Apoptosis in the equine small intestine following experimental ischemia-reperfusion injury

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ABSTRACT

This study was aimed at characterizing the apoptotic response equine small intestine subjected to experimental ischemia-reperfusion injury and determining if use of an angiotensin converting enzyme inhibitor (enalaprilat) would ameliorate the apoptotic response. It was designed to determine if mucosal epithelial cells undergo apoptosis during the ischemia phase and also examined if apoptosis is significantly exacerbated by reperfusion. It also investigated whether administration of enalaprilat decreased reperfusion injury secondary to reduced enterocyte apoptosis. Injury was induced using a low flow model of I-R. During celiotomy a single loop of jejunum was isolated and arterial flow decreased to 20% of baseline for one hour and complete occlusion for a second hour. Reperfusion was monitored for 3 hours. A control group (n=6) were not treated while the treatment group (n=6) received 0.5 mg/kg enalaprilat in 0.9% NaCl immediately following ischemia. Jejunal samples were taken prior to the induction of ischemia, immediately post-ischemia and at 1, 2 and 3 hours of reperfusion. Samples were evaluated for gross tissue pathology with standard hematoxylin and eosin staining, the presence of apoptotic cells via TUNEL staining, and gene expression of three apoptosis related genes (bax, bcl-2, p53) using qPCR. Serum enalaprilat and ACE concentrations were determined from blood samples drawn concurrent with jejunal sampling using HPLC/MS and standard HPLC. Plasma enalaprilat concentrations were comparable to previous reports in awake horses. Enalaprilat appeared to have no effect on serum ACE concentrations, however a significant spike in ACE concentration occurred in the treatment group at 1 hour of reperfusion (P=0.0001). Grade of mucosal damage was not significantly different between control and treatment groups at any time point. Subjectively apoptotic index appeared to be higher in the treatment group at end ischemia and during reperfusion. There were no changes in expression of p53 or bcl-2 in either group. Bax expression was significantly decreased (P= 0.02) in the control group at 2 hours of reperfusion. Based on our data administration of an ACE inhibitor during anesthesia in horses with an ischemic segment of intestine confers no protective benefit and may be associated with increased intestinal injury and apoptosis. Lack of expression of p53, bax and bcl-2 suggests another apoptotic mechanism in equine ischemic intestine.

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ATTRIBUTION

Several colleagues and coworkers aided in the writing and research behind this thesis. A brief description of their background and their contributions are included here.

Nathaniel A. White II, DVM, MS, DACVS- is the primary Advisor and Committee Chair. Drs. White and Crisman were responsible for the development of the research project and the acquisition of grant money. Dr. White developed the surgical model used during the study and assisted during the surgical procedures undertaken at the Marion Dupont Scott Equine Medical Center. Dr. White also was responsible for reading and interpretation of the TUNEL stained tissue sections.

Mark V. Crisman, DVM, MS, DACVIM- is co-Chair and secondary advisor. Dr. Crisman also provided extensive administrative and laboratory support.

Jolynne R. Tschetter, Ph.D.- is the principal supervisor for all the laboratory work and was responsible for the development of various methods used during the study.

Laura Jill McCutcheon DVM, DACVP, Ph.D.- is responsible for evaluating and grading the histology slides.

Bernard Jortner, VMD, MS, DACVP, Ph.D.- is responsible for developing the grading system used for evaluating the histology slides and also for interpreting the TUNEL slides.

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LIST OF ABBREVIATIONS

SISO: small intestinal strangulating obstruction
SIRS: systemic inflammatory response
MODS: multi-organ dysfunction syndrome
DIC: disseminated intravascular coagulation
I-R: ischemia-reperfusion
ROS: radical oxygen species
OH: hydroxyl radical
$ONOO^-$: peroxynitrite
H_2O_2: hydrogen peroxide
NO: nitric oxide
eNOS: endothelial nitric oxide
iNOS: inducible nitric oxide
nNOS: neuronal nitric oxide
O_2^-: superoxide radical
SOD: superoxide dismutase
GPX: glutathione peroxidase
GSH: glutamylcysteinylglycine
R-S-NO: S-nitrosothiol
ATP: adenosine triphosphate
XD: xanthine dehydrogenase
XO: xanthine oxidase
AcH: acetylcholine
PAF: platelet activating factor
ICAM-1: intercellular adhesion molecule-1
VCAM-1: vascular cell adhesion molecule-1
TNF-α: tumor necrosis factor-alpha
IL-1β: interleukin-1beta
DED: death effector domain
CARD: caspase recruitment domain
DD: death domain
PARP: poly(ADP-ribose) polymerase
IAP: inhibitor of apoptosis proteins
FADD: Fas-associated death domain
DISC: death-inducing signaling complex
TNF-R1: TNF receptor-1
NF-κB: nuclear factor kappa-B
MOMP: mitochondrial outer membrane permeabilization
IMS: intermembrane space
PUMA: p53-up-regulated modulator of apoptosis
Apaf-1: apoptosis protease-activating factor
PTP: permeability transition pore
BH: bcl-2 homology
ER: endoplasmic reticulum
IEL: intra-epithelial lymphocytes
ACE: angiotensin converting enzyme
ACE-I: ACE inhibitor
PAF-AH: PAF-acetylhydrolase
RAS: renin-angiotensin system
ANG I: angiotensin I
ANG II: angiotensin II
JG: juxtaglomerular
BK: bradykinin
AT-1R: angiotensin-1 receptor
AT-2R: angiotensin-2 receptor
BK-R2: bradykinin-2 receptor

INTRODUCTION

Gastrointestinal disease is the number one cause of death in horses following old age and injury.[1] Small intestinal strangulating obstruction (SISO) represents one of the most devastating forms of colic and despite continuing advances in surgical and anaesthetic management, case mortality remains high. It is well recognized that horses with simple obstructions of the small intestine have a better prognosis than those with strangulating lesions, although evidence exists that even horses with non-strangulating forms of colic may still suffer from ischemic intestinal damage.[2,3] Evaluation of the pattern of post-operative survival also shows that the highest mortality is associated with the first few post-operative days with continuing mortality rates at a much lower level.[4,5] The most common complications reported in the immediate post-operative period consist of ileus (18.2%), persistent pain (32.1%) and endotoxic shock (13.9%).[6]

It is also evident that horses suffering from SISO are at significantly increased risk for post-operative complications secondary to the development of the systemic inflammatory response syndrome (SIRS), multiorgan dysfunction syndrome (MODS), disseminated intravascular coagulation (DIC) and non-responsive shock.[7] The exact mechanisms underlying the development of this uncontrolled systemic inflammation remain unclear but both systemic endotoxemia and intestinal ischemia-reperfusion (I-R) injury are believed to play a role.

In adult horses primary sepsis is rare. The inciting causes of shock in adult equine patients are usually related to intestinal inflammation or ischemia due to the effects of primary gastrointestinal disease.[8] Historically the initiation of these processes in the post-SISO patient has been attributed to compromise of intestinal barrier function that leads to translocation of bacterial endotoxin from the intestinal lumen into the systemic circulation. Circulating bacterial endotoxin then mediates the activation of local and systemic leukocytes as well as the production of a variety of inflammatory cytokines.[8] Neutrophil activation may then

lead to generalized activation of humoral inflammatory mediators that ultimately provoke uncontrolled systemic inflammation.

It is currently accepted that bacterial endotoxins can cross the compromised intestinal mucosa, enter the systemic circulation and provoke shock. However, the role of ischemia-reperfusion injury and its involvement in the development of systemic shock during naturally occurring gastrointestinal disease in horses is still unclear. Although the underlying mechanisms of I-R injury in laboratory animals have been well documented, controversy still exists as to whether these same events or other alternative pathophysiologic processes are responsible for the effects of equine I-R injury. Small intestinal ischemia in horses is usually secondary to entrapped bowel resulting in direct arterial and venous occlusion, or volvulus that increases venous backpressure on capillaries.[9] The intestinal mucosal epithelium is highly energy dependent and reduced blood supply and oxygenation results in rapid injury and death. If blood flow returns to tissue before irreversible cell death certain biochemical events occur immediately on reperfusion. Reperfusion injury is then initiated by several mechanisms that create an inflammatory response.[9]

OBJECTIVES/HYPOTHESES

1. Establishing that apoptosis occurs in the equine small intestine following experimental I-R injury

2. Use of an ACE-I during experimental ischemia-reperfusion will ameliorate enterocyte apoptosis and reduce intestinal damage

3. Characterizing the apoptotic response in treated and untreated intestine following I-R injury will help determine if treatment with an ACE-I can facilitate recovery of equine mucosa

4. Identifying expression levels of some apoptosis related genes will lead to a clearer understanding of some of the molecular events involved in I-R injury

LITERATURE REVIEW

ISCHEMIA-REPERFUSION INJURY

Ischemia is defined as tissue hypoxia secondary to decreased blood flow. The resulting decrease in oxygen delivery causes a depletion of cellular energy stores, disrupts tissue homeostasis and initiates a cascade of metabolic events that are then activated during reperfusion. Reperfusion injury is the damage sustained by tissues on restoration of blood flow following a period of ischemia. The severity of damage depends on both the degree and duration of the ischemic event. During partial ischemia of moderate duration, known as low-flow ischemia, the majority of damage occurs following reperfusion. After complete ischemia or prolonged duration such massive injury is sustained during the ischemic phase that additional damage during reperfusion becomes inconsequential. In horses, 3-4 hours of complete arteriovenous occlusion results in irreversible mucosal necrosis. Although still not completely elucidated, current understanding of I-R injury is based on the premise that it is a complex cascade of interrelated pathophysiologic events that is initiated by endothelial dysfunction and includes production and release of radical oxygen species (ROS) from endothelial cells, the damaged intestinal mucosa and resident and circulating neutrophils. Studies performed in rats show that production of ROS leads to lipoperoxidation and the activation of neutrophils, platelets, cytokines, the coagulation system, vascular endothelium and the xanthine-oxido-reductase enzyme system.[10-12] Programmed cell death, or apoptosis, is a form of cellular elimination without uncontrolled release of cellular contents and subsequent inflammatory response. There is increasing evidence that apoptosis of intestinal mucosal epithelial cells may represent another significant source of intestinal damage following I-R injury.[13,14]

Historically, intestinal I-R injury has been largely attributed to cellular necrosis but recent evidence also implicates apoptosis. Studies in experimental models of intestinal I-R injury in rats and mice have demonstrated that the number of apoptotic cells significantly increases after reperfusion and peaks at 3

hours in all intestinal layers.[10] However, the relationship between I-R induced apoptosis in intestinal cells and injury mechanisms involved in signal transduction are still unclear.[15] Apoptotic cells have been recently identified in the mucosa, circular muscle, longitudinal muscle and serosa in clinical cases of equine strangulating obstruction.[9]

There are several proposed events underlying equine small intestinal I-R injury, the first of which involves an influx of calcium into cells and redistribution of intracellular calcium stores. This results in an increase in free, biologically active, calcium that then alters mitochondrial respiratory function and energy metabolism. Derangement of the electron transport chain and depletion of ATP in the absence of available oxygen work in conjunction with free calcium to generate ROS, trigger lipoperoxidation and thus destroy membrane structures, including plasma and mitochondrial membranes. The "no reflow" phenomenon is also believed to play a significant role not only in direct damage to the endothelium, but also in the perpetuation of vascular dysfunction that can then lead to further ischemia. I-R injury is known to induce upregulation of intercellular adhesion molecules on both activated neutrophils and endothelial cells. Neutrophil adherence to the endothelium then leads to further vascular insult setting up a vicious cycle of microvascular disturbance. Research in both rats and horses indicates that the most likely scenario involves some combination of all of these mechanisms.[9,11]

Radical oxygen species are believed to play a central role in reperfusion injury. A free radical is a molecule with one or more unpaired electrons in its outer shell that is capable of reacting with all biological molecules including nucleic acids, proteins, carbohydrates and lipids, with lipids as the most frequent target.[12] Under normal biological conditions the major source of cellular ROS is leakage from the mitochondrial electron transport chain. Normally oxygen is reduced to water via tetravalent reduction by cytochrome c, a small heme protein found in the mitochondria. One to two percent of oxygen escapes this path and undergoes univalent reduction, generating ROS.[11] This leakage is kept to a minimum by the high

efficiency of electron transfer and sequestration of metal ions. Within the cell separate microenvironments exist for the mitochondria, lysosomes and peroxisomes with each containing a ROS generating system that is coupled immediately adjacent to antioxidant defense mechanisms. This compartmentalization may be the most important factor in the cell's endogenous defense against free radicals.[12] During reperfusion excessive concentrations of ROS develop and elicit oxidative stress.[11]

During oxidative stress, free radicals react with non-radicals in the cell triggering loss of an electron from the non-radical which is then itself transformed into a free radical. Interaction of these ROS and lipids in the presence of free iron initiates a chain reaction that causes lipid peroxidation. Lipid peroxidation not only damages cell membranes, but also deranges enzyme systems and receptors, alters ion channels, increases membrane permeability to calcium and other ions, initiates inflammation, causes inactivation of thiol-containing enzymes and triggers apoptosis.[12]

There are 2 major radicals that initiate lipid peroxidation, the hydroxyl radical (OH) and peroxynitrite ($ONOO^-$). The hydroxl radical is formed when hydrogen peroxide (H_2O_2) combines with metals (usually iron). Peroxynitrite forms when nitric oxide (NO) combines with the superoxide radical (O_2^-).[12] Cells have 3 primary mechanisms of antioxidant defense. The first line of defense is thiol-reducing glutathione peroxidase, a sulfur containing tripeptide that is synthesized in all mammalian cells and reduces H_2O_2 to water using glutamylcysteinylglycine (GSH) as a substrate. Oxidative stress is known to be associated with depletion of cellular GSH. Superoxide dismutase (SOD) represents the second line of defense. SOD is an oxidoreductase that contains copper, zinc or manganese at its active site and is present in the cytosol, mitochondria and on the extra-cellular surface. The final major antioxidant defense mechanism is catalase, a heme protein located in peroxisomes that converts H_2O_2 to water and oxygen. Catalase functions in conjunction with SOD, whereby SOD converts superoxide to H_2O_2 and then catalase converts H_2O_2 to water and oxygen.[12]

In both rat and mouse models, radical oxygen species are directly implicated in intestinal apoptosis following I-R injury. Several mechanisms of ROS induced apoptosis have been proposed but no integrated model currently exists. One proposed theory is that H_2O_2 acts on the mitochondria, disrupts membrane potential and triggers release of cytochrome c. A second possibility is that H_2O_2 induced apoptosis involves upregulation of the Fas/FasL system. Lastly H_2O_2 may directly modulate some transcription factors. For example, H_2O_2 could cause nuclear translocation of p53 that, once activated, may result in transcription of pro-apoptotic genes or expression of inhibitors of survival-related proteins.[16]

Although free radicals can induce apoptosis it is apparent that various levels of the same compounds may cause different outcomes. Apoptosis can be switched to necrosis by two possible mechanisms, either through inactivation of cellular caspases due to oxidation of their active thiol group or alternatively S-nitrosylation can lead to necrosis-like death in fatally damaged cells. Caspases contain an active site cysteine nucleophile that is vulnerable to oxidation or thiol alkylation. Caspases function best in a reducing environment and any change in these conditions in an injured cell could negatively affect caspase activity and render them inactive.[16] S-nitrosylation is the transfer of the NO group to a cysteine sufhdryl to form an S-nitrosothiol (R-S-NO). S-nitrosylation regulates the physiological and biological activity of many proteins such as enzymes, ion channels, G-proteins and transcription factors. Thus NO may function as a molecular switch to control protein function via reactive thiol groups. Exposure to NO or activation of inducible NOS (iNOS) is also reported to inhibit apoptosis in some cell types including, lymphocytes, endothelial cells, eosinophils and some neurons. NO mediated inhibition of apoptosis is probably due to direct inhibition of caspase activity through S-nitrosylation of the active site cysteine.[16]

During ischemia in horses, numerous biochemical events occur that set the stage for reperfusion injury on restoration of blood flow (Figure 1). The severity of this injury is determined by the magnitude and length of the initiating ischemic insult.[12] Ischemic cells cannot generate adequate ATP and enter into anaerobic metabolism. During anaerobic metabolism cells degrade existing ATP to adenosine which is then converted to inosine and finally hypoxanthine. Hypoxanthine accumulates within the cell and intracellular pH decreases secondary to accumulation of lactate and the hydrogen ion. Decreased ATP also inactivates membrane pumps and allows efflux of potassium and influx of sodium, calcium and chloride all resulting in acute cellular swelling.[12]

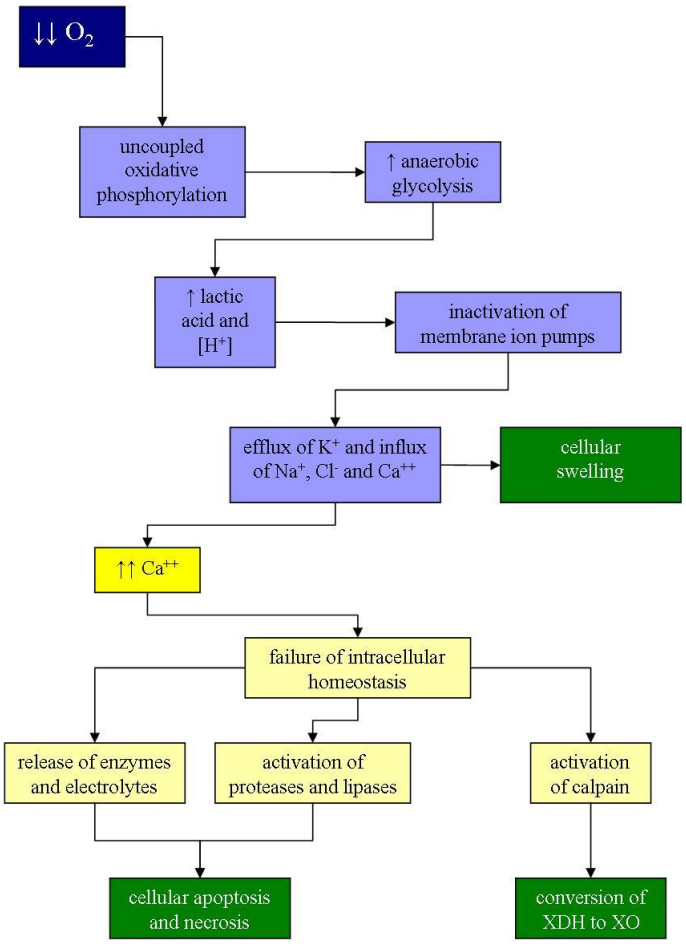

Figure 1: Early intracellular events during ischemia

An increase in intracellular calcium is one of earliest events in I-R injury and can serve as the trigger for both apoptosis and necrosis. This increase in calcium activates the protease calpain which then converts xanthine dehydrogenase (XD) to xanthine oxidase (XO). Xanthine dehydrogenase does not require oxygen and under normal conditions converts hypoxanthine to xanthine and then xanthine is converted to uric acid and superoxide. During ischemia activation of calpain triggers the conversion of XD to XO. Xanthine oxidase is an oxygen dependent enzyme and cannot function in the face of ischemic oxygen deprivation. This essentially creates a roadblock with the resultant accumulation of XO and hypoxanthine.[12]

The equine endothelium and small intestinal villus mucosa have the highest concentration of XO in the body and are highly susceptible to I-R injury [12]. XO activation appears to be a key event in post-ischemic mucosal injury in the small intestine.[9] Following reperfusion oxygen combines with XO and water and converts hypoxanthine to uric acid and superoxide. This burst of ROS formation occurs within 10-30 seconds after reperfusion. The superoxide molecule itself is not very damaging but is a source of H_2O_2. Endogenous SOD converts superoxide to H_2O_2 which can then combine with free iron to form the hydroxyl radical. Although iron is normally sequestered by binding with hemoglobin and myoglobin in muscle, transferrin in plasma and ferritin within the cell, during ischemia intracellular iron bound to ferritin is released and H_2O_2 mobilizes iron from heme. The hydroxyl radical is then generated from the interaction of superoxide and H_2O_2 via the iron dependent Haber-Weiss reaction ($O_2^- + H_2O_2 \rightarrow OH + O_2 + OH^-$) which cannot occur in biological systems without a metal catalyst (Figure 2).[12] The hydroxyl radical is highly destructive and one of the most potent oxidizing agents known. It is capable of reacting with almost every molecule of the living cell. Formation of OH^- initiates a chain reaction of lipid peroxidation that causes loss of selective membrane permeability, damage to DNA, degradation of structural proteins and loss of membrane-bound enzyme activity.[12]

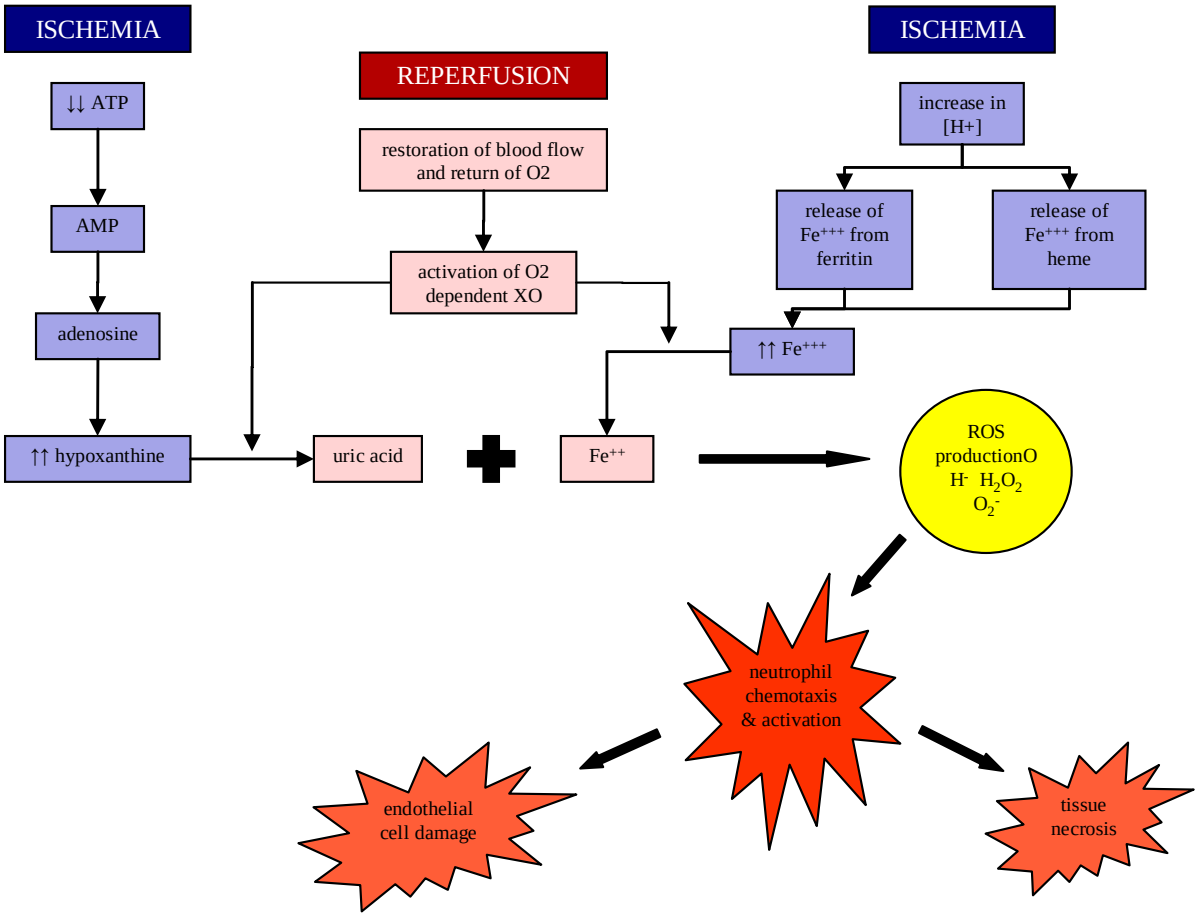

Figure 2: Cellular consequences of ischemia-reperfusion injury

Research from numerous species including rats, mice and felines indicate that endothelial cells are the primary initiator of reperfusion injury.[9] Reperfusion is associated with severe endothelial dysfunction that is probably secondary to ROS damage, decreased NO release from endothelial cells and increased endothelin causing marked vasoconstriction that further impairs blood flow to tissues (Figure 3). Thus, although reperfusion is necessary for cellular salvage it paradoxically causes more injury than ischemia.[12]

Endothelial cells have numerous specialized functions involving regulation of blood pressure, vascular permeability, vascular tone, inflammatory cell adhesion, coagulation and platelet aggregation. The endothelium regulates blood flow and tissue perfusion by modifying smooth muscle tone through a balance of endothelial derived vasoconstrictors and vasodilators. The response of vascular smooth muscle depends on the agent, the condition of smooth muscle and a functional endothelium. For example, acetylcholine (AcH) stimulates muscarinic receptors on intact endothelial cells to stimulate release of NO, causing

vasodilation. The endothelium can also release prostacyclin to cause vasodilation and inhibit platelet aggregation or it can release endothelin and other eicosanoids to trigger vasoconstriction.[9]

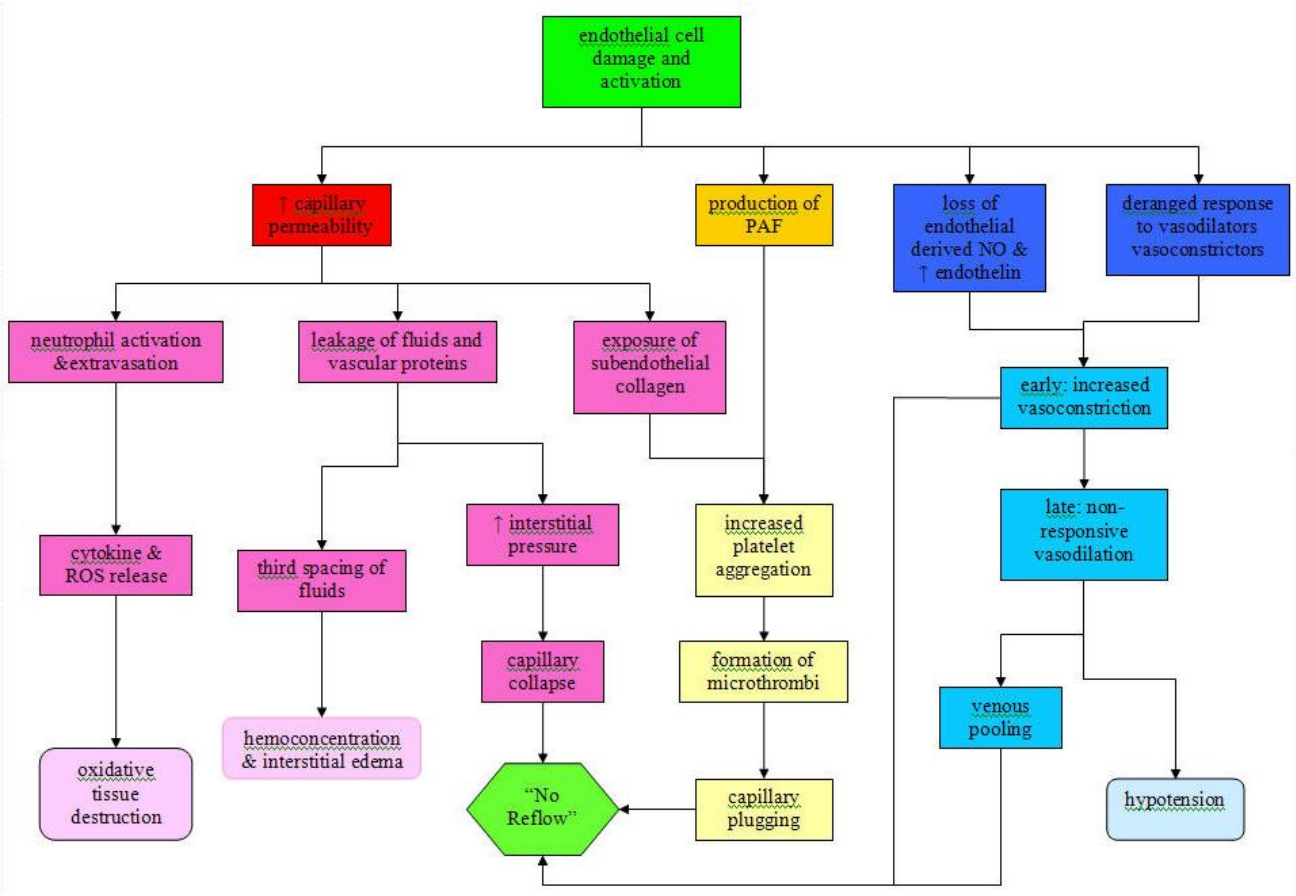

Figure 3: Consequences of endothelial cell injury resulting from ischemia-reperfusion

Activated or injured endothelial cells swell and cause disruption of tight cell junctions and leakage of red blood cells and neutrophils. This allows protein and fluid leakage into the interstitium creating increased interstitial pressure and eventual capillary collapse. In conjunction with endothelial swelling this results in decreased blood flow to tissue and contributes to the so called "no reflow" phenomenon that leads to further ischemia.[9]

In species such as cats and mice, reperfusion injury is also mediated by endothelial production of NO and endothelin, a highly potent vasoconstrictor. During ischemia endothelial transcription of endothelin

is upregulated. NO is the end product of nitric oxide synthase (NOS) and has several isoforms, inducible (iNOS), endothelial (eNOS) and neuronal (nNOS). Release of NO in arteries can reverse the vasoconstrictive effects of endothelin but has the opposite effect in veins.[12] Thus NO exhibits paradoxical functions where it may be beneficial or toxic depending on conditions. In health, low doses of NO cause vasodilation, decrease platelet aggregation and leukocyte adhesion, neutralize ROS and have anti-microbial and anti-apoptotic effects. Molecular oxygen is required for NO synthesis, thus during hypoxia transcription of iNOS occurs but due to the oxygen deficit NO is not produced despite high iNOS concentrations. Re-introduction of oxygen during reperfusion then results in high and sustained increases in NO.[12] Under these conditions NO becomes cytotoxic and causes severe non-responsive vasodilation. NO and superoxide can also combine to form peroxynitrite, a highly reactive oxygen species that interacts with proteins, lipids and DNA.[15]

In horses, neutrophil infiltration is thought to be a crucial component of the I-R injury cascade and may mediate the majority of mucosal and microvascular injury. Xanthine oxidase, platelet activating factor (PAF) and ROS can all initiate neutrophil chemotaxis and infiltration. During neutrophil migration cell adhesion molecules on the neutrophil surface bind to ligands on endothelial cells resulting in neutrophil rolling, adhesion and extravasation from the microvasculature.[12] In the normal intestine of mice and rats it is recognized that intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are constitutively expressed at low levels on vascular endothelium. In response to I-R injury they are markedly upregulated in vascular endothelium, connective tissue, lamina propria and inflammatory cells. Upregulation leads to increased leukocyte-endothelial cell adhesion and promotes leukocyte migration into the intestine. Mass adhesion of neutrophils in capillaries also results in occlusion of the microcirculation and contributes to the “no reflow” phenomenon.[11] While this occurs in the vascular lumen, activated perivascular cells such as macrophages and mast cells release inflammatory mediators like

histamine, leukotriene B4 and various cytokines that further promote expression of CAMs on endothelial cells and leukocytes.[17] In addition to direct endothelial damage from diapedesis, tissue injury occurs secondary to oxidants generated by proteolytic enzyme release from resident and invading neutrophils.[12]

The "no-reflow phenomenon" describes absent or diminished blood flow to tissue after relief of vascular occlusion, and longer periods of ischemia are more likely to result in this phenomenon. Work in cats suggests that neutrophils probably play a major role in the development of no reflow. The damage caused by the adherence of neutrophils to the endothelium causes endothelial swelling that contributes to the generation of inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), platelet activating factor (PAF), complement and chemokines, all of which are potent chemoattractants and contribute to even greater neutrophil accumulation. Platelets rapidly adhere to subendothelium exposed by vascular injury and recruit more neutrophils. These accumulated neutrophils adhere to the platelets, promoting fibrin deposition and thrombus formation resulting in capillary plugging and "no-reflow" that then creates a vicious cycle of further ischemia.[12]

NORMAL INTESTINAL MUCOSAL FUNCTION

In an effort to understand the intestinal damage sustained during I-R injury it is first necessary to have a strong knowledge of normal intestinal function and repair mechanisms, including the role that apoptosis plays during these events. Enterocytes are highly specialized and very metabolically active columnar cells with apical microvilli and lateral tight junctions with adjacent cells. These mucosal epithelial cells have both hydrolytic and absorptive functions and are the primary mediators in the degradation and absorption of nutrients.[18] The small intestinal mucosa has a high rate of proliferation and cell loss that takes place in a well-defined and polarized topographical system. In this structural context cellular "age" can be assessed by position of the cell along the crypt-villus axis.[19,20] Crypt stem cells in the murine small intestinal mucosa give rise to approximately 1200 new epithelial cells per day on each villus, which is equal to roughly one gram of tissue or 10^9 cells every 5 days. Complete turnover of the mucosal epithelium requires roughly 3 days and is a process of rapid cellular generation and death. Normal crypt maintenance during steady state requires about 4-6 stem cells per villus and differentiation is considered complete when cells reach the crypt-villus junction (Figure 4).[18,21,22] Crypt stem cells are surrounded by a rich microvascular supply and numerous intraepithelial lymphocytes as well as a layer of pericryptal fibroblasts that migrate upward in conjunction with the differentiating epithelial cells. These fibroblasts contain well developed endoplasmic reticulum, differentiate immediately below the progenitor cells of the mucosal epithelium and exert inductive actions on epithelial development.[23] Enteric

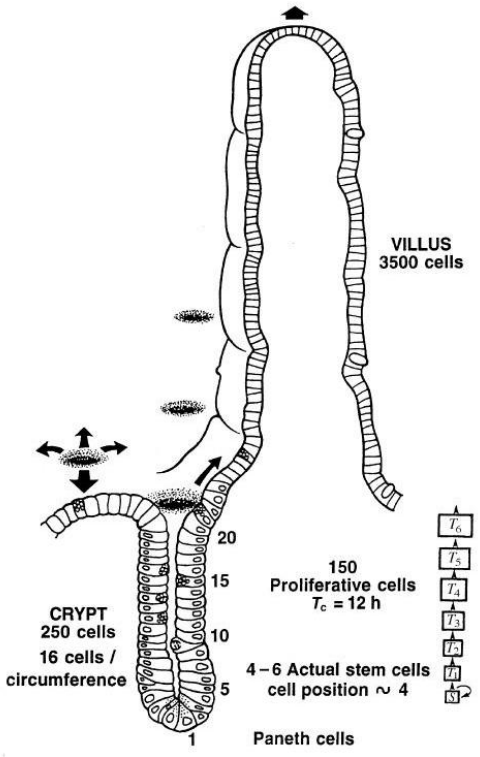

Figure 4: Normal architecture of a small intestinal villus used with permission of The Royal Society (Potten CS: Stem cells in gastrointestinal epithelium: numbers, characteristics and death. Philos Trans R Soc Lond B Biol Sci 353:821-830, 1998.)

neurons extend projections close to the epithelium of the crypt and stimulate proliferation of stem cells committed to the absorptive cell lineage through the secretion of enteroendocrine cell-produced glucagon-like peptide. Cell-cell and cell-extracellular matrix interactions precisely control epithelial cell renewal.[23]

Stem cells are defined as relatively undifferentiated proliferative cells that maintain their numbers while also producing a variety of differentiated progeny. Stem cells have a high proliferative rate and morphology characterized by a large nucleus, diffuse chromatin, scant cytoplasm and few organelles. Epithelial stem cells in the small intestine are found roughly 4-5 cell positions up from crypt base directly above differentiated Paneth cells. These crypt cells produce 4 distinct cell types: enterocytes (~80% of all intestinal epithelial cells), enteroendocrine cells, Paneth cells and goblet cells. Strict control of stem cell numbers within the crypt is critical to maintain steady cellular output from the crypt onto the villus. Small intestinal stem cells are extremely efficient in detecting the increase or decrease of a single cell. Increase in one cell immediately induces spontaneous apoptosis while a decrease in one cell stimulates cell proliferation.[18,22]

In normal adult murine intestine there is a persistent low rate of crypt apoptosis or "spontaneous apoptosis" and it is estimated that approximately one apoptotic cell is seen per every 5th longitudinal crypt section. Apoptotic cells are usually seen in the lower regions of crypt and represent less than 1% of total crypt cell numbers. Deletion of excess epithelial cells at the villus tip may be responsible for removing exhausted non-productive cells and roughly one apoptotic cell per villus in a 3um tissue section is considered normal. Thus, in normal intestine, healthy cells undergo apoptosis based on their position and signals encountered in the extracellular environment as they move toward the villus tip. Following intestinal damage, a regenerative burst in proliferation overshoots normal steady-state levels to initiate mucosal repair and this leads to increased crypt cell numbers. Following re-establishment of mucosal integrity, the

epithelium then decreases its level of proliferation in correlation with an observed increase in apoptosis. This is probably the mechanism for reducing stem cell numbers back to steady-state.[19,20]

Evidence from multiple species including deer, seals, horses, mice and humans shows that intestinal epithelium is highly polarized and intact cells have tight junctions that surround each cell, separating the plasma membrane into apical and basolateral domains. Through this mechanism a tight epithelial barrier is preserved and luminal solutes must cross via a transcellular route. Tight junctions are maintained as effete cells are extruded so that there are no epithelial discontinuities. Despite the relatively rapid turnover of mucosal cells, 2-7 days depending on species, only trace amounts of macromolecules leak across the normal epithelial barrier. Non-epithelial cells are involved in this process including mononuclear phagocytes, large granular lymphocytes and intraepithelial lymphocytes. Mononuclear cells within the villus appear phagocytically active and do not visibly extend processes into the epithelium. Signs of cell death and extrusion are usually seen near villus tip, and since it is accepted that intact tight junctions are necessary in limiting paracellular transfer of nutrients, cell extrusion mechanisms that retain tight junction integrity have functional significance.[24]

Three types of cell extrusion are recognized and there is some species variation involved. In two of these types, tight junction integrity is maintained and anucleate apical fragments are shed into the intestinal lumen. Type 1 extrusion is the mechanism that is known to occur in horses. During this process large intercellular spaces are created that extend from the lamia propria to a debris containing apical cap. Intraepithelial lymphocytes are responsible for targeting exhausted enterocytes in the lamina propria, while mononuclear phagocytes interiorize the basal portion below the tight junction level. The anucleate apical portion, including its tight junctions, is temporarily retained until normal surrounding enterocytes create new tight junctions. Following the establishment of these new tight junctions the apical fragment is lost into the lumen. Deer and seals demonstrate type II cell extrusion where there is gradual shrinkage of individual

cells followed by degeneration of nucleated subapical portions while cell fragments are confined to intercellular spaces between adjacent healthy cells. The removal mechanisms involved with this type of extrusion are unknown but intraepithelial lymphocytes are located nearby and may be involved. Type III cellular extrusion resembles necrosis and is accompanied by breaks in epithelial continuity and total or subtotal degradation of organelles and membranes.[24]

APOPTOSIS

Molecular mechanisms involved in apoptosis have been investigated in many species, most extensively in rats and mice. There are 3 major recognized pathways for cell death, apoptosis (type I), autophagy (type II) and necrosis (type III). Autophagy occurs during normal embryonic development and is a catabolic process that involves the degradation of a cell's own cytoplasmic material via the lysosomal machinery. It is a tightly regulated process that helps to maintain homeostasis between synthesis, degradation and recycling of cellular components. Autophagy is also the mechanism through which a starving cell reallocates nutrients from less critical functions to more crucial processes. Necrosis is an energy dependent catastrophic response to pathophysiologic inductors and is the consequence of ATP depletion resulting in rapid disintegration of organelles, rupture of the plasma membrane, leakage of lysosomes and release of pro-inflammatory molecules and cell contents.[25]

Apoptosis or programmed cell death is a biologically conserved asynchronous, irreversible process. The mechanisms for programmed cell death consist of a complex network of biochemical pathways that mediate normal homeostasis between cell proliferation and turnover in almost all tissues. Apoptosis plays major role in a wide variety of physiological events including embryonic development, tissue renewal, hormone-induced tissue atrophy, removal of inflammatory cells, wound repair and transformation of granulation tissue into scar tissue and this type of cell death typically does not cause inflammation or tissue scarring. There is a distinct apoptotic cell morphology that includes: 1) cytoplasmic shrinkage, 2) disappearance of the nucleolus, 3) chromatin condensation at the periphery of the nuclear membrane, 4) blebbing of the plasma membrane, 5) cellular detachment from surrounding cells and 6) cellular and nuclear fragmentation into small membrane bound vesicles that are then phagocytosed by local mononuclear cells. There are also several important biochemical markers of apoptosis including nuclear DNA fragmentation, activation of caspases and cell surface exteriorization of phosphatidylserine residues in the plasma

membrane. In vivo, apoptosis is a rapid process and clearance of apoptotic bodies takes a few hours at most. This means that identification of only a few apoptotic bodies in a tissue section may represent a considerable degree of cumulative cell loss. Thus numerically small differences in apoptotic indices can be of significant biological and medical importance. Deregulation of normal apoptosis can lead to several diseases.[19,20,26]

Apoptosis is the end-point of a bioenergetic cascade initiated by death-inducing stimuli and can be grouped into 4 overlapping phases. The initiation phase is triggered when a stimulus provokes the apoptotic response. This stimulus may be an external signal delivered through cell surface receptors or may originate inside the cell secondary to the effects of a drug, toxin, radiation, etc. After initiation there is a signal transduction phase where detection of an external trigger or a change in the interior metabolic state is communicated to the death cell effector machinery. This signal transduction is followed by the effector phase where relevant proteases and their positive and negative feedback regulators are activated. Finally there is a post-mortem phase during which chromatin and DNA are degraded and dying cells are recognized and phagocytosed.[20,25]

Two major pathways lead to the induction of programmed cell death, the extrinsic (receptor mediated) pathway and the intrinsic (mitochondrial mediated) pathway. A fundamental component to both is activation of the caspase cascade.[25] Cell death may occur in the absence of caspases but the distinctive morphological features that define apoptosis depend on caspase activation and cleavage of specific cellular proteins or "death substrates" within the cell. Thus apoptosis may be viewed, in biochemical terms, as a caspase-mediated form of cell death.[26]

Caspases are a family of highly conserved cysteine-dependent aspartate-specific acid proteases that are the main mediators of the regulation and execution of apoptosis.[25,27] Caspases are synthesized as inactive zymogens containing a prodomain followed by p20 and p10 subunits. These proenzymes must be

proteolytically processed in order to become activated. Induction of apoptosis triggers cleavage of the zymogens to active enzymes.[27] Upstream initiator caspases are believed to undergo autocatalytic activation, while downstream effector caspases are only activated through cleavage by the initiator caspases. The end result of caspase activation is an intracellular cascade of proteolytic activity that disassembles the dying cell.[25,26]

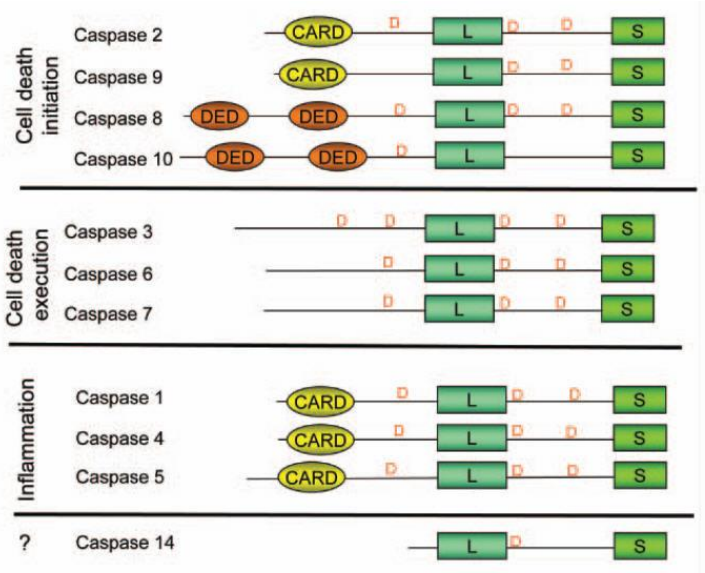

Figure 5: Internal domains of mammalian caspases used with permission of Landes Bioscience (Jin Z, El-Deiry WS: Overview of cell death signaling pathways. Cancer Biol Ther 4:139-163, 2005.)

Currently there are 14 identified caspases with more than 250 known cellular substrates. All caspases are synthesized as a single polypeptide chain with 3 common domains, a 20-kDa central large internal domain (p20) that contains the active site/death effector domain (DED), a 10-kDa small C-terminal domain (p10) called the caspase recruitment domain (CARD) and a NH2-terminus pro-domain called the death domain (DD)(Figure 5).[25-27]

Caspases are divided into 3 groups:

1. Group I inflammatory caspases: 1, 4, 5, 11, 12, 13, 14
2. Group II apoptotic initiator caspases have a long pro-domain containing either a death effector domain (DED), caspases 8 & 10, or a caspase activation and recruitment domain (CARD), caspases 2 & 9, and the DED or CARD mediates interaction with upstream adaptor molecules
3. Group III apoptotic effector caspases are the executioner class (caspases 3, 6, 7) characterized by a short pro-domain – typically processed and activated by

upstream caspases and then carry out downstream executioner steps by cleaving multiple cellular substrates

Caspases function as signaling mediators that coordinate execution paths by cleaving certain sets of cellular proteins. They have more than 100 known targets that are subdivided into 4 categories. There are the proteins responsible for regulating apoptosis as well as the structural proteins that dismantle the intracellular actin filament network, nuclear laminin and cell adhesion molecules. The third category of caspases contains cellular DNA repair proteins such as DNA dependent protein kinase and poly(ADP-ribose) polymerase (PARP). Lastly there are the cell cycle proteins such as Cdc27 and Wee1. Caspase activity is tightly controlled and apoptosis is not usually dependent on de novo protein synthesis. Caspases can be regulated through post-translational modifications while regulation of gene expression can influence cellular sensitivity to apoptosis. The main regulators of the caspases are the inhibitor of apoptosis proteins (IAP), the bcl-2 family of proteins, the TNF family of proteins and p53.[25-27]

The two apoptotic pathways are elicited by separate mechanisms that ultimately converge on a final common path. The extrinsic pathway plays a fundamental role in maintenance of tissue homeostasis, especially in the immune system.[26] Binding of death molecules to cell surface receptors induces the receptor mediated pathway.[27] There are 2 major signaling subtypes in the extrinsic pathway, the Fas-mediated and TRAIL receptor mediated, however Fas receptors are capable of activating either the receptor mediated or mitochondrial mediated pathways.[25] In the extrinsic pathway, ligand stimulation leads to oligomerization of the death receptor and recruitment of the adaptor protein Fas-associated death domain (FADD) and caspase-8 to form the death-inducing signaling complex (DISC). Autoactivation of caspase-8 at the DISC is followed by activation of effector caspases-3, 6 and 7 that then function as downstream mediators of the cell death program.[25]

Tumor necrosis factor (TNF) is a soluble, pleiotropic cytokine involved in cell proliferation, immunomodulation and inflammation that can also function as a death inducing ligand to trigger apoptosis. The TNF receptor-1 (TNF-R1) mediates transduction of the death signal via its cytoplasmic death domain and triggers apoptosis by activating caspase-8 alone, activating caspase-3, or through activation of a mitochondrial dependent amplification loop.[25] The TNF mediated pathway, unlike Fas and TRAIL signaling, does not spontaneously induce cell death. TNF activated nuclear factor kappa-B (NF-κB) mediates a strong pro-survival path and latent cytotoxicity is only manifested when NF-κB activation is blocked. Thus, in vivo, the death-inducing ability of TNF is normally masked by concurrent activation of NF-κB.[26,27]

Fas (CD95) and Fas ligand (FasL) are members of the TNF superfamily and have an important role in maintaining homeostatic and cytotoxic effector functions in T-cells.[25] Fas is a 45-kDa type I transmembrane protein that contains a number of cysteine-rich repeats in its extracellular domain and is ubiquitously expressed on a variety of normal cells. FasL is a 40-kDa type II cell surface glycoprotein with limited expression on activated T cells and natural killer (NK) cells.[28,29] FasL binding causes receptor trimerization that results in intra-cellular clustering of death domains (DD). The receptor is then internalized into the endosomal pathway where adaptor protein FADD associates with the receptor via interaction between homologous DDs on both molecules. FADD also contains a death effector domain (DED) that initiates recruitment and binding of procaspase-8 to the FADD complex. Clustering of FADD and procaspase-8 in the DISC leads to

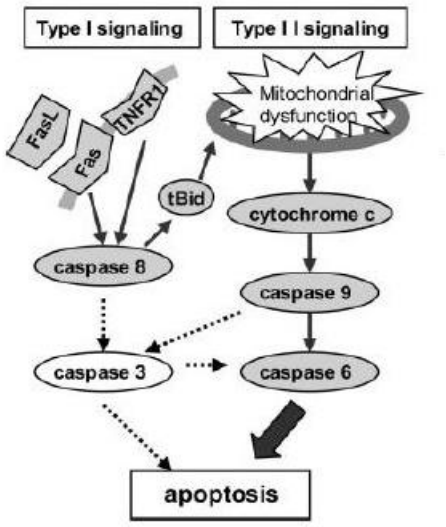

Figure 6: Apoptotic signaling in Type I and Type II cells used with permission of the American Physiological Society (Fujise T, Iwakiri R, Wu B, et al: Apoptotic pathway in the rat small intestinal mucosa is different between fasting and ischemia-reperfusion. Am J Physiol Gastrointest Liver Physiol 291:G110-116, 2006.)

autoproteolytic cleavage of procaspase-8 to active caspase-8 through dimerization from induced proximity and triggers the release of active proteases. Caspase-8 is then released from the DISC and apoptosis occurs via the extrinsic pathway.[26,27,30]

At this step cells can be divided into 2 types (Figure 6) according to their requirement for mitochondrial pathway induced apoptosis. In type I cells, processed caspase-8 is sufficient for direct activation of other caspases that then act on substrates leading to the execution phase. These cells are refractory to bcl-2 inhibition of mitochondrial signaling. In type II cells, activation of effector caspases depends on an amplification loop that relies on caspase-8-mediated cleavage of the bcl-2 family protein bid (Figure 7). Bid translocates to the mitochondria and there is subsequent release of mitochondrial proapoptotic factors, IAP antagonists such as SMAC/DIABLO or cytochrome c. This drives formation of the caspase-9 activating apoptosome through which active caspase-9 activates caspase-3. Caspase-3 then activates capspase-8 and completes the feedback loop.[27]

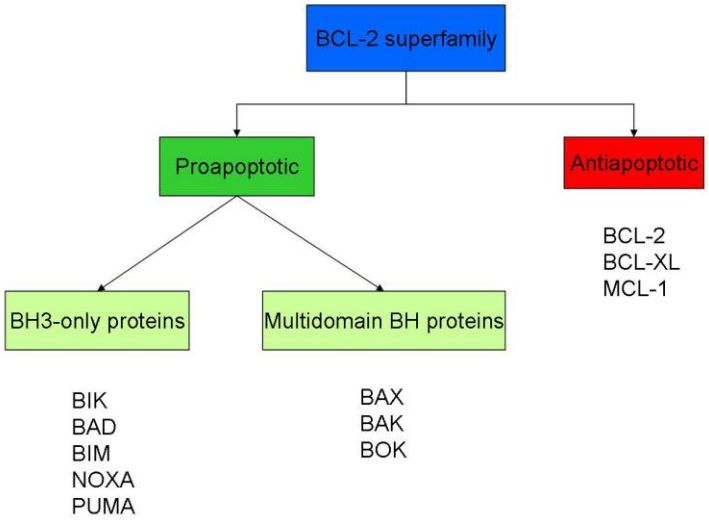

Figure 7: Bcl-2 superfamily of proteins

In contrast to the receptor mediated pathway, extracellular signals or internal insults such as DNA damage can act as initiators of the intrinsic pathway through diverse "stress" stimuli that converge at the mitochondria. The mitochondria is the central regulator in the path and activation occurs inside the cell. These mitochondrial events may or may not trigger apoptosis but seem to be the critical mediators when a signal becomes pro-apoptotic.[25] The central event in induction of apoptosis is mitochondrial outer membrane permeabilization (MOMP), which is primarily

regulated by the bcl-2 family of proteins. Once MOMP occurs it triggers cell death through release of various apoptotic molecules or loss of mitochondrial functions essential for cell survival.[27] MOMP can cause cell death via 2 mechanisms, the release of soluble mitochondrial intermembrane space (IMS) proteins like cytochrome c or disruption of essential mitochondrial functions.[26,31]

An intrinsic death signal creates MOMP through induction of bcl-2 family pro-apoptotic proteins such as bad, bax, bid, and p53-up-regulated modulator of apoptosis (PUMA). These facilitate assembly of bcl-2 family bad and bak proteins into pores that form large voltage gated ion channels in the mitochondrial membrane and allow leakage into the cytosol of apoptosis inducing factors such as cytochrome c. These channels are termed the mitochondrial permeability transition pore (PTP) and function in the regulation of calcium, membrane potential and pH. PTP formation is the integration point for multiple pro-apoptotic pathways and pro-apoptotic messengers such as Ca^{2+} ions, ROS and p53-induced changes in cellular redox potentials facilitate opening of these pores. Once mitochondrial membrane permeabilization occurs cells die either by necrosis or apoptosis. Apoptosis is triggered when cytochrome c enters the cytosol through the PTP and binds to the cytosolic adaptor protein apoptosis protease-activating factor (Apaf-1) which then recruits procaspase-9 in the presence of dATP/ATP. This allows for self processing and activation of procaspase-9. The caspase-9/Apaf-1 complex is known as the "apoptosome" and it mediates cleavage of caspase-3. Following caspase-3 activation the downstream events of the intrinsic pathway are then the same as the extrinsic pathway.[25,26]

Bcl-2 proteins constitute a critical checkpoint in the intrinsic path and there are both pro- and anti-apoptotic constituents of this family. The ratio of pro-apoptotic to anti-apoptotic components acts like "rheostat" to regulate cellular sensitivity to apoptotic stimuli, and mitochondrial permeability is determined by this ratio.[26,27] There are twenty known bcl-2 proteins in mammals that are categorized into 3 groups all containing at least one of four conserved bcl-2 homology (BH) domains (Figure 8). The first group contains

anti-apoptotic members bcl-2, bcl-Xl, bcl-w, A1 and Mcl-1 that contain all 4 homology domains (BH1, BH2, BH3, BH4) and promote cell survival. Group 2 consists of the pro-apoptotic multi-BH members bax, bak and bok that share 3 common domains with bcl-2 (BH1, BH2, BH3). Finally there are the pro-apoptotic proteins bid, bad and bim that contain only the BH3 domain; this domain alone is probably sufficient for induction of apoptosis.[27]

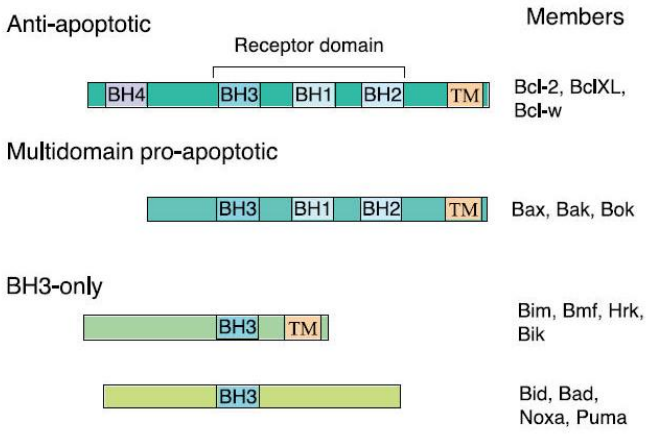

Figure 8: Bcl-2-homology domains used with permission of Blackwell Publishing (Chan SL, Yu VC: Proteins of the bcl-2 family in apoptosis signalling: from mechanistic insights to therapeutic opportunities. Clin Exp Pharmacol Physiol 31:119-128, 2004.)

In the absence of death signals, bcl-2 proteins are localized to discrete intracellular compartments. In healthy cells the inactive form of bax is primarily restricted to the cytosol or loosely attached to membranes. Anti-apoptotic members are usually integral membrane proteins localized to the mitochondria, ER and nuclear membranes. These proteins form ion channels in the mitochondrial membrane and help maintain mitochondrial integrity. Bcl-2 family members can inhibit cell death by sequestering or neutralizing the BH1, BH2, BH3 molecules, while BH3-only proteins serve as sentinels for damage to specific intracellular structures and respond by triggering apoptosis.[26,27]

After receiving death stimuli, BH3-only proteins induce oligomerization of pro-apoptotic BH1, BH2, BH3 molecules like bax and bak. These proteins then undergo pre- and post-translational modifications, change conformation and integrate into the outer mitochondrial membrane causing membrane permeabilization. BH3-only proteins act as sentinels for intracellular damage and are inactive without stimulation from a variety of mechanisms. They are controlled primarily at the level of transcription and can be transactivated by p53 in response to DNA damage. BH3 only proteins can also act indirectly by releasing

BH1, BH2, BH3 proteins from bcl-2 members that normally sequester them in the cell. Anti-apoptotic bcl-2 has an important role in the "commitment phase" of apoptosis when cleavage results in removal of its BH4 domain converting it into a pro-apoptotic molecule.[25-27,32]

Intracellular p53 mediation of the intrinsic pathway can function independently of death receptor stimulation. p53 is a stress response 53-kDa nuclear protein that accumulates in the cytoplasm during the G1 phase of the cell cycle and migrates to the nucleus at start of the S phase. It is a well described transcription factor capable of catalyzing MOMP by inducing expression of numerous pro-apoptotic gene products that function in oxidative stress, endoplasmic reticulum stress, caspase activation and regulation of pro-apoptotic bcl-2 family proteins. Activation of p53 is controlled via its biosynthesis and a large number of post-transcriptional modifications with subcellular relocalization. p53 can also mediate apoptosis by transcriptionally regulating other genes such as bax, Apaf-1 and Fas. A transcriptionally independent pathway also exists that involves localization of p53 to the mitochondrial membrane followed by binding and inhibition of bcl-xL and/or bcl-2 or direct activation of bax .[25-27]

Another non-receptor mediated inducer of the intrinsic pathway is the endoplasmic reticulum (ER) stress activated path. Cellular stress, such as oxidative stress, can cause misfolding of proteins and disrupt calcium homeostasis. The ER is a major store of intracellular calcium and efflux from the ER is usually followed by uptake into the mitochondria. This coordinates an amplification loop between the mitochondria and ER where a small amount of cytochrome c is released from the mitochondria, diffuses to the ER and triggers more calcium release. Increased cytosolic calcium induces a mass efflux of cytochrome c from the mitochondria that elicits caspase activation. Bcl-2 proteins are also involved in the ER stress response. Some bcl-2 protein is present in the ER membrane and normally interrupts crosstalk between the ER and mitochondria during stress. Thus changes in bax or bak are required for stress induced ER apoptosis and appear to play a role in calcium homeostasis of the ER.[25-27]

APOPTOSIS: NORMAL INTESTINE

The mechanisms involved in regulating apoptosis in the normal small intestine are still incompletely understood. It is known that in mice there are important regional differences in p53 and bcl-2 expression between the large and small intestine.[19,20] In the small intestine p53 has an important role in damage surveillance but degree of expression is normally very low. Levels of spontaneous apoptosis in the intestine of normal and p53 knockout mice are very similar, suggesting that p53 has little involvement in controlling removal of excess, but undamaged, cells. However, following DNA damage, expression is upregulated, p53 is translocated to nucleus where it binds to DNA and then regulates transcription of numerous genes including *p21/waf1, mdm2,* and *bax.*[19,20]

Bcl-2 expression in the small intestine is somewhat controversial. Some research has demonstrated apoptosis that is associated with bcl-2 staining in a wide variety of cell types along the entire length of the small intestinal villus, suggesting that enterocytes undergo apoptosis after terminal differentiation.[33] This group found that bcl-2 is virtually undetectable in the crypt epithelium of normal adult mice but levels markedly increase at the crypt-villus junction and are sustained throughout migration to villus tip.[10,20,34] Other studies in mice found that morphological evidence of apoptosis along villus is extremely rare.[35] In vivo immunohistochemical results showed that bcl-2 expression was almost completely absent in the small intestine.[35-37] Evaluation of cellular bcl-2 protein (mouse and human) showed a consistent pattern with little expression in any cell type except for strong staining in lymphocytes of the Peyer's patches, some mesenchymal cells and occasional intraepithelial lymphocytes. These findings are supported by the fact that there is no change in spontaneous apoptosis in small intestinal crypts in bcl-2 null mice as compared to wild-type mice.[35]

Despite the controversy over bcl-2 it is generally accepted that there is some bax expression at the crypt base, and bad, bax and bcl-xL/S are all expressed to some level along the villus.[22] The distribution of

bax is concentrated in the cytoplasm of basal crypt epithelial cells, intercalating cells and Paneth cells with lower levels in enterocytes and goblet cells at the villus tip. The presence of high levels of bax in the proliferative compartment of the crypt indicates that it may have a role in controlling basal apoptotic rate.[10,20,38]

APOPTOSIS: ABNORMAL INTESTINE

Apoptosis of intestinal cells in the mouse is part of the normal homeostatic mechanism involved in maintenance of the mucosal epithelium, however apoptosis also plays a role in a number of pathological intestinal processes. Recognized pathophysiologic initiators of apoptosis in the small intestine include radiation damage, cytotoxic drugs, carcinogens, bacterial toxins, intestinal resection and ischemia-reperfusion injury. Radiation, some chemotherapeutic drugs, some mutagens and some carcinogens can induce stem cell apoptosis in the small intestine approximately 3-6 hours after damage occurs. Although it is known that spontaneous apoptosis in the normal villus is p53 independent, this early "damage associated apoptosis" is totally p53 dependent. Research in p53 knockout mice has confirmed that deletion of p53 gene completely ameliorates intestinal cell apoptosis after radiation damage.[22] In direct contrast, following intestinal I-R injury lack of p53 did not abolish enterocyte apoptosis indicating that apoptosis was p53 independent.

In mice, massive small bowel resection initiates an adaptive response in the remaining intestine that stimulates cellular proliferation and signals for increased enterocyte apoptosis. This apoptotic response is probably a physiologic mechanism in re-establishing homeostasis between cell proliferation and death.[21] Interestingly, during post-resection mucosal restitution there is a marked difference in enterocyte apoptosis rates between the proximal and distal regions of the small intestine. Analysis of gene and protein expression of some components in the intrinsic and extrinsic apoptotic pathways shows that expression patterns vary greatly along the oral-aboral axis of the small bowel. This suggests that different mechanisms may be responsible for regulation of apoptosis in ileal vs. jejunal sections of intestine.[39]

In the mouse, one proposed mechanism of intestinal growth following massive resection is thought to involve the action of intra-epithelial lymphocytes (IEL). IEL secrete a variety of cytokines and growth factors including IFN-γ, TNF-α and IL-12 that all play an important role in sustaining enterocyte growth and

structure. It is thought that IEL may directly influence regulation of intestinal turnover of enterocytes.[40] Post-resection mice have increased small intestinal concentrations of angiotensin converting enzyme (ACE) at 1 week post-surgery that immediately precede observed apoptotic changes. The mechanism of ACE induced apoptosis is unknown but seems to involve multiple pathways. The use of ACE inhibitors (ACE-I) triggers changes in cytokine expression in lymphocytes, suggesting that ACE mediated alterations in IEL derived cytokines may be a factor in inducing enterocyte apoptosis.[40] It is also known that following small intestinal resection bax deficiency prevents increased post-resection enterocyte apoptosis during a one month adaptation period. Interestingly however, attenuation of apoptosis does not augment mucosal adaptation to massive intestinal loss.[41] This research indicates that the bcl-2 family of cytoplasmic proteins may play a role in regulation of enterocyte apoptosis during adaptation.[21]

Enterocyte apoptosis is known to occur following I-R injury. Studies in mice show that the percentage of fragmented DNA in jejunal and ileal mucosa increases just after ischemia, peaks at 1 hour of reperfusion and by 6 hours of reperfusion returns to baseline. Histology of jejunal mucosa reveals substantial destruction at 1 hour of reperfusion but mucosal integrity is partially restored at 6 hours post-injury.[14] Most TUNEL positive cells are found in detached mucosal cells with a few detectable in the toward the base of villi, however enterocytes at the villus tip are not TUNEL positive after 75 minutes of reperfusion. This suggests that if attention is not paid to detached epithelial cells damage may be missed in the reperfusion phase.[13]

It is notable that in rats, induction of apoptosis begins during the ischemic phase and continues into early reperfusion. It is known that following I-R injury apoptosis can appear within a few minutes after an insult and is often completed in 1-4 hours. In this model, the peak at 1 hour of reperfusion and its return to baseline by 6 hours suggests that induction of apoptosis and mucosal recovery are rapid processes.[14] The kinetics of the induction of apoptosis and restoration of the intestinal mucosa is still unclear. There may be a

time-dependent increase in apoptosis-promoting factors during ischemia and early reperfusion that rapidly declines with prolonged reperfusion. Concurrent with this decline in pro-apoptotic factors there may be a simultaneous induction of apoptotic inhibitors as well as promoters of tissue repair.[14]

Multiple studies in mice show that ROS can stimulate apoptosis, however other reports report a lack of association between ROS and enhanced apoptosis.[14,42,43] Cells undergo oxidative stress when levels of ROS exceed the counter-regulatory anti-oxidant capacity of the cell. Oxidative stress is known to induce apoptosis in a variety of other cell types by activating the intracellular cell death cascade. Paradoxically, it is also known to trigger some signaling pathways that protect against cell death.[44] Murine studies indicate that iNOS, inflammatory cytokines and platelet-activating factor promote I-R induced apoptosis via a FasL mediated caspase-9 pathway.[30,44] NO is recognized as an important mediator of numerous physiological and pathophysiological processes. High concentrations of iNOS are capable of inducing apoptosis in intestinal epithelial cells, macrophages, thymocytes, smooth muscle cells and neuronal cells, but the overall role of NO in apoptosis is still controversial. While NO can induce caspase mediated apoptosis in variety of cell types it also prevents apoptosis in some cells by suppressing increases in caspase activity and the release of cytochrome c. In vivo research demonstrates that increases in I-R induced mucosal apoptosis are reduced by pretreatment with NO inhibitors and the degree of histological damage is also decreased. However, apoptosis was not completely abolished indicating that other mechanisms are also active.[45]

In addition to the Fas mediated pathway intestinal I-R injury in mice can induce apoptosis through expression of TNF-R1, cytosolic cytochrome c, cleaved caspase-9 and caspase-6, all of which are known initiators the extrinsic pathway. In this model I-R injury also induced mitochondrial respiratory dysfunction.[44] Following 60 minutes ischemia without reperfusion there was a slight increase in fragmented DNA in the mucosa but there was no effect on expression of cleaved caspase-9 and cytochrome c indicating that damage is potentiated during reperfusion. Evaluation at various time points during the reperfusion

period (0-24 hours) showed that FasL was found in the villus epithelium and stroma following ischemia. After 3 hours of reperfusion there was a marked increase of FasL in the stroma but no change in epithelial cell expression. Fas was identified in the cytoplasm of stromal cells and crypt epithelial cells after the ischemic phase and was significantly increased in the stroma by 3 hours of reperfusion. At this same time point, expression of Fas, FasL and activated caspase-3 were all markedly increased in the stroma and apoptotic cells were increased in all layers of the intestine.[46] Cytochrome c staining was still present in villus epithelial cytoplasm after 1 hour of ischemia but disappeared by 3 hours of reperfusion and then reappeared after 24 hours. These findings suggest that apoptotic triggers may vary depending on cell type/location.[10]

In rats, platelet activating factor (PAF) is also involved in cellular apoptosis through the Fas/FasL pathway in numerous cell types. PAF is a pro-inflammatory lipid mediator that can activate leukocytes, platelets, macrophages and intestinal epithelial cells and is deactivated by PAF-acetylhydrolase (PAF-AH) induced deacetylation. Evidence suggests that PAF plays an important role in the initiation and amplification of I-R injury. During intestinal I-R injury PAF activity is increased secondary to inhibition of PAF-AH activity in the small intestinal mucosa, increased mucosal pro-inflammatory IL-6 concentration and inhibition of anti-inflammatory IL-10 synthesis. This results in an increase in FasL on intestinal mucosal cells. FasL binding to Fas leads to cleavage of bid and release of cytochrome c from the mitochondria to the cytosol to activate caspase-9 in the intestinal mucosa.[30]

In some rat tissues, increased expression of bcl-2 or bcl-xL blocks translocation of cytochrome c following a death inducing stimulus whereas increased expression of bax promotes translocation. This indicates that bcl-2 regulates the commitment phase of apoptosis and forced expression of bcl-2 might delay commitment to apoptosis or inhibit cellular competency to progress to the execution phase. After I-R injury, forced expression of bcl-2 in the small intestine partially suppresses p53 independent apoptosis. This

suggests that the apoptotic mechanism involved in enterocyte apoptosis secondary to I-R injury involves a commitment phase that is sensitive to bcl-2 and thus bcl-2 mediated reduction of apoptosis is probably due to a delay in commitment. These findings suggest that interventions to increase bcl-2 levels or enhance its function could be useful for reducing I-R induced damage. However, the general lack of bcl-2 expression in the small intestine may make this untenable in clinical disease.[30]

NORMAL RENIN-ANGIOTENSIN SYSTEM

It is now generally accepted that the renin-angiotensin system (RAS) plays a role in apoptotic processes secondary to ischemia-reperfusion in numerous organs including the heart, liver, kidney and brain. Whether the RAS also influences apoptosis in the gastrointestinal tract following I-R injury is still largely unknown. In humans, the RAS has historically been viewed only as a means of controlling systemic blood pressure, however recent research has demonstrated that it also influences numerous pathological processes in a variety of diseases. The RAS is peptidergic system with endocrine characteristics and some of its key components include renin, angiotensinogen, angiotensin I (ANG I), angiotensin II (ANG II) and angiotensin converting enzyme (ACE).[47,48]

Angiotensin II is a potent vasoconstrictor; one millionth of a gram can increase arterial pressure in humans by 50 mm Hg or more. It constricts small arterioles and when present in an isolated tissue area can cause local ischemia by severely depressing regional blood flow. ANG II is generated via renin from the kidney, circulating angiotensinogen, ANG I and pulmonary ACE. Renin is a protein enzyme released by the kidney in response to a decrease in systemic blood pressure. It is synthesized and stored in its inactive form (prorenin) in the juxtaglomerular (JG) cells of the kidneys. JG cells are modified smooth muscle cells located in walls of the afferent arterioles immediately proximal to the glomeruli. A drop in systemic pressure causes prorenin molecules in JG cells to split and release renin which then enters the systemic circulation. Renin is an enzyme, not a vasoactive substance, and acts on the plasma protein angiotensinogen (ANG) to release 10-amino acid peptide angiotensin I (ANG I). Within a few seconds to minutes ANG I splits into 2 more amino acids to form the 8-amino acid peptide ANG II. This conversion occurs almost entirely in the lungs and is catalyzed by ACE located in the endothelium of pulmonary vessels.[47,49]

ACE is an ectoprotein anchored in the plasma membrane of cells through a hydrophobic domain near its carboxy-terminal region. Although historically believed to be isolated to the pulmonary endothelium

and involved solely in vascular homeostasis it is now known that tissue forms of ACE also exist. There are 2 different isozymes of ACE that are expressed in a tissue specific manner, a larger one synthesized by vascular endothelial cells, kidney epithelial cells and several other tissues as well as a smaller isozyme synthesized exclusively by testicular sperm cells. The catalytic and kinetic properties are similar for all forms and sources of the enzyme with respect to both substrates and inhibitors. All enzyme forms can hydrolyze physiologic substrates regardless of tissue source and there are no significant differences in catalytic or kinetic properties of membrane bound or soluble ACE. This would indicate that ACE tissue types are catalytically similar at least to the degree that tissue localization or source cannot be determined by measurement of kinetic parameters.[49]

Irrespective of tissue localization, ACE is primarily a cell-associated enzyme, although soluble ACE is found in various body fluids including plasma and peritoneal fluid [50]. Somatic ACE can also be produced by macrophages, GI epithelium and renal tubular epithelium and is found in both membrane bound and soluble forms. The soluble form is derived from membrane bound ACE through the action of specific secretases.[49] It is now recognized that in addition to its function in the cleavage of ANG I to ANG II, ACE can also hydrolyze a variety of circulating peptides. It can remove dipeptide residues from substance P, ANG 1-9, Ac-SDKP, cholecystokinin, hemopressin, amyloid beta-protein, [Met^5]enkephalin and kallidin.[47,51] ACE also cleaves bradykinin (BK) to inactive fragments thus contributing to the vasoconstrictive response. Normally the vasodilator effects of BK are very potent due to its activation of 3 important endothelium-derived vasodilator autocoids: NO, prostacyclin and endothelium-derived hyperpolarizing factor. Cleavage of BK into inactive fragments prevents this response.[51]

ANG II persists in blood for only 1-2 minutes and is then rapidly inactivated by blood and tissue enzymes called angiotensinases.[52] The actions of ANG II result from its binding to angiotensin-1 (AT-1R) and angiotensin-2 (AT-2R) receptors that are classified by their differential affinities for various non-

peptide agonists.[53] The AT-1R is a 7 transmembrane receptor of the heterodimeric G protein family that activates multiple signaling pathways.[54] The AT-2R couples to heterotrimeric proteins and was originally thought only to oppose actions of the AT-1R, inhibit cell growth and induce apoptosis, however recent findings indicate that in some circumstances the AT-2R has growth stimulatory and proinflammatory actions similar to the AT-R1.[53]

Importantly it is now known that ACE is not the only enzyme capable of converting ANG I to ANG II. Data indicates that mast cell derived chymase and neutrophil derived cathepsin G are capable of rapid conversion of ANG I to ANG II at rates significant enough to be of biologic significance. Thus some release of ANG II is likely in the presence of either enzyme, especially at inflammatory sites where both neutrophils and mast cells are likely to be sequestered.[55] It is of note that this ANG II generating system would be operable in the presence of an ACE inhibitor (ACE-I).[55]

The RAS is best known for its role in blood pressure regulation and fluid homeostasis and ANG II function is crucial to cardiovascular equilibrium, mediating vasoconstriction to increase arterial pressure. ANG II directly and indirectly controls vasoconstriction by stimulating the AT-1R on vascular endothelium , increasing sympathetic tone and triggering arginine vasopressin release.[53] It functions in long-term maintenance of systemic pressure by stimulating central thirst centers in brain and aldosterone release from the adrenal cortex to increase water intake and sodium and fluid retention in the kidney respectively.[54] The AT-R2 is thought to oppose the vasoconstrictive actions of the AT-1R by mediating vasodilation attributable to activation of the NO pathway.[48,53] ANG II plays an important role in modulating the balance between NO and ROS in the endothelium and thereby maintaining homeostasis of the vascular wall, but the relative contributions of the AT-1R and the AT-2R in this regard are currently unknown.[48] The renin-angiotensin vasoconstrictor system requires roughly 20 minutes to become fully activated and thus is somewhat slower than sympathetic system responses.[52]

ANG II is now also known to exert direct effects at the cellular level that can influence cell growth and differentiation and may also have a role in apoptosis. These are universal paracrine and autocrine actions that are important in many organ systems and can mediate important physiologic stimuli.[48] The cellular effects of ANG II are primarily mediated by plasma membrane AT-1 receptors and are generally opposed by the AT-2R.[54] At the tissue level the RAS displays intracrine characteristics which, by definition, are mediated via a peptide hormone or factor that acts in the intracellular space either after internalization or retention in its cell of synthesis. In some cases one isoform is secreted and acts as an intercellular signaling molecule while a second isoform acts in its cell of synthesis in a manner either similar or dissimilar to that of the secreted hormone.[56] ANG I and ANG II can also be taken up by tissues with subsequent enzymatic conversions occurring locally. While it is accepted that ANG II is a known intracrine signaling peptide, ACE, angiotensinogen, ANG 1-7 and renin appear to be as well.[57]

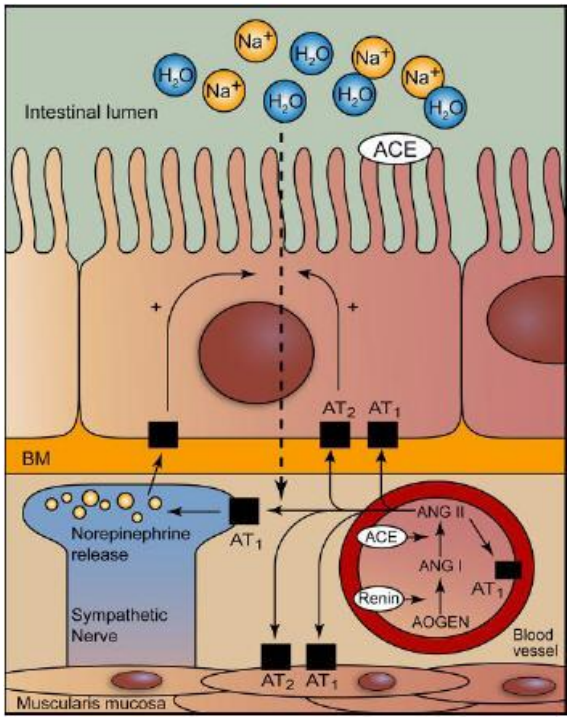

Figure 9: Functions of the renin-angiotensin system in the small intestine used with permission of the American Physiological Society (Paul M, Poyan Mehr A, Kreutz R: Physiology of local renin-angiotensin systems. Physiol Rev 86:747-803, 2006.

In addition to its enzymatic function, membrane bound ACE can also act as a "receptor" in that, on binding a specific substrate it couples to activation of signaling events to elicit a particular physiologic response from the cell. There is considerable evidence that local synthesis of components of the RAS can lead to ANG generation in a variety of tissues.[57] Functional intracellular AT-1-like ANG II receptors have been detected in the sarcolemma, T-tubules and nuclei of rat cardiomyocytes and appear to be coupled to gene transcription.[51] Intracellular delivery of ANG II leads to a rapid increase in intracellular calcium and growth

of cardiomyocytes and vascular smooth muscle cells. These effects are suppressed by an AT-1R antagonist that is present intracellularly but not extracellularly.[54]

The local RAS seems to exert diverse actions in many organs. In some tissues it appears to be regulated independently of the plasma RAS, while in other organ systems there appears to be close cross-talk between the local and plasma RAS. The common denominator of the physiologic role of local RAS seems to be maintenance of homeostasis at the tissue level between opposing effects mediated by the system. The dual action of ANG II on its AT-1 and AT-2 receptors is probably the basis for this balance, however there may also be alternative pathways using different substrates or receptors. If this balance is disturbed by over expression of some RAS components or inhibition of others, the RAS can become a mediator of pathophysiological stimuli. It appears that in regulatory processes, the plasma RAS has the role of an acute "response unit" while tissue based ANG II formation is more linked to subacute and chronic modulation.[48]

Based on work in rats, it is currently accepted that the RAS functions at the tissue level in the gastrointestinal tract (Figure 9). The brush border of the small intestine has high levels of ACE, and although it is similar in most respects to vascular, kidney and lung ACE, under normal conditions it functions independently of the RAS. There is no local production of other components of the RAS and in the jejunum ACE acts as an important dipeptidyl carboxypeptidase that participates in digestion and assimilation of dietary peptides.[58] The greatest concentration of ACE mRNA and protein is proximal to midregion of small intestine and activity is highest in proximal jejunum and decreases towards the ileum suggesting that ACE expression is controlled at the level of mRNA.[48] This is opposite to the few other membrane hydrolases with similar patterns of expression in the intestine where regional differences appear to be regulated at level of translation.[58]

AT-1 and to lesser extent AT-2 receptors are found in rat intestine and while no ANG II binding is seen in the submucosa and muscularis, specific binding is moderately abundant in the mucosa and muscularis of the jejunum and ileum. ANG II causes a contractile response in the longitudinal smooth muscle of the ileum that is mediated by the AT-R1 suggesting that it plays a role in inducing peristaltic waves.[48]

ABNORMAL RENIN-ANGIOTENSIN SYSTEM

In addition to its role in numerous physiologic processes the RAS is also involved in the pathophysiology of numerous disorders including hypovolemia, shock, endotoxemia and ischemia-reperfusion injury. In human disease states soluble ACE can be found in fluids other than plasma such as cerebrospinal and peritoneal.[51] The RAS is highly activated during hypovolemic shock and may contribute to deteriorated splanchnic circulation. ANG II appears to play a critical role in burn and endotoxin induced intestinal I-R injury and contributes to increases in intestinal permeability and bacterial translocation.[48] The splanchnic vasculature of rats normally expresses high numbers of the AT-1 receptor compared to other vascular beds and in response to hypovolemia blood levels of ANG II are higher in splanchnic circulation compared to systemic blood.[59] I-R injury elicits up-regulation of the AT-1R in the vasculature of several tissues perfused by splanchnic circulation including the small intestine, colon, liver and mesentery as well as the lung. I-R injury also increases ACE gene expression in the colon and increases plasma levels of ANG II.[60] Gastrointestinal I-R injury not only increases systemic ANG II levels but also increases mRNA for ACE in postischemic gut tissue.[59] It is known that ANG II dose-dependently increases leukocyte rolling and adhesion in colonic venules during intestinal I-R injury in mice. Inhibition of ACE and the AT-1R significantly reduces I-R induced leukocyte adhesion demonstrating that ANG II has pro-inflammatory effects in colonic microcirculation and inhibition of ANG II expression or function partially ameliorates these effects.[60]

Historically, ACE has been considered the major generator of ANG II but mast cell chymase is increasingly recognized as another significant enzymatic source of ANG II production in the vessel wall, especially at sites of vascular injury. In normal mouse tissues chymase is sequestered in mast cells and ACE is the primary source of ANG II formation. However in injured tissues mast cells degranulate and release chymase and this can become the predominant source of ANG II. Following myocardial infarct, chymase

can account for as much as 80-90% of ANG II formation in the heart and is also a significant source of ANG II in damaged arterial vessels.[38] Mast cell density is very high in intestinal tissues and plays a prominent role during the inflammatory response to I-R injury.[61] ANG II derived from mast cell chymase may induce inflammation in post-ischemic venules of the small intestine via an AT-1 and AT-2 receptor dependent mechanism.[62]

There is growing evidence that AT-1R activation is an important determinant of the pro-thrombogenic events that take place in the microvasculature during I-R injury in mice.[59] It is likely that pro-inflammatory actions of ANG II include increased expression of adhesion molecules, increased chemokine expression and ROS generation, all of which may result in vascular injury and organ damage (Figure 11).[60] Studies indicate that ACE inhibition post I-R injury completely prevents leukocyte rolling while chymase inhibition largely abolishes both rolling and adhesion. This suggests that chymase derived ANG II may be of greater influence in the cellular changes that promote leukocyte adhesion while both ACE and chymase generated ANG II are important for post-injury leukocyte rolling.[62] There is growing evidence that ANG II inhibition protects against I-R induced tissue injury in the heart, brain and liver but its possible role in intestinal I-R is still unknown.[60]

ACE mRNA is expressed at very low levels in the normal mouse colon but following I-R injury gene expression and plasma ANG II concentration are greatly increased suggesting that ACE is up-regulated in response to colonic I-R. The GI mucosa is particularly vulnerable to even mild hypovolemia and the splanchnic vasculature has a high concentration of ANG II receptors. The increase in ANG II during hypovolemia is much more prominent in the splanchnic circulation compared to the systemic circulation. This suggests that ANG II is relatively more important in the GI system during critical situations that help to ensure adequate allocation of blood to organs such as the heart and brain.[60]

The RAS also mediates some NO dependent events during I-R injury. During I-R injury in rats, vascular endothelial dysfunction leads to reduced NO activity that may provoke enhanced leukocyte/endothelial cell interactions and increased microvascular permeability. This indicates that constitutive NO has a crucial role in maintaining the functional integrity of the endothelium and that loss of endothelial NO may significantly contribute to the development of inflammation. Research shows that when mice are subjected to NOS inhibition, pre-treatment with an AT-1 receptor blocker or an ACE-I dramatically attenuates rolling flux, adhesion and migration in post-capillary venules. Use of ACE inhibitors also decreases arteriolar leukocyte adhesion and causes down regulation of P-selectin, E-selectin, ICAM-1 and VCAM-1.[63]

Another function of ANG II is cleavage of bradykinin into inactive metabolites. BK is an endogenously produced, NO dependent neuropeptide vasodilator that exerts powerful inflammatory effects, increases chemotactic activity, induces expression of P-selectin, causes formation of interendothelial gaps and mediates extravasation of plasma proteins. However, there is growing evidence that experimental BK treatment in mice prevents tissue injury induced by prolonged mesenteric I-R injury and can inhibit leukocyte adhesion by inducing prostacyclin. It is hypothesized that these divergent effects are concentration dependent. Low doses of BK have anti-inflammatory and vasodilatory effects via NO production, while high doses have proinflammatory effects via increased leukocyte adhesion through a mechanism involving release of PAF.[64] This contradiction may be due to fact that when administered at high concentrations BK induces production of PAF or PAF-like lipids by endothelial cells. The low dose vasodilatory effects of BK are not affected by COX inhibitors but are completely blocked by an NO inhibitor suggesting that BK induced vasorelaxation in the mesentery is completely mediated by NO and is independent of prostacyclin.[64]

The RAS can also contribute to oxidative stress during I-R injury. ANG II increases vascular and neutrophilic superoxide production through a NADPH oxidase-dependent mechanism in rats and mice. The proinflammatory effect of the AT-1R appears to involve activation of NADPH oxidases expressed by endothelial cells, vascular smooth muscle cells, adventitial fibroblasts and leukocytes during early reperfusion.[62] ANG II also induces ROS generation in leukocytes and upregulation of CXC chemokines; blockade of NADPH induced ROS prevents both rolling and adhesion of leukocytes. Administration of SOD reduces ANG II induced leukocyte adhesion by 70% but inhibition of xanthine-oxidase does not change ANG II provoked leukocyte-endothelial interactions.[60] Production of ROS via ANG II NAD(P)H oxidase is an important mechanism linking activation of the RAS to inflammation and apoptotic changes in response to ANG II in the vascular wall have also been described.[48]

ANG II is reported to have a bifunctional role in apoptosis. It appears to be pro-apoptotic in fibroblasts, cardiac myocytes, and vascular smooth muscle cells but anti-apoptotic in smooth muscle cells, neuronal cells, muscle cells of the aortic media and the subpericardium of the heart. Thus the role of ANG II in apoptosis seems to depend on context. What role ANG II may play in post-IR intestinal damage and apoptosis is currently unknown.[65,66]

Ischemia-reperfusion induced apoptosis occurs in numerous organs including the heart where apoptosis is the early and predominant form of cell death following myocardial infarction (MI). It has been demonstrated that in the house heart Bcl-2 and bax concentrations are important in protection against or acceleration of apoptosis in cardiomyocytes post-infarct and the ratio of these proteins is important in determining whether a cell dies in response to a death signal. I-R injury triggers increases in both bax and bcl-2 in cardiomyocytes with a proportionally greater increase in bax, and these effects are potentiated by administration of exogenous ANG I. Cardiomyocyte apoptosis may be influenced by ANG II as well as ANG I. ANG II binds the AT-1R on cardiomyocytes, enhances intracellular calcium and stimulates calcium

dependent endogenic endonucleases that cause DNA laddering, cell shrinkage and formation of apoptotic bodies. Little is known about modulating the mechanisms of apoptosis in cardiac ischemia-reperfusion injury but the influence of p53 and p53 dependent genes in the onset of apoptosis has been ruled out in vivo.[67]

ACE INHIBITION

Research demonstrates that ACE inhibitors can positively influence outcome during hemorrhagic shock in pigs and has beneficial effects in ulcerative colitis, small intestinal resection and decreasing the production of ROS in the liver of mice. The usefulness of ACE inhibition during I-R injury of the liver, heart, endothelium and brain in multiple species is well established and recently a small number of studies have begun to evaluate the effects of ACE-I during intestinal I-R injury in mice and rats.[68-73] The exact role of the RAS in apoptosis and ischemic disease is still unclear but there is significant interest in using ACE inhibitors to ameliorate apoptosis following I-R injury. In vivo experiments in rats and mice demonstrate that use of ACE inhibitors and AT-R blockers provides anti-apoptotic benefits following an ischemic insult to the heart, brain and kidney. There is also increasing interest in the effects of ACE inhibition on intestinal apoptosis secondary to hypovolemia, shock, resection and I-R injury.[65,66,74-77]

ACE inhibitors are primarily used in the treatment of cardiovascular and renal disease. The various inhibitors differ in their affinity for tissue bound ACE and bioavailability and are classified into 3 groups according to the chemical structure of their active site. The oldest group consists of the sulfhydryl containing inhibitors such as captopril and these drugs may also have properties other than ACE inhibition, such as free radical scavenging and effects on prostanoids. Fosinopril is currently the only phosphinyl containing inhibitor. Lastly, there are the carboxyl containing inhibitors including enalapril, which was the first of the dicarboxylate-containing ACE inhibitors.[65,66]

The primary function of an ACE inhibitor is to alter the balance between the vasoconstrictive, salt-retentive and hypertrophic properties of ANG II and the vasodilatory and natriuretic properties of bradykinin. They are also capable of altering the metabolism of a number of other vasoactive substances. ACE inhibitors exert their effects on cardiovascular homeostasis by decreasing the concentration of ANG II and the degradation of bradykinin. The result of these actions is a decrease in systemic vascular resistance

with little change in heart rate. In general, ACE inhibitors lack adverse metabolic effects and they are known to cause regression of left ventricular hypertrophy and reduce mortality in patients with congestive heart failure and diabetic nephropathy.[65,66]

One postulated mechanism for the anti-apoptotic effects of ACE-I following I-R injury is via scavenging of free radicals, but numerous studies in both mice and rats have yielded conflicting results. It has been hypothesized that ACE-I might alter the pro-oxidant/anti-oxidant balance through enhancement of antioxidant enzymatic defenses to protect cells from ROS damage. Although it has been generally accepted that only thiol containing ACE-I are capable of scavenging free radicals, this was not the case following experimental cerebral ischemia in mice. The anti-apoptotic effects of two non-thiol containing ACE inhibitors, enalapril and moexipril, were evaluated and it was demonstrated that administration of an ACE-I one hour prior to ischemia decreased cerebral infarct size. Both drugs were potent ROS scavengers and it was postulated that these effects could be due to a direct chemical interaction of the ACE inhibitor and ROS or that modulation of intracellular antioxidant pathways might be involved.[71]

Research into the ability of chronically administered enalapril or captopril to enhance enzyme defenses related to SOD, GPX and catalase following I-R injury to the liver, kidney, heart and brain of mice found that both ACE inhibitors increased activity of SOD and GPX during a 4-11 week time period, but neither drug had any effect on catalase activity. Acute administration of these inhibitors was not able to modify activity of any enzyme. This model found that the scavenging abilities of these ACE inhibitors during long term use were mediated through an increase in both enzymatic and nonenzymatic antioxidant defenses in various tissues. Interestingly, both enalaprilat and captopril were able to influence anti-oxidant defenses through alterations in endogenous enzymatic and non-enzymatic systems but only captopril had direct scavenging effects.[68]

It has been suggested that the radical scavenging ability of captopril is due to its thiol group. Studies in cultured human T-cells investigated the effects of thiol-containing versus non-thiol-containing ACE inhibitors on apoptosis during oxidative stress. Thiol-containing inhibitors were able to almost completely inhibit Fas mediated apoptosis in human peripheral T cells but non-thiol antioxidants did not confer protection against apoptotic signals. This suggests that the anti-apoptotic effects were secondary to sulphydryl redox regulation of critical molecules in the apoptotic cascade.[78] Other groups have postulated that the thiol group on captopril may not only be essential to its ability to inhibit Zn2+ dependent ACE, but may also enable the drug to block other enzymes with transition metals at their active sites. Thus it must be considered that inhibition of metal-dependent enzymes other than ACE could be responsible for the effects of captopril on T cell activation.[79]

However, the radical scavenging ability of ACE inhibitors is controversial. Some of these drugs have been shown to improve endothelial function in numerous tissues and in cultured human endothelial cells. Despite these findings, evaluation of the effects of enalaprilat and captopril found that both inhibitors decreased glutathione peroxidase and SOD in cultured endothelial cells, had no effect on lipoperoxidation and were unable to protect against apoptosis.[80] This suggests that improvement in endothelial function secondary to administration of these drugs is not due to modulation of intracellular radical scavenging systems and that these drugs are not effective in preventing apoptosis in vitro.

In vivo studies in a murine model of cardiac I-R injury provides evidence that ACE inhibitors may modulate levels of a number of pro- and anti-apoptotic proteins. Treatment of these mice with an ACE-I suppressed the disappearance of Bcl-xL in the area where most apoptotic cells were found but expression of bax, bcl-2 and bad in this region were the same with or without ACE inhibition. Thus it was concluded that the anti-apoptotic effect of ACE-I was probably secondary to the bax/bcl-xL ratio. Since concurrent administration of a bradykinin-2 receptor (BK-R2) antagonist significantly decreased the protective effects

of ACE inhibition it was suggested that these protective effects are partially mediated by a BK-R2 dependent pathway.[70]

The effects of enalapril on colonic mucosal apoptosis during ulcerative colitis demonstrated that ACE inhibition significantly reduced intestinal epithelial cell apoptosis, decreased mucosal expression of TNF-α and attenuated colitis. Enalaprilat treated mice showed increased expression of both bax and bcl-2 but the relative changes showed a marked increase in bcl-2. This suggests that expression of these factors is influenced by ACE inhibition, and that enalapril mediated decreases in apoptosis may be due to a relative increase in bcl-2 expression.[73]

It is known that intestinal intraepithelial lymphocytes play a role in intestinal adaptation following massive small bowel resection. ACE is present in human, swine, rat and mouse intestinal mucosal epithelium and a recent study demonstrated that use of an ACE-I significantly reduced mucosal cell apoptosis rates following small bowel resection. This action of ACE inhibition on lowering the rate of apoptosis varied with location along crypt-villus axis with a significant decline seen in both the crypt and lower 1/3 of villus and only a slight decline in the top 1/3. There was also a significant post-resection increase in IEL-derived ACE mRNA expression in mice not treated with an ACE-I concomitant with a rise in TNF-α mRNA expression. Use of an ACE-I ameliorated increases in both TNF-α and ACE mRNA expression concurrent with a decline in mucosal epithelial cell apoptosis. This suggests that the TNF-α mediated apoptotic pathway is involved in mucosal apoptosis following small bowel resection. Results were confirmed in TNF-null mice, indicating that TNF-α is essential for ACE mediated mucosal cell apoptosis. This study found no significant changes in Fas or FasL mRNA after resection nor did ACE-I administration lead to any changes in their expression implying that the Fas/FasL extrinsic pathway of apoptosis is not involved in resection associated mucosal cell apoptosis.[81]

There is limited information regarding the pharmacokinetic and clinical effects of ACE inhibitors in horses. Intravenous administration of enalapril at a dosage of 0.5mg/kg resulted in plasma concentrations consistent with those known to achieve therapeutic levels in other species but there is extremely low bioavailability of enalapril administered to horses per os.[82] The effect of intravenous enalaprilat in exercising horses at a dose of 0.5mg/kg is an immediate and significant reduction in serum ACE activity that is maintained for approximately 2 hours post-administration and does not return to baseline for 24 hours. Despite this substantial ACE inhibition enalaprilat does not have any effect on pulmonary or systemic hemodynamics during intense exercise.[83] The effects of enalaprilat on horses under general anesthesia are currently unknown, as is their potential use in gastrointestinal I-R injury.

Apoptosis in the equine small intestine following experimental ischemia-reperfusion injury

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INTRODUCTION ii

Ischemia-reperfusion injury following a strangulating obstruction of the equine small intestine is recognized as a significant contributor to post-operative complications such as SIRS, MODS, DIC, non-responsive shock and death.[6,7] Small intestinal ischemia in horses is usually secondary to entrapped bowel resulting in direct arterial and venous occlusion, or volvulus that increases venous backpressure on capillaries.[9] The intestinal mucosal epithelium is highly energy dependent and reduced blood supply and oxygenation results in rapid injury and death. If blood flow returns to tissue before irreversible cell death certain biochemical events occur immediately upon reperfusion. Reperfusion injury is then initiated by several mechanisms that create an inflammatory response.[9]

Although still not completely elucidated, current understanding of I-R injury is based on the premise that a complex cascade of interrelated pathophysiologic events that is initiated by endothelial dysfunction that includes production and release of ROS from endothelial cells, the damaged intestinal mucosa and resident and circulating neutrophils. Studies performed in rats show that production of ROS leads to lipoperoxidation and the activation of neutrophils, platelets, cytokines, the coagulation system, vascular endothelium and the xanthine-oxido-reductase enzyme system.[10-12] There is also increasing evidence that apoptosis of intestinal mucosal epithelial cells may represent another significant source of intestinal damage following I-R injury.[13,14]

Recent work in other species has established that the RAS can play a significant role in apoptosis following an ischemic event in numerous organs including the heart, kidney, lungs and intestine. Furthermore, apoptosis was detected in intestine from horses with natural obstruction and strangulating obstruction.[84] Evidence exists that the use of angiotensin-converting enzyme inhibitors may confer benefit in ameliorating apoptosis following I-R injury. A number of mechanisms for the beneficial effects of ACE-I

during I-R injury have been proposed including scavenging of radical oxygen species and alterations in expression of various apoptosis related genes.[60,68,74-77]

The objective of this study was to characterize the apoptotic response in equine small intestine subjected to experimental ischemia-reperfusion injury and determine if use of an angiotensin-converting enzyme inhibitor (enalaprilat) would ameliorate apoptosis. Specifically, the experiment was designed to determine if mucosal epithelial cells undergo apoptosis during ischemia, and if apoptosis is exacerbated by reperfusion.

MATERIALS AND METHODS

Horses

Twelve healthy horses (age 5-15 years, weight 400-600kg) were used in the study. All horses received routine vaccinations, prophylactic anthelmintic treatment, foot care, and Coggins testing during a 2-week quarantine period. Horses were housed at the quarantine facility for an additional 2-4 months prior to use in the study, and randomly assigned to one of two groups (6 horses/group). Five days before scheduled research surgery, each horse was transported to the College of Veterinary Medicine Laboratory Animal Resources facility and housed individually in a stall and provided with free-choice grass hay until the night before surgery. Water was available at all times. The experimental protocol was approved by the Virginia Tech Animal Care and Use Committee prior to acquisition of the horses.

Research Surgery

On the morning of surgery two jugular catheters were placed, one in each vein. Horses were sedated with xylazine (0.2 to 0.5 mg/kg IV) and general anesthesia induced with guaifenesin (50 mg/kg, IV) and ketamine hydrochloride (2.0 mg/kg, IV). The horses were positioned in dorsal recumbency and anesthesia maintained with isoflurane vaporized in oxygen using intermittent positive pressure ventilation. Mean blood pressure was continuously measured and monitored by direct arterial catheterization. Blood pressure was maintained at >70mm Hg by dobutamine infusion and lactated Ringer's solution was administered at 5-10ml/kg/hr IV. The $PaCO_2$ was maintained at < 30-40mm Hg. Group I (n=6) horses were randomly assigned to receive enalaprilat (0.5mg/kg IV in 1L 0.9%NaCl, Sigma Aldritch, St. Louis, MO) by continuous rate infusion for one hour beginning 15 minutes prior to the end of ischemia. Group 2 (n=6) horses received an equal volume of saline (placebo).

Horses underwent a routine ventral midline celiotomy. Beginning at the distal jejunal arcade, a 30cm intestinal segment supplied by one jejunal artery and vein was identified and circumferential latex rubber

tubing was placed at each end of the intestinal segment occluding the lumen and extramural vasculature. The jejunal segment was placed on a plastic sheet overlying a warm heating pad (37^0C). Intestine was kept continually moist with sterile lactated Ringer's solution and covered with plastic sheeting to prevent tissue dehydration.

The mesenteric artery and vein supplying the segment was isolated and a Doppler ultrasonic probe applied to the jejunal artery to monitor continuous arterial blood flow to the isolated segment. Systemic arterial pressure and mesenteric arterial blood flow were continually monitored. Jejunal blood flow was then reduced to 20% of normal flow for one hour, and zero blood flow for an additional 60 minutes by application of a jejunal artery vascular clamp. Following this ischemic period the artery clamp was released to initiate reperfusion according to the technique described by Dabareiner et al.[2]

Reperfusion was observed for 3 hours and then at the completion of that time the horses were euthanatized under general anesthesia with an overdose of sodium pentobarbitol (100mg/kg IV) at the conclusion of the study. Blood flow was recorded every 15 minutes during reperfusion. Full thickness jejunal segments were taken from the isolated segment prior to bowel manipulation (control sample: time 0), at the end of ischemia (time 2), and after 1 (time 3), 2 (time: 4) and 3 (time 5) hours of reperfusion. Specimens were pinned to a board and covered in buffered neutral formalin prior to immersion and storage for histologic evaluation.

Additional mucosal biopsy samples for gene expression studies were obtained and were immediately stored in an RNA preserving fluid (RNAlater, Ambion, Austin, TX), refrigerated overnight and were then transferred to -80°C until analyzed. Blood samples were taken from the jugular venous catheter and collected in ACT and serum clot tubes at the same time as the intestinal biopsies. Samples were immediately centrifuged and plasma or serum aliquoted into 5mL tubes and stored at -80°C for future plasma ACE and enalaprilat determination.

Histology

Paraffin imbedded, formalin fixed jejunum was cut into five micrometer sections on a microtome and then stained with hematoxylin and eosin. Degree of injury or regeneration was determined via light microscopy and scored using a modification of a previously reported grading system (Table 1).[85] Slides were evaluated independently by three investigators (JM, BJ, ADN)[1] who were blinded to both group and time point. Sections that showed variable grades of damage were scored based on the average score of the 3 investigators.

Table 1: Histological Grading System[2]

Grade	Criteria
I	***Subepithelial edema:*** mild-small focus confined to region of the villus tip ***Epithelial cell loss:*** minimal-focal loss of a few cells at villus tip ***Villus blunting:*** none ***Hemorrhage in debris:*** none
II	***Subepithelial edema:*** moderate-(between I & II) ***Epithelial cell loss:*** mild-loss of most cells at villus tip ***Villus blunting:*** mild ***Hemorrhage in debris****:* scattered erythrocytes in debris
III	***Subepithelial edema:*** marked-extensive edema, lifting of large sheets of epithelium from villus ***Epithelial cell loss:*** moderate-loss of up to 25% of villus epithelial cells ***Villus blunting:*** moderate ***Hemorrhage in debris:*** moderate-between I & II
IV	***Epithelial cell loss:*** marked-extension of cell loss involving 25-50% of villus epithelial cells ***Villus blunting:*** marked ***Hemorrhage in debris:*** small clots and/or significant # erythrocytes in cellular debris

[1] JM: Jill McCutcheon, BJ: Bernard Jortner, AND: Amy Dae Nagy

[2] White NA, Moore JN, Trim CM: Mucosal alterations in experimentally induced small intestinal strangulation obstruction in ponies. Am J Vet Res 41:193-198, 1980.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling method (TUNEL)

Five micrometer sections of formalin fixed jejunum were used to assess apoptosis in all layers of intestine via the terminal deoxynucleotidyl transferase (TdT) dUTP-nick end labeling method (TUNEL) using (*In Situ* Cell Death Detection Kit, Roche, Mannheim, Germany) according to manufacturer's instructions. In brief, tissue sections were deparaffinized by heating at 55^{o} C for 30 minutes and then washed and rehydrated in graded solutions of xylene and ethanol. Tissue was permeabilized in proteinase K for 30 minutes followed by incubation in blocking solution of 3% hydrogen peroxide in methanol for 10 minutes to quench endogenous peroxidases. TUNEL reaction mixture was used at a dilution of 2:25 (TdT:buffer), followed by rinsing and incubation with anti-fluoroscein antibody conjugated with horse-radish peroxidase (POD). 3,3'-Diaminobenzidine (DAB)(Sigma, Saint Louis, Missouri) was added to precipitate peroxidase and finally slides were lightly stained with hematoxylin. Positive and negative controls were run in parallel with each batch of slides.

Isolation of RNA

Individual samples of jejunal mucosa preserved in RNAlater were thawed, homogenized and RNA was isolated from tissue using a silica gel based method of RNA isolation (Mini Kit, Qiagen GmbH, Hilden, Germany). RNA was quantitated using a fluorescence based method (Quant-iT Ribrogreen, Invitrogen, Carlsbad, CA)in a fluorescent plate reader (Tecan Genios, MTX Lab Systems Inc, Vienna, VA. cDNA was generated using MoMLV reverse transcriptase, dNTPs,and a mix of random hexamers and oligo dT primers (iScript, Bio-Rad, Hercules, California) using 100ng total RNA per reaction.

Primers for bax, bcl-2 and p53 were designed using primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MD, USA) (Table 2).[86] The p53 primer sequence was based on a partial equine sequence. Bax was designed using a consensus sequence derived from homo sapiens, mus musculus,

bos Taurus, and ovis aries. Bcl-2 was created from a consensus sequence derived from homo sapiens, bos taurus and ovis aries.

Table 2: Primer sequences

Gene	Forward	Reverse
Bax	AACATGGAGCTGCAGAGGAT	CGTCCCAAAGTAGGAGAGGA
Bcl-2	GGTGGAGGAGCTCTTCAGG	TTCAGAGACAGCCAGGAGAAA
P53	GCCATGGCATCTACAAGAA	TCCTTCTTGCGGAAGTTTTC

PCR product was generated using Platinum PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. In brief, 0.5ul forward primer (200ng), 0.5ul reverse primer (200ng) and 4ul cDNA were added to 45ul Supermix and the reaction was run as follows: 95C for 30 seconds, 55C for 30 seconds, 68C for 1 minute per kb for a total of 30 cycles. PCR product was evaluated via gel electrophoresis and purified using QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). Purified PCR product was then cloned using a TA cloning vector and chemically competent E. coli (Transforming One Shot TOP10 kit, Invitrogen, Calrsbad, CA). Plasmid DNA was purified with a silica gel based plasmid isolation technique (QIAprep Miniprep Kit Qiagen GmbH, Hilden, Germany) and quantified using Hoechst 33258 (Invitrogen, Carlsbad, CA) using a fluorimeter and submitted to the Virginia Bioinformatics Institute (Virginia Tech, Blacksburg, VA) for sequencing to ensure correct product. Gene sequences, homology to gene of interest and E-values provided in Appendix 1.

Specific primers for qPCR were designed using the same Primer 3 software as used above with the equine specific sequences as determined by the cloned sequences (Table 3).

Table 3: Primer sequences for qPCR

Gene	Forward	Reverse
Bax	ATGGAGCTGCAGAGGATGAT	GGCAAAGTAGAAAAGGGCAAC
Bcl-2	ATGTGTGTGGAGAGCGTCAA	GCCGGTTCAGGTATTCAGTC
p53	GGAGTCTTCCAGGGTGATGA	TGGCTCTGACTGTACCACCA

cDNA was then generated as previously described and the product of the reverse transcriptase reaction was used for qPCR to determine expression of apoptosis related genes. qPCR was performed using SYBRGreen (SYBR-490, Invitrogen, Carlsbad, CA) in a Bio-Rad iCycler (Certified GeneTool, Milpitas, CA) using equine 18s as the housekeeping gene.

HPLC

Quantification of ACE was performed via a previously validated liquid-chromatographic assay.[87]

Table 4: Reagents for HPLC

Substrate	8 mg of hippuryl-L-histidyl-L-leucine dissolved in 1mL of 20mmol/L NaOH and then added to 4mL of PBS. pH adjusted to 8.3 and refrigerated for 24 hours prior to use
Standard	100mg *n*-benzoyl-glycine in 1 L H_2O
Sulfamic Acid	100g sulfamic acid in 1 L H_2O
Extraction Solvent	30mg β-hydroxyethyl-theophylline in 1 L of dichloromethane/*n*-amyl alcohol solution (2/1 by volume)

Sample preparation:

100uL aliquots of substrate were placed in 1.5mL centrifuged tubes and equilibrated at 37C. 25uL of serum added to tubes and incubated at 37C for 10 minutes. 25uL of sulfamic acid was then added to tubes to stop the reaction. 100uL of extraction solvent was then added to the centrifuge tubes and vortexed for 10 seconds. Tubes were centrifuged at 9000xg for 120 seconds and then a 25 uL aliquot was extracted from the bottom layer for injection onto the column.

Separation and quantification of angiotensin converting enzyme (ACE) was performed via 1050 Agilent (Palo Alto, CA) HPLC system equipped with quaternary pump, Diode Array UV Detector, Autosampler and oven heater using a Phenomenex LUNA C_{18} Column (250 x 4.6mm- 5μm dp)

(Phenomenex, Torrence, CA) with an injection volume of 10 μL and UV detection at 230 nm. The mobile phase A consisted of 26 mM NH_4OAc, adjusted to pH 4 using acetic acid and mobile phase B was CH_3CN. All separations were obtained using gradient elution (Table 5) at a flow rate of 1 mL/min. Aliquots of plasma supplemented with 6.25-100ug/mL to establish the linearity of the chromatographic measurement.

Table 5: Gradient elutions for HPLC

Gradient Elution Conditions		
Time	%A	%B
0	95	5
10	70	30
12	95	5
14	95	5

HPLC/Mass Spec

Enalaprilat was extracted using a method previously developed and validated and analyzed by LC-MS/MS.[88] Extracts were diluted to place concentration of enalaprilat within the range of the LC-MS/MS calibration curve: 10ng/mL-125ng/mL. All HPLC separations were obtained using an Agilent (Wilmington, DE USA) HPLC 1100 series equipped with diode array detector, column heater, and Thermo Survey (San Jose, CA USA) auto-sampler. A solution (20 μL) of each enalaprilat standard or extract was then injected onto the column via the auto-sampler. The column for all HPLC separations was an Agilent (Wilmington, DE USA) silica based-C_{18} (150 x 2.1mm, 5 μm, dp). Mobile phase A consisted of 1% aqueous formic acid; while mobile phase B contained 1% (v/v) formic acid in acetonitrile. The mobile phase was delivered to the HPLC column at a flow rate of 0.2 mL/min and the gradient mobile phase elution program (Table 6) was as follows:

Table 6: Gradient elutions for HPLC/MS

Gradient Elution Conditions		
Time	%A	%B
0	85	15
4	40	60
7	20	80
11	20	80
15	85	15

For mass spectral analysis, the HPLC column effluent was pumped directly without any split into the Thermo Instrument TSQ triple quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA), equipped with ESI source, which was used in the positive ion MS/MS modes. The instrument was calibrated with a solution of polytyrosin according to the manufacture's recommendation. Tuning was performed for enalaprilat by direct infusion of a solution (1 ng/μL) at a rate of 5 μL/min into mobile phase (50/50% A/B) at flow rate of 0.2 mL/min. Table 6 shows the tuning parameters for enalaprilat. Table 1 also shows the parent ion and product ion transition for analyte when operated in the single reaction monitoring (SRM) mode.

Table 7: MS/MS Conditions for Analysis of Different Drugs

Analyte	Parent Ion (amu)	Product Ions (amu)	Collision Energy (eV)	Q2 Collision Gas Pressure (mTorr)	Analysis Mode
Enalaprilat	349	206	27	0.9	+

Statistics

Horse to horse variability in baseline ACE concentrations was assessed using a coefficient of variation. Baseline ACE concentrations were compared between the treatment groups using a 2-sample t-test. Effects of group (or treatment) and time on percent change from baseline in ACE concentration were

assessed using repeated measures analysis of variance. Interactions between group and time were investigated using the slice option of proc glimmix followed by the tukey's procedure for multiple comparisons. Statistical significance was set at alpha = 0.05. All analyses were performed using SAS version 9.1.3 (Carry NC, USA).

Statistical Methods for Histology Scores

Within groups, the median change in histology scores from baseline up to each of the time points was assessed for statistical significance using the Wilcoxon signed rank test (Wilcoxon one sample test). To compare the groups for median change (from baseline) in histology scores at each of the time points, a Wilcoxon rank sum test (Wilcoxon 2 sample test) was performed. Statistical significance was set alpha = 0.05. All analyses were performed using SAS version 9.1.3 (Cary, NC, USA).

Statistical methods for Gene data

After normalizing BAX and P53 starting quantities to the house keeping gene (rRNA), horse to horse variability in baseline quantities was assessed using a coefficient of variation. Subsequently, the normalized quantities were log (base e) transformed and effects of group and time assessed using repeated measures analysis of variance. Because expression at each of the time points was assessed relative to baseline, the Dunnett procedure was applied to correct for multiple comparisons. Interactions between group and time were investigated using the slice option of proc glimmix. Statistical significance was set at alpha = 0.05. All analyses were performed using SAS version 9.1.3 (Carry NC, USA).

RESULTS

Histopathology

When compared to control tissues (Figure 10), ischemic tissue showed substantial epithelial damage with no obvious evidence of restitution during reperfusion. Grade of mucosal damage was 0 at control time in all samples in both groups. Histologic grade increased to grade I or II by the end of ischemia in both groups. Although there was significant variability in grade of damage between individual horses within each group (P=0.03) (Table 8), when results were pooled there were no significant differences between groups at the end of ischemia (P=0.54) (Table 10). Grade of damage at all reperfusion time points remained significantly greater than controls but never increased beyond grade III (Figure 11). Similar to post-ischemic samples, there was significant variability between individual horses within groups (P=0.03) except for hour 3 of reperfusion in the treatment horses (P=0.06) (Table 9). However, due to a poor biopsy sample one horse in the treatment group had no mucosal tissue for evaluation and could not be scored, resulting in fewer samples for pooled data at this time point. Pooled comparisons between groups showed no significant differences between treatment and controls at any time point.

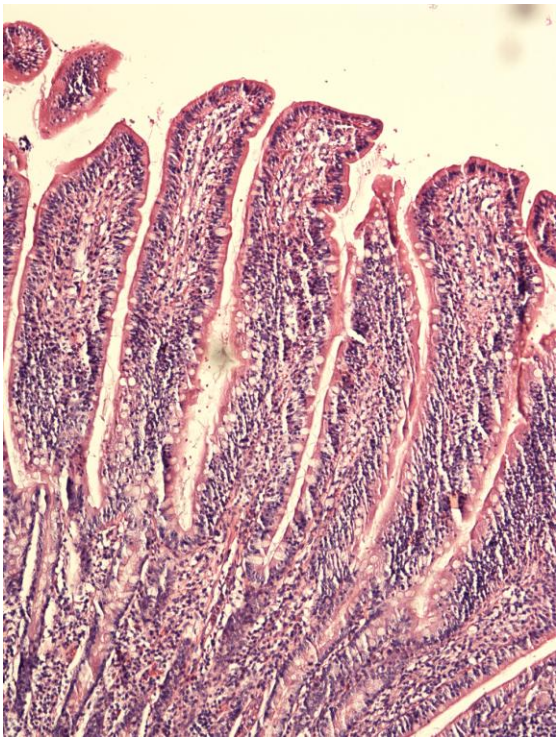

Figure 10: Photomicrograph of normal mucosa from pre-ischemia taken at 40x magnification.

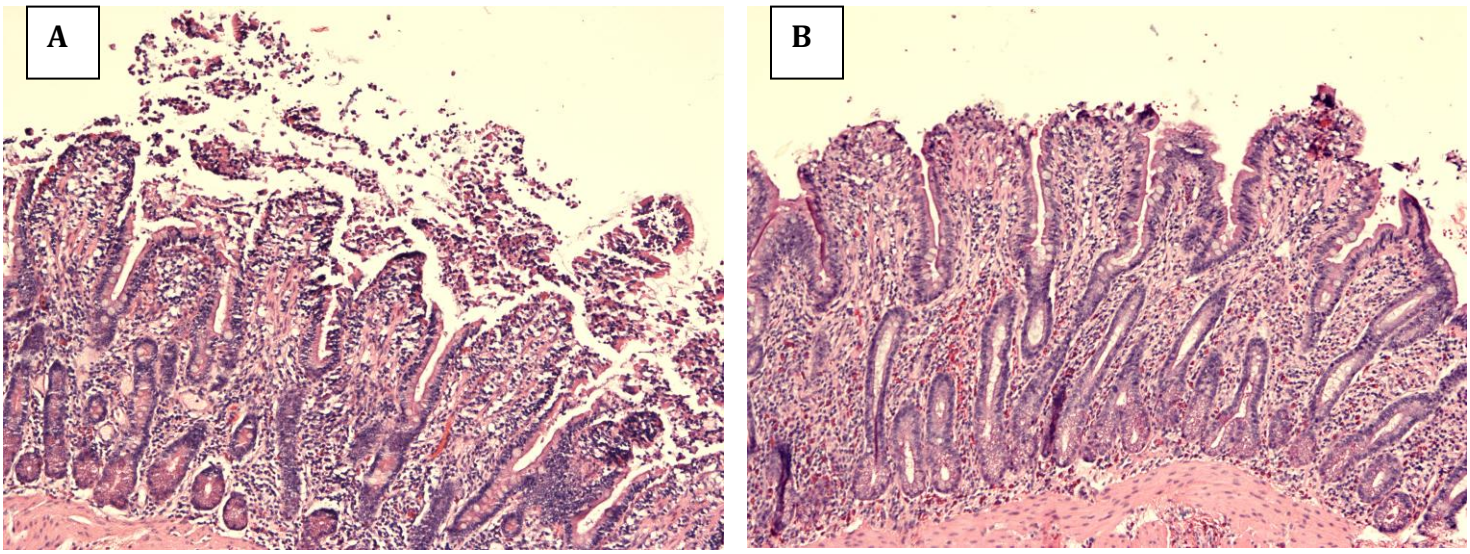

Figure 11: Photomicrographs of post-ischemic and post-reperfusion small intestine. The slide on left (A) is from immediately post-ischemia. The slide on right (B) is from 3 hours reperfusion; both show Grade II mucosal damage. Note the substantial villus blunting and loss of approximately 25% of epithelial cells at villus tip. Also note the variability in the presence of cellular debris between tissue sections. The photomicrographs were both taken at 40x magnification

Table 8: Histology: Comparison Within Groups – Control Group

Time (hours)	Mean & Standard Deviation Control Group	P Value	Mean & Standard Deviation Treatment Group	P Value
0	0	0	0	0
2	1.33 ± 0.52	0.03	1.67 ± 0.82	0.03
3	1.67 ± 0.52	0.03	2.00 ± 0.63	0.03
4	1.67 ± 0.52	0.03	1.67 ± 0.52	0.03
5	1.50 ± 0.55	0.03	1.40 ± 0.55	0.06

Grade of mucosal damage in pooled samples from control and treatment group horses is shown for each time point. P values represent the change in grade at each time point as compared to pre-ischemia (0 = pre-ischemia, 2 = post-ischemia, 3 = 1 hour reperfusion, 4 = 2 hours reperfusion, 5 = 3 hours reperfusion). One sample from the 3 hour time point in the treatment group contained no mucosa for evaluation. This may be responsible for the lack of significance at this time point as compared to previous times.

Table 9: Histology – Comparison Between Groups

Time	Group	P Value
0	Control	1
	Treatment	
2	Control	0.54
	Treatment	
3	Control	0.41
	Treatment	
4	Control	1
	Treatment	
5	Control	0.84
	Treatment	

P values for comparison of control and treatment groups showing lack of significant difference between groups at all time points (0 = pre-ischemia, 2 = post-ischemia, 3 = 1 hour reperfusion, 4 = 2 hours reperfusion, 5 = 3 hours reperfusion).

TUNEL

Quantitative assessment of apoptotic index could not be determined from TUNEL stained slides due to substantial variability in staining (Figures 12 and 13). Subjectively however, apoptosis was present in specific intestinal layers in individual horses at various time points. Apoptotic cells could be identified in all layers except serosa but location varied from horse to horse. In general it appeared that apoptosis was most prominent in the muscularis with more nuclear staining seen at the end of 3 hours reperfusion in the treatment group horses.

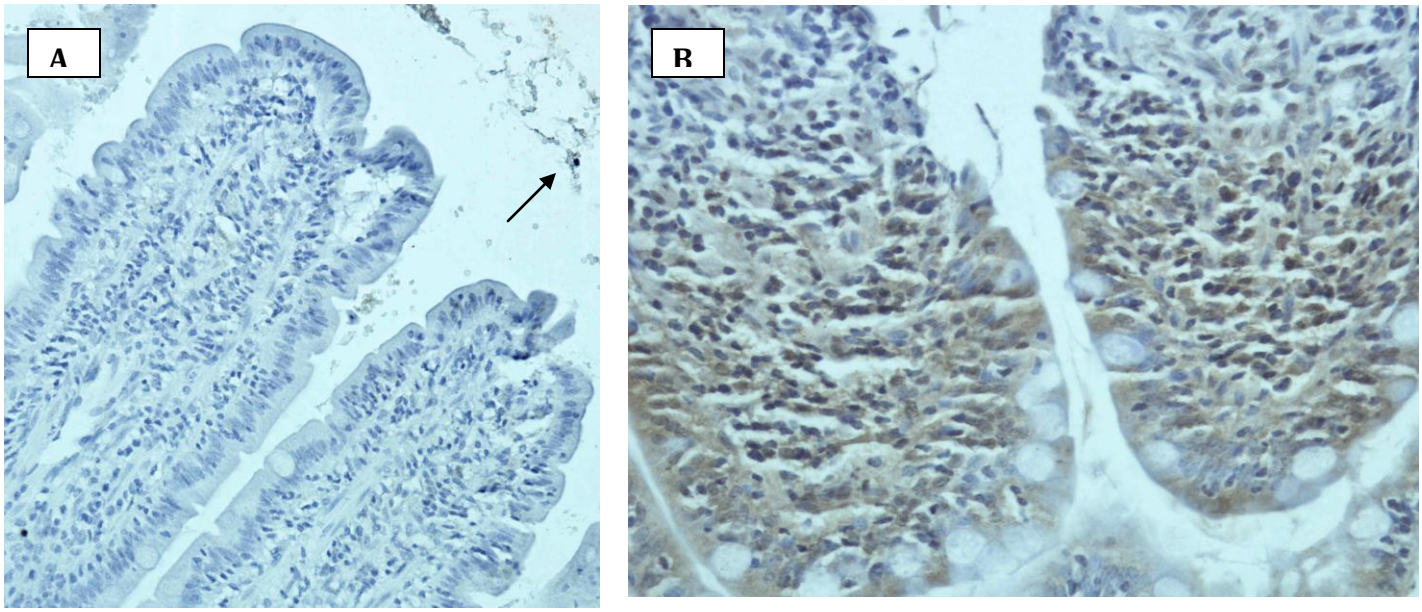

Figure 12: TUNEL staining of mucosa from two horses in the treatment group. The samples were from the same time point, 2 hours of reperfusion. Tissue from both horses showed a similar degree of pathology and slides were stained concurrently. Note the marked differences in uptake of stain. The slide on the left (A) shows good uptake of stain and there is a single cell in the luminal debris (arrow) showing dark nuclear staining and typical apoptotic morphology. The slide on the right (B) was stained at the same time and shows marked background staining as well as diffuse cytoplasmic staining in numerous morphologically normal cells. Many darkly stained nuclei are also present in slide B, but do not show typical morphology associated with apoptosis.

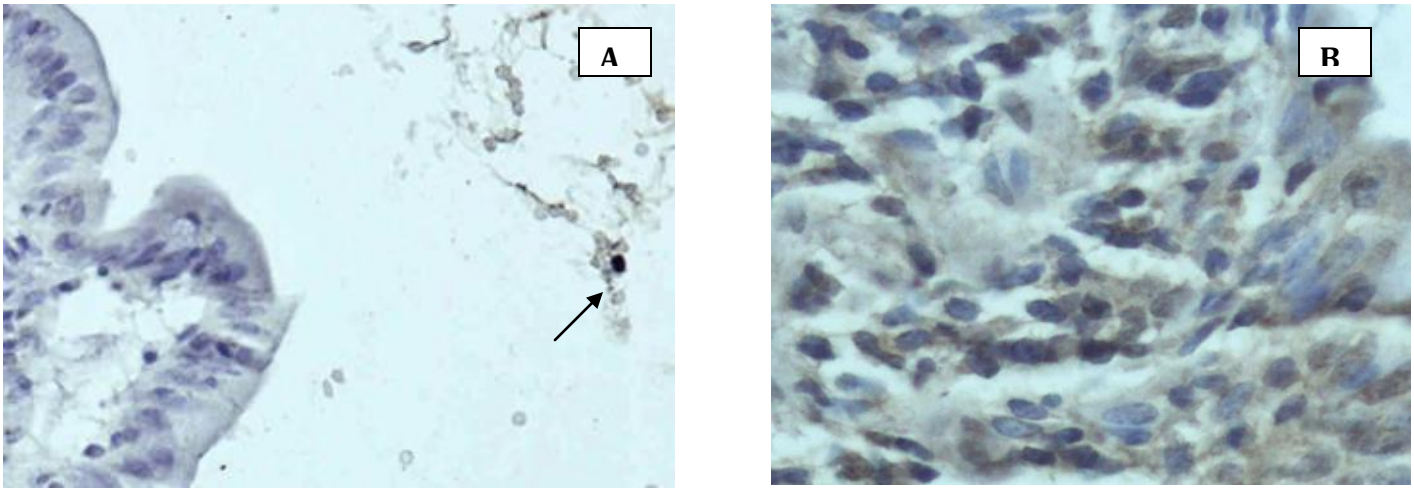

Figure 13: Detail of slides from Figure 12. In slide on the left a single apoptotic cell is apparent in the cellular debris. The slide on the right shows the marked diffuse background staining and many morphologically normal cells with both cytoplasmic and nuclear staining. No apoptotic cells are present

Enalaprilat

Serum enalaprilat concentrations demonstrated considerable variability between horses at each time point. Continuous rate infusion in two horses was initiated less than 15 minutes prior to the end of ischemia and these horses both had no detectable concentration of enalaprilat in serum at hour 2 (immediately post-ischemia). This was most likely due to timing of the infusion. Late initiation of the infusion resulted in inadequate serum concentration at hour 2 and thus enalaprilat could not be detected in serum until 1 hour of reperfusion (hour 3). All concentrations at all time points remained above previously reported therapeutic concentrations (Figure 14 and Table 10).

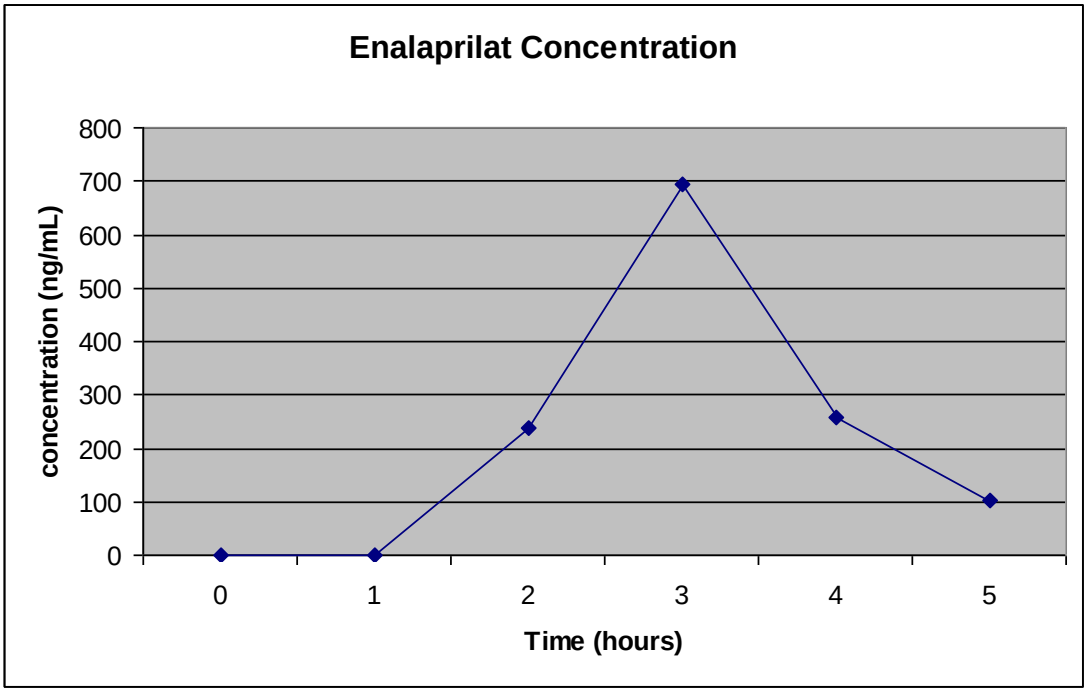

Figure 14: Serum concentration of enalaprilat at each time point in treatment group horses (0 = pre-ischemia, 2 = post-ischemia, 3 = 1 hour reperfusion, 4 = 2 hours reperfusion, 5 = 3 hours reperfusion). Values used are the mean from pooled samples. Enalaprilat was administered as a one hour continuous rate infusion beginning 15 minutes prior to the end of ischemia (hour 2). Peak serum concentrations occurred approximately one hour after the end of infusion at 1 hour reperfusion (hour 3).

Table 10: Serum Concentration of Enalaprilat in Treatment Horses

Time (in hours)	Mean & Standard Deviation	Range
0	0	0
2	85.4 ± 26.3	58.7 – 121.7
3	129.3 ± 57.6	63.8 – 229.7
4	49.3 ± 26.5	30.6 – 95.1
5	20.4 ± 8.2	10.2 – 29.3

Mean, standard deviation and range for serum concentration of enalaprilat in treatment group horses at each time point. Concentrations remained above previously reported therapeutic levels (in humans) at all time points (0 = pre-ischemia, 2 = post-ischemia, 3 = 1 hour reperfusion, 4 = 2 hours reperfusion, 5 = 3 hours reperfusion).

ACE

There was significant variation between all horses (treatment and control) pre-ischemia (coefficient of variation=22.8). ACE concentrations decreased over time within both groups except for a significant spike in ACE activity in the treatment group at 1 hour of reperfusion (P=0.0001) (Figures 15 and 16). There was no significant difference in ACE activity between the groups at any time point except at 1 hour of reperfusion (P=0.009) (Table 11 and Figure 17). There was no apparent correlation between serum concentration of enalaprilat and serum ACE concentration (Figure 18).

Table 11: ACE activity

Time	Control Group		Treatment Group	
	Mean & Std Dev	% decrease	Mean & Std Dev	% decrease
0	46.5 ± 9.0	0	44.5 ± 12.9	0
2	36.3 ± 6.3	22.0	30.3 ± 9.8	32.0
3	35.5 ± 9.3	23.7	39.6 ± 9.8	11.1
4	33.8 ± 10.3	27.4	26.3 ± 14.7	41
5	28.8 ± 6.0	38.0	20.6 ± 9.8	53.8

Mean, standard deviation and % decrease of ACE activity at each time point in both control and treatment groups (means are in ug/mL). Percent change was decreased at all time points in both groups (0 = pre-ischemia, 2 = post-ischemia, 3 = 1 hour reperfusion, 4 = 2 hours reperfusion, 5 = 3 hours reperfusion).

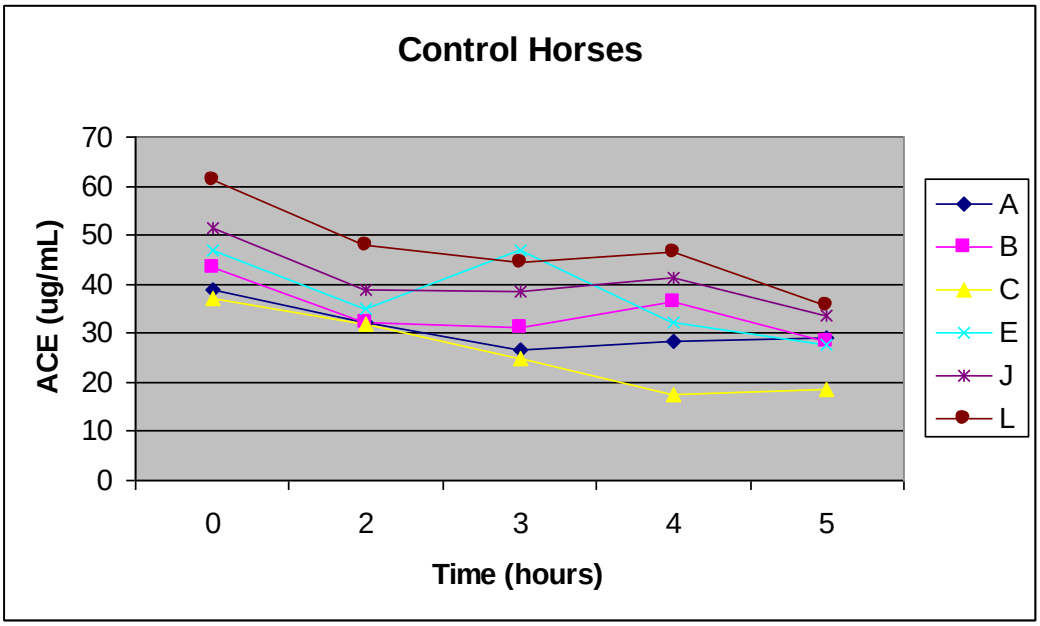

Figure 15: Concentration of ACE in serum from the control group (0 = pre-ischemia, 2 = post-ischemia, 3 = 1 hour reperfusion, 4 = 2 hours reperfusion, 5 = 3 hours reperfusion). Each letter represents an individual horse.

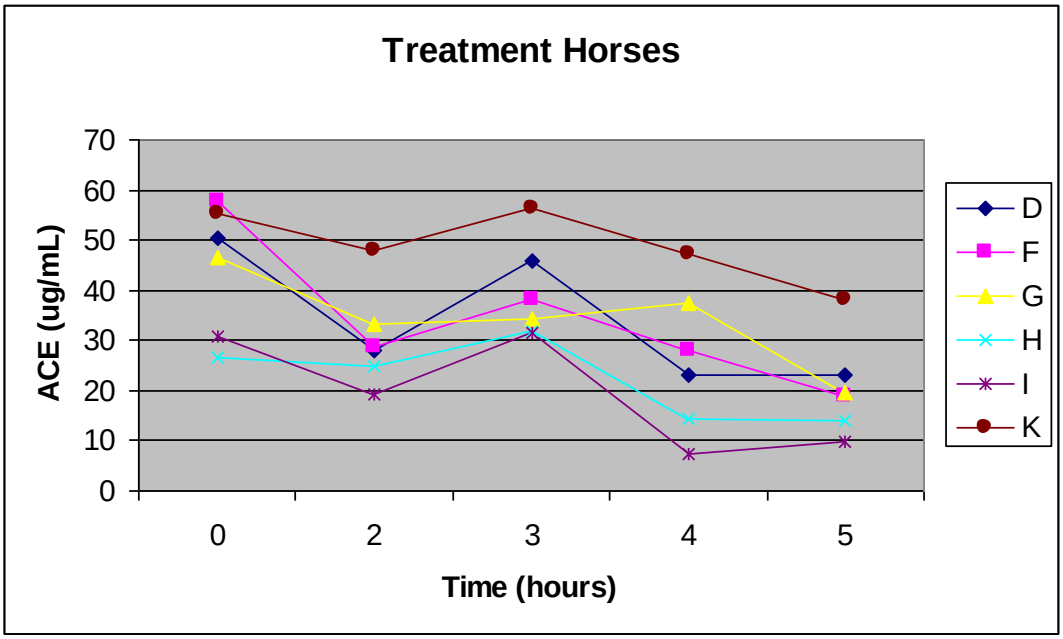

Figure 16: Concentration of ACE in serum from the treatment group horses (0 = pre-ischemia, 2 = post-ischemia, 3 = 1 hour reperfusion, 4 = 2 hours reperfusion, 5 = 3 hours reperfusion). Each letter represents an individual horse.

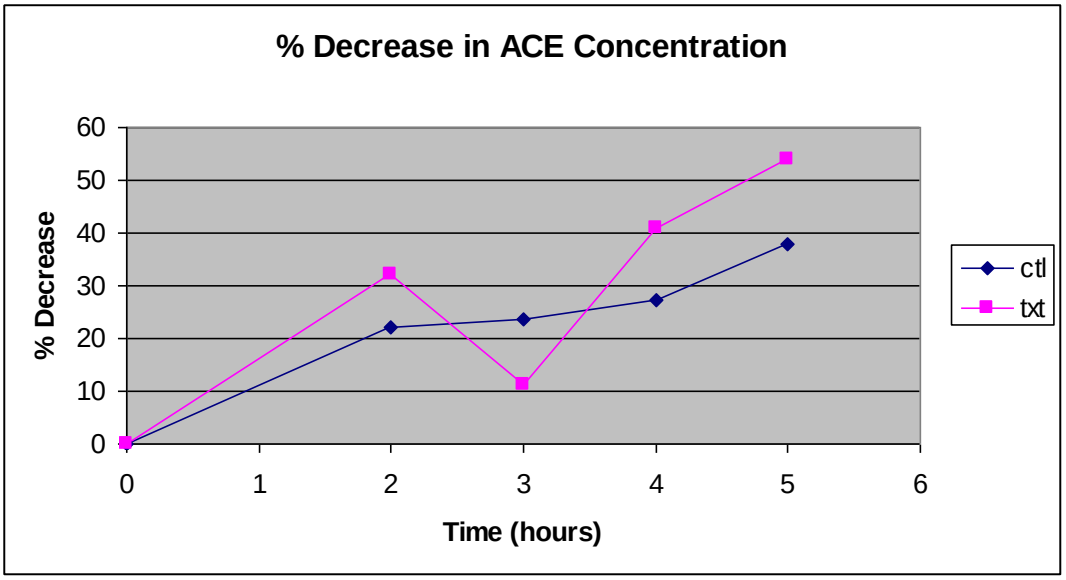

Figure 17: Percent decrease in ACE activity from baseline in both groups using pooled data (0 = pre-ischemia, 2 = post-ischemia, 3 = 1 hour reperfusion, 4 = 2 hours reperfusion, 5 = 3 hours reperfusion). The pink line represents data from treatment group horses and the blue line is data from control group horses.

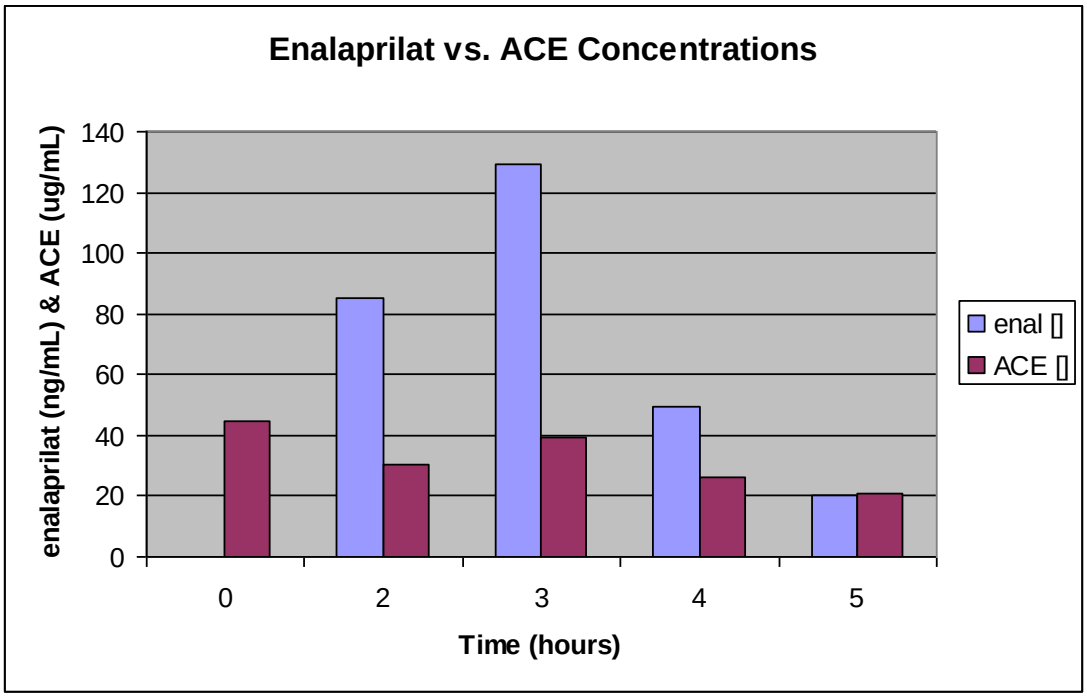

Figure 18: Concentrations of enalaprilat (ng/mL) and ACE (ug/mL) over time (0 = pre-ischemia, 2 = post-ischemia, 3 = 1 hour reperfusion, 4 = 2 hours reperfusion, 5 = 3 hours reperfusion). The maroon bars represent ACE concentration in ug/mL and blue bars represent enalaprilat concentration in ng/mL. Note that despite large increases in enalaprilat concentration at time points 2 and 3 there was little change in ACE concentration from the previous sampling time.

Gene Expression

There was no expression of Bcl-2 in either group at any time point. There was significant variation in both Bax and p53 expression between all horses pre-ischemia (CV=186.9, CV=313.2 respectively) (Tables 12 and 13) (Figures 19 and 20). Control horses demonstrated no significant changes from baseline in Bax expression at all time points except at 2 hours of reperfusion (P=0.02). Expression of Bax was decreased at this time point compared to baseline values. There was no significant change in Bax expression in any of the treatment horses at any time point. There was no significant difference in Bax expression between groups at any time point. p53 demonstrated no significant change in expression within either group at any time point. There was a significant difference between the control and treatment groups at 3 hours of reperfusion (P=0.05) where p53 showed a decrease in expression in the treatment group compared to the control group.

Table 12: p53 data normalized to control

A: CTL			G: TXT		
0	1		0	1	
2	0.260938	0.26 fold decrease	2	0.107818	0.11 fold decrease
3	1.0375	1.04 fold increase	3	0.498804	0.50 fold decrease
4	0.0625	0.06 fold decrease	4	0.006725	0.01 fold decrease
5	0.68125	0.68 fold decrease	5	0	no change

The table shows a comparison of change of expression in p53 between one horse from each group (0 = pre-ischemia, 2 = post-ischemia, 3 = 1 hour reperfusion, 4 = 2 hours reperfusion, 5 = 3 hours reperfusion). Note that due to the high degree of variation in baseline expression of p53 that data from a single horse is not representative of pooled results.

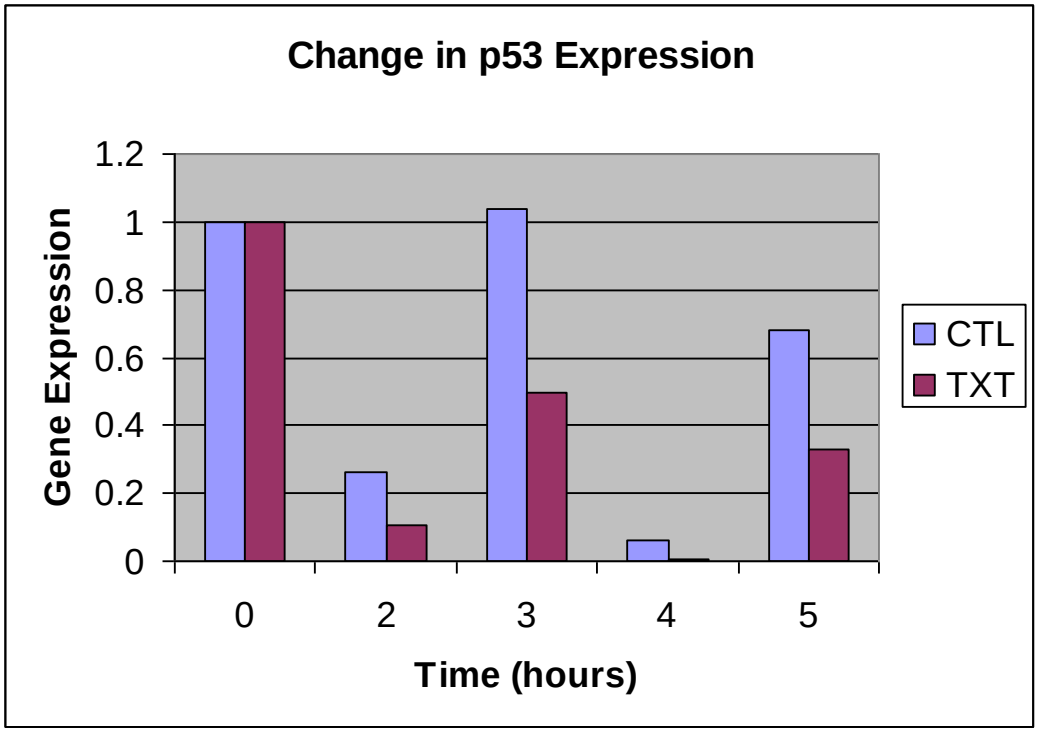

Figure 19: Comparison of change in expression in p53 between one horse from each group (0 = pre-ischemia, 2 = post-ischemia, 3 = 1 hour reperfusion, 4 = 2 hours reperfusion, 5 = 3 hours reperfusion). The blue bars represent data from control group horses and maroon bars represent treatment horses. Note that due to the high degree of variation in baseline expression of p53 that data from a single horse is not representative of pooled results.

Table 13: Bax data normalized to control

L: CTL				I: TXT			
Time	0	1		Time	0	1	
	2	0.234465	0.23 fold decrease		2	0.709684	0.71 fold decrease
	3	0.537128	0.54 fold decrease		3	1.275217	1.28 fold increase
	4	0.228056	0.23 fold decrease		4	0.941604	0.94 fold decrease
	5	0.297009	0.30 fold decrease		5	0.88653	0.89 fold decrease

The table shows a comparison of change of expression in bax between one horse from each group (0 = pre-ischemia, 2 = post-ischemia, 3 = 1 hour reperfusion, 4 = 2 hours reperfusion, 5 = 3 hours reperfusion). Note that due to the high degree of variation in baseline expression of bax that data from a single horse is not representative of pooled results.

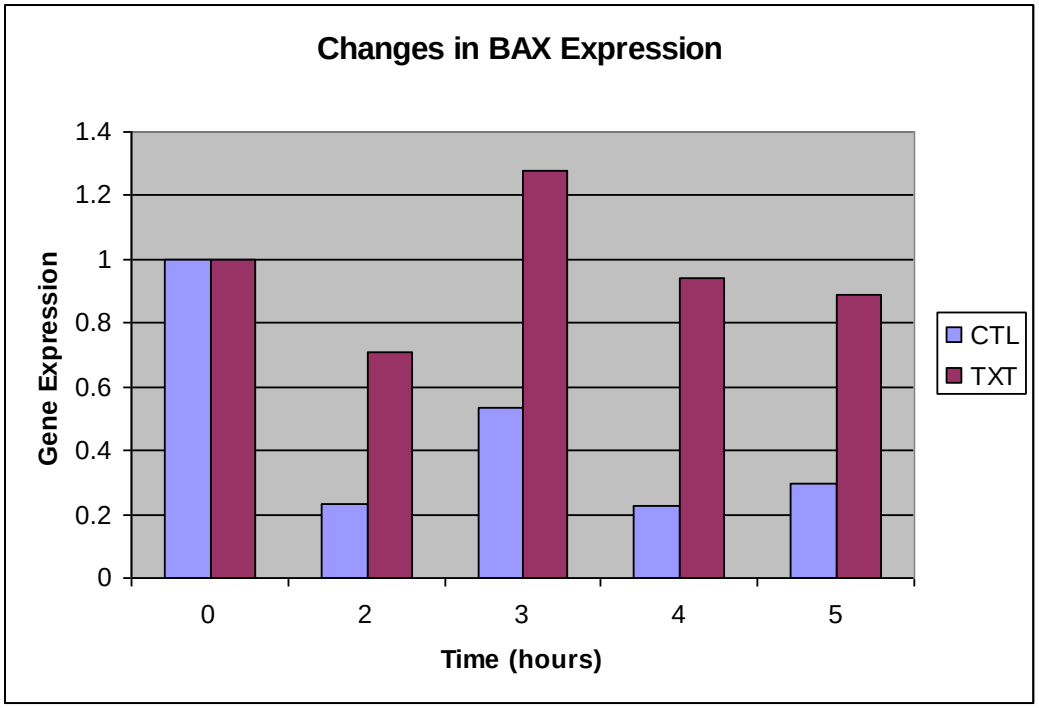

Figure 20: Comparison of change in expression in bax between one horse from each group (0 = pre-ischemia, 2 = post-ischemia, 3 = 1 hour reperfusion, 4 = 2 hours reperfusion, 5 = 3 hours reperfusion). The blue bars represent data from control group horses and maroon bars represent treatment horses. Note that due to the high degree of variation in baseline expression of bax that data from a single horse is not representative of pooled results.

DISCUSSION

The model used in the current study consisted of one hour of low-flow ischemia (20% of baseline arterial flow) followed by one hour of complete ischemia. It should be noted that throughout the study there were intermittent technical problems with our Doppler flow-probe that may have resulted in inaccurate or variable reduction of flow during the first hour of ischemia. Thus only the second hour of complete ischemia can be confirmed in all horses with potentially variable amounts of ischemia during the first hour. It is generally accepted that the degree of damage during the reperfusion phase is directly related to the severity and duration of the initial ischemic insult. Thus it is conceivable that the severity of ischemia in the current study was insufficient to initiate significant increases in pathology secondary to reperfusion.

Previous studies of I-R induced small intestinal damage in horses have yielded conflicting results regarding degree of histologic mucosal pathology. All reports generally agree that there is a significant increase in mucosal damage following various time periods of ischemia. Post-ischemic damage has been observed following as little as 60 minutes of low flow ischemia up to 3 hours of complete arteriovenous occlusion.[48,82] It is widely accepted that complete arteriovenous occlusion of longer than 4 hours duration results in irreversible mucosal necrosis.[35,48] Results of histologic evaluation of post-reperfusion pathology are extremely varied, with some groups reporting no change in damage compared to end-ischemia and other studies demonstrating significant increases concurrent with increasing duration of reperfusion. Such conflicting results are likely due to differences in models of I-R injury and use of differing grading schemes to evaluate mucosal damage. These variations in model and grading system make direct comparisons between studies extremely challenging.

Horne et al. evaluated a model of venous occlusion as compared to a model of complete arteriovenous occlusion. Samples were taken after 3 hours of ischemia, 4 hours of ischemia and 3 hours of ischemia followed by 1 hour of reperfusion. The grading system used was very similar to the one utilized in the

current study. This group found that 3 hours of ischemia resulted in grade III damage in both groups. Grade of injury increased to grade IV following 3 hours of ischemia plus 1 hour of reperfusion in both groups.[89] This suggests that low flow ischemia is capable of inducing reperfusion injury, however the ischemic time period was significantly longer than that used in the current study.

Vatistas et al. reported a significant increase in damage at the end of reperfusion in a low flow model of jejunal I-R. Two hours of low flow ischemia were followed by 2 hours of reperfusion and grade of damage increased from the end of ischemia to the end of the second hour of reperfusion. It should be noted however that the grading system was substantially less stringent compared to that used by Horne et al and the one used in the current study.[90] This same group also used a model that compared the effects of 2 hours of low flow or 1 hour of complete arteriovenous occlusion followed by 2 hours of reperfusion. The grading scheme evaluated mucosal volume, mucosal surface area and submucosal volume as opposed to morphological changes. They reported no decrease in mucosal volume or surface area from the end of ischemia to the end of reperfusion in the complete AVO model. In the low flow group surface area and mucosal volume worsened during the reperfusion phase. However, once again the method for assessing histologic damage was substantially different than those used in other studies.[91]

Evaluation of full thickness tissue sections via light microscopy showed no morphologic abnormalities in any of the horses in either group prior to ischemia. Mucosal damage increased as much as 2 grades by the end of the ischemic. Following ischemia there was no significant increase in grade of mucosal damage at any time point in either group. Pooled results from horses within each group at all sampling times did not reveal any significant differences in grade of histologic damage between treatment and control animals. This indicates that treatment with enalaprilat had no effect on intestinal injury following I-R.

There was substantial variability in TUNEL staining between samples even from concurrently stained slides. Excessive background staining was present in a large proportion of samples and dense nuclear

staining was present in numerous cells lacking classic apoptotic morphology. It appeared that apoptosis was genuinely present to some degree in all horses in various intestinal layers except serosa, but an accurate estimate of apoptotic indices could not be made. Qualitatively, apoptotic staining was most obvious in the muscularis with the most intense staining seen at the end of the 3 hour reperfusion period in the treatment group.

There is still some debate regarding the timing of apoptosis in the small intestine following I-R injury. Rat studies by Noda et al in 1998 found that mucosal cell apoptosis increased immediately after ischemia and peaked at 1 hour of reperfusion.[14] Similar work in rats performed by An et al in 2005 looked at all intestinal layers and found that apoptosis peaked at 3 hours of reperfusion.[10] However this study only assessed overall rate of apoptosis and did not differentiate between changing indices in individual cell layers. Therefore it remains unclear whether such contradictory results are due to actual differences in rate of apoptosis, or if discrepancies are related to inconsistencies in method. It is conceivable that rates of apoptosis differ between cell types during I-R injury and that evaluation of indices in each layer would reveal peak levels of apoptosis at varying time periods depending on cellular location or lineage.[10,14]

Determination of specific apoptotic index is likely of critical importance given the low rate of spontaneous apoptosis in normal small intestine. Since only one apoptotic cell is seen in every 5th longitudinal crypt section and one cell per villus in a 3um tissue section from normal mouse and human small intestine, even small increases in number of apoptotic cells could be clinically relevant.[19,20] Accurate estimates of apoptosis may also be hindered by the rapid nature of the process. In rats, mice and humans, in vivo clearance of apoptotic bodies takes a few hours at most. Following I-R injury apoptosis can appear within minutes after an insult and is frequently completed in 1-4 hours.[14] Thus sampling time becomes critical in determining accurate apoptotic indices. Such studies also indicate that the majority of TUNEL positive intestinal cells are found in the detached epithelial mucosa at the villus tip and by 75 minutes of

reperfusion remaining epithelial cells are not TUNEL positive. Therefore if these detached cells are missing in any given section gross underestimates of overall apoptotic rate are possible.[13] Many tissue sections in the current study lacked the remnants of sloughed epithelium or epithelial debris and thus considerable apoptosis may have been present but gone unrecognized. It must also be considered whether these detached mucosal cells are an important factor in small intestinal I-R damage. /Once these sheets of cells are separated from the basement membrane due to subepithelial edema the mucosal barrier is breached and the animal is susceptible to bacterial translocation and resultant endotoxemia.[13,14,19,20]

Studies of apoptotic gene expression in the mouse small intestine also indicate that considerable regional differences in apoptosis exist. Levels of pro-apoptotic gene expression steadily decrease aborally and are much lower in the distal jejunum and ileum. It is not known if similar regional differences exist in the equine small intestine, but given that our samples were taken from the most distal portion of the small intestine it is possible that degree of apoptosis is much lower in this region. Samples taken further proximally might have revealed higher numbers of apoptotic cells.

Other groups have also reported numerous problems and inaccuracies associated with TUNEL staining. One study designed to evaluate TUNEL specificity in well-defined models of apoptosis, necrosis and post-mortem autolysis in the rat liver found that DNA fragmentation was common to all forms of cell death and that it's detection in situ could not be considered a specific marker for apoptosis.[92] Additionally, use of TUNEL staining in 2 separate studies utilizing a murine model of small intestinal I-R showed that there was an overestimation of the extent of apoptosis when compared to cellular morphology with standard H&E methods. As with our results from the current study, it was found that villus cells with no morphologic evidence of apoptosis were positive to TUNEL staining. It was concluded that it is probably necessary to utilize a combination of methods to more accurately assess apoptosis due to significant non-specific staining associated with the TUNEL method.[14,19,33]

Due to these concerns multiple investigations of apoptosis in normal rat small intestine were conducted and found similar problems associated with TUNEL staining. Apoptotic cell morphology was not concomitant with positive TUNEL staining, but this group postulated that it may have been due to sub-optimal fixation of tissues. It was discovered that number, intensity and extent of TUNEL labeling increased with delayed fixation.[30,45] Our method of harvesting biopsy samples for histologic staining included stretching and pinning samples to a fixed surface and applying formalin to the stretched tissue prior to full immersion. This was done to insure that samples would not curl excessively during the fixation process and would be easier to orient when imbedded in paraffin, allowing for more consistent sectioning and making eventual evaluation of slides more accurate. However, due to possible variations in length of time that samples remained stretched prior to full immersion in formalin it is likely that there were inconsistencies in fixation. This may have resulted in sub-optimal fixation of some samples and could account for our difficulty in obtaining consistent results even within concurrently stained tissue sections.

In the future, more specific techniques for identifying apoptosis could also be employed. Since apoptosis is essentially a caspase mediated form of cell death, use of caspase-3 immunohistochemistry is frequently employed to detect the presence of active apoptosis in tissue. Definitive identification of apoptotic bodies and clear visualization of cellular morphology is possible with electron microscopy and would differentiate between true apoptotic cells and any TUNEL associated non-specificity. Staining for cytochrome c, Fas/FasL, TNF-α and PAF may assist in both identifying the presence of apoptotic cells and also provide insight into specific molecular pathways activated following I-R injury.[10,26,46] This may be especially relevant since both PAF and TNF-α are known to promote I-R induced intestinal cell apoptosis in other species such as rats and mice.[30,44,45]

Bcl-2 expression was undetectable in all small intestinal samples from both groups at any time point. This finding was in agreement with the majority of data from similar studies in mouse, rat and human small

intestine.[48,60,66] Although mouse and human small intestine do demonstrate some staining in the lymphocytes within the Peyer's patches and also in intraepithelial lymphocytes, positive results were for the presence of preformed bcl-2 protein rather than de novo gene expression.[35] It is possible that similar results would be seen in equine small intestine with the use of techniques specific for proteins as opposed to gene expression studies. However, based on results from studies of I-R related small intestinal apoptosis in bcl-2 knockout mice this seems unlikely. It was found that lack of bcl-2 had no effect on rate of apoptosis in these mice. Lack of identifiable bcl-2 in horses from the current study suggests that any I-R induced apoptosis was also through a bcl-2 independent pathway. It should also be considered that gene expression in this study only evaluated mucosal homogenates and this would have excluded the presence of gene expression in other intestinal layers.

In contrast to bcl-2, expression of both p53 and bax was detectable in all control samples prior to initiation of ischemia. Interestingly, there was extreme variability in this basal level of expression between individual horses for both genes (CV=313.2, CV=186.9 respectively). None of the treatment horses showed any changes in bax expression at any time point in the study. Control horses exhibited a significant decrease in bax expression at 2 hours of reperfusion (P=0.02) but expression at all other time points was not significantly different than baseline. The reason for the decrease at this time point in the control group is unknown. Theoretically a decrease in bax expression should result in anti-apoptotic effects as opposed to pro-apoptotic effects, although it is generally believed that changes in bax expression alone are not an accurate predictor of apoptotic activity. Rather it is the ratio between expression of bax and bcl-2 that regulates changes in apoptosis. Since bcl-2 is essentially undetectable in the small intestine of multiple species there is considerable debate regarding the mechanisms that influence apoptosis in the post-ischemic jejunum. Evidence exists that other bcl-2 family members such as bcl-Xl may play the primary role in small intestine as opposed to bcl-2. Future work should evaluate the possible presence of other anti-apoptotic

members of the bcl-2 superfamily. Most groups agree that other factors must play the predominant role in regulating small intestinal apoptosis following I-R injury.[26,27] However, even if bax expression is involved in apoptotic regulation in the small intestine, without accurate results from TUNEL staining to evaluate concurrent levels of apoptosis no correlations can be made. It is possible that downregulation of bax expression at this time point would not result in detectable morphologic evidence of changes apoptosis until later in the reperfusion period. Since samples were only obtained for one additional hour of reperfusion, changes in apoptosis could have occurred and gone undetected.

Bcl-2 proteins are known to be involved in the intestinal adaptive response following resection and anastomosis. After resection bax deficiency prevents increases in enterocyte apoptosis during a one month adaptation period. Thus sustained decreases in bax may be more important during this long term adaptive phase as opposed to the acute reperfusion phase.[27] This seems unlikely however given that decreases in bax expression were no longer evident by hour 3 of reperfusion.

Individual horses in both groups demonstrated no changes in p53 expression at any time point during the study. Pooled results showed a decrease in p53 expression at 3 hours of reperfusion in the treatment group horses when compared to control animals (P=0.05). It should be noted that since the P value cutoff was set at 0.05, this difference between groups just reached the level of significance. Given the lack of change in expression between individual horses in both groups at this same time point, the significance of these results should be interpreted with caution.

In mice and rats, p53 expression is very low in normal small intestine and does not appear to have any role in spontaneous apoptosis. Although changes in p53 expression in some species are known to be involved in increased apoptosis following other types of small intestinal damage this is not believed to occur following I-R injury. Upregulation of p53 is known to correlate with increased intestinal apoptosis following radiation damage and chemotherapeutic insults but results of studies in p53 knockout mice show

that intestinal apoptosis following I-R injury occurs via a p53 independent pathway.[19,20] This would support the lack of change in p53 expression found in the current study and indicate that the slight decrease in p53 in the treatment group horses at hour 3 of reperfusion may have been a spurious result.[19-22] This interpretation of the results would indicate that a p53 independent path is responsible for I-R associated apoptosis in the equine small intestine.

Little research exists regarding the use of ACE inhibitors in horses and currently all available data has been acquired in standing or exercising horses. Dosage for the pro-drug enalapril has been extrapolated from human doses and evaluated both intravenously and orally in horses. Enalaprilat is only available in an intravenous form and dosages in horses are the same as for the pro-drug enalapril. To date, one pharmacodynamic/pharmacokinetic study has been done for enalapril in awake horses using the same dose employed in the current study (0.5mg/kg IV). Serum concentrations of enalapril were measured after both intravenous and oral dosing. Intravenous administration resulted in serum concentrations comparable to what are considered therapeutic in humans. Oral administration showed that bioavailability in horses is poor at this dose and serum concentrations were undetectable in all but one animal. Concurrent ACE activity was only measured after oral administration and no data is available regarding the effect of intravenous enalapril on serum ACE activity at this dosage.[27]

A more recent study evaluated the effects of enalaprilat on various cardiovascular and hematologic values in both standing and exercising healthy horses. Enalaprilat was administered at the same dosage (0.5mg/kg i.v.) and ACE activity was measured along with a variety of cardiorespiratory values. Unfortunately no concurrent measurements of serum enalaprilat concentrations were undertaken. This group found that intravenous administration of enalaprilat as a bolus resulted in immediate and pronounced decreases in serum ACE activity.[83] They found a 75% reduction in ACE activity for 2 hours after administration that did not return to baseline values for 24 hours. There were no associated changes in

pulmonary or systemic hemodynamics in either exercising or resting horses.[83] To our knowledge no data exists that has concurrently measured serum concentrations of both enalaprilat and ACE activity.

In the present study serum enalaprilat concentrations demonstrated considerable variability between horses at each time point. Concentrations at all time points were significantly lower than those previously reported by Gardner et al.[82] However previous data was measured for the pro-drug enalapril administered as a bolus as opposed to the metabolite enalaprilat as a continuous rate infusion. Despite these differences concentrations at all time points remained above previously reported therapeutic concentrations for enalapril. Dosage used in our study was the same as that used in the work done by Muir et al that resulted in significant decreases in ACE activity.[82,83] Similar to the previous findings in both resting and exercising horses, our study found no obvious changes in any cardiopulmonary parameters under general anesthesia and no complications occurred in any of the treated horses.

Baseline concentration of ACE varied significantly in all 12 horses prior to the initiation of ischemia (CV=22.8). Although changes in ACE activity were measured in the previous study by Muir et al they did not report any quantitative values for ACE concentration in serum. ACE activity was reported as percent change from baseline values. In the current study we report actual serum concentrations for ACE. To our knowledge this is the first time that concurrent values for serum enalaprilat and ACE concentrations have been reported. It is also the first report of enalaprilat administration and ACE activity in horses under general anesthesia. According to our data there was no apparent correlation between enalaprilat concentration and ACE concentration at any point in the study. ACE concentrations were similar in both groups and decreased over time except at 1 hour of reperfusion in the treatment group. At this time there was a significant increase in ACE activity in all treatment horses (P=0.009).

The reason for the spike in ACE concentration in the treatment group at this time point is unknown. One possible explanation is that during the ischemic period ACE concentrations could increase in the

isolated loop of intestine and remain sequestered there until reperfusion is initiated. It is known that ANG II is locally increased in the splanchnic vasculature during hemorrhagic shock in pigs but whether this is secondary to increases in ACE activity and also occurs following I-R injury is unknown.[89] If increases in ACE activity are responsible then upon reperfusion this accumulated ACE would enter the systemic circulation resulting in a transient spike in serum concentration. However this explanation would apply to both treatment and control horses and does not account for the fact that the spike only occurred in the treatment group.

Intestinal ACE is essentially a brush border enzyme involved in digestion and thus predominantly located in the intestinal lumen.[48] One additional possibility is that following I-R luminal ACE is able to translocate across the damaged intestinal barrier and enter the splanchnic circulation. This would be initiated immediately following ischemia and concentrations would be expected to increase during the early reperfusion phase. It is conceivable that levels would peak in the systemic circulation within the first hour of reperfusion and then equilibrate and return to baseline by hour two. However this explanation also fails to account for the lack of increase in the control group at the comparable time point. Since grade of mucosal damage was similar in both groups at the end of ischemia it is unlikely that treatment horses would have been more susceptible to such a phenomenon.

Hypothetically ACE concentrations should be decreased in the treatment group at all time points as compared to the control group, however this effect was not seen. Results from the control group indicate that there is no significant change in ACE activity in horses under general anesthesia during experimentally induced small intestinal I-R. Although a general trend toward a decrease in concentration was seen it did not reach the level of significance. ACE concentrations in the treatment group were comparable to those seen in the control horses prior to ischemia and immediately post-ischemia. Following the large spike in ACE concentration at hour 1 of reperfusion levels returned to those seen at similar times in the control group.

This would suggest that ACE activity is not significantly altered during experimental small intestinal I-R in horses and that at the dose used in this study enalaprilat has no suppressive effect on ACE activity under these conditions.

CONCLUSIONS

Subjective results from TUNEL staining and lack of increased expression in the apoptosis related genes evaluated in this work would suggest that no significant degree of apoptosis occurs in experimental I-R injury to the equine small intestine in horses under general anesthesia using isoflurane in oxygen. However, it should be considered that the lack of significant increase in mucosal injury during the reperfusion phase in both treatment and control groups may indicate that the model used in this study does not generate enough damage to accurately assess the occurrence of apoptosis during I-R. This potential problem, in combination with the inconsistencies in TUNEL staining, makes an accurate assessment of apoptosis based on our data challenging. Planning for future research should include consideration of a different model and use of alternative techniques to assess apoptosis.

If apoptosis does play a significant role in reperfusion damage to the equine small intestine, lack of changes in the genes tested in this research does necessarily mean that they are not involved in the apoptotic process. It is possible that preformed protein or regulation at the post-translational level is occurring rather than de novo gene expression. Future work should include evaluation of these factors as well as investigating other apoptosis related genes. Alternative techniques for measuring apoptosis in situ should also be employed in further studies. Use of immunohistochemical techniques, electron microscopy or staining for components of the apoptotic cascade such as cytochrome c would all represent alternative methods for evaluating apoptosis following I-R injury.

The current study did not assess for the presence of ROS activation with or without ACE-inhibitor treatment, but the lack of difference between treatment and control groups indicates that any potential scavenging effects of enalaprilat were insufficient to confer clinical benefit. Other than direct measurement of ACE concentrations in the systemic circulation, additional components of the RAS were not assessed in our study. Thus the potential role of ANG II in our model of equine small intestinal I-R remains

unelucidated. Based on the evaluated parameters there was no positive effect of treatment with an ACE-I during intestinal I-R. There was an apparent lack of ACE suppression despite enalaprilat treatment and this could be responsible for the ineffectiveness of treatment. However there were no increases in ACE concentration in the control group at any time point indicating that ACE activity is not altered during experimental equine intestinal I-R. If ACE activity is unchanged during I-R in horses it is reasonable that use of an ACE-I would confer no benefit apart from its possible radical scavenging effects.

This does not preclude the involvement of the RAS in I-R related damage to the equine small intestine. The ability of both mast cell chymase and neutrophil derived cathepsin G to activate ANG II provides an alternative mechanism for the activation of the RAS. Neutrophilic infiltrate following equine I-R injury is well documented and large numbers of mast cells are also present in the stroma of the small intestine. Use of an ACE-I would have no effect on ANG II derived from these sources. Under these conditions it is possible that the use of a specific AT-1 receptor antagonist might have protective benefits. Future studies should not only utilize additional specific markers for apoptosis but should also attempt to document whether the RAS is stimulated during I-R in horses. ANG II involvement in ileal contractions could potentially contribute to post-operative ileus and measurements of ANG II may be beneficial not only during the acute stages of I-R but also in the 48-72 hours immediately post-surgery. If connections can be established between increases in ANG II, activation of ROS, increased apoptosis and post-operative ileus then further studies employing receptor antagonists may be warranted.

Ultimately the presence and potential significance of apoptosis in experimental equine I-R injury are still unclear, as is the possible association with the RAS. Future efforts should focus on additional markers of apoptosis and may need to utilize other models of I-R injury. Investigation of other components of the RAS during experimental intestinal I-R in horses will help to establish the potential role of RAS associated pathology. If increases in apoptosis and derangements of the RAS can be documented then further studies

should evaluate whether alterations in the RAS are directly correlated with changes in apoptosis. Alternatively, RAS associated pathology may be secondary to other factors such as local vasoconstriction and exacerbation of local or systemic inflammation. RAS induced increases in leukocyte rolling and adhesion may also be a significant contributor to I-R related endothelial dysfunction that could potentiate bacterial translocation and systemic endotoxemia.

APPENDIX 1

GENE SEQUENCING RESULTS:

Gene Sequence for Equine bax: (vector sequences removed)

CATGGAGCTGCAGAGGATGATTGCGGCCGTGGACACGGACTCCCCCCGCGAGGTCTTTTT
CCGAGTGGCAGCTGAGATGTTTTCCGACGGCAACTTCAACTGGGGCCGGGTTGTTGCCCT
TTTCTACTTTGCCAGCAAATTGGTGCTCAAGGCCCTGTGCACCAAGGTGCCCGAGCTGAT
CAGGACCATCATGGGCTGGACACTGGACTTCCTTCGAGAGCGGCTGCTGGGCTGGATCCA
GGACCAGGGTGGTTGGGACGGCCTCCTCTCCTACTTTGGGACGA

BLAST results for bax sequence

Description		Max Identity	E value
Gene	Genus species		
Bax	Cervus elaphus	96%	2e-128
Bax	Ovis aries	96%	1e-126
Bax	Canis lupus	95%	2e-123
Bax	Felis catus	95%	2e-123
Bax	Bos taurus	95%	2e-123

Gene Sequence for Equine p53: (vector sequences removed)

TTCCTTCTTGCGGAAGTTTTCTTCCTCTGTGCGCCGGTCTCTCCCAGGACAGGCACAAACA
CGCACCTCAAAGCTGTTCCGTCCCAGCAGATTACCACTGGAGTCTTCCAGGGTGATGATA
GTGAGGATGGGCCGCCGGTTCATGCCGCCCGTGCAGGAGCTATTACACATGAAGTTGTAG
TGGATGGTGGTACAGTCAGAGCCAACCTCAGGTGGCTCGTAGGGCACCACCACGCTATGT
CGAAAAGTGTTTCTGTCATCCAAATACTCAGCACGCAGATTCCCTTCCACCCGGATGAGA
TGCTGAGGAGGGGCCAGACCATCGCTACTGTCAGAGCAGCGTTCATGGTGGGGGCAGCG
CCTCACGACCTCCGTCATGAACTCTGACTTCTTGTAGATGGCCATGGC

BLAST results for p53

Description		Max Identity	E value
Gene	Genus species		
p53	Equus caballus	99%	0.0
p53	Equus caballus	99%	0.0
p53	Delphinapteras leucas	94%	9e-174
p53	Sus scrofa	92%	4e-162
p53	Homo sapiens	92%	1e-161

Gene Sequence for Equine bcl-2: (vector sequences removed)

GGTGGAGGAGCTCTTCAGGGATGGGGTGAACTGGGGAAGGATTGTGGCCTTCTTTGAGTT
CGGTGGGGTCATGTGTGTGGAGAGCGTCAACCGGGAGATGTCGCCCCTGGTGGACAACA
TCGCCCTGTGGATGACTGAATACCTGAACCGGCACCTGCACACCTGGATCCAGGATAACG
GAGGCTGGGACGCCTTTGTGGAACTGTACGGCCCCAGCATGCGGCCGCTGTTTGATTTCT
CCTGGCTGTCTCTGAA

BLAST results for bcl-2

Description		Max Identity	E value
Gene	Genus species		
Bcl-2	Equus caballus (predicted)	99%	7e-128
Bcl-2	Homo sapiens	98%	9e-122
Bcl-2	Sus scrofa	96%	9e-117
Bcl-2	Sus scrofa	97%	1e-115
Bcl-2	Canis familiaris	96%	4e-115

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