Effect of large colon ischemia and reperfusion on concentrations of calprotectin and other clinicopathologic variables in jugular and colonic venous blood in horses

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Objective—To determine the effect of large colon ischemia and reperfusion on concentrations of the inflammatory neutrophilic protein calprotectin and other clinicopathologic variables in jugular and colonic venous blood in horses.

Animals—6 healthy horses.

Procedures—Horses were anesthetized, and ischemia was induced for 1 hour followed by 4 hours of reperfusion in a segment of the pelvic flexure of the large colon. Blood samples were obtained before anesthesia, before induction of ischemia, 1 hour after the start of ischemia, and 1, 2, and 4 hours after the start of reperfusion from jugular veins and veins of the segment of the large colon that underwent ischemia and reperfusion. A sandwich ELISA was developed for detection of equine calprotectin. Serum calprotectin concentrations and values of blood gas, hematologic, and biochemical analysis variables were determined.

Results—Large colon ischemia caused metabolic acidosis, a significant increase in lactate and potassium concentrations and creatine kinase activities, and a nonsignificant decrease in glucose concentrations in colonic venous blood samples. Values of these variables after reperfusion were similar to values before ischemia. Ischemia and reperfusion induced activation of an inflammatory response characterized by an increase in neutrophil cell turnover rate in jugular and colonic venous blood samples and calprotectin concentrations in colonic venous blood samples.

Conclusions and Clinical Relevance—Results of this study suggested that large colon ischemia and reperfusion caused local and systemic inflammation in horses. Serum calprotectin concentration may be useful as a marker of this inflammatory response. (Am J Vet Res 2013;74:1281–1290)

S trangulation obstruction of the large colon is one of the most severe types of colic in horses; that problem is typically associated with a high mortality rate attributable to progressive ischemic damage to the intestinal wall, dysfunction of the intestinal epithelial barrier, and septic shock.\textsuperscript{1,2} Intestinal epithelial barrier dysfunction during ischemia is caused by various biochemical, metabolic, and ultrastructural changes of epithelial cells that result in cell death.\textsuperscript{3,4} Consequently, local innate immune cells such as macrophages, neutrophils, eosinophils, and mast cells are activated and release proinflammatory mediators, proteins that cause toxic effects, and radicals.\textsuperscript{3} During reperfusion, additional reactive metabolites are generated that can further intensify the inflammatory response and exacerbate mucosal damage.\textsuperscript{3,6} Because of passage of bacterial toxins and inflammatory mediators through the compromised epithelial and endothelial barriers, intestinal inflammation attributable to IR is often accompanied by a systemic inflammatory response resulting in multiple organ failure.\textsuperscript{7,8} This acute response of the immune system is characterized by systemic activation of neutrophils and other leukocytes and by the generation and release of various inflammatory, regulatory, and metabolic proteins and enzymes into the circulation.\textsuperscript{9,10} Release of cell contents and stimulation of various activities of target cells and

<table>
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<th>ABBREVIATIONS</th>
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organs cause hematologic and biochemical alterations in the blood of affected humans and animals.

Results of biochemical and hematologic assays of blood samples are typically used to assess disease severity after intestinal IR.11–15 Few studies have been conducted to determine local hematologic and biochemical alterations in colonic venous blood of horses with IR.16–19 Other investigators6 found that horses with colon IR have changes in colonic venous blood oxygenation and lactate and pyruvate concentrations but no alterations in values for systemic venous blood samples.

In other studies,16–19 neutrophilic proinflammatory molecules and enzymes (such as lactoferrin, neutrophil elastase, lysozyme, MPO, and calprotectin) were assayed to evaluate the activation status of neutrophils during intestinal IR and inflammation. Calprotectin is a proinflammatory protein that is a sensitive marker of acute and chronic inflammatory conditions,19–22 but it also has anti-inflammatory and protective functions including oxidant scavenging, antimicrobial activity, stimulation of tissue repair, and regulation of the anti-inflammatory response of macrophages.23 Calprotectin is a cytoplasmic protein mainly produced in neutrophils, but it is also found in circulating monocytes, keratinocytes, osteoclasts, and chondrocytes. Calprotectin is typically released after cell disruption and death.24,25 Because calprotectin expression in monocytes decreases during extravasation from blood and differentiation in tissues, resting macrophages in tissues do not generate calprotectin.26 Calprotectin can be measured by means of radioimmunoassay or ELISA.28–31 Mucosal calprotectin expression is associated with neutrophil infiltration and an inflammatory response in equine large colon tissues during IR.26 Thus, quantification of neutrophil activity by evaluation of calprotectin concentrations in local and systemic venous blood could be a useful method of assessing colonic inflammation in an experimental model of IR in horses.

The objective of the study reported here was to develop an ELISA for detection of equine calprotectin and to measure hematologic and biochemical variables and calprotectin concentrations in jugular and colonic venous blood samples after colon IR in horses. We hypothesized that short-term colon IR in horses would cause detectable alterations in various variables in colonic venous blood samples, and that activation of a local inflammatory response in colonic mucosa during IR would be associated with increased concentrations of calprotectin and changes in other markers of inflammation and cell damage in such blood samples.

Materials and Methods

Animals—Six horses of various breeds with a median age of 14 years and a median body weight of 535 kg were used in this study. The horses were donated for research purposes and were free of gastrointestinal tract diseases as determined by means of thorough clinical examinations performed at the beginning of the study. The study was performed with approval and under guidelines of the University of Florida Institutional Animal Care and Use Committee. Horses were fed grass hay (amount, 2% of body weight/d), and water was provided ad libitum. Horses were routinely dewormed and vaccinated and were allowed to adapt to the diet and environment for at least 1 week before the start of the study.

Experimental procedures—A 14-gauge, 13.3-cm polyethylene catheter was inserted into the left and right jugular veins of each horse for administration of anesthetic drugs and isotonic fluids and collection of venous blood samples. Horses were sedated with xylazine (0.3 mg/kg), butorphanol (0.02 mg/kg), or a combination of both drugs IV. Anesthesia was induced with diazepam to effect (approx 0.02 mg/kg) followed by ketamine (2.0 mg/kg; administered as a bolus) IV. Anesthesia was maintained with isoflurane (1% to 2%) in 100% oxygen. Horses were mechanically ventilated (6 breaths/min). Horses were positioned in dorsal recumbency and aseptically prepared for surgery, and a ventral midline celiotomy was performed as described in another report.32 The large colon was exteriorized and placed on a plastic drape on the ventral aspect of the abdomen. To induce ischemia, a 40-cm segment of colon at the pelvic flexure was subjected to transmural compression by use of intestinal clamps placed at each end of the selected segment, 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 of comparable size (dorsal and ventral) and associated vasculature 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 exteriorized and 1 of the 2 ischemic segments (determined with a randomization procedure) was resected for histologic evaluations and in vitro experiments for another study.32 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 this period). Blood samples were collected from jugular veins through catheters before induction of anesthesia (preanesthetic control samples), before ischemia (preischemic control samples), 1 hour after the start of ischemia (after 1 hour of ischemia), and 1, 2, and 4 hours after the start of reperfusion (after 1, 2, and 4 hours of reperfusion). Colonic blood samples were collected before ischemia, after 1 hour of ischemia, and 1, 2, and 4 hours of reperfusion; samples were alternately collected from the left dorsal and ventral colonic veins (these vessels provided venous drainage from the ischemia-injured part of the pelvic flexure). For collection of colonic venous blood samples, a 22-gauge needle was inserted into the venous branch of the colonic vein on the wall of the ischemic colonic segment. Heparinized blood samples were analyzed immediately after collection to determine values of blood gas variables and hemoglobin, PCV, I-lactate, glucose, sodium, Ca++, and potassium concentrations. In addition, a blood smear was prepared and examined microscopically for differentiation of leukocytes. Blood samples were kept on ice for <1 hour and then centrifuged for 10 minutes at 2,000 × g, serum was collected,
and samples were stored at –80°C until further analyses. After collection of tissue and blood samples, horses were euthanized with an overdose of sodium pentobarbital (88 mg/kg, IV) while they were anesthetized. The same investigator (DEF) performed all surgeries and collected all colonic venous blood samples.

**Calprotectin ELISA**—For quantification of calprotectin in equine serum samples, a noncompetitive sandwich ELISA was developed on the basis of a procedure used for analysis of calprotectin in human serum, plasma, and urine samples. The assay was designed by use of 2 commercially available monoclonal anti-human antibodies with cross-reactivity to equine calprotectin subunits A100A8 and A500A9, which were capture and detection antibodies, respectively. Human recombinant calprotectin subunits S100A8 and S100A9 were used as standard samples for ELISA.

**Development and validation of calprotectin ELISA**—The ELISA was performed at room temperature (23°C to 24°C) in 96-well high protein-binding capacity polystyrene ELISA plates. The coating antibody (mouse anti-human monoclonal IgG1 [clone 3h2617]) was determined to have cross-reactivity with equine calprotectin (verified for equine serum samples by means of western blot analysis). The detection antibody was a customized biotinylated mouse anti-human monoclonal IgG1 (clone MAC387) that was used in another study to detect mucosal calprotectin in equine large colonic tissues by means of immunohistochemical analysis. Streptavidin–horseradish peroxidase and tetramethylbenzidine peroxidase substrate were used as detection indicators that produced a blue-colored product. The plates were analyzed with a microplate reader operating at 650 nm and a microplate reader software program. The human recombinant large (13.2 kDa; S100A8) and small (10.8 kDa; S100A9) calprotectin subunits were used as calprotectin standards for the assay.

Optimal concentrations of coating and detection antibodies and assay standards were determined by means of antibody titration grid experiments with signal-to-noise ratios > 5 and background values < 0.2 OD. An optimal standard curve was determined for 250, 125, 62.5, 31.2, 15.6, 7.8, and 3.9 ng/mL of equal aliquots of S100A8 and S100A9 subunits that were pre-incubated in 1 mmol of CaCl2 solution at room temperature before further dilution (1:15 dilution in blocking buffer). Results of another study indicate that incubation of both calprotectin subunits with CaCl2 increases the assay signal intensity.

A venous blood sample collected from the dorsal venous arch of the left hand of 1 healthy person (AG) was used as a positive control sample, and background values were determined with blocking buffer (dilution, 1:15) only. Positive control and equine serum samples were centrifuged for 10 minutes at 2,000 × g and 4°C, and supernatant was collected and diluted in blocking buffer (dilution for positive control samples, 1:100 and 1:150; dilution for equine serum samples, 1:20, 1:150, 1:200, and 1:300 [depending on calprotectin concentrations]) immediately before addition to assay wells. Performance of the ELISA had been assessed by means of evaluation of the precision and reproducibility (intra- and interassay variabilities, respectively), sensitivity (minimum detectable concentration = zero standard concentration [B0] + 3 SD; n = 20), and linearity of the assay.

**ELISA protocol**—The ELISA plates were incubated with 100 µL of a coating antibody (1.0 µg/mL diluted in 10 mM PBS solution [pH 7.4])/well overnight at 4°C. After coating the wells with the antibody, plates were allowed to warm at room temperature for 15 minutes and washed 4 times with a commercially available wash solution (300 µL/well) and then residual liquid in wells was emptied by tapping the contents out. Plates were then blocked with a commercially available blocking solution containing bovine serum albumin (300 µL/well) for 1 hour on a plate shaker. Wells were emptied and tapped out; 100 µL of standard samples, equine serum samples, and positive control samples diluted in blocking solution were added in triplicate to wells; and plates were incubated for 1 hour on a shaker. After washing the plates 4 times, 100 µL of biotinylated detection antibody (0.1 µg/mL [diluted in 1:15-diluted blocking solution]) was added to each well and incubated for 1 hour on a plate shaker. Unbound detection antibody was removed with another wash step, and the plate was incubated with streptavidin–horseradish peroxidase diluted in blocking solution (1:10,000 dilution; 100 µL/well) for 1 hour on a plate shaker. After washing, tetramethylbenzidine peroxidase substrate (100 µL) was added to each well. Color development was allowed for 40 minutes in the dark on a plate shaker, and then OD was determined with the microplate reader at 650 nm. Concentrations of calprotectin were determined with standard curves by use of the microplate reader software program. Calprotectin concentrations were expressed as nanograms per milliliter of serum. Because of the high variation in absolute serum calprotectin concentrations among horses of the study, calprotectin values were also expressed as a percentage relative to the preschismic control sample for each horse.

**Blood gas, hematologic, and serum biochemical analyses**—The jugular and colonic venous blood sample values for blood gas (pH and Pco2), hematologic (PCV and hemoglobin concentration), and biochemical (sodium, potassium, Ca2+, glucose, and lactate concentrations) analyses were determined immediately after sample collection. Values for bicarbonate concentration and base excess were calculated with plasma pH and Pco2 values by use of the Henderson-Hasselbalch equation. Because Pco2 values in colonic venous blood samples collected after 1 hour of ischemia were higher than the upper measurable limit of the analyzer (115 mm Hg) for some horses, a value of 115 mm Hg was used for calculations for such horses. Leukocyte differentiation (determination of percentages of lymphocytes, segmented neutrophils, band [nonsegmented] neutrophils, necrotic [ie, dead] neutrophils, eosinophils, basophils, and monocytes) for jugular and colonic venous blood samples was performed with Wright-Giemsastained blood smears with light microscopy by use of a 40X objective. Values of other serum biochemical analysis variables (TP, albumin, and...
creatinine concentrations and alkaline phosphatase and CK activities) in serum harvested from jugular and colonic venous blood samples were determined with an analyzer.

Statistical analysis—Statistical tests were performed with computer software. Data were tested for normality with the Kolmogorov-Smirnov test. Because values of variables for colonic and jugular venous blood samples were not normally distributed within sample collection times (control samples and samples collected 1 hour after ischemia and 1, 2, and 4 hours after reperfusion), nonparametric tests were used for further statistical analyses. Data for preanesthetic and preischemic jugular venous blood control samples were compared with the related-samples Wilcoxon test. Data for each collection time for jugular and colonic venous blood samples were compared with the Kruskal-Wallis test. Whenever a significant \( P < 0.05 \) value was identified, the Mann-Whitney U test was used for pairwise comparison of data for each sample collection time. Data for jugular and colonic venous blood samples for each collection time were compared with related-samples Wilcoxon test. Data were expressed as median and interquartile range values. Values of \( P < 0.05 \) were considered significant. Adjustments of \( P \) values for multiple comparisons were performed by means of Bonferroni corrections.

Results

The lactate (median, 1.20 mmol/L; interquartile range, 1.13 to 1.28 mmol/L) and glucose (median, 7.80 mmol/L; interquartile range, 6.53 to 8.02 mmol/L) concentrations in preischemic jugular venous blood control samples were significantly higher than the lactate (median, 0.40 mmol/L; interquartile range, 0.40 to 0.48 mmol/L) and glucose (median, 6.02 mmol/L; interquartile range, 5.56 to 6.56 mmol/L) concentrations.
in preanesthetic jugular venous blood control samples. The Ca²⁺ concentration (median, 1.42 mmol/L; interquartile range, 1.40 to 1.43 mmol/L) and base excess (median, 7.40 mmol/L; interquartile range, 7.10 to 7.85 mmol/L) in preischemic jugular venous blood control samples were significantly lower than the Ca²⁺ concentration (median, 1.53 mmol/L; interquartile range, 1.51 to 1.56 mmol/L) and base excess (median, 8.90 mmol/L; interquartile range, 8.10 to 9.70 mmol/L) in preanesthetic jugular venous blood control samples. All other hematologic and serum biochemical analysis variables determined for jugular venous blood samples were not significantly different between preanesthetic and preischemic sample collection times.

After 1 hour of ischemia, metabolic acidosis was detected in colonic venous blood samples, which was characterized by significantly lower pH, bicarbonate concentration, and base excess and significantly higher PaCO₂ and lactate concentration, compared with values for preischemic colonic venous blood control samples (Figure 1). Glucose concentrations were lower (results not significant) and potassium concentrations and CK activities were significantly higher in colonic venous blood samples obtained after 1 hour of ischemia, compared with values for preischemic colonic venous blood samples (Figure 2). The pH, PaCO₂, base excess, and lactate, glucose, sodium, and potassium concentrations in colonic venous blood samples were significantly different from values of those variables in jugular venous blood samples after 1 hour of ischemia.
The pH, pCO₂, base excess and bicarbonate, glucose, and potassium concentrations and CK activities in colonic venous blood samples decreased to values similar to preischemic values after 1 hour of reperfusion (Figures 1 and 2). Lactate concentrations in colonic venous blood samples were lower after 1, 2, and 4 hours of reperfusion than they were after 1 hour of ischemia (results were significant only for samples obtained 4 hours after the start of reperfusion), but were significantly higher after 1 and 4 hours of reperfusion versus preischemic colonic venous blood control sample values. For jugular venous blood samples, lactate concentrations were significantly higher after 1, 2, and 4 hours of reperfusion versus preischemic colonic venous blood control sample values. For jugular venous blood samples, lactate concentrations were significantly higher after 1, 2, and 4 hours of reperfusion than they were after 1 hour of ischemia (results were significant only for samples obtained 4 hours after the start of reperfusion).

Table 2—Precision and reproducibility (intra- and interassay variabilities, respectively), sensitivity (minimum detectable concentration), and linearity of an ELISA for detection of equine calprotectin.

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<tr>
<th>OD</th>
<th>SD</th>
<th>CV (%)</th>
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<tr>
<td>Intraassay variability (n = 8)</td>
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<tr>
<td>Sample 1</td>
<td>2.134</td>
<td>0.033</td>
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<tr>
<td>Sample 2</td>
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<td>Sample 3</td>
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<td>Interassay variability (n = 8)</td>
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<tr>
<td>Day 1</td>
<td>2.134</td>
<td>0.033</td>
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<tr>
<td>Day 2</td>
<td>2.339</td>
<td>0.155</td>
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<td>Day 3</td>
<td>2.162</td>
<td>0.050</td>
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<td>Sensitivity (B₀ + 3SD)*</td>
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<tr>
<td>n = 20</td>
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<td>0.031</td>
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<td>Linearity†</td>
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<tr>
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<tr>
<td>1:20 dilution</td>
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<tr>
<td>1:30 dilution</td>
<td>0.425</td>
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<tr>
<td>1:40 dilution</td>
<td>0.382</td>
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<tr>
<td>Sample 2</td>
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<tr>
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<tr>
<td>1:50 dilution</td>
<td>0.347</td>
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<tr>
<td>1:60 dilution</td>
<td>0.315</td>
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*The minimum detectable concentration of calprotectin was 6.5 ng/mL. †Calprotectin concentrations determined with the ELISA in plasma samples obtained from healthy humans (Table 3). High variations in calprotectin concentrations were detected among horses of the present study (Table 3; Figure 3), and the median absolute concentration of calprotectin was not significantly different between jugular and colonic venous blood samples or among sample collection times during IR. However, for calprotectin concentrations expressed as a percentage of the concentrations in preischemic control samples, values were significantly higher for colonic venous blood samples after 1 hour of ischemia and after 1 and 4 hours of reperfusion, compared with values for jugular venous blood samples at those times. The median percentage of changes of calprotectin concentrations in colonic venous blood samples did not differ substantially during the time of IR; after 1 and 4 hours of reperfusion they were in preischemic control samples.

After 1 hour of ischemia, the percentages of band neutrophils in jugular and colonic venous blood samples were significantly higher than those in preischemic blood samples; a further increase in the percentage of band neutrophils was detected after 4 hours of reperfusion (Table 1). The percentage of necrotic neutrophils in colonic venous blood samples was significantly higher than it was in jugular venous blood samples after 2 hours of reperfusion. The percentages of eosinophils in colonic venous blood samples were decreased after 4 hours of reperfusion, compared with control samples and samples obtained during ischemia. The percentages of segmented neutrophils, lymphocytes, basophils, and monocytes in jugular and colonic venous blood samples and the percentages of eosinophils in jugular venous blood samples did not change significantly during IR, and there were no significant differences in percentages of each type of cell between jugular and colonic venous blood samples at any time during IR.

The precision and reproducibility (intra- and interassay variabilities, respectively), sensitivity (minimum detectable concentration), and linearity of the calprotectin ELISA were summarized (Table 2). Positive control serum samples (n = 6) contained 3,387.8 to 4,799.8 ng of calprotectin/mL (minimum and maximum concentrations, respectively). Median calprotectin concentrations in positive control serum samples obtained from horses of the present study were higher (3,794.9 ng/mL; coefficient of variation, 14.2%) than the reference interval (<3,000 ng/mL) for plasma samples obtained from healthy humans (Table 3). High variations in calprotectin concentrations were detected among horses of the present study (Table 3; Figure 3), and the median absolute concentration of calprotectin was not significantly different between jugular and colonic venous blood samples or among sample collection times during IR. However, for calprotectin concentrations expressed as a percentage of the concentrations in preischemic control samples, values were significantly higher for colonic venous blood samples after 1 hour of ischemia and after 1 and 4 hours of reperfusion, compared with values for jugular venous blood samples at those times. The median percentage of changes of calprotectin concentrations in colonic venous blood samples did not differ substantially during the time of IR;
similarly, median percentage of changes of calprotectin concentrations in jugular venous blood samples did not differ substantially during the time of IR.

Discussion

Results of the present study indicated ischemia caused reversible metabolic acidosis in the affected part of the large colon as measured in blood samples obtained from venous branches draining the ischemic segments. Metabolic acidosis was accompanied by disturbances in energy homeostasis (increased lactate and decreased glucose concentrations) and signs of cellular damage (increased potassium concentrations and CK activities). Reoxygenation of tissues resulted in a return of most of these variables to concentrations similar to baseline values; however, a mild increase in lactate concentrations persisted during colon reperfusion. Results of another study indicate pH decreases and lactate concentration increases in colonic venous blood of horses after 30 minutes of colon ischemia; values of those variables returned to within reference intervals within 5 minutes after reperfusion. Results of another study indicate 3 hours of experimentally induced colon ischemia in horses causes significantly decreased pH, P\(_{\text{CO}_2}\), and glucose concentration and significantly increased lactate concentration in colonic venous blood samples of horses, and values of blood gas variables and lactate concentrations in such samples are correlated with the severity of colonic mucosal damage. Additionally, ischemia induced an inflammatory response in colons of horses in the present study that was characterized by activation of neutrophils (release of calprotectin) and increased neutrophil turnover (increased percentage of band neutrophils) that further progressed during reperfusion. Changes in values of measured variables in venous blood samples obtained from veins that drained ischemic parts of the colon were of interest to us as objective measures of intravascular changes in horses with experimentally induced short-term IR in a small segment of the colon. Similar changes may be detected in jugular venous blood samples (systemic circulation) of horses with clinical colonic ischemia that have extensive and severe colonic injury. Such changes were not detected in the jugular venous blood samples obtained from horses of the present study, possibly because of dilution of the small volume of colonic venous blood in the systemic circulation and because the methods used in this study did not induce severe and extensive changes similar to those detected for horses with clinical disease attributable to problems such as large colon volvulus.

The findings of the present study were similar to those expected in accordance with the pathophysiologic mechanisms of ischemia. Cellular hypoxia is associated with a decrease in pH and an increase in P\(_{\text{CO}_2}\) and causes local accumulation of lactic acid generated by anaerobic glycolysis. An increase in venous or extracellular potassium concentration is typically detected after ischemia-induced acidosis and could be caused by the release of intracellular potassium in exchange for intracellular H\(^+\) ions to maintain electroneutrality and acid-base homeostasis. However, an increase in potassium concentrations in venous blood could also be caused by loss of intracellular potassium attributable to ischemic cell necrosis. In addition, necrosis of colonic mucosal cells or intravascular cells might have caused an increase in CK activities in colonic venous blood samples affecting.
ter 1 hour of colon ischemia of horses in the present study. Although most gastrointestinal tract CK is found in the seromuscular layer of the intestines, it is also found in the cytoplasm of intestinal epithelial and endothelial cells. This enzyme has been detected in physiologically normal small and large intestines of dogs, and release of CK into circulation is detected within the first 3 hours after colon ischemia in dogs and rabbits. Results of other studies indicate CK activity increases in jugular and colonic venous blood and peritoneal fluid in horses with intestinal ischemia; these results are probably attributable to CK leakage from damaged cells. The explanation that potassium and CK are released by damaged intestinal cells after ischemia can be supported by histologic examination results of other studies that single cell necrosis and small epithelial defects are detected in colonic tissues after 1 hour of ischemia.

Similar to results of another study, colonic ischemia in horses of the present study did not affect values of variables measured for systemic (jugular venous) blood samples. However, evidence of continuous generation of lactate and activation of neutrophils during reperfusion can be detected in systemic blood samples following colon ischemia. The decreased TP concentration detected in jugular venous blood samples of horses in the present study after IR could have been attributable to circulatory volume expansion after administration of IV fluids, protein loss through damaged vascular endothelium during IR, or both of these factors.

Most of the biochemical abnormalities in colonic venous blood samples of horses in this study resolved after reperfusion, probably because of the rapid restitution of mucosal epithelium and improvement of mucosal barrier integrity that develops after 4 hours of reperfusion. However, the findings of high lactate concentrations, activation of neutrophils, and increased neutrophil turnover during reperfusion could indicate the persistence of a mild systemic inflammatory response, as detected during IR in humans and animals of other species.

Band neutrophil counts were increased in jugular and colonic venous blood samples after colon IR of horses in this study, indicating increased rate of cell turnover attributable to loss of activated mature neutrophils from the circulation. However, the absolute leukocyte numbers in jugular and colonic blood samples were not evaluated. Neutrophils accumulate in submucosal venules after 1 and 2 hours of ischemia and after 30 minutes and 1 and 2 hours of reperfusion. Neutrophils migrate through endothelium into the colonic lamina propria during reperfusion, and they move toward damaged epithelium, which is accompanied by a decrease in neutrophil numbers in submucosal venules. The increased turnover rate of neutrophils in colonic and jugular venous blood of horses in the present study after IR may have been attributable to such factors: this suggested that neutrophils infiltrated colonic mucosa and caused inflammation after IR.

Recruitment and activation of neutrophils are induced by microbial moieties that enter the damaged epithelial barrier and chemotactic mediators, such as interleukins 8 and 17, tumor necrosis factor, interferon-γ, and granulocyte-macrophage colony-stimulating factor released by mucosal immune and nonimmune system cells. In addition, cell necrosis is commonly detected after IR as indicated by the increased percentage of necrotic neutrophils in colonic venous blood samples obtained from horses of the present study after 2 hours of reperfusion, compared with values for jugular venous blood samples obtained at that time. Cell necrosis could be responsible for activation of a systemic inflammatory response by means of the release of cellular danger molecules (ie, molecules that are released by damaged or dying cells) and other signals.

Calprotectin is generated in activated neutrophils and monocytes and is released during inflammation. Calprotectin concentrations did not change significantly in blood samples of horses in the present study during colon IR, likely because of the high variation in calprotectin concentrations among horses. To adjust for this high variation in calprotectin concentrations, values were expressed as a percentage of the preischemic control value for each horse; results of analysis of such data indicated a significant increase in calprotectin in colonic venous blood samples after IR, compared with values in jugular venous blood samples. This finding was consistent with the findings of other studies that neutrophils are activated in ischemia-damaged colonic tissue during IR.

Detection of calprotectin release is a sensitive indicator of cytotoxicity. Activated neutrophils release this protein into the interstitium or blood actively by means of secretion or passively during necrosis. Detection of necrotic neutrophils after IR of horses in the present study was consistent with passive secretion of calprotectin during necrosis. Although results were not significant, calprotectin concentrations decreased in jugular venous blood samples during colon IR of horses in this study, likely because of leukocyte margination and compartmentalization in the injured segment of the colon. Alternatively, systemic inflammatory stimuli might not have been potent enough to cause release of calprotectin by circulating neutrophils. Longer IR times than those used in this study would likely result in greater changes in serum calprotectin concentrations in horses during IR. Serum calprotectin concentration is a useful biomarker for the detection of idiopathic inflammatory bowel disease in dogs, which suggests its potential value as a biomarker for horses.

Although the monoclonal antibodies used in this study detect calprotectin released from monocytes, the predominant cell type that releases calprotectin in venous blood and large colon tissues of horses is activated neutrophils. Results of another study indicate most of the calprotectin-stained cells in submucosal vessels of colonic tissues of horses in the present study could be directly identified as neutrophils, distinct from monocytes. This finding would be expected because the percentages of neutrophils in blood samples of horses in this study (median values for jugular and colonic venous blood samples, 57.5% and 51.5%, respectively) were higher than the percentages of monocytes in such samples (2.0% and 1.5%, respectively). In addition, calprotectin is the most abundant cytosolic protein in neutrophils (30% to 60% of cytosolic proteins; esti-
mated cytosolic calprotectin concentration, 5 to 15 mg/mL), whereas calprotectin expression in monocytes does not exceed 1% of cytosolic proteins. Thus, the concentration of calprotectin released from monocytes into circulation in horses of the present study was expected to be negligible.

Results of other studies indicate neutrophils are activated after colon IR in horses on the basis of cytologic and immunologic properties of neutrophils and increased mucosal and plasma MPO activities. Myeloperoxidase is a granulocyte-specific enzyme that generates hypochlorous acid from hydrogen peroxide and chloride anion. This process is used by granulocytes and monocytes to kill microorganisms with polysaccharide-containing capsules that protect such organisms from granulocyte-derived proteolytic and hydrolytic enzymes. However, available assays do not distinguish MPO activities among types of cells, and eosinophils are an important source of this marker.

Because equine colonic mucosa is replete with eosinophils during healthy conditions and such cells are not an important source of calprotectin, calprotectin should be more useful than MPO as a marker of neutrophil activity in colons of horses.

Development of an ELISA for detection of equine calprotectin allowed us to quantify this pro-inflammatory neutrophilic protein; this was the first study in which equine calprotectin concentrations were measured, to the authors’ knowledge. The ELISA protocol we developed would be suitable for measurement of calprotectin in serum and other body fluids of horses. To optimize the quality of the test, the standard protein used in this study (human recombinant calprotectin) should be replaced with a purified equine calprotectin protein, and anti-horse calprotectin antibodies should be developed and used as capture and detection antibodies. Results of the ELISA used in this study indicated the median calprotectin concentration in control blood samples of horses was higher than it was in the human calprotectin positive control samples. This finding could have been attributable to cross-reaction of human calprotectin antibodies with other proteins in equine serum. That finding could also have been attributable to species-specific characteristics or activation of neutrophils during induction of anesthesia and the beginning of the surgical procedure in horses of this study. Analysis of blood samples obtained from awake horses and anesthetized horses that did not undergo surgery might have aided determination of a conclusion regarding such factors and may have allowed lowered variability in data.

As has been determined for humans and dogs, calprotectin concentrations in jugular venous blood samples were variable among horses of the present study. Calprotectin concentrations in serum samples obtained from healthy pet dogs range from 76 to 1,292 ng/mL (reference interval, 92 to 1,221 ng/mL). Because results of this study indicated high variation in serum calprotectin concentrations among horses, single measurements of calprotectin without the use of appropriate control values may not allow determination of the severity of an inflammatory response in such animals. Further studies would be required to determine reference values for equine serum calprotectin concentrations and to determine the importance of calprotectin in horses with local or systemic inflammation.

Results of the present study indicated concentrations of calprotectin and other measures of tissue acidosis and metabolic energy disturbance in colonic venous blood samples were useful objective markers of tissue injury in horses with experimentally induced large colon ischemia. Results also indicated that such colonic metabolic changes typically resolved after reperfusion; this finding was consistent with histologic evidence of colon tissue recovery after IR was determined in another study conducted by personnel in our laboratory. These findings suggested that serum calprotectin concentrations could be evaluated in further studies as a marker of neutrophil activation and inflammation after colon IR in horses.

References


