TREATMENT OF ISCHEMIC EQUINE JEJUNUM WITH TOPICAL AND INTRALUMINAL CAROLINA RINSE

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Abstract

Carolina Rinse (CRS) has been shown to be effective in decreasing vascular permeability and neutrophil infiltration in reperfused equine small intestine. The objective of this study was to show that CRS applied topically and intraluminally could prevent immediate reperfusion injury after low flow ischemia or distention in the equine jejunum.

Materials & Methods: Two groups of 5 horses were used to evaluate CRS treatment after low-flow ischemia (Group 1) and intraluminal distention (Group 2) of distal jejunum. Mesenteric blood flow, osmotic reflection coefficient (ORC), wet weight to dry weight ratios (WW/DW), and neutrophil accumulation in the serosa were measured. ORC is defined as the lymph protein concentration to plasma protein concentration ratio subtracted from one (1- Cl / Cp) at maximal lymph flow. The ORC from baseline values and at 60 minutes after initiating reperfusion was compared between Groups 1 and 2. Pair wise comparisons were made for mesenteric blood flow, tissue volume, neutrophil number, and WW/DW proximal control and CRS treated jejunal segments were made using a Mann Whitney U test ($P < 0.05$)..

Results: The mean ORC of bowel treated topically and intraluminally with CRS was similar to that recorded in normal bowel or ischemic intestine treated with CRS by arterial perfusion. The ORC after distention and decompression increased and was similar to that reported in untreated intestine. The WW/DW after both ischemia and distention increased compared to the proximal control segments. There was no difference in neutrophil number in either ischemic or distended intestine compared to the proximal control segments.

Discussion: Carolina CRS was effective in preventing alterations in microvascular permeability during reperfusion afterischemia but not distention. Neutrophil migration curtailed in both groups suggesting that combined topical and intraluminal application of CRS to ischemic intestine may reduce the acute inflammatory responses during reperfusion thereby decreasing complications after ischemia or distention.

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DEDICATION

Dedicated to my wife Anita and son Hamilton
for their never ending love and support

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CHAPTER 1
INTRODUCTION

Intestinal ischemia during intestinal strangulation obstruction has been shown to cause damage to the mucosa and serosa of small intestine and large colon of the horse (Meschter *et al.* 1986), (Snyder 1989). Mucosal changes can be seen on histologic section after 30 minutes of experimental ischemia, and three hours of jejunal ischemia causes irreversible morphologic damage(White *et al.* 1980), which is associated with permanent intestinal scarring (Sullins *et al.* 1985). Changes during reperfusion of ischemic intestine in experimental models include increased microvascular permeability, mucosal cell sloughing, endothelial cell swelling, neutrophil infiltration, loss of the serosal mesothelial layer, and lymphatic dilation (Dabareiner *et al.* 1994c; Moore *et al.* 1994; Dabareiner *et al.* 1995). After surgical correction of ischemic strangulation/obstruction, the inflammatory response, which occurs during reperfusion ultimately leads to excessive scarring and adhesions in the equine small intestine (Dabareiner *et al.* 1993a).

In naturally occurring strangulation obstruction of the small intestine there is accumulation of gas, fluid, and ingesta in the lumen proximal to the obstruction (Allen and Tyler 1990). The degree of distention was found to correlate with survival in naturally occurring small intestinal obstruction (Allen *et al.* 1986). In foals, experimental intraluminal distention caused postoperative peritoneal adhesions (Lundin *et al.* 1989). During experimental intestinal distention in adults, blood flow decreased with distention and increased during reperfusion similar to the response seen after low flow ischemia (Dabareiner *et al.* 2001). Reperfusion after distention was accompanied by increases in microvascular permeability similar to that following low-flow ischemia and reperfusion (Dabareiner *et al.* 1995).There are few treatments that successfully treat or prevent all the alterations which occur during reperfusion injury. To try and combat the numerous biochemical events, which occur during reperfusion, Carolina Rinse (CRS) was developed as a organ transplant rinse solution. Carolina Rinse is comprised of antioxidants (allopurinol, glutathione, and desferroxamine), ATP substrates (glucose and fructose), a dihydropyridine-type calcium channel blocker (nicardipine), a vasodilator

(adenosine), and a cytoprotectant (glycine) at a pH of 6.5. Caroline Rinse has been shown to increase liver transplant survival in rats and is reported to decrease cytokine production, improve blood flow, reduce leukocyte adhesion and migration, and preservation of endothelial cell integrity (Lemasters and Thurman 1997). Several components of the rinse have been shown to be effective when applied topically (Lemasters and Thurman 1997).

The arterial infusion of CRS to an isolated ischemic segment of equine jejunum 10 minutes prior to reperfusion prevented increases in capillary permeability and resulted in fewer adhesions and less mesenteric contracture when compared to controls at 10 days (White *et al.* 1996). Carolina Rinse also attenuated serosal neutrophil infiltration after reperfusion. The purpose of this study was to show that CRS, applied topically and intraluminally, would maintain normal microvascular permeability and prevent tissue inflammation following low-flow ischemia and distention of the equine jejunum.

The CRS used in this experiment was different from the original formula; the hydroxyethyl starch component being replaced with the cytoprotective glycine. In one study looking at cell death in transplanted livers, glycine plus CRSwas shown to reduce cell death to virtually the same level as that of unstored control livers (Currin *et al.* 1996).

CHAPTER 2

LITERATURE REVIEW

Equine Small Intestinal Strangulation Obstruction

Causes

Intestinal strangulation obstruction is defined as simultaneous luminal obstruction and vascular occlusion. Hemorrhagic strangulation obstruction by definition is luminal obstruction with venous occlusion, and ischemic strangulation obstruction is luminal obstruction with simultaneous venous and arterial occlusion (Snyder 1989). Intestinal strangulation usually occurs as the result of incarceration of bowel in an intra-abdominal location such as a mesenteric rent or epiploic foramen or in an extra-abdominal location such as the scrotum or diaphragm. Alternatively, the obstruction can occur as the result of the twisting of the mesentery associated with volvulus (Moore 1999).

Incidence / Mortality / Fatality

Small intestinal strangulation obstruction accounts for approximately 21% of referral colic cases seen at university veterinary teaching hospitals (White 1990a). In one study, 51% of 140 horses that had surgery for small intestinal strangulation died during the initial post-operative period and only 24% were still alive 36 months later (MacDonald *et al.* 1989). The low survival rate is largely due to the inability to identify and surgically remove all of the compromised small intestine. The resulting post-operative complications include continued intestinal necrosis, peritonitis, ileus, and adhesion formation (Snyder 1989). Recent work by Freeman et al. 1999 reports a survival rate of near 70% for short-term survival. This improved survival appears to reflect more rapid identification of cases and improved care during the

perioperative period. Even with the improved survival, complications observed by Freeman include ileus, adhesions, and shock.

Pathology

During hemorrhagic strangulation obstruction, arterial flow continues in spite of venous occlusion resulting in increased venous and capillary hydrostatic pressure. This increase in the vascular pressure leads to increased leakage water, protein and red blood cells to the interstitial space. This transmural edema and hemorrhage appears grossly as dark red to purple serosa and purple to black the mucosa. With ischemic strangulation, arterial flow is occluded thus transmural edema and hemorrhage are usually absent. During obstruction of both the arteries and veins, serosa is blue grey and mottled with the mucosa ranging from normal appearance to dark red (Tedder *et al.* 1995).

During ischemia of the small intestine, ultrastructural morphologic changes are detectable within minutes of ischemia (Allen and Tyler 1990). Mucosal epithelial cells slough beginning at the villous tips and progressing to the crypts (White *et al.* 1980). Accumulation of edema in the intercellular space at the level of the basement membrane causes detachment of epithelial cells (Allen and Tyler 1990). By three hours, epithelial cells have sloughed from the villi and by five hours crypt epithelium become necrotic (Allen and Tyler 1990). Experimentally, three hours of ischemia of the small intestine results in irreversible morphologic damage (White et al. 1981, Sullins et al. 1986).

In most clinical situations of small intestinal strangulation obstruction (hemorrhagic form), venous blood supply is occluded with continued arterial flow(White 1990b). The intestinal obstruction results in accumulation of gas, fluid, and ingesta in the lumen of the bowel proximal to the obstruction (Allen and Tyler 1990). This resulting distention compresses the transmural venules causing increased capillary hydrostatic pressure that leads to reduced blood flow particularly to the serosal layer (Dabareiner *et al.* 1993a). Injury to the serosa later leads to intra-abdominal adhesion formation (Snyder 1989),(Lundin *et al.* 1989).

Surgical correction of the strangulated intestine is further compounded by the additional cellular damage which occurs when the obstruction is relieved and the ischemic tissue is reperfused. It has been shown experimentally that reperfusion causes increased capillary

permeability and morphologic including additional mucosal cell sloughing, endothelial cell swelling, neutrophil infiltration , los(already gone with ischemia), and lymphatic dilation (Dabareiner *et al.* 1994c; Moore *et al.* 1994; Dabareiner *et al.* 1995).

Reperfusion Injury

A vast body of evidence indicates that this reperfusion injury is initiated by the formation of oxygen free radicals (Flaherty and Weisfield 1988). Under normal cellular conditions ATP is metabolized into adenosine, inosine, and hypoxanthine. Xanthine dehydrogenase, an enzyme found in endothelial cells, converts hypoxxanthine to xanthine and uric acid, which is then eliminated by the kidneys (Cohen 1989). During ischemia, the lack of oxygen reduces ATP production and the metabolism of the existing ATP results in a build up of hypoxanthine within endothelial cells (Cohen 1989). The lack of available energy also leads to decreased function of the Na^+ - K^+ ATPase pumps (Cohen 1989). These pumps are necessary to maintain proper intracellular and extracellular ion balance and their failure results in an influx of calcium into the cells (Cohen 1989). The increased calcium initiates the conversion of xanthine dehydrogenase to xanthine oxidase by the calcium- dependent protease calpain (Cohen 1989). Upon reperfusion, the reintroduction of oxygen catalyzes the oxidation of the accumulated hypoxanthine by xanthine oxidase to xanthine and uric acid with the formation of a superoxide radical (Flaherty and Weisfield 1988). Endogenous superoxide dismutase is capable of converting superoxide radicals to hydrogen peroxide, which is then converted by catalase to water (Flaherty and Weisfield 1988). However, the production of superoxide and hydrogen peroxide after an ischemic event overwhelms these endogenous antioxidant systems and the highly toxic hydroxyl radicals are formed from hydrogen peroxide through the iron dependent Haber-Weis reaction (Flaherty and Weisfield 1988).

Oxygen-free radicals induce cellular damage directly by the peroxidation of cell membrane phospholipids with resultant cell lysis (Enster 1988). Additionally, cellular enzymes are inactivated and DNA strands are broken due to oxidation by oxygen-free radicals (Enster 1988). Hydroxyl radicals alter cell membranes resulting in cell death.

The increased intracellular calcium also activates phosolipase A_2 and the resultant arachidonic acid cascade leads to increased levels of prostaglandins, leukotriens, and platelet

activating factor (Zimmerman *et al.* 1990). These inflammatory mediators induce neutrophil activation and recruitment (Zimmerman *et al.* 1990). Neutrophil interaction with endothelial cells is mediated by adhesion molecules (Tedder *et al.* 1995). Selectin molecules located on the neutrophil and endothelial cell surfaces create loose attachments between the cells thereby slowing the neutrophils as they roll along the endothelilal cell surface. This rolling effect allows for the firm attachment of neutrophils to the endothelium via the neutrophil adhesion receptor (CD18) and the endothelial cell adhesion receptor (ICAM-1) (Tedder *et al.* 1995). Neutrophil infiltration from the vasculature into the interstitium requires the release of elastase from the neutrophil (Zimmerman *et al.* 1990). Elastase degrades the components of the restrictive barriers between endothelium and the interstitium allowing neutrophil infiltration (Moore *et al.* 1995). Once activated, neutrophils release oxygen radicals and form hypochlorous acid, which can also cause cell membrane damage. The release of proteolytic enzymes in tissue causes a breakdown of the ground substance and the resultant inflammation.

The importance of neutrophils in reperfusion injury was demonstrated in studies in the small intestine of rats. Ninety minutes of ischemia followed by 60 minutes of reperfusion with whole blood produced severe damage and that this damage was attenuated when the intestine was instead perfused with a leukocyte- free perfluoro-chemical solution. These protective effects were negated when leukocytes were added to the solution (Brown *et al.* 1990). In another study using a cat small intestine ischemia model, the administration of monoclonal antibody that prevented the adherence of neutrophils significantly attenuated the increased microvascular permeability that occurs during reperfusion (Hernandez *et al.* 1987b).

Following 70 minutes of complete vascular occlusion in the equine jejunum, microangiographic evaluation revealed that intramural perfusion was redistributed away from the mucosal and serosal layers and toward the submucosa and muscularis.(Dabareiner *et al.* 1993b). Following 60 minutes of reperfusion , the jejunal segments had a transmural hyperemic response and previously unfilled capillaries were reperfused in all intestinal layers except the serosa (Dabareiner *et al.* 1993b). Following reperfusion, mucosal injury progressed and neutrophil infiltration and edema formation were noted in the serosa (Dabareiner *et al.* 1993b). These findings support the role of reperfusion contributing to the increased inflammatory response that ultimately leads to excessive healing (adhesion formation) in the equine small intestine following surgical correction of ischemic strangulation obstruction (Dabareiner *et al.* 1993b).

Because microvascular permeability is a sensitive indicator of endothelial cell integrity experiments have been designed to measure microvascular permeability during ischemia and reperfusion(Granger *et al.* 1980; Dabareiner *et al.* 1995). Jejunal intestinal segments were isolated and instrumented to create an ischemic period followed by reperfusion. In these studies, arterial blood flow was reduced to 25% of baseline to produce ischemia. Catheterization of one of the lymphatic vessels draining the intestinal segment allowed collection of lymph to determine flow rate and protein content. Studies in cats showed that ischemia followed by reperfusion of jejunum results in an increase in the capillary filtration coefficient (Granger *et al.* 1980). This coefficient is defined as the ratio of lymph protein to plasma protein (Cl /Cp) at maximum lymph flow rates (Granger *et al.* 1980). Low- flow ischemia without reperfusion of an isolated segment of feline small intestine for one hour resulted in a doubling of the microvascular permeability (Granger *et al.* 1980; Granger 1988). The same one-hour period of ischemia followed by reperfusion increased the microvascular permeability fivefold (Granger 1988). In the equine jejunum, 60 minutes of ischemia at 25% baseline flow followed by reperfusion significantly increased microvascular permeability (Dabareiner *et al.* 1995). In the same study, submucosal and serosal tissue volume (edema) increased during reperfusion suggesting changes in microvascular permeability (Dabareiner *et al.* 1995).

Horses with small intestinal obstruction often have luminal distention proximal to the obstruction. The degree of distention was found to correlate with survival in naturally occurring small intestinal obstruction. Horses that survived had a mean intraluminal pressure of 6.3 cm of H_2O compared with 15 cm of H_2O in the ones that died (Allen *et al.* 1986). Intraluminal distention of foal jejunum produced enough serosal injury to stimulate postoperative peritoneal ashesions (Lundin *et al.* 1989). In a study involving adult horses in which a jejunal segment was distended to 25 cm of water (intestine within the abdomen) for 120 minutes followed by 60 minutes of decompression; perfusion improved in all layers during decompression, but the overall number of perfused vessels was less than that in control specimens (Dabareiner *et al.* 1993b).

In a subsequent experiment designed to look at changes in blood flow resulting from jejumal distention followed by decompression, Dabareiner et al found that blood flow decreased maximally within the first 15 minutes of distention to 46% of baseline flow. Flow then progressively increased to 93% of baseline by 120 minutes of distention (Dabareiner *et al.* 2001).

Within 5 minutes of decompression, mesenteric blood flow increased to 303% of baseline and then gradually decreased to 84% of baseline after 180 minutes of decompression (Dabareiner *et al.* 2001). This study showed that blood flow responds to intraluminal distention and subsequent decompression in a similar manner as it does to low flow ischemia and reperfusion in the equine small intestine (Dabareiner *et al.* 2001). Microvascular permeability was found to increase as evidenced by a decrease in the ORC (defined as 1- Cl /Cp) to 0.64 after 120 minutes of decompression compared to an ORC of 0.81 for normal equine jejunum (Dabareiner *et al.* 2001). This finding is similar to the microvascular permeability changes seen following low-flow ischemia followed by reperfusion in the equine jejunum (Dabareiner *et al.* 1995).

Attenuation of Reperfusion Injury

Several treatments have been used in an attempt to halt the morphologic and permeability changes which occur during reperfusion. Intraluminal oxygen was shown to prevent progressive mucosal sloughing during the initial period of reperfusion (Moore et al. 1981) Other treatments include systemic treatment with compounds which inhibit the sequence of oxygen radical production or the adhesion and migration of neutrophils.

Allopurinol

Xanthine oxidase inhibitors such as allopurinol have been shown to prevent injury due to reperfusion of ischemic intestines in laboratory animals (Grisham *et al.* 1986). However; intravenous administration of allopurinol to ponies immediately after correction of small intestinal ischemia failed to reduce tissue injury (Horne *et al.* 1994). It is possible that the pathophysiological processes started by xanthine oxidase derived oxygen free radicals were already in effect before the allopurinol was administered (Moore *et al.* 1995).

Superoxide Dismutase

Superoxide dismutase is an endogenous cellular antioxidative enzyme. This enzyme can also be administered parenterally. Administration of SOD to cats and rats before ischemia has been shown to reduce microvascular and mucosal injury during the reperfusion period (Parks

et al. 1982; Parks *et al.* 1984; Granger *et al.* 1986). Superoxide dismutase did provide protection from reperfusion injury to the mucosa of hypoxic equine small intestine in vitro (Johnston *et al.* 1991). However; no protection against reperfusion injury has been shown in the horse in vivo. (Freeman and Johnston 1992) .

DMSO

DMSO is a hydroxyl radical scavenger and has been shown to reduce the increased microvascular permeability associated with IR in the small intestine of rats and cats (Parks *et al.* 1984; Sekizuka *et al.* 1989; Zimmerman *et al.* 1990). Although Horne et al concluded that DMSO was not effective in preventing mucosal reperfusion injury in the equine jejunum (Horne *et al.* 1994), Sullins et al found DMSO administered intravenously to be beneficial in preventing adhesion formation following small intestinal surgery in foals (Sullins *et al.* 1991).

Iron Chelators

Desferoxamine, an iron chelator, inhibits hydroxyl radical formation from hydrogen peroxide by inhibiting the iron dependent Haber-Weiss reaction (Flaherty and Weisfield 1988). Pretreatment of small intestine in cats with desferoxamine prevented microvascular injury and neutrophil infiltration associated with ischemia- reperfusion (Hernandez *et al.* 1987a; Zimmerman *et al.* 1990).

Calcium Channel Blockers

Treatment with calcium channel blockers has been associated with an increase in survival time for liver transplants in laboratory animals (Takei *et al.* 1990). In a recent study, the effects of verapamil (a calcium channel blocker) on intestinal ischemia –reperfusion injury were evaluated in a rat model. Magnetic resonance spectroscopy was used as a marker of energy metabolism. Recovery rates of energy metabolism after ischemia-reperfusion were improved by verapamil either administered before ischemia or during reperfusion. Also, tissue edema was significantly reduced with verapamil. Presumably by reducing intracellular calcium flux, verapamil results in improved recovery of energy metabolism and cellular preservation (Kimura *et al.* 1998). Us e of verapamil in horses after intestinal ischemia did not alter adhesion formation (Baxter *et al.* 1993).

Hydroxyethylstarch

Hydroxyethylstarch administered intravenously has been shown to limit microvascular permeability associated with ischemia-reperfusion and thus limit interstitial edema (Zikria *et al.* 1990).

Carolina Rinse

No single drug has been identified that completely prevents reperfusion injury (Moore *et al.* 1995), however; multimodal therapy has been used successfully to limit reperfusion injury in transplanted organs (Dabareiner *et al.* 1994a). Originally made as a rinse used prior to reperfusion in experimental liver transplants, Carolina Rinse was originally comprised of the antioxidants (allopurinol, glutathione, and desferroxamine) with ATP substrates (glucose and fructose), a dihydropyridine- type calcium channel blocker (nicardipine), Hydroxyethyl starch and a vasodilator (adenosine), at a pH of 6.5. Kaminski and Proctor showed that topical use of adenosine caused vasodilation in rat intestine and prevented the no reflow phenomenon in the serosa and mucosa (Kaminski and Proctor 1989). The same study concluded that topical adenosine attenuated most of the morphologic damage attributed to reperfusion injury.

Under physiological conditions, nitric oxide plays a crucial role in maintaining vascular integrity as well as mucosal barrier function (Kurose *et al.* 1994). Studies have shown that nitric oxide reduces mucosal injury after ischemia-reperfusion of the intestine(Kubes 1993) and that administering nitric oxide inhibitors greatly exacerbates intestinal injury and increases mucosal barrier dysfunction associated with ischemia-reperfusion (Ishida and al 1997). Recent evidence has shown that exposing tissues to a short period of ischemia (ischemic preconditioning) prior to the ischemia-reperfusion event resulted in reduced tissue injury. Leukocyte post ischemic adhesion was prevented by maintaining the bioavailibility of nitric oxide and this nitric oxide synthesis may be up regulated by adenosine or adenosine A_1 receptors (Takei *et al.* 1990).

In an initial study in the horse, intravenous infusion of Carolina Rinse solution during halothane anesthesia did not cause untoward effects[1] In a subsequent study, the same authors looked at arterial infusion of an isolated ischemic segment of jejunum 10 minutes prior to reperfusion (White *et al.* 1998). The authors concluded that treatment with Carolina Rinse prevented increases in capillary permeability during the immediate period of reperfusion and resulted in fewer adhesions and less mesenteric contracture as compared with controls 10 days later. Carolina Rinse treatment also attenuated some of the tissue inflammation as determined by serosal neutrophil infiltration. However, the intestinal wall thickness and serosal fibrosis observed at necropsy at 10 days was not significantly reduced as compared to controls. In the authors opinion, although Carolina Rinse appears to attenuate some of the immediate reperfusion injury, treatment should still include non-steroidal anti-inflammatory drugs and antibiotics which are known to reduce intestinal damage during the postoperative period (White *et al.* 1998).

[1] Donaldson, L L, White, N.A, Dabareiner, R.M., White, N.A. and Donaldson, L.L. *The effects of intravenous infusion of Carolina Rinse in dorsally recumbent, halothane anesthetized horses.* p. Unpublished data.

CHAPTER 3
MATERIALS AND METHODS

A physical examination, a complete blood count, liver profile and coagulation were normal in ten horses used in the experiment. Horses were sedated with xylazine (0.2 - 0.5 mg/kg, IV) and general anesthesia was 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 halothane in oxygen using intermittent positive pressure ventilation. Mean blood pressure measured, monitored continuously by direct arterial catheterization, was maintained $\geq$ 70mm Hg by dobutamine infusion. Lactated Ringer's solution was administered at 5-10 ml/kg/hr IV. The end tidal CO_2 was maintained at 30-40 mm Hg.

Low-flow ischemia was created in the jejunal segment of 5 horses (Group 1) and increased intraluminal distention created in the remaining horses (Group 2). All ten horses were instrumented using aseptic conditions. A ventral midline celiotomy was performed. After entering the abdomen, a full thickness biopsy was collected from the jejunum at a site proximal to the jejunal segment to be isolated. The enterotomy site was sutured, with 2-0 polygalactin using a continuous Lembert suture pattern. Beginning at the distal jejunal vascular arcade, a 30-cm intestinal segment supplied by one jejunal artery and vein was identified. Circumferential latex rubber tubing was placed at each end of the intestinal segment occluding the lumens and extramural vasculature to prevent collateral circulation. The jejunal segment was placed on a plastic sheeting overlying a warm water-heating pad (37 C). The intestine was kept moist with sterile lactated Ringer's solution and covered with plastic sheeting to prevent tissue dehydration. Once the jejunal segment was isolated the jejunal artery and vein were isolated proximal to their branching in the mesenteric arcade and a No. 4 Doppler ultrasonic probe[2] applied to the jejunal artery; a 22-gauge intravenous catheter [b] was

[2] Transonic, Inc., Ithaca, NY.
[b] Deseret – Medical Inc. Sandy, Utah

inserted in the mesent eric vein. Clamps [c] were applied to the artery and vein proximal to the blood flow probe and the venous catheter. The arterial clamp was adjusted to alter arterial blood flow. The intravenous catheter was connected to a mercury manometer[d] to monitor venous pressure and was used to collect plasma samples. A lymphatic vessel draining the jejunal segment was cannulated with a 24-gauge catheter and used for lymph sample collection.

Mesenteric arterial blood flow (ml/min), lymph protein concentration (Cl) and plasma protein concentration (Cp) were measured prior to ischemia or distention. In Group 1 mesenteric blood flow in the instrumented jejunal segment was reduced to 25% of baseline for 60 minutes and subsequent reperfusion observed for one hour. Ten minutes prior to reperfusion the bowel lumen of the isolated segment was filled but not distended with CRS (1 ml/kg body weight-intraluminal pressure less than 3 mm Hg). The segment was also immersed in 1 liter of CRS within the plastic sheet covering the heating pad (Figure 5). In Group 2 horses the instrumented jejunal segment was distended with lactated Ringer's solution to an intraluminal pressure of 18 cm H_2O. Lactated Ringer's infusion was continued, as needed, to maintain that pressure for two hours, at which time the intraluminal fluid was replaced with CRS (1 ml/kg body weight), filling but not distending the lumen, and the segment was immersed in 1 liter of Carolina Rinse for 60 minutes (Figure 6). Euthanasia was completed while all horses were still anesthetized at the end of the experiments.

Blood flow was recorded at 5-minute intervals for the duration of the experiment in all horses. After 15-20 minutes of reperfusion the venous return was partially occluded to maintain a venous pressure of 30 mm Hg to maximize lymph flow (Dabareiner *et al.* 1995). Three corresponding lymph and plasma samples were collected during maximal lymph flow.

Experimental segments were collected and weighed after 60 minutes of reperfusion (group 1) or decompression (group 2). Full thickness biopies were fixed in 10% BNF, paraffin sectioned, and stained with hemotoxylin and eosin. Tissue sections were evaluated for morphologic alterations in the mucosa, submucosa, muscle, and

[c] Wolliner Vascular Clamp, Davis, Calif.
[d] Model CCQ PM-2A, Honeywell Inc, Hayward, Calif.

serosa. The mucosa injury was graded using a previously reported grading system (White *et al.* 1980). Serosal thickness was measured using a micrometer at three locations and a relative thickness based on the micrometer units recorded. The mean number of neutrophils in a 0.01-mm^2 area of the serosal layer just below the mesothelial border was determined by averaging the counts from 5 adjacent high power fields.

Ten centimeter segments were collected from the experimental jejunum and an undisturbed proximal jejunum. The segments were weighed and then dried at 60 degrees C until there was no change in the tissue weight. Wet-to-dry weight ratios (WW/DW) were calculated.

Lymph volumes were measured in calibrated micropipettes[e]. Lymph and plasma samples were centrifuged to remove blood cells and protein concentration measured by the modified biuret method (Cornall *et al.* 1949). The microvascular permeability was determined by estimates of the osmotic reflection coefficient (ORC) as determined from lymph to plasma protein concentration ratio (Cl/Cp) at maximal lymph flow. The ORC (1-Cl/Cp) from baseline values and at 30 minutes after initiating reperfusion was compared between Groups 1 and 2. Comparisons between baseline, and CRS treated jejunal segments were made using a Mann Whitney U test. Pair wise comparisons were made for mesenteric blood flow, ORC, tissue volume, neutrophil number, and WW/DW with $p<0.05$.

[e] Ultramicro Pipetman, Ranin Instrument Co. Emeryville, Calif.

CHAPTER 4
RESULTS

Ischemic intestine

The baseline mean arterial blood flow of 34.6 +/- 6.53 ml/min (mean ±SEM) increased significantly to 95.4 +/- 10.15 ml/min 5 minutes after reperfusion (Figure 1)((p= 0.03). After 22-25 minutes flow decreased to 38 +/- 3.59 ml/min but subsequently increased to 45.4 +/- 4.64 ml/min at the end of the 60 minute reperfusion period. The mean ORC at maximal lymph flow rate during reperfusion was 0.75 +/-.06.

The mean serosal thickness in the pre-ischemia sample was 1.57 +/- 0.18 mm. After 60 minutes of ischemia followed by 60 minutes of reperfusion there was no significant difference in the serosa thickness between the experimental segment (2.60 +/- 0.26 mm) and that of the proximal control segment (2.59 +/- 0.29 mm.) (Figure 3) with p = 0.8. After ischemia and reperfusion, the WW/DW in the proximal control sample (3.98 +/-0.18) was significantly less than that of the experimental segment (5.14 +/- 0.15) with p= 0.02.

There was a significantly greater mean number of neutrophils in the experimental segment (1.84 +/- 0.35/HPF) compared to the pre-ischemic control sample (.02 +/- 0.09 /HPF)(p = 0.02, but there was no difference when compared with the proximal control segment collected at the end of the experiment (1.04 +/- 0.41/HPF) (Figure 4)with p=0.6. The grade of mucosal injury after reperfusion was zero.

Distended intestine

The m ean baseline arterial blood flow of 48.2 +/- 9.63 ml/min decreased to 23.8 +/- 7.36 ml/min in the distended intestine. At the end of 120 minutes of distention, the blood flow had increased to 33.8 +/- 10.36 ml/min. After decompression, the mean blood flow increased to 124.2 +/- 24.99 ml/min, subsequently decreased to 112.0 +/- 30.52 ml/min at 25 minutes and then increased to 137.2 +/- 36.49 ml/min at the end of 60 minutes (Figure 2). The mean ORC in group 2 at maximal lymph flow rate during decompression was 0.653 +/- 0.06.

The mean serosal thickness of the pre-distention control jejunum was 2.162 +/- 0.584 mm. After 120 minutes of distention and 60 minutes of decompression, the serosal thickness significantly increased to 3.914 +/- 0.572 mm (p=0.04), which was greater than that of the proximal control jejunal segment sampled at the same time (2.144 +/- 0.158 mm). The WW/DW of the proximal control segment was significantly less (3.584 +/- 0.279 than the experimental segment (5.324 +/- 0.5) with p = 0.04.

The mean number of serosal neutrophils was greater after distention and decompression compared to the baseline sample (0 vs. 3.2+/- 1.17 per HPF), but was not significantly different from that of the proximal control segment (2.4 +/- 1.53 /HPF) with p = 0.8. Mucosal injury was not apparent with an injury grade of zero.

CHAPTER 5
DISCUSSION

Ischemia Reperfusion

Several of the components of CRS have been shown to decrease reperfusion injury in experimental models of intestinal ischemia in other species. Pretreatment with desferoxamine, an iron chelator , (Zimmerman *et al.* 1990) (Hernandez *et al.* 1987a) , calcium channel blockers (Kimura *et al.* 1998), adenosine (Kaminski and Proctor 1989) , glycine (Bachmann *et al.* 1995) ,and allopurinol (Grisham *et al.* 1986) prevented changes in capillary permeability and provided protection from reperfusion injury. Also, it has been shown that the naturally occurring acidosis of ischemia greatly retards the onset of cell death(Gores *et al.* 1988). The critical injury causing graft failure after prolonged storage of rat livers involves reperfusion induced killing of sinusoidal endothelial cells (Currin *et al.* 1996). In one study, the only component of CRS preventing endothelial cell death was acidic pH of 6.5 (Currin *et al.* 1996).

The results from this study suggest that microvascular permeability was preserved during the first hour of reperfusion with topical and intraluminal CRS treatment. Using the same protocol for instrumentation as used in this group of horses, Dabareiner et al. found that the ORC for normal equine jejunum was 0.81 +/- 0.06 (Dabareiner *et al.* 1993b), which is similar to that reported for normal feline jejunum (0.87 to 0.92) (Granger *et al.* 1979) . The equine model of 60 minutes of low-flow ischemia with 180 minutes of reperfusion resulted in an ORC of 0.52 +/- 0.05 (Dabareiner *et al.* 1995), again, comparable to that of feline small intestine following 60 minutes of low-flow ischemia and 60 minutes of reperfusion (0.59+/- 0.01) (Granger *et al.* 1979). The ORC in Group I horses (0.75 +/- .06) was comparable to the ORC after intra-arterial CRS (0.72 +/- 0.03) which was significantly different from experimental intestine treated with lactated Ringer's solution (Dabareiner *et al.* 1994b).

Interstitial edema formation compromises small intestinal function by increasing interstitial fluid pressure and decreasing absorptive function (Granger and Barrowman 1983). Edema of the intestinal wall has previously been reported in ischemia-reperfusion studies in the horse (Dabareiner *et al.* 1995) (Dabareiner *et al.* 1993a) (Meschter *et al.* 1986). Some edema formation also has been reported in instrumented control segments presumably as a result of tissue handling and maintaining an open abdominal cavity during the procedure. However, the tissue edema in the intestinal segments exposed to ischemia-reperfusion was significantly greater than controls (Dabareiner *et al.* 1995). Increased serosal thickness, reported after ischemia/reperfusion was prevented by intra-arterial [b], as well as topical, and intraluminal CRS. Although we did find a significant difference in the WW: DW ratio in the proximal control compared to the treated ischemic intestine, the bowel appeared to be protected from fluid retention by intra-arterial and topical/intraluminal CRS treatment when compared to wet to dry weight ratios seen in untreated intestine exposed to ischemia-reperfusion.

Dabareiner et al. observed neutrophil infiltration in the mucosal and serosal layers of jejunum exposed to 60 minutes of ischemia followed by reperfusion (Dabareiner *et al.* 1995). Depletion of neutrophils or blocking neutrophil adherence prevented the increased microvascular permeability caused by ischemia-reperfusion in feline small intestine (Hernandez *et al.* 1987b). Since inflammatory response after reperfusion has been associated with increased intestinal injury, scarring, and adhesions (Snyder 1989); limiting this immediate reperfusion response should decrease the inflammation attributed to neutrophils migration. Treatment with CRS intra-arterially (Dabareiner *et al.*1994a) significantly reduced serosal neutrophil infiltration as compared with untreated ischemic intestine. The lack of increased serosal neutrophil accumulation in intestine treated topically and intraluminally supports use of CRS locally during surgery to decrease inflammation during reperfusion. Since leukocyte post ischemic adhesion has been shown to be prevented by the maintenance of the bioavailibility of nitric oxide and this nitric oxide synthesis may be up regulated by adenosine or adenosine A_1 receptors (Takei *et al.* 1990); it is likely that adenosine in the CRS was key in preventing the accumulation of serosal neutrophils in this study.

Distention-decompression

Increased intraluminal pressure is known to cause mural ischemia and is associated with decreased survival in the horse (Allen *et al.* 1986). Increased intraluminal pressure is transmitted through the interstitium to the veins resulting in venous outflow obstruction. Theoretically this leads to increased capillary hydrostatic pressure and an increase in capillary filtration. The edema formation and continued increase in interstitial pressure ultimately produce sufficient back pressure to compromise arteriolar blood flow (Dabareiner *et al.* 2001) (Moore *et al.* 1988).

The experimental protocol for producing distention and compression in the present study was the same as that used by Dabareiner et al. except the response to decompression was only evaluated for 60 minutes compared to 180 minutes.(Dabareiner et al. 2001) The increase in blood flow measured after decompression in the initial model (Dabareiner et al. 2001) was higher than that observed in topically treated intestine. The subsequent increased in blood flow after 60 minutes may have been due to persistence of the CRS in the lumen of the bowel. This pattern contrasts with that of untreated distention-decompression study where there was a persistent drop in blood flow during 2 hours of decompression. (Dabareiner et al. 2001) The maintainence of increased flow may be beneficial to the reperfused tissue by preventing the no-reflow phenomena observed in both ischemic and distended bowel in the immediate reperfusion period (Kaminski and Proctor 1989). The ORC after decompression of equine jejunum distended to 18cm H_2O was reported to be 0.64 +/-.07 indicating a 21% increase in microvascular permeability (Dabareiner *et al.* 2001). In our present study, the calculated ORC was similar (0.65+/- 0.06) indicating that topical CRS treatment did not prevent the increase in microvascular permeability produced by intraluminal distention and decompression.

Dabareiner found distention and subsequent decompression resulted in a 2.7 fold greater serosal thickness as compared to control (Dabareiner *et al.* 2001). In the present study, the same period of distention followed by decompression with CRS treatment resulted in a 1.8 fold greater serosal layer thickness as compared to controls.

Additionally, **t**he WW/DW for the experimental jejunal segments treated with CRS were similar to those from the untreated experimental segments in the previous study (Dabareiner *et al.* 2001). This suggests that topical/intraluminal CRS treatment does not prevent distention induced edema formation. We speculate that the return of blood flow prior to decompression resulted in the initiation of reperfusion injury prior to CRS treatment. Furthermore, the increased intramural pressure most likely causes sufficient post capillary pressure to initiate capillary leakage during the early stages of distention. In effect the CRS treatment was applied too late to prevent endothelial cell damage and maintain capillary integrity.

In the present experiment the mean number of serosal neutrophils per HPF in the CRS treated segment was 1.3 times greater than that of the control specimen collected at the same time. In the previous study, which had a longer period of reperfusion, there was a forty-fold increase in neutrophils in the non-treated experimental segment versus controls after 180 minutes of reperfusion. The results suggest that CRS, applied topically, reduces neutrophil infiltration after distention and decompression, and therefore, may be beneficial as a treatment to prevent intestinal postoperative inflammation. Hypothetically preventing damage to the serosa could decrease the risk of adhesions, which is known to be increased after small intestinal obstruction (Snyder 1989).

Conclusion

The benefit of administering CRS to ischemic equine small intestine has been shown by several experiments, including this study. The decrease in the inflammatory response and preservation of vascular integrity, suggests that CRS acts during the early phase of reperfusion to prevent the formation or actions of oxygen radicals. Because endothelial cells are protected, the chemotaxis for neutrophils is prevented or decreased, thereby limiting the secondary release of reactive oxygen metabolites when neutrophils migrate into the interstitium. Assuming this mechanism of action, CRS can only be effective if it is administered in a timely fashion enabling it to prevent the biochemical cascade, which initiates the subsequent inflammatory response. Even with this limitation,

CRS appears to be a valid treatment for equine small intestine that has been subjected to obstruction or strangulation obstruction.

CHAPTER 6
SUMMARY

In summary Carolina Rinse Solution applied to the serosal and mucosal surfaces was effective in preventing alterations in microvascular permeability after jejunal ischemia reperfusion but not after jejunal distention. CRS was effective in reducing neutrophil migration in equine jejunum under both conditions and did not appear to create morphologic changes in the mucosal in the experimental segments. These results suggest that combined topical and intraluminal CRS is a clinically applicable treatment to prevent immediate inflammatory changes during reperfusion.

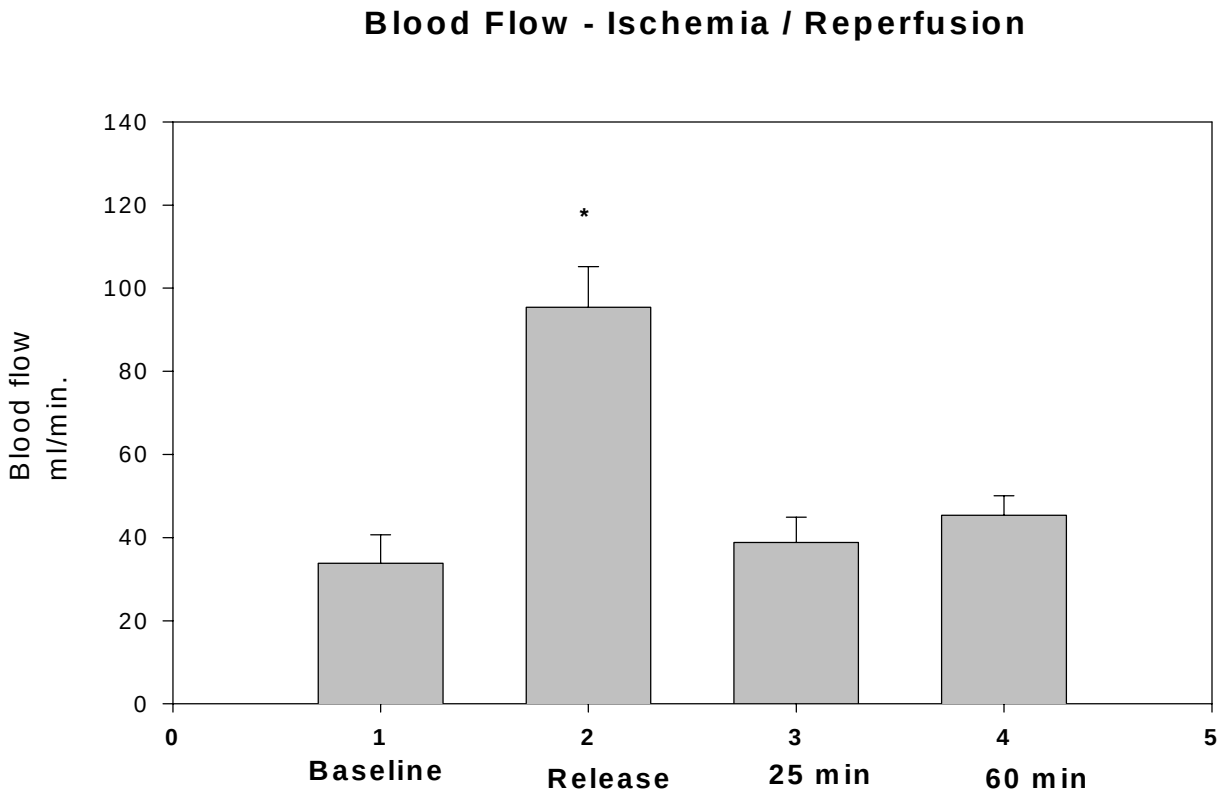

Figure 1: Blood Flow. Ischemia / Reperfusion. 1) Baseline blood flow. 2) Blood flow following release after 60 minutes of ischemia (25% baseline flow). 3) Blood flow 25 minutes post release. 4) Blood flow 60 minutes post release. * denotes significant difference from baseline with $p < 0.05$.

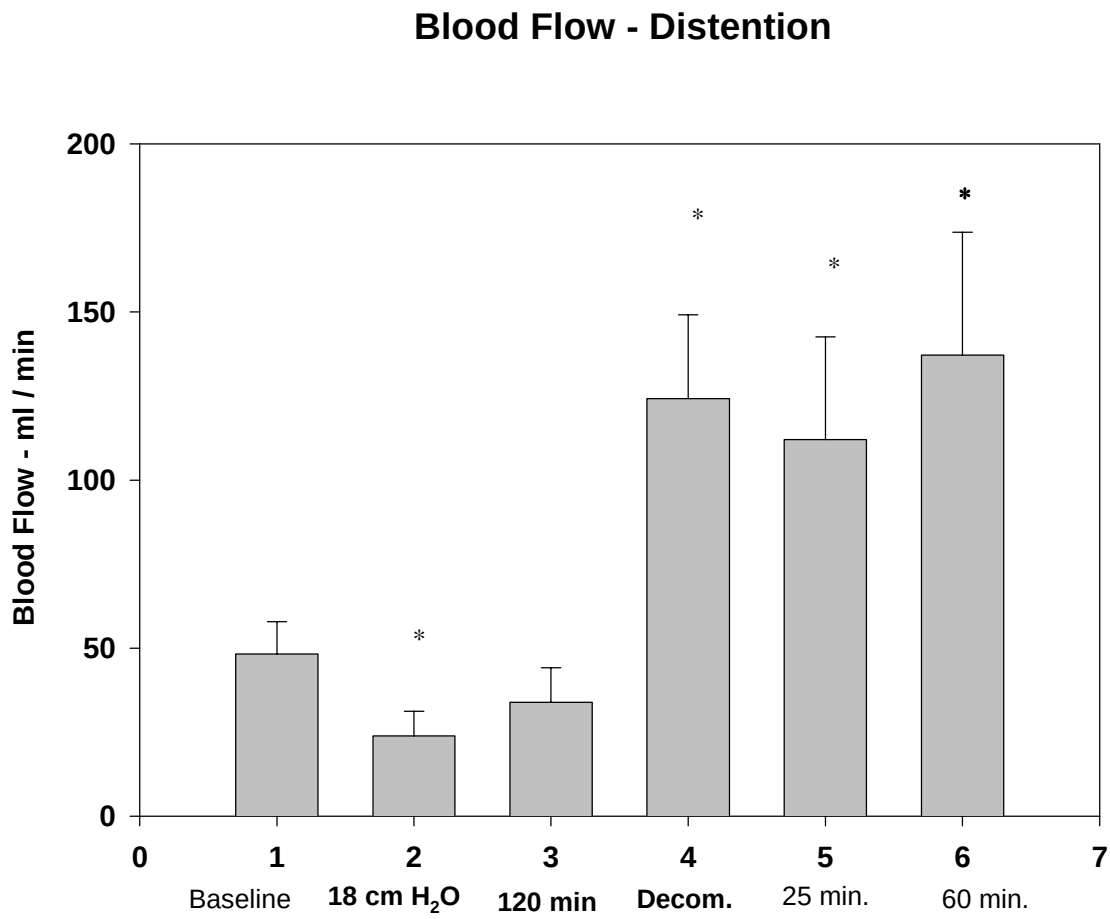

Figure 2: Blood Flow. Distention / Decompression. 1) Baseline intestinal blood flow. 2) Blood flow after luminal distention to 18 cm of water pressure. 3) Blood flow at 120 minutes of distention. 4) Blood flow following luminal decompression. 5) Blood flow 25 minutes post decompression. 6) Blood flow 60 minutes post decompression. * denotes significant difference from baseline with $p < 0.05$.

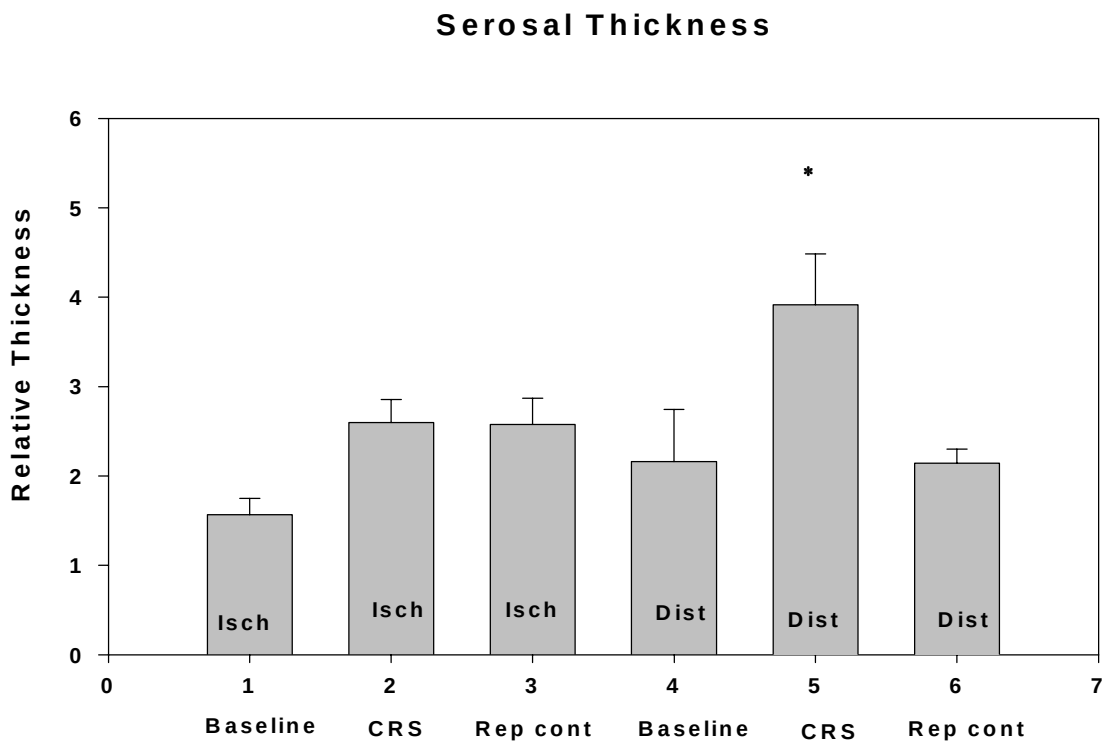

Figure 3: Serosal Thickness. 1) Relative thickness of baseline jejunal serosal layer for ischemia / reperfusion (Group 1). 2) Relative thickness of topical /intraluminal CRS treated jejunum (Group 1). 3) Reperfusion control sample for Group 1. 4) Relative thickness of baseline serosal layer for distention / decompression (Group 2). 5) Relative thickness of distention /decompression CRS treated jejunum (Group 2). 6) Reperfusion control sample for Group 2. * denotes significant difference from baseline with $p < 0.05$.

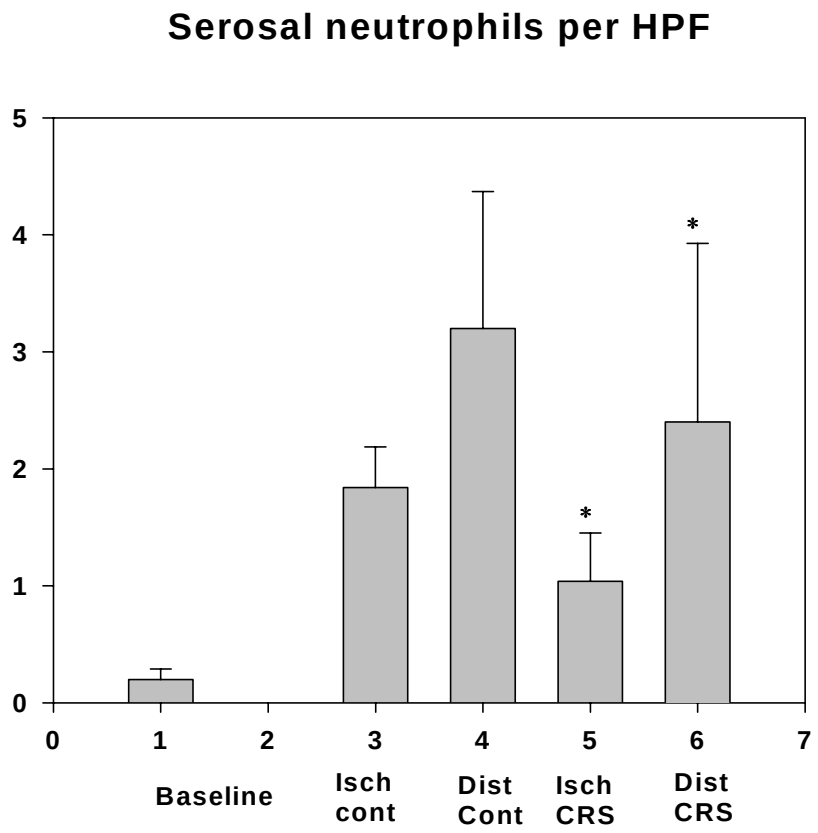

Figure 4: Serosal Neutrophils. 1) Baseline serosal neutrophil count per high power field (HPF) for ischemia /reperfusion (Group 1). 2) Baseline serosal neutrophils per HPF for distention / decompression (Group 2). 3) Serosal neutrophils per HPF for reperfusion control (Group 1). 4) Serosal neutrophils per HPF for reperfusion control (Group 2). 5) Serosal neutrophils per HPF for CRS treated Group 1. 6) Serosal neutrophils per HPF for CRS treated Group 2. * denotes significant difference from baseline with $p < 0.05$.

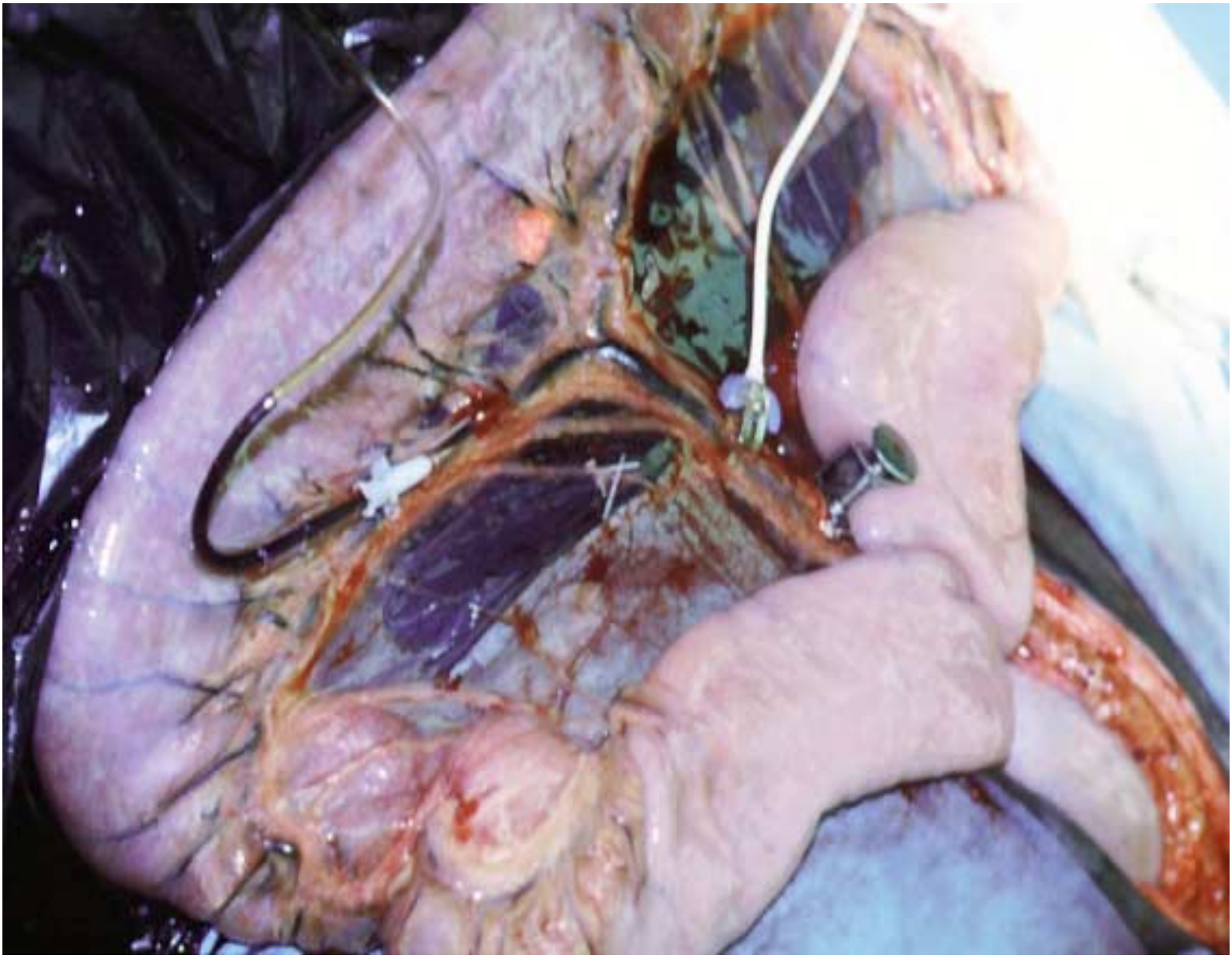

Figure 5: Set Up for Experimental Ischemia.
Thirty cm jejunal segment. Note No. 4 Doppler ultrasonic probe, 22 gauge intravenous catheter connected to a mercury monometer (not shown), vascular clamp, and lymphatic catheterization with a 24 gauge intravenous catheter.

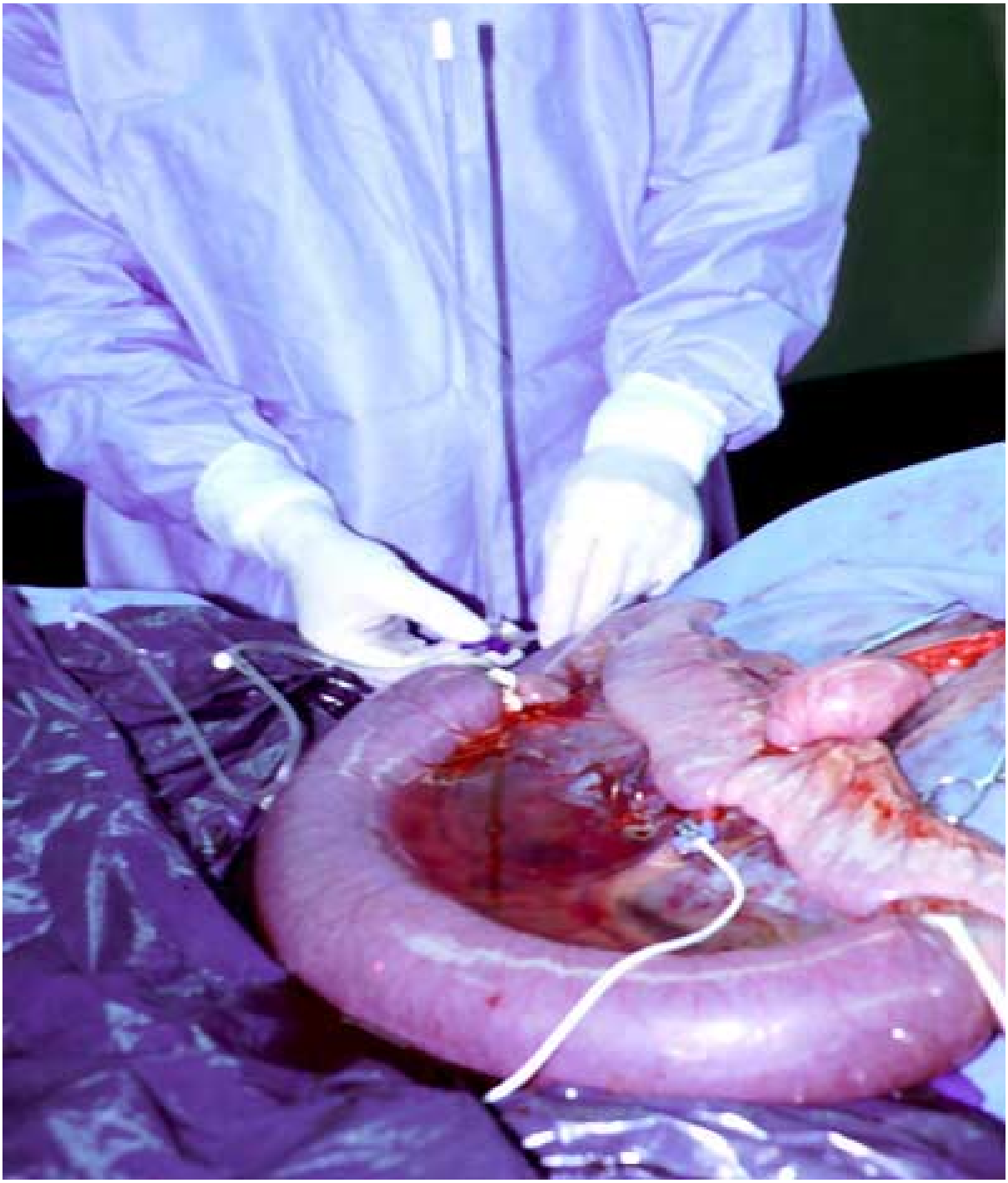

Figure 6: Set Up for Experimental Distention. Thirty cm jejunal segment distended to 18 cm of water pressure with Lactated Ringer's Solution. Note No. 4 ultrasonic probe and water monometer.

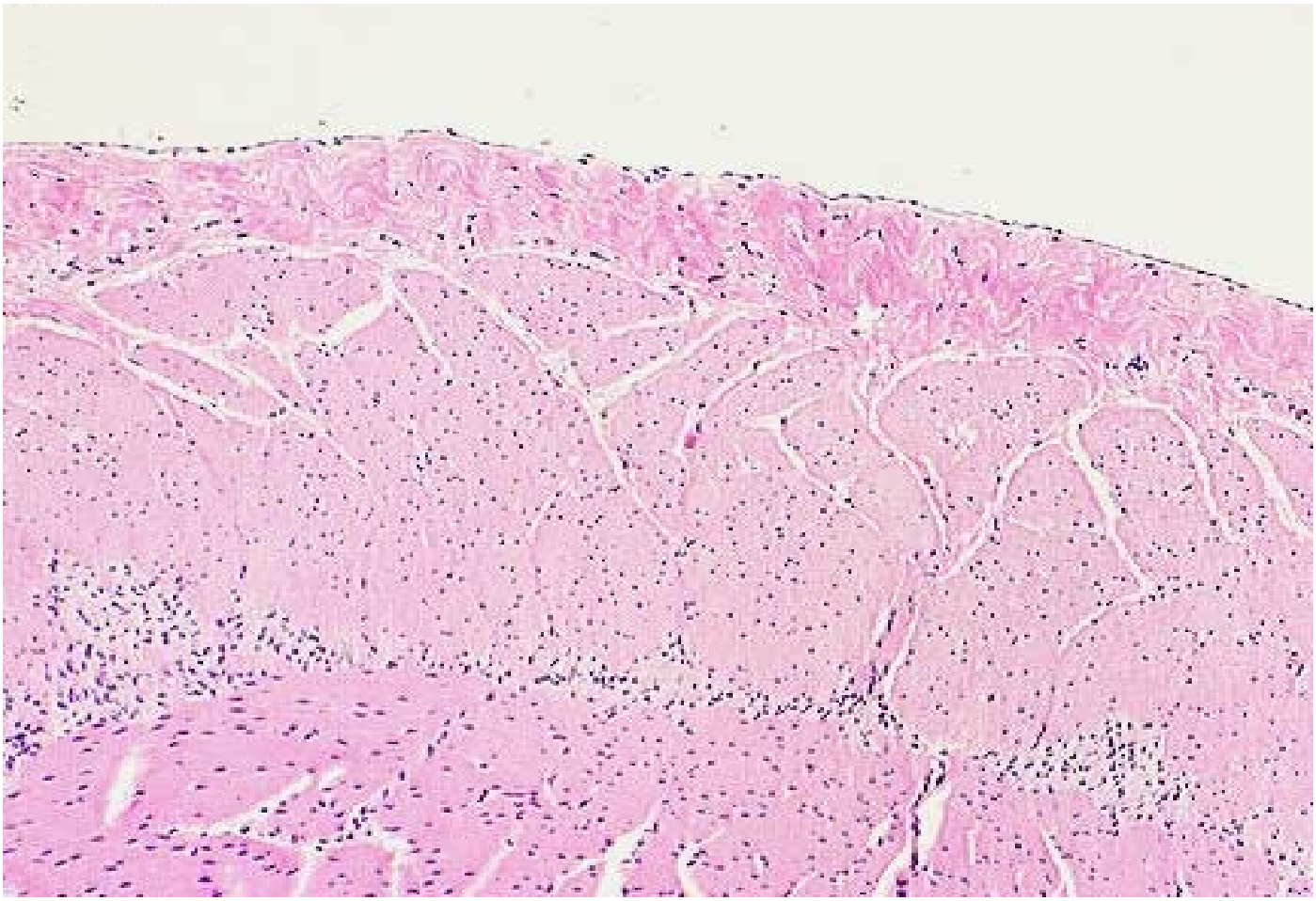

Figure 7: Histology H&E. Jejunal serosa and muscle layers. Baseline (10X). Normal serosa. Note intact mesothelial layer, lack of serosal edema, and few serosal neutrophils.

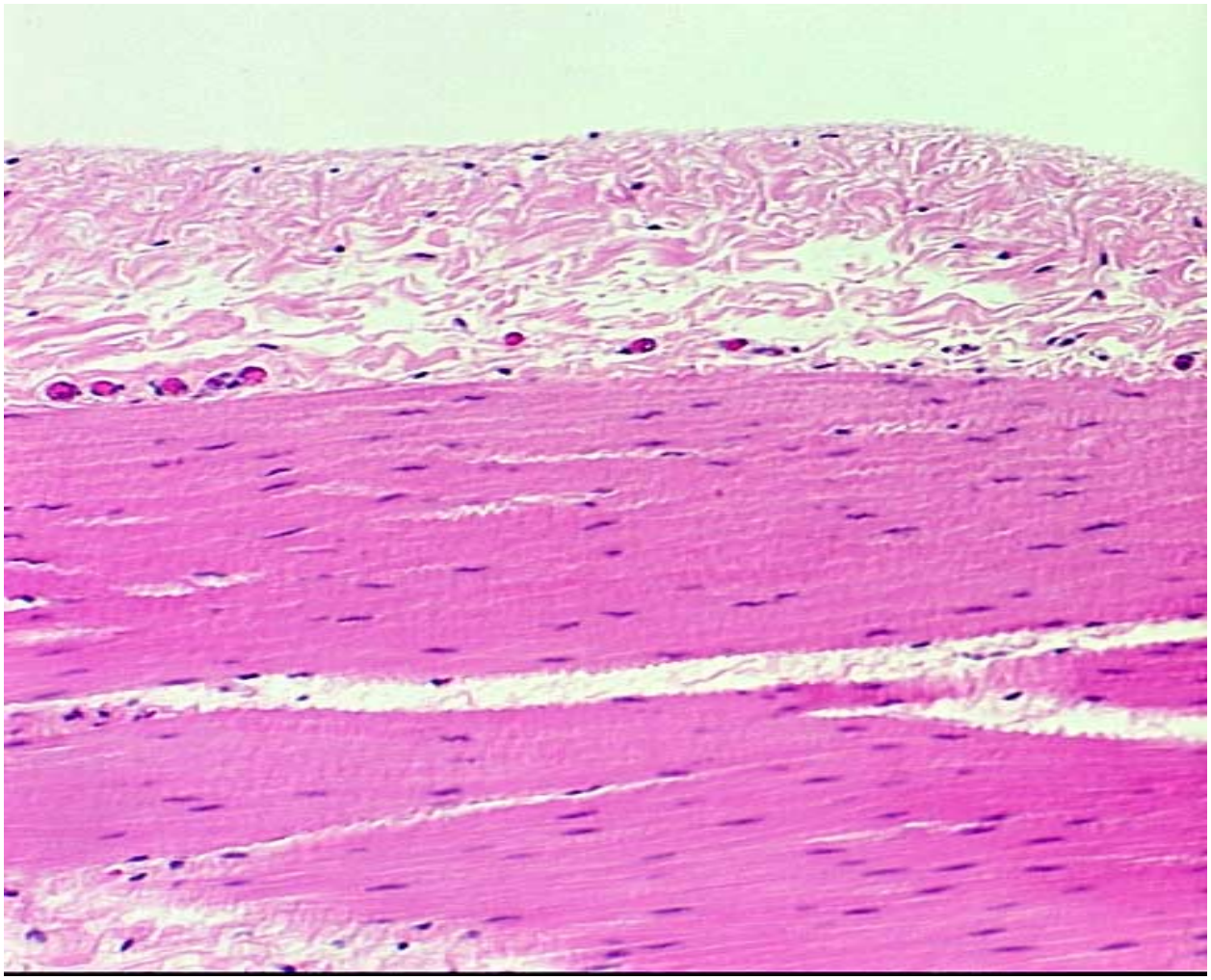

Figure 8: Histology H&E (10X). Experimental from Ischemia. Jejunal serosal and muscle layers. Note loss of the mesothelial cell layer, serosal edema, and increased neutrophil numbers within the serosa compared to baseline (Figure 7).

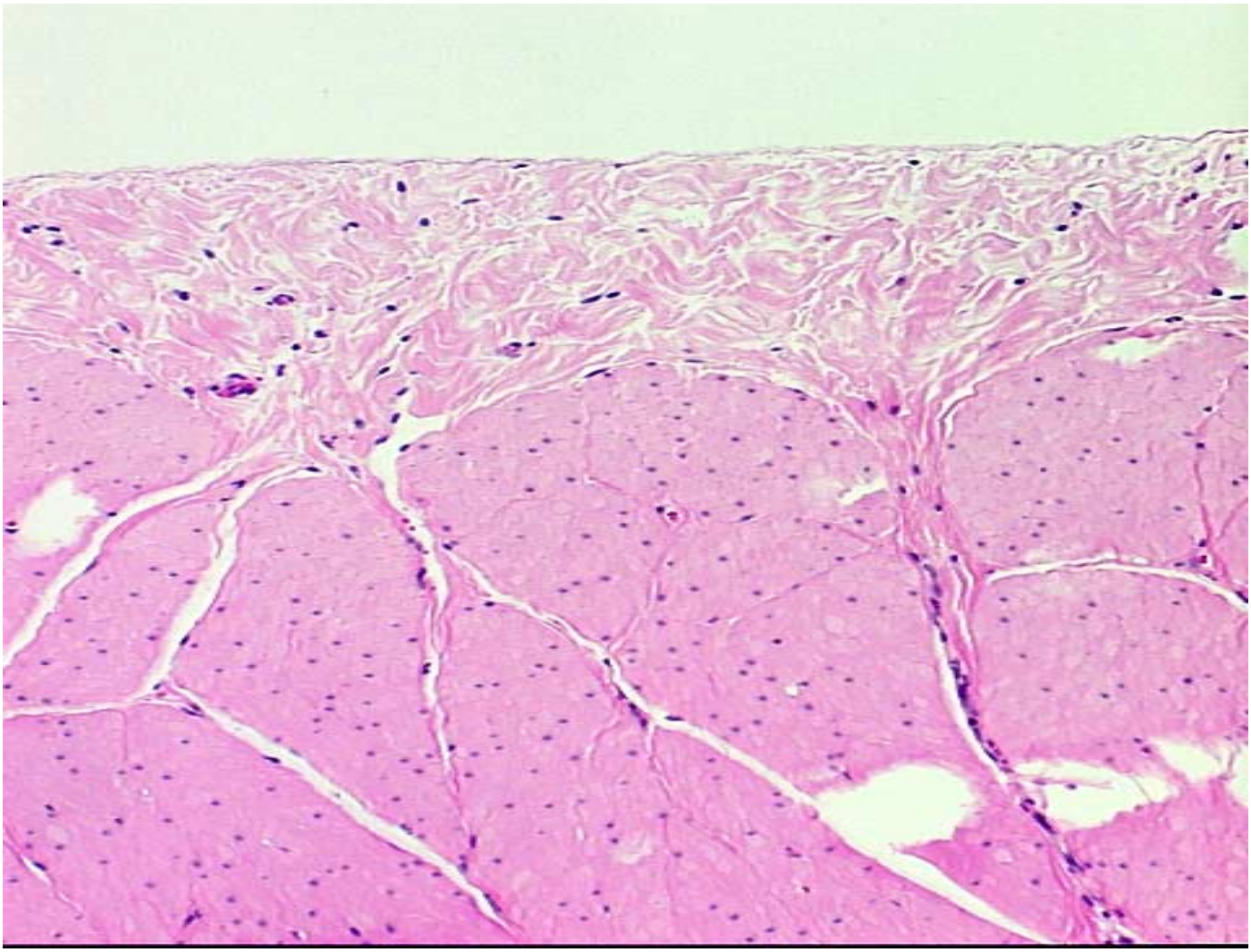

Figure 9: Histology H&E (10X). Experimental from Ischemia. Proximal Control. Jejunal serosal and muscle layers. The mesothelial cell layer has been lost. There is moderate serosal edema and more serosal neutrophils than in the baseline segment (Figure 7).

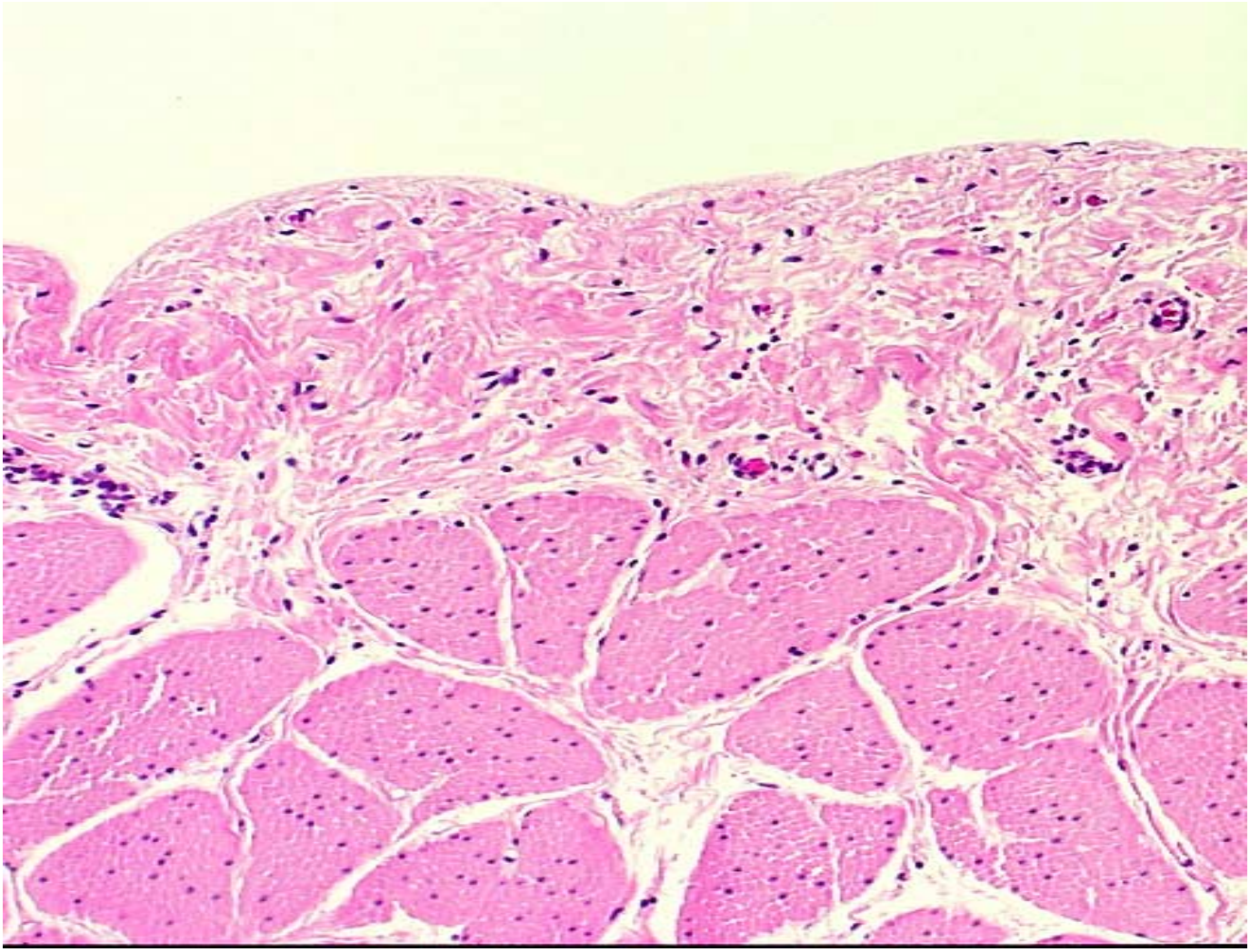

Figure 10: Histology H&E (10X). Experimental from Distention. Jejunal serosal and muscle layers. Note the absence of the mesothelial cell layer, serosal edema, and increased serosal neutrophils compared to baseline (Figure 7).

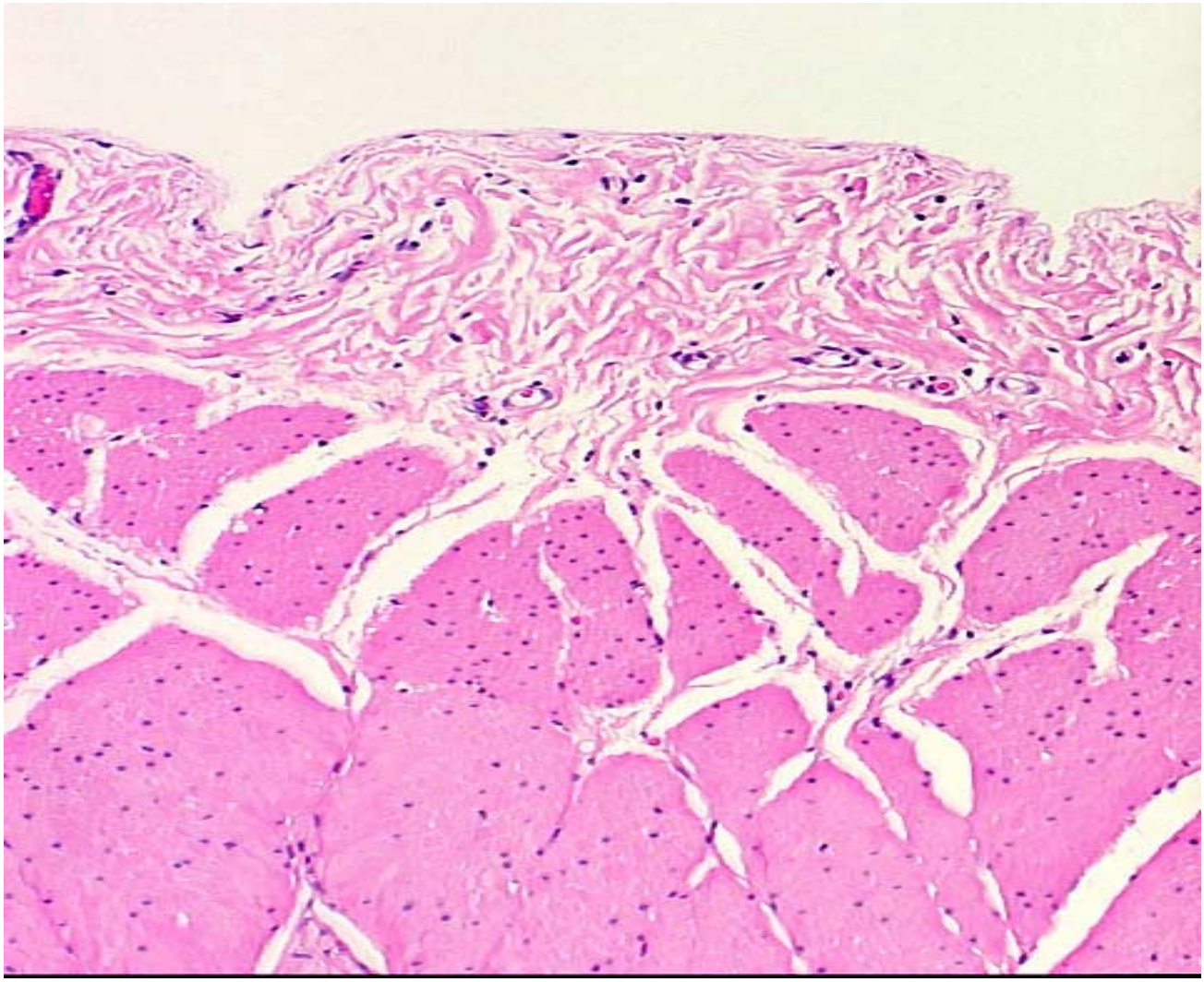

Figure 11: Histology H&E (10X). Experimental from Distention. Proximal control. Jejunal serosal and muscle layers. Note loss of the mesothelial cell layer and serosal edema formation but fewer serosal neutrophils than the corresponding experimental segment (Figure 11).

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VITA

Byron Leslie Young was born in Christiansburg, Virginia. He graduated from Christiansburg High School and entered Virginia Polytechnic Institute and State University in 1973 with a major in biology. He was accepted to Auburn University School of Veterinary Medicine in 1977 and graduated with the degree of Doctor of Veterinary Medicine in 1981.

Following graduation, he practiced large animal medicine and surgery in southwest Virginia for three years followed by a one year clinical instructor position in ambulatory field service at the Virginia- Maryland Regional College of Veterinary Medicine. From 1986 – 1997, he practiced equine and small animal medicine and surgery in southern Maryland. In 1998, he was accepted into the Virginia -Maryland Regional College of Veterinary Medicine Graduate School and Residency Program in Equine Surgery at the Marion duPont Scott Equine Medical Center in Leesburg, Virginia. Completion of the American College of Veterinary Surgeons approved residency program and graduation from the Graduate School with the degree of Masters of Science in Veterinary Medicine is anticipated in June of 2001.