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Large colon ischemia-reperfusion injury in the horse

Moore, Rustin MacArthur, Ph.D.

The Ohio State University, 1994
LARGE COLON ISCHEMIA-REPERFUSION INJURY IN THE HORSE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree of Philosophy in the Graduate School
of The Ohio State University

By

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*****

The Ohio State University

1994

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DEDICATION

To My Parents, Daniel and Roylene Moore
and My Great Aunt, Evelyn Zinn
ACKNOWLEDGEMENTS

I extend my gratitude to Dr. William Muir for his guidance and support while serving as my thesis adviser. I express my sincere thanks to Dr. Alicia Bertone for her technical assistance and for the countless hours of histopathologic evaluation. I thank Dr. Warren Beard and Dr. Paul Stromberg for their advice and encouragement. I extend thanks to Dr. D. Neil Granger and the Department of Physiology and Biophysics at Louisiana State University School of Medicine in Shreveport for their hospitality and unselfish assistance. The studies reported here could not have been completed without the splendid technical support provided by Angela Doerres-Phillips, Margaret Cawrse, and Linda Bednarski. I sincerely appreciate the technical assistance provided by Heather Burkhart, Sue Cross, Kristin Garabian, Lori Kipp, Andrea O’Connor, and Mary Wilkins. I appreciate the support of the faculty, residents, and technicians in the equine section during my concurrent clinical training. I acknowledge Dr. Jean Powers and Dr. Joanne Hardy for providing statistical analyses and advice. I commend John Swartz and Tim Vojt for their assistance with photomicrographs and computer illustrations and graphics respectively. I recognize the American Quarter Horse Association, The Ohio State University College of Veterinary Medicine Equine Research Group, and American College of Veterinary Surgeons for their financial support of these studies.

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<td>APTT</td>
<td>activated partial thromboplastin time</td>
</tr>
<tr>
<td>ATIII</td>
<td>antithrombin III</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BE</td>
<td>base excess</td>
</tr>
<tr>
<td>BL</td>
<td>baseline</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium</td>
</tr>
<tr>
<td>CₐO₂</td>
<td>arterial oxygen content</td>
</tr>
<tr>
<td>CₐvO₂</td>
<td>colonic venous oxygen content</td>
</tr>
<tr>
<td>CI</td>
<td>cardiac index</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>chloride</td>
</tr>
<tr>
<td>CₘvO₂</td>
<td>mixed venous oxygen content</td>
</tr>
<tr>
<td>CO</td>
<td>cardiac output</td>
</tr>
<tr>
<td>CV</td>
<td>colonic venous</td>
</tr>
<tr>
<td>DAP</td>
<td>diastolic arterial pressure</td>
</tr>
<tr>
<td>DIC</td>
<td>disseminated intravascular coagulation</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMTU</td>
<td>dimethylthiourea</td>
</tr>
<tr>
<td>DO₂ colon</td>
<td>colonic oxygen delivery</td>
</tr>
<tr>
<td>FDP</td>
<td>fibrin degradation product</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>ferric iron</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>ferrous iron</td>
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<tr>
<td>Hb</td>
<td>hemoglobin</td>
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<tr>
<td>HCO₃⁻</td>
<td>bicarbonate</td>
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<td>HETAB</td>
<td>hexadecyltrimethyl ammonium bromide</td>
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<td>HETE</td>
<td>hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>HETOH</td>
<td>hexadecyltrimethyl ammonium hydroxide</td>
</tr>
<tr>
<td>HOCl</td>
<td>hypochlorous acid</td>
</tr>
<tr>
<td>H₂O</td>
<td>water</td>
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<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HPETE</td>
<td>hydroperoxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>HR</td>
<td>heart rate</td>
</tr>
<tr>
<td>I:C</td>
<td>mucosal interstitial: crypt ratio</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin 1</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin 6</td>
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<tr>
<td>IL-8</td>
<td>interleukin 8</td>
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<tr>
<td>I-R</td>
<td>ischemia-reperfusion</td>
</tr>
<tr>
<td>LH</td>
<td>polyunsaturated fats</td>
</tr>
<tr>
<td>LOOH</td>
<td>lipid hydroperoxides</td>
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<tr>
<td>LTA₄</td>
<td>leukotriene A₄</td>
</tr>
<tr>
<td>LTB₄</td>
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<td>LTC₄</td>
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LTD₄ - leukotriene D₄
MAP - mean arterial pressure
mPAP - mean pulmonary artery pressure
MPO - myeloperoxidase
mRAP - mean right atrial pressure
MnCl₂ - manganese chloride
MV - mixed venous
NADPH - nicotinamide adenine dinucleotide phosphate
NO - nitric oxide
NSAID - nonsteroidal antiinflammatory drugs
OFRs - oxygen free radicals
OH· - hydroxyl radical
OH⁻ - hydroxide ion
OSPT - one stage prothrombin time
O₂ - oxygen
O₂⁻ - superoxide radical
PAF - platelet activating factor
PLA₂ - phospholipase A₂
PO₂ - partial pressure of oxygen
PCO₂ - partial pressure of carbon dioxide
PCV - packed cell volume
PGE₂ - prostaglandin E₂
PGF₂α - prostaglandin F₂α
PGG₂ - prostaglandin G₂
PGH₂ - prostaglandin H₂
PGI₂ - prostacyclin
Qₜ₁₀ - colonic blood flow
SAP - systolic arterial pressure
SEM - standard error of the mean
%SO₂ - percent hemoglobin saturation with oxygen
SOD - superoxide dismutase
SV - systemic venous
TP - total plasma protein
TXA₂ - thromboxane A₂
TXB₂ - thromboxane B₂
VO₂ₜ₁₀ - colonic oxygen consumption
XDH - xanthine dehydrogenase
XO - xanthine oxidase
6kPG - 6-keto prostaglandin F₁α
⁹⁹mTc - technetium 99
INTRODUCTION

Colic is the leading cause of death in horses. Large colon volvulus of $\geq 360^\circ$ is a relatively common cause of colic in horses and is universally fatal without surgical correction. Despite surgical correction and intensive medical care 60-80% of horses die subsequent to volvulus. The high mortality is associated with severe mucosal necrosis occurring during the volvulus and the ensuing mucosal injury sustained after surgical correction. Clinically, serosal color normalizes upon surgical correction of a large colon volvulus; these horses frequently, however, deteriorate post-operatively. This is believed to occur secondary to a phenomenon known as reperfusion injury.

Reperfusion injury is that component of tissue damage sustained upon restoration of blood flow after a period of ischemia. Reperfusion injury is believed to be principally initiated by the generation of toxic oxygen free radicals produced by the reintroduction of oxygenated blood during reperfusion. Oxygen free radicals cause lipid peroxidation leading to cell membrane damage and dysfunction. Oxygen free radical-induced tissue injury is also perpetuated by phospholipid-derived mediators and neutrophils.

Although gastrointestinal reperfusion injury has been repeatedly demonstrated in laboratory animals, only one study has reported the occurrence of reperfusion injury in the equine large colon upon re-establishing blood flow after a period of ischemia. Since most studies evaluating complete arteriovenous occlusion of the large colon have failed to demonstrate reperfusion injury and because gastrointestinal reperfusion injury has most consistently been demonstrated in laboratory animals following low flow arterial ischemia we proposed to investigate and characterize the effects of low flow arterial ischemia and
reperfusion of the equine large colon.

The principal hypothesis for this series of studies was that restoration of colonic blood flow after low flow arterial ischemia would produce colonic mucosal reperfusion injury. The specific aims of the studies reported were to characterize the histomorphologic and histomorphometric changes in the large colon mucosa, and the hemodynamic, metabolic, inflammatory, and hemostatic alterations in colonic and systemic venous blood during low flow ischemia and reperfusion of the equine large colon. The administration of drugs directed against oxygen free radical mediated injury was evaluated for their protection against colonic mucosal reperfusion injury.

The long term objective of these studies was to increase knowledge of the pathophysiologic processes occurring during low flow ischemia and reperfusion of the equine large colon. Ultimately, the goal of these studies was to generate knowledge with the potential to improve survival of horses with naturally occurring large colon volvulus by reducing the colonic injury sustained following surgical correction.
CHAPTER I
REVIEW OF PERTINENT LITERATURE

Large Colon Volvulus in the Horse

Colic is the leading cause of death in horses and it accounts for 1 out of 3 mortality insurance claims. Seven insurance companies paid over $16 million in 1983 for mortality claims in which death was directly attributable to colic. The large colon is involved in approximately 28% of all equine colics. In a recent study, a large colon abnormality was identified in approximately 56% of the horses that underwent colic surgery. Large colon lesions account for approximately 50% of all equine deaths due to colic. The large colon is mobile, and thus is predisposed to twisting around its mesentery (volvulus). Large colon volvulus usually occurs in a dorsomedial or counter-clockwise direction at the attachment of the cecocolic fold. The colon may be twisted from 270° to 720°. One study revealed that approximately 88% of large colon volvulus were ≥360°; this magnitude of twisting results in vascular compromise. Large colon volvulus is diagnosed in 7 - 40% of the horses requiring abdominal exploration.

The large colon vascular supply arises from branches of the cranial mesenteric artery. The right colic artery supplies the dorsal colon and the colic branch of the ileocolic artery supplies the ventral colon. The colonic arteries enter the mesocolon through the attachments of the cecum to the dorsal body wall, travel in the mesentery and anastomose at the pelvic flexure. Venous drainage from the large colon exits via the colonic veins, which lie in close proximity to the colonic arteries. The colonic vessels thus enter and exit
the large colon at the site where volvulus commonly originates.9

The microvascular circulation of the equine large colon has been studied extensively by Snyder, et al..13 Arteries branch from the mesenteric colic vessels toward the colonic wall approximately every 2 cm. After branching, these arteries form an anastomotic plexus known as the colonic rete and then continue into the colonic tissue. The mesenteric lymph nodes are supplied by a second vascular network that originates from the colonic rete. A submucosal vascular network is formed from arteries that penetrate the tunica muscularis 3 to 4 cm toward the antimesenteric border. Submucosal terminal arterioles either continue without branching or more commonly divide into 3 to 4 precapillary arterioles at the base of the colonic glands and supply mucosal capillaries. Multiple circular constrictions of precapillary arterioles exist and may represent precapillary sphincters. Mucosal vessels lack smooth muscle suggesting that there are no mucosal arterioles. Mucosal capillaries surround the colonic glands and anastomose at the luminal surface forming a superficial vascular plexus. Sparsely distributed venules drain the superficial plexus. There is no evidence of arteriovenous anastomoses. Larger submucosal veins have a circumferential helical distribution of smooth muscle that gives it a sacculated appearance. The majority of intestinal blood is located in the capacitance vessels (veins) and thus this helically distributed smooth muscle may allow for increased distension and storage of blood. This smooth muscle may also function to direct blood flow through a low pressure system.

A relatively large area of mucosa is supplied by a small number of capillaries and is drained by sparsely distributed venules. These venules are not parallel with the majority of arterioles and capillaries.13 Obstruction within a submucosal arteriole that supplies the mucosa may result in ischemia to a larger luminal area in the colonic mucosa than for a similar obstruction in a small intestinal villus.
With the onset of colonic volvulus, the vasculature is compressed by twisting and stretching of the vessels in the mesentery. In some instances, venous drainage is occluded first, while the thicker walled arteries resist compression thus maintaining some arterial flow. This results in a low flow phenomenon typical of ischemia-reperfusion models. Venous obstruction results in increased capillary pressures, which causes congestive hyperemia and mural edema, characteristic of hemorrhagic strangulation obstruction (HSO). In other cases, the arterial supply may be occluded nearly simultaneous with venous drainage resulting in ischemic strangulation obstruction (ISO).

Naturally occurring large colon volvulus is associated with injury and thrombosis of the colonic vasculature. Thrombosis of the colonic vessels may occur due to the twisting and stretching during volvulus or due to a combination of factors such as low blood flow, endothelial injury, platelet activation, and activation of the hemostatic system. Microangiographic evaluation of the large colon from horses with naturally occurring volvulus has demonstrated vascular obstruction in submucosal vessels at the level of the muscularis mucosae. Thrombosis was present within some mesenteric arteries around which the colonic rete was patent. This may represent a mechanism for circumventing obstruction of a short segment of a mesenteric artery and may help to maintain colonic blood flow and viability if the thrombosis is not extensive.

The overall survival rate for horses with large colon strangulation obstruction is only 21-42%. Without surgical intervention, all horses with strangulating obstruction of the large colon (> 180°) die rapidly due to endotoxemia and hypovolemia. The prognosis is dependent upon the degree and duration of the volvulus prior to surgical intervention. The overall high mortality is likely due to a number of factors. Three to four hours of severe ischemia induced by transmural compression, combined with venous and arterial occlusion is conjectured to result in irreversible damage to the colonic mucosa.
Strangulating obstruction of the intestine results in increased intraluminal bacteria. Loss of mucosal integrity secondary to ischemia allows bacteria and endotoxins to be absorbed into the systemic circulation, which exacerbates the shock occurring from severe fluid and blood loss into the bowel wall, bowel lumen and peritoneal cavity during volvulus and contributes to the rapid deterioration of the patient's metabolic and cardiovascular status.

Due to the origin of colonic volvulus at the area of the cecocolic ligament the majority of the colon is affected. If the magnitude and duration of volvulus are enough to cause mucosal necrosis a large surface area is available for absorption of luminal bacteria and endotoxin. A technique for resection of up to 95% of the large colon has been developed, however, it is technically demanding and can be associated with a high complication rate. The degree of ischemia and infarction associated with most cases of large colon volvulus makes successful resection and anastomosis difficult. It is important to try to determine if the colon is viable at the time or surgery in order to decide whether or not to attempt surgical resection of the colon or to correct the volvulus and treat the horse medically. Numerous methods have been used to assess colonic viability. These include visual and tactile assessment, fluorescein fluorescence patterns, doppler flow, surface oximetry, and histopathologic evaluation of frozen colon biopsies. The most promising of these techniques appears to be a combination of surface oximetry and histopathologic examination of colonic biopsies. The normal surface oximetry is approximately 55 mm Hg (± 20%) and if it is < 20 mm Hg horses do not survive. Histopathologic criteria for nonviable colon are ≥ 50% loss of crypt depth, ≥ 97% loss of surface epithelium, and an interstitial:crypt ratio ≥ 3:1.

Surgical correction of a naturally occurring large colon volvulus restores blood flow and the serosal color generally returns to a normal pink. This often gives a false sense of hope because a large number of these horses subsequently die despite surgical correction and
intensive medical care. This is likely a reflection of the severe damage sustained by the colonic mucosa during the ischemic period and the continuing damage that occurs following restoration of blood flow. Surgical correction of a naturally occurring large colon volvulus restores blood flow and thus automatically produces a reperfusion period with the potential for subsequent injury. Therefore, the horse serves as a natural model for intestinal ischemia and reperfusion.

**Overview of Ischemia-Reperfusion (I-R) Injury**

Maintenance of adequate blood flow and tissue perfusion are necessary for preserving cellular metabolic pathways, delivering oxygen and nutrients and removing waste products. Ischemia is a reduction in tissue perfusion that can compromise oxygenation of tissues. Cellular viability is dependent upon the maintenance of an adequate blood flow in order to meet metabolic demands. The severity and duration of blood flow reduction (complete or partial) ultimately determine cellular function and longevity. Due to cellular energy reserves and the ability to increase oxygen extraction most tissues can withstand a substantial reduction in blood flow without compromising cellular metabolism and energy production severely enough to alter structure or function. If the metabolic rate is higher or the reduction in blood flow is below that necessary to maintain cellular viability then changes in structure and function are inevitable. Brief periods (1-3 minutes) of complete ischemia, for example, are tolerated by most tissues, however, longer periods (>3 minutes) of ischemia (complete or partial) begin to produce significant alterations in cellular energy production, membrane and cytoplasmic regulatory processes, and cellular function and structure. Restoring blood flow and oxygen supply to ischemic tissues are thus necessary in order to return the ischemic tissue to normal function and to repair damaged components. Restoration of tissue perfusion and oxygenation, however, is capable of producing and initiating deleterious biochemical reactions that may contribute to further
tissue damage. This phenomenon, termed reperfusion injury, is defined as that component of tissue injury, which is sustained or produced during reoxygenation following an episode of ischemia.

Numerous experimental studies in a variety of organs (especially intestine) and species have demonstrated that a substantial portion of ischemic related tissue injury actually occurs during the period of reperfusion. The reintroduction of oxygenated blood to ischemic intestine has been shown to initiate a cascade of events leading to the production of oxygen-derived free radicals, which produce tissue injury through lipoperoxidation chain reactions. These pathophysiologic processes are likely to occur in horses during naturally occurring intestinal ischemia and reperfusion following the correction of intestinal strangulation. Pharmacologic disruption of these pathologic processes may reduce intestinal ischemia-reperfusion injury and improve survival.

Pathogenesis of Ischemia

Ischemia is a critical reduction in blood supply due to either a functional constriction or mechanical obstruction of blood vessels and leads to an inadequate tissue perfusion and oxygenation. Significant reductions in blood flow disrupt normal cellular metabolic and bioenergetic pathways. Oxygen is essential for normal cellular function and ultimately cell survival because of its role in energy production. The majority of cellular oxygen consumption occurs in the mitochondria where oxygen is reduced to water by the electron transport chain. This process is coupled with the synthesis of adenosine triphosphate (ATP), the major energy source in most cells. Oxygen deprivation leads to a failure of oxidative phosphorylation and a reduction in ATP production which is necessary in order to meet the metabolic demands of the cell. Reduced blood supply in the intestinal tract results in rapid injury and death of the highly energy-dependent mucosal epithelial cells.
When oxygen becomes depleted at the cellular level, a series of steps are initiated that leads to cellular damage and eventually cell death. Initially, oxygen stores are depleted and there is a reduction of stored energy in the form of creatine phosphate. As the oxygen level continues to fall, ATP levels decrease and anaerobic glycolysis occurs in an attempt to maintain cell function. These changes cause the intracellular pH to fall due to the accumulation of lactate and hydrogen ions from ATP hydrolysis. The accumulation of lactate causes intracellular acidosis which inhibits ATP production; thus cellular energy stores become diminished. As energy stores become exhausted cellular swelling occurs due to the inability of energy-dependent membrane pumps to maintain normal ionic balance. An influx and accumulation of calcium ions alters many cellular reactions. Increased intracellular calcium, for example, activates cell associated phospholipase A2 and the subsequent degradation of membrane phospholipids to free fatty acids leading to both intracellular and cell membrane structural alterations. As cell membranes become swollen they become ineffective in maintaining intracellular homeostasis; thus leakage of enzymes and other cellular elements occur. With continued ischemia, intracellular pH continues to fall due to hydrogen ion accumulation leading to a loss of intracellular negative charges, which alters the structure and function of enzymes and regulatory proteins. Eventually there is lysosomal release of proteases, lipases and other degradative enzymes, which initiates autolytic destruction of cellular organelles. With these changes, cell damage becomes irreversible and cell death occurs.

The Production of Oxygen-Derived Free Radicals (OFRs)

Under normal circumstances, cellular oxidative phosphorylation involves a four electron reduction of oxygen to water. This occurs as a series of reactions that begins with a one electron reduction of oxygen to form superoxide anion, which is reduced by
another electron to form hydrogen peroxide. Further reduction of hydrogen peroxide leads to formation of the hydroxyl radical, which is reduced by a fourth electron to water (Figure 1.1). The superoxide anion and the hydroxyl radical are highly reactive free radicals that potentially can react with cell membranes causing loss of cellular integrity and tissue damage. Hydrogen peroxide is not as potent an oxidizing agent as are superoxide and hydroxyl radicals. Hydrogen peroxide can be converted to a more reactive hydroxyl radical by ferrous iron, which is made available from the superoxide mediated conversion of ferric iron to ferrous iron. Oxygen-derived free radicals can initiate lipoperoxidation of cell membranes, destroy enzymes, and cleave DNA strands. These radicals can react with cell membrane lipids resulting in chain reactions of lipoperoxidation.

Naturally occurring antioxidants exist which serve to protect tissues from the OFRs formed during normal cellular metabolism. Endogenous antioxidants such as enzymatic antioxidants (superoxide dismutase, catalase, and glutathione peroxidase) and nonenzymatic antioxidants (alpha tocopherol, ascorbate, and beta carotene) function to protect against oxidant injury. Superoxide dismutase catalyzes the dismutation of superoxide anions to hydrogen peroxide. Catalase catalyzes the reduction of hydrogen peroxide to water, which prevents secondary generation of hydroxyl radicals. Glutathione peroxidase also reduces hydrogen peroxide to water, using glutathione as the substrate. Two forms of glutathione peroxidase exist: an selenium containing enzyme which catalyzes the conversion of both hydrogen peroxide and lipid peroxides and a non-selenium containing enzyme that catalyzes reduction of only hydrogen peroxide. During reoxygenation of ischemic tissues rapid production of these radicals can overwhelm these protective mechanisms resulting in further injury.

Reoxygenation of ischemic tissues can result in further tissue injury due to a series of reactions that are believed to be initiated by oxygen free radicals. Any molecular species
During normal oxidative phosphorylation oxygen (O\(_2\)) is sequentially reduced by four electrons to form water. Reduction of O\(_2\) by one electron (e\(^-\)) yields a superoxide radical (O\(_2^\cdot\)), which is further reduced by a second electron to form hydrogen peroxide (H\(_2\)O\(_2\)). A third electron reduction results in formation of a hydroxyl radical (OH\(^\cdot\)), which undergoes a final one electron reduction to form water (H\(_2\)O).
Figure 1.1

\[ \text{O}_2 \overset{1e^{-}}{\rightarrow} \text{O}_2^- \overset{1e^{-}}{\rightarrow} \text{H}_2\text{O}_2 \overset{1e^{-}}{\rightarrow} \text{OH}^- \overset{1e^{-}}{\rightarrow} \text{H}_2\text{O} \]
with an unpaired electron in its outer orbit is considered a radical. Oxygen is a natural radical because it has two unpaired electrons in different orbits. If a radical reacts with a nonradical another radical is produced. Free radicals are generally unstable and highly reactive. Formation of OFRs can occur in chain reactions by passing an electron along in oxidation-reduction reactions within biologic systems. If cellular hypoxia occurs, uncoupled oxidative phosphorylation results in a drop in cellular energy. Electrons from these uncoupled reactions can leak from the cytochrome system within mitochondria. When oxygen is again available, these cells attempt to resume oxidative metabolism, but instead, the oxygen acts as a substrate for the generation of OFRs such as superoxide radicals.

Several pathways exist for the generation of superoxide radicals; some pathways involve enzymatic production while others are non-enzymatically formed. Xanthine oxidase (XO), an enzyme present in the cytosol of most animal cells, in the presence of oxygen catalyzes the oxidation of hypoxanthine to xanthine and uric acid with the generation of a superoxide radical. The majority of tissue XO is present as xanthine dehydrogenase (XDH), which is linked to NAD+. Conversion of XDH to XO occurs by the Ca\(^{2+}\)-dependent protease, calpain, and by sulfhydryl oxidation. Neutrophils can produce superoxide radicals via the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system, an enzyme which acts as a catalyst for the reduction of oxygen to superoxide anion and hydrogen peroxide (Figure 1.2). This phenomenon is known as the “respiratory burst” and is a major mechanism by which neutrophils phagocytize and destroy microorganisms. The superoxide radical leads to the generation of a hypochlorous acid (HOCl) from hydrogen peroxide and chloride ions by myeloperoxidase (MPO). This enzyme is normally inactive, but activation occurs upon adherence to bacteria. Superoxide production can occur as a by-product of mitochondrial respiration.
During ischemia hypoxanthine accumulates in endothelial cells and intestinal mucosal cells simultaneous with conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO). Upon reperfusion, hypoxanthine is metabolized by XO to a superoxide radical (O$_2^-$) and water; O$_2^-$ is further metabolized to hydrogen peroxide (H$_2$O$_2$). The O$_2^-$ and H$_2$O$_2$ react in the presence of an iron catalyst (Fe$^{3+}$) to form hydroxyl radicals (OH$^-$). The OH$^-$ radical initiates peroxidation of polyunsaturated fats (LH) to form lipid hydroperoxides (LOOH). The net result of this toxic oxygen metabolite production is cell membrane damage via lipoperoxidation and release of phospholipid-derived mediators (LTB$_4$ and PAF) that are chemoattractants for neutrophils. Upon adhesion to the endothelium, neutrophils release elastase and lactoferrin which promote extravasation. Oxygen is converted to neutrophils by a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system into O$_2^-$, which is further metabolized to H$_2$O$_2$. Myeloperoxidase (MPO) catalyzes the reaction of H$_2$O$_2$ and a chloride ion (Cl$^-$) resulting in the formation of a very toxic substance, hypochlorous acid (HOCI). Illustration courtesy of Dr. D. Neil Granger.
Figure 1.2
via oxidation of semiquinones; however, this represents only a small proportion of the total superoxide formed.\textsuperscript{20,27} Superoxide is formed during the conversion of prostaglandin $G_2$ to prostaglandin $H_2$ and can occur through the oxidation of catecholamines.\textsuperscript{20} The production of superoxide associated with intestinal ischemia and reperfusion largely results from xanthine oxidase present in the endothelium\textsuperscript{29} and mucosal epithelium\textsuperscript{30}; however, neutrophil-derived superoxide likely contributes to the oxidant injury.

**Role of OFRs in Reperfusion Injury**

Under normal conditions, XDH, an enzyme present in endothelial cells, converts hypoxanthine to xanthine and uric acid, which is eliminated by the kidneys.\textsuperscript{31} During ischemia several events occur that set the stage for oxygen radical generation upon reperfusion. Adenosine triphosphate production is curtailed during ischemia, and the metabolism of existing stores results in a build-up of hypoxanthine.\textsuperscript{31} Removal of calcium from the cytosol requires ATP; therefore, calcium accumulates intracellularly due to decreased function of the Na$^+$-K$^+$ ATPase pumps.\textsuperscript{31} Disruption in calcium homeostasis results in high intracellular Ca$^{2+}$, which in conjunction with calpain, convert XDH to XO.\textsuperscript{31} The hypoxanthine that accumulates during ischemia provides a large quantity of substrate for superoxide production upon reoxygenation. Superoxide radicals are converted into hydrogen peroxide by endogenous superoxide dismutase.\textsuperscript{20} The production of superoxide and hydrogen peroxide during reperfusion of ischemic tissues overwhelms the endogenous antioxidant defense mechanisms and hydroxyl radicals are formed through the iron-dependent Haber-Weis reaction. The over abundance of OFRs produced during reperfusion leads to oxidant-induced tissue injury.\textsuperscript{20}
Oxygen-free radicals can exert direct effects on cellular structure and function as well as secondary effects through disruption of intracellular calcium homeostasis. Direct effects of OFRs result from the peroxidation of membrane phospholipids which causes cell damage through disruption and lysis of cells and their organelles. This lipoperoxidation reaction requires the presence of iron and is believed to be initiated by an iron radical. Once initiated, this lipoperoxidation can be propagated, thus amplifying the extent of tissue injury. Other direct effects of OFRs are enzyme inactivation and DNA strand breaks due to hydroxyl radical-induced oxidation of proteins and nucleic acids. Indirect tissue injury initiated by OFRs involves disruption of intracellular calcium homeostasis. Calcium accumulates due to release from the endoplasmic reticulum and mitochondria and from influx of extracellular Ca\(^{2+}\) into the cytosol due to inactivation of cell membrane associated Ca\(^{2+}\)-ATPase. Increased cytosolic calcium occurs during ischemia due to a reduction in cellular ATP. Increased cytosolic calcium activates calpain, a Ca\(^{2+}\)-dependent protease, and activates phospholipase A\(_2\), both of which can lead to further structural damage. The net result of OFR generation is structural damage to tissue caused by lipoperoxidation of cell membranes and their organelles and production of phospholipid mediators due to activation of phospholipase A\(_2\).

**Role of OFRs in Intestinal Reperfusion Injury**

Xanthine-oxidase-derived OFRs play an integral role in the microvascular and mucosal injury induced by intestinal ischemia and reperfusion. Ischemia and reperfusion of cat small intestine results in increased microvascular permeability and structural damage to the intestinal mucosa. Pretreatment with superoxide dismutase, catalase, xanthine oxidase inhibitors (allopurinol), hydroxyl radical scavengers (dimethyl sulfoxide), or an iron chelator (deferoxamine) attenuates or prevents the increase in microvascular
permeability and morphologic mucosal injury associated with reperfusion of ischemic
intestine. Granger et. al., demonstrated that reperfusion of ischemic cat intestine
decreases mucosal thickness, villus height, and crypt depth and results in lifting of
epithelium along the sides of the villi beginning at the tips and extending to the lamina
propria. Reperfusion of ischemic stomach and small intestine results in increased mucosal permeability. Local intra-arterial infusion of hypoxanthine and XO caused increased microvascular permeability; this was attenuated by the administration of either superoxide dismutase or dimethyl sulfoxide. These studies provide evidence that xanthine oxidase-derived superoxide radicals, hydrogen peroxide, and hydroxyl radicals play a role in this I-R injury.

Recent evidence suggests nitric oxide (NO), also known as endothelium-derived relaxation factor, serves as an endogenous modulator of intestinal epithelial permeability. It appears the effect of NO is independent of blood flow or neutrophil adherence to the endothelium. Nitric oxide is inactivated by superoxide; it is believed that inhibition of NO by superoxide radicals could contribute to increased intestinal mucosal permeability. A reduction in endogenous NO production may predispose to vasospasm, platelet deposition, and increased neutrophil adherence all of which could exacerbate intestinal injury. Administration of a NO donor (C87-3754) attenuated endothelial dysfunction and improved short term survival associated with two hours of splanchnic artery occlusion followed by two hours of reperfusion in cats.

Role of Neutrophils

Xanthine oxidase-derived OFRs appear to initiate recruitment and activation of granulocytes in post-ischemic intestine; neutrophil-derived OFRs then can mediate and/or exacerbate the intestinal mucosal injury (Figure 1.2). Using intravital microscopy (in vivo
microscopic evaluation), ischemia of cat small intestine for one hour has been demonstrated to cause significant adherence to and extravasation of neutrophils in mesenteric venular endothelium. Maximal adherence occurs ten minutes following reperfusion. Superoxide dismutase and allopurinol prevent the increase in neutrophil adherence and extravasation during reperfusion of ischemic intestine. Neutrophil infiltration in cat intestinal mucosa following reperfusion is largely prevented by pretreatment with allopurinol, superoxide dismutase, catalase, and deferoxamine. The increase in microvascular permeability following reperfusion of ischemic intestine is significantly attenuated by pretreatment with XO inhibitors, neutropenia and prevention of neutrophil adherence to microvascular endothelium by administration of a monoclonal antibody directed against the CD18 neutrophil adhesion molecule or by the administration of dimethyl sulfoxide. The attenuation of reperfusion-mediated leukocyte adherence and extravasation by superoxide dismutase, allopurinol, and dimethyl sulfoxide further supports the role of OFRs in initiating the infiltration of leukocytes during reperfusion of ischemic intestine. The oxidants derived from XO may mediate the mucosal injury directly or indirectly through their effect on neutrophils; it is believed that the neutrophil-derived oxidants mediate the majority of mucosal and microvascular injury associated with reperfusion of ischemic intestine. Pretreatment of cats with elastase inhibitors (Elgin C and L658, 758) prevent the increased leukocyte adherence and extravasation associated with ischemia and reperfusion. This suggests that elastase is essential for the migration of neutrophils from the microvasculature to the interstitium following intestinal ischemia and reperfusion. Elastase, released from activated neutrophils, can degrade components of the restrictive barriers between the endothelium and the interstitium thus allowing neutrophil infiltration (Figure 1.2).
Intravascular leukocytes appear to be an important mediator of intestinal ischemia-reperfusion injury.\textsuperscript{53} Severe damage was observed in the small intestine of rats following 90 minutes of ischemia and 60 minutes of reperfusion with whole blood.\textsuperscript{53} A significant attenuation of intestinal damage occurred when the intestine was reperfused with a leukocyte free perfluorochemical solution. The protective effects of leukocyte free perfusate were negated when neutrophils were added to the solution. Administration of anti-neutrophil serum or a monoclonal antibody that prevents neutrophil adherence significantly attenuates the increased microvascular permeability occurring upon reperfusion in the cat small intestine.\textsuperscript{47} Leukocyte depletion ameliorated intestinal reperfusion injury in the cat\textsuperscript{47} and has been shown to reduce the size of myocardial infarcts in dogs.\textsuperscript{54} Recent evidence suggests that resident interstitial granulocytes in the intestine contribute more to the increased mucosal permeability associated with ischemia and reperfusion than do those cells newly recruited from the vascular space.\textsuperscript{49} It appears that the newly recruited cells do have a role in altering fluid exchange in the microvasculature.\textsuperscript{49}

The XO-derived oxidants do not directly stimulate neutrophil chemotaxis; however, they may initiate the generation of other substances such as leukotriene B\textsubscript{4}, complement C5a, and platelet-activating factor, which promote chemotaxis, adherence, and extravasation of leukocytes following reperfusion.\textsuperscript{46,55,56} Neutrophil adherence to the microvascular endothelium is necessary for emigration or diapedesis. This neutrophil-endothelial adhesion occurs through the interaction of a neutrophil adhesion receptor complex (CD18) with an intercellular adhesion molecule-1 (ICAM-1) and the endothelial leukocyte adhesion molecule-1 (ELAM-1).\textsuperscript{57-64} The CD18 receptor complex is composed of three subunits: CD11a, CD11b, and CD11c. The ICAM-1 serves as a ligand for the binding of the CD11a and CD11b subunits. The neutrophil receptor for the ELAM-1 is currently unknown. There are at least two mechanisms for the adherence of
neutrophils to endothelial cells. The first mechanism involves neutrophil stimulation by chemoattractants such as LTB₄, complement C5α, or platelet-activating factor. These chemotactic agents result in increased activity of the CD18 receptor complex, but have no effect on the activity of endothelial cell adhesion molecules. The second mechanism involves stimulation of endothelial cells with subsequent expression of ICAM-1 and ELAM-1. Cytokines are necessary for this mechanism of adhesion and it is dependent upon protein synthesis.

Role of Monocyte-Derived Cytokines

There are a number of cytokines released from monocytes or tissue macrophages and lymphocytes that may be chemotactic for neutrophils. The term interleukin is used for describing those cytokines which act on leukocytes. There are a number of these interleukins; however, only interleukin 1 (IL-1) and interleukin 8 (IL-8) are chemotactic for neutrophils. Tumor necrosis factor (TNF) is another monocyte-derived cytokine that has a variety of actions, including stimulating neutrophil infiltration.

Interleukin 1 is a potent stimulant of neutrophils in vivo. Intraperitoneal injection of recombinant human IL-1 resulted in a rapid influx of large numbers of neutrophils into the peritoneal cavity of mice. Neutrophil infiltration was detectable within one hour, peaked at two hours, and declined by 24 hours. Intradermal administration of recombinant IL-1 results in extensive neutrophil infiltration in rabbits. Interleukin 1 and TNF are synergistic in their stimulatory effects on neutrophil infiltration. Interleukin 1 directly and selectively increases the adhesiveness of vascular endothelial cell surfaces for neutrophils. Interleukin 1 may also stimulate synthesis of phospholipases which result in the release of arachidonic acid from cell membrane phospholipids. Arachidonic acid metabolism via the 5-lipoxygenase pathway
results in the production of LTB₄, which is a potent chemoattractant for neutrophils.⁵⁵,⁷¹

Interleukin-8 is a glycoprotein produced predominantly by monocytes and tissue macrophages, but also by endothelial cells and fibroblasts. Interleukin-1 and TNF stimulate the release of IL-8 from monocytes and macrophages. Interleukin-8 is a potent chemoattractant specific for neutrophils.⁶⁵ In addition, IL-8 causes neutrophil degranulation; release of myeloperoxidase, elastase, and other enzymes from neutrophils; and increases neutrophil adherence to endothelium. Intraperitoneal or subcutaneous injection of IL-8 stimulates neutrophil infiltration. Although the role of IL-8 in stimulating neutrophil infiltration during ischemia and reperfusion has not been investigated, it could be a potential mediator of the tissue injury caused by neutrophil adherence, extravasation, and degranulation during reperfusion of ischemic tissue.

Tumor necrosis factor (cachectin) is a polypeptide produced by monocytes and tissue macrophages in response to a variety of stimuli, notably endotoxin.⁶⁶ Tumor necrosis factor has a multitude of physiologic actions. Most notably, it is believed to be a critical proximal mediator of the deleterious effects of endotoxemia.⁷² Recently, TNF has been shown to activate neutrophils resulting in their adhesion to endothelial cell surfaces and enhancement of their phagocytic activity.⁶⁶ Tumor necrosis factor is a potent chemoattractant for monocytes and neutrophils.⁷³

Tumor necrosis factor has a variety of effects on neutrophil function. Neutrophil adherence to endothelial cells is caused, in part, by a direct effect of TNF on neutrophils.⁷⁴ Additionally, TNF may increase the susceptibility of endothelial cells to neutrophil mediated attack, and induce neutrophils to produce superoxide anion and hydrogen peroxide, which damage cell membranes via lipoperoxidation.⁷⁵-⁷⁷ Tumor necrosis factor is direct, but weak stimulant of the respiratory burst and degranulation of neutrophils.⁷⁶ Tumor necrosis factor enhances LTB₄ generation, which is chemotactic for neutrophils.⁷⁸
Endothelial cells produce a number of cytokines (IL-1, IL-8, and platelet activating factor) in response to TNF, which may secondarily increase neutrophil infiltration. Intestinal ischemia induced by superior mesenteric artery ligation of rats for 120 minutes resulted in a slight elevation of systemic plasma levels of TNF; however, within 15-30 minutes of reperfusion TNF levels increased 5-10 fold respectively. The fact that TNF levels increase within minutes following reperfusion lends support to the hypothesis that reactive oxygen metabolites stimulate TNF release. Intestinal mucosal injury following ischemia and reperfusion allows endotoxin absorption into the systemic circulation, which could result in the increase in TNF. However, studies have shown that TNF increases in the absence of detectable circulating endotoxin.

Role of Phospholipid-Derived Mediators

Phospholipase A₂, an enzyme located in the cell membrane, plays a central role in several biochemical reactions and cellular functions. It is responsible for the release of arachidonic acid from cell membrane phospholipids and serves to regulate the formation of lysophospholipids such as lysophosphatidylcholine and platelet-activating factor (Figure 1.3). Activation of phospholipase A₂ (PLA₂) is believed to be initiated by increased intracellular calcium, which accumulates during ischemia due to malfunction of the membrane pumps which normally maintain ionic homeostasis. Under physiologic conditions, activation of phospholipase A₂ appears to have a membrane sparing effect whereby it catalyzes the elimination of lipid peroxides that would otherwise propagate lipid peroxidation.

Two hours of complete ischemia of the rat ileum followed by 5 minutes of reperfusion results in increased mucosal PLA₂, increased mucosal malondialdehyde (MDA) concentration, decreased mucosal activity of lysophospholipase, and an increased ratio of
Increased cytosolic Ca\(^{2+}\) activates phospholipase A\(_{2}\) (PLA\(_{2}\)) which causes release of a number of biologically active phospholipids from cell membranes; these include platelet activating factor (PAF), arachidonic acid metabolites (LTs, PGs), and lysophosphatidyl choline. Arachidonic acid is enzymatically (enzymes italicized) metabolized along one of two pathways. Metabolism along the lipoxygenase pathway results in the formation of 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and 12-hydroperoxyeicosatetraenoic acid (12-HPETE) intermediates; these are further metabolized to leukotrienes (LTA\(_{4}\), LTB\(_{4}\), LTC\(_{4}\), LTD\(_{4}\), and LTE\(_{4}\)) and hydroxyeicosatetraenoic (HETE) respectively. Metabolism along the cyclooxygenase pathway results in the formation of prostaglandins (PGD\(_{2}\), PGE\(_{2}\), PGF\(_{2\alpha}\), and PGI\(_{2}\)) and thromboxane (TXA\(_{2}\)). Some of these phospholipid metabolites exert effects on neutrophils (PAF, LTB\(_{4}\), TXA\(_{2}\)), platelets (PAF, PGI\(_{2}\), TXA\(_{2}\)), and vascular tone (PGI\(_{2}\), TXA\(_{2}\)). Phospholipid metabolites thus appear to play an integral role in injury associated with tissue ischemia and reperfusion.
Figure 1.3
lysophosphatidylcholine to phosphatidylcholine. Additionally, there was increased mucosal permeability to sodium fluorescein, increased mucosal N-acetyl-glucosaminidase release, and increased mucosal myeloperoxidase (MPO) activity. There are concurrent increases in portal venous PLA$_2$, MDA, and MPO following intestinal ischemia and reperfusion. Pretreatment of these rats prior to the development of intestinal ischemia with quinacrine, a PLA$_2$ inhibitor, prevented increases in the above parameters; protection against morphologic injury of the mucosa also occurred. This supports the contention that activation of PLA$_2$ and the accumulation of lysophosphoglycerides might be involved in mediating the mucosal injury caused by small intestinal ischemia. Concurrent with this effect, PLA$_2$ activity may serve to initiate membrane repair; the resulting lysophosphoglycerides produced could then be used for synthesis of new membrane diacylphosphoglycerides.

Activation of PLA$_2$ leads to the formation of lysophosphatidylcholine, platelet-activating factor (PAF), and arachidonic acid metabolites (prostaglandins and leukotrienes). There is evidence that at least lysophosphatidylcholine and PAF contribute to mucosal damage after intestinal ischemia. Comparison of the effects of PLA$_2$, PAF, cyclooxygenase, and lipoxygenase inhibition revealed that the above mentioned alterations occurring in the small intestine following ischemia and reperfusion were prevented by inhibiting PLA$_2$ and PAF, but not cyclooxygenase or lipoxygenase pathways. Cyclooxygenase inhibition caused an even greater increase in mucosal permeability than ischemia and reperfusion suggesting that selective prostaglandins may have a protective role in the intestinal mucosa during ischemia. Another possibility is that cyclooxygenase inhibition causes shunting of arachidonic acid into the lipoxygenase pathway resulting in the production of various leukotrienes.
Increased mucosal MPO activity demonstrates neutrophil accumulation in the injured mucosa; these neutrophils may have been recruited secondary to lipoperoxidation and activation of PLA$_2$. There was a high correlation between mucosal MPO and MDA suggesting that the lipoperoxidation-induced increase in MDA may have been due to the infiltration of neutrophils.\textsuperscript{86} Phospholipase A$_2$ activation can generate a number of inflammatory and potentially toxic lipid mediators in leukocytes such as arachidonic acid metabolites, lysophospholipids and PAF.

Platelet-activating factor is derived from phospholipid membranes by PLA$_2$ activation; it is released from a number of cell types including endothelial cells, platelets, neutrophils, and macrophages.\textsuperscript{87} Several studies have recently demonstrated a potential role for PAF in acute inflammation, such as I-R injury. Intestinal venous \textsuperscript{88} and mucosal\textsuperscript{56} PAF concentrations significantly increase upon reperfusion of ischemic intestine. Local arterial infusion of PAF into the rat stomach produces macroscopic mucosal hemorrhage and necrosis, which could be prevented by pretreatment with a PAF antagonist.\textsuperscript{89} Systemic administration of PAF did not cause similar damage in the gastric mucosa.\textsuperscript{89} Local superior mesenteric arterial infusion of PAF in cats promoted increased microvascular permeability as evidenced by increased filtration of fluid and protein across intestinal capillaries.\textsuperscript{90} This was prevented by administration of monoclonal antibody directed against the CD11/CD18 leukocyte adhesive glycoprotein complex, which suggests that the effects of PAF on the microvasculature are mediated in part by leukocytes.\textsuperscript{56,90} In vitro experiments have documented that PAF increases adherence, degranulation, and superoxide production of cat neutrophils.\textsuperscript{91} Intra-arterial infusion of PAF into the superior mesenteric artery of cats caused increases in the adherence of leukocytes to mesenteric venular endothelium and increased vascular protein leakage.\textsuperscript{91}
Platelet-activating factor appears to promote the extravasation of circulating leukocytes through interactions that are both dependent and independent of the adherence of leukocytes to endothelium. Endothelial cell injury induced by anoxia and reoxygenation is mediated in part by neutrophils; this injury is associated with an increased release of elastase from neutrophils. It is possible that PAF may contribute to leukocyte extravasation by stimulating the release of proteases (elastase) and oxidants from neutrophils, which may enable these cells to cross the endothelium into the interstitium. The increased leukocyte adherence and extravasation associated with ischemia and reperfusion of feline small intestine is prevented by pretreatment with PAF antagonists. This protective effect may be due to the binding of these antagonists to PAF receptors on either the leukocyte or endothelial cell membrane that regulate leukocyte-endothelial adhesion.

It is believed that oxidants formed during reperfusion of ischemic intestine leads to the production of PAF which ultimately results in leukocyte adherence and extravasation. Small intestinal necrosis induced by aortic injection of PAF into rats is attenuated by pretreatment with superoxide dismutase/catalase or allopurinol, which suggests oxygen free radicals are involved in the pathogenesis of PAF induced intestinal injury associated with ischemia and reperfusion. Release of oxygen free radicals upon reperfusion of ischemic intestine could stimulate the production of PAF through activation of PLA2; PAF could then mediate the injury by stimulating the generation of toxic oxidants in mucosal neutrophils, which injures the microvascular endothelium.

Other membrane phospholipids can undergo metabolism secondary to I-R. Arachidonic acid is metabolized via cyclooxygenase and lipoxygenase pathways; the resultant prostaglandins and leukotrienes may play a role in blood flow regulation, platelet adhesiveness, and neutrophil chemotaxis and adhesion (Figure 1.3). Prostacyclin (PGI2)
has direct vasodilating effects, inhibits platelet aggregation, and stabilizes microvascular membranes. Thromboxane A$_2$ (TXA$_2$), on the other hand, is a potent vasoconstrictor, stimulates platelet aggregation and enhances microvascular permeability. Canine intestinal mucosal TXB$_2$, the stable hydrolysis product of TXA$_2$, and 6-ketoprostaglandin F$_1$ alpha (the stable hydrolysis product of PGI$_2$), and LTB$_4$ increased 365% and 97% respectively following 3 hours of ischemia and one hour of reperfusion, but were not affected by ischemia alone. Thromboxane appears to mediate early neutrophil adherence and emigration during reperfusion of ischemic tissues through activation of CD18 neutrophil adhesion receptors which then interact with ICAM-1 normally expressed by the endothelium. There are a number of leukotrienes produced from the effects of lipoxygenase on arachidonic acid. Leukotriene B$_4$ (LTB$_4$) has been shown to increase in the small intestine of cats (200%) and dogs (687%) following 3 hours of ischemia and 1 hour of reperfusion, but not by ischemia alone. This increased mucosal LTB$_4$ production was attenuated by lipoxygenase inhibition. Lipoxygenase inhibition and LTB$_4$ antagonism prevented the infiltration of neutrophils during reperfusion of ischemic intestine. Administration of an LTB$_4$ antagonist prevented the increased intestinal myeloperoxidase activity associated with superior mesenteric arterial occlusion in rats and inhibited the adherence of rat neutrophils to isolated superior mesenteric artery endothelium. These findings suggest LTB$_4$ mediates neutrophil infiltration in the intestine secondary to ischemia and reperfusion.

**Effects of Intestinal I-R on Distant Organs**

Intestinal I-R injury may lead to multiorgan failure and death due to the release of a variety of endogenous inflammatory mediators; it has been stated that the "gut is the motor
of multiple organ failure". The myocardium and pulmonary parenchyma are well documented sites of distant organ damage associated with intestinal I-R, although, other distant organs, such as the liver, could potentially be affected. The above organs are prime targets for injury secondary to intestinal I-R because they are the first organs that circulating toxins and inflammatory products contact after traversing the portal system. Intestinal I-R results in acute lung injury characterized by increased microvascular permeability, morphologic injury of the alveolar endothelial cells, lung tissue ATP depletion, and accumulation of neutrophils in pulmonary tissue. These effects were observed following reperfusion, but not during the ischemic period. Endotoxin and TNF are increased in systemic blood following reperfusion of ischemic rat intestine. Pretreatment with anti-TNF antibody attenuated the increased microvascular permeability, but did not prevent the sequestration of neutrophils in pulmonary tissue. This suggests that TNF plays a role in neutrophil activation, but not neutrophil adherence. Administration of quinacrine (PLA2 inhibitor) to rats prior to the induction of intestinal ischemia prevented the increased pulmonary microvascular permeability that occurs upon reperfusion. This suggests phospholipase A2 activation is a proximal step in the pathogenesis of distant organ damage following intestinal I-R. Activation of intestinal PLA2 appears to stimulate neutrophils possibly through the generation of phospholipid-derived mediators. Dysfunction of myocardial contraction and relaxation occurred within 2 hours of intestinal I-R in rats and persisted for 12-16 hours. These effects were prevented by enteral administration of allopurinol for four days prior to the intestinal ischemia, suggesting that XO-derived OFRs contribute to myocardial dysfunction.
Experimental Models of Intestinal I-R

Numerous models of intestinal ischemia have been used to study the effects of reperfusion. Models of intestinal ischemia are categorized based upon the creation of either complete cessation or partial reduction in blood flow. The degree of intestinal injury is directly proportional to the both duration and severity of ischemia. The magnitude of change in mucosal architecture and permeability is directly related to the duration of the ischemic event with both complete and partial arterial occlusion. Partial ischemia is capable of causing the same degree of injury as complete ischemia if the duration of partial ischemia is prolonged. Most investigators prefer partial intestinal ischemia for studying the effects of reperfusion injury because complete ischemia causes such severe injury that any injury sustained during reperfusion is inconsequential.

Clinical Implications of Intestinal I-R in the Horse

Strangulating obstruction of the small intestine and large colon are common causes of acute abdominal disease in the horse. Small intestinal strangulations frequently result from either a volvulus or incarceration at a variety of intra-abdominal locations whereas volvulus is the only clinically significant cause of large colon strangulation. Mortality associated with intestinal strangulation in horses is quite variable, but usually ranges between 50-80%. Strangulation obstruction is defined as simultaneous luminal obstruction and vascular occlusion. Strangulation obstruction occurs in two forms: hemorrhagic and ischemic. The most common type of strangulation occurring in both the small intestine and large colon of horses is hemorrhagic strangulation obstruction (HSO). Hemorrhagic strangulation obstruction is characterized by venous occlusion, however, some arterial flow often continues. Fluid accumulates in the intestinal wall, lumen, and mesentery. The
serosa becomes dark purple, the mucosa dark red to black, and severe intestinal wall thickening occurs due to congestion, hemorrhage, and edema following the onset of intestinal HSO. These changes are due to the increased hydrostatic pressure associated with venous obstruction and the increased microvascular permeability caused by inflammatory mediators released in response to intestinal ischemia. The large colon mucosa becomes dark red to purple within 30 minutes and is black by four hours when subjected to HSO. Ischemic strangulation obstruction (ISO) involves simultaneous occlusion of the intestinal venous and arterial vasculature resulting in blanched and cyanotic serosa. The large colon mucosa progresses from bright red to dark red within one hour and becomes black by four hours when subjected to ISO.

During small intestinal HSO or ISO, fluid sequesters in the subepithelial space and the epithelium begins to loosen from its underlying attachment to the basement membrane at the villus tip. This separation progresses downward along the villus toward the crypts. Epithelial cells slough before they develop severe intracellular alterations; the cells remain attached to one another and slough in sheets. The villi are completely denuded of epithelium by 3 hours and the villi contract to the level of the crypts. Complete necrosis of the mucosal epithelium extending to the base of the crypt occurs by 4-5 hours and by 6-7 hours the degeneration has progressed outward beyond the muscular layers. The morphologic alterations occurring in the large colon during complete ischemia include cellular necrosis of three to five surface epithelial cells, which then loosen from their attachments at their base and from neighboring cells. This results in sloughing of small clusters of surface epithelial cells rather than sheets of cells as observed in the small intestine. Ultrastructural evaluation of the large colon following arteriovenous occlusion reveals cellular degeneration becomes irreversible before epithelial sloughing occurs. It appears that 3-4 hours of complete ischemia caused by arteriovenous occlusion and
transmural compression results in irreversible damage to the large colon mucosa. Sloughing of 100% of the surface epithelium and ≥ 50% of the glandular epithelium were associated with death in naturally occurring large colon volvulus.

Progression of mucosal epithelial injury may lead to complete mucosal necrosis. Disruption of the mucosal barrier thus allows migration of luminal bacteria and endotoxin into the systemic circulation resulting in endotoxemia. Additionally, sequestration of large quantities of fluid in the intestinal wall, lumen, and mesentery result in hypovolemia. Left untreated, these horses succumb to hypovolemic and endotoxic shock. Even with surgical intervention, the overall prognosis for long term survival of horses with strangulating intestinal lesions is guarded. Of 140 horses that underwent small intestinal resection for a strangulating lesion only 11% were still alive in three years. The reported survival rate in horses with large colon volvulus ranges from approximately 20-40%. The prognosis is obviously dependent upon the degree and duration of ischemia prior to surgical intervention. Successful resection and anastomosis of naturally occurring large colon volvulus in horses depends on the severity of the ischemia and infarction and on the origin of the volvulus. In many instances, the area involved is so extensive that surgical resection is not possible. Therefore, the horses are either euthanatized or are aggressively managed with intensive medical therapy and supportive care following detorsion. Many horses subsequently die during the post-operative period despite surgical correction, intensive medical therapy and supportive care. This is likely a reflection of the severe damage the colon sustains during the ischemic period and the further injury that occurs upon reperfusion.

If horses survive the initial period of mucosal necrosis and sloughing, and if there are viable enterocytes present, the mucosa can regenerate by migration of these cells. The mucosa will be covered by epithelium within 12-24 hours.
oral to the strangulation often undergo mucosal degeneration; this degeneration has been observed at the villus tips in some biopsies harvested from portions of the intestine oral to the strangulation in horses with naturally occurring strangulations. The injury sustained by mucosal cells can either heal or can progress leading to further mucosal injury. Intestinal injury incurred during strangulation may continue after detorsion or release of the incarceration due to irreversible cellular alterations that occurred during the ischemic period or due to exacerbation of the cellular injury as a result of the cascade of events initiated by reperfusion.

Experimental Intestinal I-R in the Horse

Several studies have evaluated the effects of ischemia and reperfusion in the gastrointestinal tract of horses. These studies are based upon histopathologic, biochemical, and hemodynamic alterations following intestinal ischemia with or without prior treatment with pharmacologic agents directed at reperfusion injury. There is conflicting evidence that I-R injury occurs in the equine small intestine. One hour of complete ischemia followed by one hour of reperfusion resulted in moderate to severe mucosal injury; this injury was typified by disrupted attachment of enterocytes from the basement membrane to the lamina propria. This detachment is believed to have occurred due to the accumulation of subepithelial fluid, resulting in epithelial separation. The administration of dimethylsulfoxide failed to protect the mucosa from this I-R injury. Prichard et. al., documented a significant level of XO/XDH in normal, non-ischemic, equine small intestine. One hour of intestinal ischemia caused significant conversion of XDH to XO, which suggests this enzyme system may be involved in I-R injury of the small intestine. In vitro, superoxide dismutase provided protection of the equine small intestinal mucosal cells during reoxygenation following a period of anoxia. However, when superoxide
dismutase was administered to ponies 5 minutes before or 30 minutes after induction of small intestinal ischemia, or immediately prior to reperfusion, no protection against mucosal injury was observed.\textsuperscript{113}

Limited studies have evaluated the effects of reperfusion of ischemic large colon in the horse. One study concluded that reperfusion injury occurs in the pony large colon based on morphologic and ultrastructural assessment\textsuperscript{114}; however, due to the experimental design (small number of experimental animals, use of two different models of ischemia, and lack of controls) it is impossible to discern if reperfusion injury actually occurred. Two other studies failed to demonstrate reperfusion injury in the equine large colon following 1 to 2 hours of complete arteriovenous ischemia.\textsuperscript{115,116} Reeves et al., utilized a one hour ischemic period followed by two hours of reperfusion and evaluated histopathology, immunohistochemical staining for albumin, and mucosal and serum reduced and oxidized glutathione levels. There was no evidence of reperfusion injury using these markers and the administration of dimethylsulfoxide exerted no protective effects. Wilkins et al., used a two hour ischemic period followed by one hour of reperfusion of the large colon in ponies. There was no morphologic evidence to support reperfusion injury in the one hour or 48 hour post-reperfusion biopsies. Measurement of mucosal XO/XDH concentrations were inconsistent and present in low levels. The authors concluded that XO/XDH may not contribute to post-ischemia reperfusion injury in the equine large colon. Neutrophils, not XO, have been shown to be the source of OFR generation in the colon of rabbits.\textsuperscript{117}

Colonic microvascular injury following I-R was evaluated by measuring the ratio of lymph-to-plasma protein.\textsuperscript{119} Two hours of complete arteriovenous occlusion followed by 30 minutes of reperfusion resulted in significant increases in microvascular permeability in the equine large colon.\textsuperscript{119} This study was unable to differentiate the effects of ischemia-reperfusion versus the effects of ischemia alone on the observed increase in
microvascular permeability because there was no comparison to a comparable period of ischemia alone.

Lipid peroxidation occurs in the large colon of horses during complete arteriovenous ischemia. Colonic lipoperoxidation was quantitated by measuring tissue malondialdehyde concentrations. Malondialdehyde is a stable byproduct of cell membrane lipid peroxidation and is frequently used as an indicator of intestinal injury following I-R. A decrease in the activity of superoxide dismutase, an endogenous anti-oxidative enzyme, accompanied the increased tissue MDA during ischemia. Decreased SOD activity may predispose the colonic mucosa to OFR-mediated damage.

Provost et. al., studied the effects of one hour ischemia followed by one hour of reperfusion using an experimental model of 720° large colon volvulus in ponies. The potential beneficial effects of heparin treatment prior to surgical correction were also investigated. Colonic tissue pH progressively decreased during the ischemic period, but within 10 minutes of detorsion (reperfusion) it had returned to baseline values. The colon blood flow was reduced to 0 ml/min upon creation of the 720° volvulus and remained significantly reduced until 50 minutes following detorsion. Colonic vascular resistance was significantly increased during the first 50 minutes following reperfusion. The mean arterial blood pressure was significantly decreased within 10 minutes of reperfusion. Using this model, Stick et. al., demonstrated an increased level of colonic venous 6-keto prostaglandin F2 alpha, and thromboxane B2 immediately upon reperfusion. The TXB2 returned to baseline values by one hour of reperfusion; however, the 6-keto prostaglandin F1 alpha remained increased throughout the reperfusion period. The concentration of these prostaglandins were higher in portal venous blood than in the pulmonary arterial or jugular venous blood which suggests they are generated in the splanchnic circulation during and following detorsion of a large colon volvulus and are cleared by the liver. Intravenous
administration of heparin (80 IU/kg body weight) 30 minutes prior to detorsion prevented the hypotension, increased colonic vascular resistance, and increased TXB2 concentration associated with detorsion; it also effectively increased the colon blood flow for 40 minutes during reperfusion.121

Potential Therapies Directed Toward Reperfusion Injury

A number of exogenous chemicals and pharmacologic agents have been used experimentally in laboratory animals to prevent or attenuate gastrointestinal injury associated with I-R (Table 1.1). Many of these agents are used as pharmacologic tools to further elucidate the pathophysiologic mechanisms involved in reperfusion injury. These agents utilize mechanisms similar to the endogenous antioxidants; prevent OFR generation, scavenge OFRs, augment endogenous antioxidant defense mechanisms, and prevent amplification of tissue damage by neutrophils (Figure 1.4). These agents may effectively prevent or reduce tissue injury associated with reperfusion if administered before irreversible tissue injury is sustained due to ischemia. A limited number of these substances have been studied in intestinal I-R in horses.

Xanthine oxidase inhibition:

Xanthine oxidase inhibitors, such as allopurinol have been shown to prevent injury associated with reperfusion of ischemic intestine.39,43,45,50 Allopurinol and its active metabolite, oxypurinol, are structural analogues of hypoxanthine and thus competitively inhibit xanthine oxidase-catalyzed conversion of hypoxathine to uric acid.123 Folic acid and pterin aldehyde are additional competitive inhibitors of XO.35,124 Pretreatment of cats with allopurinol prevented the increase in microvascular permeability and neutrophil adherence and infiltration associated with I-R.43,45,50 Morphologic mucosal injury is not
Table 1.1

Pharmacologic agents and chemical that have potential to reduce ischemia-reperfusion injury.  
XO = xanthine oxidase, NADPH = nicotinamide adenine dinucleotide phosphate, 
SOD = superoxide dismutase, O$_2^-$ = superoxide radical, H$_2$O$_2$ = hydrogen peroxide, H$_2$O = water, LOOH = lipid hydroperoxides, HOCl = hypochlorous acid, OH$^-$ = hydroxyl radical, DMSO = dimethyl sulfoxide, DMTU = dimethylthiourea, PLA$_2$ = phospholipase A$_2$, PAF = platelet activating factor, LTB$_4$ = leukotriene B$_4$. 
Table 1.1

<table>
<thead>
<tr>
<th>Drug Class</th>
<th>Mechanism</th>
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<tr>
<td><strong>XO inhibitors</strong></td>
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<tr>
<td>Allopurinol</td>
<td>Inhibits XO activity</td>
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<tr>
<td>Oxypurinol</td>
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<td>Folic Acid</td>
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<tr>
<td>Pterin Aldehyde</td>
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<td><strong>NADPH Oxidase Inhibitors</strong></td>
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<td></td>
<td>Inhibits NADPH oxidase in neutrophils</td>
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<tr>
<td><strong>SOD and SOD-mimetics</strong></td>
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<tr>
<td>Bovine SOD</td>
<td>Promotes dismutation of O$_2^-$ to H$_2$O$_2$</td>
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<tr>
<td>Human Recombinant SOD</td>
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<td>Manganese</td>
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<tr>
<td><strong>Catalase</strong></td>
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<td></td>
<td>Catalyzes conversion of H$_2$O$_2$ to H$_2$O</td>
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<tr>
<td><strong>Nonenzymatic OFR Scavengers</strong></td>
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<tr>
<td>Albumin</td>
<td>Scavenges LOOH and HOCl</td>
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<tr>
<td>Mannitol</td>
<td>Scavenges O$_2^-$</td>
</tr>
<tr>
<td>DMSO</td>
<td>Scavenges OH$^-$</td>
</tr>
<tr>
<td>DMTU</td>
<td>Scavenges OH$^-$ and H$_2$O$_2$</td>
</tr>
<tr>
<td>21-Aminosteroids</td>
<td>Scavenges LOOH and O$_2^-$</td>
</tr>
<tr>
<td><strong>Inhibits Iron Redox Cycling</strong></td>
<td></td>
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<tr>
<td>Deferoxamine</td>
<td>Chelates Fe$^{3+}$ which inhibits iron-catalyzed conversion of H$_2$O$_2$ to OH$^-$</td>
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<tr>
<td><strong>Phospholipase A$_2$ Inhibitors</strong></td>
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<tr>
<td>Quinacrine</td>
<td>Inhibit PLA$_2$ activity which decreases release of phospholipid metabolites from cell membrane</td>
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<tr>
<td>Dexamethasone</td>
<td></td>
</tr>
<tr>
<td><strong>Anti-neutrophil Agents</strong></td>
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<tr>
<td>CD11/CD18 Monoclonal Ab PAF Antagonists</td>
<td>Block CD11/CD18 neutrophil adhesion molecules</td>
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<tr>
<td>WEB 2086</td>
<td>Block PAF-dependent neutrophil adhesion molecules</td>
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<tr>
<td>BN 52021</td>
<td></td>
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<tr>
<td>LTB$_4$ Antagonist</td>
<td></td>
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<tr>
<td>LY-255283</td>
<td>Prevent neutrophil chemotaxis and adherence</td>
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<tr>
<td>SC-41930</td>
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</tr>
<tr>
<td>Lipoxygenase Inhibitor</td>
<td></td>
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<tr>
<td>Northydroguaiaretic acid L663, 536</td>
<td>Prevent formation of LTB$_4$ and secondary neutrophil chemotaxis and adherence</td>
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<tr>
<td>Adenosine</td>
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<tr>
<td>Elfin C</td>
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<tr>
<td>L658,758</td>
<td>Interferes with neutrophil adherence and extravasation</td>
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<tr>
<td>Elfin C</td>
<td>Inhibits elastase which prevents neutrophil extravasation</td>
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<td>Neutropenic Agents</td>
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<td>Anti-neutrophil serum Hydroxyurea</td>
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<tr>
<td>Cyclooxygenase Inhibitors</td>
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<td></td>
<td>Block production of prostaglandins and thromboxane</td>
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Figure 1.4

Schematic representation of the pathways involved in ischemia-reperfusion injury and the specific location where pharmacologic agents and chemicals can either inhibit or catalyze these pathways. XDH = xanthine dehydrogenase, XO = xanthine oxidase, O$_2^-$ = superoxide radical, H$_2$O$_2$ = hydrogen peroxide, OH$^-$ = hydroxyl radical, SOD = superoxide dismutase, Mn = manganese, DMSO = dimethyl sulfoxide, DMTU = dimethylthiourea, PAF = platelet activating factor, LT = leukotriene, PG = prostaglandin, NADPH = nicotinamide adenine dinucleotide phosphate, NSAIDS = nonsteroidal anti-inflammatory drugs, HOCl = hypochlorous acid. Therapeutic agents represented by italics contained in black boxes. These therapeutic agents reduce ischemia-reperfusion injury of the intestinal mucosa by altering oxygen free radical production and metabolism, neutrophil activation, and phospholipid metabolism.
worsened following reperfusion of ischemic intestine when rats are pretreated with allopurinol.\textsuperscript{125} These beneficial effects of allopurinol in preventing intestinal I-R injury have been documented after prolonged enteral administration. It is believed that the lack of an effect with intravenous allopurinol is due to ineffective inhibition of XO; with longer time periods between allopurinol administration and the onset of I-R the allopurinol is converted to oxypurinol, a more effective inhibitor of XO, and XO inhibition is more complete.\textsuperscript{126} Some studies have suggested that the beneficial effects of allopurinol in I-R are due to the scavenging of free radicals\textsuperscript{127}; however, Zimmerman et. al., refute this contention.\textsuperscript{128} Enteral allopurinol administration is not practical for horses with acute abdominal disease associated with ischemic intestine. Horses were pretreated with intravenous allopurinol prior to the induction of endotoxic shock.\textsuperscript{129} Allopurinol appeared to attenuate the severity of signs associated with endotoxemia. A dose of 5 mg/kg was apparently more effective than 50 mg/kg.

\textbf{Enzymatic free radical scavengers:}

Superoxide dismutase (SOD) is an endogenous cellular anti-oxidative enzyme that catalyzes the conversion of superoxide radicals to hydrogen peroxide. There are two forms of this enzyme: the first is located in the cytosol and contains copper and zinc and the second is located in the mitochondria and contains manganese. Exogenous sources of this enzyme exist and can be administered parenterally. The commercially available bovine form of SOD and the recombinant human SOD are equipotent, but both have a short circulating half-life. Administration of SOD to cats\textsuperscript{32,33} and rats\textsuperscript{130} prior to the onset of intestinal ischemia has been shown to attenuate the microvascular and mucosal injury associated with reperfusion. Adherence and infiltration of neutrophils into the interstitium upon reperfusion of ischemic intestine is reduced by SOD.\textsuperscript{43,45} Pretreatment of rats with
SOD prior to reperfusion following one hour of superior arterial occlusion decreased lipoperoxidation and prolonged survival. Superoxide dismutase provided protection of anoxic equine small intestinal mucosa from the effects of reoxygenation in vitro, however, no protection in the equine jejunum or colon in vivo has been demonstrated. The use of superoxide dismutase is cost-prohibitive in the adult horse.

Catalase is a hemoprotein bound to peroxisomes which catalyzes the conversion of hydrogen peroxide to water. Catalase administration prior to ischemia of the cat small intestine reduced neutrophil infiltration into the intestinal mucosa upon reperfusion.

Glutathione peroxidase is a selenium-dependent, cytosolic enzyme that catalyzes conversion of hydrogen peroxide to water or organic peroxides to water and alcohols. Glutathione tissue levels are depleted during I-R.

**Nonenzymatic free radical scavengers:**

A number of nonenzymatic free radical scavengers exist that do not have SOD activity; mannitol, albumin, dimethyl sulfoxide, and dimethylthiourea. Mannitol and DMSO scavenge hydroxyl radicals, albumin scavenges lipid hydroperoxides, and dimethylthiourea (DMTU) scavenges hydroxyl radicals, hydrogen peroxide, and hypochlorous acid. Dimethyl sulfoxide (DMSO) is an exogenous agent that possesses hydroxyl radical scavenging properties. Pretreatment with DMSO attenuates the increased microvascular permeability and increased neutrophil adherence and infiltration associated with ischemia and reperfusion of the cat and rat small intestine. The administration of DMSO to horses prior to ischemia of the large colon or small intestine fails to produce any beneficial effects on mucosal morphology or permeability. Dimethyl sulfoxide may be toxic to cells at concentrations required to scavenge hydroxyl radicals. Other potentially toxic metabolites, such as methyl and methylperoxy radicals,
may form secondary to the reaction between DMSO and the hydroxyl radical. These toxic metabolites could result in tissue injury even though they are less reactive than the hydroxyl radical. Currently, there is no documented evidence that DMSO is beneficial in intestinal ischemia-reperfusion in the horse. Intravenous administration of concentrated DMSO (>10-20%) can cause hemolysis due to its hyperosmolality. There are few other known toxic side effects of DMSO.

Nonenzymatic free radical scavengers exist which mimic the action of SOD. Manganese chloride is a simple inorganic manganous salt that demonstrates superoxide dismutase-like activity. Simple manganese salts have been shown to function effectively as in vivo superoxide scavengers. They are relatively nontoxic and on a per weight basis are nearly as effective as superoxide dismutase in scavenging superoxide anions. Recall that the first step in oxygen radical production is the synthesis of superoxide radicals which are further converted into hydrogen peroxide and hydroxyl radicals. Superoxide anion and hydrogen peroxide are not considered especially toxic to cells; however, the hydroxyl radical is highly reactive and results in lipoperoxidation of cell membrane lipids resulting in cell injury and death. The manganese (Mn) ion acts both as an electron acceptor and electron donor in a two step oxidation, reduction, and re-oxidation of Mn from Mn(III) to Mn(II) and back to Mn(III). The net reaction converts two superoxide anions into hydrogen peroxide and oxygen. Manganese chloride may prevent the injury associated with ischemia and reperfusion of the equine large colon by scavenging superoxide anion and halting the production of hydroxyl radicals. Manganese-mediated superoxide dismutation is less efficient than using a similar molar quantity of superoxide dismutase; however, due to the widespread availability, low cost, and low toxicity of MnCl₂ this may prove to be a useful alternative for scavenging superoxide anions associated with reperfusion injury. This compound is inexpensive compared to a
comparable dose of a superoxide dismutase enzyme preparation.

Intravenously administered MnCl$_2$ to horses resulted in dose-related increases in plasma superoxide scavenging ability for one hour following infusion.$^{137}$ There were minimal to no adverse effects of MnCl$_2$ administration on systemic cardiovascular parameters and hypotension did not develop in these horses. Some of the horses demonstrated defeation of watery feces, pawing, hyperexcitability, flank watching, and sweating; however, these effects were short lived and were easily controlled with butorphanol tartrate (analgesic). A dose of 10 mg/kg resulted in a higher peak plasma Mn concentration and resulted in a greater superoxide scavenging ability than did a 5 mg/kg dose.$^{137}$ Clinical use of MnCl$_2$ for the treatment of intestinal ischemia-reperfusion in horses awaits further study.

Endogenous nonenzymatic antioxidants include alpha-tocopherol (vitamin E), which scavenges lipid peroxy radicals; ascorbic acid (vitamin C); and beta-carotene. Endogenous sources of these substances can be supplemented by administering exogenous sources. The effect of increasing these endogenous antioxidants against intestinal I-R is unknown, however, horses should have adequate levels if consuming a good quality feed.

Iron chelation or sequestration:

Deferoxamine, an iron chelator, inhibits the iron-catalyzed formation of hydroxyl radicals from hydrogen peroxide (Haber-Weiss reaction).$^{20}$ Deferoxamine does not interfere with the oxygen transport properties of hemoglobin; it is water soluble and has low toxicity.$^{138}$ Pretreatment with deferoxamine attenuates microvascular injury and neutrophil infiltration associated with ischemia and reperfusion of the cat small intestine.$^{37,46}$ Endogenous iron-binding proteins (transferrin, ferritin) sequester iron so its is unavailable to participate in these deleterious reactions.
21-aminosteroids:

The 21-aminosteroids, "lazaroids", are a novel group of compounds developed by modification of the structure of glucocorticoids. They have amino groups substituted on the 21-carbon of the steroid nucleus, which inactivates the corticosteroid receptor activity of these compounds. They were designed to localize in cell membranes and inhibit lipoperoxidation without the deleterious side effects of glucocorticoid or mineralocorticoid activity. These compounds are beneficial in attenuating the deleterious effects of hemorrhagic, traumatic and splanchnic arterial occlusion shock. These compounds scavenge superoxide radicals and lipid hydroperoxides, which inhibits iron-catalyzed lipid peroxidation and arachidonic acid release. The 21-aminosteroid U74006F has been shown to reduce malondialdehyde concentrations following reperfusion of surgically induced gastric dilatation volvulus in dogs indicating it functions to inhibit reperfusion-mediated lipoperoxidation. Administration of U74006F after 60 minutes of ischemia resulted in maintenance of small intestinal villus height in rats. Intravenous administration of aminosteroid U-74389G at 3 mg/kg and 10 mg/kg to horses 15 minutes prior to reperfusion following 2 hours of complete arteriovenous occlusion prevented the reperfusion-induced decrease in colonic mucosal surface area. The reperfusion-induced increase in colonic mucosal myeloperoxidase activity was partially attenuated by administration of 3 mg/kg of U-74389G. There are no known untoward effects of 21-aminosteroids, however, their origin from corticosteroids suggests caution should be used with these compounds because of the possibility of inducing or aggravating laminitis. There are no reports of the effect of 21-aminosteroids on horses.
Nitric oxide:

Nitric oxide (NO), also known as endothelium-derived relaxant factor, is a soluble gas produced by the endothelium from L-arginine. Nitric oxide functions in modulating basal vascular tone via cGMP-mediated vasodilation. It also functions to inhibit platelet and neutrophil aggregation and scavenges superoxide radicals. Endothelial damage, such as might occur with ischemia, results in decreased NO production. Inhibition of NO synthesis results in a neutrophil-independent increase in intestinal mucosal permeability. Decreased NO can result in further tissue injury due to decreased blood flow (vasoconstriction), platelet and neutrophil aggregation, and OFRs. Nitric oxide appears to protect the intestinal microvasculature and mucosal epithelium from deleterious effects of ischemia and reperfusion. Nitric oxide is consumed during scavenging of superoxide radicals; thus NO depletion can occur during I-R. The extremely short half-life of NO renders it ineffective when administered itself, however, other pharmacologic agents (nitroprusside, inorganic and organic nitrites, nitrate esters, L-arginine) can either donate NO or result in its synthesis.

Inhibition of phospholipid-derived mediator activity:

Pharmacologic or chemical manipulation of selected pathways of phospholipid metabolism have been shown to protect against reperfusion injury. Phospholipase A₂ inhibition prevents the accumulation of malondialdehyde and myeloperoxidase in intestinal mucosa, and prevents morphologic mucosal injury associated with intestinal I-R in rats. Phospholipase A₂ inhibitors documented to have protective effects include quinacrine and nordihydroguaiaretic acid. Corticosteroids have PLA₂ inhibitory effects thus blocking the liberation of arachidonic acid. Lipocortin is a protein released from cells upon exposure to anti-inflammatory doses of corticosteroids, and binds to phospholipase...
A2 to prevent release of arachidonic acid. Pretreatment of cats with methylprednisolone did not attenuate the increased microvascular permeability associated with small intestinal ischemia and reperfusion. Dexamethasone pretreatment attenuated the increases in microvascular permeability associated with I-R of the rat cremaster muscle. Other corticosteroids may have some protective effects against intestinal I-R injury, but the likelihood of inducing laminitis may outweigh their potential benefit in horses. A more plausible approach may be to inhibit prostaglandin and leukotriene synthesis or antagonize the effects of PAF.

Cyclooxygenase and lipooxygenase inhibitors may provide some protection from mucosal injury associated with I-R: cyclooxygenase inhibition alone exacerbated the increase in mucosal permeability associated with intestinal I-R. When arachidonic acid metabolism via the cyclooxygenase pathway is inhibited it may be shunted to the lipooxygenase pathway resulting in a further increase in leukotriene production. Lipooxygenase inhibition as well as LTB4 antagonism prevented neutrophil infiltration associated with reperfusion of ischemic intestine. Simultaneous cyclooxygenase and lipooxygenase inhibition may be beneficial in reducing I-R injury of the intestine.

Platelet-activating factor plays a role in the increased microvascular permeability, and increased neutrophil adherence and infiltration, following reperfusion of ischemic intestine. Administration of a PAF antagonist attenuates the microvascular injury and the adherence and extravasation of neutrophils in the intestine associated with I-R injury. Administration of a PAF antagonist (WEB 2086) to anesthetized ponies prevented the hypotension, leukopenia, in vitro platelet aggregation, and death associated with PAF infusion. When administered to ponies with experimental large colon volvulus of one hour duration this PAF antagonist prevented some of the cellular responses associated with ischemia and reperfusion, however, it did not prevent the hemodynamic
Interference with neutrophil chemotaxis, adherence or activation:

Inhibiting secondary tissue injury caused by neutrophils can be achieved by blocking chemotactic cytokines, inhibiting neutrophil-endothelial adhesion, suppressing NADPH oxidase activity, or by causing neutropenia. Lipoxygenase inhibitors, LTB4 antagonists, and PAF antagonists reduce intestinal neutrophil infiltration and tissue injury. Monoclonal antibodies directed against the CD11/CD18 glycoprotein adhesion molecule reduce neutrophil-endothelial adhesion and intestinal injury. Local anesthetics, calcium channel blockers, and nonsteroidal anti-inflammatory agents interfere nonspecifically with the NADPH oxidase system. Induction of neutropenia with anti-neutrophil serum reduces intestinal I-R injury in cats.

Macromolecule-mediated decreased microvascular permeability:

Hydroxyethylstarch (HES) macromolecules have been demonstrated to reduce microvascular permeability and tissue injury associated with I-R. Ischemia causes increased microvascular permeability by altering endothelial cell junctions, particularly in post-capillary venules. Increased microvascular permeability has been demonstrated in the equine large colon following I-R. This alteration in permeability allows albumin and other molecules to escape from the vascular space into the interstitium. The accumulation of albumin and other substances draws water and electrolytes into the interstitium creating a "third space" accumulation of fluid (edema). This edema can biophysically exacerbate tissue injury by impairing diffusion of oxygen and nutrients from the vascular space to the cells and the removal of carbon dioxide and other waste products from the cells. This third space accumulation of fluid can lead to a vicious cycle of increased tissue pressure due to
lymphatic collapse, increased venous pressure, and decreased arteriolar circulation. The combination of mechanical and metabolic alterations in the interstitium can result in tissue infarction and necrosis. It has been shown that certain sizes and shapes of macromolecules are more effective in preventing the increased microvascular permeability associated with I-R.\textsuperscript{156} These molecules decrease microvascular permeability by sealing the endothelial separations.\textsuperscript{154} Although there appear to be differences in the size and shape of molecules which will effectively seal endothelium in different organs and species, molecules in the range of 100,000-300,000 daltons have been shown to be more effective in attenuating the increased microvascular permeability associated with I-R.\textsuperscript{152,153,155,156}

No single drug has yet been identified that completely prevents the injury associated with reperfusion of ischemic tissue. Combination therapy may provide more protection by blocking sequential steps in the pathophysiology of reperfusion injury. The observed effect of these agents is variable due to several factors. A number of different models of I-R have been used to evaluate these drugs which makes comparisons difficult; the effect of antioxidant drugs likely depend on the type and duration of ischemia. The pharmacokinetics of these agents are likely not uniform across species. The time of drug administration relative to ischemia and reperfusion is probably one of the most important factors that determine if an antioxidant drug protects against reperfusion injury. The total tissue injury sustained secondary to ischemia actually reflects the combined effects of ischemia and reperfusion. Following short periods of ischemia, the degree of tissue injury is so insignificant that the effect of antioxidant treatment is undetectable. Conversely, following prolonged periods of ischemia, the injury sustained by the tissue due to ischemia is so severe that the reperfusion component is inconsequential. The theoretical therapeutic window is that period after which the majority of total tissue injury occurs due to reperfusion (Figure 1.5).\textsuperscript{133}
Figure 1.5

Graphic illustration of the contribution of both the ischemic component and reperfusion component to total tissue injury. Following short periods of ischemia neither component causes significant injury. With continued ischemia, tissue injury can occur due to the effects of ischemia or due to the biochemical processes occurring upon reperfusion. As the ischemic period progresses, the reperfusion component may contribute significantly to total tissue injury. Following prolonged periods of ischemia the injury occurring due to the ischemic component becomes so severe that it overwhelms the reperfusion component; the reperfusion component of injury thus becomes inconsequential. Antioxidant therapy has little beneficial effect on tissue injury if administered following short periods of ischemia or after prolonged ischemia. A “therapeutic window” exists where antioxidant therapy can have a dramatic effect in ameliorating reperfusion injury. Modified with permission Bulkley GB, Br J Cancer, 1987, 55 Suppl. VIII, 66-73.
Figure 1.5
Summary

Restoration of blood flow following a period of intestinal ischemia is necessary to maintain cell function and viability, however, the reintroduction of oxygen can initiate a cascade of events that exacerbates tissue injury. Intestinal I-R injury is manifest as increased microvascular and mucosal permeability and mucosal necrosis. Reperfusion injury begins with xanthine oxidase catalyzing the conversion of hypoxanthine to superoxide radicals. Superoxide radicals are further reduced to highly reactive hydroxyl radicals which initiate lipid peroxidation. Lipoperoxidation causes functional and structural alterations in cell membrane lipids and can liberate numerous inflammatory mediators which exacerbate tissue damage. Neutrophils are recruited into tissues during ischemia and upon reperfusion; the neutrophils then undergo degranulation and release destructive products (proteases and oxygen free radicals) which mediate further tissue injury. A limited number of experimental studies in the gastrointestinal tract of horses have demonstrated I-R injury. Additional studies are necessary to further elucidate and sequence the precise pathophysiologic mechanisms occurring in the equine intestine during ischemia and reperfusion. Therapy should be focused on prevention of I-R injury by pharmacologically or chemically inhibiting or modifying these pathophysiologic pathways. Selected pharmacologic agents, such as xanthine oxidase inhibitors, free radical scavengers, cyclooxygenase/ lipoxygenase inhibitors, PLA$_2$ inhibitors, and PAF antagonists may offer novel, scientifically relevant and yet practical approaches to alleviating intestinal I-R injury in horses. This should ultimately improve survival of horses with naturally occurring intestinal strangulation obstruction.
List of References


33. Parks DA, Shah AK, Granger DN. Oxygen radicals: effects on intestinal vascular


88. Filep J, Dahinden C. Increased levels of platelet activating factor in blood following intestinal ischemia in the dog. *Biochem Biophys Res Commun* 1989.


113. Freeman DE, Johnston JK. Failure of superoxide dismutase to mitigate changes caused by ischemia and reperfusion in horse intestine. 27th Annual ACVS Scientific Meeting 1992;32.


CHAPTER II
A PILOT STUDY EVALUATING FOUR MODELS OF EQUINE LARGE COLON ISCHEMIA AND REPERFUSION

Summary

Four models of large colon ischemia-reperfusion were evaluated in horses. The models evaluated included 1) 50% reduction in colonic arterial flow with transmural compression; 2) complete venous occlusion with no transmural compression; 3) complete arteriovenous occlusion with transmural compression; and 4) 80% reduction in colonic arterial blood flow with transmural compression. One horse (4 horses total) was used to evaluate each model of colonic ischemia. Colonic blood flow was reduced for 3 hrs using each model and then blood flow was restored and maintained for 1 hr. Full-thickness biopsies were harvested from the left ventral colon at 30 min intervals during the experiment and processed for light microscopic evaluation. Reduction of arterial flow to 20% of baseline and complete arteriovenous occlusion combined with transmural compression produced the most mucosal injury upon reperfusion. This injury was characterized by increased surface mucosal loss, increased depth of mucosal loss, and mucosal hemorrhage. The two other models evaluated did not produce mucosal loss. The low flow model which produced a reduction in colonic arterial flow to 20% of baseline was chosen to evaluate and characterize reperfusion injury in the equine large colon.
Introduction

Reperfusion injury is that component of tissue damage that occurs following re-establishment of blood flow after a period of ischemia.\textsuperscript{1,2} Gastrointestinal reperfusion injury has been thoroughly documented in laboratory animals upon restoration of blood flow following ischemia.\textsuperscript{3} Reperfusion injury has most consistently been observed following partial or low flow arterial occlusion.\textsuperscript{4} Reperfusion injury has not been documented in the equine large colon, but only a limited number of studies have evaluated colonic ischemia-reperfusion (I-R) in horses.\textsuperscript{5,6} The majority of these studies have used complete arteriovenous model of vascular occlusion and others have used either complete arterial or complete venous occlusion.\textsuperscript{5,6} There have been no studies to evaluate partial or low flow arterial ischemia in the equine large colon. We hypothesize that reperfusion injury occurs in the large colon of horses. We believed that low flow colonic arterial occlusion would yield detectable mucosal injury following re-establishment of blood flow and was likely an appropriate model of colonic ischemia to evaluate reperfusion injury in the equine large colon. Therefore, the purpose of this pilot study was to evaluate and compare four different models of large colon ischemia for producing reperfusion injury following 3 hrs of ischemia. The models evaluated included 1) 50\% reduction in colonic arterial flow with transmural compression; 2) complete venous occlusion with no transmural compression; 3) complete arteriovenous occlusion with transmural compression; and 4) 80\% reduction in colonic arterial blood flow with transmural compression.

Materials and Methods

This project was approved and performed under the guidelines of the Institutional Laboratory Animal Care and Use Committee of The Ohio State University. One horse was used to evaluate each of the following models of large colon ischemia: (1) 50\% reduction in
colonic arterial flow with transmural compression, (2) complete colonic venous occlusion without transmural compression, (3) complete arteriovenous occlusion with transmural compression, and (4) 80% reduction in colonic arterial flow with transmural compression. Horses were instrumented with a jugular venous catheter* for administration of anesthetic drugs and isotonic, polyionic fluids¹. The horses were induced to general anesthesia with xylazine* (0.5 mg/kg IV), guaifenesin¹ (30 mg/kg IV), and thiamylal sodium* (4 mg/kg IV) and were maintained under general anesthesia with halothane¹ (1-3%) in oxygen. Horses were mechanically ventilated at 6-12 breaths per minute at a maximum inspiratory pressure of ≤ 25 cm H₂O. Mean arterial blood pressure was maintained ≥ 70 mm of Hg by increasing the isotonic fluid infusion rate and/or decreasing anesthetic depth. Horses were placed in dorsal recumbency and the large colon was exteriorized through a ventral midline celiotomy. A doppler ultrasonic blood flow probe¹ was placed externally on the colonic artery supplying the ventral colon in horses 1, 3, and 4. The colonic vein draining the ventral colon in horse 2 was transected and a flow probe was inserted in the vein between the two transected ends for measurement of colonic venous blood flow. Horses remained anesthetized for the duration of the study and were euthanatized at its conclusion with an overdose of sodium pentobarbital¹.

Full-thickness colonic biopsies (2 x 4 cm) were harvested from the left ventral colon and immediately placed in 10% formalin. The biopsies were cut into 4 um sections, processed routinely and stained with hematoxylin and eosin. Colon sections were evaluated by two investigators for % surface mucosal loss, % depth mucosal loss, and mucosal hemorrhage.¹

Hemodynamic measurements were recorded and colonic biopsies were harvested at baseline, at 30 min intervals throughout the study and at 185, 190, and 195 min, which corresponded to 5, 10, and 15 min of reperfusion. Following collection of baseline data,
colonic ischemia was induced. Transmural compression of colonic blood flow was produced in horses 1, 3 and 4 by placing a separate 20 cm segment of latex tubing tightly around the circumference of both the dorsal and ventral colon. Rummel tourniquets were placed around either the colonic arteries, veins or both (depending on the model) and tightened until the desired blood flow reduction was achieved. Colonic blood flow was continuously monitored. Colonic blood flow was restored after 3 hrs of ischemia by releasing the vascular tourniquets and transmural compression tourniquets and the colon was reperfused for 1 hr.

Results

There was minimal or no detectable mucosal loss in the horse with 50% reduction in colonic arterial blood flow or in the horse with complete venous occlusion, but there was significant mucosal loss in the 2 horses with either 80% reduction in arterial flow or complete arteriovenous occlusion (Figures 2.1 and 2.2). There was significant mucosal hemorrhage in horses 2-4 (Figure 2.3). The magnitude of surface mucosal loss and crypt necrosis were similar in the horses with 80% reduction in colonic arterial flow and complete arteriovenous occlusion.

Discussion

Transmural colonic compression combined with a reduction of colonic arterial blood flow to 20% of baseline or complete arteriovenous occlusion produced dramatic mucosal injury following 3 hrs of ischemia and 1 hr of reperfusion. The other two models (50% reduction in arterial flow, complete venous occlusion) did not produce mucosal loss. Intestinal reperfusion injury has been demonstrated most consistently following low flow ischemia. The degree of surface mucosal loss and crypt necrosis with low flow ischemia was similar to that observed with complete arteriovenous occlusion. Complete
Figure 2.1

Percent surface mucosal disruption in the large colon of horses subjected to 3 hrs of either (1) 50% reduction in colonic arterial flow with transmural compression, (2) complete venous occlusion with no transmural compression, (3) complete arteriovenous occlusion with transmural compression, and (4) 80% reduction in colonic arterial blood flow with transmural compression, followed by 3 hrs of reperfusion.
Figure 2.1

Mucosal Surface Disruption (%)
Figure 2.2

Percent depth of mucosal loss in the large colon of horses subjected to 3 hrs of either (1) 50% reduction in colonic arterial flow with transmural compression, (2) complete venous occlusion with no transmural compression, (3) complete arteriovenous occlusion with transmural compression, or (4) 80% reduction in colonic arterial blood flow with transmural compression, followed by 1 hr of reperfusion.
Estimated Depth Mucosal Loss (%)
Figure 2.3

Mucosal hemorrhage in the large colon of horses subjected to 3 hrs of either (1) 50% reduction in colonic arterial flow with transmural compression, (2) complete venous occlusion with no transmural compression, (3) complete arteriovenous occlusion with transmural compression, and (4) 80% reduction in colonic arterial blood flow with transmural compression, followed by 3 hrs of reperfusion.
Mucosal Hemorrhage

Figure 2.3

Model

0 1 2 3 4
arteriovenous occlusion of the large colon produces irreversible mucosal injury after 3-4 hrs of ischemia. The degree of colonic ischemia achieved with the low flow model used in this study produced detectable colonic mucosal injury, however, the mucosa was not completely lost nor irreversibly damaged. Differentiating between the tissue injury being caused by the reintroduction of blood flow versus only becoming manifest after blood flow is restored requires that the model of ischemia being used does not produce irreversible mucosal injury. The potential for increased mucosal hemorrhage during longer periods of reperfusion with complete arteriovenous occlusion could possibly interfere with the ability to detect slight differences in mucosal injury. Previous studies using complete arteriovenous models of colonic ischemia were unsuccessful in demonstrating large colon reperfusion injury in horses.

Based upon the results of this pilot study and reports of unsuccessful attempts at demonstrating reperfusion injury following complete arteriovenous occlusion we chose to use the low flow model of ischemia, which was produced by reducing colonic arterial flow to 20% of baseline flow, to evaluate and characterize reperfusion injury in the equine large colon. Although this model does not produce blood flow changes identical to those in naturally occurring large colon volvulus, it does mimic the arterial component of these vascular alterations. The lack of irreversible mucosal injury after 3 hrs of low flow ischemia is necessary to evaluate reperfusion injury. Although it does not mimic naturally occurring disease, minimizing mucosal hemorrhage should enable our detection of subtle histopathologic alterations indicative of reperfusion injury. We conclude that the low flow ischemia is the most suitable of the models evaluated in this study for further evaluating and characterizing large colon reperfusion injury. Subsequent investigations were completed comparing the effects of 3 hrs of low flow ischemia and 3 hrs of reperfusion to 6 hrs of ischemia alone.
Footnotes

Angiocath®, Becton Dickinson Vascular Access, Sandy, Utah.

Lactated Ringer’s, Baxter Healthcare Corporation, Deerfield, IL.

Rompun®, Mobay Corporation, Animal Health Division, Shawnee, KS.

Guailaxin®, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.


Halothane U.S.P., Halocarbon Laboratories, North Augusta, SC.

Drager AV Model NELAC-E, Anesthesia Ventilator, Telford, Pa.

Model T201, Transonic Systems, Inc., Ithaca, NY.

Beuthanasia®-D Special, Schering Plough Animal Health Corporation, Kenilworth, NJ.

List of References


7. Parks DA, Bulkley GB, Granger DN, et al. Ischemic injury in the cat small intestine:


CHAPTER III
CHARACTERIZATION OF THE HEMODYNAMIC AND METABOLIC ALTERATIONS IN THE EQUINE LARGE COLON DURING LOW FLOW ISCHEMIA AND REPERFUSION

Summary
The effects of low flow ischemia and reperfusion of the large colon on systemic and colonic hemodynamic and metabolic parameters were determined in horses. Twenty-four adult horses were randomly allocated to three groups: 1) sham-operated (n=6), 2) 6 hrs ischemia (n=9), and 3) 3 hrs ischemia and 3 hrs reperfusion (n=9). Low flow ischemia was induced in groups 2 and 3 by reducing colonic arterial blood flow to 20% of baseline. Heart rate, arterial blood pressures, cardiac index, pulmonary artery pressure, right atrial pressure, and colonic blood flow ($Q_{\text{colon}}$) were monitored. Arterial, mixed-venous, and colonic venous blood gas and oximetry analyses, whole blood lactate and pyruvate, and packed cell volume and total plasma protein were measured. Data were recorded and blood samples collected at baseline and at 30 min intervals for 6 hr; additionally, data were collected at 185, 190, and 195 min (corresponding to 5, 10, and 15 min of reperfusion in group 3). There were no differences among groups at baseline nor across time for any systemic hemodynamic or metabolic parameter. Colonic blood flow did not change across time in group 1. Colonic blood flow significantly decreased to 20% of baseline upon induction of ischemia in groups 2 and 3 and remained significantly decreased throughout the ischemic period in groups 2 (6 hrs) and 3 (3 hrs). Colonic blood flow significantly increased above baseline by 5 min of reperfusion in group 3. Colonic oxygen delivery and
oxygen consumption, and colonic venous pH, PO$_2$, % saturation of hemoglobin, and oxygen content were significantly decreased within 30 min after induction of ischemia in groups 2 and 3; colonic venous PCO$_2$, colonic oxygen extraction ratio, and lactate and pyruvate were significantly increased by 30 min of ischemia. These alterations continued throughout ischemia, but within 5 min of reperfusion in group 3, these parameters either returned to baseline (pH, PCO$_2$, lactate, pyruvate), significantly increased above baseline (PO$_2$, oxygen content, % saturation of hemoglobin) or significantly decreased below baseline (colonic oxygen extraction ratio). Colonic oxygen consumption remained decreased during reperfusion in group 3. Colonic mucosal ischemia-reperfusion injury observed in this model of ischemia was associated with local colonic hemodynamic and metabolic alterations in the presence of systemic hemodynamic and metabolic stability. Reactive hyperemia occurred upon restoration of colonic blood flow in group 3 and persisted during reperfusion. Colonic venous metabolic alterations were corrected upon reperfusion indicating an adaptation of the colon to the return of blood flow and oxygen delivery with a resultant decrease in anaerobic metabolism. The early alterations in these parameters may simply represent a washout of metabolic by-products.

Introduction

Colic is the leading cause of death in horses.$^1$ Intestinal ischemia commonly occurs in horses with colic, with small intestinal volvulus or incarceration$^{2,3}$ and large colon volvulus$^{4-7}$ being common causes. Volvulus of the large colon is fatal without timely surgical intervention.$^{4-7}$ Despite surgical correction and intensive medical therapy many horses die during the post-operative period reflecting the severe injury sustained by the colonic mucosa during ischemia and reperfusion. Restoration of intestinal blood flow following surgical correction of intestinal strangulation permits reperfusion of ischemic
intestine, reestablishing oxygen and nutrient delivery. Reperfusion, however, can initiate deleterious biochemical reactions that contribute to further tissue injury.\textsuperscript{8-12} This phenomenon, termed reperfusion injury, is that component of tissue injury sustained during reoxygenation following ischemia. Reperfusion injury occurs as a result of a complex series of cellular metabolic events that are initiated during the ischemic period and are activated upon reperfusion. During ischemia, tissue hypoxia develops, cellular energy stores become depleted, and tissue homeostatic mechanisms are disrupted. Upon reperfusion, oxygen free radicals and cytokines are produced\textsuperscript{13} resulting in lipoperoxidation of cell membranes and disruption of cellular organelles.\textsuperscript{8,12,14} Neutrophils and other inflammatory mediators amplify the reperfusion injury.\textsuperscript{10,15,16}

Local intestinal hemodynamic and metabolic alterations occur during intestinal ischemia and reperfusion.\textsuperscript{17-23} These alterations in intestinal blood flow, vascular resistance, oxygenation, and metabolism have been documented in dogs, cats, and rats, but few studies exist that evaluate these parameters in horses.\textsuperscript{23,24} Reestablishing blood flow after ischemia can result in either a period of continued underperfusion, a return to baseline flow, or reactive hyperemia, an increase in intestinal blood flow above baseline levels.\textsuperscript{25} Experimentally, the incidence of reactive hyperemia is dependent on the model of ischemia and the species being studied.

Systemic hemodynamic alterations occur following reperfusion of ischemic intestine in dogs, cats, and rats;\textsuperscript{19,26,27} superior mesenteric artery occlusion is frequently used as a model of inducing shock experimentally in laboratory animals.\textsuperscript{28} Hypotension or decreased cardiac output could exacerbate intestinal ischemic injury due to reduced perfusion despite surgical correction of the intestinal strangulation. It seems logical that systemic hemodynamics should be maintained within a physiologic range in order to determine the direct effects of experimental intestinal ischemia-reperfusion (I-R) on
Several models of large colon ischemia have been used to study I-R injury in the large colon of horses and ponies. Reperfusion injury was not observed in the large colon of horses using complete arteriovenous occlusion or complete venous occlusion models. Reperfusion injury was reported in the large colon of ponies; however, it is difficult to apply the results of this study to horses because of the small number of subjects, and the models of ischemia used. Studies in laboratory animals have demonstrated gastrointestinal I-R injury most consistently using partial or low flow arterial ischemia models. We believe low flow arterial ischemia mimics the arterial blood flow alterations that occur in naturally occurring large colon volvulus; arterial blood flow is generally reduced, but not completely occluded during volvulus. We could find no reports on hemodynamic and metabolic alterations occurring in the equine large colon during low flow arterial ischemia and reperfusion. We have documented histopathologic evidence of reperfusion injury using this model of low flow ischemia. The purpose of this study was to characterize the local colonic hemodynamic and metabolic alterations.

Materials and Methods

This project was performed with approval and under the guidelines of the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

Animals - Twenty-four horses of various breeds (9 Thoroughbreds, 8 Standardbreds, 3 Quarter Horses, 2 Saddlebreds, and 2 Hanoverians), ranging in age from 1-10 years and body weight from 350-570 kg were used. There were 11 females, 5 intact males, and 8 geldings. Horses were fed 1-2% of their body weight in grass hay per day and were adapted to their diet and environment for at least one week prior to the experiment; water was provided ad libitum. Food, but not water, was withheld for 24 hrs
prior to the experiment to decrease the colon contents.

**Instrumentation** - Horses were sedated with xylazine\(^*\) (0.5 mg/kg IV). Subcutaneous infiltration of mepivacaine hydrochloride\(^b\) was used to desensitize the skin for intravascular catheter insertion. A 14-gauge 13.3 cm teflon catheter\(^c\) was inserted into the left jugular vein for administration of anesthetic drugs and isotonic fluids. A balloon tipped, flow directed thermodilution catheter\(^d\) was inserted into the right jugular vein and advanced until the distal port was positioned in the pulmonary artery. This catheter was used to measure cardiac index (CI), mean pulmonary artery pressure (mPAP), and for the collection of mixed-venous blood samples. Polyethylene tubing\(^f\) (I.D. 1.57 mm) was inserted into the right jugular vein and advanced until the tip was positioned in the right atrium for measurement of mean right atrial pressure (mRAP). Catheter positions were documented by characteristic pressure waves. General anesthesia was induced with guaifenesins\(^g\) (50 mg/kg IV) and thiamylal\(^h\) (4.4 mg/kg IV) and was maintained with halothane\(^i\) (1-3%) in oxygen; horses were mechanically ventilated\(^j\) at 6-12 breaths per minute at a peak inspiratory pressure of \(\leq 25\) cm H\(_2\)O. Isotonic polyionic fluids\(^k\) were administered IV at a rate of 5-10 ml/kg/hr. Arterial blood pressure was monitored continuously following percutaneous placement of a 20-gauge 5.1 cm teflon catheter\(^l\) in the facial artery.

All horses were positioned in dorsal recumbency and prepared for aseptic surgery. A ventral midline celiotomy was performed, the large colon was exteriorized, placed on a warm water heating pad\(^m\) and instrumented (Figure 3.1). A thermocouple\(^n\) was inserted into the seromuscular layer of the colon to monitor local colonic temperature. A heat lamp was directed on the colon to maintain colonic temperature between 34\(^o\) and 38\(^o\) C. Evaporative heat and moisture loss were prevented by placing cellophane over the colon. A doppler ultrasound flow probe\(^o\) was placed externally around the colonic artery supplying
Figure 3.1

Instrumentation of large colon. RVC = right ventral colon; LVC = left ventral colon; PF = pelvic flexure; LDC = left dorsal colon; RDC = right dorsal colon; CA = colonic artery; CV = colonic vein; CVC = colonic venous catheter; CBFP = colonic artery blood flow probe; TC = colonic transmural compression; L=arterial ligatures; and T = thermocouple.
Figure 3.1
the ventral colon and colonic blood flow \( (Q_{\text{colon}}) \) was measured continuously and recorded. A 14-gauge, 5.1 cm (non-occluding) teflon catheter was placed in the colonic vein draining the ventral colon for sampling colonic venous blood. Systolic, diastolic, and mean arterial pressures (SAP, DAP, MAP), mRAP, and mPAP were measured and recorded.

**Experimental Design** - Twenty-four horses were randomly assigned to one of three groups. Group 1 horses \((n=6)\) served as sham-operated controls. The large colon was exteriorized through a ventral midline celiotomy and instrumented, but no colonic ischemia was induced. Group 2 horses \((n=9)\) served as ischemic controls. These horses underwent the same procedures as group 1 horses and 6 hrs of low flow ischemia. Following 6 hrs of ischemia in group 2, the colon was reperfused for 15 min to determine if \(Q_{\text{colon}}\) rebounded above baseline. Group 3 horses \((n=9)\) were subjected to 3 hrs of low flow ischemia followed by 3 hrs of reperfusion.

Low flow ischemic strangulation obstruction of the large colon was produced in groups 2 and 3 by reducing colonic arterial blood flow to 20% of baseline. Occlusion of transmural colonic blood flow was achieved by placing a 20 cm segment of latex tubing tightly around the circumference of the right dorsal and ventral colon (Figure 3.1). Umbilical tape ligatures were placed around the colonic artery supplying the dorsal and ventral colon, passed through a 2 cm segment of plastic tubing and tightened as a Rummel tourniquet until blood flow in the colonic artery supplying the ventral colon had been reduced to 20% of baseline. Mean arterial blood pressure was maintained \(\geq 70\) mm of Hg by increasing the IV fluid administration rate, decreasing the inspired halothane concentration, and if necessary infusing dobutamine \((1-5 \mu\text{g/kg/min})\).

Baseline data was recorded approximately 1 hr after induction of anesthesia (time required for surgery and instrumentation). Hemodynamic parameters were recorded and
blood samples collected at baseline and at 30 min intervals. Data were collected at 185, 190, and 195, min which corresponded to 5, 10, and 15 min of reperfusion in group 3. The horses were euthanatized at the conclusion with an overdose of sodium pentobarbitals (100 mg/kg IV).

**Hemodynamic Measurements and Calculations** - We monitored heart rate, HR (beats/min); SAP (mm of Hg); DAP (mm of Hg); MAP (mm of Hg); mRAP (mm of Hg); mPAP (mm of Hg); cardiac index, CI (ml/min/kg); and \( Q_{co} \) (ml/min).

**Metabolic Measurements and Calculations** - Samples of arterial, mixed venous, and colonic venous blood were collected for measurement of total plasma protein, TP (g/dl); packed cell volume PCV (%); blood gas analyses; oximetry; lactate (mmol/L) and pyruvate (mmol/L) concentrations. Systemic arterial, mixed venous, and colonic venous blood samples (2 ml each) were anaerobically collected into separate heparinized syringes and stored on ice until analyzed for pH, partial pressure of carbon dioxide, PCO\(_2\) (mm of Hg); partial pressure of oxygen, PO\(_2\) (mm of Hg); base excess, BE (mEq/L); bicarbonate concentration, HCO\(_3\) (mEq/L); hemoglobin concentration, Hb (g/dl); and percent oxygen saturation of hemoglobin, %SO\(_2\) (%). The blood gas\(^1\) and oximetry analyses\(^4\) were corrected for each horse's core temperature, measured by the flow directed thermodilution catheter\(^5\). All samples were analyzed within 1 hr of collection. One ml of the blood was placed in a plastic microcentrifuge tube, immediately immersed in liquid nitrogen, and stored at -70° C until analyzed for whole blood lactate. Thawing of these blood samples resulted in lysing of the red blood cells to allow measurement\(^5\) of whole blood lactate. The remaining blood was used to measure PCV via a microhematocrit method\(^6\) and TP using a refractometer\(^7\). Three ml of blood was collected from each of the arterial, mixed-venous, and colonic venous catheters and immediately placed in cold, heparinized tubes containing 6 ml of 8% perchloric acid. The tubes were thoroughly mixed and placed on ice until
complete protein precipitation had occurred. The tubes were then centrifuged at 400 g for
10 min and the supernatant saved and kept refrigerated at 4 °C until analyzed for pyruvate
within 4 weeks of collection. Whole blood pyruvate concentration was measured using a
colorimetric assay for the enzymatic conversion of pyruvate to lactatey.

Arterial, C_aO_2; mixed-venous, C_mvO_2; and colonic venous, C_cvO_2 oxygen contents
(ml/dl) were calculated as the sum of oxygen bound to hemoglobin and oxygen dissolved
in plasma ((Hb x %SO_2 x 1.36) + (PO_2 x 0.003)).35 Oxygen delivery, DO_2 colon (ml/min)
to the large colon was estimated as the product of arterial oxygen content and colon blood
flow (DO_2 colon = C_aO_2 x Q colon).35 Large colon oxygen consumption, VO_2 colon
(ml/min/m^2) was estimated as VO_2 colon = Q colon (C_aO_2 - C_cvO_2), where Q colon is the
measured blood flow in the left ventral colon.36 Colonic oxygen extraction ratio was
calculated as the difference between arterial and colonic venous oxygen content divided by
arterial oxygen content (O_2 extraction ratio colon = (C_aO_2 - C_cvO_2)/ C_aO_2).

Statistical Analyses: All data are expressed as mean ± standard error of the mean.
The mean age and body weight of the three groups were compared using a one-way
analysis of variance (ANOVA). Hemodynamic and metabolic data were analyzed using a
two-way ANOVA with repeated measures. A Tukey’s multiple comparison test was
applied to determine specific differences among and within groups. The level of
significance was set at P < 0.05 for all statistical tests.

Results

The mean age (2.75 ± 0.47 years) and body weight (442 ± 36 kg) did not differ
among groups. Core body temperature and local colonic temperature did not differ among
or within groups. Three horses (one from each group) required dobutamine to maintain
MAP ≥ 70 mm of Hg. Measured or calculated systemic hemodynamic and metabolic
parameters did not differ among or within groups (Table 3.1 and 3.2). Arterial and mixed venous blood gas and oximetry analyses, and lactate and pyruvate concentrations did not vary among or within groups. Arterial, mixed venous, and colonic venous PCV and TP did not differ among groups, but these parameters significantly decreased over time in all groups. Colonic hemodynamic or metabolic parameters did not differ among groups at baseline; colonic venous BE, HCO$_3^-$, and Hb did not vary among or within groups.

Colonic blood flow was significantly decreased by 30 minutes of ischemia in groups 2 and 3 (Figure 3.2). Induction of ischemia produced a decrease in $Q_{\text{colon}}$ to 19.5% and 20.7% of baseline in groups 2 and 3 respectively; this reduction in $Q_{\text{colon}}$ was significant throughout the ischemic period in both groups. Following 6 h of ischemia in group 2, $Q_{\text{colon}}$ significantly increased above baseline within 5 min of reperfusion and remained increased for the 15 min in which it was measured. The $Q_{\text{colon}}$ significantly increased above baseline (217%) within 5 min of reperfusion in group 3 and remained increased throughout reperfusion (190%).

Colonic venous pH did not change with time in group 1. The pH significantly decreased within 30 min of ischemia in groups 2 and 3 and remained decreased throughout the ischemic period in both groups (Figure 3.3). The pH returned to baseline within 5 min of reperfusion in group 3.

Colonic venous PCO$_2$ ($P_{\text{cv}}CO_2$) significantly increased to 72.52 and 62.84 mm of Hg by 30 min of ischemia in groups 2 and 3 respectively (Figure 3.4). Hypercarbia persisted throughout the course of the experiment in group 2 and throughout the ischemic period in group 3. The $P_{\text{cv}}CO_2$ returned to baseline values within 5 min of reperfusion in group 3.

Baseline $P_{\text{cv}}O_2$, $C_{\text{cv}}O_2$, and $\%S_{\text{cv}}O_2$ were not different among groups at baseline (Figure 3.5). The $P_{\text{cv}}O_2$ and $C_{\text{cv}}O_2$ were significantly increased by 3 hrs of ischemia and
Table 3.1

Mean ± standard error of the mean (SEM) for baseline systemic hemodynamic parameters and core and colon temperatures. There were no significant differences among groups or within groups over time for these parameters.
Table 3.1

<table>
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<th>Parameter</th>
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<th>Ischemia &amp; Reperfusion</th>
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<tr>
<td>HR (beats/min)</td>
<td>38.00 ± 3.50</td>
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<td>SAP (mm of Hg)</td>
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<td>DAP (mm of Hg)</td>
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<td>MAP (mm of Hg)</td>
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<tr>
<td>mRAP (mm of Hg)</td>
<td>3.17 ± 1.64</td>
<td>4.56 ± 1.45</td>
<td>6.00 ± 1.35</td>
</tr>
<tr>
<td>mPAP (mm of Hg)</td>
<td>13.00 ± 2.48</td>
<td>13.17 ± 0.85</td>
<td>14.00 ± 1.62</td>
</tr>
<tr>
<td>CI (ml/min/kg)</td>
<td>47.38 ± 9.02</td>
<td>48.77 ± 5.73</td>
<td>37.07 ± 2.90</td>
</tr>
<tr>
<td>Core Temperature (°C)</td>
<td>36.3 ± 0.7</td>
<td>37.6 ± 0.4</td>
<td>37.0 ± 0.2</td>
</tr>
<tr>
<td>Colon Temperature (°C)</td>
<td>32.1 ± 0.7</td>
<td>33.8 ± 0.3</td>
<td>33.8 ± 0.4</td>
</tr>
</tbody>
</table>

HR=heart rate, SAP=systolic arterial pressure, DAP=diastolic arterial pressure, MAP=mean arterial pressure, mRAP=mean right atrial pressure, mPAP=mean pulmonary artery pressure, and CI=cardiac index.
Table 3.2

Mean ± SEM for baseline arterial and mixed venous metabolic parameters in which there were no significant differences among groups or within groups over time.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Arterial</th>
<th>Control Mixed Venous</th>
<th>Ischemia Arterial</th>
<th>Ischemia Mixed Venous</th>
<th>Ischemia &amp; Reperfusion Arterial</th>
<th>Ischemia &amp; Reperfusion Mixed Venous</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.44 ± 0.02</td>
<td>7.38 ± 0.02</td>
<td>7.38 ± 0.02</td>
<td>7.33 ± 0.01</td>
<td>7.40 ± 0.02</td>
<td>7.35 ± 0.02</td>
</tr>
<tr>
<td>$\text{PO}_2$ (mm of Hg)</td>
<td>282.17 ± 57.82</td>
<td>35.17 ± 2.71</td>
<td>247.74 ± 42.75</td>
<td>32.97 ± 1.45</td>
<td>216.20 ± 36.67</td>
<td>30.59 ± 1.35</td>
</tr>
<tr>
<td>$\text{PCO}_2$ (mm of Hg)</td>
<td>35.99 ± 1.66</td>
<td>43.58 ± 2.57</td>
<td>41.16 ± 1.38</td>
<td>49.92 ± 1.10</td>
<td>36.86 ± 1.66</td>
<td>45.66 ± 1.76</td>
</tr>
<tr>
<td>$\text{HCO}_3^-$ (mEq/L)</td>
<td>25.47 ± 1.00</td>
<td>25.47 ± 0.73</td>
<td>25.16 ± 0.41</td>
<td>25.16 ± 0.48</td>
<td>24.53 ± 0.60</td>
<td>24.60 ± 0.76</td>
</tr>
<tr>
<td>BE (mEq/L)</td>
<td>0.61 ± 1.28</td>
<td>1.07 ± 0.98</td>
<td>0.25 ± 0.50</td>
<td>1.22 ± 0.57</td>
<td>-0.59 ± 0.77</td>
<td>0.42 ± 0.87</td>
</tr>
<tr>
<td>$\text{C}_a\text{O}_2$ (ml/dl)</td>
<td>17.33 ± 0.93</td>
<td>11.99 ± 0.72</td>
<td>17.71 ± 0.82</td>
<td>9.97 ± 0.88</td>
<td>19.00 ± 0.93</td>
<td>10.86 ± 0.97</td>
</tr>
<tr>
<td>$%\text{SO}_2$ (%)</td>
<td>99.67 ± 0.14</td>
<td>69.67 ± 2.28</td>
<td>98.37 ± 1.31</td>
<td>57.76 ± 1.83</td>
<td>99.45 ± 0.34</td>
<td>58.20 ± 3.07</td>
</tr>
<tr>
<td>Lactate (mmol/l)</td>
<td>2.083 ± 0.309</td>
<td>2.192 ± 0.187</td>
<td>1.361 ± 0.153</td>
<td>1.406 ± 0.153</td>
<td>1.578 ± 0.104</td>
<td>1.583 ± 0.153</td>
</tr>
<tr>
<td>Pyruvate (mmol/l)</td>
<td>0.063 ± 0.020</td>
<td>0.051 ± 0.010</td>
<td>0.079 ± 0.011</td>
<td>0.067 ± 0.007</td>
<td>0.064 ± 0.009</td>
<td>0.066 ± 0.012</td>
</tr>
</tbody>
</table>

* indicates either “a” for arterial or “mv” for mixed venous oxygen content; $\text{PO}_2$ = partial pressure of oxygen; $\text{PCO}_2$ = partial pressure of carbon dioxide; $\text{HCO}_3^-$ = bicarbonate concentration; BE = base excess; $\text{C}_a\text{O}_2$ = oxygen content; $\%\text{SO}_2$ = percent hemoglobin saturation.
Figure 3.2

Mean ± SEM for colon blood flow ($Q_{\text{colon}}$) during low flow ischemia and reperfusion. Solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group. The letter “b” is significantly ($P < 0.05$) different from “a”.
Figure 3.2

- Control
- Ischemia
- Ischemia & Reperfusion

Q colon (ml/min)

Time (hrs)
Figure 3.3

Mean ± SEM for colonic venous pH during low flow ischemia and reperfusion. Solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group. The letter “b” is significantly (P<0.05) different from “a”.
Figure 3.3

Colonic Venous pH

- Control
- Ischemia
- Ischemia & Reperfusion

Time (hrs)
Figure 3.4

Mean ± SEM colonic venous partial pressure of carbon dioxide (PCO$_2$) during low flow ischemia and reperfusion. Solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group. The letter "b" is significantly (P<0.05) different than "a".
Figure 3.4
Figure 3.5

Mean ± SEM for A) colonic venous partial pressure of oxygen (PO₂), B) colonic venous oxygen content (CvO₂), and C) colonic venous percent saturation of hemoglobin during low flow ischemia and reperfusion. Solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group. The letter “b” is significantly (P<0.05) different than “a”.

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remained increased throughout the experimental period in group 1. The \( P_{cv}O_2 \) was significantly decreased from baseline in groups 2 and 3 by 30 min of ischemia (54.1% and 53.8% of the baseline values respectively) and continued to be decreased throughout the ischemic period. Induction of ischemia resulted in a significant decrease in \( C_{cv}O_2 \) of 43.3% and 38.7% within 30 min in groups 2 and 3. The \( P_{cv}O_2 \) significantly increased above baseline by 5 min of reperfusion (168%) in group 3 and remained increased throughout reperfusion (194%). The \( C_{cv}O_2 \) was significantly decreased throughout the ischemic period in groups 2 and 3; it was significantly increased (117%) above baseline within 5 min of reperfusion in group 3 and remained increased throughout reperfusion (138%). There was a significant increase in \( %S_{cv}O_2 \) by 3 h in group 1 and it remained increased. Induction of ischemia resulted in a significant decrease in \( %S_{cv}O_2 \) by 30 min of ischemia, 27.8% and 31.8% in groups 2 and 3 respectively; this persisted throughout the ischemic period in both group 2 (52.5%) and 3 (44.5%). A significant increase in \( %S_{cv}O_2 \) above baseline occurred by 5 min of reperfusion (91.1%) and remained increased during reperfusion (92.0%) in group 3.

The colonic oxygen extraction ratio significantly increased from a baseline value of 21% and 27.5% to 66.9% and 71.7% by 30 min of ischemia in groups 2 and 3 respectively (Figure 3.6). The colonic extraction ratio remained increased during ischemia in both groups; it significantly decreased below baseline within 5 min of reperfusion (8.4%) and remained decreased throughout reperfusion (5.1%) in group 3.

Colonic venous lactate concentration significantly increased above baseline by 30 min of ischemia in groups 2 (180%) and 3 (181%) and remained increased throughout the ischemic periods in groups 2 (203%) and 3 (196%) (Figure 3.7). Colonic venous lactate decreased to baseline values within 5 min of reperfusion and remained at baseline values throughout reperfusion in group 3. Colonic venous pyruvate concentration was not
Figure 3.6

Mean ± SEM colonic oxygen extraction ratio during low flow ischemia and reperfusion. Solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group. The letter “b” is significantly (P<0.05) different than “a”.
Figure 3.6

- Control
- Ischemia
- Ischemia & Reperfusion

Colonic Oxygen Extraction Ratio vs Time (hrs)
Figure 3.7

Mean ± SEM colonic venous lactate concentration during low flow ischemia and reperfusion. Solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group. The letter “b” is significantly (P<0.05) different than “a”.
Figure 3.7

Colonic Venous Lactate (mmol/L)

Time (hrs)

1
2
3
4
5

0
1
2
3
4
5

Control
Ischemia
Ischemia & Reperfusion

a
b
different among groups at baseline (Figure 3.8). Pyruvate concentrations were significantly increased by 30 min of ischemia in groups 2 (225%) and 3 (238%) and remained increased through the ischemic periods in groups 2 (205%) and 3 (215%). The pyruvate concentration returned to baseline values by 5 min and remained in the normal baseline range throughout reperfusion in group 3.

Colonic oxygen delivery (DO2colon) did not change with time in group 1 (Figure 3.9). Oxygen delivery was significantly decreased by 30 min of ischemia in groups 2 (24.5%) and 3 (23.6%) and remained decreased during ischemia. Oxygen delivery significantly increased above baseline within 5 min (219%) of reperfusion in group 3 and remained increased throughout reperfusion. There were no differences among groups in baseline VO2colon (Figure 3.10) and no changes over time in group 1. Colonic VO2 significantly decreased within 30 min of ischemia in groups 2 and 3 and remained decreased in both groups.

Discussion

We have shown that reperfusion exacerbates colonic mucosal injury in the large colon of horses using a low flow model of ischemia. There have been few reports on local hemodynamic and metabolic alterations occurring in the intestine of horses subjected to ischemia and reperfusion. This is the first report of local hemodynamic and metabolic alterations that accompany colonic I-R injury in the horse; this reperfusion injury occurred in the presence of controlled systemic hemodynamics and systemic metabolic stability.

Our study was designed to maintain systemic hemodynamic stability (MAP > 70 mm of Hg) to minimize variability that might confound the presence or absence of reperfusion injury. All systemic hemodynamic parameters remained stable over the course
Figure 3.8

Mean ± SEM colonic venous pyruvate concentration during low flow ischemia and reperfusion. Solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group. The letter “b” is significantly (P<0.05) different than “a”.

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Figure 3.8

Colonic Venous Pyruvate (mmol/L)

Time (hrs)

- Control
- Ischemia
- Ischemia & Reperfusion
Figure 3.9

Mean ± SEM colonic oxygen delivery ($DO_2_{\text{colon}}$) during low flow ischemia and reperfusion of the equine large colon. Solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group. The letter “b” is significantly (P<0.05) different than “a”.
Figure 3.9
Figure 3.10

Mean ± SEM colonic oxygen consumption ($VO_{2\,\text{colon}}$) during low flow ischemia and reperfusion of the equine large colon. Solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group. The letter "b" is significantly ($P<0.05$) different than "a".
of the experiment, and were similar among the three experimental groups. Maintenance of stable systemic hemodynamics is important because changes in MAP or CI during the experiment could influence colonic injury. Dobutamine infusion was required to maintain MAP > 70 mm of Hg in only three horses (one horse from each experimental group). Dobutamine was chosen to augment cardiac output and arterial blood pressure because it lacks dopamine (DA$_1$) receptor activity. Unlike dopamine or dopexamine, dobutamine does not increase intestinal blood flow secondary to DA$_1$-mediated vasodilation.

Systemic arterial blood pressure rapidly decreases upon reperfusion of the small intestine, stomach, and large colon in cats, rats, dogs, and ponies respectively. Arterial blood pressure did not change with time in our sham-operated controls or during I-R in groups 2 and 3. This is different from previous studies in ponies where MAP decreased within 10 min of correcting a 1 hr 720° colon volvulus and remained unchanged until 2-3 h after detorsion. The influence of these systemic changes on colonic physiology is not known. Twenty minutes of superior mesenteric artery occlusion in rats resulted in a decrease in HR, but no change in MAP; reperfusion resulted in a transient decrease in MAP, but no change in HR. The hemodynamic variables returned to baseline in these rats within 2 hrs of reperfusion and remained constant for 24 hrs. Systemic hemodynamics remained stable in a rat model similar to ours. This observation is similar to our study in where systemic hemodynamic and metabolic parameters were maintained within the physiologic range during large colon ischemia and reperfusion.

Colon blood flow was decreased to approximately 20% of baseline flow in groups 2 and 3. This degree of ischemia was chosen based upon studies consistently demonstrating reperfusion injury when intestinal blood flow was reduced to 20-30% of baseline. The Q$_{\text{colon}}$ gradually increased from approximately 20% upon induction of ischemia to about 25% of baseline by 3 hrs of ischemia in groups 2 and 3. In group 3,
Increased blood flow, termed reactive hyperemia, occurred in each group 3 horse and occurred in each group 2 horse upon reperfusion following 6 hrs of ischemia. The presence of reactive hyperemia upon reperfusion depends upon the species and organ studied and the type, degree, and duration of ischemia. Additional factors such as systemic hemodynamic status, anesthetic drugs, and tissue metabolism may disrupt vasoregulation and alter the blood flow response associated with I-R.

Reactive hyperemia is the overshoot in blood flow that occurs following reperfusion after periods of arterial occlusion in several tissues, including both the small and large intestine. Reactive hyperemia has been demonstrated in the small intestine in dogs and cats. The magnitude and duration of the hyperemic response is related to the duration of the arterial occlusion. Intestinal blood flow usually increases to approximately 2-3 times baseline upon reperfusion following a period of 1 hr of ischemia. Increasing the duration of skeletal muscle ischemia leads to a prolonged hyperemic response upon reperfusion rather than an increase in the peak blood flow. A relatively uniform increase in blood flow occurs throughout the intestinal wall with occlusions of short duration (1 min), but with increased duration there is a disproportionate increase in blood flow to the muscular layers. The greater increase in blood flow to the muscularis with occlusions longer than one minute is believed to be partially due to increases in intestinal motility.

The capillary recruitment and vasodilation that occur secondary to arterial occlusion lead to an overshoot in intestinal blood flow, tissue \( \text{PO}_2 \), and arteriovenous \( \text{O}_2 \) difference and a decrease in oxygen extraction upon reperfusion. The increased blood flow represents a relative overperfusion that contributes to repayment of the oxygen debt incurred during arterial occlusion. A reduction in tissue \( \text{PO}_2 \) or an accumulation of
vasodilator substances (e.g., prostacyclin or nitric oxide) results in active vasodilation during arterial occlusion. Release of the occlusion allows blood flow to rebound above baseline. If the intestine is reperfused at a constant blood flow following arterial occlusion the oxygen debt is repaid by increased oxygen extraction (increased capillarity) rather than an increase in blood flow (vasodilation).22 The hyperemia gradually declines to baseline representing a washout or metabolism of vasodilator substances. Reactive hyperemia typically does not occur following intestinal venous occlusion, but rather oxygen extraction increases.20 The reason for the absence of reactive hyperemia with venous occlusion is believed to be due to increased vascular transmural pressure causing reflex stimulation of vascular smooth muscle constriction, which leads to increased vascular resistance and decreased blood flow.25 If reactive hyperemia occurs following venous occlusion it is usually of low magnitude and short duration. The peak oxygen extraction and the duration of increased oxygen extraction are proportional to the duration of venous occlusion.20

Reactive hyperemia is not consistent following reperfusion after intestinal vascular occlusion. Reactive hyperemia was absent after release of superior mesenteric artery occlusion in rats40 and correction of large colon volvulus in ponies23. Small intestine blood flow during reperfusion reached approximately 60% of baseline within 15 min and only 70-75% by 1 hr of reperfusion in rats.40 Colon blood flow was reduced to zero immediately upon creating a 720 degree volvulus of the large colon in ponies and remained zero through 1 hr of volvulus. The Q colon remained significantly reduced (approximately 40% of baseline flow) for 50 min following restoration of blood flow.23

The absence of reactive hyperemia in the study in ponies 23 is likely due to the increased colonic vascular resistance during the first 50 min of reperfusion. Although we did not measure colonic vascular resistance, the reactive hyperemia was probably due at least in part to vasodilation (decreased colonic vascular resistance). Differences in colonic
vascular resistance between ponies with large colon volvulus and our horses with partial colonic arterial ischemia may account for the differential response of the colonic vasculature. The difference in $Q_{\text{col}}$ during reperfusion between our study and the study in ponies may also be related to model selection, anesthetic drugs, differences between horses and ponies in control of blood flow, or other unknown factors. Portal venous $\text{TXB}_2$ was significantly increased within minutes of correcting experimental colonic volvulus in ponies and returned to baseline for the remainder of the experimental period.\textsuperscript{39} Portal venous 6-keto prostaglandin $F_{1\alpha}$ was significantly increased by 1 hr of colonic volvulus and remained increased during 3 hrs of reperfusion.\textsuperscript{39} Increased portal $\text{TXB}_2$ probably represents increased colonic thromboxane production during early reperfusion, contributing to the absence of reactive hyperemia.\textsuperscript{23} The increased 6-keto prostaglandin $F_{1\alpha}$ concentrations in the portal venous system result from increased production by the colonic tissue during ischemia and reperfusion. The 6-keto prostaglandin $F_{1\alpha}$ did not result in vasodilation of the colonic vasculature as evidenced by increased colonic vascular resistance and absence of reactive hyperemia.\textsuperscript{23} Creation of the large colon volvulus likely led to either venous or both arteriovenous occlusion rather than arterial occlusion alone; the venous component of the vascular occlusion could have prevented a hyperemic response.\textsuperscript{20}

Reperfusion of intestine following an ischemic episode is often associated with systemic hemodynamic instability\textsuperscript{19,26,27}; MAP decreased dramatically upon reperfusion of ischemic rat small intestine and pony large colon.\textsuperscript{23,27} Failure of $Q_{\text{col}}$ to rebound in ponies following correction of a 720 degree colon volvulus may have been due in part to a significant decrease in MAP.\textsuperscript{23} The rebound in $Q_{\text{col}}$ in our study was associated with hemodynamic stability. Reactive hyperemia did not occur in ketamine anesthetized rats following 2 h of "low flow" ischemia despite hemodynamic stability.\textsuperscript{40} Ketamine has been
shown to reduce intestinal blood flow up to 50% in swine.\textsuperscript{41} Volatile anesthetic agents, such as halothane, may inhibit or abolish autoregulatory mechanisms in regional vascular beds by inhibiting the release of endothelium derived relaxation factor (nitric oxide).\textsuperscript{42} Intestinal blood flow in dogs\textsuperscript{43,44}, ponies\textsuperscript{45}, and swine\textsuperscript{46} was decreased by 50-60% during halothane anesthesia. The percentage of the cardiac output perfusing the gastrointestinal tract was unchanged by halothane anesthesia.\textsuperscript{46} The decrease in intestinal blood flow during halothane anesthesia is likely due to its cardiovascular depressant effects.\textsuperscript{46} Halothane anesthesia was used in the colon volvulus study in ponies and no rebound in $Q_{\text{colon}}$ was observed perhaps because of the immediate and sustained decrease in MAP.\textsuperscript{23} The maintenance of stable systemic hemodynamics in our study during halothane anesthesia may have contributed to the effect on increased $Q_{\text{colon}}$ observed upon reperfusion. It is unlikely that halothane had a major effect on autoregulation of blood flow in the colonic vasculature because $Q_{\text{colon}}$ remained stable in our sham-operated control horses.

Possible explanations for the sequence of events occurring in the large colon in the horses in our study includes: 1) metabolic feedback signals from the colonic tissue (decreased $O_2$ or increased vasodilator substances) to the microvasculature causing vasodilation and thus an increased pressure differential from the conduit arteries to the capillaries; 2) the larger pressure gradient would effectively result in an increased colonic blood flow; and 3) the increased blood flow could cause further vasodilation due to the flow-induced release of nitric oxide from the endothelium.\textsuperscript{47} Another plausible explanation is that local regulatory function within the colonic circulation was impaired consequent to endothelial damage incurred during ischemia. Healthy intact endothelium, through its production and release of vasoactive substances, is necessary to maintain vasoregulation.\textsuperscript{48} Colonic blood flow may rebounded above baseline during reperfusion due to loss of
vasoregulation.

An alternative hypothesis based upon extensive investigation of the vasodilatory response of the coronary circulation to myocardial oxygen depletion is that the reduced tissue oxygen stimulates coronary vasodilation via activation of ATP-dependent potassium channels. Hypoxic coronary vasodilation and coronary reactive hyperemia are attenuated by glibenclamide, a specific blocker of ATP-dependent channels. It has been proposed that reduced oxygen tension impairs the ability of vascular smooth muscle to generate ATP. Decreased intracellular ATP concentration activates ATP-dependent potassium channels on the smooth muscle cell membrane, resulting in vascular relaxation. Relaxation of vascular smooth muscle decreases the resistance to blood flow which facilitates reactive hyperemia following release of arterial occlusion. During ischemia, accumulation of lactate or other metabolic by-products may also activate these ATP-dependent potassium channels. The increased colonic venous lactate observed during low flow ischemia in this study may have contributed to reactive hyperemia by activating potassium channels.

The changes in colonic venous blood during the ischemic period in groups 2 and 3 suggest the development of tissue hypoxia and anaerobic metabolism. The decreases in \( P_{cv}O_2 \), \( C_{cv}O_2 \), and \( %S_{cv}O_2 \) are indicative of reduced colonic blood flow and oxygen delivery. Intestine can partially adapt to a reduced oxygen delivery by increasing the extraction of oxygen from hemoglobin. This was demonstrated by a decreased \( %S_{cv}O_2 \) and an increased oxygen extraction ratio during ischemia. The trend toward a decrease in oxygen extraction ratio over the experimental period in group 1 suggests that colonic metabolism may have decreased with time.

Colonic \( DO_2 \) remained stable in group 1, decreased during ischemia in groups 2 and 3, and increased in group 3 upon reperfusion. Colonic \( VO_2 \) decreased coincident with
the reduction in colonic DO$_2$. Oxygen consumption becomes delivery or supply dependent once a critical DO$_2$ value is reached.$^{56}$. The increase in colonic venous lactate observed during ischemia in our study confirms the critical DO$_2$ was reached. The critical DO$_2$ value represents the transition from aerobic to anaerobic metabolism and is manifested by lactate production.$^{56,57}$ Estimated oxygen consumption following arterial occlusion of the small intestine suggests a decreased oxygen demand$^{20}$; this would be represented by a reduction in intestinal oxygen consumption. Colonic VO$_2$ remained decreased throughout reperfusion despite an increase in DO$_2$; reperfusion injury may have been severe enough that cellular viability or metabolic capability was impaired; dead cells do not respire and therefore do not utilize oxygen.

The colonic venous acidemia, hypercarbia, hypoxia, and lactic and pyruvic acidosis were corrected within 5 min of reperfusion. The colonic venous pH rapidly increased to baseline by 5 min of reperfusion in group 3, but remained decreased throughout the second 3 h of ischemia in group 2. The changes in colonic venous pH are similar to the changes observed in a study in ponies where colonic tissue pH progressively decreased during 1 hr following a 720 degree volvulus, but returned to baseline values within 10 min after restoring blood flow.$^{23}$ The P$_{cv}$CO$_2$ rapidly decreased by 5 min of reperfusion. The colonic tissue had adapted to the change in oxygen delivery by decreasing oxygen extraction from hemoglobin; oxygen extraction dramatically decreased by 5 min of reperfusion. The reduced oxygen extraction ratio was accompanied by an increase in $\%S_{cv}O_2$ at 5 min of reperfusion, which remained reduced throughout reperfusion. The $\%S_{cv}O_2$ and oxygen extraction ratio stabilized at approximately 40% and 0.5 respectively during the second 3 hrs of ischemia in group 2. The P$_{cv}$O$_2$ increased above baseline by 5 min of reperfusion in group 3, but it remained reduced throughout ischemia in group 2. The change in the colonic venous parameters upon reperfusion represent adaptations of the
colon to the return of blood flow and oxygen delivery with a resultant decrease in anaerobic metabolism. The early alterations in colonic venous metabolic parameters may simply represent a “washout” of metabolic by-products that accumulate during ischemia.

The lack of any change among and within groups for blood gas and oximetry analyses, and lactate and pyruvate concentration in arterial or mixed-venous blood samples provides further evidence of the systemic metabolic stability of this model. The decreases in PCV and TP likely represent time dependent dilutional effects due to fluid administration. Although there were significant changes in some parameters in colonic venous blood, the systemic arterial and mixed-venous metabolic parameters were not altered.

Our model of low flow I-R of the horse large colon produces extensive colonic mucosal injury, and alterations in colonic hemodynamic and metabolic parameters in the presence of controlled systemic hemodynamic and metabolic stability. This suggests the reperfusion injury occurred subsequent to alterations in local colonic blood flow and metabolism. This model may be useful for differentiating and evaluating the effects of colonic I-R and for determining insults on distant organs, such as the heart and lungs. Our model can be used to study drugs which prevent or reduce I-R injury; this may lead to the identification of drugs that are potentially beneficial in horses with naturally occurring intestinal ischemia.

Footnotes

aRompun®, Mobay Corporation, Animal Health Division, Shawnee, KS.
bCarbocaine®-V, The Upjohn Company, Kalamazoo, MI.
cAngiocath®, Becton Dickinson Vascular Access, Sandy, Utah.
Cardiomax II Model 85 and Chart Recorder Model CMXR1S, Columbus Instruments, Columbus, Ohio.

IntramedicR, Clay Adams, Division of Becton Dickinson and Company, Parsippany, NJ.

GuailaxinR, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.


Halothane U.S.P., Halocarbon Laboratories, North Augusta, SC.

Drager AV Model NELAC-E, Anesthesia Ventilator, Telford, Pa.

Lactated Ringer’s, Baxter Healthcare Corporation, Deerfield, IL.

AngiocathR, Becton Dickinson Vascular Access, Sandy, Utah.

Aquamatic K Module, Gorman-Rupp Industries, Bellville, Ohio.


Model T201, Transonic Systems, Inc., Ithaca, NY.

Quik-CathR, Baxter Healthcare Corporation, Deerfield, IL.

Simultrace Recorder Model VR-12, Electronics for Medicine/PPG Biomedical Systems, Pleasantsville, NY.

Dobutrex Solution, Eli Lilly & Co., Indianapolis, IN.

BeuthanasiaR-D Special, Schering Plough Animal Health Corporation, Kenilworth, NJ.

ABL 500-K pH and Blood Gas Analyzer, Radiometer-Copenhagen, Copenhagen, Denmark.

OSM™ 3 Hemoximeter, Radiometer-Copenhagen, Copenhagen, Denmark.

YSI Model 23L Lactate Analyzer, Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio.

Critocaps™, Micro-Hematocrit Capillary Tube Reader, Monoject Scientific, St. Louis, Mo.

10436 Veterinary Refractometer, Cambridge Instruments, Buffalo, NY.

Pyruvate Assay Kit, Sigma Diagnostics, Sigma Chemical Company, Ltd., St. Louis, Mo.
List of References


CHAPTER IV
HISTOPATHOLOGIC EVIDENCE OF REPERFUSION INJURY IN THE EQUINE LARGE COLON FOLLOWING LOW FLOW ISCHEMIA

Summary

The effects of low flow ischemia and reperfusion of the large colon on mucosal architecture were determined in horses. Twenty-four adult horses were randomly allocated to three groups: 1) sham-operated controls (n=6), 2) 6 hrs ischemia (n=9), and 3) 3 hrs ischemia and 3 hrs reperfusion (n=9). Low flow ischemia was induced in groups 2 and 3 by reducing colonic arterial blood flow to 20% of baseline values. Full-thickness biopsies were harvested from the left ventral colon for histomorphologic and morphometric examination at baseline and at 30 min intervals for 6 hrs; additional biopsies were collected at 185, 190, and 195 min. These additional biopsies were harvested from all 3 groups of horses and corresponded to the 5, 10, and 15 min period of reperfusion in group 3. There were no differences among groups at baseline or across time in group 1 for any of the histopathologic parameters. There were significant (P<0.03) increases in percent surface mucosal disruption, estimated and measured percent depth mucosal loss, mucosal hemorrhage, mucosal edema, and cellular debris index during 0 to 3 hrs compared to baseline and from 3 to 6 hrs as compared to 3 hrs in groups 2 and 3. Estimated percent depth mucosal loss and cellular debris index were significantly (P<0.05) greater in group 3 compared to group 2 during the interval from 3 to 6 hrs. There were trends toward greater percent surface mucosal disruption and mucosal edema during the early phase of reperfusion (3-4 hrs) and greater mucosal hemorrhage, measured percent depth mucosal
loss, and mucosal interstitial:crypt ratio during the late phase (4-6 hrs) of reperfusion in group 3 versus group 2. In summary, re-establishment of colonic arterial blood flow after low flow ischemia caused greater mucosal injury than a comparable period of continued ischemia. Thus, reperfusion injury was demonstrated in the large colon of horses following low flow arterial ischemia. The serial mucosal alterations that developed in the colon were comparable in groups 2 and 3, however, reperfusion exacerbated the colonic mucosal injury.

Introduction

Reperfusion injury is defined as that tissue damage sustained upon restoration of blood flow following a period of ischemia.\(^1\) Intestinal reperfusion injury is believed to be principally initiated by the accumulation of hypoxanthine and its conversion by xanthine oxidase (XO) to oxygen free radicals (OFRs) and amplified by neutrophil-derived OFRs.\(^2\)-\(^5\) Intestinal ischemia is a common cause of acute abdominal pain in horses. Large colon volvulus, a condition which produces ischemic injury, accounts for between 7-20% of those lesions in horses with colic that require surgical exploration.\(^6\)-\(^8\) The survival rate for horses following surgical correction of the colon is only 20-40%\(^6\)-\(^8\). Many investigators have hypothesized that ischemia-reperfusion (I-R) injury plays a role in the high mortality rate following large colon volvulus, however, reperfusion injury has not been previously demonstrated in the large colon of horses.\(^9\) Models of complete arteriovenous, complete arterial, and complete venous occlusion have not demonstrated reperfusion injury in the large colon of horses and ponies.\(^9,10\) Meschter et al., however, did report reperfusion injury occurs in the large colon of ponies.\(^11\) These results are controversial because of the low numbers of experimental animals in the study, because of the models of ischemia used, and because colonic ischemia and reperfusion were not compared to an equivalent period of...
ischemia without reperfusion.

Low flow models of intestinal ischemia are preferred by many investigators for studying the effects of reperfusion injury because complete ischemia causes such severe necrosis that any additional injury sustained during reperfusion is inconsequential. The majority of the studies in laboratory animals that have documented intestinal reperfusion injury have utilized partial or low flow arterial ischemia. Low flow arterial ischemia has not been evaluated as a model for detecting reperfusion injury in the equine large colon. Although venous occlusion is a major component of large colon volvulus, some colonic arterial flow is generally maintained. Similarities between the arterial component of vascular occlusion in colonic volvulus and models of low flow arterial ischemia likely exist.

Comparison of the effects of I-R to a similar period of ischemia without reperfusion is necessary to determine if restoration of blood flow actually produces greater injury than ischemia alone. Based upon this premise, we predicted that reperfusion injury would be detectable by histopathologic examination of the large colon mucosa and that the colonic mucosal injury sustained in the early phase of reperfusion would be detectable by different histopathologic parameters than the injury observed during later phases of reperfusion. The purposes of this study were to 1) describe the histopathologic alterations occurring in the large colon mucosa during low flow arterial ischemia and reperfusion, 2) compare the severity of mucosal injury between horses subjected to ischemia and reperfusion and those subjected to a comparable period of ischemia alone, and 3) determine which histopathologic parameters are altered in the colonic mucosa during early versus late phases of reperfusion.

Materials and Methods

This project was approved and performed under the guidelines of the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.
Animals: Twenty-four horses of various breeds (9 Thoroughbreds, 8 Standardbreds, 3 Quarter Horses, 2 Saddlebreds, and 2 Hanoverians), ranging in age from 1-10 years and body weight from 350-570 kg were used. There were 11 females, 5 intact males, and 8 geldings. Horses were determined to be free from cardiopulmonary, gastrointestinal, or other systemic disease by a thorough physical examination and a complete blood count and fibrinogen. Horses were fed 1-2% of their bodyweight in grass hay per day and water was provided ad libitum. Horses were adapted to their diet and environment for at least one week prior to the experiment. Food, but not water, was withheld for 24 hrs prior to the experiment to decrease the colon contents.

Instrumentation: Horses were sedated with xylazine (0.5 mg/kg IV) and placed in stocks. Subcutaneous mepivacaine hydrochloride was used to desensitize the skin to facilitate transcutaneous catheter placement. A 14-gauge 13.3 cm teflon catheter was inserted into the left jugular vein for administration of anesthetic drugs and isotonic fluids. General anesthesia was induced with guaifenesin (30 mg/kg IV) and thiamylal (3-4 mg/kg IV) after placement of the vascular catheters. Anesthesia was maintained with halothane (1-3%) in oxygen. Horses were mechanically ventilated at 6-12 breaths per min to a peak inspiratory pressure of approximately 25 cm H₂O. Isotonic polyionic fluids were administered intravenously at a rate of 5-10 ml/kg/hr. Mean arterial blood pressure was monitored following percutaneous placement of a 20-gauge 5.1 cm teflon catheter in the facial artery.

Horses were positioned in dorsal recumbency and prepared for an aseptic ventral midline celiotomy. The abdominal cavity was explored, the large colon was exteriorized and placed on a warm water heating pad. A heat lamp was used to maintain colon temperature between 34-38° C, which was monitored by a thermocouple inserted into the seromuscular layer of the left ventral colon; this provided an estimate of colonic wall
temperature to ensure the temperature remained in a physiologic range and that it was similar among all groups. Polyionic fluid was applied to the colonic serosa to maintain a moist surface. Evaporative heat and moisture loss were prevented by placing cellophane over the colon. A doppler ultrasound flow probe was placed externally around the colonic artery supplying the ventral colon and colonic blood flow was measured continuously and recorded. Duration of instrumentation was approximately 1 hr (range 40-65 min); once instrumented 15-30 min were allowed for the colonic blood flow to stabilize before collecting baseline data. The horses remained anesthetized an additional 6 hrs during the combined ischemia and reperfusion periods.

Experimental Design: Twenty-four horses were randomly assigned to one of three groups. Group 1 (n=6) served as sham-operated controls. These horses were subjected to a ventral midline celiotomy and the large colon was exteriorized and instrumented, however, no colonic ischemia was induced. Group 2 (n=9) served as ischemic controls; these horses were subjected to the same procedures as group 1 and 6 hrs of low flow ischemia. Group 3 horses (n=9) were subjected to 3 hrs of low flow colonic ischemia followed by 3 hrs of reperfusion.

Low flow ischemic strangulation obstruction of the large colon was induced by reducing colonic arterial blood flow to 20% of baseline following recording of baseline hemodynamic parameters, and harvesting of colonic biopsies. Transmural compression of colonic blood flow was achieved by placing a separate 20 cm segment of latex tubing tightly around the circumference of both the right dorsal and right ventral colon. An umbilical tape ligature placed around the colonic artery supplying the dorsal colon and passed through a 1.5 cm sections of plastic tubing was tightened by sliding the tubing down the ligature (Rummel tourniquet) to occlude colic arterial blood flow. Several ligatures were placed around the colonic artery supplying the ventral colon and tightened until the colonic blood flow had been reduced to approximately 20% of baseline. Blood
flow in the ventral colonic artery was continuously monitored using the doppler ultrasound flow probe. Mean arterial blood pressure was maintained ≥ 70 mm of Hg by increasing the intravenous fluids, decreasing the inspired halothane concentration, and if necessary infusing dobutamine (1-5 ug/kg/min) in order to eliminate systemic hemodynamic effects on colonic I-R injury. The horses were euthanatized at the conclusion of the experiment with sodium pentobarbital (100 mg/kg IV).

Data were collected and colonic biopsies harvested at baseline (time = 0) and at 30 min intervals throughout the six hour experimental period. Data were also collected at 185, 190, and 195 min, which corresponded to 5, 10, and 15 min of reperfusion in group 3 horses.

Colonic Biopsies: Full-thickness 2 cm x 5 cm sections of the left ventral colon were harvested; sampling variation was minimized by collecting biopsies from similar locations and identical patterns from all horses. Biopsies were gently rinsed in a buffered electrolyte solution and immediately placed in 10% buffered formalin for histopathologic evaluation.

Histopathology: Full-thickness formalin-fixed sections of the left ventral colon were embedded in paraffin, cut in 4 um sections, processed and stained with hematoxylin & eosin.

Qualitative Morphology: Time dependent alterations in the colonic mucosa were assessed. Cytoplasmic and nuclear alterations in the surface mucosal cells and the accumulation of subepithelial fluid, cells, and cellular debris were evaluated. Surface mucosal cell detachment from the basement membrane and adjacent cells was assessed. The mucosa was observed for progression of necrosis, crypt collapse, and separation of the crypts by edema and hemorrhage.
**Semi-Quantitative Morphology:** All histomorphologic parameters were estimated or scored independently and blindly by two investigators using 10x and 40x ocular objectives. All areas of the colonic tissue sections on each slide were analyzed in arriving at the estimate or score for all histomorphologic parameters. The two investigators' scores were averaged for each horse and time period. The % surface mucosal disruption was determined by visual estimate of the proportion of the length of the colonic sections which had surface mucosal epithelium separated from the basement membrane. The % depth of mucosal loss was determined (Figure 4.1) by visual estimate of the depth of the crypts that were intact (B) subtracted from the full depth of the mucosa (A), divided by A, and then multiplied by 100%; \((A-B)/A \times 100\). This was estimated in areas where colonic crypts were cut in longitudinal section. The mucosa was separately scored from 0 to 4 for the presence of hemorrhage and edema. A score of zero indicated there was no hemorrhage or edema observed and a score of 1 indicated slight hemorrhage or edema after close inspection of the tissue section. Scores of 2, 3, and 4 were indicative of mild (readily observable, but not extensive), moderate (extensive, but not distorting normal architecture), and marked (distorting normal architecture) hemorrhage or edema, respectively. The basilar, middle, and surface one-thirds of the colonic mucosa were individually scored from 0 to 4 for the presence of cellular/nuclear debris; the scale used to score debris was similar to the scale used for hemorrhage and edema. The cellular debris index was calculated as the sum of the average scores for each of the three areas.

**Quantitative Morphometry:** Measurements of the % depth of mucosal loss (measured % depth mucosal loss) and mucosal interstitial area:crypt area (I:C) ratio were made using a microscope with a calibrated cursor coupled to a microcomputer-based image analysis system. The % depth of mucosal loss was measured as the difference between the full-thickness mucosa and the portion of the mucosa with no obvious evidence of
A photomicrograph of the large colon mucosa illustrating measurement of percent depth of mucosal loss and mucosal interstitial:crypt (I:C) ratio. Percent depth mucosal loss was estimated or measured by subtracting the depth of intact crypt (B) from the total mucosal depth (A): percent depth mucosal loss = [(A-B)/A] x 100. Mucosal I:C ratio = total grid area - crypt area. LP = lamina propria and MM = muscularis mucosae. Bar = 100 μm.
Figure 4.1
necrosis (Figure 4.01). Thirty measurements were made for each horse and time period and the average % mucosal loss was used for statistical analyses. A 1.0 mm² grid was situated in the base of the mucosa adjacent to the muscularis mucosa and was used to calculate the total mucosal area in that region (Figure 4.1). The area within this square that was occupied by crypts (crypt area) was measured and summed. The difference between the total area of the box and the area occupied by crypts was the interstitial area. The I:C ratio was calculated as the ratio of the interstitial area to the crypt area (Figure 4.1). This parameter provides a more quantitative measure of mucosal hemorrhage and edema than histomorphologic scoring. These measurements were made in 10 areas for each horse and time period in areas where the crypts were cut in cross-section. The submucosal thickness was measured as the distance between the muscularis mucosa and the circular muscular layer as an indicator of submucosal edema and hemorrhage. This was measured in 15 adjacent areas for each horse and time period.

Statistical Analyses: All data were expressed as mean ± standard error of the mean (SEM). Histomorphologic (scored) and histomorphometric (quantitative) data were analyzed using a two-way analysis of variance (ANOVA) for repeated measures. Dunnett’s t-test was used to compare back to baseline and a Tukey’s test was used for all pairwise comparisons. Two-way ANOVA was used to compare the intervals from 0 to 3 hrs and 3 to 6 hrs between groups 2 and 3 for all histomorphologic and histomorphometric parameters. Additionally, two-way ANOVA was used to compare smaller intervals within the 3 to 6 hr interval between groups 2 and 3 to evaluate changes in the early (3-4 hrs) and late phases (4-6 hrs) of reperfusion. The level of significance was set at P<0.05 for all tests. Agreement between the two investigators’ estimate of % depth mucosal loss was assessed by calculating Pearson Correlation Coefficients at all time periods.
Results

Systemic hemodynamic and metabolic parameters were maintained constant across time in all groups and within the normal physiologic range. There were no differences in local colonic hemodynamic or metabolic parameters during the first 3 hrs between groups 2 and 3 indicating that a similar magnitude of ischemia was achieved in both groups. The hemodynamic and metabolic data for these horses is presented in an accompanying manuscript. The differences in the histopathologic evaluation presented below are therefore due to differences in local colonic hemodynamic and/or metabolic alterations occurring during the 3 to 6 hr period in groups 2 and 3.

Qualitative Morphologic Assessment:

There were no detectable mucosal alterations at baseline among groups (Figure 4.2) nor were there any detectable mucosal alterations noted across time in group 1. During the 0 to 3 hr period of low flow ischemia minimal morphologic or morphometric changes were detected in groups 2 or 3 and no differences occurred between the two groups. The histopathologic alterations in the mucosa progressed similarly in groups 2 and 3, but the magnitude of change was greater in group 3. In general, changes included development of nuclear alterations (karyorrhexis, pyknosis) and cytoplasmic vacuolization in the surface mucosal epithelial cells. Mild hemorrhage and edema fluid as well as inflammatory cells accumulated in a subepithelial location and neutrophils infiltrated the lamina propria. No difference was observed in the type of histopathologic alterations occurring with continued ischemia (group 2) or reperfusion (group 3) during the 3 to 6 hr interval. Differences did exist regarding the magnitude of change as outlined in the semi-quantitative and quantitative assessments below. During the 3 to 6 hr period, there was progressive subepithelial fluid accumulation, neutrophil infiltration, and detachment of clusters of surface epithelial cells from the basement membrane and adjacent cells resulting
Figure 4.2

A photomicrograph of the large colon mucosa at baseline. Note the surface mucosal cells are intact and there are no detectable alterations in the surface mucosal epithelium (SME), lamina propria (LP), or crypt areas (C). Bar = 50 μm.
in progressive disruption and necrosis of the colonic surface epithelium (Figure 4.3). Extrusion of mucosal cells, inflammatory cells, red blood cells and cellular and nuclear debris on the luminal surface occurred with disruption of the basement membrane (Figure 4.4). Necrosis proceeded toward the base of the mucosa with collapse of the crypts. Hemorrhage and edema increased in the lamina propria causing disruption of the normal colonic architecture. The space between colonic crypts became wider as edema fluid (Figure 4.5) and hemorrhage continued to accumulate. Sloughed mucosa, cellular debris, and inflammatory cells appeared to be washed away from the luminal surface of the mucosa during later periods of reperfusion. A single layer of mucosal cells appeared to align parallel to the luminal surface during the later stages of reperfusion period (Figure 4.6).

Semi-Quantitative Morphologic Assessment:

There were no differences in baseline values among groups or across time in group 1 for all semi-quantitative histomorphologic parameters. Surface mucosal disruption (%) increased significantly from zero at 0 hrs to 21.4% and 28.0% at 3 hrs in groups 2 and 3 respectively (Figure 4.7). There was no difference between groups 2 and 3 during this period. There was a significant increase in % surface mucosal disruption during the interval from 3 to 6 hrs in groups 2 and 3. The % surface mucosal disruption was 87.8% and 95.6% at 6 hrs in groups 2 and 3 respectively. There was no difference between groups 2 and 3 during the entire 3 to 6 hr interval, but there was a trend toward greater surface epithelial disruption in group 3 compared to group 2 during the early reperfusion period (P=0.104).

There was a significant increase in the estimated % depth mucosal loss during the 0 to 3 hr period of ischemia in groups 2 and 3, but there was no difference between the two groups (Figure 4.8). There was a significant increase in % depth of mucosal loss above that observed at 3 hrs for both groups 2 and 3 during the interval from 3 to 6 hrs. There
A photomicrograph of the large colon mucosa demonstrating increased subepithelial accumulation of edema fluid, hemorrhage, inflammatory cells, and cellular debris. Clusters of surface mucosal epithelial cells have become physically detached from the basement membrane and adjacent cells. Bar = 50 μm.
Figure 4.3
Figure 4.4

A photomicrograph of the large colon mucosa during the early reperfusion phase illustrating disruption of the basement membrane and extrusion of mucosal cells and inflammatory cells onto the luminal surface. Bar = 100 μm.
Figure 4.4
Figure 4.5

A photomicrograph of the large colon mucosa from an ischemia & reperfusion horse during early reperfusion period. Note the increased edema fluid accumulation in the lamina propria; the edema fluid has distorted the normal architecture causing increased width between adjacent crypts (i.e. increased mucosal I:C ratio). Bar = 50 μm.
Figure 4.6

A photomicrograph of the large colon mucosa from an ischemia & reperfusion horse following 3 hrs of ischemia and 3 hrs of reperfusion. Note that severe mucosal necrosis has occurred. A single layer of mucosal cells remaining in the crypts have aligned parallel to the surface (arrows). Bar = 50 μm.
Figure 4.7

Mean ± SEM for percent surface mucosal disruption in equine large colon during low flow ischemia and reperfusion. The solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group.
Figure 4.7

Surface Mucosal Disruption (%)

Control

Ischemia

Ischemia & Reperfusion

Time (hrs)
Figure 4.8

Mean ± SEM for estimated percent depth of mucosal loss in the equine large colon during low flow ischemia and reperfusion. The solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group. The letter "b" is significantly (P<0.05) different from "a". The solid bar over the 3 to 6 hr interval indicates that the ischemia & reperfusion group had significantly (P<0.05) greater mucosal loss than the ischemia group across this interval.
Figure 4.8
was significantly greater depth of mucosal loss in group 3 (Figure 4.9) compared to group 2 (Figure 4.10) during the interval from 3 to 6 hrs. The % depth mucosal loss was 19.4% vs. 42.4% at 5 hrs and was 27.5% vs. 43.2% at 6 hrs in groups 2 and 3 respectively. The Pearson Correlation Coefficients, representing agreement between the two investigators' estimates of % depth mucosal loss at different time periods, ranged from 0.922 to 0.996 (R² = 0.848 to 0.992).

There was a significant increase in the hemorrhage score during the 0 to 3 hr interval in groups 2 and 3 compared to baseline (Figure 4.11), but there was no difference between groups 2 and 3 during this period. There was a significant increase in hemorrhage above that at 3 hrs in groups 2 and 3 during the 3 to 6 hr interval. There was a trend toward greater hemorrhage in group 3 compared to group 2 during the late phase of reperfusion (P=0.115).

There was a significant increase in the mucosal edema score in groups 2 and 3 during 0 to 3 hr period (Figure 4.12). There was no difference in the edema score between the two groups during this interval. There was a significant increase in the edema score above that at 3 hrs in groups 2 and 3 during the interval from 3 to 6 hr with a trend toward greater mucosal edema in group 3 versus group 2 during the early phase of reperfusion (P=0.092).

There was a significant increase from baseline in cellular debris index in groups 2 and 3 from 0 to 3 hrs (Figure 4.13). There was no difference between groups 2 and 3 during this period. Debris index was significantly increased above that at 3 hrs during the 3 to 6 hr interval in groups 2 and 3. There was a significantly greater debris index in group 3 versus group 2 during the 3 to 6 hr period.
Figure 4.9

A photomicrograph of the large colon mucosa from an ischemia & reperfusion horse following 3 hrs of low flow ischemia and 3 hrs of reperfusion. Note that approximately 40-50% of the depth of the mucosal has undergone necrosis and crypt collapse. Bar = 50 µm.
Figure 4.10

A photomicrograph of the large colon mucosa from an ischemia horse following 6 hrs of low flow ischemia. Note that approximately 20-30% of the mucosal depth has undergone necrosis and crypt collapse. Bar = 50 μm.
Figure 4.11

Mean ± SEM for mucosal hemorrhage in the equine large colon during low flow ischemia and reperfusion. The solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group.
Figure 4.11

- Control
- Ischemia
- Ischemia & Reperfusion
Figure 4.12

Mean ± SEM for mucosal edema in the equine large colon during low flow ischemia and reperfusion. The solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group.
Figure 4.12
Figure 4.13

Mean ± SEM for mucosal cellular debris index in the equine large colon during low flow ischemia and reperfusion. The solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group. The solid bar over the 3 to 6 hr interval indicates that the ischemia & reperfusion group is significantly (P<0.05) greater than the ischemia group across this interval.
Figure 4.13
Quantitative Histomorphometric Assessment:

There was no difference in the measured % depth mucosal loss or mucosal I:C ratio among groups at baseline or across time in group 1.

There was a significant increase in measured % depth mucosal loss compared to baseline in groups 2 and 3 during the 0 to 3 hr period (Figure 4.14). There was no difference between groups 2 and 3 during the 0 to 3 hr period. Measured % depth mucosal loss was significantly increased above that at 3 hrs in groups 2 and 3 during the 3 to 6 hr interval. There was no difference between groups 2 and 3 during this period. Measured % depth mucosal loss was 25.8% vs. 40.7% at 5 hrs in groups 2 and 3 and was 37.3% vs. 42.4% by 6 hrs. There was a trend toward greater measured % depth mucosal loss in group 3 compared to group 2 during the 3 to 6 hr period (P=0.110).

The mean baseline I:C ratio ranged from 2.96 to 3.35 among the three groups. There was a trend toward an increase in the I:C ratio during the 0 to 3 hr period in groups 2 and 3 (P=0.079) compared to baseline, but there was no difference between the two groups (Figure 4.15). There was an increase in the I:C ratio in groups 2 and 3 compared to that observed at 3 hrs during the 3 to 6 hr interval. The I:C ratio increased to 6.53 and 7.27 at 6 hrs in groups 2 and 3 respectively. There was a trend toward a greater I:C ratio in group 3 compared to group 2 during the late phase of reperfusion (P=0.114).

Mean submucosal thickness at baseline ranged between 0.66 and 0.98 mm for all groups. There were no differences in submucosal thickness at baseline or across time among groups.

Discussion

Restoration of colonic arterial blood flow following low flow ischemia caused exacerbation of colonic mucosal injury. This injury was manifested as significantly greater estimated % depth of mucosal loss and increased cellular/nuclear debris. Trends (0.05 < P
Figure 4.14

Mean ± SEM for measured percent depth of mucosal loss in the equine large colon during low flow ischemia and reperfusion. The solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group.
Figure 4.14
Figure 4.15

Mean ± SEM for mucosal interstitial:crypt (I:C) ratio in the equine large colon during low flow ischemia and reperfusion. The solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group.
Figure 4.15
< 0.12) toward greater % surface mucosal disruption and mucosal edema during the early phase of reperfusion and greater mucosal hemorrhage, measured % depth mucosal loss, and I:C ratio during the late phase of reperfusion support this finding. Additionally, we have shown that neutrophils accumulate in the lamina propria of the mucosa upon reperfusion.\textsuperscript{20} Even though only 2 of the parameters were significantly different between groups 2 and 3 we believe that the % depth mucosal loss is one of the most clinically important; Snyder, et al. reported that horses with large colon volvulus having > 50% crypt necrosis were not likely to survive.\textsuperscript{8} In summary, colonic mucosal injury was worse following 3 hrs of ischemia and 3 hrs of reperfusion than a matched period (6 hrs) of ischemia alone. To our knowledge, this is the first report that demonstrates histopathologic evidence of reperfusion injury in the large colon of horses. If reperfusion injury had not occurred, we would expect equal or greater mucosal injury with 6 hrs of ischemia (group 2) compared to 3 hrs of ischemia followed by reperfusion (group 3). If 3 hrs of ischemia caused complete, irreversible necrosis of the full depth of the colonic mucosa then we would expect similar histopathologic injury by the end of 6 hrs in groups 2 and 3. The fact that % depth mucosal loss reached approximately 40% at 2 hrs of reperfusion and did not increase further during the third hour of reperfusion suggests that complete necrosis of the mucosa did not occur following 3 hrs of low flow ischemia and that mucosal damage peaked at 2 hrs of reperfusion. We believe this model of colonic ischemia is suitable to evaluate reperfusion injury.

Other models of large colon ischemia including complete arteriovenous occlusion\textsuperscript{9,10}, complete arterial occlusion, and complete venous occlusion\textsuperscript{9} have not demonstrated reperfusion injury. Complete intestinal ischemia may cause such severe injury that further injury upon reperfusion is undetectable or indistinguishable from the ischemic injury.\textsuperscript{12} To our knowledge, this study is the first to evaluate a model of partial
or low flow arterial ischemia in the equine large colon.

The vascular alterations associated with naturally occurring large colon volvulus typically include compromised venous flow and a partial reduction in arterial flow, which creates hemorrhagic strangulation obstruction. Marked transmural hemorrhage and edema occur during large colon volvulus. This hemorrhage and edema occurs as a result of the increased capillary hydrostatic pressure associated with venous occlusion. The colon is usually characterized by mild to moderate transmural edema with minimal hemorrhage after experimental complete arteriovenous occlusion. We chose not to use a venous occlusion model of ischemia in order to minimize edema and hemorrhage, which could disrupt the mucosal architecture and make subtle alterations associated with reperfusion difficult to detect. We believe our low flow model of ischemia blood flow changes similar to the arterial component of the vascular alterations occurring during colonic volvulus.

Compared to other models of altered intestinal blood flow, intestinal reperfusion injury has been observed most consistently with low flow arterial ischemia. The contributions of ischemia and reperfusion to intestinal injury depend upon the degree and duration of the ischemia. If ischemia is partial and of moderate duration (1-3 hrs) the majority of the intestinal injury occurs upon reperfusion. If intestinal vascular occlusion is complete and/or prolonged the tissue damage that occurs during the ischemic period becomes the predominant injury and the injury incurred by the tissue upon reperfusion may be inconsequential. In horses, 3 to 4 hrs of complete arteriovenous occlusion of the large colon causes irreversible mucosal necrosis. Our low flow model represented a suitable duration of ischemia in which irreversible mucosal necrosis did not occur and reperfusion injury was detectable.
Many studies in laboratory animals evaluating intestinal I-R \(^2,3,22\), and all equine studies evaluating large colon I-R \(^9-11,18\) have not compared the effects of I-R to a similar period of ischemia without reperfusion. It is possible that the tissue injury observed in these studies was a latent effect of ischemia alone and not worsened by reperfusion. Mucosal injury produced by 3 hrs of low flow ischemia of the cat small intestine and 1 hr of reperfusion was more severe than 4 hrs of ischemia without reperfusion.\(^{21}\) We demonstrated greater colonic mucosal injury occurred upon reperfusion as compared to a similar period of ischemia alone. We can thus rule out the possibility that the greater mucosal injury observed upon reperfusion was simply due to a manifestation of ischemic injury during this period.

Systemic hemodynamic and metabolic status was purposefully controlled and maintained stable in these horses throughout the course of our experiments.\(^{19}\) We believe it was imperative to maintain systemic hemodynamic and metabolic stability throughout the course of the experiment and to assure that local colonic hemodynamics and metabolism were not different between groups 2 and 3 during the 0 to 3 hr period. This ensured that histopathologic differences observed during the 3 to 6 hr period between groups 2 and 3 were due to local colonic hemodynamic and metabolic alterations occurring during this period.

Upon re-establishment of colonic blood flow in group 3, a series of biochemical reactions typical of reperfusion injury were likely initiated resulting in further mucosal injury and stimulating neutrophil infiltration.\(^1\) Intestinal reperfusion injury is classically initiated by XO-derived OFRs and amplified by neutrophil-derived OFRs.\(^2,13-17,23\) The small intestine of laboratory animals is a rich source of XO\(^24\), however, the pony large colon does not have high quantities of XO.\(^{25}\) Quantitation of XO has not been reported in horse large colon. There are other systems capable of generating OFRs in the colon. For
example, OFRs can be generated in rabbit colon via aldehyde oxidase. We demonstrated neutrophil accumulation in the large colon of these horses during low flow ischemia and further accumulation upon reperfusion. The exact mechanism of reperfusion injury in the equine large colon is unknown, however, it is probably similar to the mechanisms in tissues of other species.

Complete arteriovenous ischemia and reperfusion of the ascending colon of horses causes significant increases in microvascular permeability as measured by the osmotic reflection coefficient. Microvascular permeability was not measured directly in this study via the osmotic reflection coefficient, but increases in the mucosal I:C ratio, and hemorrhage and edema scores suggest increased microvascular permeability. Increases in these parameters could also occur with increased capillary hydrostatic pressure or decreased capillary oncotic pressure. There was no venous occlusion nor was there any evidence of microvascular thromboses that would account for increased hydrostatic pressure. A low plasma oncotic pressure could lead to edema, but it would be unlikely to lead to hemorrhage. Total plasma protein decreased over time in all groups, however, it was not likely low enough to lead to edema. Therefore, we speculate the increases in hemorrhage and edema scores and in the mucosal I:C ratio across time in groups 2 and 3 are indicative of increased microvascular permeability associated with low flow I-R. We defined the mucosal I:C ratio as the ratio of the area of the mucosa occupied by the interstitium to the area occupied by the crypts. The normal I:C ratio was < 3. This is different from the I:C ratio previously reported in the ascending colon of horses. Snyder et al., defined the I:C ratio as the ratio of the width of the interstitium between adjacent crypts to the width of the crypts and a normal I:C ratio ≤ 1. In our study, the areas occupied by the interstitium and the crypts were determined by actual measurement with a computer-based image analysis system. Visual estimation of the I:C ratio was performed by Snyder et al.
Despite increased mucosal edema in groups 2 and 3, there were no increases in submucosal thickness in any group. This may be due to the extreme variation in the thickness among horses and within horses across time. This variation was partly due to artifactual separation of the colonic wall at the submucosal-muscularis junction due to harvesting, handling, and processing of the biopsies. Additionally, submucosal thickness may have varied within a horse due to contraction of colonic smooth muscle. Regardless of the reason for no increase in colonic submucosal thickness, we believe this is not a useful or reliable parameter to evaluate colonic interstitial edema.

Histomorphologic scoring and estimation of mucosal injury appeared to correspond to the more objective measures of mucosal injury as determined by histomorphometric evaluation. The estimated and measured % depth mucosal loss were similar. Likewise, the changes in hemorrhage and edema scores were similar to the changes in the measured I:C ratio. Histomorphologic evaluation of entire and multiple sections of colon had the advantage of providing an overall or global assessment of mucosal injury. The potential for bias and subjectivity in the histomorphologic scoring was minimized by using a semiquantitative scoring system and by having two investigators evaluate the colonic biopsies independently and blindly. Histomorphometric evaluation provided a more objective and quantitative assessment of mucosal injury, however, since entire sections cannot be evaluated, this may not provide the most representative measure of overall mucosal injury. We believe that the two types of histopathologic evaluation of colonic mucosal injury compliment each other and both should be used to assess colonic mucosal injury.

The mucosal alterations that developed during low flow ischemia are similar to those alterations reported in the large colon subjected to complete arteriovenous occlusion.\textsuperscript{11,18} The mucosal alterations in our study worsened with either continued ischemia or reperfusion during the 3 to 6 hr period. Clusters of surface epithelial cells
became detached from the underlying mucosa resulting in disruption and sloughing of the colonic surface epithelium. Necrosis and sloughing proceeded toward the base of the mucosa with collapse of the crypts. Hemorrhage and edema increased in the lamina propria causing distortion of the normal colonic architecture and the space between colonic crypts became greater as edema fluid and hemorrhage accumulated. The single layer of mucosal cells that aligned parallel to the luminal surface during the later phase of reperfusion has also been reported following complete arteriovenous occlusion of the large colon.18

We conclude that reperfusion injury occurs in the large colon of horses following the re-establishment of colonic arterial blood flow after 3 hrs of low flow arterial ischemia. The alterations that develop in the colonic mucosa are comparable to those previously reported with colonic ischemia, however, reperfusion exacerbates colonic mucosal injury.

Footnotes

aRompun®, Mobay Corporation, Animal Health Division, Shawnee, KS.
bCarbocaine®-V, The Upjohn Company, Kalamazoo, MI.
cAngiocath®, Becton Dickinson Vascular Access, Sandy, Utah.
dGuailaxin®, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.
fHalothane U.S.P., Halocarbon Laboratories, North Augusta, SC.
gDrager AV Model NELAC-E, Anesthesia Ventilator, Telford, Pa.
hLactated Ringer’s, Baxter Healthcare Corporation, Deerfield, Il.
iAngiocath®, Becton Dickinson Vascular Access, Sandy, Utah.
jAquatic K Module, Gorman-Rupp Industries, Bellville, Ohio.
List of References


27. Henninger DW, Snyder JR, Pascoe JR, et al. Microvascular permeability changes in
CHAPTER V
NEUTROPHIL ACCUMULATION IN THE EQUINE LARGE COLON
DURING LOW FLOW ISCHEMIA AND REPERFUSION

Summary

Histomorphology/ morphometry, leukocyte scintigraphy, and myeloperoxidase activity were used to determine if neutrophils accumulate in the large colon of horses during low flow ischemia and reperfusion. Twenty-four adult horses were assigned to one of 3 groups: 1) sham-operated (n=6), 2) 6 hrs ischemia (n=9), and 3) 3 hrs ischemia and 3 hrs reperfusion (n=9). Low flow ischemia of the large colon was induced in groups 2 and 3 by reducing colonic arterial blood flow to 20% of baseline. Radiolabeled $^{99m}$Tc autogenous neutrophils were injected at 175 min, which corresponded to 5 min prior to reperfusion in group 3. Full-thickness biopsies of the left ventral colon were harvested at baseline and at 30 min intervals for 6 hrs; a portion of the biopsy was placed in formalin for histopathology and the remainder was used to measure mucosal radioactivity and myeloperoxidase activity. There were no differences in baseline mucosal neutrophil index, mucosal neutrophil number, submucosal venular neutrophil number, mucosal radioactivity, or mucosal myeloperoxidase activity among groups, or over time in group 1. Neutrophils accumulated in the colonic mucosa during ischemia and further increased upon reperfusion as demonstrated by neutrophil index (morphology) and mucosal neutrophil number (morphometry); mucosal neutrophil index was significantly greater in group 3 during reperfusion than at the corresponding periods of ischemia in group 2. Neutrophils were significantly increased in submucosal venules at 10 min of reperfusion in group 3 and were
significantly greater in group 3 than in group 2 during the interval from 3 to 6 hrs. Mucosal radioactivity significantly increased upon reperfusion in group 3; there was a trend (P=.076) toward greater mucosal radioactivity in group 3 compared to group 2 throughout the 3 to 6 hr interval. There were no differences in mucosal myeloperoxidase activity among or within any of the 3 groups over time.

Neutrophils accumulate in the equine large colon during low flow ischemia and reperfusion. Neutrophil infiltration was demonstrated with histopathology and leukocyte scintigraphy, but not with myeloperoxidase activity. The accumulation of neutrophils that occurred during ischemia and the further neutrophil infiltration that occurred during reperfusion suggest that neutrophils may contribute to reperfusion injury of the large colon.

Introduction

Neutrophils are key components of normal defense mechanisms and inflammation, and play an important role in the pathogenesis of several gastrointestinal disorders. Neutrophil-mediated endothelial injury and tissue damage have been well documented. Stimulated neutrophils undergo chemotaxis, adherence to vascular endothelium, and migration into adjacent tissues. Neutrophil activation results in degranulation and the release of mediators of tissue injury, such as proteases and oxygen-derived free radicals (OFRs). Neutrophils passively injure the endothelium by adhering to and migrating across the vessel wall and releasing injurious substances that cause increased microvascular permeability. Neutrophil infiltration into tissues during ischemia and reperfusion (I-R) is a time dependent process. Significant neutrophil infiltration may require several hours if vascular occlusion is complete whereas neutrophil infiltration occurs rapidly if the tissue is reperfused.
Reperfusion injury is tissue injury that occurs following restoration of blood flow after an episode of ischemia; it has been repeatedly demonstrated in intestinal tissues of rats, cats, and dogs.\textsuperscript{1,12-18} Reperfusion injury is believed to be principally initiated by the generation of OFRs.\textsuperscript{12,13,17} Superoxide radicals, produced from hypoxanthine in the presence of xanthine oxidase (XO), are further metabolized to hydrogen peroxide and hydroxyl radicals.\textsuperscript{19} These OFRs stimulate neutrophil chemotaxis and cause cell membrane damage via lipoperoxidation. Neutrophils also generate superoxide anions via the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system.\textsuperscript{11} Intestinal injury associated with I-R is initiated by XO-derived OFRs and amplified by neutrophil-derived OFRs.\textsuperscript{20} Cytokines released during intestinal I-R cause neutrophil chemotaxis and adhesion to post-capillary venular endothelium.\textsuperscript{21-25} Neutrophil-endothelial adhesion and migration through the post-capillary venular endothelium associated with intestinal I-R is reduced by pretreatment with allopurinol (a xanthine oxidase inhibitor), superoxide dismutase (a superoxide radical scavenger), catalase,\textsuperscript{26} deferoxamine,\textsuperscript{26} and platelet activating factor antagonists,\textsuperscript{27} further demonstrating the interaction between xanthine oxidase-derived OFR and neutrophils in I-R injury. Administration of monoclonal antibodies directed against the CD11/CD18 neutrophil adhesion receptor complex\textsuperscript{7} or dimethylsulfoxide\textsuperscript{28} prevents neutrophil adherence and infiltration and increased microvascular permeability associated with intestinal I-R. Depletion of circulating neutrophils also attenuates the increased microvascular permeability induced by intestinal I-R,\textsuperscript{6,7} providing evidence that neutrophils play an important role in I-R injury.

Intestinal neutrophil accumulation has been evaluated in rats and cats using histopathology, leukocyte scintigraphy, myeloperoxidase activity, and intravital microscopy.\textsuperscript{8} Histopathologic examination has been the most frequently used method of
detecting and quantitating intestinal neutrophils. Histomorphologic examination provides a qualitative and semi-quantitative assessment of infiltration, but histomorphometry provides a more quantitative method. These histopathologic techniques have served as the standard, but they are time consuming because they require examination of numerous tissue sections, and they could be inaccurate if the neutrophil infiltration is not homogeneous throughout the tissue.

Tissue neutrophil infiltration can be identified and semiquantitated by leukocyte scintigraphy. White blood cells are labeled in vitro with a radionuclide then injected intravenously. Tissue radioactivity is measured; increased radioactivity signifies leukocyte infiltration. Neutrophil scintigraphy is more precise, but requires ex vivo manipulation during collection, separation, and labeling. Manipulation could alter structure and/or function of neutrophils which may alter response to chemotactic stimuli, endothelial adhesion and migration.

Myeloperoxidase (MPO) is a membrane bound enzyme contained in leukocytes, especially granulocytes. Myeloperoxidase activity is used as a marker of tissue neutrophil infiltration, however, its use in the intestine of horses has not been previously reported. The MPO assay provides an average value for neutrophil infiltration in tissues where neutrophil accumulation is not homogeneous.

Our low flow model of ischemia causes greater mucosal necrosis, edema, and hemorrhage following ischemia and reperfusion than after a comparable period of ischemia alone. To our knowledge, there have been no studies in the horse specifically quantitating neutrophil accumulation in the intestine during ischemia or reperfusion. The purpose of this study was to determine if neutrophils accumulate in the large colon of horses during low flow ischemia and if neutrophil accumulation is greater following reperfusion. Three methods of assessing neutrophil accumulation in the large colon during
I-R were evaluated; histomorphology/ morphometry, leukocyte scintigraphy and mucosal MPO activity.

**Materials and Methods**

This project was approved and performed under the guidelines of the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

*Animals* - Twenty-four dewormed horses of various breeds (9 Thoroughbreds, 8 Standardbreds, 3 Quarter Horses, 2 Saddlebreds, and 2 Hanoverians), ranging in age from 1-10 years and body weight from 350-570 kilograms (kg) were used. There were 11 females, 5 intact males, and 8 geldings. Horses were fed 1-2% of their body weight in grass hay per day and water was provided ad libitum. Horses were adapted to their diet and environment for at least one week prior to the experiment. Hay, but not water, was withheld for a 24 hrs prior to the experiment.

*Instrumentation* - Horses were sedated with xylazine\(^a\) (0.5 mg/kg IV). Mepivacaine hydrochloride\(^b\) was infiltrated subcutaneously to facilitate intravascular catheter placement. A 14-gauge 13.3 cm teflon catheter\(^c\) was inserted into the left jugular vein for administration of anesthetic drugs, isotonic fluids, and \(^{99m}\)Tc radiolabeled autogenous white blood cells. General anesthesia was induced with guaifenesin\(^d\) (30 mg/kg IV) and thiamylal\(^e\) A (3-4 mg/kg IV) and maintained with halothane\(^f\) (1-3%) in oxygen. Horses were mechanically ventilated\(^g\) at 6-12 breaths per minute at peak inspiratory pressure of \(<\) 25 cm H\(_2\)O. Isotonic polyionic fluids\(^h\) were administered IV at 5-10 ml/kg/hr. Mean arterial blood pressure was monitored following percutaneous placement of a 20-gauge 5.1 cm teflon catheter\(^i\) in the facial artery.

Horses were positioned in dorsal recumbency and prepared for aseptic surgery. Following a ventral midline celiotomy, the abdomen was explored and large colon was
exteriorized and placed on a warm water heating pad. A thermocouple was inserted into the seromuscular layer of the colon to monitor local colonic temperature. Colon temperature was maintained between 34° and 38° C with a heat lamp. Evaporative heat and moisture loss were prevented by placing cellophane over the colon. A doppler ultrasound flow probe was placed externally around the colonic artery supplying the ventral colon and colonic blood flow was measured continuously and recorded.

Experimental Design - Twenty-four horses were randomly assigned to one of three groups. Group 1 (n=6) served as sham-operated controls. These horses were subjected to general anesthesia and a ventral midline celiotomy. The large colon was exteriorized and instrumented, however, colon ischemia was not induced. Group 2 (n=9) served as ischemic controls. These horses were subjected to the same procedures as group 1 and 6 hrs of low flow colonic ischemia. Group 3 horses (n=9) were subjected to 3 hrs of low flow colonic ischemia followed by 3 hrs of reperfusion.

Low flow ischemic strangulation obstruction of the large colon was produced by reducing colonic arterial blood flow to 20% of baseline following recording of baseline hemodynamic parameters and harvesting of colonic biopsies. Briefly, transmural compression was achieved by placing a 20 cm segment of latex tubing tightly around the circumference of the right dorsal and ventral colon. Umbilical tape ligatures were placed around the colonic arteries supplying the dorsal and ventral colon, passed through a 2 cm segment of plastic tubing and tightened as a Rummel tourniquet until the colonic blood flow was reduced to 20% of baseline. Blood flow in the ventral colonic artery was continuously monitored using the doppler ultrasound flow probe. Mean arterial blood pressure was maintained ≥ 70 mm Hg. Horses were euthanatized at the conclusion of the experiment with an overdose of sodium pentobarbital (100 mg/kg IV).
Following surgery and colonic instrumentation (approximately 1 hr), hemodynamic parameters were recorded and colon biopsies harvested at baseline (time = 0) and at 30 min intervals. Additionally, data were collected at 185, 190, and 195 min, which correspond to 5, 10, and 15 min of reperfusion in group 3.

**Colonic Biopsy** - Full-thickness 2 cm x 5 cm sections of the left ventral colon were harvested. Biopsy samples were gently rinsed in buffered electrolyte solution and were divided in half. A 2 cm x 1 cm section was immediately placed in 10% formalin for histopathologic evaluation. Mucosa was separated from the submucosa in the remaining portion of the biopsy sample. Mucosal radioactivity was measured (see leukocyte scintigraphy section below) and the mucosa was placed in aluminum foil and immediately frozen by submersion in liquid nitrogen. The mucosal sample was stored at -70°C until analyzed for myeloperoxidase activity.

**Histopathology** - Biopsy specimens were embedded in paraffin, cut in 4 um sections, processed and stained with hematoxylin & eosin. Basilar, middle, and surface one-thirds of the colonic mucosa were histomorphologically scored from 0 to 4 for the presence of neutrophils and eosinophils; grade 0 was assigned when these cells were absent, grade 1 was assigned if these cells were observed after careful inspection, and grades 2, 3, and 4 were assigned if neutrophil and eosinophil accumulation were mild, moderate or marked. Histomorphologic evaluation was performed independently by 2 blinded investigators; the scores were averaged for each horse and time period. The mucosal neutrophil index and eosinophil index were calculated as the sum of the average scores for each of the three areas for both cell types. Histomorphometric evaluation including quantitation of the number of neutrophils in the colonic mucosa and in submucosal venules was performed with the investigator blinded to the animal and time period. The numbers of neutrophils in a 0.01 mm² area were counted in the base of the
mucosa using oil immersion on a microscope equipped with an ocular grid; the grid was positioned adjacent to the muscularis mucosa in the base of the mucosa. The numbers of neutrophils were counted in 10 adjacent fields for each horse and time period. Ten submucosal venules were identified and the cross sectional areas were measured using a microscope equipped with a calibrated cursor coupled to a microcomputer-based image analysis system; the numbers of neutrophils contained within each venule were counted and recorded. The number of neutrophils per cross-sectional area was used as an index of the accumulation or adhesion of neutrophils in post-capillary venules.

**Leukocyte Scintigraphy** - Jugular venous blood (50 ml) was collected into a 60 ml syringe containing acid-citrate-dextrose (1 ml per 10 ml of blood). The syringe was allowed to stand upright for 60 min to allow red blood cell sedimentation. The leukocyte rich plasma (supernatant) was slowly expressed through a 19 gauge butterfly needle into 2 sterile, pyrogen free polypropylene centrifuge tubes (approximately 15 ml volume). The tubes were centrifuged at 400g for 5-8 min at 22°C. The leukocyte poor plasma (supernatant) was removed and placed in sterile centrifuge tubes. Preparation of 99mTc HM-PAO involved mixing sterile saline and HM-PAO, adding 99mTcO₄⁻, and incubating it for 5 min. Approximately 1.0 mCi of 99mTcO₄⁻ was added to the leukocyte pellet. The suspension was incubated and gently agitated for 10 min. The leukocyte-HM-PAO preparation was centrifuged at 400g for 5 min at 22°C. The supernatant was removed and the leukocyte pellet was resuspended in the autogenous leukocyte poor plasma (total volume of 5 ml). Labeling efficiency of leukocytes with 99mTc was determined using a dose calibrator and the percent viability of the labeled cells was determined using 0.1% trypan blue dye exclusion. Resuspended radiolabeled leukocytes were injected through the jugular venous catheter at 175 min (5 minutes prior to reperfusion in group 3) in all groups. Radioactivity was measured on the mucosa from a 2 x 3 cm section of large colon.
using a portable gamma counter. Mucosal radioactivity was measured as counts per 10 seconds and standardized by determining the percent of the injected dose after correcting for radioactive decay.

_Myeloperoxidase Assay_ - Myeloperoxidase activity was determined in colonic mucosa using 2 variations of a modification of the assay methodology of Grisham. The assays are similar with the exception of the detergent used to disrupt granules and solubilize the peroxidase enzymes. One assay utilized hexadecyltrimethyl ammonium bromide (HETAB) and the other hexadecyltrimethyl ammonium hydroxide (HETOH) to disrupt the granules. A 10% weight/volume mucosal homogenate was prepared using a solution of 20mM potassium phosphate buffer (KPi) and 1.0 mM EDTA. The suspension was homogenized using a motorized tissue homogenizer for approximately 20 seconds or until it was completely homogenized (no visible tissue particles remaining). One ml of the homogenate was added to 9.0 ml of 20 mM KPi (pH=7.4); the mixture was vortexed and centrifuged at 8,000 g and 4°C for 20 min.

_HETAB Assay_ - The supernatant was removed and the pellet was resuspended with 1.0 ml of a solution containing 50 mM KPi (pH=6.0), 0.5% hexadecyltrimethyl ammonium bromide (HETAB), and 10 mM EDTA. The suspension was homogenized for approximately 10 sec. A 50 ul sample was then diluted in 450 ul of double distilled water (1:10 dilution) prior to performing the assay. A 50 ul aliquot of the sample was transferred with a pipette into a test tube containing 445 ul of a cocktail solution containing 6 ml of double distilled water, 0.8 ml of 1.0 mM KPi (pH=5.4), 1.6 ml of 10 mM tetramethylbenzidine diluted in N,N-dimethylformamide (TMB-DMF), and 0.5 ml of 10% HETAB.

_HETOH Assay_ - The supernatant was removed and the pellet resuspended in 1.0 ml of a solution containing 50 mM glacial acetic acid and 0.5% HETOH (pH=6.0). The
samples were then rehomogenized for 10 sec. A 50 ul aliquot was added to 445 ul of a cocktail solution containing 7.1 ml double distilled water, 500 ul 1.0 M acetic acid (pH=5.4), and 1.4 ml TMB-DMF.

Test tubes containing the sample and cocktail solution were placed in a 37° C water bath for 5 min. Five ul of 30mM hydrogen peroxide was added to the mixture to start the reaction. The reaction was allowed to proceed for exactly 3 min and was immediately stopped by the addition of 10 ul of catalase (300 ul/ml). Tubes were gently shaken, placed on ice and 2.0 ml of 0.2 M glacial acetic acid was added. Absorbances were read on a spectrophotometer at 655 nm within 15 min of stopping the reaction. Absorbance of a blank sample (450 ul cocktail solution and 50 ul double distilled water) was subtracted from each sample absorbance. Myeloperoxidase activity (units/ml of extract) was determined by subtracting the blank absorbance from the sample absorbance, then multiplying by the dilution factor, and finally dividing by 3 min, the time the reaction was allowed to proceed. The measured MPO activity was then standardized to the mucosal protein concentration as determined by a dye binding method.

Circulating Neutrophil Myeloperoxidase Activity-Standard Curves - Myeloperoxidase activity of isolated equine circulating neutrophils was determined using blood collected from 7 healthy adult horses. Jugular venous blood was collected into heparinized tubes. Five ml of the blood was added to 20 ml Hank’s balanced salt solution in conical tubes and vortexed. Ten ml of sodium diatrizoate (lymphocyte separation media - LSM) was pipetted into the bottom of the tubes using a long needle. The mixture was then centrifuged for 20 min at 400 g and 22° C. The Hank’s solution, LSM, and mononuclear cell layers were pipetted and discarded. Red blood cells were lysed by resuspending the pellet in 5-7 ml of Hank’s solution and 0.87% NH₄Cl (3 times the volume of resuspension). The solution was then centrifuged for 5 min at 200 g at 22° C
and the supernatant discarded. The leukocyte pellet was resuspended in 2.0 ml phosphate buffered saline. The number of leukocytes per ml was counted using a Coulter counter. A 1.5 ml sample was placed in a microcentrifuge tube and centrifuged at 400 g for 10 min. Supernatant was discarded and the pellet was frozen at -70°C. The total number of leukocytes was calculated by multiplying the number of leukocytes per ml x 1.5 ml. A differential count of the leukocytes was determined following cytocentrifugation of an aliquot of the sample. The % viability of the isolated leukocytes was determined by 0.1% trypan blue dye exclusion.\textsuperscript{32}

The pellet was resuspended in either HETAB (n=4) or HETH (n=3); 500 ul were added and the suspension was sonicated for 10 to 15 sec and then an additional 500 ul of the detergent were added and the suspension was vortexed. Serial dilutions of the suspension were made by placing aliquots of the sample in double distilled water to bring the total volume to 1.0 ml. Serial dilutions of the parent neutrophil suspension (1 x 10^6 cells/ml) were assayed for MPO activity. A 50 ul sample of the diluted suspensions was assayed with HETAB (n=4 horses) or HETH (n=3 horses) using the assays above. A standard curve was constructed for MPO activity versus neutrophil number for both HETAB and HETH.

\textit{Statistical Analyses} - All data are expressed as mean ± standard error of the mean (SEM). Data were analyzed using a two-way analysis of variance (ANOVA) with repeated measures. A Dunnett’s t-test was used to compare back to baseline and a Tukey’s test was used for all pairwise comparisons. Two way ANOVA was used to compare the entire time interval from 3 to 6 hrs between groups 2 and 3 for mucosal neutrophil index, mucosal neutrophil number, submucosal venular neutrophils, and mucosal leukocyte scintigraphy. Level of significance was set at $P<0.05$ for all statistical tests.
Results

**Histopathology** - There was no difference in the mucosal neutrophil index among groups at baseline nor over the course of the experiment in group 1 (Figure 5.1). Significant increases in mucosal neutrophil index occurred in groups 2 and 3 during the first 3 hrs. Neutrophil index remained significantly increased during the 3 to 6 hr interval, but did not increase further during this time in group 2. Neutrophil index significantly increased from 3 hrs of ischemia to 0.5 hrs of reperfusion in group 3 and remained increased throughout the reperfusion period. The neutrophil index was significantly greater in group 3 than in group 2 during the 3 to 6 hr period.

There was no difference in mucosal neutrophil numbers at baseline or during the 0 to 3 hr period among groups nor were there increases over time in group 1 (Figure 5.2). Mucosal neutrophil number was not different between groups 2 and 3. Significantly greater mucosal neutrophil numbers occurred in groups 2 and 3 versus group 1; significant increases in neutrophil number occurred at 15 min of reperfusion in group 3, and at 4 hrs of ischemia in group 2. Neutrophils accumulated in the lamina propria of the colonic mucosa during reperfusion in group 3 (Figures 5.3 and 5.4).

Submucosal venular neutrophil numbers were not different at baseline among groups nor over time in groups 1 and 2 (Figure 5.5). Neutrophil numbers significantly increased in submucosal venules at 10 min of reperfusion in group 3 (Figures 5.6 and 5.7). Significantly more neutrophils were present in submucosal venules in group 3 compared to group 2 during the 3 to 6 hrs interval.

There was no difference in the eosinophil index at baseline among groups or across time in group 1 (Figure 5.8). Eosinophil index significantly increased at 3.25 and 4 hrs of ischemia in group 2 and at 15 min of reperfusion in group 3.

**Leukocyte Scintigraphy** - Baseline mucosal radioactivity did not vary among groups (Figure 5.9). Additionally, mucosal radioactivity did not change over time in
Figure 5.1

Mean ± SEM mucosal neutrophil index in the equine large colon during low flow ischemia and reperfusion. The solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group. The letter "b" is significantly (P < 0.05) different from "a" and "c" is different from "b". Solid line extending across top of graph from 3 to 6 hrs indicates neutrophil index is greater in ischemia & reperfusion group compared to ischemia group across the entire interval.
Figure 5.1
Figure 5.2

Mean ± SEM mucosal neutrophil number in the equine large colon during low flow ischemia and reperfusion. The solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group. The letter "b" is significantly (P<0.05) different from "a". Solid line extending across top of graph from 3 to 6 hrs indicates mucosal neutrophil number is greater in ischemia & reperfusion group compared to ischemia group across the entire interval.
Figure 5.2
Figure 5.3

Photomicrograph of section of left ventral colon illustrating intense neutrophil infiltration (grade 4 neutrophil index) in the lamina propria of the large colon mucosa in an ischemia & reperfusion horse during reperfusion. Bar = 25 μm.
Figure 5.3
Figure 5.4

Photomicrograph of section of large colon from an ischemia & reperfusion horse during reperfusion. Note the neutrophil accumulation in a vessel (arrow) that extends from the submucosa (SM) into the mucosa (M); some neutrophils have migrated out of vessels into the lamina propria. Bar = 25 μm.
Figure 5.5

Mean ± SEM for submucosal venular neutrophils in the equine large colon during low flow ischemia and reperfusion. The solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group. The letter "b" is significantly (P<0.05) different from "a". Solid line extending across top of graph from 3 to 6 hrs indicates mucosal neutrophil number is greater in ischemia & reperfusion group compared to ischemia group across the entire interval.
Submucosal Venular Neutrophils (neutrophils/mm² area)

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Control</th>
<th>Ischemia</th>
<th>Ischemia &amp; Reperfusion</th>
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<td>2500</td>
<td>0</td>
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<td>1750</td>
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<td>1900</td>
<td>2500</td>
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</table>

Figure 5.5
Figure 5.6

Photomicrograph of a section of large colon from an ischemia & reperfusion horse at 10 minutes of reperfusion. Note marked neutrophil accumulation in submucosal venules (arrows), M=mucosa and SM=submucosa. Bar = 50 μm.
Figure 5.7

Higher magnification of the photomicrograph of the section of large colon shown in figure 5.6. Note the accumulation of neutrophils in submucosal venules (arrows); many of the neutrophils are located eccentrically as if undergoing adherence to the venular endothelium. Bar = 25 μm.
Figure 5.8

Mean ± SEM mucosal eosinophil index in the equine large colon during low flow ischemia and reperfusion.
Figure 5.8

- Control
- Ischemia
- Ischemia & Reperfusion

Eosinophil Index vs. Time (hrs)
Figure 5.9

Mean ± SEM mucosal radioactivity in the equine large colon during low flow ischemia and reperfusion. Open arrow at 5 min prior to 3 hrs indicates time of injection of $^{99m}$Tc-radiolabeled autogenous neutrophils. The solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group. Dashed line extending across 3 to 6 hr interval indicates a trend ($P=0.076$) toward a greater mucosal radioactivity in ischemia & reperfusion group versus ischemia group during this interval.
Figure 5.9

Mucosal Radioactivity (% injected dose)

- ▲ Control
- ■ Ischemia
- ○ Ischemia & Reperfusion

Time (hrs)

Figure 5.9
groups 1 or 2 during the 3 to 6 hrs period. Mucosal radioactivity significantly increased upon reperfusion in group 3, increasing by 1 hr of reperfusion and remaining increased throughout the reperfusion period. There was a trend (P=.076) toward greater mucosal radioactivity in group 3 compared to group 2 during the interval from 3 to 6 hrs. Mean circulating white blood cell count at the time of blood collection for leukocyte labeling was 8,490/ul with 63% (range: 32-78) segmented neutrophils. Mean labeling efficiency was 67% (range: 38-87) and the mean % viability was 94% (range: 91-97).

*Myeloperoxidase Activity* - Baseline mucosal MPO activity did not vary among groups or change within a group over time (Figure 5.10); no differences were detected using either the HETAB or HETOH assay. Mean circulating total white blood cell count at the time of blood collection for neutrophil isolation was 7,740/ul with 70% segmented neutrophils. Following granulocyte isolation and resuspension of the leukocyte pellet, the mean number of leukocytes/ml was 1.62 x 10^7 with 94% neutrophils.

*Neutrophil-MPO Standard Curves* - Standard curves for MPO activity and neutrophil numbers were constructed; curves were constructed for neutrophils isolated from the peripheral circulation using both HETAB and HETOH detergents (Figures 5.11 and 5.12). Neutrophil number can be predicted from the linear regression equations for the HETAB MPO (y = 0.59263 + 3.2452e^-5x, R^2=.947) or from the HETOH MPO activity (y = 1.0694e^-2 + 5.8371e^-6x, R^2=.995).

**Discussion**

To our knowledge there have been no previous reports on quantitation or semi-quantitation of neutrophil accumulation in the equine gastrointestinal tract associated with experimental I-R. Colonic neutrophil infiltration associated with I-R has been described in horses, however, it was not determined if neutrophil accumulation occurs
Figure 5.10

Mean ± SEM mucosal myeloperoxidase (MPO) activity in the equine large colon during low flow ischemia and reperfusion. The solid arrow at 3 hrs indicates time of reperfusion in ischemia & reperfusion group.
Figure 5.10
Figure 5.11

Standard curve for neutrophil myeloperoxidase (MPO) activity as measured by hexadecyltrimethyl ammonium bromide (HETAB). Linear regression equation for estimating the number of neutrophils from MPO activity and the correlation coefficient between MPO and neutrophil number are given.
Figure 5.11

y = 0.59263 + 3.2452e-5x  
$R^2 = 0.947$
Figure 5.12

Standard curve for neutrophil myeloperoxidase (MPO) activity as measured by hexadecyltrimethyl ammonium hydroxide (HETOH). Linear regression equation for estimating the number of neutrophils from MPO activity and the correlation coefficient between MPO and neutrophil number are given.
HETOH MPO Activity (units/min)

Figure 5.12

$y = 1.0694e^{-2} + 5.3716e^{-6}x$

$R^2 = 0.995$
primarily during ischemia or reperfusion. We report for the first time a semi-quantitation of neutrophil accumulation in the large colon of horses during experimental I-R. Large colon mucosal neutrophils increased during ischemia and increased further upon reperfusion. Neutrophil accumulation was demonstrated in the colonic mucosa during low flow I-R using histomorphology/morphometry and leukocyte scintigraphy; mucosal myeloperoxidase activity did not increase during ischemia or reperfusion. Neutrophils have been repeatedly demonstrated to accumulate in the intestinal wall of laboratory animals during I-R.1,7,9,10,20,25,26,34

Reperfusion injury has been demonstrated in the large colon of horses using this low flow model of ischemia.31 Systemic hemodynamic or metabolic parameters did not differ among groups at baseline or within a group over time.35 Local colonic hemodynamic and metabolic alterations were similar in groups 2 and 3 indicating the degree of ischemia was the same in both groups.35 This is important when comparing mucosal injury and neutrophil accumulation between these two groups; if the degree of ischemia were different it would be inappropriate to compare neutrophil accumulation between the two groups.

Neutrophil accumulation increased in the colonic mucosa during low flow ischemia and further increased upon reperfusion. Neutrophil accumulation, as shown by the neutrophil index, occurred in the lamina propria throughout the depth of the mucosa during ischemia with greater accumulation upon reperfusion. Increased submucosal venular neutrophils were observed within 10 min of reperfusion, providing an estimate of neutrophil adherence to post-capillary endothelium. This correlates with the increased adherence of neutrophils to post-capillary venular endothelium observed in feline small intestine subjected to I-R; maximal neutrophil adherence, as observed with intravital microscopy, occurred at 10 min of reperfusion.34
Histomorphology/ morphometry are useful for measuring intestinal neutrophil infiltration and localizing the accumulation to a particular region of the intestinal wall (i.e. mucosa vs submucosa), but there are disadvantages to these techniques. These procedures are time consuming because multiple sections must be evaluated to obtain an accurate assessment of tissue neutrophil infiltration, especially if neutrophil infiltration is heterogeneous. Inherent disadvantages of histomorphology include subjectivity of scoring and bias; this was minimized using a semiquantitative scoring system and having two investigators score the slides blindly and independently. Histomorphologic evaluation provides a global or overall assessment of neutrophil infiltration throughout the tissue section, not just a few focal areas as measured with histomorphometric techniques. Therefore, the combination of histomorphologic and morphometric evaluation provide complementary information regarding neutrophil infiltration.

Leukocyte scintigraphy demonstrated colonic mucosal neutrophil accumulation, providing a semi-quantitative measurement of neutrophil accumulation. Increased mucosal radioactivity occurred in group 3 during reperfusion. The increase in mucosal radioactivity is indicative of neutrophil accumulation. Although unlikely, the increased radioactivity could potentially represent accumulation of free radiopharmaceutical or other radiolabeled cells. Radiopharmaceutical elution from equine leukocytes has been reported; radiopharmaceutical was eluted more rapidly from granulocytes labeled with $^{99m}$Tc versus $^{111}$In during the first 4 hours, but was not different at 6 hrs. Renal excretion is the major route of removal of $^{99m}$Tc. If elution of $^{99m}$Tc from the leukocytes occurred in our horses it would be unlikely it would have been detected in the colonic mucosa, particularly in only one group of horses. The sensitivity and specificity of this technique of semi-quantitation of intestinal neutrophils are unknown. The fact that mucosal radioactivity was greater in group 3 during reperfusion than in either the sham-operated control or
ischemic control horses at a similar experimental time combined with greater neutrophil accumulation demonstrated during reperfusion using histopathology suggests the increased radioactivity was due to increased mucosal neutrophil infiltration.

Leukocyte scintigraphy demonstrated neutrophil accumulation, but a number of potential disadvantages should be noted. The experimental animal or carcass is radioactive thus requiring a quarantine. The in vitro labeling process could potentially modify the structure and function of neutrophils which could alter chemotaxis, adherence, migration, or phagocytosis. Studies have not evaluated the effect of radiolabeling on equine neutrophil chemotaxis, adherence or emigration. Phagocytic function of labeled neutrophils was not evaluated in the present study, however, phagocytic function following labeling of granulocytes with $^{99m}$Tc in horses has been evaluated by use of a phagocytic assay. No significant difference between granulocytes labeled with $^{99m}$Tc and $^{111}$In during the first 4 hrs post-labeling was reported, but by 6 hrs $^{99m}$Tc labeled cells had greater phagocytic function. Granulocytes labeled with both radiopharmaceutical agents maintained approximately the same phagocytic function as nonlabeled cells.

Labeling characteristics and viability of equine granulocytes radiolabeled with $^{99m}$Tc and $^{111}$In were compared for 6 hrs post-labeling. Significantly greater labeling efficiency was present for granulocytes labeled with $^{111}$In (62%) versus $^{99m}$Tc (32%). The labeling efficiency of neutrophils in our study was approximately 67%, which is greater than reported by Daniel et al. Mean viability was approximately 94% for radiolabeled neutrophils from all horses in our study, which compares favorably with granulocyte viability previously reported. We believe it is unlikely that labeling neutrophils with $^{99m}$Tc caused significant alterations in neutrophil structure, function, or viability over the short period (3-4 hrs) of the experiment following labeling and reinjection. With refinements in quantitating mucosal radioactivity we believe leukocyte
scintigraphy could be a feasible method for semi-quantitation of mucosal neutrophils in the equine large colon.

Measurement of MPO activity did not demonstrate mucosal neutrophil accumulation. Myeloperoxidase activity was high in all groups at baseline and remained unchanged throughout the course of the experiment; variability in MPO measurements was high. Myeloperoxidase activity has been used extensively in studies of intestinal I-R in the cat\(^8,9,20,25,26\) to document neutrophil adherence to small intestinal post-capillary venules and neutrophil migration into the interstitium upon reperfusion. Assays for MPO activity are reported to be sensitive for the detection of neutrophils\(^20\), however, they often lack specificity for neutrophil peroxidase activity. These assays do not distinguish between peroxidase contained in neutrophils, monocytes and eosinophils.\(^8\) In the feline small intestine there are few resident eosinophils and therefore MPO serves as a specific marker of tissue neutrophil accumulation\(^8,20\), however, the mucosa of the equine large colon has an abundant population of resident eosinophils, which likely contributed to high baseline MPO activity. Eosinophils may generate up to 10 times the peroxidase activity of neutrophils in the presence of bromide ion (HETAB)\(^37,38\); stimulation by bromide ions cause peroxidase-catalyzed oxidation to hypobromous acid which oxidizes tetramethylbenzidine (the substrate in the MPO assay)\(^39\). Therefore, the contribution of eosinophil peroxidase to total peroxidase activity is greater when HETAB is used as the detergent. To compensate for this effect of bromide on measurement of eosinophil peroxidase we also used HETOH to disrupt the granules and release the MPO enzyme; non-bromide detergents (OH\(^-\) and Cl\(^-\)) do not maximize the contribution of eosinophil peroxidase to total peroxidase activity\(^39\). Myeloperoxidase activity may not have increased during I-R because the peroxidase activity from resident eosinophils contributed to high baseline MPO activity; further increases in peroxidase activity due to neutrophil infiltration,
therefore, did not significantly increase MPO activity.

Construction of standard curves and linear regression equations for MPO activity versus neutrophil number would have been useful to predict or estimate the number of neutrophils in colonic mucosa as measured from MPO activity. The abundant resident eosinophils precluded actual quantification of colonic mucosal neutrophils based on measurement of MPO activity. In order to minimize the effect of eosinophil peroxidase on total measured MPO activity colonic mucosal MPO activity was measured using HETOH in addition to the HETAB detergent; HETOH is believed to oxidize eosinophil peroxidase less than neutrophilic peroxidase. Although not useful for quantifying neutrophils in the colonic mucosa, these standard curves could potentially be useful in horses to quantify neutrophil infiltration in tissues where there are not a large number of resident eosinophils.

Although we observed increased numbers of neutrophils in both the lamina propria of the mucosa and in submucosal venules we can only speculate that these cells contributed to both mucosal and microvascular injury. More severe mucosal injury occurred during reperfusion; this is also the period where greatest neutrophil accumulation occurred. We cannot determine from our study if increased neutrophil infiltration led to further colonic injury or if the increased colonic injury caused chemoattraction of greater neutrophils into the injured mucosa. Kubes et al., demonstrated that the resident population of neutrophils in the feline small intestine plays a more important role in mucosal injury than newly recruited neutrophils, however, microvascular injury is associated with adherence of neutrophils to the endothelium and their subsequent activation.

In conclusion, neutrophils accumulate in the large colon mucosa of horses during low flow ischemia and further accumulate upon reperfusion. Leukocyte scintigraphy and histopathology were useful for detecting and documenting neutrophil infiltration in the colonic mucosa, but measurement of MPO activity was not. Leukocyte scintigraphy and
histopathology may be useful methods to further study colonic neutrophil infiltration and to evaluate the effect of pharmacologic agents on large colon I-R in horses.

Footnotes

*aRompun®, Mobay Corporation, Animal Health Division, Shawnee, KS.
*bCarbocaine®, The Upjohn Company, Kalamazoo, MI.
*cAngiocath®, Becton Dickinson Vascular Access, Sandy, Utah.
*dGuilainax®, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.
*fHalothane U.S.P., Halocarbon Laboratories, North Augusta, SC.
*gDrager AV Model NELAC-E, Anesthesia Ventilator, Telford, Pa.
*hLactated Ringer’s, Baxter Healthcare Corporation, Deerfield, Il.
*iAngiocath®, Becton Dickinson Vascular Access, Sandy, Utah.
*jAquamatic K Module, Gorman-Rupp Industries, Bellville, Ohio.
*kModel 520, Frigitonics of Connecticut, Inc., Shelton, Ct.
*lModel T201, Transonic Systems, Inc., Shelton, Ct.
*mSimultrace Recorder Model VR-12, Electronics for Medicine/PPG Biomedical Systems, Pleasantville, NY.
*nBeuthanasia®-D Special, Schering Plough Animal Health Corporation, Kenilworth, NJ.
*pNeoprobe 1000, Neoprobe®, Columbus, Ohio.
*qHexadecyltrimethyl ammonium bromide, Sigma Chemical Company, St. Louis, Mo.
*rHexadecyltrimethyl ammonium hydroxide, Sigma Chemical Company, St. Louis, Mo.
*sModel PT10/35, Brinkman Tissue Homogenizer, Brinkman Instruments, Westbury, NY.
List of References


CHAPTER VI
SYSTEMIC AND COLONIC VENOUS EICOSANOIDS AND ENDOTOXIN
CONCENTRATION, AND COLONIC VENOUS TUMOR NECROSIS
FACTOR AND INTERLEUKIN-6 ACTIVITY DURING LOW FLOW
ISCHEMIA AND REPERFUSION OF THE EQUINE LARGE COLON

Summary

Twenty-four horses were randomly allocated to 3 groups. Horses were
anesthetized, subjected to a ventral midline celiotomy and the large colon was exteriorized
and instrumented. Group 1 served as sham-operated controls. Group 2 was subjected to 6
hrs of low flow colonic arterial ischemia and group 3 was subjected to 3 hrs of ischemia
and 3 hrs of reperfusion. Baseline samples were collected and then low flow ischemia was
induced by reducing ventral colonic arterial blood flow to 20% of baseline. All horses
were followed for 6 hrs after baseline data were collected. Blood was collected from the
colic vein (CV) and main pulmonary artery (SV) for measurement of endotoxin, 6-keto
prostaglandin F1α (6-kPG), thromboxane B2 (TXB2), and prostaglandin E2 (PGE2).
Tumor necrosis factor (TNF) and interleukin-6 (IL-6) were measured in CV blood
samples. Data were analyzed using a two way analysis of variance and post-hoc
comparisons were made using Dunnett’s and Tukey’s tests. Statistical significance was
considered at P<0.05. Endotoxin was not detected in CV or SV blood at any time. There
was no detectable TNF or IL-6 activity in CV samples at any time. There were no
differences at baseline among groups for colonic or systemic venous 6-kPG, PGE2, or
TXB$_2$ nor were there any changes across time in group 1. Colonic venous 6-kPG increased during ischemia in groups 2 and 3. It peaked at 3 hrs in group 3 and then decreased during reperfusion, but remained increased through 6 hrs in group 2. Systemic venous 6-kPG increased during reperfusion in group 3, but there were no changes in group 2. Colonic venous PGE$_2$ increased during ischemia in groups 2 and 3, remained increased for the first hr of reperfusion in group 3 and for the 6 hr duration of ischemia in group 2. There were no temporal alterations in SV PGE$_2$ concentrations. There was no difference in CV or SV TXB$_2$ among or within groups across time. Eicosanoid concentrations in SV blood were significantly lower than in the CV blood. Prostaglandin E$_2$ and 6-kPG concentrations were approximately 3 to 8 and 5 to 10 times greater respectively in CV versus SV blood. The increased concentrations of 6-kPG and PGE$_2$ in CV blood were likely due to their accumulation secondary to colonic ischemia. The increased levels of these vasodilator eicosanoids may play a role in the reactive hyperemia observed during reperfusion. The increased 6-kPG concentrations in SV blood may represent spillover from the colonic vasculature, but more likely reflects systemic production.

**Introduction**

Intestinal ischemia and reperfusion (I-R) results in a series of biochemical reactions initiated by the accumulation of hypoxanthine and conversion of xanthine dehydrogenase to xanthine oxidase (XO) during the period of low oxygen availability. Subsequently, the reintroduction of oxygen results in the production of oxygen free radicals (OFRs) due to XO-induced conversion of hypoxanthine to superoxide radicals. This OFR-mediated injury is perpetuated by neutrophils, phospholipid-derived mediators, and cytokines. Disruption of the intestinal mucosal barrier following I-R allows translocation of bacteria and toxins.
Endotoxin is the lipopolysaccharide component of the outer cell wall of gram-negative bacteria and is present in extremely high concentrations in the intestinal lumen. The large intestine is heavily populated with both obligate anaerobic and gram negative enteric bacteria; the concentration of endotoxin in the large intestine is characteristically higher than in the small intestine. As much as 80 µg/ml of endotoxin have been measured in the lumen of the equine cecum. Protective mechanisms exist to prevent movement of luminal endotoxin across the mucosal barrier. Strangulation obstruction leads to mucosal necrosis which enables endotoxin to traverse the damaged barrier. Endotoxin gaining access to the intestinal circulation is typically cleared by the monocyte phagocytic system of the liver. The monocyte phagocytic system can be overwhelmed if large quantities of endotoxin enter the portal circulation, which results in endotoxin spilling over into the systemic circulation. When endotoxin gains access to the circulation it initiates an inflammatory cascade resulting in the synthesis and release of tumor necrosis factor, interleukins, and eicosanoids. These mediators can perpetuate tissue injury, alter blood flow regulation, and cause shock and multiple organ failure.

Tumor necrosis factor (TNF) is produced by monocytes and macrophages upon exposure to endotoxin. An inflammatory cascade is initiated by TNF resulting in the production of other inflammatory mediators such as the interleukins. Interleukin-6 is also produced by monocytes and macrophages exposed to either endotoxin or TNF; IL-6 stimulates the hepatic acute phase response. This inflammatory cascade often culminates in endotoxic shock and multiple organ system failure. Increased systemic blood levels of endotoxin, TNF, IL-6, prostacyclin and thromboxane have been documented in horses during intestinal strangulation obstruction or with experimental models of endotoxemia.
During intestinal ischemia, intracellular calcium accumulates due to dysfunction of cell membrane transport pumps; this increased intracellular calcium activates phospholipase A2 causing the release of arachidonic acid from cell membranes.\textsuperscript{21} Arachidonic acid is metabolized by one of two enzyme systems (cyclooxygenase and lipoxygenase) resulting in the synthesis of prostaglandins and leukotrienes.\textsuperscript{22,23} Prostacyclin and thromboxane are produced predominantly by the endothelium and platelets, however, other cells are capable of their synthesis.\textsuperscript{22,23} Prostacyclin causes vasodilation and inhibits platelet aggregation while thromboxane causes vasoconstriction and stimulates platelet aggregation.\textsuperscript{22,23} Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) is produced in many organs including the intestine and results in vasodilation.\textsuperscript{22,23}

We have previously documented reperfusion injury in the equine large colon upon restoration of blood flow after a three hour period of low flow ischemia.\textsuperscript{24} Additionally, a reactive hyperemic response occurs in the large colon during the reperfusion period.\textsuperscript{25} Reactive hyperemia following an ischemic episode is principally caused by vasodilator substances (prostacyclin, PGE\textsubscript{2}, nitric oxide, and adenosine) that accumulate during the ischemic period.\textsuperscript{26}

The purpose of this study was to measure endotoxin and eicosanoid concentrations and TNF and IL-6 activity in systemic and colonic venous blood during low flow ischemia and reperfusion of the equine large colon.

\textbf{Materials and Methods}

This project was performed with approval and under the guidelines of the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

\textit{Animals} - Twenty-four horses of various breeds (9 Thoroughbreds, 8 Standardbreds, 3 Quarter Horses, 2 Saddlebreds, and 2 Hanoverians), ranging in age from
1-10 years and weighing from 350-570 kg were used to complete this study. There were 11 females, 5 intact males, and 8 geldings. Horses were fed 1-2% of their body weight in grass hay per day and were adapted to their diet and environment for at least one week prior to the experiment; water was provided ad libitum. Food, but not water, was withheld for 24 hrs prior to the experiment to decrease the colon contents which facilitated its manipulation.

**Instrumentation** - Horses were instrumented as we previously described.\(^{24,25}\) Briefly, horses were sedated with xylazine\(^a\) (0.5 mg/kg IV), induced to general anesthesia with guaifenesin\(^b\) (30 mg/kg IV) and thiamylal\(^c\) (4.4 mg/kg IV) and maintained with halothane\(^d\) (1-3%) in oxygen. Horses were mechanically ventilated\(^e\) 6-12 breaths per minute to a peak inspiratory pressure of \(\leq 25\) cm H\(_2\)O. A balloon tipped, flow directed thermodilution catheter\(^f\) was positioned in the pulmonary artery for the collection of mixed-venous blood samples. Isotonic polyionic fluids\(^s\) were administered IV at a rate of 5-10 ml/kg/hr. Arterial blood pressure was monitored continuously and was maintained \(\geq 70\) mm of Hg.

Horses were positioned in dorsal recumbency and prepared for aseptic surgery. A ventral midline celiotomy was performed, the large colon was exteriorized, placed on a warm water heating pad\(^h\) and instrumented.\(^{24,25}\) A doppler ultrasound flow probe\(^i\) was placed externally around the colonic artery supplying the ventral colon and colonic blood flow was measured continuously and recorded. A 14-gauge, 5.1 cm (non-occluding) teflon catheter\(^l\) was placed in the colonic vein draining the ventral colon for collection of colonic venous blood.

**Experimental Design** - The 24 horses were randomly assigned to one of three groups. Group 1 horses (n=6) served as sham-operated controls. The large colon was exteriorized through a ventral midline celiotomy and instrumented, but colonic ischemia
was not induced. Group 2 horses (n=9) served as ischemic controls. These horses underwent the same procedures as group 1 horses, but were subjected to 6 hrs of low flow ischemia. Group 3 horses (n=9) were subjected to 3 hrs of low flow ischemia followed by 3 hrs of reperfusion.

Low flow arterial ischemia of the large colon was produced in groups 2 and 3 horses by reducing colonic arterial blood flow to 20% of baseline. Baseline data was recorded approximately 15-30 minutes after instrumentation (approximately 1 hr was required for surgery and instrumentation following induction of anesthesia). Hemodynamic parameters were recorded at baseline and at 30 min intervals. Data was also collected at 195 min, which corresponded to 15 min of reperfusion in group 3. Systemic venous (SV) and colonic venous (CV) blood samples were collected for endotoxin, TNF, IL-6, and 6-keto prostaglandin F\textsubscript{1\alpha} (6-kPG), thromboxane B\textsubscript{2} (TXB\textsubscript{2}), and PGE\textsubscript{2} at the times designated below. Horses were euthanatized at the conclusion with an overdose of sodium pentobarbital (100 mg/kg IV).

**Endotoxin Assays:** Ten ml of CV and SV blood were aseptically collected into sterile, pyrogen-free, heparinized glass test tubes at time 0 hrs (baseline), 3 hrs (3 hrs ischemia in groups 2 and 3), and at 6 hrs (3 hrs reperfusion in group 3). The samples were immediately cooled on ice and then centrifuged at 2,000 rpm for 5 min. The plasma was aseptically transferred into a storage tube where it was diluted 1:10 with sterile, pyrogen-free water. The diluted plasma sample was heated to 75\(^\circ\)C for 10 min, allowed to cool to room temperature, and then stored in a -70\(^\circ\)C freezer until analyzed for endotoxin using a quantitative chromogenic limulus amebocyte lysate test. This assay is sensitive for measurement of plasma endotoxin concentrations in the range of 0.1 to 1.0 EU/ml.
**Tumor Necrosis Factor Assay:** Colonic venous TNF activity was estimated in serum samples collected at 0, 3, 3.25, 4, and 6 hrs by modification of an in vitro cytotoxicity bioassay\textsuperscript{27}, using WEHI 164 clone 13 murine fibrosarcoma cells.\textsuperscript{m} The WEHI cells were suspended in RPMI 1640 media containing 10\% fetal bovine serum and 1 \(\mu\)g of actinomycin D/ml then placed in 96-well microtitration plates (7.0 \(\times\) 10\(^4\) cells/well) for 4 hrs at 37\(^\circ\) C, 5\% CO\(_2\).\textsuperscript{27} Serum samples were diluted 10-fold; a two-fold dilution of the diluted serum was combined with human recombinant TNF-alpha in triplicate and incubated with the cells for 18 hrs. Cell survival was estimated colorimetrically after 4 hrs of incubation with the tetrazolium salt MTT. The optical densities of the wells were measured (570 nm) on a semi-automated micro-ELISA reader system.\textsuperscript{27} A standard curve was constructed from the regression of human recombinant TNF concentration vs optical density (OD). Linear regression was performed for each sample dilution vs OD, and the sample concentration (pg/ml) of TNF activity was estimated from that dilution that produced the mean OD on the standard TNF curve.\textsuperscript{27}

**Interleukin-6 Assay:** Interleukin-6 activity was estimated in CV blood samples collected at time 0, 3, 3.25, 4, and 6 hrs. Serum IL-6 activity was determined by use of a bioassay \textsuperscript{16} containing the murine hybridoma cell line B13.29 clone B.9, which is dependent on IL-6 for survival.\textsuperscript{m} Serial dilutions of previously heat-inactivated (56\(^\circ\) C, 30 min) serum samples were added to duplicate wells of flat-bottom microtitration plates containing 3.5 \(\times\) 10\(^3\) B.9 cells/well. Serum was initially diluted 1:10, then twofold dilutions were made. Cells and samples were diluted in RPMI 1640 medium containing L-glutamine, 0.03\% sodium bicarbonate, and 0.5\% gentamicin. After culture for 92 hrs at 37\(^\circ\) C in humidified 5\% CO\(_2\) in air, cell numbers were estimated colorimetrically by their ability to reduce the chromogen. Cells were incubated with the chromogen for 4 hrs, after which the supernatant was removed, the cells were lysed with 0.006N HCl in 2-propanol.
(100 ul/well), and an equal volume of distilled, deionized H$_2$O was added. Cell numbers were directly related to the optical density (OD) of the well (570 and 690 nm) as measured with a dual-channel semi-automated micro-ELISA plate reader. Recombinant murine IL-6 activity per ml was defined as the dilution of the sample required to produce half-maximal B.9 proliferation.\(^{16}\)

**Prostaglandin Assays:** Ten ml of CV and SV blood were collected at time 0, 3, 3.07 (5 min of reperfusion in group 3), 3.25, 4, and 6 hrs into plastic test tubes containing cold 100mM EDTA (pH=7.4) and 10 mM meclofenamic acid (1:20 with blood). The samples were stored on ice, centrifuged at 1,500 g for 10 min and the plasma was stored at -70° C until assayed. Plasma 6-kPG and TXB$_2$, the active metabolites of prostacyclin and thromboxane A$_2$ respectively, and PGE$_2$ were quantitated (pg/ml) by competitive binding radioimmunoassay using standard techniques.\(^{20}\) The percent recovery of the eicosanoids ranged between 74-81% for 6-kPG and between 77-81% for PGE$_2$ and TXB$_2$.

**Statistical Analyses:** All data are expressed as mean ± standard error of the mean. Data was analyzed using a two-way ANOVA with repeated measures. Post-hoc comparisons were made with Dunnett’s and Tukey’s tests. The level of significance was set at P < 0.05 for all statistical tests.

**Results**

There was no detectable endotoxin in the CV or SV plasma in any group at any time period. There was no measurable TNF or IL-6 activity in CV serum.

There was no significant difference among groups at baseline for CV 6-kPG (mean = 1,933 pg/ml) nor were there changes across time in group 1. There were significant increases in CV 6-kPG during ischemia in groups 2 and 3 (Figure 6.1). Colonic venous 6-kPG peaked at 3 hrs in group 3, decreased during reperfusion, and was not different
Figure 6.1

Mean ± SEM for A) colonic venous and B) systemic venous 6-ketoprostaglandin F$_{1\alpha}$ during low flow ischemia and reperfusion of the equine large colon. The solid arrow at 3 hrs indicates time of reperfusion in the ischemia & reperfusion group. The letter "b" is significantly (P<0.05) different than "a".
Figure 6.1
from the baseline value by 6 hrs. Colonic venous 6-kPG continued to increase throughout the 6 hrs of ischemia in group 2. There was no significant difference among groups for SV 6-kPG, but there was a significant increase in group 3 during reperfusion (Figure 6.1).

Mean baseline CV PGE$_2$ was 408 pg/ml and there were no significant changes across time in group 1 nor during the first 3 hrs in groups 2 and 3 (Figure 6.2). There were no significant differences between groups 2 and 3. Significant increases in CV PGE$_2$ were measured in groups 2 at 185 min. The PGE$_2$ concentrations remained increased during the first hr of reperfusion in group 3 and throughout the duration of the study in group 2. By 6 hrs, PGE$_2$ had returned to a level not different from baseline in group 3. Mean baseline SV PGE$_2$ was 115 pg/ml among all groups; there were no differences among or within groups across time (Figure 6.2).

The mean baseline TXB$_2$ concentration in CV plasma was 194 pg/ml for all groups. There were no differences among or within groups across time (Fig 6.3). Mean baseline SV TXB$_2$ was 126 pg/ml and there were no differences among or within groups (Figure 6.3).

Eicosanoid concentrations in systemic venous blood were significantly lower than in the colonic blood. Prostaglandin E$_2$ and 6-kPG concentrations were approximately 3 to 8 and 5 to 10 times greater respectively in CV versus SV blood.

Discussion

This is the first report to characterize and quantify endotoxin, cytokine, and eicosanoid levels in colonic and systemic venous blood during experimental low flow arterial ischemia and reperfusion of the large colon in horses. The absence of measurable endotoxin in colonic and systemic venous blood combined with no colonic venous TNF or IL-6 activity nor systemic venous TXB$_2$ suggests that endotoxin did not traverse the
Figure 6.2

Mean ± SEM for A) colonic venous and B) systemic venous prostaglandin E2 during low flow ischemia and reperfusion of the equine large colon. The solid arrow at 3 hrs indicates time of reperfusion in the ischemia & reperfusion group. The letter "b" is significantly (P<0.05) different than "a".
Figure 6.2

A

Colonic Venous Prostaglandin E₂ (pg/ml)

Control
Ischemia
Ischemia & Reperfusion

Time (hrs)

B

Systemic Venous Prostaglandin E₂ (pg/ml)

Control
Ischemia
Ischemia & Reperfusion

Time (hrs)
Figure 6.3

Mean ± SEM for A) colonic venous and B) systemic venous thromboxane B$_2$ during low flow ischemia and reperfusion of the equine large colon. The solid arrow at 3 hrs indicates time of reperfusion in the ischemia & reperfusion group. The letter "b" is significantly (P<0.05) different than "a".
Figure 6.3

A

Colonic Venous Thromboxane B₂ (pg/ml)

- Control
- Ischemia
- Ischemia & Reperfusion

Time (hrs)

B

Systemic Venous Thromboxane B₂ (pg/ml)

- Control
- Ischemia
- Ischemia & Reperfusion

Time (hrs)
colonic mucosa during prolonged ischemia or I-R. These findings contrast with reports of increased concentrations of endotoxin\textsuperscript{11,28} and TNF\textsuperscript{29,30} in systemic venous blood of horses with naturally occurring or experimental intestinal strangulating obstructions. Likewise the absence of IL-6 activity and increased systemic TXB\textsubscript{2} in systemic venous blood is dissimilar to reports of increased levels of these substances during experimental endotoxemia in horses.\textsuperscript{16,20,31} Alternative explanations for the absence of endotoxin may be because of its rapid clearance from the circulation or due to the assay methodology used. The increased concentrations of 6-kPG and PGE\textsubscript{2} measured in colonic venous blood in groups 2 and 3 likely reflect increased production of these eicosanoids in the colonic vasculature subsequent to colonic ischemia and reperfusion. These findings support the results of a previous study that reported increased concentrations of 6-kPG and TXB\textsubscript{2} in portal venous blood subsequent to an experimental 720° large colon volvulus in ponies.\textsuperscript{19}

Following intestinal I-R, endotoxin can potentially gain access to the circulation by one of three routes.\textsuperscript{12} Luminal endotoxin can traverse the mucosal barrier and enter the colonic venous circulation, which then empties into the portal venous system. Endotoxin can also enter colonic lymphatics after crossing the mucosal barrier; endotoxin within the lymphatics empties into the systemic venous system via the thoracic duct. If endotoxin or bacteria traverse full-thickness intestinal wall it enters the peritoneal cavity where it can either enter into the lymphatic system or the systemic or portal venous circulation.\textsuperscript{12} We did not quantitate peritoneal fluid endotoxin concentrations because the colon was positioned extra-abdominally throughout the entire experimental period. It is possible that endotoxin traversed the full thickness colonic wall following I-R, however, we believe this is unlikely since there was no measurable colonic or systemic venous endotoxin.

Horses are exquisitively sensitive to endotoxin and even small circulating quantities induce profound cardiopulmonary alterations.\textsuperscript{10,32} Some of the hemodynamic and
Pulmonary effects of endotoxin in conscious horses include tachycardia, pulmonary hypertension, arterial hypoxemia, and eventual systemic hypotension. Hemoconcentration accompanies these cardiopulmonary abnormalities. The absence of hemodynamic and metabolic alterations typical of endotoxemia in the horses of this study provides further evidence that endotoxin did not gain access to the systemic circulation. We do realize that the horses in this study were anesthetized, breathing 100% oxygen, and their MAP was maintained > 70 mm of Hg by adjusting anesthetic depth and administering IV fluids. This could possibly confound interpretation of the cardiopulmonary effects of endotoxin that had gained access to the systemic circulation. The lack of pulmonary hypertension supports our finding of undetectable endotoxin in colonic or systemic venous blood. Maintenance of MAP > 70 mm of Hg with IV fluids would have only exacerbated the endotoxin-induced pulmonary hypertension if endotoxin had entered the systemic circulation.

Endotoxin gaining access to the portal venous circulation is rapidly cleared by the Kupffer cells of the liver unless large quantities of portal endotoxin overwhelm this monocyte phagocytic system. Removal of endotoxin by this system could have accounted for the absence of endotoxin in systemic venous blood, but would not account for the absence of colonic venous endotoxin. The most plausible explanation for the absence of colonic venous endotoxin is that it did not traverse the colonic mucosa and thus was not absorbed into the colonic circulation. Another possibility for the absence of endotoxin is that the assay used in this study failed to detect endotoxin that was present. This is unlikely because of the high sensitivity of the assay for lipopolysaccharide. The endotoxin assay used in this study measures unbound circulating endotoxin. If small quantities of endotoxin had gained access to the circulation it could have become bound to albumin or other substances in plasma and therefore would not have been detected with this
chromogenic limulus amebocyte assay.

Endotoxin stimulates monocytes/macrophages to produce TNF, IL-1, and IL-6. These mediators initiate and perpetuate a cascade of inflammatory events that can lead to endotoxic shock and multiple organ failure. The absence of colonic venous TNF and IL-6 activity provides further evidence that endotoxin did not gain access to the circulation. Another possibility for the absence of colonic venous TNF or IL-6 activity could be that there was insufficient time after exposure of monocytes/macrophages to endotoxin for synthesis and peak release of these cytokines. Although peak serum TNF and IL-6 activity occur approximately 1.5 and 3-4 hrs, respectively, after endotoxin exposure in horses some activity should have been detected in the horses of this study if endotoxin actually entered the circulation.

The increased colonic venous eicosanoid concentrations during ischemia and reperfusion is in agreement with other studies demonstrating intestinal eicosanoid synthesis during I-R. Intestinal mucosal 6-kPG, TXB₂, and PGE₂ increased after I-R, but not after ischemia alone. Intracellular calcium increases during ischemia causing activation of phospholipase A₂, which results in the release of arachidonic acid. Arachidonic acid is metabolized into prostaglandins and leukotrienes via the cyclooxygenase and lipoxygenase pathways. Prostacyclin, produced by endothelial cells, causes vasodilation and inhibits platelet aggregation both of which tend to enhance intestinal blood flow. Thromboxane is produced predominantly by platelets and causes vasoconstriction and platelet aggregation both of which would tend to decrease intestinal blood flow.

Both 6-kPG and PGE₂ increased in colonic venous blood during ischemia and remained increased for the duration of the ischemic periods, however, they gradually decreased throughout reperfusion in group 3. The reperfusion associated decreases in colonic venous 6-kPG and PGE₂ may have been due to either decreased colonic synthesis
or to dilution or “washout” by the hyperemic flow. Reactive hyperemia is the increased blood flow observed following reestablishment of blood flow after an episode of ischemia. Reactive hyperemia is caused, in part, to vasodilator substances, such as prostacyclin and PGE₂, that accumulate during ischemia. Despite the decrease in 6-kPG and PGE₂ levels during reperfusion, colonic blood flow remains significantly increased above baseline flow throughout 3 hrs of reperfusion.²⁵

One explanation for the increased systemic venous 6-kPG levels observed in this study may be due to spillover from the colonic vasculature. The colonic circulation is the primary site of eicosanoid production during experimental 720° large colon volvulus in ponies.¹⁹ Pulmonary artery, jugular venous, and especially portal venous 6-kPG increase during a 1 hr 720° experimental colon volvulus in ponies.¹⁹ Immediately upon reperfusion, sustained increases in portal venous 6-kPG occurred and 6-kPG increased dramatically in pulmonary artery and jugular venous blood.¹⁹ Combined, these studies suggest the sustained increases in systemic venous 6-kPG is due to a process other than “washout” from the colonic vasculature. It is possible that restoring blood flow to the colon after ischemia results in synthesis and release of substances from the injured colon that subsequently enter the systemic circulation and trigger the production of prostacyclin.

During colonic I-R, colonic venous 6-kPG and PGE₂ concentrations increased approximately 2-5 fold from baseline values and were significantly higher than in systemic venous blood. The horses became significantly hemodiluted during the course of the experiment due to IV fluid administration.²⁵ In the absence of hemodilution, the magnitude of increase in these eicosanoid concentrations would likely have been even greater. The greater eicosanoid concentrations in colonic versus systemic venous blood supports the results of a previous study demonstrating higher plasma eicosanoid concentrations in the portal vein than in the pulmonary artery or jugular vein blood during experimental colonic
volvulus in ponies.\textsuperscript{19} The liver appears to clear some of these portal venous eicosanoids, which reduces the quantity gaining access to the systemic circulation.\textsuperscript{19}

The absence of increased colonic or systemic venous TXB\textsubscript{2} upon reperfusion contrasts those findings in ponies following correction of experimental volvulus. Portal and jugular venous TXB\textsubscript{2} increased immediately following correction of a 1 hr experimental 720° large colon volvulus in ponies and gradually returned to concentrations not different from pre-ischemic values by the end of the first hr of reperfusion.\textsuperscript{19} The differences between these two studies could be associated with the differences inherent to the models. Another plausible explanation is that the hemodilution\textsuperscript{25} observed in the horses of our study may have masked any significant increase that may have occurred. Although not statistically significant, both the colonic and systemic venous TXB\textsubscript{2} concentrations graphically (Figure 6.03) appeared to be increasing at 15 min of reperfusion in group 3.

The increased jugular venous TXB\textsubscript{2} and 6-kPG and pulmonary artery 6-kPG concentrations observed following correction of a 1 hr experimental volvulus lead the authors to speculate that these increased plasma eicosanoids were associated with endotoxin that gained access to the circulation during volvulus and its subsequent correction.\textsuperscript{19} Thromboxane and 6-kPG increase during experimental endotoxemia in horses.\textsuperscript{31} The alterations in plasma eicosanoids following correction of experimental colon volvulus in ponies were associated with hypotension, increased colonic vascular resistance, and an absence of colonic hyperemia following correction of the volvulus.\textsuperscript{38} These hemodynamic alterations are different from those reported with the low flow colonic ischemia used in this study and the differences are likely due to the models of ischemia. It is possible that during colon volvulus venous occlusion resulted in the accumulation of large volumes of blood and fluid in the colonic wall and lumen resulting in hypovolemia.\textsuperscript{39-41} It is also possible that during experimental colonic volvulus endotoxin gains access to the circulation resulting
in endotoxemia. The lack of colonic hyperemia upon correction of the experimental volvulus was associated with increased colonic vascular resistance. Increased thromboxane production could have accounted for increased colonic vascular resistance. Colonic microvascular thrombosis occurs with naturally occurring volvulus and experimental arteriovenous or venous occlusion. Development of thromboses in the colonic microcirculation may have occurred during experimental volvulus resulting in the obstruction of blood flow; this could have contributed to the lack of colonic hyperemia. The development of hypotension upon correction of experimental volvulus could also explain the absence of hyperemia during reperfusion.

In summary, low flow I-R of the equine large colon is not associated with detectable colonic or systemic venous endotoxin concentration or colonic venous TNF or IL-6 activity. The increased concentrations of 6-kPG and PGE$_2$ in colonic venous blood likely occur subsequent to reduced colonic oxygen availability; accumulation of these eicosanoids may contribute to the hyperemia following re-establishment of blood flow by causing vasodilation. The increased 6-kPG concentration measured in systemic venous blood may represent spillover from the colonic vasculature, but more likely reflects systemic production. The absence of significant increases in systemic venous eicosanoids provides further evidence that endotoxin did not enter the circulation.

Footnotes

aRompun®, Mobay Corporation, Animal Health Division, Shawnee, KS.
bKluailaxin®, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.
dHalothane U.S. P., Halocarbon Laboratories, North Augusta, SC.
eDrager AV Model NELAC-E, Anesthesia Ventilator, Telford, Pa.


gLactated Ringer's, Baxter Healthcare Corporation, Deerfield, Il.

hAquamatic K Module, Gorman-Rupp Industries, Bellville, Ohio.

iModel T201, Transonic Systems, Inc., Ithaca, NY.

jAngiocath®, Becton Dickinson Vascular Access, Sandy, Utah.

kBethanasia®-D Special, Schering Plough Animal Health Corporation, Kenilworth, NJ.

QCL-1000 Limulus Amebocyte Lysate, Bio-Whitaker, Walkersville, Md.

mDepartment of Large Animal Medicine, The University of Georgia, Athens, Ga.

List of References


CHAPTER VII
SYSTEMIC AND COLONIC VENOUS HEMOSTATIC ALTERATIONS
DURING LOW FLOW ISCHEMIA AND REPERFUSION OF THE LARGE
COLON IN THE HORSE

Summary

Twenty-four horses were randomly allocated to three groups. All horses underwent a ventral midline celiotomy and the large colon was exteriorized and instrumented. Group 1 served as sham-operated controls, group 2 underwent 6 hrs of colonic ischemia, and group 3 was subjected to 3 hrs of ischemia and 3 hrs of reperfusion. Baseline blood samples were collected and then low flow colonic ischemia was induced in groups 2 and 3 by reducing colonic arterial blood flow to 20% of baseline. All horses were monitored for 6 hours. Citrated systemic venous (SV) blood samples were collected from the main pulmonary artery and colonic venous (CV) samples were collected from the colonic vein draining the ventral colon. Samples were collected at 0, 2, 3, 3.25, 4, and 6 hrs for determination of one stage prothrombin time (OSPT), activated partial thromboplastin time (APTT), antithrombin III (ATIII) activity, and fibrinogen concentration. There were significant decreases in all hemostatic parameters by 2 hrs in both SV and CV in all 3 groups of horses, but there were no differences among the 3 groups for any of these parameters. These hemostatic alterations could have been secondary to a hypercoagulable state or to fluid therapy-induced hemodilution. Colonic ischemia was not likely the cause of these alterations since they also occurred in the sham-operated control horses. Significant temporal alterations existed even after

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accounting for the hemodilution. The most plausible explanation for these alterations is that a hemostatic response was incited by the celiotomy and manipulation of the colon during exteriorization and instrumentation. Comparison of paired SV and CV samples for each hemostatic parameter revealed significant differences for the absolute values of OSPT and fibrinogen concentration, but not for APTT or ATIII activity. This indicates that monitoring SV hemostatic parameters does not necessarily provide an accurate assessment of hemostatic function in regional vascular beds. Colonic ischemia and/or reperfusion did not appear to exacerbate hemostatic dysfunction.

Introduction

Large colon volvulus is a common and often fatal cause of colic in horses.1-3 The high mortality rate (58-79%) associated with large colon volvulus is likely a reflection of the severe colonic damage sustained during ischemia and the further injury that occurs upon reperfusion.1-5 Thrombosis of the colonic submucosal microvasculature develops during naturally occurring large colon volvulus.6 These thrombi may obstruct blood flow and limit perfusion of the colonic mucosa upon surgical correction of the volvulus; this may perpetuate or accentuate the mucosal injury sustained during ischemia. Hemostatic alterations are present in horses with naturally occurring colic7-11 and in those horses subjected to experimental intestinal strangulation12. Typical abnormalities observed on plasma hemostatic profiles in horses with colic are prolongation of the one stage prothrombin time (OSPT) and activated partial thromboplastin time (APTT), increased fibrinogen and fibrin degradation product (FDP) concentrations, and decreased antithrombin III (ATIII) activity, suggesting active coagulation and fibrinolysis. The most severe changes occur in horses that subsequently die and in those with the most severe forms of intestinal disease, such as ischemic or inflammatory disorders.10
We have recently developed a repeatable model of low flow ischemia of the equine large colon, where colonic arterial blood flow is reduced to 20% of baseline. This model produces blood flow alterations similar to the arterial component of the vascular abnormalities in naturally occurring large colon volvulus. Using this low flow model, we have documented histologic evidence of reperfusion injury.

Increased microvascular permeability is a consistent finding in intestinal ischemia-reperfusion (I-R). Increased endothelial permeability occurs due to a combination of oxygen free radicals (OFRs), neutrophil adhesion, phospholipid-derived mediators such as platelet activating factor, and endotoxin. According to the traditional scheme of coagulation physiology, endothelial cell damage exposes subendothelial collagen, which activates coagulation via the intrinsic coagulation pathway. Ischemic tissue injury causes release of tissue factor, which activates the extrinsic coagulation pathway. Tissue factor is also produced by macrophages in response to endotoxin exposure. The net effect of activation of both coagulation pathways is the generation of thrombin; circulating thrombin converts fibrinogen into fibrin, which forms the fibrin clot in the coagulation cascade. Antithrombin III, the most abundant inhibitor of procoagulant activity, primarily functions to inhibit thrombin; decreased ATIII activity is suggestive of consumption coagulopathy associated with disseminated intravascular coagulation (DIC) with decreased AT III production in the liver, or with ATIII loss. Under normal circumstances, fibrinolysis and coagulation are activated simultaneously. The role of fibrinolysis is to prevent tissue ischemia caused by excess fibrin deposition in the microcirculation and to biodegrade clotting factors at the site of tissue injury.

The effect of intestinal ischemia or endotoxin administration on systemic hemostasis has been evaluated in horses; prolonged OSPT and APTT, decreased fibrinogen concentration, increased FDP concentration, and decreased ATIII activity have been
documented in horses with intestinal ischemia, suggesting the development of DIC. To our knowledge, there is no information available in laboratory animals or horses regarding alterations in coagulation parameters in the venous effluent directly draining ischemic or reperfused intestine. Intestinal I-R injury likely causes activation of coagulation within the intestinal vasculature, which can lead to microvascular thrombosis. Blood flow stasis secondary to microvascular thrombosis decreases clearance of activated coagulation factors, which can lead to a vicious cycle. Intestinal microvascular thrombosis may obstruct microcirculation causing tissue ischemia; this can amplify local intestinal injury, or result in multiorgan failure or distant organ injury to the heart, lungs, kidneys, or the sensitive laminae of the hoof.21,25,26

The purposes of this investigation were 1) to determine if colonic and systemic venous hemostatic alterations occur during low flow I-R of the equine large colon, 2) to compare the effects of I-R to ischemia alone, and 3) to compare the hemostatic changes between colonic and systemic venous blood.

Materials and Methods

This project was performed with approval and under the guidelines of the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

Animals - Twenty-four horses of various breeds (9 Thoroughbreds, 8 Standardbreds, 3 Quarter Horses, 2 Saddlebreds, and 2 Hanoverians), ranging in age from 1-10 years and in body weight from 350-570 kg were used. There were 11 females, 5 intact males, and 8 geldings. Horses were fed 1-2% of their body weight in grass hay per day and were adapted to their diet and environment for at least one week prior to the experiment; water was provided ad libitum. Food, but not water, was withheld for 24 hrs prior to the experiment.
**Instrumentation** - Horses were instrumented as we have previously described.\(^{13,14}\) Briefly, horses were sedated with xylazine\(^a\) (0.5 mg/kg IV); general anesthesia was induced with guaifenesin\(^b\) (50 mg/kg IV) and thiamylal sodium\(^c\) (4.4 mg/kg IV), and maintained with halothane\(^d\) (1-3%) in oxygen. Horses were mechanically ventilated\(^e\) at 6-12 breaths per minute at a peak inspiratory pressure of \(\leq 25\) cm H\(_2\)O. Isotonic polyionic fluids\(^f\) were administered IV at a rate of 5-10 ml/kg/hr. A balloon tipped, flow directed thermodilution catheters was positioned in the pulmonary artery for collection of mixed-venous blood samples. Arterial blood pressure was monitored continuously and maintained \(\geq 70\) mm of Hg. Arterial and jugular venous catheters were flushed periodically with heparinized saline, which was prepared by adding 3,000 IU of sodium heparin to 500 ml of 0.9% NaCl.

Horses were positioned in dorsal recumbency and prepared for aseptic surgery. A ventral midline celiotomy was performed, the large colon was exteriorized, placed on a warm water heating pad and instrumented.\(^{13,14}\) A doppler ultrasound flow probe\(^h\) was placed externally around the colonic artery supplying the ventral colon, and colonic blood flow was measured continuously and recorded. A 14-gauge, 5.1 cm (non-occluding) teflon catheter\(^i\) was placed in the colonic vein draining the ventral colon for collection of colonic venous blood.

**Experimental Design** - Twenty-four horses were randomly assigned to one of three groups. Horses in group 1 (n=6) served as sham-operated controls; the large colon was exteriorized through a ventral midline celiotomy and instrumented, but colonic ischemia was not induced. Horses in group 2 (n=9) served as ischemic controls; these horses underwent the same procedures as horses in group 1 plus 6 hrs of low flow ischemia. Horses in group 3 (n=9) were subjected to 3 hrs of low flow ischemia followed by 3 hrs of reperfusion. Low flow arterial ischemia of the large colon was produced in horses in
groups 2 and 3 by reducing colonic arterial blood flow to 20% of baseline.\textsuperscript{13,14}

Baseline systemic and colonic venous blood samples were collected approximately 15-30 minutes after instrumentation (approximately 1 hr was required for surgery and instrumentation following induction of anesthesia) and at 2, 3, 3.25, 4 and 6 hrs. Horses were euthanatized at the conclusion of the experiment with an overdose of sodium pentobarbital (100 mg/kg IV).

**Hemostasis Assays:** Citrated blood samples (4 ml) were collected from the pulmonary artery (systemic venous) and colonic vein; samples were centrifuged and the plasma supernatant was stored at -70° C until assayed. One stage prothrombin time, APTT, ATIII activity, and fibrinogen concentration were measured using an automated coagulation laboratory system\textsuperscript{k}. Antithrombin III activity was expressed as the percent of activity of pooled normal equine plasma; plasma from normal horses was pooled and used as the standard for the ATIII assays. Samples from each horse were assayed together to minimize the effects of any inter-assay variation.

All hemostatic data for each time period were also expressed as the percent of the baseline value for each horse to minimize the inherent variability in these parameters. The hemostatic parameters were multiplied by the ratio of the total plasma protein concentration at each time period to the baseline total plasma protein concentration to correct for hemodilution of coagulation factors associated with isotonic fluid administration.

**Statistical Analyses:** All data are expressed as mean ± standard error of the mean. Data were analyzed using a two-way ANOVA for repeated measures. Within group post-hoc comparisons were made using a Student Newman Keuls test. A paired-t test was used to compare the absolute colonic and systemic venous values for all hemostatic parameters. Significance was set at $P < 0.05$ for all statistical tests.
Results

Baseline values for all colonic and systemic venous hemostatic parameters are listed in Table 7.1. Colonic venous OSPT was significantly decreased from baseline by 2 hrs and remained decreased throughout the experiment in all groups, but there were no differences among groups (Figure 7.1). Systemic venous OSPT was decreased from baseline values at 2 hrs and remained decreased through 6 hrs in all groups (Figure 7.1). There were no differences among groups for systemic venous OSPT.

Colonic venous APTT was decreased at 2 hrs of ischemia compared to baseline and there was a trend (P=0.10) for it to be decreased at 3 hrs in all groups; it had returned to a value not different from baseline by 3.25 hrs (Figure 7.2). There were no differences in colonic venous APTT among groups. Systemic venous APTT was decreased at 2 hrs compared to baseline and remained decreased through 4 hrs (Figure 7.2). There were no differences among groups for systemic venous APTT.

Colonic venous ATIII activity was decreased in all groups by 2 hrs of ischemia and remained decreased through 6 hrs in all groups, but there were no differences among groups (Figure 7.3). Systemic venous ATIII activity was decreased from baseline at 2 hrs and remained decreased throughout the experiment in all groups (Figure 7.3). There were no differences among groups for systemic venous ATIII activity.

Colonic venous fibrinogen concentration was decreased in all groups at 2 hrs of ischemia and remained decreased throughout the experiment; there were no differences among groups (Fig 7.4). There was a trend (P=0.09) toward a difference between groups 1 and 2 and a trend (P=0.06) toward a difference between groups 2 and 3 for colonic venous fibrinogen; these trends were apparent at baseline. Systemic venous fibrinogen concentration was decreased at 2 hrs compared to baseline and remained decreased through 6 hrs in all groups (Figure 7.4). There was a significant difference between groups 1 and 2 and a trend (P=0.11) toward a difference between groups 2 and 3.
Table 7.1

Mean ± SEM for the absolute values for baseline colonic venous (CV) and systemic venous (SV) one stage prothrombin time (OSPT), activated partial thromboplastin time (APTT), antithrombin III activity (ATIII), and fibrinogen concentration.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Ischemia</th>
<th>Ischemia &amp; Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV-OSPT (sec)</td>
<td>14.3 ± 0.8</td>
<td>13.60 ± 0.3</td>
<td>13.06 ± 0.4</td>
</tr>
<tr>
<td>CV-APTT (sec)</td>
<td>57.9 ± 0.6</td>
<td>54.7 ± 3.4</td>
<td>50.4 ± 5.8</td>
</tr>
<tr>
<td>CV-ATIII(%)</td>
<td>113.3 ± 3.7</td>
<td>98.2 ± 2.3</td>
<td>90.0 ± 8.0</td>
</tr>
<tr>
<td>CV-FIB (mg/dl)</td>
<td>220 ± 29*</td>
<td>303 ± 20b</td>
<td>259 ± 33*</td>
</tr>
<tr>
<td>SV-OSPT (sec)</td>
<td>14.4 ± 0.4</td>
<td>13.6 ± 0.3</td>
<td>14.0 ± 0.4</td>
</tr>
<tr>
<td>SV-APTT (sec)</td>
<td>58.7 ± 2.0</td>
<td>60.1 ± 5.2</td>
<td>56.7 ± 2.6</td>
</tr>
<tr>
<td>SV-ATIII (%)</td>
<td>97.2 ± 4.4</td>
<td>94.7 ± 3.1</td>
<td>94.3 ± 5.7</td>
</tr>
<tr>
<td>SV-FIB (mg/dl)</td>
<td>222 ± 16a</td>
<td>277 ± 21b</td>
<td>250 ± 21*</td>
</tr>
</tbody>
</table>

Control = sham-operated, Ischemia = 6 hrs low flow colonic ischemia, Ischemia & Reperfusion = 3 hrs low flow colonic ischemia and 3 hrs reperfusion, CV=colonic venous, OSPT=one stage prothrombin time, APTT=activated partial thromboplastin time, ATIII=antithrombin III, FIB=fibrinogen, SV=systemic venous. The letter "a" is different from "b".
Figure 7.1

Mean ± SEM for A) colonic venous and B) systemic venous one stage prothrombin time (OSPT) during low flow ischemia and reperfusion of the equine large colon. The solid arrow at 3 hrs indicates time of reperfusion in the ischemia & reperfusion group. The letter “b” is significantly (P<0.05) different from “a”.
Figure 7.1

A

Colonic Venous OSPT (%)

- Control
- Ischemia
- Ischemia & Reperfusion

Time (hrs)

B

Systemic Venous OSPT (%)

- Control
- Ischemia
- Ischemia & Reperfusion

Time (hrs)
Figure 7.2

Mean ± SEM for A) colonic venous and B) systemic venous activated partial thromboplastin time (APTT) during low flow ischemia and reperfusion of the equine large colon. The solid arrow at 3 hrs indicates time of reperfusion in the ischemia & reperfusion group. The letter "b" is significantly (P<0.05) different from “a”. 
Figure 7.2

A

Colonic Venous APTT (%)

- Control
- Ischemia
- Ischemia & Reperfusion

Time (hrs)

B

Systemic Venous APTT (%)

- Control
- Ischemia
- Ischemia & Reperfusion

Time (hrs)
Figure 7.3

Mean ± SEM for A) colonic venous and B) systemic venous antithrombin III activity (ATIII) during low flow ischemia and reperfusion of the equine large colon. The solid arrow at 3 hrs indicates time of reperfusion in the ischemia & reperfusion group. The letter "b" is significantly (P<0.05) different from "a".
Figure 7.3
Figure 7.4

Mean ± SEM for A) colonic venous and B) systemic venous fibrinogen concentration during low flow ischemia and reperfusion of the equine large colon. The solid arrow at 3 hrs indicates time of reperfusion in the ischemia & reperfusion group. The letter “b” is significantly (P<0.05) different from “a”.

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Figure 7.4
for systemic venous fibrinogen concentration; these differences were apparent at baseline.

There were significant differences between colonic and systemic venous values for OSPT and fibrinogen concentration, but not for APTT or ATIII activity.

Discussion

To our knowledge, this is the first report of hemostatic alterations occurring in the colonic venous effluent from horses subjected to large colon I-R. Interestingly, hemostatic alterations developed in horses from all three groups during the experimental period. These hemostatic alterations did not exhibit temporal differences or differences in magnitude between the sham-operated controls and either the ischemia or ischemia-reperfusion groups. Since similar alterations occurred in the sham-operated controls, these hemostatic changes did not appear to be caused or exacerbated by colonic ischemia and/or reperfusion, and are more likely associated with the surgical model used, or with factors common to all groups of horses.

Shortened colonic and systemic venous OSPT and APTT occurred in all groups suggesting a hypercoagulable state. The decrease in ATIII activity could have occurred secondary to this hypercoagulability (i.e. consumption) or due to fluid therapy-induced hemodilution, however, antithrombin III activity was decreased even after accounting for hemodilution. The decreases in colonic and systemic venous fibrinogen concentration may also represent consumption due to hypercoagulability, however, the decreases were present at 2 hrs, which may have been too short for fibrinogen to decrease secondary to consumption. Increased fibrinogen concentration usually occurs secondary to an inflammatory stimulus due to the hepatic acute phase response, which is stimulated by interleukin-6.27

Temporal alterations in systemic venous OSPT, APTT, ATIII activity, or fibrinogen concentration paralleled those alterations in colonic venous blood. These data
indicate that either the hemostatic perturbations occur globally within the body or that local hemostatic changes occurring within an organ or region of the body secondarily alter systemic hemostasis. The alterations in all hemostatic indices were present by 2 hrs in both the colonic and systemic venous blood. Since hemostatic parameters were not measured until 2 hrs, we were unable to determine any time-related differences between the development of colonic versus systemic venous hemostatic alterations. Determination of the hemostatic parameter values prior to 2 hrs may have given insight into the temporal development of and the relationship between colonic and systemic venous alterations. The absolute values for colonic and systemic venous APTT and fibrinogen concentration were different, which suggests that monitoring systemic venous hemostatic parameters does not necessarily provide an accurate assessment of hemostatic function in regional vascular beds.

Although general anesthesia with nitrous oxide and enflurane did not alter platelet function, coagulation, or fibrinolysis, balanced anesthesia with enflurane and alfentanil caused moderate activation of coagulation in human patients undergoing elective ophthalmic surgery. Additionally, ATIII activity was depressed immediately after total hip replacement in human patients, however, it remained decreased significantly longer in patients subjected to general anesthesia than in those receiving epidural anesthesia. To our knowledge, the mechanisms by which general anesthesia affect hemostatic function are unknown. The same anesthetic protocol using halothane was used for all horses of this study. In a previous study, halothane-anesthetized horses did not develop abnormalities in plasma or peritoneal fluid coagulation or fibrinolytic parameters. Additionally, plasma ATIII activity did not change in response to halothane anesthesia in horses. Although possible, it is unlikely that the hemostatic alterations observed in the present study were associated with halothane anesthesia.
All horses received sterile, isotonic, polyionic fluids at a similar rate, which resulted in hemodilution. Although hemodilution could account for at least part of the decrease in ATIII activity and fibrinogen concentration, it would probably prolong rather than shorten the OSPT and APTT values. The heparinized saline used to flush jugular venous and intra-cardiac catheters has the potential to alter hemostasis, however, heparin should also prolong rather than shorten the OSPT and APTT values. The dose of heparin in the saline received by each horse was also unlikely to affect hemostatic function. The mere presence of catheters in the colonic vein and main pulmonary artery could also have activated coagulation pathways. This would have resulted in an artificial shortening of OSPT and APTT, and possibly ATIII activity and fibrinogen concentration. Unfortunately, the only feasible method of obtaining multiple samples from these sites was via catheters.

Hemostatic function in the horses of this study could have been affected by the surgical model. All horses underwent a ventral midline celiotomy. The large colon was exteriorized from the abdomen and remained in an extra-abdominal position for the duration of the study. Manipulation of the colon during exteriorization and instrumentation could have initiated hemostatic alterations. The serosal surface was covered with plastic and was kept moistened with isotonic polyionic fluids. Colonic seromuscular temperature was maintained between 34-38° C with a heat lamp and a circulating hot water heating pad. Despite these efforts to maintain homeostasis, fibrin deposition on the serosal surface of the colon occurred in horses of all groups. Moreover, it has previously been shown that fibrin accumulates on the surface of intestine subjected to venous strangulation obstruction. Fibrinogen concentration was not increased in the peritoneal fluid of horses with naturally occurring intestinal strangulation or in horses subjected to an experimental exploratory laparotomy. Likewise, fibrinogen was not detected in the fluid surrounding intestinal segments subjected to venous strangulation obstruction. These studies suggest that
fibrinogen either does not accumulate in the peritoneal fluid or that it is rapidly converted to fibrin.

All hemostatic alterations were present by 2 hrs of the experimental period. Accounting for the time required for preparation, surgery, and colonic instrumentation this corresponded to a total of approximately 3-3.5 hrs. Hemostatic abnormalities were not detected in plasma 6 hrs after an experimental exploratory laparotomy in horses. Plasma plasminogen activity was increased 24 hrs post-laparotomy, but all other plasma coagulation/fibrinolysis parameters monitored for 144 hrs post-laparotomy were not different from anesthetized control horses.

Endotoxin exerts profound effects on the hemostatic system by numerous mechanisms. These endotoxin-mediated hemostatic alterations can lead to the development of DIC, which can manifest initially as hypercoagulability or later as hypocoagulability. Infusion of endotoxin to horses results in prolonged OSPT and APTT values and increased concentration of FDPs. These changes occur within the first hr after endotoxin infusion. In rabbits and dogs, endotoxin results in a hypercoagulable state beginning within 1 hr, peaking at 2 hrs, and returning to baseline values by 3.5 hrs after infusion. Experimentally, equine peripheral blood monocytes and rat peritoneal macrophages exposed to endotoxin express procoagulant activity. Additionally, the procoagulant activity of peripheral blood monocytes isolated from horses with colic was significantly greater than that of clinically normal horses and the OSPT and APTT were prolonged in horses not surviving colic. The finding of prolonged OSPT and APTT in horses exposed to endotoxin was opposite to the hemostatic changes observed in the horses in this study. This provides further support of our earlier finding that endotoxemia does not occur with this model of low flow colonic ischemia and reperfusion.
Hemostatic alterations occur in horses with naturally occurring colic\textsuperscript{7-11,39} and in horses subjected to experimental intestinal strangulation\textsuperscript{12}. Typical abnormalities observed on plasma hemostatic profiles in horses with colic are prolonged OSPT and APTT, increased fibrinogen and FDP concentrations, and decreased ATIII activity, suggesting active coagulation and fibrinolysis in horses with colic. The most severe changes occur in nonsurvivors and in those with the most severe forms of intestinal disease, such as ischemic or inflammatory disorders.\textsuperscript{10} Most hemostatic abnormalities were apparent during the first 2 days after hospitalization rather than being present at admission.\textsuperscript{10} Horses with colic develop decreased ATIII activity\textsuperscript{7,39}, and horses that did not survive an episode of colic had significantly lower ATIII activity than survivors.\textsuperscript{39} Decreased ATIII activity occurred during the immediate post-operative period and returned to normal within 4 to 5 days in survivors, however, ATIII activity remained low in nonsurvivors.\textsuperscript{7} Ponies subjected to 6 hrs of strangulating obstruction of the small intestine have prolonged OSPT and APTT values, and increased fibrinogen and FDP concentrations. The prolongation in the APTT and OSPT were observed 12 and 18 hrs, respectively, after releasing the strangulation, while the increase in fibrinogen concentration was not observed until 36 hrs.\textsuperscript{12} It is possible that if our horses had been recovered from anesthesia and monitored for 24-48 hrs we may have observed alterations typical of horses with colic or endotoxemia.

In summary, temporal alterations in colonic and systemic venous OSPT, APTT, ATIII activity, and fibrinogen concentration occurred in all groups following ventral midline celiotomy and instrumentation of the large colon. The changes in systemic venous blood paralleled those in colonic venous blood, however, the absolute values were different between systemic and colonic venous OSPT and fibrinogen. Colonic ischemia and/or reperfusion did not appear to exacerbate hemostatic dysfunction.
Footnotes

- Rompun®, Mobay Corporation, Animal Health Division, Shawnee, KS.
- Guailaxin®, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.
- Halothane U.S. P., Halocarbon Laboratories, North Augusta, SC.
- Drager AV Model NELAC-E, Anesthesia Ventilator, Telford, Pa.
- Lactated Ringer’s, Baxter Healthcare Corporation, Deerfield, Il.
- Swan-Ganz®, American Edwards Laboratories, Anasco, Puerto Rico.
- Model T201, Transonic Systems, Inc., Ithaca, NY.
- Angiocath®, Becton Dickinson Vascular Access, Sandy, Utah.
- Beuthanasia®-D Special, Schering Plough Animal Health Corporation, Kenilworth, NJ
- ACL 200, Instrumentation Laboratory, Lexington, Ma.

List of References


CHAPTER VIII
MURAL BLOOD FLOW DISTRIBUTION IN THE EQUINE LARGE COLON DURING LOW FLOW ISCHEMIA AND REPERFUSION

Summary

Six horses were subjected to 3 hrs of low flow ischemia and 3 hrs of reperfusion of the large colon. Following induction of anesthesia, the large colon was exteriorized through a ventral midline celiotomy. Colonic blood flow was measured continuously with doppler ultrasonic flow probes placed on the colonic arteries supplying the dorsal and ventral colon and was allowed to stabilize for 15-30 min following instrumentation. Colonic mucosal, seromuscular, and full-thickness blood flow were determined on a tissue weight basis by injecting colored microspheres proximally into the colonic artery supplying the ventral colon. Reference blood samples were obtained at a known flow rate from the colonic artery and vein at a site more distal to the site of injection. Left ventral colon biopsies were harvested at baseline (BL), 3 hrs of ischemia, and at 15 min of reperfusion. Blood and tissue samples were digested and filtered to collect the microspheres and dimethylformamide was added to release the colored dyes. Dye concentration in blood and tissue samples was measured by spectrophotometry and tissue blood flow was calculated. Data was analyzed using 2-way ANOVA for repeated measures; statistical significance was set at P < 0.05. Doppler blood flow decreased to approximately 20% of BL while microsphere blood flow ranged between 13.7-15.5% of BL flow at 3 hrs of ischemia. Doppler determined blood flow increased immediately upon restoration of blood flow, reached 183% of BL flow at 15 min of reperfusion, and remained at or above BL
throughout 3 hrs of reperfusion. This reactive hyperemia was also demonstrated using the colored microspheres; blood flow increased to 242% and 327% of BL at 15 min of reperfusion in the mucosal and seromuscular layers respectively. Mucosal blood flow was not different from seromuscular flow at any time indicating relatively equal distribution of blood flow between these two layers. As determined from the venous reference samples, there was no evidence of arteriovenous anastomoses.

Introduction

Restoration of intestinal blood flow after a period of arterial occlusion frequently leads to reactive hyperemia, which is an overshoot in blood flow above that present before vascular occlusion.\(^1\) The occurrence, magnitude, and duration of reactive hyperemia depends upon the type, degree and duration of vascular occlusion.\(^1\) The reactive hyperemic response to arterial occlusion in the small intestine is greater than combined arterial and venous occlusion or venous occlusion alone.\(^2,3\) Reactive hyperemia secondary to arterial occlusion is explained by both the metabolic and myogenic theories of blood flow regulation.\(^1\) The metabolic theory relates the accumulation of vasodilator substances during ischemia and the subsequent decrease in vascular resistance to the increased blood flow during reperfusion;\(^1\) nitric oxide, prostacyclin, prostaglandin E\(_2\), and adenosine are a few of the substances that cause vasodilation. The myogenic theory relates vascular transmural pressure and vascular resistance to blood flow.\(^1\) During arterial ischemia vascular transmural pressure decreases, which causes decreased vascular resistance; this usually leads to reactive hyperemia following release of the arterial occlusion.\(^1\) During venous occlusion vascular transmural pressure increases, which causes increased vascular resistance due to reflex arteriolar smooth muscle contraction.\(^1\) This secondarily reduces microvascular hydrostatic pressure by limiting blood flow through the affected capillary
beds. Hyperemia does not occur following release of venous occlusion because of the increased vascular resistance, however, blood flow does gradually return to pre-occlusion values as vascular transmural pressure declines.1

The post-occlusion period in the large intestine of laboratory animals is characterized by a hypoemia rather than hyperemia.4 This hypoemic response is characterized by an increase in blood flow above ischemic, but below baseline flow. Following correction of a one hour 720° experimental large colon volvulus in ponies, colonic blood flow increased above ischemic flow, but remained below the pre-occlusion flow rate for 50 minutes.5 We have recently demonstrated a profound reactive hyperemia in the equine large colon upon restoration of blood flow following a three hour period of partial arterial occlusion.6 Colonic blood flow increased within 5 minutes after release of the arterial occlusion and was maintained above baseline for 3 hours after restoring blood flow.

Distribution of blood flow to the intestinal wall is dependent upon a number of factors including motility, luminal pressure, and metabolic activity. Under normal physiologic conditions, approximately 80% of small intestinal blood flow is distributed to the mucosa due to high metabolic demands.1 Luminal pressure increases with intestinal distension and may cause shunting of blood flow away from the mucosa when luminal pressure ≥ mean mucosal capillary pressure. Following short periods (60 seconds) of arterial occlusion, there is a relatively uniform increase in blood flow to the mucosa and muscularis, but a longer duration of arterial occlusion leads to a preferential increase in blood flow to the muscularis due to increased motility.

Numerous methods have been used to quantitate intestinal blood flow.7 Many of these techniques enable determination of total blood flow, but do not allow for determination of blood flow distribution to the different layers of the intestinal wall.
Colored microspheres offer the advantages of quantitating blood flow on an intestinal weight basis and determining mural blood flow distribution.8

The purpose of this study was to characterize the distribution of mural blood flow in the large colon of horses before and after low flow ischemia.

Materials and Methods

This project was approved and performed under the guidelines of the Institutional Laboratory Care and Use Committee of The Ohio State University.

Animals: Six horses of various breeds (3 Thoroughbreds, 2 grade, 1 Arabian), ranging in age from 2-15 (8.2 ± 2.5) years and in body weight from 345-515 (412 ± 28) kg were used. Horses were determined to be free of cardiopulmonary, gastrointestinal, and other systemic disease by a thorough physical examination, a complete blood count and plasma fibrinogen determination. Horses were fed 1-2% of their body weight in grass hay per day and water was provided ad libitum. Food, but not water, was withheld for 24 hrs prior to the experiment.

Instrumentation: Horses were instrumented as we have previously described.6,9 Horses were sedated with xylazinea (0.5 mg/kg IV) and induced to general anesthesia with guaifenesinb (30 mg/kg IV) and thiamylal sodiumc (4 mg/kg IV). Following anesthetic induction, a loading dose of sodium pentobarbitald (7.5 mg/ kg) was administered and general anesthesia was maintained with a continuous infusion of sodium pentobarbitald (5-15 mg/ kg/hr). Horses were mechanically ventilatede at 6-12 breaths per minute to a peak inspiratory pressure of approximately 25 cm H2O. Isotonic polyionic fluidsf were administered at a rate of 5-10 ml/ kg/hr. Mean systemic arterial blood pressure was monitored and maintained ≥ 70 mm of Hg.
Horses were positioned in dorsal recumbency and prepared for an aseptic ventral midline celiotomy. The abdominal cavity was explored, the large colon exteriorized and instrumented. Doppler ultrasound flow probes were placed externally around the colonic arteries supplying both the dorsal and ventral colon and colonic blood flow was measured continuously and recorded (Figure 8.1). The duration of surgery and colonic instrumentation was approximately 1 hr. Three catheters were placed in the ventral colonic vessels. A 20-gauge 5.1 cm teflon catheter was placed proximal to the flow probe on the colonic artery supplying the ventral colon and a second 20-gauge catheter was inserted into the colonic artery distal to the flow probe. Additionally, a third 20-gauge catheter was inserted into the colonic vein draining the ventral colon distal to the flow probe.

**Experimental Design:** Baseline hemodynamic parameters were recorded after allowing a stabilization period of 15-30 min following colonic instrumentation. Low flow arterial ischemia was induced by creating transmural compression of the right dorsal and ventral colon and by tightening Rummel tourniquets placed on the colonic arteries supplying the dorsal and ventral colon until ventral colon arterial blood flow was reduced to 20% of the baseline value and dorsal colon arterial blood flow was decreased to zero. Blood flow in the colonic arteries was continuously monitored with the doppler ultrasound flow probes. Colonic blood flow was restored after 3 hrs of ischemia. Horses were euthanatized at the conclusion of the experiment with sodium pentobarbital (100 mg/kg IV).

Hemodynamic data were collected at baseline (time=0) and at 30 min intervals throughout the 6 hr experimental period. Data were also collected at 3.25 hrs, which corresponded to 15 min of reperfusion. Colonic blood flow was measured with colored microspheres at baseline (time = 0 hrs), 3 hrs of low flow ischemia (time = 3 hrs), and 15 min of reperfusion (time = 3.25 hrs).
Figure 8.1

Instrumentation of the large colon for measurement of doppler blood flow, injection of colored microspheres, and for collection of venous and arterial reference samples used in calculating microsphere determined colonic blood flow. RVC = right ventral colon, LVC = left ventral colon, PF = pelvic flexure, LDC = left dorsal colon, RDC = right dorsal colon, CA = colonic artery, CV = colonic vein, TC = transmural compression, RT = Rummel tourniquet, PAC = proximal colonic arterial catheter for microsphere injection, DAC = distal colonic arterial catheter for arterial reference sample withdrawal, VC = colonic venous catheter for reference sample withdrawal, and DBFP = doppler ultrasound blood flow probe.
Colonic blood flow measurements using colored microspheres: Colonic blood flow was quantitated per gram of tissue using colored polystyrene/divinylbenzene microspheres. The microspheres measure 15.1 μm in diameter and are purchased as a suspension in saline, 0.01% polyoxyethylene sorbitan monooleate (Tween 80), and thimerosal. Each ml of the microsphere suspension contains 3 x 10^6 microspheres. The microspheres were thoroughly dispersed by vortex mixing for 30 seconds, and 1 ml was then suspended in 3 ml of saline containing 0.02% Tween 80, mixed thoroughly, and rapidly injected into the colonic artery supplying the ventral colon through the catheter placed proximal to the ultrasound flow probe. Blood flow was calculated by collecting reference colonic arterial blood samples from the artery through the catheter placed distal to the flow probe. A reference colonic venous blood sample was collected from the venous catheter for detection of arteriovenous anastomoses. Reference blood samples were withdrawn for 60 seconds with a Harvard continuous flow pump set at a rate of 10.89 ml/min beginning 10 seconds before microsphere injection. The withdrawal rate of the pump was verified at the end of each experiment. To ensure a desired microsphere count of 400 to 1,000 spheres per tissue sample, 3 x 10^6 microspheres were injected at each time period. This was estimated based on the percent weight of an average colonic tissue sample of 2 g, and the weight of an evacuated colon distal to the arterial injection portal of approximately 8,600 g. The total number of microspheres to inject was calculated so that each tissue sample would contain the desired number of spheres. The number of microspheres to inject was calculated as follows:

\[
\text{# microspheres to inject} = \frac{\text{weight of colon} \times \text{number of spheres/sample}}{\text{weight of sample}}
\]

The number of microspheres needed to inject was determined as 8,600 g · 1,000/2 = 4.3 x 10^6. Since one ml of the microsphere solution contains 3 x 10^6 spheres it was determined that injection of 1 ml should result in the desired number (400 to 1,000) of microspheres.
per tissue sample. Three different colored (red, blue and yellow) microspheres were used to measure blood flow; one color was used for each of the three measurement times.

Full-thickness sections of the left ventral colon were harvested at the conclusion of the experiment. A portion of full-thickness colon was placed in a plastic vial and the mucosa and seromuscular layers were separated in the remaining portion; each layer was placed in a separate plastic vial. All tissues were assayed in triplicate. All tissue samples were weighed (0.5-2.0 g), placed in glass tubes, and digested with 7 ml of 4 M KOH containing 2% Tween 80. Tubes were closed and placed in a 37°C water bath overnight. The samples were then mixed with a teflon coated magnetic stirring bar and placed on an 8 μm diameter pore polyester filter with a burette within a high grade steel vacuum chamber. The test tube and burette were rinsed with 2% Tween 80 and 70% ethanol and then filtered to ensure retrieval of all spheres. For each 10 ml reference blood sample, 4 ml of 2% Tween 80, 4.4 ml of 16M KOH and 2 ml 20% Tween 80 were added. Following filtration of digested tissue and blood samples, 100 μl N,N-dimethylformamide was added to release the dye from the microspheres. The tubes were then vortex mixed for 30 seconds and centrifuged for 3 min at 2,000 g.

Prior to each assay, calibration was made with 9 standard dye solutions to develop the standard matrix. A matrix inversion technique was used to correct for overlap of composite spectra. Samples were diluted when necessary to maintain absorbance of each wavelength under 1.3 absorbance units (AU) to ensure linearity of the absorption-concentration curve according to the Lambert-Beer law. All tissue samples containing less than 400 spheres were discarded from analysis. Dye concentration was measured spectrophotometrically at the appropriate wavelength for each color: red-530 nm, blue-672 nm, and yellow-448 nm. Dimethylformamide was used as the blank.
Tissue blood flow was calculated as follows:

\[
\text{Tissue blood flow (ml/min) = \frac{AU \text{ of tissue sample} \times \text{reference blood flow (ml/min)}}{AU \text{ of reference sample}}}
\]

and was divided by the weight (g) of the tissue sample. Tissue blood flow was expressed as ml-min\(^{-1}\).g\(^{-1}\) of tissue.

**Statistical Analysis**: Data are expressed as mean ± standard error of the mean (SEM). Blood flow data were analyzed using a two-way repeated measures analysis of variance (ANOVA). A Student Newman Keuls was used for post-hoc comparisons. Statistical significance was considered if \(P<0.05\).

**Results**

Doppler baseline (BL) blood flow to the ventral colon was 274.9 ± 47.7 ml/min and was decreased to 56.1 ± 9.2 ml/min (20% of BL) at 3 hrs of ischemia (Figure 8.2). Blood flow to the ventral colon increased to 505.8 ± 132.3 ml/min (183% of BL) following release of the ligatures.

Full-thickness colonic wall blood flow when measured with colored microspheres was 116.83 ± 35.15 ml·min\(^{-1}\).g\(^{-1}\) tissue (Figure 8.3). Blood flow decreased to 16.02 ± 4.91 ml·min\(^{-1}\).g\(^{-1}\) (13.7% of BL) of baseline at 3 hrs of ischemia and increased to 241.47 ± 64.77 ml·min\(^{-1}\).g\(^{-1}\) (207% of BL) at 15 min of reperfusion.

Colonic mucosal blood flow was 142.38 ± 48.30 ml·min\(^{-1}\).g\(^{-1}\) at baseline (Figure 8.3). At 3 hrs of ischemia mucosal blood flow was decreased to 22.13 ± 5.84 ml·min\(^{-1}\).g\(^{-1}\) (15.5% of BL). Mucosal blood flow was increased to 344.01 ± 92.33 ml·min\(^{-1}\).g\(^{-1}\) (242% of BL) at 15 min of reperfusion.

Blood flow to the seromuscular layer was 117.95 ± 36.84 ml·min\(^{-1}\).g\(^{-1}\) at BL (Figure 8.3). Seromuscular blood flow decreased to 17.12 ± 4.53 ml·min\(^{-1}\).g\(^{-1}\) (14.5%
Figure 8.2

Mean ± SEM for colonic blood flow (ml/min) as measured by the doppler ultrasonic flow probe on the colonic artery supplying the ventral colon. Time 0 hrs = baseline; time 3 hrs = 3 hrs low flow ischemia; and time 3.25 hrs = 15 min of reperfusion. The percents located at time 3 and 3.25 hrs are the % of baseline blood flow.
Figure 8.2
Figure 8.3

Mean ± SEM for left ventral colonic blood flow (ml/min/gram) as measured with colored microspheres. Full thickness, mucosal, and seromuscular blood flow at 0 hrs (baseline), 3 hrs (3 hrs low flow ischemia), and 3.25 hrs (15 min of reperfusion). The percents located at time 3 and 3.25 hrs are the % of baseline blood flow.
Figure 8.3

Colonic Blood Flow (ml/min/gram)

- Full thickness
- Mucosal
- Seromuscular

Time (hrs)

0 3 3.25

13.7% 15.54% 14.5%

242% 207% 327%
of BL) at 3 hrs of ischemia and increased to 386.07 ± 97.22 ml·min⁻¹·g⁻¹ (327% of BL) at 15 min of reperfusion.

There were no differences in the distribution of full-thickness, mucosal, or seromuscular blood flows at baseline, 3 hrs of ischemia, or 15 min of reperfusion.

Microspheres were not detected in the colonic venous reference blood samples.

Discussion

The selective administration of colored microspheres facilitated quantitation of colonic blood flow on a tissue weight basis and enabled the determination of blood flow distribution to the different layers of the colonic wall. This is the first report to describe and quantitate mural blood flow distribution in the large colon of horses during and after prolonged periods of ischemia. Using a model of low flow colonic arterial ischemia, we have previously reported colonic blood flow significantly increases within 5 min of reperfusion and remains significantly increased throughout 3 hrs of reperfusion. This contrasts with the blood flow response following correction of a one hr experimental 720° large colon volvulus in ponies where blood flow remained below pre-occlusion values for 50 min and never increased above baseline flow. The differences in the blood flow responses are likely due to the type and magnitude of vascular occlusion produced in these studies. Reactive hyperemia generally occurs following intestinal arterial occlusion, however, it is not typical of venous or combined arteriovenous occlusion. Venous occlusion results in increased vascular transmural pressure, which stimulates a reflex vascular myogenic response characterized by smooth muscle constriction. This secondarily increases vascular resistance, which leads to decreased blood flow. Experimental 720° volvulus in ponies undoubtedly creates venous occlusion, which could explain the absence of reactive hyperemia following correction of the volvulus.
Alternatively, lack of reactive hyperemia following correction of experimental volvulus may have been associated with increased vascular resistance due to thromboxane production\textsuperscript{5,11} or hypotension\textsuperscript{5}. Vascular thrombosis\textsuperscript{12} occurs in naturally occurring large colon volvulus and there is ultrastructural evidence of microvascular thrombi following experimental large colon arteriovenous occlusion.\textsuperscript{13} The presence of thrombi could obstruct microvascular blood flow thus preventing or limiting the hyperemic response. Another potential explanation for the difference between arterial occlusion versus venous occlusion in producing reactive hyperemia is that either venous occlusion does not interfere with the generation of ATP to the same degree as arterial occlusion or that a substance is produced that inactivates ATP-dependent potassium channels. Based upon the duration of ischemia, the first argument is unlikely. During ischemia, the depletion of tissue ATP or the accumulation of metabolic by-products may activate ATP-dependent potassium channels.\textsuperscript{14-16} Activation of ATP-dependent potassium channels causes vascular smooth muscle relaxation\textsuperscript{17}, which may contribute to the reactive hyperemia observed upon reperfusion after low flow arterial ischemia of the large colon. Although this mechanism seems feasible it has not been verified and requires further investigation.

The blood flow response reported following correction of experimental volvulus in ponies\textsuperscript{5} is similar to the hypoemia observed in the large intestine of laboratory animals\textsuperscript{4}. The presence of hyperemia in the equine large colon after the low flow arterial ischemia used in the present study provides evidence that hypoemia is not the only vascular response of the colon following restoration of blood flow. Therefore, the blood flow response of the equine large colon following restoration of blood flow after ischemia appears to be related to the type and extent of vascular occlusion produced.

Colonic blood flow was not as disproportionately distributed to the mucosa as is typical of small intestinal blood flow.\textsuperscript{1} Approximately 80% of small intestinal blood flow
has been reported to be distributed to the more metabolically active mucosa in laboratory animals. Colonic mucosal blood flow was not different from seromuscular flow before, during, or after low flow ischemia indicating relatively equal distribution between the mucosal and seromuscular layers. The hyperemic response was distributed to all layers of the colon wall upon reperfusion. We have no explanation for the absence of preferential shunting of blood flow to the mucosa during ischemia or to the seromuscular layer during reperfusion.

Colonic blood flow measured with the doppler ultrasound flow probe qualitatively paralleled blood flow measured with the colored microspheres. The sensitivity and variability inherent to these blood flow measurement techniques may account for any quantitative differences. During ischemia, colonic blood flow was reduced to approximately 20% of baseline as measured with the ultrasound flow probe. Blood flow measured by the colored microspheres decreased to approximately 15% of baseline; blood flow reduction was similar in the mucosal and the seromuscular layers. Upon reperfusion, ultrasonic blood flow increased to 183% of the baseline flow. Blood flow measured with colored microspheres increased between 207% and 327% of baseline; hyperemia occurred in both the mucosal and seromuscular layers. Similarly, restoration of small intestinal blood flow after short duration (<60 sec) arterial occlusion leads to uniform increases in blood flow to the mucosa and seromuscular layers. Increased seromuscular blood flow has been reported following longer periods of arterial occlusion; this is believed to occur secondary to increased smooth muscle contraction associated with increased colonic motility. We did not observe this preferential hyperemia in the seromuscular layer; perhaps this is due to a difference in the motility patterns in these studies. Particles, such as microspheres, used for blood flow measurements more nearly approximate red blood cell flow than total blood or plasma flow; doppler blood flow measurements more likely
approximate total blood flow. Because tissue oxygen delivery is proportional to red blood cell flow, rather than plasma flow, microsphere determined blood flow likely provides a more accurate assessment of tissue oxygen delivery.\textsuperscript{18}

We were not able to determine from this study if colonic manipulation and its extra-abdominal location significantly disturbed blood flow distribution to the colonic wall. We have previously demonstrated that manipulation of the colon and its extra-abdominal presence did not cause time related alterations in colonic blood flow or colonic histopathologic parameters.\textsuperscript{6-9} Since colonic blood flow was allowed to stabilize after instrumentation, combined with the absence of changes in colonic blood flow for 6 hrs following colonic manipulation and instrumentation\textsuperscript{6}, it is unlikely that mural blood flow distribution is altered in horses subjected to a ventral midline celiotomy and colonic manipulation and instrumentation.

The profound reactive hyperemia\textsuperscript{6} occurring upon restoration of blood flow following low flow colonic arterial ischemia in the horse corresponded to increased 6-keto prostaglandin F\textsubscript{1α} and prostaglandin E\textsubscript{2} concentrations in colonic venous blood.\textsuperscript{19} The hyperemia continued throughout the 3 hr period of reperfusion despite a gradual decrease in the concentration of these vasodilator eicosanoids.\textsuperscript{19} This suggests that eicosanoids may contribute to the reactive hyperemia, but are not solely responsible for it. Other mechanisms likely exist and may contribute to this hyperemic response. During low flow colonic arterial ischemia colonic venous lactate concentration increases.\textsuperscript{6} Lactate or other metabolic by-products may activate ATP-dependent potassium channels\textsuperscript{14}, and may contribute to the reactive hyperemic response in the large colon.

The absence of microspheres in the colonic venous effluent following injection of microspheres into the colonic artery provides evidence that arteriovenous anastomoses either are not present in the equine large colon or at least were not functional. The 15.1 μm
diameter microspheres we used should not traverse the capillary microcirculation and if present in venous blood would be suggestive of arteriovenous anastomoses. Extensive investigation of the microvascular circulation of the equine large colon has not provided microangiographic or microscopic evidence of mucosal arteriovenous anastomoses. The results of our study supports this previous conclusion.

Historically, the “gold standard” for measuring tissue blood flow has been the use of radioactively-labeled particles. More recently, colored microspheres have been used successfully to measure tissue blood flow. Thus far, this technique has been used most extensively for measurement of regional myocardial blood flow in experimental cardiology. Regional myocardial blood flow measured simultaneously with radioactive and colored microspheres exhibit excellent correlation. Blood flow determination using colored microspheres has several advantages. First, the expense and inconvenience of disposal of radioactive carcasses and waste materials are unnecessary. Colored microspheres are stable in vitro for up to 1 year and in vivo for up to 48 hrs whereas radioactive materials undergo decay. The use of multiple different colored microspheres allows measurement of tissue blood flow at several time periods within the same organ or animal. The use of spectrophotometry and matrix inversion resolution of composite spectra allows nearly complete recovery of each dye with only minimal interaction between different dyes of a composite spectrum.

The presence of colonic mucosal hyperemia upon reperfusion was documented using colored microspheres. Clinically, reactive hyperemia is frequently observed by normalization of the serosal color (bluish-gray to pink) immediately following surgical correction of a large colon volvulus. The mucosal color, however, does not improve as dramatically as the seromuscular color. Following surgical correction of naturally occurring large colon volvulus, perfusion to the seromuscular layers may improve,
however, mucosal perfusion may be limited by thrombi developing in submucosal vessels at the level of the muscularis mucosae. Ultrastructural evaluation of the large colon has demonstrated plugging of capillaries with platelets, proteinaceous material, and cellular debris by 2.5 hrs of either complete venous or combined arteriovenous ischemia. Thrombi within the colonic microvasculature may cause obstruction to mucosal blood flow and could cause shunting of blood away from the mucosa following surgical correction. The low flow model of ischemia used in our study is seemingly not characterized by significant thrombosis of the colonic vasculature.

Determining intestinal viability is one of the most important, and often most challenging, aspects of clinical gastrointestinal surgery. Numerous techniques have been developed and applied to assist the equine clinician in predicting viability and in determining whether or not to resect affected intestine. The occurrence of increased mucosal blood flow within 15 min of reperfusion in this study demonstrates mucosal hyperemia occurs at least following low flow arterial ischemia. Therefore, measuring the rate of return and degree of mucosal oxygenation could be helpful clinically to determine colonic mucosal viability. Surface oximetry and doppler ultrasound have been used to evaluate intestinal viability following experimental or clinical intestinal strangulation. Surface oximetry ($S_{P}O_2$) employs the use of an electrode placed on the serosa for measuring oxygen partial pressure at the surface of visceral organs. It has been evaluated as a tool for predicting large colon viability following experimental ischemia and naturally occurring large colon volvulus. The value in normal equine colon is approximately 55 ($\pm$ 20%) mm of Hg and it has been shown to be different between survivors (> 30 mm of Hg) and nonsurvivors (< 20 mm of Hg) of colonic volvulus. The $S_{P}O_2$ measurements obtained with the surface electrode are more likely a reflection of seromuscular versus mucosal perfusion. Determination of intestinal mucosal viability should correlate more
closely with survival. Perhaps measurement of mucosal SpO₂ at the time of surgical correction of naturally occurring volvulus may provide an index of mucosal oxygen delivery and thus prognostic information regarding colonic viability and patient outcome.

In summary, colonic blood flow was quantitated on a tissue weight basis and was approximately equally distributed between the mucosal and seromuscular layers. Mucosal blood flow was not different from seromuscular flow at baseline, during ischemia, or during reactive hyperemia associated with reperfusion. Arteriovenous anastomoses are either not anatomically present or were nonfunctional in the large colon of horses during low flow ischemia and reperfusion. Colored microspheres should be useful for measuring blood flow in future gastrointestinal studies in horses. The rate of return of colonic blood flow or mucosal SpO₂ may provide useful prognostic information in horses with naturally occurring large colon volvulus.

Footnotes

*Rompun®, Mobay Corporation, Animal Health Division, Shawnee, KS.
*Guialaxin®, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.
*Sodium Pentobarbital Injection, The Butler Company, Columbus, Ohio.
*Drager AV Model NELAC-E, Anesthesia Ventilator, Telford, Pa.
*Lactated Ringer’s, Baxter Healthcare Corporation, Deerfield, Il.
*Model T201, Transonic Systems, Inc., Ithaca, NY.
*Angiocath®, Becton Dickinson Vascular Access, Sandy, Utah.
*Beuthanasia®-D Special, Schering Plough Animal Health Corporation, Kenilworth, NJ
Tween® 80, Fisher Chemical, Fisher Scientific, Fair Lawn, NJ.

Model 940 Infusion/Withdrawal Pump, Harvard Apparatus Co., Dover, Ma.

Membra-Fil®, Costar Corporation, Cambridge, Ma.


Model DU®-70 Spectrophotometer, Scientific Instruments Division, Beckman Instruments, Inc., Fullerton Ca.

List of References


CHAPTER IX

EFFECT OF DIMETHYL SULFOXIDE, ALLOPURINOL, 21-AMINOSTEROID U-74389G, AND MANGANESE CHLORIDE ON LOW FLOW ISCHEMIA AND REPERFUSION OF THE HORSE LARGE COLON

Summary

Thirty horses were randomly assigned to one of 5 groups. All horses were anesthetized, subjected to a ventral midline celiotomy, and the large colon was exteriorized and instrumented. Colonic arterial blood flow was reduced to 20% of baseline and was maintained for 3 hrs. Colonic blood flow was then restored and the colon was reperfused for an additional 3 hrs. One of 5 drug solutions were administered IV via the jugular vein 30 min prior to colonic reperfusion: group 1 (0.9% NaCl), group 2 (dimethyl sulfoxide; 1 g/kg), group 3 (allopurinol; 25 mg/kg), group 4 (21-aminosteroid U-74389G; 10 mg/kg), and group 5 (manganese chloride; 10 mg/kg). Hemodynamic parameters were monitored and recorded at 30 min intervals. Systemic arterial, pulmonary arterial (SV), and colonic venous (CV) blood samples were collected for blood gas, oximetry, lactate, packed cell volume, and total protein measurements. The eicosanoids 6-keto prostaglandin F1α (6-kPG), prostaglandin E2 (PGE2), and thromboxane B2 (TXB2) were measured in CV blood and endotoxin was measured in CV and SV blood. Full-thickness biopsies were harvested from the left ventral colon for histopathologic evaluation and determination of wet weight:dry wet (WW:DW) ratios. Data were analyzed using a 2-way analysis of variance for repeated measures and statistical significance was set at P < 0.05. Manganese chloride
(MnCl$_2$) infusion increased heart rate (HR), mean arterial pressure (MAP), and cardiac output (CO); the HR and CO remained increased throughout the experiment, but MAP returned to baseline values within 30 min after completion of MnCl$_2$ infusion. No other drug-induced changes were significant. There were significant increases in mean pulmonary artery (mPAP) and mean right atrial (mRAP) pressures at 2 and 2.5 hrs in all groups, but no other changes across time nor differences among groups were observed. Mean PAP remained increased through 6 hrs in all groups, but mRAP had returned to baseline values at 3 hrs. Mean colonic arterial pressure was significantly decreased at 30 min of ischemia and remained decreased through 6 hrs, however, by 3.25 hrs it was significantly higher than at 3 hrs of ischemia. Colonic arterial resistance decreased during ischemia and remained decreased throughout reperfusion in all groups; there were no differences among groups for colonic arterial resistance. Colonic venous PO$_2$, oxygen content, and pH decreased and PCO$_2$ and lactate increased during ischemia, but returned to baseline values during reperfusion. Compared to the baseline values, colonic oxygen extraction ratio was increased from 0.5 to 3 hrs. By 15 min of reperfusion, colonic oxygen extraction ratio had decreased from the baseline value in all groups and either remained decreased or returned to values not different from baseline through 6 hrs. Colonic venous 6-kPG and PGE$_2$ increased during ischemia, but returned to baseline upon reperfusion; there were no changes in TXB$_2$ among or within groups. No endotoxin was detected in CV blood after ischemia or reperfusion. There were no differences among or within groups for these parameters. Low flow ischemia and reperfusion (I-R) of the large colon caused mucosal injury as evidenced by increases in % surface mucosal loss, % depth mucosal loss, mucosal hemorrhage, mucosal edema, mucosal interstitial:Crypt ratio, mucosal neutrophil index, submucosal venular neutrophils and mucosal cellular debris index during ischemia and reperfusion. There was a trend (P=0.06) toward greater %
depth mucosal loss at 6 hrs in horses treated with DMSO compared to the vehicle control solution. There were no differences for the remainder of the histopathologic parameters among groups. Full-thickness and mucosal WW:DW ratios increased with colonic I-R, but there were no differences among groups. There was a trend (P=0.09) toward neutrophil accumulation, as measured by lung myeloperoxidase activity, in the lung following colonic I-R, but there were no differences among groups. There was no change in lung WW:DW ratio following colonic I-R. There were no demonstrable beneficial effects of drugs directed against oxygen free radical-mediated damage on colonic mucosal injury associated with low flow I-R. No deleterious drug-induced hemodynamic effects were observed in this study.

Introduction

Restoration of blood flow to the equine large colon following low flow arterial ischemia exacerbates mucosal injury. Gastrointestinal reperfusion injury is believed to be principally initiated by the accumulation of hypoxanthine during ischemia and its conversion by xanthine oxidase (XO) to superoxide anion upon restoration of blood flow. Injury initiated by XO-derived oxygen free radicals (OFRs) is perpetuated by synthesis of phospholipid-derived mediators and chemoattraction and infiltration of neutrophils. Numerous drugs directed against OFR-mediated lipoperoxidation have been demonstrated experimentally to reduce or prevent gastrointestinal reperfusion injury. Dimethyl sulfoxide (DMSO) is an anti-inflammatory agent, which scavenges hydroxyl radicals and prevents leukocyte adherence to endothelium resulting in decreased lipoperoxidation and neutrophil infiltration. Administration of DMSO attenuates intestinal mucosal injury, microvascular permeability, and neutrophil infiltration. Dimethyl sulfoxide did not attenuate large colon mucosal injury in a complete arteriovenous
model of occlusion.\(^8\) Allopurinol is a xanthine oxidase inhibitor, which reduces the production of superoxide anion; it has been shown to decrease intestinal injury associated with ischemia and reperfusion (I-R).\(^9,10\) The 21-aminosteroids are a group of compounds developed by modification of glucocorticoids.\(^11,12\) These compounds retain the therapeutic properties of corticosteroids, but do not have the deleterious side effects. The mechanism of action of 21-aminosteroids involves prevention of iron-dependent lipoperoxidation.\(^11,12\) Administration of 21-aminosteroids have been shown to reduce injury associated with I-R of the stomach in dogs\(^13\), small intestine in rats\(^14\), and large colon in horses\(^15\). Manganese chloride (MnCl\(_2\)) is a simple inorganic manganous salt that demonstrates superoxide scavenging capabilities.\(^16\)

The purpose of this study was to determine the effect of clinically recommended doses of dimethyl sulfoxide, allopurinol, 21-aminosteroid U-74389G, and MnCl\(_2\) on systemic hemodynamic and metabolic parameters, and on the severity of colonic mucosal injury associated with low flow ischemia and reperfusion of the large colon in the horse.

**Materials and Methods**

This project was performed with approval and under the guidelines of the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

*Animals* - Thirty horses of various breeds, ranging in age from 2-15 years of age and in body weight from 350-500 kg were used. Horses were fed 1-2% of their body weight in grass hay per day and were adapted to their diet and environment for at least one week prior to the experiment; water was provided ad libitum. Food, but not water, was withheld for 24 hrs prior to the experiment.

*Instrumentation* - Horses were instrumented similar to our previous descriptions.\(^1,17\) Briefly, horses were sedated with xylazine\(^a\) (0.5 mg/kg IV) and a 14-
A 30-gauge 13.3 cm teflon catheter\textsuperscript{b} was inserted into the left jugular vein for administration of anesthetic drugs and isotonic fluids. A balloon-tipped, flow directed thermodilution catheter\textsuperscript{c} was inserted into the right jugular vein and advanced until the distal port was positioned in the pulmonary artery for measurement of cardiac output and pulmonary artery pressure and for collection of mixed-venous blood samples. A third catheter\textsuperscript{d} was positioned in the right atrium for measurement of right atrial pressure. General anesthesia was induced with guaifenesin\textsuperscript{e} (30 mg/kg IV) and thiamylal\textsuperscript{f} (4.4 mg/kg IV). Immediately after induction, a bolus of sodium pentobarbitals (7.5 mg/kg IV) was injected and the anesthesia was maintained with a continuous IV infusion of sodium pentobarbital (5-15 mg/kg/hr). Horses were mechanically ventilated\textsuperscript{h} at 6-12 breaths per minute at a peak inspiratory pressure \leq 25 cm H\textsubscript{2}O. Isotonic polyionic fluids\textsuperscript{i} were administered IV at a rate of 5-10 ml/kg/hr. Arterial blood pressure was monitored via a 20-gauge 5.1 cm teflon catheter\textsuperscript{j} placed in the facial artery, and mean arterial pressure was maintained \geq 70 mm of Hg.

All horses were positioned in dorsal recumbency and prepared for aseptic surgery. A ventral midline celiotomy was performed, the large colon was exteriorized, placed on a warm water heating pad and instrumented (Figure 9.1).\textsuperscript{1, 17} Doppler ultrasound flow probes\textsuperscript{k} were placed externally around the colonic arteries supplying the dorsal and ventral colon and colonic blood flow was measured continuously and recorded. A 14-gauge, 5.1 cm teflon (non-occluding) teflon catheter\textsuperscript{l} was placed in the colonic vein draining the ventral colon for measurement of mean colonic venous pressure and for collection of colonic venous blood. A 20-gauge, 5.1 cm catheter\textsuperscript{j} was inserted into the colonic artery supplying the left ventral colon distal to the flow probe for measurement of mean colonic arterial pressure.
Instrumentation of the equine large colon used during low flow ischemia and reperfusion.
RVC = right ventral colon, LVC = left ventral colon, PF = pelvic flexure, LDC = left dorsal colon, RDC = right dorsal colon, CA = colonic artery, CV = colonic vein, TC = transmural compression, RT = Rummel tourniquet, CAC = colonic arterial catheter, CVC = colonic venous catheter, DBFP = Doppler ultrasound blood flow probe.
Figure 9.1
**Experimental Design** - All horses underwent 3 hrs of low flow ischemia and 3 hrs of reperfusion. All drugs or the vehicle control solution (0.9% NaCl) were administered IV 30 min prior to reperfusion. Thirty horses were randomly assigned to one of five groups of six horses each. The groups were as follows: group 1 (0.9% NaCl), group 2 (1 gram/kg DMSO), group 3 (25 mg/kg allopurinol), group 4 (10 mg/kg 21-aminosteroid U-74389GP), and group 5 (10 mg/kg manganese chloride).

Baseline data was collected and low flow colonic arterial ischemia was induced by reducing ventral colon arterial blood flow to 20% of baseline while reducing dorsal colon blood flow to zero. Blood flow in the colonic arteries was continuously monitored using the doppler ultrasound flow probes. Colonic blood flow was restored after 3 hrs of ischemia by releasing the ligatures and transmural compression and the colon was reperfused for 3 hrs. The horses were euthanatized at the conclusion of the experiment with sodium pentobarbital (100 mg/kg IV). The vehicle control solution (0.9% NaCl) and all drugs were administered aseptically as a 5 liter volume administered as a continuous infusion over 30 min. Drug infusion was started at 2.25 hrs and completed by 2.75 hrs.

Cardiovascular parameters were recorded and blood samples collected for metabolic parameters at 30 min intervals and at 195 min (corresponding to 15 min of reperfusion). Cardiovascular parameters that were monitored included heart rate (HR; beats/min); systolic, diastolic, and mean arterial pressures (SAP, DAP, MAP; mm of Hg); mean right atrial pressure (mRAP; mm of Hg); mean pulmonary artery pressure (mPAP; mm of Hg); cardiac output (CO; L/min); colon blood flow (Qcolon; ml/min); and mean colonic venous (mCVP; mm of Hg) and colonic arterial (mCAP; mm of Hg) pressures. Colonic arterial resistance was calculated by dividing the mean colonic arterial pressure by colonic blood flow.
**Metabolic Measurements and Calculations** - Samples of arterial, mixed venous, and colonic venous blood were collected for measurement of total plasma protein, TP (g/dl); packed cell volume PCV (%); blood gas and oximetry analyses; and lactate concentration (mmol/L) at 30 min intervals. Systemic arterial, mixed venous, and colonic venous blood samples (2 ml each) were anaerobically collected into separate heparinized syringes and stored on ice until analyzed for pH, partial pressure of carbon dioxide, PCO₂ (mm of Hg); partial pressure of oxygen, PO₂ (mm of Hg); base excess, BE (mEq/L); bicarbonate concentration, HCO₃ (mEq/L); hemoglobin concentration, Hb (g/dl); and percent oxygen saturation of hemoglobin, %SO₂ (%). The blood gas and oximetry analyses were corrected for each horse's core temperature, measured by the flow directed thermodilution catheters. All samples were analyzed within 1 hr of collection. One ml of the blood was placed in a plastic microcentrifuge tube, immediately immersed in liquid nitrogen, and stored at -70° C until analyzed for whole blood lactate. Thawing of these blood samples resulted in lysing of the red blood cells to allow measurement of whole blood lactate. The remaining blood was used to measure PCV via a microhematocrit method and TP using a refractometer.

Arterial (CₐO₂) and colonic venous (CᵥO₂) oxygen contents (ml/dl) were calculated as the sum of oxygen bound to hemoglobin and oxygen dissolved in plasma \{(Hb x %SO₂ x 1.36) + (PO₂ x 0.003)\}. Colonic oxygen delivery, DO₂哥伦 (ml/min), was estimated as the product of arterial oxygen content and colon blood flow (DO₂哥伦=CₐO₂ x Q哥伦). Large colon oxygen consumption, VO₂哥伦 (ml/min/m²), was estimated as VO₂哥伦 = Q哥伦(CₐO₂ - CᵥO₂), where Q哥伦 is the measured blood flow in the left ventral colon.
Prostaglandin Assays: Ten ml of colonic and mixed venous blood were collected separately into plastic test tubes containing cold 100mM EDTA (pH=7.4) and 10 mM meclofenamic acid (1:20 with blood) at 0, 3, 3.25, 4 and 6 hrs. The samples were stored on ice, centrifuged at 1,500 g for 10 min and the plasma was stored at -70° C until assayed. Plasma 6-keto prostaglandin F$_{1\alpha}$ (6-kPG) and thromboxane B$_2$ (TXB$_2$), the active metabolites of prostacyclin and thromboxane A$_2$ respectively, and prostaglandin E$_2$ (PGE$_2$) were quantitated (pg/ml) by competitive binding radioimmunoassay using standard techniques. The percent recovery of all colonic venous eicosanoids ranged between 79-88%.

Endotoxin Assays: Ten ml of colonic and mixed venous blood were aseptically collected into sterile, pyrogen-free, heparinized glass test tubes at 0, 3 and 6 hrs. The samples were immediately cooled on ice and then centrifuged at 2,000 rpm for 5 min. The plasma was aseptically transferred into a storage tube where it was diluted 1:10 with sterile, pyrogen-free water. The diluted plasma sample was heated to 75° C for 10 min, allowed to cool to room temperature, and then stored in a -70° C freezer until analyzed for endotoxin using a quantitative chromogenic limulus amebocyte lysate test. The test used for measuring endotoxin was sensitive to detect from 0.1 to 1.0 EU/ml.

Colonic Biopsies: Full-thickness 2 cm x 5 cm sections of the left ventral colon were harvested; sampling variation was minimized by collecting biopsies from similar locations and identical patterns from all horses. Biopsies were gently rinsed in a buffered electrolyte solution and divided into three portions. One portion was immediately fixed in 10% formalin for histologic evaluation. The remaining samples were used to measure wet weight:dry weight (WW:DW) ratios on full-thickness colon and on the colonic mucosa. The samples were weighed immediately and again after 48 hrs in a 80° C oven. The WW:DW ratio was used as an estimation of colonic tissue edema.
Histopathology: Full-thickness formalin-fixed sections of the left ventral colon were embedded in paraffin, cut in 4 μm sections, processed and stained with hematoxylin & eosin using standard techniques.

Semi-Quantitative Morphology: All histomorphologic parameters were estimated or scored independently and blindly by two investigators using 10x and 40x ocular objectives. All areas of the colonic tissue sections on each slide were analyzed in arriving at the estimate or score for the histomorphologic parameters. The two investigators’ scores were averaged for each horse and time period. The % surface mucosal disruption and % depth of mucosal loss were estimated as previously described. 1 The mucosa was separately scored from 0 to 4 for the presence of hemorrhage and edema. 1 A score of zero indicated there was no hemorrhage or edema observed and a score of 1 indicated slight hemorrhage or edema after close inspection of the tissue section. Scores of 2, 3, and 4 were indicative of mild (readily observable, but not extensive), moderate (extensive, but not distorting normal architecture), and marked (distorting normal architecture) hemorrhage or edema, respectively. 1 The basilar, middle, and surface one-thirds of the colonic mucosa were individually scored from 0 to 4 for the presence of cellular/nuclear debris and for neutrophils; the scale used to score these parameters was similar to the scale used for hemorrhage and edema. The cellular debris index and neutrophil index were calculated as the sum of the average scores for each of the three areas. 1,22

Quantitative Morphometry: Measurements of interstitial area:crypt area (I:C) ratio were made using a microscope with a calibrated cursor coupled to a microcomputer-based image analysis system. 1 A 1.0 mm² grid was situated in the base of the mucosa adjacent to the muscularis mucosa and was used to calculate the total mucosal area in that region. The area within this square that was occupied by crypts (crypt area)
was measured and summed. The difference between the total area of the box and the area occupied by crypts was the interstitial area. The I:C ratio was calculated as the ratio of the interstitial area to the crypt area.\textsuperscript{1} This parameter provides a more quantitative measure of mucosal hemorrhage and edema than histomorphologic scoring. These measurements were made in 10 areas for each horse and time period in areas where the crypts were cut in cross-section. Histomorphometric evaluation of neutrophils included quantitation of the number of neutrophils in colonic submucosal venules.\textsuperscript{22} Ten submucosal venules were identified and the cross sectional areas were measured using a microscope equipped with a calibrated cursor coupled to a microcomputer-based image analysis system; the numbers of neutrophils contained within each venule were counted and recorded. The number of neutrophils per cross-sectional area was used as an index of the accumulation or adhesion of neutrophils in post-capillary venules.\textsuperscript{22}

**Lung WW:DW Ratio and Myeloperoxidase Activity** - A lung sample was harvested from 6 horses at baseline and from all horses at 6 hrs using a linear automatic stapling device\textsuperscript{e} via a transdiaphragmatic approach through the celiotomy incision. The lung sample was divided into three subsamples. One sample was used to measure the WW:DW ratio as an indicator of lung tissue edema. The lung was weighed before and 48 hrs after drying in a 80\textdegree C oven. The second portion was placed in aluminum foil and immediately immersed in liquid nitrogen. It was stored at -70\textdegree C until assayed used for myeloperoxidase (MPO) activity, which was used an indicator of pulmonary neutrophil accumulation. The MPO assay was performed using a technique similar to the method used to quantitate intestinal MPO activity.\textsuperscript{22,23} The third portion was placed in 10% formalin, processed, and stained with hematoxylin and eosin for light microscopic quantitation of lung neutrophils. The average number of pulmonary neutrophils per high power (hpf) field was determined by counting neutrophils in 10 fields per sample using a
40x ocular objective.

**Statistical Analyses** - All data were expressed as mean ± standard error of the mean (SEM). Hemodynamic, metabolic, histomorphologic and histomorphometric data were analyzed using a two-way analysis of variance (ANOVA) for repeated measures. Among and within group post-hoc comparisons were made using a Student Newman Keuls test. Two-way ANOVA was used to compare the intervals from 0 to 3 hrs and 3 to 6 hrs among groups for all histomorphologic and histomorphometric parameters. A paired t test was used to compare lung MPO and neutrophil number at baseline to 6 hrs. The level of significance was set at P<0.05 for all tests.

**Results**

There were no significant differences among or within groups for SAP or DAP. There were no significant differences across time for HR, MAP, or CO in groups 1-4, but these parameters increased during the infusion of MnCl₂ in group 5 (Figure 9.2). There was also a tendency for HR to increase in groups 1-4 from 4.5 to 6 hrs. Heart rate and CO were increased in group 5 at 2.5 hrs and remained increased through 6 hrs. Mean arterial pressure was increased in group 5 at 2.5 hrs, but returned to values not different from baseline within 30 min (2.75 hrs) of completion of MnCl₂ infusion. There was a significant increase in mPAP and mRAP at 2 and 2.5 hrs in all groups, but no other changes across time nor differences among groups were observed (Figure 9.3). Mean PAP remained increased through 6 hrs in all groups, but mRAP had returned to values not different from baseline by 3 hrs.

Colonic blood flow was significantly decreased during the 3 hrs of ischemia, increased to a level greater than baseline within 5 min of restoring blood flow, and remained at or above baseline flow during the 3 hr period of reperfusion in all groups (Figure 9.4). There were no significant differences among groups for mCAP. Mean
Figure 9.2

Mean ± SEM for A) heart rate B) mean arterial pressure, and C) cardiac output during 3 hrs low flow ischemia and 3 hrs reperfusion of the equine large colon. Arrow at 2.25 hrs indicates time of intravenous drug infusion. The letter “b” indicates significantly different from “a”. The * indicates there was a difference between the MnCl₂ and 0.9% NaCl groups.
Figure 9.2
Figure 9.3

Mean ± SEM for A) mean right atrial pressure and B) mean pulmonary artery pressure during 3 hrs low flow ischemia and 3 hrs reperfusion of the equine large colon. Arrow at 2.25 hrs indicates time of intravenous drug infusion. The letter “b” indicates significantly different from “a”.
Figure 9.4

Mean ± SEM for A) colonic blood flow ($Q_{\text{col}}$), B) mean colonic arterial pressure, and C) colonic arterial resistance during 3 hrs low flow ischemia and 3 hrs reperfusion of the equine large colon. Arrow at 2.25 hrs indicates time of intravenous drug infusion. The letter “b” indicates significantly different from “a”.

CAP was significantly decreased at 30 min of ischemia and remained below baseline until 5.5 hrs, however, by 3.25 hrs (15 min of reperfusion) it was significantly higher than at 3 hrs of ischemia (Figure 9.4). Colonic arterial resistance decreased during ischemia and remained decreased throughout reperfusion in all groups; resistance was decreased at 3.25 hrs compared to 3 hrs, but had returned to a value not different from the 3 hr value at 4.5 hrs (Figure 9.4). There were no differences among groups for colonic arterial resistance. There were no significant differences among or within groups across time for mCVP.

Colonic venous PO\textsubscript{2} and C\textsubscript{vO\textsubscript{2}}, and DO\textsubscript{2colon} were decreased in all groups during ischemia and either returned to a level not different from baseline or increased above baseline during reperfusion (Figure 9.5). The colonic oxygen extraction ratio was not different among groups at baseline and was increased from 0.5 to 3.0 hrs in all groups (Figure 9.6). Colonic oxygen extraction was significantly decreased from the baseline value in all groups at 15 min of reperfusion (3.25 hrs), remained decreased through 6 hrs in groups 1, 4, and 5; however, it returned to a value not different from baseline by 30 and 60 min in groups 2 and 3 respectively. Colonic venous pH decreased and colonic venous lactate and PCO\textsubscript{2} increased during ischemia; all of these parameters returned to values not different from baseline during reperfusion (Figure 9.7). Arterial, mixed venous, and colonic venous total plasma protein and packed cell volume were significantly decreased in all groups by 2.5 hrs and remained decreased over the course of the experiment, but there were no differences among groups. Neither lactate concentrations or any of the other blood gas or oximetry parameters measured in arterial or mixed venous blood were significantly different among or within groups across time.

There were no differences at baseline among groups for colonic venous 6-kPG, PGE\textsubscript{2}, or TXB\textsubscript{2} (Figure 9.8). Colonic venous 6-kPG increased during ischemia and was greater in group 5 at 3 hrs compared to group 1. Colonic venous 6-kPG returned to values
Figure 9.5

Mean ± SEM for A) colonic venous PO$_2$, B) colonic venous oxygen content, and C) colonic oxygen delivery (DO$_{2\text{Colon}}$) during 3 hrs low flow ischemia and 3 hrs reperfusion of the equine large colon. Arrow at 2.25 hrs indicates time of intravenous drug infusion. The letter “b” indicates significantly different from “a”.
Figure 9.5

A. Colonic Venous $O_2$ Content (ml/dl)

B. Colonic Venous $PO_2$ (mm Hg)

C. $DO_2_{colon}$ (ml/min)

Legend:
- $\Delta$ NaCl
- ■ DMSO
- ● Allopurinol
- ○ Aminosteroid
- □ MnCl$_2$

Time (hrs)

Figure 9.5
Mean ± SEM A) for colonic oxygen extraction ratio during 3 hrs low flow ischemia and 3 hrs reperfusion of the equine large colon, and B) depicts a different scale of the oxygen extraction ratio during the 3 to 6 hr interval, which serves to better illustrate the patterns followed by each group of horses during this period. Arrow at 2.25 hrs indicates time of intravenous drug infusion. The letter “b” indicates significantly different from “a”. The oxygen extraction ratio patterns followed by each group of horses are represented by lines extending across top of graph: (-------) for NaCl, Aminosteroid, and MnCl₂; (--- ---) for DMSO; and (- - - -) for Allopurinol.
Figure 9.7

Mean ± SEM for colonic venous A) pH, B) lactate, and C) PCO₂ during 3 hrs low flow ischemia and 3 hrs reperfusion of the equine large colon. Arrow at 2.25 hrs indicates time of intravenous drug infusion. The letter “b” indicates significantly different from “a”.
Figure 9.8

Mean ± SEM for colonic venous A) 6-ketoprostaglandin F$_1$-alpha, B) prostaglandin E$_2$, and C) thromboxane B$_2$ during 3 hrs low flow ischemia and 3 hrs reperfusion of the equine large colon. Arrow at 2.25 hrs indicates time of intravenous drug infusion. The letter “b” indicates significantly different from “a”. The * indicates there was a difference between the MnCl$_2$ and 0.9% NaCl groups.
Colonic Venous

Thromboxane B2 (pg/ml) Prostaglandin E2 (pg/ml) Prostaglandin F2α (pg/ml)

Figure 9.8

Time (hrs)

Colonic Venous

Colonic Venous 6-Keto Prostaglandin (pg/ml)
not different from baseline in groups 1-4 by 3.25 hrs, but remained increased above baseline in group 5 through 6 hrs. Prostaglandin E₂ was not different among groups at baseline and was unchanged through 3 hrs of ischemia in groups 1-4. Prostaglandin E₂ was increased above baseline by 3 hrs in group 5 and during reperfusion in groups 1, 2 and 5. Prostaglandin E₂ was greater in group 5 compared to group 1 from 3 to 6 hrs. There was a trend (P=0.06) for a difference in colonic venous TXB₂ between group 5 and groups 1-4 beginning at baseline. Thromboxane B₂ was increased above baseline between 3.25 and 4 hrs in all groups, but there was no difference among groups. There was no difference in these eicosanoid concentrations among groups. Endotoxin was not detected in colonic or systemic venous blood in any group at any time.

There was significant surface mucosal loss and estimated depth mucosal loss across time in all groups, but there was no difference among groups for % surface mucosal loss (Figures 9.9). There was a trend (P=0.06) toward greater estimated % depth mucosal loss at 6 hrs in horses treated with DMSO compared to those administered 0.9% NaCl. There were significant differences across time in all groups for mucosal hemorrhage, edema, and I:C ratio (Figure 9.10). There was significant mucosal hemorrhage at 15 min of reperfusion in all groups, but no difference among groups. There was significant mucosal edema present in all groups at 3 hrs of ischemia, but no difference between vehicle control and drug-treated groups. Mucosal I:C.area was increased by 3 hrs of ischemia in all groups, but there was no difference among groups. Mucosal cellular debris index, mucosal neutrophil index, and submucosal venular neutrophils were significantly increased within all groups, but there were no differences among groups (Figure 9.11). Mucosal neutrophil index increased during ischemia and increased further upon reperfusion in all groups; there was no difference between vehicle control and drug-treated horses. Submucosal venular neutrophils increased by 3 hrs of ischemia and increased further at 15 min of reperfusion in
Figure 9.9

Mean ± SEM for large colon A) percent surface mucosal disruption and B) percent estimated depth mucosal loss during 3 hrs low flow ischemia and 3 hrs reperfusion of the equine large colon. Arrow at 2.25 hrs indicates time of intravenous drug infusion. The letter “b” indicates significantly different from “a”. The † indicates a trend toward greater % depth mucosal loss in horses treated with dimethyl sulfoxide compared to 0.9% NaCl.
Figure 9.9
Figure 9.10

Mean ± SEM for large colon A) mucosal hemorrhage, B) mucosal edema, and C) mucosal interstitial: crypt ratio during 3 hrs low flow ischemia and 3 hrs reperfusion of the equine large colon. Arrow at 2.25 hrs indicates time of intravenous drug infusion. The letter “b” indicates significantly different from “a”.

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Figure 9.10

- A: Mucosal Hemorrhage
- B: Mucosal Edema
- C: Mucosal I:C Ratio

Graphs showing the effects of NaCl, DMSO, Allopurinol, Aminosteroid, and MnCl₂ on mucosal hemorrhage, edema, and I:C ratio over time.
Mean ± SEM for large colon A) mucosal cell debris index, B) mucosal neutrophil index, and C) submucosal venular neutrophils during 3 hrs low flow ischemia and 3 hrs reperfusion of the equine large colon. Arrow at 2.25 hrs indicates time of intravenous drug infusion. The letter "b" indicates significantly different from "a".
Submucosal Venular Neutrophils (neutrophils/mm² area)

Mucosal Neutrophil Index

Mucosal Cellular Debris Index

Time (hrs)

Figure 9.11

Submucosal Venular Neutrophils (neutrophils/mm² area)

Mucosal Neutrophil Index

Mucosal Cellular Debris Index

NaCl

Allopurinol

MnCl₂

Aminosteroid

DMSO

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all groups; there were no differences among groups.

Colonic mucosal and full-thickness WW:DW increased by 3 hrs of ischemia (Figure 9.12) Colonic mucosal WW:DW increased further at 1 hr of reperfusion, and remained increased above baseline throughout the experiment in all groups. Mucosal WW:DW was different for groups 1 and 5 compared to groups 2-4; this difference was apparent beginning at baseline. There was no difference among groups for colonic full-thickness WW:DW.

Data for lung WW:DW, MPO activity, and neutrophils/hpf are presented in Table 9.1. Lung WW:DW did not change across time in any group, but was significantly lower in group 5 compared to group 1 after colonic I-R. There was no difference in lung MPO activity between groups 2-5 and the vehicle control group, but there was a trend (P=0.09) toward an increase in lung MPO after colonic I-R in all groups. There was no difference in the number of lung neutrophils/hpf across time or among groups.

Discussion

This is the first study to be reported that evaluated the effects of drugs directed against OFR-mediated injury of the equine large colon subjected to low flow arterial ischemia and reperfusion. Unfortunately, there were no significant effects produced by these drugs on I-R injury of the large colon mucosa. On the other hand, with the exception of DMSO, these drugs did not appear to worsen systemic hemodynamics, metabolic parameters or colonic mucosal injury.

There are numerous potential reasons for why we were unable to demonstrate drug-induced attenuation of colonic mucosal injury in this study. First of all, the drugs were administered after 2.5 hrs of low flow arterial ischemia, which may have been too late in the pathophysiologic process to exert a beneficial effect. Drug-induced attenuation of intestinal mucosal I-R injury has typically been demonstrated when administered prior to
Figure 9.12

Mean ± SEM for large colon A) mucosal and B) full-thickness wet weight:dry weight ratio during 3 hrs low flow ischemia and 3 hrs reperfusion of the equine large colon. Arrow at 2.25 hrs indicates time of intravenous drug infusion. The letter “b” indicates significantly different from “a”.
Full Thickness Colonic Wet Weight: Dry Weight Ratio

Mucosal Wet Weight: Dry Weight Ratio
Table 9.1

Lung wet weight: dry weight (WW:DW) ratio, myeloperoxidase (MPO) activity, and neutrophils/ high power field in horses subjected to low flow ischemia and reperfusion of the large colon. A) Comparisons among horses treated with drugs directed against oxygen free radical-mediated tissue damage and horses treated with a saline control solution. B) Temporal comparisons made before and after low flow ischemia & reperfusion.
### Table 9.1

#### A

<table>
<thead>
<tr>
<th>Group</th>
<th>WW:DW</th>
<th>MPO (activity/gram DW)</th>
<th>Neutrophils/ hpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>5.66 ± 0.14</td>
<td>162.56 ± 53.40</td>
<td>38.33 ± 1.45</td>
</tr>
<tr>
<td>DMSO</td>
<td>5.27 ± 0.11</td>
<td>92.52 ± 13.98</td>
<td>38.17 ± 2.89</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>5.49 ± 0.09</td>
<td>68.35 ± 17.35</td>
<td>36.87 ± 0.72</td>
</tr>
<tr>
<td>21-Aminosteroid U-74389G</td>
<td>5.13 ± 0.13</td>
<td>67.70 ± 9.24</td>
<td>34.25 ± 1.69</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>4.97 ± 0.29*</td>
<td>182.40 ± 44.12</td>
<td>38.20 ± 1.98</td>
</tr>
</tbody>
</table>

*significantly (P<0.05) different from NaCl group

#### B

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>WW:DW</th>
<th>MPO (activity/gram DW)</th>
<th>Neutrophils/ hpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.22 ± 0.11</td>
<td>61.78 ± 18.19</td>
<td>36.67 ± 1.99</td>
</tr>
<tr>
<td>6</td>
<td>5.48 ± 0.24</td>
<td>179.03 ± 55.16†</td>
<td>41.25 ± 2.66</td>
</tr>
</tbody>
</table>

†trend (P=0.09) toward difference between 0 and 6 hrs
the induction of ischemia. Pre-treatment with these drugs is probably a more effective method of attenuating intestinal I-R injury, however, this does not represent the clinical course of therapy. We chose to administer the drugs IV 30 min prior to reperfusion because this should allow adequate time for the drug to reach the colonic tissue before OFR generation upon reperfusion. Additionally, it is a time that more closely mimics drug administration to horses with a naturally occurring large colon volvulus prior to surgical correction. It is certainly possible that even if the horses had been pre-treated prior to colonic ischemia that the drugs used in this study may not have had a protective effect on the colonic mucosa.

A second potential reason for failure of these drugs to exert a demonstrable beneficial effect on the colonic mucosa is the drug dosage chosen. Since pharmacokinetic data are not well established for these drugs, the dosages chosen were based upon clinical recommendations reported for horses, and extrapolated from dosages used in studies involving experimental animals other than horses. A dose of 1 gram/kg of DMSO was chosen because this is the most commonly used clinical dosage. Other doses of DMSO have been used in horses and it may have been appropriate to evaluate other dosage regimens. The 1 gram/kg dosage of DMSO was also ineffective in preventing colonic mucosal injury associated with complete arteriovenous ischemia. A lower dosage of DMSO (20 mg/kg IV) has been demonstrated to produce a protective effect on the mucosa following intestinal I-R in cats. A dose of 25 mg/kg allopurinol was extrapolated from studies evaluating intestinal I-R in laboratory animals and endotoxemia in horses. Several studies in laboratory animals have used a dose of 50 mg/kg orally for 2 days prior to the intestinal ischemia or 50 mg/kg IV 30 min prior to intestinal arterial occlusion. The effects of both 5 mg/kg and 50 mg/kg doses of allopurinol on experimental endotoxin shock in horses has been reported. The 5 mg/kg dose provided
superior protection against the effects of endotoxin. A dose of 25 mg/kg was chosen for use in this study because it was approximately in the middle of these two dosage regimens that were beneficial in attenuating intestinal I-R injury and endotoxin shock. A dose of 10 mg/kg of 21-aminosteroid U-74389G was chosen based upon the manufacturer's recommendation. Similar dosage regimens for 21-aminosteroid U-74006F has demonstrated protection against hemorrhagic shock, splanchnic artery occlusion shock, and intestinal I-R. The 10 mg/kg dose of MnCl₂ was chosen because 5 grams (10 mg/kg) of MnCl₂ administered to 450-500 kg horses produced greater superoxide scavenging activity in plasma than did 2.5 grams (5 mg/kg). It should be noted that the hemodilution occurring secondary to IV fluid administration could have resulted in plasma drug concentrations too low to reach an effective drug concentration in the colonic tissue.

Failure of colonic mucosal perfusion upon restoration of blood flow could have prevented the drugs from reaching the mucosa in therapeutic concentrations. We did not quantitate plasma or mucosal drug concentrations and therefore we do not know if adequate drug levels were achieved in the mucosa. Colonic blood flow rebounded to levels significantly higher than baseline during reperfusion, which suggests that mucosal blood flow likely increased as well. Thrombi have been documented in the submucosal microvasculature in naturally occurring large colon volvulus and in experimental large colon I-R in horses. These thrombi could obstruct microvascular blood flow and thus shunt blood away from the mucosa; this could limit mucosal perfusion upon surgical correction of the volvulus or restoration of blood flow after experimental colonic ischemia. Based upon histologic evaluation, microvascular thrombi were not apparent in the colonic mucosa or submucosa during or subsequent to low flow ischemia and, therefore, should not have prevented mucosal perfusion. We have also demonstrated that colonic mucosal perfusion returns following restoration of blood flow after low flow arterial ischemia.
because we have demonstrated reactive hyperemia in the mucosa using colored microspheres.  

Another possibility for our inability to demonstrate a drug related beneficial effect is that the pathophysiologic mechanisms of colonic injury during low flow ischemia and reperfusion are not completely interrupted by the drugs chosen for use in this study. The drugs were selected based upon their potential to interfere with the production or metabolism of OFRs upon reperfusion. Classically, intestinal reperfusion injury is believed to be principally initiated by the accumulation of hypoxanthine and the conversion of XDH to XO during ischemia. Upon restoration of blood flow, the accumulated hypoxanthine is converted to superoxide anions by XO in the presence of oxygen. The superoxide anion is further metabolized via the iron-dependent Haber-Weis reaction to hydroxy radicals, which are potent mediators of cell membrane lipoperoxidation. Lipoperoxidative damage to cell membranes disrupts cellular integrity and function resulting ultimately in cell death. Each of the drugs evaluated in this study has the potential to interfere with this OFR-mediated pathway of tissue injury. Dimethyl sulfoxide scavenges hydroxyl radicals, allopurinol inhibits XO and thus the production of superoxide radicals, 21-aminosteroids scavenge lipid hydroperoxyl radicals generated when OFRs react with membrane lipids, and MnCl scavenges superoxide radicals. The small intestinal mucosa has a rich supply of XO, which makes it vulnerable to the effects of I-R. The large intestine, on the other hand, is not a rich source of XO and some believe is therefore not as susceptible to I-R. Xanthine oxidase activity is low in the large colon of ponies, but measurement of this enzyme has not been reported in the large colon of horses. We can assume, however, that colonic mucosal XO activity would be similar between horses and ponies. Therefore, OFR production by the XO route may not be important in equine large colon I-R injury. Aldehyde oxidase, an enzyme with similar
function to XO, has been demonstrated to be present in high concentrations in the colon of rabbits.\textsuperscript{38} It is possible that aldehyde oxidase is also present in the large colon mucosa of horses and could initiate the generation of OFRs. Neutrophils generate OFRs via their NADPH oxidase system and are a known source of OFRs during colonic ischemia in rabbits.\textsuperscript{39} We have also demonstrated neutrophil accumulation in the large colon of horses during I-R.\textsuperscript{22}

It is also possible that numerous pathophysiologic pathways interact to produce the colonic mucosal injury following low flow ischemia and reperfusion. Each horse in this study received only one drug. It is possible that even if the drug had interrupted a particular pathway an alternate pathway could have contributed to colonic injury. Another question that should be considered is could administration of the drugs have had a negative or deleterious effect on the colonic mucosa? We did demonstrate a tendency toward greater estimated \% depth mucosal loss in horses administered DMSO versus the vehicle control solution. The reaction of DMSO with hydroxyl radicals can lead to the generation of methyl radicals and methylperoxy radicals; these radicals are less potent than the hydroxyl radical, but do react with membrane lipids.\textsuperscript{40} High concentrations of DMSO can cause lipid peroxidation regardless of its hydroxyl radical scavenging effects.\textsuperscript{40} Because of the absence of a demonstrable benefit and the tendency to worsen mucosal injury, we do not recommend administering DMSO at a dose of 1 gram/kg to horses with gastrointestinal ischemia. To our knowledge, there are no reported studies of deleterious effects on tissues subjected to I-R of the other drugs used in this study, however, since no beneficial effects were demonstrated we do not recommend their use clinically.

The possibility exists that we did not demonstrate an effect of any of the drugs we investigated because of the power of our statistical tests to detect a difference.\textsuperscript{41} The power to detect a difference is affected by the variability in the measurements being made,
the sample size, the magnitude of the change that one wishes to detect, and the desired level of significance. We had a relatively small number (n=6) of horses in each group, which when combined with the variability in histopathologic parameters among horses may have caused our power to be too low to detect an effect of the drugs even if it were present. The P-values for some group effects were between 0.05 and 0.15 suggesting a possible trend toward a difference among groups. It is possible that by increasing our sample size within each group that we may have detected significant differences among groups.

There were no apparent deleterious effects of this model or the drugs on systemic hemodynamics. This is obviously an important consideration when evaluating a drug that may be used in clinical patients. Stable systemic hemodynamics is a characteristic of this low flow model. The increases in mRAP and mPAP across time in all groups in this study is different from the earlier report. Although we maintained MAP > 70 mm of Hg in horses of both studies we did not observe increases in mRAP or mPAP in the previous study. This may be associated with the administration of maintenance IV fluids combined with the 5 liters administered during drug infusion causing an increased intravascular volume. In order to minimize the hyperosmotic effects of 1 g/kg DMSO it was diluted in 5 liters 0.9% NaCl for administration. The remainder of the drugs were administered in 5 liters 0.9% NaCl to ensure similar experimental conditions among all groups.

Intravenous administration of MnCl\textsubscript{2} resulted in significant increases in HR, MAP, and CO. The MAP returned to baseline values within 30 min after the infusion was completed, but HR and CO remained increased above baseline. The hemodynamic effects of IV MnCl\textsubscript{2} infusion are suggestive of increased sympathetic discharge causing a B-adrenergic increase in sinus node discharge and increases in myocardial contractility. The heart contracted with such force during MnCl\textsubscript{2} infusion that the horse's entire torso moved forcefully in unison with each heart beat. This effect gradually diminished after
infusion was completed. Similar hemodynamic effects of MnCl₂ have been reported in standing conscious horses administered either 5 or 10 mg/kg IV over a 1 hr period.¹⁶ Although the hemodynamic effects of MnCl₂ did not appear to cause deleterious effects, it is possible that the increased HR and contractility could worsen hemodynamic status in horses with already compromised cardiovascular function. During circulatory shock intravascular volume may decrease secondary to plasma, water or blood losses and myocardial function may become compromised due to the generation and release of depressant substances.⁴³ Increases in HR and contractility increase myocardial oxygen consumption.⁴⁴,⁴⁵ Therefore, the chronotropic and inotropic effects of MnCl₂ has the potential to cause deterioration of myocardial function and contribute to hemodynamic collapse in horses in circulatory shock.

Colonic venous blood gases, oximetry values, and lactate concentrations followed similar patterns to a previous report of low flow colonic arterial ischemia and reperfusion.¹⁷ These alterations reflect anaerobic metabolic processes occurring during ischemia and a return to aerobic metabolism upon reperfusion. Colonic blood flow and DO₂ values were decreased to similar degrees during the ischemic period in all groups and these parameters were increased upon restoration of blood flow due to a reactive hyperemic response. The fact that the magnitude and temporal pattern of the colonic hemodynamic and metabolic alterations were similar among all groups ensured similar degrees of colonic ischemia. The decrease in colonic arterial resistance during ischemia and reperfusion may be indicative of vasodilation in the colonic vasculature subsequent to increased production of eicosanoids or other vasodilator substances.

The trend toward increased lung MPO activity after colonic I-R is supported by other studies that have documented pulmonary neutrophil accumulation with intestinal I-R in rats.⁴⁶,⁴⁷ A number of substances generated at the site of tissue injury gain access to
the circulation and are distributed to other regional vascular beds, including the pulmonary vasculature. Some of these factors including cytokines, arachidonic acid metabolites, and platelet activating factor can cause chemotaxis, adherence and diapedesis at distant target sites such as the endothelium of the pulmonary vascular bed. We did not detect an increase in lung \text{WW:DW} ratio secondary to colonic I-R. Increased pulmonary microvascular permeability has been documented following intestinal I-R in rats. The model of intestinal I-R in rats is associated with increased portal venous endotoxin levels. Endotoxin is known to exert an effect on endothelial cells resulting in increased permeability and neutrophil adherence. These effects are likely mediated by cytokines, such as tumor necrosis factor and interleukin-1, produced by monocytes and macrophages exposed to endotoxin. Endotoxin and TNF were not detected in colonic or systemic venous blood using the low flow model of colonic I-R used in this study.

The absence of detectable pulmonary neutrophil accumulation or lung edema subsequent to colonic I-R may be due to a true absence of such phenomena with this model. Endotoxin and TNF have not been detected in colonic or systemic venous blood in our model of low flow I-R. Absence of these substances could account for the lack of effects of colonic I-R on the lung. Alternatively, the absence of detectable alterations in the lung could be associated with the methodology used to quantitate the pulmonary parameters evaluated in this study. Neutrophils were quantitated in 10 high power fields, which represents an extremely small area of the entire lung. Likewise, MPO and \text{WW:DW} ratios were determined on small samples of the lung. It is possible these small samples were not representative of the entire lung. There are regional differences in pulmonary blood flow that become accentuated with the horse anesthetized and placed in dorsal recumbency; this could affect the number of neutrophils that are delivered to and accumulate in the lung parenchyma. It is possible that if the entire lung could have been extirpated and these
parameters analyzed on the entire lung parenchyma, as is done in laboratory animals\textsuperscript{46}, a
difference in lung edema or pulmonary neutrophils may then have been detected.

In summary, we were unable to demonstrate a beneficial effect of DMSO,
allopurinol, 21-aminosteroid U-74389G, or MnCl\textsubscript{2} in attenuating mucosal injury associated
with low flow ischemia and reperfusion of the equine large colon. Other than the tendency
for greater estimated % depth mucosal loss in horses treated with DMSO versus the vehicle
control solution, there were no deleterious effects of any of the drugs on systemic
hemodynamics, metabolic status, or colonic mucosal injury. Manganese chloride infusion
resulted in increased HR, MAP, and CO suggestive of increased sympathetic discharge.
Although these hemodynamic effects of MnCl\textsubscript{2} did not appear deleterious in the horses of
this study, it is possible that increasing HR and myocardial contractility could be
detrimental to horses in circulatory shock. Studies using larger sample sizes or different
drugs may be necessary to further evaluate the effects of these or similar drugs on large
colon I-R injury. Because numerous pathophysiologic pathways may be involved in
colonic I-R injury multimodal therapy may be required to attenuate colonic mucosal I-R
injury.

Footnotes

\textsuperscript{a}Rompun\textsuperscript{R}, Mobay Corporation, Animal health Division, Shawnee, KS.
\textsuperscript{b}Angiocath\textsuperscript{R}, Becton Dickinson Vascular Access, Sandy, Utah.
\textsuperscript{c}Swan-Ganz\textsuperscript{R}, American Edwards Laboratories, Anasco, Puerto Rico.
\textsuperscript{d}Intramedic\textsuperscript{R}, Clay Adams, Division of Becton Dickinson and Company, Parsippany, NJ.
\textsuperscript{e}Guailaxin\textsuperscript{R}, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.
\textsuperscript{f}Bio-tal\textsuperscript{R}, Bio- Ceutic Division, Boehringer Ingelheim Animal Health, Inc., St. Joseph, Mo.
Sodium Pentobarbital Injection, The Butler Company, Columbus, Ohio.

Drager AV Model NELAC-E, Anesthesia Ventilator, Telford, Pa.

Lactated Ringer's, Baxter Healthcare Corporation, Deerfield, Ill.

Angiocath®, Becton Dickinson Vascular Access, Sandy, Utah.

Model T201, Transonic Systems, Inc., Ithaca, NY.

Quik-Cath®, Baxter Healthcare Corporation, Deerfield, Ill.

0.9% Sodium Chloride Injection USP, Baxter Healthcare Corporation, Deerfield, Ill.

Domosor®, Syntex Animal Health, Division of Syntex Agribusiness, Inc., West Des Moines, Iowa.

Allopurinol, Sigma Chemical Company, St. Louis, Mo.

U-74389G, The Upjohn Company, Kalamazoo, Mi.

Manganese Chloride, Sigma Chemical Company, St. Louis, Mo.

Beuthanasia®-D Special, Eli Lilly & Co., Indianapolis, In.

Model 7562-00 Masterflex®, Cole-Parmer Instrument Company, Chicago, Ill.

ABL 500-K pH and Blood Gas Analyzer, Radiometer-Copenhagen, Copenhagen, Denmark.

OSM™ 3 Hemoximeter, Radiometer-Copenhagen, Copenhagen, Denmark.

YSI Model 23L Lactate Analyser, Yellow Springs Instruments Co., Inc., Yellow Springs, Ohio.

10436 Veterinary Refractometer, Cambridge Instruments, Buffalo, NY.

QCL 1000, Biowhitaker, Walkersville, Md.


60 Proximate Linear Stapler, Ethicon, Inc., Cincinnati, Ohio.
List of References


15. Vatistas NJ, Snyder JR, Hildebrand SV, et al. Effects of the 21-aminosteroid U-


SUMMARY

Intestinal reperfusion injury has been thoroughly documented in laboratory animals following variable periods of low flow arterial ischemia. Previous studies in the equine large colon following complete arteriovenous occlusion have failed to convincingly demonstrate reperfusion injury. The studies reported here document the occurrence of reperfusion injury in the large colon of horses following low flow arterial ischemia. Furthermore, these studies characterize the histopathologic, hemodynamic, metabolic, inflammatory, and hemostatic alterations that occur subsequent to low flow ischemia and reperfusion.

We demonstrated histomorphologic and histomorphometric evidence of reperfusion injury after three hours of low flow arterial ischemia and 3 hours of reperfusion. Large colon ischemia and reperfusion caused more severe mucosal injury and greater mucosal neutrophil accumulation than 6 hours of ischemia alone. In other words, re-establishing colonic arterial blood flow after 3 hours of ischemia exacerbated colonic mucosal injury. During low flow ischemia anaerobic metabolism occurs due to the low oxygen availability resulting in abnormalities in colonic venous blood gases, pH, and oximetry values; these abnormalities are reversed within 5 minutes of reperfusion. Reactive hyperemia occurs during the first 5 minutes after restoring blood flow and is maintained for up to 3 hours. The histopathologic and colonic venous alterations occur in the presence of systemic hemodynamic and metabolic stability; this suggests mucosal reperfusion injury occurs subsequent to alterations in colonic blood flow and metabolism.
Low flow ischemia & reperfusion of the equine large colon is not associated with detectable increases in systemic or colonic venous endotoxin concentration or colonic venous tumor necrosis factor or interleukin-6 activity. Eicosanoids accumulate in the colonic venous blood subsequent to reduced oxygen availability and may contribute to the reactive hyperemia following re-establishment of blood flow by causing vasodilation. Increased concentrations of systemic venous 6-keto prostaglandin \( \text{F}_1\alpha \) may represent spillover from the colonic vasculature, but more likely reflects systemic production.

Temporal alterations in colonic and systemic venous hemostatic parameters occurring in horses subjected to ischemia, ischemia & reperfusion, and even sham-operated controls indicates that factors common to the surgical model likely activate coagulation. Colonic ischemia & reperfusion does not appear to exacerbate hemostatic dysfunction. The alterations in colonic venous blood parallel those in systemic venous blood, however, the absolute values for the hemostatic indices are not the same. This indicates that monitoring systemic venous hemostatic parameters may not provide an accurate assessment of hemostatic function in regional vascular beds.

Colonic blood flow is approximately equally distributed between the mucosal and seromuscular layers at baseline, during low flow ischemia, and during reactive hyperemia associated with reperfusion. Arteriovenous anastomoses are either not anatomically present or are nonfunctional in the large colon of horses during low flow ischemia and reperfusion. Colored microspheres are useful for measuring colonic mural blood flow.

Administration of dimethyl sulfoxide, allopurinol, 21-aminosteroid U-74389G, or manganese chloride IV 30 min prior to reperfusion does not protect the colonic mucosa from the effects of ischemia & reperfusion. On the other hand, other than a tendency for greater estimated percent depth mucosal loss in horses treated with dimethyl sulfoxide versus the vehicle control solution, there were no demonstrable detrimental effects on the
colon. These drugs did not cause any observable deleterious cardiopulmonary effects, however, manganese chloride administration caused an increased heart rate, mean arterial blood pressure and cardiac output, suggesting increased sympathetic discharge.

The low flow model described in these studies should be useful to further study and characterize the pathophysiologic processes occurring during large colon ischemia and reperfusion and to determine the effects of other drugs on these processes.
BIBLIOGRAPHY


483.


69. Filep J, Dahinden C. Increased levels of platelet activating factor in blood following intestinal ischemia in the dog. *Biochem Biophys Res Commun* 1989.


298. Williamson LH. Studies on antithrombin III and protein C activity in the plasma and peritoneal fluid of horses with abdominal pain. University of Georgia, 1990:.


