Strangulating volvulus of the large colon is one of the most severe forms of colic and can account for 11% to 27% of horses requiring surgical correction of colic in referral hospitals.1–3 Survival after surgery for correction of large colon volvulus is dependent on the degree of ischemic injury to the colon and severity of the systemic response. The survival rate without surgical resection has been reported to be as low as 34.7%,2 but resection improved the survival rate of affected horses in 2 studies (57.7%4 and 74%,5 respectively). In most horses that undergo colon resection, some ischemic tissue remains. Thus, survival in these horses can be correlated with loss of the epithelial barrier in the remnant portion, which allows transmucosal leakage of endotoxins, bacterial chemotactic peptides, and bacteria.6 Therefore, rapid repair of the ischemic-injured epithelium is crucial to recovery of a horse after large colon volvulus but could be impaired by reperfusion injury.7–12

The biochemical pathway responsible for reperfusion injury starts with accumulation of products that build up during ischemia and ROS generated on reperfusion.7–10 Neutrophil infiltration is a time-dependent process that occurs mostly during the reperfusion period,8,11 and activation of these cells results in degranulation and additional release of inflammatory mediators, such as cytokines, ROS, proteases, and other regulatory proteins.12 Although ischemia is a major pathophysi-
The amino acid l-arginine is an indirect precursor of nitric oxide, and therefore has the potential to hasten tissue healing and modulate the inflammatory response. The amino acid l-glutamine can influence several metabolic pathways during intestinal stress and was protective in an in vitro evaluation of chemical damage to equine colonic mucosa.\(^{20}\) Metabolism of arginine and glutamine is closely related, and these amino acids could result in an additive effect on several biochemical pathways.\(^{18}\) Acetylcysteine can protect mucosa through its antioxidant properties\(^{22,23}\) and by providing sulfhydryl groups required for replenishment of glutathione, which is an important intracellular antioxidant.\(^{23}\) In equine colonic mucosa, acetylcysteine can prevent mucosal damage and reduce eosinophil migration after chemical damage in vitro.\(^{23}\)

Our hypothesis was that experimentally induced ischemia of short duration would cause mild to moderate reversible injury to equine colonic mucosa and that this injury would be exacerbated during reperfusion. The purpose of the study reported here was to evaluate mucosal damage of the equine colon caused by ischemia and reperfusion, as determined on the basis of histomorphometric changes; determine TER and permeability to mannitol in tissues subjected to ischemia and reperfusion, compared with results for control tissues and tissues subjected to ischemia only; and evaluate in vitro effects of components of an O2PS on functional and morphological measurements of mucosal recovery.

## Materials and Methods

Horses—Six healthy adult horses (3 Thoroughbreds, 2 Quarter horses, and 1 warmblood) were included in the study. Horses were 11 to 26 years old (mean, 16 years) and weighed 440 to 660 kg (mean, 548 kg). Inclusion criteria were that the horses were in good health, were free of gastrointestinal tract disease, and required euthanasia for conditions that rendered them unsuitable for use. Horses were housed on pasture with grass hay and water provided ad libitum and did not receive any medications for the 2-week period preceding the study. The Institutional Animal Care and Use Committee of the University of Florida approved the study protocol.

**Surgical procedure**—Horses were sedated with xylazine (1 mg/kg, IV). A 14-gauge, 13.3-cm polytetrafluoroethylene catheter was inserted into each of the jugular veins; the left jugular vein was used for administration of anesthetic drugs and isotonic fluids, and the right jugular vein was used for collection of blood samples. Anesthesia was induced with ketamine (2.2 mg/kg, IV) and diazepam (0.1 mg/kg, IV). Orotracheal intubation was performed and horses were positioned in dorsal recumbency. Anesthesia was maintained with isoflurane (1% to 3%) in oxygen via mechanical ventilation. Isotonic polyionic fluids were continuously infused IV at a rate of 2.5 to 5 mL/kg/h. Mean arterial blood pressure was monitored through a 20-gauge, 5.1-cm polytetrafluoroethylene catheter in the facial artery and was maintained at ≥ 60 mm Hg. Monitoring performed during anesthesia included electrocardiography, arterial blood gas analysis, capnography, and direct blood pressure measurement.

The ventral abdomen of each horse was prepared and draped for aseptic surgery, and ventral midline celiotomy was performed. The large colon was exteriorized and positioned on a sterile drape on the ventral abdomen. To induce ischemia, a 40-cm segment of colon at the pelvic flexure was subjected to transmural compression via intestinal clamps placed at each end, and venous and arterial occlusion was achieved with umbilical tape ligatures. After induction of ischemia, the colon, colonic vasculature, and associated mesentery were surgically divided at the pelvic flexure so that 2 segments of colon comparable in size (dorsal and ventral) did not communicate. The colon was then replaced in the abdomen, and the abdominal incision was closed temporarily with towel clamps. After a 1-hour period of ischemia, the colon was again exteriorized and 1 of the 2 ischemic segments (ischemic tissues) was resected for histologic evaluations and in vitro experiments in Ussing chambers. Ischemic tissues were not harvested for Ussing chamber experiments from the first 2 horses; thus, tissues from only 4 horses were used for this phase of the study. At the same time, the clamps and ligatures were removed from the other segment of ischemic colon; this segment was replaced in the abdomen to allow resumption of blood flow (reperfusion) for 4 hours (horses remained anesthetized during reperfusion). Small (1- to 2-cm\(^2\)) mucosal biopsy specimens were collected before ischemia, after a 1-hour period of ischemia (time 0 for ischemic tissues), and after reperfusion for 1, 2, and 4 hours. After reperfusion for 4 hours, the reperfused segment of colon (reperfused tissues) and an adjacent nonischemic-nonreperfused segment of colon (control tissues) were removed (time 0 for control and reperfused tissues) for histologic evaluation and in vitro experiments in Ussing chambers. After the control and reperfused tissues were harvested, the anesthetized horses were euthanized with an overdose of sodium pentobarbital (88 mg/kg, IV).

**Ussing chamber experiments**—A solution of KRB (112mM NaCl, 25mM NaHCO\(_3\), 10mM glucose, 5mM KCl, 3mM sodium acetate, 3mM sodium butyrate, 2.5mM CaCl\(_2\), 1.2mM MgSO\(_4\), 1.2mM KH\(_2\)PO\(_4\), and 0.01mM mannitol [pH, 7.4]) was prepared. After removal, full-thickness tissue sections from ischemic, reperfused, and control colon were immediately placed in cold (4°C) KRB solution and transported to our labo-
The timing of sample collection and mounting of tissues in Ussing chambers for the in vitro experiments was such that experiments were completed on the ischemic tissues before the same experiments were performed on control and reperfused tissues. Mucosal sheets from each colon segment (control, ischemic, and reperfused tissues) were removed and mounted in Ussing chambers as described elsewhere. Three treatment solutions were used to incubate the mucosal tissues: KRB alone; KRB with 70 mg of NACa/L (KRB-NAC treatment); and KRB with 5 mg of l-arginine/L, 70 mg of NACa/L, and 10 mg of l-glutamine/L (KRB-MOTS treatment). Each tissue incubation was performed in duplicate.

The short-circuit current was recorded in each chamber by use of voltage clamps through silver–silver chloride electrodes connected to 4% agar bridges in KRB solution. Junction potentials of electrodes and fluid resistance were measured before mounting of the tissues to allow continuous correction for any effects that these factors might have on the low potential differences generated by the tissues. When the tissues were mounted in the chambers, the voltage clamp could then automatically correct for junction potentials of electrodes and fluid resistance and reduce their effects on the recordings. Throughout incubation, the short-circuit current was continuously applied to the tissues, except for a brief period at 15-minute intervals when the potential difference of the tissue was measured. The TER was calculated by use of Ohm's law, whereby resistance is equal to the potential difference of the tissue divided by the short-circuit current. Resistance was used as a measure of integrity of colonic mucosa and permeability of the paracellular pathway to ions.

The unidirectional flux of tritium-labeled mannitol from the mucosal to the serosal solution was an additional measure of colonic mucosa permeability. For scintillation counting, fluid samples were collected from both sides (mucosal and serosal) at 45, 73, 105, and 240 minutes after addition of radiolabeled mannitol to the mucosal side. Transmucosal flux of mannitol was expressed as the percentage of the initial scintillation counts detected on the mucosal side of the tissue that was detected on the serosal side of the tissue at the 4 time points. The interval from tissue collection to mounting of the tissue in the first chamber was approximately 10 minutes and to first recording of TER and addition of tritium-labeled mannitol was approximately 40 minutes. Total incubation time for each tissue in the Ussing chamber was sufficient to record TER and mucosal flux for 240 minutes. After Ussing chamber experiments were completed, the tissues were removed and placed in neutral-buffered 10% formalin for histologic evaluation and histomorphometric measurements.

Histologic evaluation and histomorphometric measurements—Biopsy specimens of colonic mucosa obtained in vivo before induction of ischemia (time 0), after a 1-hour period of ischemia, and after reperfusion for 1, 2, and 4 hours and in vitro at the end of Ussing chamber experiments were fixed in neutral-buffered 10% formalin. Tissues were embedded in paraffin, cut into 5-µm-thick sections, placed on silane-coated glass slides, and stained with H&E for examination via light microscopy. For histomorphometric assessment of mucosal damage via light microscopy, a computer-based imaging-analysis program was used and 3 fields from each tissue were examined as described elsewhere. One investigator (AG), who was unaware of the treatment group for each biopsy specimen, performed all histologic evaluations.

The length of denuded epithelium was measured and expressed as a percentage of the total mucosal length in the section. The epithelium was defined as lifted when > 5 epithelial cells were separated from the basement membrane but still attached to adjacent cells. The length of epithelium affected by lifting was expressed as a percentage of the total surface length. Detached cells were defined as cells that appeared morphologically normal but were separated from the basement membrane in groups of ≥ 5 cells and completely detached from adjacent epithelium. The length of detached epithelium was measured and expressed as a percentage of the total surface length of the mucosa. Restituted cells were defined as cells that were flattened in appearance but had intact attachments to the basement membrane and to adjacent cells. The length of epithelium consisting of restituted epithelial cells was measured and expressed as a percentage of the total surface length of the mucosa. The number of sloughed cells was counted for each field; the mean number of sloughed cells/0.1 mm of surface length was calculated. Sloughed cells were not counted in tissue samples incubated in the Ussing chambers because there were no sloughed cells present or the sloughed cells were of undetermined origin.

Statistical analysis—The tissues obtained from each horse yielded 2 sets of observations for each experimental condition in the Ussing chambers. The mean of the data was calculated and expressed as least squares mean ± SEM, and a statistical software program was used for analysis. Repeated-measures ANOVA was performed on the TER for each of the 3 tissue groups. Whenever there was a significant result of an F test for treatment, time, or the treatment-by-time interaction, appropriate Bonferroni-adjusted P values were used for each family of comparisons. To determine the effect of ischemia and reperfusion on tritium-labeled mannitol flux after incubation in the Ussing chambers for 240 minutes, a 1-way ANOVA was performed. Statistical analysis of histologic data was performed by use of a Kruskal-Wallis test to determine significant differences in histomorphometric measurements between the treatment groups. Post hoc analysis was performed by use of the Mann-Whitney U test. Values of P < 0.05 were considered significant for all statistical analyses.

Results

Ussing chamber experiments—The TER was not significantly changed by the Ussing chamber treatments (KRB, KRB-MOTS, and KRB-NAC) within each of the 3 tissue groups (control tissues [n = 6 horses], ischemic tissues [4], and reperfused tissues [6]). The TER of the control tissues decreased significantly with time during the incubation period, whereas there was no
no effect of time on the ischemic or reperfused tissues. The TER in each control tissue was significantly greater than the TER in each ischemic tissue from 15 minutes until 120 minutes of incubation and from 180 minutes to 225 minutes of incubation (Figure 1). The TER of each reperfused tissue was significantly greater than the TER of each ischemic tissue at 90, 120, and 180 to 240 minutes of incubation in the Ussing chambers.

Ussing chamber treatments did not affect the transepithelial resistance (TER) in each control tissue (KRB, KRB-NAC, and KRB-MOTS) for 4 hours. Values did not differ significantly among treatments at any time within the control, ischemic, or reperfused tissue groups. Values for all reperfused tissues differed significantly from values for all ischemic tissues, except when comparing control and reperfused tissues, ischemic and reperfused tissues, respectively, and epithelial cells were wider and flatter than were control epithelial cells, which suggested that mucosal restitution during reperfusion was observed in the mucosa of control mucosal samples obtained after 1 hour of ischemia-reperfusion (control tissue [solid line and white symbols]). Each time point represents the mean value for samples (evaluated in duplicate) obtained from 6 horses (control and reperfused tissues) or 4 horses (ischemic tissue). Time 0 = end of 1 hour of ischemia (ischemic tissues) or end of 4 hours of reperfusion (control and reperfused tissues). Immediately after collection, tissues were mounted in Ussing chambers and incubated with KRB (circles), KRB and MOTS (KRB-MOTS treatment [squares]), or KRB and NAC (KRB-NAC treatment [triangles]) for 4 hours.

Histomorphometric examination—A 1-hour period of ischemia caused edema, purple discoloration of the serosa, and serosal petechiation. By 10 to 15 minutes after the ischemia-inducing clamps and ligatures were removed, the appearance of the reperfused segment was similar to that of the adjacent control segment.

Histomorphometric examination of in vivo tissue samples—Histologic changes in the in vivo mucosal samples obtained after a 1-hour period of ischemia were suggestive of cell injury, and lifting of the surface epithelial cells confirmed mucosal disruption (Figure 3). Mucosa collected after reperfusion for 4 hours had evidence of an intact epithelial lining. Significant differences were observed in the mucosa of control tissues and ischemic tissues with respect to mucosal height ($P < 0.001$), epithelial height ($P < 0.001$), epithelial width ($P < 0.001$), lifted epithelium ($P = 0.006$), degenerated epithelium ($P = 0.001$), and the number of necrotic or sloughed cells ($P < 0.001$). There was no significant difference in the amount of denuded epithelium or the amount of restituted epithelium (Table 1). There were significant differences between ischemic and reperfused mucosal samples obtained after 1 hour of ischemia-reperfusion. The control and reperfused mucosa differed in that reperfused epithelial cells were wider and flatter than were control epithelial cells, which suggested mucosal restitution during reperfusion rather than during exacerbation of ischemic injury.

Histomorphometric examinations of Ussing chamber tissues—Histologic changes observed in the mucosa after incubation in Ussing chambers for 240 minutes were similar to those observed for the in vivo samples (Table 2). Compared with the results for the control and reperfused tissues, ischemic tissues had a significantly decreased mucosal height ($P = 0.002$), epithelial height ($P < 0.001$ and $P = 0.001$ for the control and reperfused tissues, respectively), and epithelial cell injury.
lial width \((P = 0.001)\). There also was a significant \((P < 0.001)\) increase in the percentage of denuded epithelium for the ischemic tissues, compared with the percentage for the control and reperfused tissues. Compared with results for the control tissues, ischemic tissues had a significantly greater percentage of denuded epithelium \((P < 0.001)\) and greater epithelial restitution \((P = 0.003)\). Ischemic tissues also had a greater percentage of epithelial restitution than the reperfused tissues; however, these values did not differ significantly \((P = 0.05)\). Results for the reperfused tissues were not significantly different from results for the control tissues with respect to any of the variables examined.

There was no significant difference in the percentage of lifted epithelium or degenerated epithelium among any of the groups. There was no significant effect of Ussing chamber treatments (KRB, KRB-NAC, or KRB-MOTS) on the histomorphometric measurements; therefore, all statistical analyses were performed for the tissues incubated in KRB. When the in vivo mucosal biopsy specimens were compared with the in vitro biopsy specimens, the most notable difference was evidence of epithelial cell restitution in the ischemic tissues after incubation in the Ussing chambers for 240 minutes (Figure 3). Predictably, all tissues had a reduced intensity of staining in the lamina propria after incubation in the Ussing chambers for 240 minutes.

**Discussion**

In the present study of experimentally induced ischemia in the equine large colon, reperfusion did not exacerbate the ischemic damage but appeared to allow mucosal recovery, as determined on the basis of histologic and functional measurements of tissue integrity. The ischemia experimentally induced in the colon of horses in the study reported here caused mild injury that should have allowed some expression of reperfusion injury over the next 4 hours.26 A more severe ischemic injury may not have allowed a sufficient number of viable cells to survive and cause an obvious reperfusion injury. However, further studies are required to determine whether 1 hour is an optimum ischemic period to allow generation of ROS and the associated tissue damage in equine colonic mucosa.

In the original experimental design, responses of interest were to be examined in 2 separate groups of horses that would be assigned by use of a randomization procedure to an ischemia group and an ischemia-reperfusion group. However, it was evident after the procedure was performed on the first 2 horses in the ischemia-reperfusion group that the experimental procedures allowed sufficient time for investigators to mount the ischemic tissues in the Ussing chambers, in-

![Figure 3](image-url)
cubate the tissues, and then empty and rinse the chambers in time for the reperfused tissues to be mounted. In this manner, each horse could be used to provide tissues subjected to ischemia and to reperfusion, and the difference between the 2 tissues was sufficient such that only 4 sets of ischemic tissue were required to yield significant differences.

When the function of the mucosa was examined in Ussing chambers, the ischemic tissues performed significantly worse than did the control and reperfused tissues, as measured on the basis of TER. There was no significant differences.

Histomorphometric examination also revealed restitution in reperfused tissues with almost complete mucosal recovery after 4 hours in vivo. This early recovery of mucosal epithelium was mainly attributable to migration of surviving epithelial cells over the damaged surface and not by cell proliferation, which would require a considerably longer observation period in vivo.

Complete arteriovenous occlusion for 1 hour was used in the present study to closely mimic large colon volvulus in horses. A period of 4 hours of reperfusion was chosen as a reasonable amount of time in which observations could be made with horses remaining anesthetized. In other studies, in which investigators used similar ischemic methods for the equine colon and jejunum, a reperfusion period of 18 hours had been used, which necessitated recovery of the horses from anesthesia. Because mucosal restitution and tightening of paracellular pathways are critical for recovery, the time period chosen for examination of the effects of reperfusion should be appropriate to evaluate these aspects of early repair. The findings in the study reported here also are consistent with findings in another study conducted by our research group in which we found that a more severe mucosal injury followed by reperfusion for 18 hours also allowed mucosal recovery, although to a lesser extent than in the present study. Although only 4 horses were used in the ischemic group in the present study, changes were identical to those reported for this duration of ischemia in tissues of the pelvic flexure of horses.

Selected components (NAC and MOTS) of a novel OPS solution were added to Ussing chambers to determine whether these components could improve recovery in vitro. These components were chosen rather than the OPS solution described elsewhere because, if effective, they could be administered systemically in a clinical setting at low cost. Some benefit has been proposed for combining arginine and glutamine, and inclusion of acetylcysteine is warranted on the basis of salutary effects in horses and rats with chemically induced mucosal injury. There was no evidence of any improvement in recovery from mucosal injury in vitro for the conditions of the present study, although the changes induced by this reversible ischemia method would have seemed appropriate to test such an effect.

The MOTS components were applied directly to the mucosal and serosal surfaces to improve access to epithelial and inflammatory cells in the mucosa. This design could only be expected to detect an effect on epithelial cells and on neutrophils already present in the tissues in vitro, without an opportunity to evaluate

Table 1—Mean ± SEM histomorphometric values for control (n = 4 horses), ischemic (6), and reperfused (6) colonic tissues after incubation with KRB, KRB-MOTS, or KRB-NAC in Ussing chambers for 4 hours.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Ischemic</th>
<th>Reperfused</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucosal height (µm)</td>
<td>401.59±13.74</td>
<td>390.15±16.74</td>
<td>406.61±22.87</td>
</tr>
<tr>
<td>Epithelial height (µm)</td>
<td>27.23±1.72</td>
<td>25.75±0.94</td>
<td>27.47±1.26</td>
</tr>
<tr>
<td>Epithelial width (µm)</td>
<td>4.84±0.20</td>
<td>4.96±0.12</td>
<td>4.86±0.13</td>
</tr>
<tr>
<td>Lifted epithelium (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Denuded epithelium (%)</td>
<td>0.02±0.02</td>
<td>0.05±0.05</td>
<td>0.13±0.13</td>
</tr>
<tr>
<td>Degenerated epithelium (%)</td>
<td>71.91±7.10</td>
<td>32.97±11.06</td>
<td>67.28±21.13</td>
</tr>
<tr>
<td>Restituted epithelium (%)</td>
<td>4.88±4.88</td>
<td>4.48±4.48</td>
<td>4.48±4.48</td>
</tr>
<tr>
<td>Necrotic or sloughed cells/0.1 mm</td>
<td>0.11±0.03</td>
<td>2.97±0.96</td>
<td>2.20±1.15</td>
</tr>
</tbody>
</table>

*Within a row, values with different superscript letters differ significantly (P < 0.05).

Table 2—Mean ± SEM histomorphometric values for control (n = 4 horses), ischemic (6), and reperfused (6) colonic tissues after incubation with KRB, KRB-MOTS, or KRB-NAC in Ussing chambers for 4 hours.

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</tr>
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<tbody>
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<td>Mucosal height (µm)**</td>
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*Within a row, values with different superscript letters differ significantly (P < 0.05). See Table 1 for remainder of key.
any putative antioxidant effect on neutrophil infiltration. Therefore, further investigation into the potential clinical use of these components on the inflammatory response in reperfused mucosa might be better conducted in vivo. The rapid recovery of barrier function during reperfusion, as measured on the basis of TER, can be attributed to increased expression of tight junction proteins (occludin and ZO-1) during mucosal recovery.36 Application of prostaglandin E2 to ischemia-injured ileal mucosa stimulates increases in TER intestinal epithelium.45 This amount of permeability could provide a paracellular pathway for nutrient absorption in jejunum epithelium. After injury,31 the difference of TER values throughout incubation in vitro than does the control jejunum.32 These findings can be explained by populations of tight junctions throughout the jejunum that are more permeable in control conditions but that become recruited into the intense and widespread process of tight junction closure after injury.31 The difference in expression of this process between jejunum and colon could be explained by the importance of a permeable paracellular pathway for nutrient absorption in jejunal epithelium.32 This amount of permeability could be lacking in the colon, which has a limited capacity to absorb nutrients24 and a greater need to have a tight epithelial barrier against noxious luminal contents.35

The lack of reperfusion injury in the present study was consistent with results of other studies46 in horses and raises concerns about the relevance of this process in the equine intestines.47 However, results reported for a low-flow method of ischemia in the equine colon14,48 revealed a marked influx of neutrophils associated with continued mucosal degeneration during reperfusion. In clinically affected horses with large colon volvulus, the relative roles of low-flow ischemia and complete ischemia are unknown, so the most suitable method for investigation of this disease has not been established. However, the nature of the lesions would suggest that almost complete cessation of blood flow, as was induced in the present study, should be the most likely vascular change.49

In equine colonic mucosa subjected to periods of ischemia similar to that used in the present study, but with shorter and longer intervals of reperfusion, rapid and intense neutrophil influx was evident throughout reperfusion.4 Although neutrophil influx was not measured in the present study, marked increases in neutrophil numbers were observed after reperfusion for 4 hours, a time point at which tissues had recovered from ischemic damage. For most methods of intestinal reperfusion injury, influx of neutrophils of such magnitude would be expected to exacerbate the ischemic injury.11,48 The role of neutrophils in reperfusion injury is further complicated by evidence that reperfusion-induced mucosal damage in equine jejunum can be independent of neutrophils.30

We concluded for the experimental methods of the study reported here that complete ischemia of equine colonic mucosa for 1 hour followed by reperfusion for 4 hours did not result in functional or morphological evidence of reperfusion injury. Therefore, in horses that undergo surgery for large colon volvulus, efforts to hasten mucosal recovery probably should have precedence over aggressive efforts to treat reperfusion injury.

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