Can measurement of urine isoprostanes predict survival in horses with colic?

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ABSTRACT

Approximately 4% of horses suffer from one colic episode per year. The outcome is fatal in 11% of cases. F2-isoprostanes are the "gold standard" for assessment of oxidative stress *in vivo* and have been used extensively to quantify lipid peroxidation in association with risk factors in various diseases in humans. Because horses with colic may have intestinal ischemia and/or inflammation characterized by oxidative stress and increased production of isoprostanes, measurement of isoprostane concentrations in colicky horses may be of clinical value.

The purpose of this study was to gather preliminary data on the feasibility of using urine isoprostane concentrations as an early screening tool for the severity of colic and to determine the need for surgery. The long term goal of this investigation is to reduce the number of deaths due to colic by developing a stall-side test capable of identifying horses needing surgery as early as possible and expediting their timely referral. We hypothesized that urine isoprostanes and isoprostane metabolites would be significantly higher in horses with colic compared to normal horses and that they can be used an indicator for the need for surgical intervention.

Urine samples were collected from 42 normal horses and 38 horses with colic (21 medical and 22 surgical). Urine isoprostane and isoprostane metabolite concentrations were measured by mass spectrometry and normalized by urine creatinine (Cr) concentrations. Statistical analysis was performed using a one way ANOVA (Tukey's post-hoc comparison) and a 2 sample t-test. Significance was set at $P<0.05$.

Mean (± SD) concentrations of isoprostanes and isoprostane metabolites were significantly higher in urine samples of horses with colic (2.94 ± 1.69 ng/mg Cr and 0.31 ± 0.22 ng/mg Cr, respectively) compared to healthy horses (1.89 ± 1.39 ng/mg Cr and 0.22 ± 0.08 ng/mg Cr, respectively). Urine isoprostane metabolite concentrations were significantly higher in horses undergoing surgery (0.38 ± 0.28 ng/mg Cr) compared to healthy control horses and medically treated colic horses (0.26 ± 0.11 ng/mg Cr). Non-survivors had significantly higher mean urine isoprostane metabolite concentrations (0.47 ± 0.39 ng/mg Cr) compared to healthy control horses and surviving colic horses (0.29 ± 0.24 ng/mg Cr).

Since urinary concentrations of isoprostane metabolites are increased in horses suffering from colic and in non-survivor colic horses, the measurement of urine concentrations of isoprostane metabolites may be an important prognostic indicator in equine colic.

Dedicated to my son,

William

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1. Equine Colic Syndrome

1.1. Definition

Acute equine colic syndrome has been recognized for centuries and outlines all pathological conditions showing clinical signs of a painful abdomen (Mulder 1991). Originating from the Greek word "κωλικο", which means abdominal pain, the term colic was later converted into Latin as "colico" translating into spasmodic occurring pain of the abdomen and its organs. For example, during the Greek classical period of science, Aristotle (384-322 B.C.) described in his "Historia animalium" a condition affecting horses and referred to it as "eilós" (ileus) (Baranski 1886).

Colic is one of the most prevalent and challenging diseases encountered by equine veterinarians. It has a complex, multifactorial nature and is an important cause of mortality and morbidity in domesticated horses (Kaneene et al. 1997b; Mair 2002; Reeves 1997; Tinker et al. 1997a). Determining the prognosis for survival in horses suffering from colic is challenging for the reason that many different diseases and pathophysiologic processes can cause abdominal pain (Archer *et al.* 2006; Dukti and White 2009; Reeves 1997). Over the past several decades, treatment of horses with colic has improved considerably. Fatality can still be high due to delayed recognition of clinical signs, the time delay inherent in receiving veterinary care, and the

lack of effective treatment for the more severe cases (Dukti and White 2009). According to a recent national survey, approximately 4% of horses suffer from at least one colic episode per year (Proudman et al. 2002b; White 1990). The outcome is fatal in 11% of cases (Proudman et al. 2002b). Intensive case management and surgery for critically ill horses can be expensive and emotionally draining for owners. Therefore, providing an accurate prognosis is a critical component of the decision making process necessary for accurate case management (Archer *et al.* 2006; Dukti and White 2009). The availability of a simple, precise, and affordable test that could reliably quantify the severity of colic could possibly lead to more immediate and appropriate treatment of clinical cases. The results of such a test would be useful as a prognostic indicator. The long term goal of this study is to reduce the number of deaths due to colic by developing a stall-test capable of identifying horses that need surgery as early as possible.

1.2. Incidence and categories of colic

The reported incidence rate of colic varies in different horse populations from 3.5 to 10.6 colic episodes per 100 horses per year (Hillyer *et al.* 2001; Kaneene *et al.* 1997a; Tinker *et al.* 1997a). Incidence rates can vary considerably within a horse population or between farms from 0 to 30 episodes per 100 horses per year (Tinker *et al.* 1997b). In many cases of colic, the precise cause of gastrointestinal pain is unknown. Most horses showing colic (80%–85%) either spontaneously recover with no specific diagnosis identified or they respond to medical therapy (Proudman 1992; Tinker et al. 1997a; White 1990). Horses with colic that show no specific signs and resolve with minimal medical care have an excellent prognosis (Proudman 1992; Tinker et al. 1997b; White N. 2009). More serious cases suffering from obstructing or strangulating disease requiring

surgical intervention represent approximately 2% to 4% of colic cases (Hillyer et al. 2001; White 1990). In some populations the incidence has been reported to be as high as 10% (Allen *et al.* 1986; Hillyer *et al.* 2001; Tinker *et al.* 1997b). Ten to 15% of horses having had a previous colic incident will experience future episodes of abdominal pain (Tinker *et al.* 1997b). Overall, reports of estimated fatality rates as a result of colic vary from 6.7% to 15.6% depending on the population studied and the type of lesion diagnosed (Archer and Proudman 2006; Hillyer *et al.* 2001; Kaneene *et al.* 1997a; Tinker *et al.* 1997a). In a study conducted by Kaneene and colleagues, medical colics were reported to have a fatality rate of 9% compared to 31% in horses with surgical lesions. This underlines the importance of preventing colic, particularly those forms that may require surgical intervention, and also of identifying those horses with surgical lesions in a timely fashion (Kaneene *et al.* 1997b).

Colic refers only to signs of abdominal discomfort and can therefore be caused by pathology in systems other than the gastrointestinal tract that manifest with signs of abdominal pain. In fact, infections elsewhere in the body, such as the urogenital or respiratory tracts, as well as pregnancy-related diseases, may present symptoms of colic (Archer and Proudman 2006). There are many causes and types of colic. For example, factors such as sudden changes in the weather, feeding (frequency, quantity or quality of feed) and water intake, poor dentition, blood loss or overexertion can lead to signs of colic (Archer and Proudman 2006).

Colic can be categorized into the following groups: obstruction (strangulating and non-strangulating), spasms, distention, ulcerations, enteritis/colitis, infarction and peritonitis. Spasmodic colic is caused by severe contractions of the intestines (Ducharme and Fubini 1983).

Normally, contractions of smooth muscle cells in the wall of the intestine occur in a well-coordinated manner, moving the ingesta aborally along the gastrointestinal tract. In contrast, abnormal, uncoordinated contractions, otherwise known as spasms, may cause a horse to experience abdominal pain. In these instances, the blood supply to the intestine is normal, and there is no obstruction to the movement of ingesta. Presumably, spasms may occur either in the small intestine or the large colon (Navarre and Roussel 1996; Ross *et al.* 1989).

Intestinal obstruction creates a severe condition resulting in extreme pain. An obstruction should be diagnosed as early as possible, as these lesions can be life-threatening and may require surgery. Typically, an obstruction occurs when the normal movement of ingesta is restricted or prevented but no change occurs in the blood supply to the intestine (Allen *et al.* 1986). Simple obstructions of the intestinal lumen typically involve an intra-luminal mass composed of ingesta or foreign material, such as bailing twine, a piece of hay net or rubber fencing. Most often obstructions occur when the mass fails to move from a portion of the bowel having a large diameter into a portion with a smaller diameter (White 2009). For example, an enterolith formed in the ascending colon may pass into the small colon, where it may fully occlude the lumen, and begin to induce mural pressure necrosis, ultimately leading to intestinal rupture in some cases (Hassel *et al.* 1999).

Strangulating obstructions occur when both the flow of ingesta and the intestinal blood supply are interrupted. This can occur if the intestine moves through an opening, such as a tear in the mesentery, or if the intestine twists enough to occlude the lumen and vasculature. The affected intestine becomes edematous and ischemic, and the intestine proximal to the lesion distends.

Examples of strangulating obstructions include large colon volvulus, strangulating lipoma, inguinal hernia, and incarceration of small intestine through a mesenteric rent.

Distention of the intestine occurs when excess gas in the intestinal lumen stretches the wall of the intestine. When the stomach is involved, the condition is called dilation; when the cecum or colon is involved, it is referred to as tympany. The most common examples of distention are cecal tympany and gastric dilation (Moore 2008). Pain can result from the stimulation of visceral afferent neurons by local inflammatory mediators, particularly prostanoids, produced in response to mucosal injury and inflammation. Nociceptors within the gastrointestinal mucosa detect mechanical, chemical, and thermal stimuli and relay this information to the central nervous system. Low-sensitivity fibers monitor normal physiologic functions and high-sensitivity fibers respond only to noxious stimuli, such as severe distension (Al-Chaer and Traub 2002; Cervero and Laird 1999).

Ulceration is referred to as loss of mucosal epithelial cells down to the submucosa of the intestine. This may result in bleeding into the intestinal lumen and even perforation of the intestinal wall. The initial stages typically involve localized mucosal inflammation, but this frequently progresses to widespread systemic activation of the inflammatory cascade. Many of the sequelae of this condition, such as laminitis and multiple organ failure, are related to a systemic inflammatory response. The most common examples of conditions involving ulceration are gastric ulcer disease and right dorsal colitis (Al Jassim and Andrews 2009; White 2009).

Enteritis refers to inflammation of the small intestine. This inflammation results in thickening of the intestinal wall, secretion of fluid into the intestinal lumen, distention of the intestine with gas and fluid, and may lead to ileus. Colitis refers to inflammation of the colon. The flux of fluid, electrolytes, and protein into the interstitium, in combination with damage resulting from colonic inflammation, leads to the development of interstitial edema. The fluid and protein within the interstitium may then enter the intestinal lumen by way of the damaged and dysfunctional epithelium, resulting in intestinal fluid losses and protein-losing enteropathy (White 2009). Although proximal (duodenal and jejunal) enteritis is the only clinical disease that results in enteritis in adult horses apart from verminous enteritis, there are numerous causes for colitis, such as salmonellosis and clostridial enterocolitis (Moore 2008).

Inflammation of the inner lining of the peritoneal cavity is called peritonitis. Commonly peritonitis occurs secondary to strangulated or severely inflamed intestine and results in the migration of large numbers of white blood cells into the peritoneal cavity. Examples of conditions that cause peritonitis include strangulating obstruction of the small intestine by a lipoma pendulans, abdominal abscess, uterine tear or rupture, rectal tear, metastatic disease, intestinal perforation (i.e. by a foreign body). Peritonitis can also be induced during an abdominal paracentesis (Dyson 1983).

In contrast, non-strangulating infarctions are characterized by a segmental loss of blood supply to part of the intestine in the absence of a displacement or incarceration leading to ischemia of the affected tissues. This condition may affect the small intestine, cecum or colon, and it may simultaneously affect more than one of these regions of the intestine (White 2009). Regardless of

which area is affected, these cases need immediate surgical attention (resection and anastomosis) in order to survive (Hardy *et al.* 2000; Prange *et al.* 2010; White 2009).

1.3. Diagnostic and prognostic parameters

Useful information for assessing colic patients is gained with the following procedures: obtaining a thorough history and physical examination, blood work (complete blood cell count, serum chemistry, serum lactate), nasogastric intubation, abdominocentesis, and palpation per rectum. Abdominal ultrasonography is a useful diagnostic tool to determine intestinal luminal distention, intestinal wall thickness, and presence of intestinal motility in colicking horses. At the same time, abdominal radiography is used in some cases to identify enteroliths or sand accumulation in the intestines (White 2009).

Various studies have attempted to evaluate the relationship between survival and physical examination findings, revealing an association between cardiovascular status and pain with fatal outcome (Furr *et al.* 1995; Garcia-Seco *et al.* 2005; Hinchcliff *et al.* 2005; Mair and Smith 2005b; Proudman *et al.* 2006; Proudman *et al.* 2005; Thoefner *et al.* 2000; van der Linden *et al.* 2003). In the past, blood lactate concentration, oral mucous membrane refill time, as well as indices of cardiovascular function, such as systolic pressure, have had the best prognostic value to predict the outcome of colic in horses (Moore *et al.* 1976; Parry *et al.* 1983b; Orsini *et al.* 1988; Morris *et al.* 1991; Seahorn *et al.* 1994). Physical examination parameters including borborygmi, mucous membrane character, and temperature have not been consistently associated with mortality (van der Linden *et al.* 2003). A study by Proudman and coworkers found no

association between heart rate and survival (Proudman *et al.* 2002a), whereas another study found no association between packed cell volume (PCV) and survival (van der Linden *et al.* 2003). Inconsistent results were found when duration until admission and its possible negative effect on survival were evaluated (Mair and Smith 2005a; Proudman *et al.* 2002a; van der Linden *et al.* 2003).

Laboratory variables gained from blood or peritoneal samples appear to be most useful in determining prognosis. Routinely measured electrolytes have been shown to have some prognostic value in several studies. The anion gap appears to be a useful prognostic indicator (Moore *et al.* 1976), where the probability of survival decreased from 81% survival with an anion gap of less than 20 mEq/L, to 47% survival with an anion gap of 20 to 24.9 mEq/L, and 0% survival in horses with an anion gap of greater than or equal to 25 mEq/L (Bristol 1982). Another study looked at low pre-operative serum ionized magnesium and subnormal serum ionized calcium concentrations and found that low ionized calcium and magnesium were associated with strangulating lesions (Garcia-Lopez *et al.* 2001; Johansson *et al.* 2003), and low ionized magnesium was associated with post-operative ileus (Proudman *et al.* 2005). Other factors that can affect magnesium and calcium concentrations include inadequate alimentary intake, alterations in the acid-base balance and serum protein concentrations, intravenous administration of fluids without magnesium and calcium supplementation, intestinal resection, endotoxemia, sepsis, and severe trauma (Cortes and Moses 2007; Dart *et al.* 1992). Increased activity of serum tumour necrosis factor may also be associated with increased mortality (Morris *et al.* 1991). In the past, cortisol, epinephrine, norepinephrine, lactate, electrolyte concentrations, and acid-base variables have been evaluated (Hinchcliff *et al.* 2005), revealing that higher

plasma epinephrine, plasma lactate, and serum cortisol concentrations were significantly associated with fatality; however, plasma norepinephrine concentration was not. An elevation in activity of alkaline phosphatase in the peritoneal fluid was reported to be a useful predictor for surgery, while serum alkaline phosphatase was not (Saulez *et al.* 2004). In critically ill humans, glucose homeostasis is dysregulated resulting in hyperglycemia and decreased survival (Crandall *et al*. 2009). A recent study confirmed prior reports that hyperglycemia is common in horses presenting with abdominal crisis and is associated with a worse prognosis for survival to hospital discharge (Hassel et al. 2009). In this study, 16.7% (38 of 228) of horses with acute abdominal disease had severe hyperglycemia (<195 mg/dL). Factors associated with increased odds of failure to survive included glucose, severity of pain at admission, heart rate, PCV, anion gap, and diagnosis. The model used in this study correctly classified outcome for 92.5% of horses.

In humans and animals, plasma lactate concentrations have been used clinically and experimentally as a sensitive indicator of mesenteric ischemia (de Papp *et al.* 1999; Latson *et al.* 2005; Liao *et al.* 1995; Muraki *et al.* 2003). Anaerobic cellular metabolism of glucose generates lactate and is normally produced in metabolically active tissues at a rate of 1mmol/kg/h (Johnston *et al.* 2007; Marino 1997). Lactate is the end product of anaerobic glycolysis and is most commonly elevated due to poor tissue perfusion and circulatory shock (De Backer *et al.* 2003). During oxygen deprivation, aerobic oxidation of glucose by the tricarboxcylic acid cycle is blocked, causing a shift in the equilibrium to favor the production of lactate and other organic acids (Marino 1997). As cellular pH decreases during intestinal ischemia with the accumulation of lactate, inorganic phosphates, and organic acids, cell membrane permeability is altered and lactate is released into the extracellular fluid / peritoneal fluid whereby it gains access to the

systemic circulation (Liao *et al.* 1995; Marino 1997). Therefore, the detection of these biochemical markers in blood or peritoneal fluid assists in the early recognition of ischemic bowel injury in clinical cases.

Plasma lactate concentrations have also been shown to be of prognostic value in critically ill human patients (Marino 1997; Weil and Afifi 1970). There is an association between hyperlactatemia and decreased survival of human patients with a variety of diseases including hypovolemic and septic shock, traumatic injury, mesenteric infarction, and acute myocardial infarction (Liao *et al.* 1995; Marino 1997; Muraki *et al.* 2003; Weil and Afifi 1970). Previous studies have evaluated peripheral blood lactate as a pre-operative prognostic and diagnostic tool in both equine (Donawick *et al.* 1975; Furr *et al.* 1995; Moore *et al.* 1976; Parry *et al.* 1983a; Thoefner *et al.* 2000) and human cases (DeLaurier *et al.* 1994). In veterinary medicine, a correlation between hyperlactatemia and survival has been established. A study in dogs with gastric dilation–volvulus reported a strong association between plasma lactate concentration, gastric necrosis, and outcome (de Papp *et al.* 1999). Numerous studies have documented an association between both elevations in plasma and peritoneal fluid lactate and intestinal ischemia in equine acute abdomen patients and survival (Donawick *et al.* 1975; Bristol 1982; Parry et al. 1983c; Furr *et al.* 1995; Latson *et al.* 2005; Moore *et al.* 1976; Nappert and Johnson 2001; Orsini *et al.* 1988; Saulez *et al.* 2005). Latson and coworkers have shown that a logistic regression model using peritoneal fluid lactate, chloride, pH values, and peritoneal fluid gross appearance can be used to calculate a prognostic index for the presence of intestinal ischemia secondary to strangulating obstruction accurately (Latson *et al.* 2005). Increased peritoneal and plasma lactate values have a direct, predisposing association with the need for surgery in clinical

colic cases, but are not useful if isolated from other correlating clinical and laboratory data (Thoefner *et al.* 2000).

Nevertheless, plasma lactate still appears to be the most useful single prognostic parameter in horses with colic (Niinisto *et al.*). Colic severity scores based on plasma lactate and other markers have been developed by several investigators (Orsini *et al.* 1988; Furr *et al.* 1995). A more recent study found plasma lactate concentration to be highly accurate in predicting survival of horses with ascending colon volvulus (Johnston *et al.* 2007). In this study, plasma lactate of less than 6 mmol/L had a positive predictive value of 96%, and no horses with lactate greater than 10 mmol/L survived. Stall-side portable lactate sampling is a simple, quick and reliable technique for measuring plasma and peritoneal fluid lactate (Delesalle *et al.* 2007). For large colon volvulus, specifically, plasma lactate is an accurate and invaluable preoperative prognostic tool. For other gastrointestinal lesions, plasma lactate is valuable (Kelmer 2009); however, peritoneal fluid lactate has a better correlation with survival rates, and neither plasma nor peritoneal fluid lactate values predict survival if the lesion is not strangulating (Delesalle *et al.* 2007). To determine the strangulating nature of the lesion is often impossible preoperatively in small intestinal lesions, therefore one must use caution when using lactate to predict survival in horses with these types of lesions (Latson *et al.* 2005; Kelmer 2009).

Given the high cost and emotional toll that can be involved in colic surgery, providing owners with an accurate prognosis is crucial to the decision making process. Reviewing the literature and continuing to focus on future diagnostic and prognostic indicators should improve the ability to educate clients on the likelihood of survival (Dukti and White 2009). None of the currently

used predictors works satisfactory for all colic cases including mild non-strangulating surgical cases affected by displacement or impaction, suggesting that there remains a need for a reliable predictor for the need for surgery.

The determination of the need for surgery is often performed in emergency situations where limited diagnostic tools are accessible. Though the decision is based best on clinical diagnosis, a specific diagnosis can rarely be accurately determined. In particular, non-strangulating lesions of the gastrointestinal tract, such as impactions or displacements, may be treated medically initially but may eventually need surgery if no progress is made using conservative measures. For these cases, a reliable stall-side test would be needed to help decide if the case needs to be referred and if it needs surgery or not. As of now, no reliable predictor has been found in order to determine the necessity of surgery in milder non-strangulating cases of equine colic.

2. Oxidative stress and Isoprostanes

2.1 Introduction

The term oxidative stress was first introduced as the title of a book (Sies 1985), in which it was defined as a disturbance in the pro-oxidant/anti-oxidant balance that may lead to tissue damage. An array of analytical tools is available for lipid peroxidation products, including thiobarbituric acid-reactive substances, lipid hydroperoxides, hydroxylated fatty acids, volatile hydrocarbons, aldehydes and cholesterol autooxidation products, protein carbonyls, aldehyde-modified proteins, oxidatively damaged DNA, and total anti-oxidant capacity for the accurate *in vitro*

measurement of oxidative stress (de Zwart *et al.* 1999; Halliwell and Whiteman 2004; Niki and Yoshida 2005). However, when the above-mentioned methods are applied to *in vivo* samples problems arise in accurately quantifying oxidative stress.

A key step towards the accurate *in vivo* measurement of lipid peroxidation products was made with the discovery of F2-isoprostanes two decades ago (Morrow *et al.* 1990a). While the other products of the isoprostane pathway may also be of interest, they are not as stable as F2-isoprostanes and thus are not ideal candidates for measurement as a marker of lipid peroxidation (Roberts and Morrow 2000).

2.2. Physiology

Numerous studies revealed that, like proteins, carbohydrates, and nucleic acids, lipids are a target of various reactive nitrogen species (RNS) and reactive oxygen species (ROS). During this process RNS and ROS oxidize (undergo a chemical reaction with oxygen) lipids to release a diverse array of products. Extensive *in vitro* studies have revealed the mechanisms, dynamics, and products of lipid peroxidation, but the physiological concentrations and biological effects of lipid peroxidation and its products have yet to be elucidated (Niki 2009). It has been shown that lipid peroxidation induces disturbance of the structures, alteration of integrity, fluidity, permeability, and functional loss of biomembranes. It also has been shown to modify low density lipoprotein to pro-atherogenic and pro-inflammatory forms, and generate potentially toxic products (Greenberg *et al.* 2008). Products of lipid peroxidation have been shown to be carcinogenic and mutagenic (West and Marnett 2006). Also, secondary products of lipid

peroxidation, may modify biologically essential molecules such as proteins and DNA bases (Esterbauer *et al.* 1991; Poli *et al.* 2008). Thus lipid peroxidation has been implicated *in vivo* as the causal mechanism in numerous disorders such as cardiovascular diseases, cancer, neurological disorders, and aging. These studies show the association between the concentrations of lipid peroxidation product increases in the progression of oxidative stress-related diseases (Basu 2008; Chen *et al.* 2007; Dalle-Donne *et al.* 2006; Lee *et al.* 2009; Polidori *et al.* 2007; Yin 2008; Yoshida *et al.* 2009), although it is not clear yet whether lipid peroxidation is important as a consequence or a cause. Also, lipid peroxidation products may play an important role as cellular regulators and signaling messengers as shown for angiogenesis (Niki 2009). In order to exert physiologically important functions as a regulator of gene expression and mediator of cellular signaling, the formation of lipid peroxidation products must be tightly controlled and programmed (Janssen-Heininger *et al.* 2008; Murphy 2009). It has been demonstrated that oxidants are used in physiological settings as signaling molecules with important regulatory functions controlling cell division, migration, contraction, and mediator production (Janssen-Heininger *et al.* 2008; Murphy 2009). These physiological functions are carried out in an exquisitely regulated and compartmentalized manner by mild oxidants, through subtle oxidative events that involve targeted amino acids in proteins (Janssen-Heininger *et al.* 2008; Murphy 2009). It is still not elucidated how the time, site, and amount of free radical formation, the selectivity and specificity of free radical-mediated lipid peroxidation reactions, and the formation of lipid peroxidation products are controlled.

More recently, the definition and role of oxidative stress have been a matter of hot debate (Azzi 2007; Davies 1999; Halliwell 2007; Hensley and Floyd 2002; Jones 2008; Kemp *et al.* 2008;

Packer and Cadenas 2007). The classical definition of oxidative stress by Sies was an imbalance between the production of oxidants and antioxidants in favor of the oxidants, potentially leading to tissue damage (Sies 1985). A new understanding of the action of ROS, RNS, and lipid peroxidation products in signal transduction signifies a paradigm shift in the concept of oxidative stress. In the near future, there may be different definitions of oxidative stress established that may define oxidative stress as a signal that induces oxidative reaction and/or affects redox balance, resulting in either stimulation of defense capacity or induction of deleterious tissue damage. Selye, a pioneer in the field of biological stress, predicted the term oxidative stress and lipid peroxidation may turn to either positive stimulus, eustress, or deleterious insult, distress (Selye 1998). In any case, it is surmised that aerobic organisms have created a fine defense network and sophisticated adaptive system during the course of evolution to cope with, or even utilize, moderate concentrations of lipid peroxidation products. Excessive unregulated lipid peroxidation leads to degenerative and pathological consequences and, in such cases, the radical scavenging antioxidants may exert beneficial effects. Many issues have been elucidated on lipid peroxidation in the last three decades, but many issues still remain unanswered and await to be addressed in future studies (Niki 2009).

An important type of oxidation is enzymatic lipid peroxidation. It is well known that lipoxygenase (LOX) and cyclooxygenase (COX) oxidize arachidonic acid to hydroperoxyeicosatetraenoic acid, prostaglandins, prostacyclin, thromboxane, and leukotrienes. Cyclooxygenases and LOX oxidize lipids in a region-, stereo-, and enantiomere-specific manner (Schneider *et al.* 2007). The term *prostanoids* includes two classes of eicosanoids: prostaglandins (PGs) and thromboxanes (Cha *et al.* 2006). Prostaglandins and thromboxanes are hormone-like

fatty acids and include several subtypes. They are ubiquitously distributed in most mammalian tissues and organs, and have an equally wide and diverse range of biological effects. For example, prostanoids initiate physiological and pathological activities, including smooth muscle contraction, inflammation, and blood clotting (Komoto *et al.* 2004). They are generated by cells throughout the body, especially during times of illness, stress or injury. Prostaglandins influence the nervous, reproductive, gastrointestinal, and renal systems, as well as the regulation of body fluids and temperature, and have profound effects on the body's defense mechanisms (Garfield 1984).

Isoprostanes are a group of lipid peroxidation products that were discovered in 1990 by Morrow and Roberts (Morrow *et al.* 1990c). Isoprostanes are formed by the free radical-mediated peroxidation of arachidonic acid, independent of cyclooxygenase (Morrow *et al.* 1990c). Numerous studies have been performed on these interesting lipid peroxidation products.

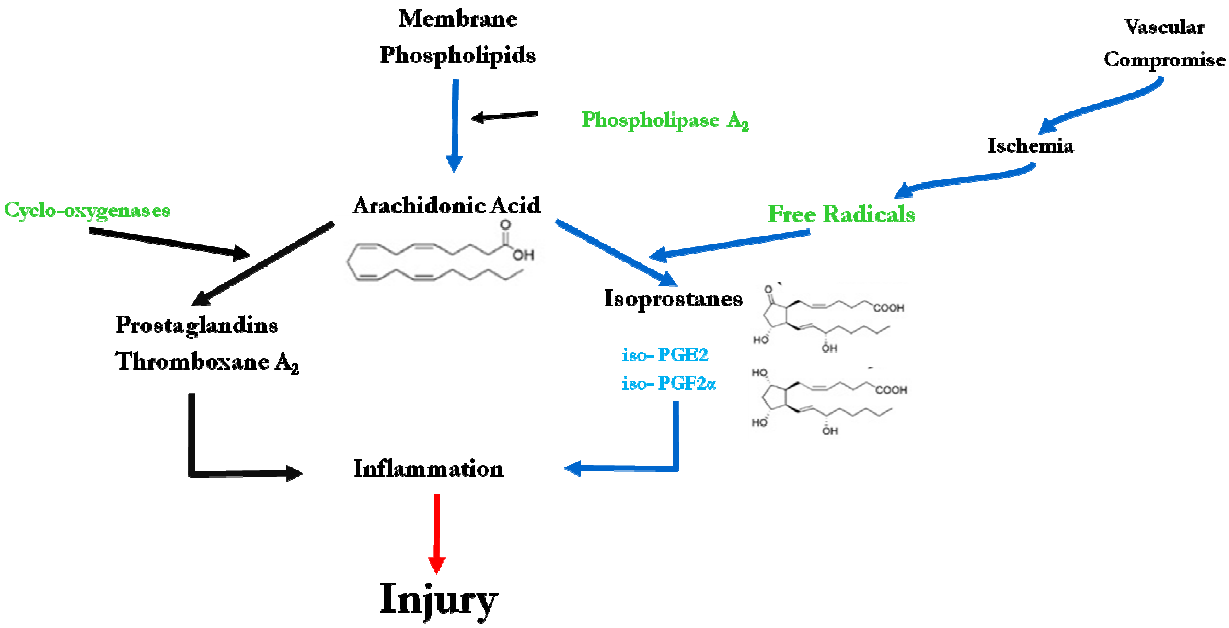

Fig 1.1 Pathophysiology of isoprostane generation during states of colic.

2.3. Isoprostanes

Eicosanoids are oxygenated hydrophobic compounds that largely function as paracrine mediators. “Eicosa” (Greek for the word ‘twenty’) denotes the number of carbon atoms in arachidonic acid (Cook 2005), the precursor of all eicosanoids. Arachidonic acid is a fatty acid derivative that is hydrolyzed from membrane phospholipids by phospholipases A_2 and C (Smith 1992). Isoprostanes, discovered more than a decade ago, are a family of prostaglandin-like compounds generated non-enzymatically *in vivo* by reactive oxygen species and free radical catalyzed-peroxidation of arachidonic acid (Liu *et al.* 1999; Morrow *et al.* 1990b). The formation of F2-isoprostanes has been shown to increase dramatically in well-established animal models of oxidant injury (Marnett *et al.* 1985). Several of the isoprostanes possess potent biological activity and thus may be mediators of tissue injury secondary to oxidative stress. Isoprostanes are not

only biological markers of oxidative stress *in vitro* and *in vivo* (Morrow and Roberts 1999a), but they also evoke important biological responses on most types of cells (Morrow and Roberts 1997). The potent vascular properties of isoprostanes, their potential role in equine laminitis (Noschka *et al.* 2009), together with their increased release in many diseases, serve as reasons to investigate their roles in equine colic syndrome where we expect gastrointestinal and vascular compromise.

Within the isoprostane family, F2- isoprostanes containing F-type prostane rings have been studied most extensively (Jahn *et al.* 2008; Lawson *et al.* 1999; Milne *et al.* 2008). F-type isoprostanes are denoted as 5-, 8-, 12-, and 15-series regio-isomers depending on the carbon atom to which the side chain hydroxyl is attached (Taber *et al.* 1997). The 8-, 9-, 11- and 12-peroxyl radicals, but not 5- and 15-peroxyl radicals, derived from arachidonic acid undergo two consecutive intra-molecular cyclizations, oxygen addition, and hydrogen abstraction to form prostaglandin G2-like compounds that are reduced by keto-reductase to generate F2-isoprostanes. An essential part in this pathway of forming isoprostanes is the endoperoxide intermediates that are reduced to F-ring isoprostanes. An important structural distinction between isoprostanes and cyclo-oxygenase-derived prostaglandins is that the former contain side chains that are predominantly oriented *cis* to the prostane ring, whereas the latter possess exclusively *trans* side chain (Morrow *et al.* 1990c). The prostaglandin H2-like bicyclic endoperoxide intermediates isomerize to yield D2- and E2- isoprostanes that are unstable and spontaneously dehydrate to yield J2- and A2- isoprostanes (Fessel *et al.* 2002), chemically reactive electrophilic cyclopentenones. The oxidation of eicosapentaenoic acid and docosahexaenoic acid by similar mechanisms form F3-isoprostanes and F4-neuroprostanes (Roberts *et al.* 2005; Song *et al.* 2008).

In addition to isoprostanes, isofurans having a substituted tetrahydrofuran ring structure have been identified (Fessel *et al.* 2002). Interestingly, increasing oxygen concentration favors the formation of isofurans over isoprostanes (Fessel *et al.* 2002). Several isoprostanes have been discovered to exert potent and important biological activities. These activities involve both receptor-mediated actions, e.g., vasoconstriction in the case of F2- isoprostanes and E2-isoprostanes (Cracowski 2004a, b) and biological effects due to their inherent chemical reactivity (Brame *et al.* 1999; Chen *et al.* 1999; Roberts and Morrow 1997). The generation of isoprostanes due to oxidative stress and their potential biological effects make an involvement in the pathophysiology of equine colic syndrome likely since there is evidence of gastrointestinal and vascular compromise in these cases.

2.3.1. Subtypes, Structure and Biosynthesis

There are four possible subfamilies of F2-isoprostanes that are formed as a result of free radical attack at the arachidonic acid carbon atoms C7, C10 and C13. Series 5 (Type VI) F2-isoprostanes are derived from free radical attack at C7. Series 8 (Type V) and series 12 (Type IV) result from free radical attack at C10. Series 15 (Type III) is derived from free radical attack at C13. Each subfamily comprises 16 diastereoisomers, since the hydroxy group on the cyclopentane ring can be arranged in eight different configurations. Overall, 64 F2-isoprostane isomers can be formed during peroxidation (Milne *et al.* 2008; Niki and Yoshida 2005). Of these, 8-epi-PGF2α (also known as 8-iso-PGF2α or 15-F2t-isoprostane) has received the most attention because it has been shown to possess certain adverse biological activities such as thrombosis, apoptosis, and cell death (Khasawneh *et al.* 2008; Morrow *et al.* 1992a; Morrow *et al.* 1992b;

Tang *et al.* 2005). Circulating 8-epi-PGF2α is mainly present bound to phospholipids and released by the local action of phospholipase A2 on cell membranes (Joy and Cowley 2008).

2.3.2. Vascular Effects and Receptor Distribution of Isoprostanes

Once released from cell membranes by phospholipases, the isoprostanes may induce vasoconstriction, platelet aggregation, and cellular proliferation (Crosswhite and Sun 2010; Giannarelli *et al.* 2010). F2-isoprostanes are released at sites of free radical-induced tissue injury and then enter the circulation. Local concentrations of isoprostanes may be sufficiently high to induce regional vasoconstriction via G-protein-coupled prostanoid receptors, but their effects depend on the type of tissue-specific vascular beds, species of mammal, and blood vessel type (arterial, venous or lymphatic) (Cracowski *et al.* 2001). In addition to their vasomotor properties, F2-isoprostanes may promote DNA synthesis and endothelial cell proliferation (Mohler *et al.* 1996; Yura *et al.* 1999), although the pathophysiological role of such processes remains unclear. Whether E2-isoprostanes induce systemic or local vasoconstrictor effects in a clinical setting has not yet been determined. However, E2-isoprostanes also may cause pronounced vasomotor effects *in vivo*, via activation of thromboxane prostanoid receptors. Consequently, E2- and F2-isoprostanes may play similar roles in the pathogenesis of vascular diseases (Cracowski *et al.* 2001). In addition to the F2 and E2-isoprostanes, isothromboxanes have recently been described (Morrow *et al.* 1996). Although the mechanism involved in the conversion of isoprostane endoperoxides to isothromboxanes remains undetermined, A2-isothromboxanes are formed and become rapidly metabolized to stable B2-isothromboxanes. Because thromboxane A_2 is a very potent vasoconstrictor (Cracowski *et al.* 1999), isothromboxanes may possess similar potent

biological activities. Isoprostanes exert their effects on platelet function and vascular tone *in vivo* by acting as incidental/secondary ligands at membrane thromboxane prostanoid receptors rather than via a distinct isoprostane receptor (Audoly et al. 2000). A specific isoprostane receptor has not yet been identified; however, it has been proposed (Khasawneh *et al.* 2008; Longmire *et al.* 1994a; Noschka *et al.* 2007; Sametz *et al.* 2000).

Activation of thromboxane prostanoid receptors by isoprostanes may be of importance since at least two potential ligands are able to bind to the same receptor, causing the same response. This may be of particular significance in syndromes where COX activation and oxidant stress coincide, such as in atherosclerosis and reperfusion after tissue ischemia. Due to their profound vasoconstrictor actions and ability to elicit robust intracellular responses in vascular smooth muscle and endothelium (Habib and Badr 2004), the isoprostanes are emerging as important mediators in vascular pathology. Therefore isoprostanes may contribute to ischemia and reperfusion injury in general but also in our colic patients where intestine and its vasculature are compromised.

2.4. Oxidative Stress Measurement Using F2-isoprostane Quantification

There are several approaches to the enrichment and final determination of F2-isoprostanes in biological samples. These include chromatographic separation involving solid-phase extraction or affinity chromatography with or without thin layer chromatography followed by final determination by gas chromatography-mass spectrometry, liquid chromatography mass spectrometry, radioimmunoassay or enzyme immunoassay (Nourooz-Zadeh 2008). The

preferred method for the quantifying F2-isoprostanes is gas chromatography-mass spectrometry negative-ion chemical ionization employing stable isotope dilution. This technique combines the high resolution of gas chromatography separation on fused silica capillary columns with the specificity and sensitivity of mass spectrometry (Nourooz-Zadeh 2000).

2.4.1. Techniques and Considerations

To assess endogenous production of F2-isoprostanes, several approaches can be utilized, each of which has certain advantages and drawbacks. Generally the measurement of free unmetabolized isoprostanes in biological fluids involves the use of plasma or urine although other biological fluids can also be used in special situations, e.g., cerebrospinal fluid. However, the use of plasma is not ideal as isoprostanes can be falsely elevated/artifactually produced by autooxidation of plasma arachidonic acid if samples are not processed and stored properly. Plasma samples must not be stored at -20°C because autooxidation may occur during storage at this temperature. However, it was found that plasma can be stored at -70°C for at least 6 months without the of generation of F2-isoprostanes by autooxidation. Since urine does not have a high lipid content, autoxidation is not a concern (Morrow *et al.* 1990c). Theoretically, concentrations of F2-isoprostanes in plasma can provide a useful index of total endogenous production of isoprostanes because concentrations in plasma presumably are derived from all tissues in the body. Local formation of F2-isoprostanes in the kidney due to dehydration may confound interpretation of urinary concentrations of unmetabolized F2-isoprostanes (Roberts and Morrow 1997; Taber *et al.* 1997). Depending on the experimental objectives, timing of sampling of blood for determination of F2-isoprostanes may not be critical. The measurement of F2-isoprostanes in a

single sample of blood only provides an index of isoprostane formation during a relatively short period of time since the elimination half-life of isoprostanes in the circulation is relatively short (within approximately 16 minutes) (Gniwotta *et al.* 1997). In chronic disease states there may be a relatively steady rate of formation and release of isoprostanes from phospholipids into the circulation. In these studies, timing of sampling of blood may not be as critical. On the other hand, in some chronic disease states there may be significant intra-day fluctuations in the formation of isoprostanes. In dynamic situations in which there is an oxidant insult for only a relatively short period of time, e.g., ischemia-reperfusion injury, multiple sequential sampling of blood is necessary to assess the full magnitude of the increase in isoprostane generation during rapidly changing rates of production over time (Richelle *et al.* 1999). If large alterations occur in the production of lipid peroxides over a long period (several hours to days in colic cases), measurement of F2- isoprostane concentrations in a single plasma sample will not provide an accurate integrated assessment of oxidative stress. Therefore, the quantification of urinary F2-isoprostanes has been proposed to be superior to the measurement of circulating F2- isoprostane concentrations, as it is believed to represent a more accurate systemic index of oxidative stress (Roberts and Morrow 2000). In addition, due to the logistics of drawing blood and the requirement that the plasma be rapidly isolated and stored at -80°C, the use of plasma for determination of F2-isoprostanes, although only minimally invasive, is frequently not suitable for clinical studies.

It is more feasible to collect urine samples; however, the interpretation of measurement of un-metabolized F2-isoprostanes in urine as an index of total endogenous isoprostane production can be confounded by the potential contribution of local isoprostane production in the kidney. A

study was conducted by the Morrow group to identify a urinary metabolite of F2-isoprostanes to circumvent these problems (Roberts and Morrow 2000). In the prostaglandin research community it has been well established that measurement of the urinary excretion of metabolites of prostaglandins represents the most reliable approach to assess total endogenous production of prostanoids (Dong *et al.* 2009; Higashi *et al.*; Kekatpure *et al.* 2009; Roberts *et al.* 1996). Consequently, it was presumed that measurement of the urinary excretion of an F2-isoprostane metabolite should also afford an accurate measure of endogenous production of isoprostanes due to a lower lipid and cellular content. Advantages of this approach are that blood does not have to be obtained and also that the measurement of the concentration of an isoprostane metabolite in urine collected over many hours can provide an integrated index of isoprostane production over time.

It was revealed that a single metabolite predominated in the profile of derivatives produced from metabolism of 15-F2t-isoprostane. This metabolite was identified by mass spectrometric analysis as 2,3-dinor-5,6-dihydro-15-F2t-isoprostane (Morrow *et al.* 1999). This metabolite has been synthesized and converted for use as an internal standard to develop a stable isotope dilution negative ion chemical ionization gas chromatography-mass spectrometry method for its analysis (Reilly *et al.* 1996). It was established that urine concentrations of this metabolite in healthy humans range between 0.39 ± 0.18 ng/mg creatinine (Morrow *et al.* 1999). A study measuring the excretion of 15-F2t- isoprostane in patients with polygenic hypercholesterolemia revealed an increase of 2.5-fold while these increases were suppressed by a mean of 54% following 8 weeks of treatment with a combination of vitamin E, vitamin C, and β-carotene (Roberts and Morrow 2000). These data suggest that quantification of the urinary excretion of 2,3-dinor-5,6-dihydro-

15-F2t-isoprostane contributes to the ability to reliably assess free radical–induced lipid peroxidation *in vivo* and provides an approach that should be applicable to clinical studies.

Additionally, concentrations have been demonstrated to be modulated by exogenous administration of antioxidant agents and by endogenous antioxidant status, e.g., vitamin E and selenium (Burk 1983; Longmire *et al.* 1994b; Richelle *et al.* 1999). Lastly, there may be a concern that concentrations of F2-isoprostanes may be influenced by lipid content in the diet that can contain isoprostanes as a result of oxidation of dietary arachidonic acid. Nevertheless, Morrow and coworkers demonstrated previously that urinary concentrations of F2-isoprostanes in subjects ingesting a normal diet were unchanged after switching to a low lipid diet consisting of only glucose polymers for 4 days (Morrow *et al.* 1990c). In another study by Richelle and collaborators, it was also confirmed that the lipid content of the diet has no effect on isoprostanes concentrations (Marnett *et al.* 1985).

To date, the gold standard for measurement of F2-isoprostanes is gas chromatography negative ion chemical ionization mass spectrometry due to its high specificity and sensitivity; however, this method is impractical as it is expensive and time consuming and not widely available (Morrow and Roberts 1999b). Enzyme-linked immunosorbent assay (ELISA) kits are available for measurement of F2-isoprostanes by several commercial vendors. Nevertheless, immunoassays for F2-isoprostanes are associated with the same potential shortcomings that have been recognized with immunoassays for prostaglandins for several decades (Granstrom and Kindahl 1978). Potential problems are primarily related to substances such as triacylglycerol, conjugated-bilirubin, and hemoglobin in biological fluids interfering with the assay via non-

specific binding (Kitano et al. 2006; Mas et al.). Immunoassays in general work very well in buffer systems that do not contain large amounts of biological substances; however, biological fluids and tissues are complex and interfering substances are frequently encountered (Proudfoot et al. 1999). Most often, biological samples have to be purified to some extent before performing the immunoassay. Simple partial purification procedures such as chemical extraction may actually concentrate interfering substances, thus requiring more extensive purification by thin-layer chromatography or high-performance liquid chromatography. An investigation by Proudfoot and coworkers comparing measurement of F2-isoprostanes in urine by ELISA and mass spectrometry found considerable inconsistencies between the two measurement methods (Proudfoot et al. 1999). Thus, at present, measurement of F2-isoprostanes by mass spectrometry remains the method of choice.

A recent study compared enzyme immunoassays with gas chromatography–mass spectrometry in domestic animal species for the measurement of urine isoprostanes as markers of *in vivo* lipid peroxidation (Soffler et al. 2010). This study demonstrated that enzyme immunoassays are not reliable for the determination of isoprostane concentrations in plasma or urine of horses. The more stable isoprostane metabolites; however, may be more suitable for an enzyme immunoassay such as the SNAP-test used for quantification of serum IgG concentrations in equine neonates. An ELISA for the isoprostane metabolite is currently under commercial development (Roberts and Morrow 2000). If the accuracy of the ELISA assay for the metabolite in urine can be validated by gas chromatography-mass spectrometry, this may potentially lead to widespread availability and use by many investigators and clinicians. The ability to reliably measure urine isoprostane metabolite concentrations using such a test could be an excellent

clinical application as an aid in determining the need for surgical intervention in horses with colic.

3. Conclusion

The most common cause of death as a direct result of equine colic is due to acute circulatory failure secondary to intestinal ischemia or infarction (Mair and Smith 2005a, b; Parry 1987; White 1990). Early and accurate recognition of an ischemic segment of bowel is essential to decrease complications and increase patient survival (Arden and Stick 1988). Availability of abundant free cell membrane-derived arachidonic acid (Moore *et al.* 1995) and hypoxemia-induced oxygen radicals (Shebani *et al.* 2000b) make the involvement of isoprostanes in the pathogenesis of colic likely since not only strangulating obstructions but also non-strangulating conditions lead to cellular injury and the release of arachidonic acid. Increased peritoneal and plasma lactate values have a direct, predisposing association with the need for surgery in clinical colic cases (Thoefner *et al.* 2000), but are not useful if interpreted in isolation from other correlating clinical and laboratory data (e.g. heart rate, rectal examination, naso-gastric intubation). Peritoneal fluid lactate, however, has a better correlation with survival rates. It appears that plasma lactate is less valuable for gastrointestinal lesions other than large colon volvulus, (Kelmer 2009) and lactate values cannot predict survival if the lesion is not strangulating (Delesalle *et al.* 2007). Nevertheless, not one of the currently used predictors works satisfactorily for all colic cases including mild non-strangulating surgical cases affected by displacement or impaction, suggesting that there is a need for a reliable predictor for the need for surgery. The intend of the following work was to gather preliminary data regarding the

feasibility of using urine F2-isoprostane and isoprostane metabolite concentrations as an early screening tool for the presence of gastrointestinal disease requiring surgical intervention in horses. We believe that oxidative stress is an integral part of the equine colic syndrome independent of its originating pathologic condition potentially reflecting severity and duration of associated tissue damage. Once the relation between colic and the formation of isoprostane is established it is our long term goal to reduce the number of deaths due to colic by developing a stall-side test capable of identifying horses that need surgery to provide the equine practitioner an additional tool to guide them in their decision process of referral.

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Chapter 2

Implications of urine F2-isoprostane metabolite concentration in horses with colic and its potential use as a predictor for surgical intervention.

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1. Summary

Reasons for performing this study: F2-isoprostanes are the "gold standard" for assessment of oxidative stress in vivo and have been used extensively to quantify lipid peroxidation in association with risk factors in various diseases. Horses with colic may have intestinal ischemia and/or inflammation characterized by oxidative stress and increased production of isoprostanes.

Objectives: To gather preliminary data regarding the feasibility of using urine F2-isoprostane and isoprostane metabolite concentrations as an early screening tool for the presence of gastrointestinal disease requiring surgical intervention in horses. The long term goal of this investigation is to reduce the number of deaths due to colic by developing a stall-side test capable of identifying horses that need surgery as early as possible and expediting their timely referral.

Methods: Urine samples were collected from 42 healthy control horses and 43 horses with gastrointestinal pain or colic. Concentrations of urine isoprostane and isoprostane metabolite were determined by mass spectroscopy and normalized to urine creatinine (Cr) concentrations.

Results: Horses with colic were treated either medically (n=21) or surgically (n=22). Mean (± SD) concentrations of urine isoprostane and isoprostane metabolite were significantly higher in horses with colic (2.94 ± 1.69 ng/mg Cr and 0.31 ± 0.22 ng/mg Cr, respectively), when compared to healthy control horses (1.89 ± 1.39 ng/mg Cr and 0.22 ± 0.08 ng/mg Cr, respectively). Mean urine isoprostane metabolite concentrations were significantly higher in horses undergoing surgery (0.38 ± 0.28 ng/mg Cr) compared to healthy control horses and medically treated colic horses (0.26 ± 0.11 ng/mg Cr). Non-survivors had significantly higher mean urine isoprostane metabolite concentrations (0.47 ± 0.39 ng/mg Cr) compared to healthy control horses and surviving colic horses (0.29 ± 0.24 ng/mg Cr).

Conclusions: Based on the finding that urine concentrations of isoprostane metabolite are significantly increased in horses with gastrointestinal disease and in horses that required surgery or failed to survive, measurement of urine isoprostane metabolite concentration may be a useful prognostic indicator in equine colic.

Potential Relevance: Urine isoprostane metabolite may potentially aid in early recognition of surgical colic horses once a reliable stall-side test is available. Isoprostanes may be a potential therapeutic target to prevent further systemic and gastrointestinal tissue injury in horses with colic.

2. Introduction

Colic or abdominal pain is one of the most prevalent and challenging diseases encountered by equine veterinarians (Dukti and White 2009; Tinker *et al.* 1997). Approximately 4% of horses experience at least one colic episode per year and a fatal outcome occurs in 11% of cases (Proudman *et al.* 2002). While the majority of horses with abdominal pain can be treated successfully on the farm, some horses require rapid referral for intensive medical therapy or surgical intervention. Horse owners are increasingly aware of the various diseases that result in abdominal pain in horses and expect veterinarians to provide information regarding the prognosis prior to referral and surgery.

A variety of parameters, including heart rate, packed cell volume, and blood or peritoneal fluid lactate concentrations, have been evaluated as potential prognostic aids (Moore *et al.* 1976; Parry *et al.* 1983a; Puotunen-Reinert 1986; Orsini *et al.* 1988; Morris *et al.* 1991; Seahorn *et al.* 1994; Furr *et al.* 1995; Thoefner *et al.* 2000; Proudman *et al.* 2002; van der Linden *et al.* 2003; Garcia-Seco *et al.* 2005; Hinchcliff *et al.* 2005; Mair and Smith 2005b; Niinisto *et al.* 2010; Proudman *et al.* 2005; Proudman *et al.* 2006). Although the results of one study indicated that an increased blood lactate concentration was of similar use as increased heart rate and packed cell volume (Parry *et al.* 1983b), blood lactate appeared to accurately predict survival only for horses with large colon volvulus (Johnston *et al.* 2007; Latson *et al.* 2005). Increased peritoneal fluid and plasma lactate values correlate with the need for surgery in clinical colic cases, but are not useful as a sole indicator for predicting survival in non-strangulating lesions (Thoefner *et al.* 2000; Delesalle *et al.* 2007). A recent study confirmed prior reports that hyperglycemia is common in

horses with colic and is associated with a worse prognosis for survival to hospital discharge (Hassel *et al.* 2009). Peritoneal fluid D-lactate (a specific bacterial metabolite) and plasma d-lactate were significantly elevated in horses with septic peritonitis or gastrointestinal rupture compared to controls, but was not useful in identifying intestinal ischemia in horses presenting for colic (Sawsan *et al.* 2010). Also, plasma D-dimer concentrations, a sensitive test for assessing coagulopathies, have been used as a predictor for non-survival in horses with colic with a cut-off value of 4 ng/mL (Cesarini et al. 2010). Nevertheless, not one of the currently used predictors works satisfactorily for all colic cases including mild non-strangulating surgical cases affected by displacement or impaction, suggesting that there is a need for a reliable predictor for the need for surgery.

Acute circulatory failure secondary to intestinal ischemia or infarction has been proposed as the most common cause of death in horses with gastrointestinal disease (Mair and Smith 2005a, b; Parry 1987; White 1990). To decrease the occurrence of complications and increase patient survival, early and accurate recognition of an ischemic segment of bowel is essential (Arden and Stick 1988; Mair and Smith 2005a). Intestinal ischemia includes all conditions in which the blood supply to the gastrointestinal tract is not adequate for its metabolic demands (Paterno *et al.* 2008). Hence, some large colon displacements and impactions may lead to mild intestinal ischemia, secondary luminal distension and edema, and subsequent production of cytotoxic oxygen radicals (Paterno *et al.* 2008). There are currently no reports in the literature describing changes in oxygen radicals in these milder non-strangulating colics.

Isoprostanes are a group of prostaglandin-like compounds formed *in vivo* by non-enzymatic free radical–induced peroxidation of arachidonic acid. Their discovery in 1990 uncovered a novel facet of free radical biology (Morrow *et al.* 1990). Isoprostanes have been reported clinically and experimentally to be sensitive indicators of oxidative stress and ischemia in humans and animals (Marnett *et al.* 1985) and to provide an objective assessment of disease progression and response to therapeutics (Morrow *et al.* 1990). Isoprostanes are present in increased concentrations in various pathophysiological states, including ischemia-reperfusion injury, atherosclerosis, diabetes, experimentally-induced oxidative stress, and inflammation (Bailey *et al.* 2004; Ishii *et al.* 2010; Kaviarasan *et al.* 2009; Loke *et al.* 2010; Wu *et al.* 2010). Isoprostanes can be measured in a variety of biological fluids; however, plasma and urine are the most commonly used. Isoprostanes are highly unstable in plasma and can become falsely elevated by autooxidation of plasma arachidonic acid if plasma samples are not processed and stored properly. For accurate isoprostane determination, blood must be processed using a complex time-sensitive protocol involving collection into pre-cooled tubes, completion of plasma isolation in less than 10 minutes (all at less than 4°C), and storage at -80°C. As it is frequently difficult to achieve this goal in a clinical setting, plasma isoprostane concentrations are not well-suited for use on clinical cases. Peritoneal fluid is a potentially ideal body fluid for isoprostane quantification generated during hypoxic conditions involving the gastrointestinal tract; however, due to high lipid and cell content, it would require treatment similar to plasma. Since urine does not have a high lipid content, autooxidation is not a concern (Morrow et al. 1990). Measurement of isoprostanes in urine samples from clinical cases is more practical than plasma or peritoneal fluid samples.

Isoprostanes are not only biological markers of oxidative stress, but also evoke biological responses such as vasoconstriction and enhanced neutrophil extravasation via increased adhesion to endothelial cells (Sametz *et al.* 1999; Zahler and Becker 1999; Basu and Helmersson 2005; Comporti *et al.* 2008). In addition, they are responsible for inflammation in such diseases as inflammatory bowel disease in humans (Stucchi *et al.* 2000). Therefore, isoprostane production may be a target to decrease in order to alleviate these adverse biological responses.

In horses, isoprostanes have only been investigated for their role in recurrent airway obstruction and during the development of laminitis (Kirschvink *et al.* 1999; Kirschvink *et al.* 2002a, b, c; Noschka *et al.* 2009). Kirschvink and coworkers found support for the use of isoprostanes as a marker of oxidative stress and airway inflammation in bronchio alveolar lavage fluid (Kirschvink *et al.* 1999; Kirschvink *et al.* 2001). Increased concentrations of isoprostanes and evidence of associated potent venoconstricting properties in laminar tissue of horses with experimentally induced laminitis contributed to the development of equine laminitis (Noschka *et al.* 2009). As such, isoprostanes may represent viable targets for the development of more effective therapeutic strategies for equine laminitis (Noschka *et al.* 2009) and other related diseases associated with oxidative stress. Although elevated concentrations of isoprostanes have been detected in tissues and urine from animals and humans with various conditions associated with oxidative stress (de Zwart *et al.* 1999), their presence in horses with gastrointestinal disease has not been explored.

The metabolism of F2-isoprostanes results in the production of an entire profile of derivatives, however, a single metabolite predominates, 2,3-dinor-5,6-dihydro-15-F2t-isoprostane (referred to hereafter as isoprostane metabolite). The increased stability and longer half-life of this

metabolite compared to the parent compound (Roberts and Morrow 2000), suggests that the metabolite may provide a more accurate measurement in determining the need for surgical intervention. Periods of hypovolemia and dehydration that often occur in seriously ill colic patients result in oxidative stress in renal tissues. Renal oxidative stress may result in the production of additional isoprostanes and lead to falsely elevated plasma or urine isoprostane concentrations. Because isoprostane metabolites are only generated in the liver during the metabolism of plasma isoprostanes, urine isoprostane metabolites are believed to represent a more accurate systemic index of oxidative stress than urine isoprostanes (Roberts and Morrow 2000).

The purpose of this prospective clinical study was to compare urine concentrations of isoprostane and its predominant metabolite in a population of horses with colic to those in healthy, control horses. Our aim was to determine the feasibility of using urine isoprostane and isoprostane metabolite concentrations as an early screening tool for the severity of colic and to determine the need for surgery. We hypothesized that urine concentrations of isoprostane and its metabolite could be used as prognostic indicators for the outcome of clinical cases of equine colic syndrome and as predictors for the need for surgical intervention. The long term goal of this investigation is to reduce the number of deaths due to colic by developing a stall-side test capable of identifying colic horse in need of surgery as early as possible and expediting their timely referral.

3. Materials and Methods

Horses - This prospective study included 42 clinically healthy (control) horses and 43 horses with colic (21 medical and 22 surgical) admitted to the Veterinary Medical Teaching Hospital at the Virginia-Maryland Regional College of Veterinary Medicine and fitted the inclusion criteria of gastrointestinal pain and urine collection within 6 hours of admittance of a live horse. Healthy control horses were selected from the teaching herd or from horses attending a local horse show. All samples were collected between September 2008 and August 2009. All procedures were approved by the Institutional Animal Care and Use Committee and the Hospital Board. Informed consent was obtained from the owner for each horse included in the study.

An initial physical examination was performed on all horses presented as colic cases. Individual patient data recorded included age, sex, breed, heart and respiratory rates, body temperature, capillary refill time, and results of abdominal auscultation, *per rectum* examination, and nasogastric intubation at the time of admission. Venous blood was submitted for complete blood cell count and serum chemistry. The history, duration of colic signs prior to presentation, location and length of intestinal lesion, specific diagnosis, presence of a strangulating obstruction at either surgery or necropsy, ideal treatment recommended at the time of urine collection, necessity for surgical intervention for resolution of the problem (medical vs. surgical), treatment elected by owner to pursue, complications, and outcome (discharged from the hospital, died, or euthanatized) were recorded.

Urine sample collection - Urine samples were collected from a total of 85 horses by free catch whenever possible; however, if horses had not urinated within the first six hours following hospitalization, urine was collected by sterile catheterization (n=8). Samples were aliquoted into sterile tubes and immediately frozen and stored at -80˚C for mass spectroscopy and urine creatinine determination.

Assay for isoprostanes - Samples were shipped on dry ice via overnight courier as one batch to the Eicosanoid Core Laboratory at Vanderbilt University Medical Center for quantification of urine isoprostane and isoprostane metabolite and urine creatinine concentrations. Free urine F2-isoprostane (15-F2t-isoprostane or 8-iso-PGF2α) and isoprostane metabolite concentrations were quantified by selected ion monitoring gas chromatography negative ion chemical ionization/mass spectrometry employing [2H4]8-iso-PGF2a as an internal standard (Agilent GC/MS system, Palo Alto, CA) using stable isotope dilution methodology. Compounds were analyzed as pentafluorobenzyl ester, trimethylsilyl ether derivates monitoring the M-181 ions, m/z 569 for endogenous F2-isoprosanes and m/z 573 for 8-isoPGF2a. The F2-isoprostanes elute as a series of chromatographic peaks during a 20-s interval and quantification is based on the primary peak eluting at the same time as the internal standard (Morrow and Roberts 1999). Data were expressed in ng/ml and the detection limit of the assay was 2 pg. Isoprostane and isoprostane metabolite values were normalized to urine creatinine concentration to adjust for the specific hydration status and inter-subject differences in renal function of each horse at the time of collection and results are expressed in ng/mg urine creatinine.

Statistical analysis - Normalized urine isoprostane and isoprostane metabolite concentrations from clinically healthy horses were used to establish normal ranges for the horse. Data from control horses were compared to colic horses using one way ANOVA and Tukey-Kramer post-hoc comparison. Comparisons included treatment (medical or surgical), survival, and presence of a strangulating lesion. A 2 sample t-test was used to compare control horses to all colic horses. Statistical analysis was performed using commercial software (SAS/STAT® SAS Institute Inc. Cary, NC, USA). Significance was set at $P<0.05$.

4. Results

Of the 43 horses admitted for colic, 21 horses met the inclusion criteria for surgical colic and 22 for medical colic. Median age of the study horses was 9.5 years (range 0.4–26 years) with no significant difference between groups. Twenty-seven horses were geldings, 12 were mares, and 4 were intact males. A wide variety of breeds were represented and consistent with the general population of horses admitted to the hospital (11 Warmbloods, 7 Quarter Horses, 3 Thoroughbreds, 3 American Paint Horses, other miscellaneous breeds [1-2 each]: Arabians, Standardbreds, Saddlebreds, Haflinger, Tenneesee Walking Horse, Welsh Pony, Paso Fino, Belgian Draft, Arab-Saddlebred mix, mixed breed). Five horses were identified during the statistical analysis as outliers (1 control, 1 medical, and 3 surgical colics) and excluded from further statistical analysis, leaving a total of 41 control and 38 colic horses (20 medical and 18 surgical).

Clinical signs of gastrointestinal pain were first observed 4 to 168 hours (median 8 hours) before admission to the hospital. All horses with gastrointestinal disease had been treated at least one time with analgesic drugs (e.g., flunixin meglumine, xylazine hydrochloride, detomidine hydrochloride, or butorphanol tartrate) or other medications (e.g., laxatives and fluids) prior to urine sample collection. Among the 38 colic horses, 9 had gastrointestinal inflammation (4 enteritis/colitis, 1 endotoxemia, 1 mesenteric abscess, 2 gastric ulceration, 1 intestinal rupture), 6 had gastrointestinal strangulation (3 pedunculated lipoma/mesenteric rent entrapment, 3 large colon torsion), and 23 had non-strangulating gastrointestinal lesions (19 impactions, 9

displacements, 1 spasmodic colic, 1 mesenteric hematoma, 1 Potomac Horse Fever, and 1 unknown origin).

Of 38 colic horses, 32 were discharged (84.2%). Mean (± SD) urine isoprostane and isoprostane metabolite concentrations were significantly higher in the overall colic horse population (2.94 ± 1.69 ng/mg creatinine; and 0.31 ± 0.22 ng/mg creatinine) compared with control horses (1.89 ± 1.39 ng/mg creatinine, P=0.003 and 0.22 ± 0.08 ng/mg creatinine, P=0.008).

Urine isoprostane concentrations were significantly higher in medically treated colics (3.31 ± 1.97 ng/mg creatinine) compared to controls (1.89 ± 1.39 ng/mg creatinine) (P=0.004); however, there were no significant differences between surgical colics (2.52 ± 1.23 ng/mg creatinine) and controls or medical colics. Mean urine isoprostane metabolite concentrations were significantly higher in surgical colics compared to control horses or medical colics (P=0.002) (Fig 1a).

Survival

Exploratory celiotomy was performed in 17 horses, 12 of which survived to hospital discharge (70.6%). All medical colics were discharged alive. Necropsy, including histopathologic evaluation, was performed in all horses that were euthanized. One horse was euthanized without undergoing surgery for economic reasons. Four horses were euthanatized intra-operatively because of a poor prognosis and the high expense of resection and anastomosis (1 each: jejunal rupture, large colon impaction/eosinophilic enteritis, pedunculated lipoma, and mesenteric abscess). One horse recovered from surgery but died 4 hours following surgery for undetermined reasons. Of 18 horses diagnosed with large colon impaction, 12 were treated medically and 6

surgically. One of the impactions treated surgically had a concurrent large colon volvulus and was euthanized during surgery while all other horses diagnosed with an impaction survived until discharge. Of the 9 horses suffering from a right dorsal displacement of the large colon, 5 (presumed right dorsal displacements) were treated medically and 4 surgically. All horses treated for a displacement survived until discharge. All 3 horses diagnosed with a large colon volvulus were treated surgically. One of these horses was euthanized during surgery due to the associated cost and prognosis. The other two cases recovered and were discharged.

Urine isoprostane concentrations were significantly higher in surviving colic horses (3.00 ± 1.69 ng/mg creatinine) compared with controls (P=0.012); however, there were no significant differences between non-survivors (2.61 ± 1.76 ng/mg creatinine) and controls or survivors. Urine isoprostane metabolite concentrations were significantly higher in non-survivors than controls or survivors (P=0.001) (Fig 1b).

Strangulation

Urine isoprostane concentrations were significantly higher in non-strangulating (n=31) colic horses (3.03 ± 1.74 ng/mg creatinine) compared with control horses (P=0.011). Urine isoprostane concentrations were not significantly different in strangulating (n=7) colics (2.55 ± 1.46 ng/mg creatinine) compared to either controls or non-strangulating colics. Mean urine isoprostane metabolite concentrations were significantly different between groups based on whether the lesions were strangulating or not (P=0.020) (Fig 1c).

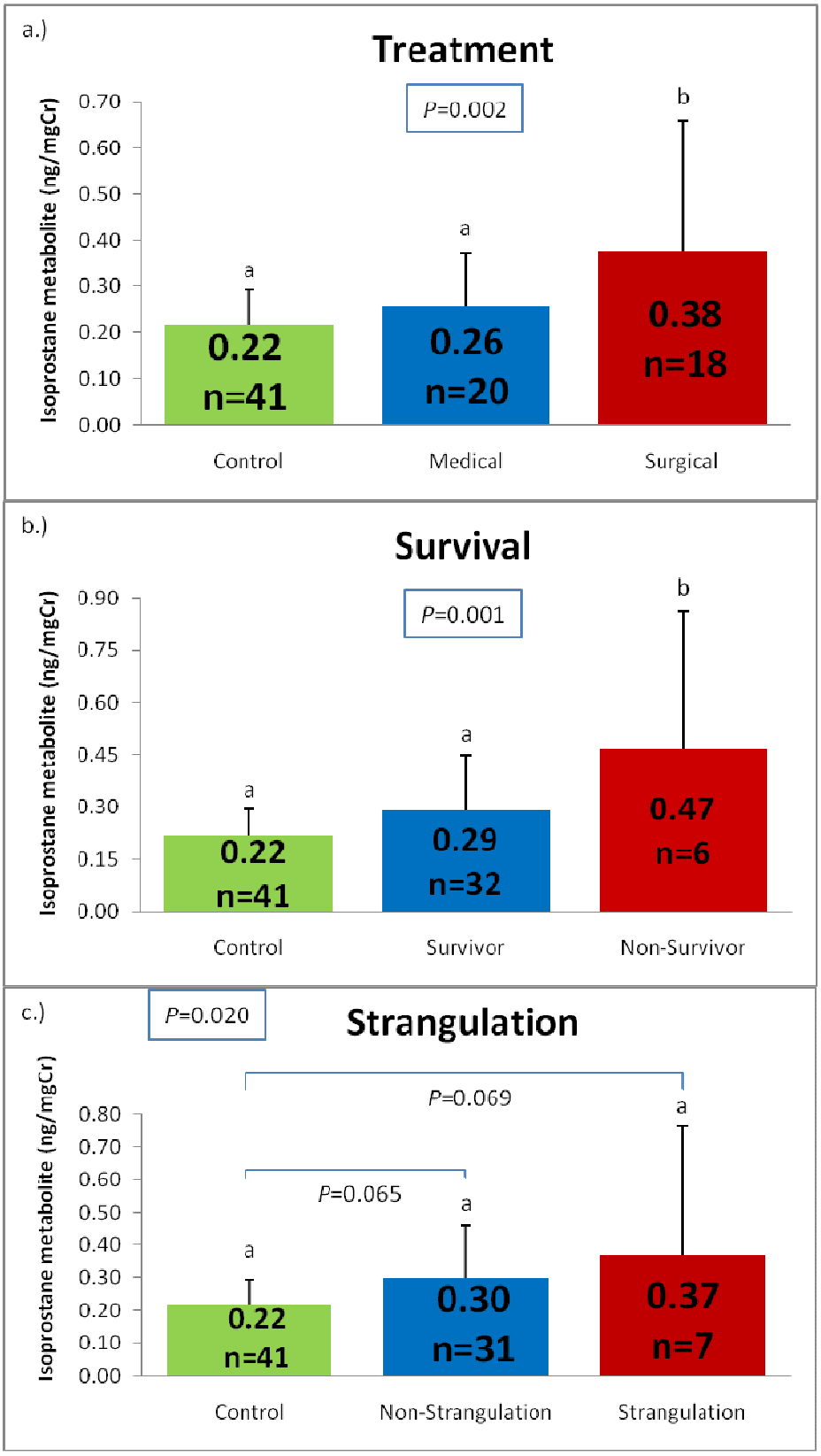
a.)
Treatment
P=0.002
Isoprostane metabolite (ng/mgCr)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
a
a
b
0.22
n=41
0.26
n=20
0.38
n=18
Control
Medical
Surgical
b.)
Survival
P=0.001
Isoprostane metabolite (ng/mgCr)
0.90
0.75
0.60
0.45
0.30
0.15
0.00
a
a
b
0.22
n=41
0.29
n=32
0.47
n=6
Control
Survivor
Non-Survivor
c.)
P=0.020
Strangulation
P=0.069
P=0.065
Isoprostane metabolite (ng/mgCr)
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
a
a
a
0.22
n=41
0.30
n=31
0.37
n=7
Control
Non-Strangulation
Strangulation

Fig 1: Urine isoprostane metabolite concentrations normalized by urine creatinine (Cr) concentrations from control horses and horses presenting for colic grouped by a) Treatment; b) Survival; or c) Strangulation. Superscript letters indicate significant differences between groups. Mean ± SD.

5. **Discussion**

Isoprostane biosynthesis, secretion, and excretion are regulated in part by the availability of precursors required for isoprostane synthesis, such as dietary and tissue arachidonic acid, as well as tissue oxygen concentration, and the generation of various free radical species (Basu and Helmersson 2005). Segmental blood supply to the intestinal tract in horses with gastrointestinal disease may be reduced due to either a functional constriction or mechanical obstruction of blood vessels. Reduced blood supply can lead to inadequate tissue perfusion and oxygenation resulting in rapid injury and death of the highly energy-dependent mucosal epithelial cells and to an increased release of arachidonic acid from injured cells and lipoproteins in blood (Moore *et al.* 1995). Non-steroidal anti-inflammatory drugs are the first line treatment for horses with colic because these drugs control pain by reducing arachidonic acid products involved in inflammation and pain (Moore *et al.* 1981). Availability of abundant free cell membrane-derived arachidonic acid (Moore *et al.* 1995) and hypoxemia-induced oxygen radicals (Shebani *et al.* 2000b) make the involvement of isoprostanes in the pathogenesis of colic likely since not only strangulating obstructions but also non-strangulating conditions lead to cellular injury and the release of arachidonic acid. In this study, concentrations of isoprostane and isoprostane metabolite in the urine were significantly higher in horses with colic than in healthy horses. This is not surprising since studies on ileal stasis in rats have shown elevated isoprostane concentrations (Shebani *et al.* 2000b) and the type of intestinal damage seen in this model may have similarities to certain types of non-strangulating causes of equine colic syndrome, e.g. impactions of the ileum or large colon. In these milder cases of non-strangulating obstructions, locally generated isoprostanes may contribute to vasoconstriction, worsening ischemia in the bowel secondary to edema

formation. Concentrations of isoprostane metabolite were significantly higher in horses requiring surgical intervention than in horses treated medically, as well as in horses that failed to survive. These preliminary findings suggest that urine concentrations of isoprostane metabolites may be useful as an indicator of prognosis and the need for surgical intervention in horses with colic.

There are several factors that could account for the differences between the pattern of changes of isoprostane and isoprostane metabolite concentrations in urine, including isoprostane metabolism, excretion, the techniques used to measure them, and type of colic. Because isoprostane metabolite is more stable and has a longer half-life than the parent compound, it may be more accurate in determining the need for surgical intervention (Roberts and Morrow 2000). Values for isoprostane metabolite in the healthy horses were tightly grouped with a small standard deviation, whereas the deviation for the isoprostane concentrations was quite large. This difference in standard deviation substantiates the concept that isoprostane metabolite may more closely reflect systemic lipid peroxidation independent of any influence of hydration status. Because of the increased stability and lack of renal oxidation, the remainder of the discussion will focus solely on isoprostane metabolite.

Urine isoprostane metabolite concentrations were significantly higher in horses requiring surgical intervention than in control horses or those treated medically. The group of horses requiring surgery included both strangulating and non-strangulating obstructions. Most surgical conditions including more severe impactions and displacements of the large bowel are potentially characterized by ischemia-induced oxidative stress due to decreased gastrointestinal perfusion, unregulated lipid peroxidation, and tissue damage. Increased urine isoprostane

metabolite concentrations may reflect the duration and severity of the condition. Values for the medically treated colic horses and healthy control horses were nearly indistinguishable, suggesting that the horses requiring medical therapy were not as severely compromised in their gastrointestinal perfusion status as the horses requiring surgery.

The results of this study suggest that there may indeed be ischemia-induced oxidative stress occurring in non-strangulating lesions. Non-strangulating conditions have not typically been thought of as related to oxidative stress and ischemia in the past. However, it appears that surgically corrected impactions or displacements of the large colon induced sufficient isoprostane metabolite concentrations in order to statistically distinguish them from the less severe medically treated colics, suggesting that isoprostane metabolite may be helpful in distinguishing medical from surgical cases of non-strangulating obstructions. A study in rats found increased urine isoprostane concentrations in animals suffering from intestinal stasis caused by a surgically created U-pouch of the terminal ileum, signifying an association with oxidative stress (Shebani et al. 2000a). Further evidence for the role of free radical species in the formation of isoprostanes is that dietary antioxidant intervention with vitamin E, a potent free radical scavenger, and allopurinol, an inhibitor of xanthine oxidase, reduced myeloperoxidase activity and isoprostane production and ameliorated the early inflammatory events associated with intestinal stasis in rats (Shebani et al. 2000a). The effectiveness of antioxidants also points out the potential use of isoprostanes as a therapeutic target.

The results of our study identify the important role that isoprostane and its metabolite may have in the pathophysiology of colic since their vasoactive properties may mediate edema formation,

ischemia, and oxidative stress within the bowel wall. At the same time, isoprostane may represent a viable target for treatment with antioxidants that inhibit the formation of free radicals, even in milder forms of surgical colic cases. Isoprostane may exacerbate oxidative stress in the inflamed colon by inducing mucosal ischemia secondary to their vasoconstricting properties (Stucchi et al. 2000). These findings support the use of antioxidant or direct cell-protecting treatments such as dimethylsulfoxide, lidocaine, tocopherol, ascorbic acid, and allopurinol (not only as part of the Carolina Rinse) as adjunctive treatments in surgical colics (Schoenberg and Beger 1993; de Moffarts et al. 2005; Guschlbauer et al. 2010). The routine use of a lidocaine continuous rate infusion started intraoperatively, even in milder colic cases, may diminish post-operative oxidative stress and ileus, and enhance the speed of recovery. Measurement of isoprostane and isoprostane metabolite concentrations could not only be useful pre-operatively, but as a serial assay post-operatively to monitor disease progression and/or resolution similar to the ultrasonographic measurement of large colon wall thickness following correction of large colon volvulus (Sheats *et al.* 2010).

Although there were relatively few non-survivors in the colic horse population in this study, urine concentrations of isoprostane metabolite were significantly higher in these horses when compared to either survivors or healthy control horses. Further studies are indicated to strengthen these findings and further assess the sensitivity and specificity of predicting survival in the aim of identifying a cut-off value that would help determine prognosis or the need for surgery.

Urine isoprostane metabolite concentrations in horses with strangulating obstructions were higher than those in horses with non-strangulating obstructions or in healthy control horses;

however, this difference did not reach statistical significance. This could, in part, be due to the low number of horses with strangulating lesions. The relatively large standard deviation within this group may make it difficult to find a cut-off value; however, strangulating lesions are easier to diagnose pre-operatively than some milder forms of colic requiring surgery such as large colon displacements and impactions making the need for an objective value less urgent. Delineation of the need for surgery in non-strangulating lesions may provide a more useful application for measurement of urine isoprostane metabolite concentrations than in horses with strangulating lesions.

The use of isoprostane metabolite concentrations as an additional screening tool to complement the measurement of lactate may not only detect oxidative stress associated with strangulating lesions but also milder ischemic conditions accounting for the severity of non-strangulating lesions. Lactate seems to be more useful in predicting survival in strangulating lesions, but is less accurate in non-strangulating lesions. Coupling the use of lactate and urine isoprostane metabolites may provide accurate determination of prognosis or the need for surgery in a wider range of diagnoses. A larger multi-center study involving more horses in each category would help further define the potential value of isoprostane metabolite concentrations in distinguishing horses with strangulating obstructions from those with non-strangulating obstructions and to provide an accurate cut-off value.

Isoprostane plasma concentrations can provide a useful index of total endogenous production of isoprostane because concentrations in plasma presumably are derived from all tissues in the body. A potential contribution of local formation of isoprostane in the kidney may confound

interpretation of urine concentrations of unmetabolized isoprostane (Roberts and Morrow 1997) (Taber et al. 1997). Despite the high specificity and sensitivity of isoprostane for oxidative stress, their quantification in the circulation will only offer information regarding a discrete point in time, since it is cleared rapidly from the circulation (within approximately 16 minutes) (Morrow *et al.* 1990). Consequently, if large alterations occur in the production of lipid peroxides over a longer period (several hours to days in colic cases), measurement of isoprostane concentrations in a single plasma sample will not provide an accurate integrated assessment of oxidative stress. In dynamic situations in which there is an oxidant insult for only a relatively short period of time, (e.g., ischemia- reperfusion injury), multiple sequential samplings of blood would be necessary to assess the full magnitude of the increase in isoprostane generation during rapidly changing rates of production (Richelle et al. 1999). Measurement of the more stable metabolized isoprostanes in urine as an index of total endogenous isoprostane production may circumvent the potential contribution of local isoprostane production in the kidney during states of dehydration and provide a more accurate measure of the systemic oxidative stress of a patient over time. Considering all of these aspects, it appears more practical to collect urine samples than plasma samples for the evaluation of isoprostane metabolite from clinical colic cases.

The usefulness of isoprostane metabolite concentrations as a clinically useful predictive indicator for colics is dependent in part in the ability to develop a stall-side assay. A recent study compared enzyme immunoassays with gas chromatography–mass spectrometry in domestic animal species for the measurement of urine isoprostanes as markers of in vivo lipid peroxidation and demonstrated that enzyme immunoassays are not reliable for the determination of isoprostane concentrations in plasma or urine of horses (Soffler *et al.* 2010). The more stable

isoprostane metabolite; however, may be more suitable for an enzyme immunoassay that could be developed as a stall-side assay, such as the SNAP-test used for quantification of serum IgG concentrations in equine neonates. The ability to reliably measure urine isoprostane metabolite concentrations using a simple stall-side test would be an excellent clinical application as an aid in determining the need for surgical intervention in horses with colic.

The results of this study provide justification for a large multi-center study on horses with colic to further develop the predictive value of isoprostane metabolites in the need for surgery and to prognosticate during the post-surgical period. This contribution to the knowledge of the formation of isoprostanes and their metabolites in horses suffering from colic may potentially aid in the reduction of isoprostane generation through early surgical intervention based on a stall-side test or the prevention of further systemic and gastrointestinal tissue injury in horses with colic by timely referral and surgery.

6. References

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