogs were treated with 1 tablet (n=6) or 2 tablets (n=6) of 30 mg zinc-L-carnosine/ 30 IU vitamin E q12h PO, or a placebo (n=6). On days 7 – 35, all dogs were given 25 mg/kg buffered aspirin q8h PO. Endoscopy was performed on Days -1, 14, 21, and 35, and GI lesions (hemorrhages, erosions, or ulcers) were scored using a 12-point grading scale. Repeated measures ANOVA was used for statistical evaluation. The significance level was set at p ≤ 0.05. Zinc-L-carnosine was well tolerated, but all dogs developed mucosal lesions. Treatment had no significant effect on gastric (p=0.31) or duodenal lesions scores (p=0.067). Mean gastric lesions score increased
significantly on Days 14 (mean ± SD: 28.39 ± 3.7), 21 (30.22 ± 2.5) and 35 (28.36 ± 4.0), compared to Day -1 (4.33 ± 0.57; p <0.001). Duodenal lesions scores increased significantly between Days -1 (1.0 ± 0.0) and 14 (1.0 ± 0.0) compared to Days 21 (3.2 ± 1.8) and 35 (4.3 ± 3.2; p <0.001) in the placebo group only. In conclusion, zinc-L-carnosine at the 30 or 60 mg dose is well tolerated but did not prevent gastric mucosal lesions in an aspirin-induced gastritis model. The role for zinc-L-carnosine as a gastroprotectant in the clinical setting needs further investigation.
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CHAPTER 1

INTRODUCTION

The gastrointestinal tract is exposed to a wide range of noxious stimuli such as bacterial products capable of causing local and systemic inflammatory reactions, cytotoxic agents, detergents, mechanical damage, and large variations in temperature, pH and osmolarity. Defense mechanisms in the stomach such as high acid content, luminal immunoglobulins and digestive enzymes, and a thick surface mucus layer are crucial in the prevention of bacterial, viral and fungal colonization of the gastrointestinal tract. The net result of the stomach’s defense mechanisms is that the stomach and remainder of the gastrointestinal tract are protected from infection and other noxious stimuli.

The mucosa of the stomach consists of a superficial layer of columnar epithelial cells, underneath which glandular lamina propria and musularis mucosae layers are present. Three types of gastric glands are recognized in the dog, which differ in the types of cells they contain, and thus in the nature of their secretions. The cardiac glands are found in a narrow zone around the cardia and also scattered along the lesser curvature. The fundic glands are
found in the corpus and fundus of the stomach. They are absent from the cardiac and the pyloric regions. The pyloric glands are present in the pyloric region.  

Four types of glandular exocrine cells are found within the glands of the stomach. The chief cells, or zymogenic cells, produce, store and secrete pepsinogen, the precursor molecule for pepsin. The parietal, or oxyntic, cells are responsible for gastric acid secretion and intrinsic factor production.  

The mucous neck cells, located in the neck of the gastric glands, fill the spaces between the parietal cells and produce mucus. The glands of the cardiac and pyloric regions function mainly to produce mucus. Those in the corpus and the fundus produce hydrochloric acid and pepsin.  

The surface epithelial cells throughout the stomach also have the ability to secrete mucus.

The gastric mucosal defense consists of local factors within the gastric mucosa and its surface, and of neurohormonal mechanisms. Locally, the first line of defense is formed by an unstirred mucus layer, secretion of bicarbonate at the epithelial surface, and rapid turnover and restitutio...
weight trefoil factor family proteins (TFFs), which also increase the viscosity of gastric mucin. Prostaglandin E2 is a major stimulus of gastric mucus production. Gastrointestinal hormones such as gastrin and secretin, and cholinergic agents also stimulate mucus production.

The mucus layer contains phospholipids and is coated on the luminal side with surface-active phospholipids that form a hydrophobic layer, which protects the epithelium from back diffusion of acid.

Bicarbonate is produced in moderate amounts at the surface of the epithelium. The gastric mucus layer retains the bicarbonate ions, and thus allows for maintenance of a neutral pH at the apical cell surface. In the stomach, prostaglandins stimulate bicarbonate secretion via the EP₁ receptor. Its production also is stimulated by luminal acid, corticotrophin-releasing factor, melatonin, uroguanylin and orexin A. For each hydrogen ion generated and secreted at the apical membrane of the gastric epithelial cells, a bicarbonate ion is secreted at the baso-lateral surface. These bicarbonate ions are transported by capillary blood flow to the surface of the gastric epithelium, where they contribute to the unstirred layer of mucus and bicarbonate.

The mucosal epithelial cells are bound by tight junctions that prevent back diffusion of acid, and they produce a variety of protective substances, such as mucus, bicarbonate, heat shock proteins, TFFs, and cathelicidins. Heat shock proteins are released during cellular stress. They prevent protein denaturation and protect against cellular injury. TFFs stabilize the mucus layer and play a role
in regulation of reepithelialization. Cathelicidins function as part of the innate immune system by preventing mucosal bacterial colonization. When the epithelial cells are damaged, they release mucus that forms a mucus cap over the denuded basal membrane, providing a first line of protection against the stomach’s acid. Within minutes of injury, epithelial cells adjacent to the defect stretch and migrate to cover the denuded area. Under influence of transforming growth factor-α (TGF-α) and insulin like growth factor-1 (IGF-1), the gastric epithelial layer is continuously being renewed, replacing the gastric surface epithelium every 3 to 5 days. Prostaglandin E2 and gastrin also exert a trophic effect.

Perfusion within the gastric mucosa is regulated by locally-produced vasodilatory factors, and a neurohormonal reflex arc. The endothelial cells of the capillary vessels produce the vasodilators prostaglandin I2 (prostacyclin) and nitric oxide in response to exposure to an irritant or back-diffusion of acid. In addition, stimulation of afferent nerve endings located just below the epithelial surface leads to release of neurotransmitters that work to decrease the tone of the submucosal arterioles. Together, these mechanisms increase mucosal blood flow and protect the mucosa from damage by dilution and removal of noxious agents.

Prostaglandins play a crucial role in the defense mechanisms of the stomach, and almost all of them are either stimulated or facilitated by prostaglandin production. Maintenance of the mucosal integrity is dependent on
the continuous generation of PGE2 and PGI2. As described above, prostaglandins stimulate mucus, bicarbonate, and phospholipid production, and they increase mucosal blood flow. In addition, prostaglandins inhibit acid secretion by attaching to an inhibitory G–protein-coupled receptor on the basolateral membrane of the parietal cells. This interaction decreases adenylate cyclase activity and cAMP formation, with a subsequent decrease in acid production. In addition, prostaglandins are potent inhibitors of leukocyte adherence to the vascular endothelium.

Prostaglandins accelerate ulcer healing in experimental animals and in humans. They trigger the release of vascular endothelial growth factor and increase blood flow to the ulcer margin. In addition, prostaglandins inhibit gastric acid and stimulate mucus and bicarbonate secretion.

Despite this intricate system of gastric mucosal defense, subclinical gastrointestinal injury is common and often associated with use of nonsteroidal anti-inflammatory drugs (NSAIDs). NSAIDs are used for the management of pain and inflammation in dogs with osteoarthritis and other painful diseases. Aspirin and other NSAIDs frequently lead to adverse gastrointestinal effects such as inappetence, nausea, vomiting, diarrhea and gastrointestinal hemorrhage associated with irritation, erosion and ulceration of the gastrointestinal epithelium.
1.1 MECHANISM OF ACTION OF NSAIDS

Tissue damage elicits an inflammatory response, which is characterized by the cardinal signs of inflammation: rubor, tumor, calor, dolor and functio laesa (redness, swelling, heat, pain, and loss of function). This inflammatory response is caused by the release of signaling molecules from damaged cells, which trigger the immune system and initiate the healing process. As a result, the invading organisms are eliminated and normal tissue function is restored. Despite the protective effect of the acute inflammatory response, the process is accompanied by activation of pain receptors and may, by itself, contribute to substantial morbidity. In addition, an inflammatory response that persists chronically is detrimental to the patient. Therefore, suppression of the inflammatory response is an important therapeutic goal. Anti-inflammatory drugs help to achieve that goal.

When cells are damaged, the enzyme phospholipase A2 releases arachidonic acid from the phospholipid component of their membranes. Arachidonic acid serves as a substrate for lipoxygenases and cyclooxygenases, and is converted to leukotriene and prostaglandin inflammatory mediators, respectively.6 NSAIDs inhibit cyclooxygenase. In the 1990s, it was discovered that 2 types of cyclooxygenases are present, cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2).7

In healthy tissues, COX-1 is constitutively expressed, and produces prostaglandins that play an important role in normal body functions.6 COX-1
expression has been detected in canine platelets, mucosal cells of the gastrointestinal tract, endothelial cells, and in the renal medullary collecting ducts and interstitium. Although present at low concentrations in healthy tissues, COX-2 expression is markedly increased in inflammation, and its products are associated with negative effects of the inflammatory response. Initially, much attention was given to the development NSAIDs that specifically inhibited the action of COX-2. These efforts resulted in development of COX-2-specific NSAIDs such as deracoxib, firocoxib, and, most recently, robenacoxib.

More recently, it has become clear that there is an incomplete division between the roles of COX-1 (i.e., housekeeping activity) and COX-2 (i.e., inflammation). The function of these enzymes is more complex, and both play a role in gastrointestinal mucosal defense. Indeed, in the normal mucosa combined inhibition of COX-1 and COX-2 is necessary to induce injury. Whereas COX-1-derived prostaglandins contribute importantly to the maintenance of a neutral pH at the mucosal surface and basal mucosal blood flow, COX-2 derived prostaglandins are important for the maintenance of blood flow during ischemia reperfusion injury. In addition, COX-2-derived prostaglandins are potent inhibitors of leukocyte adherence to the endothelium of the mesenteric venules, thereby down-regulating the inflammatory response. COX-2 is induced at sites of gastric injury or ulceration, and the use of COX-2-selective inhibitors increases the amount of time required for gastric ulcers to heal.
In rats and dogs, administration of a low dose of the COX-1-selective inhibitor aspirin results in increased COX-2 expression in the stomach and duodenum, respectively, but does not cause gastrointestinal ulceration.\textsuperscript{14,15} Up-regulation of COX-2 expression during inflammation appears to be a defensive response aimed at protecting the gastrointestinal mucosa.\textsuperscript{1}

Exposure of the normal rat gastric mucosa to hydrochloric acid does not induce lesions.\textsuperscript{9} When rats were pre-treated with a COX-1 inhibitor, dose-dependent mucosal lesions developed after an acid challenge. In contrast, pre-treatment with a COX-2 inhibitor did not result in lesions in the acid-challenged stomach mucosa. When a selective COX-2 inhibitor is co-administered during COX-1 inhibition, extensive tissue damage ensues.\textsuperscript{14} These results indicate that COX-1-derived prostaglandins play a major role in the mucosal defense against luminal noxious agents. Imminent injury induces the expression of COX-2, which then assists COX-1 in the maintenance of mucosal integrity.\textsuperscript{9}

Inhibition of COX-2 only, without inhibition of COX-1 activity, does not result in mucosal lesions unless one of the other components of the gastric defense system also is attenuated.\textsuperscript{9} Combined inhibition of nitric oxide production and COX-2 activity resulted in severe dose-dependent damage in the acid-challenged mucosa.\textsuperscript{9} Similarly, inhibition of the afferent mucosal nervous system in combination with COX-2 caused marked mucosal damage.

In summary, these findings indicate that COX-2 inhibition during an acid challenge only results in mucosal injury when one of the other factors involved in
gastric mucosal defense is impaired, whereas COX-1 inhibition in the face of an acid challenge is ulcerogenic regardless of the presence of normal nitric oxide production or normal function of the afferent neural system.\textsuperscript{9}

The mechanism by which NSAIDs produce damage in the stomach can be subdivided into local (topical) and systemic actions.\textsuperscript{1,4,16} Some NSAIDS, particularly those that are acidic in nature, can directly injure epithelial cells locally. NSAIDs decrease mucus and bicarbonate secretion, thereby impairing the ability of the mucosa to maintain a neutral pH at the epithelial surface. NSAIDs also disrupt the layer of surface-active phospholipids on the luminal side of the mucosal surface, making it easier for dissolved substances to reach the epithelium.\textsuperscript{1} These effects are independent of the effects on prostaglandin synthesis. NSAIDs inhibit epithelial growth factor pathways, thereby inhibiting epithelial proliferation and epithelial repair. These topical mechanisms may play a more important role in the small intestine, where enterohepatic circulation results in repeated exposure of the enterocytes to these medications.

The second mechanism for gastric mucosal injury is systemic. Parenterally-administered or enteric-coated NSAIDs produce just as much gastric ulceration as do orally-administered NSAIDs. By their negative effect on prostaglandin production, NSAIDs render the mucosa more susceptible to the damaging effects of luminal agents including, in some cases, the NSAID itself.\textsuperscript{1}

NSAIDs decrease mucosal blood flow primarily by suppression of COX-1 activity. They also trigger the adherence of leukocytes to the vascular
endothelium, resulting in endothelial injury by the release of inflammatory mediators. Rats rendered neutropenic by treatment with an anti-neutrophil antibody did not exhibit endothelial damage after NSAID administration.\textsuperscript{17,18}

COX-1 suppression by NSAIDs increases gastric acid secretion. Evidence to support the contention that gastric acid plays a role in the development of NSAID-induced ulceration comes from clinical trials with proton pump inhibitors.\textsuperscript{19-22} The incidence of ulceration in patients receiving placebo plus an NSAID or selective COX-2 inhibitor was 20.5\%, whereas the incidence was only 7.4\% in the group receiving omeprazole.\textsuperscript{21} Furthermore, ulcer bleeding is enhanced by NSAID actions on platelets. Thromboxane produced by platelets is a potent stimulus for platelet aggregation and a potent vasoconstrictor. Synthesis of thromboxane occurs via COX-1.

Impairment of ulcer healing by NSAIDs is due in part to effects on platelets. The beneficial effects of platelets on ulcer healing are likely related to the release of vascular endothelial growth factor (VEGF), which is a potent stimulus of new blood vessel growth (angiogenesis), and an essential element in the ulcer healing process. COX-2 derived prostaglandins stimulate VEGF release from gastric fibroblasts.\textsuperscript{23,24}

1.2 MECHANISM OF ACTION OF ASPIRIN

Aspirin was developed in 1899 by Bayer in Germany.\textsuperscript{13} It is a nonselective COX inhibitor that binds irreversibly to the COX enzymes. Aspirin prevents
arachidonic acid from accessing the core of the COX enzymes and thus inhibits the production of prostaglandins.\textsuperscript{13} Therapeutic serum salicylate concentrations are reported to range from 5 mg to 30 mg/dL.\textsuperscript{25,26} These concentrations can be achieved by oral dosing at 25 mg/kg three times daily.\textsuperscript{27} At much lower dosages (0.5 mg/kg q12h), aspirin inhibits platelet aggregation by inhibition of thromboxane formation, and as such it is a potent anticoagulant.\textsuperscript{28} Aspirin is metabolized mostly by the hepatic enzyme glucuronyl transferase. Because cats lack this enzyme, they are predisposed to salicylate intoxication, especially after repeated administration.\textsuperscript{13} Salicylate and its metabolites are excreted in the urine both by filtration and active tubular secretion.

Concurrent administration of corticosteroids increases the risk of damage to the gastrointestinal epithelium. Concurrent use of alkalinizing agents increases the excretion of aspirin, and carbonic anhydrase inhibitors may cause an increase in the transport of aspirin into the central nervous system. Conversely, urinary acidifiers decrease the rate of aspirin excretion. Drugs that increase cytochrome p450 enzyme activity in the liver (barbiturates), may be associated with a decrease in the half-life of aspirin. The dosage of aspirin for dogs is 10-25 mg/kg PO q8-12h hours, and for cats is 10 mg/kg PO q48h.\textsuperscript{26}

Several studies have investigated aspirin’s selectivity for COX and its effects on the production of prostanoids in the dog.\textsuperscript{15,29-31} \textit{In vitro}, using a canine whole blood assay, aspirin was determined to be a relatively nonselective COX inhibitor, with a predisposition towards COX-1 inhibition.\textsuperscript{29,31}
Administered at 25 mg/kg PO q12h, aspirin was shown to significantly decrease prostaglandin E2 production in blood, gastric mucosa and synovial fluid at 7 and 21 days after the start of treatment, indicating activity against COX-1. Thromboxane 2 concentrations in blood at days 7 and 21 were suppressed, as also predicted from aspirin’s action on COX-1. Lipopolysaccharide-induced PGE2 production, an indicator of COX-2 activity, was decreased in blood and synovial fluid at 7 days. However, concentrations rebounded to above baseline 21 days after the start of treatment. These results indicate a nonselective inhibition of both COX-1 and COX-2 by aspirin, but with COX-1 inhibition being more pronounced.

When administered at 10 mg/kg PO q12h, aspirin did not have an effect on COX-1 and COX-2 protein expression in the pyloric mucosa, but did significantly increase COX-2 expression in the duodenum compared with the effects of deracoxib and carprofen. This finding was surprising, because aspirin was not expected to affect protein expression, and it may indicate a compensatory up-regulation of COX-2 expression secondary to aspirin-induced COX-1 inhibition, as was previously noted in rats. Aspirin treatment did, as expected, significantly decrease prostaglandin and thromboxane concentrations in the stomach and the duodenum, indicating efficient inhibition of COX activity.
1.3 ELEMENTAL ZINC IN THE LITERATURE

Zinc is an essential trace mineral that is a cofactor of many enzymes. For example, it is required for the catalytic activity of carbonic anhydrase, and, together with copper, it is a main component of superoxide dismutase. It also plays an important role in DNA replication, RNA transcription, pancreatic insulin secretion, signal transduction, enzymatic catalysis, and cell proliferation, differentiation and apoptosis. Approximately 85% of the body’s zinc is found in muscle (~52%) and bones (~33%), with the remainder found primarily in skin and the liver (11%). Plasma zinc concentrations represent <0.1% of whole body zinc. In plasma, zinc is primarily protein bound.

Zinc concentrations in the body primarily are regulated by changes in zinc absorption and excretion in the gastrointestinal tract. A small amount of zinc is lost daily in urine, and in hairs, nails, sweat and semen. There is no true storage of zinc within the body, and a marked decrease in dietary zinc intake is quickly followed by early signs of zinc deficiency (e.g., decreased food intake, growth failure). When zinc intake is low, absorption is upregulated by an increased rate of carrier-mediated transport of zinc across the mucosa of the gastrointestinal tract. Metallothioneins are thought to mediate this response by binding zinc in the lumen and facilitating its transport across the membranes. Intestinal excretion of zinc is regulated independently of absorption, and, in humans with chronic inadequate zinc intake, a decrease in fecal zinc excretion is more important in the prevention of total body zinc depletion than is an increase.
Renal losses of zinc are low, and remain constant over a wide range of intake. Only with extremely low or high intake of zinc, does renal zinc transport contribute to zinc homeostasis. In dogs, glucagon infusions have been shown to increase and insulin infusion to decrease urinary zinc excretion independently of glomerular filtration rate, suggesting a possible hormonal influence on renal tubular zinc transport. With a prolonged decrease in dietary zinc intake, zinc is slowly released from bones, liver and testes, which become depleted, while muscle zinc concentrations are maintained at their expense, contributing further to the maintenance of zinc homeostasis.

Zinc is essential for the normal function of the immune system, and both innate and adaptive immunity are affected by zinc deficiency. Zinc deficiency induces T and B cell apoptosis, resulting in a lymphopenia, impaired natural killer cell function and decreased phagocytic function. In addition, zinc deficiency affects the secretion of cytokines such as INFγ, TNFα, and IL-2, which are essential for a well-regulated immune response.

During inflammation and infection, serum zinc concentrations decrease as a result of zinc sequestration in the liver where it is used for the production of acute phase proteins. Bacterial pathogens require zinc, and the decreased plasma zinc concentrations induced by the acute phase response could be protective by limiting zinc availability to bacteria. Conversely, zinc is part of the enzyme copper-zinc-superoxide dismutase, one of the most important enzymes
in the protection against free radicals that are formed as a consequence of oxidative damage.\textsuperscript{33}

Zinc is required for normal proliferation and regeneration of skin epithelial cells. Zinc is an essential co-factor for methalloproteinases, a group of enzymes that break down the extracellular matrix and prepare wounds for re-epithelialization.\textsuperscript{33} Zinc supplementation stimulates integrins, cell adhesion molecules that mediate cell migration. Consequently, zinc deficiency results in impaired epithelialization and poor wound healing. Gastrointestinal wound healing has also been shown to improve in animal models of bowel anastomosis.\textsuperscript{33}

Zinc deficiency in humans is common, especially in children growing up in underdeveloped countries where nutritional deficiency is caused by high cereal protein intake. This protein source is rich in phytate, which impairs the gastrointestinal absorption of zinc.\textsuperscript{35} Gastrointestinal bleeding secondary to hookworm infection, and loss of zinc due to excessive sweating in a hot climate also may contribute to deficiency.\textsuperscript{36} In developed countries, zinc deficiency usually occurs in the elderly, where it is associated with poor socio-economic status leading to greater consumption of inexpensive foods deficient in micronutrients, with loss of appetite, poor dental health, decreased energy requirement and intestinal malabsorption.\textsuperscript{37} Absorption of zinc also can be affected by other trace minerals, such as copper, magnesium, calcium, nickel, cadmium and iron.\textsuperscript{38} In addition, gastric acidity enhances zinc absorption in
people, especially absorption of zinc oxide. Gastric acidity is commonly decreased in geriatric patients. Disease syndromes associated with zinc deficiency include malabsorption syndrome, total parenteral nutrition without zinc supplementation, and hyperzincuria as seen in liver cirrhosis and sickle cell disease.

In humans with zinc deficiency, growth retardation, infertility, dermatitis, delayed wound healing, alopecia, poor pregnancy outcomes, teratology, anorexia, diarrhea, and increased susceptibility to infectious diseases caused by bacterial, viral and fungal pathogens may occur. The classic zinc deficiency syndrome acrodermatitis enteropathica is caused by a mutation in an intestinal zinc transport protein. The clinical signs that develop with this syndrome are completely reversible with zinc supplementation. Zinc-responsive dermatoses also have been described in dogs, a red wolf and two goats. Zinc supplementation is used in Wilson’s disease, an autosomal recessive disorder of copper metabolism, resulting in copper storage disease in people. Therapy with high doses of zinc impairs intestinal absorption of copper and results in improvement of clinical signs. Similarly, zinc supplementation has been used in the treatment of copper storage hepatopathy in Bedlington terriers and West Highland White terriers.

Zinc supplementation has been shown to be beneficial in the prevention and treatment of diarrhea, pneumonia and possibly malaria in children living in underdeveloped countries. Also, a beneficial effect was noted with zinc
supplementation in shigellosis, leprosy, tuberculosis and acute cutaneous leishmaniasis in human patients.\textsuperscript{38} Local effects of zinc in the gastrointestinal tract are suggested by a correlation between decreased gastric mucosal zinc concentrations and the severity of inflammation in Helicobacter pylori-infected patients.\textsuperscript{46} A decreased immune response associated with zinc deficiency has been noted with advancing age, and it improves with zinc supplementation. Zinc supplementation also may decrease oxidative damage that occurs in the diabetic state. Conflicting results have been noted with respect to glycemic control in these patients. Overall, zinc seems to be helpful against oxidative stress in diabetic patients, but the effects of zinc supplementation on glucose metabolism in humans require additional investigation.\textsuperscript{47}

Despite its beneficial effects in selected diseases, zinc supplementation has not been shown to be unequivocally advantageous. Zinc has contradictory effects on antibody formation after vaccination, where it seems to enhance the response to cholera bacteria, but decreases the response to cholera toxin. No effect was found of zinc supplementation on the response to influenza vaccination. Zinc supplementation has been investigated in the treatment of the common cold, AIDS, and viral hepatitis. Reports on efficacy are conflicting, and supplementation may only be beneficial in zinc-deficient patients.

Zinc supplementation is not beneficial in zinc-replete individuals, and it may even have some deleterious effects.\textsuperscript{47} Oversupplementation of orally-administered zinc (>100 mg of elemental zinc per day) may result in severe
anemia, leukopenia, and neutropenia secondary to interference with copper uptake.\textsuperscript{48} Zinc toxicity associated with metallic gastric foreign bodies in dogs can result in acute severe intra- or extra-vascular hemolytic anemia.\textsuperscript{49}

Total body zinc stores are difficult to assess, because serum or plasma zinc concentrations do not reflect total body stores. Bioavailability of zinc supplements varies depending on the form.\textsuperscript{38} Zinc can be complexed with oxide or a metal, or with an organic anion, such as acetate, histidine, methionine, or sulfate.\textsuperscript{38}

1.4 ZINC-L-CARNOSINE IN THE LITERATURE

Several studies have shown the potential beneficial effects of zinc-L-carnosine, or polaprezinc, on the prevention and healing of gastrointestinal mucosal lesions. The compound is thought to have anti-oxidant, anti-inflammatory, protective and healing properties. It has been approved for use as an anti-ulcer drug in Japan. Zinc-L-carnosine has been investigated in several cell culture lines and experimental animal models. Zinc-L-carnosine is marketed in combination with vitamin E as a food supplement in dogs to "reduce flatulence, bowel discomfort and unpleasant gassy odors" (GastriCalm™; IVX Animal Health Inc, St Joseph, MO). To the author’s knowledge, no studies on its efficacy in dogs have been reported in the English literature to date.

One of the earliest repair responses after epithelial injury is the migration of surviving cells over a denuded area to re-establish the integrity of the
epithelial layer, which is followed by cellular proliferation to restore the thickness of the layer. Because it is very difficult to study the migration process in vivo, a cell culture model is used, in which a monolayer of cells is disrupted by scraping a pipette tip across the cells in a dish. Using such a model, it was noted that zinc-L-carnosine significantly stimulates epithelial cell migration, an effect that was not seen by treatment with equimolar concentrations of zinc sulfate, and thus could not be attributed to zinc alone. Zinc-L-carnosine also increased cellular proliferation in cell culture, as measured by an increase in tritiated-thymidine uptake by the proliferating epithelial cells. This effect was partially seen in cells supplemented with zinc sulfate.

In a rat model of gastric injury, using the prostaglandin synthesis inhibitor indomethacin and Bollman-type restraint cages, zinc-L-carnosine was shown to cause a significant dose-dependent decrease in the amount of injury, measured as surface area of gastric lesions and microscopic assessment of ulceration. Gastric pH was not affected. The protective effect of the higher dose of zinc-L-carnosine was similar to the one exerted by the cytoprotective agent epidermal growth factor (EGF), the positive control.

Zinc-L-carnosine (10, 30 and 100 mg/kg) also showed a protective effect on gastric injury in an aspirin/ hydrochloric acid-induced rat model, as measured by a dose dependent decrease in the total surface area of erosions, and a decrease in the concentration of thiobarbituric acid-reactive substances and myeloperoxidase activity, measures of lipid peroxidation and neutrophil
accumulation, respectively. At 30 and 100 mg/kg, zinc-L-carnosine significantly impaired the increase in TNF-\( \alpha \) production after aspirin administration.

Indomethacin-induced apoptosis of gastric epithelial cells was inhibited by suppression of caspase-3 activation. In another gastric cell culture model, TNF\( \alpha \)- and IL-1\( \beta \)-induced production of the proinflammatory cytokine interleukin 8 was significantly attenuated by zinc-L-carnosine. Zinc-L-carnosine also decreased inflammation and formation of gastric lesions in Mongolian gerbils with Helicobacter pylori-induced gastritis.

Exposure to zinc-L-carnosine significantly increased expression of 72kDa heat shock protein (HSP72), an endogenous cytoprotectant, in gastric and colonic cell cultures. In rats, induction of HSP72 in the stomach or colon also was noted after zinc-L-carnosine administration, and a decrease in acid-induced gastric or colonic lesions was shown with concomitant zinc-L-carnosine administration as compared with controls.

In a mouse model of intestinal injury, using a single injection of indomethacin, zinc-L-carnosine was shown to decrease wet weight gain and the degree of villous shortening and blunting as measures of mucosal injury as compared with the positive control group. In both the zinc-L-carnosine and positive control groups, crypt cell proliferation was significantly increased after indomethacin treatment, indicating that there was no additional proliferative effect of zinc-L-carnosine. Zinc-L-carnosine did not influence the growth of the gastrointestinal tract mucosa under non-damaged conditions, indicating that
administration of zinc-L-carnosine under non-damaged conditions had no added benefit.\textsuperscript{50}

In healthy human volunteers, zinc-L-carnosine prevented gastrointestinal epithelial injury, as measured by gut sugar permeability, during a challenge with the prostaglandin synthesis inhibitor indomethacin.\textsuperscript{50}

In some studies the beneficial effects of zinc-L-carnosine seem to be mediated predominantly by the zinc portion of the compound with little activity of the carnosine portion \textsuperscript{52,53}, whereas others have noted a possible synergistic effect in combination with carnosine.\textsuperscript{56,60,61} The zinc-L-carnosine complex adheres to ulcerated sites within the stomach, thus prolonging duration of action in the stomach.\textsuperscript{62}

In the study described in this thesis, zinc-L-carnosine was used in combination with vitamin E (GastriCalm or polaprezinc). The drug was administered at a total dose of 60 to 120 mg polaprezinc per dog per day for 35 days. Tissue zinc concentrations and risk for zinc toxicity with polaprezinc administration have been investigated previously.\textsuperscript{63} Beagle dogs showed significantly increased zinc concentrations in almost all tissues after treatment with 300 mg/kg/day for 13 weeks. At 50 and 120 mg/kg/day for 13 weeks, concentrations were increased in the liver and kidney as compared with controls. All concentrations returned to normal after a 5-week withdrawal period. Long-term toxicity was evaluated at 8, 20 or 50 mg/kg/day for 52 weeks.\textsuperscript{63} A transient
increase in serum zinc concentrations was noted at 13 weeks, but returned to almost normal at week 52. No other abnormalities were noted.

From this data, it may be concluded that a total dose of 30 to 60 mg of polaprezinc (equivalent to 6.7 to 13.4 mg of zinc) twice daily, as was used in the study described here (a maximal dosage of 7.1 mg/kg/day), would not have a substantial effect on systemic zinc concentrations.

In addition, adverse effects from polaprezinc treatment were investigated. In a single high dose (200 mg/kg) toxicity study, zinc-L-carnosine was shown to cause vomiting and mucosal lesions in the stomach and proximal small intestine. Additional toxicity studies showed mild adverse effects in Beagle dogs treated with 50, 120 or 300 mg/kg/day for 13 weeks. These adverse effects consisted of emesis, mild diarrhea and salivation. In female dogs that were treated with 300 mg/kg/day for 13 weeks, decreased appetite and associated weight loss were noted. No changes were noted on evaluation of the complete blood count. Serum biochemistry profiles showed a very mild increase in alkaline phosphatase activity as compared to controls. In addition, urine specific gravity decreased significantly as compared to controls. However, even after treatment for 13 weeks, urine specific gravity was 1.028 or higher in all dogs, with a median of 1.045 in the treated groups. On necropsy of the animals in the high dose group, hyaline degeneration or swelling of tubular epithelium in the kidney was noted, as well as interstitial fibrous proliferation in the pancreas. These findings were not noted in dogs that were necropsied after a withdrawal period
of 5 weeks. No adverse effects were seen in animals treated with 8 or 20 mg/kg/day for 13 weeks compared to placebo. Evaluation of dogs treated with 8, 20 or 50 mg/kg/day for 52 weeks revealed occasional vomiting in the middle (week 15-30) of the study in the 50 mg/kg/day dosing group only. This group also showed a transient decrease in food consumption. Serum biochemistry revealed transient, mild changes in serum concentrations of bilirubin (+60%), urea (+120%), creatinine (+40%), sodium, potassium and chloride (-5 to -15% for each) at week 26 of the study. All changes had returned to normal by week 52. Necropsy and histopathology findings were unremarkable.

1.5 VITAMIN E IN THE LITERATURE

Vitamin E is a lipid soluble vitamin. It exists as tocopherol (α, β, γ, δ) and tocotrienol forms (α, β, γ, δ), which differ in their biological activity. Dietary vitamin E consists of γ-tocopherol mostly, even though α-tocopherol is the most biologically active form in mammals. Regulation of vitamin E absorption is closely related to lipid and lipoprotein homeostasis. Hydrophobic dietary vitamin E is incorporated into micelles composed of lipids and bile salts that aid in absorption. Recent studies have indicated that vitamin E absorption by intestinal cells may be regulated by a specialized receptor (i.e. the scavenger receptor class B type I; SR-BI) rather than being a function of passive diffusion through the enterocyte membrane. Inside the enterocytes, vitamin E is incorporated into the microsomal membranes, after which it is repackaged into chylomicrons.
intestines do not discriminate between tocopherol forms, and both α and γ tocopherols are similarly absorbed. Intestinal vitamin E absorption thus is mostly dependent on chylomicron formation and has been shown to correlate with oleic acid availability for triglyceride synthesis. However, studies in chylomicron-deficient patients revealed that an alternative pathway for vitamin E absorption can be used by direct secretion of vitamin E from epithelial cells into high-density lipoproteins (HDLs), a process mediated by ATP-binding cassette transporters (ABC transporters). Oral bioavailability of vitamin E varies greatly (20-80%), and may be associated with expression of SR-BI and ACB transporter activity. In the blood, vitamin E is carried by lipoproteins. Lipoprotein lipase plays a regulatory role in the delivery and uptake of α tocopherol in peripheral tissues such as the liver, adipose tissue and skeletal muscle. The liver is the main storage site for vitamin E, and selectively releases α tocopherol back into the circulation mediated by α-tocopherol transport protein (α-TTP; associated with VLDL production) or ABC transport proteins (HDLs). Excretion of α-tocopherol and other vitamin E compounds occurs in bile. Some vitamin E may enter an enterohepatic cycle. Uptake of vitamin E into the tissue cells occurs by lipoprotein receptor- and SR-BI receptor-mediated mechanisms.

Vitamin E compounds, and α-tocopherol in particular, play an important role as lipid-soluble antioxidants for the protection of cellular membranes against oxidative damage. It is also thought to inhibit smooth muscle cell proliferation, endothelial dysfunction, and platelet aggregation.
Vitamin E deficiency in people most often occurs secondary to fat malabsorption syndromes, such as cystic fibrosis, chronic liver disease, abetalipoproteinemia, and intestinal resection. It can also occur secondary to some hematological disorders (e.g., β thalassemia major, sickle cell anemia, and glucose-6-phosphate dehydrogenase deficiency).

Signs of vitamin E deficiency are a result of increased oxidative damage to the tissues. Red blood cells in patients deficient in vitamin E have a decreased life span and show acanthocytosis, which is characteristic of oxidative stress.\(^{65}\) Chronic vitamin E deficiency results in neurological abnormalities, blindness and dementia.\(^{65}\)

In dogs, decreased serum vitamin E concentrations have been detected in immune-mediated hemolytic anemia (IMHA), suggesting a decreased antioxidant reserve.\(^{67}\) A case series of 15 English Cocker Spaniels with vitamin E deficiency revealed ataxia, proprioceptive deficits, abnormal spinal reflexes and muscle weakness, in addition to blindness secondary to retinal pigment epithelial dystrophy.\(^{68}\) Thirteen of the dogs were treated with vitamin E supplementation (60-90 IU/kg q12h), which did not improve their visual function. Four of the 9 dogs that had presented with neurologic deficits did show marked improvement within a few weeks of treatment.
1.6 AIM OF THE STUDY

Despite their beneficial effects, NSAIDs are a common cause of injury to the gastrointestinal mucosa, resulting in adverse effects such as inappetence, nausea, vomiting, diarrhea, and gastrointestinal hemorrhage or perforation. Lesions and adverse effects were most severe when NSAIDs were used at an inappropriate dosage, or when one NSAID was used in close temporal association with another NSAID or with glucocorticosteroid therapy.\(^{69-71}\) However, occasional severe adverse effects can occur even at appropriate therapeutic dosages.\(^{72}\)

Data on the efficacy of gastro-protectants in the prevention of gastrointestinal lesions secondary to NSAID use is dogs are sparse. Jenkins et al reported a consistently lower, but not statistically significant, mucosal lesion score in dogs treated with either omeprazole or cimetidine compared to untreated controls, in an aspirin-induced gastritis model.\(^{73}\) No data is available in the English literature on the effect of zinc-L-carnosine administration on the development of gastrointestinal lesions in dogs. The aim of this study was to investigate the mucosal protective effects of a commercially-available chelated formulation of zinc-L-carnosine in combination with the antioxidant vitamin E as alpha-tocopheryl acetate (GastriCalm™) on the development and healing of gastrointestinal lesions in dogs receiving daily doses of aspirin. In addition, the clinical implications of its use were evaluated.
1.7 REFERENCES


Eighteen random source mixed breed dogs (10 intact females, 8 intact males), ranging in age from 1 to 2 years and in body weights from 16.8 to 23.4 kg, were studied. All dogs had an acclimation period of at least 3 weeks before the start of the study (Day 0). Dogs were included in the study based on normal physical examination, unremarkable complete blood count and serum biochemistry panel; and negative evaluations for gastrointestinal parasites by fecal flotation and fecal Giardia antigen immunoassay testing. The study protocol was approved by the Institutional Animal Care and Use Committee of The Ohio State University.

Before the start of the study, all dogs were treated with two three-day courses of fenbendazole (Panacur granules, 222 mg/g; 50 mg/kg PO q24h; Patheon Inc, Toronto, Ontario, Canada). Fecal flotations (sugar and zinc sulfate centrifugation technique) and Giardia antigen immunoassay testing (SNAP® Giardia; IDEXX Laboratories, Westbrook, Maine) were performed in the
acclimation period before and after the first course of fenbendazole. A single dog (No 3005; 1X treatment group), testing positive for whipworms on the second fecal, had a third fecal flotation performed after the second three-day course of fenbendazole. Fecal samples (flotation and antigen testing) from all dogs were negative between 7 and 11 days before the start of the study (Day 0). Fecal flotation (zinc centrifugation technique), and Giardia antigen immunoassay testing were repeated on Day 35. Seven dogs from the same source, noted to have fleas on acquisition, were treated with praziquantel for tapeworms (Droncit, 34 mg tablets, 3 or 4 tablets according to weight; Bayer HealthCare LLC, Animal Health Division, Shawnee Mission, Kansas, USA) and nitenpyram for fleas (Capstar, Novartis Animal Health US, Inc, Greensboro, NC).

Dogs were housed in individual indoor runs with a 12-hour on/off lighting schedule. Dogs were given 5 cups of a dry commercial dog food once daily (Iams Chunks, The Iams Company, Cincinnati, Ohio, USA), and water was available ad libitum through an automatic watering system. In preparation for each gastroduodenoscopic procedure, dogs were fasted for approximately 22 hours. Blood samples were collected by jugular venipuncture before Day -1 (baseline) and on Days 14 and 35 of the study. Samples were chilled immediately after collection, the serum was separated, and samples were processed the same day. Complete blood counts and serum biochemistry profiles were performed on automated analyzers (CellDyn 3500; Abbott Laboratories, Abbott Park, IL, and
Hitachi 911; Linco Research, St Charles, MO), which were calibrated daily.

Treatments (aspirin, test/placebo) and observations (clinical observations, fecal scores, endoscopy) were performed by separate members of the research team. All were blinded to the treatment groups throughout the study. From Day 0 until Day 35 of the study, dogs were treated with 1 tablet (1X group, n=6) or 2 tablets (2X group, n=6) of 30 mg zinc-L-carnosine/30 IU vitamin E (GastriCalm™; IVX Animal Health Inc, St Joseph, MO) q12h PO, or a placebo (0X group, n=6) q12h PO. On days 7 to 35, all dogs were given approximately 25 mg/kg buffered aspirin q8h PO (rounded to the nearest 0.25 of a 325-mg tablet), for the induction of gastrointestinal lesions. Chewable test and placebo tablets were offered without a meatball initially, and upon rejection disguised in a meatball. If still refused, the tablets were force fed. After administration, the oral cavity was inspected to ensure the animal had swallowed the tablets. Any tablets spit up within 30 minutes after administration were re-dosed.

Dogs were monitored once daily for food intake and trice daily for vomiting and diarrhea. Body weight was measured once weekly. Food intake was estimated as a percentage of the total daily food allotment rounded to the nearest 5%. Fecal consistency was graded using a 5-point grading scale (Table 1). A qualitative test for the presence of fecal occult blood was performed weekly (Hemoccult SENSA; Beckman Coulter Inc, Brea, CA, USA).
<table>
<thead>
<tr>
<th>Fecal Consistency Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Greater than two-thirds of the feces in a defecation are liquid. The feces have lost all form, appearing as a puddle or squirt.</td>
</tr>
<tr>
<td>2</td>
<td>Soft-liquid feces are intermediate between soft and liquid feces. Approximately equal amounts of feces in a defecation are soft and liquid.</td>
</tr>
<tr>
<td>3</td>
<td>Greater than two-thirds of the feces in a defecation are soft. The feces retain enough form to pile but have lost their firm cylindrical appearance.</td>
</tr>
<tr>
<td>4</td>
<td>Firm-soft feces that are an intermediate between the grades of firm and soft. Approximately equal amounts of feces in a defecation are firm and soft.</td>
</tr>
<tr>
<td>5</td>
<td>Greater than two-thirds of the feces in a defecation are firm. They have a cylindrical shape with little flattening.</td>
</tr>
</tbody>
</table>

Table 2.1: Fecal consistency grading scale.

Gastroduodenoscopy was performed under general anesthesia on Days -1, 14, 21 and 35. Dogs were premedicated with acepromazine (0.1 mg/kg IM); general anesthesia was induced with thiopental 10 mg/kg and maintained with isoflurane in oxygen. Gastroduodenoscopy was performed (by MB) in left lateral recumbency. During the procedure 5 ml/kg/h of NaCl 0.9% was administered intravenously. Blood pressure during anesthetic procedures was monitored every 10 minutes. Videoendoscopic images were recorded digitally (Sony RDR GX355 DVD recorder; Sony Corp, Japan), and independently scored at a later time by two of the authors (SJ, RS). The endoscopist and both scorers were blinded as to
treatment.

Gastroduodenal mucosal scoring was performed as described previously.¹ In short, the procedure was as follows: the stomach and duodenum were divided endoscopically into 5 anatomical regions (A through E): A, pylorus and pyloric antrum; B, angularis incisura, extending along the lesser curvature; C, greater curvature from the cardia to the pyloric antrum; D, cardia, extending from the greater curvature region to the lesser curvature that was not included with the angularis incisura; and E, proximal duodenum to the major duodenal papilla.

The endoscope was passed down the esophagus, through the gastro-esophageal sphincter, and into the body of the stomach. The stomach was insufflated with air to distend the rugal folds and facilitate viewing of the mucosal surfaces. The angularis, antrum, pylorus, and duodenum were visualized as the scope was advanced. The endoscope then was retroflexed to view the cardia, the fundus, and lesser curvature. If mucus, bile, hair, or food particles obscured the gastric or duodenal mucosa, distilled water was infused through the operating channel of the endoscope to clear the material and allow visualization.

Each region was systematically evaluated and images were recorded without inducing iatrogenic trauma from the endoscope. Lesions that bordered 2 regions were assigned to the most appropriate region and were not counted twice when assessing the adjacent region. Each of the 5 regions was assessed individually and assigned a numerical value based on a 12-point scale described
previously. An erosion was defined as a superficial discontinuation of the mucosal epithelium. An ulcer was defined as a lesion producing wide discontinuation of the mucosa with a central defect and a raised margin. Each score was assigned based on the most severe lesions present in each region. Scores for all 5 regions were summed for a total endoscopy score, and scores for the 4 stomach regions were summed to form a total gastroscopy score.

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
<td>7</td>
<td>3-5 erosions</td>
</tr>
<tr>
<td>2</td>
<td>1 mucosal hemorrhage</td>
<td>8</td>
<td>&gt;5 erosions</td>
</tr>
<tr>
<td>3</td>
<td>2-5 mucosal hemorrhages</td>
<td>9</td>
<td>1 ulcer</td>
</tr>
<tr>
<td>4</td>
<td>&gt;5 mucosal hemorrhages</td>
<td>10</td>
<td>2 ulcers</td>
</tr>
<tr>
<td>5</td>
<td>Diffuse mucosal hemorrhages</td>
<td>11</td>
<td>3 or more ulcers</td>
</tr>
<tr>
<td>6</td>
<td>1-2 erosions</td>
<td>12</td>
<td>perforating ulcer</td>
</tr>
</tbody>
</table>

Table 2.2: Endoscopy scoring scale.
2.1 STATISTICAL ANALYSIS

Eighteen dogs were randomly assigned to three treatment groups (0X, 1X, 2X) within three phases (n=6 per phase), or two dogs per treatment group per phase, resulting in a randomized complete block design.

The endoscopy scores data set was visually inspected for normality, which was subsequently assumed given the minimal differences between mean and median. For the purpose of the analysis, stool consistency was treated as continuous and normality was assumed. In addition, analysis was performed on the number of days with diarrhea and presence of any diarrhea (1 = diarrhea on at least one day during days 1 -35, 0 = absent on all days). Food consumption was estimated throughout the study. Food was withheld on some days. For each dog, days with food withheld or where consumption was not reported were not included in the analysis. For the purpose of the analysis, food consumption was treated as continuous and normality was assumed.

The endoscopy variables (total endoscopy score, total gastroscopy score, regional endoscopy scores), stool consistency score, food consumption, and body weights were considered for analysis by repeated measures analysis of variance (ANOVA). Baseline values were investigated as covariates. If there was insufficient variability in the covariate, then repeated measures ANOVA was used. The analyses were performed using PROC MIXED of the SAS system, version 9.1. The statistical models included treatment, day, and the treatment by day...
interaction as fixed effects and phase and phase by treatment as random effects.

To determine the variance-covariance structure, a model including the treatment by day interaction and a term for unequal covariate slopes for the treatment by day interaction was fitted. The following variance-covariance structures were investigated, fitting both equal and unequal group variances: compound symmetry, heterogenous compound symmetry, first order autoregressive, Toeplitz, heterogeneous Toeplitz, first-order ante-dependent, first order banded unstructured, and unstructured. Structures with smaller Akaike’s information criteria are considered to have better fit. Once the variance-covariance structure was selected, the final form of the covariate was determined by first testing the equality of slopes for the treatment by day interaction. If no significant difference was detected then equality among treatment slopes and among day slopes was tested. If no significant differences were detected, then a test for common slope equal to zero was performed. If the common slope did not differ significantly from zero, then the covariate was eliminated from the model and a repeated measures ANOVA was performed. If there was a significant difference among slopes for treatment by day, then pairwise comparisons were performed at the mean of the covariate. Kenward-Rogers degrees of freedom were used in all hypothesis testing involving means.

The mean Day 0 weights were considered as a covariate in the repeated measures analysis of body weights. The treatment groups’ variances differed
significantly, so the model was fitted allowing different treatment groups variances. No significant difference among treatment groups means was detected, so no adjustment was made to the baseline values before use as a covariate.

Presence of vomiting (present = 1, absent = 0) was assessed for each day of the study. Analysis was performed on the number of days with vomiting and the presence of any vomiting (1 = vomiting on at least one of days 1-35, 0 = absent on all days). Stool consistency was scored (1 to 5) throughout the study. Scores were ordinal, and scores ≤ 3 were considered diarrhea. If no stool was present, the score was set to missing. Fecal occult blood (1 = positive, 0 = negative) was assessed once weekly throughout the study. Analysis was performed on the number of positive responses and the presence of positive response (1 = positive response on at least one of days 7, 14, 21, 28, or 35; 0 = absent).

Number of days with vomiting, number of days with diarrhea, and number of days positive for fecal occult blood were analyzed using the Cochran-Mantel-Haenszel test, testing the equality of mean scores between treatments. Phase was used as a stratification variable. The analyses were performed using PROC FREQ of the SAS system, version 9.1. Presence of vomiting on at least one day, presence of diarrhea on at least one day and presence of fecal occult blood on at least one occasion were analyzed using exact logistic regression, with phase as a
stratification variable. The binary regression option to LogXact from Cytel Studio 7 was used to perform the analyses.

Hematology and biochemistry variables were analyzed using ANOVA.

Significance level was set at p < 0.05.

2.2 REFERENCES

CHAPTER 3

RESULTS

All dogs completed the study. Two dogs developed minor footpad lesions and were treated with a topical betadine cleanser for 4 to 7 days. Another dog developed a minor pressure point sore at the lateral hock, which was treated with silver sulfadiazine ointment and betadine topical cleanser for 10 days. A fourth dog developed scrotal dermatitis, likely a contact allergy, and was treated with chlorhexidine topical, betadine topical cleanser, silver sulfadiazine ointment, and No-Bite topical. These findings were considered incidental to the study drugs or procedures, and all dogs recovered without complications.

Mean (± SD) of body weight is depicted in Figure 3.1. Throughout the study, body weight was maintained within 1 kg of Day 0 in all but 3 dogs. Two dogs, one in the placebo group and one in the 2X treatment group lost 6.5 and 10.8% of their body weight, respectively. One dog in the 1X treatment group gained 13.6% of her body weight. There was no significant difference in mean body weight between the treatment groups on any of the days. No significant
differences among slopes were detected for the treatment day interaction, treatment, and time.

Figure 3.1: Body weight (mean ± SD) in dogs treated with 1 tablet (1X group, n=6) or 2 tablets (2X group, n=6) of 30 mg zinc-L-carnosine/30 IU vitamin E q12h PO, or a placebo (0X group, n=6) q12h PO on Days 0 – 35. Gastrointestinal lesions were induced between Days 7 – 35 with 25 mg/kg buffered aspirin q8h PO.

Daily estimated food intake varied throughout the study for most dogs, regardless of treatment or day of the study (range 0 -100 %, data not shown). Three dogs (two in the 1X and one in the 2X treatment groups) ate 100% of their food on almost all days. There was no significant difference between treatment groups at any time during the study, except for Days 11 and 31 in the
placebo and 1X treatment group (p = 0.0419 and 0.0457, respectively), and Days 30 and 33 in the 1X and 2X treatment groups (p = 0.0330 and 0.0161, respectively). Estimated daily food intake decreased significantly over time (p = 0.0001). Estimated daily food intake on Day 0 was 87.5 ± 10.84% (mean ± SD), 89.2± 18.55%, and 96.7 ± 8.16 % for group 0X, 1X and 2X, respectively. On Day 34, food intake had decreased to 60.9 ± 8.99%, 60.4 ± 9.28%, and 53.6 ± 9.28%, in group 0X, 1X and 2X, respectively.

Vomiting occurred on at least one day during the study in 3 of 6 dogs (50%) from the placebo group, in 3 of 6 dogs (50%) from the 1X treatment group, and in 4 of 6 dogs (66.7%) from the 2X treatment group (Table 3.1). These differences were not significant. No significant difference in mean number of days with vomiting was observed among any of the groups. Dogs in the 2X treatment group tended to experience more vomiting days than for 0X and 1X treatment groups (0X vs 2X, p = 0.076; 1X vs 2X group, p = 0.090). This was primarily attributed to 2 dogs in the 2X treatment group that vomited 7 and 8 days. The most vomiting days of any dog in the placebo group was 5 days, and in the 1X group was 3 days.
<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Total # of dogs with vomiting</th>
<th># Days with vomiting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>1X</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2X</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1: Frequency of vomiting.

Table 3.2 shows the frequency of days with diarrhea (i.e. a stool consistency score ≤3) for each treatment group. Scores for stool consistency were 4 or 5 for most days in all dogs. Diarrhea was observed on one or more days in 2 of 6 dogs (33.3%) in the placebo group, and in 3 of 6 dogs (50%) in both the 1X and 2X treatment groups. There was no significant difference among treatment groups in the proportion of animals with diarrhea present on at least one day. There was no significant difference in the number of days with diarrhea among any of the treatment groups (0X vs 1X, p = 0.23; 0X vs 2X, p = 0.083; 1X vs 2X, p = 0.37).
## Table 3.2 Frequency of diarrhea

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Total # of dogs</th>
<th># Days with fecal score ≤ 3 (Diarrhea)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fecal score ≤ 3</td>
<td>0 1 2 4 5 6 8 10</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>4 1 1 0 0 0 0 0</td>
</tr>
<tr>
<td>1X</td>
<td>3</td>
<td>3 1 0 0 1 0 0 0</td>
</tr>
<tr>
<td>2X</td>
<td>3</td>
<td>3 0 0 1 0 0 1 1</td>
</tr>
</tbody>
</table>

Anesthesia time ranged from 15 minutes to 1 hour in all dogs but one (1X group, 1 ¾ h), in which foreign body material – pieces of soft rubber chewed off a floor mat that was lining the run – was removed from the stomach on Day -1. No gastric lesions were noted in this dog (total mean gastric score 4). Mean arterial blood pressure was maintained at or above 60 mmHg at all times during the anesthetic procedure, with systolic arterial blood pressures at or above 80 mm Hg. Recovery was uneventful in all cases.

Upon endoscopic evaluation on Day -1, none of the dogs had erosions or ulcerations. Mean total endoscopy scores, total gastroscopy scores, and regional gastric scores for regions A, B, C, D and E were not significantly different on Day -1 among groups. All dogs developed lesions during the study.

Mean total gastroscopy score in each treatment group was not significantly different on Day -1 (mean ± SD: placebo, 4.4 ± 0.8; 1X treatment, 4.4 ± 0.6; 2X treatment, 4.2 ± 0.3; p = 0.55). Mean gastroscopy scores in all three treatment groups increased significantly between Day -1 and Days 14, 21,
and 35 (p < 0.0001; Figure 3.2). On Day 35, mean total gastroscopy scores had increased to 29.2 ± 5.2, 27.3 ± 3.7, and 28.6 ± 3.3 in the placebo, 1X, and 2X treatment groups, respectively. The mean gastroscopy scores were not significantly different among treatment groups on any of the days (p = 0.61).

![Total Gastroscopy Score](image)

Figure 3.2: Total gastroscopy score (mean ± SD) of mucosal lesions in dogs treated with 1 tablet (1X group, n=6) or 2 tablets (2X group, n=6) of 30 mg zinc-L-carnosine/ 30 IU vitamin E q12h PO, or a placebo (0X group, n=6) q12h PO on Days 0–35. Gastrointestinal lesions were induced between Days 7–35 with 25 mg/kg buffered aspirin q8h PO. There is a significant difference between Day-1 and Days 14, 21, and 35 in all treatment groups, but no significant difference among treatment groups at any time point.
Total mean duodenal scores (region E) were not significantly different on Day -1 among groups (mean ± SD: 1 ± 0.0, 1 ± 0.2, 1 ± 0.0 for the placebo, 1X and 2X treatment groups, respectively; p = 0.44). There was no significant effect of treatment on the duodenal lesion score (p = 0.23), but there was a significant interaction between treatment and time (p = 0.020). The mean duodenal score increased significantly at Days 21 and 35 compared with Day -1 and 14 in the placebo group (p ≤ 0.0073, Figure 3.3). In both treatment groups (1X and 2X), there was no significant difference between the treatment days. On Day 35 of the study, the mean duodenal score in the placebo group was significantly higher than in either treatment group (p = 0.0009 with 1X and p = 0.0414 with 2X).
Figure 3.3: Endoscopy score of duodenal mucosal lesions (region E; mean ± SD) in dogs treated with 1 tablet (1X group, n=6) or 2 tablets (2X group, n=6) of 30 mg zinc-L-carnosine/ 30 IU vitamin E q12h PO, or a placebo (0X group, n=6) q12h PO on Days 0 – 35. Gastrointestinal lesions were induced between Days 7 – 35 with 25 mg/kg buffered aspirin q8h PO. The duodenal score increased significantly at Days 21 and 35 compared to Days -1 and 14 in the placebo group, but not in either treatment group. There was a significant interaction between treatment and time. Treatment had no significant effect on lesion scores.

Regional endoscopy scores for the stomach are depicted in Figure 3.4. Compared with Day -1, mean scores were significantly increased on all other days for total endoscopy score (data not shown), and regions A, B, C, D for all three treatment groups (p <0.0002 for each comparison). Treatment had no significant effect on gastric regional scores (p ≥ 0.13).
Figure 3.4: Regional endoscopy score of mucosal lesions (mean ± SD) in the pylorus/ pyloric antrum (A), the angularis incisura (B), corpus (C), and cardia (D) of the stomach in dogs treated with 1 tablet (1X group, n=6) or 2 tablets (2X group, n=6) of 30 mg zinc-L-carnosine/ 30 IU vitamin E q12h PO, or a placebo (0X group, n=6) q12h PO on Days 0 – 35. Gastrointestinal lesions were induced between Days 7 – 35 with 25 mg/kg buffered aspirin q8h PO. There is a significant difference in all treatment groups between Day -1 and Days 14, 21, and 35 (P < 0.0002 for each) for each of the 4 regions. Treatment had no significant effect on lesion scores in any of the 4 gastric regions at any time.

There were no other significant differences among any of the treatments or any of the days for any of the regions scored.
Total gastroscopy scores were significantly correlated between scorer 1 and scorer 2 \((r= 0.9473; 95\% \text{ CI}: 0.9169 \text{ to } 0.9668. \text{ Figure 3.5, A})\). Regional endoscopy scores for the duodenum also were significantly correlated \((r= 0.6343; 95\% \text{ CI}: 0.4719 \text{ to } 0.7550. \text{ Figure 3.5, B})\).

![Correlation between Scorers](image)

**Figure 3.5**: Correlation between scorers \((n=2)\) for total gastroscopy score (A) and regional duodenal score (B) in dogs treated with 1 tablet \((1\times \text{ group, } n=6)\) or 2 tablets \((2\times \text{ group, } n=6)\) of 30 mg zinc-L-carnosine/ 30 IU vitamin E q12h PO, or a placebo \((0\times \text{ group, } n=6)\) q12h PO on Days 0 – 35. Gastrointestinal lesions were induced between Days 7 – 35 with 25 mg/kg buffered aspirin q8h PO.

Complete blood count results at baseline were within the reference range in 8 dogs, and deviated minimally from this range in one of the tests in 10 dogs. None of the dogs was anemic. None of the changes were considered clinically relevant. In the 0X group, 3 dogs had a mildly decreased MCV \((61-63 \text{ fL}; \text{ normal}\)
range, 64-75). In the 1X group, one dog had slight thrombocytosis (435 x10^9/L; normal range, 106-424 x10^9/L), and one dog had a mildly decreased MCV (61 fl). In the 2X group, one dog had 0.1 /100 WBCs nucleated red blood cells (normal, 0/100 WBCs), two dogs had a moderate increase in plasma protein concentration (8.2 and 8.0 g/dL; normal range, 5.7-7.2), and 2 dogs had mild eosinophilia (1.8 and 1.6 x10^9/L; normal range, 0.1-1.2 x10^9/L). Both of these dogs had evidence of flea infestation upon arrival, and one tested positive for gastrointestinal parasites on initial evaluation. These parasites were treated before the start of the study. At baseline, there was no statistical difference in hematological results between the groups.

Mean hematocrit and hemoglobin concentration are depicted in Figure 3.6 A and B, respectively. Between Day < -1 and Day 14 of the study, hematocrit decreased significantly in all groups, but remained within the normal reference range in all dogs except one dog in the 1X group (34% on Day 14; normal range, 36-54). Between Day < -1 and Day 14 of the study, hemoglobin concentration also decreased in groups 1X (p < 0.05) and 2X (p < 0.01). On Day 35, hematocrit and hemoglobin concentration had partially recovered and were not significantly different from results on Day < -1 in any of the three treatment groups. There was no significant effect of treatment on hematocrit and hemoglobin concentration (p = 0.47 for both).
Figure 3.6: Hematocrit (A) and hemoglobin concentration (B; mean ± SD) in dogs treated with 1 tablet (1X group, n=6) or 2 tablets (2X group, n=6) of 30 mg zinc-L-carnosine/ 30 IU vitamin E q12h PO, or a placebo (0X group, n=6) q12h PO on Days 0 – 35. Gastrointestinal lesions were induced between Days 7 – 35 with 25 mg/kg buffered aspirin q8h PO. There is a significant difference in the hematocrit (Hct) between Day -1 and Day 14 (p < 0.05, < 0.05 and <0.01 for the 0X, 1X and 2X groups, respectively). There is a significant difference in the hemoglobin concentration (Hb) between Day -1 and Day 14 (p < 0.05 and <0.01 for 1X and 2X groups, respectively). Treatment had no significant effect on hematocrit or hemoglobin concentration. Horizontal dashed lines indicate the normal reference range for hematocrit and hemoglobin concentration.

Serum biochemistry profile results at baseline were within the reference range in 7 dogs and deviated minimally from this range in 11 dogs. None of these changes were considered clinically relevant. In the 0X and 1X groups, 3 dogs and 1 dog, respectively, had mild increases in BUN (23-30 mg/dL; normal range, 5-20 mg/dL), two of which showed signs of mild dehydration such as increased serum sodium concentration or increased plasma osmolality on their biochemistry profile. Three dogs (one in each group) had mild increases in their
serum globulin concentrations (3.3 – 3.6 g/dL; normal range, 2.2 – 2.9 g/dL). One dog (1X group) had a slight decrease in serum globulin concentration (2.1 g/dL). One dog (2X group) had slight increases in ALT and AST activities (ALT 75 U/L, normal range, 10-55 U/L; AST 47 U/L, normal range 12-40). One dog (0X group) had a slight hypochloremia (107 mEq/L; normal range, 109-120 mEq/L), and slightly increased serum bicarbonate concentration (25.6 mEq/L; normal, range 16-25 mEq/L). At baseline, there was no significant difference in serum biochemistry results between the groups.

Serum potassium concentrations were minimally below the reference range in 5 of 18 dogs at baseline (Day < -1; one each in the 0X and 2X groups, and 3 in the 1X group; 3.9 – 4.1 mEq/L; normal range, 4.2-5.4 mEq/L. Figure 3.7). Mean serum potassium concentrations decreased significantly over time (p < 0.0001). On Days 14 and 35, 11 of 18 and 15 of 18 dogs, respectively, had mild hypokalemia, ranging from 3.3 to 4.1 mEq/L. Serum potassium concentrations decreased to slightly below the reference range at Day 14 in 2 dogs in group 0X, 2 dogs in group 1X, and 1 dog in group 2X; and at Day 35 in 5 dogs in group 0X, 4 dogs in group 1X, and 2 dogs in group 2X; however, treatment had no significant effect on serum potassium concentrations (Figure 3.7).
Figure 3.7: Serum potassium concentration (mean ± SD) in dogs treated with 1 tablet (1X group, n=6) or 2 tablets (2X group, n=6) of 30 mg zinc-L-carnosine/30 IU vitamin E q12h PO, or a placebo (0X group, n=6) q12h PO on Days 0 – 35. Gastrointestinal lesions were induced between Days 7 – 35 with 25 mg/kg buffered aspirin q8h PO. Serum potassium concentrations decrease significantly over time (p < 0.0001). Treatment had no significant effect on serum potassium concentrations. Horizontal dashed lines indicate the normal range for serum potassium (4.2-5.4 mEq/L).

Serum globulin concentration was decreased minimally on Day 14 or 35 in 2 dogs in group 0X (1.6 and 1.9 g/dL), 3 dogs in group 1X (1.7, 1.7, and 1.8 g/dL), and 2 dogs in group 2X (1.5 and 2.1 g/dL).

All dogs were negative for fecal occult blood at the start of the study (Day 0), and after 7 days of treatment with placebo or test product only (Day 7). After the start of aspirin treatment, once weekly fecal hemoccult testing was positive in 2 dogs in the 0X treatment group, in 3 dogs in the 1X treatment group (one positive fecal in 2 dogs, and 3 positive fecals in one dog) and in 4 dogs in the 2X
treatment group (one dog was positive twice). There was no significant difference in the proportion of animals with a positive fecal occult blood test between the treatment groups. There was no significant difference in the number of days with a positive fecal occult blood test between any of the treatment groups (0X vs 1X, p = 0.38; 0X vs 2X, p = 0.23; 1X vs 2X, p = 1.00).

Fecal flotation on Day 35 was positive for *Capillaria* in two dogs (one in the 1X and one in the 2X treatment group). The fecal immunoassay for Giardia was negative in all dogs.
CHAPTER 4

DISCUSSION

The zinc-L-carnosine/vitamin E product was well tolerated. Throughout
the study, body weight either increased or maintained within 1 kg of Day 0 in
almost all dogs, despite a significant decrease in food intake during the course of
the study. Due to the management practices in the housing facility, all dogs were
given the same amount of food each day (5 cups), regardless of their weight.
Food intake was estimated as a percentage of the total allotment. As a result,
the significantly decreasing food intake during the study period was not reflected
in a decrease in body weight, as most dogs were still ingesting enough to
maintain their body weight.

Vomiting occurred infrequently in all groups, but a slightly higher
frequency of vomiting was observed in the 2X treatment group. Fecal
consistency scores were 4 or 5 for most days in all dogs, and there was no
significant difference among the groups. Safety studies with zinc-L-carnosine
have shown vomiting and diarrhea as possible adverse effects of treatment.¹
Dogs given zinc-L-carnosine at 50, 120 or 300 mg/kg/day for 13 weeks showed
an increase in vomiting and mild diarrhea with increased dosages.\textsuperscript{1} No such side effects were noted at 8 or 20 mg/kg/day.\textsuperscript{1} The doses of zinc-L-carnosine used in our study are much lower than those reported in this toxicity study; thus a significant effect of zinc-L-carnosine on the frequency of vomiting or diarrhea would not be expected.

The use of many different NSAIDs has been associated with gastrointestinal injury in dogs.\textsuperscript{2-6} Aspirin reliably induces gastrointestinal mucosal lesions\textsuperscript{7-11}, and therefore often is used as a model for gastrointestinal injury.

All dogs developed multiple mucosal erosions or ulcers during the course of the study. The total gastroscopy scores at the end of the study were 29, 27, and 28 in the placebo, 1X, and 2X treatment groups, respectively. No significant differences in total or in regional gastric scores were noted among the placebo group and either of the treatment groups throughout the study. Therefore, zinc-L-carnosine/vitamin E administration at 1X or 2X dosing was not found to be effective for prevention of gastric lesions in this model of gastrointestinal injury. This is in contrast with misoprostol, which significantly reduced mucosal injury using the same model.\textsuperscript{10} The dose of zinc-L-carnosine used in this study, 30 or 60 mg/ dog twice daily ($\sim$ 2.6 – 7.1 mg/kg/day) was based on the manufacturer’s recommended dosage in the product package insert. However, this dosage is markedly lower than the range of 10 – 100 mg/kg reported to have a beneficial protective effect on the gastrointestinal mucosa of rats.\textsuperscript{12}
The use of aspirin for the induction of gastrointestinal mucosal lesions in dogs has been reported previously; however, the dosages of aspirin used in these studies varied, and a standardized protocol for the evaluation of mucosal lesions was not used. This makes comparison between studies difficult. While not identical, four studies that used similar aspirin dosages and endoscopic scoring systems will be discussed here.

The aspirin dosage and the grading scale for endoscopic lesions used in this study are modeled after a study reported previously by Sennello and Leib. These authors investigated the effects of deracoxib and buffered aspirin on the gastric mucosa of healthy dogs. Similar to the effects of aspirin on the gastric mucosa noted in our study, total gastric lesion scores increased significantly between the pretreatment evaluation, and after 6, 14, and 28 days of aspirin administration. After the initial increase, there was no significant difference in lesion scores during aspirin administration. The aspirin group had significantly higher gastric lesion scores than dogs receiving deracoxib or placebo on days 6, 14, and 28. The median total gastroscopy scores for the aspirin group in our study ranged from approximately 21 to 30 (approximate range, 11 – 35). These values are somewhat lower than the total gastroscopy scores found in the aspirin-only group of our study (median, 30 – 32; mean, 29 – 32; range, 20 – 37), suggesting a more increased ulcerogenic effect of aspirin on the stomach mucosa of the dogs in our study.
In another study, Johnston et al investigated the protective effects of the prostaglandin analogue misoprostol on aspirin-induced gastro-duodenal lesions in dogs.\textsuperscript{11} These authors used an identical 12-point scoring scale, but administered a higher dose of aspirin (35 mg/kg PO q8h) than was used in our study (25 mg/kg PO q8h). A mean total endoscopy score (total of 5 regions evaluated) of 26, 24, and 21 on study days 5, 14, and 30, respectively, was reported in this study. Despite a higher aspirin dosage, these lesion scores are again somewhat lower than the total endoscopy scores in the aspirin-only group of our study; 30 ± 5 (mean + SD) after 7 days of aspirin treatment (Day 14), 35 ± 3 after 14 days of aspirin treatment (Day 21), and 33 ± 8 after 28 days of aspirin treatment (Day 35). Again, the effects of aspirin on the dogs in our study seemed more pronounced.

Another study by Ward et al used an 11-point grading scale to evaluate a 25 mg/kg q8h aspirin model.\textsuperscript{10} In this study the median total gastric lesion scores were 16, 19, and 21 on study days 5, 14, and 28, respectively. Despite a 1 point difference in the scoring scale, these lesion scores also are lower than the total gastroscopy scores in the aspirin-only group of our study; 31 (20 – 32; median, range) after 7 days of aspirin treatment (Day 14), 32 (28 – 36) after 14 days of aspirin treatment (Day 21), and 30 (22 – 37) after 28 days of aspirin treatment (Day 35).

In the last study, Reimer et al used 16.5 mg/kg q12h of aspirin for the
induction of gastro-duodenal mucosal lesions and a modified 11-point scoring system. The severity of gastro-duodenal lesion scores with aspirin was significantly higher than in any of the other groups (carprofen, etodolac, and placebo). Median scores for dogs in the aspirin group were 27, 26, and 27 on days 5, 14, and 28, respectively, with a range not exceeding 30.

Overall, the mucosal effects of the aspirin model used in these four studies were similar but less severe than observed in our dogs based on comparable scoring systems. The higher lesion scores in our study likely represent variation in observer application of the subjective scoring criteria, but could also be attributable to variable responses in separate dog populations. It is noteworthy that the scoring for the two blinded scorers in our study correlated well, especially for the stomach.

All dogs in our study developed multiple erosions or ulcerations. Prolonged anesthesia and associated hypotension were unlikely to have contributed to the development of gastrointestinal ulcerations. Even though some of the animals were under general anesthesia for up to one hour, all animals received intravenous fluids during the procedure and their blood pressure was monitored every 10 minutes. At no time, was clinically relevant hypotension detected.

The severity of the model may have affected our ability to evaluate more subtle preventative or healing effects of zinc-L-carnosine/ vitamin E in the dogs of our study. It is unclear why the animals used in this study seemed more
sensitive to the effects of aspirin compared to other reports. Perhaps, higher endogenous cortisol concentrations secondary to the stress of parasitic infections or helicobacter infections played some role, but plasma cortisol concentrations were not measured in our dogs.

In dogs, the prevalence of helicobacter-like organisms (HLO) in the gastrointestinal tract is high among healthy dogs and in dogs with gastrointestinal signs (100% of random source dogs, 76-100% of clinically healthy pet dogs and 100% of laboratory beagles and shelter dogs infected).\textsuperscript{16,17} Currently, the role of HLO as a cause of gastrointestinal signs and lesions in dogs is unclear. No clear association has been made between a HLO infection and the severity of gastritis or the presence of clinical signs.\textsuperscript{18,19} The presence of HLO was not evaluated in this study since the prevalence in random source research dogs approaches 100%, the available diagnostic tests are imprecise, and the pathogenic significance of HLO in dogs is inconclusive.\textsuperscript{7,8,20,21}

Our study showed a tendency for a lower lesion score in the duodenum in the treated groups, which could indicate a preferential protective effect of zinc-L-carnosine/ vitamin E for the intestine. However, most of the intestine was not evaluated with endoscopy during this study and it is not possible to draw any conclusions about the severity of lesions within the more distal intestines. In addition, scores in the duodenum were generally low while the variation was large.
In addition, most non-neoplastic gastrointestinal ulcers in dogs occur in the stomach (~74%) rather than in the proximal duodenum (~33%). Of dogs receiving NSAIDs, most had ulcers located in the stomach (n = 15), whereas only five had duodenal ulcers. One dog had ulcers in multiple sites.

Aspirin in particular may have a preferential ulcerogenic effect on the stomach rather than the intestinal mucosa. In a group of healthy dogs treated with aspirin, Reimer et al reported significantly lower duodenal lesion scores than gastric lesion scores. On necropsy of 5 dogs treated with high dosages of aspirin (101-310 mg/kg/day for 4 days to 3 weeks), Lev et al found the most severe lesions in the stomach (e.g., ulcers, perforating ulcer). Two of 5 dogs had erosions in the small intestines. This predilection of the stomach to aspirin-induced mucosal lesions has been reported in people, in which gastric ulcers are approximately 4 times more common than duodenal ulcers in patients taking NSAIDs.

This preferential ulcerogenic effect of aspirin on the gastric mucosa may be explained by a higher constituitive activity of cyclooxygenases in the stomach. Wooten et al showed that cyclooxygenase expression (both COX-1 and COX-2) and prostaglandin concentration in dogs were significantly greater in the pyloric mucosa, compared with the duodenal mucosa. In addition, the thromboxane concentration was significantly higher in the pyloric mucosa compared with the duodenum.
Evaluation of gastrointestinal lesions was performed by endoscopic assessment and fecal occult blood testing. Unexpectedly, only 12 of 90 hemoccult tests (13%) during the study were positive. The presence of fecal occult blood was identified in only 9 of 18 dogs (50% of the dogs in each treatment group) at any time despite the presence of multiple gastric erosions or ulcerations in all dogs. Thus, fecal occult blood testing was an unreliable indicator of gastrointestinal mucosal injury in this study. This result is in accordance with previous studies that reported poor sensitivity of fecal occult blood testing for the detection of gastrointestinal lesions in dogs.\textsuperscript{25,26} Perhaps, intermittent rather than continuous bleeding from the ulcerated sites, as was observed endoscopically, explains the inconsistently positive fecal occult blood tests.

Overall, hematologic and serum chemistry results varied minimally from baseline during the study and generally remained within or near the normal reference ranges for each parameter. The hematocrit and hemoglobin concentration decreased from baseline at Day 14 or 35 in almost all dogs, but remained within the reference range in all dogs except one. The most likely explanation for the decrease in erythrocyte parameters was subclinical blood loss associated with bleeding from gastrointestinal mucosal erosions and ulcers seen in all dogs. The eosinophil count was above the normal range in 4 dogs before the study and in one dog in each of the 3 treatment groups at Day 14, Day 35,
or both. Eosinophilia was attributable to endoparasites or fleas that were identified and treated during the acclimation period. All dogs were free of parasites before the treatment phase began.

At the end of the study (Day 35), 2 dogs with eosinophilia were unexpectedly found to be positive for Capillaria. Infection was not detected in previous fecal examinations, and the short courses of fenbendazole before the start of the study would likely not have adequately eliminated this parasite. This was considered to be an incidental infection. At no time during the study did the dogs become clinical for this respiratory nematode and it would not be expected to have affected the results of our study, but it could explain the eosinophilia in these 2 dogs.

The hypokalemia noted during the study was most likely normal physiologic variation and was not considered clinically relevant. Hypokalemia could be associated with increased loss of potassium through vomiting, diarrhea, or renal excretion. However, the development of hypokalemia did not seem to correlate with the presence of vomiting, diarrhea, or other recognizable mechanisms of potassium loss.

A few dogs in each treatment group had serum globulin concentrations that were above or below the reference range. Globulins reflect past and current exposure to antigenic stimuli, such as vaccines, viruses, gastrointestinal parasites, and ectoparasites (fleas). The variation in globulins seen in this
population of young dogs likely represents normal physiologic variation rather than any factor associated with the study, and this would not be expected to have any impact on the results.

This study was designed to investigate the gastroprotective effects of zinc-L-carnosine/ vitamin E in an aspirin-induced gastritis model. Gastrointestinal lesions and clinical parameters in two treatment groups were compared with those in a group receiving aspirin only. The study did not include a group of dogs without any treatment or a group of dogs treated with zinc-L-carnosine/ vitamin E only. Including these groups would have allowed for evaluation of the development of gastrointestinal lesions over time, and the effect of zinc-L-carnosine/ vitamin E on the gastrointestinal mucosa. A toxicity study in dogs treated with 50, 120, or 300 mg/kg/day of zinc-L-carnosine for 13 weeks, or with 8 or 20 mg/kg/day for 52 weeks revealed no gastrointestinal mucosal lesions on necropsy in any of the dogs (n=4-6 dogs per group). Mucosal lesions were only detected in a single dog after a single dose of 200 mg/kg. Hemorrhage, erosion, ulceration, and an inflammatory cell infiltration were noted. It is unlikely that zinc-L-carnosine/ vitamin E at the dose used in our study would have contributed to the development of gastrointestinal lesions.

The number of dogs in this study was small, affecting the statistical power of the analysis and perhaps limiting the ability to detect smaller effects of the treatment. However, all dogs developed lesions that were considered to be
clinically significant (multiple erosions or ulcerations). Despite the severity of their gastrointestinal lesions, the dogs showed surprisingly little signs of gastrointestinal disease or discomfort. Food intake decreased significantly during the course of the study, but vomiting and diarrhea occurred only occasionally. The dogs were monitored three times daily for clinical signs and behaviors of discomfort, but abnormalities were rarely observed. However, the dogs were always aware of the presence of the observer, and pain behaviors could have been more apparent had the dogs been observed remotely with a video camera system.

Despite their benefits, NSAIDs are a common cause of injury to the gastrointestinal mucosa, resulting in adverse effects such as inappetence, nausea, vomiting, diarrhea, and gastrointestinal hemorrhage or perforation. Lesions and adverse effects are most severe when NSAIDs are used at an inappropriate dosage, or when they are used in close temporal association with another NSAID or glucocorticosteroid therapy.\textsuperscript{25,27,28} However, occasional severe adverse effects can occur even at appropriate therapeutic dosages.\textsuperscript{6}

This study aimed to investigate the gastroprotective effects of zinc-L-carnosine/ vitamin E on the development of gastrointestinal mucosal lesions in an aspirin-induced gastritis model in dogs. Zinc-L-carnosine/ vitamin E at 30 or 60 mg is well tolerated but did not prevent gastric mucosal lesions in our gastritis model. The gastroprotective effect of zinc-L-carnosine/ vitamin E in other models
of gastric injury or in the clinical setting (e.g., naturally occurring gastritis) cannot be extrapolated from these results. In clinical practice, it is rare to detect such severe gastrointestinal mucosal lesions. Therefore, the role for zinc-L-carnosine as a gastroprotectant in the clinical setting needs further investigation.

4.1 REFERENCES


Lonnerdal B. Dietary factors influencing zinc absorption. J Nutr 2000;130:1378S-1383S.


Wallace JL. Prostaglandins, NSAIDs, and gastric mucosal protection: why doesn't the stomach digest itself? Physiol Rev 2008;88:1547-1565.


