DETECTION OF APOPTOTIC CELLS IN INTESTINE FROM HORSES WITH AND WITHOUT GASTROINTESTINAL DISEASE

by

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In

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(ABSTRACT)

A study was performed to identify apoptotic cells in the equine intestine and to determine if the occurrence of apoptosis is affected by gastrointestinal disease and tissue layer of intestine. Samples of intestine were collected from 38 horses that underwent surgery or were humanely destroyed for small or large bowel obstruction, strangulation or distension. Samples were also taken from 9 horses which were humanely euthanized for reasons other than gastrointestinal disease or systemic disease. Specimens were collected at surgery from intestine involved in the primary lesion, distant to the primary lesion, or at necropsy from several sites including the primary lesion. Tissues were fixed, serially sectioned and stained with hematoxylin and eosin (H&E) and for apoptosis by the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) technique. The number of apoptotic cells per high power field were counted in the mucosa, circular muscle, longitudinal muscle and serosa for each sample of intestine. Apoptotic staining nuclei were seen in all layers of intestine. An increased number of apoptotic cells were found in the circular muscle of the intestine from horses with simple obstruction.
Intestine distant from the primary strangulating lesion had higher numbers of apoptotic cells than intestine distant from a simple obstruction lesion or intestine taken at the site of a strangulating or simple obstructive lesion. Intestine from horses with obstructing or strangulating lesions in the small intestine and large colon has increased numbers of apoptotic cells. Further investigation is required to determine whether increased apoptosis affects intestinal function.
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Christopher and Anne Rowe

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LIST OF ABBREVIATIONS

ATP = adenosine triphosphate

O2-. = superoxide free radicals

OH-. = hydroxyl radical

NADPH = nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system

TNF-a = tumor necrosis factor alpha

IL-1ß = interleukin-1-beta

IL-6 = interleukin-6

Ca++ = calcium ion

FADD = fas associated death domain

TNFR1 = tumor necrosis factor receptor

TRADD = TNFR1 associated death domain

RIP = receptor interacting protein

Apaf-1 = apoptosis protease activating factor

ADP = adenosine diphosphate

DNA = deoxyribonucleic acid

PT = mitochondrial permeability transition

NO = nitric oxide

H2O2 = hydrogen peroxide

iNOS = inducible NO synthase

TUNEL = terminal deoxynucleotidyl transferase mediated dUTP nick end labeling

3-OH = 3-hydroxy

PI = propidium iodide
LPS = lipopolysaccharide

IGF-1 = insulin like growth factor

EMAP-11 = endothelial monocyte-activating polypeptide II

Hg2+ = mercury ion

NSAID = non-steroidal anti-inflammatory drugs

PG = prostaglandin

PGE2 = prostaglandin E2

ISNT = in situ nick translation

H & E = hematoxylin and eosin

PBS = phosphate buffered saline

TdT = terminal deoxynucleotidyl transferase

DAB = diaminobenzidine
INTRODUCTION

Gastrointestinal disease is the cause of high morbidity and mortality in the horse. Experimental evidence suggests that one reason for morbidity and mortality in horses with obstructive diseases of the gastrointestinal tract is reperfusion injury.\textsuperscript{1,2} In the horse experimental ischemia has been shown to cause mucosal and serosal damage, which is exacerbated during reperfusion\textsuperscript{2,3}. These studies have been performed with and without treatments directed at interrupting the inflammatory pathways associated with reperfusion injury and have yielded conflicting results.\textsuperscript{4,5} Intestinal ischemia-reperfusion injury has been largely attributed to cellular necrosis. Apoptosis, a distinct form of cell death, has recently been recognized as having an important role in both ischemia and reperfusion injury.\textsuperscript{6,7} Apoptosis is a natural physiologic mechanism to allow death of unwanted cells with minimal tissue inflammation. Apoptosis has an important role in embryogenesis, tissue homeostasis, lymphocyte development and function and tumor regression.\textsuperscript{8}

In the gastrointestinal tract apoptosis can be associated with the maintenance of homeostasis and shedding of cells from the villous tips in the small intestine, or with pathological states such as the development of intestinal adenocarcinomas, the pathogenesis of inflammatory bowel disease and radiation induced gut damage.\textsuperscript{9} The apoptotic response appears to be activated by membrane changes in the mitochondria with subsequent transcription of specific genes which affect individual cells of a specific cell type\textsuperscript{10}. Apoptosis has been observed following experimental ischemia-reperfusion injury to the brain, heart, adrenals, liver, and kidneys of other species.\textsuperscript{7} Apoptosis has found to be important an important source of inflammation in the kidney\textsuperscript{11,12} and muscle injury\textsuperscript{6}. In human medicine non-steroidal anti-
inflammatory drugs have been found to induce apoptosis in the colon with sufficient magnitude to disrupt the epithelial barrier causing inflammation. 13

Apoptosis is thus part of the homeostatic processes of the body but if it is present in excess in certain cell types or if it is delayed in inflammatory cells, this form of cell death can create inflammation. This inflammation has been documented with increased apoptosis in ischemia reperfusion injury of the muscle and kidney and therefore may be important in ischemia-reperfusion injury in the equine intestine. To date experimental and clinical investigations into therapeutic intervention in reperfusion injury have been at time conflicting and unrewarding. Apoptosis can be controlled and thus may have important therapeutic interventions in certain disease states including ischemia reperfusion. To our knowledge apoptosis has not been investigated following ischemia-reperfusion of the equine intestine.

Apoptosis can be examined using the TUNEL ( terminal deoxynucleotide transferase mediated dUTP nick end labeling ) technique. This technique distinguishes apoptotic from necrotic cells by specifically detecting DNA cleavage associated with apoptosis. This technique has been shown to be effective in the detection of apoptosis in the formalin fixed samples of human large intestine. 14

The objectives of this study were to 1) identify apoptosis in the equine intestine using the TUNEL method, 2) to identify the type(s) of cells undergoing apoptosis, 3) determine whether the occurrence of apoptotic cells was affected by gastrointestinal disease and tissue layer of the intestine. We hypothesized that equine intestine directly or indirectly affected by naturally occurring simple or strangulation obstruction would have increased numbers of apoptotic cells.
LITERATURE REVIEW

GASTROINTESTINAL DISEASE IN THE EQUINE

IMPORTANCE

Acute abdominal pain (colic) in the horses is one of the most frequent problems encountered by veterinarians in large animal practice\(^1\) and these diseases causing colic have a great economic impact due to the high case fatality rate\(^2\). The annual national incidence of colic has been estimated to be 4.2 colic events/100 horses per year with a case fatality rate 11%. The annual cost of colic in the United States has been estimated to be $115,300,000\(^2\).

Strangulating obstruction of the small intestine and large colon is a frequent cause of this acute abdominal disease requiring surgery.\(^3\)-\(^5\) In a study with 2,385 horses referred to 14 veterinary teaching hospitals with signs of abdominal pain, 18.4% had strangulating intestinal obstruction, with overall mortality of 79.9%\(^6\). Recent reports have documented improved survival with surgery for strangulation of the large and small intestine. However, intestinal strangulation still has the highest case fatality of diseases that cause colic, and it is associated with the highest rate of post-operative complications. In a recent study the long-term survival rates of horses recovered from anesthesia after surgery for a small intestinal lesion at 7 months and greater than 12 months following surgery, were 75% and 68% respectively.\(^7\) Original reports suggested survival rates for horses with a strangulating obstructing lesion of the large colon are lower than for small intestinal strangulation with recovery rates expected between 21-42% after surgery.\(^8\) However, recent short term survival rates of 83% and 93% have been reported for survival after surgery for large colon volvulus.\(^9,10\)
Any interference of the aboral flow of ingesta through the gastrointestinal tract has the potential to cause bowel distention and severe pain. There are many conditions which can result in an acute abdomen and these can be grouped into categories: 1. primary gastrointestinal tympany, 2. simple obstruction, 3. strangulation obstruction, 4. nonstrangulating infarction, and 5. inflammatory (enteritis and peritonitis). Primary gastrointestinal tympany is the result of accumulation of gas within the lumen of the intestine or stomach. The gas accumulation causes distention and abdominal pain due to excessive stretch of enteric nerves.

Simple obstruction occurs commonly in both the small and large intestine. It occurs when there is occlusion of the intestinal lumen by intraluminal obturation, an intramural mass, or by external compression. Simple obstruction of the small intestine can be caused by lesions within the bowel wall that constrict the lumen, obstruction of the lumen by an intraluminal food mass or foreign body, or by external compression by a mass such as an adhesion, abscess or neoplasm. Failure of the small intestinal musculature to contract which may occur with a physical blockage, surgical manipulation of the bowel, or which may be secondary to peritonitis will also result in an obstruction. Functional obstructions may also be produced by spastic contractions which can occur during spasmodic colic.

When small intestinal occlusion then prevents the aboral movement of ingesta, fluid and gas, there is subsequent increase in osmolality of the intestinal content and water is then drawn into the lumen from the mucosal vasculature. If the distention worsens the increased pressure compromises venous drainage and the mucosa will become edematous and congested. If the obstruction is present for a prolonged period of time blood is shunted away from the mucosa and serosa resulting in ischemia.
The two most common cause of simple obstruction in the large colon are impactions and nonstrangulating displacements. Large colon impactions cause approximately 30% of all colics diagnosed at referral centers. Large colon impactions will often occur at sites where the lumen diameter is narrowed, for example at the pelvic flexure or just proximal to the transverse colon in the right dorsal colon. The mechanism of these impactions is not completely understood, but poor quality feed, old age, debilitation, poor dentition, parasites, overeating, inadequate water intake, motility disorders, and limited exercise have all been reported as possible causes of this condition. Impactions develop slowly over time (several days to weeks) unless there is an enterolith or foreign body causing an acute obstruction. There is progressive distention of the large colon proximal to the obstruction, but in the early stages some ingesta and gas can still pass the mass. If the obstruction becomes complete, ingesta and gas accumulate more rapidly and marked distention occurs oral to the obstruction. The distention may become so great that it exerts pressure against the diaphragm and vena cava which results in impaired pulmonary function and venous return. This will ultimately cause hypovolemic and hypoxemic shock. Impactions also occur in the cecum. Prolonged distention of the cecum or large colon may impair mucosal perfusion resulting in the mucosa becoming devitalized and eventually rupturing, which is usually fatal. Total blockages of the large intestinal lumen with an enterolith or foreign body are usually located at the pelvic flexure or transverse colon. Stretching of the mass over the bowel wall will cause pain and impairment of venous drainage. If there is severe ischemia the mucosa will degenerate from pressure necrosis. The left ventral and dorsal colons are freely movable in the abdomen and can become displaced or twisted. The cause of the abnormal positioning is not known but predisposing factors may include the horse rolling from abdominal pain, motility changes with
stasis and hypermotility of segments of the large colon, and excessive and rapid gas production
with rotation of the colon due to lifting of the gas-filled segments. The large colon may also
rotate 90° or 180° without causing vascular occlusion but obstructing the passage of gas and
ingesta to cause a simple obstruction. Lymphatic drainage distal to a non-strangulating
displacement results in edema of the bowel wall and mesentery eventually leading to intestinal
compromise.

Strangulating obstruction of the intestines involves simultaneous vascular occlusion and
luminal obstruction. This type of obstruction can be the result of incarceration of bowel in an
intra-abdominal (mesenteric rent, epiploic foramen) or extra-abdominal (scrotum, diaphragm)
location or from the twisting of the mesentery associated with volvulus. Strangulating
obstruction of the colon is usually the result of torsion along its long axis. The rotation has to be
greater than 180° to produce venous occlusion and more than 270° to obstruct arterial flow.
There are two types strangulating obstruction; hemorrhagic strangulating obstruction and
ischemic strangulating obstruction. In the horse most cases of strangulating obstruction are
hemorrhagic. During hemorrhagic strangulating obstruction there is venous occlusion with
continued arterial flow resulting in congestion, hemorrhage, and edema of the bowel wall.
With the venous occlusion there is an increase in the hydrostatic pressure and the microvascular
permeability also increases in response to inflammatory mediators. The other type of
strangulation, ischemic strangulation involves simultaneous arterial and venous occlusion. In
this case there is no increase in hydrostatic pressure because the arterial supply is also constricted
and there is less hemorrhage and edema compared with the hemorrhagic form. During this form
of ischemia the serosa becomes blanched and cyanotic.
During hemorrhagic or ischemic strangulating obstruction in the small intestine fluid sequesters in the subepithelial space causing the epithelium to loosen from the underlying basement membrane at the villus tip. The epithelial cells slough in sheets with the cells attached to each other. This separation continues along the villus into the crypts. Loss of the entire villus epithelium occurs after 3 hours of experimentally induced total arteriovenous occlusion. After 4-5 hours of occlusion there is complete necrosis of the mucosal epithelium extending to the base of the crypts. The degeneration progresses so that after 6-7 hours necrosis has extended beyond the muscular layers.

Complete ischemia of the large colon induces cellular necrosis of groups of surface epithelial cells which loosen from their basilar attachments and from the neighboring cells. Compared to the small intestinal mucosa, cell degeneration becomes irreversible before epithelial sloughing occurs. Arteriovenous occlusion and transmural compression causing complete ischemia for a period of 3-4 hours results in irreversible damage to the mucosa of the large colon. Sloughing of 97% of the surface epithelium and at least 50% of the glandular epithelium is associated with death in the naturally acquired large colon volvulus.

A nonstrangulating infarction results from reduced blood flow to a segment of bowel caused by an intravascular occlusion. In the horse the occlusion is usually due to a thrombus or embolus and occurs most frequently in the ileoceccolic branch of the cranial mesenteric artery. Damage and thrombosis of these vessels is caused by migration of the Strongylus vulgaris larvae. Reduced blood flow and tissue perfusion result from verminous thrombosis which leads to focal or multifocal infarction of the ileum, cecum or colon. Emboli can break from the thrombus and can lodge in the peripheral branches of the ileoceccolic artery. In some cases the emboli or thrombi are small and the resulting ischemia is only transient as
the circulation is re-established by collateral circulation. However, when the ischemic lesion is extensive and cannot be reached by the collateral circulation an infarct develops, and the affected intestinal segment becomes necrotic.

Inflammatory conditions of the bowel or enteritis can be caused by bacterial or viral infections, or may result from parasites, trauma (surgical) or toxins. The mucosa is the most susceptible layer of the intestine to this type of injury as it is the most metabolically active. In the small intestine inflammation extending to the submucosa and into the muscularis causes ileus with the accompanying intestinal distention and gastric reflux. Conversely in the large colon any increase in water delivered by the small intestine, or any alteration in the absorptive capabilities of their mucosa due to enteritis, results in the accumulation of large amounts of water in the lumen and subsequent diarrhea. Very large quantities of water and electrolytes may be lost from the body when oral or aboral loss occurs, resulting in dehydration and hypovolemia.

Endotoxemia and/or septicemia may also occur when damage to the mucosa is severe enough to allow bacteria and endotoxin to pass through the lamina propria into the circulating blood. The development of the endotoxemia is enhanced when the endotoxin and bacteria leaks into the peritoneal cavity through the bowel wall. Inflammation of the peritoneum is usually caused by ischemia or bacterial contamination through part of the gastrointestinal tract which is devitalized, perforated or ruptured. Surgical procedures that involve entry into the abdomen or accidental perforation of the abdominal wall may also cause peritonitis. Extension of enteric infections into the peritoneal cavity is a common cause of peritonitis in the horse where as blood-borne infections are rare.
ISCHEMIA

Ischemia is a deficiency in blood flow to an organ or tissue. A functional constriction or mechanical obstruction of blood vessels supplying intestines can lead to ischemia causing inadequate tissue perfusion and oxygenation. Distention of the intestines also causes ischemia—this occurs in obstruction of the small intestine, resulting in severe distention proximal to the obstruction, or distention of the cecum due to dysfunction. As the intraluminal pressure increases, the capillaries and venules are compressed and the subsequent decrease in perfusion and increased accumulation of interstitial fluid causes further vascular compromise.

Cell viability is dependant on the maintenance of an adequate blood flow for the delivery of oxygen and the removal of waste products. Cells have the capability to increase oxygen extraction if there is a substantial decrease in blood flow and they continue to function with energy reserves. However, if the blood flow is decreased below the amount necessary to maintain cellular viability or if the cellular metabolic rate and oxygen consumption increase, the cell metabolism is compromised and there is change in both cellular structure and function. Different cells in the intestine have different metabolic demands and susceptibility to ischemia. As an example mucosal epithelial cells of the intestine are highly energy dependant and if there is a reduction in blood supply and decreased oxygenation of the tissues, rapid injury and death occur.

When ischemia is continuous, the cell metabolism goes through several alterations which eventually lead to cell necrosis. In the cell, oxygen is consumed in the mitochondria where it is reduced to water by the electron transport chain which is coupled with the synthesis of adenosine triphosphate (ATP). When the oxygen supply to a tissue decreases, both cellular oxygen stores and stored energy are decreased initiating anaerobic glycolysis. Anaerobic
Glycolysis results in an accumulation of lactic acid within the cell causing an intracellular acidosis, which inhibits the ATP production. The synthesis of ATP is relatively inefficient during anaerobic glycolysis and can result in increases in lactate and proton concentrations in the bloodstream which have been associated with a poor prognosis in horses with ischemia-reperfusion injury of the large colon. The cell membrane and cytosol remain intact until the energy dependant pumps, which maintain normal ionic balance within the cell, fail causing an influx of calcium into the cell. The accumulation of calcium within the cell activates proteases which cause cell membrane damage and nuclear clumping. Calcium uptake within the mitochondria is increased and this inhibits oxidative phosphorylation. As the cell membranes deteriorate intracellular homeostasis fails, resulting in swelling of the cell and the leakage of cellular elements such as enzymes and electrolytes into the interstitium. If the ischemia continues hydrogen ions increase and the cellular proteins and structures are permanently altered. Eventually proteases, lipases, and other degradative enzymes initiate autolytic destruction of organelles resulting in irreversible injury and death.

Cell structure may change within 5 minutes of ischemia, with changes in the mitochondria including swelling and disorganization of the cristae. These mitochondrial changes occur before the cytoplasmic and membrane changes which occur in the first 30 minutes by activation of phospholipases, cytokine production and accumulation of arachidonic acid. Microscopic changes are evident following 30 minutes of ischemia when the mucosal epithelial cells and serosal mesothelial cells separate from their basement membranes creating a space at the tip of the villus in the small intestine called Grunehagan’s space. The separation appears to be caused by water accumulation in the subepithelial space. Alterations in the basement are
caused by activation of metalloproteinases. Concurrent with the sloughing of the mucosal cells toward the crypts, cell degeneration progresses in the capillary tuft and the lamina propria.

The change in the large colon is similar, but the sloughing of epithelial cells off the surface of the mucosa is slower as it proceeds to the crypts. The serosa also reacts with the mesothelial cells lifting off the basement membrane before there is visible cell membrane or cytoplasmic change. There is minimal change in the architecture of the supporting tissues in the mucosa or the serosa for the first 60 minutes of total ischemia. After 180 minutes of ischemia the lamina propria and mucosal vascular tuft has lost its architecture and the tissue is necrotic with lack of definition and cell structure.

REPERFUSION

If oxygen and energy return to the tissue just prior to membrane and organelle failure the cells can still survive. However if the blood flow returns when the cells are still viable, a cascade of events is set in motion by the delivery of oxygen to the previously ischemic tissue. This resuscitation can be destructive and the resultant injury is called reperfusion injury. Reperfusion injury relies on renewed oxygen in the tissue with participation of endothelial cells and afferent receptors to create the inflammatory response which ensues. If cells have been damaged the reperfusion injury can result in inflammation, apoptosis and necrosis, thereby delaying healing. Reperfusion injury is observed more commonly following partial or low flow ischemia but can be seen following complete ischemia unless severe cell injury does not allow the cells to respond to reperfusion.

Initially during reperfusion there is a reactive hyperemia due to increased bloodflow. The response of the blood vessels’ smooth muscle to these substances depends on the particular agent, the smooth muscle condition and whether there is an intact or functional endothelium.
The bloodflow and tissue perfusion is regulated by the endothelium which alters the smooth muscle tone by releasing a balance of endothelium derived vasoconstrictors and vasodilators. \(^{70}\) Vasodilatory mechanisms include the stimulation of the muscarinic receptors on intact endothelial cells by acetylcholine which results in the release of nitric oxide from the endothelium which causes vasodilation. \(^{71}\) The endothelial cells synthesize nitric oxide from L-arginine via NO synthase and this synthesis can be blocked by NO synthase inhibitors. \(^{72-74}\) Prostacyclin is released from the endothelium and contributes a little to the endothelium dependent relaxation in blood vessels and also inhibits platelet aggregation. \(^{75}\) The endothelium also releases vasoconstrictive substances including endothelin and eicosanoids. \(^{76}\) During physiologic and pathologic conditions the large colon can release other agents that cause arterial constriction including angiotensin, histamine, serotonin and norepinephrine. Serotonin and histamine stimulate smooth muscle contraction via the release of endothelium-derived substances such as endothelin and therefore require a functional endothelium to work. \(^{77}\)

Damage to the endothelium of the arteries and veins has been thought to result in loss of endothelium derived vasodilatory agents such as nitric oxide and thus an increased sensitivity to vasoconstrictive agents. The contractile force in the vessels of the large colon are the strongest with intact endothelium, less strong with a nitric oxide synthase inhibitor (N\(_{\text{w}}\)-Nitro-L-Arginine methyl ester, L-NAME) and the least strong with a denuded endothelium. Contractile agents such as thromboxane and vasopressin usually cause a minor response in the endothelium when the vessel is damaged but their effect is enhanced if the endothelium has been damaged. \(^{78}\) If the vessels of the large colon are damaged endothelium derived contractile agents may be lost which may contribute to the decreased colonic bloodflow and the pooling of blood in the venous circulation. \(^{77}\)
The hyperemic response during immediate reperfusion in the large colon is equally distributed from the mucosa to the seromuscular layer. Mechanical or biochemical response to reperfusion exacerbates the mucosal edema, hemorrhage and necrosis. Enteric neuropeptides, which increases during reperfusion of the large colon including calcitonin gene-related peptide and substance P may be responsible for the vascular smooth muscle relaxation.

At the cellular level reperfusion initiates immediate formation of oxygen metabolites, which occurs immediately on reperfusion. Free radicals have an unpaired electron in their outer orbit and are formed during chain reactions when electrons are released as part of oxidation-reduction reactions within. When there is a decrease in blood flow severe enough to cause cellular hypoxia, the oxidative phosphorylation is uncoupled and there is a decrease in ATP. The electrons formed during the uncoupled reactions leak from the mitochondria and react with oxygen when blood flow returns during reperfusion. There is a rapid generation of oxygen free radicals during reperfusion, such as the superoxide free radicals ($O_2^-$). Oxygen free radicals are produced during normal cellular metabolism and can damage cells, but antioxidants normally present in cells protect against injury. The endogenous anti-oxidants are both enzymatic (superoxide dismutase, catalase and glutathione peroxidase) and nonenzymatic (alpha tocopherol, ascorbate and betacarotene). These protective anti-oxidants are overwhelmed during reperfusion injury allowing the free radicals and their metabolites to cause injury to the cells.

Free radicals are also produced from intracellular reactions- the most frequently reported reaction involves xanthine oxidase, an enzyme present in the cytosol of most animal cells. Xanthine oxidase is retained as xanthine dehydrogenase which is linked to nicotinamide dinucleotide. During ischemia the ATP decreases causing the cell to metabolize existing stores
which results in the accumulation of hypoxanthine. As the Na+K+ATPase pump fails calcium accumulates within the cell and the high intracellular calcium concentration and calpain converts xanthine dehydrogenase to xanthine oxidase. The xanthine oxidase catalyzes the conversion of hypoxanthine to xanthine which when oxygen is present results in superoxide production. The superoxide radical is normally converted into hydrogen peroxide by endogenous superoxide dismutase, which is then further degraded to water. However, during reperfusion, the endogenous mechanisms are overwhelmed and hydroxyl radicals (OH) are formed through the iron-dependant Haber-Weis reaction. Hydroxyl radicals are active against cell membranes and cause further perturbations in cell metabolism. The importance of the xanthine oxidase in postischemic injury has been demonstrated with pretreatment of animals with superoxide dismutase or allopurinol. Detectable levels of xanthine oxidase/xanthine dehydrogenase have been measured in the horses following one hour of small intestinal ischemia causing conversion of xanthine dehydrogenase to xanthine oxidase. However, Vatistas et al. did not find conversion of this enzyme system during reperfusion after low-flow ischemia. The large colon of horses or other species has low levels of xanthine oxidase and increased levels of aldehyde oxidase another enzyme which generates free radicals. The cells in the lamina propria of the feline large colon have been shown to have xanthine oxidase activity and the subepithelial cells can also produce free radicals. Oxygen free radicals damage the tissue by causing lipid peroxidation of cell membranes and intracellular organelles with subsequent production of phospholipid mediators due to the activation of phospholipaseA₂. Malondialdehyde and conjugated dienes, which are byproducts of cell membrane lipid peroxidation, have been detected in intestinal reperfusion injury in cats, rats and dogs.
One of the primary initiators of reperfusion is the endothelial cell. \(^{55}\) Endothelial cells are metabolically active and they have specialized functions including the regulation of blood pressure, vascular permeability, vascular tone, inflammatory cell adhesion, coagulation and platelet aggregation. \(^{99}\) During reperfusion injury when the endothelial cells are activated or injured they swell, and there is disruption of the tight cell junctions which results in extravasation of erythrocytes and neutrophils. \(^1\) Increased permeability allows fluid and protein move into the interstitium causing an increased interstitial pressure with capillary collapse. \(^1, 55\) The vascular collapse and endothelial swelling decreases the blood flow to the tissue causing further ischemia. When stimulated by oxygen radicals or platelets, endothelial cells generate cytokines, which attract and activate neutrophils during reperfusion injury. \(^55, 5\) Studies in the feline intestine have demonstrated that the neutrophil infiltration during reperfusion can be prevented with pretreatment with allopurinol (inhibits xanthine oxidase) \(^{100}\), superoxide dismutase (promotes the dismutation of \(O_2^-\) to \(H_2O_2\)) \(^{100}\), catalase (catalyzes conversion of \(H_2O_2\) to \(H_2O\)) \(^{101}\) and deferoxamine (inhibits conversion of \(O_2^-\) to \(OH^-\)) \(^{101}\). Neutrophil migration and accumulation in the interstitium can cause a second phase of reperfusion injury with a decrease in reduced glutathione and superoxide dismutase in the feline intestine. \(^{100}\) Pretreatment with allopurinol or administration of superoxide dismutase prevents the neutrophil influx and the decrease in reduced glutathione levels \(^{100}\) which suggests a relationship between the oxygen free radicals and neutrophil migration as a cause of mucosal damage. This neutrophil response is seen in the intestine affected by ischemia and also in the bowel adjacent or at a distant site to it. \(^{102, 103}\)

When neutrophils are activated they degranulate and release free radicals, proteases and hypochlorous acid which cause the vascular and mucosal injury seen in reperfusion injury. \(^5, 94, 96\) Neutrophils also produce damaging superoxide free radicals via the nicotinamide adenine
dinucleotide phosphate (NADPH) oxidase system, which catalyzes the reduction of oxygen to the superoxide anion and hydrogen peroxide. The production of these oxidative compounds is the main mechanism by which the neutrophils phagocytize and subsequently destroy microorganisms and this process is called the “respiratory burst”. 

White blood cells contain an enzyme called myeloperoxidase which is inactive in the dormant neutrophil. Various infectious or inflammatory conditions trigger the priming response which activates neutrophils and subsequent release of myeloperoxidase into extracellular fluids. A significant increase in myeloperoxidase activity in the large colon has been demonstrated during reperfusion following a period of ischemia. Myeloperoxidase catalyzes the enzymatic conversion of hydrogen peroxides and chloride ions to hypochlorous acid. Hypochlorous acid is a potent oxidant and reacts with other compounds to form other potent oxidants, such as the lipophilic N-chloramines. Hypochlorous acid can cause tissue damage by inactivating alpha-antiproteases and activating gelatinase and collagenases and has the ability to disrupt cell membranes. When applied in vitro to the equine right ventral colon hypochlorous acid can affect the permeability of the mucosa and can cause cellular damage which suggests that neutrophils cause significant damage to the large colon during reperfusion injury. Neutrophils have been shown to increase in the large colon lamina propria and mucosa during low-flow ischemia with a subsequent increase during reperfusion. Neutrophils increase significantly in the serosa of small intestine during reperfusion an event associated with subsequent scarring. In a recent study the role of the neutrophil seemed less clear when a filter in an extracorporeal circuit, which depleted leukocytes, failed to attenuate reperfusion injury in the small intestine. It is speculated that resident rather than circulatory
neutrophils mediate reperfusion injury\textsuperscript{94} and/or that the production of free radicals alone may be as important as a cause of tissue damage in the small intestine.

Proteases are enzymes that cleave peptide bonds and they contribute to reperfusion injury by causing tissue damage. Granulocytes contain at least three serine proteases including elastase, neutral proteases, and chymotrypsin-like proteases (cathepsin G), which are release during phagocytosis.\textsuperscript{5} These serine proteases catalyze the degradation of proteins or polypeptides\textsuperscript{112}. For example elastase released from activated neutrophils, degrades the intracellular barriers between the endothelial cells which allows the migration of the neutrophils from the microvasculature to the interstitium.\textsuperscript{5} Lysosomes are also a source of proteases and are small intracellular organisms found in the cytoplasm of most cells. They degrade or digest cellular debris.\textsuperscript{112} Normally endogenous inhibitors protect against this type of injury\textsuperscript{112} and mucosal injury during reperfusion has been prevented experimentally with protease inhibitors in the feline intestine\textsuperscript{113}. Granulocytes and lysosomes appear to be the most important source of proteases in the large intestine.\textsuperscript{5} In addition to these sources the pancreas is also a very important protease source in the small intestine\textsuperscript{112}. The pancreatic endoproteases can maintain some enzymatic activity when they bind to the intestinal mucosa and thus may cause injury by proteolytic destruction of the mucosal cells.\textsuperscript{5}

Intestinal ischemia-reperfusion injury has been largely attributed to cellular necrosis, however recent research indicates another form of cell death known as apoptosis may be involved. Cellular necrosis is characterized by changes resulting from lack of oxygen, where as apoptosis results from an intracellular signaling which initiates the transcription of specific genes.\textsuperscript{10} Cellular necrosis normally affects groups of cells or entire organs, while apoptosis affects individual cells of a highly specific cell type.\textsuperscript{10} Apoptosis has been recognized in several
species as having an important role in ischemia-reperfusion injury. Apoptosis has been observed following ischemia-reperfusion injury to the brain, heart, adrenals, liver and kidneys in experimental studies and has been recognized in several species as having a role in exacerbating reperfusion injury.

**INTESTINAL DISTENTION**

Distended intestine often appears to have a normal color and motility after decompression, however the intestine has sustained damage which may not be evident grossly. During intestinal distention the intraluminal pressure increases causing collapse of the veins and capillaries, even when the blood pressure is normal. It has been demonstrated in clinical disease when the small intestinal pressure is increased at 18cm of water there is a decrease in mesenteric blood flow to the intestine by 50% resulting in low flow ischemia. There is an increase in capillary back pressure but the arterial pressure is maintained which alters the Starling forces within the intestinal wall and subsequent secretion of fluid from the vasculature. Water and protein escape into the interstitium causing submucosal and serosal edema. At the higher intraluminal pressures there is more secretion than absorption of water and thus the intraluminal pressure increases further after the bowel compliance has reached its limit. Eventually fluid and protein leak into the peritoneal cavity and if the pressure is maintained the intestinal compliance allows blood flow to gradually increase. The serosa and submucosal edema progresses, mucosal and serosal lymphatics dilate and red blood cells and white blood cells migrate into the serosa and submucosa. However in clinical cases with simple obstruction of the small intestine or colon there is minimal mucosal injury.

If the bowel is decompressed there is a hyperemic response as is seen after low flow or total ischemia. The blood flow can initially double but then will decrease below normal.
The mucosa is relatively resistant to short term distention, however in the serosa the edema causes capillary closure and increased vascular permeability. Neutrophils migrate into the serosa and cause destruction of collagen and ground substance. The intestinal smooth muscle is also affected by edema and neutrophil migration into the fascial planes around the myenteric plexi. The bowel can heal after this type of insult, however the serosa is frequently thickened by fibrous tissue with the possibility of adhesion formation and permanent mesenteric constriction. It is rare that prolonged distention causes bowel necrosis. Bowel necrosis is more common when focal distention is caused by foreign bodies or impactions.

The small intestine is more susceptible to distention injury compared to the large colon. Ileus is common in horses with previously distended small intestine and the severity can be predicted by the bowel pressure measured at surgery. Clinical measurement of the intraluminal pressure of the large colon during obstruction indicates that the colon can withstand at least twice the distention pressure with no adverse effects. The correlation of increased intraluminal pressures and survival indicates that intestinal distention is an important cause of the injury that is seen with those conditions of the acute abdomen causing distention, in addition to the peritoneal changes and failure of the cardiovascular system.

Intestinal distention also effects the enteric nervous system. Impaction of the colon or cecum resulting in chronic obstruction has been associated with a significant decrease in the number of neurons in the myenteric plexi but with similar numbers of myenteric plexi as normal horses. The change in neuron number was associated with increased thickness of the longitudinal muscle in the pelvic flexure or both circular and longitudinal muscle hypertrophy in the cecum. This may be similar to the pseudo-obstruction in humans and to the experimental denervation of intestinal segments in rats. The lack of nervous inhibition has been
hypothesized to allow constant and uncoordinated muscular contractions which result in the hypertrophy and eventually the poor transit of ingesta. Affected myenteric plexuses have also been shown to have an increase in the number of glial cells. This may be an inflammatory response by the enteric nervous system to the intestinal distention, or the inflammation and decrease in neurons may be responsible for the colon dysfunction in the affected horses due to colon obstruction.

**INFLAMMATION OF THE PERITONEUM**

The serosal layer of the intestine has the important function of maintaining a lubricated barrier at the bowel surface which is vital for normal intestinal motility. The serosa is made up of a single layer of mesothelial cells that attaches to a basement membrane which is adjacent to an elastic layer. Some of the mesothelial cells are short with channels linking the peritoneal surface to the serosa and others have long microvilli which help trap fluid on the surface of the peritoneum, thus maintaining lubrication. The mesothelial cells react to circulating or intraperitoneal lipopolysaccharide, infection and surgery by releasing TNF-a, IL-1β, IL-6 and macrophage inflammatory protein resulting in the attraction and migration of neutrophils into the serosal connective tissue. During an ischemic process the mesothelium is rapidly lost with serosal swelling and edema. With reperfusion the serosal vascular permeability increases and white blood cells migrate through capillaries or venules and infiltrate into the serosa connective tissue layer. Neutrophils accumulate at the basement membrane around vessels and within lymphatics and fibrin accumulates within the serosa and at the surface. The neutrophils release lysozymes with apparent disruption of the collagen and the denuded serosal surface becomes covered with a fibrin clot. There is a massive accumulation of cells, predominately neutrophils, in the serosa and at the surface within 24-48 hours. The larger the inflammatory
reaction within the serosa and on the surface, the more fibroplasia which delays the resurfacing of the mesothelium and increases the chance of scarring and adhesion formation. The severity of the adhesions has been experimentally correlated with increasing concentrations of TNF-α in the peritoneal fluid and TNF-α antibodies have been shown to decrease adhesion formation.125,126

Following small intestinal ischemia there is an initial hyperemia in most of the bowel but actually a reduction of perfusion to the serosa.55 The same process occurs during bowel distention and is greater when the distention is relieved following decompression. There is immediately edema formation in distended bowel which hypothetically increases serosal pressure.55 The increased serosal pressure exerts extravascular pressure and closes capillaries and venules.114,127 This process continues after decompression and a reduction in the distention and thus during reperfusion there is ischemic injury, serosal endothelial cell swelling, and capillary plugging.55 This may explain adhesions involving bowel which was distended and was proximal to an obstruction or strangulating lesion but not actually involved in the primary ischemic lesion.55 Adhesion formation due to septic peritonitis also occurs in response to the massive inflammatory response in the serosa with neutrophil migration and fibrin deposition in and on the serosa.128 In some cases the inflammatory response is so great that it is possible that lysozymes prevent adhesions by breaking down the adhesions.55

APOPTOSIS

APOPTOSIS-PROGRAMMED CELL DEATH

Cellular death can occur in two biochemically and morphologically distinct ways—cellular necrosis and apoptosis. Cellular necrosis refers to the light microscopic changes resulting from enzymatic degradation of the nucleus and cytoplasm and is characterized by
biomechanical and morphologic alterations resulting from anoxia.\textsuperscript{10} Necrosis typically occurs in response to acute cellular dysfunction and is a passive and destructive process which results in the release of intracellular contents into the extracellular environment. There is a subsequent activation of a marked inflammatory response which exacerbates the condition.\textsuperscript{129} Apoptosis is, in contrast, a highly regulated process characterized by cell shrinkage. Cells’ contents are subsequently packaged into apoptotic bodies that are subsequently engulfed by macrophages thereby preventing an inflammatory response.\textsuperscript{129} Apoptosis results from activation and transcription of specific genes and affects individual cells of a highly specific cell type in contrast to cellular necrosis in which there are large numbers of all cell types affected in tissue or entire organs.\textsuperscript{10} There is evidence that protein molecules, which are involved in the induction of apoptosis, are constitutively expressed in mammalian cells and are normally inactivated by antiapoptotic proteins.\textsuperscript{10} These protein molecules are synthesized in the same cell in response to certain survival signals including growth factors, extracellular matrix or fluid or a variety of hormones.\textsuperscript{10} Loss of these protective factors\textsuperscript{10} or activation of the harmful proteins induced by genotoxic stimuli\textsuperscript{130} can initiate apoptotic pathways which can ultimately result in the demise of the cell.\textsuperscript{131}

Apoptosis is characterized by cellular shrinking, condensation and margination of the chromatin and ruffling of the plasma membrane which is called budding.\textsuperscript{137} The cell eventually becomes divided into the apoptotic bodies which consist of cell organelles and/or nuclear material surrounded by an intact plasma membrane.\textsuperscript{137} These apoptotic cells are mostly engulfed by neighboring cells, particularly macrophages.\textsuperscript{132} The macrophage recognizes the apoptotic cell fragments by their expression of phosphatidylserin on the outside of the plasma membrane.\textsuperscript{138} Other mechanisms of phagocytosis include expression vitronectin receptors or
stimulation by certain carbohydrates. Apoptotic cells are not always recognized by phagocytes and then they may undergo secondary or apoptotic necrosis. Necrosis can be thought of as the end stage of any cell death process.

Apoptosis is a physiological cell death that has a critical role in the development and tissue homeostasis. Apoptosis eliminates superfluous or potentially harmful cells. It is crucial for an organism to remove apoptotic cells before they become secondarily necrotic and release intracellular contents, causing tissue damage or an inflammatory reaction. Macrophages are the key cells in this process, using a number of receptors to recognize surface changes appearing as cells die. In addition to removing potentially inflammatory and immunogenic cell debris, macrophages also inhibit inflammation by engulfing apoptotic cells through the release of transforming growth factor β and other inflammatory mediators. Therefore as apoptosis is part of normal cellular turnover, the clearance by macrophages and other phagocytes plays a critical role in tissue homeostasis. Macrophages have also been found to induce apoptosis. Apoptotic cell engulfment has an important role in various diseases such as cancer, virus-induced pathologic conditions, autoimmune diseases, and other inflammatory states.

Apoptosis is involved with embryogenesis, tissue homeostasis, lymphocyte development and function, and tumor regression, resulting from DNA damage induced by various factors. Apoptosis is an energy-dependant, active form of cell death, which not only occurs normally during development and in the immune system but also in response to injury. Normal cells will typically die by undergoing apoptosis or by entering a state of irreversible growth arrest, termed replicative senescence, at the end of the lifespan. The regulation of apoptosis has a direct influence on the ability of a cell to survive cellular insult from both external and internal
sources and thus the genes involved in apoptosis influence cellular longevity. A variety of molecules have the ability to regulate apoptosis. Inhibitory molecules include heat shock proteins, anti-apoptotic members of the Bcl2 family and anti-oxidant molecules. Molecules which can promote apoptosis include pro-apoptotic members of the Bcl2 family and p53. Almost every tissue can undergo apoptosis when provided with an appropriate stimulus and thus many apoptotic genes are present in all tissues. However, the exact compositions of the apoptosis regulators vary in tissues and this variation contributes to different responses of different cells and tissues to different apoptotic stimuli.

Mitochondria play a central role in both apoptosis and necrosis. Mitochondria provide a key amplification step in the apoptotic pathway by releasing apoptogenic proteins in the cytosol of the cells. Mitochondria have a multiprotein complex formed at the contact site between the inner and outer mitochondria membranes which is called the mitochondria permeability transition (PT) pore. The PT pore is involved in the regulation of matrix Ca++, voltage, pH, transmembrane potential and volume and functions as a Ca++, voltage, pH and redox-gated channel with several levels of conductance and little or no ion selectively. Certain proteins in the cell, when activated, move from the cytoplasm or nucleus to the mitochondrial membranes, where they interact with mitochondrial receptors such as hsp70, BckXL, PTPC, Bax and Bcl2. When these proteins are translocated to the mitochondrial membranes they promote permeabilization of the membranes with the consequent release of caspases or nuclease activators from the mitochondrial intermembrane space. One of the most critical proteins in this process is cytochrome c which in the presence of ATP and dATP, forms a complex with apoptosis activating factor 1 and pro-caspase 9. This induces cleavage of pro-caspase 9 resulting in the release of active caspase 9 which then cleaves and activates
procaspase 3. Active caspase 3 then induces proteolytic cleavage of a range of target proteins. These target proteins then cause changes in the cytosol, nucleus and plasma membrane that are the characteristic of apoptosis.

There are three major mechanisms through which apoptosis can be induced by the activation of caspases. These include a receptor-ligand binding with activation of caspase-8; a mitochondrial mechanism with activation of caspase-9; a process involving the endoplasmic reticulum with activation of caspase-12. The first mechanism involves the binding of a ligand on a specific receptor which is located on the cell membrane. This binding activates the conversion of procaspases to caspases. Two well known ligands are the Fas ligand, which has Fas as the receptor, and TNF-a which binds to TNFR$_1$. Both of these receptors contain an analogous cytoplasmic domain called the ‘death domain’, which is responsible for the signal transduction in apoptosis. There are two molecules which are linked to the death domain- the Fas associated death domain (FADD) for Fas, and the TNFR$_1$ associated death domain (TRADD) for TNFR$_1$. TRADD binds to FADD and thus both receptors use FADD to transducer the death signal. Procaspase 8 binds with its death domain to FADD and becomes activated to caspase 8. Activated caspase 8 results in the conversion of procaspase-3 into the effector caspase 3 which seems essential for apoptosis. TNF- alpha can induce apoptosis by the binding of receptor interacting protein (RIP) to the TRADD which leads to the activation of caspase 2. There is also a highly specialized form of Fas mediated apoptosis caused by cytotoxic T lymphocytes. Other receptor-ligand mediated forms of apoptosis are known but their exact mechanisms are not yet understood. The second mechanism involves the mitochondria. Certain cytotoxic agents such as nitrogen monoxide and radiation can cause apoptosis which involves the mitochondrial protein cytochrome c. Cytochrome c is
localized on the outside of the inner mitochondrial membrane and in the intermembrane space and has an important function in the intracellular electron transport chain for the production of ATP. Cytochrome c is released into the cytosol during apoptosis and then it binds to the apoptosis protease activating factor (Apaf-1). The cytochrome c and the Apaf-1 form a complex together with dATP which then activates procaspase 9 to caspase 9. This results in the activation of procaspase-3 into caspase-3 which leads to the known characteristic morphological consequences of apoptosis. Most agree that the release of cytochrome c into the cytosol is regulated by the proteins coded by the genes of the Bcl2 family. Bcl2 and BclxL are both apoptosis inhibitors and Bid and Bax are two pro-apoptotic proteins. The proteins of the Bcl2 family are mainly localized in the outer mitochondrial membrane and the ratio of Bax transcripts to the bcl2/bcl-xL transcripts will determine whether cytochrome c is release or not. The mechanism of release of cytochrome c by Bax is not understood as yet. Some authors believe that these proteins regulate the mitochondrial permeability via the PT pore. The opening of this channel results in the influx of ions which results in swelling of the mitochondrion and breaks in the outer membrane while the inner membrane remains intact due to its larger surface. Cytochrome c then breaks through the outer membrane and enters the cytosol. This ion influx explains the decrease in transmembrane potential of the inner mitochondrial membrane which has been documented in apoptotic cells. Recent studies suggest that the mitochondrial mechanism and the receptor-ligand mechanism are not completely independent. Caspase 8 can activate the protein Bid. Activation of Bid leads to the release of cytochrome c and activation of caspase 9. Another recent study showed that the outer membrane permeabilization by Bcl2 family proteins does not require the mitochondrial matrix, the inner mitochondrial membrane or other proteins. Bid, or its BH3-domain peptide,
activated monomeric Bax to produce membrane openings that allowed passage of very large (2 megadalton) dextran molecules which explains the translocation of large mitochondrial proteins during apoptosis. This process requires cardiolipin and is inhibited by antiapoptotic BclxL. A third mechanism in the apoptotic process has also been demonstrated in transgenic mice. Agents that stress the endoplasmic reticulum such as tunicamycin and thapsigargin cause much apoptosis in embryonic fibroblasts of normal mice but not caspase-12/- knock-out mice. This process appears to be independent of both former pathways described above.

In vascular smooth muscle cells it has been shown that cytochrome c activates K+ channels before inducing apoptosis. In vascular smooth muscle cells, K+ is the dominant cation and Cl- is the dominant anion in the cytoplasm. Cytoplasmic K+ at a normal concentration (~140mM) suppresses apoptosis by inhibiting caspase activation. In a cell-free system with only isolated nuclei, a decrease in K+ from 140-180mM caused a 1.6-fold increase of apoptosis induced by the apoptosis-inducing factor. Efflux of K+ through K+ channels, therefore, plays an important role in initiating the apoptotic volume decrease and apoptosis. The cytochrome c-mediated decrease in cytoplasmic K+ resulting from opened Kv channels, in addition to the volume decrease which occurs during apoptosis, may contribute to induction of DNA fragmentation and apoptosis by reducing the inhibitory effect of cytoplasmic K+ on the cytoplasmic caspases and the internucleosomal DNA cleavage nuclease. It is possible that apoptosis inducers such as staurosporine, may also open K+ and Cl- channels through cyt-c independent mechanisms.

**IMMUNOHISTOCHEMICAL STAINING TO DETECT APOPTOSIS**

In many of the pathways that are initiated in apoptosis, permeabilization of the mitochondrial membrane is a critical event that results in release of various molecules from the
mitochondrial intermembrane space. \textsuperscript{131} These molecules include procaspases (enzymes), cytochrome c (a caspase activator), Smac/Diablo (a caspase coactivator) and an apoptosis-inducing factor. \textsuperscript{131} The apoptosis inducing factor activates the nucleases that cleave the DNA into small fragments. \textsuperscript{131} The DNA strand breaks that result from the endonuclease(s) activation can be labeled in situ, in individual fixed, permeabilized cells or tissues in sections, by the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) technique \textsuperscript{187,188}. In this technique, the 3’-OH termini which are generated as a result of the DNA fragmentation during apoptosis, are labeled with modified nucleotides by terminyl deoxynucleotidyl transferase. \textsuperscript{189} This enzyme selectively detects apoptotic rather than necrotic cells and is more specific than DNA polymerase. \textsuperscript{189} The new ends that are generated upon DNA fragmentation are typically localized in the morphologically identifiable nuclei and apoptotic bodies.

There is a high correlation between TUNEL staining and apoptosis. \textsuperscript{189,190} When compared to another technique, the ISNT (in situ nick translation), the TUNEL technique was more sensitive. \textsuperscript{191} A major advantage of the TUNEL technique is the ability to reveal early DNA breaks during apoptosis which occur before histological changes characteristic of apoptosis can be detected. The TUNEL technique is very successful in detecting apoptotic lymphocytes and neutrophils in the lamina propria of fixed human intestinal samples. \textsuperscript{14} Dexamethasone is known to induce apoptosis in the murine thymus \textsuperscript{192,193,194} ApopTag® Peroxidase kits are used to successfully stain apoptotic rat thymus lymphocytes treated in vitro with dexamethasone. \textsuperscript{195,187}

A potential concern of detecting apoptosis in formalin fixed tissue was that of random DNA damage with long term fixation, resulting in nonspecific labeling. This effect has been
investigated in rat testicular tissue. Results indicated that formaldehyde is able to replace protons in the purine and pyrimidine bases but does not react with sugar hydroxyls or phosphate ester groups in nucleic acids.\(^{196}\) It is possible that with prolonged fixation any single-stranded regions of DNA, or ‘free ends’ are locked up and they are thus inaccessible to the polymerase.\(^{196}\) Any damage resulting from fixation or cytopathological mechanisms such as necrosis would thus not be detectable with this technique.\(^{196}\) Therefore the test has a risk of a false negative result rather than a false positive result.

**APOPTOSIS AND THE INTESTINE**

Apoptosis has been suggested to play a complementary and opposite role to mitosis in the regulation of animal cell populations.\(^{132}\) In the gastrointestinal tract apoptosis is important in physiologic cell renewal.\(^{197,198}\) Apoptosis in the gastrointestinal tract is associated with the shedding of cells from the villous tips in the small intestine, and with the development of intestinal adenocarcinomas, the pathogenesis of inflammatory bowel disease, and radiation induced gut damage.\(^9\) The apoptotic targeting of damaged stem cell populations, early response for apoptotic removal of DNA-damaged cells, and early repair of DNA-damaged cells are considered major indicators of differences in levels of tumorigenesis in the intestine.\(^{199}\) Enterocyte apoptosis is increased in patients with untreated celiac disease which results in the formation of villous atrophy.\(^{200}\) Thus apoptosis plays an important role in the maintenance of normal gastrointestinal homeostasis and in the induction of gastrointestinal disease.\(^{201,202}\)

Endothelial and epithelial barrier functions protect against potential pathogenic invasion.\(^9\) An intact intestinal barrier prevents invasion of potential pathogens \(^{203}\) and macromolecules which are not normally permeable through the barrier may pass to variable degrees under pathologic conditions.\(^{204}\) The number of apoptotic cells in the colon in human patients with
diseases such as untreated inflammatory bowel disease, non-steroidal anti-inflammatory drug-related colitis, and active celiac disease. In one study a model of intestinal epithelial apoptosis was induced by injecting doxycycline into the peritoneum of rats. When the endothelial and epithelial permeability and the amount of apoptotic cells were measured there was a significant increase in gut water content, albumin flux, and bidirectional clearance of albumin with a significantly higher apoptotic index. This data indicates that occasional apoptotic cells which appear in normal intestine may not cause alterations in intestinal barrier integrity, whereas increased intestinal apoptosis causes increased endothelial and epithelial cell permeability.

Both the glycolytic and protein synthetic pathways may play a crucial role in the formation of mucosal endothelial apoptosis and dysfunction. Chemotherapeutic agents can induce apoptosis in the intestinal epithelium and cause an increase in barrier permeability. An increase in barrier permeability enables pathogenic bacteria and/or their toxins to pass through the intestinal barrier which can result in gut-origin sepsis and other complications. The inflammatory response associated with the non-steroidal anti-inflammatory induced apoptosis in the human colon indicates that apoptosis achieves sufficient magnitude to disrupt the epithelial barrier and cause inflammation.

Morphological and biochemical evidence suggests that glutamine deprivation induces apoptosis in rat small intestinal epithelial cells. Glutamine is rapidly absorbed and metabolized by enterocytes from intraluminal or arterial sources and is the most abundant amino acid in the blood. In human patients treated with sustained parenteral nutrition which does not contain glutamine, have decreased mucosal weight and DNA and protein content. Gut atrophy induced by glutamine starvation is caused in part by an increased rate of apoptosis in
intestinal epithelial cells which affects the homeostatic balance between cell proliferation and cell death. Glutamine prevents apoptosis in the gut by supplying an essential energy source to maintain cellular adenosine triphosphate (ATP) concentrations.\textsuperscript{206} Administration of antioxidants such as dimethyl sulfoxide prevents glutamine deprivation-induced apoptosis in HuH-7 cells which may indicating that in some cells glutamine prevents apoptosis by maintaining glutathione levels rather than ATP.\textsuperscript{206}

Recently apoptosis has been found to be a major mode of cell death in the destruction of rat small intestinal epithelial cells induced by ischemia and ischemia/reperfusion injury.\textsuperscript{210} The intestinal mucosa is one of the most sensitive tissues to ischemia/reperfusion injury.\textsuperscript{210} Apoptosis has been characterized in rat jejunum and ileum following occlusion of the superior mesenteric artery for 15 or 60 minutes followed by a period of reperfusion.\textsuperscript{211} Apoptosis does not occur in the duodenum as blood flow is not decreased after occlusion of the artery.\textsuperscript{211} The percentage of fragmented DNA increases during ischemia and is maximal after 1 hour of reperfusion.\textsuperscript{211} The magnitude of DNA fragmentation is directly proportional to the duration of the ischemic insult with a significantly higher percentage of fragmented DNA after 60 minutes of ischemia compared with 15 minutes of ischemia.\textsuperscript{211} Because maximal DNA fragmentation occurs at 1 hr postreperfusion followed by a return to baseline values by 6 hours, induction of intestinal apoptosis and mucosal recovery appear are rapid processes.\textsuperscript{211} There may be a time-dependent increase in apoptosis-promoting factors during ischemia and early phases of reperfusion which rapidly decreased with prolonged reperfusion.\textsuperscript{211} There may also be a simultaneous induction of inhibitors of apoptosis as well as promoters of tissue repair during the later phase of reperfusion.\textsuperscript{212,213} The exact mechanism for the induction of apoptosis is not known. However studies have shown that free radicals can cause apoptosis\textsuperscript{212,213} which is
consistent with the increase in free radical production during ischemia/reperfusion injury.\textsuperscript{212,213} Certain authors have reported alterations in cell adhesion molecules or substratum along the crypt-villous axis, and used this to support the idea of cell loss by shedding.\textsuperscript{215,210} However, the regulation of intestinal cell death and proliferation is highly complex and is most likely controlled by a variety of factors.\textsuperscript{214} It has been proposed that altered expression of $\beta_1$ integrins along the crypt-villous axis facilitates cell loss.\textsuperscript{215} Recently the interaction between extracellular matrix materials and cells has been proposed as a critical role in causing apoptosis, with the cell death occurring when the contacts are disrupted; a phenomenon is known as anoikis.\textsuperscript{216} Affected epithelial cells lose their contact with the villous stroma indicating apoptosis is induced by ischemia and ischemia/reperfusion could represent anoikis, however in this study not all the detached cells demonstrated positive staining with the TUNEL method and some cells had evidence of necrotic cell death when examined by DNA binding fluorochromes PI (propidium iodide).\textsuperscript{210}

The intestine is susceptible to stress during surgery and surgical trauma induces oxidative stress and damage through increased production of reactive oxygen species.\textsuperscript{217} Surgical stress increases intestinal permeability and induces bacterial translocation in the systemic circulation.\textsuperscript{218} There are profound metabolic changes, organ dysfunction, and immunosuppression during gastrointestinal surgery which form part of the surgical stress response to trauma.\textsuperscript{219} The intestine is highly susceptible even to surgery at remote locations. Mild handling of the intestine following laparotomy caused oxidative stress in the enterocyte, accompanied by functional alterations in the intestine.\textsuperscript{220} Furthermore during surgical stress there is increased activity of xanthine oxidase and increased oxygen free radicals which activate proteases in the enterocyte mitochondria.\textsuperscript{221} This protease activation was not seen in xanthine oxidase deficient animals.
Increased mitochondrial protease activity may be responsible for the induction of the mitochondrial permeability transition and mitochondrial dysfunction seen during surgical stress. Surgical stress also activates cytosolic proteases, which are modulated by thiol redox status and are maximally activated by 60 min after surgical stress and return to normal by 24hours.

APOPTOSIS AND ISCHEMIA/REPERFUSION INJURY

Pro-apoptotic stimuli will stimulate cells to undergo apoptosis. However intracellular ATP concentration is a determining factor in the reactions causing apoptosis. Decreased ATP levels will favor necrosis over apoptosis and an increased ATP provided by glycolytic substrates has the opposite effect. The concept that the mitochondria can trigger apoptosis when caspases are activated and necrosis when such proteases fail to act is compatible with the finding that many drugs induce necrosis at high doses and apoptosis at lower doses. Induction of the mitochondrial PT pore in a smooth protracted fashion allows for the activation and action of proteases before ATP depletion causes cell death. Alternatively when the PT pore is induced in a massive, rapid fashion, heavily compromising cellular metabolism then necrosis will occur before apoptogenic proteases come into action. Several apoptosis-inducing agents such as pro-oxidants and nitric oxide can act on the critical cysteine residues of caspases provoking their inactivation. Low doses of these compounds induce the PT pore and cause apoptosis, whereas high doses induce the PT pore but inhibit caspases causing necrosis. Therefore the intensity of the PT-inducing stimulus may determine which among the two major consequences of PT will dominate over the other resulting in either a bioenergetic catastrophe culminating in cytolysis, or the activation/action of apoptogenic caspases.

The susceptibility to either apoptosis or necrosis is also influenced by the ratio between glycolytic and respiratory ATP generation, which is differentially affected by the disruption of
mitochondrial function. This explains why lymphocytes in which glycolytic ATP generation dominates tend to undergo apoptosis, whereas neurons in which oxidative ATP generation dominates tend to undergo necrosis in response to stress. 145 The existence of an alternative ATP source to oxidative phosphorylation may be a critical factor allowing cells to progress to apoptosis. 168 For example if the onset of the mitochondrial permeability transition occurs in a subpopulation of mitochondria as would occur during the early stages of TNFa-induced apoptosis in hepatocytes, then the remaining functional mitochondria may produce sufficient ATP to permit apoptosis to proceed. 168 In ischemic tissue pH decreases rapidly due to anaerobic glycolysis and the hydrolysis of ATP. During reperfusion normal pH is restored. While the naturally occurring acidosis is protective against loss of cell viability during ischemia, the restoration of a normal pH during reperfusion can accelerate cell death. A pH below 7 inhibits conductance through the PT pore of the mitochondria so it is inhibited during ischemia. However an increasing pH has been shown to trigger the onset of the mitochondrial permeability transition which depolarizes mitochondria with uncoupler-stimulated mitochondrial ATPase activation.169 170 If the rise in pH is prevented after reperfusion the mitochondrial permeability transition is blocked and there is improved ATP recovery and inhibition of cell death. 171 Cyclosporin A has also been shown to prevent cell death by blocking the mitochondrial permeability transition without blocking recovery of pH.171 Because acidosis and cyclosporin A both act to block onset of the mitochondrial permeability transition, accelerated ATP hydrolysis caused by onset of the mitochondrial permeability transition is likely to be the basis for continued ATP depletion and subsequent cell death. 171
ROLE OF NITRIC OXIDE IN APOPTOSIS

Nitric oxide (NO) is an important molecule with diverse roles including the modulation of apoptosis. Nitric oxide can either induce or inhibit apoptosis depending on the cell type and coexisting metabolic or experimental conditions. Nitric oxide can inhibit caspases by S-nitrosylation, by inhibiting Fas induced apoptosis, or by increasing anti-apoptotic proteins such as Bcl2. Some studies indicate that NO may be an important protective molecule against ischemia/reperfusion injury, while other studies demonstrate that NO is involved in the breakdown of the intestinal mucosa after an ischemia/reperfusion insult. Further tissue NO generation increases during ischemia/reperfusion of the rat small intestine when adenosine is added to the preservation solution and decreases when the adenosine receptors are blocked with theophylline, even in the presence of exogenous xanthine. The adenosine administration increases NO generation that is concomitant with an increase in caspase-3-like activity and DNA fragmentation. These findings indicated that adenosine is related to NO production in ischemia/reperfusion injury and there is a direct role of NO in the activation of apoptosis during the ischemia/reperfusion of the rat small intestine. This finding contraindicates the NO inhibition of apoptosis previously found in rat gastric mucosa cells, but is in agreement with previous results demonstrating that NO is required for the activation of apoptosis. DNA fragmentation and morphologically recognizable apoptotic injury in small intestinal tissue occurs with the addition of exogenous xanthine which increases caspase-3-like activity. The protective effects of blocking adenosine function by theophylline are reversed by further addition of xanthine. The administration of NO donors to the xanthine-treated group induces the same effect on apoptotic parameters as that produced by the administration of theophylline. This indicates a NO-independent effect of xanthine on apoptosis which involves reactive oxygen
species. The role of xanthine in the development of apoptosis during ischemia/reperfusion is supported by the production of apoptotic tissue injury with the addition of exogenous xanthine even when adenosine receptors are blocked.

The effect of NO on mitochondria has been examined in other studies. Short-term exposure to low levels of NO results in a completely reversible inhibition of respiration (at cytochrome oxidase), but longer-term exposure to high levels of NO, or exposure to NO in the presence of reactive oxygen species, leads to irreversible inhibition of respiration. The conversion from the reversible to the irreversible phase of inhibition is mediated by the following factors a) accumulating inhibition by peroxynitrite or NO, b) increased reactive-oxygen-species production by mitochondria, c) increased H$_2$O$_2$ levels due to inhibition of catalase, d) reaction of H$_2$O$_2$ and NO with superoxide dismutase to produce peroxynitrite, and e) secondary effects of cytochrome oxidase inhibition, for example glutamate release from nerve terminals. Nitric oxide has specific interactions with mitochondria at the level of both cytochrome c oxidase and complex III. One study demonstrated NO restoration of the mitochondrial PT pore at high release rates in excess of those to be encountered in vivo, even in a pathologic situation with maximal stimulation of inducible NO synthase. This study identified inhibition of mitochondrial PT pore opening as a novel site of action for NO signaling in apoptosis. The mechanism involves the depolarization of the mitochondrial membrane and inhibition of calcium accumulation and favors partial inhibition of mitochondria.

Nitric oxide can induce necrotic or apoptotic cell death. Nitric oxide-induced necrosis in the absence of glucose is due to the inhibition of mitochondrial respiration and subsequent ATP depletion. However when glucose, nitric oxide donors and mitochondrial inhibitors are available, apoptosis is induced. This demonstrates that necrosis versus apoptosis induced by
either NO or mitochondrial inhibitors depends critically on the glycolytic capacity of the cell. Nitric oxide-induced apoptosis has been shown to be mediated by caspase activation and specifically the caspase-3 and caspase-3-processing-like proteases. Nitric oxide also induces cell death in several other cell types including cardiac myocytes and smooth muscle cells. Inducible NO synthase (iNOS) expression and NO production is associated with an increase in caspase-3 activity and apoptotic cells death in adult cardiac fibroblasts. The cardiac fibroblast apoptosis in response to IL-1β is attenuated by the caspase-3 inhibitor. In the smooth muscle cells and cardiac myocytes, the proapoptotic effect of cytokines is usually mediated by the induction of iNOS protein which only occurs in pathological conditions when there are high levels of NO production. Nitric oxide as discussed before can have a proapoptotic or antiapoptotic effects- low levels of NO generated by endothelial NOS may have a protective effect, whereas the higher levels produced by iNOS may exert a proapoptotic effect.

**APOPTOSIS AND INFLAMMATION**

Apoptosis has been shown to be an important source of inflammation in the kidney which is an important documentation of the fact that apoptosis is not only a benign process causing minimal tissue damage. Apoptosis of glomerular endothelial cells may contribute to the development of glomerulosclerosis during severe forms of glomerulonephritis. Tumor necrosis factor-a and lipopolysaccaride (LPS) induce apoptosis of bovine glomerular endothelial cells which is characterized by early mitochondrial cytochrome c release, mitochondrial permeability transition, Bak protein upregulation, BckXL protein downregulation and caspase-3 activation. Co-treatment of bovine glomerular endothelial cells with 10nM dexamethasone and TNF- α or LPS has been demonstrated to block approximately 90% of apoptotic cell death. Using a murine model of ischemia and reperfusion Daeman demonstrated administration of
the anti-apoptotic agents IGF-1 and ZVAD-fmk (a caspase inactivator) prevented the early onset of not only renal apoptosis and also inflammation and tissue injury. When these agents were added after the onset of apoptosis the effects were completely attenuated. The presence of apoptosis in this study was directly correlated with posttranslational processing of the endothelial monocyte-activating polypeptide II (EMAP-II) which could explain apoptosis-induced influx and sequestration of leukocytes in the reperfused kidney. These results indicate that apoptosis is a crucial event that initiate the inflammation observed in ischemia/reperfusion injury of the bovine kidney and subsequent tissue injury. The potent nephrotoxin Hg2+ at cellular concentrations does not compromise cellular energy production, yet impairs activation of NF-kB. This increases the sensitivity of the kidney cells to the apoptosis-inducing effects of other toxicants and infectious agents to which kidney cells are otherwise resistant. Apoptosis is thought to play an important role in the pathogenesis of renal failure associated with toxicant injury to tubular epithelial cells.

Recently muscle injury (measured by creatinine kinase) caused by aortic clamping was reduced with a pancaspase inhibitor. In this study the muscle injury resulting from 90-120 minutes of ischemia was reduced by the pancaspase inhibitor z-VAD. The pancaspase inhibitor leads to a blockade of apoptosis which reduced inflammation and subsequent injury. Thus in this study apoptosis was associated with inflammation in the muscle which could be reduced with a caspase inhibitor.

**APOPTOSIS AND NON-STEROIDAL ANTI-INFLAMMATORY DRUGS**

Nonsteroidal anti-inflammatory drugs (NSAIDs) are associated with gastrointestinal inflammatory damage and aggravation of gut inflammatory conditions in human medicine and veterinary science. In human medicine NSAIDs have been shown to exert a
protective effect against colon cancer due to an increased colon cell apoptosis.\textsuperscript{226} NSAIDs have also been shown to modulate the release of colony stimulating factors in some cells. In one study reduced secretion of granulocyte-colony stimulating factor and macrophage-colony stimulating factor and increased HT-29 cell apoptosis was observed with the highest concentrations of nonselective NSAIDs.\textsuperscript{226} NSAID administration in rats induces systemic release of TNF-a and drives gastric epithelial cells to apoptosis activating the pro-apoptotic cascade of caspases.\textsuperscript{227} In response neutrophils are recruited into the gastric microcirculation through a process which involves activation of the adhesion molecules.\textsuperscript{227} In the gastric mucosa in vivo PGE\textsubscript{2} is one of the most important protective factors and NSAID’s are thought to damage the gastric mucosa by inhibiting cyclooxygenase and decreasing the amount of PGs at the level of the gastric mucosa. It has been demonstrated that PGE\textsubscript{2} shows cytoprotective effects on cultured gastric mucosal cells in relation to apoptosis but not with respect to necrosis.\textsuperscript{228} PGE\textsubscript{2} has also been shown to inhibit spontaneous apoptosis in vitro.\textsuperscript{229} It appears that the target of PGE\textsubscript{2} as an inhibitor of apoptosis is a protein such as caspase 3 which is common to both apoptotic pathways.\textsuperscript{228} The antiapoptotic effect of PGE\textsubscript{2} in human colon cancer cells is due to an increase in the expression of Bcl2\textsuperscript{230} and therefore PGE\textsubscript{2} may inhibit the cytochrome c-dependent activation of caspase-9 through an increase in the expression of Bcl2 to inhibit gastric irritant-induced apoptosis.\textsuperscript{228} An increase in apoptotic bodies in equine right dorsal colon tissue incubated with an NSAID compared with controls has been recently documented.\textsuperscript{231} Possible causes for this increased apoptosis in the presence of NSAID could be reactive oxygen metabolites and proteases in addition to inhibition of prostaglandin synthesis.\textsuperscript{231} In this study it was postulated that even after extensive cell loss through apoptosis barrier function may be preserved by rearrangement of tight junctions and desmosomes and by flattening and spreading
neighboring cells over the defect. \textsuperscript{232} The inflammatory response associated with NSAID-induced apoptosis in human colon suggests that apoptosis can achieve sufficient magnitude to disrupt the epithelial barrier and cause inflammation so it is reasonable to suggest that the same pathology may occur in the equine intestine and that intestinal function could be altered. \textsuperscript{13}
REFERENCES CITED


105. Smith JA. Neutrophils, host defense, and inflammation: a double-edged sword. 


122. Schusser GE, White NA. Morphologic and quantitative evaluation of the myenteric plexuses and neurons in the large colon of horses. *Journal of the American Veterinary Medical Association* 1997;210:928-34.


207. Windmueller HG, Spaeth AE. Intestinal metabolism of glutamine and glutamate from the lumen as compared to glutamine from the blood. *Arch Biochem Biophys* 1975;171:662-72.


Detection of apoptotic cells in intestine from horses with and without gastrointestinal disease.

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Abstract

Objective: To identify apoptosis in the equine intestine and determine if occurrence of apoptosis is affected by gastrointestinal disease and tissue layer of intestine.

Animals: Samples of intestine were collected from 38 horses that underwent surgery or were humanely euthanized for small or large bowel obstruction, strangulation or distension. Samples were also taken from 9 horses which were humanely euthanized for reasons other than gastrointestinal disease or systemic disease.

Procedure: Specimens were collected at surgery from intestine involved in the primary lesion, distant to the primary lesion, or at necropsy from several sites including the primary lesion. Tissues were fixed, serially sectioned and stained with hematoxylin and eosin (H&E) and for apoptosis by the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) technique. The number of apoptotic cells per high power field was counted in the mucosa, circular muscle, longitudinal muscle and serosa.

Results: Apoptotic staining nuclei were seen in all layers of intestine. An increased number of apoptotic cells were found in the circular muscle of the intestine from horses with simple obstruction. Intestine distant from the primary strangulating lesion had higher numbers of apoptotic cells than intestine distant from a simple obstruction lesion or intestine taken at the site of a strangulating or simple obstructive lesion.

Conclusion: Intestine from horses with obstructing or strangulating lesions in the small intestine and large colon has increased numbers of apoptotic cells, possibly due to ischemic cell injury and subsequent inflammation. Further investigation is required to determine whether increased apoptosis affects intestinal function.
Introduction

Gastrointestinal disease is the cause of high morbidity and mortality in the horse. Strangulating obstruction of the small intestine and large colon is a frequent cause of the acute abdominal disease requiring surgery. Even with surgical intervention, the overall prognosis for long-term survival of horses with a strangulating lesion of the small intestine or large intestine is guarded, with long-term survival rates of 68% and 24%. Experimental evidence suggests that one reason for morbidity and mortality in horses with obstructive diseases is reperfusion injury. Mucosal and serosal damage which occurs during ischemia is exacerbated during reperfusion. To date, intestinal ischemia-reperfusion injury has been largely attributed to cellular necrosis, although apoptosis has recently been recognized as having an important role in both ischemia and reperfusion injury.

Apoptosis, a distinct form of cell death, is a natural physiologic mechanism for the removal of cells which are not needed, with minimal tissue inflammation. Apoptosis has an important role in embryogenesis, tissue homeostasis, lymphocyte development and function and tumor regression.

Apoptotic cells have been observed following experimental ischemia-reperfusion injury to the brain, heart, adrenals, liver, and kidneys of other species. In the gastrointestinal tract, apoptosis can be associated with the maintenance of homeostasis and shedding of cells from the villous tips in the small intestine, or with pathological states such as intestinal adenocarcinomas, inflammatory bowel disease and radiation induced gut damage. Apoptotic cells can be detected using the terminal deoxynucleotide transferase mediated dUTP nick end labeling (TUNEL) technique. This technique distinguishes apoptotic from necrotic cells by specifically detecting DNA cleavage associated with nuclear change during apoptosis. This technique has
been shown to be effective in the detection of apoptosis in the formalin fixed samples of human large intestine.\textsuperscript{13}

The objectives of this study were to 1) determine if apoptosis could be identified in the equine intestine using the TUNEL method, 2) to identify the type(s) of cells undergoing apoptosis and, 3) to determine whether the occurrence of apoptotic cells was affected by gastrointestinal disease and tissue layer of the intestine. We hypothesized that equine intestine directly or indirectly affected by naturally occurring simple or strangulating obstruction would have increased numbers of apoptotic cells.

**Materials and Methods**

Samples of intestine from thirty eight horses (gastrointestinal disease group) that underwent abdominal surgery or were humanely euthanized for small or large bowel strangulation, obstruction or distension were collected. Intestine from 5 horses (musculoskeletal group) which were humanely euthanized, due to acute or chronic orthopedic conditions including capital physis fracture (1), chronic bilateral degenerative suspensory desmitis (1), neoplasia of the right mandible (1), cervical vertebral malformation (1), and comminuted second phalangeal fracture (1) was sampled for comparison. Samples of small intestine, large colon, cecum and small colon were also obtained from 4 normal horses (control group) which had no clinical signs of gastrointestinal disease, had not received systemic medication for at least 6 months, and were donated for other reasons including behavioral problems (2), osteochondrosis of the left tarsocrural joint and behavioral problems (1), or cutaneous sarcoids (1).

Intestinal specimens were collected at surgery or at necropsy and consisted of one sample per designated region. Samples collected at surgery were full thickness biopsies from resected
bowel, from the enterotomy site in the small intestine or large colon or from the edge of bowel
segments that were incorporated in an anastomosis. When possible samples were taken at the
primary lesion and at sites distant to the lesion. Samples collected at necropsy were collected
immediately after death (within 5 minutes post lethal injection with concentrated pentobarbital)
from the primary lesion and at various sites proximal and distal to the lesion. For the nine horses
with no gastrointestinal disease, samples (one specimen from each site) were taken from the
duodenum, mid jejunum ileum, cecum, large colon and small colon. All samples were
immediately placed in 10% neutral buffered formalin, fixed for a minimum of 48 hours,
dehydrated, and then embedded in paraffin. Serial sections of each specimen were cut and
stained with hematoxylin and eosin (H&E) and with the TUNEL technique for apoptotic cells.

Apoptotic cells were detected by in situ DNA end labeling (TUNEL method) using the
Apoptag® In Situ Apoptosis Detection Kit (Intergen Co., Purchase, NY). All reagents used were
contained in the Apoptag® kit unless otherwise specified. Unstained tissue intestinal sections
were deparaffinized and transferred to phosphate buffered saline (PBS) for 5 minutes. The tissue
sections were pretreated with 20µg/ml Proteinase K (Sigma, St. Louis, MO), washed in dH2O,
quenched with hydrogen peroxide (3.0%), covered in 75µg/ml equilibration buffer and then
incubated with 55µg/ml terminal deoxynucleotidyl transferase (TdT enzyme) for 3 hours in a
humidified chamber. The reaction was terminated with stop wash buffer and the tissue sections
were incubated with 65µg/ml anti-digoxigenin conjugate for 30 minutes in a humidified chamber
and then washed with PBS. The tissue sections were stained with 75µg/ml diaminobenzidine
(DAB), washed and then counterstained with methyl green and mounted.

As a detection control for the TUNEL technique, sections of thymus were taken from
untreated (negative control) and mice treated with dexamethasone (positive control mice).
Dexamethasone is known to induce apoptosis in the thymic lymphocytes.\textsuperscript{14-16} Thymus samples were stained simultaneously with intestinal samples to ensure the staining technique was effective and that apoptotic cells were being detected. Stained slides were examined in a blinded manner and scored for the presence or absence of apoptotic cells (see below). Hematoxylin and eosin stained sections were reviewed to identify the specific cell types undergoing apoptosis in the various locations by comparison with TUNEL stained slides.

In each specimen the apoptotic cells located in the mucosa, circular muscle, longitudinal muscle and serosa were counted in three high power fields at 400X magnification. The mean number of apoptotic cells in each tissue layer was calculated for all samples. Samples were assigned to one of four diagnostic categories for statistical analysis- simple obstructed, strangulated, musculoskeletal and control. The data for the samples was log transformed to stabilize the variances. The data for the samples was back transformed for presentation. The MIXED procedure of the SAS system (ver. 8.2, SAS Institute Inc., Cary, NC 27513) was used to perform a split plot analysis of variance to test for effects of colic, intestine and tissue layer and their interactions. Gastrointestinal disease (colic) was the whole plot factor, type of intestine (small intestine or large intestine) was the subplot factor and tissue layer (mucosa, circular muscle, longitudinal muscle and serosa) was the subplot factor in the analysis of variance. Significant interactions were further investigated using the SLICE option to test for simple main effects. Simple contrasts were used to compare means with simple main effects. Small colon and cecum samples were not included in the analysis, because the number of these horses recovered from anesthesia did not include a representative number of these intestine types across all categories (including simple obstructed, strangulated, musculoskeletal and control categories).
Results

Samples were collected from 43 clinical cases and from four horses donated to the Veterinary Teaching Hospital. One intestinal sample was taken in 22 horses, and a sample from more than one location in the intestinal tract was taken from 25 of the horses. Of 106 samples collected, 50 were from the small intestine, 38 from the large intestine, 10 from the cecum, 4 from the small colon and 4 from the stomach. The numbers of intestinal sections examined in the disease categories are as follows: simple obstruction (14), peritonitis (4), strangulation obstruction (23), distant to strangulation (different intestinal segment) (12), distant to strangulation (same intestinal segment) (6), no gastrointestinal disease (chronic musculoskeletal) (8), distant simple obstructed (other intestinal segment) (2), adjacent to strangulated lesion (4), inflammatory (same intestinal segment) (1), no gastrointestinal disease (other) (6), control (no systemic medication) (24).

Control slides for the TUNEL procedure reacted properly and the procedure was considered to provide valid and reproducible staining of equine intestinal samples. Apoptotic cells were evident in all 4 layers of intestine, except in five horses with no clinical evidence of gastrointestinal disease (one in the musculoskeletal category and four in the control category). One slide was unreadable due to the very poor condition of the devitalized intestine. Apoptotic nuclei were observed in both the capillaries and connective tissue of the lamina propria, predominately at the tip of intestinal villi, and in the mucosal epithelial cells (Figures 1 & 2). Apoptotic nuclei were observed in circular (Figures 3 & 4) and longitudinal muscle. Apoptotic nuclei were also identified in the connective tissue of fascial planes between groups of muscle fibers. Schwann cells, glial cells or activated satellite cells and neurons were also apoptotic in five of the samples of intestine in horses with gastrointestinal disease(Figure 5); however the
number of affected cells in the ganglia was not counted. Apoptosis was rare in the serosa but did occur in the endothelial cells in the small vessels and in the mesothelial cells. (Figure 6) Apoptotic neutrophils in the serosa and other regions of intestine were uncommon. In the musculoskeletal group no apoptotic cells were identified in one horse with a left hind second phalanx fracture, but apoptotic cells were identified in the other four horses of this group. The horse with severe degenerative suspensory desmitis had generalized apoptosis; the horse with a chronic capital physis fracture had apoptosis only in the cecum; the horse with cervical vertebral malformation had apoptosis predominately in the large colon; whereas and one with a tumor on the mandible had apoptosis predominately in the small intestine. Very small numbers of apoptotic cells (no more than 10% of the number of apoptotic cells present in the horses with gastrointestinal disease) were identified in the samples from the four control horses.

Horses with gastrointestinal disease (colic) had a significantly greater number of apoptotic cells (P<0.007) when compared with the horses with musculoskeletal disease and the control horses. There was a statistically significant difference in the number of apoptotic cells in the different tissue layers (P<0.004) over all the categories. There was a significant three way interaction between colic, tissue and intestine was found (P<0.003). The test for simple main effects revealed a significantly higher number of apoptotic cells in the circular muscle of the intestine in horses with simple obstruction compared to the other tissue layers in the strangulation obstruction, musculoskeletal and control categories (P<0.009). (Figure 7) There was also a significantly higher number of apoptotic cells in the large colon compared with the small intestine for all categories (P<0.001). A significantly increased number of apoptotic cells were found in the intestine at a distant site to the primary lesion in the horses with strangulation obstruction compared to those with simple obstruction and compared to the intestine taken at the
primary strangulating obstruction lesion or simple obstruction lesion. (P< 0.019)(Figure 8) A trend for a higher number of apoptotic cells in the circular muscle compared to the other tissue layers was found in the horses with a simple obstruction or strangulating lesion of the intestine. (Figure 9)

**Discussion:**

Cellular death occurs in two biochemically and morphologically distinct ways; cellular necrosis and apoptosis. Apoptosis results from activation and transcription of specific genes. In many of the pathways that are initiated in apoptosis, permeabilization of the mitochondrial membrane is a critical event that results in release of various molecules from the mitochondrial intermembrane space. These molecules include procaspases (enzymes), cytochrome c (a caspase activator), Smac/Diablo (a caspase co activator) and an apoptosis-inducing factor. The apoptosis inducing factor activates the nucleases that cleave the DNA into small fragments. The DNA strand breaks that result from the endonuclease(s) activation can be labeled *in situ*, in individual fixed, permeabilized cells or tissues in sections, by the terminal deoxynucleotide transferase mediated dUTP nick end labeling (TUNEL) technique. In this technique, the 3’-OH termini that are generated as a result of the DNA fragmentation during apoptosis are labeled with modified nucleotides by terminyl deoxynucleotide transferase. This enzyme selectively detects apoptotic rather than necrotic cells and is more specific than DNA polymerase. There is a high correlation between TUNEL staining and apoptosis. The TUNEL technique had greater sensitivity when it was compared to the in situ nick translation (ISNT). A major advantage of the TUNEL technique is the ability to reveal early DNA breaks during apoptosis which occur before the light microscopic changes characteristic of apoptosis. The TUNEL
technique using the same kit as was used in this study has been very successful in detecting apoptosis of lymphocytes and neutrophils in the lamina propria of fixed human intestinal samples.\textsuperscript{13}

A potential concern of detecting apoptosis in formalin fixed tissue was that of random DNA damage with long term fixation, resulting in nonspecific labeling. This effect has been investigated in rat testicular tissue. Results indicated that formaldehyde is able to replace protons in the purine and pyrimidine bases but does not react with sugar hydroxyls or phosphate ester groups in nucleic acids.\textsuperscript{23} It is possible that with prolonged fixation any single-stranded regions of DNA, or ‘free ends’ are locked up and they are inaccessible to the polymerase.\textsuperscript{23} Any damage resulting from fixation or cytopathological mechanisms such as necrosis would not be detectable with this technique.\textsuperscript{23} Therefore there is the risk of a false negative result rather than a false positive result. Apoptotic cells may have been missed in this study, but the positively stained cells are unlikely to be the result of any mechanism other than apoptosis.

There were significantly higher numbers of apoptotic cells in the circular muscle from the intestine in horses with simple obstructive gastrointestinal disease compared to the control and musculoskeletal groups. The difference in the pathophysiology of simple obstruction and strangulation may explain increased apoptosis found in intestine during simple obstruction. The mitochondrial PT pore can initiate apoptosis or necrosis, and the induction of the pore in a sequential fashion, allows for the activation and action of proteases which will stimulate apoptosis before ATP depletion results in cell death.\textsuperscript{24} However when the pore is activated rapidly, as in ischemia, the cellular metabolism may be severely compromised and necrosis will occur before the apoptosis-inducing proteases are activated.\textsuperscript{24} Caspase activation and the intracellular ATP content are critical factors which determine whether a cell will undergo
ATP is required for activation of cytochrome c which induces apoptosis.\textsuperscript{24} If ATP is decreased, thereby inhibiting caspases which activate the apoptotic process, cells will undergo necrosis.\textsuperscript{24} If glycolytic substrates can provide an alternative source of ATP, then apoptosis is likely to occur. Though speculative, intestinal hypoxia during strangulation may cause this intracellular mechanism to induce more cells to undergo necrosis, whereas in the case of simple obstruction, inflammatory mediators may induce apoptosis when there is sufficient ATP. This is not an all or nothing event as renal apoptosis after ischemia is induced by hypoxia\textsuperscript{25} and ATP depletion\textsuperscript{26}.

Apoptosis is a physiologic mechanism that allows cells to be eliminated without associated tissue inflammation. In the intestine apoptosis plays a role in the maintenance of normal gastrointestinal homeostasis\textsuperscript{27} and apoptotic cells are ingested by phagocytic cells or are shed into the lumen\textsuperscript{28}. While apoptosis occurring as part of the intestine’s normal function should not cause inflammation, excessive apoptosis has been shown experimentally to cause inflammation. For example in the rat, occasional apoptosis in the intestine does not cause an alteration in the intestinal barrier integrity, however when intraperitoneal doxycycline is administered there is an increase in mucosal apoptosis and a concomitant increase in epithelial and endothelial permeability.\textsuperscript{12} Apoptosis is considered an important source of inflammation in the kidney.\textsuperscript{29} During severe forms of glomerulonephritis apoptosis of the glomerular endothelial cells may contribute to the development of glomerulosclerosis.\textsuperscript{30} In a murine model administration of antiapoptotic agents at the time of reperfusion of the kidney following ischemia prevented the early onset of apoptosis, inflammation and tissue injury.\textsuperscript{10} Glutamine deprivation induced apoptosis in the rat intestinal epithelial cells and this subsequent disruption in homeostatic balance between cell proliferation and cell death was proposed to contribute to gut
atrophy. The significant increase in numbers of apoptotic cells in the circular muscle of intestine in horses with simple obstruction, and in intestine distant to a primary strangulating obstruction lesion, suggests the apoptosis may be associated with intestinal inflammation.

It was interesting that there were significantly higher numbers of apoptotic cells in the circular muscle of the intestine taken distant to the site of a strangulating lesion compared to that taken distant to a simple obstruction lesion or at the site of either type of lesion. As an example high numbers of apoptotic cells were present in the large colon of horses with a strangulating obstruction of the small intestine. Increases in apoptotic cells at distant sites may be a result of systemic inflammatory mediators resulting from the inflammation during ischemia and reperfusion. Many mediators such as cytokines, growth factors, chemotactic peptides, hypoxia, and acidosis which can activate leukocytes and can initiate cell apoptosis. For example in human patients following elective surgery the interleukin 6 (IL-6) was notably increased at 24 hours after surgery and at this concentration inhibited neutrophil apoptosis. This effect was attenuated with the addition of recombinant human interleukin 10 (IL-10). The authors from this study concluded that imbalances of pro-inflammatory and anti-inflammatory cytokine release affected the apoptotic capacity of the plasma. Ischemia/reperfusion following occlusion of the superior mesenteric artery in the rat small intestine has been shown to induce apoptosis in the rat jejunum and ileum. The exact mechanism of ischemia/reperfusion induced apoptosis in this rat small intestine was not explained as the regulation of intestinal cell death and proliferation is highly complex and is likely to be controlled by a variety of factors. Since free radicals have been shown to cause apoptosis, it is reasonable to hypothesize that free radicals produced during reperfusion in the equine intestine may stimulate apoptosis.
Another possible explanation of the significantly increased apoptotic cells distant to a strangulating lesion in intestine from horses undergoing surgery may be the effect of surgical manipulation of the intestine. The intestine is known to be susceptible to stress at surgery and studies have demonstrated that surgical trauma induces oxidative stress and damage through enhanced production of reactive oxygen species.\(^{38}\) Surgical stress induced in the rat intestine (induced by opening the abdominal wall and handling the intestine as done during exploratory laparotomy) was shown to induce and activate protease activity in the villus and crypt cells.\(^{39}\) Both the mitochondrial and cytosolic proteases were activated in this study and free radicals generated by xanthine oxidase were proposed as possible mediators of protease activation after surgical stress in the intestine.\(^{39}\) The increased protease activity observed in the mitochondria may be responsible for the mitochondrial permeability transition and mitochondrial dysfunction leading to apoptosis.\(^{39}\) The intestine is highly susceptible to surgical manipulation at remote locations and mild handling of intestine following laparotomy has been shown to cause oxidative stress in the enterocyte and functional alterations in the intestine.\(^{40}\)

There was trend for there to be higher numbers of apoptotic cells in the circular muscle of the intestine from horses with either a simple obstructive or strangulating lesion. If the apoptosis is associated with an increased inflammatory response in the muscle, it could affect the contractility of the muscle. Contractility of equine intestine has been reduced experimentally by inhibition of the circular muscle with nitric oxide in both jejunum\(^ {41}\) and ventral colon. Recently muscle injury (measured by creatinine kinase) resulting from 90-120 minutes of ischemia was reduced by the pancaspase inhibitor z-VAD.\(^ {10}\) This suggests that apoptosis which is blocked by pancaspase inhibitor, is responsible for or associated with muscle inflammation and subsequent injury.\(^ {10}\) A better understanding of the relationship of intestinal muscle cell apoptosis and
intestinal inflammation may help development of new therapy to attenuate apoptosis in intestinal muscle.

The locations of the different types of apoptotic cells in the intestine were recorded; however the number of cells in the specific regions of the four intestinal layers was not quantitated. Apoptotic cells were observed in the mucosa and large numbers were located within the connective tissue of the lamina propria at the tip of the intestinal villi. Apoptosis has been proposed to be associated with the shedding of the cells from the villus tips in the small intestine and has been induced in the mucosa of the ileum and jejunum of rats following occlusion of the superior mesenteric artery.\textsuperscript{34} In these rats apoptotic cells were most evident at the villus tips.\textsuperscript{34} The apoptosis, which increased during the ischemic period in the rat small intestine, was maximal after one hour of reperfusion and then significantly decreased after only 6 hours indicating, it was a transient event.\textsuperscript{34} Apoptosis was also observed in the muscle fibers of the longitudinal muscle and within the fascial planes between the groups of muscle fibers. Further investigation is required to determine whether this apoptosis in the muscle is detrimental to motility.

Apoptosis of schwann cells, glial cells or activated satellite cells and neurons in the myenteric plexus also suggests a possible relationship of apoptosis and bowel function. Since the myenteric plexus is important for functional motility and for other gastrointestinal functions such as secretion, apoptosis of cells in the enteric nervous system may alter intestinal function.\textsuperscript{42}

Few apoptotic cells were observed in the serosa even though ischemia and reperfusion, and the intraluminal distention and decompression, have been reported to cause severe morphologic changes in the equine jejunal serosa.\textsuperscript{7} Both mesothelial cells and endothelial cells were apoptotic in the serosa but appeared to be few in number compared to other layers. In this
study both the musculoskeletal and gastrointestinal disease groups of horses had large numbers of apoptotic cells compared with the control group. Three of the five horses in the musculoskeletal group had been treated with phenylbutazone for greater than or equal to 14 days prior to euthanasia. Because phenylbutazone has been shown to cause an increase in apoptosis \textit{in vitro} in the equine right dorsal colon \cite{43}, the apoptosis present in this musculoskeletal group of horses may have been related to the systemic inflammation or medication. The horses with gastrointestinal disease in this study all received flunixin meglumine at variable times prior to sample collection. Though it may be possible for flunixin meglumine to induce apoptosis in the intestine of treated horses, this does not explain the differences in the number of apoptotic cells observed between simple obstructed and strangulated intestine or those differences observed cells from the primary lesion to those at a distant site. Further investigation is required to determine the effect of non steroidal anti-inflammatory drugs on apoptosis in the equine intestine.

In summary the TUNEL technique is an effective method for detecting apoptotic cells in the equine intestine. The large numbers of apoptotic cells in the intestine of horses with gastrointestinal disease may be important in the initiation of inflammation and subsequent intestinal injury. The significant increase in apoptotic cells in intestine distant to strangulating lesions may be very important clinically in understanding the post-operative response of horses and possible complications following diseases causing ischemia. Specifically the increased apoptosis in circular muscle and enteric nervous tissue in intestine which is not removed at surgery may be important in post-operative bowel function. However further investigation is required to understand the apoptotic events during gastrointestinal injury and to determine if
apoptosis is related to changes in intestinal function and inflammation seen with ischemia and reperfusion.

References:


8. White NA. Pathophysiology and principles of therapy: Pathophysiology of obstruction, strangulation and strangulation/obstruction ischemia. In: P. T. Colahan, I. G.


Figure legends:

Figure 1: Photomicrograph of the mucosa in the small intestine from a horse with gastrointestinal disease. All dark staining nuclei represent apoptotic cells. Healthy or necrotic cells did not stain. Diaminobenzidine (DAB) stain and methyl green counterstain; bar equals 50µ.

Figure 2: Photomicrograph of the mucosa in the small intestine from a horse in the control group. No apoptotic nuclei are present. Diaminobenzidine (DAB) stain and methyl green counter stain; bar equals 50µ.

Figure 3: Photomicrograph of the circular muscle in the small intestine from a horse with gastrointestinal disease. All dark staining nuclei represent apoptotic cells. Healthy or necrotic cells did not stain. Diaminobenzidine (DAB) stain and methyl green counterstain; bar equals 50µ.

Figure 4: Photomicrograph of the circular muscle in the small intestine from a horse in the control group. No apoptotic nuclei are present. Diaminobenzidine (DAB) stain and methyl green counter stain; bar equals 50µ.

Figure 5: Photomicrograph of a myenteric plexus in the large colon. All dark staining nuclei represent apoptotic cells. Healthy cells or necrotic cells did not stain. Neurons, glial cells and Schwann cells were apoptotic in this myenteric plexus. Diaminobenzidine (DAB) stain and methyl green counterstain; bar equals 50µ.
Figure 6: Photomicrograph of the serosa from the large colon. All dark staining nuclei are apoptotic mesothelial cells on the surface of the serosa. Healthy cells or necrotic cells did not stain. Diaminobenzidine (DAB) stain and methyl green counterstain; bar equals 50µ.

Figure 7: Graph of the mean numbers of apoptotic cells in the intestine from horses in all 4 categories - simple obstruction, strangulation, musculoskeletal, control. A significant increase in apoptotic cells was present in the simple obstruction category (P< 0.0088) which is indicated by the star (*). Columns represent the geometric mean number of apoptotic cells while error bars represent the 95% confidence intervals.

Figure 8: Graph of the mean number of apoptotic cells in intestine taken from the primary lesion or at a distant site in horses with simple obstruction and strangulation. A significant increase in apoptotic cells was present at a distant site to a strangulation lesion (p< 0.0186) which is indicated by the star (*). Columns represent the geometric mean number of apoptotic cells while error bars represent the 95% confidence intervals.

Figure 9: Graph of the mean number of apoptotic cells in the four tissue layers from horse with simple obstruction or strangulation. Columns represent the geometric mean number of apoptotic cells while error bars represent the 95% confidence intervals.
FIGURES

Figure 1: Jejunal mucosa with apoptotic cells from horse with GI disease

Figure 2: Jejunal mucosa with no apoptosis from control horse
Figure 3: Jejunal circular muscle with apoptotic cells from horse with GI disease

Figure 4: Jejunal circular muscle with no apoptosis from control horse
Figure 5: Apoptotic cells in myenteric plexus from horse with GI disease

Figure 6: Apoptotic cells in serosa from horse with GI disease
Figure 7: Graph of number of apoptotic cells in intestine from horses in the 4 categories of disease.
Figure 8: Graph of number of apoptotic cells in intestine taken from the primary site and distant to the site of strangulating and simple obstructive lesions.
Figure 9: Graph of number of apoptotic cells in the 4 layers of intestine over all disease categories.
Figure 10: Thymus stained for apoptotic cells from mouse not treated with dexamethasone

Figure 11: Thymus stained for apoptotic cells from mouse treated with dexamethasone
Figure 12: Jejunal circular muscle stained for apoptosis with DAB on the left and with H&E on the right to allow identification of apoptotic nuclei.
TABLES

Table 1: The ANOVA for split-plot with colic, intestine, and tissue as factors.
Results of Type 3 Tests of Fixed Effects.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colic</td>
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<tr>
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Table 2: The ANOVA for split-plot with colic, intestine, and tissue as factors.
Results of The Mixed Procedure Tests of Effect Slices.

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<th>Intestine</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr&gt;F</th>
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</thead>
<tbody>
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<td>Mucosa</td>
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<tr>
<td></td>
<td>Simple obstructed</td>
<td>Circular muscle</td>
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<td>160</td>
<td>7.04</td>
<td>0.0088</td>
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<tr>
<td></td>
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<td>Longitudinal muscle</td>
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<td>2.47</td>
<td>0.1180</td>
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<tr>
<td></td>
<td>Simple obstructed</td>
<td>Serosa</td>
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<td>Small intestine</td>
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<td>1.06</td>
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<tr>
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<td>Simple obstructed</td>
<td>Large intestine</td>
<td>3</td>
<td>160</td>
<td>5.99</td>
<td>0.0007</td>
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</table>
Table 3: The ANOVA for split-plot with affected (code for intestine at primary site of the lesion compared with intestine at a distant site), colic, intestine, and tissue as factors. Results of the Mixed Procedure Tests of Effect Slices.

<table>
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<th>Den DF</th>
<th>F Value</th>
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<td>1</td>
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CONCLUSION

Apoptotic cells were detected in the equine intestine using the TUNEL method. This method was successful in detecting the apoptotic nuclei which stained a distinctive brown color. Horses with gastrointestinal disease had a greater number of apoptotic cells compared to horses with no gastrointestinal disease. Horses with simple obstructive disease had a higher number of apoptotic cells in the circular muscle compared to the other tissue layers in horses with strangulation or no gastrointestinal disease. There was also a higher number of apoptotic cells in the large colon compared to the small intestine for all the samples. There was a trend for a higher number of apoptotic cells in the circular muscle of intestine over all. This finding of increased apoptosis in the presence of disease may be of clinical importance as apoptosis has been shown to be associated with inflammation and tissue injury in other species both in the gastrointestinal system and other body systems.

There were a significantly higher number of apoptotic cells found in intestine distant to the primary lesion in horses with strangulating obstruction compared to those with simple obstruction and compared to the intestine taken at the primary strangulating obstruction lesion or simple obstruction lesion. This indicated that intestine which is not directly affected by a lesion has increased apoptosis which may be the result of systemic inflammatory mediators or free radical production. This is may be important clinically as the intestine which remains following a resection anastomosis for a strangulating lesion has increased apoptosis based on this finding. Further investigation is required to determine if increased apoptosis causes inflammation and tissue injury and affects intestinal function. As apoptosis can be controlled at the mitochondrial level there may be potential therapeutic implications for the future. Apoptosis and the associated inflammation has been inhibited in human smooth muscle and bovine and murine kidney.
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