LEAKY GUT SYNDROME IN HORSES

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ABSTRACT

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Leaky gut syndrome involves the non-mediated passage of harmful substances through the intestinal barrier causing inflammation and other undesirable effects. This can be caused by non-steroidal anti-inflammatory drugs (NSAIDs) which have also been linked to causing equine gastric ulcer syndrome (EGUS). The gastric permeability probe, sucrose, has correlated the severity of EGUS and the amount of gastric permeability present. Preliminarily, our laboratory confirmed that NSAIDs caused ulceration associated with a level of intestinal permeability in a 2-horse crossover study. This second study examined organic mineral capability in decreasing the severity of EGUS caused by NSAIDs and their combined effects on leaky gut syndrome in horses. Eighteen Arabian horses were divided into two treatment groups supplemented with either organic (OM) or inorganic (IM) minerals for 63 days. The variables packed cell volume (PCV), total solids (TS), BW, BCS, and ulcer grade were measured. Phenylbutazone was administered on day 42 (4.4 mg/kg BW, PO, 2x per d for 7 days) at which point sucrose (1 g/kg BW, PO) was given and urine and blood was collected thereafter. No treatment differences resulted but multiple day differences between the measured variables were observed. Ulcer grade and TS increased following the cessation of NSAID treatment ($p<0.0001$). Sucrose concentration in urine and plasma increased from baseline ($p<0.0001$). Based on this study and prior work completed by this laboratory, we can conclude that NSAIDs substantially contribute to EGUS and an unknown level of gastric permeability but no difference between organic and inorganic minerals in the prevention or healing rate of EGUS was detected.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Leaky gut syndrome, also referred to as increased intestinal permeability, has been demonstrated in the literature to occur in many species including humans, pigs and rats. In humans, increased intestinal permeability is linked to various diseases such as Crohn's Disease, Celiac Disease, and Irritable Bowel Syndrome. In pigs and rats, heat stress and colitis have been associated with a certain level of permeability. When a situation of leaky gut occurs, harmful substances are "leaked" through the intestinal barrier into the body's internal environment. This syndrome starts a cascade of events that can lead to many complications such as inflammation, distress during exercise, and even organ failure.

Many different factors have been examined as causation agents for this syndrome including physical and physiological stress, strenuous exercise, hyperthermia, and non-steroidal anti-inflammatory administration (NSAID). In particular to the research conducted for this thesis, NSAID administration is of interest due to its known ulcerative effects on gastric mucosal which can also be referred to as equine gastric ulcer syndrome. There is potential for intestinal permeability and ulceration to be interrelated.

Leaky gut syndrome can be evaluated by administering non-invasive probes, which are then quantified in urine and/or plasma. Preventatives for this syndrome have been suggested through pharmaceutical, physiological and nutritional means. Of further interest to our research is organic mineral supplementation, specifically zinc as it is known for its immune system support, gut health integrity and other associated health benefits.

CHAPTER 1. Review of the literature

Introduction

Increased gastrointestinal (GI) permeability is a topic of interest to many in the human medical and exercise physiology related fields. It has been linked to Crohn's Disease and Irritable Bowel Syndrome, as well as other various conditions in human medicine (Meddings and Swain, 2000). In human physiology, increased GI permeability has been shown to be an effect of non-steroidal anti-inflammatory drug (NSAID) use, exhaustive physical stress such as prolonged exercise, and physiological factors such as heat stress (Lambert, 2010). Increases in GI permeability are made possible because of a disruption to the intestinal barrier whose function is to mediate diffusion of certain molecules. When this barrier loses integrity, a situation of non-mediated diffusion of potentially harmful molecules ensues (Lambert, 2009). This condition is commonly referred to as "leaky gut syndrome". Leaky gut syndrome has many potential altercations, some of which affect exercise performance. The ways in which increased GI permeability, or leaky gut syndrome, can be accessed include the oral administration of non-invasive markers such as carbohydrate probes, ^{51}chromium labeled ethylenediamine-tetra-acetic acid (^{51}Cr-EDTA), fluorescent probes such as fluoroisothiocyanate (FITC)-dextrans and horseradish peroxidase. Leaky gut has been examined in various ways in humans, and multiple interventions for prevention have been proposed, but, to date, no research has demonstrated leaky gut in horses and, thus, no scientifical prevention has been shown to eliminate the syndrome in horses.

GI Permeability

The gastrointestinal tract is a complex system comprised of multiple organs and tissues that all work in unison to sustain life by the breakdown of foodstuff in order to provide nutrients to the body for survival. The way in which this complex system functions determines peak performance during slow, continuous, or strenuous exercise (Lambert, 2011). The GI tract contains many harmful substances including food antigens, bile, hydrolytic enzymes, bacteria, and bacterial components (Lambert, 2010). These substances can cause harm if they leave their designated place of residence within the body.

Intestinal Barrier

The intestinal barrier, a single-cell epithelial layer, lines the GI lumen and, along with contributing to the role of digestion, functions as a barrier between the GI tract and the body's internal environment (Soderholm and Perdue, 2001). This barrier is made up of enterocyte membranes, tight junctions, secreted mucus, and immunological factors (Lambert, 2009). It functions to regulate transport and provide defense at the interface of the internal structure via transcellular and paracellular fluxes that are controlled by membrane pumps, ion channels, and tight junctions (Baumgart and Dignass, 2002). This barrier is the body's way of restricting foreign, potentially harmful content, from entering into and disturbing the rest of the body, including the bloodstream. Transcellular transport of molecules involves the processes of diffusion, active transport, and endocytosis of smaller substances (Pals et al., 1997). Normally permitted molecules that are larger in size, are moved via paracellular transport through tight junctions into the interstitial space. From here, the molecules can now enter systemic circulation from between the enterocytes. The tight junctions, through whom these molecules pass through,

function to maintain cell polarization and prevent unlawful substances from passing through the intestinal barrier into the interstitial fluid. Increased permeability to larger, normally restricted, molecules results from the dysfunction of these tight junctions (Pals et al., 1997).

Dysfunction of the intestinal barrier and its associated tight junctions increases GI permeability and allows normally restricted molecules from within the intestinal lumen to enter the internal environment and blood (Lambert, 2009). A certain level of low permeability is always present, but a large increase can lead to damaging, local and systemic inflammatory reactions. In the case of increased permeability, the body's normal immune function is not able to keep certain pathogens contained (Lambert, 2009). Thus far, research has been primarily conducted on humans to look at leaky gut syndrome and its various causes, evident by the amount of literature available.

Assessment of GI Permeability

Various non-invasive methods of assessing intestinal permeability have been utilized in both human and animal models by orally administering specific markers. Such markers include ^{51}Cr-EDTA, fluorescent probes such as FITC-dextrans, horseradish peroxidase and carbohydrate (sugar) probes. These substances are quantified by the amount excreted in urine and/or plasma. This amount will depend on the integrity of the intestinal mucosa. Other researchers have also measured the amount of an endotoxin, known as lipopolysaccharide (LPS), in plasma to determine the extent of intestinal permeability.

^{51}Cr-EDTA is a type of isotopic tracer that can be used to measure unidirectional flux from the intestinal lumen to the vascular compartment (Lorenzo-Figueras and Merritt, 2008). This tracer is readily measured in blood but its radioactive character limits its application in

many environmental settings. Fluorescent probes such as FITC-dextrans can be infused into a subject to measure the intestinal area of interest while measuring plasma concentrations over a specific period of time (Lambert, 2009). This probe comes in a variety of molecular weights and, thus, can provide an idea as to the size of the intestinal epithelial opening. Horseradish peroxidase is another probe used to assess intestinal barrier integrity and can be used in a manner similar to fluorescent probes. It is a common enzyme used in immunohistochemistry to label antigens and their antibodies.

Lastly, carbohydrate probes, also known as sugars, are commonly used to measure intestinal integrity because they are very adaptable to various environmental settings. Disaccharides, such as sucrose, lactulose, and sucralose, and monosaccharides such rhamnose, xylose, 3-*O*-methyl-D-glucose, and mannitol are the most commonly cited sugars that can assess paracellular and transcellular transit respectively, across the intestinal barrier. In other words, monosaccharides show the permeation through aqueous pores and disaccharides reflect the permeation of the intercellular pathway (Davies, 1998). The urinary excretion of these probes can be expressed as a percent of the ingested dose recovered (Pals et al., 1997). The ratio of a disaccharide to a monosaccharide can also be used as an index of the relative permeability present (Davies, 1998).

Sucrose is naturally metabolized by the enzyme sucrase in the small intestine. Since sucrase is not present in the gastric region, excretion of sucrose found in urine and/or plasma indicates intestinal permeation of the stomach mucosa only (Lambert, 2009). Davies et al. (1998) state that sucrose's exclusivity to the stomach was confirmed in a study that used balloon pyloric occlusion in which it was accurately able to measure the level of gastric permeability. Small intestinal permeability can be assessed by examining the excretion of lactulose as this

carbohydrate is degraded by the bacterial flora of the colon (Meddings and Gibbons, 1998). Both sucrose and lactulose are large molecules and can therefore only be excreted if they pass through the weakened tight junctions or damaged mucosa caused by leaky gut syndrome giving them a paracellular route of travel (Lambert, 2010). Often a solution made up of a monosaccharide, such as rhamnose (or mannitol), and a disaccharide, such as lactulose, is administered to evaluate small intestinal permeability. This combination relies on the assumption that rhamnose will cross the small intestinal epithelium via a transcellular route and the larger lactulose molecule will follow a paracellular route through the tight junctions (Pals et al., 1997). The urinary excretion rate of the two sugars, expressed as a ratio, will eliminate factors altering urinary excretion. Such factors could include gastric emptying, intestinal transit time, and renal function, and each would affect both sugars the same. Again, this ratio of recovered sugars would be expressed as a percentage of the ingested dose.

Sucralose is a chlorinated molecule of sucrose (Meddings and Gibbons, 1998). This sugar probe can be absorbed across either the small or large intestine. For this reason, sucralose can be expressed in a ratio with lactulose excretion to analyze which part of the GI tract has increased permeability, as lactulose only indicates small intestinal dysfunction (Meddings and Swain, 2000). Sucralose is noted to be an indicator of whole gut permeability because it passes through the entire GI tract undigested before it begins to be degraded (Lambert, 2009).

The range of recovery of such sugars in urine has been demonstrated to be between 72.1% to 97.3% (Meddings and Gibbons, 1998). The majority of the probes remained intact in the urine collection. Meddings and Gibbons (1998) demonstrated the discrimination of sugar probes within a given region of the GI tract in order to identify the precise location of diseases such as celiac disease, which can affect the proximal or distal colonic regions. It would be

difficult to assess which portion of the colon is affected in such diseases if it were not for the degradation selectivity of sugar probes within the GI tract. The concentrations of each probe at a specific GI site during a specific period of time using rats as a model was conducted by Meddings and Gibbons (1998). These results validate the use of sucrose, lactulose, mannitol, and sucralose as probes indicative of increased intestinal permeability specific to their respective areas of influence within the GI tract.

Causes of Increased Permeability

Increased GI permeability, or leaky gut syndrome, has been linked to many human diseases such as Crohn's Disease, Celiac Disease, and Irritable Bowel Syndrome in many scholarly articles. Intestinal barrier function can also be disrupted by physical or psychological stress, hyperthermia, reduced fluid intake during exercise, and non-steroidal anti-inflammatory drugs.

Physical Stress. Strenuous exercise can result in a decreased blood flow to the GI tract and, in turn, can reduce the gut barrier integrity. Brock-Utne et al. (1988) first observed that prolonged physical exercise could result in increased intestinal barrier permeability. This study concluded ultra-marathoners had increased LPS in their plasma after a long distance race. Lipopolysaccharide is considered the endotoxin of the highly immunogenic component of the wall of gram-negative bacteria and its increased levels can be associated with decreased GI integrity (Lambert, 2011). Baker et al. (1988) concluded similar findings in a study that examined endotoxemia in racehorses following a race. Researchers in this study state there is a possibility that training induced stress could lead to the release of LPS into systemic circulation, indicating a certain level of increased GI permeability. Upon completion of the 1998 Ironman

Triathlon in Hawaii, Lambert et al. (1999) analyzed data collected from select competitors that ingested a permeability probe solution after completion of the event. Subjects were found to have a high lactulose to rhamnose ratio when compared to controls that did not compete. The authors of this review concluded that long endurance events could increase intestinal permeability. There is also evidence for intestinal barrier dysfunction for shorter bouts of exercise under similar circumstances. Running for 60 minutes at a fast pace (80% maximal oxygen uptake), as compared with running at a slower pace (40 to 60% maximal oxygen uptake) for the same amount of time, resulted in a similar increase of small intestinal permeability (Pals et al., 1997).

In contrast to the numerous strenuous exercise studies conducted on humans, certain researchers have examined racing Alaskan sled dogs for intestinal permeability following the 2003 Iditarod dog sled race. These researchers found a high lactulose to rhamnose ratio and concluded that the results were suggestive of increased intestinal permeability (Davis et al., 2005).

Psychological Stress. Meddings and Swain (2000) examined GI permeability effects of psychological stress placed upon rats through means of restraint and also a combination of psychological-physical stressors by means of forced swimming. The result of such tests was an induction of increased epithelial permeabilty in all regions of the GI tract examined. This response seemed to be mediated by adrenal corticosteroids as the increase in permeabilty disappeared after adrenalectomy or pharmacological blockade of glucocorticoid receptors. This group of researchers also found an increase in GI permeabilty after transportation of the rats, as well as when administered dexamethasone. Both appear to mimick the effects of stress on the GI tract and allow for luminal constituents to access the mucosal immune system.

Lambert (2009) cites Saunders et al. (1997, 2002) as showing that psychological-induced GI permeabilty is related to release of acetylcholine and corticotropin-releasing hormone. Also, various research groups have shown that mast cells may play an important roll in the stress-related changes affecting the function of the intestinal barrier (Soderholm and Perdue, 2001). Mast cells can often be found near neurons and are activated by specific neurotransmitters. Stress-related migraine headaches have been associated with the activation of such mast cells. The activation, pathways taken, and chemical mediators leading to the action of mucosal mast cells during stress is not clearly understood (Soderholm and Perdue, 2001).

Hyperthermia. Another condition that has shown to provoke increased GI permeability is heat stress. The body's best attempt to combat heat stress is to dissipate excess heat by splanchnic vasoconstriction to supply increased peripheral blood flow. By doing so, blood flow to other vital organs is diminished. A rise in core body temperature combined with a decreased blood flow to the GI tract is likely to produce tissue hyperthermia (Lambert, 2010). This can result in tissue hypoxia, acidosis, ATP depletion, and oxidative stress, which can all lead to the disruption of intestinal barrier and tight junction dysfunction (Lambert, 2010).

Hall et al. (2001) demonstrated a significant increase in LPS concentration of portal blood in anesthetized rats whose core temperature was raised to 41.5° C. This was proven again by Lambert et al. (2002), as he discovered that by using FITC-dextrans, significant increases in small intestinal permeabilty were found in both anesthetized rats and everted rat intestinal sacs that had a temperature greater than ~ 41.5° C (Lambert, 2010). In vitro studies using caco 2 cell lines demonstrated prolonged hyperthermia of 41° C for 24 hours increased paracellular permeability and reduced epithelial resistance (Lambert, 2009).

The harmful result of reduced intestinal blood flow can occur during heat stress, exercise stress, or a combination of both. The diversion of blood flow away from the splanchnic region is what leads to a disruption within the intestinal barrier. This reduction in blood flow and hyperthermia can also result in oxidative and nitrosative stress which leads to cell membrane damage and weakening of tight junctions (Lambert, 2009). Together, significant intestinal mucosal damage is produced and the un-mediated passage of substances increases across the intestinal barrier.

Reduced Fluid Intake. To combat increased hyperthermia, it is important to replace fluid loss during exercise. Lambert et al. (2008) investigated the effects of fluid loss as it pertained to GI permeability. He concluded that fluid restriction during one hour of steady-state exercise at 70% VO2max led to increased gut permeabilty. Replacing fluid during exercise is likely important in maintaining gut barrier integrity by helping to support normal gut blood flow and reducing hyperthermia through increased sweating and/or skin blood flow (Lambert, 2011). Furthermore, Lambert et al. (2001) concluded that the intake of fluids containing energy substrates for the enterocytes may be more beneficial to combat increased intestinal permeability. However, all fluid losses can not be entirely replenished as humans do not match their fluid intake with fluid losses and, thus, some level of dehydration will continue during prolonged exercise (Lambert, 2011).

Non Steroidal Anti-Inflammatory Drugs. The GI tract is severely affected by NSAIDs but their effect may not be limited to the stomach mucosa and ulcer causation. In humans, studies have demonstrated their harmful effect on small bowel inflammation (Bjarnason and Takeuchi, 2009) and, in horses, significant effects upon the right dorsal colon causing colitis (Marshall and Blikslager, 2011). The most common reason to administer NSAIDs is to alleviate

pain. In order to do so, NSAIDs inhibit the cyclooxygenase (COX) enzyme. This enzyme is important for the conversion of arachidonic acid to prostaglandin (PG) H2. Prostaglandin H2 is then converted into a variety of prostanoids (Marshall and Blikslager, 2011). Blikslager et al. (1997) concluded that PG I2 and E2 are required to restore mucosal barrier function after ischemic injury by increasing intracellular cAMP and Ca+2 which then enables a signal for cytoskeletal-mediated tight junction closure. Prostaglandins protect the intestinal mucosa by promoting blood flow, mucus, and bicarbonate secretion. Thus, administration of NSAIDs may not only cause adverse GI effects but they may also inhibit necessary restoration of the mucosal barrier after injury. One specific NSAID, flunixin meglumine, has shown to inhibit barrier function recovery of the small intestine, as well as incease permeabilty to LPS (Marshall and Blikslager, 2011). Throughout these conditions, the intestinal barrier function is disrupted and permeabilty to harmful substances into the internal environment is likely. There is also evidence for NSAIDs to have an inhibitory effect on contractile activity of the GI tract (Marshall and Blikslager, 2011).

An early study performed by Bjarnason et al. (1986) demonstrated, in humans, an increase in urine excretion of ^{51}Cr-EDTA after the administration of either aspirin, ibuprofen, or indomethacin, with indomethacin being the most potent inhibitor of cyclooxygenase. Indomethacin increased urine ^{51}Cr-EDTA excretion the most and also decreased glomerular filtration rate. The conclusion was made that NSAIDs disrupt the intestinal barrier of humans and the damage may reside in the intercellular junctions. A follow-up Medline review conducted by Bjarnason and Takeuchi (2009) searched through all papers that described increased intestinal permeability in humans. All studies agreed that conventional NSAIDs increase intestinal permeability in humans within 24 h of administration. Particular to horses, a study conducted by

D'Arcy-Moskwa et al. (2012) concluded that adminstration of phenylbutazone significantly increased gastric permeability to sucrose.

Research has also shown exercise and NSAIDs in combination produce increased intestinal permeability (Lambert et al., 2007). During a period of 24 hours prior to an exercise test, subjects consumed a measured amount of aspirin, ibuprofen, or placebo every 6 h. Urine analysis of excreted sugar probes indicated that with prolonged running, GI permeability increases if the chosen NSAID is taken prior to moderate intensity exercise. In this study, aspirin created the greatest gastro-duodenal permeability.

Consequences of Increased GI Permeability

Intestinal barrier dysfunction can cause high amounts of LPS to leak into the internal environment. This event can cause a severe inflammatory response, increasing the production of pro-inflammatory cytokines. These cytokines come from immunological cells such as monocytes and macrophages. This response initiates a vicious cycle of events and is likely to cause further inflammation of the intestinal epithelium. Such events have been shown to occur in human and animal models of severe heat stress (Lambert, 2009). The cytokines released during the inflammatory response of GI permeabilty include tumor necrosis factor-α, interleukin-6, interleukin-1β, and interferon-γ. This type of systemic inflammation can lead to sepsis-like conditions and possible organ failure (Lambert, 2010). The cytokine cascade effect can also occur and contribute to GI distress during and after exercise (Pals et al., 1997). Lambert (2011) also states that the combination of exercise and heat stress resulting in increased intestinal permeabilty and inflammation can lead to other GI symptoms such as dehydration, reduced exercise performance, greater risk of heat injury and, again, mutiple organ failure.

Improvement of Intestinal Barrier Integrity

Given that increased intestinal permeability during exercise, heat stress, NSAID treatment, or other types of stress have been shown to invoke significant serious consequences, research into potential interventions is warranted. In contrast to the multiple studies conducted on the physiology of leaky gut syndrome that have gathered all the known evidence of the syndrome, no single preventative measure has been identified. There are, however, many theories that have been examined as to their effectiveness of enhancing intestinal barrier integrity. Such methods include the use of pharmaceutical, nutritional, and environmental treatments.

Pharmaceutical. Hyperthermia and decreased blood flow can result in increased oxidative stress to the body. A reduction in oxidative stress could prove to be helpful in dealing with increased intestinal permeability. Hall et al. (2001) administered allopurinal to anesthetized rats that had a heated core temperature of 41.5° C. Allopurinal inhibits the activity of the enzyme xanthine, which is responsible for producing superoxide anion and hydrogen peroxide. In this study, allopurinal was able to reduce portal LPS concentrations, which was likely accomplished by reducing the activity of xanthine and the oxidative effects induced by heat stress on the intestinal tight junction's integrity and enterocyte viability (Lambert, 2009).

Recent findings indicate glucagon-like peptide-2, an intestinotrophic growth hormone, to be effective at decreasing chronic physiological stress-induced intestinal barrier dysfunction. Cameron and Perdue (2005) subjected mice to 10 days of water avoidance stress and administered glucagon-like peptide-2 four hours before each bout of water avoidance stress. The administration of glucagon-like peptide-2 eased the increase of intestinal permeability to horseradish peroxidase across jejunal, ileac, and colonic tissues mounted in Ussing chambers.

Nutritional. A number of potential nutritional interventions have been considered to reduce the severity of leaky gut syndrome such as glutamine, antioxidants, bovine colostrum, goat milk powders, water and zinc.

Glutamine, a nonessential amino acid, is used as a primary fuel source by enterocytes of the GI tract (Lambert, 2010). It is the main respiratory substrate for enterocytes and is also significant to immune cells and intestinal metabolism (Amasheh et al., 2009). Oral supplementation of glutamine (0.65 g/kg BW twice daily for 5 d) to heat-stressed rats by Singleton and Wischmeyer (2006) resulted in reduced gut permeability to FITC-dextrans. This effect also produced a decrease in plasma LPS concentrations, increased intestinal heat shock protein (HSP)-70 expression, increased heat shock factor-1 activation, and improved survival of the rats from heat stress. Thus, if one can combat the ill effects of heat stroke, gut permeability could remain unchanged from such a stressor. Demirkan et al. (2008) found the oral treatment of glutamine (1 g/kg BW/d for 4 d) reduced increased gut permeability, but not plasma LPS levels, in rats suffering from intestinal disruption from ischemia-reperfusion.

The addition of dietary antioxidants may provide protection against oxidative stress that can cause increased GI permeability. Ashton et al. (2003) found that exercise-induced increases in plasma LPS could be attenuated by ingesting 1,000 mg of ascorbic acid two hours prior to exercise. Antioxidants such as flavonoids, namely quercetin, have been shown to enhance the tight junction barriers *in vitro* as it is related to the increased expression of claudin-4, a tight junction protein (Amasheh et al., 2008).

Both bovine colostrum (1.7 g/kg BW) and goat milk powder (1.7 g/kg BW) supplementation have been shown to attenuate heat stress-induced increases in GI permeability in rats (Prosser et al., 2004). These researchers measured GI permeability by the accumulation

of ^{51}Cr-EDTA in the blood of rats whose core temperatures had been raised to 41.5° C. When the standard diet was supplemented by either bovine colostrum or goat milk powder, significant reduction in intestinal permeability from heat stress was observed. The protective effect of these nutrients is attributed to their ability to modulate tight junction permeability.

Water is an important nutrient regardless of body condition but it is especially important in thermoregulation. If dehydration occurs during heat and/or physical stress, the body loses its ability to dissipate heat efficiently, which could lead to a greater core GI temperature. Gastric blood flow may also be compromised during dehydration stress. Research has shown that fluid restriction during exercise can lead to increased GI permeability and thus, fluid intake is suggested to combat the negative side effects of its loss and help maintain GI integrity (Lambert et al., 2008).

Various minerals may also play a role in GI protection and healing. One mineral in particular is zinc. Zinc is an essential mineral to biology and health and has been shown to protect the GI tract from increased permeability. Patients in remission from Crohn's Disease that were supplemented with oral zinc sulfate (110 mg 3 times/d for 8 wk) experienced a decrease in their intestinal permeability (Sturniolo et al., 2001). Further research by Sturniolo et al. (2002) supplemented rats experiencing colitis with zinc acetate (2 or 30 mg/kg BW). These researchers found paracellular permeability to improve by means of examining the lessened number of opened tight junctions with zinc supplementation. Zhang and Guo (2009) found that zinc supplementation (zinc oxide or tetrabasic zinc chloride, 200 mg/kg BW for 14 d) in weanling piglets reduced GI permeability, increased tight junction mRNA, and protein expression when compared with control piglet's lactulose to mannitol ratio. Piglets that were supplemented with zinc had an increased expression of occludin and zona occludens protein-1 (ZO-1). These

transmembrane proteins are located in the tight junction strands of the intestinal epithelial cells (Amasheh et al., 2009).

Zinc has also been found to attenuate the effects of NSAIDs. In a human model of intestinal permeability, researchers administered indomethacin (50 mg 3x/d) or indomethacin in combination with zinc carnosine (37.5 mg 2x/d) to different groups of subjects and measured GI permeability. Oral zinc administration decreased gastric permeability by 75% and small intestinal permeability by 50%. Indomethacin caused a three-fold increase in permeability as well as an increased lactulose to rhamnose ratio. However, when indomethacin was co-administered with zinc, no significant increase in permeability was observed (Mahmood et al., 2007). Sharma et al. (2003) was able to demonstrate a significant reduction in ulcers using a zinc complex of naproxen compared with administration of naproxen alone in rats. Equally as valid, Opoka et al. (2010) was able to conclude that chelated compounds of zinc exert a beneficial influence on ulcer healing as well, again in rats.

Physiological. There is evidence that prior heat exposure can produce an adaptation for improved intestinal barrier function upon exposure to successive heat stress. Rats exposed to heat stress produced significant increases in heat shock protein 72 (HSP72) induction in gut tissue (Ruell et al., 2004). HSP72 has been shown to be related to thermal tolerance and an increased ability to withstand lethal levels of hyperthermia. Dokladny et al. (2006) and Moseley et al. (1994) have also demonstrated an increase in HSP72 in response to heat stress. However, this effect has only been known to last 96 hours and it is undetermined if the increased HSP concentrations can be maintained for a prolonged period of time.

Equine Gastric Ulcer Syndrome

Equine gastric ulcer syndrome (EGUS) is a condition in horses, which is characterized by ulceration of the non-glandular and glandular regions of the stomach and proximal portion of the duodenum (Videla and Andrews, 2009). The horse is a continuous secretor of gastric acid. One acid in particular that is secreted by the parietal cells in the gastric glandular mucosa is hydrochloric acid (HCl). The epithelium of the gastric glandular region possesses many physiological features to prevent the mucosa from being injured by HCl including epidermal growth factors, bicarbonate buffering, mucosal blood flow, mucus secretion from prostaglandin production, and cellular repair mechanisms (Bell et al., 2007). In contrast, the non-glandular, or squamous part of the stomach, has traditionally been thought to lack these defense mechanisms, thus leaving the mucosa vulnerable to acid exposure (Bell et al., 2007). However, there are a few recent investigations for the potential of a surface barrier component of protection within the squamous mucosa but the role of the proposed mechanisms is still unknown. However, this squamous portion of the stomach does seems to be the most afflicted by EGUS as Videla and Andrews (2009) state that 80% of ulcers occur in the non-glandular region, while only 20% occur in the glandular portion of the mucosa.

Throughout the past three decades much research has been dedicated to this disease in regards to its pathology, prevalence, and treatment options. The prevalence of ulcers has been reported as ranging from 53 to 100% of horses in training (Bell et al., 2007) to as little as 11% in university riding horses (Nadeau and Andrews, 2009). Nieto et al. (2004) concluded that 67% of endurance horses had gastric ulcers at the end of a 50 or 80 km ride. This was similar to the prevalence of ulcers observed in show horses by McClure et al. (1999) when they reported gastric ulceration in 58% of horses examined (n=50). Researchers have also examined pregnant

and non-pregnant mares that were similarly managed on pasture and found an occurrence of 66.6% and 75.9%, respectively (le Jeune et al., 2006).

The gold standard for diagnosing EGUS is via endoscopic evaluation after an overnight fast but also taken into account is the horse's history, clinical signs, and response to treatment (Bell et al., 2007). Potential indicators of EGUS exhibited by horses have been documented to include a decrease in body condition score, changes in appetite, and fecal consistency, as well as decreases in packed cell volume (PCV) and total solids (TS) in plasma/serum (Cate et al., 2012). However, this same study found no relation of gender, age, body condition score or performance history to the ulcer scores of 40 Standardbred horses studied in Michigan, USA (Cate et al., 2012). In contrast to these conclusions, some studies have demonstrated a slightly higher percentage of gastric ulceration in geldings, but age and temperament still lack support as being indicative of EGUS in the literature (Videla and Andrews, 2009). Murray et al. (1989) demonstrated significantly greater non-glandular ulceration in horses exhibiting clinical signs when compared with horses not exhibiting clinical indications of EGUS. However, there are still many horses that do not exhibit clinical signs of EGUS and, upon endoscopic examination, indeed do have ulceration of the gastric mucosa. In humans, Davies (1998) has reported that as many as one-third of patients who demonstrate endoscopic evidence of ulcers, are asymptomatic. In addition to endoscopic and clinical evaluations for the diagnosis of EGUS, recent literature has also demonstrated the use of sucrose excretion rates in urine and plasma as indicative of a positive correlation to the level of ulceration present in the gastric mucosa (Hewetson et al., 2006; O'Connor et al., 2004).

The causes of EGUS are just as varied as their prevalence and include factors such as intense training, racing, intermittent feeding, stall confinement, high starch diets, and NSAID

treatment (Videla and Andrews, 2009). While many of the above factors have been shown to initiate non-glandular and glandular ulcers alike, NSAIDs in particular have a trend to cause greater glandular ulceration compared with non-glandular ulceration because of their effect on prostaglandin inhibition (Videla and Andrews, 2009). The results of prostaglandin inhibition include a decrease in mucosal blood flow and mucus secretion in combination with an increase in HCl secretion. The decrease in blood flow is thought to be the main reason for NSAIDs more potent effect on glandular mucosa because adequate blood flow is necessary to remove the hydrogen ions that diffuse through the mucus layer and allow for adequate buffering of acid. When this process is not homeostatic, cell damage may occur as a result of cellular acidosis and lead to necrosis (Videla and Andrews, 2009).

MacAllister et al. (1993) compared different NSAIDs (phenylbutazone, flunixin meglumine, and ketoprofen) against a control treatment receiving saline. None of the horses in the control group had glandular ulcers however, all horses in the various NSAID groups developed severe ulceration in the glandular portion of their stomachs. In stark contrast, Orsini (2000) states that the role of NSAIDs is controversial and thus should not be considered a major cause of EGUS since EGUS primarily occurs in the squamous mucosa. In agreement with Orsini (2000), Vastistas et al. (1999) also speculated that NSAID contribution to squamous ulceration was not an important factor in many studies examining horses in training. This does coincide with what has been reviewed by Videla and Andrews (2009) when they stated that 80% of ulceration occurs in the non-glandular, squamous mucosa compared with only 20% occurring in the glandular region.

Similar to the extensive research conducted into the prevalence and causation of ulcers in horses, there is also a long list of various treatments available for EGUS that have been promoted

in the literature with varying degrees of success. The main goal in the pharmacological treatment of EGUS in horses is to suppress acid secretion and increase stomach pH. The list of potential treatments includes H2-receptor antagonists such as ranitidine, proton pump inhibitors such as omeprazole and other antacids, coating or binding agents such as sucralfate, and synthetic prostaglandins such as misoprostol (Bell et al., 2007; Videla and Andrews, 2009). Once the pharmacological treatment has been discontinued and the ulceration has been healed, Videla and Andrews (2009) point to the necessity of management changes to ensure that EGUS does not recur. Changes in environment, exercise schedule, nutrition, and dietary management can be made to help facilitate healing and prevent reoccurrence. Some ways described by Reese and Andrews (2009) include the following: modification of exercise intensity and duration, increase pasture turnout, increase forage and fiber, decrease size and increase frequency of concentrate feedings, antibiotic and probiotic therapy, and lastly, various dietary supplements such as seabuckthorn berry extract and corn and rice bran oils.

Conclusions

While there is evidence of leaky gut syndrome in humans and other species, research in horses is limited. It is believed that intestinal barrier dysfunction begins by the trigger of systemic stress. The main causes of this stress can be attributed to physical or physiological stress, hyperthermia, reduced fluid intake during exercise, and non-steroidal anti-inflammatory drugs. Upon disruption, the intestinal barrier weakens and allows the non-mediated diffusion of harmful substances through the tight junctions via a paracellular route. The implications of increased permeability include decreased exercise performance, dehydration, sepsis-like conditions, and multiple organ failure.

The methods of assessing leaky gut syndrome include the administration of various non-invasive permeability markers including ^{51}Cr-EDTA, FITC-dextrans, horseradish peroxidase, sugar probes, and LPS. These markers are quantified in the urine and/or plasma of subjects and can then be expressed as a percent of the ingested dose. Research has shown some alleviation of the disease through pharmaceutical, nutritional, and physiological means.

Equine gastric ulcer syndrome is well studied in horses and has many known causes, treatments, and preventatives. What remains uncertain at this time is whether or not EGUS is correlated with intestinal permeability. Both share similar causes such as intense exercise and NSAID treatment. Also, an inexpensive treatment common to both is dietary considerations. If it is found that the two are interrelated, further research could lead to a cost-effective treatment and/or preventative.

While there has been continuous research conducted on leaky gut syndrome in humans and various other species, there is a lack of evidence that this disease is present in horses. This niche area of research has yet to be pursued in the equine species and thus, investigation is warranted.

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LITERATURE CITED

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CHAPTER 2. Do horses develop a leaky gut from NSAID administration? A preliminary validation project

Introduction

Leaky gut syndrome, also referred to as increased intestinal permeability, has been demonstrated in multiple species. In a situation of increased permeability, intestinal barrier integrity is diminished and the gut becomes "leaky" to normally restricted molecules (Lambert, 2009). These molecules are then allowed to move freely from the gastrointestinal (GI) tract through the lumen and into the internal environment causing potential problems such as inflammation, exercise distress, and even organ failure (Lambert, 2009). In humans, endurance exercise of high intensity, prolonged hyperthermia, non-steroidal anti-inflammatory drugs (NSAIDs) administration, and psychological stress have all been shown to cause leaky gut syndrome (Pals et al., 2007; Lambert, 2010; Bjarnason and Takeuchi, 2009).

It is therefore important to question whether GI barrier dysfunction is quantifiable following one of these above factors such as NSAID administration in horses. A commonly administered NSAID in horses is phenylbutazone (bute), which is most widely used as a pain reliever. Bute is a known cause of equine gastric ulcer syndrome (EGUS) in horses because of its inhibitory effect of prostaglandin ion production by cyclooxygenase (Videla and Andrews, 2009). Prostaglandins play an important role in protecting the intestinal mucosa by promoting blood flow, mucus secretion, and bicarbonate secretion. Based on a cumulation of research, we know that if blood flow is decreased, a situation of intestinal permeabilty is likely to develop.

The determination of GI barrier dysfunction can be assessed through the biochemical analysis of urine and blood samples for ingested carbohydrate markers, as their excretion is indicative of increased GI permeability. Excretion of ingested GI permeability probes such as

sucrose, lactulose, rhamnose, and sucralose have been validated and are analyzed for this purpose (Meddings and Gibbons, 1998). A recent publication has also made positive claims for plasma sucrose's positive correlation with intestinal permeability (Hewetson et al., 2006). Sucrose is a sugar probe that is indicative of potential gastric damage if it is excreted in large amounts as it is readily metabolized by sucrase in the small intestine (Pals et al., 1997). Lactulose excreted in high amounts can indicate an increased permeability of the small intestine as it is non-digestible in the small intestine and only begins to be metabolized in the large intestine by colonic bacteria (Lambert, 2009). Rhamnose is thought to serve as a 'control' probe as it is readily passed transcellularly across the small intestine epithelium (Pals et al., 1997). Sucralose is recognized to be indicative of whole gut permeability because it is not readily degraded within the GI tract (Lambert, 2009). Lastly, the L/R ratio is another way to evaluate small intestine permeability. Lactulose is a larger molecule than rhamnose and if "leaked" from the GI tract will follow a paracellular route of travel. Rhamnose on the other hand easily passes through the intestinal barrier via a transcellular route. When the two are taken into a ratio, one can eliminate any potential problems with excretion unrelated to intestinal permeability such as gastric emptying, intestinal transit time, and renal function and each would affect both sugars the same (Pals et al., 1997).

The purpose of this study is to examine the effects of NSAID treatment on equine intestinal permeability through the analysis of blood and urine samples for the presence, if any, of the previously mentioned GI permeability probes. Our hypothesis is that GI permeability will increase following one week of NSAID administration to horses. We also aim to quantify the level of mucosal damage caused by NSAIDs through ulcer scoring and evaluate its potential as a causation agent for EGUS.

Materials and Methods

This initial pilot study consisted of a crossover experimental design, denoted by trial 1 and trial 2, using two mature Standardbred geldings with an average weight of 519 ± 32.5 kg. All methods were approved by the Michigan State University Institutional Animal Care and Use Committee (approval number 10/11-216-00). Both horses were housed in 3.7 x 3.7 m box-stalls, received grass hay twice daily for a total of 2% of their individual body weight per day, had *ad libitum* access to water, and received turn-out when applicable in a small dry lot with access to hay. Turnout occurred on at least six of the seven days of the treatment period and was allowed for a minimum of two and a half hours each day. Horses were monitored daily for changes in attitude and eating habits.

During each of the trials, one horse received 2 g of phenylbutazone (bute) paste twice per day for a total of 4 g daily designated as treatment (B) and the other served as the control (C). Treatment lasted for a period of seven days, followed by thirty-six hours of total urine collection. Endoscopy, weight measurements, and blood sample collections (plasma and serum) were conducted on days 0, 3, and 7. A licensed veterinarian conducted all endoscopic procedures using an Olympus three-meter endoscope. During examination, the stomach was insufflated with air to allow for adequate distention in order to thoroughly evaluate both the non-glandular (squamous) and glandular (pyloric) epithelium. There was a washout period of three weeks in between trial 1 and trial 2 in which horses received omeprazole treatment of 4 mg/kg BW per day for two weeks to heal any residing ulceration (GastroGard; Merial Limited, Duluth, GA).

In preparation for endoscopy, horses were fasted 12 to 18 h prior to examination and water was removed on the morning of examination. Blood samples were drawn prior to endoscopy with a 20 G needle and vacuum tubes (BD Vacutainer®, Becton Dickenson, Franklin

Lakes, NJ), followed by a sedative dose of 0.4 mg/kg BW IV of Sedivet (romifidine hydrochloride; Boehringer Ingelheim, Ridgefield, CT) After endoscopic evaluation on day 7, horses were administered 35 g sucrose, 35 g lactulose, and 14 g rhamnose (analytical grade carbohydrate probes; Sigma-Aldrich, St. Louis, MO) in a 1-liter solution of tap water via nasogastric tube. An additional 0.5 liters of tap water was used to rinse the graduated cylinder and nasogastric tube, after the 1-liter solution was given, for a total volume of 1.5 liters administered. This was performed to ensure that the horses were given the entire sugar solution. Blood samples were taken every 45 min after probe administration until 225 min post-sugar probe administration via jugular venipuncture. At 2 h post-sugar probe administration, horses were given 2 kg of hay and provided ad libitum access to water. At 6 h, 35 g sucralose in 1 liter of tap water via nasogastric tube was administered, followed by an additional 0.5 liters that was used for rinsing the graduated cylinder to ensure full dosage of sucralose. A single blood sample was taken 120 min post-sucralose administration. Urinary catheters were inserted at 0, 6, 12, 24, and 36 h to retrieve the total volume of urine for that time period. For each catheterization, horses were sedated with 2 ml of Sedivet. To ensure that all urine from each specified time period was collected from the bladder, horses were examined rectally to confirm and/or further express all urine out of the bladder. After catheterization at 0 h, horses were outfitted with Nappies which would serve as total urine collection devices throughout the next 36 h. Total urine volume for each time period (0, 6, 12, 24, 36 h) was recorded and a well-mixed sub-sample was saved and frozen. At 8 h after initial sugar probe administration, horses received *ad libitum* access to hay.

Samples Analysis

Endoscopic procedures on days 0, 3 and 7 were recorded and scored for severity. A

grading scale of 0 to 4 was used, with 0 indicating no ulceration and 4 indicating severe lesions (Bell et al., 2007). Blood samples taken on days 0, 3 and 7 were spun in a centrifuge at 2,000 x g for 15 min. Two aliquots of both serum and plasma were saved and frozen at -80^{o} C. Before centrifugation, whole blood was placed into microcapillary tubes and spun for 3 min in a microhematocrit centrifuge to determine packed cell volume. Supernatant plasma in microcapillary tubes was then analyzed for total solids by a refractometer. Three aliquots of plasma were saved after centrifugation for each of the 45 min blood draws from sucrose/lactulose/rhamnose probe administration (time 0, 45, 90, 135, 180, 225 min). The same protocol was followed for plasma drawn 120 min post-sucralose dosage. One aliquot from each time period was further filtered to remove plasma proteins by the following procedure: 500 microliters (μL) of each aliquot were placed into an Amicon Ultra 3K filter in a microcentrifuge tube and centrifuged for 15 min at 10,000 x g and 22^{o} C (van Wijck et al., 2011). These filtered plasma samples were sent to Dr. P. Lambert at Creighton University for further analysis.

After each urine collection time, a small sample was analyzed for specific gravity using a refractometer. For each time period (0, 6, 12, 24, 36 h), two 25-ml samples were saved and sent to Dr. P. Lambert for further analysis and one 90-ml sample was saved and frozen at Michigan State University for future use. The percent of probe excreted was determined by multiplying the amount of probe detected in the sample by the corresponding urine volume from that time period to get the mass excreted in grams. This value was then divided by the sugar probe dose administered and multiplied by 100 to arrive at the percent of sugar probe excreted.

Dr. Lambert Sample Analysis. Urine and plasma samples were analyzed using a Dionex DX-500 high performance liquid chromatography (HPLC) system. All samples were diluted 5-fold and centrifuged at 2,000 x g prior to HPLC analysis. The eluent used was 50 mM sodium

hydroxide and isocratically run. Concentrations of rhamnose, sucrose, and lactulose were determined based on peak height using a linear regression equation derived from standard curves for each sugar.

Michigan State University Biochemistry Department Analysis. A mass analyzer in multiple reaction monitoring (MRM) mode for selective and sensitive identification of sucrose was employed to analyze urine and plasma. Prior to this selective MS/MS detection, an ultra-high performance liquid chromatographic separation was performed using an Acquity UPLC BEH Amide column to elute and resolve sucrose from other sugars. Analyses were conducted using a QTRAP 3200 mass spectrometer (AB/Sciex) equipped with binary LC-20AD pumps (Shimadzu), a SIL-HTc autosampler, and column oven. All mass spectrometric analyses, including data processing, were performed using Analyst v. 1.4.2 software (AB/Sciex). Sucrose was separated from other sugars using an Acquity UPLC BEH Amide column (10 cm × 2.1 mm × 1.7 µm) using a normal phase binary gradient with raffinose as the internal standard. Solvent A was 0.15% aqueous formic acid and solvent B was acetonitrile. Total solvent flow was maintained at 0.1 mL/min and gradient elution was performed using the following solvent compositions: Initial: 5%A/95%B, held for 1 min; linear gradient to 50%A/50%B at 3 min and then to 75%A/25%B at 6 min and hold till 11 min; sudden increase to 5%A/95%B at 11.01 min and a final hold at this composition until 15 minutes. Injection volume and column temperature were 5 µL and 40° C respectively. Mass spectrometer conditions were optimized for MRM detection of sucrose in electrospray negative mode. The Channel used for MRM was m/z 341 > 179.

Results

It should be noted that, due to the small number of subjects used in this preliminary study, data were not analyzed statistically to determine whether there were treatment differences. However, the following are observations that may be due to treatment.

Both horses lost weight while receiving bute (Table 1). No changes in packed cell volume or total solids were observed (Tables 2 and 3, respectively). Table 4 represents the urine volumes for the given collection times and the respective specific gravity. Again, no suggestive changes were seen in these data. Administration of bute did produce damaging stomach mucosal changes causing ulceration when compared with the control (Figures 1-8 and Table 5).

Dr. P. Lambert from Creighton University completed the analysis of the urine and plasma samples from sugar probe administration collection periods. His results are reported in Appendix A. Due to equipment failure and other uncontrollable variables, the urine excretion results from this lab should be examined with caution as to their accuracy. Dr. P. Lambert was unable to detect any of the administered sugar probes in the plasma samples. Sucralose excretion in urine samples is not reported as the HPLC methods failed to detect any recordable amounts.

Since there were confusing and confounding results from Creighton University, another laboratory was sought to analyze urine and plasma samples. Unlike the prior analysis, the Biochemistry Department at Michigan State University used mass spectrometry (MS) to analyze the samples. However, the same problem persisted with plasma and detection of sugar probes could not be determined. Also, lactulose, rhamnose, and sucralose were proving to be very difficult to separate, thus it was decided to analyze sucrose excretion in urine only.

Figure 9 demonstrates the results of sucrose excreted from 0 to 24 hours. There was a trend for sucrose excretion to increase while the horse received bute compared with the control

treatment. This could indicate an unknown level of gastric permeability due to bute administration. Table 5 illustrates sucrose concentration and percent excreted with the corresponding ulcer scores for both treatments. There was no detectable amount of sucrose found in urine samples from 36 hours.

Table 1. Body mass changes for trial 1 and trial 2 for both horses receiving bute and control treatments.

		Treatment	
Horse	**Day**	**Bute**	**Control**
		Weight, kg	
Allen	0	487	491
	3	473	479
	7	469	477
	Mean	476	482
Chaser	0	567	552
	3	562	553
	7	558	556
	Mean	562	554
Treatment Mean		519	518

Table 2. Changes in percent packed cell volume (PCV) for trial 1 and trial 2 for both horses receiving bute and control treatments.

		Treatment	
Horse	**Day**	**Bute**	**Control**
		PCV, %	
Allen	0	35	28
	3	36	32
	7	37	32
	Mean	36	30
Chaser	0	34	44
	3	36	36
	7	35	36
	Mean	35	38
Treatment Mean		35	34

Table 3. Changes in total solids (TS) for trial 1 and trial 2 for both horses receiving bute and control treatments.

		Treatment	
Horse	**Day**	**Bute**	**Control**
		TS, g/100 mL	
Allen	0	6.8	6.7
	3	7.3	7.3
	7	6.7	7.0
	Mean	6.9	7.0
Chaser	0	6.7	7.2
	3	6.5	7.0
	7	7.0	6.7
	Mean	6.7	7.0
Treatment Mean		6.8	7.0

Table 4. Total urine volume collected, amount saved and respective specific gravity for each time period of urine collection from trial 1 and trial 2 for both horses receiving bute (B) and control (C) treatments.

	Urine Variables				
Trial 1	**Urine Volume, mL Allen (B)**	**Specific Gravity (g/dl)**	**Urine Volume, mL Chaser (C)**	**Specific Gravity (g/dl)**	**Total Amount Saved, mL**
Pre 0	80	--	45	--	80/45
0 to 6 h	1,300	1.027	5,650	1.012	140
6 to 12 h	1,500	1.027	4,500	1.015	140
12 to 24 h	6,700	1.023	4,600	1.026	140
24 to 36 h	8,700	1.020	9,100	1.019	140
Total	18,280		23,895		
Trial 2	**Allen (C)**		**Chaser (B)**		
Pre 0	250	1.028	600	1.025	140
0 to 6 h	3,300	1.020	6,600	1.010	140
6 to 12 h	3,200	1.020	8,000	1.008	140
12 to 24 h	6,000	1.026	10,200	1.019	140
24 to 36 h	8,000	1.023	15,400	1.015	140
Total	20,750		40,800		

Figure 1. Bute treatment day 0, trial 1.

Ulcer score: squamous 2/4, pyloric 1/4

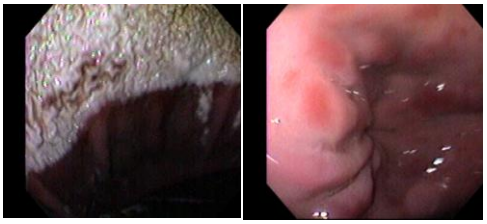


Figure 2. Control treatment day 0, trial 1.

Ulcer score: squamous 0/4, pyloric 0/4

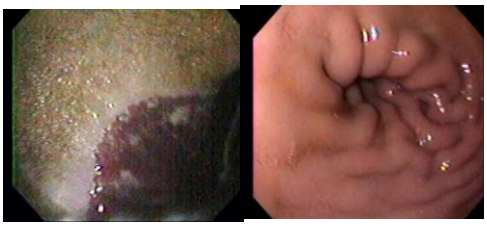


Figure 3. Bute treatment day 7, trial 1.

Ulcer score: squamous 1/4, pyloric 3/4

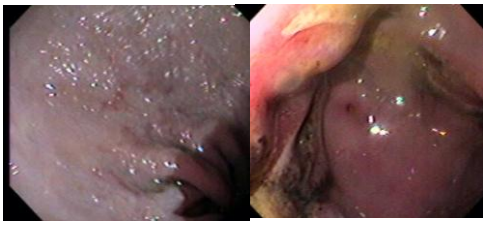


Figure 4. Control treatment day 7, trial 1.

Ulcer score: squamous 0/4, pyloric 1/4

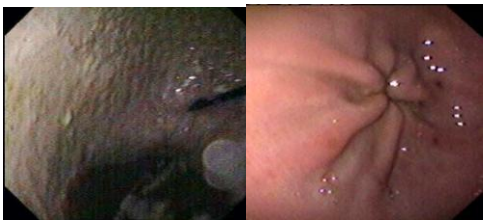


Figure 5. Bute treatment day 0, trial 2.

Ulcer score: squamous 0/4, pyloric 0/4

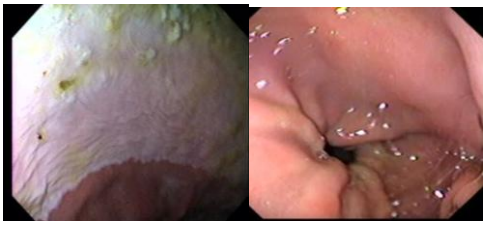
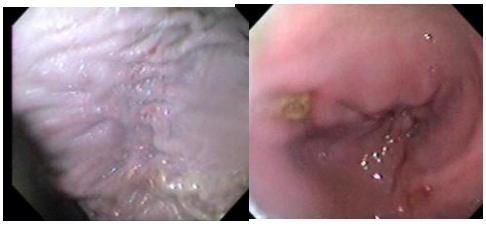


Figure 6. Control treatment day 0, trial 2.

Ulcer score: squamous 0/4, pyloric 1/4



[Note: For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.]

Figure 7. Bute treatment day 7, trial 2.

Ulcer score: squamous 2/4, pyloric 3/4

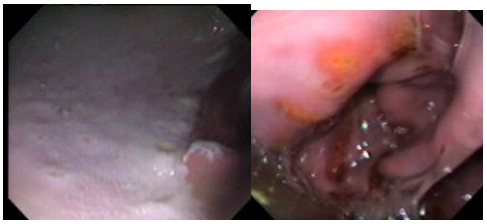


Figure 8. Control treatment day 7, trial 2.

Ulcer score: squamous 2/4, pyloric 1/4

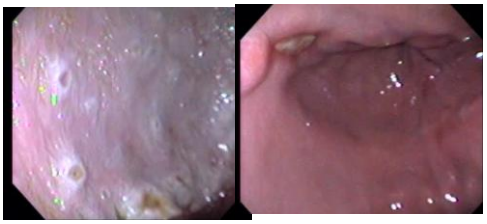


Figure 9. Percent sucrose excretion in trial 1 and trial 2 from mass spectrometry analysis.

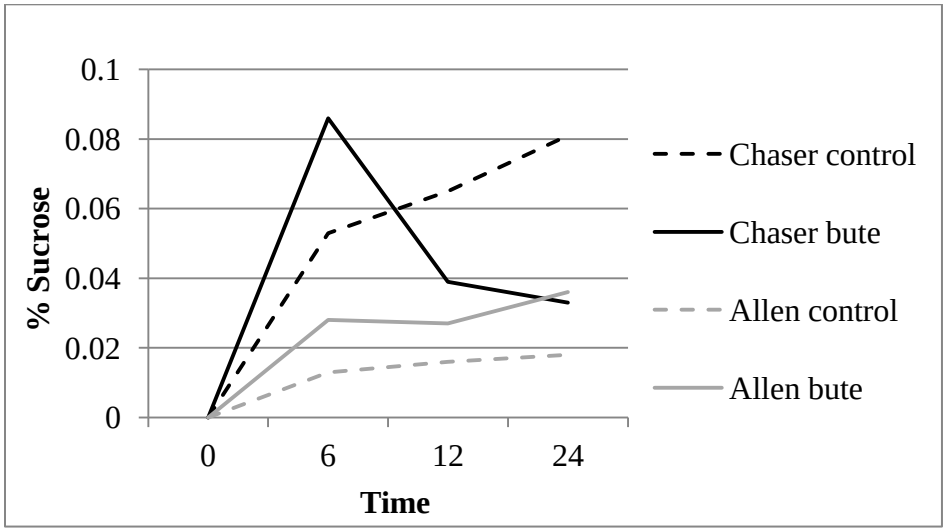


Table 5. Ulcer scores from trial 1 and trial 2 along with the corresponding sucrose concentration and percent excreted from 0 to 6 hours for both horses receiving bute and control treatments (Ulcer scores are graded from 0 to 4; Bell et al., 2007).

Horse	Day	Treatment	Trial	Ulcer Score Squamous	Ulcer Score Pyloric	6 h Urine Sucrose, g/L	Sucrose Excreted, %
Allen	0	Bute	1	2	1		
	3			1	2		
	7			1	3	0.0072	0.027
	0	Control	2	0	1		
	3			1	1		
	7			2	1	0.0012	0.011
Chaser	0	Bute	2	0	0		
	3			1	2		
	7			2	3	0.0047	0.088
	0	Control	1	0	0		
	3			1	0		
	7			0	1	0.0035	0.056

Discussion and Conclusion

The results from this pilot study are confounding. Duplicates were not run on data originally reported from Creighton University and thus, the lab re-ran the samples and reported differing data. This fact should be kept in mind when reviewing the results from Creighton University. Discussion of these results can be found in Appendix A.

Meddings and Gibbons (1998) established that the range of recovery of such sugars in an intravenous dose (sucrose, lactulose, rhamnose, and sucralose) could be expected to be between 72.1% and 97.3%. The sucrose excretion data from the MSU Biochemistry Department analysis demonstrated that while the percent of sucrose excreted was very small (less than 1%), excretion did follow a trend for GI permeability. Percent excreted was higher for the horse receiving bute when compared with the control. Part of the reason for this small excretion rate could be related to the dosage of sucrose administered. Our dosage of sugar probes administered was based off of the recommendation from researchers in this area of study, although they mainly dealt with human subjects. In future studies, increasing the amount of sugar probes administered may prove to be advantageous for more accurate detection of increased intestinal permeability.

To the author's knowledge, there are limited studies that have examined sucrose excretion in horses. O'Conner et al. (2004) collected urine for 4 hours following the administration of 454 g of sucrose to thirteen horses and reported much higher sucrose concentrations in urine compared with the results from the current study. Hewetson et al. (2006) administered 250 g of sucrose to each horse and were able to report quantifiable amounts of sucrose concentrations in plasma. Most recently in 2012, D'Arcy-Moskwa and colleagues also examined the effects of NSAIDs and their corresponding sucrose excretion rate. These researchers administered 0.5 g/kg BW of sucrose to each horse. Thus, the low dose of sucrose given in the present study could explain the reason why two different labs were unable to quantify sucrose in plasma samples. It is also possible that NSAID administration simply did not produce a detectable level of intestinal permeability by means of plasma analysis in our study as was already displayed by a low percentage quantified in urine.

O'Conner et al. (2004) was not able to induce glandular ulcers with an intermittent feeding model, however they did conclude the following upon examining the relationship between ulcer score and urine sucrose concentration: an ulcer score of 0 corresponded with sucrose ranging from 0.18 to 1.63 mg/mL, an ulcer score of 1 corresponded with a sucrose range of 0.08 to 0.36 mg/mL, an ulcer score of 2 gave way to 0.15 to 2.89 mg/mL for a range of sucrose concentration and lastly, an ulcer score of 3 corresponded with a range of sucrose concentration between 0.50 to 7.68 mg/mL. In our model of NSAID administration, we did not have grade 0 ulcer scores after treatment. However, if we compare our sucrose concentrations found in urine strictly at 6 hours after NSAID administration and grouped them with day 7 squamous ulcer scores, the following would be reported: a grade 1 ulcer had a sucrose concentration of 0.00723 mg/mL and a grade 2 ulcer score had a sucrose concentration of 0.00468 mg/mL. These sucrose concentrations are at least 10 times lower than the previously published data by O'Conner et al. (2004). Likewise, our dosage amount of sucrose was also about 13 times lower than their dosage of 454 g. This even further exemplifies that our dosage rate was probably a limiting factor in our inability to significantly link ulceration and intestinal permeability.

In this model, bute appeared to cause mucosal changes when compared with the control. Both horses on this study were from the same farm and management practices, so both underwent similar changes in environmental factors upon starting this project. Upon starting the bute treatment in either trial, each horse had an ulcer score of 2 or less in either the squamous or pyloric region. After completion of bute administration, both horses had increased ulcer scores, with significant ulceration within the pyloric region (grade 3). Compared with their control, these horses demonstrated that NSAIDs did, in fact, cause EGUS.

In conclusion, the NSAID phenylbutazone, was attributed to gastric mucosal changes in horses accompanied by a loss in body weight. A definitive level of intestinal permeability seems to be unquantifiable given the varied results of this pilot study. However, if sucrose were given in a higher dosage, more conclusive results of intestinal permeability may be seen as our data demonstrate a trend for increased sucrose excretion while receiving bute compared with controls. Further studies are warranted to investigate sugar probe dosage amount, collection time, and analytical procedures for quantifying sugar probes in urine and plasma.

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LITERATURE CITED

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CHAPTER 3. Does mineral supplementation attenuate NSAID effects and intestinal permeability in horses?

Introduction

The aim of this second study was to examine organic mineral capability in decreasing the severity of equine gastric ulcer syndrome (EGUS) caused by non-steroidal anti-inflammatory (NSAID) administration and their combined effects, if any, on leaky gut syndrome in horses.

EGUS is common in the horse with prevalence rates ranging from 11 to 100% depending on the type of activity and environment (Nadeau and Andrews, 2009). This condition is characterized by mucosal damage from decreased gastric mucus protection and subsequent acid exposure, both in the non-glandular and glandular portions of the equine stomach. The primary causes of EGUS include factors such as intense training, racing, intermittent feeding, stall confinement, high starch diets, and NSAID treatment (Videla and Andrews, 2009).

While many of the above factors have been shown to initiate non-glandular and glandular ulcers alike, NSAIDs in particular tend to cause greater glandular ulceration compared with non-glandular because of their effect on prostaglandin production via inhibition of cyclooxygenase (COX) enzyme activity (Videla and Andrews, 2009). The results of prostaglandin inhibition include a decrease in mucosal blood flow and mucus secretion along with an increased exposure to hydrochloric acid (HCl). This decrease in blood flow is thought to be the main reason for NSAIDs more potent effect on glandular mucosa as adequate blood flow is necessary to maintain the cytoprotective barrier overlying the glandular mucosa that is essential to remove the hydrogen ions that diffuse through the mucus layer of the glandular region of the stomach (Videla and Andrews, 2009). When this process does not occur, the cytoprotective barrier can be

disrupted and, along with continuous acid production, creates ideal conditions for EGUS to occur.

Along with the potential for NSAIDs to cause gastrointestinal (GI) damage, it has also been demonstrated by multiple researchers, as well as our own preliminary findings from the initial leaky gut pilot study, that NSAIDs also aid in mucosal barrier dysfunction that could lead to increased GI permeability in humans and horses (Marshall and Blikslager, 2011; Bjarnason and Takeuchi, 2009). Leaky gut syndrome, also referred to as increased intestinal permeability, is a situation characterized by the unmediated diffusion of normally restricted molecules across the intestinal barrier into the body's internal environment. A certain low level of permeability is always present, but a large increase can lead to damaging, local and systemic inflammatory reactions as the body's normal immune function is not able to keep certain pathogens contained (Lambert, 2009). Thus far, research has been primarily conducted on humans to investigate leaky gut syndrome and its various causes, evident by the amount of literature available.

One way of assessing intestinal permeability is through the use of ingested carbohydrate probes as their passage, or "leakage", through paracellular and transcellular pathways is site specific within the GI tract (Meddings and Gibbons, 1998). Sucrose is a known marker of gastric permeability and its level of excretion in urine and blood has been used to reveal a correlation between the severity of ulcers and the amount of gastric permeability present in models of naturally occurring ulceration and intermittent feeding (O'Conner et al., 2004; Hewetson et al., 2006). With this in mind, it is of interest to examine the potential effects of NSAID administration on leaky gut syndrome in greater detail. It is likely that NSAID treatment will cause some degree of EGUS based on presently reported data (Videla and Andrews, 2009) however, we are unsure of the level of intestinal permeability, if any, present due to EGUS.

Various organic minerals may have a role to play in the protection and healing of the GI tract. A process of chelation produces "organic minerals" through the binding of a trace mineral ion to an organic molecule, typically either an amino acid or carbohydrate (Kellogg et al., 2011). The thought is that chelated [organic] minerals become more bioavailable and efficiently utilized by the body. Of high interest to this study is the organic form of zinc. Sharma et al. (2003) was able to demonstrate a significant reduction of ulcers in rats using a zinc complex of naproxen. A decrease in gastric and small intestinal permeability was again observed in humans receiving indomethacin treatment in conjunction with supplemental zinc (Mahmood et al., 2007). Further, another group of researchers concluded that chelated compounds of zinc exerted a beneficial influence on ulcer healing, again in rats (Opoka et al., 2010). In addition, prior research conducted by our sponsoring company (Zinpro; unpublished) showed a trend for significance of organic mineral supplementation attenuating ulcer severity in horses.

Our hypothesis for this experiment is that NSAID administration will create a situation of increased ulceration and gastric permeability in horses. Further, the horses supplemented with organic minerals will experience a lessened ulceration as well as a decrease in gastric permeability. In addition, organic minerals may also enhance the healing rate of any potential ulceration that may have occurred as a result of NSAID administration.

Materials and Methods

The project began with 20 mature geldings (n=8) and mares (n=12) comprised of 18 Arabians and 2 Standardbreds with an average body weight (BW) of 514 ± 12 kg that were housed in 3.7 x 3.7 m stalls box stalls at the Michigan State University Horse Teaching and Research Center. All methods were approved by the Michigan State University Institutional

Animal Care and Use Committee (approval number 10/11-216-00). Horses were provided *ad libitum* access to water and hay at the rate of 1.5% BW, divided into two equal daily feedings. All horses were allowed turnout daily or were subject to exercise in a riding class. Of the 20 horses, 10 were included in weekly riding courses. Every two weeks during the course of the project, horses were weighed on a digital floor scale and assessed for body condition score (BCS) by three different researchers (Henneke et al., 1983). Horses were also monitored closely for changes in attitude and eating habits. Both an outside researcher and the farm manager, who were blinded to mineral treatment groups, briefly assigned behavioral scores to all the horses involved in a riding class. Researchers and farm staff monitored the horse's eating behavior.

All horses were examined endoscopically and blood samples were drawn on days 0, 42, 49, 56, and 63 for signs of EGUS. The following procedures were conducted for each endoscopic occurrence. Horses were subjected to an overnight fast for a minimum of 14 hours, blood samples were drawn via jugular venipuncture with a 20 G needle and vacuum tube (BD Vacutainer®, Becton Dickenson, Franklin Lakes, NJ) before the procedure and the horse was then sedated at 0.4 to 0.6 mg/kg BW with an intravenous injection into the jugular vein of 100 mg/mL xylazine HCl (Anased; Lloyd Laboratories, Shenandoah, IA). A licensed veterinarian performed all endoscopic evaluations using an Olympus (CF-100S; Olympus, Center Valley, PA) 3-m endoscope. During examination, the stomach was insufflated with air to allow for adequate distention in order to thoroughly evaluate both the non-glandular (squamous) and glandular (pyloric) epithelium. All gastric examinations were recorded and any ulceration was scored on a grading scale from 0 to 4 (Table 6; Bell et al., 2007).

After initial baseline endoscopic evaluation on day 0, horses were pair-matched after being blocked (striated) according to ulcer score, gender, breed, and age and were then randomly

assigned to either an organic mineral supplemented treatment group (OM) or a standard inorganic supplement treatment group (IM). Mineral treatments were fed at a rate of 0.2% BW mixed in with 0.2% BW of oats fed daily along 1.5% BW of hay, divided into two equal, daily allotments. Each diet was balanced and fed according to the 2007 NRC recommendations for maintenance of a mature horse (NRC, 2007). In total, horses were fed 1.9 to 2.0% of their individual BW throughout the entire project as to maintain their starting BW and BCS. Hay and concentrate feed amounts were adjusted individually throughout the project accordingly. A few horses were requested to have an increase in weight per the farm manager and thus, were fed oats at 0.3% BW. The nutrient composition of hay, mineral supplements and concentrate as fed can be seen in table 7. Hay 1 was fed to the horses from day 0 to 35. After this period, the farm needed to buy more hay to facilitate the project and consequently, hay 2 was fed to the horses from day 36 to 63. Analyzed ingredients of both mineral supplements, OM and IM, can be viewed in table 8.

Table 6. Equine gastric ulcer syndrome (EGUS) grading scale (Bell et al., 2007).

Grade	**Appearance of gastric mucosa**
0	Intact epithelium
1	Intact mucosa, evidence of hyperkeratosis or hyperaemia
2	Small, single, or multifocal lesions
3	Large, single or multifocal lesions or extensive superficial lesions
4	Extensive lesions with areas of apparent deep ulceration

On day 42, all horses underwent their second endoscopic evaluation for ulcers using the aforementioned procedures, followed by administration of the NSAID, phenylbutazone paste (bute; Vetribute; VetOne, Boise, ID) at a dosage of 4.4 mg/kg twice daily by mouth for 7 days. After completion of bute administration, another endoscopic examination was performed on day 49. Directly following this procedure, all horses were dosed with 1 g/kg BW of sucrose (Sigma-Aldrich, St. Louis, MO) dissolved in a 1.0 L tap water solution via nasogastric intubation. An additional 0.5 L of tap water was used to rinse the graduated cylinder and nasogastric tube after the 1 L solution was given, for a total volume of 1.5 L administered. This was performed to ensure that the horses were given the entire sugar solution. Urine collection commenced immediately for 4 hours (O'Conner et al., 2004). Urinary catheters were inserted at time 0 to ensure the bladder was completely empty and again at 4 hours. Both geldings and mares were monitored and urine was caught in a sanitized bucket if a horse urinated within the 4-hour collection time. Blood samples were also drawn at time 0, 15, 30, 45, 60, and 90 min post-sucrose administration via jugular venipuncture. A small, well-mixed sub-sample from each horse's 0 and 4-hour urine collection was saved and frozen for later analysis and the total volume of urine recovered at 4 hours was recorded. For these aforementioned procedures conducted on day 49, horses were put into pairs with 10 horses being examined per day. All horses completed the initial evaluations between 9 am and 12 pm which ensured there was not a day or time effect to any of the measured variables.

After these procedures on day 49, horses continued to receive mineral treatments OM and IM. All horses were then endoscopically evaluated twice more in the following 2 weeks to monitor the gastric mucosal integrity and evaluate the effects of mineral supplementation on the healing process of gastric ulcers, if any, on days 56 and 63. At the completion of the study on

day 63, any horses with ulceration greater than or equal to a score of 2 were prescribed omeprazole paste (GastroGard; Merial Limited, Duluth, GA) according to the manufacturer's recommendations of 4 mg/kg BW per day to heal any residing ulcers. One of the Standardbred geldings was not able to complete the entire study under the prescribed protocol as he quit eating after day 49. The decision was made to remove him from the study to allow him to go home and have access to pasture to ensure no ill effects would last long-term. Because of his termination, the decision was made to remove the remaining Standardbred's data from the results as well so that "breed" could be removed as a variable.

Sample Analysis

Blood samples (both serum and plasma) taken on days 0, 42, 49, 56 and 63 were spun in a centrifuge at 2,000 x g for 15 min. Two aliquots each were saved and frozen. Before centrifugation, whole blood was placed into microcapillary tubes and spun for 3 min in a microhematocrit centrifuge to determine packed cell volume (PCV). Supernatant plasma in microcapillary tubes was then analyzed for total solids (TS) by a refractometer. Additionally, two aliquots of plasma were saved and frozen after centrifugation for each of the blood draws during sucrose administration (0, 15, 30, 45, 60, 90 min). Later, these samples were then thawed and put into a 1:2 solution of plasma and acetonitrile and centrifuged at 10,000 x g for 45 minutes to remove plasma proteins. The supernatant was then saved for further analysis of sucrose. Serum samples also collected on days 42 and 49 were submitted for serum biochemical analysis.

For both 0 and 4-hour urine samples, a small sample was analyzed for specific gravity using a refractometer. Further urine reagent strip analyses were conducted on time 0 samples for evidence of blood, protein, and other elements. A preservative, sodium azide (Sigma-Aldrich,

St. Louis, MO), was added to all 4-hour urine samples at 0.1 g/mL to inhibit bacteria that may metabolize sucrose (O'Conner et al., 2004). One other method of urine preservation using a 10% solution of thymol (Sigma-Aldrich, St. Louis, MO) in isopropanol was also used to preserve urine on three horses' samples to determine if there was any difference between urine preservation methods compared with each other and a control (no preservative) (McOmber et al., 2010). This information could be useful on future projects examining sugar probes in urine.

Table 7. Analyzed forage composition of hay 1 and 2 (Equi-Analytical Laboratories, Ithaca, NY, USA), analyzed composition of select nutrients in mineral supplements (OM = organic mineral and IM = inorganic mineral; Kaufmans Animal Health, Lebanon, PA, USA) and oat (Equi-Analytical Laboratories, Ithaca, NY, USA) concentrate all on an as fed basis.

Nutrients	Hay 1	Hay 2	Zinpro Performance Pellet (OM)	Zinpro Sulfate Pellet (IM)	Oats
Dry matter, %	92.2	91.2	90.6	90.1	87.8
DE, Mcal/kg	1.76	1.98	2.7	2.9	3.1
Crude protein, %	7.6	14.3	33	33	9.8
Crude fat, %	2.0	3.8	2.3	2.3	4.9
Ca, %	0.41	0.92	3.0	2.8	0.05
P, %	0.19	0.20	1.8	1.8	0.28
Mg, %	0.21	0.31	0.3	0.3	0.09
K, %	1.37	1.61	1.7	1.4	0.39
Na, %	0.044	0.031	0.6	0.6	0.01
Fe, ppm	144	160	291	237	35
Zn, ppm	18	23	440	438	21
Cu, ppm	8	9	124	124	5
Mn, ppm	87	83	434	430	33

Table 8. Composition of mineral supplement ingredients (Kaufmans Animal Health, Lebanon, PA, USA; OM = organic mineral and IM = inorganic mineral) on an as fed basis.

Ingredients	Zinpro Performance Pellet (OM)	Zinpro Sulfate Pellet (IM)
Dry matter, %	90.6	90.1
Soybean meal 48%	1,111	1,200
Alfalfa, Dehy, 17%	271	0
Wheat middlings	211	185
Dical, Dynafos	132	132
Molasses, cane	100	100
Limestone	59	59
ZINPRO ®	8.0	0
MANPRO ®	10	0
COPRO ®	2.0	0
CuPLEX ®	0.5	0
Salt	20	20
Corn oil	20	20
Lysine, HCl	13	13
Threonine, L 98.5%	8.5	8.5
dl-Methionine 98%	21	21
K's Selenium 0.06%	4.5	4.6
Vitamin E 50%	2.7	2.7
Mang sulfate 32%	0	2.5
Zinc sulfate 36%	0	2.2
Copper sulfate 25.2	0	0.79
Cobalt sulfate 33%	0	0.04
Ferrous sulfate 30%	1.0	1.0
Biotin 2% (K's)	0.50	0.50
K's Rbo 60g/lb pmx	0.48	0.50
Niacin 99.5%	0.30	0.30
Thiamine, mono 91.7	0.14	0.14
Vit A 1000 KIU/G	0.08	0.09
K's Folic 50 100296	0.07	0.08
Pyridoxine HCl, usp	0.02	0.02
Vit D-3 500k IU/g	0.02	0.02
Eddi, 79.5% Iodine	0.01	0.01
K's Cal Pan 160 pmx	0.01	0.02
FF Power sweet, replac	1.5	1.5

Urine samples were then centrifuged at 10,000 x g for 45 minutes. Finally, 10 μL of a 100 μM raffinose (Sigma-Aldrich, St. Louis, MO) solution was added to 490 μL of centrifuged urine and plasma from sucrose administration. Raffinose was added to these samples to serve as an internal standard for mass spectrometry (MS) analysis. These urine and plasma samples were then sent to the MSU Biochemistry Department for complete analysis in accordance with the same procedure described for MS analysis in Chapter 2 of this thesis.

The percent of probe excreted in urine samples was determined by multiplying the amount of probe detected in the sample by the corresponding urine volume from that time period to get the mass excreted in grams. This value was then divided by the sugar probe dose administered and multiplied by 100 to arrive at the percent of sugar probe excreted. Results of sucrose in plasma were simply recorded as a concentration, not a percent.

Statistical Analysis

Data from variables PCV, TS, BW, BCS, ulcer grade, squamous ulcers (ulcerS), and pyloric ulcers (ulcerP) was analyzed using SAS 9.2 (SAS Institute Inc., Cary, NC, USA) with repeated measures in Proc MIXED using Tukey-Kramer adjustment. The model had fixed effects of horse, treatment, and day. The Shapiro-Wilks test for normality was conducted on the data and ulcer scores were not normally distributed. However, this was expected, as some variables are discrete. Further, analysis such as chi-square tests were conducted on ulcer score data to examine it categorically, these efforts provided data that were not different from using Proc MIXED. A test of homogeneity of variances was conducted successfully. A test for possible correlations was also conducted using Proc CORR. A separate analysis of blood parameters before and after bute was conducted using Proc MIXED with Tukey-Kramer

adjustment, which again included fixed effects of horse, treatment, and day. Data from sucrose analysis were analyzed using Proc MIXED with fixed effects of horse, treatment, and time. The trapezoidal method was used to determine the area under the curve for plasma sucrose over time (Shiang, 2004). Multiple tests for correlations using Proc CORR were conducted for both urine and plasma sucrose along with other measured variables. Means are reported with their SEM. Significance was considered at $p \leq 0.05$. Again, the two Standardbred geldings were not included in the statistical analysis.

Results

No differences were seen between the two treatments, OM and IM, nor between the interaction of treatment and day for the measured variables PCV, TS, ulcer grade, squamous ulcers (ulcerS), pyloric ulcers (ulcerP), BW, or BCS (Table 9). Also, no significant correlations were found between the two treatments or amongst the days. However, as illustrated in table 10, there were day differences for the variables PCV, TS, BW, ulcer grade, ulcerS, and ulcerP (treatment groups are combined and horses are viewed as one group $n=18$; $p < 0.0001$). The variable BCS was not measured on days 49 and 63 as it was measured only every other week to aid in adjusting feed intake if necessary. Results of urine analysis from the samples collected at time 0 can be viewed in table 11 of Appendix B along with discussion of renal effects of NSAIDs. Also, results of blood analyses from day 42 and 49 for blood urea nitrogen, creatinine, chloride, total protein and albumin concentrations can be viewed in tables 12 and 13 of Appendix B. Urine preservation methodology results can be viewed in table 14 of Appendix C.

No changes were observed in the behavior of these horses during the study (not statistically analyzed). Most horses were exercised in riding classes during the study and no

decrease in performance was noted. Only two horses were reported to be slightly irritated or apathetic during their riding classes. Towards the last few days of bute administration, three horses had a decrease in appetite. In all, five different horses displayed these riding and eating behavior changes. Upon the cessation of bute, horses resumed normal behavior over the subsequent few days. These behavior changes did not appear related to ulcer grades on perusal of the data; however, statistical analysis for potential association was not performed.

Packed cell volume was different on day 0 compared to all other days measured during the study, with the mean being highest on day 0 (36.4 ± 0.7%; $p < 0.0001$) and lowest on day 56 (31.1 ± 0.7%) which corresponds with one week post-bute administration. However, day 56 was not different from days 42, 49 or 63. Total solids decreased from day 0 to 42 ($p = 0.005$), decreased again from day 42 to 49 ($p = 0.05$), remained low through day 56, and then increased from day 56 to 63 ($p = 0.003$). The mean body weight was 457 kg on day 0, decreased to 450 kg on day 42 ($p = 0.005$), and remained unchanged until another slight decrease from day 42 to 63 ($p = 0.03$). The lowest mean BW occurred on day 63 of the study (448 ± 7.4 kg).

The variable ulcer grade corresponds to the highest ulcer score of the two sections of the stomach, the non-glandular and glandular regions, which were graded separately within each individual horse. Ulcer grade increased from 0.6 on day 0 to 2.4 at day 49 ($p < 0.0001$) and then improved to 1.7 by day 56 ($p = 0.01$). Ulcer grade mean on day 49 was greater (2.4 ± 0.2) than all other days and corresponds to the day directly following the cessation of bute administration.

Upon examining the two subsections of ulcer grade, ulcerS and ulcerP, differences can again be seen amongst the days as well. UlcerS mean increased from 0.2 on day 0 to 1.1 on day 49 ($p = 0.0005$) and remained unchanged at 1.2 ± 0.17 through day 63. The mean of UlcerP

increased from 0.5 on day 0 to 2.4 at day 49 ($p < 0.0001$), improved to 1.2 by day 56 ($p < 0.0001$) and ended at 0.8 on day 63, which was not different from day 0.

The overall prevalence of ulcers was as follows: 50% on day 0, 78% on day 42, 100% on day 49, and 94% on days 56 and 63. All horses had gastric mucosal changes after bute administration. If we limit the observation to grade 2 or greater ulceration in these horses (as grade 1 ulcers are likely an insignificant clinical problem), the prevalence was, 11% on day 0, 33% on day 42, 94% on day 49, and 67 and 61% on days 56 and 63, respectively.

There was no effect of treatment or treatment and time interaction for urine sucrose concentration ($p = 0.76$ and 0.69, respectively). Urine sucrose concentration did increase from 1.4 mg/L ± 4.0 at 0 hours to 31.5 mg/L ± 4.0 at 4 hours ($p < 0.0001$). When taking into account urine volume and the amount of sucrose given, the actual percent of sucrose excreted in the urine was very low with an average of 0.012 ± 0.007% for the organic mineral and 0.023 ± 0.007% for inorganic mineral supplemented horses but no difference was observed between these two groups ($p = 0.27$). Percent sucrose excretion in urine showed no correlation to the measured variables PCV, TS, ulcerP, ulcerS, or ulcer grade.

Plasma sucrose concentrations were not different between treatments OM and IM ($p = 0.53$), however a difference in time was demonstrated as time 0 was less than all other times measured ($p < 0.0001$). Area under the curve for plasma sucrose represented the entire amount of sucrose excreted for each horse for all of the given time measurements. Similarly, there were no differences among the treatments for this measurement ($p = 0.52$). Time to peak was also analyzed for plasma sucrose and was demonstrated at 72 min ± 24 and no differences were detected between treatments as to rate of reaching peak sucrose. No correlation between ulcerS, ulcerP, or ulcer grade was made for time or treatment. Plasma sucrose excretion for day 49 can

be seen in figure 10. No relationship was found between plasma sucrose and urine excretion ($p = 0.86$).

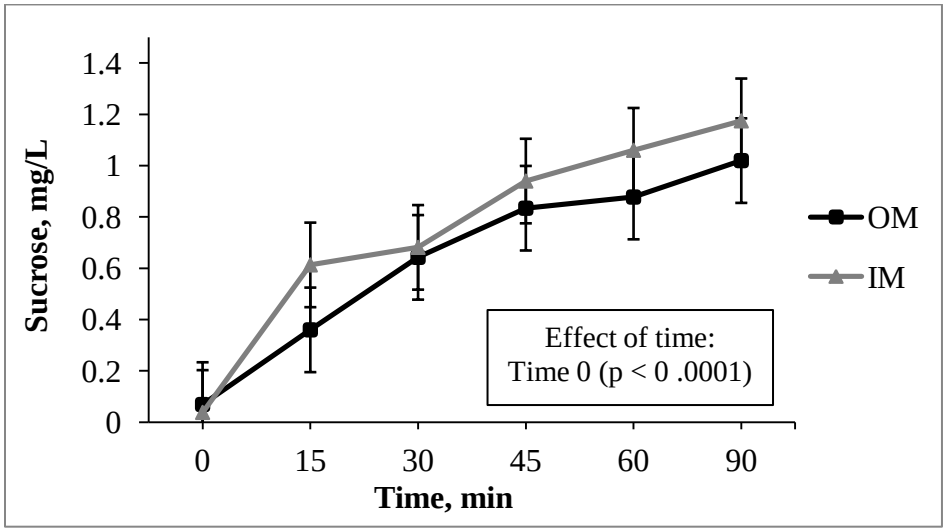
Table 9. Treatment and treatment*day effects for each of measured variables, packed cell volume (PCV), total solids (TS), ulcer score (Ulcer Grade), squamous ulcers (UlcerS), pyloric ulcers (Ulcerp), body weight (BW), and body condition score (BCS) for both the organic mineral (OM) and inorganic mineral (IM) groups.

	Day												
Variable	**0**		**42**		**49**		**56**		**63**			**Effects, p-value**	
	OM	IM	OM	IM	OM	IM	OM	IM	OM	IM	SEM	Trt	Trt*day
PCV, %	36.5	36.2	31.0	32.9	31.1	33.7	30.8	31.4	30.9	33.4	0.98	0.15	0.27
TS, g/dL	6.67	6.57	6.18	6.12	5.89	5.67	5.88	5.79	6.40	6.24	0.16	0.45	0.97
Ulcer Grade	0.56	0.67	1.00	1.11	2.56	2.33	1.67	1.78	1.56	1.67	0.24	0.84	0.91
UlcerS	0.22	0.11	0.44	0.78	1.00	1.11	1.67	1.56	1.00	1.44	0.25	0.57	0.56
UlcerP	0.44	0.56	0.89	0.78	2.56	2.33	1.33	1.11	0.89	0.78	0.27	0.70	0.92
BW, kg	464.6	449.8	458.8	442.1	455.3	436.7	453.6	440.0	455.0	434.4	10.4	0.26	0.34
BCS	6.3	5.9	6.2	6.1	NA	NA	6.0	6.0	NA	NA	0.19	0.44	0.11

Table 10. Combined means for both the treatment groups (organic and inorganic minerals) for each of the measured variables, packed cell volume (PCV), total solids (TS), ulcer score (Ulcer Grade), squamous ulcers (UlcerS), pyloric ulcers (Ulcerp), body weight (BW) and body condition score (BCS) on days 0, 42, 49, 56 and 63 of the study (BCS not measured on days 49 and 63). [abcd] Means not sharing similar superscripts are different ($P \leq 0.05$).

	Day						Effect, p-value
Variable	0	42	49	56	63	SEM	Day
PCV, %	36.4[a]	31.9[b]	32.4[b]	31.1[b]	32.2[b]	0.70	<.0001
TS, g/dL	6.62[a]	6.15[bc]	5.78[d]	5.83[cd]	6.32[ab]	0.12	<.0001
Ulcer Grade	0.61[d]	1.06[cd]	2.44[a]	1.72[b]	1.61[bc]	0.17	<.0001
UlcerS	0.17[c]	0.61[bc]	1.06[ab]	1.61[a]	1.22[a]	0.17	<.0001
UlcerP	0.50[c]	0.83[bc]	2.44[a]	1.22[b]	0.83[bc]	0.19	<.0001
BW, kg	457[a]	450[b]	446[bc]	447[bc]	445[c]	7.0	<.0001
BCS	6.1	6.2	NA	6.0	NA	0.1	0.29

Figure 10. Plasma sucrose concentrations for organic mineral (OM) or inorganic mineral (IM) treatments for the measured time increments of 0, 15, 30, 45, 60, and 90 minutes post-sucrose administration on day 49.



Discussion and Conclusion

It was the goal of researchers to maintain an average BCS of 6 along with day 0 BW throughout the entire study. Since horses were weighed and scored every two weeks, feed could be adjusted accordingly. However, significant BW changes were observed from day 42 compared with the last day of this study. At the beginning of this study, horses were not involved in a riding class as school was not in session. Classes began about two weeks into this study but the first few riding labs focus on grooming and tacking-up. With this knowledge, it is likely that these horses began exercising close to day 42. This could account for some of the weight loss by day 63. Also, a few horses on this study were being prepped for a sale. These sale horses were body clipped in the middle of the project and were also subjected to a greater exercise load. All of these factors, combined with bute administration could account for a drop

in BW. Nonetheless, no factor was substantial enough to have a significant effect on BCS. Also, no apparent relationships were observed between attitude, appetite or ulcer score, although these data were not compared statistically. In fact, the one horse that had the most severe ulcer score (grade 4 out of 4) did not outwardly display any obvious adverse clinical signs at all.

Packed cell volume on day 0 was different from all other measured days. This is likely due do the increased level of excitement of the horses at the start of the project. They had just been brought in from being kept on pasture and put into stalls. It may also be probable that the decrease in PCV after day 0 is indicative of blood loss through the means of ulcerated mucosa in horses following bute treatment (Bueno et al., 2000). However, there was no difference in PCV between any of the days after day 0 including day 42, at which point no bute had been administered yet, but mucosal ulceration did increase from day 0 to 42. Thus the blood loss and lower PCV as explained by Bueno et al. (2000) could still be a valid explanation as to why PCV was highest on day 0.

Total solids were found to be the lowest on day 49, but days 49 and 56 were not different from one another. Day 49 directly followed the 7-day bute administration period and also had the greatest ulcer grade. Previous publications have similarly demonstrated a decrease in TS after NSAID administration (Snow et al., 1981; Hough et al., 1999). Total solids are representative of the total amount of plasma protein in a given blood sample and can also indicate hydration status of an animal. The administration of NSAIDs has been documented to cause right dorsal colitis in horses (RDC; Bueno et al., 2000). This condition is often characterized by hyoproteinemia, which can be attributed to the loss of protein in the GI tract through the inflamed and ulcerated mucosa (Bueno et al., 2000). It is possible that a few of our horses were exhibiting signs of this condition as evident by their TS measurement. One of the

Standardbreds that was dropped from the study had very low TS on day 49 (4.0 g/dL) along with a poor appetite. For these reasons, he was removed from the study and turned out on pasture to avoid further complications to his health. Of note, this horse's ulcer grade was a 3 out of 4. One of the Arabian mares also exhibited lower TS on days 49 and 56 (3.8 and 4.1 g/dL, respectively). Unlike the gelding, this mare continued to eat, which ensured sufficient gut motility and was therefore kept on the study protocol. This mare's ulcer grade on day 49 was a 2 out of 4. Also of note, these two horses with low TS were on different treatments. Evident from the higher TS mean on day 63, TS had increased from day 49 and were not different from days 0 or 42. This suggests that a drop in TS, caused by bute administration, can recover to pre-bute administration levels within two weeks of discontinuing the NSAID treatment indicating a transient effect.

Even though there were no treatment differences observed between OM and IM groups, the day differences between ulcer grade, ulcerS, and ulcerP can provide helpful information to the equine industry. Ulcer grade was increased on day 49, directly after bute administration, with a mean score of 2.4 ± 0.2. By only looking at this finding, one may suggest that bute caused EGUS. However, there was also a difference in ulcer grade between day 0 and the start of bute administration on day 42. One reason for this difference could be related to the change in housing of these horses. This group of horses was used to living on pasture and one of the known causes of EGUS is stall confinement (Videla and Andrews, 2009). Therefore, this could have been a cofactor for the observed increase in ulcer grade from day 0 to day 42. It should be noted however, that these horses were allowed adequate turnout (at least 5 h/day weather permitting) throughout this study and were thus not confined to stalls 100% of the time. Another confounding factor could have been the increase in exercise of these horses. While not dramatic, it may have created a desirable situation for EGUS to occur as noted by the literature.

Also, prior to the start of this project, horses received similar concentrate and hay amounts so a change in feeding was an unlikely contributor.

According to Cate et al. (2012), ulcers that are scored a grade 2 or greater are considered clinically significant and may impact performance or clinical findings. Grade 1 is considered clinically insignificant and pharmacological treatment is often not recommended. In this study, 11% of horses had grade 2 ulceration or greater on day 0 and 33% on day 42 before NSAIDs were given. Bute administration increased this prevalence to 94% on day 49. These dramatic changes in percentages further exemplify that, while there may be other factors that can also be attributed to an increase in ulceration occurrence, bute seems to remain a powerful element as well.

Upon breaking down the ulcer grade into its two subunits, squamous ulcers and pyloric ulcers, we demonstrated a significant increase in ulcerS and ulcerP on day 49 compared with day 0 but ulcerS is not different from day 42 (pre-bute) until day 56 which is one week post-bute. Bute has been known to cause increasing pyloric, or glandular, ulcers when compared with its effects on the squamous mucosa (Orsini, 2000; Vastistas et al., 1999). A known attribute of NSAIDs is their ability to inhibit the COX enzyme, which is important for the conversion of arachadonic acid to prostaglandin. Prostaglandins protect the intestinal mucosa by promoting blood flow, mucus production, and bicarbonate secretion (Videla and Andrews, 2009). When blood flow is diminished and bicarbonate fsecretion is hindered, the removal of excessive hydrogen ions from within the glandular mucosa cannot be completed efficiently. Thus, a more acidic environment ensues causing substantial ulceration. This effect was certainly occurring in horses by day 49 of this study. Current literature does not understand why this effect of NSAIDs is not concurrently assimilated in the squamous region following NSAID use. Radi (2009) does

illustrate that the COX enzymes are mainly expressed in the jejunum of the small intestine in the horse. Perhaps this anatomical placement gives reason for the greater glandular effect of NSAIDs. Also, Bueno et al. (2000) describes one theory for the greater severity of NSAID effects seen in the large intestine, specifically the right dorsal colon, by means of NSAIDs extensive absorption by roughage and its subsequent release into the large intestine during forage fermentation. Also, the RDC is the sole site of net fluid secretion in the GI tract (Cohen, 2002).

In light of this knowledge, the results of this study do propose that the squamous region is in fact affected by NSAIDs, just at a slower rate. We observed pyloric ulcers to occur rapidly; within one week of bute administration, 89% of horses had an ulcer of grade 2 or greater. Squamous ulcer grade did not increase in significance from day 42 until day 56 (days 42 and 49 were not different from one another but they were different from 0), which was one-week post-bute administration. On day 49, 28% of the horses had acquired squamous ulcers of a grade 2 or greater. By day 56, 61% of the horses had a grade 2 or greater ulcer in the non-glandular mucosa. Total solids were also found to be lower on both days 49 and 56 and were not different from one another. These findings would be important to include in future literature regarding EGUS because as it stands now, NSAIDs are viewed as a minor contributor to this condition as they have not been shown to significantly effect the squamous portion of the stomach (Orsini, 2000; Vastistas et al., 1999). One could also argue that the increase in squamous ulceration one-week post bute was not a direct effect of NSAID administration but rather a result from the mild inappetence observed in some horses during the last few days during bute treatment and post-bute administration. Conversely, it would be difficult to distinguish between the two factors and their relative weight in causing squamous ulceration. Also, very few horses exhibited a notable decrease in appetite.

This may also explain why other studies have not found squamous ulceration when administering NSAIDs to horses as it is appears that current research has only examined the subjects endoscopically directly following NSAID administration. No references can be found regarding NSAIDs affect on gastric epithelium any greater than a few days post-NSAID administration. Our project was designed to enable researchers to assess the gastric mucosa up to two weeks post-bute. Because of this, we were also able to demonstrate the healing time for ulcers upon the cessation of bute. Day 63 ulcer grade was not different from pre-bute ulcer grade on day 42 which suggests that in this given population of horses receiving bute at 4.4 mg/kg BW twice daily, induced EGUS and its associated mucosal damage was able to heal within two weeks of stopping bute administration without prescribing any other pharmacological aid such as omeprazole; barring no effect of minerals.

Given all of this information, it does stand to reason that bute did, in fact, cause adverse mucosal changes in these horses, as evident from the large jump in the percent of horses exhibiting clinically significant ulcer scores (2 or greater) at day 42 (33%) compared with day 49 (94%). We can also look at results from our initial pilot study conducted in the fall of 2011 where we also utilized bute as a model for gastric permeability and EGUS induction. Similar to these results, the horses in the crossover designed pilot study had increased ulcer scores, compared with their control, after receiving bute for one week at the same dosage.

The absence of a treatment difference between OM and IM in regards to sucrose excretion was in opposition to the hypothesis of this study. While sucrose excretion, both in plasma and urine, increased from their respective time 0 measurements, there was no difference between the excretion rate of sucrose for the horses on organic minerals compared with inorganic. It does stand to reason that we demonstrated a certain level of increased gastric

permeability, as sucrose was detectable in both urine and plasma indicating that it had "leaked" out through the gastric mucosal barrier before being degraded to sucrase in the small intestine. This may indicate that mineral supplementation was not effective at attenuating gastric permeability. The urine sucrose excretion was very small, less than 1%, similar to that which was observed in a past pilot study conducted by our laboratory (unpublished data). It is interesting that dosage of sucrose was increased to over 10-fold greater in this study compared with 35 g given in the 2-horse crossover pilot study and yet, percent urine sucrose excretion is still roughly the same. The two Standardbreds that were eliminated from this current study were utilized in this previously conducted pilot study. The average urine sucrose concentration for these horses while on bute treatment during the pilot study was 11.9 mg/L and average percent excretion was 0.06%. If included in the data analysis for this current study, their average urine sucrose concentration would have been 50.2 mg/L and average percent excreted was 0.02%. The lower percent excreted could demonstrate that minerals were potentially efficacious in decreasing gastric permeability due to NSAIDs.

We were also unable to correlate ulcer grade with plasma and urine sucrose excretion. This is in contrast to data published by O'Conner et al. (2004) and Hewetson et al. (2006), in which both were able to correlate ulcer severity and sucrose excretion in horses. Only O'Conner et al. (2004) quantified urine sucrose concentrations but they did not report percent sucrose excreted, therefore, a true comparison is hard to complete. D'Arcy-Moskwa et al. (2012) had a dissimilar conclusion when looking at serum sucrose permeability due to NSAID administration in horses. D'Arcy-Moskwa and colleagues' study concluded that NSAID administration did increase gastric permeability to sucrose as evident by increased peak serum sucrose concentrations after NSAID treatment compared with a control and observed sucrose

concentrations ranging from 0.19 to 1.58 mg/L. The plasma sucrose concentrations found by our methodology, excluding time 0, ranged from 0.13 to 2.62 mg/L, indicating closely related results from two studies examining gastric permeability to sucrose as caused by NSAIDs. Based off of this information, we could conclude that these current data appear to demonstrate increased gastric permeability due to NSAID administration, though a control group not receiving NSAIDs would have been helpful in evaluating this fully. Of note, when conducting research on horses, it is hard to take into account the genetic variability of the subjects and thus, this could be another source for the variation observed between our results and other published data. Also, worthy of mentioning was the difference in plasma/serum sucrose time to peak found in our study of 72 min ± 24 compared with that reported by D'Arcy-Moskwa et al. (2012) of 51.2 min ± 8.8 and Hewetson et al. (2006) of 45 minutes.

Unfortunately, we were not able to detect any differences between treatments and have no evidence that minerals, organic or inorganic, have any affect on attenuating ulcer prevalence and/or healing rate. Of the many possible reasons for this result, the greatest is likely the lack of control group present in this study. Of particular interest to us was zinc and we are unable to say that it did or did not have any effect based on our results. It is possible that if we had included a control (unsupplemented) group, we may have seen worse ulcer scores in those non-supplemented horses and could then make justifiable conclusions regarding minerals and ulcer scores. The lack of control group could also account for the fact that we did not see a treatment difference in sucrose excretion for urine or plasma. It is possible that both treatments were gastro-protective and thus, neither group had a sizable increase in gastric permeability over the other. The main reason for not including a positive control was the lack of subject availability. This project would have needed to include 30 horses, which we could not produce easily. Also,

in order to have a true control group, horses could not be supplemented with any minerals. Our hay alone did not meet most of the nutritional requirements for a mature maintenance horse; it was deficient in both zinc and copper. Hence, if we did not add any inorganic to the controls, we would be comparing supplemented to deficient. Upon completion of the study, a power analysis was conducted on the data from ulcer grade for OM and IM treatments and greater than 100 horses per treatment would be necessary to see a difference at $p \leq 0.05$. For a difference between treatments in regard to total solids, 28 horses per treatment would be needed. Keeping in mind this is a specific analysis for our population of horses, a study using a different genetic variation of horses could yield different results.

The 2007 NRC recommended minimum daily requirement for zinc in a 500 kg mature maintenance horse is 400 mg. If we assume an average BW of 500 kg for these horses as well, OM supplemented horses received 623 mg of zinc on hay 1 and 668 mg when fed hay 2, while IM supplemented horses received 621 mg on hay 1 and 666 mg with hay 2. Essentially, these horses received one and a half times the recommended minimum amount of zinc, just in two different forms. Opoka et al. (2010) found that a significant amount of endogenous zinc was more available when rats were supplemented with zinc hydroaspartate (organic) and this was able to produce an increase in gastric healing. During healing, researchers found the gastric mucosa to be deficient in zinc but when highly supplemented with zinc, luminal and mucosal zinc content increased along with blood flow, which was attributed to helping the mucosa heal faster. There is the potential that the horses in this study were not supplemented with enough zinc to provide a difference between treatments and type of mineral. However, it still stands to reason as stated above, that if horses had not received any mineral supplementation, the potential for delayed ulcer healing could have been observed. At this point, we can only speculate and are

not certain as to the role and/or difference organic and inorganic minerals play in gastric mucosal healing in horses following EGUS.

In conclusion, we observed no differences in horses treated with organic or inorganic minerals in regards to ulcer prevalence and/or healing rates. Ulcer grade increased directly after NSAID administration. Squamous ulcers increased further one-week post- NSAID administration (and after the increase in pyloric ulceration) within this population of horses. Sucrose excretion both in urine and plasma, was not different between the two treatments but did increase from time 0 in all horses to a level closely resembling other published works. Based on these findings, we are unable to conclude that mineral supplementation attenuated EGUS or gastric permeability. However, we can demonstrate the harmful effects of NSAID administration on the stomach mucosa both in the glandular and non-glandular regions and can conclude it is a contributor to EGUS and potentially gastric permeability to sucrose in horses. We have also demonstrated that TS and ulcer grade can return to pre-bute measurements within two weeks post-bute administration with specific medication for treatment of ulcers.

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LITERATURE CITED

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CHAPTER 4. Overall discussion and conclusions

With any study, there will always be limitations and various factors that, upon completion, researchers wish they had changed or added. This thesis is no exception. At the start of this research back in the summer of 2011, all of us had fairly limited knowledge of leaky gut syndrome, nevertheless the role it may play in horses. The past two years have been a learning process for all involved, including myself, in regards to what and how this syndrome may present itself in horses. Two of the most substantial limitations we encountered were the inability to accurately measure and understand all of the carbohydrate probes used in the pilot study (Chapter 2) and then, in the larger study (Chapter 3), our lack of a control group. Understanding and discussing these limitations is important for further research conducted into this subject matter in future endeavors.

The first limitation we were confronted with after completing the 2011 fall pilot study that utilized two horses, was the inability to accurately measure all the sugar probes administered in urine and plasma. The actual probes given and their dosage rate was based off the advising we received from a researcher at Creighton University who had published numerous works on human intestinal permeability. In hindsight, we were quick to think the information was easily transferred to horses as was evident by the confusing results of sugar probes found in urine along the different time points. We did not understand what to resolve of sugar excretions increasing at 6 hours, decreasing at 12 hours and then increasing again at 24 hours. Especially with sucrose as it is degraded in the small intestine of the horse and certainly none would be expected to be excreted much past 12 hours. This remains to be an area of uncertainty and should be taken into consideration with future research. Also, another limitation concerning these sugar probe analyses was the laboratory work itself. The laboratory that conducted these analyses provided

us with multiple different sets of data. We had no way of confirming which numbers were correct. Furthermore, neither Creighton University nor the Michigan State University Biochemistry Department was able to quantify any of the sugar probes in plasma, nor sucralose in either urine or plasma. This led us to spend 5 months working in a lab at the Diagnostic Center for Population and Animal Health utilizing HPLC. This was a unit that had not been working for a few years and unfortunately, had operational impediments that we were not aware of at the start. After many unsuccessful attempts to regain the system's operational capacity, the manufactures' support team for the machine suggested it be sent out to their repair facility but had no inclination of the time or money it may take to get it back up and running. At this point, we began searching for another option and found the MSU Biochemistry Department who specialized in analyzing carbohydrates via mass spectrometry. This laboratory was finally able to re-run our samples from the pilot study which resulted in much lower concentrations than previously reported. This laboratory did have a problem separating all of the sugars from one another, thus we decided to just look at sucrose as this was a probe other equine researchers had used and we would then be able to justify and understand its excretion rate. Also, we still have not been able to quantify any of the carbohydrate probes in plasma, which has been demonstrated by multiple other researchers. Finding a laboratory and getting accurate analyses proved very difficult for us during this research. In future studies, ensuring this factor would be extremely valuable to the end result. Validating the methodology for the analysis of sugar probes in plasma would make future research much easier by eliminating the need to collect urine on horses as well.

One chief limitation in the final study (Chapter 3) was not including a control group. During the planning stages of this study, discussions took place regarding this decision. In a

previous unpublished project, a group of researchers demonstrated a trend for organic mineral supplementation to attenuate EGUS in horses and it was decided that this previous work lacked power and needed more subjects to detect differences between OM and IM treatments. In light of this, it was decided to not have a control group and we would use 20 horses with 10 per treatment (the prior study had 14 horses with 7 per treatment).

While the confidence of the company in their product is commendable and well intended, the results of this study tell us that there was no difference in mucosal damage and/or healing between organic and inorganic minerals supplemented to horses medicated with phenylbutazone. Had we included a control group, we would have had a greater chance of determining if zinc was efficacious in preventing or healing ulcers in horses. With this current study, we cannot say whether minerals did or did not assist in lessening NSAID effects because they actually may have done so but, without a control, we cannot see the supplements true effects. This confirms that having a control group is part of a better study design and will never hurt the quality of a project though finding sufficient subject numbers will always remain a limiting factor in equine research.

One last limitation associated with lacking a control group in this 20-horse study was our inability to say for certain that bute caused EGUS. We can surmise this because of the effects of bute found by the crossover design in the fall pilot study but the larger study alone cannot conclude that bute was the sole cause for EGUS's occurrence. At best, we can say that it aided in the observed mucosal damage but one can definitely argue other factors contributed. A group of un-treated horses would have been helpful to demonstrate phenylbutazone's significance. Also, a control group would have been beneficial in determining an exact level of gastric permeability present in these horses. In essence, the ideal study would have included two

treatments receiving the various minerals, one treatment receiving no mineral supplementation (to determine whether zinc played a role in the prevention and healing of ulcers), and another control group that did not receive bute (to confirm that it was bute that caused the increase in gastric ulceration seen at d 49), and with all groups dosed with sucrose. However, to have sufficient power in each treatment group, the total number of horses in the study would have been 40 – a number of that would not have been possible. So while it is easy to reflect back as to what would have been ideal, there are practical limitations to conducting the ideal study.

Potential areas of interest for future studies could include investigating and developing accurate methodology for sugar probe excretion and analysis in horses; how similar/dissimilar is it to other species data. One may also want to further investigate the potential role of minerals, specifically zinc, in protecting/healing the GI mucosa. There is published research to support a positive effect but none to our knowledge in horses. Also, it is possible that a feed deprivation model may elicit different results as many of the EGUS induction studies utilize this method to induce ulcers primarily in the squamous mucosa. Another future direction could be in the form of researching exercise and/or endurance horses for signs of leaky gut. In humans, a lot of the research surrounds strenuous exercise, which is often combined with heat stress and this produces significant intestinal permeability. An endurance race may be a great model for demonstrating any potential intestinal permeability in horses, as this event is similar to a marathon race, which has been associated with leaky gut in humans. Lastly, sucralose may be a probe of interest when determining right dorsal colitis due to NSAID administration. Sucralose is considered to be a whole gut permeability probe as it is not degraded in the GI tract and would be the only probe able to pass through the intestines in their entirety. It has potential to evaluate the level of intestinal damage in the right dorsal colon. There are many factors that would need

to be considered before this could occur such as the passage rate of sucralose and also its detection accuracy in urine or plasma samples of horses.

Overall, this thesis has been a great learning adventure and has provided us with many challenges and successes along the way, as any good research should do. Securing our understanding of sugar probe excretion rates and concentration amounts would be paramount for our next project along with including a control when attempting to demonstrate intestinal permeability in horses. All the challenges presented with this project have provided us with many answers but have also led us into valuable future research directions which will help increase all researchers' understanding of leaky gut syndrome, NSAIDs, and mineral gastro-protectivity. Understanding these factors will help to improve the equine industry and all of its constituents with the greater knowledge gained in regards to the physiology and welfare of horses.

APPENDICES

Appendix A. Pilot study urine analysis, Creighton University

Results

Results reported from Creighton University's analysis only contain excretion rates for sucrose, lactulose, and rhamnose from urine. No excretion data is available for sucralose as the HPLC methods described above in the sample analysis section of chapter 2 were not able to accurately detect meaningful amounts in any of the urine or plasma samples. Also, no concluding results of specific sugar concentrations could be made from any of the plasma samples sent to Dr. Lambert. It appeared to be a detection problem for the equipment used.

Figure 11 displays the percent sucrose excreted by the two horses while either receiving bute or control treatment. Sucrose excretion was higher for Chaser while receiving bute treatment compared to his control treatment between 0 and 6 hours. Allen however, had a decrease in sucrose excretion while receiving bute treatment compared with the control treatment between 0 and 6 hours. The data also demonstrate a decrease in sucrose excretion at 12 hours followed by a spike in sucrose excretion 24 hours for both horses, which is puzzling.

The percent lactulose excreted by both horses during trial 1 and trial 2 is illustrated in Figure 12. Similar to his sucrose excretion, Chaser exhibited increased lactulose excretion after receiving bute treatment compared with the control treatment. Allen, however, exhibited the opposite effect. Lactulose excretion was found to increase at 24 hours in both horses, similar to sucrose excretion and, again, is not fully explainable at this time.

Figure 11. Percent sucrose excretion for trial 1 and trial 2 for both horses receiving bute and control treatments.

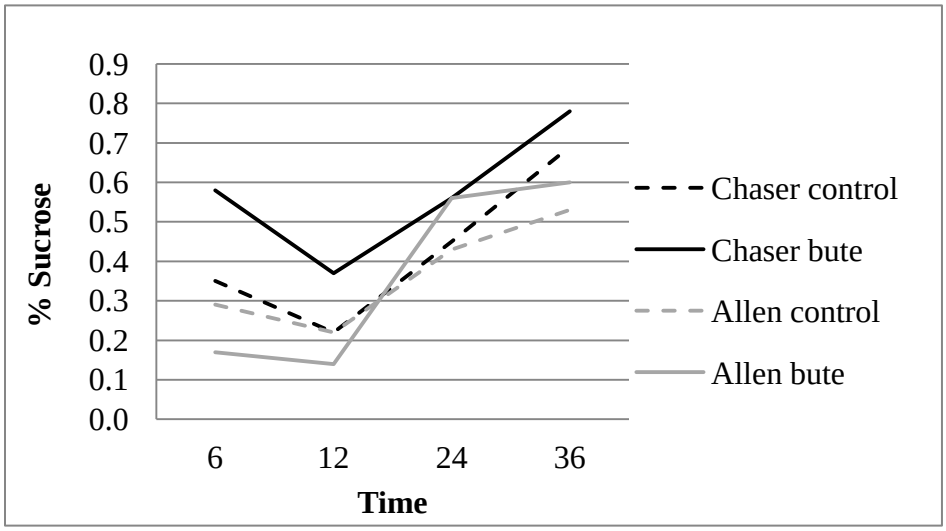


Figure 12. Percent lactulose excretion from trial 1 and trial 2 for both horses receiving bute and control treatments.

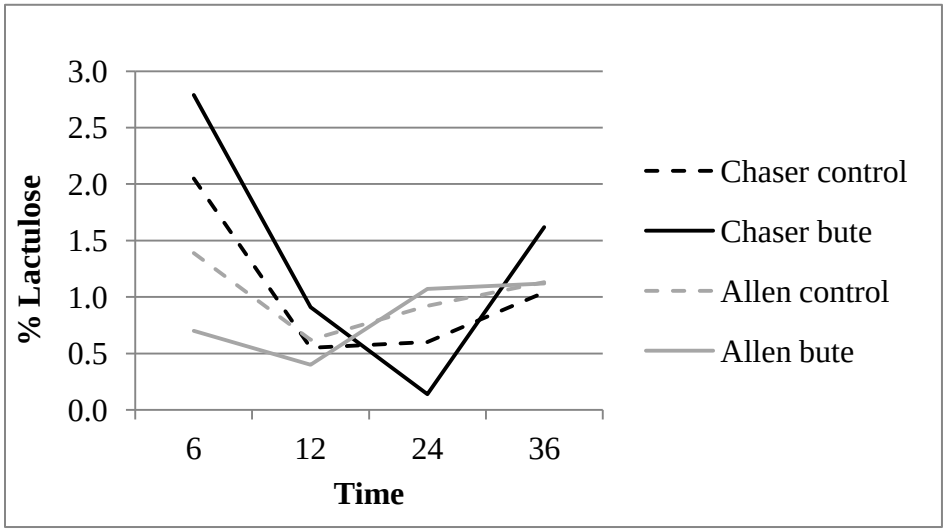


Figure 13 shows the percent rhamnose excreted. Rhamnose is thought to serve as a 'control' probe as it is readily passed transcellularly across the small intestine epithelium (Pals et al., 1997). The decline in excretion after 12 hours could indicate the maximum retention time in horses, as there is little rhamnose left to be excreted. While Allen was receiving bute treatment, rhamnose excretion increased slightly again after 12 hours. This is not explainable. Looking at Allen's sucrose and lactulose excretion, it appears that he follows an opposite trend of what we had expected to see, however, at 24 hours, he excretes more of each sugar while receiving bute treatment. The reason behind this is unknown.

The results of the lactulose to rhamnose (L/R) ratio from 6 to 12 hours of excretion post-sugar administration are depicted by Figure 14. By taking the ratio of rhamnose and lactulose, one can eliminate physiological factors affecting excretion (Pals et al., 2007). From the graph in Figure 13, it can be seen that small intestinal permeability did increase for both the bute and control treatments with the bute treatment increases seemingly greater than the control.

Figure 13. Percent rhamnose excretion from trial 1 and trial 2 for both horses receiving bute and control treatments.

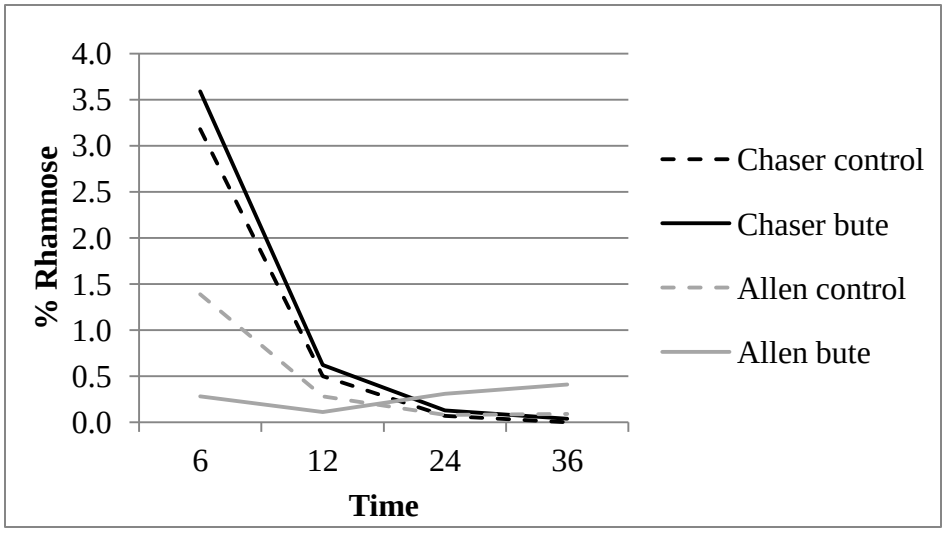
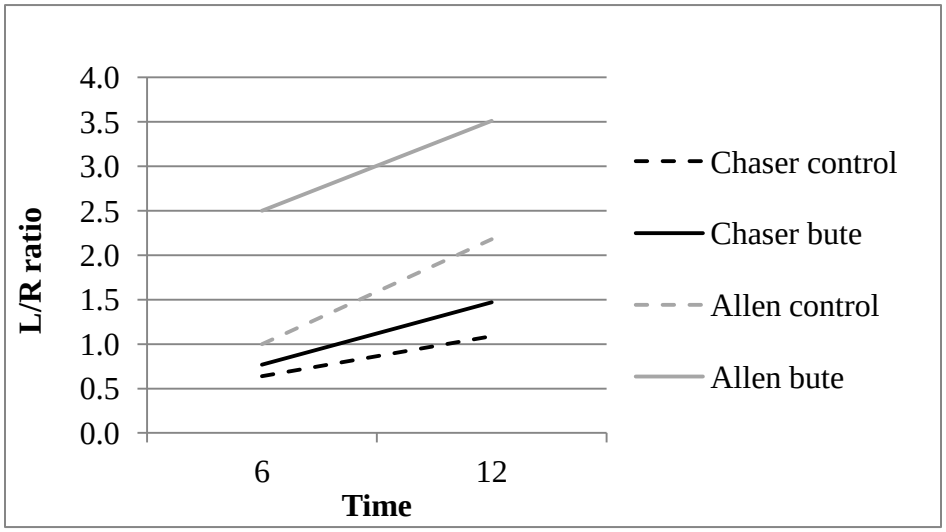


Figure 14. Lactulose/rhamnose excretion from 6 to 12 h in trial 1 and trial 2 for both horses receiving bute and control treatments.



Discussion

For all of the sugar excretion data, results after 12 h are still puzzling and need further investigation as to their importance. One explanation for the increased excretion of sugars past this point may be because of increased urine output, thus a higher percentage of the probe would have been present. For example, Chaser produced 15,400 ml of urine at 36 h, which is more than double the amount he produced at 6 hours. One may also argue that the clearance rate of these sugars should have been such that they would be found at greater concentrations during early hours of collection and thus, the amount of urine produced would not directly affect the amount of probe excreted. In theory, the liquid phase gastric emptying time ranges from approximately 27.9 to 87.3 minutes (Hewetson et al., 2006). Liquids pass through the small intestine fairly quickly, reaching the cecum in about 2 to 8 hours after ingestion (Lewis, 2005). Within another 5 hours, most of the liquid is completely transferred into the colon. Passage rate through the colon takes the longest and occurs over the period of 36 to 48 hours (Lewis, 2005). With this knowledge, we might expect to see sucrose excretion highest in the 6-hour collection, lactulose highest by 12 hours and sucralose being highest around 24 to 36 hours. This, however, is not the trend we see. Sucrose excretion is initially high but then decreases at 12 hours, only to increase again at 24 and 36 hours. Lactulose excretion also follows a similar trend and, unfortunately as stated previously, no excretion data is available for sucralose. This unexplainable result could benefit from further research with a greater number of subjects to evaluate whether or not data past 12 hours is significant.

Along with GI transit time, kidney clearance rate may also play a role in how quickly the probes are absorbed and excreted but in theory, such effects should be zeroed out by looking at

the ratio of lactulose to rhamnose (Pals et al., 1997). Which according to our results, the L/R ratio appeared to model increased permeability of bute treated horses compared with controls.

For the most part, these trends were not mutually observed in the results received from the analysis done by the MSU Biochemistry Department. Unfortunately, there's no good way to understand which data are truly correct and we only have a second set of results for sucrose as MSU was not able to accurately separate lactulose, rhamnose and sucralose.

Appendix B. Urine and blood analyses following bute administration

Table 11. Time 0 urinalysis results for the presence of blood, protein and level of specific gravity from 18 mature Arabians following one week of bute administration for treatment groups OM (organic mineral) and IM (inorganic mineral).

		Urine Variables		
Horse #	**Treatment**	**Blood**	**Protein**	**Specific Gravity, g/dl**
2	OM	++	+	1.029
3	OM	neg	+	1.019
4	IM	neg	+	1.015
6	IM	trace- hemolysed	trace	1.011
7	IM	neg	++	>1.035
8	OM	trace- hemolysed	+	1.013
9	IM	trace- hemolysed	trace	1.018
10	IM	neg	trace	1.017
11	IM	neg	++	1.029
12	OM	neg	++	>1.035
13	IM	neg	trace	1.018
14	OM	neg	trace	1.015
15	IM	neg	+	1.019
16	OM	neg	+	>1.035
17	IM	neg	trace	1.012
18	OM	neg	++	>1.035
19	OM	+	+	1.031
20	OM	neg	+	1.029

As discussed in previous sections, NSAIDs work by inhibiting the COX enzyme. This enzyme actually has two isoforms, COX-1 and COX-2. The COX-2 isoform is highly induced by pro-inflammatory mediators in the event of inflammation, pain, or injury and thus, non-selective NSAIDs work to effectively blunt this response (Radi, 2009; Whelton, 1999). The COX-1 isoform is predominantly expressed in the normal GI and the consequences of its blockade include decreased gastric mucosa cytoprotection and enhanced acid secretion, of which causes ulceration, one of the many problems caused by NSAIDs (Whelton, 1999).

A decrease in prostaglandin secretion and the blockade of COX-1 may be an important contributor to the nephrotoxic effects seen with NSAID administration. COX-derived prostanoids can serve to modulate renal blood flow and glomerular filtration (Radi, 2009). Also, both COX isoforms have been shown to reduce portal vein pressure and visceral blood flow volume of portal vein and gastric mucosa in rats (Rahi, 2009). In the face of diminished renal perfusion, local prostaglandins do work to counter regulate the effects of NSAIDs but the regulation of renal hemodynamics and function is a highly complex, multifactorial process beyond the scope of this discussion. Essentially, NSAIDs decrease blood flow and help intensify renal ischemia.

Clinically, this chain of events can lead to acute renal papillary necrosis and has been documented to occur in horses. Gunson and Soma (1983) demonstrated that a decrease in water intake accompanied by NSAID administration could lead to necrosis. Bute alone did not result in renal dysfunction in this study. The authors stated that the combination of the two led to a decrease in blood flow because of the diminished prostaglandin activity and this, in turn, led to a reduction in urine production, output, and flow which ultimately decreased blood flow further

leading to acute renal papillary necrosis. Whelton (1999) also states a similar summary for water deprivation and its additive affects with NSAID use.

Out of the eighteen horses used in our study, there were few that exhibited a decrease in appetite and water consumption was hard to measure. Upon looking at table 11, we could conclude that many of the specific gravities for each of the horses does demonstrate a level of dehydration, some greater than others. This might be expected as some of these horses had been without water for a few hours due to endoscopy and other procedures being performed on the day of urine collection. The protein levels in horse numbers 7, 11, 12, and 18 may suggest proteinuria and slight nephrotic effects due to NSAID administration. However, in a clinical setting these horses may be considered in the normal range because of their highly concentrated sample as indicated by their respectively higher specific gravities. Only two horses (numbers 2 and 19) had evidence of more than trace elements of blood in their urine, which could be indicative of some kidney damage. However, these two horses did not have high protein levels but did have greater specific gravity of the urine. The horse mentioned in Chapter 3 as having very low TS on day 49 and 56 did not demonstrate any of these adverse effects measured in urine. Also, upon looking at the ulcer scoring data from day 49 of the study, ulcer grade does not seem to associate 100% with urinalyses. Some of the above horses that exhibited adverse conditions did not have worse ulcer grades.

Table 12. Blood analysis results from day 42 and 49 for the variables blood urea nitrogen (BUN), creatinine (CR), chloride (Cl), total protein (TP) and albumin (Alb) from 18 mature Arabians receiving either organic mineral (OM) or inorganic mineral (IM) treatments.

			Variables				
Horse #	**Treatment**	**Day**	**BUN, mg/dL**	**CR, mg/dL**	**Cl, mmol/L**	**TP, g/dl**	**Alb, g/dL**
2	OM	42	18	1.1	88	5.8	3.0
		49	23	1.2	99	6.0	3.3
3	OM	42	19	1.0	93	5.5	2.9
		49	21	1.2	96	5.2	2.8
4	IM	42	16	1.0	89	5.6	3.2
		49	37	1.5	94	5.7	3.3
6	IM	42	20	1.1	98	5.3	3.0
		49	21	1.1	98	5.0	2.9
7	IM	42	20	1.1	98	5.0	3.0
		49	21	1.1	97	5.2	3.1
8	OM	42	23	1.1	97	5.6	3.0
		49	25	1.2	96	5.5	2.9
9	IM	42	19	1.1	97	6.0	3.0
		49	21	1.1	101	5.3	2.7
10	IM	42	15	1.0	91	5.8	3.1
		49	17	0.9	100	5.9	3.3
11	IM	42	24	1.1	98	5.4	2.9
		49	26	1.1	97	5.0	2.6
12	OM	42	17	1.1	95	5.7	3.2
		49	21	1.1	101	4.8	2.8
13	IM	42	17	1.1	93	6.0	3.0
		49	23	1.1	100	5.0	2.5
14	OM	42	17	0.8	94	5.8	3.2
		49	18	0.9	95	5.9	3.3
15	IM	42	18	1.0	97	5.4	3.1
		49	21	1.0	97	5.2	3.0
16	OM	42	18	1.0	95	6.1	3.3
		49	22	1.1	100	5.8	3.2
17	IM	42	22	0.9	96	5.4	3.1
		49	18	0.7	99	3.4	1.8
18	OM	42	18	0.9	97	5.8	3.2
		49	19	0.8	103	5.2	2.8
19	OM	42	17	0.9	94	6.3	3.2
		49	22	0.8	100	5.5	2.8
20	OM	42	21	0.9	99	5.7	3.1
		49	22	1.0	100	5.7	3.1

Table 13. Statistical analysis for blood analysis results from day 42 and 49 for the variables blood urea nitrogen (BUN), creatinine (CR), chloride (Cl), total protein (TP) and albumin (Alb) from 18 mature Arabians receiving either organic mineral (OM) or inorganic mineral (IM) treatments.

	Day							
Variable	**42**		**49**			**Effects, p-value**		
	OM	IM	OM	IM	SEM	Trt	Day	Trt*Day
BUN, mg/dL	18.7	19.0	21.4	22.8	1.20	0.49	0.01	0.68
CR, mg/dL	0.98	1.04	1.03	1.07	0.05	0.32	0.44	0.74
Cl, mmol/L	94.7	95.2	98.9	98.1	0.96	0.91	0.009	0.49
TP, g/dL	5.8	5.5	5.5	5.1	0.15	0.03	0.02	0.59
Alb, g/dL	3.1	3.0	3.0	2.8	0.09	0.14	0.05	0.51

Clinicians often utilize these various variables measured in serum to better understand the physiological state of the animal and level of disease present, if any, due to adverse conditions such as those caused by NSAID (phenylbutazone) administration. From these results seen in tables 12 and 13, we are able to demonstrate changes due to treatment and mineral supplementation.

Blood urea nitrogen (BUN), chloride (Cl), total protein (TP), and albumin (Alb) were all different after the administration of phenylbutazone (bute) on day 49 compared with day 42 prior to bute treatment. Urea is produced in the liver from the breakdown of protein. If the kidneys are not functioning correctly, BUN can increase, as it is not being excreted properly. Increased intestinal absorption of protein can also increase BUN as excess protein that is not used will be broken down into urea. Ulceration present in the stomach can exacerbate this process with blood passing from the stomach into the small intestine. In this study, there was significant ulceration on day 49 so an increased BUN could be expected. Similarly, TP and Alb decrease after bute

($p = 0.02$ and $p = 0.05$, respectively) administration could reflect the hydration status of the animal, inflammation, and protein loss, all of which were likely occurring conditions. Cohen (2002) states that in a condition of right dorsal colitis (RDC), typical serum protein concentrations would be 4.0 to 5.5 g/dl, and albumin concentrations between 1.5 to 2.0 g/dl. In a state of equine gastric ulcer syndrome (EGUS), protein loss through the GI tract can be expected. Albumin is a main component of TP, but is smaller in size, so if hypoproteinemia is seen it is likely due to the loss of Alb within the GI tract (Bueno et al., 2000). The increase in chloride before and after bute is interesting but at this time, no conclusion can be made for this finding ($p = 0.009$).

There were no treatment by day interactions but a treatment difference was noticed for total protein ($p = 0.03$). The TP values both pre and post-bute administration was below the normal range, which is 6.0 to 8.7 g/dl. Inorganic mineral (IM) treatment resulted in a lower TP of 5.1 g/dl after bute administration on day 49 compared with organic mineral (OM) treatment TP of 5.5 g/dl. Based on all of our other measurements, we can not say that this is physiologically significant as there was no difference in ulcer grade, healing rate, total solids or packed cell volume between the two treatments. There were a few horses that exhibited increased protein in urine after NSAID administration but only one of them corresponds to a horse having low TP in blood (horse number 12). However, it may warrant more investigation as to why there was this small, but significant resulting difference between OM and IM. The high level of variability between horses should also be taken into account when assessing horses clinically, as it appears that there is not a clear relationship between ulcer score and the variables measured in urine and plasma.

Appendix C. Urine preservation methodology results

Table 14. Statistical analysis of the three different urine sucrose preservation methods used, no preservative (none), sodium azide, and thymol for three different horses.

	Urine Sucrose, mg/L			
Horse	**None**	**Sodium Azide**	**Thymol**	**P-value**
Allen	48.8	51.2	101.8	Trt
Chaser	56.3	49.3	70.0	
Sera Star	22.4	12.4	24.9	
Mean	42.5	37.6	65.6	0.13
SEM	15.9	15.9	15.9	