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Effects of ischemia and reperfusion on production of nitrotyrosine, activation of eosinophils, and apoptosis in the large colonic mucosa of horses

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Objective—To assess the effects of ischemia and reperfusion on indicators of oxidative stress, activation of eosinophils, and apoptosis in the large colonic mucosa of horses.

Animals—40 horses.

Procedures—In 1 or two 20-cm-long segments of the pelvic flexure, ischemia was induced for 1 or 2 hours followed by no reperfusion or 30 minutes and 18 hours of reperfusion in anesthetized horses. Mucosal specimens were collected before (controls; n = 20 horses) and after each period of ischemia, and full-thickness tissue samples were collected after each period of reperfusion. Sections of colonic tissues were stained for histomorphometric analysis or assessment of eosinophil accumulation. Nitrotyrosine was identified immunohistochemically, and severity of apoptosis was determined via the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling method.

Results—Numbers of mucosal eosinophils were similar before induction of ischemia, after ischemia, and after ischemia-reperfusion. Eosinophil nitrotyrosine production increased significantly during ischemia and continued through 30 minutes of reperfusion; production was decreased at 18 hours of reperfusion but remained greater than that of the controls. In other leukocytes, nitrotyrosine generation peaked at 1 hour of ischemia and again at 18 hours of reperfusion. Compared with control findings, epithelial apoptosis increased gradually at 1 through 2 hours of ischemia with no further progression after reperfusion.

Conclusions and Clinical Relevance—Results suggested that resident eosinophils in the large colon of horses react to mucosal injury from ischemia and reperfusion and may undergo oxidative stress under those conditions. Epithelial apoptosis could contribute to tissue damage. (Am J Vet Res 2012;73:53–61)
they are abundant in the colon of healthy horses, can generate an array of destructive mediators and toxic proteins, and contribute to pathological changes in humans with asthma and other hyperesoinophilic diseases. Equipped with enzymes that may cause oxidative damage to biological targets, activated eosinophils can also induce the respiratory burst that generates reactive oxygen species and peroxynitrite. Increased intestinal accumulation of eosinophils in horses with experimentally induced acute colitis, ischemia-reperfusion injury, eosinophilic enteritides, or parasitism has been described.

Cell death as a result of necrosis and apoptosis is initiated by mediators released during ischemia and reperfusion. Cells that die as a result of necrosis release their intracellular contents and generate an intense inflammatory reaction. In contrast, apoptotic cell death is a controlled, energy-dependent event that regulates the physiologic cell turnover in tissues undergoing cell replication without stimulation of the immune system. Although apoptosis is essential for the maintenance of normal intestinal epithelial function, dysregulated apoptosis is associated with several pathological conditions in the gastrointestinal tract. Apoptosis could disrupt the intestinal barrier function, or it might control tissue damage, maintain a defensive barrier, reduce inflammation, and initiate repair.

Results of previous studies have indicated that reperfusion causes neutrophil infiltration into equine colonic mucosa and that the respiratory burst produced by neutrophils might be responsible for exacerbation of tissue damage after reoxygenation. However, we are not aware of studies to assess activation of eosinophils, oxidative stress, and cell death in the colon of horses over a range of periods of ischemia and reperfusion time. The purpose of the study reported here was to assess the effects of ischemia and reperfusion on indicators of oxidative stress, activation of eosinophils, and apoptosis in the large colonic mucosa of horses. To investigate the potential role of eosinophils, nitrotyrosine generation by those cells in the equine colon after colonic ischemia and reperfusion was evaluated. Our hypothesis was that reperfusion would induce inflammation, as indicated by the activity of eosinophils, and concurrently cause changes in oxidative stress and apoptosis patterns over the various periods of ischemia and reperfusion evaluated.

Materials and Methods

Animals—Forty horses of mixed breeds with a mean age of 9.2 years and mean body weight of 469 kg were used in the study. The horses were donated for research purposes and were free of gastrointestinal tract diseases. These horses were involved in several overlapping studies on ischemia and reperfusion that were performed with approval and under guidelines of the Institutional Animal Care and Use Committee of the University of Florida. Horses were fed grass hay (2% of body weight/d), and water was provided ad libitum. Horses were adapted to their diet and environment for at least 1 week before commencement of the study.

Study design—Horses were randomly assigned to undergo 1 of 5 conditions of colonic ischemia alone or colonic ischemia and reperfusion. In 13 horses, ischemia was induced in a 20-cm-long segment of pelvic flexure for 1 hour; tissue samples were collected immediately after the period of ischemia. In 6 horses, ischemia was induced in a 20-cm-long segment of pelvic flexure for 2 hours; tissue samples were collected before ischemia (control samples) and immediately after the 2-hour period of ischemia. In 6 horses, ischemia was induced for 2 hours in a 20-cm-long segment of pelvic flexure; tissue samples were collected before ischemia, immediately after a 1-hour period of ischemia, and immediately after a second 1-hour period of ischemia. In 6 horses, ischemia was induced for 1 or 2 hours in each of two 20-cm-long segments of pelvic flexure, after which reperfusion was allowed for 30 minutes; tissue samples were collected from both segments before ischemia, from 1 segment immediately after the 1-hour period of ischemia and again after the following 30-minute period of reperfusion, and from the other segment immediately after the 2-hour period of ischemia and again after the subsequent 30-minute period of reperfusion.

Anesthesia and monitoring—A 14-gauge, 13.3-cm-long polytetrafluoroethylene catheter was inserted into the left jugular vein of each horse for administration of anesthetic drugs and isotonic fluids. Xylazine hydrochloride (0.3 mg/kg), butorphanol tartrate (0.02 mg/kg), or a combination of those drugs was administered IV to provide sedation, and then anesthesia was induced via IV administration of diazepam (0.02 mg/kg) to effect followed by a bolus IV injection of ketamine hydrochloride (2.0 mg/kg). Anesthesia was maintained with isoflurane (1% to 2%) in 100% oxygen. Each horse was mechanically ventilated at a rate of 6 breaths/min. Isotonic polyionic fluids were infused continuously (5 to 10 mL/kg/h, IV). Mean arterial blood pressure was monitored through a 20-gauge, 5.1-cm-long polytetrafluoroethylene catheter placed in a facial artery and was maintained at ≥ 70 mm Hg. Other monitoring tools used during anesthesia included ECG, blood gas analysis, capnography, and pulse oximetry.

Surgical procedures—Each horse was positioned in dorsal recumbency and aseptically prepared for a ventral midline celiotomy. The large colon was extirpated and placed on a plastic drape on the ventral aspect of the abdomen. To induce ischemia, a 20-cm-long segment of the medial part of the pelvic flexure...
was selected. Transmural compression was achieved by placement of intestinal clamps at each end of the selected segment, and combined venous and arterial occlusion was achieved with umbilical tape ligatures. In 6 horses, 2 pelvic flexure segments approximately 20 cm apart were selected to undergo ischemia for 1 or 2 hours, after which reperfusion was allowed for 30 minutes. In these horses, a 2-hour period of ischemia was commenced in 1 of the 2 pelvic flexure segments. One hour after ischemia was started in the first segment, a 1-hour period of ischemia was commenced in the other pelvic flexure segment. In this manner, reperfusion (30 minutes) of both segments was instituted at the same time and full-thickness samples of tissues could be obtained from both segments simultaneously.

After induction of ischemia, the colon was replaced in the abdomen and the abdominal incision was closed temporarily with suture. At the end of the predetermined period of ischemia in horses for which tissue reperfusion was not planned, the colon was exteriorized and full-thickness colon tissue samples from the ischemic-injured and adjacent nonischemic colon were harvested. At the end of the predetermined period of ischemia in horses for which tissue reperfusion was planned, the intestinal clamps were removed and the colon was replaced in the abdomen. During reperfusion of pelvic flexure segments for 30 minutes, each horse remained anesthetized. To facilitate reperfusion of pelvic flexure segments for 18 hours, the horse’s abdomen was closed in a routine manner and the horse was allowed to recover from anesthesia. Each horse undergoing reperfusion for 18 hours received butorphanol (0.05 mg/kg, IV) as a supplement. At the end of the predetermined period of ischemia, the horse’s abdomen was closed in a routine manner and the horse was allowed to recover from anesthesia.

Histologic examinations—All mucosal biopsy specimens and full-thickness tissue samples were fixed in neutral-buffered 10% formalin for 36 hours, subsequently embedded in paraffin, and cut into 4- to 5-µm-thick sections. Adjacent sections were mounted on several silane-coated glass slides. After deparaffinization and rehydration, slides were stained with H&E and Luna stains in a routine manner for histomorphometric evaluation and assessment of the accumulation of eosinophils within the colonic mucosa. For evaluation of the images obtained by use of light microscopy, a computer-based image analysis program was used.

Histomorphometric examinations included determination of the ICR and evaluation of the severity of mucosal hemorrhage. By use of the computer-based image analysis program, the widths of 10 randomly selected crypts and the distances of the lamina propria between these crypts were measured by use of a 10X objective lens. The ICR was calculated as the ratio of the lamina propria occupied by the interstitium, compared with the width of the crypts. The ICR for healthy equine colonic mucosa (ie, the value in colons without ischemia-reperfusion injury) is defined as ≤ 1.41 The degree of erythrocyte accumulation within the mucosa was classified on the basis of the distribution and severity of interstitial hemorrhage by use of a scoring system from 0 to 3. A score of 0 was assigned when no erythrocytes were observed within the lamina propria. Scores from 1 to 3 indicated focal and mild, multifocal and moderate, or diffuse and extensive hemorrhage within the tissue, respectively.

The number of eosinophils was determined during examination of Luna-stained tissues. Three fields of view within each section from each tissue sample were randomly selected; each selected field (length, 866 µm [equal to the length of 1 image obtained by use of the 10X objective lens]; full height of the mucosa) was photographed. The number of eosinophils within each region of the mucosa was counted with the aid of the computer-based image analysis program, and the mean number of eosinophils per square millimeter of mucosal area was calculated. Based on previous evidence that eosinophils can migrate from the lower lamina propria to the lumen of the colon, independent of vessels, a system was used to detect such migration. Specifically, in each photographed region, mucosal eosinophils were counted in each of 5 mucosal zones that cumulatively spanned the full height of the mucosa from the muscularis mucosa to the surface epithelium to detect population changes in zones that could indicate migration. Four lines were drawn at intervals of one-fourth of the mean distance from the muscularis mucosa to the luminal surface (delineating zones M1 up through M4); the surface of the epithelial cells was designated as zone M5. The number of eosinophils within each mucosal zone was counted, and the mean number of eosinophils per square millimeter of mucosal area of 3 fields of view was used for further statistical analysis.

Immunohistochemical analysis and TUNEL staining—After tissue sections were deparaffinized and rehydrated, nitrotyrosine was detected by use of 1:30 monoclonal mouse anti-human nitrotyrosine antibody and a commercially available avidin-biotin-complex detection kit with horseradish peroxidase–3-amin-9-ethylcarbazole as the chromogen. The detection of...
Apoptotic cells was determined on the basis of a TUNEL method, which was provided in a commercially available apoptosis detection kit with 3,3′-diaminobenzidine as the chromogen, according to a protocol of Rowe et al. apoptotic bodies identified in H&E-stained tissue sections with a 40X objective lens.

For antigen retrieval, all tissue samples underwent heat pretreatment by use of a pressure cooker (125°C for 30 seconds and 90°C for 10 seconds) and a retrieval buffer (pH, 6.0). After cooling and washing with Dulbecco PBS solution, sections were processed according to manufacturer instructions. When the desired stain intensity developed with 3,3′-diaminobenzidine as the chromogen, counterstained with Mayer's hematoxylin stain in a routine manner before processing for mounting. Instead of primary antibodies (nitrotyrosine) or terminal deoxynucleotidyl transferase enzyme (apoptosis), only Dulbecco PBS solution was added to experimental samples as negative controls. Tissue sections were examined via light microscope by use of a 40X objective lens and the image analysis program. A positive reaction was evidenced by cells that appeared red (nitrotyrosine) or had brown nuclei (apoptotic cells).

The number of nitrotyrosine-containing eosinophils (characterized by their prominent intracytoplasmic granules) and the number of other stained leukocytes (neutrophils, macrophages, and lymphocytes) were counted manually. These cells were identified by their shape, size, and location but could not be distinguished from each other because the staining pattern obscured identifying features. Nitrotyrosine-positive cells were counted in 3 randomly selected fields of view of 2 mucosal areas (217.5 × 162.5 μm [equal to the size of 1 image obtained by use of the 40X objective lens]). One such area was adjacent to the muscularis mucosa (lower lamina propria), and another was adjacent to the epithelium (upper lamina propria). Nitrotyrosine-containing eosinophils and other leukocytes in 1 submucosal area (217.5 × 162.5 μm) adjacent to muscularis mucosa were also counted in triplicate. The mean number of nitrotyrosine-positive cells per mucosal area in 3 fields of view was used for further statistical analysis.

The severity of apoptosis was determined (number of apoptotic cells/mm² of mucosa) as described for assessment of eosinophil accumulation and as the apoptotic index. The apoptotic index was defined as the number of apoptotic cells/1,000 epithelial cells, which were counted manually with the 40X objective lens. Histomorphometric examinations, quantification of eosinophils, and detection of nitrotyrosine- and TUNEL-positive cells within the tissues were performed in a blinded manner by 1 investigator (AG).

Statistical analysis—Data are expressed as means ± SEM. A statistical software program was used for analyses. Values of P < 0.05 were considered significant. The Kruskal-Wallis test was performed to compare the nonparametric data during the ischemia and reperfusion periods and between experimentally treated tissues and controls. Whenever a significant P value for ischemia and reperfusion was identified, the Mann-Whitney U test was used for pairwise comparison. To assess the correlation among eosinophils, nitrotyrosine generation, and apoptosis on tissue dam-

Figure 1—Mean ± SEM ICR (A) and mucosal hemorrhage score (B) in colonic tissue samples obtained from 40 anesthetized horses that underwent 1 of 5 conditions of colonic ischemia alone or colonic ischemia and reperfusion. In 1 or 2 pelvic flexure segments in each horse, ischemia was induced for 1 or 2 hours followed by no reperfusion (1h [25 horses] and 2h [24 horses], respectively) or 30 minutes or 18 hours of reperfusion (1h+30minR [6 horses], 2h+30minR [8 horses], and 2h+18hR [7 horses], respectively). One mucosal specimen was collected before (controls; n = 20 horses only) and after each period of ischemia, and 1 full-thickness tissue sample was collected after each period of reperfusion. Sections of the tissue samples were examined microscopically in 3 randomly chosen fields of view. To calculate the ICR, the widths of 10 randomly selected crypts and the distances of the lamina propria between these crypts were measured by use of a 10X objective lens; the ICR was calculated as the ratio of the lamina propria occupied by the interstitium, compared with the width of the crypts. The severity of mucosal interstitial hemorrhage was scored on a scale from 0 to 3 representing no hemorrhage, focal and mild, multifocal and moderate, or diffuse and extensive hemorrhage within the tissue, respectively. Different letters above the bars represent significant (P < 0.05) differences between conditions.
Figure 2—Mean ± SEM number of eosinophils per square millimeter of colonic mucosa and the number of eosinophils in each of 5 mucosal zones in tissue samples collected from the horses in Figure 1 that underwent 1 of 5 conditions of colonic ischemia alone or colonic ischemia and reperfusion. As illustrated in the photomicrograph, the distribution of eosinophils was determined in 5 mucosal zones that cumulatively spanned the full height of the mucosa from the muscularis mucosa to the surface epithelium. Four lines were drawn at intervals of one-fourth of the mean distance from the muscularis mucosa to the luminal surface (delineating zones M1 up through M4); the surface of the epithelial cells was designated as zone M5. Eosinophils were counted in each zone in 2 fields of view (each 866 µm in length) of each tissue sample, and the mean number of eosinophils per square millimeter of mucosa was calculated. Luna stain; bar = 100 µm.

<table>
<thead>
<tr>
<th>No. of cells/ mm² of mucosa (SEM)</th>
<th>Control</th>
<th>1h</th>
<th>2h</th>
<th>1h+30minR</th>
<th>2h+30minR</th>
<th>2h+18hrR</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>645.4 ± 63.9</td>
<td>520.5 ± 45.5</td>
<td>494.0 ± 45.9</td>
<td>610.4 ± 91.9</td>
<td>482.6 ± 55.6</td>
<td>370.5 ± 55.6</td>
</tr>
</tbody>
</table>

Figure 3—Mean number of nitrotyrosine-positive eosinophils (A) and other leukocytes (C) within the submucosa (SM), lower lamina propria (LLP), and upper lamina propria (ULP) in sections of tissue samples collected from the horses in Figure 1 that underwent 1 of 5 conditions of colonic ischemia alone or colonic ischemia and reperfusion and representative photomicrographs of a negative control section (B) and a section in which nitrotyrosine-positive mucosal eosinophils are present (D). Sections were processed by use of an avidin-biotin-complex detection system with 3-amino-9-ethylcarbazole. Mayer’s hematoxylin counterstain; bar = 20 µm.

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age, the Spearman rank correlation coefficient (R) was calculated.

**Results**

In 13 horses, ischemia was induced in a pelvic flexure segment for 1 hour, after which tissue samples were collected. Mean age of these horses was 8.2 years (age range, 1 to 19 years), and mean weight was 467.7 kg (weight range, 350 to 530 kg); there was 1 stallion, 6 mares, and 6 geldings. In 6 horses, ischemia was induced in a pelvic flexure segment for 2 hours, and tissue samples were collected before and immediately after the 2-hour period of ischemia. Mean age of these horses was 8.3 years (age range, 2 to 20 years), and mean weight was 479.2 kg (weight range, 390 to 605 kg); there was 1 stallion, 3 mares, and 2 geldings. In 6 horses, ischemia was induced for 2 hours in a pelvic flexure segment, and tissue samples were collected before ischemia, immediately after a 1-hour period of ischemia, and immediately after a second 1-hour period of ischemia. Mean age of these horses was 9.2 years (age range, 1 to 20 years), and mean weight was 486.7 kg (weight range, 445 to 520 kg); there was 1 stallion, 3 mares, and 2 geldings. In 6 horses, ischemia was induced for 1 or 2 hours in each of two 20-cm-long segments of pelvic flexure, after which reperfusion was allowed for 30 minutes; tissue samples were collected from both segments before ischemia, from 1 segment immediately after the 1-hour period of ischemia and again after the following 30-minute period of reperfusion, and from the other segment immediately after the 2-hour period of ischemia and again after the subsequent 30-minute period of reperfusion. Mean age of these horses was 6.3 years (age range, 2 to 18 years), and mean weight was 438.3 kg (weight range, 300 to 545 kg); there were 2 stallions, 3 mares, and 2 geldings. In 2 horses, ischemia was induced in a pelvic flexure segment for 2 hours, after which reperfusion was allowed for 30 minutes; tissue samples were collected before ischemia, immediately after the 2-hour period of ischemia, and again after the subsequent 30-minute period of reperfusion. These horses were a 21-year-old 500-kg mare and a 32-year-old 500-kg gelding. In 4 horses, ischemia was induced in a pelvic flexure segment for 2 hours, after which reperfusion was allowed for 18 hours; tissue samples were collected immediately after the 2-hour period of ischemia and again after the subsequent 18-hour period of reperfusion. Mean age of these horses was 8.0 years (age range, 2 to 15 years), and mean weight was 478.8 kg (weight range, 420 to 513 kg); there was 1 stallion, 1 mare, and 3 geldings. In 3 horses, ischemia was induced in a pelvic flexure segment for 2 hours, after which reperfusion was allowed for 18 hours; tissue samples were collected immediately after the 18-hour period of postischemia reperfusion. Mean age of these horses was 11.0 years (age range, 6 to 20 years), and mean weight was 476.7 kg (weight range, 390 to 330 kg); there was 1 mare and 2 geldings. For all horses, mean age was 9.2 years and mean weight was 468.75 kg.

Compared with control findings, the ICR and the mucosal hemorrhage score increased gradually and significantly as the period of ischemia increased (Figure 1). There was no further significant change in either variable after reperfusion. The overall number of mucosal eosinophils per square millimeter of mucosa appeared to generally decrease as a result of ischemia and reperfusion, compared with the control tissue value, but the changes were not significant (Figure 2). After 1- and 2-hour periods of ischemia, nitrotyrosine production by eosinophils had significantly increased from the control value; however, the longer period of ischemia did not result in further increase in production. After either period of ischemia, production was maintained through 30 minutes of reperfusion, although the value declined significantly after 18 hours of reperfusion in samples that had previously undergone 2 hours of ischemia (Figure 3). In contrast, ischemia and reperfusion resulted in increased nitrotyrosine production by mucosal and submucosal leukocytes, compared with the control value. A substantial increase in production was evident after 1 hour of ischemia, which decreased somewhat as the duration of ischemia increased. The 30-minute period of reperfusion after 1 or 2 hours of ischemia had little effect on nitrotyrosine production by mucosal and submucosal leukocytes, but there was a significant peak in production after 18 hours of reperfusion in samples that had previously undergone 2 hours of ischemia.

Changes in the extent of apoptosis among mucosal epithelial cells were detected as a result of ischemia.
reperfusion injury (Figure 4). Apoptosis (as indicated by the number of apoptotic cells/mm² of mucosa) increased significantly from the control findings during ischemia, although the value after 2 hours of ischemia did not differ significantly from that after 1 hour of ischemia (Table 1). A peak value was detected in samples that underwent 1 hour of ischemia followed by 30 minutes of reperfusion. With regard to the apoptotic index, ischemia resulted in significantly more apoptotic epithelial cells, compared with the control value; the index increased as the duration of ischemia increased. Following either period of ischemia, the index remained unchanged at the end of the 30-minute period of reperfusion; similarly, following the 2-hour period of ischemia, the index remained unchanged at the end of the 18-hour period of reperfusion.

Table 1—Mean ± SEM apoptotic index (apoptotic epithelial cells and number of apoptotic cells per square millimeter of mucosa in colonic tissue samples obtained from 40 anesthetized horses that underwent 1 of 5 conditions of colonic ischemia alone or colonic ischemia and reperfusion.

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>No. of apoptotic cells/mm² of mucosa</th>
<th>Apoptotic index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>545.4 ± 68.8</td>
<td>98.2 ± 13.1</td>
</tr>
<tr>
<td>1h</td>
<td>905.4 ± 97.3</td>
<td>199.9 ± 18.5</td>
</tr>
<tr>
<td>2h</td>
<td>1,139.9 ± 135.5</td>
<td>286.4 ± 23.4</td>
</tr>
<tr>
<td>1h+30minR</td>
<td>2,084.2 ± 480.1</td>
<td>192.9 ± 19.1</td>
</tr>
<tr>
<td>2h+30minR</td>
<td>1,719.1 ± 331.0</td>
<td>339.4 ± 58.3</td>
</tr>
<tr>
<td>2h+18R</td>
<td>953.8 ± 102.6</td>
<td>270.0 ± 33.7</td>
</tr>
</tbody>
</table>

Note: Within a variable, different superscripted letters represent significant (P < 0.05) differences between conditions.

Discussion

Results of the present study indicated that oxidative stress is involved in the inflammatory process initiated in equine colon tissues during ischemia and reoxygenation, as evidenced by increased nitrotyrosine generation. This response was localized in resident eosinophils and other mucosal leukocytes. Nitrotyrosine is considered to be a potential marker of peroxynitrite generation in tissues, although it is also generated by other molecules such as nitrate, nitric oxide, nitrogen dioxide, or nitryl chloride. Therefore, detection of nitrotyrosine should be regarded as evidence of generated reactive nitrogen species rather than as a specific marker of peroxynitrite.

In the colonic tissue samples assessed in the present study, eosinophils and other leukocytes (neutrophils, macrophages, and lymphocytes) that accumulated within the mucosa and submucosa represented the main sources of nitrotyrosine; therefore, they could have a role in the pathogenesis of ischemia-reperfusion injury in the colon of horses. Eosinophils are multifunctional proinflammatory leukocytes involved in numerous inflammatory processes. Armed with toxic mediators, including granule proteins, oxygen metabolites, lipid mediators, and proteases, eosinophils can contribute to intestinal epithelial dysfunction. Eosinophils are involved in tyrosine nitration as well as the formation of peroxynitrite and peroxidase-catalyzed reactive nitrogen species, which are capable of inflicting cell damage. However, the biological role of eosinophils in equine colonic mucosa during ischemia and reperfusion is unknown.

Whereas eosinophils accumulate within the gastrointestinal tract of humans with gastrointestinal disorders, eosinophilic granulocytes are resident in the gastrointestinal lamina propria in healthy horses. Most of the eosinophils in the colon of horses are found near the muscularis mucosae and are rarely located close to the surface of the mucosa, consistent with results of the present study. The number of eosinophils per square millimeter of mucosa did not change during 18 hours of reperfusion, suggesting that eosinophil adhesion molecules might not be activated during a period of reperfusion of that duration and that resident eosinophils in equine colonic mucosa only respond to the ischemic insult. These results indicated that resident eosinophils in the colon of horses could contribute to the inflammatory reaction initiated in the early stage of ischemia-reperfusion injury.

It has been reported that nitrotyrosine staining in mucosal leukocytes of horses with naturally acquired small intestinal strangulation obstructions is increased, compared with findings in horses without gastrointestinal tract diseases, which could reflect the presence of peroxynitrite subsequent to increased nitric oxide and superoxide production. A possible cytotoxic role of nitric oxide in small intestinal strangulation obstructions was proposed. Administration of peroxynitrite into the colon of rats resulted in widespread injury and inflammation that are similar to findings in rats with inflammatory bowel disease, which provides evidence that peroxynitrite could initiate intestinal inflammation and tissue damage. Although peroxynitrite has limited extracellular stability and diffusion range, it could be cytotoxic toward invading pathogens and act as a potent microbialid compound. The second peak production of nitrotyrosine by submucosal leukocytes after 18 hours of reperfusion in the present study could be a defense mechanism against transmucosal passage of bacteria or bacterial products during this stage of reperfusion.

In a previous study performed by our group, the number of neutrophils in equine colonic mucosa increased during ischemia and reperfusion in a time-dependent manner starting at 2 hours of ischemia and progressing after reperfusion. Accumulation of neutro-
phils within the mucosa was significantly correlated with mucosal damage, consistent with the role of the neutrophil influx in reperfusion injury in the colon of horses. The peak tissue accumulation of neutrophils in that study was at 18 hours after ischemia, when repair of damaged tissue commenced. Thus, neutrophil influx into the tissue and production of reactive nitrogen species at that time could be possible reactions of the innate immune system as a defense against invading pathogens crossing the damaged epithelial barrier.

Although other studies have revealed that cellular necrosis is the principal effect of ischemia on epithelial cells, the results of the present study support the hypothesis that apoptosis could contribute to cell death during ischemia and reperfusion in equine colonic mucosa. Recently, it has been shown that ischemic-injured intestinal tissue responds by undergoing programmed cell death rather than necrosis and the severity of apoptosis depends on the differentiation stage of the enterocytes. In the present study, apoptotic cell death started during the first hour of ischemia in the epithelium, in the lamina propria and crypt cells, with further progression after 2 hours of ischemia in the epithelium and after 1 hour of ischemia followed by 30 minutes of reperfusion in the mucosa. Although studies have revealed an activation of apoptosis during intestinal ischemia with further exacerbation during reperfusion, the role of programmed cell death in tissue damage and dysfunction remains controversial. Failure to eliminate unnecessary and damaged cells by apoptosis will prolong inflammation through continued release of toxic agents, and repair after tissue damage requires the elimination of proliferative mesenchymal and inflammatory cells from the sites of injury. On the other hand, pronounced apoptosis can contribute to leaks in the epithelial barrier and to permeability defects of the bowel, which could be accompanied by proteolytic cleavage of tight junction proteins.

In the present study, ischemia and reperfusion in the colon of horses caused activation of eosinophils and other leukocytes, and production of nitrogen radicals by these cells might be involved in the inflammatory process. Additionally, apoptosis in the injured colonic mucosa appeared to be a prominent cause of cell death during ischemia and reperfusion. Although the role of eosinophils or apoptosis in injury or repair remains to be elucidated in future studies, results of the present study indicated that resident eosinophils in equine colonic tissues are subjected to oxidative stress in the early stage of ischemia and reperfusion, thereby favoring their contribution to this and other disease processes in the colon of horses.

References

6. Beckman JS, Beckman TW, Chen J, et al. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci U S A 1990;87:1620–1624.