To my parents
ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

1hI One hour ischemia
4hR Four hours reperfusion
30minR 30 minutes reperfusion
ABC Avidin-biotin-complex
ALI Acute lung injury
ALP Alkaline phosphatase
AKI Acute kidney injury
ATP Adenosine triphosphate
Ca\(^{2+}\) Calcium
CaCl\(_2\) Calcium chloride
CB Colonic venous blood
CCL5 Chemokine ligand-5
CD Cluster of differentiation
CI Confidence interval
CINC1 Cytokine-induced neutrophil chemoattractant-1
COX Cyclooxygenase
CPK Creatine phosphokinase
CRI Continuous rate infusion
CRP C reactive protein
CXCL1 Chemokine ligand-1
DAB Diaminobenzidine
DAMP Danger-associated molecular pathogen
DNA Deoxyribonucleic acid
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<td>Endoplasmic reticulum</td>
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<tr>
<td>FABP</td>
<td>Fatty acid binding protein</td>
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<td>GI</td>
<td>Gastrointestinal</td>
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<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
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<td>GX</td>
<td>Glycinexylidide</td>
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<tr>
<td>HCO$_3$</td>
<td>Bicarbonate</td>
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<td>H&amp;E</td>
<td>Hematoxylin-eosin</td>
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<td>HIF1-α</td>
<td>Hypoxia-inducing factor-1 alpha</td>
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<td>HMGB1</td>
<td>High mobility box protein-1</td>
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<td>HOCl</td>
<td>Hypochlorous acid</td>
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<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
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<td>I-kappa-B protein complex</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>iNOS</td>
<td>Inducible nitric oxide synthesis</td>
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<td>IR</td>
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<td>IRI</td>
<td>Ischemia-reperfusion injury</td>
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<td>K$^+$</td>
<td>Potassium</td>
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<tr>
<td>KRB</td>
<td>Krebs ringer bicarbonate</td>
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<td>LCT</td>
<td>Loose connective tissue</td>
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<td>LCV</td>
<td>Large colon volvulus</td>
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<td>LM</td>
<td>Light microscopy</td>
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<td>MadCAM</td>
<td>Mucosal vascular addressin cell adhesion molecule</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>MCP1</td>
<td>Monocyte chemotactic protein-1</td>
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<tr>
<td>MEGX</td>
<td>Monoethylglycinexylidide</td>
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<tr>
<td>MIP1</td>
<td>Macrophage inflammatory protein-1</td>
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<td>MODS</td>
<td>Multiple organ dysfunction syndrome</td>
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<td>MOF</td>
<td>Multiple organ failure</td>
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<td>MPO</td>
<td>Myeloperoxidase</td>
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<td>MRP</td>
<td>Macrophage inhibitory related protein</td>
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<tr>
<td>Na⁺</td>
<td>Sodium</td>
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<td>NAC</td>
<td>N-acetylcysteine</td>
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<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<td>NaOH</td>
<td>Sodium hydroxide</td>
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<tr>
<td>NFkB</td>
<td>Nuclear factor kappa beta</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>PAF</td>
<td>Platelet-activating factor</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
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<tr>
<td>pCO₂</td>
<td>Partial pressure of carbon dioxide</td>
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<tr>
<td>PG</td>
<td>Prostaglandin</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide-3-kinase</td>
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<tr>
<td>POI</td>
<td>Post-operative ileus</td>
</tr>
<tr>
<td>POR</td>
<td>Post-operative reflux</td>
</tr>
<tr>
<td>pO₂</td>
<td>Partial pressure of oxygen</td>
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<td>rER</td>
<td>Rough endoplasmic reticulum</td>
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<td>RNS</td>
<td>Reactive nitrogen species</td>
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<td>ROM</td>
<td>Reactive oxygen metabolites</td>
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<td>Abbreviation</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SB</td>
<td>Systemic venous blood</td>
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<td>Small intestine strangulation</td>
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<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
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<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
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<td>TER</td>
<td>Transepithelial electrical resistance</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>TJ</td>
<td>Tight junction</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>XDH</td>
<td>Xanthine dehydrogenase</td>
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<tr>
<td>XO</td>
<td>Xanthine oxidase</td>
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<tr>
<td>ZO-1</td>
<td>Zonula occludens-1</td>
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REMOTE ORGAN RESPONSES TO INTESTINAL ISCHEMIA AND REPERFUSION INJURY IN THE HORSE

By

Julia Elizabeth Daggett

August 2017

Chair: David E. Freeman
Major: Veterinary Medical Science

Intestinal ischemia reperfusion (IR) injury can activate inflammatory cells and release mediators in the primary intestinal site, but the effects on remote organs (kidney, liver, and lung) have received little attention. The objective of this study is to evaluate the effects of experimentally induced intestinal IR injury in the kidney, liver, and lung of the horse.

Fourteen anesthetized horses were randomly treated with either lidocaine or saline, and selected inflammatory mediators or ischemia byproducts [cyclooxygenase-1 and 2 (COX-1, COX-2), fibrin, hypoxia inducible factor-1α (HIF-1α), and calprotectin] were analyzed by immunohistochemistry in biopsies obtained after 30 minutes of jejunal or 1 hour of colonic ischemia, and 4 hours of reperfusion. Tissues sampled were the kidney, liver, and lung. Seven non-anesthetized horses were used as controls. Data was analyzed by the Kruskal-Wallis test.

Although inflammatory mediators were identified in the liver and lung after IR, the most prominent changes were in kidney samples. Fibrin deposits were found in the upper medulla near transition from cortex; HIF1-α positive cells were found in distal convoluted tubules; calprotectin-positive cells were found in the glomerulus; COX-1
positive cells were found only in collecting ducts; and COX-2 positive cells were found in the macula densa and dispersed in the glomerulus. Lidocaine had an insignificant therapeutic effect only on fibrin in the kidney, liver, and lung.

These findings demonstrate that remote responses can develop in liver and lung, but particularly in the kidney, of horses within a brief period of IR and could account for some of the complications experienced after severe intestinal injury in clinical cases.
CHAPTER 1
INTRODUCTION

According to a national survey performed in the USA in 1998 by The United States Department of Agriculture’s (USDA) National Animal Health Monitoring System (NAHMS, 1998), the national incidence of colic was 4.2 events/100 horses per year. Of reported cases 1.4% of colic events required surgical intervention, and the case fatality rate for all colic events was 11.0% (NAHMS, 1998). In a follow-up study performed in 2015, NAHMS found that colic remained the leading cause of death in horses after old age (NAHMS, 2015). Survival of horses undergoing surgery for strangulating obstruction has improved in recent years, most likely the result of many factors including improved surgery, anesthesia, and early referral. However, strangulating obstructions that cause irreversible intestinal ischemia remain the most dreaded types of colic, with high fatality and complication rates. In addition to surgical treatment of these diseases, usually through resection of the ischemic segments, equine surgeons also seek pharmacologic methods of modifying pathologic changes in intestine during the post-ischemic period. Such efforts are intended to prevent potentially fatal postsurgical complications, such as postoperative ileus (POI), adhesions, and endotoxemia.

One aspect of post-ischemic damage to equine small intestine that has garnered much interest and generated some controversy is reperfusion injury. This is the paradoxical exacerbation of tissue damage that occurs when ischemic tissue becomes reoxygenated, and has been documented in several organs, including the gastrointestinal tract. The clinical relevance of reperfusion injury is poorly established, but the development of pharmacological methods to control the biochemical events directly responsible for the tissue damage are of great interest. The attention given to
reperfusion injury by equine surgeons also rests in the possibility that this mechanism could explain many complications of gastrointestinal tract surgery in horses, such as POI, postoperative endotoxemia, and adhesions. While the tissue consequences of reperfusion injury to the colon and small intestine may be similar, different clinical complications are expected for each. For example, the predominant changes in the colon would be those associated with endotoxemia and sepsis, whereas POI and adhesions would be the predominant consequences after small intestinal ischemia.

**Small Intestinal Strangulation**

Strangulating intestinal obstruction is one of the leading causes of death in horses (White, 1990). Small intestinal strangulation comprises 85% of all small intestinal obstructive diseases in horses, and 68% of these require resection of the strangulated bowel (Freeman et al. 2002). In 32% of horses with strangulating lesions of the small intestine, resection might not be deemed necessary or possible, and then ischemic intestine is left in situ in the hope that it can recover fully (Freeman et al. 2014). The concern with such cases relates to the ability of affected intestine to heal and regain function without causing severe postoperative complications such as endotoxemia, hypovolemic shock, and death. A repeat celiotomy can be required if the intestine that was deemed viable during the primary celiotomy is not able to recover.

**Large Colon Volvulus**

Large colon volvulus is a severe, acute abdominal crisis in the horse, with short-term survival rates ranging from 35%-86% (Barclay et al., 1980; Harrison, 1988; Emberton et al, 1996; Johnston et al, 2007; Gonzalez et al, 2015). Large colon volvulus accounts for 10–20% of horses that present for colic and undergo exploratory celiotomy. Long-term survival rates are ranked lowest among surgical cases of colic,
with a recent report of 48.3% and 33.7% postoperative survival after one and 2 years, respectively (Suthers et al, 2013). Horses of any breed, age, or sex can develop large colon volvulus, but taller horses and broodmares 1-3 months post foaling are at a substantially higher risk (Snyder et al. 1989a; Embertson et al. 1996; Suthers et al, 2013).

Volvulus typically develops at the origin of the right dorsal colon near the mesenteric attachment to the dorsal body wall, where the colon is twisted on its longitudinal axis up to 720°, typically in dorsomedial direction (Harrison, 1988; Snyder et al, 1989a; Gibson and Steel, 1999). Large colon volvulus has features typical of a hemorrhagic strangulating obstruction, which is characterized by luminal occlusion and compromised venous drainage (Snyder et al. 1989a). Continuous flow of arterial blood into the tissue causes low-flow ischemia, which results in severe interstitial hemorrhage and edema accompanied by compartmentalization of large amounts of fluid, followed by gradual disruption of mucosal tissue architecture (Gibson and Steel, 1999; Blikslager, 2009). Occasionally, large colon volvulus will cause ischemic strangulation from simultaneous venous and arterial occlusion, causing a sudden loss of oxygen supply to the organ and rapid degeneration of the mucosa and its epithelium (Meschter et al, 1986; Gibson and Steel, 1999; Moore et al, 1995; Lopes, 2009; Sheats et al, 2009).

The severity of intestinal injury is directly proportional to both the duration and severity of ischemia, and the magnitude of change in mucosal architecture and permeability is directly related to the duration of the ischemic event (Chiu et al, 1970; Parks et al, 1982; Moore et al, 1995). Prolonged partial ischemia can cause the same degree of damage as complete ischemia (Parks et al, 1982; Moore et al, 1995). Partial
ischemia of short duration in low-flow models is preferred by researchers as a means of inducing the intestinal changes of interest, as it leaves a mild enough injury to progress to a more severe injury during reperfusion (Parks et al, 1982; Kviety et al, 1989; Moore et al, 1995). Three to 4 hours of a 360° volvulus resulted in irreversible damage of the entire intestinal wall followed by direct contact of luminal contents with subepithelial structures (Snyder et al. 1988). Thus, death in horses with large colon volvulus is mostly attributed to hypovolemic and endotoxic shock due to loss of extracellular fluid into the lumen of the colon and translocation of bacterial toxins across the ischemia-damaged mucosa into the blood stream respectively (Snyder et al. 1989a,b; Hardy 2009). Abdominal compartment syndrome develops from the progressive colonic distention with fluid and gas and exacerbates cardiovascular derangements and hypovolemic shock (Munsterman et al. 2013).

Pathophysiology of Intestinal Ischemia and Reperfusion Injury

Reperfusion injury is the exacerbation of tissue damage that occurs when ischemic tissue is reoxygenated. Ischemia/reperfusion (I/R) injury has been well documented in the mammalian gastrointestinal tract (Freeman et al, 2012).

Ischemia

Ischemia reperfusion (IR) injury is a complex cascade of precise synchronized cellular and molecular events that are thought to play a crucial role in the pathophysiology of large colon volvulus (Meschter et al. 1986; Moore et al. 1995a; Mallick et al. 2004; McMichael and Moore 2004). It starts with progressive ischemic damage caused by reduced arterial inflow to the affected tissue. The resulting tissue hypoxia induces intestinal barrier dysfunction through various biochemical, metabolic, and ultrastructural changes in epithelial cells that cause cell death (Snyder, 1989;
Moore et al, 1995; McAnulty et al, 1997; Gibson et al, 1999). In addition, ischemia also activates the local innate immune cells, such as macrophages, mast cells, neutrophils, and eosinophils, all of which release pro-inflammatory cytokines, free radicals, and proteins that produce toxic effects on otherwise healthy tissue (Eltzschig et al, 2011). Consequently, ischemia interrupts the cellular aerobic metabolism, which sharply decreases intracellular ATP and pH levels, causing an accumulation of toxic metabolites and lactate generated by anaerobic glycolysis (Moore et al. 1995a; Rowe and White 2002; McMichael and Moore 2004).

**Reperfusion**

Restoration of blood flow to an ischemic organ is crucial for prevention of irreversible cellular injury. However, reperfusion with oxygenated blood can paradoxically exacerbate the tissue injury produced by ischemia alone (Parks and Granger 1986). Many theories exist to explain the progression of tissue damage after reoxygenation, and all of them depend on biochemical changes that occur during ischemia (Soffler 2007).

Reactive oxygen species (ROS), or metabolites (ROM), and reactive nitrogen species (RNS), such as superoxide anions, hydroxyl radicals, hypochlorous acid (HOCl), and nitric oxide-derived peroxynitrite are generated rapidly in the presence of oxygen delivered during reperfusion (Carden and Granger, 2000; Collard and Gelman, 2001; McMichael and Moore, 2004). While the superoxide radical alone is not viewed as highly cytotoxic, it is responsible for generation of secondary ROMs that are toxic. Hydrogen peroxide produced by reduction of oxygen or from dismutation of the superoxide radical is very lipophilic and can readily cross cell membranes. Both the superoxide radical and hydrogen peroxide react with intracellular transition metals, such
as iron or copper, through the Haber-Weiss or the superoxide-driven Fenton reactions, to produce the highly reactive hydroxyl radical. In addition, the superoxide radical can also react with nitric oxide or HOCl to yield the hydroxyl radical. While the hydroxyl radical is very short-lived, it is extremely reactive with virtually all-known biomolecules in the site where it forms, and is likely responsible for the deleterious effects of hydrogen peroxide on DNA. Consequently, it also causes structural damage and generation of phospholipid-derived mediators in cell membranes through lipid peroxidation.

Oxidants promote chemotaxis, activate leukocytes, and initiate cytokine gene expression through interactions with extracellular fluid, cell membrane lipids, and polyunsaturated fatty acids (Granger and Parks, 1983; Granger and Korthuis, 1995; Dröge, 2002). They are also responsible for the stimulation of cellular adhesion molecule expression, which is a crucial step in IR that allows leukocytes to rapidly enter the injured tissue. The most current explanation for vascular endothelial injury is disruption of the glycocalyx on the luminal surface of the venular endothelial cells, which then exposes the adhesion molecules to circulating leukocytes. The resulting endothelial cell disruption leads to tissue changes, including tissue edema and cell disruption.

Peroxynitrite, an active member of RNS, can damage tissue through lipid peroxidation, oxidation of protein sulphhydryl groups, and nitration of aromatic amino acids (Radi et al., 1991; Beckman and Koppenol, 1996). Peroxynitrite formation has been demonstrated in both activated leukocytes (Ischiropoulos et al. 1992; Gagnon et al., 1998; Takemoto et al., 2007) and endothelial cells (Kooy and Royall, 1994), and is a possible mediator of cytokine-induced epithelial hyperpermeability (Chavez et al., 1999).
Intestinal Ischemia-Reperfusion in Horses

Many studies on IRI in the horse have been published, although inconsistent results have been reported, due in part to the use of different experimental IR models [low-flow (hemorrhagic strangulation) versus complete vascular occlusion (ischemic strangulation)], different IR times, and different intestinal segments (Reeves et al. 1990, Meschter et al. 1991, Moore et al. 1994b, Wilkins et al. 1994, Wilson et al. 1994, Darien et al. 1995, Laws and Freeman 1995, Moore et al. 1995a, Moore et al. 1996, 1998a,b; Moore 1997, Rowe and White 2002, Soffler 2007). The true clinical relevance of intestinal injury after reperfusion is unknown in the horse, or if it differs from the progression of cell death that was initiated under previous hypoxic conditions (Snyder et al. 1988, Blikslager et al. 1997a, Rowe and White 2002). The pathogenesis of small intestinal IRI in horses appears to involve activation of the xanthine-oxidoreductase system during ischemia followed by generation of toxic radicals that contribute to intestinal damage sustained after reoxygenation (Prichard et al. 1991, Soffler 2007). Equine small and large intestines behave differently in response to IR (Kooreman et al. 1998, Dabareiner et al. 2001, Rowe and White 2002), and evidence suggests that reperfusion injury does develop in the equine small intestine (Moore et al. 1995a Dabareiner et al. 1995, 2001; Van Hoogmoed et al. 2001), with low-flow ischemic models displaying the most compelling evidence of postischemic damage (Laws and Freeman 1995, Blikslager et al. 1997a, Kooreman et al. 1998, Van Hoogmoed et al. 2001). In these cases, the equine small intestine appears to follow the classic pathway of IRI characterized by generation of ROS and RNS as described above (Prichard et al. 1991, Soffler 2007). Xanthine oxidase is present in the small intestine and is proven to be significantly increased after ischemic strangulation of the jejunum (Prichard et al.
1991). However, other mechanisms have the capacity to exacerbate intestinal damage after reperfusion and may be involved in small intestinal IRI as well (Laws and Freeman 1995, Vatistas et al. 1996, Vatistas et al. 1998, Dabareiner et al. 2001).

Despite some similarities in IRI between the small and large intestine (Moore et al. 1995a), the true relevance of colonic IR is not clear. Reperfusion injury was demonstrated in the large colon after 3 hours of low-flow ischemia followed by 3 hours of reperfusion (Moore et al. 1994b). Furthermore, mucosal lesions progressively worsened after twisting the colon for 2 or 3 hours followed by a 2-hour-reperfusion time period (Meschter et al. 1991; Darien et al. 1995). Moreover, IR resulted in significant damage to the vasculature in the equine colon after 2 hours of ischemia (Henninger et al. 1992), but remained unchanged after complete ischemic strangulation for 70 minutes followed by 1 hour of reperfusion (Dabareiner et al. 1993). In contrast, several studies on equine large colonic IR failed to detect reperfusion injury (Snyder et al. 1988, Reeves et al. 1990, Wilkins et al. 1994, Dabareiner et al. 2001, Matyjaszek et al. 2007, 2009; Morton et al. 2009, Grosche et al. 2011a,b). Furthermore, the classic model of IRI which depends on XO is not likely to contribute to postischemic damage in the large colon (Kooreman et al. 1998), because the enzyme is not present or in very low concentrations in the equine colonic mucosa (Wilkins et al. 1994, Moore et al. 1995a, Blikslager et al. 1997a, Kooreman et al. 1998). Thus, other mechanisms, such as alternative oxidants, mitochondrial dysfunction, failure of mucosal postcapillary microcirculation, collateral tissue damage by infiltrating neutrophils, uncontrolled activation of an innate immune response, or simply a time-related continuation of injury initiated during ischemia, might be responsible for colonic mucosal damage after

**Systemic Inflammatory Response Syndrome (SIRS)**

The systemic response to disruption of the mucosal lining in the small or large intestine is attributed to shock caused by endotoxin derived from intraluminal gram-negative organisms and by other toxins and bacteria. These septic mediators enter the circulation through the mucosal breach caused by IR to become disseminated to various organs, where they activate monocytes that release potent mediators. This process was once categorized as endotoxemia, although a more appropriate label is Systemic Inflammatory Response Syndrome, or SIRS, to include multiple other potential causes of systemic sepsis (Bone et al. 1996). SIRS is usually followed by remote organ injury or multiple organ dysfunction syndrome (MODS), as described below.

**Endotoxemia**

Endotoxemia has been regarded as the leading cause of death in horses with severe mucosal injury, such as that inflicted by ischemia alone or exacerbated by reperfusion injury. It is currently regarded as a component of SIRS. Endotoxins are components of the outer cell envelope of enteric Gram-negative bacteria that were identified more than 100 years ago (Pfeiffer 2003). Chemical studies determined that endotoxins are lipopolysaccharides that are composed of hydrophobic and hydrophilic regions, and this amphipathic feature of endotoxins causes them to form aggregates when they enter plasma (Morrison et al. 1987). The hydrophilic portion of endotoxin consists of the O-specific polysaccharides and a core region. The O-specific polysaccharides provide endotoxins with their well-recognized serotype specificities,
whereas the hydrophobic region contains the toxic "lipid A" region that produces the deleterious effects of endotoxins (Bishop 2005).

Endotoxins normally exist in the lumen of the horse’s intestine, and are largely restricted to that site by a very efficient intestinal mucosal barrier composed of the mucosal epithelial cells, their secretions, and resident bacteria (Moore 1981). Occasionally, small numbers of endotoxin molecules cross the barrier, enter the portal circulation, and are removed by Kupffer cells in the liver. However, horses are exquisitely sensitive to endotoxin so only a small amount needs to enter the circulation to cause dramatic clinical signs.

When the intestinal barrier is damaged in some gastrointestinal diseases, principally those characterized by intestinal hypoperfusion, ischemia, or inflammation, endotoxins gain access to the general circulation and initiate a series of inflammatory responses that cause the clinical signs recognized as endotoxemia. As an alternative route, endotoxins may enter the peritoneal cavity and are absorbed into the systemic circulation. In one study, endotoxins were more commonly detected in peritoneal fluid than in plasma of horses with colic (Barton et al. 1999). There also is histologic evidence that the mucosal barrier is damaged in “healthy intestine” left in horses after resection of devitalized small intestine (Meschter 1986). These findings may account for the persistence of endotoxemia in horses recovering from surgery after removal of the devitalized intestine, as well as in horses with severely inflamed intestine as with enteritis or colitis.

**Bacterial Translocation**

Bacterial translocation is the invasion of indigenous intestinal bacteria through the gut mucosa to normally sterile tissues and the internal organs (Vaishnavi 2013),
thereby contributing to SIRS. Bacterial translocation occurs more frequently in patients with intestinal obstruction and in immunocompromised patients and is the cause of subsequent sepsis. The three primary mechanisms promoting bacterial translocation in animal models are identified as: (a) disruption of the ecologic gastrointestinal GI equilibrium to allow intestinal bacterial overgrowth, (b) increased permeability of the intestinal mucosal barrier, and (c) deficiencies in host immune defenses (Berg 1999). Bacterial translocation occurs through the transcellular and the paracellular pathways and can be measured both directly by culture of mesenteric lymph nodes and indirectly by using labeled bacteria, peripheral blood culture, detection of microbial DNA or endotoxin and urinary excretion of non-metabolizable sugars of bacterial origin (Vaishnavi 2013). Bacterial translocation may be a normal phenomenon occurring on a frequent basis in healthy individuals without any deleterious consequences, but when the immune system is compromised, there may be septic complications at different sites remote to the site of primary infection. The bacteria released from the gut and carried in the mesenteric lymphatics, but not in the portal blood, may cause MODS or multi-organ failure. Thus, bacterial translocation may be an initiator of sepsis.

**Mechanisms of Remote Organ Injury**

The profound cardiovascular changes and the widespread failure of other organs in response to SIRS is called MODS, and could explain deaths from gastrointestinal diseases in horses and human patients. MODS could also explain remote effects from intestinal reperfusion injury in organs such as the lungs in horses (Montgomery et al. 2014, Faleiros et al. 2008) and laboratory animals (Simpson et al. 1993). Intestinal disease is not the only cause of SIRS, and other causes are sepsis in other organs and extensive tissue injury, such as burns, fractures, wounds and trauma. Remote organ
damage is considered to be a consequence of an IRI event or other form of severe body injury and causes MODS. In human patients, MODS is mainly evident as acute kidney injury (AKI), acute lung injury (ALI), myocardial IR and the effect of stroke. However, the mechanism of damage caused by the inflammatory cascade in humans does not differ from that described in animal models.

ALI is typically associated with increased neutrophil infiltration, increased vascular permeability, and increased tissue edema, all of which are characteristic features of an inflammatory process (Jin et al. 2017). Both human studies and animal models of ALI invariably report increased production of systemic and local cytokines (IL-1β, IL-6, IL-8, IL-10, and TNF-α) and chemokines (CXCL1, CINC1, MIP1, and MCP1). Other immune mediators are also possibly involved. ALI occurs without endotoxin in remote organ injury caused by trauma, ischemia and tissue transplantation (Xiang et al. 2014, Potocnik et al. 2014, Fan et al. 2015, Chi et al. 2016). The inflammatory mediators are thought to play a critical role in the pathogenesis of ALI, because eliminating various parts of the inflammatory cascade alleviates the severity of lung injury (Chun et al. 2010, Guzel et al. 2012, You et al. 2012, Altemeier et al. 2013, Chi et al. 2015). Tissue injury, whether of the lung or other remote organ, leads directly to cell injury and necrosis. In this process, TNF-α and IL-1β are released (Hempel et al. 1996). TNF-α increases vascular permeability, which increases recruitment of neutrophils and macrophages. It can inactivate IkB thorough phosphorylation, thus removing the inhibiting effect of NF-kB, which further upregulates the expression of IL-1β (Miyamoto et al. 1996, Lawrence 2009). Increased levels of TNF-α and IL-1β recruit more macrophages and neutrophils, in addition to promoting their survival. IL-1β is also able
to upregulate the production of acute phase proteins, such as C reactive protein (CRP) and complement, as well as promoting the expression of other cytokines, chemokines and adhesion molecules (Dinarello 2009).

Cytokines upregulated by TNF-α and IL-1β include IL-6 and IL-8. IL-6, which is produced mainly by endothelial cells and modulates the immune response by altering the expression of neutrophil, macrophage, and T cell chemokines, including CXCL1, MIP1, MCP1 and CCL5. All of these are reported to be associated with the pathogenesis of ALI. Also adhesion molecules, such as selectin ICAM-1 and VCAM-1, are upregulated. IL-8, a chemoattractant produced by macrophages, induces neutrophil chemotaxis, and upregulates the phagocytic function of neutrophils (Scheller 2011). Although increased IL-10 is reported by a number of studies in association with ALI, it is actually an anti-inflammatory cytokine, which inhibits TNF-α, IL-1β, and NF-kB, as well as reducing adherence of macrophages (Pierson 2010). It is likely that its role in ALI is more regulatory in nature. This is difficult to prove, however, as most studies to date demonstrate an increased level of IL-10 with ALI mimetics and a reduced level of IL-10 with ALI treatment (Fortis et al. 2012).

Toll like receptors are pattern recognition receptors, which are known for their affinity to bind bacterial endotoxins. Studies have shown that knockout of toll like receptor 4 (TLR4), and its adaptor protein MyD88, is associated with reduction in ALI severity in apparently aseptic conditions (Chun et al. 2010, Altemeier et al. 2013, Chi et al. 2015). More recent studies have alluded to the possibility that as well as bacterial molecular patterns, TLR4 is also able to identify endogenous ligands associated with tissue injury (Tsan et al. 2004). The TLR4/MyD88 complex acts through downstream
molecule IKK, which phosphorylates and inactivates IkB, thus lifting inhibition on NF-kB (Tsan et al. 2004, Ray et al. 2010). Vascular endothelial growth factor (VEGF) is a group of growth factors that promote angiogenesis and has some chemotactic functions. It has been demonstrated that VEGF expression is upregulated by various inflammatory mediators described above, including IL-1β, IL-6, and TNF-α (Nagineni et al. 2012, Maloney et al. 2015). It has been reported that administration of VEGF increases lung vasculature permeability, and VEGF inhibition alleviates lung injury (Maloney et al. 2014, Sato et al. 2016, Yu et al. 2016); however, VEGF attenuation of lung injury has also been reported (Song et al. 2015, Yang et al. 2015). It is possible that the effect of VEGF depends on timing in relation to the injury.

Numerous animal models of ALI secondary to remote organ injury and other conditions have reported an altered activity of the PI3K/Akt/mTOR pathway, a series of signaling molecules which play a vital role in cell proliferation (Jin et al. 2017). General anesthetic agents exert their cytoprotective effects at least in part by upregulating this pathway (Akahori 2006). PI3K and mTOR are reported to inhibit the expression of NF-kB; mTOR could regulate downstream molecules like HIF1-α (Zhao et al. 2015), which upregulates the expression of antioxidative enzymes, promotes cell survival, and encourages angiogenesis; however, it may have a secondary role depending on tissue levels, as it has been associated with both partial resolution and deterioration of lung injury (Liu et al. 2015, Zhao et al. 2015). One of the downstream effects of HIF1-α is to inhibit HMGB1 release from the nucleus. HMGB1 is a chromatin protein that acts as a damage associated molecular pattern (DAMP). It can interact with the TLR4/MyD88 complex, which upregulates NF-kB and MAPK (Weber et al. 2015).
Animal studies of transplant related ALI consistently reported increased serum levels of cytokines such as IL-1β and TNF-α; these cytokines are also found to be increased in lung tissue (Chi et al. 2015, Zhao et al. 2015, Ning et al. 2016). Studies have also shown that disabling part of the inflammatory pathway, such as TLR knockout, NF-kB inhibition and preventing leukocyte adhesion can reduce the extent of ALI and reduce cytokines in the lungs (Chi et al. 2015, Hashimoto et al. 2016). This suggests that cytokines released from remote organ injury could spread to the lungs through the blood supply, where they activate pro-inflammatory pathways in the lungs and cause ALI. In addition to circulating cytokines, remote organ injury can also cause the release of proinflammatory damage associated molecular pattern such as HMGB1 into the circulation, which can also activate the proinflammatory pathways in the lungs (Weber et al. 2015).

Kidney IRI, like ALI, develops in various clinical settings including shock, sepsis, organ transplantation, and vascular surgery. Much of the mortality associated with AKI can be attributed to the onset of SIRS and progression to MOF. Kidney dysfunction directly contributes to the onset of remote organ injury. For example, increased kidney ischemia time during complex aortic surgery is associated not only with acute and chronic renal failure, but also with an increased incidence of remote organ injury and death (Svensson et al. 1993, Kashyap et al. 1997, Back et al. 2005). Clinical and translational laboratory studies have demonstrated the relevance of interactions between the injured kidney and distant organs, and complex mechanisms of crosstalk between injured kidneys and remote organs such as the lungs, liver, heart, gut, brain, and hematologic system have been identified. Recent data highlights the importance of
both the innate and adaptive immune response, activation of proinflammatory cascades, and an alteration of transcriptional events in remote organs during ischemic AKI.

Kidney IRI activates an inflammatory response that causes endothelial cell activation, leukocyte adhesion and entrapment, and compromised microvascular blood flow (Bonventre et al. 2004). Inflammation in the postischemic kidney triggers the upregulation of leukocyte adhesion molecules, toll-like receptors, and downstream transcription factors, which all contribute to disruption of the integrity of the renal vascular endothelium (Jang et al. 2009). Adhesion molecules such as integrins and selectins along with proinflammatory cytokines propagate cellular injury not only locally in the renal tubular epithelial cells but also travel to remote organs where genomic markers of injury are upregulated and phenotypic injury occurs (Hassoun et al. 2009). Unfortunately, selective inhibition of cytokines and adhesion molecules such as TNF-α and intercellular adhesion molecule (ICAM-1) have failed to demonstrate global attenuation of both local and remote organ injury during experimental models of ischemic AKI (Kelly et al. 1996, Donnahoo et al. 1999, Yoshidome et al. 1999, Deng et al. 2004). However, α-melanocyte-stimulating hormone (α-MSH), a cytokine with broad anti-inflammatory, anticytotoxic, and antiapoptotic properties, has been used with success in treating the inflammatory phenotype in rodents. In this model, treatment with α-MSH has attenuated both renal and pulmonary injury during ischemic AKI (Chiao et al. 1997, Deng et al. 2004). The largely unsuccessful effort to ameliorate MOF with specific anti-inflammatory therapeutics highlights the complexity of the systemic response to kidney IRI. From these early experimental studies, it is possible that the multiple
inflammatory pathways activated in each organ system represent a unique response to ischemic AKI.

Despite a scarcity of data, several different pathophysiologic responses to ischemic AKI in remote organs have been identified. In the heart, increased expression of TNFα and interleukin-1 (IL-1) are associated with myocyte apoptosis. In the brain, increased expression of chemokines including keratinocyte chemoattractant (KC, a brain IL-8 homologue) and granulocyte colony-stimulating factor (G-CSF) are seen with increased vascular permeability.

The innate immune system plays an important role in mediating the inflammatory response during ischemic AKI. Traditionally, innate immunity elicits an immediate, preprogrammed response to tissue injury that lacks immunologic memory. It is composed of plasma proteins (complement), cells (neutrophils, macrophages, and natural killer cells), and physical barriers. Proposed initiators of the innate immune response during IRI include the activation of toll-like receptors (TLRs) and the release of reactive oxygen species (nitric oxide and superoxide anion) and mitochondrial products (Chakraborti et al. 2000). The complement system, particularly the alternative pathway, is activated, stimulating release of cytokines and subsequent activation of neutrophils, endothelium, and macrophages (Rabb et al. 2002).

Ischemic AKI is also implicated in oxidative stress, inflammation, apoptosis, and tissue damage in hepatocytes. Hepatic stellate cells (HSCs) regulate leukocyte trafficking and the secretion of chemokines such as IL-8, and crosstalk between HSCs likely occurs through a c-Jun N-terminal kinase pathway (Schwabe et al. 2001). Oxidative stress during ischemic AKI causes hepatic malondialdehyde, an index of lipid
peroxidation, to increase while total glutathione, an antioxidant, decreases (Golab et al. 2001). Pro-inflammatory cytokine TNFα expression and hepatic cellular apoptosis is also evident during ischemic AKI (Golab et al. 2001).

Previous investigators and clinicians have labeled the gut as the ‘motor’ of MOF because of its ability to amplify the SIRS response in the setting of shock and gut hypoperfusion (Carrico et al. 1986, Hassoun et al. 2001, Clark et al. 2007). These mechanisms include increased intestinal permeability, interactions between host and bacterial pathogens, and propagation of toxins to distant organs via the lymphatic system (Fink et al. 2005, Clark et al. 2007) and could potentially play a role during ischemic AKI.

Role of Immune Cells and Inflammatory Response in Ischemia-Reperfusion

The intestinal mucosa contains many immune cells, such as lymphocytes, macrophages, mast cells, neutrophils, and eosinophils, with intestinal lymphocytes and macrophages representing the largest pool of these cells in the body under healthy conditions (Lloyd 2000; Schenk and Mueller 2008). These diverse inflammatory cells respond through well-coordinated mechanisms to maintain a delicate balance between protection against infectious agents and injury, and tolerance of the abundant antigens in the intestinal lumen (Sansonetti 2004; Artis 2008; Pasparakis 2009; Turner 2009). Therefore, disrupted epithelial integrity could induce an intense and uncontrolled inflammatory response through these immune cells that could play a critical role in the pathogenesis of intestinal IRI (Wallace and Ma 2001; Platt and Mowat 2008; Schenk and Mueller 2008; Laskin et al. 2011).

Macrophages

The innate immune system is the first line of defense to provide protection during

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2008, Laskin et al. 2011). Thus, the macrophages could play an important role in the
pathophysiology of intestinal IR injury and also subsequent repair processes.

**Neutrophils**

Intense leukocyte recruitment and activation is a crucial and early step in the IR
cascade, and much of the tissue injury that occurs upon reperfusion is attributed to
neutrophilic radicals and proteolytic enzymes (Blikslager et al. 1997, Gayle et al. 2000,
Souza et al. 2004). Neutrophils residing in the interstitium might be as important as
recruited neutrophils in generation of reperfusion injury, based on a study in cat small
intestine (Kubes et al. 1992). In addition, most methods that induce ischemia in the
experimental setting, even those intended to cause complete vascular occlusion, can
permit sufficient circulation to allow neutrophil recruitment and infiltration during
ischemia. Such inflow would also be likely during the early stages of a naturally
occurring intestinal strangulation (Snyder 1988). Circulating neutrophils recruited to
sites of ischemic injury roll along the endothelium, and become adhered to endothelium
in postcapillary venules before they migrate across it to the inflamed tissue (Witko-
facilitated by neutrophil integrins and endothelial ICAM-1 (Chamoun et al. 2000, Witko-
Sarsat et al. 2000) and loss of the glycocalyx that normally shrouds these components
of the endothelial lining (Chin and Parkos 2007). Migration of neutrophils into the
mucosal lamina propria during reperfusion assists in recognizing and ingesting cell
detritus and destroying invading bacteria (Chamoun et al. 2000, Witko-Sarsat et al.

Neutrophils contribute to the development of endothelial injury (Granger et al.
1986, Hernandez et al. 1987, Kurose et al. 1994, Cooper et al. 2004) and mucosal
damage during intestinal IR through an arsenal of pro-inflammatory factors, enzymes, antibacterial proteins and other toxic molecules (Granger 1988, Grisham and Granger 1988, Grisham et al. 1990, Schoenberg et al. 1991, Kubes et al. 1992, Friedman et al. 1998, Gayle et al. 2002, Chin and Parkos 2007). The two enzymes, NADPH oxidase and MPO, could contribute to formation of ROS during reperfusion (Bhaskar et al. 1995, Granger and Korthuis 1995). Neutrophils produce a small amount of hydroxyl radical but are also an important source of HOCl from MPO and hydrogen peroxide (Granger 1988, Grisham and Granger 1988, Grisham et al. 1990). Neutrophils can secrete proteases, such as elastase, collagenase, and gelatinase, into the extracellular fluid, where they can degrade the basement membrane and interstitial matrix of the endothelial cell (Granger 1988, Grisham and Granger 1988, Grisham et al. 1990).

Most of the damage mediated by neutrophils is called collateral or bystander damage, because it involves incidental contact of toxic molecules with surrounding non-target cells (Grisham and Granger 1988, Witko-Sarsat et al. 2000) or physical damage while migrating to the site of injury (Milks et al. 1986, Moore et al. 1995, Gayle et al. 2000, Nathan 2006, Chin and Parkos 2007). Neutrophils increase intestinal permeability as they traverse intercellular spaces in restituted epithelium and this effect can be prevented by blocking neutrophil adhesion or scavenging superoxide (Gayle et al. 2002).

One of the antibacterial proteins produced by neutrophils is calprotectin, an endogenous pro-inflammatory molecule (Yui et al. 2003, Foell et al. 2007) that is found to a lesser extent in monocytes and macrophages (Johne et al. 1997). Although the exact biological role of calprotectin is unknown, evidence suggests it can modulate
inflammation (Johne et al. 1997) and aids in host defense against pathogens via the binding of manganese and zinc (Damo et al. 2013). In high concentrations for a long period, calprotectin can cause local tissue destruction (Yui et al. 2003). Because of its strong association with neutrophil movement and location, calprotectin can be used as a marker of acute and chronic inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease and intestinal IR (Foell et al. 2004, Striz and Trebichavsky 2004). Although its presence in macrophages and monocytes diminishes its specificity as a marker of neutrophils, its association with neutrophil influx as determined by hematoxylin and eosin staining is so closely correlated that the non-neutrophil sources become negligible (Grosche et al. 2012).

**Eosinophils**

Eosinophils reside in abundance in the lamina propria of the equine colon and small intestine under healthy states, but their role in gastrointestinal health and disease is unresolved (Rötting et al. 2008a; Rötting et al. 2016). Eosinophils are a poorly understood inflammatory cell, although they are widely accepted to be pro-inflammatory leukocytes that can generate a mixture of destructive mediators (radicals, lipid mediators, and proteases) and toxic granule proteins (major basic protein, eosinophil peroxidase, eosinophil-derived neurotoxin, and eosinophil cationic protein) (Walsh 1997, Hogan et al. 2008). Eosinophils have been implicated in pathological changes and tissue damage in patients with asthma, eosinophil myalgia syndrome and other hypereosinophilic diseases (Wardlaw 1996). They are equipped with enzymes that cause oxidative damage to biological targets and undergo a respiratory burst similar to that in activated neutrophils, followed by generation of ROS and RNS such as peroxynitrite (Van Dalen et al. 2006, Takemoto et al. 2007, Lotfi et al. 2009).
Furthermore, eosinophilic major basic protein stimulates IL-8 release, a potent hemoattractant and activator of neutrophils (Moy et al. 1990, Page et al. 1999). This basic protein also promotes neutrophil respiratory burst in eosinophil-associated inflammation (Moy et al. 1990, Haskell et al. 1995). Additionally, a number of cytokines are synthesized by eosinophils, increasing the range of their potential functions including wound healing and fibrosis (TGF-α, TGF-β), antigen presentation (IL-1) and autocrine stimulation (IL-5, IL-3, GM-CSF) (Rothenberg et al. 2001, Munitz and Levi-Schaffer 2004). Inappropriate accumulation of eosinophils and eosinophil-derived granules in tissues are considered as biomarkers of the severity of the pathological event, and they also evoke cellular and tissue damage. Furuta et al. (2005) demonstrated a deleterious effect of major basic protein on colonic epithelial barrier function in mice. Moreover, increased intestinal accumulation of eosinophils has been described in horses with experimentally induced acute colitis, IR injury and parasitism (Moore et al. 1994a, McConnico et al. 1999, Edwards et al. 2000, Archer et al. 2006, Rötting et al. 2008b). They are also implicated in different gastrointestinal tract syndromes in horses that affect the large and small intestines and, in one type of disease, skin and other organs (Schumacher and Legere 2017).

**Mast Cells**

Mast cells are potent immunomodulatory cells that can promote and increase inflammation, tissue injury and remodeling and they can also play an important protective role in mucosal defense (Kanwar and Kubes 1994b, Penissi et al. 2003, Galli et al. 2008, Shea-Donohue et al. 2010). Activated by pathogens, danger signals or inflammatory mediators, mast cells can clear pathogens by phagocytosis or by secretion of anti-microbial peptides, degrade potentially toxic endogenous peptides, and release

Mast cells can be harmful to intestinal barrier integrity in certain pathologic situations (Penissi et al. 2003, Marshall 2004). Mast cells are recruited into the tissue during colonic IR in the rat (Sand et al. 2008), and they are thought to be important mediators of IR-induced mucosal and microvascular dysfunction in the mouse intestine (Kanwar et al. 1998). Although mast cells are sensitive to intestinal ischemia (Boros et al. 1995) and they cause mucosal permeability alterations during reperfusion in canine small intestine, their role in IR-induced structural changes might be minor (Szabo et al. 1997). Mast cell release of histamine contributes most to the severity of mucosal damage compared with other toxic mast cell metabolites (Kimura et al. 1998, Boros et al. 1999a). However, mast cell degranulation before ischemia can be protective in the small intestine and can decrease IR injury in the dog (Boros et al. 1999b).

Calprotectin

As a key component of the early inflammatory response, neutrophils play an important role in intestinal mucosal defense, but can also contribute to loss of mucosal integrity, cell death and tissue damage under certain pathological situations (Gayle et al. 2000, 2002; Nathan 2006). Neutrophils produce a large array of pro-inflammatory factors, enzymes, antibacterial proteins and other toxic molecules that are originally directed against invading microorganisms and infectious agents. One of these
antibacterial proteins is calprotectin, a member of endogenous pro-inflammatory molecules of the innate immune system that signals tissue and cell damage, and has antimicrobial and antifungal functions (Johne et al. 1997, Foell et al. 2007, Lotze et al. 2007).

Calprotectin is a heterodimeric complex that is highly associated with inflammatory reactions (Foell et al. 2004). The complex belongs to the S100 superfamily of proteins, and is also referred to as S100A8/A9, L1, or MRP-8/14. The 36kDa heterodimer consists of one light chain (S100A8; 10.8 kDa), and two heavy chains (S100A9; 13.2 kDa). Calprotectin is a calcium- and zinc-binding protein that is primarily present in the cytoplasm and on plasma membranes of neutrophils, monocytes and macrophages (Johne et al. 1997, Kerkhoff et al. 1998). In neutrophils, calprotectin constitutes 5% of total proteins and approximately 60% of the cytosolic protein fraction (Fagerhol et al. 1980a, 1990). Each neutrophil cell contains 5 to 25 pg calprotectin, whereas in monocytes, calprotectin accounts for approximately 1.6% of the total protein content (Fagerhol et al. 1980a, Berntzen et al. 1988, Fagerhol et al. 2005). Several research groups have stated the possibility of an extracellular secretion of calprotectin from stimulated neutrophils, but calprotectin is also released as a result of cell disruption or death (Dale et al. 1985, Hetland et al. 1998, Voganatsi et al. 2001). Although the exact biological role of calprotectin is still unknown, available evidence suggests calprotectin can modulate inflammatory reactions through activities that inhibit growth and induce apoptosis in fibroblasts or other cell types (Johne et al. 1997, Nisapakultorn et al. 2001, Yui et al. 2003). Furthermore, there is strong evidence that calprotectin is a potent stimulator of neutrophils, and it is involved in neutrophil migration to inflamed
tissues when released at the site of cell damage (Roth et al. 2003, Ryckman et al. 2003, Vandal et al. 2003, Simard et al. 2010). If present in an excess amount for a long period, calprotectin can cause local tissue destruction (Yui et al. 2003).

Calprotectin is one of the pro-inflammatory proteins that is used as a sensitive marker of acute and chronic inflammatory conditions such as systemic infections, pneumonia, rheumatoid arthritis, laminititis, dermatitis, inflammatory bowel disease and intestinal IR in human beings, horses and dogs (Eckert et al. 2004, Foell et al. 2004, Striz and Trebichavsky 2004, Little et al. 2005, Grosche et al. 2008, Faleiros et al. 2009). Because of its distribution in various cells, tissues and body fluids, calprotectin is emerging as a valuable marker for diagnosis, monitoring, and prognosis of gastrointestinal diseases, and it may serve as a marker for any diseases associated with increased neutrophil or monocyte/macrophage activity (Johne et al. 1997, Poullis et al. 2003).

Calprotectin is resistant to bacterial degradation in the gut and is stable in feces for up to 1 week at room temperature (Roseth et al. 1992). The half-life of calprotectin in human plasma is 5 hours (Fagerhol et al. 2005). It is readily quantified by ELISA or RIA, and over the last decade, improved assays have been developed to increase the specificity and sensitivity of its detection (Fagerhol et al. 1980b, Ivanov et al. 1996, Ton et al. 2000, Heilmann et al. 2008b). Normal values of 2 to 897 μg/g feces have been determined in humans, with fecal calprotectin concentrations of up to 6850 μg/g feces in patients with active inflammatory bowel disease (Konikoff and Denson 2006). The excretion of calprotectin in feces seems to be related to the flux of neutrophils and mononuclear cells into the intestinal wall, their turnover and their migration into the gut.
lumen as shown by a correlation between excreted labeled neutrophils and fecal calprotectin (Roseth et al. 1997, 1999; Tibble et al. 2000). Reference concentrations for human plasma calprotectin has been assessed at 0.1 to 0.9 μg/L. Up to 40-fold increased concentrations have been found in plasma in a variety of inflammatory conditions which seems to reflect an increased leukocyte turnover or possible release of calprotectin by activation or cell death (Sander et al. 1984, Johne et al. 1997, Striz and Trebichavsky 2004). Serum calprotectin values of up to 11, 15 and 46 μg/L were associated with systemic bacterial infections, after major surgeries or rheumatoid arthritis in humans, respectively (Johne et al. 1997).

Plasma and fecal calprotectin have been used routinely to assess disease activity and therapeutic progress or relapse in patients with inflammatory bowel disease (Aadland and Fagerhold 2002, Foell et al. 2004, Konikoff and Denson 2006, Sutherland et al. 2008). Plasma calprotectin was also increased up to 5 days after abdominal surgeries in humans. This response was related to an increase of CRP and endotoxin following surgery, possibly due to translocation of bacterial toxins from the gastrointestinal tract that triggered a post-operative acute phase reaction and activation of monocytes and neutrophils (Berger et al. 1997). In horses, calprotectin expression correlated significantly with neutrophil infiltration in colonic tissues, and calprotectin expression increased during colonic IR in horses (Grosche et al. 2008, 2011b; Matyjaszek et al. 2009).

Recently, canine calprotectin has been purified and characterized, and a RIA has been developed for quantification of calprotectin in serum and feces from dogs with reference values of 92 to 1121 μg/L serum, and 29 to 1375 μg/g feces (Heilmann et al.
Since there is no immunoassay available for horses, an ELISA for quantification of equine calprotectin in blood and fecal samples is needed for this species, as a clinical marker for inflammatory conditions in the gastrointestinal tract and for any systemic or local inflammation.

**Cyclooxygenase**

Prostaglandins (PGs) are synthesized by COX enzymes in many cell types, including those in the intestinal lamina propria (Krause and DuBois 2000, Morton et al. 2009, Hilton et al. 2011), and can play a critical role in inflammation (Krause and DuBois 2000). Phospholipase A2, an enzyme in the cell membrane, is activated during ischemia by increased cytosolic calcium, and causes release of biologically important cell membrane phospholipids, such as platelet-activating factor (PAF) and arachidonic acid. Arachidonic acid metabolites are leukotrienes produced by the lipoxygenase pathway (LOX) and prostaglandins produced through COX. Prostacyclin (PGI$_2$) is a vasodilator and it also inhibits platelet aggregation, whereas thromboxane A$_2$ is a potent vasoconstrictor, promotes platelet aggregation, and enhances neutrophil adherence and chemotaxis. Both leukotriene (LTB$_4$) and thromboxane A$_2$ are considered to mediate neutrophil recruitment and microvascular dysfunction (Krause and DuBois 2000, Morton et al. 2009, Hilton et al. 2011). In equine colon, the reactive oxygen metabolites, superoxide anion, hydrogen peroxide, hypochlorous acid, and the hydroxyl radical increase chloride secretion through a mechanism mediated by mucosal prostaglandins (Inoue et al. 2007).

The COX enzyme is represented by two isoforms in the gastrointestinal tract, COX-1 and COX-2. COX-1 is the constitutive form, responsible for housekeeping activities that relate to health and function of the tissue, such as intestinal fluid and
electrolyte transport (Argenzio and Liacos 1990), transepithelial permeability (Blikslager et al. 1997), cell proliferation (DeRubertis 1985), crypt cell survival in damaged intestinal epithelium (Cohn 1997), blood flow, mucosal immunology, mucus and bicarbonate secretion (Wallace 1997), and epithelial restitution (Zushi 1996). COX-2 is the inducible isoform and it plays an active role in inflammation in response to various forms of tissue injury (see above).

Some overlap in function of these COX isoforms can arise, as evident in the observation that COX-2 seems to be constitutive in equine small intestine (Cook et al. 2008a, Bauck et al. 2017). Also, COX-2 seems to play an important role in mucosal repair (Shifflett et al. 2004) and in healing of anastomoses in human colon (Reisinger et al. 2017). These issues are vitally important in treating patients with gastrointestinal diseases and their side effects, because the resulting injury could be exacerbated or resolved by using a specific inhibitor of COX-2 or a nonspecific inhibitor of both isoforms (Cook and Blikslager 2008a).

**HIF1-α**

HIF1-α is used as a marker of tissue ischemia, because its production is upregulated in cells during periods of tissue hypoxia (Brooks 2010). Although most tissues subjected to hypoxia are clearly identifiable on gross and microscopic examinations, cells distant to the site of primary injury could be subjected to diminished circulation and transient hypoxia during maldistributive shock from any cause, and affected cells could be identified by staining for this marker. Blood flow to tissues can affect expression of HIF1-α, a transcription factor composed of an inducible α unit and a constitutive β unit (Wang et al. 1995). During periods of tissue hypoxia, the α and β
units form a complex that initiates the transcription of oxygen-regulated genes that improve survival of hypoxic cells (Hirota 2002). It has been examined in intestine from horses with colic as a marker of intestinal ischemia (De Ceulaer et al. 2011) and could be a valuable marker of tissue injury remote to intestinal ischemia.

**Fibrin**

Fibrin is produced from fibrinogen under the influence of thrombin. Fibrinogen is a protein marker of inflammation produced from the liver in response to an inflammatory process in any part of the body, with normal levels in the horse ranging from 1.0-4.0 g/L. Research from Gilliam et al. (2011) shows that fibrin plays an important role in the inflammatory response and development of rheumatoid arthritis in humans. Low fibrinogen levels are indicative of systemic activation of the clotting system, with consumption of clotting factors occurring faster than synthesis which is consistent with clinical signs of disseminated intravascular coagulation (DIC). DIC is a syndrome characterized by excessive formation of microvascular fibrin deposits, which can lead to ischemic tissue lesions, MOF, and death (Katsumura et al. 1995, Morris 2002, Zimmel 2003). Previous studies have demonstrated that the incidence of DIC in horses with septicemia and in those with ischemic or inflammatory gastrointestinal disorders may be high (Holland et al. 1986, Johnstone et al. 1986, Henry et al. 1991, Welles et al. 1991, Monreal et al. 2000, Dolente et al. 2002, Cotovio et al. 2017). In these cases, endotoxemia may be a predisposing factor for the development of hypercoagulation and massive production and accumulation of fibrin (King et al. 1988, Morris 2002, Oikawa et al. 2003, Lohmann et al. 2004, Sellon 2004). DIC is frequently present in horses with guarded gastrointestinal prognoses, and since DIC is such a dynamic process, clinical signs are variable and results of coagulation tests may change rapidly, which makes...
confirmation of this syndrome challenging (Stokol 2010). Histologic studies in people with DIC have shown that excessive production and massive deposition of fibrin can be observed in capillaries of several organs, such as the kidneys, lungs, liver, spleen, heart, and brain (Hafter et al. 1977, Wilde et al. 1988, Hermida et al. 1999, Montes et al. 2000), while the most frequently affected organs are the lungs, kidneys, and liver (Kawasaki et al. 1987, Katsumura et al. 1995, Scherer et al. 1995, Montes et al. 2000, Montes et al. 2002). In veterinary medicine, the detection of microvascular fibrin deposition in 1 or more organs is also considered essential for a histopathological diagnosis of DIC (Bateman et al. 1995). Cotovio et al. (2007) demonstrated that horses with severe gastrointestinal disorders have fibrin deposits in the lung, liver, and kidney consistent with capillary microthrombosis, multiorgan failure, and DIC. Researchers found a marked increase in fibrin deposits in the ischemic group, which confirmed that severe hypercoagulation and DIC occurred frequently in strangulated obstructions (Cotovio et al. 2007). These results confirm previous studies which found that clinically and experimentally induced intestinal ischemia and endotoxemia in horses is associated with activated coagulation and thrombosis (Meyers et al. 1982, Pablo et al. 1983, Morris et al. 1988, Welles et al. 1991). DIC occurred in up to 55% of horses with ischemic disorders (Monreal et al. 2000). Ischemia-associated fibrin deposition is speculated to result from ischemia-induced changes in the microvascular environment, including diminished aerobic metabolism, accumulation of waste products, and activation of the inflammatory response (Schoots et al. 2003). The resulting generation of fibrin and inadequate removal produces intravascular fibrin deposition in the microcirculatory beds of different organs (Cotovio et al. 2007).
Effects of Ischemia-Reperfusion on Intestinal Epithelial Cells

The first histologic evidence of epithelial damage begins within 20 minutes of total small intestinal ischemia, and is characterized by lifting of small clusters of epithelial cells, their detachment from the basement membrane, and subsequently death by apoptosis and necrosis (Snyder et al. 1988, Meschter et al. 1991, Moore et al. 1995, Kong et al. 1998). Many inflammatory stimuli in response to ischemia can increase leakiness of the epithelial barrier by disassembly of apical junctions (Ivanov et al. 2010).

Cell death by necrosis or apoptosis is triggered during IR (Cummings et al. 1997, Ramachandran et al. 2000, Festjens et al. 2006, Zong and Thompson 2006, Rock and Kono 2008). Both types of cell death can be seen simultaneously in response to the same stimulus, but the intensity of the stimulus determines which predominates (Bonfoco et al., 1995; Leist et al. 1997). Unlike necrosis, apoptotic cell death is a controlled, programmed, energy-dependent event that does not stimulate the immune system (Hall et al. 1994, Kono and Rock 2008). It is associated with normal cell turnover in the gastrointestinal tract (Hall et al. 1994) and other tissues. The defining characteristic of apoptosis is cell shrinkage, chromatin condensation and margination, membrane blebbing, and segmentation into apoptotic bodies that can be phagocytized within several hours (Taylor et al. 2008, Kroemer et al. 2009). Apoptotic cell loss might be a means to control tissue damage, maintain a defensive barrier, regulate inflammation and hasten epithelial repair (Ramachandran et al. 2000, Maderna and Godson 2003, Serhan et al. 2008, Maniati et al. 2008). Although it typically affects single cells and does not induce an inflammatory response, pronounced apoptosis can contribute to leaks in the epithelial barrier (Shah et al. 1997, Abreu et al. 2000, Gitter et
Necrosis can involve sheets of cells and an intense inflammatory response, so that barrier function is lost at the site of ischemia.

**Lidocaine**

Lidocaine has been used in horses that undergo colic surgery (van Hoogmoed et al. 2004) based on evidence that it can treat or prevent POI (Cohen et al. 2004; Malone et al. 2006; Torfs et al. 2009). In a multicenter study of horses with nasogastric reflux attributed to POI or proximal enteritis, 65% of horses treated with lidocaine ceased to reflux within 30 hours, compared with 27% of horses treated with saline (Malone et al. 2006). Lidocaine also reduced the hourly volume of reflux during and after the infusion, which decreased the duration of hospitalization (Malone et al. 2006). In another study, lidocaine reduced the prevalence of POI in horses with small intestinal surgery and improved short-term survival (Torfs et al. 2009). Because lidocaine does not appear to act as a prokinetic drug in normal horses (Nieto et al. 2000, Milligan et al. 2007, Rusiecki et al. 2008), a potential benefit for prevention or treatment of POI by lidocaine could be mediated through an anti-inflammatory effect (Cook et al. 2009) or an ability to enhance mucosal repair (Cook 2013).

**Hypothesis and Objectives**

Hypothesis states that organs remote to the gastrointestinal tract undergo inflammatory changes after ischemia and reperfusion injury in the equine colon and jejunum. Researchers believe lidocaine will ameliorate this inflammation. The objectives of this study were to assess the effects of intestinal ischemia reperfusion injury to the remote organs (kidney, liver, lung), and to examine the effects of a continuous rate infusion (CRI) of lidocaine on inflammation in remote organs.
CHAPTER 2
MATERIALS AND METHODS

Horses and Anesthesia

This study was approved by the Institutional Animal Care and Use Committee of the University of Florida. Fourteen adult horses of different breeds, aged 3 to 15 years (median age of 6 years), weighing 400 to 575 kg (median weight of 522 kg), and without signs of gastrointestinal tract disorders, were used in the ischemia phase of the study under general anesthesia. These horses had not received a nonsteroidal anti-inflammatory drug within 3 weeks before the study and did not have laminitis or a history of gastrointestinal disease. They were kept on pasture, fed grass hay (2% of body weight/day) and had free choice water for 1 week before the study.

Horses were randomly assigned to 2 groups: one group (n=7) received lidocaine throughout anesthesia, and the other group (n=7) received an equivalent volume of a physiologic saline solution in addition to the polyionic fluids they received as part of their anesthesia regimen. Horses were sedated with xylazine (0.5 mg/kg, IV) and butorphanol (0.02 mg/kg, IV) and anesthesia induced with diazepam (0.1 mg/kg, IV bolus) and ketamine (2.2 mg/kg, IV). Following anesthetic induction and tracheal intubation, horses were positioned in dorsal recumbency and general anesthesia was maintained with isoflurane (1-3%) in oxygen. Mean arterial blood pressure, heart rate, respiratory rate, fraction of inspired oxygen, and inspiratory isoflurane concentrations were monitored continuously. Isotonic polyionic fluids were infused continuously IV at 5 ml/kg/hr and mean arterial blood pressure was maintained at or above 70 mmHg using a dobutamine CRI and adjusting the flow of isoflurane.
Control horses (n = 7) were normal horses that were donated for musculoskeletal disease and were otherwise in good health. They were not anesthetized and did not receive lidocaine or saline and the organs of interest were harvested immediately after euthanasia.

**Lidocaine Infusion**

Lidocaine (2%) was given to horses assigned to receive it as a bolus of 1.3 mg/kg IV over 15 minutes after induction of anesthesia, followed by a CRI of 0.05 mg/kg/min throughout anesthesia (Malone et al. 2006). The control group received physiologic saline solution (sterile 0.9% sodium chloride) in equivalent volumes on a body weight basis as a bolus and CRI. Surgeons responsible for inducing ischemia and manipulating the jejunum were blinded as to whether the horse received saline or lidocaine.

**Ischemia and Reperfusion**

Following standard aseptic preparation and draping of the abdomen, a 25-cm ventral midline approach was made. The jejunum, starting at least 4 arcades proximal to the ileum; three equal portions were assigned to a treatment and sampling group as described below. In the middle of each of the two most distal portions of jejunum, a 40-cm segment was rendered ischemic by venous and arterial occlusion for 30 minutes as described previously (Freeman et al. 1988). At the same time, ischemia was induced for 1 hour in two 20-cm long segments of colon at the pelvic flexure (Graham et al. 2011). To simulate intestinal manipulation, a 1-meter segment of jejunum in the most proximal portion was ligated at each end with umbilical tape. An approximately 30 cm by 7 cm fluid column was massaged proximally and distally between the ligatures for a total of 10 strokes.
Horses were humanely euthanized at the end of anesthesia with a 100 ml overdose of pentobarbital sodium with phenytoin (Beuthanasia solution). Immediately after euthanasia, the left kidney was digitally separated from its perirenal attachments and was drawn ventrally to expose its vascular attachments and ureter for transection. The edge of the caudate lobe of the liver or of the right liver lobe was digitally separated from the remainder of the liver to obtain as large a segment as possible (>6 x 10 cm). An incision was made in the diaphragm close to the rostral end of the abdominal incision and a segment of the ventral edge of a lung lobe was digitally separated from the remainder and incised with scissors to yield as large a segment as possible (>6 x 10 cm). Sections of the lung, kidney and liver of each horse were sharply excised from the removed portions, remote to the transected edges, and were fixed in 10% neutral buffered formalin, embedded in Tissue Prep® paraffin. They were then cut into 4 to 5 µm sections and mounted on silane-coated glass slides. After deparaffinizing 3 times with xylene for 5 minutes and rehydration 3 times with 100% ethanol, twice each with 95% and 70% ethanol, and once with deionised water for 5 minutes, slides were stained with H&E in a routine manner to assess the adhesion and accumulation of neutrophil granulocytes and fibrin deposits. Paraffin-embedded sections were also stained for immunohistochemistry. The kidney specimens included both cortex and medulla, while the pulmonary and the hepatic samples were taken randomly. Cytosolic COX-1/2, and HIF1-α within submucosal venules and infiltration into the colonic mucosa were analyzed under light microscopy following immunohistochemistry staining.
Immunohistochemistry

Calprotectin

Immunolabeling was performed with a 1:100 dilution of mouse monoclonal anti-human macrophages (MAC387; Serotec, Raleigh, NC, USA) which is known to have cross reactivity with equine calprotectin, and a commercially available biotin-free detection kit (Histar Detection System; Serotec, Raleigh, NC, USA), using a modified staining procedure for equine jejunum (Little et al, 2005).

Antigen retrieval was performed by placing the tissue slides in a sodium citrate buffer solution (0.1M; Electron Microscopy Sciences) in a 50 mL centrifuge tube, and subsequently immersing the tubes into a water bath for 20 minutes. Specimens were allowed to cool at room temperature for 20 minutes and then washed in PBS for 5 minutes. Tissues were incubated in 0.03% hydrogen peroxide for 15 minutes to quench endogenous peroxidase, and after rinsing 3 times with PBS for 5 minutes, slides were incubated with 5% bovine serum albumin for an additional 15 minutes to block non-specific binding. Specimens were then incubated with the primary antibody (mouse anti-human MAC 387) 1:100 diluted in PBS for 45 minutes at room temperature in a humidified chamber. After blocking the antigen-antibody reaction with Special Block Solution (Serotec, Raleigh, NC, USA) for 20 minutes at room temperature followed by rinsing 2 times with PBS for 5 minutes, slides were incubated with a biotin-free secondary antibody, which was polymerized directly with horseradish peroxidase, for 30 minutes. Slides were washed 2 times in PBS for 5 minutes, then stain intensity was developed with 2.5% DAB as peroxidase substrate for 3 to 5 minutes under microscopic control. Tissues were rinsed well and incubated with distilled water for 1 minute. Slides were then counterstained with a 1:1 dilution of water and hematoxylin VS (Vector
Laboratories, Burlingame, CA, USA) for 45 seconds and rinsed in tap water 2 times for
2 minutes to develop color. After dehydration with 70%, twice with 95%, and 3 times
with 100% ethanol and xylene for 3 minutes, respectively, sections were mounted in
Permount™ Mounting Medium (Fisher Scientific, Pittsburgh, PA, USA) and covered with
glass cover slips.

**Cyclooxygenase-1 and 2**

Immunolabeling was performed with a 1:100 dilution of goat anti-human
polyclonal antibody (Santa Cruz Biotech., Santa Cruz, CA, USA), and a commercially
available streptABC kit (Santa Cruz Biotech., Santa Cruz, CA, USA). Cytosolic COX-1
and 2 were stained according to a protocol by Morton et al. (2009) with deparaffinization
and rehydration techniques as stated above.

**Hypoxia-inducing factor 1-alpha**

After sections were deparaffinized and rehydrated as described above, cytosolic
HIF1-α was detected with a 1:100 dilution of polyclonal goat anti-human antibody (Santa
Cruz Biotech., Santa Cruz, CA, USA), and a commercially available ABC kit (Santa
Cruz Biotech., Santa Cruz, CA, USA).

**Fibrin**

Tissues were deparaffinized and rehydrated, then immunolabelled with a 1:200
dilution of polyclonal rabbit anti-human fibrinogen/fibrin antibody (Dakocytomation,
Glostrup, Denmark) with a streptavidin biotin complex method (streptABC) (Santa Cruz
Biotech., Santa Cruz, CA, USA). Staining was completed using a protocol adapted from
Cotovio et al. (2007).
Lidocaine Concentration

Serum concentrations (μg/mL) of lidocaine and two lidocaine metabolites, monoethylglycinexylidide (MEGX) and glycinexylidide (GX), were determined by high-performance liquid chromatography (HPLC) with ultraviolet absorbance detection. Duplicate 1mL samples of serum from untreated or lidocaine-treated horses were extracted following alkalinisation with 1N NaOH (100μL) by mixing with dichloromethane (4mL) and low-speed centrifugation for 20 min. The organic layer was removed and the extraction process was repeated three times before all portions of the dichloromethane extracts were combined and dried under a nitrogen stream at 60 ºC. Control serum samples were spiked with standard solutions of lidocaine, MEGX and GX prior to extraction in order to determine recoveries. All dried extracts were reconstituted in mobile phase (30mM phosphate buffer, pH 3.7 and acetonitrile in a 92:8 volumetric mixture) and filtered through 0.45micron syringe filters prior to analysis. Analyte separation and quantification was performed on an HPLC system using a C-18 analytical column (150 x 4.6mm, 5 micron particle size) with a mobile phase flow rate of 1 mL/min and UV detector wavelength of 205nm. Peak areas for lidocaine, MEGX and GX in samples from lidocaine-treated horses were compared with standard curves that were constructed for each analyte (0.05 to 5 μg/mL) by fortifying equine serum with known amounts of reference standards. Each standard curve had a coefficient of determination of 0.99 or above and extracted spiked samples were within ±15% of predicted concentrations across the standard curve range.

Histological Evaluation

Samples were evaluated under light microscopy after completion of IHC and hematoxylin and eosin staining. Sections stained with H & E were examined to assess
any tissue injury, to identify any specific anatomical structures associated with the
stained cells, to determine the cell type that correlated with the immunostained, to
determine if immunostained areas correlated with the appropriate cell type in the tissue,
and if the fibrin deposition were premortem or postmortem. For this distinction, fibrin
unattached in the lumen of vessels was considered a postmortem event whereas fibrin
intimately attached or occluding the lumen was considered an antemortem finding.

Positive staining cells were quantified using Nikon Instruments Series (NIS)
Objects were counted by pixel color where positive cells were stained various shades of
brown and negative cells were stained with hematoxylin. Sixteen photos were captured
at a 20x objective in a systematic pattern for each slide of tissue.

![Figure 2-1. Schematic of fields selected for image capture on tissue histology slides. Captured photos highlighted in blue.](image)

Pixel color is easily customizable as positive cells may stain slightly differently on
each tissue. Colors were selected for the stained cells versus the nonstained cells and
background to provide the sharpest contrast between the two by colors selected. This
allowed the program to measure the stained section as a fraction of the nonstained area and thereby obtain an objective assessment of the proportion of the section examined that was occupied by stained cells.

**Statistical Analysis**

All samples were analysed using statistical software (SPSS Statistics 24®, SPSS Inc., Chicago, IL, USA). Data were expressed as means ± SD and values of P < 0.05 were considered significant. Kruskal-Wallis test was used to compare binary fraction area of positive-staining cells in different tissues (kidney, liver, lung) with different treatments (calprotectin, COX-1, COX-2, HIF1-α, fibrin).
CHAPTER 3
RESULTS

Serum Lidocaine Concentrations

Serum lidocaine concentrations of all horses in this study were within the reported target steady state of 1-2 µg/ml (Malone et al. 1999) within 15 minutes after start of administration and remained within that range throughout anesthesia. The active metabolites MEGX and GX continued to accumulate throughout the experimental period.

![Graph](image)

Figure 3-1. Serum lidocaine concentrations of all horses in this study were within the reported target steady state of 1-2 µg/ml within 15 minutes after start of administration and remained within that range throughout anesthesia. Active metabolites monoethylglycinexylidide and glycinexylidide continued to accumulate throughout the experimental period.

Colon and Jejunal Ischemia and Jejunal Manipulation

Ischemia caused edema, purple discoloration of the serosa, and serosal petechiation that resolved by 10 to 15 minutes after the clamps and ligatures were
removed. Histological evidence of mucosal injury, inflammation, and repair and functional effects on barrier integrity (transepithelial resistance and mannitol fluxes in vitro) in the intestinal segments were reported separately (Bauck et al. 2017).

**Tissue Staining**

**Kidney**

Calprotectin-stained cells were scattered throughout the glomerulus, while COX-1 staining cells were only in the collecting ducts. COX-2 positively stained cells were dispersed in the glomerulus with clusters of positive cells in the macula densa. HIF1-α staining showed positive cells in the distal convoluted tubules, with a higher prevalence in the cortical labyrinth near the distal cortex. Positively staining cells were also found clustered near the medullary ray closer to the medulla. In the medulla, positive cells were present in collecting ducts, distal tubules, and near small vessels. Fibrin-stained positive cells were displayed in the upper medulla near transition from the cortex.

**Liver**

Calprotectin stained cells were evenly dispersed throughout the tissue, with slightly heavier concentrations in and around vessels. COX-1 staining cells were evenly scattered throughout the tissue with slight increases around vessels. In COX-2 staining, positive cells were found mainly near vessels. HIF1-α staining was evident in cells around vessels. Cells positively stained for fibrin were evident throughout the tissue in the lumen of small vessels predominantly and adhered to the endothelium.

**Lung**

Calprotectin stained cells were near smaller vessels and bronchioles. In COX-1 staining, positively stained cells were distributed evenly throughout the tissue with higher concentrations around capillaries. COX-2 stained cells were mostly near vessels.
in loose connective tissue (LCT). HIF1-α positive cells were primarily in venules and capillaries. Fibrin stained cells were in the arteries and arterioles, and in venules and veins near the bronchi and bronchioles; however, the capillaries were virtually free of fibrin.
Figure 3-2. Remote organ tissue in response to Calprotectin (20x objective) A-C: Control tissue in the kidney, liver, and lung, respectively, having been treated with neither lidocaine, saline, or anesthesia. D-F: Experimental IR tissue in the kidney, liver, and lung, respectively, having been treated with lidocaine under anesthesia. Control tissues were from normal horses that were not anesthetized and did not receive lidocaine and were harvested immediately after euthanasia. Experimental refers to those tissues taken immediately after euthanasia that followed ischemia in the small intestine (0.5 hours) and large colon (1 hour) and 4 hours of reperfusion in anesthetized horses in dorsal recumbency. Experimental horses were treated with saline or lidocaine but not distinguished as such because there was no difference between these treatments. Positively stained cells (discrete brown dots) are more abundant in the experimental tissues than in control tissues.
COX-1

Control

![Control tissue in the kidney, liver, and lung, respectively, having been treated with neither lidocaine, saline, or anesthesia.](image)

Experimental

![Experimental IR tissue in the kidney, liver, and lung, respectively, having been treated with lidocaine under anesthesia.](image)

Figure 3-3. Remote organ tissue in response to COX-1 (20x objective). A-C: Control tissue in the kidney, liver, and lung, respectively, having been treated with neither lidocaine, saline, or anesthesia. D-F: Experimental IR tissue in the kidney, liver, and lung, respectively, having been treated with lidocaine under anesthesia. Control tissues were from normal horses that were not anesthetized and did not receive lidocaine and were harvested immediately after euthanasia. Experimental refers to those tissues taken immediately after euthanasia that followed ischemia in the small intestine (0.5 hours) and large colon (1 hour) and 4 hours of reperfusion in anesthetized horses in dorsal recumbency. Experimental horses were treated with saline or lidocaine but not distinguished as such because there was no difference between these treatments. Positively stained cells (discrete brown dots) are more abundant in the experimental tissues than in control tissues.
COX-2

Control

Experimental

Figure 3-4. Remote organ tissue in response to COX-2 (20x objective). A-C: Control tissue in the kidney, liver, and lung, respectively, having been treated with neither lidocaine, saline, or anesthesia. D-F: Experimental IR tissue in the kidney, liver, and lung, respectively, having been treated with lidocaine under anesthesia. Control tissues were from normal horses that were not anesthetized and did not receive lidocaine and were harvested immediately after euthanasia. Experimental refers to those tissues taken immediately after euthanasia that followed ischemia in the small intestine (0.5 hours) and large colon (1 hour) and 4 hours of reperfusion in anesthetized horses in dorsal recumbency. Experimental horses were treated with saline or lidocaine but not distinguished as such because there was no difference between these treatments. Positively stained cells (discrete brown dots) are more abundant in the experimental tissues than in control tissues.
HIF1-α

Control

Experimental

Figure 3-5. Remote organ tissue in response to HIF1-α (20x objective). A-C: Control tissue in the kidney, liver, and lung, respectively, having been treated with neither lidocaine, saline, or anesthesia. D-F: Experimental IR tissue in the kidney, liver, and lung, respectively, having been treated with lidocaine under anesthesia. Control tissues were from normal horses that were not anesthetized and did not receive lidocaine and were harvested immediately after euthanasia. Experimental refers to those tissues taken immediately after euthanasia that followed ischemia in the small intestine (0.5 hours) and large colon (1 hour) and 4 hours of reperfusion in anesthetized horses in dorsal recumbency. Experimental horses were treated with saline or lidocaine but not distinguished as such because there was no difference between these treatments. Positively stained cells (discrete brown dots) are more abundant in the experimental tissues than in control tissues.
Fibrin

Control

![Control tissue in the kidney, liver, and lung, respectively, having been treated with neither lidocaine, saline, nor anesthesia.](image)

Experimental

![Experimental IR tissue in the kidney, liver, and lung, respectively, having been treated with lidocaine under anesthesia.](image)

Control tissues were from normal horses that were neither anesthetized nor received lidocaine and were harvested immediately after euthanasia. Experimental tissues were taken immediately after euthanasia that followed ischemia in the small intestine (0.5 hours) and large colon (1 hour) and 4 hours of reperfusion in anesthetized horses in dorsal recumbency. Experimental horses were treated with saline or lidocaine but not distinguished as such because there was no difference between these treatments. Positively stained cells (discrete brown dots) are more abundant in the experimental tissues than in control tissues.

Figure 3-6. Remote organ tissue in response to fibrin (20x objective). A-C: Control tissue in the kidney, liver, and lung, respectively, having been treated with neither lidocaine, saline, nor anesthesia. D-F: Experimental IR tissue in the kidney, liver, and lung, respectively, having been treated with lidocaine under anesthesia. Control tissues were from normal horses that were not anesthetized and did not receive lidocaine and were harvested immediately after euthanasia. Experimental refers to those tissues taken immediately after euthanasia that followed ischemia in the small intestine (0.5 hours) and large colon (1 hour) and 4 hours of reperfusion in anesthetized horses in dorsal recumbency. Experimental horses were treated with saline or lidocaine but not distinguished as such because there was no difference between these treatments. Positively stained cells (discrete brown dots) are more abundant in the experimental tissues than in control tissues.
Figure 3-7. Image of a section of liver to show a cluster of cells stained positively for calprotectin with some scattered positive cells throughout the section.
Figure 3-8. Same image but with the color change produced by the Nikon Instruments Series (NIS) Advanced Research software programming (Nikon Instruments Inc., Melville, NY). The calprotectin-positive cells stained cells are now transformed to a maroon color and the nonstained cells and background are green, to produce a sharp contrast between the cells that the system can recognize and generate a binary fraction measurement.
Table 3-1. Median of binary area fraction in positively staining kidney cells. Controls different from lidocaine and saline groups, P<0.05.

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<tr>
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<th>Saline (n=7)</th>
<th>Kruskal-Wallis (Lido vs. Saline)</th>
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<td>0.060</td>
<td>0.075</td>
<td>P&lt;0.474</td>
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<tr>
<td>COX-1</td>
<td>0.168</td>
<td>0.114</td>
<td>P&lt;0.568</td>
</tr>
<tr>
<td>COX-2</td>
<td>0.053</td>
<td>0.041</td>
<td>P&lt;0.886</td>
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<tr>
<td>HIF1-α</td>
<td>0.104</td>
<td>0.092</td>
<td>P&lt;0.775</td>
</tr>
<tr>
<td>Fibrin</td>
<td>0.184</td>
<td>0.429</td>
<td>P&lt;0.116</td>
</tr>
</tbody>
</table>

Table 3-2. Median of binary area fraction in positively staining liver cells. Controls different from lidocaine and saline groups, P<0.05.

<table>
<thead>
<tr>
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<td>P&lt;0.562</td>
</tr>
<tr>
<td>HIF1-α</td>
<td>0.234</td>
<td>0.328</td>
<td>P&lt;0.482</td>
</tr>
<tr>
<td>Fibrin</td>
<td>0.094</td>
<td>0.301</td>
<td>P&lt;0.085</td>
</tr>
</tbody>
</table>
Table 3-3. Median binary area fraction of positively staining lung cells. Controls different from lidocaine and saline groups, P<0.05.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Lidocaine (n=7)</th>
<th>Saline (n=7)</th>
<th>Kruskal-Wallis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calprotectin</td>
<td>.081</td>
<td>.101</td>
<td>P&lt;0.834</td>
</tr>
<tr>
<td>COX-1</td>
<td>.176</td>
<td>.163</td>
<td>P&lt;0.866</td>
</tr>
<tr>
<td>COX-2</td>
<td>.088</td>
<td>.113</td>
<td>P&lt;0.527</td>
</tr>
<tr>
<td>HIF1-α</td>
<td>.207</td>
<td>.328</td>
<td>P&lt;0.461</td>
</tr>
<tr>
<td>Fibrin</td>
<td>.178</td>
<td>.487</td>
<td>P&lt;0.075</td>
</tr>
</tbody>
</table>

There was no significant difference between lidocaine-treated and saline groups in tissue production of calprotectin, COX-1, COX-2, and HIF1-α in liver, lung and kidney (Tables 3-1 to 3-3). There was a trend to lidocaine reducing the fibrin deposition in all organs examined but this was not significant. All organs from anesthetized horses were significantly different from control horses.
CHAPTER 4
DISCUSSION

This study was designed to investigate a possible relationship between IR injury in the equine colon and jejunum with injury in remote organs of the body (kidney, liver, lung). Many studies have explored the effects of IR injury on the intestine itself, but few have investigated the effects of IR injury on a systemic level. Our hypothesis stated that a catastrophic injury in the gastrointestinal tract should have deleterious effects on the entire body due to the involved and complex nature of the inflammatory response. Our results demonstrated significant differences between control groups (no ischemia, no anesthesia and no lidocaine) and the anesthetized groups (jejunal and colonic ischemia, general anesthesia) treated with lidocaine or saline. These findings suggest that a substantial IR injury in the gastrointestinal tract can affect remote organs of the body, which could explain a multitude of postoperative complications such as endotoxemia, bacterial translocation, SIRS, or MODS. Any of these could cause patients to succumb to secondary disease after a successful surgery for an ischemic lesion.

This study found no significant difference between lidocaine-treated and saline groups in tissue production of calprotectin, COX-1, COX-2, and HIF1-α in liver, lung and kidney. This is similar to our reported findings with lidocaine in the jejunum and colon of horses at sites remote to those subjected to ischemia, reperfusion and manipulation (Bauck et al. 2017). However, it did demonstrate a trend to ameliorating remote organ production of fibrin, but this was not statistically significant in any tissue. The cause of this is unknown, but could be caused by an interaction between the clotting cascade and the innate immune system. Platelet activating factor and thromboxane A₂ are responsible for activating platelets which release granules containing G-protein coupled
receptor-activating factors. These factors increase calcium in the cytosol of platelets, which activate protein kinase C, in turn, activating phospholipase A2. This modifies integrin membrane glycoprotein IIb/IIIa, thus increasing its affinity to bind to fibrinogen.

Based on findings of this study, a drug other than lidocaine should be sought to reduce the effects of IR because lidocaine seems to have a limited effect on the inflammatory response. This is in agreement with a study that demonstrated failure of lidocaine to ameliorate neutrophilic inflammation in horses with recurrent airway obstruction, as demonstrated through bronchoalveolar lavage cytology (Wilson et al. 2012). The effects of intestinal IR on hoof lamina should be investigated in the future in a study such as ours because laminitis is a rare but severe complication of colic. However, lidocaine has not demonstrate an antiinflammatory effect in either the laminae or skin in horses with laminitis induced using black walnut extract (Williams et al. 2010).

Contrary to our results, another study on remote lung injury after experimentally induced intestinal IR (70 minutes ischemia and 60 minutes reperfusion) in anesthetized horses did demonstrate a benefit with lidocaine infusion (Montgomery et al. 2014). Neutrophil numbers, but not MPO concentrations, were significantly decreased and macrophage numbers were higher in the IR group that received lidocaine than in horses that received lactated Ringer's solution (LRS) as a control infusion (Montgomery et al. 2014). The authors concluded that intestinal IR causes remote lung injury with recruitment of inflammatory cells and expression of inflammatory molecules in horses, and lidocaine could ameliorate the lung inflammation following intestinal IR (Montgomery et al. 2014). However, they concede that their results did not provide conclusive evidence of the role of lidocaine in IR-associated lung inflammation.
(Montgomery et al. 2014). The MPO and TNFα concentrations, which were used as surrogate markers for tissue neutrophils, were not different between the LRS and lidocaine-treated IR horses (Montgomery et al. 2014). This is in contrast to the findings that endotoxemia–induced changes in clinical scores and in plasma and peritoneal fluid TNFα were reduced by lidocaine infusion (Peiro et al. 2010).

In the study by Montgomery et al. on remote lung inflammation, an anesthetized control group was also examined and these horses had similar findings as in non-anesthetized controls, and did not develop the inflammation seen after IR (Montgomery et al. 2014). However, our study lacked an anesthetized control group, which should be used to rule out a possible increase in HIF1-α from isoflurane anesthesia-induced hypoperfusion in abdominal organs (Manohar et al. 1987). This was one of the more critical findings of our study, evidence that all remote tissues suffered from some degree of hypoxia, which was not measured by Montgomery et al (2014).

A limitation of our study is the types of control horses used. These were healthy horses with no evidence of any systemic disease and were mostly donated because of musculoskeletal problems that interfered with their athletic performance. The present study controlled for many variables to make groups as comparable as possible, although it was impossible to obtain control tissues at the start of the study, before ischemia. The limited access to the organs in the live horse would have required a very invasive approach and caused traumatic injury and possibly death shortly after tissue removal. Consequently, control tissue was collected post-mortem from euthanized horses that were free from gastrointestinal disease. Although these horses were not anesthetized, they were euthanized with an overdose of barbiturate anesthetic. They
differed from the ischemia and reperfusion horses by not undergoing long anesthesia in dorsal recumbency and the positioning alone could have altered blood flow to many organs, but especially the lungs. In our horses with induced intestinal ischemia, blood flow to many intra-abdominal organs, including the liver and kidneys would be reduced under the influence of isoflurane anesthesia. This was clearly demonstrated in a study that used different concentrations of isoflurane in isocapnic ponies (Manohar et al. 1987). The results of our measurements with H1F-α demonstrate that all 3 tissues that we studied suffered from a hypoxic event. This alone could have caused sufficient tissue damage to induce a robust inflammatory response at the end of the 4 hour reperfusion period. The method for assessing the role of anesthesia in this development would be to examine a similar group of horses in which only anesthesia was performed and a sham surgery was also performed but without inducing any intestinal damage through either manipulation or ischemia. The lungs are also the organs that receive and absorb the inhalant anesthetic (isoflurane) constantly during anesthesia. We used donated horses that were probably free of even a low-grade systemic illness or other ailments that may invoke an inflammatory response. Every effort was made to use healthy horses donated only for musculoskeletal injury (lameness other than laminitis). Tissues were harvested shortly after euthanasia because delays could increase HIF1α nuclear immunoreactivity, as evident in a study on slaughterhouse tissue, probably because of the delay between slaughter and sampling (De Ceulaer et al. 2011).

The NIS Elements Advanced Research system was used to quantify positive staining as a simpler and more objective method than the 0-3 subjective scale previously used in our laboratory (Grosche et al. 2008). In previous studies from our
laboratory, calprotectin-stained cells were counted (Grosche et al. 2008). In that study, the number of calprotectin-positive cells within submucosal venules and within the colonic mucosa correlated significantly with the accumulation of neutrophils within the corresponding tissue segments. Within the submucosal venules, both calprotectin-positive cells and H & E-stained neutrophils increased with duration of ischemia and peaked after 30 min of reperfusion. In the scoring system described previously, evaluation of images obtained by light microscopy was performed using Image-Pro Express 5.01. Three randomly defined areas from each tissue with a length of 866 μm (equal to the length of one image using the 10x objective) were examined. Colonic mucosa was scored from 0 to 3 for presence of neutrophils using a 40x objective. The mean number of calprotectin-positive cells within 45 randomly identified circular- or oval-shaped submucosal venules were counted and computed as described for H&E.

The NIS methodology used for cell counting is a newer system than those described above for quantifying tissue neutrophils (Grosche et al. 2008) and may introduce an unknown error. The success of the method is dependent on accurate identification of the color signal from stained cells. Since we quantified cells by pixel color, it is unknown if varying shades of brown cells are positive neutrophils or monocytes. However, counting methods are labor-intensive and are not error-free, and also have an element of subjectivity. The scoring system for neutrophils is also prone to error from subjective assessment. Ideally, two methods should be used as internal checks for the accuracy of each one. We did not check the NIS method against counting stained cells or against the scoring system, although this might be warranted as a means of internal validation.
In this study, we were able to demonstrate remote organ effects that could be analogous to MODS in human patients after they have developed SIRS. In a rat model of 1 hour complete ischemia and 4 hours of reperfusion, neutrophils and their products mediated most of the associated injury in lung and liver during reperfusion, but did not contribute to intestinal injury, despite a marked increase in intestinal neutrophils (Simpson et al. 1993). In horses that were subjected to distension of the small colon by a latex balloon surgically implanted in the lumen and inflated to a pressure of 40 mm Hg for 4 hours, similar observations were made (Faleiros et al. 2008). The mucosa was not affected by luminal distension, but neutrophil accumulation and edema were observed in the distension group, as well as hemorrhage, fibrin deposition, and increased MPO activity in the seromuscular layer. Similarly, there was greater accumulation of neutrophils in the lung samples from the distension group than in those from a sham-operated group, based on light microscopy and MPO assay (Faleiros et al. 2008).

In a study on small intestinal IR alone, without any manipulation of the colon, an eosinophilic and neutrophilic inflammatory response was observed in the colon after small intestinal reperfusion (Hopster-Iversen et al. 2011). These results support our finding of a remote response, possibly secondary to SIRS. However, by contrast, 1 hour of induced large colon torsion in ponies followed by 3 hours of reperfusion caused extravascular neutrophil accumulation in all sections of colon and cecum, but not in liver and lung (Wilson et al. 1994). These differences could be attributed to differences in measuring inflammation and flaws in measuring only tissue neutrophils.

The intestinal lesions that were inflicted in the anesthetized horses in this study were generally considered of short duration and mild compared with other experimental
lesions (Cook et al. 2013) and clinical cases (Meschter et al. 1986). In most clinic cases, longer segments of both small and large intestine would be involved instead of the short segments (<45 cm long) used in the current study. In addition, the duration of strangulation could be longer than in this study, because many cases may not become clinically evident until the disease is at an advanced stage in its development. Large colon volvulus has features typical of a hemorrhagic strangulation obstruction, which is characterized by luminal occlusion and compromised venous drainage (Snyder et al. 1989a). Continuous flow of arterial blood into the tissue causes low-flow ischemia, which causes severe interstitial hemorrhage and edema accompanied by compartmentalization of large volumes of fluids and gradual disruption of mucosal tissue architecture (Gibson and Steel 1999, Blikslager 2009). Occasionally, large colon volvulus will cause ischemic strangulation from simultaneous venous and arterial occlusion, causing a sudden loss of oxygen supply to the organ and rapid degeneration of the mucosa and its epithelium (Meschter et al 1986, Gibson and Steel 1999, Moore et al 1995, Lopes 2009, Sheats et al 2009). However, the model we used was effective in creating a distinct and consistent mucosal defect and loss of mucosal lining in both the small and large intestines (Bauck et al. 2017). During the reperfusion period in both segments, mucosal repair is initiated shortly after reperfusion starts and reaches a reasonably advanced stage at the end of 4 hour reperfusion period. This is consistent with results of other studies with similar durations of ischemia (Graham et al. 2011, Freeman et al. 1989). However, during that time and subsequently, the intestinal mucosa will be damaged, with loss of superficial epithelial cells and a breach in the mucosal lining that would allow bacterial toxins, endotoxin, and bacteria can be
translocated and enter the peritoneal cavity or the systemic circulation. During the reperfusion period, these intestinal products will be transferred through the portal vein to the liver, where they could be detoxified to some extent by the Kupffer cells that line the sinusoids in the liver. These are specialized macrophages that form part of the mononuclear phagocyte system.

The results of our study clearly demonstrated that even a mild injury causes an inflammatory response in remote organs. However, the severity of that response and its clinical relevance in clinical cases are unknown. Although strangulating diseases of the small and large intestines are common in horses, failure of the organs we examined in this study, such as the kidney, lungs, and liver, are rare after ischemia-reperfusion injury more severe than we created in our research subjects. This finding raises concerns about the impact of this study. However, the results confirm that MODS could develop in horses and could play a role in those that do succumb to the most severe forms of intestinal strangulation, of long duration and with sufficient lengths of intestine, to cause SIRS. Clinical cases of equine small intestinal inflammation and cell injury caused by proximal enteritis can have elevated hepatic enzymes, either secondary to SIRS associated with this disease or bile duct obstruction secondary to small intestinal distention and stasis (Davis et al. 2003).

The liver plays an important role in the acute-phase response, a well-documented nonspecific phenomenon of rapid onset incited by different stimuli related to infection, severe trauma, neoplasia, inflammation, or stress (Belgrave et al. 2013). The liver also responds to these and to gastrointestinal tract injury through the production of inflammatory markers or acute phase proteins, such as haptoglobin, C-
reactive protein, ceruloplasmin, fibrinogen and serum amyloid A (Belgrave et al. 2013). This provides evidence that the liver receives some signal about injury at a site remote to it, such as the intestine in our hypothesis. Horses with colic can also develop cardiac muscle injury, as evident by elevated blood troponin concentrations (Diaz et al. 2014), further evidence that horses can develop some degree of MODS. Cardiac troponin I is a sensitive and specific marker for myocardial injury in human beings and dogs (Díaz et al. 2014). Therefore, postoperative assessment and treatment of horses that undergo surgery for intestinal strangulation should also recognize the importance of systemic illness as well the intestinal problems. We did not examine troponin production in our study, but we did include lung, which was examined in previous studies similar to ours (Faleiros et al. 2008, Montgomery et al. 2014), because it would provide a suitable indicator to compare methods and findings under similar conditions.

Renal disease is recognized as a possible complication of severe colic in horses (Cook and Blikslager 2015). It is usually attributed to hypovolemic shock and decreased renal perfusion. It can be exacerbated by the administration of nonsteroidal anti-inflammatory drugs (NSAIDs), such as flunixin meglumine (FM), especially when administered to the hypovolemic horse (Cook and Blikslager 2015). The mechanism of action of these drugs is probably different to the inflammatory response described in this study. However, cyclooxygenase plays an important role in renal function in horses and could contribute to some of the problems that are noted during fluid therapy in these cases. Sodium retention, for example, could be enhanced by elevated levels of renal COX-1 and COX-2 (Cook and Blikslager 2015). Azotemia, which is manifested by an elevated serum creatinine, is a well-recognized but uncommon complication in horses.
before colic surgery and during the postoperative period. In most cases, a favorable response is achieved by aggressive intravenous fluid therapy, because this is largely a prerenal azotemia, typically caused by hypovolemia and reduced renal blood perfusion.

In this study, both COX enzymes were increased in kidneys, which should increase renal prostaglandin production and thereby affect renal function. PGE$_2$ acts on the thick ascending loop of Henle to decrease sodium and water reabsorption (Cook and Blikslager 2015). This should increase urinary excretion of sodium and water, which are already increased after colic surgery by infusion of high volumes of sodium-rich replacement solutions. Crystalloid fluids that are administered long-term to horses are replacement solutions that have a relatively high concentration of sodium compared to maintenance fluids. Such infusions increase sodium intake in excess of daily needs and induce diuresis that causes a loss of other critical plasma electrolytes. However, most horses after colic surgery are treated with FM, and inhibition of PGE$_2$ production by this NSAID should increase the retention of sodium in the body. The significance of these offsetting effects, crystalloid-induced sodium wasting and FM-induced sodium retention, is unknown. Therefore, horses receiving NSAIDs and large amounts of crystalloids could be at risk of clinically significant sodium retention and edema. Increased COX activity in the kidney might offset the NSAID-induced effect and decrease sodium reabsorption.

In this study, we found that intestinal ischemia and reperfusion increased COX-1 and COX-2 in all the organs examined. Elevation of COX-2 is not surprising because this is an inducible enzyme, known to increase in tissues and in blood during an inflammatory process. However, COX-1 is considered constitutive and its increase was
not expected in response to our ischemia and reperfusion injury. The most likely explanation is that COX-1 is also inducible in these tissues, as was demonstrated in study on FM and firocoxib effects on equine ischemic jejunum (Cook et al. 2009). Our study also raised the possibility, because of the high levels of COX-2 in tissues from control horses, that COX-2 could also be constitutive in some equine tissues. This was also demonstrated in the study on FM and firocoxib effects on equine ischemic jejunum (Cook et al. 2009). This is highly relevant because of evidence that COX-2 is actually required in some tissues and especially for healing of colonic anastomosis in human patients and laboratory animal models (Reisinger et al. 2017). Therefore, it can be concluded that the assignment of COX-1 and COX-2 to the constitutive and inducible role may not be entirely accurate. Also, COX-2–preferential agents would be likely to have adverse renal effects similar to those for nonselective COX inhibitors in volume-depleted horses because both enzymes seem to play similar roles in this tissue (Raidal et al. 2014)

Endotoxin is typically considered to be responsible for release of mediators that initiate inflammatory responses in remote organs, and it does so by stimulating an intense response from tissue macrophages that release pro-inflammatory mediators. These amplify the cellular response. However, other bacterial-derived toxins and bacteria themselves could also play a role. In response to the inflammation in the primary organ, TNF-α and IL-1β are inflammatory mediators produced from macrophages and other cells. These can recruit more macrophages and neutrophils, in addition to promoting their survival. IL-1β is also able to upregulate the production of acute phase proteins, such as CRP and complement, and can increase the expression
of other cytokines, chemokines and adhesion molecules (Dinarello 2009). Cytokines upregulated by TNF-α and IL-1β include IL-6 and IL-8.

Bacterial translocation is the movement of viable bacteria across the intestinal barrier to extraintestinal sites, such as the portal blood stream, mesenteric lymph nodes, and peritoneal fluid. Gastrointestinal organisms are abundant, especially in the large intestine of the horse, and ischemic lesions in the mucosa caused by strangulation or infarction are the most likely mechanisms that allow dissemination to extraintestinal sites. Mucosal lesions in jejunum and small colon did not allow bacteria to leak from the lumen to the peritoneal fluid or into the intestinal wall, at least based on culture results (Ruggles et al. 1993). In one study, bacteria were isolated in similar numbers from the mesenteric venous blood and from the mesenteric lymph tissue in horses with small intestinal strangulation as in a control group with normal intestinal tracts (Hurcombe et al. 2012). There was no association between lesion duration, type, or location, or antimicrobial administration and the chance of a horse with small intestinal strangulation having a positive culture (Hurcombe et al. 2012). *Escherichia coli* was the predominant organism detected in horses with small intestinal strangulation (Hurcombe et al. 2012). The low prevalence of presumptive bacterial translocation in horses with small intestinal ischemia in this study led to the conclusion that other factors could explain postoperative morbidity in some cases (Hurcombe et al. 2012). Although we did not explore the possibility that our horses had bacterial translocation across the intestinal wall, the available evidence would suggest this was unlikely (Ruggles et al. 1993; Hurcombe et al. 2012).
The cells that play a dominant role in inflammation in any organ appear to be the neutrophils. These are the predominant inflammatory cells in blood and are usually mobilized and migrate to the site of an inflammatory process. They also release mediators that increase the activity of other inflammatory cells that are resident in the tissue. The high numbers of neutrophils measured in this study in kidney, liver, and lung are consistent with an inflammatory response to our experimental conditions. Calprotectin was used to measure neutrophil influx in the tissues of interest because it has been shown to be a useful marker of intestinal inflammation in previous studies in our laboratory (Grosche et al. 2009). The major limitation of calprotectin is that it is also produced by monocytes and macrophages, but these cells are in low numbers in most tissues. We measured calprotectin to assess inflammation in the intestinal segments in the same horses used in this study and found that it was actually lower in intestinal layers from those horses treated with lidocaine (Bauck et al. 2017). This was unexpected because the neutrophil scores were similar in tissues from horses treated with lidocaine as in those treated with saline (Bauck et al. 2017). It is possible that lidocaine in some way reduces intracellular calprotectin so that the staining process is reduced in cells treated with this local anesthetic agent. This is consistent with evidence that lidocaine can stimulate activity of equine neutrophils in vitro in contrast to its effects on human neutrophils (Cook et al. 2008). If lidocaine were to activate neutrophils, tissue neutrophils would be expected to discharge the calprotectin into the interstitium, so that its value as a stain for neutrophils would be lost. In the present study, in the tissues examined, sufficient calprotectin was retained in the tissues to allow measurement of neutrophil influx. However, it is also possible that the lidocaine horses might have had
reduced intracellular calprotectin in their neutrophils, a possibility that could only be explored by scoring neutrophil infiltration in the same samples.

Unlike horses that developed strangulating lesions through natural causes, our horses were not treated specifically for any complications from the artificially induced lesions in their intestines. In most clinic cases, the effects of endotoxemia/SIRS are treated with aggressive intravenous fluid therapy and NSAIDs, such as FM. Our horses were not treated in this way before or during anesthesia although they did receive fluid therapy at rates sufficient to maintain blood pressure during anesthesia and preserve adequate tissue perfusion. Therefore, intravenous fluids were used at a standard rate to offset any cardiodepressant effects of the anesthetic agents and probably did not reach the rates of infusion required to treat endotoxemia/SIRS. Also, horses used in this study did not have a recent history of treatment with NSAIDs and did not receive these drugs during the ischemic period. In clinic cases, these drugs would be administered at regular dosing intervals as part of treatment for inflammation and pain. Therefore, the response to these drugs and the effects on inflammation in the tissues under study remain unknown. Other agents can also be used to treat endotoxemia, such as polymixin B, which binds endotoxin in plasma. Therefore, future studies with this antibiotic might be effective in determining the role of endotoxemia in inducing inflammation in lung, liver and kidney.

In conclusion, the findings of this study using experimentally induced ischemia and reperfusion in equine colon and jejunum demonstrate that intestinal injury causes inflammation in remote organs. Previous studies have shown that this ischemia model and intestinal manipulation also cause inflammation in the intestine remote to the
ischemic segments (Hopster-Iversen et al. 2011, Bauck et al. 2017). Therefore, several organs remote to the primary lesion probably become inflamed and possibly injured, which could constitute a true manifestation of MODS in horses. The changes inflicted by MODS could compound many of the systemic effects from intestinal ischemia and even cause death in horses with the severe ischemic damage seen in clinical cases. However, MODS is not clinically apparent in most clinical cases of intestinal strangulation obstruction in small or large intestine, despite a more severe lesion in many cases than the experimental model used in this study. Also, many horses can return to athletic performance at the same or higher level after surgery for these lesions as before surgery (Christophersen et al. 2011, Davis et al. 2013), evidence that residual organ damage that could impair recovery does not commonly develop. In those cases in which clinical signs of MODS are suspected, current treatment modalities aimed at reducing inflammation, improving tissue perfusion through fluid therapy, and reducing the effects of endotoxin should provide some opportunity for resolution. Resection of the ischemic intestine when irreversible changes have developed is also a critical step to minimizing the effects of SIRS and MODS.
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BIOGRAPHICAL SKETCH

Julia Daggett was born in Reston, Virginia, and raised in Fort Lauderdale, Florida. In 2010, she moved to Westerville, Ohio to attend Otterbein University, where she majored in Equine Pre-Veterinary Studies. Upon graduation in 2014, Julia returned to Fort Lauderdale, where she worked for a year as a veterinary assistant for the practice of Teigland, Franklin, and Brokken at both Gulfstream and Calder Racetracks. Her interest in research brought her to the University of Florida, where she began her Masters of Science in Veterinary Medicine in August 2015. Julia received her Master’s degree in August 2017 and began the Doctor of Veterinary Medicine program at University of Florida immediately thereafter.