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ISCHEMIA/REPERFUSION INJURY OF THE ASCENDING COLON IN PONIES: A CORRELATIVE STUDY UTILIZING MICROVASCULAR HISTOPATHOLOGY AND CORROSION CASTING

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Abstract

Volvulus of the ascending colon (ACV) in the horse results in microvascular injury and necrosis of the intestinal mucosa. This study investigated the site and type of microvascular injury which occurs within the mucosa and submucosa following ACV. Histopathology of volvulus treated ponies demonstrated mucosal necrosis with microvascular hemorrhage and thrombosis. Thrombi occurred within the subepithelial capillaries and edema and hemorrhage developed throughout the mucosa and submucosa. Vascular casts allowed 3-D viewing of samples obtained from the entire pelvic flexure and demonstrated two distinct microvascular changes: 1) disruption of the colonic glandular capillary network occurred concomitantly with the mucosal injury, and 2) extensive endothelial leakage from the submucosal microvasculature contributes to edema formation. Thus, microcorrosion casting of the equine pelvic flexure provided an effective means to characterize the location and severity of vascular leakage and visualize the extent and severity of injury to the capillary network not easily depicted by histopathology. Microvascular casting in conjunction with routine histopathology provided additional information on the pathomorphologic changes in this model of ischemia/reperfusion injury.

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Introduction

Colic is a common condition in horses, with ascending colon volvulus (ACV) the most severe large intestinal disorder. 360° volvulus of the ascending colon occurs most commonly, and results in transmural compression, increased microvascular permeability and thrombus formation and necrosis of the intestinal mucosa; the ensuing endotoxemia results in fatality rates greater than 70% (Fisher and Meager, 1985; Harrison, 1988; White, 1990). Recent reports from clinical and experimental studies have aided significantly in our understanding of bowel morphology following ACV. The chronology of pathomorphologic changes to the mucosa include tissue necrosis, microvascular thrombosis and hemorrhage; and have been well characterized in natural and experimental ACV studies (Snyder et al., 1988, 1990; Meschter et al., 1991; Darien et al., 1993a). Separation of the glandular epithelium from the basal lamina, increased microvascular permeability, and microvascular thrombosis are the cornerstone morphologic lesions of ACV (Snyder et al., 1992; Darien et al., 1993a). While the pathologic changes after ischemia/reperfusion injury have been well described, there is a paucity of information characterizing the extent of microvascular injury to the mucosa and submucosa, and correlating these changes with concomitant histopathology. Specifically, in the face of subepithelial capillary thrombosis, what is the extent and location of the microvascular injury which contributes to submucosal and mucosal edema and hemorrhage?

The present study investigated the microvascular pathology in the ascending colon after experimental ischemia/reperfusion injury by using vascular corrosion casting and scanning electron microscopy (SEM). The microvascular changes observed by SEM were evaluated relative to changes observed in tissue sections using light (LM) and transmission electron microscopy (TEM). The pathomorphologic changes of the microvasculature were the basis of this correlative study to better characterize the site, location, and extent of microvascular injury in this model of intestinal ischemia/reperfusion injury.
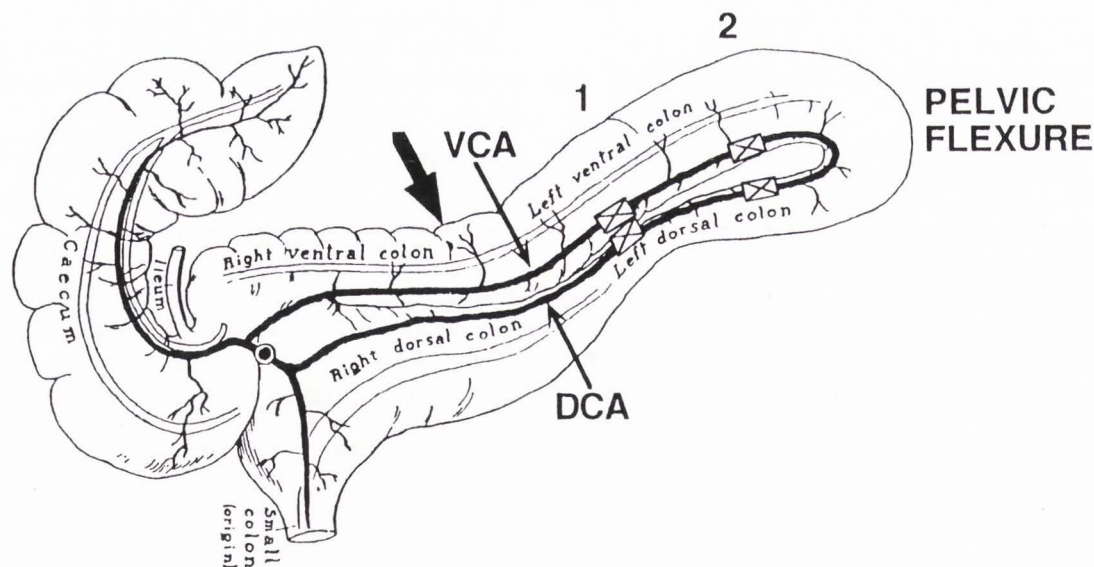
Figure 1. Schematic of equine large intestine. Ascending colon volvulus was performed cranial to the cecocolic fold (**large arrow**). Colonic arterial blood flow and pressure were recorded 30 cm from the pelvic flexure (**site 1, cross-hatch boxes**) in the left ventral and dorsal colonic arteries, **VCA** and **DCA**, respectively. The VCA was cannulated and the DCA ligated 15 cm from the pelvic flexure (**site 2, cross-hatch boxes**) for vascular perfusion and casting. Intestinal biopsies were obtained from the **pelvic flexure**. (Modified from Sisson and Grossman, 1953).

Materials and Methods

Animal preparation

Ten normal, healthy adult ponies (weighing 160-210 kg) were randomly divided into two groups of five (**Group 1**, control; **Group 2**, ACV). All ponies received a physical examination upon arrival and were dewormed 10 days prior to surgery and had a normal complete blood count and differential on the day of surgery. All experiments were performed under general anesthesia and were terminal. Anesthesia was induced with xylazine and ketamine (1.1 mg/kg and 2.2 mg/kg intravenously, respectively) and maintained with Isoflurane in oxygen. The ponies were mechanically ventilated with 100% O_2 through an endotracheal tube and administered Multisol-R® (Travenol) at 5-10 ml/kg/hr through a jugular vein catheter to help maintain circulating blood volume and pressure. Systemic arterial pressure was maintained at or above 65 mm Hg during the experimental period with IV fluids and dopamine. The large colon was exteriorized through a ventral midline laparotomy and sites for arterial cannulation, flow probe instrumentation and biopsy site were identified as follows (Fig. 1):

Left ventral colon (LVC). 30 cm proximal (oral) to the pelvic flexure a 4 mm ultrasonic transit-time flow probe (Transonic Systems Inc., Ithaca, NY) was fitted to an isolated portion of the ventral colic artery (VCA), and biopsy site identified at the pelvic flexure. The flow probe was connected to a T201 2-channel ultrasonic blood flow meter (Transonic Systems Inc., Ithaca, NY) for continuous recording of colonic blood flow.

Left dorsal colon (LDC). 30 cm distal (aboral) to the pelvic flexure the dorsal colic artery (DCA) was cannulated with PE-205 tubing (Intramedic polyethylene tubing, Clay Adams, Inc., Cleveland, OH). The cannula in the DCA was connected to a pressure transducer (model P23 1D, Gould) and direct writing oscillograph (model 2400 S, Gould Inc., Cleveland, OH) for continuous recording of colonic blood pressure. Systemic arterial pressure was similarly recorded from the right facial artery.

Experimentally induced ischemia and reperfusion

A 30 minute recovery period followed exteriorizing and instrumenting the colon. For the treatment group, the right dorsal colon was rotated 720° about the long axis of the colon, but not including the cecum, repositioned into the abdomen and maintained in that position for 120 minutes. After 120 minutes of volvulus, the colon was exteriorized, untwisted, and repositioned into the abdomen for 120 minutes of re-perfusion. The control ponies underwent 240 minutes of anesthesia following the 30 minute recovery period.

Physiology measurements. The intestinal microvasculature of the pelvic flexure (30 cm proximal and distal to the apex of the pelvic flexure was studied in this model. Blood flow to the LVC and LDC was monitored by a flow probe and pressure transducer, respectively. During the torsion experiments, the flow probe and the blood pressure recordings confirmed complete interruption of arterial blood flow during the ischemic period.

Table 1. Pathologic Criteria For Grading Ascending Colon Injury[*]

Grade	Histopathology description
0	Microvilli are numerous and well defined. Intercellular interdigitation with intact tight junctions, nuclei of the columnar epithelial cells are located basally and the basal portion of the cella are well attached to the basal lamina; the underlying lamina propria contains collagen, fibrils, and small capillaries.
1	Most of all the columnar epithelial tight junctions are intact, but there were occasional small to large gaps between epithelial cells and dilated spaces separated the basal cells from the borders of the basement membrane. Vacuolation of some enterocytes and accumulation of small foci of necrotic debris in the lamina propria.
2	Occasional sub-epithelium cleft formation, inter and intracellular swelling and vacuolation; small groups of cells are broken away from their attachment to adjacent cells and basement membranes, some cells have ruptured and extruded intracellular organelles into the colonic lumen, multifocal necrolytic debris congestion and edema of the lamina propria.
3	Progressive necrolysis and sloughing of the columnar epithelial cells and variable degeneration of glandular epithelial cells begins at the proximal aspect of the gland. In the lamina propria there is capillary engorgement and edema and hemorrhage into the interstitium. Platelets, proteinaceous material and cellular debris accumulate within the microvasculature.
4	Most of the superficial epithelium is sloughed, extending only occasionally into the crypts, with edema, necrosis, congestion and hemorrhage of the lamina propria. There is capillary plugging by platelets and proteinaceous material and RBC are present in the colonic lumen.
5	The superficial epithelium is sloughed and extends deeply into the crypts. Large vacuolar spaces beneath and between the cells exists and there is congestion, erythrodiapedesis and edema in the lamina propria. Capillaries are plugged with platelets and fibrin is deposited inside and outside of the endothelium.
6	Extensive to complete loss of the entire epithelium, sparing only the deepest parts of the crypts. Extensive congestion and erythrodiapedesis of the lamina propria. Capillaries are plugged with fibrin and platelets, and there is abundant extravascular fibrin.

[*]Modified from Meschter CL *et al.*, 1991.

Histopathology. An intestinal biopsy was obtained from each pony on the antimesenteric border of the pelvic flexure at the end of each study. The biopsy site on the colon was draped-off to assure a sterile surgical technique, obtained by scalpel excision and sutured by routine intestinal closure. Full thickness biopsy samples were fixed overnight in Bouin's fixative and then changed into 70% ethyl alcohol. Histopathologic sections for LM were prepared by routine methods (i.e., dehydrated, embedded into paraffin, cut into approximately 5 μm sections, and stained with hematoxylin and eosin). Mucosal sections were collected similarly for TEM from a site adjacent to the histopathologic sample. The mucosal samples were fixed for 2 hours at room temperature (22°C) in 2.5% Karnovsky's solution with 0.1 M cacodylate buffer and then rinsed and stored under refrigeration in 0.1 M cacodylate buffer. The tissue was routinely prepared for TEM (i.e., post-fixed in osmium tetroxide, serially dehydrated, and embedded in epon Araldite). Semi-thin sections (1.0 μm) were stained with 0.1% toluidine blue for orientation and evaluation of lesions. Thin sections (60-70 nm) were cut on a Reichert Ultracut E ultramicrotome fitted with diamond knife (Diatome), placed on copper grids (Fullam), stained with 1% uranyl acetate, followed with lead citrate, and examined on a Phillips 410 transmission electron microscope. Tissue sections were interpreted and scored by a board certified pathologist according to predetermined criteria (Table 1).

Microvascular corrosion casting. At the end of each study, the VCA was cannulated and the DCA ligated (15 cm proximal and distal to the pelvic flexure, respectively) for perfusion and vascular casting of the pelvic flexure (Fig. 1). The respective veins were isolated with vascular clamps. Doyen intestinal clamps were applied across each colonic section and the bowel excised. The vasculature was perfused with 1 liter of 0.1 M Krebs buffer (with 2 mM $CaCl_2$, 9 mM dextrose, 3 mM $NaNO_2$, 10,000 U Na heparin and 1% wt/vol bovine serum albumin; heated to 37°C and pH adjusted to 7.3 with 10% ascorbic acid and passed through a 0.45 μm Millipore filter) at a pressure that did not exceed 90

mm Hg. Thirty ml of partially polymerized methacrylate (Mercox) was mixed just before use with 30 ml methyl methacrylate (inhibited with 25 ppm hydroquinone monomethylether) plus 4.8 gm benzoyl peroxide. All perfusions were administered through the VCA via a 3-way stop cock. A manometer was used to periodically monitor perfusion pressure, which was kept below 120 cm H_2O. The 30 cm pelvic flexure was placed in a 45°C water bath for 12 hours and transferred into a 1 to 5% NaOH solution which was changed every 24 hours until the intestinal tissue was completely digested, as judged by evaluating 5 cm sections through a dissecting microscope. Specimens were fastened to aluminum studs with silver paint (Tousimis, Rockville, MD) sputter coated with a layer of gold-palladium (Bio-Rad, E5000M) about 20 nm thick, and viewed with a Hitachi S570 SEM (200 μm aperture, 10-20 kV accelerating voltage, and 15 mm working distance).

The vascular casts were evaluated for the presence of a normal vascular anatomy as well as for the presence of artifacts (Snyder *et al.*, 1989; Aharinejad *et al.*, 1991; Lametschwandtner and Lametschwandtner, 1992). Vascular cast quality was based on identifiable vascular anatomy and nuclear impressions, and the absence of extravasation of resin or "plastic strips" and impressions of erythrocytes in the vascular cast (Lametschwandtner *et al.*, 1990; Konerding, 1991; Ohtani and Murakami, 1992).

Results

Light microscopy

Morphologic alterations observed in the colon of the control ponies ranged from 0 to 2, with grade 1 being the most common. Grade 1 changes included vacuolation of some enterocytes with accumulation of small foci of necrotic debris in the lamina propria (Fig. 2a). Both control and torsion treated ponies had a non-specific increase in inflammatory cells in the lamina propria. In the torsion treated group, the histopathologic score ranged from 4 to 6, with 5 being the most common. Grade 5 was characterized by extensive loss of the superficial epithelium, sub-epithelial cleft formation extending deeply into the crypts (Fig. 2b). Edema, necrosis, congestion, and hemorrhage occurred throughout the mucosa, and edema and hemorrhage was prominent in the submucosa. Emigration of leukocytes was noted throughout the submucosa in the experimental group, and appeared most pronounced post reperfusion. Edema formation was noted in all tissue samples, but was most pronounced in the submucosa following reperfusion.

transmission electron microscopy

The microvascular ultrastructural changes observed in this model were similar to those previously reported (Meschter *et al.*, 1991; Snyder *et al.*, 1992). Electron micrographs of the subepithelial microvasculature in the control group showed an intact capillary endothelium, which occasionally was thickened but not detached from the basal lamina (Fig. 2c). Electron micrographs of the mucosa in the torsion group showed extensive to complete necrosis and sloughing of the epithelium. Subepithelial capillaries were contracted and endothelial tenting separated the cell from its basal lamina. Fibrin was attached to endothelial cells and subjacent to those which were separated from the basal lamina (Fig. 2d). Subepithelial capillaries contained numerous platelets, irrespective of an intact tight junction.

Corrosion casting

Because the microvascular injury in this model resulted in thrombosis, hemorrhage and edema, the presence of extravasated resin in the torsion treated group was evaluated in light of the overall vascular cast quality. Based on the appearance of normal vessel anatomy, the absence of artifacts other than extravascular resin, the extravasation of resin in the torsion treated group was considered to be a result of the vascular pathology and not a vascular casting artifact. The vascular casts of the control group closely resembled those reported by Snyder *et al.* (1989). The dorsal and ventral colonic arteries arose from the cranial mesenteric artery and supplied the left dorsal and ventral colon, respectively. The colonic artery gave rise to a colonic rete which extended into the plica semilunares as it coursed toward the antimesenteric border. Recognizable endothelial cell nuclear imprint patterns were used to distinguish arteries from veins within the submucosa and mucosa (Hodde *et al.*, 1990). The nuclear imprints retained in arteries were oval to elongated and oriented parallel to the long axis of the vessel, while those in veins were round to polygonal in shape with no particular orientation (Figs. 3a and b, respectively). The submucosal vasculature was an extensive network of small arteries and arterioles intertwined with small veins and venules. Small arterioles gave rise to terminal arterioles, which branched at right angles prior to passing through the muscularis mucosa. Within the mucosa, terminal arterioles gave rise to capillaries which surrounded the colonic glands, forming a "honeycomb" vascular plexus (Figs. 3b and 4a). The capillary plexus drained into a catwalk-like polygonal plexus at the most luminal aspect of the lamina propria (Fig. 3c). The "catwalk" capillaries drained into transversely oriented capillaries, which subsequently joined the submucosal venules (Fig. 3d). Submucosal veins were often noted to have band-shaped constrictions, which were most severe in the torsion treated group.

The vascular casts of the volvulus treated group retained the normal artery and vein anatomy to the level of the submucosa (Fig. 4a). However, in tissue sections with necrosis, the vascular cast demonstrated extensive injury to the microvasculature supplying the submucosa and mucosa. Necrosis to the lamina propria resulted in complete disruption to the colonic mucosal capillary network which included fragmentation and sloughing of the luminal "catwalk" capillaries and glandular "honeycomb" capillary plexus (Fig. 4b, arrowhead). Transversely oriented capillary posts were often isolated and detached from any luminal capillaries (Fig. 4b, arrow).
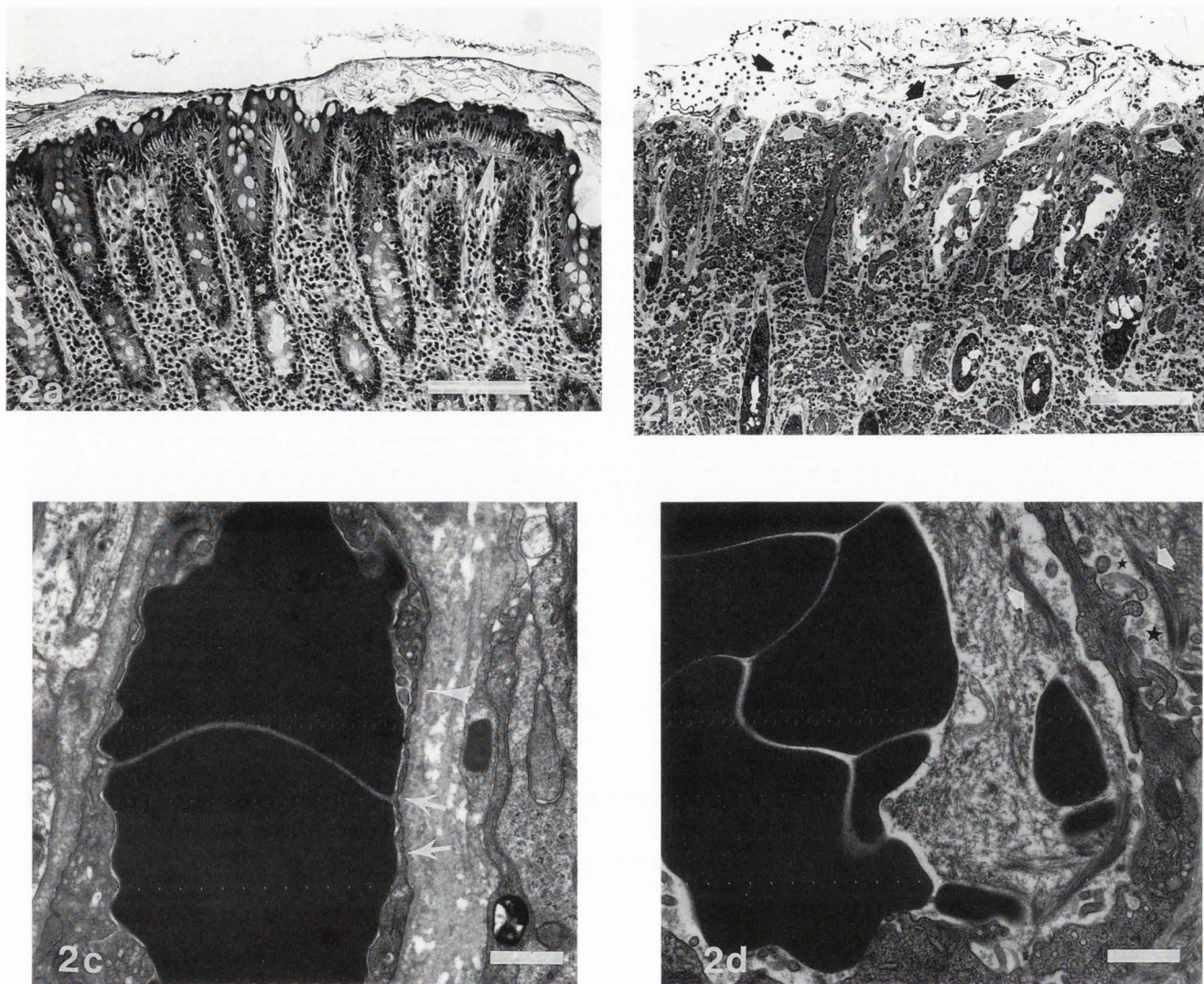
Figure 2. Light (**2a, 2b**) and transmission electron (**2c, 2d**) photomicrographs of epithelium and subepithelial cap-
illaries of the pelvic flexure colonic mucosa from control and volvulus treated ponies at T = 5 (two hours post reperfu-
sion). **a)** Control pony, the mucosa is normal with closely packed, straight, tubular glands. Nuclei of the columnar
epithelial cells are located basally and the basal portion of the cells are well attached to the basement membrane
(arrows). The subepithelial capillaries are small and not dilated (paraffin section, H&E stain). **b)** Volvulus treated
pony, there is complete loss of the epithelium, hemorrhage, and necrotic tissue in the lumen (short, black arrows) and
the basal lamina is intact. Most of the crypts have collapsed and there is marked hemorrhage in the lamina propria.
Capillaries are dilated (short, white arrows) near the surface (plastic section, toluidine blue). **c)** Control pony,
fenestrated capillaries with diaphragms (arrows) are supported by a continuous basal lamina (arrowhead). **d)** Volvulus
pony, endothelial cells are detached from the basal lamina (stars) and fibrin is abundant in the intravascular and
extravascular space (arrows) of the lamina propria. Bars = 100 μm (2a and 2b); and 1 μm (2c and 2d).

In areas devoid of a capillary network, there was exten-
sive leakage of cast material from the submucosal micro-
vasculature. Most commonly, as observed on histopa-
thology by LM, areas of mucosa without necrosis were
adjacent to areas with extensive necrosis, edema and
hemorrhage. On scanning electron micrographs, these
changes were observed as areas with minimal changes to
the colonic capillary network (vascular disruption con-
fined to the luminal capillaries and extravasation of cast
material, Fig. 4c, arrows) adjacent to areas with exten-
sive disruption of the mucosal capillary network,
vascular leakage (blebbing of casting material) and
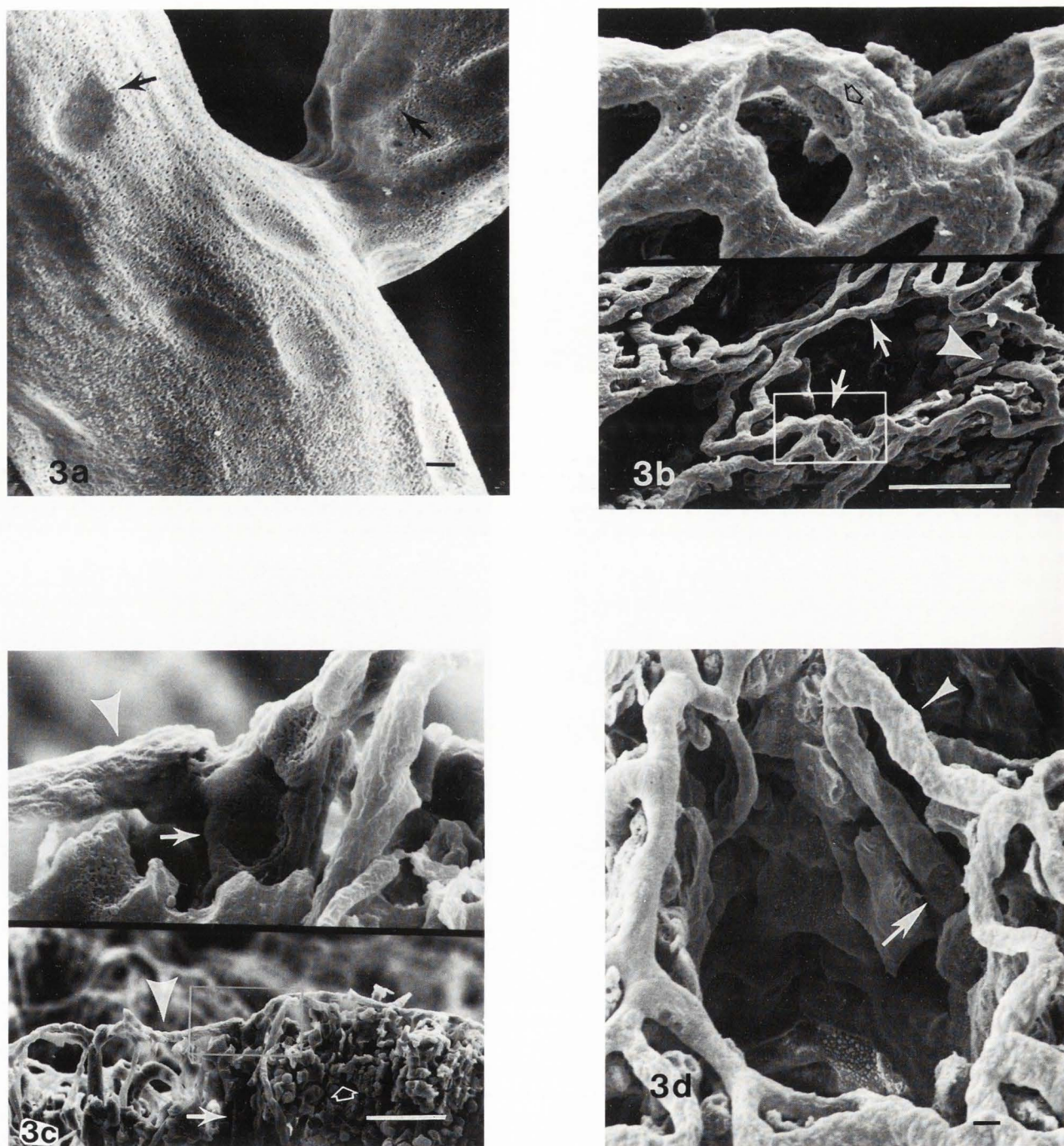embedded ingesta (Figs. 4c and 4d).

Figure 3. Vascular cast and nuclear imprints of colonic submucosal artery and mucosal capillaries. **a)** The nuclear imprints (arrowheads) retained in arteries were oval to elongated and oriented parallel to the long axis of the vessel, except at a branch. **b)** The colonic glandular capillaries (arrow head/no tail) drained into a "catwalk-like" polygonal plexus (arrow/short tail) at the most luminal aspect (boxed area, enlarged in Inset) of the lamina propria. **Inset)** Higher magnification of a nuclear imprint (open arrow/outline) in the luminal capillary plexus. **c)** Sagittal section of the mucosal microvasculature showing numerous colonic glandular capillaries (open arrow), and luminal "catwalk" plexus (arrowhead) draining into the luminal capillary (arrow). **Inset)** Higher magnification (of the boxed area in Fig. 3c) of the luminal plexus (arrowhead) which drains the mucosa via transversely oriented capillaries (arrow). **d)** Transversely oriented capillary posts (arrow) had round to polygonal shaped nuclei with no particular orientation and drained into submucosal venules. Bars = 10 μm (3a and 3d); and 100 μm (3b and 3c).
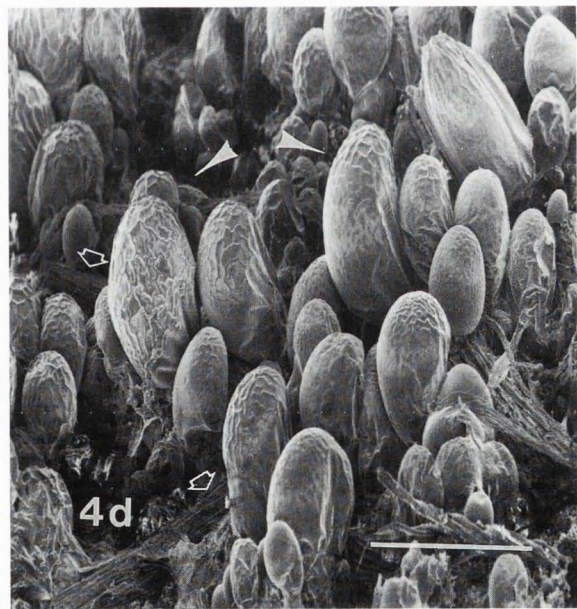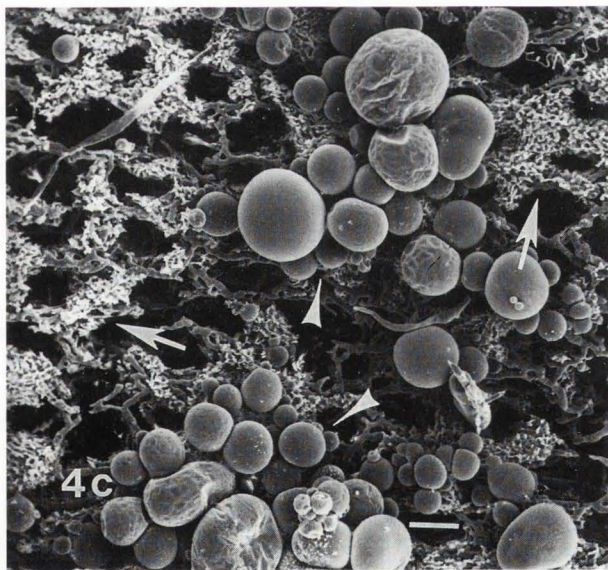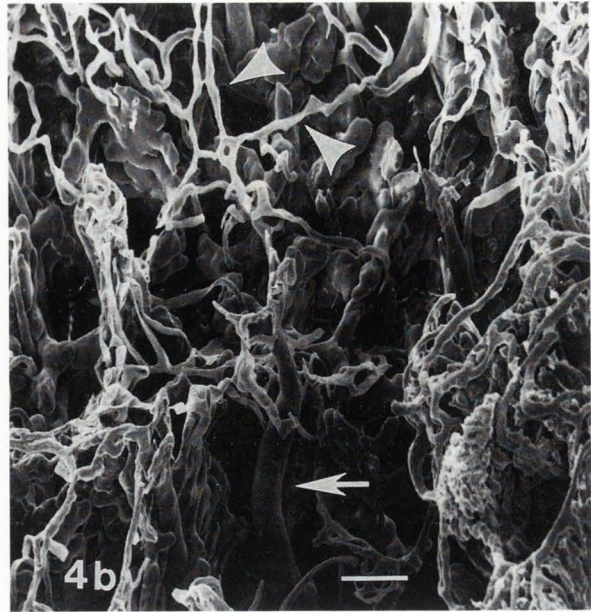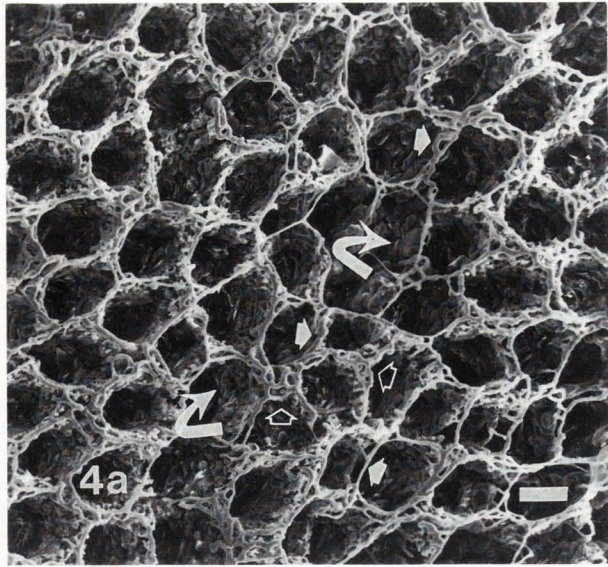
Figure 4. Vascular cast of the mucosal capillaries from control and volvulus treated ponies. **a)** Vascular cast of the mucosal surface from a control treated pony. Capillaries surround the colonic glands (curved arrows), forming the "honeycomb" vascular plexus (open arrows) and luminal "catwalk" (closed arrows). **b)** Vascular cast of the mucosal surface from a volvulus treated pony. The luminal "catwalk" is fragmented and sloughing (arrowhead) and the transversely oriented capillary posts are isolated and detached (arrow) from the luminal plexus. **c)** Glandular capillary leakage (arrows) as well as blebbing of casting material (arrowheads) from the microvasculature. **d)** Extensive vascular blebbing from the submucosa microvasculature (arrowheads). Ingesta, embedded into the submucosa, is also present (open arrows). Bar = 100 μm (4a, 4b, and 4c); and 1 mm (4d).

Discussion

An understanding of the pathomorphology of microvascular ischemia/reperfusion injury is essential to formulating a therapeutic regimen which can attenuate this pathogenic process. In this study of experimentally induced ischemia/reperfusion injury of the ascending colon in ponies, we used SEM of vascular corrosion casts to characterize the type and severity of microvascular injury and to corroborate the histopathologic changes observed by light and electron microscopy. Two different pathologic changes were observed within the microvasculature: one of microvascular thrombosis, the other of increased endothelial permeability.

Control ponies with grade 2 mucosal score had focal areas of mucosa with epithelial cell swelling and vacuolation and occasional clefts into the sub-epithelium. In the lamina propria there was multifocal necrolytic debris, congestion and edema. In volvulus treated ponies, extensive epithelial necrosis encompassed subepithelial capillaries, often resulting in their collapse. The submucosa and lamina propria had extensive edema with extravascular infiltrates of neutrophils and macrophages. Extravasation of erythrocytes was most prominent in the lamina propria, but also present in the submucosa. Subepithelial capillaries developed the characteristic changes ascribed to disseminated intravascular coagulation: endothelial cells swelling, formation of cytoplasmin projections, dilation of interendothelial junctions, adhesions of leukocytes and subsequent separation from the basal lamina (Bick, 1992; Hardaway, 1982). Platelets were attached to endothelial cells as well as subendothelial collagen and fibrin deposits were observed in the intravascular and extravascular space.

The histopathologic changes observed in this model of intestinal ischemia/reperfusion injury approximate those of previous reports using similar periods of ischemia and reperfusion. However, we did not observe the degree of hemorrhage in the lamina propria and submucosa that others have reported previously (Snyder *et al.*, 1988; Meschter *et al.*, 1991; Provost *et al.*, 1991; Henninger *et al.*, 1992). Snyder and Meschter surgically induced ischemia by separate transmural and vascular techniques and Provost manually twisted the colon, as did we, but included the cecum. Variations in inducing ischemia and time periods of ischemia and reperfusion may explain the differences observed in extravascular hemorrhage. Common to all pathologic finding were microvascular sludging with platelet and fibrin deposition, and mucosal and submucosal edema. Emigration of leukocytes was hard to appreciate with light microscopy because control ponies had non-specific increase in lamina propria inflammatory cells. Although all ponies were dewormed prior to their study date, 10 days may not have been enough time for resolution of parasite-induced inflammatory infiltrate. TEM of capillaries showed increased neutrophils in affected submucosa. Platelet and fibrin deposition may reflect an acquired hypercoagulable state. Hypercoagulation, as defined by decreasing plasma antithrombin III (AT III) activity, has been reported to correlate with survival in naturally occurring cases of equine colic (Holland *et al.*, 1986; Darien *et al.*, 1991). The hypercoagulable state is believed to be induced by endotoxemia which results in the generation and subsequent interaction of coagulation proteins, i.e., thrombin, and inflammatory mediators (cytokines), i.e., tumor necrosis factor, TNF (Esmon *et al.*, 1991; Darien 1993). Plasma AT III activity has been reported to decrease significantly at the end of the reperfusion period and coincide with the histopathologic changes of microvascular thrombosis in this model (Darien *et al.*, 1993a, b). Consequently, interrupting the generation of thrombin and tumor necrosis factor may attenuate microvascular thrombi formation.

Vascular corrosion casting of the pelvic flexure microvasculature demonstrated an arborizing plexus of capillaries which arose from submucosal arterioles and supplied a glandular capillary "honeycomb" network. The capillary plexus drained into a luminal "catwalk" plexus which drained into long, transversely oriented capillary posts which penetrated the submucosa. Vascular casts of the torsion treated group demonstrated extensive injury to the glandular capillary network. Observed on all volvulus treated ponies was sloughing of the luminal capillaries which often extended into the "honeycomb" plexus of the glandular capillaries. Scanning electron microscopy of the vascular casts demonstrated that injury to the glandular capillary network occurs concomitantly with mucosal epithelial injury.

Not explained by histopathology, but clearly demonstrated by vascular casting, was the source of edema formation within the submucosa and mucosa. Vascular permeability, was demonstrated by the "blebbing" of casting material from the submucosal microvasculature. The loss of casting material from the vasculature was not observed in the control ponies, and is most likely responsible for the intestinal edema noted during surgery in horses with ACV. Because edema formed where mucosal necrosis varied from extensive to mild, vascular permeability may be a separate pathologic change. Thrombin, TNF, platelet activating factor, and nitric oxide are generated during endotoxemia, can induce endothelial permeability, and may facilitate microvascular thrombosis as well as increased endothelial permeability (Malik and Fenton, 1992; Yi and Ulich, 1992; Kurose *et al.*, 1993).

Vascular corrosion casting of the pelvic flexure demonstrated the extent to which the glandular capillary network became abrogated during the process of mucosal necrosis. While histopathology was able to demonstrate microvascular thrombosis, it did not demonstrate the extent of pathology to the glandular capillary network nor the origin of bowel edema. Vascular casting was able to demonstrate the extensive loss of the capillary network and vascular permeability. Observing the loss of colonic glandular capillaries by SEM clearly demonstrated that mucosal and glandular capillaries undergo extensive injury during ischemia and reperfusion, and facilitates

understanding electrolyte and fluid losses which occur in naturally occurring ACV. Further selective studies are required to determine the pathogenesis of increased microvascular permeability, edema formation and microvascular thrombosis following intestinal ischemia/reperfusion injury.

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Discussion with Reviewers

S. Aharinejad: You mixed 30 ml Mercox® with 30 ml methyl methacrylate and added 4.8 gm catalyst to the mixture. According to our experience (Aharinejad S, MacDonald IC, MacKay CE, Mason-Savas A, New aspects of microvascular corrosion casting: A scanning, transmission electron, and high-resolution intravital videomicroscopic study. Microsc Res Techn **26**: In press), when 1 gm catalyst is added to 10 ml Mercox® a polymerization time of 5.5 to 7 minutes would result. The amount of catalyst you have used is apparently higher than 1 gm/10 ml Mercox® (1.6 gm/10 ml Mercox®). This would give you less than 1 minute time to inject the resin. Is this time enough for perfusion? The highest amount of catalyst we added to 10 ml Mercox® was 1.5 gm, which gave us 1 minute to inject the resin.

Authors: The ratio of catalyst to methacrylate used in this study was 4.8 gm/60 ml (or 1 gm/12.5 ml), greater than the 1 gm/10 ml which yields a polymerization time of 5.5 to 7 minutes. Additionally, half of the methacrylate was not prepolymerized (methyl methacrylate), as is the case for prepolymerized Mercox®. Thus the polymerization time following mixing would be extended beyond the time described for a perfusion mixture whose methacrylate consists entirely of Mercox®.

D.E. Schraufnagel: In most conditions that produce edema (at least in the lung), we find that the resin leaks out and fills lymphatics. Did you find lymphatic filling in animals and what forms did it take? The diagonal cast (Fig. 4a) looks like a lymphatic to me.

S. Aharinejad: Ponies undergoing bowel torsion had extensive local colonic edema. Under such conditions, one would expect to extensively see well-perfused lymphatics. Lymphatics are even visible under normal conditions when casting the blood circulation system of the gut (Aharinejad *et al.* in press, cited in question above). I miss lymphatics in your micrographs. Why?

Authors: While microvascular permeability changes during ascending colon volvulus in horses does result in increased lymph plasma protein (Henninger *et al.*, 1992), dilated lymphatics (as demonstrated by corrosion casting) has not been commonly described. I am sure there is some lymphatic filling during the perfusion process, however, the integrity of the subepithelial lymphatics must be such that they are difficult to demonstrate by corrosion casting.

O. Ohtani: The leakage of the media artificially injected with high pressure does indicate vulnerable sites of the vessels. But, this does not necessarily mean the source of edema formation. It seems to the reviewer that some other novel experiments will be needed to show the real source of edema formation.

Authors: Several factors were evaluated prior to deciding that the microvasculature was the site of edema formation: **1)** Perfusion pressure during vascular casting did not exceed the systemic arterial pressure recorded from the ventral colic artery during the study; **2)** the viscosity of our perfusate was similar to that of blood, thus we were able to fill the mucosal capillary bed while maintaining "physiologic" perfusion pressures; and **3)** serial tissue samples obtained from the colon corroborated the progression of edema formation by light microscopy, and separation of endothelial cell tight junctions by transmission electron microscopy.