EXERCISE & OXIDATIVE STRESS EFFECTS ON THE GASTROINTESTINAL BARRIER & THE CONTRIBUTION OF DIETARY PRO- & ANTI-OXIDANTS IN ALASKAN SLED DOGS

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TABLE OF CONTENTS

Chapter Page

I. INTRODUCTION 1

II. REVIEW OF LITERATURE

EXERCISE-INDUCED GI DYSFUNCTION IN ATHLETES 3

Normal GI Physiology 3
Normal GI Mucosal Barrier Structure 4
GI Dysfunction Relative to Exercise 5
Signs and symptoms 5
Barrier disruption evidence 6
Consequences of dysfunction 7
Barrier disruption causes 7
Barrier disruption due to free radicals 11
Systemic Oxidative Stress with Exercise 16
Exercise-induced oxidative stress 16
Exercise inflammatory response 16
Dietary Deficiency 17
Dietary Pro-Oxidant 18

ALASKAN SLED DOG MODEL 19

Gastroscopy 20
Serum Sugars 20
Oxidative Stress Markers 21
Antioxidant Supplement 23

HYPOTHESIS AND SPECIFIC AIMS 24

III. METHODOLOGY

EXPERIMENTAL DESIGN 25

Experiment #1: Onset of Oxidative Stress & Extent of Gastrointestinal Mucosal Dysfunction during Sustained Exercise 25
Experiment #2: Recovery from Exercise-Induced Oxidative Stress & Gastrointestinal Mucosal Dysfunction 26
Experiment #3: Effect of Dietary Antioxidant 27
Experiment #4: Dietary Lipid Peroxides 28

MATERIALS & METHODS: 29
Physical Examination 29
Sugar Permeability Assay 30
Breath Analysis 31
Glutathione Analysis 32
Gastroscopy 33
Biopsy Collection & Processing 34
Sled Dog Diet 35
Antioxidant Supplement 36
Lipid Hydroperoxide Content 36
Calculations and Statistical Analyses 37

IV. FINDINGS

Experiment #1 40
Systemic oxidative stress: Erythrocyte glutathione 40
Systemic oxidative stress: Exhaled ethane and pentane 44
Exercise-induced gastric lesions: 46
Experiment #2 49
Systemic oxidative stress: Erythrocyte glutathione 49
Gastrointestinal mucosal recovery following exercise 53
Experiment #3: Antioxidant Therapy 61
Experiment #3: Antioxidant Therapy 61
Gastrointestinal lesions and permeability: 65
Experiment #4: Dietary Lipid Hydroperoxides 68

V. CONCLUSION

Exercise-Induced Gastrointestinal Dysfunction 70
Onset and extent of exercise-induced gastric mucosal defects 70
Gastric mucosal defects 71
Gastrointestinal permeability 73
Exercise-Induced Oxidative Stress: 76
Exercise effects on glutathione 77
Exercise effects on ethane & pentane 80
Effect of flavonoid antioxidant on exercise-induced oxidative stress 83
Exercise-Induced GI Dysfunction and Oxidative Stress 85
Effect of dietary flavonoid antioxidant on GI dysfunction 86
Lipid hydroperoxide content 88
Potential mechanisms of gastric ulcers 88
Summary 92

REFERENCES 93

APPENDIX I 106

LIST OF FIGURES

Figure Page

1. Exercise effect on total glutathione in erythrocytes, all samples. 41

2. Exercise effect on total glutathione in erythrocytes, repeated samples. 41

3. Exercise effect on oxidized proportion of glutathione in erythrocytes. 42

4. Exercise effect on reduced glutathione concentration in erythrocytes. 42

5. Exercise effect on oxidized glutathione concentration in erythrocytes. 43

6. Exercise effect on exhaled ethane. 45

7. Gross assessment of gastric mucosa after 12 hours of fasting and following each indicated exercise distance. 47

8. Exercise effect on total glutathione concentration in erythrocytes. 50

9. Exercise effect on oxidized proportion of glutathione in erythrocytes. 50

10. Exercise effect on reduced glutathione concentration in erythrocytes. 51

11. Exercise effect on oxidized glutathione concentration in erythrocytes. 51

12. Exercise and rest effect on oxidized proportion of glutathione in erythrocytes. 52

13. Post-exercise recovery effect on oxidized proportion of glutathione in erythrocytes. 52

14. Post-exercise recovery of gastroscopic lesion scores. 60

15. Exercise and antioxidant effect on total glutathione concentration in whole blood... 62

16. Exercise and antioxidant effect on oxidized proportion of glutathione in whole blood. 62

17. Exercise and antioxidant effect on reduced glutathione concentration in whole blood. 63

18. Exercise and antioxidant effect on oxidized glutathione concentration in whole blood. 63

19. Ethane and pentane from expired breath before and after 150 miles of exercise while receiving either flavonoid antioxidant or placebo. 64

20. Subjective assessment of the gastric mucosa after 150 miles of exercise in dogs receiving either flavonoid antioxidant or placebo. 66

21. Effect of antioxidant on protein carbonyl content of post exercise gastric mucosal biopsies. 66

22. Exercise and antioxidant effect on serum sucrose concentration. 67

23. Exercise and antioxidant effect on ratios of lactulose to rhamnose concentrations in serum. 67

24. Lipid hydroperoxide content of four feedstuffs used to sustain sled dogs. 69

LIST OF PLATES

Plate Page

1. Effect of exercise on the gross appearance of gastric mucosa. 48
2. Normal sled dog gastric mucosal histology. 55
3. Effect of exercise on gastric mucosal histology: mild inflammation. 56
4. Effect of exercise on gastric mucosal histology: severe inflammation..... 57
5. Effect of exercise on gastric mucosal histology: severe inflammation..... 58
6. Exercise-induced gastric erosion. 59

LIST OF TABLES

Table Page

1. Gastrointestinal permeability following exercise and rest. 60

NOMENCLATURE

ATP	Adenosine Triphosphate
CRH	Corticotropin Releasing Hormone
CWSP	Cacao Liquor Water Soluble Polyphenols
$ETCO_2$	End Tidal Carbon Dioxide
fMLP	formyl-Methionine-Leucine-Phenylalanine
IFNγ	Interferon gamma
GI	Gastrointestinal
GSH	glutathione, gamma-Glutamyl Cysteinyl Glutamate, reduced
GSSG	glutathione, gamma-Glutamyl Cysteinyl Glutamate, oxidized-dimer
GSSG/TGSH	oxidized proportion of the sum of reduced and oxidized forms
LOOH	Lipid Hydroperoxide
ROS	Reactive Oxygen Species
$O_2^{\cdot -}$	Superoxide Radical
$OH^{\cdot}$	Hydroxyl Radical
H_2O_2	Hydrogen Peroxide
$ONOO^-$	Peroxynitrite
TGSH	Sum of reduced and oxidized glutathione
TNFα	Tumor Necrosis Factor alpha
VO_{2max}	Maximal oxygen uptake
ω-3 FA	Omega-3 Fatty Acids
ω-6 FA	Omega-6 Fatty Acids

CHAPTER I

INTRODUCTION

Moderate exercise has many beneficial effects, but when approaching the extremes of performance the untoward effects can outweigh the beneficial effects. Sustained strenuous exercise can cause many undesirable effects such as gastrointestinal dysfunction and/or ulceration[1], overuse injuries such as tendinopathies [2] or muscle inflammation and/or damage[3], as well as metabolic or electrolyte derangements[2]. The gastrointestinal tract in particular has been described as a non-athletic organ (i.e., gastrointestinal symptoms are highly prevalent with exercise and do not decrease with increasing capacity for exercise) especially with respect to extended duration or endurance exercise[4]. Gastrointestinal symptoms such as melena, diarrhea, cramping, and rectal bleeding are often noted in humans exercising for extended periods as in marathons or triathlons[5-7]. The cause and extent of gastrointestinal disease or damage is of particular interest due to the possibility of subsequent complications such as endotoxemia[8], malabsorption[4], and anemia[6], perhaps leading to permanent damage.

A potential mechanism of dysfunction in the mucosal barrier of the gastrointestinal tract is oxidative stress. Oxidative stress may be defined as a shift in the normal oxido-reductive state in which there is a pro-oxidant excess and/or decrease in the antioxidant system of the body with subsequent increasing damage due to free radicals. Oxidative stress occurs with endurance exercise, as illustrated by products of damage and

decreased endogenous antioxidants[9]. Reactive oxygen species (ROS) have also gained much attention for their role in ischemia/ reperfusion injury. Ischemia/reperfusion injury potentially occurs in poorly perfused gut tissues that regain normal perfusion after exercise. Originally ischemia/reperfusion injury due to ROS was described in intestines[10] and since has been applied to rat models of gastric "stress" ulcers, e.g. burns, hypovolemia, and restraint stresses. This evidence presents an opportunity to examine interactions of exercise-induced gastrointestinal dysfunction and exercise-induced oxidative stress.

It is often appropriate to find a suitable animal model displaying an extreme of a condition being studied to facilitate the study of the condition. In this case an animal model of extreme endurance is the Alaskan sled dog. Among humans and the animals studied thus far, sled dogs have the greatest known daily energy expenditure during exercise[11]. Sled dogs also exhibit many of the exercise-induced problems of elite human athletes, including diarrhea, hematochezia, melena, vomiting, gastritis, and gastric ulceration. Therefore, sled dogs are a model for the study of the greatest known extreme of endurance exercise, as well as providing a model of the associated problems therein.

CHAPTER II

REVIEW OF LITERATURE

Exercise-Induced GI Dysfunction in Athletes

Normal GI Physiology

The gastrointestinal (GI) system is an internal interface between the body and the environment. In health, this interface provides a physical barrier yet is able to selectively provide passage for nutrients needed by the body. The GI tract facilitates digestion of nutrients prior to absorption. Proper motility aids the mechanical breakdown of macromolecules, ensures mixing of digestive enzymes with nutrients, and allows sufficient contact time for mucosal surface digestion (carbohydrates and proteins) and absorption. Once digested, carbohydrates and proteins are transported into epithelial cells. Triglycerides are also broken down during luminal digestion. The resulting monoglycerides and free fatty acids must then be incorporated into micelles, along with other lipids (cholesterols and lipid soluble vitamins) and bile acids, to increase solubility. Dietary lipid then reaches the cell membrane, in micelles, to diffuse through the epithelial cell membrane. The digestion process facilitates the barrier selectivity by breaking down the macromolecules into constituents that can be recognized and transported through the mucosal barrier.

Normal GI Mucosal Barrier Structure

The selectively permeable barrier properties of the gastrointestinal mucosa are due in large part to a healthy epithelium lining the gastrointestinal tract. In turn, the health of the epithelial layer depends on both luminal contents and underlying tissues. Structurally, this barrier can be divided into the following layers (lumen to capillary)[12]:

1) an unstirred water layer that provides a barrier to hydrophobic constituents that do not get incorporated into micelles,
2) a hydrophobic phospholipid layer that is especially active in the gastric compartment for protection from aqueous acid[13],
3) an adhesive mucous gel layer that protects underlying epithelial cells from digestion, friction from luminal contents, and adhesion of bacteria,
4) epithelial cells that provide overlying non-selective barrier constituents, as well as, maintaining paracellular interactions (tight junctions) to minimize macromolecular permeation by this route,
5) underlying connective tissue that houses many of the non-epithelial cells and can become active when overlying barriers have been permeated, e.g. fibroblasts, lymphoid and myeloid immune cells,
6) an endothelium that maintains continuity of blood flow while providing signals for leukocyte homing to particular sites, and
7) active blood flow that is required for nutrient delivery to the overlying barrier constituent cells as well as providing a sink for locally injurious metabolites.

Epithelial cells are the cornerstone of the mucosal barrier. Epithelial cells have relatively rapid turnover which requires coordination of apoptosis and replication while maintaining the barrier. Turnover contributes to the barrier function in that attachment by bacteria or pathogenic modification of the cells is lost with the cellular death and release to the lumen. Maintenance of this barrier is important to the health of an athlete, as gastrointestinal disease and subsequent complications are a significant risk for the athlete.

GI Dysfunction Relative to Exercise

Signs and symptoms

A substantial proportion of endurance athletes suffer consequences of sustained exercise and signs attributed to GI dysfunction are a common finding. For example, 36% of long distance runners experienced upper GI signs (nausea, vomiting, belching, heartburn, chest pain), and 71% experienced lower GI signs (cramping, bloating, urge to defecate, defecation, diarrhea, flatulence, side ache) during exercise. In this study the prevalence of medication use with GI symptoms was 5%[7]. GI distress can be severe enough to cause race participants (7%) to quit[14]. If symptoms are taken altogether (in marathoners and triathletes) up to 81% experience some problem(s) associated with GI dysfunction[15].

No specific cause(s) of exercise-induced gastrointestinal abnormalities has been proven, but signs and symptoms are often related to intensity and/or duration of exercise and are often attributed to hypoperfusion, increased motility, and/or mechanical trauma. Motility, digestion, and absorption are not well studied with respect to exercise, but they are not likely to be adversely affected by submaximal exercise until the intensity adversely affects gut blood flow[16].

Evidence for Exercise-induced Barrier disruption

A primary mechanism of gastrointestinal dysfunction seems to arise from changes in the mucosal barrier function of the GI tract. Evidence for mucosal barrier disruption has been found in both gastric and intestinal mucosa. Gastric mucosal responses to exercise range from mild hyperemia to ulceration and hemorrhage[1;6;17]. Proximal intestinal mucosa has not been shown to suffer the same gross defects or hemorrhage as gastric mucosa and visualization of distal small intestine is difficult. However, intestinal mucosa has been shown to have increased non-selective permeability in response to exercise [1;18] and bacterial endotoxin increases in the blood of trained triathletes following an ultradistance event[8]. Derangement of the gastrointestinal mucosal barrier occurs with exercise, but more studies are needed to determine the cause(s).

The most dramatic effect of exercise on the GI tract is hemorrhage, which is most commonly attributed to gastric mucosa with colonic mucosa being the next most common site of bleeding[6]. Associated signs are typically enough to preclude continuation of exercise and GI bleeding has been reported as a cause of death in one jogger[19]. GI bleeding is most often occult, and in a review of the subject prevalence was found to range from 8-85%[6]. There appears to be some association between the prevalence of exercise-induced GI bleeding and exercise intensity[20] which may contribute to the variability among prevalence studies. The cause of bleeding has proven difficult to define and is most often attributed to visceral ischemia, but mechanical trauma cannot be ruled out. Extrinsic factors, such as medications, contribute in cases where such things as non-steroidal anti-inflammatory drugs are used.

Consequences of Gastrointestinal barrier disruption

Mucosal barrier dysfunction in the GI tract is of particular interest due to severe consequences that may occur. Endotoxemia occurs in athletes, and it is directly related to exercise intensity[8]. Endotoxemia occurring after a long-distance triathlon was correlated to increased C-reactive protein, but not interleukin 6 (IL-6) or GI complaints, although all increased significantly[14]. It could also be postulated that, with the loss of selective permeability, microbes, toxins, or antigens could exacerbate inflammation and tissue damage in the gut. GI bleeding also has a potential for severe consequences such as acute hemorrhage and hypovolemia, or anemia from chronic blood loss[6]. Thus, GI mucosal barrier dysfunction may lead to unwanted flux of constituents normally sequestered on either side of the barrier, and the consequences may be severe.

Causes of Gastrointestinal Barrier disruption

Direct disruption of the tight junction apparatus

There are many potential causes of exercise-induced GI mucosal barrier dysfunction, and many of the general mechanisms studied thus far may interact to produce the dysfunction with exercise. For example, luminal contents, inflammatory mediators, and temperature can affect the intercellular tight junctions. The intercellular tight junctions are the most apical complexes between epithelia and restrict passage of luminal contents. The tight junction apparatus is complex and dynamic. Multiple proteins interact to create a bridge from the interface through transmembrane domains and anchoring to the apical intracellular actomysosin ring[21;22]. Physiologically the barrier is modulated by intraluminal glucose such that increased paracellular diffusion of creatinine (and collected in urine) has been shown in humans drinking a glucose test solution[23]. The diffusion of creatinine illustrates the dynamic permeability of the tight

junction apparatus due to changes from fasted to fed states. Derangement of the GI mucosal barrier can occur when inflammatory cytokines are present. For example, colonic epithelial cultures have increased permeability in the presence of IFNγ, an effect which is potentiated by TNFα. The increased permeability in this study[24] appeared to be a paracellular rather than transcellular transport (suggesting a breakdown of tight junctions); and was independent of apoptosis. TNFα has also been shown to alter the structure and impair function of the tight junction in colonic cell cultures[25]. The tight junction apparatus permeability is dynamic in response to factors in the local environment, but factors external to the gut such as temperature may also play a role. The protein-protein interaction at the tight junction is sensitive to hyperthermia. Paracellular permeability increased at four hours of hyperthermia and linearly thereafter to 24 hours in colonic cell cultures exposed to 41°C. Transepithelial electrical resistance measurement revealed that this effect is fully reversible in as little as 12 hours after return to 37°C[26]. The hyperthermic effect is also illustrated in anesthetized rats on heating pads which produced small intestinal temperatures between 41.5 and 42°C. Permeability to a fluorescent labeled dextran (4,000 Da) was significantly increased (compared to control temperature of 37°C) by 45 minutes of exposure[27]. Paracellular permeability increases and recovers relatively rapidly in response to hyperthermia, further illustrating the dynamic nature of this barrier. Dynamic changes may be transient provided the effectors are removed, however, when the effectors lead to tissue damage or are more chronic in nature, mucosal permeability changes may be more difficult to recover.

Leukocyte migration

Events leading to extensive migration of leukocytes through epithelial barriers may elicit increased barrier permeability. At low migration rates the barrier function is preserved, but if stimulated for increased leukocyte migration, barrier dysfunction and epithelial erosion may occur. As illustrated in an intestinal cell culture model, stimulated neutrophil migration (fMLP applied on the opposite side of the epithelial layer) led to erosion of approximately 4% of the surface area and significantly reduced transepithelial electrical resistance[28]. Leukocyte migration may not initiate GI mucosal barrier dysfunction, as these cells require some chemotactic signal; however, this mechanism may exacerbate ongoing inflammation-induced mucosal changes subsequent to increased barrier permeability.

Stress-induced permeability

"Stress"-induced permeability is another potential derangement of barrier function, however, the mechanism has not been defined. Stress in this case refers to that which is sensed by the brain and leads to activation of the hypothalamic-pituitary-adrenal (HPA) axis. The activated adrenals release glucocorticoids. Stressors such as swimming or immobilization of rats induced an increase in gastrointestinal permeability (inert sugar by orogastric gavage, discussed below), and this effect was attenuated with both adrenalectomy and glucocorticoid antagonist RU486. The effect was restored after adrenalectomy by using the glucocorticoid dexamethesone[29]. Interestingly, the pharmacological use of glucocorticoids (prednisone) reduces colonic mucosal permeability in patients with active inflammation (diagnosed Crohn's disease). The reduction of permeability was believed to be a result of a reduction of the pro-permeability effects of TNFα[30]. Thus, stress as a cause of mucosal barrier dysfunction

remains ill-defined which may be due, in part, to the pleiotropic effects of glucocorticoids.

More recently corticotrophin releasing hormone (CRH) was found to be a link between stress and gut dysfunction. CRH is released from the hypothalamus as a stimulus for adrenocorticotrophic hormone release from the pituitary and subsequent glucocorticoid release from the adrenal glands. Receptors for CRH have been found in many different tissues and systemically applied CRH leads to inflammation upon activation of the receptor CRH-R1[31]. In particular, mast cells contain CRH-R1 and degranulate in response to CRH, increasing the macromolecular permeability of the colon mucosal barrier[32].

Stress ulcer

The gastric mucosal barrier defect commonly called a "stress" ulcer has received much attention. The stomach has a unique challenge to maintain mucosal barrier continuity while combating strong hydrochloric acid and proteases. The energy required to maintain the protective barrier is perhaps a reason that the stomach is exquisitely sensitive to deficits in energy metabolism as shown in early experiments by Menguy, Desbaillets, and Masters utilizing hypovolemia in rats and rabbits to model stress ulceration[33-36]. Acute hypovolemia from removing a blood volume equivalent to 2% of bodyweight led to extensive ATP use (25% of control values at 15min post-bleeding) in the gastric mucosa which recovered to 63% of control values 1hr after bleeding. The gastric mucosa's dependence on blood flow was reinforced by a measure of glycogen in control gastric mucosal tissues (10.572, SD 2.896 μmol/g tissue) which was 84 fold and 15 fold lower than liver and muscle, respectively[33]. Restriction of luminal nutrients also

appears to contribute to energy dependency of mucosal defects when compared to carbohydrate administration prior to a hypovolemic challenge in rabbits. The hypovolemia coupled with 24 hours of fasting produced many erosions which coincided with decreased cellular ATP as compared to animals drinking sugar water until two hours before challenges[34]. Various stressors that have the potential to decrease gastric blood flow or nutrition can thus initiate stress ulcers. No mechanism has been proven; however, models are often linked to stress-induced reduction in perfusion to the gut that leads to free radical damage of the GI mucosa.

Barrier disruption due to free radicals

Free radical damage may play a major role in acute episodes of inflammation and lead to mucosal barrier dysfunction. Free radicals may be defined as molecules with an unpaired electron that are highly reactive with surrounding molecules and covalent bonds. The radicals may take electrons to gain stability thereby producing other reactive molecules, adducts on surrounding molecules, or breaking covalent bonds. These reactions alter the function of proteins and enzymes, reduce stability of cellular membranes, and alter DNA. ROS are highly reactive oxygen centered species, such as superoxide ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2). In models of inflammatory bowel disease, inflammation (with mediators such as TNFα and IFNγ present) leads to increased mucosal permeability. When free radical scavengers or endogenous antioxidant mimics are systemically administered to the inflammatory bowel model the effects are diminished[37]. This illustrates that some forms of the known free radicals may play a role in inflammatory tissue injury of the GI mucosal barrier.

Reperfusion injury

Exercise stress is associated with mucosal damage in the gastrointestinal tract, and a postulated mechanism of damage is ischemia followed by reperfusion. A growing body of evidence suggests stress ulcers and intestinal damage are caused by ROS [10;38-40] secondary to ischemia and reperfusion injury, and the ROS provide chemotactic signals to leukocytes which may exacerbate the injury[41]. Oxidative stress occurs during exercise[9;42;43] and exercise has been shown to decrease gastric[44] and mesenteric[45] blood flow in humans. It may then be postulated that exercise may cause transient decreases in gastrointestinal perfusion leading to reperfusion injury mediated by ROS. Interestingly, much of the evidence for gastrointestinal dysfunction has been acquired after exercise. Thus, reperfusion injury is a possibility for the damage seen in sled dog gastrointestinal mucosa if there is hypoperfusion during exercise. In the intestine, the mechanism of damage during reperfusion injury has been shown to be mediated through the derangement of the xanthine dehydrogenase system[10]. The initiating insult leads to ischemia and xanthine dehydrogenase is converted to xanthine oxidase which efficiently produces superoxide radicals coupled to conversion of hypoxanthine to xanthine. Hypoxanthine is an end-product of ATP degradation which is increased during hypoxia within intestinal mucosa due to the decreased oxidative metabolism and subsequent ATP recycling during ischemia. Utilizing restraint or cold stress, Das et al., have proposed a mechanism in gastric mucosa utilizing release of redox active transition metals like iron and copper which coupled with increased superoxide radicals and hydrogen peroxide production can produce hydroxyl radicals through the Haber-Weiss reaction[38]. Superoxide radicals can oxidize the metal ions as well as combine in the presence of protons to produce hydrogen peroxide. Oxidized metal ions and hydrogen peroxide will

then produce hydroxyl radicals that have the fastest reaction rates and are non-specific, making them very damaging[46]. This mechanism is pertinent to gastric mucosa, and although it was not measured the restraint or cold stress involved was thought to yield gastric ischemia[38] with subsequent reperfusion injury.

Exercise-induced gastrointestinal ischemia

Redistribution of blood flow occurs during exercise based on metabolic needs of tissues and the need to dissipate heat. Muscle tissue requires an increased oxygen delivery during aerobic exercise. The accumulation of heat requires blood flow to be distributed to surfaces that can dissipate heat, e.g., skin in human beings or respiratory mucosa in dogs. The blood volume is fixed and an increase in the volume of a tissue bed comes at the expense of other tissue beds. Splanchnic tissues, especially, are subject to decreased blood flow during exercise.

Ischemia and potentially reperfusion may be a primary cause of injury that can be mediated by free radicals, yet studies of canine splanchnic blood flow during exercise yield mixed results. Early studies using flow probes at the cranial mesenteric artery showed little or no change in blood flow[47;48]. There are no studies of this type in dogs utilizing the celiac artery making gastric perfusion difficult to assess. Also, the many anastomoses of visceral arteries[49] make it difficult to completely assess local perfusion based on main artery flow. Contrary to the above studies, radionuclide-labeled microsphere deposition in various organs during different exercise intensities revealed that gastrointestinal blood flow is decreased, especially in the stomach and colon[50-53]. The measure was based on left atrial injection of the labeled microspheres that subsequently deposit in capillary beds proportionally to the flow received by each

capillary bed. Calculated flows were based on either a simultaneous measure of cardiac output or a reference sample during injection of microspheres. In graded treadmill exercise, mongrel dogs exhibited an approximately 50% decrease in stomach and colon blood flow from rest to 30% of maximal oxygen uptake (VO_{2max}). This trend continued to drop at a much lower rate for exercise intensities up to 100% of VO_{2max}. Small intestine flow was different in that flow remained steady until approximately 90% of VO_{2max}, upon which this flow also dropped to ~50% of resting levels[51]. The stomach was shown to receive the lowest blood flow as percent of cardiac output during rest and at exercise intensities yielding 75% max HR, as well as to exhaustion[53]. Interestingly, when the effect of training was examined in maximally exercising foxhounds there was an inverse relationship of training status to blood flow to the stomach. Blood flow decreased from 61±5 $ml \cdot min^{-1} \cdot 100g^{-1}$ tissue at rest to 33±6 $ml \cdot min^{-1} \cdot 100g^{-1}$ prior to training and 12±2 $ml \cdot min^{-1} \cdot 100g^{-1}$ after training[50]. It remains unknown why the disparities exist between studies of splanchnic flow in dogs. Differences in study parameters such as training, measurement techniques, and exercise intensities are proposed causes of the lack of agreement. Human studies have revealed a more definitive hypoperfusion during exercise using doppler assisted ultrasound[45] and gastric tonometry[44]. Endurance exercise in humans[6] can lead to bleeding (mucosal defect) in the stomach and colon while the small intestine is rarely involved. Anecdotally, sled dogs exhibit the same distribution (necropsy findings of gastritis without small intestine involvement and frank blood noted in the feces). It stands to reason that if hypoperfusion in humans is a cause of mucosal dysfunction and with the above evidence it is possible that dogs are similarly affected during exercise.

Leukocyte influx due to reperfusion injury

Leukocyte trafficking and activation may also play a role in oxidative mucosal damage. Inflammation is known to lead to activation of leukocytes which effectively produce ROS for immune defense. Also, superoxide radicals lead to chemotactic signals for neutrophils[41]. In ischemia-reperfusion models, reduction of xanthine oxidase-derived radicals reduce the granulocyte influx[54]. However, it is unclear which mechanism initiates the other during sustained exercise, i.e., whether neutrophil influx leads to local oxidative stress or if local oxidative stress leads to neutrophil influx. Regardless of the initiator, the combination appears to be present and responsible for much of the GI mucosal damage in reperfusion injury.

Nitrosative Stress

Little is known about the effects of nitric oxide (NO) on GI mucosa with respect to exercise. NO is protective against ulcerogenic stimuli[55-57] yet with overproduction it may combine with $O^{\cdot -}$ to produce a very potent free radical, peroxynitrite ($ONOO^-$). The amount of NO produced is due in large part to activities of the nitric oxide synthases (NOS), namely the constitutive form (cNOS) and the inducible form (iNOS). Inflammatory cells, such as neutrophils and macrophages, produce iNOS when recruited to the colon in an inflammatory bowel model. Excess NO production in this model was shown to lead to $ONOO^-$ production[58]. NO production in gastric ulceration modulates healing in that the presence of iNOS (due to neutrophil influx) is detrimental while cNOS promotes healing[56]. Thus, inflammation with induction of iNOS may contribute to GI mucosal dysfunction through free radical damage.

Systemic Oxidative Stress with Exercise

Exercise-induced oxidative stress

Studies have revealed that sustained exercise will produce oxidative stress[9;42;43]. Relatively little is known about the primary source of free radicals and predominant species that are important during exercise. One proposed mechanism of ROS production relative to aerobic exercise is that of mitochondrial inefficiency during respiration. Oxidative metabolism is slightly inefficient in which approximately 2% of oxygen consumed will be converted to ROS rather than water[59]. Oxygen consumption (VO_2) can increase up to 20 fold during intense exercise. If the mitochondrial electron transport chain has given inefficiency, free radical production will increase proportionally to mitochondrial recruitment and activity. However, studies of basal (state 4) versus ADP stimulated (state 3) mitochondrial respiration show that the ROS production is decreased with ADP stimulation[60]. State 3 respiration is analogous to ATP use and subsequent ADP accumulation such as in exercising muscle. Thus, although the inefficiency or proportion of ROS produced can decrease during exercise, the absolute amount of ROS will increase due to the increased recruitment of mitochondria and increased metabolic rate. It is unclear whether systemic ROS production affects GI mucosal oxidative stress; however, it is plausible that the consumption of endogenous antioxidants leads to a decreased antioxidant capacity at the GI mucosa.

Exercise inflammatory response

Macrophages and neutrophils may also be activated during exercise and in this state are able to produce and release ROS. Neutrophils and macrophages contribute ROS most often in defense against microbial invasion. However, derangement or dysfunction

of this system may lead to a pro-oxidant excess during exercise. As in disease, exercise can activate pro-inflammatory cascades[61] which can activate the leukocytes. Exercise-induced activation of inflammatory cascades may be the result of exercise-induced endotoxemia, which leads to increased TNFα[62], increased IL-6[14], or muscle damage. Neuroendocrine changes with exercise such as increased growth hormone release may also prime and activate neutrophils[63], making them more responsive to pro-inflammatory stimuli. Neutrophils infiltrate sites of inflammation and then release superoxide anions generated by myeloperoxidase and NADPH oxidase activities. Superoxide can also be converted to hydrogen peroxide by superoxide dismutase activity and then to a highly reactive hydroxyl radical catalyzed by redox-active metal ions. The proportion of oxidative damage due to exercise-induced neutrophil activation is unknown, but the mechanisms above may be potent causes of damage.

Dietary Deficiency

Oxidative stress may be initiated by dietary antioxidant deficiency, reduced endogenous antioxidant defenses, or pro-oxidant excess. In sled dogs commercial kibble which has been specifically manufactured for performance sled dogs is used as the primary sustenance. The kibble is presumed to contain micronutrients relevant to maintenance and performance and this is supplemented with other forms of macronutrients to fulfill energy requirements. It is unknown if the dietary antioxidant content is sufficient to reduce potential adverse effects of ROS in performance sled dogs. However, the pre-exercise plasma concentration of the free radical scavenger vitamin E was associated with a reduced rate of withdrawal from racing[64]. Supplementation with the antioxidants vitamin E, β-carotene, and lutein decreased oxidation of DNA and

increased the plasma antioxidant capacity after exercise in sled dogs[65]. There are no known deficiencies in the sled dog diet leading to adverse clinical conditions; however, more studies are needed to determine the adverse effects of ROS and the potential prevention with dietary antioxidants.

Dietary Pro-Oxidant

Dietary pro-oxidants affect the GI mucosa, and little is know about the pro-oxidant load in a sled dog diet. Lipid peroxides are known to alter the redox status in mucosal cells and can suppress turnover and apoptosis[66] and dietary lipid peroxides may contribute to mucosal dysfunction. The redox balance of the mucosa in racing sled dogs may be predisposed in the oxidant direction due to dietary pro-oxidants. Experiments in rats have shown that dietary lipid peroxides lead to decreased epithelial turnover and apoptosis of epithelial cells due to undefined intracellular redox signaling mechanisms[66]. Dietary lipid peroxides are detoxified in the gastrointestinal mucosa [67] and, in doing so, the antioxidant systems may be depleted relative to the peroxide intake, thus altering the cellular redox ratio. Sled dog diets are composed of approximately 65% lipid and they are often heated prior to feeding. There is evidence that heating of polyunsaturated lipids in the presence of air may increase the peroxidation of the lipids and, subsequently, peroxidized lipids may be found in biomembranes[68]. Therefore, the amounts of polyunsaturated lipid and the subsequent levels of peroxidized lipid in the sled dog diet need to be defined and corrected if needed.

Alaskan Sled Dog Model

Alaskan sled dogs are well suited for the study of sustained exertion. Sled dogs have been shown to be the leading species in terms of energy usage in sustained strenuous exercise[11]. Sled dogs are also known to suffer consequences of sustained strenuous exercise similar to humans, such as gastric ulceration[17] and increased intestinal permeability[69]. Furthermore, non-steroidal anti-inflammatory drugs are not used during exercise in this population and, therefore, will not complicate potential gastritis findings. Sled dogs are relatively homogeneous when grouped according to kennels in which they are reared. Typically kennels have few breeding lines in which many siblings and half siblings are among study groups. Management of each individual is homogeneous for each kennel as well. When exercising, sled dogs can run in teams of up to 20, which create a relatively similar workload for individuals of the same team running on a flat surface in a straight line. Alaskan sled dogs were chosen for study based on their unique ability to sustain exercise for extreme durations and the potential consequences of this exercise volume. In general exercising sled dogs are quite healthy and the most common disorders are related to foot problems and lameness. GI disorders have a relatively low prevalence. Diarrhea was found to be the primary GI disorder with 7.5% of racing dogs affected during the annual Yukon Quest (1000mile) sled dog race for years 1987-1992[70]. However, a primary concern in sled dog racing is an overrepresentation of gastric disease in dogs that die during or shortly after races. Thus, study of GI disorders in racing sled dogs will lead to preventative strategies that may also be applied to other athletic species.

Gastric Mucosal Assessment

Attempts have been made to non-invasively quantify gastric mucosal defects, but endoscopically visible lesions are the gold standard in gross mucosal assessment. Administering sucrose has been used as a gastric mucosal defect specific marker. It is assumed sucrose enters the blood via mucosal barrier defects. The sucrose that is transported to the duodenum is hydrolyzed to its constituent monosaccharides and, therefore, not a significant contributor to serum sucrose concentrations. Increased sucrose has been associated with endoscopic lesions in humans [71] and animals[72-74]. A study of exercise intensity did not show a relationship of sucrose excretion to intensity or GI signs. However, endoscopic lesions were not assessed in this study[18].

Intestinal Permeability

There has been a great interest in providing indicators of increased permeability in vivo given the difficulty in visualizing GI mucosa and the potential for the direction of permeability to be reversed, i.e., noxious agents entering the circulation. Non-invasive techniques are largely based on drinking (or gavage in animal models) solutions with constituents that have no known transcellular transport mechanism. Also, a constituent with no known transcellular transport can be coupled with another that has a robust transport which is relatively unaffected by barrier damage. The appearance of these constituents in the blood and urine then is indicative of relative permeability or barrier dysfunction. The two constituents, one not transported and one transported, are coupled as a ratio of concentrations found in blood or urine. This measure more accurately defines mucosal barrier dysfunction in that the two constituents are both affected by extra-mucosal factors such as gastric emptying and intestinal transport, yet one is

relatively insensitive to mucosal barrier dysfunction while the other is sensitive. Permeability studies using inert sugars seem to indicate that the leaky portion of the mucosa appears to be the epithelial tight junctions[75]. With disruption of this barrier, paracellular movement of noxious luminal contents may occur. Although the utility is debated, there is a significant correlation of permeability to disease states and permeation by harmful molecules[75].

Oxidative Stress Markers

Measurement of oxidative stress is difficult to perform directly and associated biomarkers have supplanted ROS quantification in vivo. Free radicals contain an unpaired electron in an outer orbital and therefore tend to abstract electrons from surrounding molecules. A shared pair of electrons creating a covalent bond is a target that can be disrupted by electron abstraction. The covalent bond disruption activity of free radicals make them particularly damaging. The unpaired electron, thus, makes the free radical very unstable in the presence of the rich cellular milieu. Due to the high reactivity (thus, short half life) of ROS, their measurement requires specialized equipment such as electron spin resonance that can identify compounds with unpaired electrons. Products of ROS reactions are easier to measure from biologic samples. For example, assays have been developed to measure the DNA adduct 8-hydroxy-2'-deoxyguanosine (8OH2'dG), protein carbonylation, and many lipid breakdown products. Commonly measured lipid products are thiobarbituric acid reactive substances (TBARS), malondialdehyde, 4-hydroxy-2-nonenal, isoprostane, ethane, pentane, and lipid hydroperoxides. Products can also be in the form of natural redox cycling of endogenous antioxidants. The largest and most ubiquitous antioxidant reserve is the glutathione pool.

With a constant cycle between reduced and oxidized forms, a measure of both forms of glutathione allows quantification of oxidative insults. A systemic measure of oxidative insult is possible by measuring the glutathione redox state of erythrocytes which carry a large proportion of the body's glutathione pool. Several studies have reported increases in oxidation of blood glutathione with exercise. Products reflective of cellular damage that result from oxidation of polyunsaturated fatty acids can also be measured as ethane and pentane in expired breath. Ethane and pentane result from oxidative liberation from omega-3 and omega-6 fatty acids, respectively. These markers of oxidative damage have been found to be elevated with exercise, but ambient hydrocarbon contamination has made quantification difficult. The expired markers are very sensitive measures, but they are non-specific as to location of oxidative damage.

We have used several markers to assess oxidative stress, free radical products of cellular damage, and effects on the gastrointestinal tract. Exhaled breath has proven to be a valuable marker of oxidative lipid damage by detecting ethane and pentane, liberated from omega-3 and omega-6 fatty acids, respectively[76]. Part of the experiments designed to assess oxidative stress will be to examine the validity of this tool as a non-invasive marker in the sled dog. Glutathione is a ubiquitous antioxidant and its ratio of reduced to oxidized form can be used to indicate the redox status of tissues, especially in erythrocytes. Due to the ubiquitous nature of glutathione, it provides an easily obtainable marker of general oxidative stress status by measurement of reduced versus oxidized glutathione in the blood[77]. Study of mucosal permeability will provide evidence for intestinal dysfunction due to sustained exercise and will also provide information on the role of oxidative stress when measured after specific dietary intervention. Gastroscopic

assessments will also provide evidence for an oxidative stress mechanism when coupled with oxidative stress status and dietary intervention.

Antioxidant Supplement

The mechanism of most dietary interventions may be either general ROS scavenging, augmenting specific endogenous antioxidant systems, or inhibiting intrinsic enzyme abilities to produce ROS. The generation of ROS is difficult to prevent, thus, limiting the damage and augmentation of recovery are the appropriate strategies for combating the effects of oxidative stress. ROS scavengers have been shown to decrease products of cellular damage due to exercise[78]. Certain vitamins have been noted for their oxidant scavenging ability and vitamin E, in particular, has gained acceptance as an antioxidant in the sled dog diet[42]. In studying the gastrointestinal mucosa, we have chosen to utilize an antioxidant believed to remain close to the mucosal membranes and, thus provide more antioxidant ability at the apical epithelial membrane. The Cacao Liquor water-soluble crude polyphenols (CWSP) show radical scavenging ability, and has also been shown to reduce ethanol-induced gastric lesions in rats[79]. Dietary supplementation with the scavenger in proximity to the gut may ameliorate the damage and signs potentially due to increased ROS produced as a result of sustained exercise.

HYPOTHESIS AND SPECIFIC AIMS

A growing body of evidence suggests oxidative stress plays a significant role in exercise-induced gastrointestinal disease; however, its role needs to be further defined. Dietary antioxidants are noted to have mixed results in alleviating the oxidative stress of exercise, and seem to be situation dependant. A dietary antioxidant that remains largely in the gastrointestinal tract may counteract oxidative stress within this compartment. Racing sled dogs are an ideal model for exercise-induced gastrointestinal disease due to their ability to exercise for extended periods at high metabolic rates and the demonstrated prevalence of exercise-induced gastrointestinal disease in these athletes. Through this model, we tested the hypothesis that dietary pro-oxidants and sustained exercise lead to oxidative stress in turn causing gastrointestinal disease, and supplemental dietary antioxidants could ameliorate those effects. To this end, the specific aims were to:

1. determine the onset, duration, and recovery from oxidative stress during sustained, multi-day exercise using biomarkers of erythrocyte redox status and lipid peroxidation products in expired breath,
2. quantify an association between oxidative stress and gastrointestinal dysfunction,
3. determine the role of oxidative stress in gastrointestinal dysfunction due to sustained exercise using a dietary antioxidant, and
4. determine the amount of lipid hydroperoxides in the normal racing sled dog diet.

CHAPTER III

METHODOLOGY

Experimental Design

Experiment #1: Onset & Extent of Oxidative Stress & Gastrointestinal Mucosal Dysfunction during Sustained Exercise

All experiments described herein were reviewed and approved by the Oklahoma State University Institutional Animal Care and Use Committee. Trained Alaskan sled dogs (n=40) aged between two and ten years were conditioned for four months to be able to sustain exercise for five days of approximately 100 miles each day. Dogs were sexually intact, and 16 dogs were female and 24 dogs were male. During the last month of conditioning, they were acclimated to the high fat diet utilized during the study (60% calories from fat, 15% from carbohydrates, and 25% from protein). The exercise challenge consisted of two teams of 18 dogs each pulling a lightly-laden sled and musher over snow in weather typical of central Alaska (10 to -20° F) during the month of January. Dogs were fed, watered, and rested after every 50 miles for approximately five hours. Prior to starting and at the end of each 100 miles, each dog completing the run was examined and expired breath and venous blood was collected. Each dog's ability to continue was assessed by the musher and veterinarian responsible for physical examination. Six dogs were randomly selected for gastric endoscopy with gastric biopsies after each 100 miles.

These dogs were removed from the study after endoscopy, and the remaining dogs continued to participate in the exercise challenge. Ten dogs remained after 500 miles of exercise. Blood and expired breath was collected from each immediately upon completion of the run, and all ten underwent gastric endoscopy with biopsies. This group of ten dogs was used for comparison of blood and breath oxidative stress indices over the course of 500 miles of exercise.

Experiment #2: Recovery from Exercise-Induced Oxidative Stress & Gastrointestinal Mucosal Dysfunction

Trained Alaskan sled dogs (n=44) aged between two and ten years were conditioned for four months to be able to sustain exercise for five days of 100 miles each day. Dogs were sexually intact, and 16 dogs were female and 28 dogs were male. During the last month of conditioning they were acclimated to the high fat diet utilized during the study (60% calories from fat, 15% from carbohydrates, and 25% from protein). Six trained, randomly selected dogs were held from exercise for nine days as an unexercised control group for oxidative stress and gastrointestinal dysfunction indices. Blood samples for GSH determination were taken from the control group followed by orogastric sugar administration, serum samples for sugar quantification (four hours post administration), and gastric endoscopy. The exercise challenge consisted of two teams of 15 dogs and one team of 14 dogs each pulling a lightly-laden sled and musher over snow in typical central Alaskan weather (10 to -20° F) during the month of January. Dogs were fed, watered, and rested after every 50 miles for approximately five hours. Each dog's ability to continue was assessed by the musher and veterinarian responsible for physical examination. Three groups of six dogs each were examined and blood was

sampled (GSH determination) after 100, 300, and 400 miles. Blood samples were taken again (GSH determination) after six hours of rest for each dog. These dogs were withdrawn from the study after these samples were obtained, and did not perform any additional exercise. A separate group of six dogs that completed 350 miles of exercise were randomly selected to receive a five sugar mixture (to determine mucosal integrity), and for blood sampling (GSH determination) at intervals of one, two, and four days following completion of the exercise. The same six dogs underwent gastric endoscopy with gastric biopsies on the fourth day. Two more groups of seven dogs each were selected to receive the five sugar mixture after 400 or 350 miles of exercise, followed by rest of one or two days, respectively, and gastric endoscopy with biopsies.

Experiment #3: Effect of Dietary Antioxidant

Trained sled dogs (n=20; sexually intact: 13 male, 7 female) were randomly divided into two groups: supplemented vs. placebo fed controls. Supplemented dogs received a proprietary dietary supplement (see appendix I for pharmacokinetic profile) of naturally-occurring antioxidants: Cacao Liquor Water Soluble Polyphenols (CWSP) developed by Mars Inc. in addition to their normal diet, every 12 hours with a meal and beginning 1.5 days prior to exercise. Control dogs received their normal diet and cornstarch placebo. Expired breath and blood was sampled two hours prior to exercise. Ambient temperatures precluded the ability to separate erythrocytes from plasma prior to freezing therefore glutathione values were based on whole blood analysis. On day one, all dogs began a 150 mile exercise test, completing this test in approximately 30 hours. Supplemented dogs continued to receive the supplement with food during the exercise trial (three more doses, every 12 hours, and every other rest period). The ability of the

dogs to continue running was determined by experienced mushers driving the teams and the veterinarian responsible for the physical assessment of the dogs. After 100 miles and at the conclusion of the run, blood and breath samples were obtained. The dogs were rested for 12 hr after completion of the exercise challenge, and then fasted for 12 hr prior to gastric endoscopy. Approximately four hours prior to endoscopy, dogs received the sugar solution used to test gastrointestinal permeability. Blood for sugar analysis was obtained from all dogs immediately prior to performing endoscopy.

Experiment #4: Dietary Lipid Peroxides

The dietary pro-oxidant load in the form of lipid hydroperoxides was determined. Racing sled dogs were fed approximately 60% of their metabolizable energy in the form of fat and it was generally heated in water prior to serving. Heated kibble samples and non-heated dry kibble were taken from the bulk preparations of feed on at least three different days. Also, samples of supplemental beef used in addition to the kibble above were taken. The samples consisted of a frozen stick of beef cuttings (fed frozen) and a beef broth made by melting the beef stick in the water for flavor. Samples were assayed for lipid hydroperoxide using a commercially available kit (Cayman Chemical Co., Ann Arbor, MI)

Materials & Methods

Physical Examination

Body weight was measured 24 hours before the start of the exercise test, at the completion of each exercise loop, at the end of exercise or when dogs are retired from the study, and then every 8 hours for 48 hours after the end of exercise. Body weight was recorded from a calibrated electronic scale. Heart rate of resting dogs was measured by thoracic auscultation or digital palpation of the femoral pulse. Hydration was estimated by skin tent and mucous membrane color and moistness. Rectal temperature was measured with an electronic thermometer immediately before exercise, at the end of each loop, at the end of exercise and then every 8 hours for 48 hours.

Fatigue was assessed by readily identified and previously established changes in physical parameters of a working sled dog as follows:

- heart rate exceeding 120 bpm at or beyond 15 minutes of rest,
- and/or dehydration equal to or greater than 5% as assessed by skin tenting at or beyond 15 minutes of rest,
- and/or capillary refill time equal to or greater than 2 seconds at or beyond 15 minutes of rest, and
- auscultation of cardiac arrhythmias, signs of hyperthermia or myoglobinuria at any time.

Sugar Permeability Assay

Dogs received 240 ml of an isotonic sugar solution by orogastric intubation. Sugar solution constituents were sucrose (960mg), lactulose (240mg), rhamnose (240mg), methyl glucose (120mg), and xylose (240mg) dissolved in tap water. Blood was drawn by jugular venipuncture after either four hours (Experiments #1 and 2) or two hours (Experiment #3) for analysis of sugar absorption. Blood was allowed to clot at room temperature for 30 minutes, then tubes were centrifuged for 10 minutes and serum was transferred to cryovials and snap-frozen in liquid nitrogen.

Serum sugar concentrations were quantified using high pressure liquid chromatography (HPLC) with pulsed amperometric detection. Serum was thawed and 900µl was mixed with 100µl of fucose (1 mg/ml) as an internal standard. This mixture was further mixed with 2 ml of ice cold acetone, gently mixed, and centrifuged for 5 minutes. The supernatant was transferred to a new tube and slowly degassed to approximately -30 in Hg for control of sample boil-over. The samples were then centrifuged under vacuum to dryness (Vacufuge, Eppendorf, Westbury, NY). Samples were reconstituted in 500 µl sodium phosphate buffer (0.05M, pH 6.0). In order to resolve sugars from the relatively larger chromatographic peak of endogenous glucose, catalase (~7800units/10µl; Sigma C30) and glucose oxidase (~75units/10µl; Sigma G0543) were added and the samples were incubated at 37°C for three hrs to reduce the amount of glucose in the sample. Samples were moved to a boiling water bath for five minutes. Further clarification of samples was achieved by passing each sample through a solid phase extraction cartridge (GracePure C-18Aq, Grace Davison, Deerfield, IL, activated by passing 2 ml of methanol followed by 2 ml of deionized, ultrapure water

through each cartridge) and a 0.2 μm nylon membrane filter in series by centrifugation. Standards were treated as above after spiking normal canine serum with each sugar at various dilutions for a standard curve. Separation of compounds was obtained using an anion exchange column (CarboPac PA-10, 4 X 250mm, 10μm, with guard column: 4 X 50mm, Dionex Corp., Sunnyvale, CA) and a linear gradient of sodium hydroxide at 1ml/min. The linear gradient used to resolve rhamnose and lactulose from other peaks was as follows: 0-12min 4mM, 12-20min 4-48mM, 20-30min 48mM, 32-47min 380mM, 47-57min 380-4mM, 57-67min 4mM. The linear gradient used to resolve methyl glucose, xylose, and sucrose was as follows: 0-25min 4mM, 25-26min 4-380mM, 26-40min 380mM, 40-41min 380-4mM, 41-51min 4mM. A post-column pump added 400 mM sodium hydroxide (0.5ml/min) which was followed by a pulsed amperometric detector (Coulachem III, ESA, Chelmsford, MA). Detection pulses and duration were as follows: 250mV for 250ms (recorded response) after an acquisition delay of 250ms; 1500mV for 10ms; 600mV for 10ms; 100mV for 40ms.

Breath Analysis

An anesthesia mask was placed over the nose and mouth and was attached to a nonrebreathing circuit. A capnograph continuously sampled the expiratory portion of the circuit. When the end-tidal CO2 reached a steady state (subjectively assessed), an aspiration pump in parallel with the capnograph was activated. The pump was programmed to sample 60 mL (over two minutes) through a proprietary adsorbent that traps volatile hydrocarbons present in the expired breath while at room temperature. Samples of ambient air (two per location) were also aspirated into sample tubes using the same pump parameters but open to atmospheric air at the sampling location. Tubes were

capped (airtight) for transportation and batch analysis. For analysis, tubes were loaded in an autosampling thermal desorption unit that upon heating the tubes causes desorption of the hydrocarbons. The autosampler (Turbomatrix ATD, Perkin Elmer, Waltham, MA) was inline with a gas chromatography system (Clarus 500, Perkin Elmer, Waltham, MA) for analysis of constituents and corresponding concentrations. Gas chromatographic separation utilized a dimethyl polysiloxane capillary column (RTX-1, 60m, 0.32mm ID, 5µm wall coating, Restek, Bellefonte, PA) with a thermal gradient and flame ionization detection. Premixed standards (Scott Specialty Gases, Supelco, Bellefonte, PA) were purchased and aspirated into the collection tubes at varying volumes of standard to obtain a standard curve using the same proprietary hydrocarbon adsorbent tubes.

Glutathione Analysis

Approximately nine ml of blood was drawn from the jugular vein of each dog, using a 20 ga, 1" Vacutainer™ needle. For Experiment #1 and #2, blood samples in EDTA were separated within one hour of collection by centrifugation (2000rpm, 5 min, room temperature). Plasma was removed and 500uL of erythrocytes were mixed 1:1 with 0.9% saline with EDTA added for 2mM concentration in saline and snap frozen in liquid nitrogen for batch analysis. Whole blood samples from Experiment #3 could not be processed as described above and were processed beginning with the following step. Immediately prior to analysis, samples were thawed and 800µL of perchloric acid (5% w/v) was added to 200µL packed red cell/EDTA mix or whole blood. Samples were mixed and incubated at room temperature for 15 min then centrifuged for 5 min at 13,000g (4°C). Resulting supernatant was transferred to a new tube and 40µL of 10M

potassium hydroxide was added, mixed, and centrifuged for 2 min at 13,000g (4°C). Resulting supernatant was then diluted 10-fold in HPLC mobile phase for analysis.

Samples were analyzed using a HPLC method with coulometric electrochemical detection (CoulArray, ESA, Chelmsford, MA). Separation was achieved using a reverse-phase column (Hypersil ODS, 4.6 X 150mm, 3µm, Thermo Scientific, Waltham, MA) at 27°C and isocratic mobile phase consisting of 10mM sodium dihydrogen phosphate (Fluka/Sigma-Aldrich 17844) adjusted to pH 2.7 and 5% v/v methanol at 1ml/min. Mobile phase was filtered with 0.2µm nylon (prior to methanol addition), degassed, and sparged with helium during use. The pre-injection mobile phase was passed through a guard cell (GuardStat, ESA, Chelmsford, MA) at a potential of 0.9V. Oxidized and reduced glutathione were both detected from a single sample injection. A pre-detection potential of 0.35V was applied to samples followed in series by detection at 0.85V.

Gastroscopy

Dogs were anesthetized using a single bolus of propofol (approximately 5 mg/kg, but titrated to effect) administered through a cephalic vein catheter. The dog was placed in left lateral recumbency, and an endotracheal tube was placed to maintain a patent airway. Pulse oximetry was used to monitor peripheral oxygen delivery, and positive pressure ventilation using room air was used to increase oxygen delivery if peripheral hemoglobin saturation decreased below 90%. Additional small boluses of propofol were administered as needed to maintain light anesthesia, based on lack of spontaneous movement. A 10 mm OD fiberoptic endoscope was advanced down the esophagus until it reached the stomach. The stomach was inflated sufficiently to visualize the gastric mucosa (no folds present) using air provided through the endoscope. The tip of the

endoscope was advanced to the pylorus and then slowly retracted while rotating 360º to visualize the condition of the gastric mucosa. The video feed from the endoscope was recorded to be reviewed later. Biopsies were collected, after a full mucosal assessment, from non-ulcerated mucosa in the body region of the stomach. The endoscope tip was withdrawn to the cardia, and the stomach was deflated using suction until the walls of the stomach collapsed around the endoscope. The endoscope was then removed, and the dog was recovered from anesthesia.

The severity of the gross lesions of gastric mucosa was reviewed and scored from recorded video endoscopies. An experienced small animal gastroenterologist, board certified by the American College of Veterinary Internal Medicine, scored lesions based on the following system: 0 = no lesions found, 1 = < 6 discolored areas, 2 = ≥ 6 discolored areas or 1 bleeding lesion, 3 = >1 bleeding lesion. As a clinical assessment, scores of 2 and 3 were considered to be clinically significant gastritis or ulceration. A score of 0 was normal while a score of 1 was typically a questionable lesion(s) in which definitive mucosal barrier involvement could not be determined.

Biopsy Collection & Processing

Biopsies were obtained for histological examination and protein carbonyl determination. Biopsies, at least two from the body region of the stomach, were taken from grossly normal mucosa in each region. Upon collection, histological specimens were preserved in buffered formalin while specimens for protein carbonyl determination were snap-frozen in liquid nitrogen. Histological specimens were embedded in paraffin, and sectioned. Histological sections were stained with hematoxylin and eosin.

Histological descriptions were performed by an anatomic pathologist certified by the American College of Veterinary Pathologists.

Gastric biopsy protein carbonyl assessment was achieved following a previously published protocol[80] utilized in the lab of Dr. Kenneth Hensley. Biopsies were homogenized in 10 mM sodium acetate buffer (pH 7.2) containing 0.1% triton X-100 and mammalian protease inhibitor cocktail (Sigma Chemical, St Louis, MO, USA). Samples were centrifuged briefly to remove particulate matter, then adjusted to 4 mg/ml total protein based on the Lowry protein assay, and diluted 1:1 with 20 mM methylethanesulfonate (MES) buffer, pH5.5. To each sample was added a 1% volume of 100 mM butylated hydroxytoluene (BHT), predissolved in ethanol, and a 10% volume of 50 mM biotin hydrazide (Pierce Chemical, Rockford, IL, USA) pre-dissolved in dimethyl sulfoxide (DMSO). Samples were incubated with gentle agitation for eight hours at room temperature. Aliquots containing 10μg of protein each were electrophoresed on 4–20% gradient polyacrylamide gels, electroblotted to polyvinylidene difluoride (PVDF) membranes and blocked overnight with 4% bovine serum albumin (BSA). Blots were probed by incubation with 0.1μg/mL horseradish peroxidase-conjugated streptavidin (Pierce Chemical), washed thoroughly, and developed using enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Sled Dog Diet

Commercial kibble (Eagle Pack Ultra Powerpack, containing 5400 Mcal/kg of kibble, 28% protein, 54% fat, 18% carbohydrates) provided the base nutrients. Diets were further supplemented with beef, and intermittently with salmon and tallow (each dog

received equal supplementation when given). Dogs were fed twice daily from individual bowls. Dogs typically received approximately 10 Mcal/day while training.

Antioxidant Supplement

Dogs received a proprietary oral cocoa flavanol extract antioxidant at a rate of 250 mg/dog/feeding (2 capsules totaling 1200mg of crude extract per feeding). The extract was detheobrominated and decaffeinated by Mars, Inc. such that the content of theobromine was 0.184% by weight and caffeine was 0.092% by weight. The cocoa flavanol extract components of interest were based on a flavan-3-ol unit and had the following breakdown of components (% of total): monomers (4.6), dimers (28.8), trimers (22.4), tetramers (17.4), pentamers (13.1), hexamers (8.5), heptamers (3.4), octamers (1.7), nonamers (1.8), and decamers (0.8). The supplement was provided as a capsule, and dogs were dosed immediately prior to serving food. Placebo-treated dogs received equal volume capsules containing cornstarch (940mg/dog/feeding). See appendix I for a pharmocokinetic profile in laboratory dogs.

Lipid Hydroperoxide Content

A commercial assay (Cayman Chemical Co., Ann Arbor, MI) was used to determine dietary lipid hydroperoxide content. Samples were homogenized in ultra pure, deionized water and four aliquots of each food type were subsequently analyzed. The lipid content of the samples was quantitatively extracted into chloroform. The assay was done in chloroform to prevent iron and hydrogen peroxide contamination. The assay utilized direct reaction of the hydroperoxides with ferrous ions to produce ferric ions.

The resulting ferric ions were then detected using thiocyanate ions as a chromagen with absorbance detected at 500nm.

Calculations and Statistical Analyses

Oxidative stress data are presented as means ± standard deviation. Glutathione measures consisted of oxidized (GSSG) and reduced (GSH) forms. Total glutathione (TGSH) was calculated as the sum of the individual components (GSSG + GSH) and oxidized proportion was the ratio of GSSG to TGSH for each individual, prior to averaging. In Experiment #1 glutathione data are from all dogs available for each distance (TGSH) and 10 dogs sampled repeatedly after 100 miles for 500 miles of exercise. Statistical analysis consisted of one-way ANOVA for TGSH, all samples, and one-way ANOVA for repeated measures with potential post-hoc testing by Tukey's test for each pair of groups. Data for exhaled ethane are also presented as means ± standard deviation. Individual measures were calculated by subtraction of ambient amount and normalized to each individual end-tidal CO_2 ($ETCO_2$). The normalization was necessary to counter the effect of ventilation pattern[81] and it was done by averaging $ETCO_2$ for each individual in the study then multiplying an individual's exhaled ethane (corrected for ambient amount) by the ratio of group mean $ETCO_2$ divided by the individual's $ETCO_2$. Exhaled ethane data were subjected to one-way ANOVA for repeated measures with potential post-hoc testing by Tukey's test for each pair of groups. Due to the high concentration of ambient ethane (sampled in a different location from post-exercise samples) in the pre-exercise time point these data were removed from the statistical analysis. Data describing the gastroscopy findings are presented as individual scores and overlaid by the median for each group. The effect of exercise on gastric endoscopy score

was evaluated using a gamma statistic derived from a contingency table with ordinal categories. Two comparisons were performed: the effect of exercise using scores from trained, rested dogs compared to scores from all exercised dogs; and the effect of distance traveled using scores from exercised dogs. Correlation of each individual's oxidative stress markers to endoscopic finding was performed using Spearman's correlation for ordinal data.

For Experiment #2 glutathione data are presented as above, but data consisted of randomly selected dogs after each distance rather than repeated measures. Statistical analysis was performed with a one-way ANOVA and post-hoc testing by Tukey's test for each pair of groups. Glutathione data for post-exercise and six hour recovery were analyzed using a paired T-test for each distance group. Four day extended recovery glutathione data was analyzed using repeated measures ANOVA for the sequential sampling while control values are presented for visual comparison as these were not the same dogs. Endoscopic data for recovery of gastric mucosa following exercise and trained, nine day rested dogs are presented as individual scores overlaid by the group median. Each group consisted of different dogs randomly selected and analysis consisted of Kruskal-Wallis one-way ANOVA for ordinal data. Post-hoc testing utilized Dunn's multiple comparison test. Correlation of each individual's oxidative stress markers to endoscopic finding was performed using Spearman's correlation for ordinal data.

For Experiment #3 glutathione and exhaled ethane and pentane data are presented and were analyzed for two criteria: exercise and treatment with a flavonoid antioxidant. Data are presented as mean ± standard deviation for each treatment group and exercise distance. Individuals were repeatedly sampled over the course of the exercise challenge.

Data were analyzed using two-ANOVA for repeated measures and Bonferonni post-test for the effect of flavonoid antioxidant treatment. The effect of flavonoid on post-exercise gastroscopy is presented as individual scores overlaid by the median group value. The effect was analyzed using a Mann-Whitney test for unpaired ordinal data. Gastrointestinal permeability data (serum sucrose and lactulose to rhamnose ratio found in serum) are presented as values for each individual and overlaid by mean ± standard deviation. Data were analyzed using two-ANOVA for repeated measures and Bonferonni post-test for the effect of flavonoid antioxidant treatment. Correlation of each individual's oxidative stress markers to endoscopic finding was performed using Spearman's correlation for ordinal data. Comparisons of oxidative stress markers (GSSG/TGSH, ethane, and pentane) to serum gastrointestinal permeability markers (sucrose and L/R ratio) were done using Pearson's correlation.

Dietary lipid hydroperoxide determinations for four feedstuffs are presented as mean ± standard deviation for each replicate determination of each feedstuff. Data are for illustrative purposes and the reported value for control diets is also presented for comparison.

All statistical analyses were performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California USA. Statistical significance was achieved for any $p \leq 0.05$.

CHAPTER IV

FINDINGS

Experiment #1: Onset & Extent

Systemic oxidative stress: Erythrocyte glutathione

Oxidative stress was not demonstrated during multiple days of exercise, as measured by oxidation of glutathione. Due to a lack of proper blood collection tubes at the pre-exercise collection location samples for glutathione determination were not obtained for this time point. Total erythrocyte glutathione content remained stable throughout the exercise challenge in repeated samples (Figure 1) but when all samples for each distance were pooled a difference was noted ($p = 0.0209$). Post hoc testing could not define which pair(s) of time points differed (Figure 2). Oxidized proportion and component concentrations were not different when all samples were pooled (GSSG/TGSH, $p=0.7596$; GSH, $p=0.9887$; GSSG, $p=0.0852$; data not shown). The proportion of oxidized glutathione varied widely among the ten individuals completing 500 miles and statistically no differences were found (Figure 3). Also, no difference was noted in erythrocyte concentrations of reduced or oxidized glutathione (Figures 4 & 5).

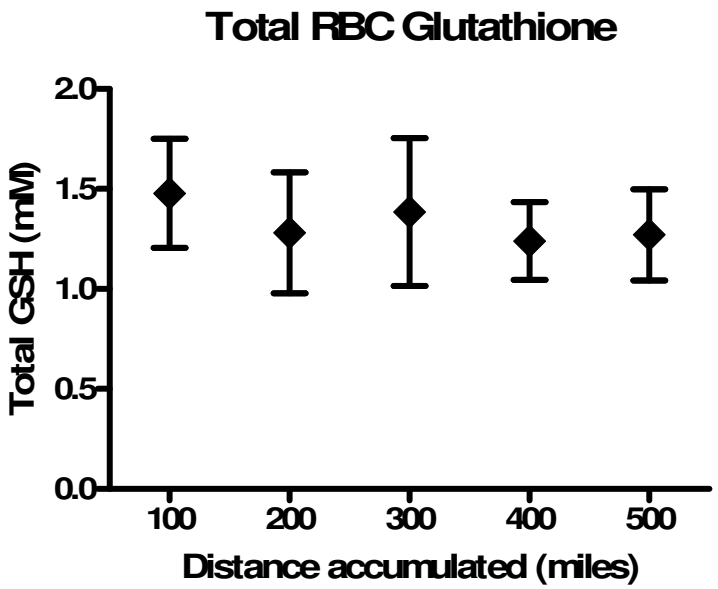

Figure 1. Exercise effect on total glutathione in erythrocytes, all samples. Samples were taken from all dogs available immediately following each indicated distance (n=34, 28, 22, 16, 10 respectively). A change in total glutathione was found ($p = 0.0209$) but post hoc testing could not further define the difference. Data are means ±SD.

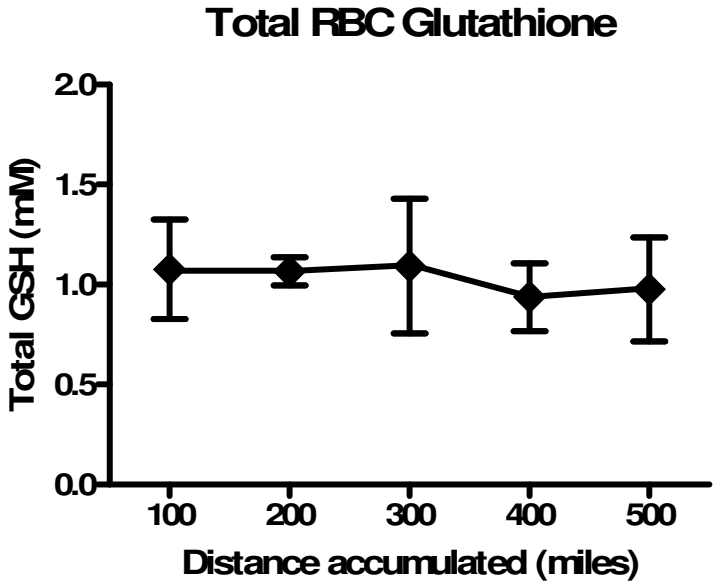

Figure 2. Exercise effect on total glutathione in erythrocytes, repeated samples. Sampling was repeated for the same ten dogs immediately following each indicated distance. Total glutathione concentration in packed red blood cells did not change with exercise ($p = 0.2197$). Data are means ±SD.

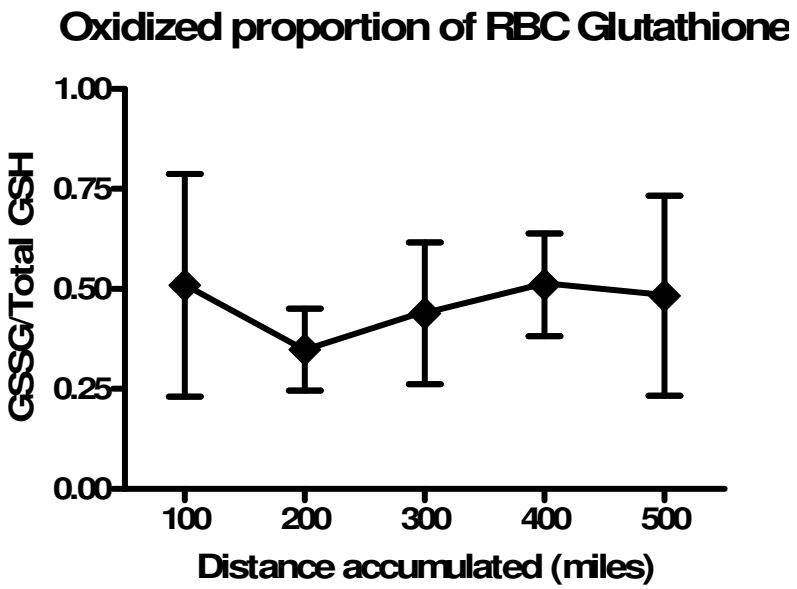

Figure 3. Exercise effect on oxidized proportion of glutathione in erythrocytes. Samples were taken from the same ten dogs immediately following each indicated distance. No change in oxidized glutathione was found ($p = 0.3798$). Data are means ±SD.

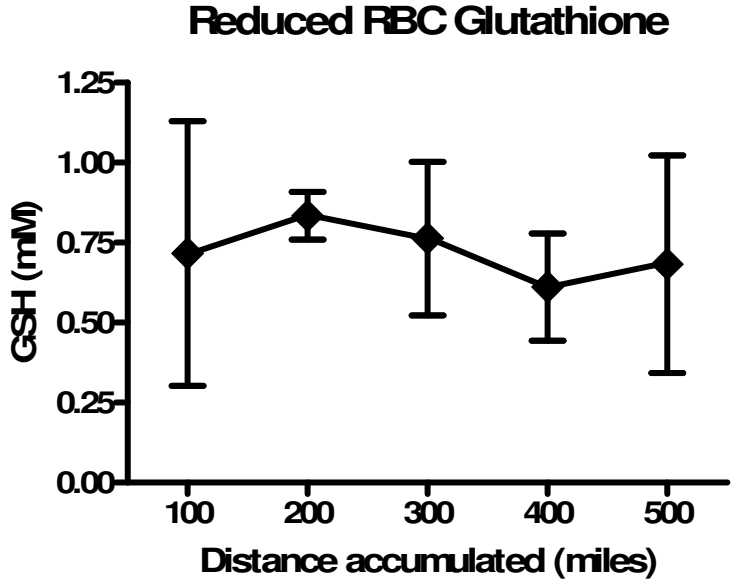

Figure 4. Exercise effect on reduced glutathione concentration in erythrocytes. Samples were taken from the same ten dogs immediately following each indicated distance. No change in reduced glutathione was found ($p = 0.3459$). Data are means ±SD.

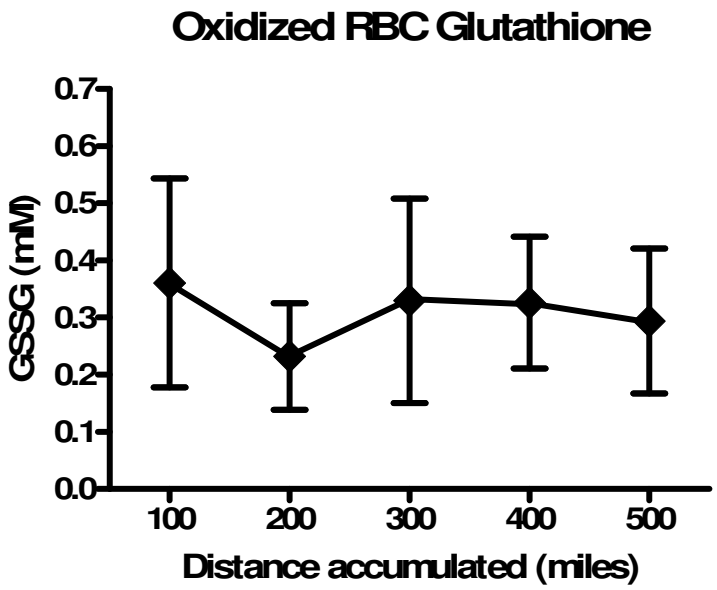

Figure 5. Exercise effect on oxidized glutathione concentration in erythrocytes. Samples were taken from the same ten dogs immediately following each indicated distance. No change in oxidized glutathione was found ($p = 0.3638$). Data are means ±SD.

Systemic oxidative stress: Exhaled ethane and pentane

Exhaled pentane was not detectable in any sample and exhaled ethane was close to ambient values. Ambient concentrations of ethane at each sampling point were : 68665.3 pmol/L (pre-exercise); 1687.6 pmol/L (100 miles); 1992.9 pmol/L (200 miles); 1926.0 pmol/L (300 miles); 2294.7 pmol/L (400 miles). Ambient samples for the last time point (500 miles) were lost due to non-gas tight closure, therefore ambient amounts were averaged for each of the previous four time points, all sampled in the same location at different times (1975.3 ± 250.0 pmol/L). Pre-exercise samples were obtained in a different location than that of post-exercise samples. Presence of ambient ethane led to highly negative values (-16697 ± 12518 pmol/L) when subtracting ambient contributions to the measurement. When only the exercise groups are compared there is a significant difference overall ($p = 0.0143$) with the 100 mile group significantly greater than both 400 mile and 500 mile groups (each $p < 0.05$) (Figure 6).

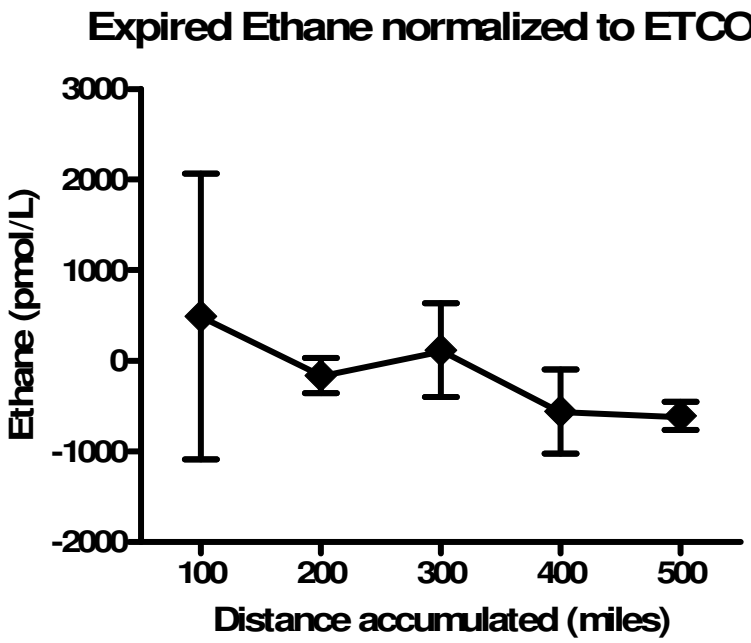

Figure 6. Exercise effect on exhaled ethane. Ethane decreased as the distance accumulated increased. Samples at 100 miles were significantly different from both 400 and 500 miles ($p<0.05$). Pre-exercise samples are excluded due to the ambient levels of ethane contributing to large differences compared to the sampling conditions for exercise groups. Ambient samples were lost from 500 mile group and previous ambient samples were averaged to provide an estimate of ambient contamination. Data are means ±SD.

Exercise-induced gastric lesions

Dogs were affected early in exercise as grossly visible lesions were apparent after the first 100 miles and continued through each subsequent 100 miles (Figure 7). Lesion severity scores were significantly different ($p < 0.0001$) from unexercised dogs, indicating an effect of exercise. However, the duration of exercise was not associated with a difference in severity scores ($p = 0.22$). Lesion severity scores did not correlate with concurrently measured indices of oxidative stress (proportion of oxidized glutathione: Spearman $r = -0.03$; $p = 0.8643$, $n=34$; or exhaled ethane: Spearman $r = 0.05257$; $p = 0.7678$, $n= 34$) over the course of the exercise challenge. Questionable (severity score = 1) lesions of two dogs in the control group may have been artifactual due to finding a rock in one stomach and linear erosions resembling endoscopic scraping of the mucosa in the other stomach.

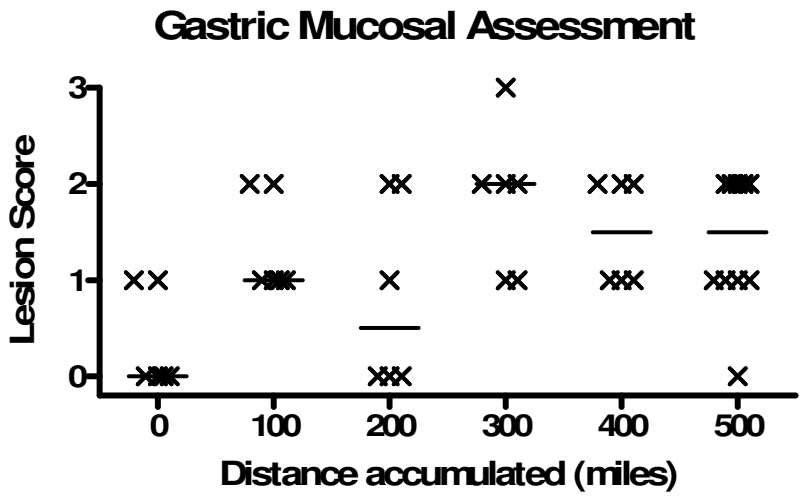

Figure 7. Gross assessment of gastric mucosa after 12 hours of fasting and following each indicated exercise distance. Abnormal gastric mucosa was apparent after the first exercise bout and continued through subsequent bouts. There was a significant effect of exercise ($p < 0.0001$) but not the duration of that exercise ($p = 0.22$). Individual scores are marked and lines indicate medians for each exercise group.

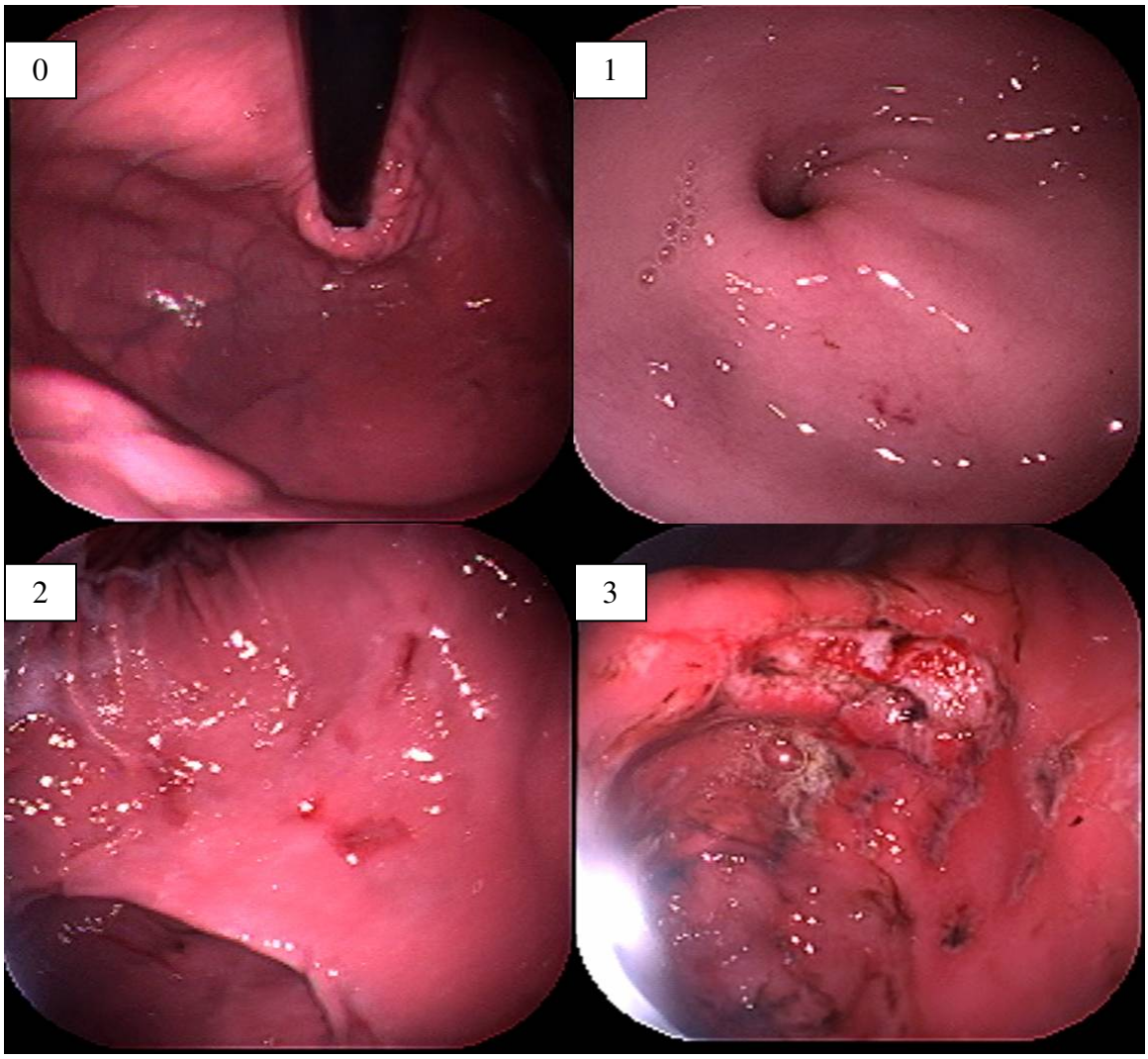

Plate 1. Effect of exercise on the gross appearance of gastric mucosa. Images are representative of the scoring system used in the studies. Annotations in upper left corners indicate the score for the respective image.

Experiment #2: Recovery

Systemic oxidative stress: Erythrocyte glutathione

Total glutathione appeared to be decreased by the initiation of exercise and approached statistical significance (Figure 8, $p = 0.0840$). Oxidative stress was not demonstrated due to exercise (Figures 9-11). However, when post-exercise samples were compared to corresponding samples obtained after six hours of rest with meals, an increase was noted following 100 miles of exercise and subsequent rest (Figure 12, A; $p = 0.0028$). Rest periods thereafter showed no difference (Figure 12, B&C). Furthermore, no difference was noted between 24, 48, and 96 hours of rest (after accumulating 350 miles of exercise) or when control dogs were compared to rested dogs in a non-repeated format (Figure 13). Total glutathione did not change during the recovery period ($p = 0.9289$, data not shown).

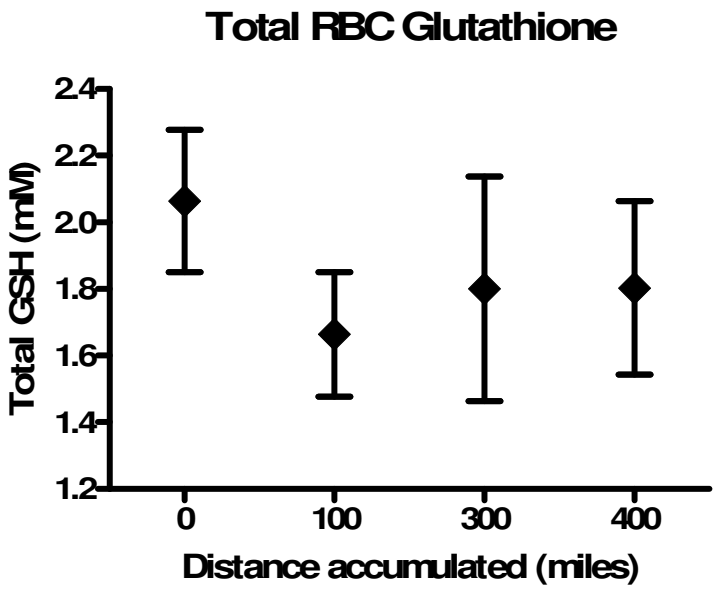

Figure 8. Exercise effect on total glutathione concentration in erythrocytes. Total glutathione change with exercise approached significance ($p = 0.0840$). Individuals were randomly selected at each blood collection (n=6). Data are means ±SD.

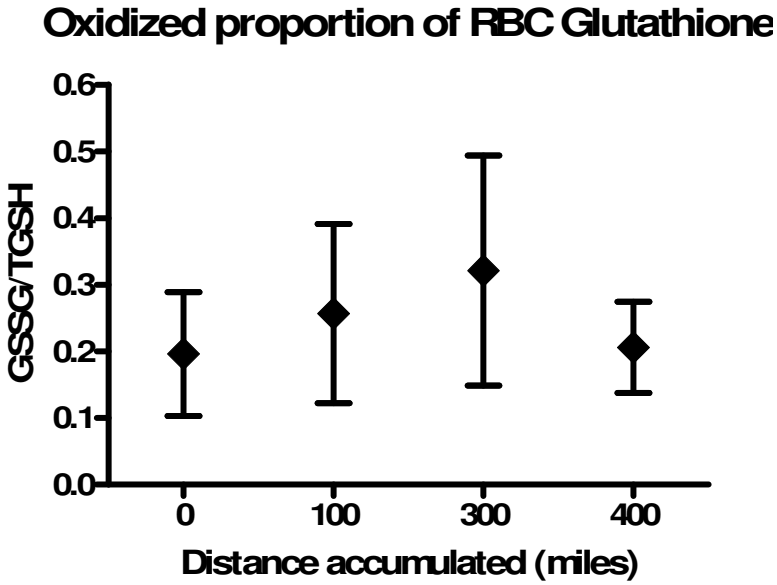

Figure 9. Exercise effect on oxidized proportion of glutathione in erythrocytes. No change in oxidized proportion was found ($p = 0.3057$). Individuals were randomly selected at each blood collection (n=6). Data are means ±SD.

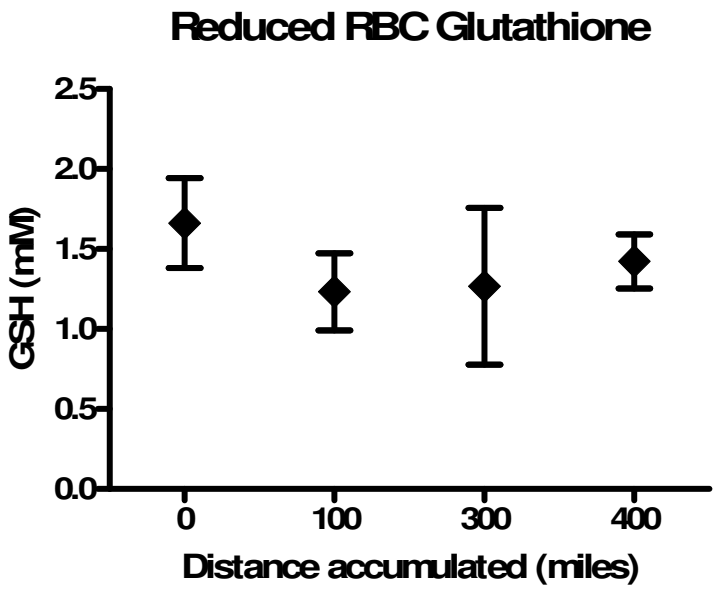

Figure 10. Exercise effect on reduced glutathione concentration in erythrocytes. Different exercise distances approached a significant difference in reduced glutathione ($p = 0.1108$). Individuals were randomly selected at each blood collection (n=6). Data are means ±SD.

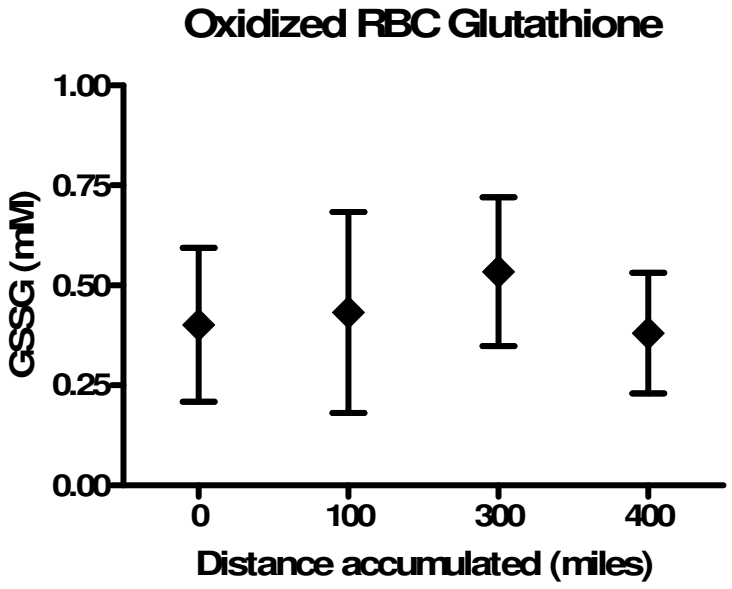

Figure 11. Exercise effect on oxidized glutathione concentration in erythrocytes. No change in oxidized glutathione was found ($p = 0.5613$). Individuals were randomly selected at each blood collection (n=6). Data are means ±SD.

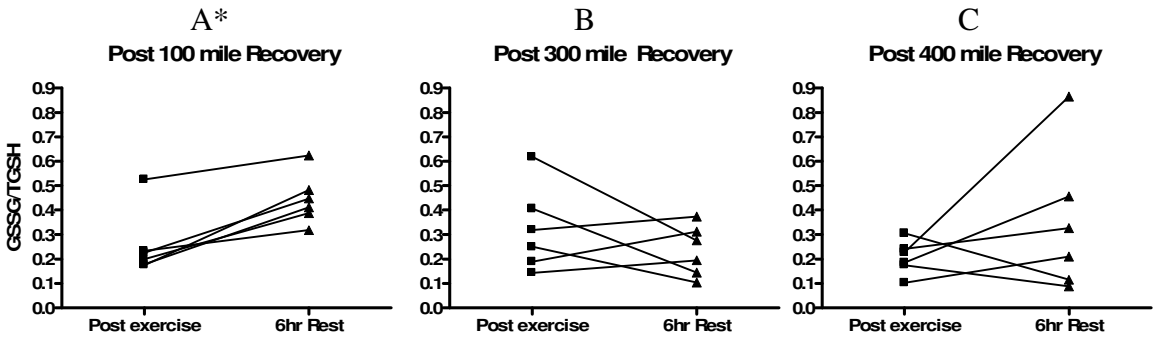

Figure 12. Exercise and rest effect on oxidized proportion of glutathione in erythrocytes. Samples were taken following exercise at each indicated distance and then after six hours of rest with meals. Recovery from 100miles of exercise produced an increased oxidized proportion of glutathione (A*: $p = 0.0028$) while post-300 and 400 mile challenges did not significantly change (B: $p = 0.3146$ & C: $p = 0.3029$, respectively). Squares and triangles represent individual measurements and lines connect each dog's pre- and post-rest measurement.

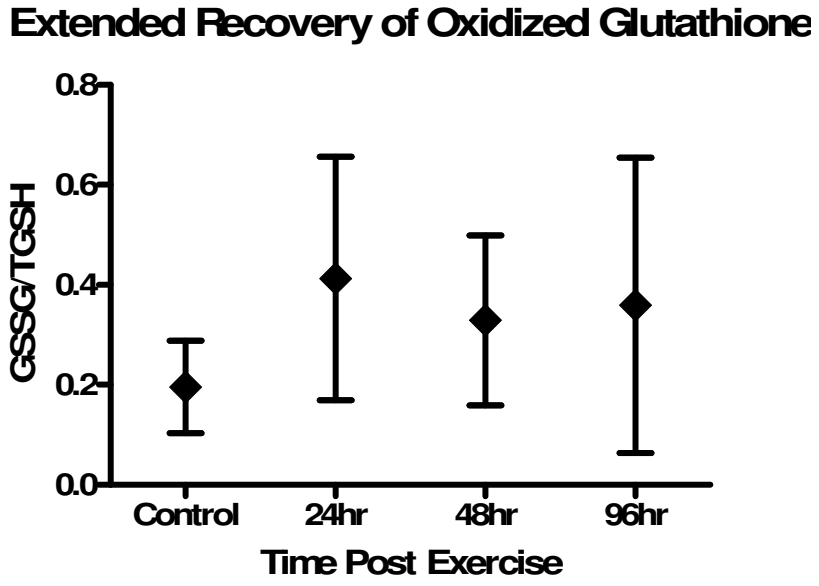

Figure 13. Post-exercise recovery effect on oxidized proportion of glutathione in erythrocytes. Samples were taken following exercise (350 miles) at each indicated time during recovery. Post-exercise samples are from the same individuals whereas control samples are different individuals. There was no difference between repeated post-exercise groups ($p = 0.8689$) or when control dogs were compared to rested dogs ($p = 0.3683$). Data are means ±SD.

Gastrointestinal mucosal recovery following exercise

Grossly visible gastric mucosal barrier defects had resolved by four days of recovery from 350 miles of exercise. Control dogs all had grossly normal gastric mucosa (6 dogs, all scoring 0). At 24hrs post exercise, 4/7 dogs had significant gastric lesions, while at 48hrs post exercise 1/7 dogs had significant gastric lesions, and at 96hrs post exercise 0/6 dogs had significant gastric lesions, although 3/6 dogs had questionable gastric mucosal lesions (score of 1, see Plate 1).

Gastric permeability was decreased from control values during post-exercise recovery. In both 24 and 48 hours of recovery, sucrose permeability was significantly less in exercised dogs than trained, rested control dogs (Table 1). After 96 hours of rest, the values were no longer significantly different from control values. Intestinal permeability (L/R measure) did not change with exercise or rest (Table 1).

The epithelium of the gastric mucosa exhibited signs of recovery similar to the gross assessment while deeper levels remained inflamed. In the gastric epithelium, 2/6 control dogs had conspicuous apoptotic debris and lymphocytic infiltrate; while at 24hrs post exercise 1/5 dogs had disseminated lymphocytic infiltrate with apoptotic debris; at 48hrs post exercise 2/4 dogs had lymphocytic infiltrate with apoptotic debris with one having multifocal effacement of gastric glands by inflammatory cell infiltrate; and at 96hrs post exercise all dogs were within normal limits. In the lamina propria, 5/6 control dogs had mild to severe lymphoplasmacytic infiltrate with 4/6 dogs accompanied by neutrophils as well; at 24hrs post exercise 3/5 dogs had lymphoplasmacytic infiltrate with one of these having severe neutrophilic influx as well; at 48hrs post exercise 3/4 dogs had lymphoplasmacytic infiltrate with one of these having disseminated inflammation with

neutrophilic influx; and at 96hrs post exercise 3/3 dogs had lymphoplasmacytic infiltrate and 2/3 dogs with neutrophilic influx.

Most (15/18 dogs) had helical bacteria associated with the epithelium. The bacteria were > 5µm and none had bacteria < 5µm (which would be consistent with the size of pathogenic Helicobacter spp.). There was one instance of helical bacteria associated with inflammation of gastric glands.

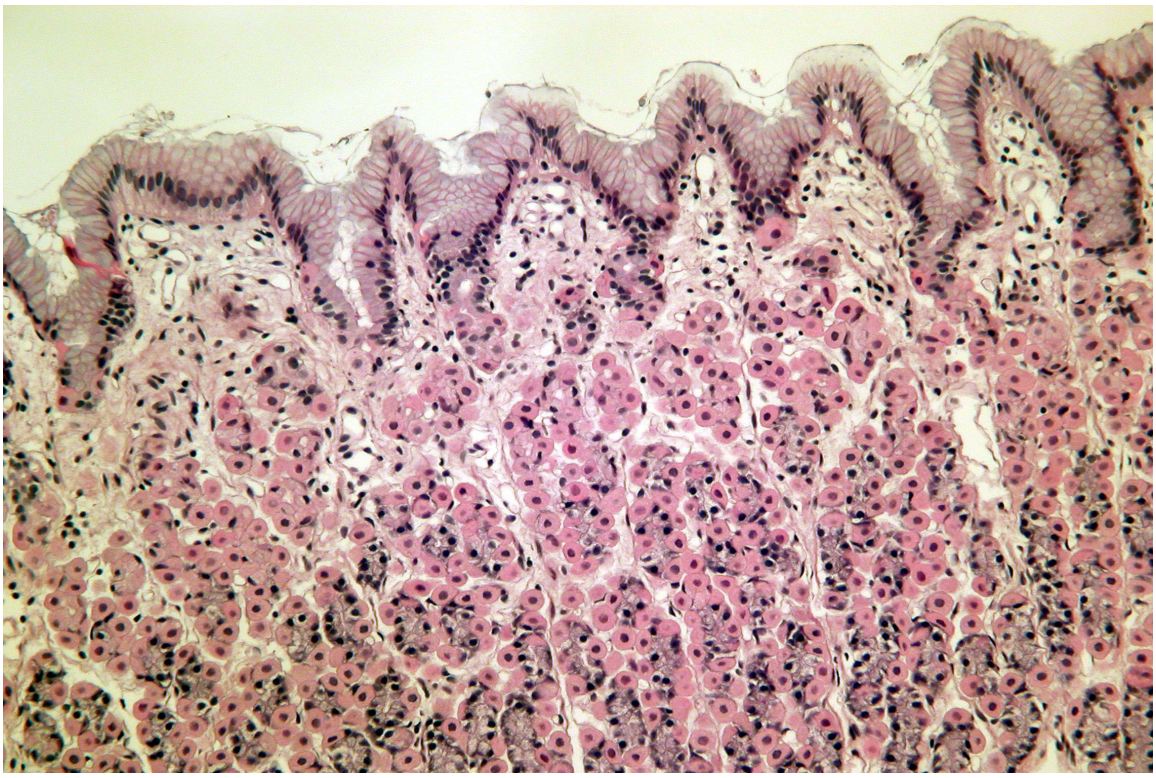

Plate 2. Normal sled dog gastric mucosal histology.

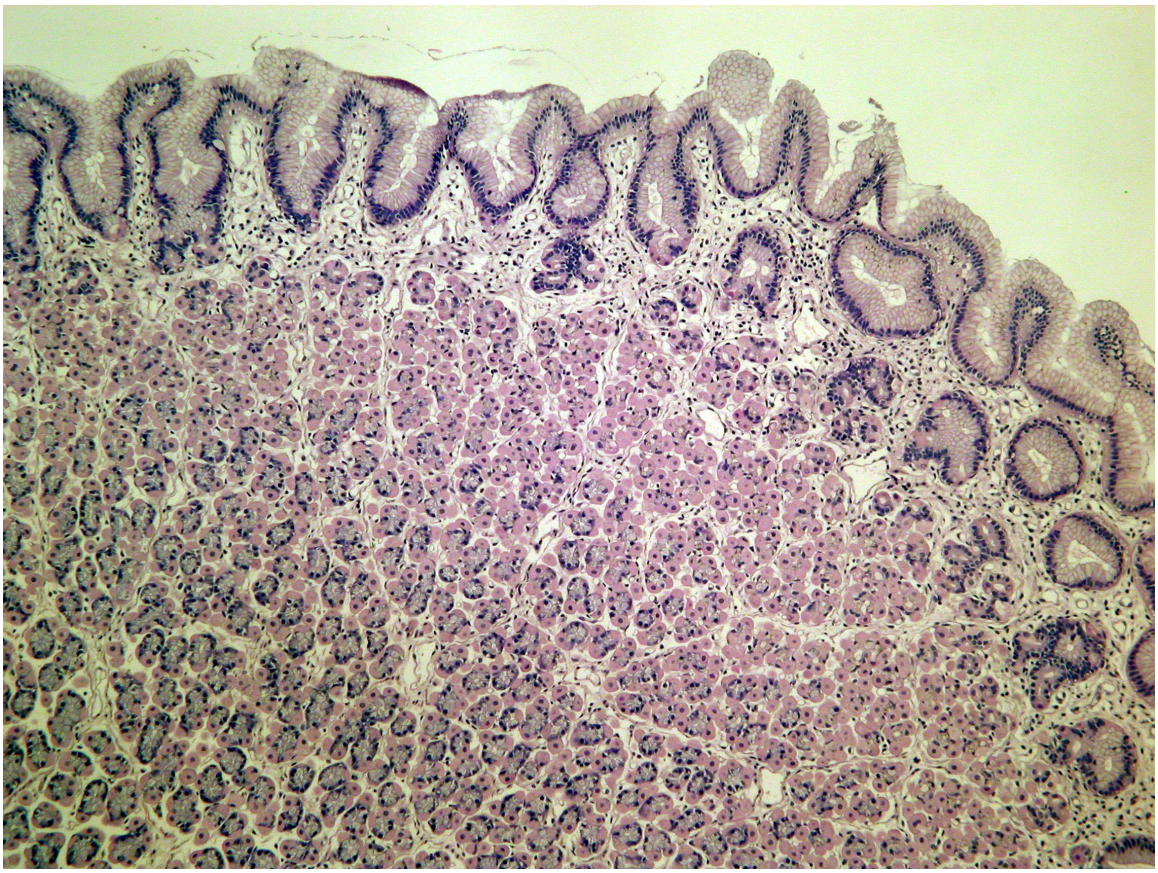

Plate 3. Effect of exercise on gastric mucosal histology: mild inflammation. Epithelium is intact. There is accumulation of lymphoplasmacytic cells in the superficial lamina propria.

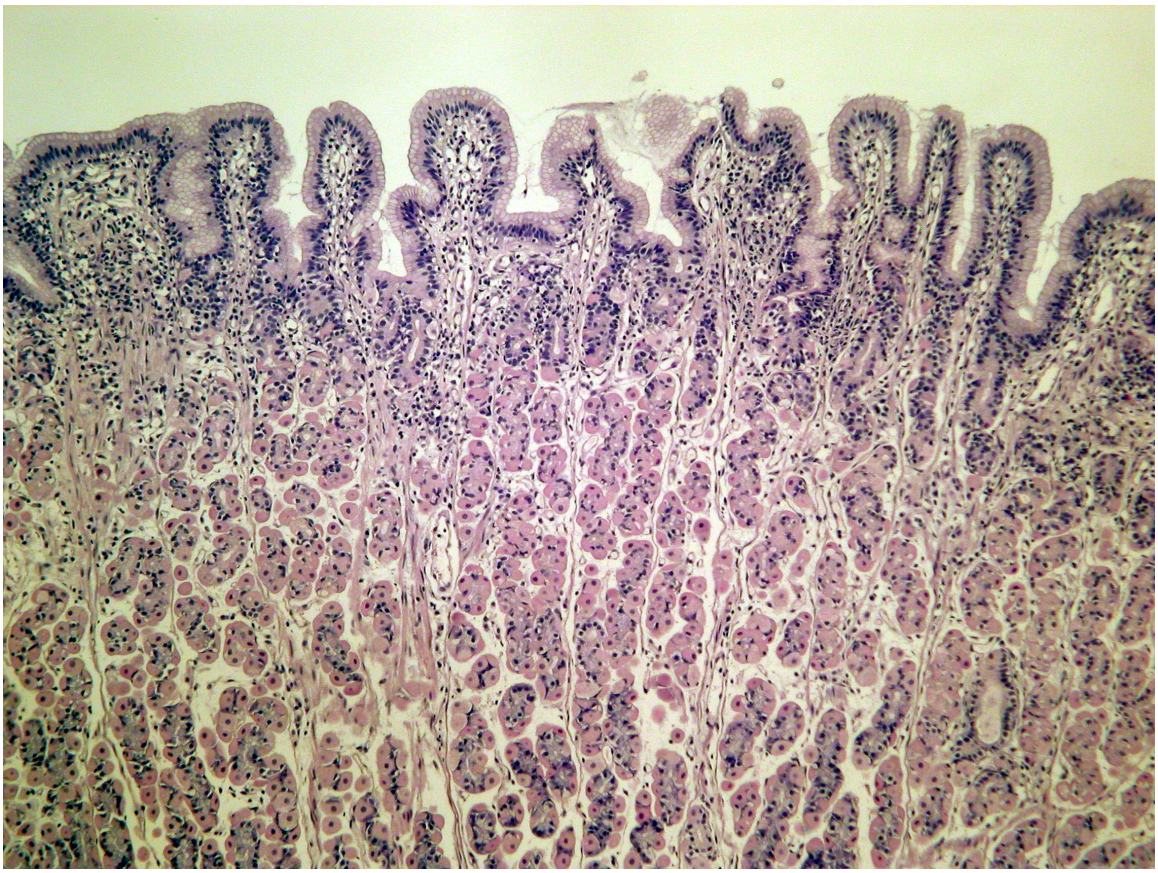

Plate 4. Effect of exercise on gastric mucosal histology: severe inflammation. Epithelium is intact. There is severe, diffuse infiltration of lymphoplasmacytic cells in the lamina propria.

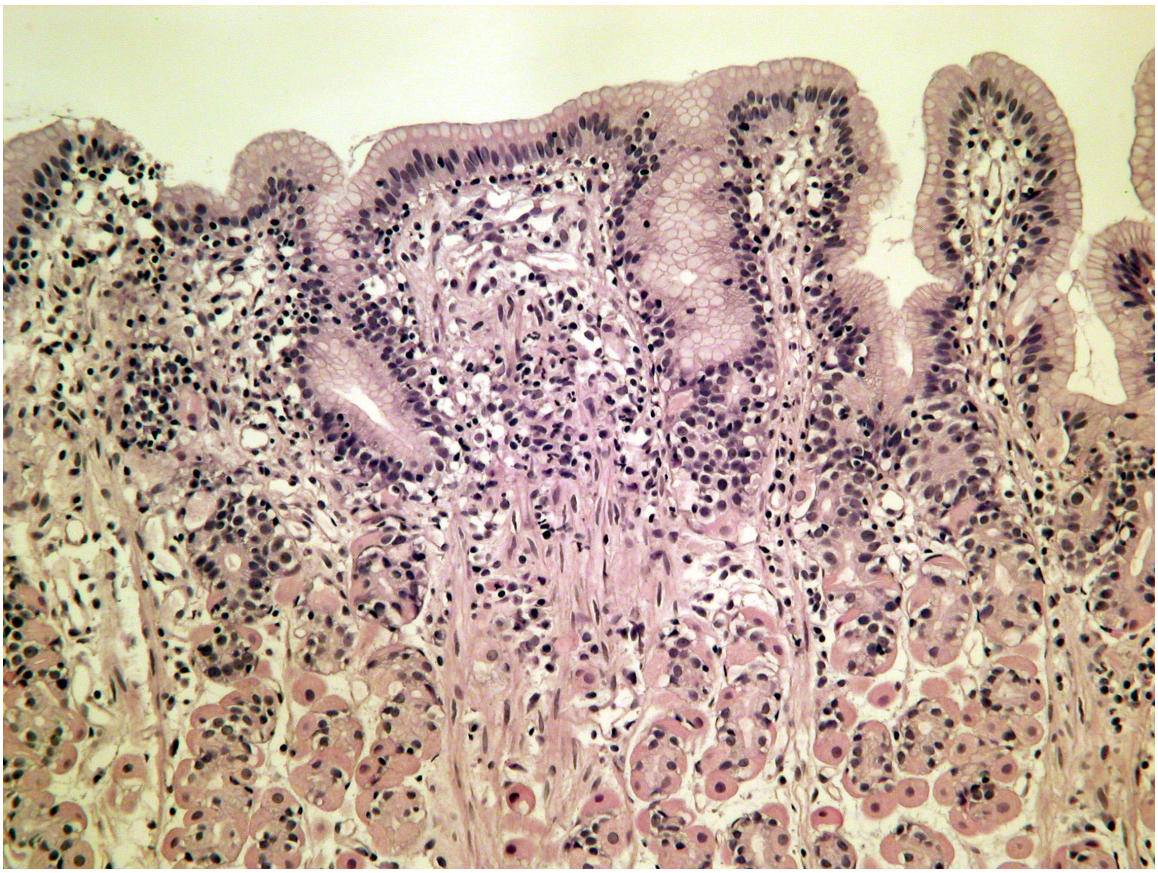

Plate 5. Effect of exercise on gastric mucosal histology: severe inflammation. Magnified view of plate 4. There is severe, diffuse infiltration of lymphoplasmacytic cells in the lamina propria and basal epithelium, with neutrophils evident.

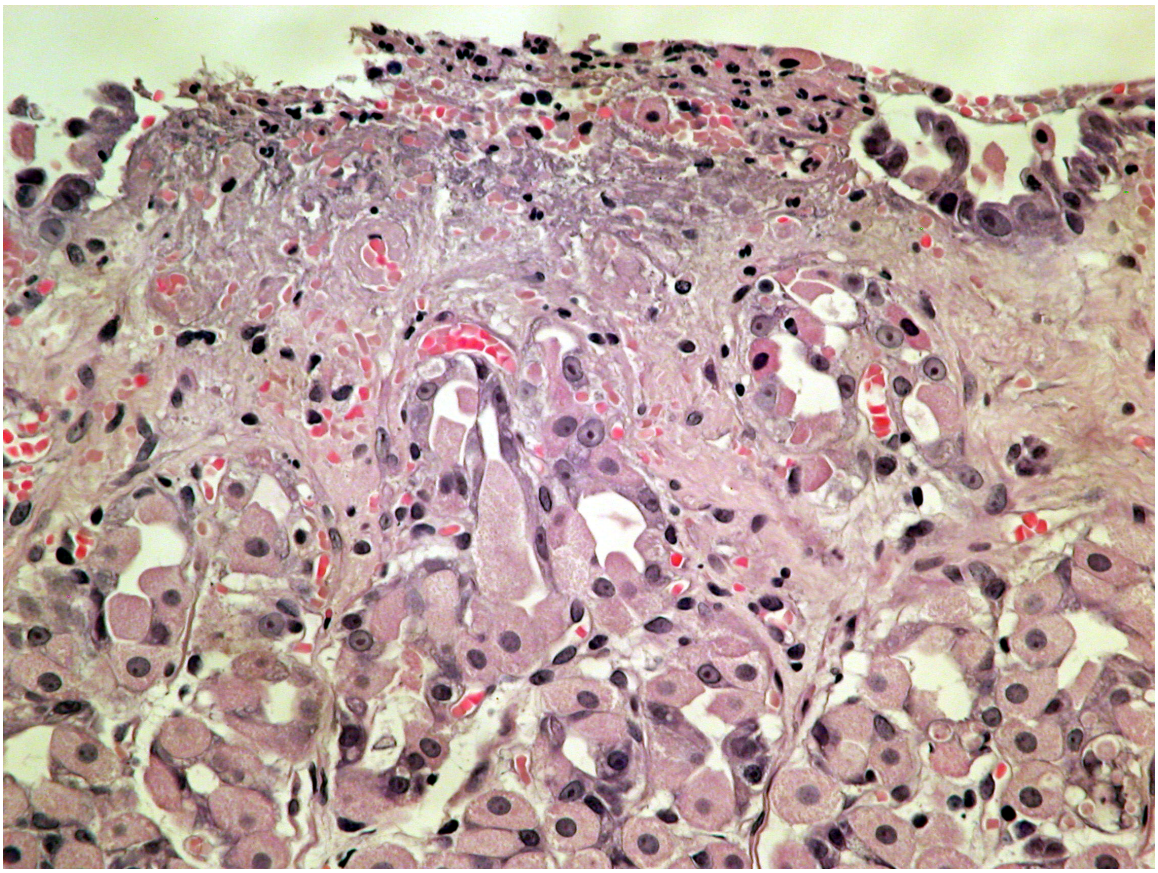

Plate 6. Exercise-induced gastric erosion. Epithelium is flattened or denuded with erythrocytes, lymphoplasmacytic cells, and neutrophils at the surface.

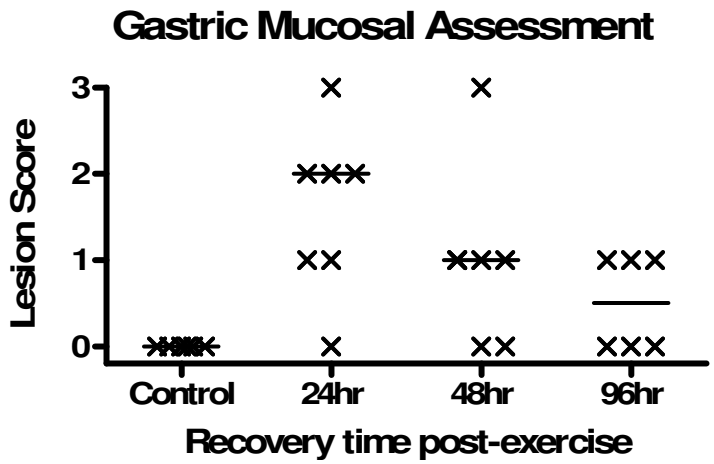

Figure 14. Post-exercise recovery of gastroscopic lesion scores. Assessments were considered clinically insignificant at four days post exercise. Post-exercise samples are from the same individuals followed over the four days (n=6) whereas control samples are different individuals (n=6). Individual scores are marked and lines indicate medians for each group.

		Unexercised	350 miles	400 miles	24hr post	48 hr post	96 hr post
Sucrose (mg/L)	mean	6.31	6.47	9.97	1.01*	1.62*	4.09
	SD	3.49	1.95	13.23	0.75	1.94	3.26
L/R	mean	0.18	0.18	0.13	0.15	0.14	0.18
	SD	0.09	0.04	0.04	0.05	0.10	0.09

Table 1. Gastrointestinal permeability following exercise and rest. Trained, rested control dogs are compared to those exercised for 350 and 400 miles and sampled four hours post exercise as well as another group exercised for 350 miles and sampled at 1, 2, and 4 days after exercise. * indicates significant difference ($p < 0.05$) from unexercised control dogs. L/R= serum concentration of lactulose divided by rhamnose for each individual.

Experiment #3: Dietary Antioxidant Therapy

A pharmacokinetic profile (analysis by Mars, Inc.) of the antioxidant supplement is provided in appendix I.

Glutathione analyses were done for whole blood due to ambient conditions leading to freezing of samples prior to erythrocyte and plasma separation. Exercise caused a decrease in the whole blood total glutathione (Figure15; $p = 0.0007$) and both components, whole blood GSH (Figure 16; $p = 0.0376$) and GSSG (Figure 17; $p = 0.0012$). Flavonoid treatment did not affect total glutathione concentrations (Figure15; $p = 0.1171$). Oxidative stress did not occur with respect to exercise; however, flavonoid-treated animals had a significantly more oxidized proportion than placebo treated animals (Figure 16). The difference due to flavonoid treatment occurred in reduced glutathione (Figure 17; $p = 0.0199$) but not in the oxidized form (Figure 18; $p = 0.3365$).

Flavonoid treatment did not affect oxidative products of cellular damage, ethane and pentane, however, both significantly decreased after exercise (Figure 19A; $p = 0.0003$; Figure 19B; $p < 0.0001$, respectively). Pentane was heavily affected by ambient levels as means were below a zero level after ambient subtraction (Figure 19, B). Ambient samples for the 100 mile time point were improperly stored, thus, samples for this time point could not be used due to unknown ambient contribution.

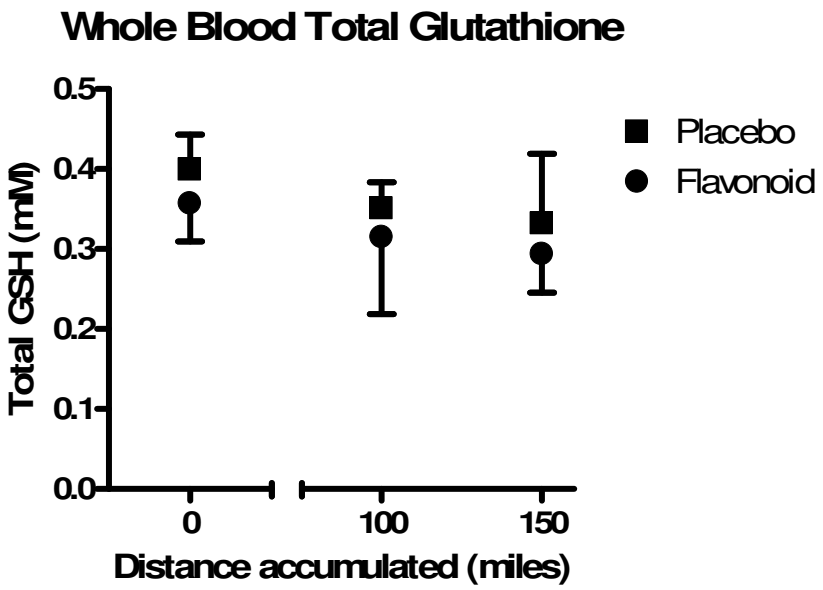

Figure 15. Exercise and antioxidant effect on total glutathione concentration in whole blood. Total glutathione changed with respect to distance ($p = 0.0007$) but not flavonoid treatment ($p = 0.1171$). Post hoc testing (Bonferonni post-test) did not determine a difference at a specific exercise distance. Circles and squares are means, and error bars represent standard deviation.

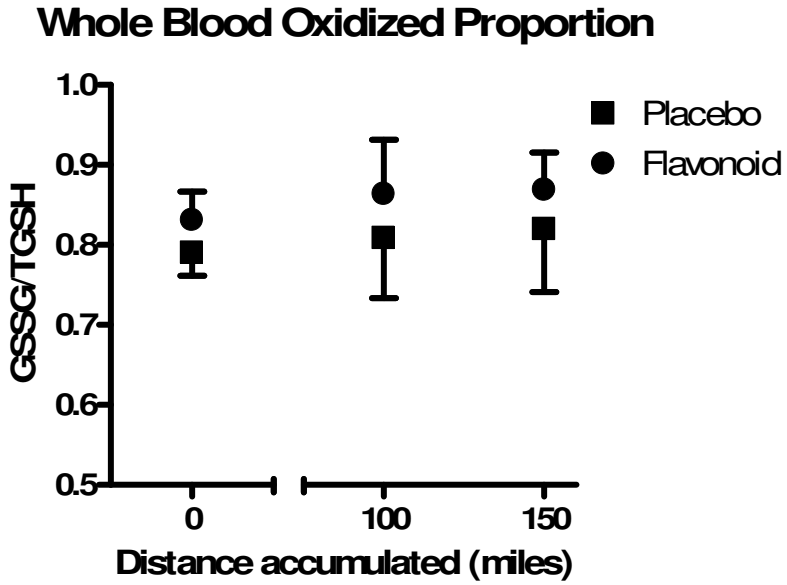

Figure 16. Exercise and antioxidant effect on oxidized proportion of glutathione in whole blood. Flavonoid treated animals were more oxidized ($p = 0.0090$) but no effect of exercise was evident ($p = 0.1693$). Post-hoc testing could not determine a specific distance at which the flavonoid effect was evident. Circles and squares are means, and error bars represent standard deviation.

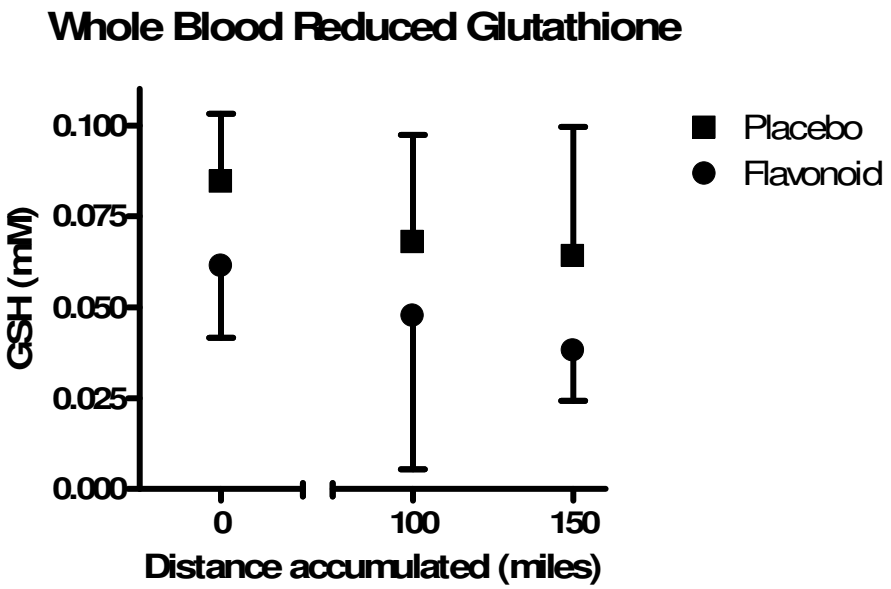

Figure 17. Exercise and antioxidant effect on reduced glutathione concentration in whole blood. Reduced glutathione was different with respect to flavonoid treatment ($p = 0.0199$) and exercise accumulated ($p = 0.0376$). The flavonoid treatment difference could not be localized to a specific distance. Circles and squares are means, and error bars represent standard deviation.

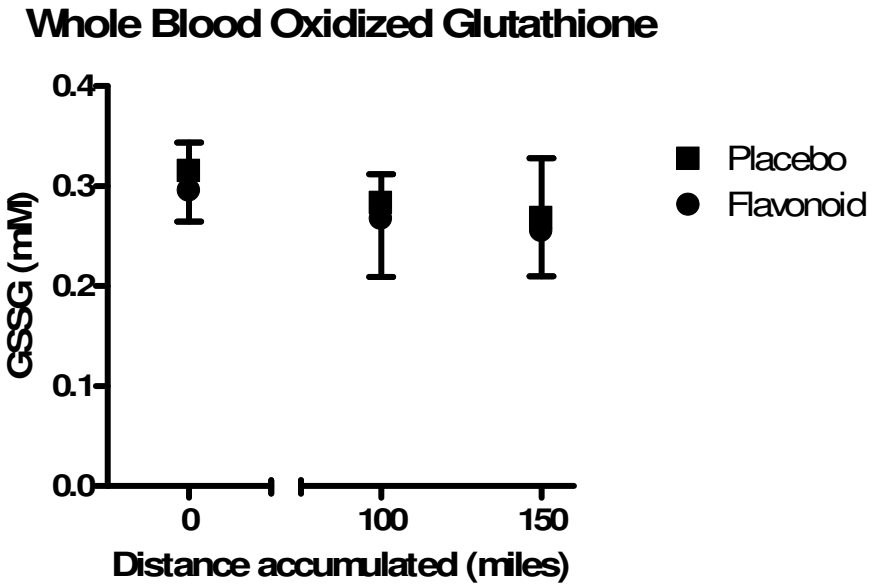

Figure 18. Exercise and antioxidant effect on oxidized glutathione concentration in whole blood. Oxidized glutathione was different with respect to exercise accumulated ($p = 0.0012$) but not flavonoid treatment ($p = 0.3365$). Circles and squares are means, and error bars represent standard deviation.

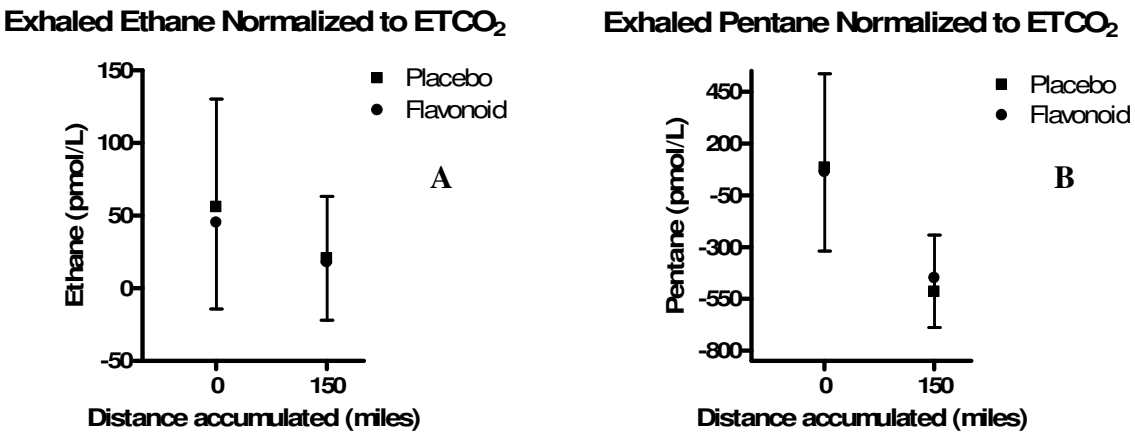

Figure 19. Ethane and pentane from expired breath before and after 150 miles of exercise while receiving either flavonoid antioxidant or placebo. Ethane and pentane represent free radical damage to ω-3 and ω-6 unsaturated fatty acids, respectively. Both ethane and pentane were different with respect to exercise accumulated ($p = 0.0003$ & $p < 0.0001$, respectively), but not flavonoid treatment ($p = 0.4238$ & $p = 0.6149$, respectively). Circles and squares are means, and error bars represent standard deviation.

Gastrointestinal lesions and permeability:

After 150 miles of exercise 60% of dogs had grossly abnormal gastric mucosa (Figure 20). Flavonoid treatment did not affect lesion scores after exercise. Flavonoid treatment reduced carbonylation of proteins in biopsy specimens obtained at endoscopy ($p=0.0493$, Figure 21). No difference occurred in serum sucrose with respect to exercise ($p = 0.2438$) or flavonoid ($p = 0.9416$) treatment (Figure 22). Furthermore ulcer score was not correlated to post-exercise serum sucrose concentrations (Spearman $r= -0.1892$, $p= 0.4243$). Likewise intestinal permeability (Figure 23) did not change with exercise ($p = 0.0821$) or flavonoid treatment ($p = 0.3513$).

Histologic assessment of gastric mucosal biopsies was not different with flavonoid treatment. In the untreated group the mucosal epithelium appeared normal in the untreated group, and 4/8 untreated dogs had normal lamina propria while the other four contained disseminated to multifocal lymphoplasmacytic infiltrates with 2/8 in this group containing neutrophilic infiltrates. One dog (1/12) in the flavonoid-treated group had a discrete area of denuded epithelium overlaid by fibrin with mild edema in the underlying lamina, with the remaining 11/12 flavonoid-treated dogs having normal histological appearance of the gastric epithelium. Four of 12 flavonoid-treated dogs had normal histological appearance of the lamina propria, while 4/12 had very few, scattered lymphoplasmacytic cells. Of the remaining 4/12, 2/4 had discrete foci of lymphoplasmacytic and neutrophilic infiltrates, 1/4 with a regional infiltrate of lymphoplasmacytic and neutrophilic infiltrate with numerous eosinophils, and 1/4 with a disseminated lymphoplasmacytic infiltrate. All samples contained helical bacteria.

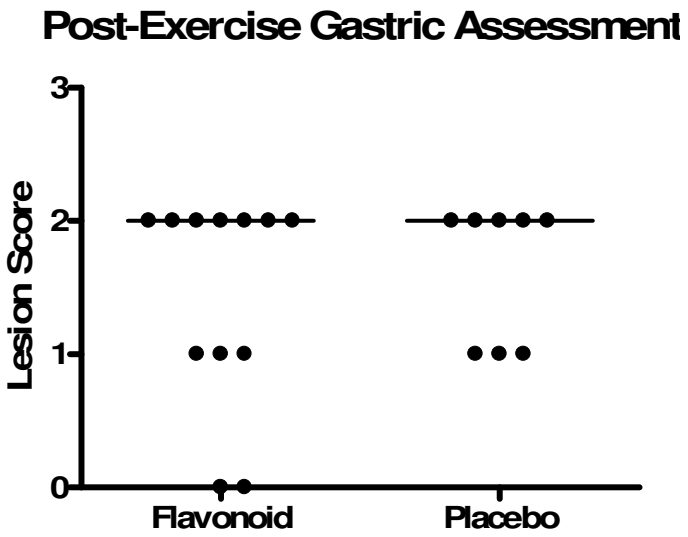

Figure 20. Subjective assessment of the gastric mucosa after 150 miles of exercise in dogs receiving either flavonoid antioxidant or placebo. No difference was found with flavonoid treatment (p = 0.7236). Individual scores are marked, and the overlaid line represents median for each group.

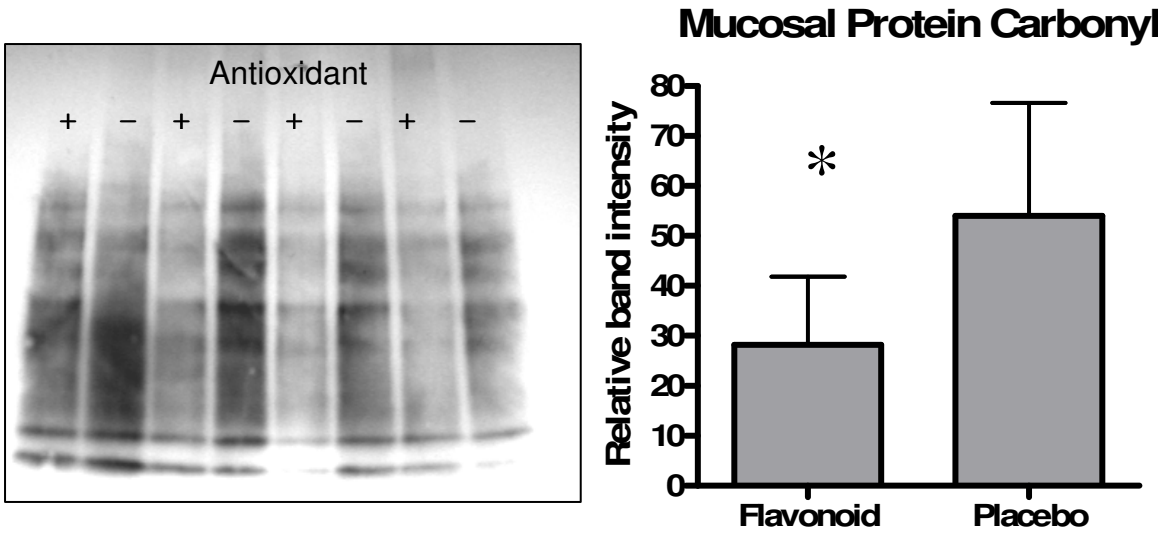

Figure 21. Effect of antioxidant on protein carbonyl content of post-exercise gastric mucosal biopsies. Flavonoid treated dogs had less protein carbonylation (p=0.0493) following 150 miles of exercise. Bars represent mean relative intensity, and error bars represent standard deviation.

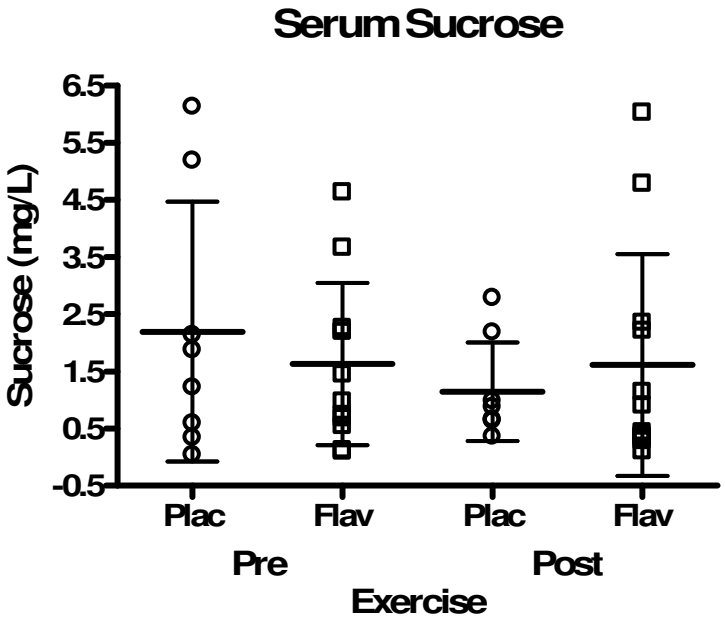

Figure 22. Exercise and antioxidant effect on serum sucrose concentration. No difference was found for either exercise (p = 0.2438) or flavonoid treatment (p = 0.9416). Plac= Placebo treated; Flav= Flavonoid treated. Circles and squares indicate individual measures and mean with bars representing standard deviation are overlaid.

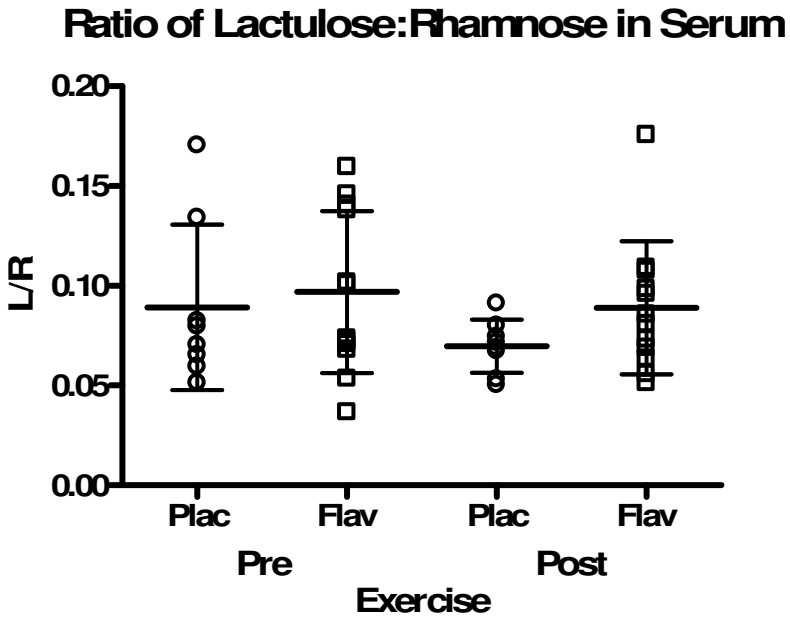

Figure 23. Exercise and antioxidant effect on ratios of lactulose to rhamnose concentrations in serum. No difference was found for either exercise (p = 0.0821) or flavonoid treatment (p = 0.3513). Plac= Placebo treated; Flav= Flavonoid treated. Circles and squares indicate individual measures and mean with bars representing standard deviation are overlaid.

Experiment #4: Dietary Lipid Hydroperoxides

Lipid hydroperoxide content in the diet varied among different dietary components, but was similar to that used in published control diets (Figure 24). Frozen beef sticks (BS) contained the highest lipid hydroperoxide content (181.1 ± 55.6 nmol/g food), followed by dry kibble (DK: 129.7 ±30.4), beef broth (BB: 94.5 ±15.3), and wet kibble (WK: 64.1 ±19.1). Control diets used in studies of mucosal cell turnover have been shown to be approximately 100nmol/g food, whereas 12-fold increases in this amount have been utilized to show modulation of turnover.

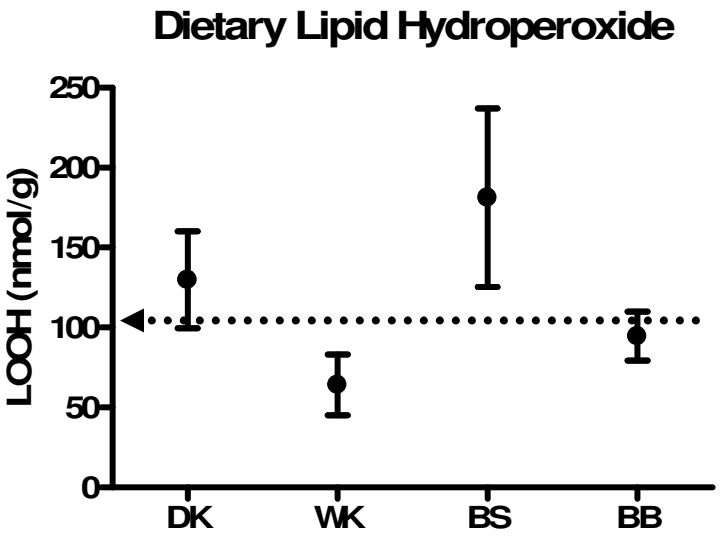

Figure 24. Lipid hydroperoxide content of four feedstuffs used to sustain sled dogs. Concentrations are per gram of food sample with wet kibble being drained prior to sampling while beef broth was not drained for solids. Dotted arrow represents the level found in control diets used in studies of lipid hydroperoxide modulation of intestinal mucosal turnover (approximately 1200nmol/g of food was used for mucosal turnover modulation). DK= Dry Kibble; WK= Wet Kibble; BS= Frozen Beef Stick used as snack; BB= Beef Broth made from adding beef sticks to drinking water. Data are group means ±SD.

CHAPTER V

CONCLUSION

Exercise-Induced Gastrointestinal Dysfunction

Onset and extent of exercise-induced gastric mucosal defects

Prevalence of gastritis/gastric ulceration was similar to previous findings in sled dogs after exercise. Previous evidence suggests approximately 50-60% of exercising sled dogs will have grossly visible gastric lesions[69;82-84] after the 1,100 mile Iditarod. Interestingly, the onset of grossly abnormal gastric mucosa occurred after the first 100 miles of exercise with the prevalence remaining relatively constant (~50%) through subsequent 100 mile bouts of exercise (Figure 7). If gastric barrier dysfunction is a function of the amount of exercise it would follow that the prevalence of ulceration would increase with the amount exercise. However, our findings indicate that gastritis/ulceration does not progress with further bouts of exercise[85]. Exercise must be considered the initiating factor as untrained or trained, rested dogs do not exhibit these gastric defects[85]. Furthermore, the initiation and maintenance or propagation of gastric defects may be mechanistically different.

Few studies in human runners have examined the effects of endurance exercise on endoscopically visible gastric lesions. Running in humans may produce a similar prevalence of gastric disease in that erosive gastritis occurred in 17 of 24 runners during a

training regimen although only one instance of ulceration was noted[86]. While reported gastrointestinal symptoms may increase exponentially with duration of exercise in humans[87], it is unknown if gastric mucosal dysfunction is duration-dependant in humans, or if it plateaus as in multi-day exercise of sled dogs (Figure 7). Gastrointestinal symptoms may occur in response to many effects of exercise on the GI tract. Direct assessment of human gastric mucosa would be required to determine the onset, extent, and duration of gastric mucosal defects due to exercise.

Recovery of exercise-induced gastric mucosal defects

Exercise-induced gastric ulceration resolved after four days of rest, but histologically the presence of inflammatory cells in the lamina propria persists to at least nine days without exercise. We could not determine the resolution of the histologic gastritis because nine days of rest represented a trained, rested control group in our study. It is difficult to follow individual dogs over the course of recovery due to the need for fasting and general anesthesia for gastroscopy. We, therefore, do not know the extent to which our sample populations for each recovery time period were affected by exercise. However, the evidence would suggest that exercise-induced gross gastric lesions will be clinically insignificant by four days of rest and grossly normal after nine days of rest (Figure 14). Biopsy specimens indicated that epithelial layers were normal after four days of rest but underlying lamina propria continued to contain lymphoplasmacytic (5/6 dogs) and neutrophilic (4/6 dogs) infiltrates for at least nine days after exercise. These findings are from dogs that all had grossly normal gastric mucosa (trained control dogs, rested nine days). It appears that exercise training leads to adhesion and chemotactic

signals for leukocytes to migrate into the gastric mucosa. Leukocyte recruitment may involve antigenic stimulation and/or tissue damage occurring due to exercise.

Causes of exercise-induced gastric mucosal leukocyte influx

The gastric mucosa may be at risk for excess antigenic stimulation as it takes as little as 100 miles of exercise to cause mucosal defects in ~50% of sled dogs. Non-selective gastric permeability increase is detectable as early as 200 miles of exercise (using orogastrically administered sucrose)[85]. Presumably, these mucosal defects allow increased absorption of microbial or antigenic constituents that would lead to our demonstrated increased influx of leukocytes.

Increased permeability and/or focal gastric mucosal defects allow gastric acid to exacerbate mucosal injury. Gastric acid suppression is associated with decreased severity of exercise-induced gastric lesions in sled dogs treated with either a proton pump inhibitor (PPI)[83] or histamine antagonist (H_2 Blocker)[88]. Interestingly, acid suppression did not decrease the prevalence of exercise-induced gastric lesions. Acid suppressors are generally believed to reduce damaging effects of gastric acid on the mucosa; however, they potentially modulate gastric mucosal injury by reducing leukocyte influx or function. For example, an H_2 blocker was shown to reduce blood granulocyte numbers in gastric ulcer patients and a PPI reduced $O_2^{\cdot-}$ production by the granulocytes[89]. It is unclear whether direct damage from gastric acid or its effects on other factors, such as leukocyte influx, are the primary mechanisms of exercise-induced gastric mucosal damage. Gastric acid contributes to gastric mucosal injury and though the mechanisms remain elusive, acid suppressors remain a primary treatment of exercise-induced

gastritis/ulceration. Future studies utilizing acid suppressors are needed to define their potential role in prophylaxis of histological gastritis.

Tissue damage and inflammation may produce the required stimulation for leukocyte influx as well. I/R induction of gastric ulcers in rats led to increases in pro-inflammatory cytokines, IL-1β, TNFα, and intercellular adhesion molecule-1 (ICAM-1) expression[90]. It is also possible that free radicals produce chemotactic signals as it has been shown that superoxide radicals lead to neutrophil influx in a gastric I/R model in rats[41]. It was later shown in this model that changes in leukocyte and endothelial adhesion molecules mediate the neutrophil influx in reperfused gastric mucosa[91]. It is unclear if ischemia/reperfusion contributes to the leukocyte influx in sled dogs. Furthermore, it is unknown to what extent the leukocytes contribute to gastric mucosal dysfunction (discussed further in 'potential mechanisms of gastric ulcers'). Sled dogs may have intermittent episodes of gastritis/ulceration during training that yield antigenic stimulation or inflammation in the gastric mucosa. Hence, a chronic gastritis may develop with lymphoplasmacytic, and sometimes neutrophilic, influx which takes some time to resolve. A state of chronic gastritis potentially modulates the development of the gross lesions noted in our study. Further studies are required to define the role of gastric mucosal leukocytes, as well as to develop strategies to reduce or eliminate the exercise-induced chronic gastritis.

Gastrointestinal permeability

Gastric permeability to sucrose did not change after exercise, but was lower than unexercised control dogs in the initial rest period following exercise (Table 1). The fact that exercise did not result in increased serum sucrose concentrations is in contrast to

reports in humans (increased urinary excretion of sucrose)[18] and sled dogs[85], in which exercise resulted in an increase in gastrointestinal permeability to sucrose. One possible explanation is that our control group had abnormally increased gastric permeability to sucrose for reasons other than recent exercise. Previous evidence in sled dogs suggests the serum sucrose concentration in trained, rested dogs is in the range of 0.0-2.5 mg/L[69], while serum sucrose concentrations in trained, rested control dogs in Experiment #2 were greater. Control dogs in Experiment #2 had histological evidence of inflammation, thus our comparison may have been biased by ongoing inflammation in the gastric mucosa of our control group (implied from the inflammatory cell populations present in the gastric mucosa of the control dogs).

The decrease in permeability observed during the recovery period may indicate normalization once the effect of exercise is removed. Concurrent gastric mucosal defects may provide the stimulus as gross mucosal defects were present in the parallel study population examined over four days of recovery (Figure 14). We have previously described an association of increased serum glucocorticoid (cortisol) and gastric mucosal defects after exercise[84]. The stress/cortisol response may serve to "tighten" tight junctions. Tightening of intestinal tight junctions due to a glucocorticoid, prednisone, has been shown in intestinal epithelium subjected to TNFα[92]. Our data also provide evidence that the temporal relationship of permeability to gross defects during recovery may be sufficiently different to produce the difficulty in correlating the two findings at a similar time point of examination[69].

The elapsed time between sugar administration and serum collection may bias our results. Since there was no increased sucrose permeability despite gross mucosal defects

it is possible that two to four hours represents sufficient time for absorption and clearance of much of the administered sucrose. A pharmacokinetic profile of sucrose absorption and elimination has been applied to equine gastric permeability. In this study the peak serum concentration occurred as soon as 45 minutes post-administration and correlated well with gastric ulcer scores. Values had decreased approximately 33% by 90 minutes post-administration, but the time to complete elimination was not assessed[73]. Thus, the peak concentration and subsequent elimination in our study may have occurred before sampling. Factors such as renal clearance and individual hydration would then affect the analysis. It may also be that visible lesions represent foci of permeability that represent small fractions of the overall mucosal permeability[69]. Studies are needed to define optimal sampling protocols for gastric permeability markers such that they may be successfully used as less invasive gastric damage assessments.

Intestinal hyperpermeability due to exercise may take multiple exercise bouts to develop in sled dogs. The markers of intestinal mucosal permeability, which we have used in our study, were increased in sled dogs completing the 1,100 mile Iditarod sled dog race[69]. There was no detectable change in intestinal permeability with up to 400 miles of exercise (Table 1). Our collection time for samples was decreased to approximately 2 hours post-administration for Experiment #3. Pharmacokinetic analysis of this procedure indicated this to be an optimal time for these markers in dogs[93]. However, we could not define an increase in the L/R ratio used in a 150 mile challenge in which an antioxidant was added to the diet (Figure 22). In cell culture models, the tight junction portion of the monolayer barrier can be disrupted within hours and likewise sealing or restitution of defects takes hours. One the other hand, chronic disease is more

likely to elicit an increased intestinal permeability[93]. The challenge in Experiment #3 took place over two days of exercise and rest or three bouts of 50 miles followed by five hours of rest. Twelve hours of rest followed the final exercise prior to fasting and sugar administration. This challenge is relatively small duration compared to the previous description of sled dogs[69] and chronic disease in dogs[93] with increased intestinal permeability. Thus, intestinal permeability may take several days to develop, even though gastric mucosal defects may occur as early as one day of exercise. Furthermore, the ability to measure a change in hyperpermeability may be confounded by the capacity for repair, given the sampling times used in these studies.

Exercise-Induced Oxidative Stress:

Exercise did not induce a significant oxidative stress as determined by blood glutathione oxidation and exhaled ethane and pentane. Previous studies in sled dogs have shown changes in various markers indicating an exercise-induced oxidative stress. Hinchcliff, et.al.[42] showed increased isoprostane, an end product of free radical attack of arachidonic acid, in sled dogs after three successive days of running 58km/d. The increased isoprostane occurred after one day and continued to day three. Serum vitamin E, a free radical scavenger, was decreased also on days one and three. However, other markers in this study did not change, i.e. plasma total antioxidant status (measure of the plasma's ability to quench an exogenous free radical-generating reaction), superoxide dismutase (SOD), glutathione peroxidase (GPX), and ceruloplasmin. In a later study, the enzyme activities of SOD and GPX in erythrocytes were decreased following a sled dog race of approximately 1600km[94]. Another study found increases in a marker of oxidative damage to DNA in the plasma of dogs running for three days[65]. These sled dog studies

indicate that the products of free radical damage increase early in multi-day exercise. Further exercise, then, causes endogenous antioxidant enzyme activities to decrease presumably due to oxidative modification. Vitamin E could not be linked to muscle damage in exercising sled dogs[95], but it was related to a reduced incidence of retirement from exercise[64]. Vitamin E has since become a widely used supplement in sled dog racing. Despite the evidence for exercise-induced oxidative stress above, in a previous study from our lab, we could not define an increase in isoprostanes after multi-day exercise of 1,100 total miles[84]. Furthermore, we could not identify post-exercise oxidative stress with multi-day exercise up to 500 miles using erythrocyte and whole blood glutathione as well as the free radical products of cellular damage, ethane and pentane, in exhaled breath. Our results could be confounded by the use of vitamin E in racing sled dogs. The apparent lack of exercise-induced oxidative stress is discussed below, with respect to the individual measure used in our studies.

Exercise effects on glutathione

Glutathione is a ubiquitous endogenous antioxidant system that was not affected by exercise in our studies. The glutathione antioxidant system may be too robust to detect exercise-induced oxidative stress in well-trained sled dogs. Factors affecting our ability to detect blood glutathione oxidation are training effects on the capacity and rate at which the glutathione system can recycle (the sampling design relative to exercise), the exercise intensity, and the effects of increased GSSG on the erythrocyte mass. In humans that trained for ironman competitions, glutathione peroxidase activity is higher than matched, untrained controls[96]. This enzyme is responsible for oxidation of glutathione while breaking down peroxides. Thus, an effect of training may be to increase this and

other enzymes in glutathione cycling, increasing the turnover efficiency. Exercise training increases the capacity to combat exercise-induced oxidative stress in humans [97] and skeletal muscle glutathione peroxidase activity and glutathione content increase with training in dogs [98]. It is unknown if the glutathione-based erythrocyte antioxidant capacity can be increased with exercise training in sled dogs.

Exercise intensity contributes to the oxidant load on the antioxidant system, as free radical products of cellular damage increase with exercise intensity[99]. In humans exercising at 65% VO_{2max}, the oxidized glutathione proportion in whole blood increased to ~80% during a 90 minute exercise test. The oxidation was resolved to near normal by 30min after cessation of exercise [100]. Studies have shown free radical products of cellular damage in sled dogs [42;65;94], but the exercise intensity required to detect an increase in the oxidized proportion of glutathione remains unknown. Furthermore, in our protocol times from cessation of exercise to snap-freezing of blood for glutathione analysis approximated one hour. Recovery of the normal redox state of blood glutathione may have confounded our efforts to detect oxidative stress after exercise if it was occurring.

Oxidation of GSH may also affect the erythrocyte mass in exercising sled dogs. We have previously seen a decreased packed cell volume in exercising sled dogs (accepted for publication in the Journal of the American Veterinary Medical Association). Erythrocyte glutathione is a well known protective factor for lipid peroxidation and subsequent hemolysis as illustrated by congenital enzyme deficiencies in animals (enzymes that feed reducing equivalents to the GSH redox system) that reduce erythrocyte survival[101]. Exercise is associated with a decrease in total erythrocyte glutathione when all samples were pooled (Figure 2), and it approached significance in

Experiment #2 (Figure 8). Exercise caused a more definitive decrease in whole blood total glutathione during our antioxidant trial (Figure 15). It is possible that erythrocytes containing more oxidized glutathione may be preferentially scavenged from circulation or hemolyzed and the remaining erythrocyte glutathione content is less oxidized. As a result, our ability to detect an effect of exercise on the oxidized proportion would be reduced. Indeed, there is evidence that excess GSSG can be preferentially ejected from erythrocytes[102].

The pronounced shift of erythrocyte glutathione oxidation during the rest period following the first 100 miles of exercise was unexpected (Figure 12A). Endogenous antioxidant mechanisms may be upregulated during exercise, but decrease more rapidly than oxidant mechanisms during the rest period. It is likely that this mechanism would be present in the 300 and 400 mile samples if this were the case, yet the later samples did not display this relationship. It is possible that ischemia/reperfusion injury occurs following the initial transition to exercise of the first 100 miles. Anecdotally, sled dogs are more excitable and work harder in this initial exercise period, and exercise intensity has been shown to be inversely related to splanchnic blood flow in dogs. For example, graded treadmill exercise revealed that blood flow to the gut may be reduced by 50-70% when approaching VO_{2max}[51]. The graded exercise took approximately 30 minutes to reach 100%. Interestingly, blood flow to the stomach and large intestine decreased approximately 50% at the first measured intensity of 30% VO_{2max}. Thus, ischemia in the stomach and large intestine may be maintained for hours, especially in the initial exercise period, with subsequent reperfusion and production of ROS upon cessation of exercise. The initial exercise period's oxidative stress may provide the signals to upregulate

antioxidant mechanisms that reduce the overall oxidant effect in later sampling periods. The presence of ischemia/reperfusion is debated, yet this is a possible example of its occurrence in exercising sled dogs.

Another possible explanation for the apparent increase in glutathione oxidation during the rest period is dietary lipid hydroperoxides. The dogs consume the majority of their daily calories during the prolonged rests, and these meals may include reactive lipids. Gastrointestinal GSH is a major detoxification pathway for lipid hydroperoxides. While it is not known to what extent GSH in gastrointestinal tissue directly influences erythrocyte GSH, dietary hydroperoxides may escape breakdown to enter the circulation. For example, humans consuming either a highly oxidized meal or simply a high fat meal (1,200 kcal meal, 55% fat) leading to hyperlipidemia have an increase in oxidized chylomicrons[103] or serum lipid hydroperoxides[104] respectively. With sled dog diets reaching 60% of metabolizable energy as fat it is plausible that erythrocytes experience an increased post-prandial oxidant load. Similar to the recovery periods after 300 and 400 miles of exercise, extended recovery periods revealed variable levels of oxidation to four days post exercise (Figure 13). The initiation of exercise appears to be a stimulus for multifaceted changes in the body's redox status that is based on individual factors and may not resolve for days following exercise.

Exercise effects on ethane and pentane

Measurement of exhaled breath markers of oxidative stress was complicated by a variety of experimental conditions, including ambient conditions that did not allow a reliable pre-exercise measure for comparison (Figure 6), the timing of marker collection relative to marker production, and possible endogenous breakdown of markers. We

could not detect pentane in Experiment #1 and levels were heavily affected by ambient conditions in Experiment #3. As many authors have encountered, ambient conditions confounded our analysis of endogenous ethane and pentane. To identify changes in the products of cellular damage of ω-3 and ω-6 fatty acids, we measured exhaled ethane and pentane as markers of ongoing damage following exercise at increasing distances. This method has a great potential due to the non-invasive nature. We reported our results with the ambient amount subtracted, then normalized to each individual's end tidal CO_2 ($ETCO_2$), expressed as a function of group $ETCO_2$ to allow comparison from dog to dog. This is a simple way to minimize variability in the concentration of exhaled markers due to differences in ventilation, and assumes that the respiratory drive is to maintain the overall average and deviations from this create proportional changes in ventilation and, thus, exhaled marker concentration. For example, given the overall average of resting individuals, a decrease from this value would indicate a dilution due to pulmonary dead space and exhaled biomarkers would proportionally decrease with $ETCO_2$. Thus, multiplying by the inverse of the dilution (unitless ratio) should normalize the resulting biomarker concentration to reduce biomarker variability induced by minor differences in ventilatory pattern. In Experiment #1, ambient ethane concentrations were much greater in the pre-exercise group due to the location chosen for sampling (as well as an inability to "pre-screen" such locations or easily transport hydrocarbon free air in large cylinders to a collection site). Determinations of equilibration with ambient ethane have been inconsistent and may be 4 minutes up to hours[81]. The variability of this equilibration period contributes to the highly negative ethane concentrations relative to other measures and confounds the statistical analysis. It was therefore excluded from the analyses in

favor of the more consistent findings in the post-exercise locations (Figure 6). Furthermore, this relatively consistent environment was extended, as the mean of previous measures, for use as the approximation of ambient ethane for the final measure (post-500 miles). This time point should be examined carefully due to this, but it could not be analyzed otherwise due to the contribution of ambient amounts. In future experiments, hydrocarbon-free air should be used in the non-rebreathing circuit due to the heavy use of various hydrocarbon fuels for heat in these field research settings. Furthermore, a continuous measure of both $ETCO_2$ and the average contribution of the mixed fraction would provide a more accurate representation of dilutional effects that could be used as the ratio for normalization of exhaled biomarkers.

Our sampling protocol could not detect ongoing damage during exercise, rather, it provided a post-exercise status of the ongoing damage during rest approximately one hour after cessation of exercise. The kinetics of ethane exhalation are faster than glutathione equilibration in that free radical attack of ω-3 fatty acids yield ethane that is exhaled as soon as it reaches the lung. Pentane may also be affected by ongoing metabolism as it does not diffuse through the lung into exhaled air as readily as ethane. Pentane is metabolized in tissues such as the liver likely by cytochrome p-450[105]. Our exercise model may have the metabolic capacity to metabolize endogenous pentane to undetectable levels or it may not be produced. It is likely the former predominates as ethane was detectable and the ratio of dietary ω-3 to ω-6 fatty acids should be reflected by about a four-fold increase in pentane relative to ethane production.

Exhaled ethane decreased as exercise progressed. Measurements of expired ethane were highest after 100 miles of exercise (Figure 6). We did not have a pre-

exercise value to compare, however, we found that expired ethane significantly decreased during the subsequent exercise bouts (Figure 6). Considering the rapid elimination kinetics of ethane, the detection of changes in expired ethane during the rest periods can be viewed as evidence of changes in oxidative stress during those rest periods, and thus this finding is comparable to our findings from erythrocyte glutathione in which the initial exercise plus rest period (Figure 12A) produces an oxidant stress. A range of reactions discussed above are likely involved in our findings of increased oxidized glutathione after exercise plus rest (Figure 12A), thus increasing the lag time from free radical production to measurable increases in oxidized glutathione. Taken together the results provide evidence that oxidative stress due to exercise may require cessation of the exercise as it is greatest in the rest period after initiation of exercise and further free radical insults occurring during exercise and/or subsequent rest periods are combated by a sufficient antioxidant capacity.

Effect of flavonoid antioxidant on exercise-induced oxidative stress

Sled dog whole blood was more oxidized with the application of a dietary antioxidant (Figure 16), but not with exercise. When examining the glutathione components of reduced and oxidized amounts, the significant effect was exhibited on the reduced glutathione (Figure 17) rather than the oxidized component (Figure 18). A finding of increased whole blood oxidized proportion may be a spurious effect of the antioxidant on the reduced GSH content in turn affecting the subsequent calculation of GSSG/TGSH. These results must be interpreted carefully. The extensive oxidation of the samples is questionable as the effect was present in the pre-exercise samples as well. This was potentially due to freezing hemolysis that was encountered during sampling. It

has been shown that oxidized glutathione in plasma increases substantially with hemolysis[106]. Plasma provides a more oxidized milieu which may be contributing to our findings. It may also be that hemolysis releases reducing equivalents and GSSG into the plasma thereby simply diluting the available constituents needed for enzymatic reduction of GSSG. Furthermore, hemolysis may result in release of redox active metals such as copper which can potentiate a pro-oxidant reaction of flavonoids[107]. Otherwise, our findings would be some of the highest known levels of oxidation at rest and after exercise.

Blood glutathione has been shown to increase with exercise presumably due to upregulation of counter-regulatory hormones. When a carbohydrate supplement was added during exercise glucose and insulin increased while blood glutathione remained unchanged. In the unsupplemented, group blood glutathione increased during exercise[108]. Flavonoid treatment reduced the amount of GSH present in each sample although the effect on TGSH was not significant (Figure 15). A potential mechanism for our findings is that a flavonoid antioxidant reduces the signaling involved in increasing glutathione in blood due to exercise. If the flavonoid contributes to a decrease in blood glutathione, then the subsequent calculation of the oxidized proportion may be spuriously affected. All other known mechanisms for flavonoid action in blood, such as decreasing hemolysis[109;110] or decreasing oxidative stress[111], would result in findings opposite of the increased oxidation found here if it is indeed what has occurred.

A direct flavonoid effect on blood parameters is difficult to assess as few indications of flavonoid bioavailability and metabolism are available[112]. It is unknown to what extent the flavonoids were absorbed and analysis of serum from our subjects by

Mars, Inc. yielded immeasurable amounts of flavonoids for which there was a known, purified standard. However, it is unknown if absorbed flavonoids may reach the liver and reduce oxidative stress in this tissue in turn reducing the liver's response which may lead to changes in availability of GSH precursors, counter-regulatory hormone effects, or reticuloendothelial clearance of erythrocytes.

Exercise-Induced GI Dysfunction Relative to Oxidative Stress

No correlation of systemic oxidative stress markers to gastrointestinal dysfunction was demonstrated. Systemic oxidative stress was not directly related to gastrointestinal dysfunction in the present study. As a general rule, free radicals are highly reactive and primarily produce their effects close to their production. Tissues such as muscle and endothelia that are heavily utilized during exercise can produce free radicals, however, their direct contribution to potential free radical mediated gastrointestinal damage is likely minimal. On the other hand, reduction of endogenous antioxidants could contribute to damage away from a site of free radical production. Our studies showed no correlation of the ubiquitous antioxidant in blood, glutathione, to the gross damage or mucosal barrier permeability markers. As discussed above, we did not demonstrate oxidative stress due to exercise in the studies utilizing blood glutathione. Other endogenous antioxidants could be involved and indeed tocopherol (vitamin E) has been shown to be decreased after exercise in sled dogs[42].

Presently, an attempt was made to describe the potential for exercise-induced oxidative stress to provide a secondary effect of exercise that may lead to gastrointestinal dysfunction. Our only evidence for this association is the oxidative stress response after 100 miles of exercise plus six hours of rest and meals (Figure 12). This coincides with

our earliest described evidence of gastric dysfunction which also occurs after 100 miles of exercise (Figure 7). Otherwise no correlation of oxidative stress markers to gastrointestinal dysfunction was found. However, there are limitations to our study in that oxidative stress is a multifactorial system of free radical production and endogenous antioxidant systems throughout the body. Furthermore, we only describe two markers of this system at a whole body level.

Effect of dietary flavonoid antioxidant on GI dysfunction

The flavonoid antioxidant used in this study did not prove efficacious in reducing or preventing gastrointestinal barrier dysfunction. The flavonoid did show a local reduction of oxidative stress in the form of reduced protein carbonyls (Figure 21). Protein carbonyls are known to be increased in gastric mucosa due to cold/restraint stress[38] and ischemia-reperfusion[113] that leads to gastric ulceration in rats. However, our demonstrated reduction in the free radical adducts was insufficient to decrease gastric ulceration if oxidative stress does contribute to gastric ulcers. These are preliminary findings in that we do not have untrained or unexercised control values for protein carbonyls present in gastric mucosal biopsies. For future studies, it will be necessary to define the normal levels of protein carbonyls in gastric mucosa prior to an exercise challenge. This information will help define any increase in this form of free radical modification to mucosal proteins due to exercise.

The flavonoid supplement did not appear to affect the histologic assessment of gastric mucosal biopsies. The presence of leukocyte infiltrates in sled dog gastric mucosa occurs with training and persists for at least nine days of rest (Experiment #2). The persistence of cellular infiltrates may confound our assessment of the effects of the

flavonoid supplement on histologic assessment of gastric mucosa. Flavonoids have been shown to decrease cytokine-induced cellular adhesion molecules in human endothelial cells[114] which would presumably decrease cellular influx. The supplement was given for a short duration, in our trial, relative to the persistence of leukocytes. Free radicals are associated with inflammatory cell influx into gastric mucosa[41], however, the ability of our dose of antioxidant to prevent free radical associated extravasation and the duration of treatment utilized may not be optimal. Future studies utilizing higher doses and/or longer durations of treatment are needed to define the potential antioxidant reduction of leukocyte influx into gastric mucosa, as well as any subsequent effect on mucosal dysfunction.

A primary consideration for these data is the adequacy of the dose to provide a demonstrable reduction in gastric ulcers. A concern for canine subjects was the toxicity of methylxanthine compounds that are present in the flavonoid extract as they occur naturally in the parent cacao extract. The dose used in the study was reduced such that toxicity could be avoided (at least 100-fold reduction in theobromine and caffeine from published toxic doses). Dogs received 500mg flavonoids per day and dogs average 50kg body weight. The dose used was much less than that previously described for reducing ethanol-induced ulcers in rats, i.e. 500 mg/kg of a crude liquor composed of 55% polyphenols was used[79]. When used at this dose, ethanol-induced ulcer surface area was decreased by 82.7%. At the dose used in our study we could not detect a decrease in gastric mucosal defects. Further studies are needed to define a dose and duration of dietary antioxidants that may affect exercise-induced oxidative stress and gastrointestinal dysfunction.

Lipid hydroperoxide content

Lipid hydroperoxides can be toxic to mucosal cells and modulate mucosal cell turnover at sub-toxic concentrations. Both markers of apoptosis and proliferation are shown to be suppressed in rat enterocytes when fed lipid hydroperoxides at 1200nmol/g food. The control diet in this case contained 100 nmol/g food. The analysis of the sled dog diet showed the lipid hydroperoxide content to be in the range of this control diet (Figure 24). It is not known what differences there may be in the "bioreactor" activity of canine stomachs especially when the diet utilized for exercise performance has 60% of the caloric content as fat. This high fat content mixed with myoglobin, hemoglobin, and/or other redox active elements in the acidic gastric environment could potentially increase the hydroperoxide delivery to the gastrointestinal tract by as much as eight-fold[115]. Hydroperoxides at this concentration may lead to modulation of the mucosal cell turnover. As mentioned above, potential hydroperoxide propagation could also lead to spillover into the circulation[103] in turn increasing oxidative stress in the blood and other tissues. Thus, further studies are needed to define the potential intragastric propagation of lipid hydroperoxides with respect to exercise.

Potential mechanisms of gastric ulcers

Exercise-induced gastric ulceration is a serious consequence of endurance exercise. The mechanisms involved have been difficult to define due to the variability in occurrence and myriad of events potentially involved. We could not define any direct relationship of oxidative stress to the mucosal defects seen in our study. Findings from our study provide some indication for mechanisms involved.

Helical bacteria were noted in most biopsy specimens from Experiment #2 and all specimens in Experiment #3. *Helicobacter pylori* is a primary cause of human gastric

ulceration, but it is rarely isolated from dogs. Studies have shown a high prevalence of infection with spiral organisms in dogs. This high prevalence confounds investigations of infectious pathology as both healthy dogs and dogs with gastric disease are equally infected. Our results indicate that there is a high prevalence of infection in sled dogs, yet in only one instance were bacteria associated with an active inflammatory lesion. This bacterial association provides reasonable evidence that helical bacterial may play a part in some instances of gastritis/ulceration, or it may be coincidental. The bacteria did not appear to be associated with inflammatory cell infiltration in other instances. It cannot be determined from this study whether or not the bacteria are involved in later stages of inflammation as we only obtained biopsy specimens from non-ulcerated gastric mucosa. Furthermore, the potential for bacterial modulation of gastric disease in this population cannot be ruled out given the high infection rate. The bacterial contribution to gastric disease requires further study.

As our studies have attempted to address, evidence exists for a free radical mechanism contributing to "stress" ulcers, however, our study could not confirm this. The stress involved in exercise has been difficult to define as a mechanism for gastric ulceration. Hypothalamic-pituitary-adrenal axis activation is commonly considered a cause as exogenous glucocorticoid dosing can produce gastric ulceration in 100% of healthy dogs[116]. Whether endogenous glucocorticoids reach concentrations to elicit this response has not been determined although there is a relationship of increased cortisol and increased ulceration in sled dogs[84]. Other stress responses may also contribute to gastric ulceration. Vasoconstriction due to local or systemic catecholamine release and potentially a subsequent ischemia-reperfusion has received criticism in the dog. Previous

studies have shown that dogs do not exhibit a reduced splanchnic flow through the cranial mesenteric artery[48]. Furthermore, urinary catecholamine metabolites were determined to be very low after exercise in a study population similar to ours[117]. On the other hand, celiac arterial flow has not been determined with respect to exercise in the dog. Radioisotope deposition in the stomach and colon has been shown to be reduced in exercising dogs with higher intensity exercise yielding lower deposition[51]. Furthermore, catecholamine spillover from splanchnic tissue has been demonstrated to be small in dogs and, thus, represents a large uptake capacity of splanchnic tissues[118]. It is unclear if this uptake provides vasoconstrictive receptor activation, however. The present antioxidant-induced reduction of gastric mucosal protein carbonyl indicates that oxidative modification or damage occurs in this tissue and an ischemic/reperfusion injury potential requires further tissue specific study. However, we provide evidence that oxidative stress occurs after 100 miles of exercise plus six hours of rest and meals. This indicates a potential reperfusion event leading to a systemic oxidative stress illustrated with erythrocyte glutathione oxidation, although it cannot be specifically localized to splanchnic tissue. One hundred miles of exercise also provides the initiation of changes in gastric barrier integrity that are non-progressive.

Inflammatory cell populations are present and may modulate gastric disease beyond the point of gross gastric mucosal barrier recovery. Free radical damage in gastric mucosa of ischemia-reperfusion ulcer models has been associated with leukocyte influx, especially neutrophils[41;91], although the extent of their contribution may be less than other free radical generating systems[119]. Since the initiation of gastric disease may differ from ongoing inflammation, biopsies were taken to survey grossly unaffected

gastric mucosa. In Experiment #2, biopsy specimens from control dogs that had been rested for nine days continued to display inflammatory cell populations in the lamina propria similar to one, two, and four days post-exercise. Our findings provide evidence for inflammatory cell-mediated damage and a persistent, ready supply in the deeper lamina propria.

Exercise training was associated with increased gastric mucosal cellular infiltrate, and the increased cellular infiltrate may modulate barrier disruption by changes in the rate of epithelial apoptosis. Intestinal inflammation leads to increased Fas-ligand mediated mucosal epithelial apoptosis[120]. However, as illustrated in colonic epithelial cells, a Fas-ligand mediated apoptosis yielding 50% cell loss did not change barrier permeability[121]. Inflammation potentially stimulates an increased gastric epithelial apoptosis. Gastric mucosa undergoing apoptosis may initially flatten and provide a barrier, but permeation to small molecules and decreased production of overlying mucous layers may not allow the surviving mucosa to combat the apoptotic insult with an intact barrier.

The initiation of gastric mucosal defects may be independent of factors that maintain or propagate them. For example, evidence suggests that gastric acid reduction reduces the prevalence of gastric ulcers, but does not prevent them[83]. This would seem to indicate that gastric acid leads to maintenance of gastric ulcers. It is difficult to believe that gastric acid initiates ulceration given the fact that the mucosa is constantly bathed in the acidic gastric juice and gastroscopy of rested sled dogs yields few indications of gastritis that cannot be explained by other factors[85]. Decreasing gastric acid would decrease the time for healing which subsequently would decrease prevalence.

Summary

The gastrointestinal barrier is sensitive to exercise and has been considered a non-athletic organ[4], i.e. its capacity to combat effects associated with exercise cannot be increased with training. Sled dogs exhibit similar effects of exercise on their gastrointestinal tract as do humans. The cause(s) of these effects remain ill-defined. In the present study, we could not define an association to oxidative stress nor could we ameliorate the gastric mucosal barrier dysfunction with a flavonoid antioxidant. Studies of in vivo free radical damage are difficult due to the extreme reactivity/short duration of existence. More studies are needed to define 1) the potential for hypoperfusion of the GI tract of sled dogs with exercise; 2) the potential of free radical-induced initiation of gastric mucosal defects; 3) long-term effects of gastric disease and potential intestinal hyperpermeability.

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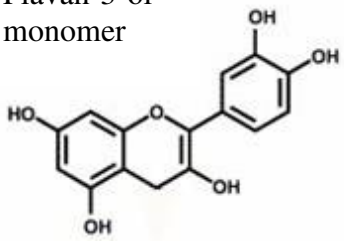
APPENDIX I

Pharmacokinetic profile for flavonoid antioxidant

A pharmacokinetic profile of the flavonoid antioxidant used in this study was performed by dosing laboratory dogs and subsequently collecting and analyzing plasma samples (analysis by Mars, Inc). The flavonoid antioxidant provided by Mars, Inc. was a detheobrominated, decaffeinated cocoa flavanol extract processed from cacao liquor water-soluble crude polyphenols. The final theobromine content was 0.184% and caffeine content was 0.092% by weight. The cocoa flavanol extract components of interest were based on a flavan-3-ol unit and had the following breakdown of components (% of total): monomers (4.6), dimers (28.8), trimers (22.4), tetramers (17.4), pentamers (13.1), hexamers (8.5), heptamers (3.4), octamers (1.7), nonamers (1.8), and decamers (0.8).

Flavan-3-ol monomer



Six purpose bred laboratory mixed breed hounds (2 years old; intact, 4 female, 2 male) were used in the study. Two capsules providing approximately 256mg of cocoa flavanols were administered to each dog after overnight fasting. No meals were givin during the sampling period. Peripheral catheters were placed in the cephalic vein of each dog prior to antioxidant administration. Blood samples were aspirated into 3cc syringes and transferred to lithium heparin blood tubes. Blood samples were acquired prior to administration and at 15min, 30min, 1hr, 2hr, 3hr, 6hr, and 12hr post-administration. Plasma was separated, stored at -80°C, and sent to Mars, Inc. for analysis. Plasma

samples were purified by acidified methanol precipitation and concentrated 5-6 fold prior to HPLC analysis.

Known compounds for which there were purified standards (epicatechin and catechin monomers and procyanidin B2 dimer) could not be identified in the plasma at any time or in any sample after dosing, out to 12 hours post-administration. Other unknown peaks appeared to change with time. A diode array detector was used which allows a simultaneous measure of the absorption spectrum of each eluting compound. Two compounds had spectral characteristics (absorption band from 250 to 285nm) of flavonoid molecules but could not be definitively identified as such. One of the compounds with flavonoid spectral characteristics appeared to be glucuronidated or sulfated as sample treatment with β-glucuronidase (also has sulfatase activity) to cleave these groups led to an increased concentration.

There was no measurable concentration of known (those for which there was a purified standard) flavonoid constituents found in the plasma of laboratory dogs. There were two peaks that qualitatively may be flavonoid compounds. This study reveals that some of the compounds are absorbed and reach measurable levels systemically, while the bulk of the extract remains in the gut and/or is heavily metabolized/recirculated by the liver. The flavonoid extract at the given dose is unlikely to have direct systemic effects, but may lead to systemic effects mediated by the liver. As the above study reveals, there is a reduction in gastric mucosal carbonylation (Figure 21). Thus, there is a potential for this antioxidant to be therapeutic in alleviating gastric damage that requires further study.

VITA

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Candidate for the Degree of

Doctor of Philosophy

Thesis: EXERCISE & OXIDATIVE STRESS EFFECTS ON THE GASTROINTESTINAL BARRIER & THE CONTRIBUTION OF DIETARY PRO- & ANTI-OXIDANTS IN ALASKAN SLED DOGS

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Title of Study: EXERCISE & OXIDATIVE STRESS EFFECTS ON THE GASTROINTESTINAL BARRIER & THE CONTRIBUTION OF DIETARY PRO- & ANTI-OXIDANTS IN ALASKAN SLED DOGS

Pages in Study: 121 Candidate for the Degree of Doctor of Philosophy

Major Field: Veterinary Biomedical Sciences

Scope and Method of Study:

Exercise can lead to untoward effects when the intensity of exercise approaches extremes of performance. Gastrointestinal (GI) dysfunction occurs due to exercise and the mechanisms are unknown. Oxidative stress is encountered with exercise and a free radical mechanism has been implicated in causes of exercise-induced GI dysfunction. Little is known about the contribution of dietary pro-oxidants to GI mucosal barrier dysfunction in athletes. Alaskan sled dogs experience oxidative stress and GI dysfunction due to exercise. Sled dogs were chosen to test the hypothesis that dietary pro-oxidants and exercise-induced oxidative stress causes GI dysfunction. During multiple days of exercise, measurement of oxidative stress parameters (blood glutathione oxidation and exhaled ethane and pentane) was coupled with post-exercise gastroscopy and analysis of mucosal biopsies and GI permeability markers. Markers were reassessed while administering a flavonoid antioxidant before and during exercise. Also, lipid hydroperoxides were measured in foodstuffs used to sustain sled dogs.

Findings and Conclusions:

Gastric dysfunction was similar to previous studies, but increased intestinal permeability and oxidative stress did not occur with exercise. Gastric mucosal defects appeared after 100 miles of exercise and did not progress. Also, oxidative stress occurred post-exercise, but only after six hours of rest with food following the first 100 miles. There was no correlation of oxidative stress to GI dysfunction. Histologically, gastric mucosal biopsies revealed persistence of lymphoplasmacytic and sometimes neutrophilic influx persisting for at least nine days of rest. Administration of a flavonoid antioxidant had no effect on oxidative stress or GI dysfunction. The flavonoid supplement reduced the protein carbonyl content of gastric mucosal biopsies (local antioxidant effect), but did not change the histologic appearance of the gastric mucosa. The lipid hydroperoxide load in sled dog foodstuffs was similar to levels in control diets used in studies of hydroperoxide modulation of mucosa. Our data indicate there is not a direct effect of oxidative stress on exercise induced GI dysfunction. However, local free radical modification (protein carbonyls) occurs and the persistent mucosal leukocytes may modulate exercise induced oxidative damage.

ADVISER'S APPROVAL: Dr. Michael S. Davis