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Carprofen-induced oxidative stress in mitochondria of the colonic mucosa of the dog

Lynne A. Snow
Louisiana State University and Agricultural and Mechanical College

CARPROFEN-INDUCED OXIDATIVE STRESS IN MITOCHONDRIA OF THE COLONIC MUCOSA OF THE DOG

A Thesis

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Lynne A. Snow
B.S., University of South Carolina, 2000
D.V.M., University of Illinois, 2004
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To my loving family.

I couldn’t have done it without you.

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ABSTRACT

Objectives

1) To measure conductance and permeability of canine colonic mucosa exposed to increasing concentrations of carprofen.

2) To compare conductance and permeability of canine colonic mucosa exposed to carprofen or 2,4-dinitrophenol (DNP) and tempol blockade.

Design

In vitro randomized block design

Animal

20 mixed breed dogs

Methods

Conductance, mannitol flux, and histology were evaluated in colonic mucosa mounted in Ussing chambers. Mucosa was first exposed to increasing concentrations of carprofen. Mucosa was then exposed to either carprofen (200 μg/ml) or DNP (0.25mM) +/- tempol (1mM) pretreatment. Conductance over time, mannitol fluxes, and frequency of histologic categories were analyzed for treatment effects. Histopathology and electron microscopy were evaluated post experiment.

Results

Mean +/- SEM conductance*time for 400 μg/ml carprofen treated colon was significantly greater than control. Mean +/- SEM conductance*time for carprofen treated colon at 200, 100 and 40 μg/ml were not significantly different from control. Mean +/- SEM conductance*time for 400 μg/ml and 200 μg/ml carprofen treated colon were not significantly different. Period 3 mannitol flux was greater than period 1 for 400 μg/ml and 200 μg/ml carprofen treated colon but not significantly different for 100 μg/ml, 40 μg/ml, and control. Period 3 flux for 400 μg/ml and 200

μg/ml carprofen treated colon were not different but were greater than control. Mean +/- SEM conductance*time for carprofen or DNP treated colon were not significantly different from control regardless of blockade. Period 3 flux for carprofen and DNP treated colon were not different but were greater than control. Period 3 flux for carprofen treated colon with tempol pretreatment was not significantly different than control. Period 3 flux for DNP treated colon with tempol pretreatment was not different than without tempol but was greater than control. Cell sloughing and erosions were observed with high carprofen concentrations. Mitochondrial damage was seen with carprofen treatment compared to DNP treatment or control. Tempol pretreatment effect on mitochondrial morphology was inconsistent.

Conclusion

Carprofen exhibits concentration dependent toxicity to canine colonic mucosa. Carprofen and DNP induce similar mucosal damage evident by changes in electrical conductance, mannitol flux, and histopathology. Carprofen damages enterocyte mitochondria.

CHAPTER 1. BACKGROUND AND REVIEW OF LITERATURE

1.1 Colon

1.1.1 Macroscopic Anatomy

The canine large intestine is separated into the cecum, colon, and rectum. The colon accounts for almost the entire length of the large intestine in the dog. It is differentiated into three anatomic regions, the ascending, transverse, and descending parts. The ascending colon begins at the ileocolic orifice and sphincter extending to the right colic flexure. It lies between the descending duodenum and the root of the mesentery.[1] The transverse colon traverses the abdomen from right to left cranial to the root of the mesentery and ventral to the left lobe of the pancreas.[1] The transverse colon terminates at the left colic flexure. The descending colon is the longest segment from the left colic flexure to the pelvic inlet. It follows along the left flank before edging medially at the pelvic inlet where it continues as the rectum.[1] The colon is suspended throughout its length by a moderately long mesocolon which allows some mobility.[1]

The arterial blood supply of the colon is supplied by the cranial and caudal mesenteric arteries (Fig 1). The cranial mesenteric artery supplies the bulk of the small intestine, the ileocolic junction, and the midpart of the colon.[1] The colic branch of the ileocolic artery supplies the ascending colon; the right colic artery courses through the mesocolon to the right colic flexure supplying the distal ascending and proximal transverse colon; the middle colic artery extends to the left colic flexure supplying the distal transverse and proximal descending colon. The left colic branch of the caudal mesenteric artery supplies the distal descending colon and the cranial part of the rectum.[1] The caudal mesenteric vein carries blood from the colon to the portal vein.[1] Autonomic innervation of the colon is supplied by the cranial and caudal mesenteric plexuses.[1]

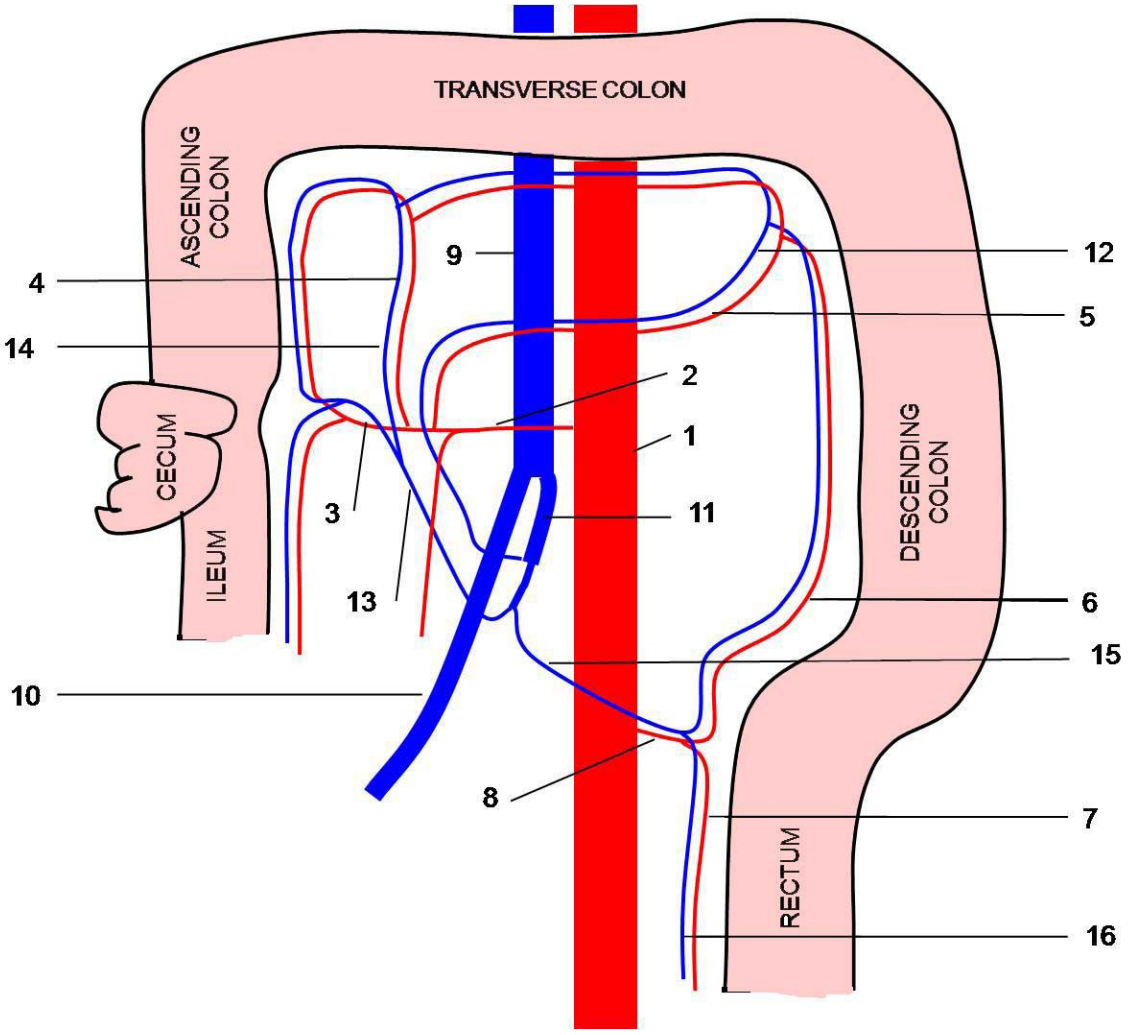


Figure 1. Arterial and venous blood supply of the colon. 1) aorta, 2) cranial mesenteric artery, 3) ileocolic artery, 4) right colic artery, 5) middle colic artery, 6) left colic artery, 7) cranial rectal atery, 8) caudal mesenteric artery, 9) portal vein, 10) cranial mesenteric vein, 11) caudal mesenteric vein, 12) middle colic vein, 13) ileocolic vein, 14) right colic vein, 15) left colic vein, 16) caudal rectal vein.

1.1.2 Microscopic Anatomy

The wall of the colon is composed of four layers: the mucosa, the submucosa, the muscularis, and the serosa (Fig 2). The mucosa is smooth and lines the luminal surface of the colon.[1;2] The mucosa is lined by a simple layer of columnar epithelial cells interspersed with goblet cells.[2] Junctional complexes between the epithelial cells prevent intestinal contents from

diffusing directly into the lamina propria.[2] The columnar epithelial cells have a basal nucleus.[2] Ultrastructurally mitochondria are most prominent near the nucleus in the basal region along with the supranuclear Golgi.[2] Rough endoplasmic reticulum and free ribosomes are located in the basal part of the cell.[2] Apical cytoplasm contains smooth endoplasmic reticulum.[2] The epithelial cells are folded on themselves to form straight tubular mucosal glands that extend away from the lumen to the lamina muscularis. The lamina muscularis is a thin layer of smooth muscle at the base of the mucosa, adjacent to the submucosa.[2] The lamina propria occupies the region between the epithelium and the lamina muscularis. It is composed of loose connective tissue containing blood and lymph vessels, leukocytes, plasma cells, mast cells, and fibrocytes.[2]

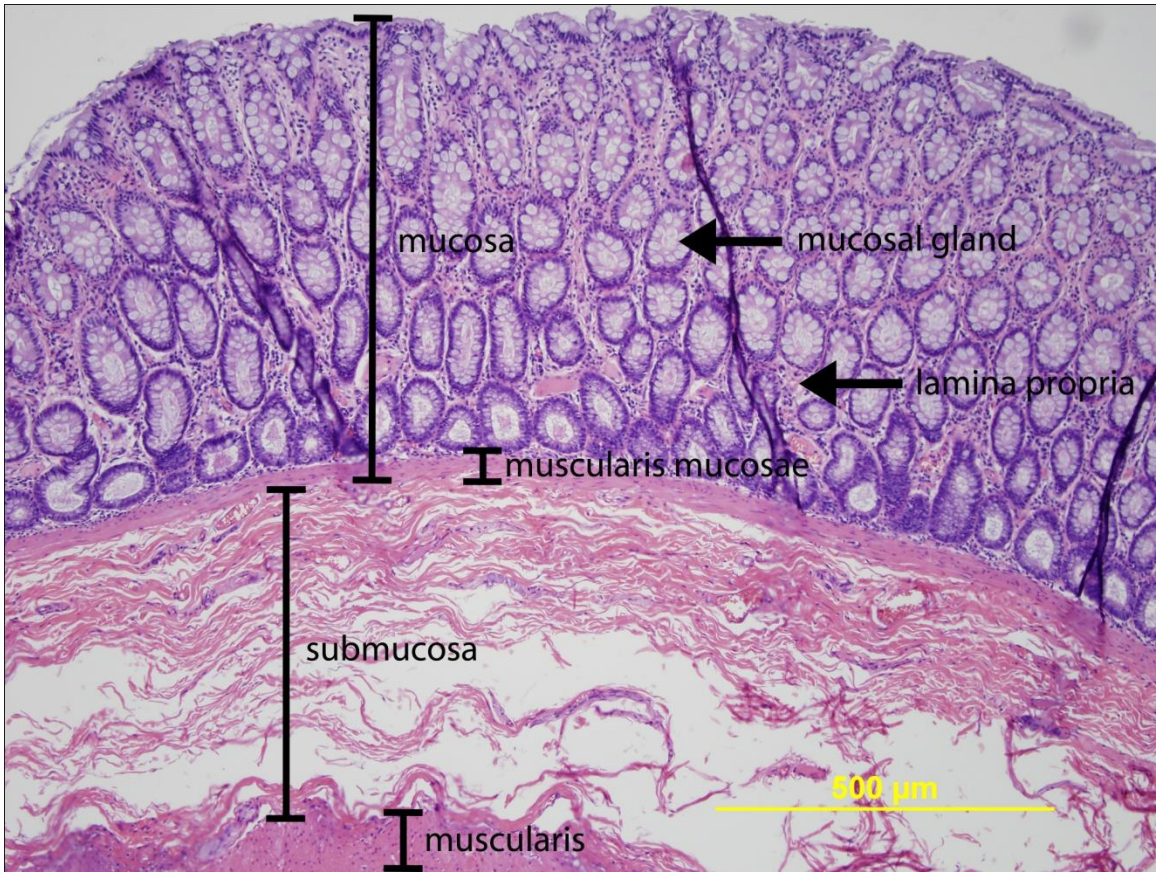


Figure 2. Light microscopy of normal colon

The submucosa is a layer of dense connective tissue. The submucosa contains lymphatic nodules, blood vessels, and nerve fiber plexuses.[2] The muscularis layer consists of an inner circular and outer longitudinal smooth muscle layer.[2] The connective tissue between the muscle layers contains the myenteric plexus.[2] The outer layer of the colon is a layer of loose connective tissue and mesothelium known as the serosa.[2]

1.1.3 Colonic Mucosal Epithelium and Ion Transport

The major cations present in the colon lumen are sodium (35 - 40 mEq/L) and potassium (90 mEq/L).[3] Chloride (15 mEq/L) and bicarbonate (30 mEq/L) are the major anions within the colon. Movement of ions through the intestinal lumen occurs via two mechanisms, through the transcellular or paracellular pathways. Through the transcellular pathways ions pass through the epithelial cells via membrane bound proteins.[3] The paracellular pathway involves the movement of ions through the tight junctions which connect epithelial cells.[3] Ions move down a concentration gradient via the paracellular pathway. Intestinal tight junctions are approximately twice as permeable to sodium and potassium as they are to chloride, therefore an electrical potential difference can be established across the epithelium.[3]

All regions of the colon absorb sodium and chloride. Restricted diffusion of sodium via mineralocorticoid dependent channels is the primary mechanism of sodium absorption.[3] This is an electrogenic process. Sodium is also absorbed through an electroneutral process by the cotransport with chloride. Most likely this electroneutral process involves the countertransport of sodium with hydrogen ions, coupled with the countertransport of chloride with bicarbonate.[3] Once in the epithelial cells, sodium may exit the basolateral membrane via one of two routes. It may passively diffuse along a concentration gradient.[3] Alternatively it is pumped out of the cell

in exchange for potassium in a 3:1 ratio by sodium-potassium adenosine triphosphatase (ATPase) pump.[3]

Sodium chloride secretion occurs within the colonic crypt cells. The primary mechanism involves the electroneutral sodium chloride cotransport into the epithelial cells along the basolateral membrane.[3] Sodium moves down an electrochemical gradient creating the driving force for chloride to diffuse into the cell against an electrochemical potential. Sodium is then transported out of the cell by the sodium potassium ATPase of the basolateral membrane[3]. This creates an electrochemical gradient with high intracellular chloride levels compared to the lumen. Chloride diffuses across the apical membrane through selective chloride channels.[3] These apical chloride channels are opened by elevations in intracellular cyclic adenosine monophosphate and calcium, gastrointestinal hormones, neurotransmitters, and prostaglandins.[3] The movement of chloride from the serosal to mucosal compartment is the major source of electrical potential difference in the colon causing the lumen to be negatively charged in reference to the serosa.[3] Sodium passes from the serosal fluid to the lumen via the paracellular pathway in response to this electrical potential difference, leading to a net secretion of sodium chloride.[3]

Potassium transport in the colon is through passive diffusion or active transport via the sodium potassium adenosine triphosphatase pump. The basolateral sodium potassium adenosine triphosphatase pump maintains high intracellular potassium levels.[3] The colonic basolateral and apical epithelial membranes are permeable to potassium and potassium will diffuse across a concentration gradient.[3]

Bicarbonate is produced in epithelial cells by the hydration of carbon dioxide by carbonic anhydrase.[3] Bicarbonate ions are secreted into the colonic lumen in connection with chloride

absorption. Colonic contents are high in bicarbonate ions (30 mEq/L) creating a relatively alkaline environment.[3]

There is no active transport of mannitol across the colonic mucosa. Mannitol, like other small molecules, diffuses by both paracellular and transcellular mechanisms.[4] Permeation of mannitol is along the concentration gradient and is therefore sensitive to differences in surface area and convective forces in the colon.[4]

1.1.4 Ussing Chamber System

The Ussing chamber was developed by Hans Ussing in the 1950's to investigate molecular conduction and permeability across a membrane. In its simplest form the system consists of a chamber separated by a membrane and connected to an electrical circuit.[5]
The Ussing chamber consists of two symmetric hemispheres which are separated by the tissue of interest (Fig 3).[5] Each hemisphere consists of a "U" shaped tube connected to an acrylic hemi-chamber by polyethylene tubing. The lumens of the hemi-chambers are separated by the membrane of interest, thus the only communication between the hemi-chambers is through the membrane being examined. Each reservoir is surrounded by a temperature controlled water jacket. The chamber is filled with a bathing solution which is circulated with air or other gasses. The gas, typically 95% oxygen and 5% carbon dioxide, serve to oxygenate the tissue as well as stir the solution in the reservoir.[5;6]

Each hemi-chamber is connected to an electrical circuit via a voltage and current electrode. The silver - silver chloride (Ag- AgCl) electrodes are connected to each hemi-chamber through a conducting medium, typically agar.[6] Each electrode is connected to a pre-amplifier. The polarity of the membrane allows electrical measurements of short circuit current and potential difference across the membrane to be measured.[5] Transport of chloride, sodium,

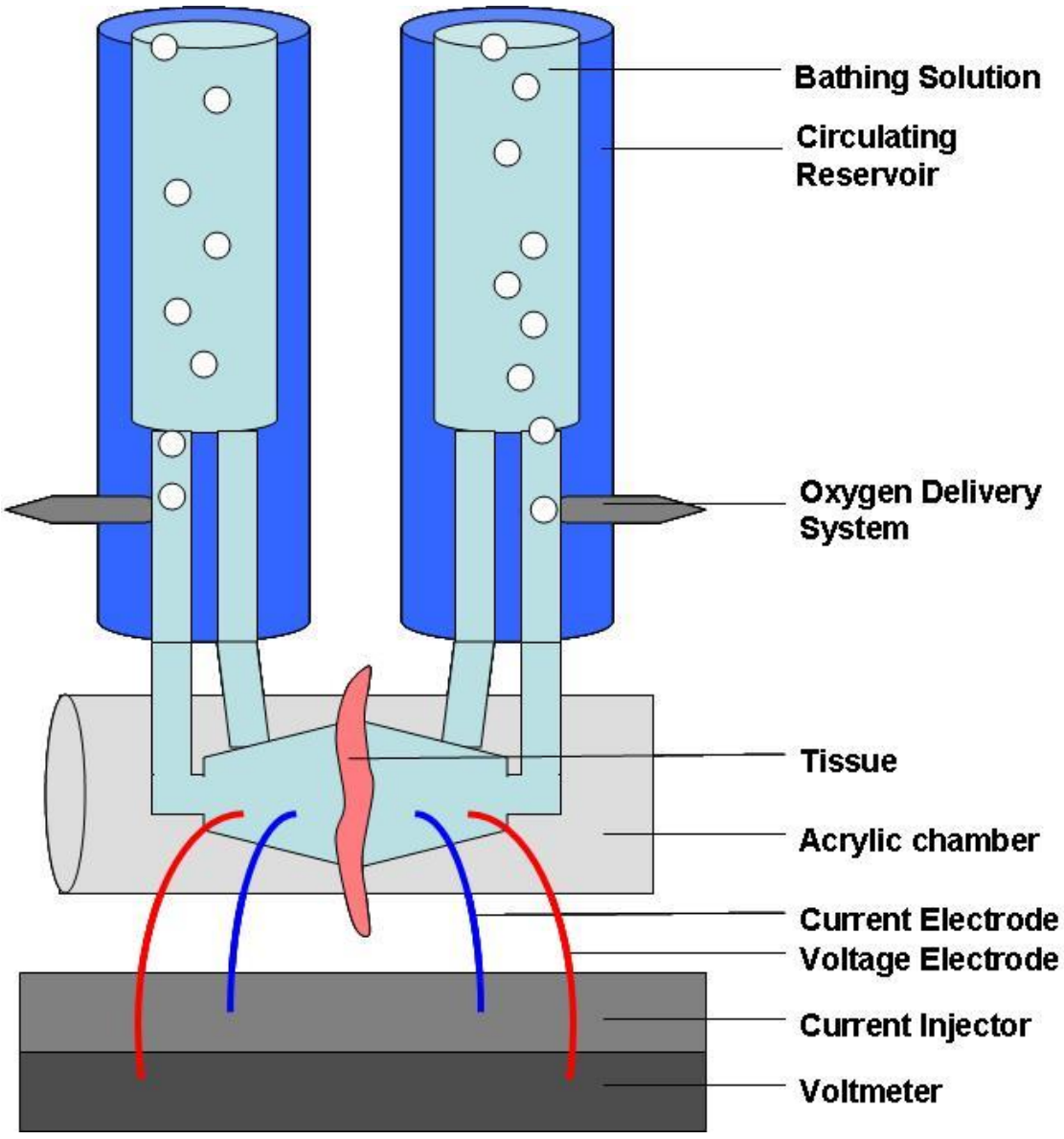


Figure 3. Ussing chamber

and potassium ions across the membrane accounts for most of the in vitro short circuit current across the colonic membrane.[3] From these values electrical conductance is calculated using Ohm's law. Ohm's law states that the current through a conductor is directly proportional to the electrical potential difference or voltage between two points of an electrical circuit, and inversely

proportional to the resistance between them.[5] Algebraic conversion allows the calculation of the electrical resistance across the membrane used in the Ussing chamber as:

$$R = V / I$$

Where, R is the electrical resistance (Ω, ohm),

V is the electrical potential difference (V, volt),

and I is the short circuit current (A, ampere).

Electrical resistance of a conductor is a function of the amount of current flowing through the membrane for a given voltage and is a measure of the degree to which a conductor resists the passage of electrical current. Therefore the integrity of a membrane is a crucial determinant of electrical resistance.[5] Electrical conductance is the reciprocal of electrical resistance:

$$G = 1 / R = I / V$$

Where, G is the electrical conductance (S, siemens),

R is the electrical resistance (Ω, ohm),

I is the short circuit current (A, ampere),

and V is the electrical potential difference (V, volt).

1.2 Non Steroidal Anti-inflammatory Drugs (NSAIDs)

1.2.1 Prostaglandin Synthesis

Arachidonic acid is liberated into the cytoplasm when the cell phospholipid membrane is damaged. Cyclooxygenase enzymes catalyze the two reactions necessary to form prostaglandins from arachidonic acid (Fig 4). Arachidonic acid is also the substrate for the production of leukotrienes via lipoxygenase.[7] Endoperoxidase synthase converts arachidonic acid into prostaglandin G_2 which is converted into prostaglandin H_2 by peroxidase.[7;8] The fate of prostaglandin H_2 is determined by the cell type and other enzymes present. Prostaglandin H_2 is

made into thromboxane A_2, prostaglandin D_2, prostaglandin E_2, prostaglandin F_2, and prostaglandin I_2.[9;10]

Two cyclooxygenase enzymes have been identified. Cyclooxygenase 1 is responsible for maintaining normal homeostasis in the body including the gastrointestinal tract, renal tissues, and platelet function.[7;8] Cyclooxygenase 2 is up regulated in inflammatory states.[7;8] Cyclooxygenase 2 also produces prostaglandins that are involved in the healing of mucosal erosions, inhibition of leukocyte adherence, and renal maturation and protection.[10]

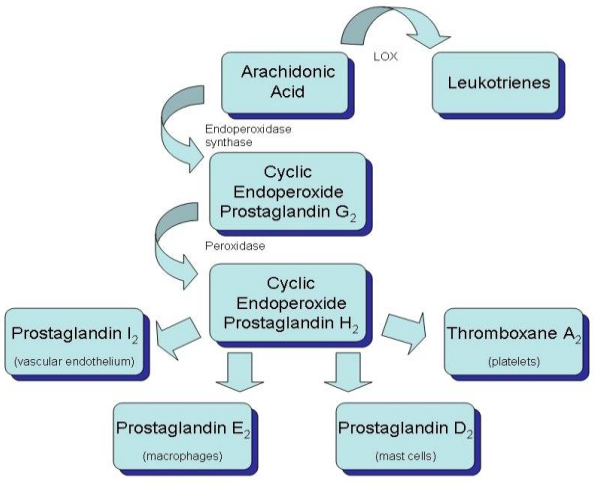


Figure 4. Arachidonic acid cascade

1.2.2 Prostaglandins and Gastrointestinal Physiology

Prostaglandins have long since been recognized for their homeostatic function in the gastrointestinal tract. Suggested actions of prostaglandins in gastric secretion include modulation of gastric acidity, secretion of mucus and bicarbonate, and augmentation of gastric blood flow.[11] In the human gastrointestinal tract the most abundant prostaglandins in the gastric mucosa are prostaglandin E_2 and $F_{2\alpha}$ with much lower levels of prostaglandins D_2 and I_2.[11] Excessive gastric acid production may lead to ulceration. The inhibition of gastric acid secretion by the administration of arachidonic acid is blocked by indomethacin thus suggesting that acid production is mediated by cyclooxygenase system.[11] Administration of prostaglandin analogues such as intravenous prostaglandin E, has been shown to decrease acid and pepsin secretion.[11]

Three populations of cyclooxygenase producing cells have been identified in the human colon. The most numerous cells in the colon staining positive for cyclooxygenase 1 protein responsible for the majority of prostaglandin synthesis are mononuclear $CD3^+$ T lymphocytes in the lamina propria.[12] Fewer numbers of intraepithelial and crypt apical cells were identified as positive for cyclooxygenase 1 protein.[12] The major prostaglandins produced by colonic epithelial cells are prostaglandin I_2 and $F_{2\alpha}$ and prostaglandin E_2 by the subepithelium.[11] Prostaglandins of the E and F series administered intraluminally to the large and small intestine stimulate chloride secretion and inhibit electroneutral sodium chloride absorption.[11]

In addition to electrolyte and acid control, prostaglandins have been shown to be integral in intestinal motility. Isolated intestinal muscle exposed to prostaglandin E_2 had increased contraction of longitudinal muscle and relaxation of circular muscle.[11] Prostaglandin $F_{2\alpha}$ stimulated contraction in both longitudinal and circular muscle layers.[11]

1.2.3 Non Steroidal Anti-inflammatory Drugs and the Colon

The mechanism of colonic damage by NSAIDs has not been investigated in the dog. There are three methods by which NSAIDs can have an adverse effect on the colonic mucosa; 1) inhibition of prostaglandins by systemic absorption 2) direct topical toxicity and 3) topical toxicity through enterohepatic recirculation. Studies in rats suggest a topical toxic effect of NSAIDs to be important, along with inhibition of prostaglandins. One study demonstrated jejunal mitochondrial damage, seen by electron microscopy, in rats given indomethacin by gavage but not in rats in which the bile duct had been ligated.[13] This suggests that the systemic effect of indomethacin at the given dose was not sufficient to affect intestinal mitochondria, where as the biliary excreted component was sufficient to cause a topical toxicity.[13] Aspirin is unique among NSAIDs since it is rapidly absorbed by the stomach while having minimal

enterohepatic recirculation. When aspirin was given by gavage no intestinal ulceration or mitochondrial changes were noted, however, when directly instilled into the duodenum, aspirin caused extensive ulcerations distal to the administration site. Mitochondrial changes seen in the intestinal segments were similar to those of indomethacin and 2,4-dinitrophenol (DNP), an uncoupler of mitochondrial oxidative phosphorylation.[13]

Somasundaram, et al. suggest that NSAID induced changes in mitochondrial energy production may be a mechanism of the topical phase of NSAID damage.[13;14] Isolated rat mitochondria exposed to indomethacin, aspirin and DNP showed a stimulation of mitochondrial respiration (uncoupled oxidative phosphorylation) followed by a progressive decrease in oxygen consumption with increasing drug concentrations, consistent with inhibition of the electron transport chain.[15] These results suggest a mechanism of inhibitory uncoupling in indomethacin and aspirin.[15] The mitochondrial morphologic changes were identical in the DNP and indomethacin treated groups with patchy swelling and elongation of the mitochondria with loss of cristae and vacuolization, which is consistent with uncoupling of oxidative phosphorylation or inhibition of the electron transport chain.[15] The relationship was explored further by giving aspirin intraperitoneally, indomethacin by gavage, and DNP instilled into the small bowel. Dinitrophenol did not change intestinal prostaglandin concentrations but did uncouple mitochondrial oxidative phosphorylation.[15] Both aspirin and indomethacin decreased prostaglandin concentrations but only indomethacin caused increased intestinal permeability and mitochondrial changes.[15] When intraperitoneal aspirin and intraluminal DNP were administered, the small intestinal changes were similar to indomethacin.[15] These results suggest both uncoupling of oxidative phosphorylation and inhibition of prostaglandins are important in the pathogenesis of NSAID induced ulceration.[15]

1.2.4 Carprofen

Carprofen is a non steroidal anti-inflammatory drug (NSAID) of the propionic acid class approved for use in dogs at a dose of 2.2 mg/kg twice daily or 4.4 mg/kg once daily. Carprofen has anti-inflammatory, analgesic, and antipyretic activity through the inhibition of cyclo-oxygenase and phospholipase A2.[14] The majority of anti-inflammatory action appears to be due to the S(+) enantiomer because the R(-) enantiomer is eliminated from the blood roughly twice as fast as the S(+) enantiomer.[7] Both enantiomers of carprofen are found in either the oral or injectable formations.[7] Following oral administration of carprofen, 90% of the drug is rapidly absorbed within 0.5 to 5 hours.[16] Plasma levels are proportional to dose when administered between the 1 mg/kg and 10 mg/kg dose range.[16] Carprofen is metabolized primarily in the liver to an ester glucuronide.[16] The majority of metabolites are excreted in the feces (70-80%) while 8-15% of an injectable dose is excreted in the urine.[16;17] Less than 5% of the drug is likely to be excreted unchanged.[16] Enterohepatic recirculation of carprofen and its metabolites has been reported.[17] Following a single oral dose of carprofen (25 mg) the mean maximum plasma concentration was 16.9 μg/ml after 0.5 to 3 hours.[14] After 7 days of oral carprofen (25 mg, per os, every 12 hours) administration, the mean maximum plasma concentration of 18.7 μg/ml was observed within 0.5 to 3 hours of the last dose.[14] Following a subcutaneous dose of carprofen (25 mg) the mean maximum plasma concentration was 8.0 μg/ml after 1.5 to 8 hours.[14] After 7 days of oral carprofen (25 mg, subcutaneously, every 12 hours) administration, the mean maximum plasma concentration of 14.7 μg/ml was observed within 1.5 to 4 hours of the last dose.[14]

Carprofen is reported to cause gastrointestinal hemorrhage and ulceration.[18;19] A previous study investigated the effects of a high concentration of carprofen on the canine colonic mucosa

in vitro. The colonic mucosa was harvested and exposed to 400 μg/ml of carprofen in an Ussing chamber. This concentration of carprofen decreased the transepithelial resistance and increased the permeability to mannitol of the colonic segments.[18]

1.3 Oxidative Stress and Anti-oxidants

1.3.1 Mitochondria and Cellular Energy

Mitochondria are considered the power houses of the cell because they are the primary site of adenosine triphosphate (ATP) synthesis. Mitochondria have an outer membrane and an inner membrane (Fig 5). The outer mitochondrial membrane is composed of 30-40% lipid and 60-70% protein.[20] The outer membrane is rich in a protein called porin allowing most ions and small molecules, up to 10,000 molecular weight, to pass freely from the cellular cytosol to the intermembrane space of the mitochondria.[20;21] The inner membrane is composed of 80% protein and is rich in unsaturated fatty acids.[20] The inner membrane is impermeable to most small ions, including H+, Na+, and K+, small molecules such as ATP, adenosine diphosphate (ADP), pyruvate, and other metabolites important to mitochondrial function.[21] In order for these ions and small molecules to be used in energy production they must be moved across the membrane via transport systems.[21] The inner membrane is involuted to form cristae which increase the surface area available for cellular energy production.[20;21] Within the inner mitochondrial membrane is a gel-like matrix containing enzymes responsible for oxidation of pyruvate, animo acids, fatty acids, and those of the tricarboxylic acid (TCA) cycle.[20;21] The matrix also contains nicotinamide adenine dinucleotide

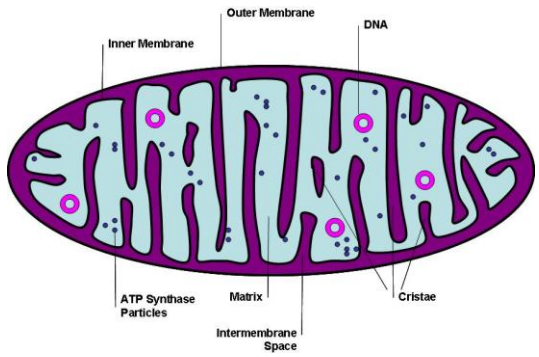


Figure 5: Mitochondrial structure

(NAD^+) and flavin adenine dinucleotide (FAD) which act as hydrogen acceptors in the electron transport chain, as well as ADP and phosphate (Pi) which are used in oxidative phosphorylation to make ATP.[21] Aside from components of cellular energy production the matrix contains mitochondrial DNA, ribosomes, and proteins necessary for transcription of mitochondrial DNA and translation of mitochondrial RNA.[20] The reactions of the electron transport chain and oxidative phosphorylation occur through the ATP synthetase complexes which are attached to the inner mitochondrial membrane.[21]

Metabolic fuels such as carbohydrates, lipids, and proteins are oxidized to produce intermediate molecules of cellular energy. Oxidation of long-chain fatty acids by β-oxidation; breakdown of carbohydrates by glycolysis; oxidation of ketone bodies, acetoacetate and β-hydroxybutyrate; oxidation of ethanol; and oxidative break down of amino acids results in the production of acetyl coenzyme A (CoA).[20] Acetyl CoA is a two-carbon unit which serves as the substrate entering the tricarboxylic acid (TCA) cycle.[20] Through the TCA cycle acetyl CoA is oxidized by four reactions to oxaloacetate (Fig 6).[20] The product of these reactions result in the transfer of electrons to either NAD^+ or FAD to result in the net production of three NADH, one $FADH_2$, one high energy bond as guanosine triphosphate (GTP), and two carbon dioxide molecules.[20] NADH and $FADH_2$ produced during

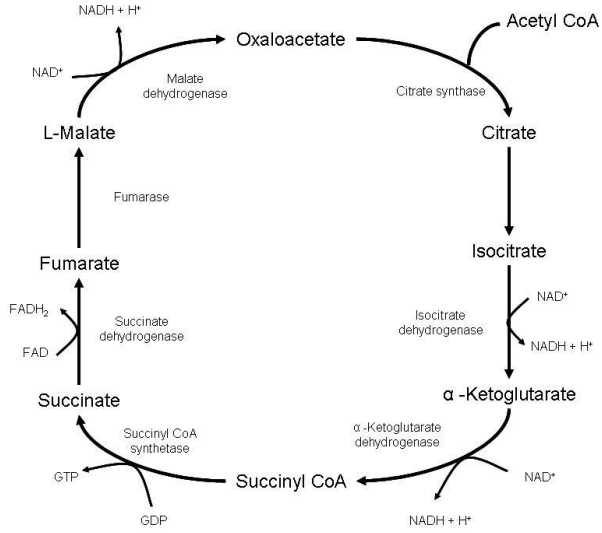


Figure 6: Tricarboxylic acid cycle

one TCA cycle are subsequently oxidized by the electron transport chain to produce nine molecules of ATP.[20]

The electron transport chain removes electrons from an electron donor (NADH or $FADH_2$) and passes them to a terminal electron acceptor (O_2) via a series of reduction-oxidation reactions (Fig 7). These reactions are coupled to creation of a proton gradient across the mitochondrial inner membrane via complexes I, III, and IV.[21] NADH is oxidized by Complex I (NADH-dehydrogenase), which transfers two electrons to ubiquinone while four protons are

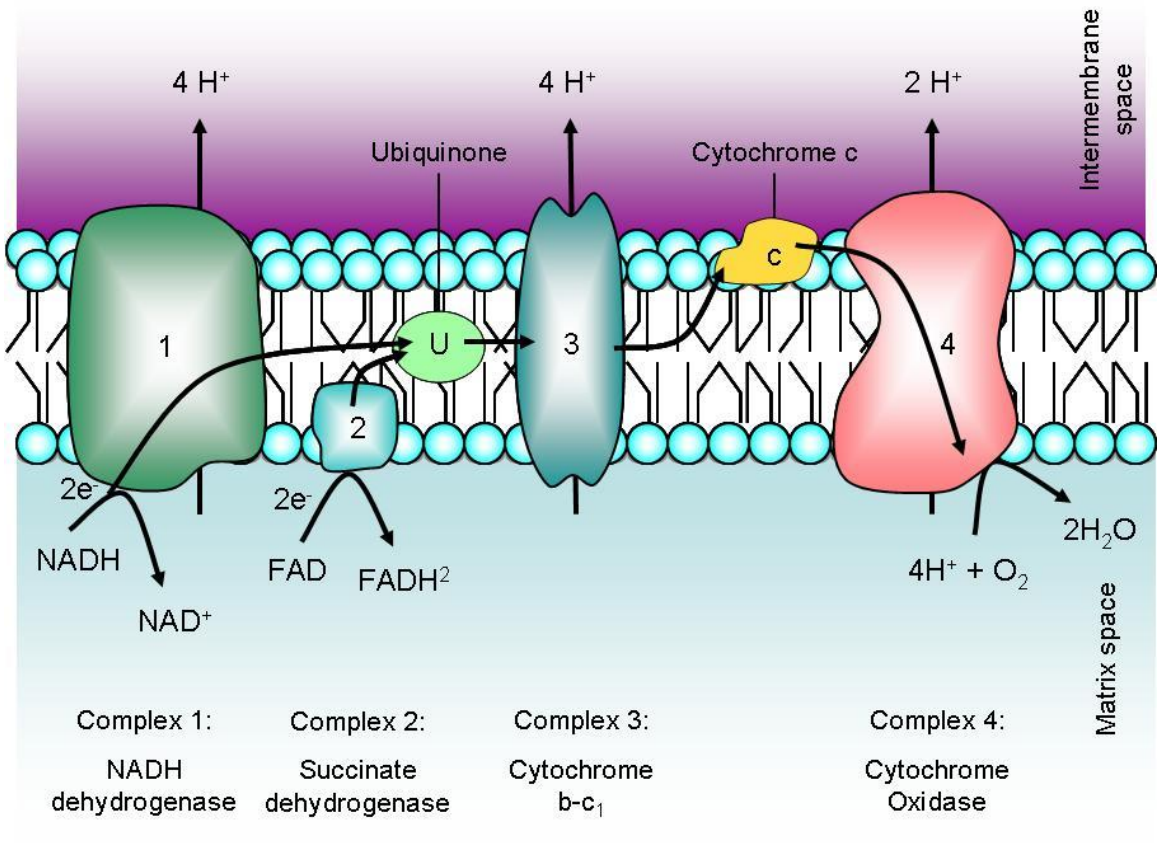


Figure 7. Electron transport chain.

transferred to the intermembrane space.[20;21] Complex II (succinate dehydrogenase) catalyzes the oxidation of succinate to fumarate thus transferring two electrons to two proton to FAD producing $FADH_2$.[20] The $FADH_2$ thus transfers electrons to ubiquinone. Ubiquinone, also known as Coenzyme Q, serves to transfer electrons to Complex III. The remaining members of the electron transport chain are cytochromes. Cytochromes contain a porphyrin ring with an iron atom, which is reversibly converted from its ferric (Fe^{3+}) form to its ferrous form (Fe^{2+}).[21] Two electrons are passed down the chain from ubiquinone through Complex III (cytochrome bc_1 complex) to cytochrome c.[20;21] This results in the translocation of four protons across the inner mitochondrial membrane to the intermembrane space.[20] Cytochrome c functions as a mobile electron carrier. Complex IV (cytochrome oxidase or cytochrome a + a_3 complex) catalyzes the transfer of electrons from cytochrome c to oxygen (O_2).[21] Complex IV contains the only heme iron electron carrier which can react directly with molecular oxygen to form water.[21] Two electrons are transferred to O_2, which is tightly bound to form a peroxide derivative of oxygen (O_2^{2-}).[20] Additionally, two electrons are transferred to the binuclear center with the concomitant uptake of four protons from the matrix thus forming water.[20] In addition to the protons required for water production Complex IV also pumps additional protons across the membrane resulting in net stoichiometry of two protons into the intermembrane space for every four protons taken up from the matrix.[20] The net result of the electron transport chain is the translocation of 10 protons from the mitochondrial matrix to the intermembrane space thus creating an electrochemical gradient for each NADH which is oxidized to water.[20;21] The electrochemical gradient subsequently provides the potential energy used in the synthesis of ATP.

Oxidative phosphorylation is the process where the energy potential created by the electron transport chain is used to drive ATP synthesis. This occurs via Complex V (ATP

synthetase). Complex V is composed of two domains (Fig 8). The F_0 domain is embedded in the inner mitochondrial membrane to provide a channel for the translocation of protons from the intermembrane space to the matrix.[20] The passage of protons through the F_0 domain results in a conformational change in the subunits comprising the F_1 domain that drives ATP synthesis.[20] The F_1 domain has binding sites for ADP + Pi and ATP and is involved in the catalytic reactions of ATP synthesis.[20] This is known as the chemiosmotic hypothesis.[21] The result of complete oxidation of NADH and $FADH_2$ (per mole) by the electron transport chain and oxidative phosphorylation results in the production of approximately 2.5 and 1.5 mol of ATP respectively.

The rate of ATP synthesis by oxidative phosphorylation is closely coupled to electron transport. When cellular energy needs are low, ATP will accumulate and the proton gradient will increase. This continues until the energy potential required to pump protons across the membrane from the matrix against the electrochemical gradient equals the energy released

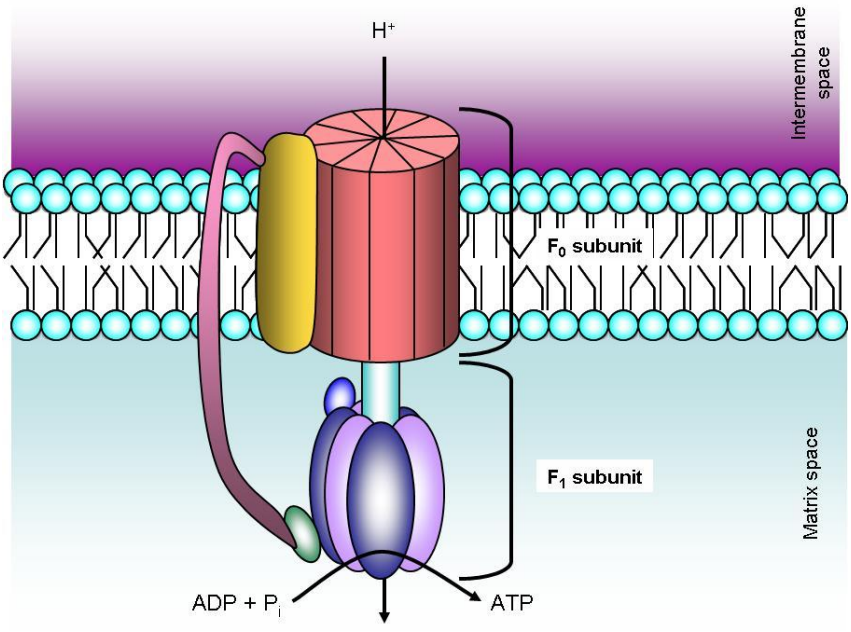


Figure 8. Adenosine triphosphate synthase used during oxidative phosphorylation.

during the transfer of electrons from NADH to O_2 thus reaching an equilibrium.[20] When cellular energy requirements are high, ADP accumulates thus stimulating ATP synthesis.[20] This results in fewer protons in the intermembrane space, allowing electron transport to proceed.[20] The increased concentrations of NAD+ and ADP stimulates the TCA cycle and fatty acid oxidation.[20] This provides tight regulation and coordination between cellular energy demands (ATP utilization) and metabolism of energy sources (carbohydrates, lipids, and proteins).

1.3.2 Oxidative Stress

Oxygen is reduced to water by Complex IV as the terminal step of the electron transport chain. During the process O_2 is tightly bound to the complex to prevent the release of toxic intermediates created during the oxidation process. The electronic structure of O_2 favors the production of oxygen radicals such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl free radical ($OH\bullet$).[20] A radical is a highly reactive molecule with an unpaired electron in an outer orbital.[20] Radicals can initiate a chain reaction by removing electrons from other molecules in order to complete its own orbit.[20] The most dangerous reactive oxygen species produced in the reduction of O_2 is hydroxyl free radical because it is involved in reactions which produce other toxic radicals.[20] Hydrogen peroxide is not a free radical however it is converted to hydroxyl radical in the presence of iron and copper, which are prevalent in cells.[20]

There are many sources of reactive oxygen species. The major source of intracellular oxygen radicals is through the electron transport chain where superoxide is produced by the transfer of one electron to O_2 during reduction of ubiquinone by Complexes I and II.[20] Toxic oxygen species are also produced by peroxisomes during fatty acid oxidation.[20] Other sources of reactive oxygen species include those released by the respiratory burst used by white blood cell to kill bacteria, cosmic radiation, ingestion of chemicals and drugs, and smog.[20]

Reactive oxygen species react with all major classes of macromolecules in cells. Reaction with phospholipids of the plasma and organelle membranes results in lipid peroxidation.[20] These lipid radicals react with O_2 to form lipid peroxyl radicals, lipid peroxide, and malondialdehyde.[20] Lipid peroxidation increases membrane permeability to calcium and other ions resulting in cell swelling and maldistribution of ions. This may trigger cell death by apoptosis.[20;22] Oxygen radicals may also damage mitochondrial or nuclear DNA resulting in mutations. Mitochondrial DNA is the more susceptible to damage because the mitochondrial electron transport chain is the major source of toxic oxygen radicals and it lacks the protective mechanisms available to nuclear DNA.[20;22]

Approximately one quarter of inhaled O_2 forms free radicals; however in unhealthy conditions this may be increased to three quarters of inhaled O_2 forming free radicals.[22] Therefore cells in aerobic environments have developed protective mechanisms against damage caused by reactive oxygen species. There are three isozymes of superoxide dismutase found in mammalian cells which catalyze the conversion of superoxide to hydrogen peroxide.[20] Hydrogen peroxide is then removed by catalase.[20] The highest concentrations of catalase are found in peroxisomes and to a lesser extent in mitochondria and cytosol.[20] Glutathione peroxidase is another enzyme that catalyzes the reduction of both hydrogen peroxide and lipid peroxides.[20] Furthermore, ingestion of oxygen scavengers such as vitamin C, vitamin E, and β-carotene may protect against reactive oxygen species.[20;22]

1.3.3 2,4 Dinitrophenol (DNP)

The electron transport chain and oxidative phosphorylation are tightly coupled and substances that inhibit one will generally inhibit both. 2,4 Dinitrophenol is a metabolic toxin that uncouples the electron transport chain reaction from oxidative phosphorylation.[21;23] DNP is a

weak acid (pKa 4.114) and forms explosive salts with alkalies and ammonia. Dinitrophenol emits toxic fumes of nitrogen oxides when heated to decomposition and is incompatible with heavy metals. In the cell, DNP acts by shuttling hydrogen ions across biological membranes such as the mitochondrial inner membrane.[21] This defeats the proton gradient created by the electron transport chain and abolishes the proton motive force which drives oxidative phosphorylation.[21] The net result is rapid energy consumption without the production of ATP. The energy of the proton gradient is instead lost as heat.[21] Dinitrophenol has been used to investigate the role of active transport of molecules by energy dependent systems. Intestinal mucosal transport of various molecules has been investigated using DNP in combination with the Ussing chamber in concentrations ranging from 0.1 mM to 1 mM on either one or both sides of the epithelium being tested.[23-25]

1.3.4 4-Hydroxy-2,2,6,6-tetra-methylpiperidine-*N*-oxyl (tempol)

4-Hydroxy-2,2,6,6-tetra-methylpiperidine-*N*-oxyl (tempol) is a stable piperidine nitroxide free radical. Nitroxides share a reducible nitroxide group as part of a six- or five-member carbon ring.[26] Tempol is a low molecular weight molecule able to permeate cell membranes freely.[26;27] It catalyzes the metabolism of hydroxyl radical to hydrogen peroxide thus acting as a superoxide dismutase mimetic.[26;28] Tempol also detoxifies redox-reactive forms of transition metal ions and reacts directly with many radicals.[28] Tempol has been shown to protect lipids, DNA, and proteins from oxidative damage.[26;28] The rate of tempol reduction in an organ is related to the production of reactive oxygen species, therefore the rate of tempol reduction can be used to assess the redox state of a tissue.[26]

Nitroxides are stable free radicals with minimal plasma protein binding. Tempol is rapidly converted to tempol-hydroxylamine by the liver microsomes, primarily by NADPH and

cytochrome c.[26] It can also be reduced by sulfhydryl groups on proteins or by ascorbate in the cell cytosol.[26] Tempol has been found in bile after intravenous injection, supporting hepatic metabolism and biliary excretion.[26] In rats tempol has been shown to immediately penetrate the colonic mucosa and persist in gastric and colonic mucosae for several hours.[29] Extremely high concentrations of tempol (100 to 1000 μM) may have a pro-oxidant effect thus enhancing toxicity.[26] In mice the lethal dose of 50% and 70% after an intraperitoneal dose were 1.6 and 2.0 mmol/kg, respectively.[26] Clinical signs of toxicity include restlessness and seizures.[26] Pharmacokinetics, metabolism, and toxicity of tempol have not been investigated in the dog.

1.4 Summary and Hypothesis for Present Studies

A large dose of carprofen, such as a ten times overdose as used in the Briere study, is not unreasonable in an acute toxicity. However, the overwhelming mucosal damage makes investigation of the pathogenesis of the damage difficult since the mucosa is obliterated. Documenting functional compromise with lesser structural damage would be more useful in understanding the pathogenesis of toxicity and also characterize the toxic effects at lower, recommended doses of NSAIDs.

If the colonic damage demonstrated with carprofen in vitro is similar to that induced by an inhibitor of mitochondrial oxidative phosphorylation, DNP, then the two drugs may have the same mechanism of toxicity. By further characterizing the mechanisms of toxicity we may be able to determine the contribution of topical effects, along with effects due to systemic mechanisms, which may provide information for treating toxicity or developing safer drugs.

This study will investigate the role of mitochondrial damage in the topical toxicity of carprofen to the colonic mucosa of normal dogs in vitro. Specifically, the effects of carprofen concentration on the colonic mucosa of normal dogs will be investigated. The hypothesis is that

carprofen will compromise the integrity and increase the permeability of the colonic mucosa of normal dogs in vitro in a concentration dependent manner. Once a concentration of carprofen which damages the colonic mucosa without severe histologic effects is determined, the effect of DNP on the colonic mucosa of normal dogs will be compared to that of carprofen. Changes in the transepithelial electrical conductance, mannitol permeability, and morphology, (by evaluation of light microscopy and transmission electron microscopy) will be documented in the colonic mucosa of normal dogs that has been exposed to carprofen or DNP in vitro. The hypothesis is that both carprofen and DNP will cause changes in the mitochondria consistent with uncoupling of oxidative phosphorylation as seen with transmission electron microscopy. In addition, carprofen and DNP treated tissue will be pretreated with tempol, an anti-oxidant free radical scavenger to further support the hypothesis that carprofen causes mitochondrial oxidative stress. Similar mitochondrial morphologic changes which are mitigated by an anti-oxidant may be an indication that the two drugs, carprofen and DNP, induce colonic injury by a similar mechanism (i.e. uncoupling of oxidative phosphorylation).

CHAPTER 2. MATERIALS AND METHODS

2.1 Objective 1. Concentration Dependent Effect of Carprofen on the Descending Colon

2.1.1 Harvesting and Preparation of Sections of Colonic Mucosa

Ten mature mixed breed dogs were used for objective 1. The dogs were placed under general anesthesia with thiopental and maintained with isoflurane. The entire colon was harvested immediately prior to euthanasia by overdose of sodium pentobarbital. Euthanasia was for reasons unrelated to the study. The colon was opened on the mesenteric boarder, rinsed with 0.9% saline, and placed in ice-cold Krebs-Ringer bicarbonate buffer solution and transported to the laboratory.

The colon was placed in stripping pans filled with 500 ml of ice-cold oxygenated (95% oxygen / 5% carbon dioxide) Krebs-Ringer bicarbonate buffer solution. The colon was divided into transverse and descending sections at the level of the middle colic vein. Only the descending colon, from the level of the middle colic vein to the pelvic inlet, was used in this study. A full thickness section of colon was placed in neutral-buffered 10% formalin and reserved for histologic examination. The colonic mucosa was separated from the seromuscular layer using blunt and sharp dissection. Ten sections of colonic mucosa were harvested from the descending colon.

2.1.2 Ussing Chamber Studies

2.1.2.1 Mounting

Each section of mucosa was randomly assigned to one of 10 Ussing chambers. The mucosa was placed in tension as a flat sheet between the two halves of the acrylic chamber with an exposed area of 3.14 cm^2. The chamber was then connected to the circulation reservoir and the Ag-AgCl agar bridges as previously described.

2.1.2.2 Solutions

Each hemi-chamber was filled with 15 ml of Krebs-Ringer bicarbonate buffer solution (pH 7.4) containing (in nM) 118 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, and 10 dextrose. The Kreb-Ringer bicarbonate buffer solution was continuously oxygenated (95% oxygen / 5% carbon dioxide). The Kreb-Ringer bicarbonate buffer solution was circulated in water-jacketed reservoirs maintained at a temperature of 37° C. Carprofen was added to both the mucosal and serosal chambers 30 minutes after mounting. Concentrations (μg/ml) of carprofen were 400, 200, 100, 40, and 0. These concentrations represent 10x, 5x, 2.5x, and 1x the maximum expected plasma concentration after a daily (4.4 mg/kg) oral dose of carprofen.[14] Each treatment was performed in duplicate in randomly assigned Ussing chamber units. The pH of each chamber was recorded 30 minutes after the addition of carprofen and at the end of the study (240 minutes).

2.1.2.3 Electrical Measurements

Transepithelial potential difference (mV) was measured using agar bridges connected to Ag-AgCl voltage electrodes. If transepithelial potential difference was between -1.0 and 1.0 mV, tissues were current clamped at 100 μA and transepithelial potential difference recorded.

Transepithelial potential difference was short-circuited through the voltage electrodes using a voltage clamp. Short-circuit current (μA) was measured using a separate pair of Ag-AgCl current electrodes and agar bridges.

Transepithelial potential difference and short-circuit current were recorded every 15 minutes for 240 minutes. Electrical conductance was calculated using Ohms law. Ohm's law states that the voltage drop (electrical potential difference) between two points of an electrical circuit is equal to the product of the current flowing through it and the electrical resistance of the

conductor. Electrical conductance (G) is the inverse of electrical resistance. Using this information the relationship G = I / V is derived, where G is the electrical conductance (S, siemens), I is the current (A, ampere), and V is the potential difference (V, volt).

2.1.2.4 Mannitol

Fifteen minutes after mounting, 200 µl tritium-mannitol (^{3}H-mannitol) as a solution containing 10 µCi/ml in 6% mannitol was added to the mucosal bathing solution. At the same time, 200 µl non-radioactive 6% mannitol was added to the serosal bathing solution. A sample (0.1 ml) was collected from the mucosal solution 30 minutes after mounting. Samples (0.5ml) were collected from the serosal solution at 60, 120, 180, and 240 minutes after mounting. Samples were assessed for β emission (counts/min) using a liquid scintillation counter (Tri-Carb 2800TR Liquid Scintilation Analyzer, Perkin Elmer, Waltham, MA). Mucosal to serosal flux of mannitol was calculated for each of three time periods: 60 to 120 minutes, 120 to 180 minutes, and 180 to 240 minutes.

2.1.3 Histologic Examination

At 240 minutes, colonic mucosal sections were removed from the Ussing chambers. The sections were placed in 10% neutral-buffered formalin for examination by light microscopy. Fixed sections of colonic mucosa were trimmed, embedded in paraffin, and cut into 5 µm thick sections. Tissue slices were mounted on glass slides and stained with hematoxyline and eosin.

Tissue slices were evaluated with light microscopy by a single, blinded evaluator. The presence of inflammation, edema, sloughing of cells from the surface epithelium, erosions, and sloughing of epithelial cells within the mucosal glands was recorded.[18] Tissue inflammation was graded according to the percentage of lamina propria infiltrated with inflammatory cells. Grades of inflammation were normal (< 20%), mild (20-40%), moderate (40-60%), and severe (> 60%).

Edema was considered to be absent if mucosal glands were not separated from each other by clear fluid, mild if less than 50 μm apart, moderate 50-150 μm apart, and severe if more than 150 μm apart. Sloughing of surface epithelial cells was graded based on percent detachment of surface epithelial cells without loss of surface epithelium continuity. Grades of surface epithelial sloughing were minimal (<10% detachment), mild (10-20% detachment), moderate (20-50% detachment), and severe (>50% detachment). Erosion of the mucosa was assessed based on percentage loss of surface epithelium continuity. Grades of erosion were absent, mild (<10%), or severe (>10%). Sloughing of mucosal gland epithelial cells was graded according to percentage of detached epithelial cells in the lumen. Grades of gland sloughing were absent, scattered, mild (<5%), moderate (5-10%), and severe (>10%).

2.2 Objective 2. Mitochondrial Oxidative Stress

Methods for objective 2 are based on results of objective 1. The methods specific to objective 2 are described.

2.2.1 Harvesting and Preparation of Sections of Colonic Mucosa

Ten mature mixed breed dogs were used for objective 2. Sections of colonic mucosa were harvested and prepared as in objective 1.

2.2.2 Ussing Chamber Studies

2.2.2.1 Mounting

Each section of colonic mucosa was randomly assigned to one of ten Ussing chamber units and mounted as in objective 1.

2.2.2.2 Solutions

Each hemi-chamber was filled with 15 ml Kreb-Ringer bicarbonate buffer solution, continuously oxygenated with 95% oxygen / 5% carbon dioxide, and circulated in water-jacketed

reservoirs as in objective 1. Carprofen (200 μg/ml) or DNP (0.25 mM) was added to both the mucosal and serosal chambers 30 minutes after mounting. The carprofen concentration was determined by objective 1 as that which compromised the colonic mucosa (based on mannitol flux and light microscopy) without profound changes in the electrical conductance. Pretreatment with tempol was achieved by adding tempol (1 mM) to each hemi-chamber at the time of mounting, followed by the addition of either carprofen (200 μg/ml) or DNP (0.25 mM) to both the mucosal and serosal chambers 30 minutes after mounting. The final treatment was a negative control without the addition of any chemicals. Each treatment was performed in duplicate in randomly assigned Ussing chambers. The pH of each chamber was recorded 30 minutes after the addition of treatment and at the end of the study (240 min).

2.2.2.3 Electrical Measurements and Mannitol

Data for electrical conductance and mucosal to serosal mannitol flux was obtained as in objective 1.

2.2.3 Histologic Examination

After 240 minutes, one of two sections of colonic mucosa from each treatment was randomly selected and removed from the Ussing chambers, then placed in 10% neutral-buffered formalin for evaluation by light microscopy as in objective 1.

2.2.4 Transmission Electron Microscopic Examination

After 240 minutes, the remaining section of colonic mucosa from each treatment was removed from the Ussing chambers, then placed in 1.25% glutaraldehyde + 2% paraformaldehyde in buffer (0.1M sodium cacodylate buffer to pH 7.3-7.4) for evaluation by transmission electron microscopy. After fixation, samples were embedded in epon-araldite resin and ultrathin sections cut. The sections were stained with uranyl acetate and lead citrate. The

samples were examined with a Joel JEM-1011 electron microscope in transmission mode. Ten images (five from the brush boarder surface and five from the lamina propria surface) at 1500X magnification were taken of each sample. All enterocyte mitochondria of a given image were evaluated for evidence of damage consistent with uncoupling of oxidative phosphorylation on a positive or negative scale.[13;15] The presence of electron dense particles within the mitochondria was considered positive if any were noted. Mitochondrial swelling and loss of cristae were considered positive if greater than 33% of the mitochondria were affected. The presence of ruptured mitochondria was positive if any were noted.

2.3 Statistical Analysis

2.3.1 Ussing Chamber Studies

2.3.1.1 Electrical Conductance

Data from 0 minute to 15 minutes were not used for analysis (equilibration period). Electrical conductance from 30 minutes to 240 minutes was graphed against time for each chamber and the area under the curve was calculated using the trapezoid method. Sections with the same treatment were considered replicates. The mean +/- SD area under the curve was evaluated for a fixed effect of carprofen concentration, DNP treatment (objective 2), and Tempol pretreatment (objective 2) using a mixed effect linear model that also included the random effect of dog across treatments. Significance was considered at $p \leq 0.05$. Post hoc comparisons across carprofen concentrations and DNP treatments were tested using Scheffe's adjustment to maintain type I error at 0.05.

2.3.1.2 Mannitol Flux

Mucosal to serosal flux of mannitol was calculated for three one hour time periods: 60-120 minutes, 120-180 minutes, and 180-240 minutes. Sections with the same treatment were

considered replicates. The mean +/- SD mannitol flux was evaluated for a fixed effect of period; and carprofen concentration, DNP treatment (objective 2), and tempol pretreatment (objective 2) using a mixed effect linear model that also included the random effect of dog across treatments. Significance was considered at $p<0.05$. Post hoc comparisons across carprofen concentrations and DNP treatments were tested using Scheffe's adjustment to maintain type I error at 0.05. (PROC MIXED)

2.3.2 Light Microscopy

Degree of inflammation, edema, sloughing of epithelial cells and erosions were evaluated with light microscopy. The frequency distribution of histologic categories from the control group and treatments were compared using a Chi square analysis. Where there was significant heterogeneity at $p<0.05$, post hoc comparisons were made using Fisher's Exact test to determine where the frequency differences were occurring. A Bonferroni adjusted p value was used for evaluation of significance. Overall significance was considered at $p<0.05$. (PROC FREQ)

2.3.3 Transmission Electron Microscopy

The results of electron microscopy assessment were reported as presence/absence of structural features. The response (Yes/No) was modeled for an effect of treatment using logistic regression, expressing the magnitude of the effect of each treatment as the estimated odds (and 95 % confidence interval) of the presence of the feature compared to its presence in controls. Estimated treatment effects where the 95% confidence interval excluded 1.0 were considered significant. Where there were significant effects compared to controls, sequential modeling was performed to compare each treatment to the others to determine which treatment-to-control effects were different. Estimated treatment effects where the 95% confidence interval excluded 1.0 were considered significant. (PROC LOGISTIC) (SAS v 9.1, SAS Institute, Cary, NC).

CHAPTER 3. RESULTS

3.1 Objective 1. Concentration Dependent Effect of Carprofen on the Descending Colon

3.1.1 Ussing Chamber Studies

The pH of the mucosal and serosal baths 30 minutes after the addition of the treatment and at the end 240 minutes was 7.5 in all chambers regardless of carprofen concentration.

3.1.1.1 Electrical Conductance

The mean +/- standard deviation electrical conductance*time for carprofen treated colon at 400 μg/ml, 200 μg/ml, 100 μg/ml, 40 μg/ml, and control were 4145.9 +/- 2031.33, 2490.8 +/- 647.36, 2486.1 +/- 1149.39, 1945.0 +/- 896.24, and 2608.1 +/- 1461 mS/cm^2*min respectively. The mean +/- standard deviation electrical conductance*time for carprofen treated colon at 400 μg/ml was significantly greater than control ($p < 0.001$). The mean +/- standard deviation electrical conductance*time for the carprofen treated colon at 200, 100, and 40 μg/ml were not significantly different from control. The mean +/- standard deviation electrical conductance*time for carprofen treated colon at 400 μg/ml was not significantly different than at 200 μg/ml.

3.1.1.2 Mannitol Flux

The mean +/- standard deviation of mucosal to serosal flux of mannitol across the colonic mucosa over time for carprofen treated colon at 400 μg/ml, 200 μg/ml, 100 μg/ml, 40 μg/ml, and control are shown in table 1. The mannitol flux for carprofen treated colon at 400 μg/ml and 200 μg/ml was significantly greater at 180 – 240 minutes than at 60 – 120 ($p < 0.001$) or 120 – 180 minutes ($p < 0.029$). The mannitol flux for carprofen treated colon at 100 μg/ml, 40 μg/ml, and control was not significantly different over time ($p = 0.167$, 0.286, and 0.831, respectively).

Table 1. Mucosal to serosal flux (μmol/cm^2*h) of mannitol for concentration dependent effects of carprofen on the mucosa of the descending colon for time periods 60 to 120 minutes, 120 to 180 minutes, and 180-240 minutes. Treatments are in micrograms of carprofen per milliliter of solvent (μg/ml).

Treatment	60 – 120 (minutes)	120 – 180 (minutes)	180 – 240 (minutes)
400	0.093 (0.033) * [a]	0.114 (0.47) * [a]	0.177 (0.073) # [a]
200	0.092 (0.034) * [a]	0.116 (0.061) * [a]	0.149 (0.089) # [a]
100	0.096 (0.041) * [a]	0.103 (0.036) * [a b]	0.121 (0.049) * [a b]
40	0.103 (0.091) * [a]	0.076 (0.051) * [b]	0.140 (0.193) * [b]
control	0.081 (0.031) * [a]	0.083 (0.038) * [b]	0.077 (0.033) * [b]

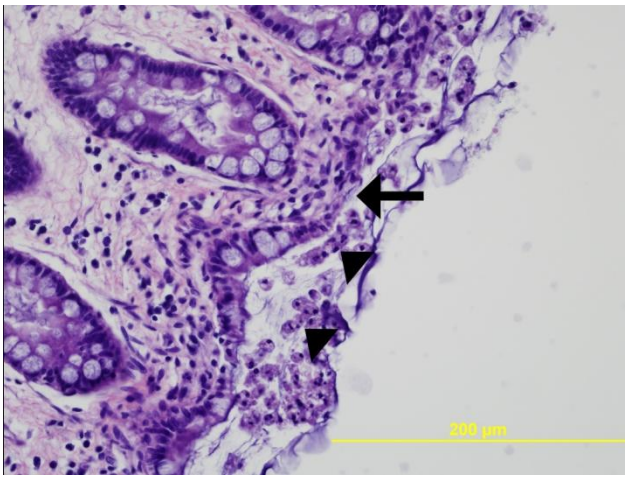
Rows with different symbols (* or #) are significantly different.
Columns with different superscripts (a or b) are significantly different.

There was no significant difference in mucosal to serosal mannitol flux between any treatment at 60 – 120 minutes. The mannitol flux at 180 – 240 minutes was not significantly different in colonic mucosa treated with carprofen at 400 μg/ml and 200 μg/ml, but both were significantly greater than control ($p = 0.003$ and $p = 0.030$, respectively). The mannitol flux at 180 – 240 minutes was not significantly different in colonic mucosa treated with carprofen at 100 μg/ml and 40 μg/ml, and both were also not significantly different from control.

3.1.2 Light Microscopy

One section of colon from each dog was examined by light microscopy prior to mucosal harvesting. Three sections had evidence of inflammation (2 mild and 1 moderate). This was characterized as lymphoplasmacytic infiltrate in all abnormal sections. The sample with moderate inflammation also had an elevated number of eosinophils. None of the sections had edema. Three sections had sloughing of surface epithelial cells (2 mild and 1 severe). One section had mild erosions. One section had scattered sloughing of epithelial cells within the mucosal glands.

The results of evaluation by light microscopy of treated sections are shown in table 2. There was no significant difference across treatment for inflammation ($p = 0.382$) or edema ($p = 0.05$). There was a significant difference in the amount of sloughing of surface epithelial cells ($p = 0.037$), the severity of surface erosions ($p < 0.001$), and the presence of sloughed epithelial cells within the mucosal glands ($p < 0.001$) (Fig 9). Sloughing of epithelial cells at 400 µg/ml, 200 µg/ml, and 100 µg/ml was significantly greater than at 40 µg/ml and control ($p < 0.05$). The amount of surface erosions at 400 µg/ml and 200 µg/ml were significantly greater than at 100 µg/ml, 40 µg/ml and control ($p < 0.05$). Sloughing of cells in mucosal glands at 400 µg/ml and 200 µg/ml was significantly greater than at 100 µg/ml, 40 µg/ml and control ($p < 0.05$). Sloughing of cells in mucosal glands at 100 µg/ml and 40 µg/ml was significantly greater than control ($p < 0.05$).

a) 

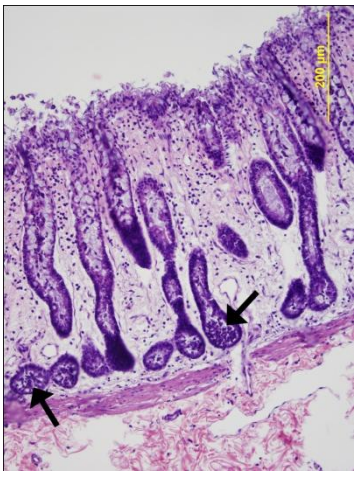
b) 

Figure 9. Light micrograph of section of colonic mucosa from carprofen treated tissue at the end of the experiment. (a) There is mild swelling, severe sloughing of surface epithelial cells (arrowhead), and erosions of the surface epithelium (arrow). x40 magnification. (b) There is severe sloughing of surface epithelial cells and sloughing of epithelial cells in colonic mucosal gland (arrow). x20 magnification.

Table 2. Histologic findings for sections of colonic mucosa after exposure to various concentrations of carprofen in an Ussing chamber. Treatments are in micrograms of carprofen per milliliter of solvent (μg/ml).

Inflammation

Treatment	Normal (<20%)	Mild (20-40%)	Moderate (40-60%)
400 *	16 (80%)	2 (10%)	2 (10%)
200 *	18 90%)	2 (10%)	0
100 *	17 (85%)	3 (15%)	0
40 *	18 (90%)	2 (10%)	0
control *	18 (90%)	2 (10%)	0

Edema

Treatment	Absent	Mild (<50μm)	Moderate (50-150μm)
400 *	15 (75%)	5 (25%)	0
200 *	7 (35%)	13 (65%)	0
100 *	9 (45%)	9 (45%)	2 (10%)
40 *	5 (25%)	12 (60%)	3 (15%)
control *	10 (50%)	9 (45%)	1 (5%)

Surface epithelial sloughing

Treatment	Minimal (<10%)	Mild (10-20%)	Moderate (20-50%)	Severe (>50%)
400 #	0	0	1 (5%)	19 (95%)
200 #	1 (5%)	1 (5%)	1 (5%)	17 (85%)
100 *	1 (5%)	2 (10%)	3 (15%)	14 (70%)
40 *	2 (10%)	4 (20%)	4 (20%)	10 (50%)
control *	5 (25%)	2 (10%)	4 (20%)	9 (45%)

Erosions

Treatment	Absent	Mild (<10%)	Severe (>10%)
400 #	2 (10.5%)	11 (57.9%)	6 (31.6%)
200 #	7 (35%)	13 (65%)	0
100 #	13 (65%)	7 (35%)	0
40 *	12 (60%)	8 (40%)	0
control *	18 (85%)	3 (15%)	0

Mucosal gland epithelial sloughing

Treatment	Absent	Scattered	Mild (<5%)	Moderate (5-10%)	Severe (>10%)
400 #	2 (10%)	2 (10%)	4 (20%)	3 (15%)	9 (45%)
200 #	4 (20%)	4 (20%)	3 (15%)	4 (20%)	5 (25%)
100 §	7 (35%)	6 (30%)	3 (15%)	3 (15%)	1 (5%)
40 §	3 (15%)	10 (50%)	5 (25%)	2 (10%)	0
control *	14 (70%)	3 (15%)	0	1 (5%)	2 (10%)

Within histologic groups, rows with different symbols (*, #, or §) are significantly different.

3.2 Objective 2. Mitochondrial Oxidative Stress

3.2.1 Ussing Chamber Studies

The pH of the mucosal and serosal baths 30 minutes after the addition of the treatment and at the end 240 minutes was 7.5 in all chambers regardless of treatment.

3.2.1.1 Electrical Conductance

The mean +/- standard deviation electrical conductance*time for carprofen treated colon at 200 μg/ml was 2425.7 +/- 1105.39 and with tempol pretreatment was 2461.8 +/- 1552.15. The mean +/- standard deviation electrical conductance*time for DNP treated colon at 0.25 mM was 3389.5 +/- 2198.34 and with tempol pretreatment was 3789.6 +/- 1776.47. The mean +/- standard deviation electrical conductance*time for control was 3572.6 +/- 5193.21. There was no significant difference between treatments ($p = 0.46$).

3.2.1.2 Mannitol Flux

The mean +/- standard deviation of mucosal to serosal flux of mannitol across the colonic mucosa over time for carprofen and DNP treated colon without and with tempol pretreatment are shown in table 3. The mannitol flux for carprofen treated colon was significantly greater at 180 – 240 minutes than at 60 – 120 or 120 – 180 minutes ($p = 0.029$). The mannitol flux for carprofen treated colon with tempol pretreatment was not significantly different at any time period ($p = 0.073$). The mannitol flux for DNP treated colon regardless of tempol pretreatment was significantly greater at 180 – 240 minutes than at 60 – 120 or 120 – 180 minutes (DNP alone $p = 0.017$, tempol pretreatment and DNP $p < 0.001$). The mannitol flux for control colon was not significantly different over time ($p = 0.153$).

There was no significant difference in mucosal to serosal mannitol flux between carprofen treated colon and control at 60 – 120 minutes and 120 –180 minutes. There was a

Table 3. Mucosal to serosal flux (μmol/cm^2*h) of mannitol for canine colonic mucosa treated with carprofen or 2,4-dinitrophenol. Time periods 60 to 120 minutes, 120 to 180 minutes, and 180-240 minutes. Treatments are 1) C = carprofen 200 μg/ml, 2) T + C = carprofen 200 μg/ml pretreated with tempol 1 mM, 3) D = 2,4 dinitrophenol 0.25 mM, 4) T + D = 2,4 dinitrophenol 0.25 mM pretreated with tempol 1 mM, and 5) control.

Treatment	60 – 120 (minutes)	120 – 180 (minutes)	180 – 240 (minutes)
C	0.072 (0.021) * [a]	0.076 (0.030) * [a]	0.109 (0.073) # [b]
T + C	0.066 (0.020) * [a]	0.075 (0.028) * [a]	0.093 (0.052) * [a]
D	0.092 (0.044) * [b]	0.115 (0.063) * [b]	0.159 (0.098) # [b]
T + D	0.099 (0.035) * [b]	0.119 (0.060) * [b]	0.186 (0.099) # [b]
control	0.058 (0.020) * [a]	0.053 (0.014) * [a]	0.048 (0.013) * [a]

Rows with different symbols (* or #) are significantly different.
Columns with different superscripts (a or b) are significantly different.

significant difference in mannitol flux between carprofen alone and carprofen with tempol pretreatment at 180 – 240 minutes ($p = 0.008$). There was a significant difference in mannitol flux between carprofen alone and control at 180 – 240 minutes ($p = 0.011$) but not between carprofen with tempol pretreatment and control. The mannitol flux in DNP treated colon, without and with tempol pretreatment, was significantly greater than control at all time periods ($p < 0.03$ all comparisons). There was no significant difference between DNP treated colon (without or with tempol pretreatment) and carprofen alone at 180 – 240 minutes.

3.2.2 Light Microscopy

One section of colon from each dog was selected prior to mucosal harvesting for later examination by light microscopy. None of the sections had evidence of inflammation or edema. Two sections had mild sloughing of surface epithelial cells. One section had a focal area of erosion. One section had scattered sloughing of epithelial cells within the mucosal glands.

The results of light microscopic evaluation of treatment sections are shown in table 4. There was no significant difference between treatments for inflammation ($p = 0.537$), edema ($p = 0.500$), or sloughing of surface epithelial cells ($p = 0.417$). There was a significant difference in

the severity of surface erosions ($p < 0.008$) and the presence of sloughed epithelial cells within the mucosal glands ($p < 0.004$). The amount of surface erosions in carprofen treated colon (without and with tempol pretreatment) and in DNP treated colon (without and with tempol pretreatment) were significantly greater than control ($p < 0.05$). Sloughing of cells in mucosal glands in carprofen treated colon (without and with tempol pretreatment) was significantly greater than DNP treated colon (without and with tempol pretreatment) and control ($p < 0.05$).

3.2.3 Transmission Electron Microscopy

The results of electron microscopic evaluation of enterocyte mitochondria are shown in table 5. The likelihood of mitochondria with electron dense particles was significantly less for all treatments compared to controls ($p < 0.001$) (Fig 10b, Table 6). The estimated odds of treatment effect (95% confidence interval) of mitochondria with electron dense particles for carprofen treated colon was 0.49 (0.28 – 0.86) and carprofen with tempol pretreatment was 0.47 (0.27 – 0.82). The estimated odds (95% confidence interval) of mitochondria with electron dense particles for DNP treated colon was 0.18 (0.09 – 0.34) and DNP with tempol pretreatment was 0.14 (0.07 – 0.27). There was no significant difference in the odds of electron dense particles in colon treated with carprofen without or with tempol pretreatment which were both greater than DNP treated colon without or with tempol pretreatment.

The likelihood of mitochondria with swelling were significantly greater for all treatments compared to controls ($p < 0.001$) (Fig 10c, Table 6). The estimated odds of treatment effect (95% confidence interval) of mitochondria with swelling for carprofen treated colon was 15.09 (6.86 – 33.20) and with tempol pretreatment was 37.99 (13.00 – 111.06). The estimated odds (95% confidence interval) of mitochondria with swelling for DNP treated colon was 3.79 (2.13 – 6.74) and with tempol pretreatment was 14.02 (6.56 – 29.99). There was no significant

Table 4. Histologic findings for sections of colonic mucosa after exposure to carprofen or 2,4 dinitrophenol in an Ussing chamber. Treatments are 1) C = carprofen 200 μg/ml, 2) T + C = carprofen 200 μg/ml pretreated with tempol 1 mM, 3) D = 2,4 dinitrophenol 0.25 mM, 4) T + D = 2,4 dinitrophenol 0.25 mM pretreated with tempol 1 mM, and 5) control.

Inflammation

Treatment	Normal (<20%)	Mild (20-40%)
C *	10 (100%)	0
T + C *	10 (100%)	0
D *	9 (90%)	1 (10%)
T + D *	9 (90%)	1 (10%)
control *	10 (100%)	0

Edema

Treatment	Absent	Mild (<50μm)	Moderate (50-150μm)
C *	10 (100%)	0	0
T + C *	9 (90%)	1 (10%)	0
D *	10 (100%)	0	0
T + D *	10 (100%)	0	0
control *	8 (80%)	1 (10%)	1 (10%)

Surface epithelial sloughing

Treatment	Minimal (<10%)	Mild (10-20%)	Moderate (20-50%)	Severe (>50%)
C *	0	0	0	10 (100%)
T + C *	0	0	0	10 (100%)
D *	0	1 (10%)	0	9 (90%)
T + D *	0	1 (10%)	0	9 (90%)
control *	0	2 (20%)	1 (10%)	7 (70%)

Erosions

Treatment	Absent	Mild (<10%)	Severe (>10%)
C #	0	4 (40%)	6 (60%)
T + C #	0	6 (60%)	4 (40%)
D #	0	5 (50%)	5 (50%)
T + D #	0	3 (30%)	7 (70%)
control *	3 (30%)	7 (70%)	0

Mucosal gland epithelial sloughing

Treatment	Absent	Scattered	Mild (<5%)	Moderate (5-10%)	Severe (>10%)
C #	0	0	0	0	10 (100%)
T + C #	0	0	0	1 (10%)	9 (90%)
D *	2 (20%)	2 (20%)	0	1 (10%)	5 (50%)
T + D *	1 (10%)	4 (40%)	1 (10%)	0	4 (40%)
control *	2 (20%)	7 (70%)	1 (10%)	0	0

Within histologic groups, treatments with different symbols (* or #) are significantly different.

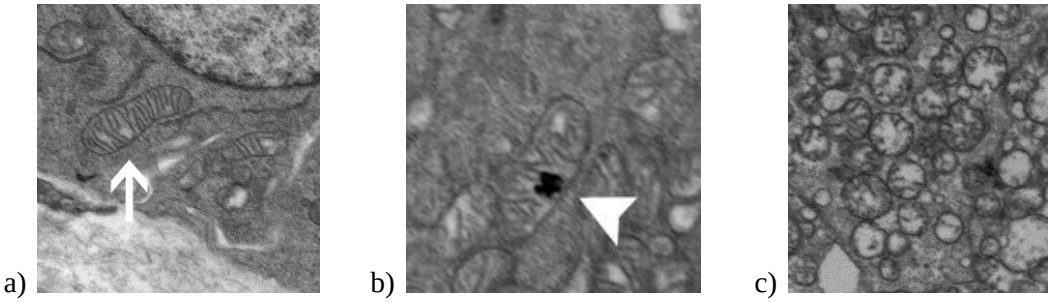


Figure 10. Transmission electron micrograph of sections of colonic mucosa at the end of the experiment. (a) normal mitochondria (white arrow). (b) electron dense particles (arrowhead) within the mitochondria. (c) swollen mitochondria with loss of cristae.

difference in the odds of mitochondria with swelling in colon treated with carprofen, carprofen with tempol pretreatment, or DNP with tempol pretreatment all of which were greater than DNP alone.

Table 5. Electron microscopy findings for sections of colonic mucosa after exposure to carprofen or 2,4 dinitrophenol in an Ussing chamber. Treatments are 1) C = carprofen 200 μg/ml, 2) T + C = carprofen 200 μg/ml pretreated with tempol 1 mM, 3) D = 2,4 dinitrophenol 0.25 mM, 4) T + D = 2,4 dinitrophenol 0.25 mM pretreated with tempol 1 mM, and 5) control. The presence of electron dense particles within the mitochondria was considered positive if any were noted. Mitochondrial swelling and loss of cristae were considered positive if greater than 33% of the mitochondria were affected. The presence of ruptured mitochondria was positive if any were noted.

Treatment	Electron Dense Particles	Swelling	Loss of Cristae	Rupture
C	36/101 (35.6%)[b]	92/101 (91.1%)[c]	90/100 (89.1%)[c]	22/101 (21.8%)[b]
T + C	37/107 (34.6%)[b]	103/107 (96.3%)[c]	99/107 (92.5%)[c]	42/107 (39.3%)[c]
D	18/106 (17.0%)[c]	77/106 (72.6%)[b]	63/97 (64.9%)[a]	7/105 (6.7%)[a]
T + D	14/104 (13.5%)[c]	95/104 (91.3%)[c]	80/101 (79.2%)[b]	1/104 (1.0%)[a]
control	55/104 (52.9%)[a]	42/103 (41.2%)[a]	49/83 (59.0%)[a]	6/104 (5.8%)[a]

Within a column, treatments with different superscripts (a, b, or c) are significantly different.

The likelihood of mitochondria with loss of cristae was significantly greater for carprofen treated colon (without or with tempol) and DNP treated colon with tempol pretreatment compared to controls but no significant difference with DNP treatment compared to control ($p < 0.001$) (Table 6). The estimated odds of treatment effect (95% confidence interval) of mitochondria with loss of cristae for carprofen treated colon was 9.18 (4.40 – 19.15) and with tempol pretreatment was 13.89 (6.14 – 31.44). The estimate odds (95% confidence interval) of mitochondria with swelling for DNP treated colon was 1.61 (0.93 – 2.77) and with tempol pretreatment was 3.59 (1.99 – 6.49). There was no significant difference in the odds of mitochondria with loss of cristae in colon treated with carprofen without or with tempol pretreatment which were both greater than DNP treated colon pretreated with tempol.

The likelihood of ruptured mitochondria were significantly greater for carprofen treated colon (without or with tempol) compared to control but no significant difference with DNP treatment (without or with tempol pretreatment) compared to control ($p < 0.001$) (Table 6). The estimated odds of treatment effect (95% confidence interval) for ruptured mitochondria for carprofen treated colon was 4.55 (1.76 – 11.76) and with tempol pretreatment was 10.55 (4.24 – 26.25). The estimated odds (95% confidence interval) for ruptured mitochondria for DNP treated colon was 1.14 (0.37 – 3.52) and with tempol pretreatment was 0.16 (0.02 – 1.33). The odds of ruptured mitochondria in carprofen treated colon was less than in carprofen treated colon with tempol pretreatment both of which were greater than DNP treated colon (without or with tempol pretreatment).

Table 6. Results of electron microscopy comparisons across treatments. Odds are expressed relative to controls. Treatments with odds excluding 1.0 are significantly different from control. Treatments are 1) C = carprofen 200 μg/ml, 2) T + C = carprofen 200 μg/ml pretreated with tempol 1 mM, 3) D = 2,4 dinitrophenol 0.25 mM, 4) T + D = 2,4 dinitrophenol 0.25 mM pretreated with tempol 1 mM.

Electron dense particles

Treatment	Odds Ratio	95% Confidence Interval
C [a]	0.49	0.28-0.86
T + C [a]	0.47	0.27-0.82
D [b]	0.18	0.09-0.34
T + D [b]	0.14	0.07- 0.27

Swelling

Treatment	Odds Ratio	95% Confidence Interval
C [a]	15.09	6.86-33.21
T +C [a]	37.99	13.00-111.06
D [b]	3.79	2.13-6.74
T + D [a]	14.02	6.56-29.99

Cristae

Treatment	Odds Ratio	95% Confidence Interval
C [a]	9.18	4.40-19.15
T + C [a]	13.89	6.14-31.44
D	1.61	0.93-2.77 NS
T + D [b]	3.59	1.99-6.49

Rupture

Treatment	Odds Ratio	95% Confidence Interval
C [a]	4.55	1.76-11.76
T + C [b]	10.55	4.24-26.25
D	1.14	0.37-3.52 NS
T + D	0.16	0.02-1.33 NS

Treatments within each feature with the same superscript are not significantly different from each other in their effect against controls. NS=not significant from controls.

CHAPTER 4. DISCUSSION

The results of objective one of this study show that carprofen has a concentration dependent toxic effect on the colonic mucosa in vitro. Electrical conductance, mannitol flux, and histopathology of the two highest concentrations of carprofen, 400 μg/ml and 200 μg/ml, were not different from each other and both indicate mucosal damage compared to control. Electrical conductance and mannitol flux of colonic mucosa treated with carprofen at 100 and 40 μg/ml were not significantly different from control. The only significant difference detected was between the 40 μg/ml carprofen group and control was minimal mucosal gland sloughing.

The majority of electrical potential difference in the colon is due to secretion of chloride into the lumen.[3] Alterations in chloride secretion into the colonic lumen will therefore affect the electrical conductance. Prostaglandins promote secretion of chloride ions into the colonic lumen leading to an increase in transepithelial conductance.[3;18] Therefore inhibition of prostaglandin synthesis, such as with NSAIDs, would decrease chloride secretion and therefore decrease electrical conductance across the membrane. In the current study carprofen increased transepithelial electrical conductance. This confirms the results of a previous study evaluating the effects of carprofen on the canine colonic mucosa.[18] The disparity between expected results if prostaglandin inhibition were the cause of colonic damage (decreased electrical conductance) and the actual findings (increased electrical conductance) suggests an alternate mechanism of colonic damage.

There are no specific transport mechanisms for mannitol in the colon and transport across the epithelium is via transcellular and paracellular mechanisms in a concentration dependent manner.[4] The majority of mannitol flux is via the paracellular pathway and increases with damage to the tight junctions.[23] Mitochondrial damage increases permeability to calcium, which

has been shown to damage tight junctions.[30] Therefore the increased mucosal to serosal flux of mannitol may in part be due to damaged tight junctions secondary to mitochondrial injury. In our study there was a significant difference in the mannitol flux with carprofen treated colon at 200 μg/ml compared to control, however there was no significant increase in electrical conductance. This may indicate that the paracellular pathway is more significantly damaged in colon treated with carprofen compared to the transcellular pathway and that mannitol flux is a more sensitive indicator of mucoal damage.

Since mannitol flux and electrical conductance are both measures of membrane integrity, any damage to the surface epithelium would cause increases in these parameters. Carprofen treated colon displayed severe erosions on histopathology indicating a loss of the epithelial barrier function. This may contribute to increased permeability of ions and molecules. It is impossible to tell the relative contribution of each of these mechanisms on mannitol flux based on this study.

The plasma concentration of carprofen after an orally administered dose of 25 mg of carprofen in Beagles is 18.7 μg/ml.[14] This dose corresponds to approximately 2.2 mg/kg, which is roughly half of the total recommended daily dose of carprofen presuming normal body weight of the Beagles in this study.[17] Although plasma concentrations cannot be directly extrapolated to that used in a tissue bath, as no additional information is available, the current pharmacokinetic data was used as a guideline to choose solution concentrations in our study. The plasma concentration of carprofen is directly proportional to the dose.[14] If the linear relationship is maintained at high doses it is presumed that the highest concentration used in this study (400 μg/ml) would represent a 10 times overdose, and the lowest concentration (40 μg/ml) would represent that expected after a single recommended daily dose. With this relationship the lack of

damage seen at 40 μg/ml may indicate that carprofen at the recommended daily dose does not cause significant loss of the barrier function of the colonic mucosa as measured in this study. It is not known however if this is the concentration the colonic mucosa is exposed to as it does not take into account unabsorbed drug and that which enters the intestinal lumen via biliary excretion. The experimental model in our study was chosen to evaluate the direct effect of carprofen on the colonic mucosa and not the systemic effect that would be expected with systemically absorbed carprofen.

The oral dosage of carprofen that consistently causes colonic toxicity in dogs is not known. The distributors of carprofen reported one incident of reddened colonic mucosa in a dog treated with carprofen at 6.6 mg/kg twice daily, a 3 times overdose, for 6 weeks.[31] The concentration of carprofen to which dog colon is exposed following an oral dose is not documented and would depend on many things including gastric and small intestinal absorption, metabolism and elimination, and colonic transit time. The concentration of carprofen to which the colonic mucosa is exposed is presumed to be significant due to hepatic metabolism and enterohepatic recirculation. In dogs, carprofen is primarily eliminated via biliary secretion. After an intravenous dose of radiolabeled carprofen 70% was excreted in the feces while only 8-15% was eliminated in the urine.[16] The exact amount of carprofen which undergoes enterohepatic recirculation in the dog is not well documented. In vivo studies evaluating colonic luminal and mucosa drug concentrations following intravenous and oral doses of carprofen may help to further describe the pharmacokinetics and metabolism of carprofen.

The mechanism of colonic damage by NSAIDs has not been investigated in the dog. There are three methods by which NSAIDs can have an effect on the colonic mucosa; 1) inhibition of prostaglandins by systemic absorption 2) direct topical toxicity of orally

administered drug which is unabsorbed and 3) topical toxicity through enterohepatic circulation via biliary excretion. Studies in rats indicate a topical toxic effect of NSAIDs to be important, along with inhibition of prostaglandins. Somasundaram et al demonstrated jejunal mitochondrial damage, seen by electron microscopy, in rats given indomethacin by gavage but not in rat in which the bile duct had been ligated.[13] This suggests that the systemic effect of indomethacin at the give dose was not sufficient to affect intestinal mitochondria, where as the biliary excreted component was sufficient to cause a topical toxicity.[13] Aspirin is unique among NSAIDs since it is rapidly absorbed by the stomach while having minimal enterohepatic recirculation. When aspirin was given by gavage no intestinal ulceration or mitochondrial changes were noted, however, when directly instilled into the duodenum, aspirin caused extensive ulcerations distal to the administration site. Mitochondrial changes seen in the intestinal segments were similar to those of indomethacin and DNP.[13;15]

Somasundaram et al. suggest that NSAID induced changes in mitochondrial energy production may be a mechanism of the topical phase of NSAID damage.[13;15] Isolated rat mitochondria exposed to indomethacin, aspirin and DNP showed a stimulation of mitochondrial respiration (uncoupled oxidative phosphorylation) followed by a progressive decreased in oxygen consumption with increasing drug concentrations, consistent with inhibition of the electron transport chain.[15] These results suggest a mechanism of inhibitory uncoupling in indomethacin and aspirin.[15] The mitochondrial morphologic changes were identical in the DNP and indomethacin treated groups with patchy swelling and elongation of the mitochondria with loss of cristae and vacuolization consistent with uncoupling of oxidative phosphorylation or inhibition of the electron transport chain.[15] The relationship was explored further by giving aspirin intraperitoneally, indomethacin by gavage, and DNP instilled into the small bowel. DNP

did not change intestinal prostaglandin concentrations but did uncouple mitochondrial oxidative phosphorylation.[15] Both aspirin and indomethacin decreased prostaglandin concentrations but only indomethacin caused increased intestinal permeability and mitochondrial changes.[15] When intraperitoneal aspirin and intraluminal DNP were administered, the small intestinal changes were similar to indomethacin.[15] These results suggest that both uncoupling of oxidative phosphorylation and inhibition of prostaglandins are important in the pathogenesis of NSAID induced ulceration.[15]

Mitochondrial energy production involves a complex interaction between the electron transport chain and oxidative phosphorylation. Each of these processes has multiple steps involving intramembrane proteins and the transport of ions and electrons. Multiple NSAIDs have been shown to negatively impact isolated rat mitochondria primarily as mitochondrial uncouplers of oxidative phosphorylation, such as nimesulide, meloxicam, piroxicam, and indomethacin.[32] Other mechanisms were appreciated including inhibition of complex 1 of the electron transport chain as with nabumetone, or direct effect on the hydrogen-ATPase as with diclofenac.[32] Naproxen did not have any effect on mitochondrial parameters measured.[32] It is therefore impossible to extrapolate the mechanism of mitochondrial damage of one NSAID to all NSAIDs. The effects of carprofen on mitochondrial respiration could be further examined by evaluating specific markers of oxidative stress such as catalase and superoxide dismutase as well as the function of each complex in the electron transport chain and oxidative phosphorylation.

The response of isolated canine hepatic mitochondria to carprofen has recently been investigated. Isolated canine hepatic mitochondria were treated with carprofen or tempol followed by carprofen, and compared to controls.[33] Tempol is a superoxide dismutase mimetic which acts as an antioxidant enzyme by scavenging superoxide radicals and reactive oxygen

species formed when the oxidative phosphorylation chain is uncoupled.[26;28] Mitochondrial swelling, reactive oxygen species, and catalase activity were significantly affected by treatment with carprofen.[33] This effect was mitigated by pretreatment with tempol.[33] Transmission electron microscopy of carprofen treated mitochondria showed diffuse structural damage with swelling and disruption of the mitochondrial membrane compared to untreated mitochondria.[33] These results provide evidence to support a topical effect of carprofen on mitochondria through uncoupling of oxidative phosphorylation.[33] In our study, the effect of DNP on the colonic mucosa of normal dogs was compared to colonic mucosa treated with carprofen. The hypothesis was that if the effects of carprofen parallel those of a known inhibitor of oxidative phosphorylation (e.g. DNP), this would provide evidence of a parallel mechanism for topical toxicity of the canine colon.

It was hypothesized that a concentration of carprofen would be determined which caused damage to the barrier function of the colonic mucosa without completely obliterating the integrity of the membrane. Two hundred micrograms per milliliter seemed to represent this transition period in objective one. Electrical conductance of colonic mucosa treated at 200 μg/ml of carprofen was not significantly different from control; however mannitol flux was significantly greater than control. The carprofen concentration for objective two was then determined (200 μg/ml) by the results of objective one as that which compromised the colonic mucosa (based on mannitol flux and light microscopy) without profound electrochemical changes.

The results of objective two verified the effect of carprofen treated colon at a concentration of 200 μg/ml in that a significant increase in mannitol flux compared to control was seen without a significant difference in transepithelial electrical conductance. The electrical

conductance of colonic mucosa treated with DNP was not significantly different from controls, however mannitol flux was significantly affected. These results further support the conclusion that mannitol flux may be a more sensitive indicator of colonic mucosal barrier function.

The mannitol flux in colonic mucosa treated with carprofen was not different from control in the first two time periods but had increased by the third time period. In comparison treatment with DNP increased mannitol flux compared to control at all time periods. The increased mannitol flux when treated with DNP at the starting time period was unexpected. This may indicate that DNP had a faster onset of tissue damage than carprofen. However, DNP is yellow and this coloration may have interfered with the scintillation counter. Any artifact that this may have contributed would be present throughout the study and so changes in mannitol flux over the course of the experiment may be more accurate than the calculated value of mannitol flux in DNP treated colon. Mannitol flux did increase over time in tissue treated with either carprofen or DNP, indicating damage progressed with continued exposure to either treatment.

The only beneficial effect of tempol in this study was seen in mitigation of increased mannitol flux of carprofen treated colon. This lends support, although not conclusive, that carprofen causes oxidative stress of colonic mucosa. It is believed that mannitol flux represents the most sensitive indicator of mucosal damage evaluated in this study. It is possible that the effects of carprofen were only able to be mitigated by tempol when the effect was more pronounced. Tempol did not mitigate the effects of DNP in this study. The lack of benefit seen in DNP treated colon was likely due to the overwhelming damage caused by DNP. This was not expected because the mechanism of action of DNP, uncoupling of oxidative phosphorylation, is known to induce oxidative stress. Tempol is well documented as a free radical scavenger and counters damage caused by oxidative stress.[26;28]

The limited effect of tempol in DNP treated colon may be due to an inappropriate concentration used. The concentration used was derived from previous in vitro studies using tempol, however, tempol has not been investigated in an Ussing chamber or with the canine colon.[33] Additionally, the concentration of DNP was extrapolated from other in vitro experiments. Dinitrophenol has been investigated with use in the Ussing chamber at various concentrations ranging between 0.1 mM on both sides of the tissue to 1 mM on just one side of the tissue.[23-25] None of these previous studies were investigating colonic mucosa or canine tissues. The concentration of DNP in this study, 0.25 mM, was an intermediate dose compared to previous studies. This DNP concentration may have been excessive and overpowered the mitigating effects of tempol. Alternatively the concentration of tempol may have been insufficient to counter the toxic effects of carprofen and DNP. Additional studies may be directed at using a higher concentration of tempol to mitigate the effects of carprofen on the colon. Determining a concentration dependent effect of DNP on the colonic mucosa would have limited benefit since this is not a drug used clinically.

In both objectives inflammation within the tissues was not significantly different among treatments (carprofen and DNP) or control. This is expected because the tissues were ex vivo so there was no source of additional inflammatory cells after the colon was removed from the dog. If inflammation were noted in the tissue sections it would change the interpretation of our results so inflammation was used as an internal control. A section of colon just after harvest was examined histologically to identify preexisting colonic disease. It is presumed that findings would represent the entire colon, however inflammatory diseases in the colon may be segmental. Since there were no significant differences in inflammation we believe that preexisting disease was not a factor in the results.

Edema is primarily a result of altered sodium concentrations and subsequent flux of water into the cells and interstitial space. Sodium concentration in the gastrointestinal epithelial cells is regulated by sodium phosphate adenosine triphosphatase and sodium hydrogen exchanger.[3;34] Mitochondrial RNA expression for these receptors was increased in an acidic environment (pH 1.5 and 4.0) in equine nonglandular stomach.[34] Both of the drugs in our study are weak acids (carprofen pKa 4.88 and DNP pKa 4.114) so edema may be seen in treated colon because the pH of the drugs caused alterations in sodium regulation. Differences in tissue edema were not appreciated in our study between either of the drugs and control. The pH of the bathing solution did not change with addition of either drug, making any effect of pH on colonic mucosal edema impossible to determine. The effect of carprofen on the colonic pH and the subsequent effect on the colonic mucosa in vivo is not known.

The most striking histologic changes seen in this study were erosions and sloughing of epithelial cells both on the surface and within the mucosal glands in tissues treated with high concentrations of carprofen. Erosions of the surface epithelium were also seen in tissue treated with DNP. Erosions of the surface epithelium would eliminate the barrier function of the mucosal epithelium allowing mannitol to pass freely from the mucosal bath to the serosal bath. In addition, electrical conductance would be affected as the barrier responsible for creating a concentration gradient necessary for ion transport would be disrupted. Histologically more damage was seen in colon treated with carprofen than with DNP. Additionally, pretreatment with tempol did not mitigate the histologic changes. These findings were confirmed with electron microscopy.

The most severe damage to enterocyte mitochondria was seen in the carprofen treated colon. Moderate changes in mitochondria were seen in the colon exposed to DNP. Carprofen

treated colon had significantly more frequent mitochondrial swelling, loss of cristae, and ruptured mitochondria when compared to control. These changes are consistent with uncoupling of oxidative phosphorylation but can also be seen with any toxic insult to the mitochondria. Morphologic changes seen in colonic mucosa treated with DNP were not as severe as those after carprofen treatment. Ruptured mitochondria were rarely seen in controls and mucosa treated with DNP, but quite commonly after treatment with carprofen. Rupture of mitochondria was the most drastic morphologic change evaluated, and indicates a severe effect of carprofen on mitochondria. It is important to remember that morphologic changes do not necessarily represent functional changes in mitochondria. Despite the lack of severe morphologic changes in the DNP treated colonic mucosa there were likely functional changes which were not evaluated in this study.

Elevated intracellular calcium is seen in response to oxidative stress.[35-37] Calcium is released as a result of oxidative stress to the mitochondria, which then serves as a second messenger to initiate apoptosis.[35] Calcium deposits are represented as electron dense spots on electron microscopy.[35;38] Intramitochondrial electron dense particles were seen most commonly in control tissues. Fewer electron dense particles were seen in both the carprofen and DNP treated colon. This was particularly unexpected in the DNP treated colon because DNP is known to induce oxidative stress and would therefore be expected to have increased calcium deposits. It appears from this study that less calcium deposition occurs in colonic mucosa treated with carprofen, however the mechanism and cellular effect is undetermined. There may be a significant variation in baseline amounts of calcium contained within the colonic mucosa. Alternatively, mitochondrial damage may have caused a release of calcium from the mitochondria into the cytosol which was not measured in this study. Quantitative evaluation of

the calcium concentration in colonic mucosa treated with NSAIDs may provide insight into the relationship of calcium metabolism in gastrointestinal injury induced by NSAIDs.

The lack of a beneficial response to tempol morphologically was surprising. Tempol is a known anti-oxidant and therefore was expected to mitigate the toxic effect to the mitochondria. Tempol has been shown to be beneficial in a variety of in vivo experimental models. Tempol improved healing of colonic anastamosis in rats in a septic model[39] and ischemia reperfusion model[40] at a dose of 30 mg/kg given intravenously before the toxic insult. Oral tempol administration in rats had immediate absorption in the colonic mucosa with persistent tissue levels achieved and was effective in reducing the severity of chemically induced colitis.[29;41] It is possible that tempol was not effective in the Ussing chamber or that the concentration was not adequate in this study. Alternatively, tempol is known to be pro-oxidant at extremely high concentrations. At a concentration of 10^{-4} to 10^{-2} M of tempol pro-oxidant effects were noted on vascular smooth muscle and endothelial cells.[26] It is possible that tempol concentrated within the colonic mucosal tissues in the Ussing chambers causing accumulation of tempol and leading to a pro-oxidant state. It would be helpful to determine tissue levels of tempol in the colonic mucosa and measure quantitative measures of oxidative stress such as catalase to determine the effect of tempol on the canine colon.

Carprofen may not have behaved identically to DNP because the damage is induced by an alternate mechanism. Ibuprofen, a common NSAID used in people, has been found to inhibit fatty acid oxidation in the human and rat colon.[42] Oxidation of fatty acids helps to maintain the epithelial barrier of the colonic mucosa by providing acetyl-CoA used in the TCA cycle. Mucosal damage may not always be due to reduced levels of prostaglandins but also due to

metabolic damage through fatty acid oxidation.[42] The effect of carprofen on fatty acid oxidation would have to be investigated to determine if this plays a role in its toxicity.

This study is restricted in its evaluation only to the descending colon. The descending colon was chosen for use in our study due to the availability of a large enough section of colonic mucosa to do multiple trials with duplicates. A significant difference in baseline electrical resistance and mannitol permeability between the transverse and descending colonic mucosa in the dog has previously been demonstrated.[18] The difference was no longer appreciated between the different segments of the colon following carprofen exposure.[18] The difference in baseline measurements indicates that there may be a difference in the response seen in the transverse and descending colon at lower concentrations of carprofen. To avoid having a physiologic difference in colonic permeability confound the response to treatment, only the descending colon was chosen for this study. A high proportion of people with colonic ulceration secondary to NSAID toxicity occur at the cecocolic region.[43] The location of colonic ulceration in dogs exposed to NSAIDs is not documented, so there is no clinical justification for investigating the effect of carprofen on either transverse or descending colon.

An additional limitation in objective 2 was in the chemical properties of DNP. Dinitrophenol has limited solubility in cold water. It is soluble in ethyl acetate, acetone, chloroform, pyridine, carbon tetrachloride, toluene, ethanol, benzene, and aqueous alkaline solutions. Ethanol was chosen as the solvent since this is the least toxic compound. Ethanol in concentrations above 30% breaks the mucosal barrier and causes diffuse colonic damage when directly instilled into the distal colon of rats. The concentration of ethanol after dilution in the Ussing chamber in this study was 0.76%, which was considered unlikely to cause a significant toxic effect.

CHAPTER 5. CONCLUSION

Carprofen increased the in vitro electrical conductance and permeability to mannitol of the colonic mucosa of the dog in a concentration dependent manner. Sloughing of epithelial cells and surface erosions were noted in a concentration dependent manner. Damage to the colonic mitochondria was evident on an ultrastructural level. These results suggest a potential mechanism of topical carprofen toxicity in the dog may be damage to colonic mitochondria, leading to compromise of the colonic mucosa and loss of the barrier function.

The exact mechanism of colonic mucosal compromise has yet to be determined. There were some similarities in electrical conductance and histologic changes of colon treated with carprofen and colon treated with DNP, an inhibitor of oxidative phosphorylation. However when samples were pretreated with tempol, a free radical scavenger, the mitochondria remained damaged. At the concentration tested DNP increased colonic mucosal permeability to mannitol without affecting the in vitro electrical conductance. Erosions and sloughing of surface epithelial cells were seen when treated with DNP. However, ultrastructural changes in colonic mitochondria were variable.

One of the limitations of the study that it relies on a comparison of colon treated with carprofen to colon treated with DNP. By looking at mitochondrial structural changes it is evident that carprofen damages the mitochondria. It is uncertain how the morphologic damage correlates to functional compromise. The primary function of the mitochondria is the production of ATP via the electron transport chain and oxidative phosphorylation. It is reasonable that morphologic changes would be seen when mitochondrial function is affected. Future studies are warranted to evaluate functional compromise of mitochondria when treated with carprofen, in order to elucidate the mechanism of toxicity.

Furthermore, there is an inherent variability in the colon with respect to various transporter mechanisms. This may be why such variation was seen in the electrical conductance of control tissues and the increased likelihood of having electron dense particles in mitochondria from control tissues. This inherent variability was minimized by only evaluating the descending colon.

The oral dosage of carprofen that consistently causes colonic toxicity is not known. The toxic dose may act systemically by prostaglandin inhibition or by a direct topical effect. The concentration of carprofen to which dog colon is exposed following an oral dose is not documented. It is presumed to be a significant amount due to hepatic metabolism and enterohepatic recirculation. If direct topical toxicity of carprofen is demonstrated in vivo it would be useful to further characterize the metabolism of carprofen, including the colonic luminal concentration of carprofen and its metabolites at the level of the colonic mucosa.

REFERENCES

1. Dyce K, Sack W, Wensing C. *Textbook of Veterinary Anatomy*, 2 ed. Philadelphia: W.B. Saunders Company, 1996.

2. Dellmann H, Eurell J. *Textbook of Veterinary Histology*, 5 ed. Piladelphia: Lippincott Williams and Wilkins, 1998.

3. Johnson L. Fluid and Electrolyte Absorption. In: Johnson LR ed. *Gastrointestinal Physiology*. 5 ed. St. Louis: Mosby, 1997;135-145.

4. Nejdfors P, Wang Q, Ekelund M et al. Increased colonic permeability in patients with ulcerative colitis: an in vitro study, *Scand.J Gastroenterol.* 1998;33: 749-753

5. Li H, Sheppard DN, Hug MJ. Transepithelial electrical measurements with the Ussing chamber, *J Cyst.Fibros.* 2004;3 Suppl 2: 123-126

6. Ussing System for investigation of epithelial transport: Instruction Manual. 1996. World Precision Instruments, Inc.

7. Curry SL, Cogar SM, Cook JL. Nonsteroidal antiinflammatory drugs: a review, *J Am Anim Hosp.Assoc* 2005;41: 298-309

8. Tomlinson J, Blikslager A. Role of nonsteroidal anti-inflammatory drugs in gastrointestinal tract injury and repair, *J Am Vet Med Assoc* 2003;222: 946-951

9. Little D, Jones SL, Blikslager AT. Cyclooxygenase (COX) inhibitors and the intestine, *J Vet Intern.Med* 2007;21: 367-377

10. Lamont LA, Mathews KA. Opioids, Nonsteroidal Anti-inflammatories, and Analgesic Adjuvants. In: Tranquilli WJ, Thurmon JC, and Grimm KA eds. *Lumb and Jones' Veterinary Anesthesia and Analgesia*. 4 ed. Ames: Blackwell Publishing, 2007;241-271.

11. Eberhart CE, DuBois RN. Eicosanoids and the gastrointestinal tract, *Gastroenterology* 1995;109: 285-301

12. McCarn K, Yursik B, Halim S et al. Peri-epithelial origin of prostanoids in the human colon, *J.Cell Physiol* 2003;194: 176-185

13. Somasundaram S, Rafi S, Hayllar J et al. Mitochondrial damage: a possible mechanism of the "topical" phase of NSAID induced injury to the rat intestine, *Gut* 1997;41: 344-353

14. Clark TP, Chieffo C, Huhn JC et al. The steady-state pharmacokinetics and bioequivalence of carprofen administered orally and subcutaneously in dogs, *Journal of Veterinary Pharmacology and Therapeutics* 2003;26: 187-192

15. Somasundaram S, Sigthorsson G, Simpson RJ et al. Uncoupling of intestinal mitochondrial oxidative phosphorylation and inhibition of cyclooxygenase are required for the

development of NSAID-enteropathy in the rat, *Alimentary Pharmacology and Therapeutics* 2000;14: 639-650

16. Rubio F, Seawall S, Pocelinko R et al. Metabolism of carprofen, a nonsteroid anti-inflammatory agent, in rats, dogs, and humans, *Journal of Pharmaceutical Sciences* 1980;69: 1245-1253

17. Fox SM, Johnston SA. Use of carprofen for the treatment of pain and inflammation in dogs, *Journal of the American Veterinary Medical Association* 1997;210: 1493-1498

18. Briere CA, Hosgood G, Morgan TW et al. Effects of carprofen on the integrity and barrier function of canine colonic mucosa, *Am J Vet Res.* 2008;69: 174-181

19. Thiefin G, Beaugerie L. Toxic effects of nonsteroidal antiinflammatory drugs on the small bowel, colon, and rectum, *Joint Bone Spine* 2005;72: 286-294

20. *Textbook of Biochemistry with Clinical Correlations*, 5 ed.: Wiley-Liss, 2002

21. Bioenergetics and Oxidative Phosphorylation. In: Champe P and Harvey R eds. *Lippincott's Illustrated Reviews: Biochemistry.* 2 ed. Philadelphia: Lippincott Williams and Wilkins, 1994;61-74

22. Mandelker L. Introduction to oxidative stress and mitochondrial dysfunction, *Vet Clin.North Am Small Anim Pract.* 2008;38: 1-30

23. Menon RM, Barr WH. Comparison of ceftibuten transport across Caco-2 cells and rat jejunum mounted on modified Ussing chambers, *Biopharmaceutics and Drug Disposition* 2003;24: 299-308

24. Gabel G, Muller F, Pfannkuche H et al. Influence of isoform and DNP on butyrate transport across the sheep ruminal epithelium, *J Comp Physiol B* 2001;171: 215-221

25. Vaghefi N, Guillochon D, Bureau F et al. The effect of cysteine and 2,4-dinitrophenol on heme and nonheme absorption in a rat intestinal model, *J Nutr.Biochem.* 2000;11: 562-567

26. Wilcox CS, Pearlman A. Chemistry and antihypertensive effects of tempol and other nitroxides, *Pharmacological Reviews* 2008;60: 418-469

27. Abdallah DM, El-Abhar HS, bdel-Aziz DH. TEMPOL, a membrane-permeable radical scavenger, attenuates gastric mucosal damage induced by ischemia/reperfusion: a key role for superoxide anion, *Eur.J Pharmacol.* 2009;603: 93-97

28. Trnka J, Blaikie FH, Logan A et al. Antioxidant properties of MitoTEMPOL and its hydroxylamine, *Free Radical Research* 2009;43: 4-12

29. Karmeli F, Eliakim R, Okon E et al. A stable nitroxide radical effectively decreases mucosal damage in experimental colitis, *Gut* 1995;37: 386-393

30. Tai YH, Flick J, Levine SA et al. Regulation of tight junction resistance in T_{84} monolayers by elevation in intracellular Ca^{2+}: a protein kinase C effect, *Journal of Membrane Biology* 1996;149: 71-79

31. Rimadyl (carprofen) Sterile Injectable Solution 50 mg/mL Non-steroidal anti-inflammatory drug . 2005. New York, NY, Pfizer Animal Health

32. Moreno-Sanchez R, Bravo C, Vasquez C et al. Inhibition and uncoupling of oxidative phosphorylation by nonsteroidal anti-inflammatory drugs: study in mitochondria, submitochondrial particles, cells, and whole heart, *Biochemical Pharmacology* 1999; 57: 743-752

33. Coutin J, Elks C, Mariappan N et al. The topical effect of carprofen on the integrity of canine mitochondria. 2008. Unpublished Work

34. Peretich AL, Abbott LL, Andrews FM et al. Age-dependent regulation of sodium-potassium adenosinetriphosphatase and sodium-hydrogen exchanger mRNAs in equine nonglandular mucosa, *Am J Vet Res.* 2009;70: 1124-1128

35. Domoki F, Bari F, Nagy K et al. Diazoxide prevents mitochondrial swelling and Ca2+ accumulation in CA1 pyramidal cells after cerebral ischemia in newborn pigs, *Brain Research* 2004;1019: 97-104

36. Fu W, Luo H, Parthasarathy S et al. Catecholamines potentiate amyloid beta-peptide neurotoxicity: involvement of oxidative stress, mitochondrial dysfunction, and perturbed calcium homeostasis, *Neurobiology of Disease* 1998;5: 229-243

37. Lee SH, Na SI, Heo JS et al. Arachidonic acid release by H2O2 mediated proliferation of mouse embryonic stem cells: involvement of Ca2+/PKC and MAPKs-induced EGFR transactivation, *J Cell Biochem.* 2009;106: 787-797

38. Boyne AF, Bohan TP, Williams TH. Effects of calcium-containing fixation solutions on cholinergic synaptic vesicles, *J Cell Biol.* 1974;63: 780-795

39. Aytekin FO, Teke Z, Aydin C et al. Effects of a membrane-permeable radical scavenger, Tempol, on healing of colonic anastomoses in the cecal ligation and puncture model of polymicrobial sepsis in rats, *Am J Surg.* 2007;193: 723-729

40. Aydin C, Teke Z, Aytekin F et al. Tempol prevents harmful effects of remote ischemia reperfusion injury on healing of experimental colonic anastomoses, *Int.J Colorectal Dis.* 2007;22: 325-331

41. Cuzzocrea S, McDonald MC, Mazzon E et al. Tempol, a membrane-permeable radical scavenger, reduces dinitrobenzene sulfonic acid-induced colitis, *Eur.J Pharmacol.* 2000;406: 127-137

42. Roediger WE, Millard S. Selective inhibition of fatty acid oxidation in colonocytes by ibuprofen: a cause of colitis?, *Gut* 1995;36: 55-59

43. Kurahara K, Matsumoto T, Iida M et al. Clinical and endoscopic features of nonsteroidal anti-inflammatory drug-induced colonic ulcerations, *American Journal of Gastroenterology* 2001;96: 473-480

APPENDIX 1. ELECTRICAL CONDUCTANCE OBJECTIVE 1

Electrical conductance (mS/cm^2) for concentration dependent effects of carprofen on the mucosa of the descending colon. Time is in minutes. Treatments are in micrograms of carprofen per milliliter of solvent (μg/ml).

Dog	Time	Treatment									
		400	400	200	200	100	100	40	40	0	0
1	0	15.5	13.5	15.1	13.7	25.8	16.4	15.0	13.6	17.3	17.9
1	15	15.4	13.3	14.1	12.6	24.3	16.3	13.9	15.0	16.3	15.0
1	30	14.7	12.6	13.6	11.7	23.9	15.6	14.4	14.4	0.2	14.1
1	45	11.7	8.8	13.2	12.8	13.7	14.2	13.6	14.1	13.4	13.1
1	60	-7.6	9.4	7.9	7.3	9.7	13.4	-17.7	8.6	12.7	12.9
1	75	1.0	8.5	8.3	9.2	13.5	-45.5	9.0	10.3	13.3	12.4
1	90	19.1	15.3	9.9	11.1	13.8	-15.9	8.7	11.0	-11.6	11.2
1	105	24.3	23.6	10.8	11.7	13.3	-12.7	8.9	-17.7	13.2	10.6
1	120	32.8	27.6	12.0	11.9	-53.1	-12.2	9.3	-14.5	11.6	10.2
1	135	37.0	30.1	12.2	11.7	-35.4	-53.1	9.3	-13.8	13.4	9.8
1	150	37.3	36.0	13.3	13.0	11.9	.	9.8	-13.3	11.1	9.3
1	165	45.1	42.9	.	13.0	-22.7	-14.5	10.0	-12.7	11.4	9.0
1	180	45.9	45.5	-0.5	14.3	-24.5	-14.5	10.2	-12.7	11.4	8.7
1	195	52.2	48.1	19.4	15.7	-24.5	-12.7	10.8	-12.2	11.1	8.6
1	210	53.5	49.1	19.9	17.7	-26.5	-12.7	-11.4	-12.2	11.6	8.3
1	225	59.5	53.1	22.3	20.2	-35.4	-12.2	11.8	-11.8	12.4	8.6
1	240	52.2	54.4	23.5	21.4	-39.8	-11.8	12.6	-11.8	11.8	8.8
2	0	10.9	18.4	-318.5	18.7	19.1	29.2	16.9	21.6	571.6	-63.7
2	15	15.8	14.2	-45.5	13.3	.	25.9	-15.5	19.5	-18.0	318.5
2	30	15.8	15.5	-45.5	0.2	14.7	21.6	0.2	18.4	178.9	159.2
2	45	16.2	-9.4	-10.0	1.5	14.2	18.8	12.9	15.3	43.7	35.4
2	60	-35.4	-7.1	-7.4	13.5	12.7	-35.4	10.2	-15.2	16.7	106.2
2	75	-22.7	18.0	-8.0	15.2	.	-24.5	9.7	-10.6	16.2	-159.2
2	90	-22.7	-10.0	-13.3	16.4	12.2	-22.7	9.6	-9.7	15.4	-39.8
2	105	-26.5	16.2	-8.4	16.6	12.4	-21.2	9.8	-9.7	14.8	-29.0
2	120	-26.5	17.2	-8.4	17.6	13.1	-26.5	10.0	-10.0	14.7	-26.5
2	135	-26.5	18.7	-10.6	18.0	13.6	-29.0	10.5	-9.7	14.3	-19.9
2	150	-24.5	19.8	-10.3	18.7	13.6	-24.5	11.0	-10.0	14.3	-18.7
2	165	-24.5	21.0	-10.6	19.1	13.9	-21.2	11.4	-10.3	14.1	-15.2
2	180	-24.5	21.7	-11.8	20.7	14.3	-19.9	12.4	-10.3	14.2	-12.7
2	195	-22.7	23.4	-11.8	21.0	14.9	-21.2	13.6	12.7	14.6	-11.4
2	210	-24.5	23.7	-12.2	21.4	15.2	-22.7	14.6	-9.7	14.8	-11.4
2	225	-24.5	25.2	-12.7	21.5	15.8	-22.7	16.3	-9.1	15.4	-10.6
2	240	-29.0	26.0	-12.7	22.1	16.7	-19.9	17.7	-8.6	16.6	-8.8
3	0	318.5	-318.5	159.2	12.7	13.9	15.6	14.6	20.1	-24.5	-29.0
3	15	20.7	79.6	17.5	9.7	13.4	12.7	9.0	-8.0	-318.5	13.0
3	30	31.8	24.4	14.7	8.6	10.0	11.1	8.2	-7.1	13.6	11.1
3	45	13.3	14.3	-35.4	-13.8	7.7	8.5	7.4	9.0	11.3	10.5
3	60	15.1	11.7	10.7	8.6	-7.2	-10.3	7.1	8.3	10.3	8.9

3	75	11.0	8.9	6.8	7.7	8.1	9.4	6.7	8.1	-39.8	-12.7
3	90	10.5	8.4	5.9	-8.4	-10.3	-13.3	10.6	7.4	9.0	-13.3
3	105	9.8	8.3	5.8	-7.8	-7.6	-9.1	6.6	7.1	8.7	-10.0
3	120	9.8	8.0	5.6	-8.4	-7.2	-7.6	-7.6	7.1	-39.8	6.9
3	135	3.6	8.5	5.8	-8.8	-6.8	-7.4	-7.4	6.9	7.8	-7.4
3	150	11.9	-17.7	6.0	-8.6	-6.9	-8.0	-8.0	6.8	7.7	-7.1
3	165	-79.6	-16.8	6.0	-9.1	-6.9	-8.0	-8.0	6.6	7.5	-6.2
3	180	-53.1	-15.9	6.4	-10.3	-6.5	-8.0	-8.0	6.6	6.9	-5.9
3	195	-45.5	-15.2	6.9	-11.0	-6.8	-8.8	-8.8	6.4	6.8	-5.7
3	210	-39.8	-15.2	7.4	-11.4	-6.8	-8.8	-8.8	6.3	6.7	-5.7
3	225	-35.4	-17.7	7.8	-12.2	-7.1	-8.2	-8.2	6.4	-11.0	-5.6
3	240	-53.1	-24.5	8.9	-12.2	-7.6	-8.2	-8.2	-2.1	-9.7	-5.7
4	0	11.9	14.3	16.2	12.2	15.5	12.4	11.4	14.0	17.3	12.7
4	15	-7.2	12.7	15.6	9.1	15.4	12.0	11.5	11.7	16.5	11.8
4	30	-7.2	11.3	12.5	8.9	13.8	10.5	10.4	11.4	14.4	11.3
4	45	9.3	11.3	10.2	11.1	11.1	-9.2	9.6	10.3	12.5	13.9
4	60	8.4	-15.2	11.0	-6.2	-21.2	-13.8	7.9	9.4	13.0	10.5
4	75	-6.9	13.3	-16.8	-6.8	-13.8	-9.4	.	9.3	11.7	11.0
4	90	9.8	12.3	11.4	-10.3	-11.4	-8.6	7.4	-18.7	13.9	9.6
4	105	8.7	-26.5	10.1	-9.4	-8.8	-8.2	-9.4	-15.9	12.3	9.9
4	120	8.3	-29.0	9.9	-7.4	-6.9	-8.6	7.1	-15.9	11.2	10.0
4	135	8.8	-31.8	10.1	-7.2	-5.5	-8.0	-10.6	-15.2	11.3	11.3
4	150	9.1	-39.8	10.1	-6.5	8.4	-9.1	-8.8	-16.8	10.6	10.7
4	165	9.3	-45.5	10.4	-6.9	8.2	-9.4	-8.8	-15.2	10.0	8.4
4	180	10.0	-45.5	10.6	-9.1	8.3	-9.7	-11.4	-13.8	9.7	-11.8
4	195	10.6	-39.8	11.0	-10.0	8.2	-10.6	-10.6	-15.2	9.8	-11.0
4	210	11.1	-29.0	11.2	-9.4	8.4	-12.7	-8.6	-16.8	8.9	-11.4
4	225	11.2	-29.0	11.4	-11.0	7.7	-13.8	-8.8	-159.2	8.5	-9.4
4	240	11.7	-26.5	11.5	-11.0	-6.1	-15.9	-8.6	-11.4	-53.1	.
5	0	14.4	9.0	12.6	63.7	9.6	14.4	12.7	11.3	11.9	11.9
5	15	11.8	10.4	11.2	17.3	10.7	13.7	11.9	10.3	11.0	9.7
5	30	11.5	10.1	11.3	15.5	11.2	12.1	12.3	10.2	10.8	9.7
5	45	7.7	7.3	7.2	3.3	10.1	7.0	6.9	7.0	10.3	9.2
5	60	10.0	7.8	6.5	13.7	6.7	6.1	5.8	5.2	9.0	8.8
5	75	7.8	8.3	8.1	11.7	6.3	6.6	-6.8	4.6	8.3	8.7
5	90	5.8	7.0	6.0	-22.7	6.3	7.1	-6.5	4.5	7.2	8.6
5	105	5.5	6.1	5.4	-18.7	6.5	6.5	4.9	4.4	6.7	8.5
5	120	5.8	6.4	5.3	-15.2	6.5	6.1	4.9	4.2	6.2	8.5
5	135	5.9	6.7	5.4	-13.8	6.4	5.7	4.9	4.3	6.2	8.6
5	150	6.2	7.0	5.3	-13.3	5.9	5.7	4.7	4.3	5.6	8.6
5	165	6.4	7.6	5.4	-12.7	5.8	5.7	4.8	4.2	5.0	8.5
5	180	6.9	8.4	5.4	-12.7	5.8	5.8	4.6	4.4	5.1	8.4
5	195	7.3	-21.2	5.5	-11.8	5.5	5.8	4.9	4.4	4.9	8.2
5	210	8.1	-22.7	5.9	-12.7	5.5	5.8	4.5	4.5	5.0	8.5
5	225	-6.2	-24.5	6.1	-13.3	5.5	5.5	4.8	4.7	5.1	8.6
5	240	-9.7	-29.0	6.1	-13.8	5.7	5.6	4.8	4.6	5.5	8.5
6	0	8.8	11.1	7.2	9.5	9.0	10.1	15.0	9.1	14.0	9.5

6	15	8.7	10.6	6.7	29.4	8.8	8.9	13.3	9.0	15.8	9.8
6	30	8.4	9.8	6.8	9.3	8.6	-8.8	12.6	8.5	13.4	9.1
6	45	9.6	14.8	6.6	7.7	8.1	7.0	9.1	7.6	13.5	8.9
6	60	7.6	12.6	6.8	7.1	7.1	7.0	7.0	6.9	13.2	8.6
6	75	7.4	9.2	7.0	7.2	7.0	7.2	6.9	6.9	12.0	8.4
6	90	6.9	8.5	6.9	7.1	7.3	7.3	7.0	7.0	12.6	8.4
6	105	6.8	9.0	7.6	7.0	7.4	7.5	6.4	7.0	11.8	8.3
6	120	7.2	10.2	9.2	7.2	7.7	8.6	6.5	7.2	11.1	7.9
6	135	7.7	10.9	10.8	7.8	8.2	9.9	6.4	7.4	11.0	7.7
6	150	8.2	11.8	12.9	8.6	8.8	12.7	6.2	7.6	10.3	7.6
6	165	8.9	13.1	14.2	10.0	9.9	14.3	6.4	7.9	10.5	7.7
6	180	9.8	14.9	13.9	11.1	11.8	16.3	6.4	8.4	10.2	8.0
6	195	10.9	15.4	13.9	12.7	13.1	17.0	6.5	9.2	8.9	7.8
6	210	12.1	159.2	15.1	-79.6	-63.7	18.1	6.6	9.8	9.0	8.0
6	225	13.2	159.2	16.1	-53.1	-53.1	17.9	6.6	10.2	8.9	8.2
6	240	14.6	159.2	18.1	-26.5	-53.1	18.5	6.7	10.7	8.6	8.4
7	0	9.9	8.4	10.0	18.5	15.5	12.7	10.4	10.2	79.6	14.2
7	15	11.9	11.5	10.2	14.8	13.9	12.1	10.6	9.5	8.2	13.1
7	30	10.7	9.3	9.2	12.9	11.6	11.8	9.9	9.0	7.1	12.1
7	45	-18.7	-12.7	8.3	11.1	11.1	9.7	9.2	8.8	8.0	10.8
7	60	-21.2	-10.3	8.0	10.3	11.2	9.3	9.1	8.4	9.7	11.7
7	75	9.6	6.9	7.8	10.0	11.3	9.1	8.3	8.1	8.5	11.4
7	90	9.1	-11.0	7.2	10.2	10.7	8.6	8.4	8.1	8.8	10.9
7	105	9.6	-12.2	7.2	10.2	10.7	8.4	8.9	-13.8	7.1	10.2
7	120	9.8	6.7	7.2	10.9	10.0	8.5	8.6	8.4	-9.1	9.7
7	135	-22.7	-17.7	7.6	-24.5	10.0	8.6	8.8	-12.2	8.7	9.2
7	150	-18.7	-21.2	7.8	-22.7	10.0	8.4	8.6	-15.2	9.4	8.9
7	165	-17.7	-21.2	-16.8	-21.2	9.9	-15.2	9.3	-13.3	9.4	9.0
7	180	-15.9	-19.9	-17.7	-21.2	9.2	-15.9	9.0	-11.8	8.8	8.5
7	195	-15.2	-17.7	-19.9	-22.7	10.7	-15.2	10.1	-14.5	10.0	8.5
7	210	-14.5	-17.7	-21.2	-24.5	10.9	-13.3	9.8	-13.3	9.4	8.4
7	225	-13.8	-17.7	-22.7	-24.5	11.1	-14.5	10.1	-8.4	9.6	8.3
7	240	-13.3	-15.9	-24.5	-26.5	12.2	-15.9	11.3	-9.7	9.3	8.2
8	0	13.7	17.6	13.9	20.7	14.6	11.2	-8.2	10.4	6.9	23.0
8	15	11.7	14.0	-6.9	14.6	11.3	-9.2	15.9	9.4	-45.5	18.7
8	30	10.2	12.7	-13.8	14.0	10.5	8.4	-12.7	8.6	10.6	16.5
8	45	10.6	13.4	-5.4	12.1	10.0	8.5	12.5	8.8	11.3	14.9
8	60	-8.0	13.6	7.1	-7.4	-5.9	-11.4	10.9	7.8	10.9	13.3
8	75	9.6	12.9	9.3	12.9	-5.1	-8.0	10.9	7.3	10.9	12.8
8	90	-10.6	10.2	7.3	-26.5	9.6	-7.4	8.4	7.3	9.7	11.3
8	105	-5.5	10.3	6.6	-7.8	-6.2	-9.4	8.8	6.9	8.8	10.5
8	120	9.1	11.1	6.6	-6.4	-5.0	-11.0	9.6	-10.6	-0.1	9.7
8	135	10.0	12.5	7.0	-6.4	7.3	-7.1	10.1	-9.7	-19.9	9.2
8	150	11.9	14.5	7.4	-6.6	7.1	-6.5	9.5	-8.4	-15.2	9.0
8	165	13.6	16.9	8.0	-6.8	6.8	-6.1	8.5	-8.0	-10.0	8.7
8	180	15.1	19.6	8.8	-7.1	7.1	-5.7	9.3	-8.0	-9.1	8.4
8	195	16.8	22.3	9.2	-7.8	7.1	-5.9	8.9	-7.2	-8.2	.

8	210	18.2	30.3	10.2	-8.0	7.4	-6.1	9.2	-6.8	-7.1	.
8	225	-8.4	34.3	11.8	-8.6	7.7	-6.2	9.7	-6.8	-6.9	.
8	240	-8.6	38.1	13.5	-8.8	7.6	-6.4	9.7	-6.8	-6.4	.
9	0	16.5	16.8	11.1	16.1	-8.6	26.8	16.9	.	8.9	16.4
9	15	14.7	12.5	10.5	11.5	12.8	63.7	15.8	.	8.7	16.3
9	30	12.9	10.3	10.2	10.2	10.9	-16.8	13.7	.	8.2	13.9
9	45	-16.8	10.1	9.2	9.3	10.8	-9.4	13.0	.	7.7	12.9
9	60	-13.3	10.0	-16.8	8.6	10.8	11.8	10.7	.	7.5	12.4
9	75	-19.9	11.1	7.8	8.1	11.0	11.9	10.1	.	7.3	12.0
9	90	-39.8	10.1	9.4	8.8	11.2	-7.4	9.8	.	7.4	11.9
9	105	-35.4	10.2	8.5	9.2	11.4	-8.4	9.6	.	7.3	11.4
9	120	-31.8	11.1	-14.5	8.5	11.6	-8.0	9.7	.	7.4	11.5
9	135	-21.2	12.7	-14.5	8.1	11.9	-7.8	9.8	.	7.5	11.5
9	150	-24.5	-79.6	-15.9	-22.7	12.4	-7.6	9.8	.	7.7	11.1
9	165	-26.5	-79.6	-17.7	-26.5	12.7	-7.8	10.0	.	7.8	11.0
9	180	-24.5	-63.7	-21.2	-31.8	13.1	-8.0	10.9	8.1	8.3	11.8
9	195	-24.5	-63.7	-21.2	-35.4	13.4	-8.2	11.7	8.2	8.8	11.4
9	210	-19.9	-79.6	-24.5	10.7	13.9	-8.4	12.7	8.3	9.4	12.4
9	225	-19.9	-53.1	-29.0	11.3	-14.1	-8.6	13.3	8.3	9.7	-53.1
9	240	-21.2	-53.1	-35.4	-45.5	14.7	-8.8	14.5	8.5	10.3	-53.1
10	0	12.7	24.8	6.8	20.5	15.5	13.0	25.6	14.2	12.8	13.3
10	15	12.5	14.3	13.0	-9.7	15.1	9.7	13.6	12.5	10.9	11.7
10	30	11.0	12.4	9.7	-7.6	14.2	9.5	12.2	12.4	10.9	11.4
10	45	11.3	10.3	12.0	10.8	11.0	9.2	10.1	10.5	10.9	11.0
10	60	10.7	13.4	7.6	11.6	10.5	9.7	9.4	10.0	10.4	10.9
10	75	10.0	9.8	10.2	12.4	11.0	9.8	10.4	10.7	10.8	10.9
10	90	9.2	9.3	8.3	12.5	11.1	9.3	10.8	10.8	10.7	10.3
10	105	9.5	9.8	7.5	12.1	10.6	9.0	9.9	10.4	10.6	10.7
10	120	10.8	11.2	7.8	11.8	11.6	8.6	8.9	11.8	11.1	10.8
10	135	11.9	12.3	9.1	11.9	11.1	8.7	10.4	11.1	10.9	-26.5
10	150	13.5	13.3	8.5	11.9	11.7	8.7	10.3	11.0	10.8	-24.5
10	165	15.9	14.9	11.4	12.3	11.4	9.0	10.3	11.9	-31.8	-24.5
10	180	18.9	16.5	7.7	13.4	12.1	9.2	9.8	12.2	-26.5	-19.9
10	195	20.9	106.2	16.9	15.1	-45.5	9.6	10.4	13.0	-26.5	-19.9
10	210	25.3	159.2	19.9	17.6	-53.1	10.1	10.7	10.8	-29.0	-19.9
10	225	29.7	.	25.3	20.8	-53.1	10.5	10.7	11.6	-31.8	-21.2
10	240	33.5	.	30.7	22.8	-63.7	11.1	9.6	11.4	-35.4	-15.2

APPENDIX 2. MANNITOL FLUX OBJECTIVE 1.

Mucosal to serosal flux (μmol/cm^2*h) of mannitol for concentration dependent effects of carprofen on the mucosa of the descending colon. Time period[a] 1 = 60 to 120 minutes, 2 = 120 to 180 minutes, 3 = 180-240 minutes. Treatments are in micrograms of carprofen per milliliter of solvent (μg/ml).

Dog	Period[a]	Treatment									
		400	400	200	200	100	100	40	40	0	0
1	1	.	.	0.15	0.11	0.13	.	.	0.06	0.08	0.07
1	2	0.18	0.21	0.27	0.16	0.14	0.15	0.09	0.06	0.07	0.06
1	3	0.29	0.37	0.38	0.19	0.14	0.09	0.94	0.05	0.07	0.07
2	1	0.14	0.14	0.12	0.15	0.12	0.24	0.45	0.19	0.10	0.16
2	2	0.14	0.15	0.16	0.12	0.14	0.03	-0.10	0.14	0.09	0.13
2	3	0.16	0.16	0.16	0.25	0.15	0.27	0.19	0.13	0.07	0.07
3	1	0.13	0.13	0.08	0.06	0.06	0.08	0.08	0.07	0.08	0.07
3	2	0.12	0.08	0.06	0.06	0.06	0.07	0.06	0.06	0.05	0.05
3	3	0.20	0.16	0.06	0.07	0.07	0.08	0.06	0.05	0.04	0.06
4	1	0.00	0.10	0.07	0.11	0.09	0.09	0.09	0.05	0.07	0.00
4	2	0.00	0.13	0.06	0.17	0.07	0.12	0.11	0.06	0.08	0.00
4	3	0.00	0.14	0.06	0.15	0.08	0.13	0.14	0.06	0.07	0.00
5	1	0.09	0.09	0.10	0.07	0.09	0.11	0.06	0.11	0.07	0.06
5	2	0.08	0.08	0.08	0.06	0.12	0.11	0.05	0.13	0.10	0.05
5	3	0.15	0.16	0.12	0.09	0.10	0.10	0.06	0.17	0.11	0.05
6	1	0.06	0.10	0.07	0.09	0.10	0.08	0.13	0.08	0.08	0.12
6	2	0.08	0.16	0.12	0.14	0.14	0.11	0.13	0.09	0.14	0.13
6	3	0.10	0.16	0.16	0.16	0.19	0.13	0.15	0.09	0.14	0.09
7	1	0.12	0.09	0.16	0.09	0.09	0.11	0.07	0.10	0.09	0.13
7	2	0.16	0.12	0.25	0.12	0.14	0.15	0.09	0.13	0.11	0.16
7	3	0.24	0.17	0.35	0.15	0.14	0.16	0.10	0.14	0.06	0.15
8	1	0.09	0.06	0.06	0.09	0.05	0.07	0.07	0.06	0.07	0.08
8	2	0.12	0.09	0.07	0.09	0.06	0.10	0.07	0.06	0.08	0.09
8	3	0.22	0.13	0.08	0.13	0.06	0.08	0.09	0.07	0.08	.
9	1	0.10	0.08	0.05	0.05	0.08	0.10	0.08	0.04	0.08	0.09
9	2	0.13	0.08	0.07	0.07	0.09	0.12	0.07	0.06	0.06	0.09
9	3	0.20	0.14	0.09	0.07	0.11	0.12	0.08	0.06	0.07	0.09
10	1	0.09	0.08	0.08	0.07	0.08	0.05	0.06	0.08	0.05	0.07
10	2	0.10	0.07	0.10	0.08	0.09	0.06	0.06	0.10	0.05	0.07
10	3	0.24	0.16	0.15	0.10	0.13	0.11	0.06	0.11	0.06	0.08

APPENDIX 3. HISTOLOGIC EXAMINATION OBJECTIVE 1

Histologic findings for sections of colonic mucosa after exposure to various concentrations of carprofen in an Ussing chamber. Treatments are in micrograms of carprofen per milliliter of solvent (μg/ml). [a]Inflammation, edema, and sloughing of cells from the surface epithelium were graded as follows: 1 = absent or minimal; 2 = mild, 3 = moderate, 4 = severe. [b]Erosion of the surface epithelium was graded as: 1 = absent, 2 = mild, 3 = severe. [c]Sloughing of epithelial cells within the mucosal glands was graded as: 1 = absent, 2 = minimal, 3 = mild, 4 = moderate, 5 = severe.

Treatment	Inflammation [a]	Edema [a]	Sloughing surface epithelial cells [a]	Erosions [b]	Sloughing mucosal gland epithelial cells [c]	Comments
Dog 1						
400	1	1	4	2	5	
400	1	1	4	2	5	
200	1	1	4	1	3	
200	1	2	4	2	4	
100	1	1	3	1	1	
100	1	2	3	2	1	scattered neutrophils and eosinophils
40	1	2	2	1	2	
40	1	1	3	1	1	
0	1	2	2	1	1	
0	1	1	4	1	1	
Dog 2						
pre	3	1	1	1	1	lymphoplasmacytic inflammation with many eosinophils
400	1	1	4	2	5	tattered looking surface epithelium
400	1	2	3	2	4	attenuated surface epithilium with tattered appearance
200	1	1	4	2	5	
200	2	2	4	2	5	lymphoplasmacytic inflammation with scattered eosinophils
100	1	1	4	1	4	thick layer of surface sloughing; mucus
100	1	1	4	1	3	mucus
40	2	2	3	1	4	lymphoplasmacytic inflammation with scattered eosinophils and neutrophils
40	1	2	2	2	4	
0	1	1	4	1	1	
0	1	2	4	2	2	
Dog 3						
pre	1	1	1	1	1	

400	1	2	4	3	1	mucus
400	1	2	4	3	3	
200	1	1	4	2	4	
200	1	2	2	2	5	scattered eosinophils
100	1	2	4	2	2	areas of flattened and attenuated epithelial cells
100	1	2	2	1	1	
40	1	3	4	1	1	
40	1	1	4	1	2	
0	1	2	1	1	1	
0	1	1	1	1	1	areas of flattened and attenuated epithelial cells
Dog 4						
pre	1	1	1	1	1	mucus
400	1	2	4	1	3	
400	1	1	4	3	2	
200	1	2	4	2	2	
200	1	2	4	1	3	some areas of attenuated surface epithelium
100	1	3	4	1	2	
100	1	1	4	1	2	
40	1	1	2	2	2	
40	1	2	4	2	2	inflammation in pyres patch
0	1	2	1	1	1	
0	1	2	4	1	1	mucus
Dog 5						
pre	2	1	2	1	1	lymphoplasmacytic inflammation with scattered neutrophils; tattered looking surface epithelium; some artifactual separation of surface epithelium
400	3	1	4	3	5	lymphoplasmacytic and neutrophilic inflammation
400	3	1	4	2	5	lymphoplasmacytic and neutrophilic inflammation within pyres patch; small sample
200	1	2	4	2	5	swollen surface enterocytes
200	2	2	4	1	5	lymphoplasmacytic inflammation with scattered neutrophils and eosinophils
100	1	2	4	1	4	
100	2	1	1	1	4	inflammation in pyres patch; small sample
40	1	3	1	1	2	
40	2	3	1	1	3	lymphoplasmacytic and eosinophilic inflammation
0	2	1	1	1	4	plasmacytic inflammation with scattered eosinophils
0	2	1	1	1	5	lymphoplasmacytic inflammation with scattered

						eosinophils
Dog 6						
pre	1	1	2	1	2	
400	1	1	4	.	4	artifactual separation of surface epithelium, unable to evaluate for erosions; small section
400	1	1	4	3	2	separation of surface epithelium
200	1	2	3	1	1	
200	1	1	4	2	1	some separation of surface epithelium
100	1	1	4	2	1	
100	1	2	4	2	1	
40	1	2	4	2	2	
40	1	2	4	1	3	
0	1	1	4	1	2	
0	1	2	4	1	1	small sample
Dog 7						
pre	1	1	4	1	1	
400	1	1	4	2	5	
400	1	1	4	2	3	
200	1	2	4	1	1	scattered neutrophils and inflammation in a pyres patch at the edge of the sample; small sample
200	1	2	4	2	2	small sample
100	1	2	4	2	2	
100	2	2	4	2	3	neutrophilic pyres patch lymphadenitis and overlying focal moderate inflammation
40	1	2	4	2	3	
40	1	1	4	2	3	
0	1	2	4	2	1	
0	1	1	3	1	1	small foci of neutrophilic inflammation in superficial mucosa
Dog 8						
pre	2	1	1	2	1	scattered lymphoplasmacytic inflammation; mucus
400	2	1	4	2	5	lymphoplasmacytic inflammation with scattered eosinophils
400	1	1	4	1	4	artifactual lifting of surface epithelium
200	1	1	1	2	4	
200	1	2	4	2	4	
100	1	1	2	1	2	mucus
100	1	1	3	1	2	
40	1	2	3	1	2	
40	1	2	2	2	2	small sample
0	1	1	4	1	2	sample artifact; surface

						sloughing primarily on one side
0	1	2	3	1	1	
Dog 9						
pre	1	1	1	1	1	mucus
400	1	1	4	2	5	separation of surface epithelium
400	2	1	4	2	5	lymphoplasmacytic inflammation with scattered eosinophils
200	1	2	4	1	3	
200	1	1	4	2	2	
100	1	3	4	2	3	
100	2	2	4	1	5	focal crypt abscesses and focal neutrophilic and eosinophilic inflammation; inflammation within pyres patch
40	1	2	4	2	2	
40	1	1	4	1	3	
0	1	1	4	1	5	
0	1	2	3	2	1	
Dog 10						
pre	1	1	1	1	1	
400	1	1	4	2	3	
400	1	2	4	3	1	small section; ballooning and isolation of surface epithelial cells
200	1	1	4	2	2	scattered neutrophils
200	1	2	4	1	1	
100	1	2	4	1	1	
100	1	1	4	1	1	
40	1	2	4	1	2	
40	1	2	3	1	1	
0	1	3	3	1	1	
0	1	1	2	1	1	

APPENDIX 4. ELECTRICAL CONDUCTANCE OBJECTIVE 2

Electrical conductance (mS/cm^2) for the effects of carprofen and dinitrophenol on the mucosa of the descending colon. Time is in minutes. Treatments are 1) carprofen (200 μg/ml), 2) carprofen (200 μg/ml) pretreated with tempol (1 mM), 3) dinitrophenol (0.25 mM), 4) dinitrophenol (0.25 mM) pretreated with tempol (1 mM), and 5) no drug.

Dog	Time	Treatment									
		1	1	2	2	3	3	4	4	5	5
1	0	-9.1	15.4	14.8	16.6	-31.8	19.8	15.1	10.4	-159.2	19.3
1	15	-7.8	14.4	11.7	14.9	15.0	-15.9	11.9	8.1	13.5	11.3
1	30	-7.8	9.7	10.7	14.4	12.2	-17.7	11.1	7.8	-21.2	9.0
1	45	13.7	9.3	10.4	14.8	22.5	21.2	18.2	13.8	14.7	15.6
1	60	11.6	10.6	9.2	14.3	19.5	-8.2	14.0	10.5	11.2	12.6
1	75	13.2	10.3	10.1	13.3	.	-7.8	12.8	10.7	11.7	12.3
1	90	13.9	9.5	-12.7	-16.8	-35.4	15.5	11.9	-19.9	11.6	0.1
1	105	13.3	9.0	-9.7	-13.3	-24.5	15.3	12.2	-14.5	11.4	13.5
1	120	12.3	9.2	-9.1	-13.8	-21.2	15.8	12.4	-13.3	11.7	12.6
1	135	12.5	10.1	-9.1	-13.8	-22.7	16.7	12.5	-12.2	11.5	9.9
1	150	12.8	11.8	-9.7	-18.7	-24.5	-5.9	13.7	-11.8	11.4	11.7
1	165	13.8	15.3	-10.6	-24.5	-19.9	20.8	11.7	-11.8	10.4	-29.0
1	180	15.2	20.6	-12.7	-29.0	-26.5	23.2	7.3	-11.8	10.7	-11.8
1	195	17.2	26.5	-14.5	-35.4	-29.0	24.8	17.8	-11.8	11.5	-8.8
1	210	20.2	32.7	-18.7	-63.7	-26.5	26.8	20.0	-12.2	11.6	-8.2
1	225	25.0	40.5	-22.7	-106.2	-35.4	30.0	22.7	-12.2	-35.4	-7.8
1	240	29.5	48.1	-26.5	-318.5	-35.4	32.0	23.4	-13.3	-63.7	15.7
2	0	20.4	21.1	12.5	.	17.9	65.3	15.1	17.4	14.4	17.0
2	15	18.4	18.7	14.1	.	19.3	13.6	12.8	15.6	18.3	13.9
2	30	14.7	14.1	11.5	.	14.7	11.6	11.0	11.8	15.5	12.4
2	45	-9.7	-9.7	9.0	.	18.3	15.2	14.2	16.3	13.8	11.5
2	60	8.9	8.6	8.5	.	12.3	9.6	10.2	12.7	12.4	10.9
2	75	-8.4	-11.0	8.0	.	11.5	10.0	10.7	16.4	12.5	11.1
2	90	7.2	-5.1	7.3	.	11.3	10.8	11.9	18.3	13.0	10.7
2	105	7.7	-5.1	7.2	.	11.4	11.6	13.2	106.2	12.9	10.1
2	120	8.0	-5.1	7.3	.	11.6	12.2	-106.2	.	13.6	9.8
2	135	-5.4	-6.2	7.4	.	12.7	13.1	-31.8	-159.2	12.2	10.0
2	150	-6.0	-7.6	7.5	.	13.5	14.2	-39.8	-318.5	-24.5	9.8
2	165	-7.8	-12.7	7.9	.	14.9	15.7	-31.8	-318.5	-11.4	-12.7
2	180	-10.0	-22.7	8.4	.	15.9	17.1	-31.8	.	-8.0	-8.0
2	195	-12.7	-31.8	9.2	.	17.5	18.5	-29.0	318.5	14.2	-6.4
2	210	-15.9	-79.6	10.4	.	19.1	20.1	-31.8	159.2	12.9	10.4
2	225	-18.7	-63.7	11.4	.	20.6	22.0	-31.8	159.2	13.2	10.0
2	240	-26.5	-159.2	12.4	.	53.1	23.4	-31.8	106.2	12.0	9.9
3	0	9.3	13.7	11.9	13.4	17.1	11.3	14.5	10.7	11.6	16.1
3	15	9.8	13.2	6.7	10.9	8.1	9.8	13.0	-106.2	13.6	15.5
3	30	9.9	11.4	11.0	8.4	23.2	17.2	10.8	12.1	13.0	14.7
3	45	6.6	7.6	9.6	8.2	20.0	18.5	13.6	15.5	0.2	14.3

3	60	6.6	7.3	-18.7	8.1	18.4	15.2	10.3	9.2	13.1	13.3
3	75	7.2	7.4	10.1	8.1	10.4	13.6	9.0	9.2	12.4	12.4
3	90	6.4	-11.8	-1.3	7.3	24.6	12.9	8.0	9.5	12.3	11.9
3	105	5.8	-10.3	-7.4	6.9	20.0	12.1	7.5	9.6	12.3	11.8
3	120	5.7	-10.3	-6.2	-10.6	8.3	11.5	7.4	9.5	12.5	30.9
3	135	5.6	-11.4	-5.9	-10.6	8.2	11.7	7.2	9.5	12.1	11.3
3	150	5.8	-13.3	-5.8	-11.0	8.9	11.4	7.0	9.9	12.4	11.3
3	165	6.0	-13.8	7.0	-11.4	5.9	9.3	7.4	7.8	9.7	11.2
3	180	6.2	-14.5	70.9	-11.4	5.5	8.0	7.3	7.0	7.2	11.7
3	195	6.2	-15.2	7.5	-10.6	5.3	7.8	7.5	6.8	6.6	11.7
3	210	6.2	-15.2	9.3	-11.0	5.2	8.3	7.8	8.4	8.4	11.5
3	225	6.5	-17.7	11.1	-10.6	8.1	11.8	7.8	11.2	10.9	11.9
3	240	6.9	-19.9	14.5	-10.3	9.0	14.1	8.9	14.5	12.8	11.4
4	0	-39.8	14.6	-13.3	14.1	-45.5	-10.3	-11.4	28.7	106.2	25.1
4	15	17.8	12.8	12.8	-31.8	15.1	-15.9	17.9	17.9	15.3	-24.5
4	30	13.1	11.4	9.4	-16.5	11.6	8.9	-17.7	12.6	12.8	-79.6
4	45	-11.4	8.3	6.2	.	12.9	10.4	10.7	15.0	9.7	11.5
4	60	-5.9	-12.2	-5.9	-7.6	7.8	-15.2	8.5	12.3	7.5	10.4
4	75	-4.4	64.7	-4.5	-14.5	6.2	-7.2	7.1	10.3	6.7	9.3
4	90	4.0	6.9	-4.0	-8.2	5.7	-5.6	6.6	9.1	6.4	9.1
4	105	-4.0	6.0	-5.4	-6.1	5.4	-5.0	5.8	8.3	6.4	9.9
4	120	-4.4	5.5	5.1	-5.5	5.1	-4.6	-8.0	8.4	6.1	8.6
4	135	7.4	5.6	7.8	-5.4	7.1	-4.4	-11.8	-18.7	10.1	9.3
4	150	-8.0	5.8	5.1	-5.5	8.1	5.5	-9.1	-17.7	9.7	-13.8
4	165	-8.0	5.8	4.9	-5.7	8.2	6.1	-8.6	-19.9	9.6	-9.1
4	180	-7.8	5.8	5.0	-6.5	8.1	6.4	-8.6	-21.2	9.3	7.2
4	195	-4.6	-11.0	-4.2	-6.0	6.9	6.6	-7.2	-22.7	7.8	7.7
4	210	-4.2	-11.8	-3.9	-6.0	5.4	6.8	-5.6	-21.2	6.2	7.8
4	225	-4.8	-13.3	-4.1	-6.6	4.9	7.9	-5.1	-26.5	5.1	7.0
4	240	-4.9	-13.8	-4.4	-6.8	4.7	8.9	-5.0	-39.8	5.3	6.7
5	0	32.9	12.9	19.7	17.8	-318.5	17.8	15.8	318.5	15.2	17.7
5	15	26.6	12.2	14.5	15.9	-35.4	13.5	12.0	.	12.5	13.3
5	30	30.0	11.2	11.3	.	-106.2	17.6	8.9	-53.1	10.7	12.2
5	45	-26.5	6.4	8.1	11.7	25.4	17.9	14.8	19.1	10.1	12.5
5	60	-63.7	8.7	11.7	14.1	19.9	17.0	12.1	12.1	9.2	14.0
5	75	-6.9	6.4	8.2	10.2	18.0	16.4	10.0	11.8	7.8	12.3
5	90	-6.1	6.0	-9.1	9.4	-318.5	15.9	-12.2	-10.8	8.0	11.0
5	105	-6.1	5.9	-8.2	9.3	-31.8	15.3	-10.6	-63.7	7.9	9.9
5	120	-6.2	6.1	-8.0	9.2	-21.2	14.9	-10.0	-31.8	7.3	9.6
5	135	6.7	6.1	-8.4	8.1	-12.7	13.1	-10.0	-13.8	7.3	9.1
5	150	-6.4	6.2	-8.2	7.5	-8.2	9.7	-10.0	-8.6	7.2	9.2
5	165	6.4	6.6	-8.8	7.5	-6.9	9.3	-9.7	-6.8	6.6	8.2
5	180	-6.2	7.2	-9.7	10.0	-10.6	13.2	-10.3	-8.8	6.9	8.1
5	195	-7.2	8.3	-11.0	10.4	-12.2	17.3	-10.6	-9.4	6.5	7.8
5	210	-8.2	9.7	-12.7	12.7	-12.2	19.6	-11.4	-9.7	6.8	7.3
5	225	-9.7	11.6	-15.2	16.7	-12.7	22.0	-11.4	-10.3	6.4	7.2
5	240	-7.6	13.7	-15.9	16.8	-9.1	16.2	-11.8	-14.5	6.1	6.9

6	0	15.9	18.8	13.9	15.3	10.1	318.5	-11.8	16.1	13.1	14.6
6	15	8.2	8.7	10.8	11.8	8.8	14.3	12.1	10.7	8.6	9.7
6	30	11.3	6.9	8.4	9.1	7.2	11.6	-24.5	8.0	7.0	8.2
6	45	8.7	5.6	8.4	10.6	15.3	15.8	19.5	16.6	6.6	7.6
6	60	8.6	4.9	6.1	7.5	10.0	10.3	18.8	11.4	6.7	7.9
6	75	10.6	6.6	7.2	6.9	9.3	9.1	13.4	8.6	6.6	8.2
6	90	10.4	4.5	5.5	7.1	9.3	8.9	11.8	7.8	6.6	7.7
6	105	5.9	5.3	5.2	5.5	9.0	8.0	11.1	7.1	6.5	7.6
6	120	8.5	5.4	5.7	4.9	-8.8	-13.8	10.0	6.9	6.6	7.6
6	135	12.7	6.2	7.2	5.4	9.0	-11.8	9.7	6.7	6.6	7.1
6	150	15.9	7.8	9.1	5.3	9.4	-11.8	9.4	6.6	6.5	6.8
6	165	18.3	-15.2	12.3	5.5	10.1	-10.3	9.4	6.6	6.5	6.5
6	180	20.1	-10.0	16.3	6.0	11.3	-9.7	10.1	6.9	6.4	6.1
6	195	20.6	17.7	20.1	7.1	12.8	-9.1	10.9	7.2	6.6	5.5
6	210	33.5	21.3	22.9	8.5	14.4	-8.8	12.4	7.7	6.6	5.3
6	225	24.4	21.0	25.7	11.3	16.5	-8.6	14.1	8.4	6.5	5.2
6	240	22.1	22.9	27.2	11.9	18.8	-8.2	16.0	9.6	6.8	4.9
7	0	-11.2	-10.5	-9.9	-12.2	-11.4	-11.9	-9.9	-15.1	-13.1	-20.2
7	15	-7.4	-21.0	-9.0	-9.5	-9.6	-8.8	-7.5	-11.4	-10.1	-10.5
7	30	-7.1	-7.9	-6.3	-6.6	-9.8	-7.9	-7.0	-9.1	-9.1	-8.6
7	45	-6.0	-6.6	-5.7	-6.9	-11.3	-10.2	-9.1	-11.1	-9.1	-8.3
7	60	-5.9	-5.7	-5.6	-5.9	-7.8	-6.1	-10.3	-9.7	-8.8	-7.7
7	75	-6.2	-6.1	-5.6	-6.7	-5.8	-5.4	-18.7	-8.8	-8.5	-7.7
7	90	-5.5	-5.2	-4.7	-5.5	-6.0	-5.0	-106.2	-8.9	-8.0	-8.0
7	105	-5.1	-4.6	-4.8	-5.1	-6.1	-5.2	-14.5	-9.0	-7.6	-8.0
7	120	-5.1	-4.5	-5.0	-5.0	-6.3	-5.2	-14.5	-10.0	-7.1	-8.2
7	135	-5.1	-4.7	-5.2	-5.1	-6.7	-5.2	-10.6	-10.6	-6.9	-7.7
7	150	-5.3	-4.9	-5.5	-5.2	-6.9	-5.6	-14.5	-11.4	-6.5	-8.2
7	165	-5.6	-5.1	-6.4	-5.3	-6.9	-5.9	-11.8	-11.8	-6.2	-7.8
7	180	-5.6	-5.6	-6.0	-5.5	-6.9	-6.0	-24.5	-12.2	-6.4	-7.7
7	195	-5.9	-5.2	-5.9	-5.6	-7.1	-6.6	-13.3	-12.7	-6.2	-6.8
7	210	-5.8	-5.5	-6.2	-5.9	-7.5	-6.8	.	-13.8	-6.0	-6.9
7	225	-6.0	-5.4	-6.7	-6.1	-7.8	-8.0	18.7	-14.5	-6.4	-6.4
7	240	-6.2	-5.6	-4.3	-7.0	-8.2	-8.5	-39.8	-15.2	-6.0	-6.4
8	0	7.8	11.6	9.9	8.8	10.4	8.5	10.1	.	9.2	10.0
8	15	8.0	9.6	9.1	8.2	9.0	7.7	7.3	.	8.8	8.9
8	30	8.0	8.8	8.4	7.3	9.2	7.5	6.6	.	8.3	8.2
8	45	5.6	-5.6	6.4	7.3	12.1	11.2	9.2	.	7.9	7.8
8	60	5.6	4.8	7.4	7.3	7.8	7.5	9.9	.	7.5	7.8
8	75	6.2	5.0	6.4	8.0	7.4	7.1	11.2	.	7.3	7.9
8	90	5.3	4.5	-5.9	6.9	7.3	7.2	11.2	.	7.1	7.6
8	105	4.8	4.5	-4.6	6.4	7.2	7.6	11.7	.	7.0	7.8
8	120	4.8	4.5	4.8	7.1	7.3	7.5	10.2	.	6.7	7.6
8	135	4.7	4.8	-4.0	7.2	7.8	7.9	10.4	.	0.7	7.8
8	150	5.1	4.8	-3.9	-11.8	8.4	8.7	-24.5	.	7.1	7.3
8	165	5.2	5.1	5.6	-13.8	9.6	10.0	-24.5	.	7.0	7.4
8	180	5.4	5.3	6.6	-12.7	11.4	11.6	-18.7	.	7.0	7.3

8	195	5.8	5.9	7.4	-15.2	14.5	14.0	-22.7	.	7.3	6.7
8	210	-8.6	6.6	8.0	-18.7	-18.6	15.7	-22.7	.	7.0	-11.8
8	225	-9.7	7.3	9.2	-16.8	23.4	20.0	-22.7	.	7.0	-9.1
8	240	-9.7	8.9	10.1	-17.7	28.9	22.0	-24.5	.	7.0	-8.4
9	0	20.2	14.9	18.3	17.8	17.1	12.5	14.6	20.9	15.0	17.8
9	15	13.5	12.3	11.1	13.9	11.5	11.4	11.2	14.9	12.4	13.2
9	30	11.0	11.3	8.6	9.4	11.0	10.0	7.9	10.4	11.0	11.2
9	45	6.4	6.8	6.9	10.4	16.7	14.4	14.5	15.6	9.8	9.6
9	60	-6.2	6.4	5.8	8.1	10.4	10.4	11.1	13.2	9.2	9.5
9	75	6.2	7.0	6.6	7.3	9.6	9.1	9.6	13.3	8.9	9.1
9	90	-5.2	6.4	7.4	8.7	-22.7	8.8	-16.8	14.1	9.0	8.8
9	105	-4.5	5.8	6.2	7.5	-10.6	8.5	-13.8	14.5	8.1	8.2
9	120	-4.6	-8.2	-9.1	7.0	-10.3	8.3	-18.7	15.3	8.2	8.2
9	135	-4.6	-8.4	-9.4	7.2	-7.1	8.4	-13.3	17.5	8.1	7.8
9	150	-4.8	-8.8	-9.4	7.5	-8.0	8.7	-21.2	20.5	8.0	8.2
9	165	-5.1	-9.1	5.8	7.6	10.6	9.0	-15.2	24.5	7.9	7.3
9	180	-5.7	-9.1	5.8	8.3	10.7	8.9	-21.2	29.1	7.7	7.4
9	195	-6.5	-11.0	6.4	9.4	12.1	9.4	-15.9	34.9	7.5	6.9
9	210	-7.1	-10.0	6.9	10.5	12.9	9.8	-17.7	42.2	8.5	6.5
9	225	-8.8	-9.7	7.5	12.0	14.2	10.4	-53.1	50.1	8.3	6.4
9	240	-9.7	-10.3	-22.7	13.6	16.1	11.5	-21.2	59.4	8.3	6.4
10	0	13.0	12.6	14.7	12.3	10.8	14.0	9.4	10.1	14.6	20.1
10	15	11.5	12.6	13.6	11.1	10.2	13.7	9.6	8.9	14.1	17.2
10	30	11.5	11.7	11.1	9.5	10.0	12.0	8.4	7.1	12.7	15.0
10	45	7.0	7.0	12.3	8.1	13.2	15.9	13.7	13.2	12.4	13.9
10	60	7.6	7.2	8.3	6.6	11.0	10.8	11.6	7.5	11.7	12.6
10	75	9.3	8.5	7.7	8.2	9.8	9.1	11.7	6.8	10.9	12.0
10	90	6.3	5.8	8.2	5.5	9.9	8.9	12.5	6.1	10.7	10.5
10	105	5.8	5.3	6.7	5.1	9.8	8.5	13.0	5.7	9.6	10.0
10	120	5.7	5.5	6.2	5.1	9.7	8.4	-63.7	-7.6	9.1	9.1
10	135	5.7	5.5	6.2	5.1	9.4	8.0	-53.1	-5.4	8.6	8.6
10	150	5.6	5.6	6.2	5.3	9.6	8.2	-31.8	-6.0	7.3	8.0
10	165	5.6	5.5	6.4	5.5	9.7	8.2	-35.4	-6.6	7.1	7.6
10	180	5.8	6.1	6.9	5.8	9.6	8.3	-35.4	-6.5	7.1	7.2
10	195	6.3	6.4	7.2	6.2	10.0	8.5	-39.8	-7.2	6.4	6.8
10	210	6.7	7.0	8.2	6.6	10.4	9.0	-45.5	-7.6	6.4	6.5
10	225	7.0	7.5	8.5	7.2	-31.8	9.4	-45.5	-8.0	6.2	6.4
10	240	7.9	8.0	10.1	7.8	-22.7	10.0	-63.7	-8.6	6.3	6.2

APPENDIX 5. MANNITOL FLUX OBJECTIVE 2.

Mucosal to serosal flux (μmol/cm^2*h) of mannitol for concentration dependent effects of carprofen on the mucosa of the descending colon. Time period[a] 1 = 60 to 120 minutes, 2 = 120 to 180 minutes, 3 = 180-240 minutes. Treatments are 1) carprofen (200 μg/ml), 2) carprofen (200 μg/ml) pretreated with tempol (1 mM), 3) dinitrophenol (0.25 mM), 4) dinitrophenol (0.25 mM) pretreated with tempol (1 mM), and 5) no drug.

Dog	Period[a]	Treatment									
		1	1	2	2	3	3	4	4	5	5
1	1	0.13	0.08	0.06	0.09	0.15	0.13	0.08	0.12	0.10	0.07
1	2	0.10	0.09	0.07	0.14	0.31	0.23	0.16	0.16	0.05	0.05
1	3	0.07	0.20	0.07	27.66	0.39	0.32	0.23	0.22	0.02	0.06
2	1	0.07	0.07	0.11	.	0.07	0.11	0.08	0.18	0.05	0.01
2	2	0.15	0.08	0.13	.	0.14	0.18	0.03	0.30	0.06	0.02
2	3	0.22	0.16	0.23	.	0.28	0.30	0.39	0.41	0.07	0.03
3	1	0.07	0.07	0.05	0.06	0.07	0.11	0.09	0.05	0.04	0.05
3	2	0.08	0.08	0.12	0.07	0.07	0.09	0.07	0.05	0.06	0.05
3	3	0.08	0.11	0.08	0.08	0.08	0.09	0.10	0.06	0.06	0.05
4	1	0.08	0.07	0.06	0.07	0.06	0.08	0.05	0.08	0.05	0.07
4	2	0.07	0.06	0.07	0.06	0.07	0.08	0.06	0.09	0.06	0.06
4	3	0.06	0.07	0.06	0.09	0.06	0.14	0.06	0.13	0.04	0.05
5	1	0.11	0.07	0.10	0.08	0.18	0.20	0.10	0.16	0.06	0.05
5	2	0.06	0.07	0.07	0.07	0.09	0.17	0.17	0.09	0.06	0.06
5	3	0.07	0.09	0.11	0.09	0.07	0.21	0.19	0.11	0.05	0.05
6	1	0.06	0.09	0.08	0.08	0.11	0.10	0.15	0.13	0.06	0.08
6	2	0.09	0.15	0.10	0.07	0.12	0.10	0.12	0.09	0.06	0.06
6	3	0.18	0.33	0.23	0.08	0.20	0.11	0.13	0.12	0.06	0.06
7	1	0.04	0.04	0.04	0.05	0.05	0.06	0.09	0.08	0.07	0.05
7	2	0.05	0.04	0.04	0.04	0.06	0.06	0.13	0.12	0.05	0.04
7	3	0.05	0.05	0.06	0.06	0.08	0.09	0.16	0.15	0.06	0.04
8	1	0.08	0.07	0.04	0.04	0.05	0.05	0.08	.	0.04	0.04
8	2	0.07	0.07	0.05	0.06	0.10	0.08	0.12	.	0.04	0.04
8	3	0.09	0.11	0.07	0.10	0.18	0.19	0.20	.	0.04	0.04
9	1	0.05	0.05	0.06	0.07	0.07	0.06	0.08	0.09	0.05	0.05
9	2	0.04	0.05	0.07	0.08	0.11	0.09	0.13	0.16	0.04	0.05
9	3	0.05	0.07	0.06	0.09	0.13	0.11	0.26	0.30	0.04	0.06
10	1	0.06	0.07	0.07	0.05	0.07	0.06	0.11	0.08	0.08	0.09
10	2	0.05	0.06	0.06	0.05	0.08	0.07	0.14	0.07	0.09	0.06
10	3	0.06	0.06	0.06	0.06	0.06	0.08	0.20	0.11	0.03	0.05

APPENDIX 6. HISTOLOGIC EXAMINATION OBJECTIVE 2

Histologic findings for sections of colonic mucosa after exposure to carprofen or 2,4 dinitrophenol with or without Tempol pretreatment in an Ussing chamber. Treatments are 1) carprofen (200 μg/ml), 2) carprofen (200 μg/ml) pretreated with tempol (1 mM), 3) dinitrophenol (0.25 mM), 4) dinitrophenol (0.25 mM) pretreated with tempol (1 mM), and 5) no drug. [a]Inflammation, edema, and sloughing of cells from the surface epithelium were graded as follows: 1 = absent or minimal; 2 = mild, 3 = moderate, 4 = severe. [b]Erosion of the surface epithelium was graded as: 1 = absent, 2 = mild, 3 = severe. [c]Sloughing of epithelial cells within the mucosal glands was graded as: 1 = absent, 2 = minimal, 3 = mild, 4 = moderate, 5 = severe.

Treatment	Inflammation [a]	Edema [a]	Sloughing surface epithelial cells [a]	Erosions [b]	Sloughing mucosal gland epithelial cells [c]	Comments
Dog 1						
pre	1	1	1	1	1	
1	1	1	4	3	5	
2	1	1	4	2	5	
3	1	1	4	3	5	
4	2	1	4	3	2	lymphoplasmacytic inflammation
5	1	1	4	2	2	focal crypt abscesses near lymphoid follicles
Dog 2						
pre	1	1	2	1	1	artifactual erosions
1	1	1	4	3	5	
2	1	1	4	3	5	
3	2	1	4	3	1	
4	1	1	4	3	5	
5	1	1	4	2	2	epithelial thinning
Dog 3						
pre	1	1	1	2	1	focal area of erosion
1	1	1	4	3	5	
2	1	1	4	2	5	mucus and fecal material, few cells per gland
3	1	1	4	2	5	few cells per gland
4	1	1	4	2	3	epithelial thinning
5	1	1	4	2	1	epithelial thinning
Dog 4						
pre	1	1	1	1	1	
1	1	1	4	3	5	epithelial thinning
2	1	1	4	2	5	epithelial thinning
3	1	1	4	3	1	epithelial thinning
4	1	1	4	2	2	epithelial thinning
5	1	1	4	2	1	scattered eosinophils
Dog 5						

pre	1	1	1	1	1	
1	1	1	4	3	5	
2	1	1	4	3	5	
3	1	1	4	2	5	few cells per gland
4	1	1	4	3	2	focal crypt abscesses near lymphoid follicles
5	1	1	2	2	2	epithelial thinning
Dog 6						
pre	1	1	1	1	2	
1	1	1	4	2	5	
2	1	1	4	2	5	few cells per gland
3	1	1	2	2	4	epithelial thinning
4	1	1	4	3	5	scattered neutrophils around submucosa/muscularis mucosa, few cells per gland
5	1	2	3	1	2	
Dog 7						
pre	1	1	1	1	1	
1	1	1	4	2	5	
2	1	1	4	2	5	
3	1	1	4	3	2	epithelial thinning
4	1	1	4	3	5	epithelial thinning, few cells per gland
5	1	1	4	1	3	some epithelial thinning
Dog 8						
pre	1	1	1	1	1	
1	1	1	4	3	5	some epithelial thinning
2	1	1	4	3	5	
3	1	1	4	3	2	epithelial thinning
4	1	1	2	3	1	epithelial thinning
5	1	1	2	2	2	focal crypt abscesses near lymphoid follicles
Dog 9						
pre	1	1	2	1	1	
1	1	1	4	2	5	
2	1	1	4	3	4	focal areas of epithelial thinning
3	1	1	4	2	5	few cells per gland
4	1	1	4	3	5	
5	1	1	4	2	2	
Dog 10						
pre	1	1	1	1	1	
1	1	1	4	2	5	
2	1	2	4	2	5	
3	1	1	4	2	5	epithelial thinning, few cells per gland
4	1	1	4	2	2	
5	1	3	4	1	2	focal crypt abscesses

APPENDIX 7. TRANSMISSION ELECTRON MICROSCOPIC EXAMINATION OBJECTIVE 2

Electron microscopic findings for sections of colonic mucosa after exposure to carprofen or 2,4 dinitrophenol with or without Tempol pretreatment in an Ussing chamber. Treatments are 1) carprofen (200 μg/ml), 2) carprofen (200 μg/ml) pretreated with tempol (1 mM), 3) dinitrophenol (0.25 mM), 4) dinitrophenol (0.25 mM) pretreated with tempol (1 mM), and 5) no drug. The presence of electron dense particles (EDP) within the mitochondria was considered positive if any were noted. Mitochondrial swelling and cristae loss were considered positive if greater than 33% of the mitochondria were affected. The presence of ruptured mitochondria was positive if any were noted.

Treatment	EDP	Swelling	Cristae Loss	Rupture	Other
Dog 1					
1	+	-	-	-	
1	-	-	-	-	
1	-	-	-	-	
1	-	-	-	-	
1	-	-	-	-	
1	-	-	-	-	
1	-	+	-	-	
1	-	-	-	-	
1	-	+	-	-	
1	-	-	-	-	
2	+	+	+	+	
2	-	+	+	-	
2	-	+	+	+	
2	-	+	+	+	
2	-	+	+	+	
2	+	+	+	+	
2	+	+	+	-	
2	-	+	+	-	
2	-	+	+	+	
2	-	+	+	-	
3	-	-		-	
3	-	-	-	-	
3	-	+	+	-	
3	-	-		-	
3	-	+	+	-	
3	-	-	+	-	
3	-	-		-	
3	-	-		-	
3	-	-	-	-	
3	-	+	+	+	
4	-	+	+	-	
4	+	+	-	-	
4	-	+	+	-	

4	-	+	-	-	
4	-	-	-	-	
4	-	+	+	-	
4	-	+	+	-	
4	-	+	+	-	
4	-	-	+	-	
4	-	+	+	-	
4	-	+	+	-	
5	-	-	+	-	
5	+	-	-	-	
5	+	+	-	-	
5	-	-	-	-	
5	-	+	+	-	
5	-	+	+	-	
5	+	-	-	-	
5	+	-		-	
5	-	+	+	-	
5	+	-		-	
Dog 2					
1	+	+	+	+	
1	+	+	+	-	
1	+	+	+	+	
1	+	+	+	+	
1	+	+	+	+	
1	+	+	+	+	
1	+	+	+	+	
1	+	+	+	-	
1	+	+	+	+	
1	+	+	+	+	
2	+	+	+	+	
2	+	+	+	+	
2	-	+	+	+	
2	+	+	+	+	
2	+	+	+	+	
2	+	+	+	+	
2	+	+	+	-	
2	+	+	+	+	
2	+	+	+	+	
2	+	+	+	+	
3	-	+	+	-	
3	-	-	-	-	
3	-	+	+	-	
3					
3	-	+	+	-	
3	-	-	+	-	
3	-	-	-	-	
3	-	-	-	-	

3	-	+	+	-	
3	-	+	+	-	
3	-	+	+	-	
4	+	+	+	-	
4	-	-	-	-	
4	-	-	-	-	
4	-	-	-	-	
4	-	+	+	-	
4	-	+	+	-	
4	-	+	+	-	
4	-	+	+	-	
4	-	+	-	-	
4	-	+	-	-	
4	-	+	+	-	
5	-	-	-	-	
5	-	-	-	-	
5	-	-	-	-	
5	-	-	-	-	
5	-			-	
5	-	-	-	-	
5	-	-	-	-	
5	-	-	-	-	
5	-	-		-	
5	-	-	-	-	
Dog 3					
1	+	+	+	-	
1	+	+	+	+	
1	+	+	+	+	
1	+	+	+	+	
1	+	+	+	-	
1	-	+	+	-	
1	-	+	+	-	
1	-	+	+	-	
1	-	+	+	+	
1	-	+	+	-	
2	+	+	+	+	
2	-	+	+	+	
2	-	+	+	+	
2	-	+	+	-	
2	+	+	+	-	
2	+	+	+	+	
2	-	+	+	+	
2	-	+	+	+	
2	-	+	+	-	
2	-	+	+	-	
2	-	+	+	-	
3	-	+	+	+	

3	-	+	+	-	
3	+	+	+	-	
3	-	+	+	-	
3	-	+	+	-	
3	+	+	+	-	
3	-	+	+	-	
3	-	+	-	-	
3	-	+	+	-	
3	-	+	+	-	
3	-	+	+	-	
4	-	+	+	-	
4	+	-	-	-	
4	-	+	+	-	
4	-	+	+	-	
4	-	+	+	-	
4	+	+	+	-	
4	-	+	+	-	
4	-	+	+	-	
4	-	+	+	-	
4	-	+	+	-	
5	-	+	+	-	
5	-			-	
5	-	+	+	-	
5	+	-	-	+	
5	+	-	+	-	
5	+	+	+	+	
5	-	+	+	+	
5	+	+	+	-	
5	+	+	+	+	
5	-	+	+	-	
Dog 4					
1	-	+	+	-	
1	-	+	+	-	
1	-	+	+	-	
1	-	+	+	+	
1	-	+	+	+	
1	-	+	+	-	
1	-	+	+	-	
1	-	+	+	-	
1	-	+	+	-	
1	-	+	+	-	
2	-	+	+	+	
2	-	+	+	+	
2	+	+	+	+	
2	+	+	+	+	
2	+	+	+	-	
2	+	+	+	+	

2	+	+	+	+	
2	-	+	+	-	
2	-	+	+	-	
2	-	+	+	+	
2	-	+	+	+	
3	-	-	-	-	
3	-	-	-	-	
3	-	-	-	-	
3	+	+	+	-	
3	-	-	-	-	
3	+	-			
3	-	+	+	-	
3	+	-		-	swollen endoplasmic reticulum
3	+	+	+	-	
3	+	+		-	
3	-	+	+	-	
3	+	+	+	-	
4	-	+	+	-	
4	-	+	+	-	
4	-	+	+	-	
4	-	+	+	-	
4	-	+	+	-	
4	-	+	+	-	
4	-	+	+	-	
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4	-	+	-	-	
4	-	+	+	-	
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4	-	+	+	-	
5	-	+	+	-	
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5	-	+	+	-	
5	-	+	+	-	
5	-	+	-	-	
5	-	+	+	-	
5	-	+	+	-	
5	+	+	+	-	
5	-	+	+	-	
Dog 5					
1	-	+	+	-	
1	+	+	+	+	
1	+	+	+	-	blebbing
1	-	+	+	-	

1	-	+	+	-	
1	+	+	+	-	blebbing
1	-	+	+	-	
1	-	+	+	-	
1	-	+	+	-	
1	-	+	+	-	
2	-	+	+	-	
2	-	+	+	-	
2	-	+	+	-	
2	-	+	+	-	
2	+	+	+	+	
2	+	+	+	+	
2	-	+	+	+	
2	-	+	+	+	blebbing
2	-	+	+	+	
2	+	+	+	-	
3	+	+	-	-	
3	+	+	-	-	
3	-	+	-	-	
3	-	+	-	-	
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5	+	+	+	-	
5	+	+	+	-	
5	+	+	+	-	
5	-	+	+	-	
Dog 6					

1	-	+	+	-	blebbing
1	-	+	+	-	
1	-	+	+	-	blebbing
1	-	+	+	-	
1	+	+	+	-	
1	-	+	+	+	
1	-	+	+	+	
1	-	+	+	-	
1	-	+	+	-	
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5	+	+		-	
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5	-	+	+	-	
Dog 7					
1	+	+	+	-	
1	+	+	+	-	
1	-	+	+	-	
1	-	+	+	+	
1	-	+	+	-	
1	-	+	+	+	
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5	-	+	-	-	
5	-	-	-	-	
5	+	-	-	-	
5	-	-	-	-	
5	-	-	-	-	
Dog 8					
1	+	+	+	-	
1	+	+	+	-	
1	+	+	+	-	
1	+	+	+	-	
1	+	+	+	-	
1	-	+	+	-	
1	+	+	+	-	
1	+	+	+	-	
1	+	+	+	-	
1	+	+	+	-	
1	-	+	+	-	
2	-	+	+	-	
2	-	+	+	-	
2	-	+	+	-	
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2	-	+	+	-	
3	-	+	+	+	
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4	-	+	+	-	
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4	+	+	+	-	
4	+	+	-	-	
4	+	-	-	-	
4	-	+	+	-	
5	+	+	+	+	
5	+	+	+	-	
5	+	-	-	-	
5	+	-	+	-	
5	+	-	+	-	
5	+	-	+	-	
5	+	-	+	-	
5	+	-	+	-	
5	+	-	+	-	
Dog 9					
1	-	+	+	-	
1	-	+	+	-	
1	-	+	+	-	
1	-	+	+	-	
1	+	-		-	
1	-	+	+	-	
1	-	+	+	-	
1	-	+	+	-	
1	-	+	+	-	
1	+	+	+	-	
2	-	+	+	-	
2	-	+	+	-	
2	+	+	+	-	
2	-	+	+	-	
2	-	+	+	-	
2	+	+	+	-	
2	-	+	+	-	
2	-	+	+	-	
2	+	+	+	-	
2	+	+	+	-	
2	-	+	+	-	
3	-	+	-	-	
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3	-	+	+	-	
3	-	+	+	-	
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3	+	+	+	-	

3	-	+	+	-	
3	-	+	-	-	
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4	-	+	+	-	
4	-	+	-	-	
4	+	+	+	-	
4	-	-	-	-	
4	-	+	+	-	
4	-	+	+	-	
4	-	+	-	-	
4					
4	-	+		-	
4	-	+	+	-	
5	-	-	-	-	
5	+	-	-	-	
5	-	-	-	-	
5	-	-	-	-	
5	+	+	+	-	
5	+	-		-	
5	-	-	-	-	
5	+	-	-	-	
5	+	+		-	
5	+	-		-	
5	+	-		-	
Dog 10					
1	-	+	+	-	
1	-	+	+	-	
1	-	+	+	-	
1	-	+	+	-	
1	+	+	+	-	
1	+	+	+	-	
1	-	+	+	-	
1	+	+	+	+	
1	-	+	+	+	
1	-	+	+	+	
2	+	+	+	+	
2	-	+	+	+	
2	-	+	+	-	
2	-	+	+	-	
2	+	+	+	-	
2	+	+	+	+	
2	+	+	+	-	
2	+	+	+	+	
2	+	+	+	+	
2	-	+	+	-	
2	-	+	+	+	

3	-	+		-	
3	+	+	+	-	
3	-	+	+	-	
3	+	+	+	-	
3	+	+	+	+	
3	-	+	+	+	
3	+	+	+	-	
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3	+	+	+	+	
4	-	+	+	-	
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4	-	+	-	-	
4	-	+	+	-	
5	+	-	+	-	
5	+	-	+	-	
5	+	+	+	+	
5	+	-	+	-	
5	+	-		-	
5	+	-		-	
5	+	-	+	-	
5	+	-	+	-	
5	+	-		-	
5					
5	+	+		-	
5	+	-		-	
5	+	-		-	

VITA

Lynne A. Snow was born in the Pittsburgh suburb of Natrona Heights, Pennsylvania. She studied marine science at the University of South Carolina where she graduated cum laude in 2000. Following completion of her Bachelor of Science degree Lynne was granted admission to the University of Illinois College of Veterinary Medicine. Lynne graduated from the veterinary curriculum in May of 2004 with high honors. She went on to complete a rotating medicine and surgery internship at Veterinary Medical and Surgical Group in Ventura, California, and a surgical internship at Animal Specialty Group in Los Angeles, California. Lynne joined the Louisiana State University School of Veterinary Medicine as a surgical fellow in 2006 and continued on as a resident in the companion animal surgery department. She will be awarded the Master of Science degree in veterinary medical sciences in May 2010 and will complete her residency training in July 2010.