Detection of apoptotic cells in intestines from horses with and without gastrointestinal tract disease

Emma L. Rowe, BVMS; Nathaniel A. White, DVM; Virginia Buechner-Maxwell, DVM; John L. Robertson, VMD, PhD; Daniel L. Ward, MS

Objective—To identify apoptosis in equine intestines and determine whether apoptosis is associated with gastrointestinal tract disease or a specific tissue layer of intestine.

Animals—38 horses that underwent surgery or were euthanatized for small or large intestine obstruction, strangulation, or distension and 9 control horses euthanatized for reasons other than gastrointestinal tract disease or systemic disease.

Procedure—Specimens were collected at surgery from intestine involved in the primary lesion and distant to the primary lesion site or at necropsy from several sites including the primary lesion site. Histologic tissue sections were stained with H&E, and apoptosis was detected by use of the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling technique. The number of apoptotic cells per hpf was counted in the mucosa, circular muscle, longitudinal muscle, and serosa.

Results—Apoptotic nuclei were seen in all layers of intestine. An increased number of apoptotic cells was found in the circular muscle of the intestine from horses with simple obstruction, compared with strangulating obstruction or healthy intestine. Intestine distant from a primary strangulating lesion had higher numbers of apoptotic cells than did intestine distant from a simple obstructive lesion or intestine taken at the site of a strangulating or simple obstructive lesion.

Conclusions and Clinical Relevance—Intestine from horses with obstructing or strangulating lesions in the small intestine and large colon had high numbers of apoptotic cells possibly because of ischemic cell injury and subsequent inflammation. Whether substantial apoptosis affects intestinal function is not yet known. (*Am J Vet Res* 2003;64:982–988)

Gastrointestinal tract disease is the cause of high morbidity and mortality rates in horses. Strangulating obstruction of the small intestine and large colon is a frequent cause of acute abdominal disease requiring surgery.[1-3] Even with surgical intervention, the overall prognosis for long-term survival of horses with a strangulating lesion of the small intestine or large colon is guarded, with long-term survival rates of 68[4] and 24%, respectively.[5] Experimental evidence suggests that 1 reason for illness and death in horses with obstructive diseases is reperfusion injury.[6,7] Mucosal and serosal damage that occurs during ischemia is exacerbated during reperfusion.[7,8] To date, intestinal ischemia-reperfusion injury has been largely attributed to cellular necrosis, although apoptosis has recently been recognized as having an important role in both ischemia and reperfusion injury.[9,10]

Apoptosis, a distinct form of cell death, is a natural physiologic mechanism for the removal of cells that are not needed, with minimal tissue inflammation. Apoptosis has an important role in embryogenesis, tissue homeostasis, lymphocyte development and function, and tumor regression.[11]

Apoptotic cells have been observed after experimental ischemia-reperfusion injury to the brain, heart, adrenal glands, liver, and kidneys of other species.[9] In the gastrointestinal tract, apoptosis can be associated with the maintenance of homeostasis and shedding of cells from the villus tips in the small intestine or with pathologic states such as intestinal adenocarcinoma, inflammatory bowel disease, and radiation-induced intestinal damage.[12] Apoptotic cells can be detected with the **terminal deoxynucleotide transferase mediated dUTP nick end labeling (TUNEL)** technique. This technique distinguishes apoptotic from necrotic cells by specifically detecting DNA cleavage associated with nuclear change during apoptosis. This technique is effective in the detection of apoptosis in formalin-fixed specimens of human large intestine.[13]

The objectives of the study reported here were to determine whether apoptosis could be identified in equine intestines by use of the TUNEL method, identify the types of cells undergoing apoptosis, and determine whether apoptotic cells were associated with gastrointestinal tract disease or tissue layer of the intestine. We hypothesized that equine intestine directly or indirectly affected by naturally occurring simple or strangulating obstruction would have increased numbers of apoptotic cells.

Materials and Methods

Specimens of intestine from 38 horses (gastrointestinal tract disease group) that underwent abdominal surgery or were euthanatized for small intestine or large colon strangulation, obstruction, or distension were collected. Intestines from 5 horses (musculoskeletal group) that were euthanatized because of acute or chronic orthopedic conditions, including capital physis fracture (n = 1), chronic bilateral degenerative suspensory desmitis (1), neoplasia of the right

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From the Marion duPont Scott Equine Medical Center (Rowe, White), the Departments of Large Animal Clinical Sciences (Buechner-Maxwell) and Pathobiology (Robertson), and the Office of Research and Graduate Studies (Ward), Virginia-Maryland Regional College of Veterinary Medicine, Virginia-Tech and University of Maryland, Blacksburg, VA 24061
Supported by the Patricia Bonsall Stuart Equine Research Award.
Address correspondence to Dr. Rowe.

mandible (1), cervical vertebral malformation (1), or comminuted second phalangeal fracture (1), were sampled for comparison. Specimens of small intestine, large colon, cecum, and small colon were also obtained from 4 clinically normal horses (control group) that had no clinical signs of gastrointestinal tract disease, had not received systemically active medication for at least 6 months, and were donated for other reasons including behavioral problems (n = 2), osteochondrosis of the left tarsocrural joint and behavioral problems (1), or cutaneous sarcoids (1).

Intestinal specimens were collected at surgery or necropsy and consisted of 1 sample/designated region. Specimens collected at surgery were full-thickness biopsy specimens from resected intestine, the enterotomy site in the small intestine or large colon, or the edge of intestinal segments that were incorporated in an anastomosis. When possible, specimens were taken at the primary lesion site and sites distant to the lesion. Specimens collected at necropsy were collected immediately after death (within 5 minutes after lethal injection with concentrated pentobarbital) from the primary lesion site and at various sites proximal and distal to the lesion. For the 9 horses with no gastrointestinal tract disease, specimens (1 specimen from each site) were taken from the duodenum, mid jejunum, ileum, cecum, large colon, and small colon. All specimens were immediately placed in neutral-buffered 10% formalin, fixed for a minimum of 48 hours, dehydrated, and embedded in paraffin. Serial sections of each specimen were cut and stained with H&E and the TUNEL technique[13] for apoptotic cells by use of a commercially available kit.[a] All reagents were contained in the kit unless otherwise specified. Unstained tissue intestinal sections were deparaffinized and transferred to PBS solution for 5 minutes. The tissue sections were pretreated with 20 µg of proteinase K/mL,[b] washed in dH_20, quenched with hydrogen peroxide (3.0%), covered in 75 µg of equilibration buffer/mL, and incubated with 55 µg of **terminal deoxynucleotidyl transferase (TdT)** enzyme/mL for 3 hours in a humidified chamber. The reaction was terminated with stop-wash buffer, and the tissue sections were incubated with 65 µg of antidigoxigenin conjugate/mL for 30 minutes in a humidified chamber and washed with PBS solution. The tissue sections were stained with 75 µg of **diaminobenzidine (DAB)**/mL, washed, counterstained with methyl green, and mounted.

As a detection control for the TUNEL technique, sections of thymus were taken from untreated (negative control) mice and mice treated with dexamethasone (positive control). Dexamethasone is known to induce apoptosis in thymic lymphocytes.[14-16] Thymus specimens were stained simultaneously with intestinal specimens to ensure the staining technique was effective and apoptotic cells were being detected. Stained slides were examined in a masked manner and scored for the presence or absence of apoptotic cells. The H&E-stained sections were reviewed to identify the specific cell types undergoing apoptosis in the various locations by comparison with TUNEL-stained slides.

In each specimen, the apoptotic cells located in the mucosa, circular muscle, longitudinal muscle, and serosa were counted in 3 hpf at 400× magnification. The mean number of apoptotic cells in each tissue layer was calculated for all specimens. Specimens were assigned to 1 of 4 diagnostic categories for statistical analysis: simple obstructed, strangulated, musculoskeletal, and control. Data for the specimens were log-transformed to stabilize the variances and back-transformed for presentation. The MIXED procedure of a software program[c] was used to perform a split-plot ANOVA to test for effects of gastrointestinal tract disease, type of intestine (small intestine or large colon), tissue layer (mucosa, circular muscle, longitudinal muscle, or serosa), and interactions among these categories. Gastrointestinal tract disease was the whole plot factor, type of intestine was the subplot factor, and tissue layer was the subplot factor in the ANOVA. Significant ($P \leq 0.05$) interactions were further investigated by use of the SLICE option to test for simple main effects. Simple contrasts were used to compare means with simple main effects. Small colon and cecum specimens were not included in the analysis, because there was not a high enough number of each to be representative across all categories (including simple obstructed, strangulated, musculoskeletal, and control categories). For all comparisons, values of $P \leq 0.05$ were considered significant.

Results

Specimens were collected from 43 horses with clinical disease and 4 horses donated to the Veterinary Teaching Hospital. One intestinal specimen was taken in 22 horses, and specimens from more than 1 location in the gastrointestinal tract were taken from 25 of the horses. Of 106 specimens collected, 50 were from the small intestine, 38 from the large colon, 10 from the cecum, 4 from the small colon, and 4 from the stomach. The numbers of intestinal sections examined in the various disease categories were simple obstruction (n = 14), peritonitis (4), strangulation obstruction (23), distant to strangulation (different intestinal segment; 12), distant to strangulation (same intestinal segment; 6), no gastrointestinal disease (chronic musculoskeletal; 8), distant simple obstructed (other intestinal segment; 2), adjacent to strangulated lesion (4), inflammatory (same intestinal segment; 1), no gastrointestinal disease (other; 6), and control (no systemic medication; 24).

Control slides for the TUNEL procedure reacted properly, and the procedure was considered to provide valid and reproducible staining of equine intestinal specimens. Apoptotic cells were evident in all 4 layers of intestine except in 5 horses with no clinical evidence of gastrointestinal tract disease (1 in the musculoskeletal category and 4 in the control category). One slide was unreadable because of the poor condition of the devitalized intestine. Apoptotic nuclei were observed in the capillaries and connective tissue of the lamina propria, predominately at the tips of intestinal villi, and

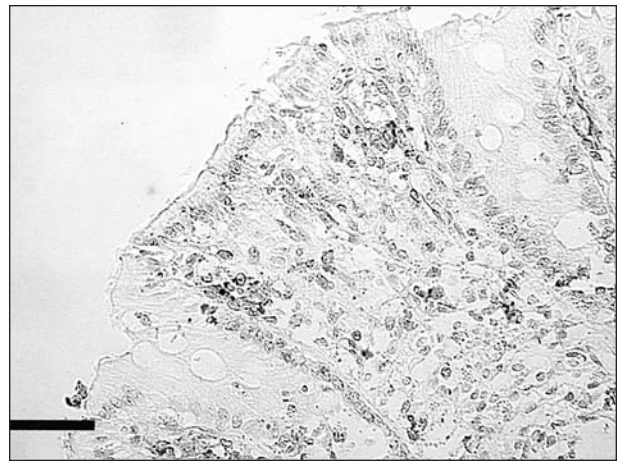

Figure 1—Photomicrograph of a section of mucosa in the small intestine of a horse with gastrointestinal tract disease. Darkly stained nuclei represent apoptotic cells. Healthy or necrotic cells are not stained. Diaminobenzidine stain and methyl green counterstain; bar = 50 µm.

in the mucosal epithelial cells (**Fig 1**). Apoptotic nuclei were observed in circular (**Fig 2**) and longitudinal muscle. Apoptotic nuclei were also identified in the connective tissue of fascial planes between groups of muscle fibers. Schwann cells, glial cells, or activated satellite cells and neurons were also apoptotic in 5 of the specimens of intestine in horses with gastrointestinal tract disease (**Fig 3**); however, the number of affected cells in the ganglia was not counted. Apoptosis was rare in the serosa but did occur in the endothelial cells in small vessels and in the mesothelial cells (**Fig 4**). Apoptotic neutrophils in the serosa and other regions of intestine were uncommon. In the musculoskeletal group, no apoptotic cells were identified in 1 horse with a left hind limb second phalanx fracture, but apoptotic cells were identified in the other 4 horses of this group. The horse with severe degenerative suspensory desmitis had generalized apoptosis; the horse with a chronic capital physis fracture had apoptosis only in the cecum, the horse with cervical vertebral malformation had apoptosis predominately in the large colon; and a horse with a tumor on the mandible had apoptosis predominately in the small intestine. Small numbers of apoptotic cells (≤ 10% of the number of apoptotic cells in the horses with gastrointestinal tract disease) were identified in the specimens from the 4 control horses.

Horses with gastrointestinal tract disease had a significantly (P = 0.007) greater number of apoptotic cells, compared with horses with musculoskeletal disease or control horses. There was a significant (P = 0.004) difference in the number of apoptotic cells in the different tissue layers among all the categories. There was a significant (P = 0.003) 3-way interaction between colic, tissue, and intestine. The test for simple

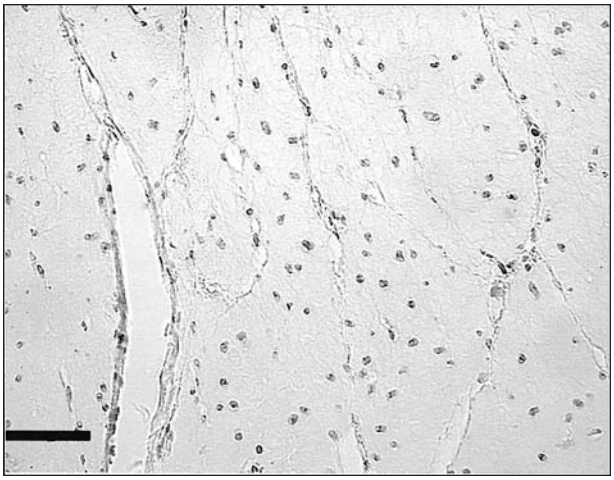

Figure 2—Photomicrograph of a section of circular smooth muscle in the small intestine of a horse with gastrointestinal tract disease. Darkly stained nuclei represent apoptotic cells. Diaminobenzidine stain and methyl green counterstain; bar = 50 μm.

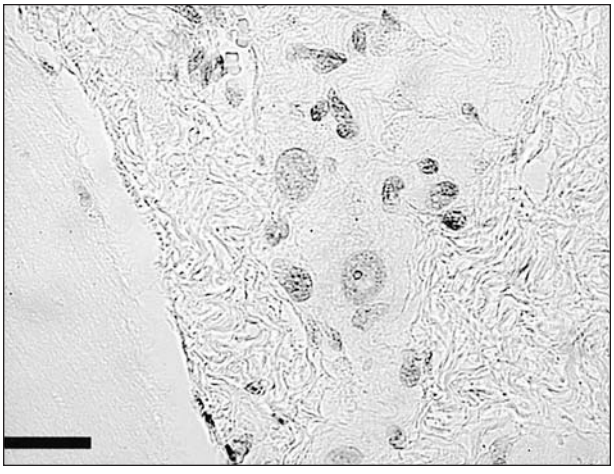

Figure 3—Photomicrograph of a myenteric plexus in the large colon of a horse with simple intestinal obstruction. Darkly stained nuclei represent apoptotic cells. Notice that neurons, glial cells, and Schwann cells are apoptotic. Diaminobenzidine stain and methyl green counterstain; bar = 50 μm.

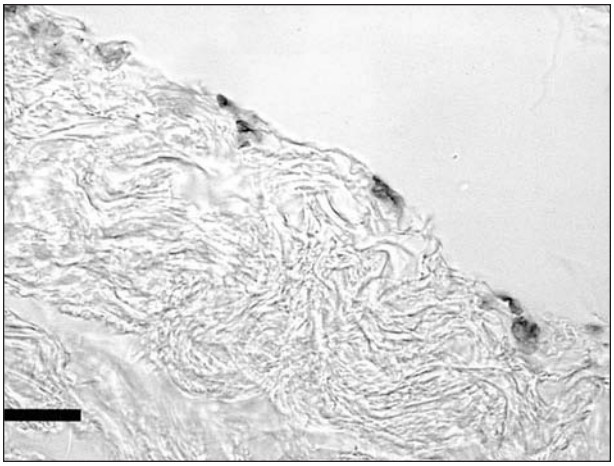

Figure 4—Photomicrograph of a section of serosa from the large colon of a horse with strangulating intestinal obstruction of the large colon. Darkly stained nuclei are apoptotic mesothelial cells on the surface of the serosa. Diaminobenzidine stain and methyl green counterstain; bar = 50 μm.

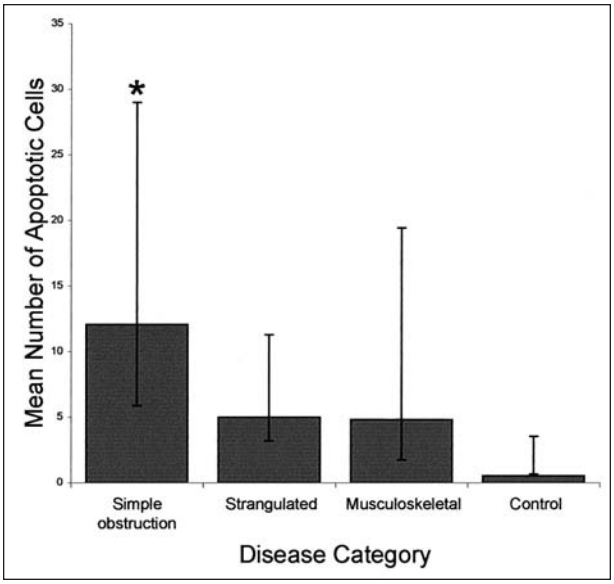

Figure 5—Geometric mean numbers of apoptotic cells in specimens of intestine from horses with simple intestinal obstruction, intestinal strangulation, musculoskeletal diseases, or no diseases (control). *Significant (P = 0.009) difference from other categories. Error bars represent 95% confidence intervals for the means.

main effects revealed a significantly ($P = 0.009$) higher number of apoptotic cells in the circular muscle of the intestine in horses with simple obstruction, compared with the other tissue layers in the strangulation obstruction, musculoskeletal, and control categories (**Fig 5**). There was also a significantly ($P < 0.001$) higher number of apoptotic cells in the large colon, compared with the small intestine for all categories. A significantly ($P = 0.019$) increased number of apoptotic cells were found in the intestine at a site distant to the primary lesion in horses with strangulation obstruction, compared with those with simple obstruction and with the intestine taken at the primary strangulating obstruction lesion or simple obstruction lesion (**Fig 6**).

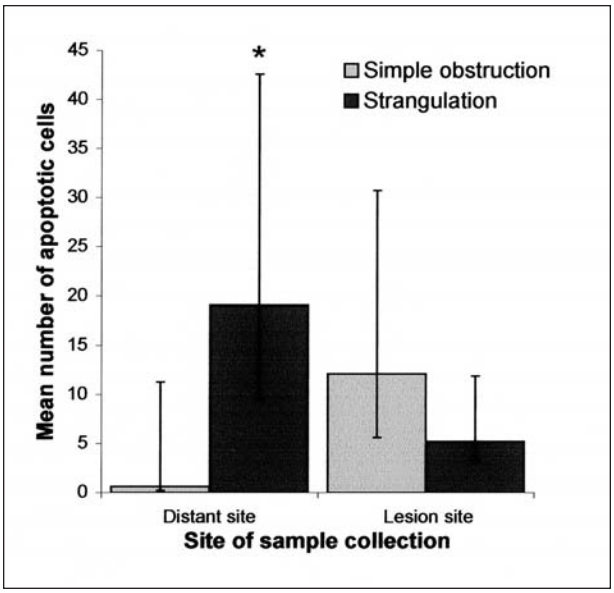

Figure 6—Geometric mean numbers of apoptotic cells in specimens of intestine obtained from the primary lesion site or a distant site in horses with simple obstruction or intestinal strangulation. *Significantly ($P = 0.019$) different from specimens obtained from horses with simple obstruction. Error bars represent 95% confidence intervals for the means.

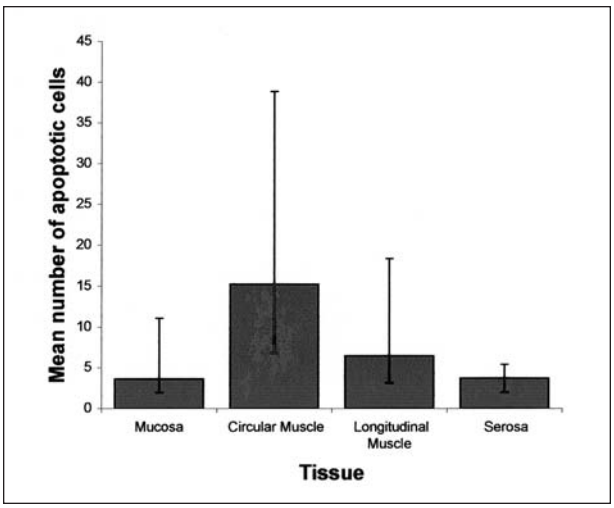

Figure 7—Geometric mean numbers of apoptotic cells in 4 tissue layers of the intestine in specimens from horses with simple obstruction or intestinal strangulation. Error bars represent 95% confidence intervals for the means.

A higher number of apoptotic cells in the circular muscle, compared with the other tissue layers, was found in certain horses with a simple obstruction or strangulating lesion of the intestine, but this difference was not significant (**Fig 7**).

Discussion

Cellular death occurs in 2 biochemically and morphologically distinct ways: cellular necrosis and apoptosis. Apoptosis results from activation and transcription of specific genes. In many of the pathways that are initiated in apoptosis, permeabilization of the mitochondrial membrane is a critical event that results in the release of various molecules from the mitochondrial intermembrane space.[17] These molecules include procaspases (enzymes), cytochrome c (a caspase activator), Smac/Diablo (a caspase coactivator), and an apoptosis-inducing factor.[17] The apoptosis-inducing factor activates the nucleases that cleave the DNA into small fragments.[17] The DNA strand breaks that result from the endonucleases' activation can be labeled in situ; in individual fixed, permeabilized cells; or tissues in sections by use of the TUNEL technique.[18,19] In this technique, the 3′-OH termini that are generated as a result of DNA fragmentation during apoptosis are labeled with modified nucleotides by terminyl deoxynucleotide transferase.[20] This enzyme selectively detects apoptotic rather than necrotic cells and is more specific than DNA polymerase.[20] There is a high correlation between TUNEL staining and apoptosis.[20,21] The TUNEL technique has greater sensitivity, compared with in situ nick translation.[22] A major advantage of the TUNEL technique is the ability to reveal early DNA breaks during apoptosis that occur before the light microscopic changes characteristic of apoptosis. The TUNEL technique performed with the same kit used in our study has been successful in detecting apoptosis of lymphocytes and neutrophils in the lamina propria of fixed human intestinal specimens.[13]

A potential concern of detecting apoptosis in formalin-fixed tissue is that of random DNA damage with long-term fixation, resulting in nonspecific labeling. This effect has been investigated in rat testicular tissue. Results indicated that formaldehyde can replace protons in the purine and pyrimidine bases but does not react with sugar hydroxyls or phosphate ester groups in nucleic acids.[23] It is possible that with prolonged fixation, any single-stranded regions of DNA (ie, free ends) are locked up and inaccessible to the polymerase.[23] Any damage resulting from fixation or cytopathologic mechanisms, such as necrosis, would not be detectable with this technique.[23] Therefore, there is the risk of a false-negative result rather than a false-positive result. Apoptotic cells may have been missed in our study, but the positively stained cells are likely not the result of any mechanism other than apoptosis.

There were significantly higher numbers of apoptotic cells in the circular muscle from the intestine in horses with simple obstructive gastrointestinal tract disease, compared with the control and musculoskeletal groups. The difference in the pathophysiology of simple obstruction and strangulation may explain increased apoptosis found in intestine during simple

obstruction. The mitochondrial permeability transition pore can initiate apoptosis or necrosis, and the induction of the pore in a sequential fashion permits activation and action of proteases, which will stimulate apoptosis before ATP depletion results in cell death.[24] However, when the pore is activated rapidly, as in ischemia, the cellular metabolism may be severely compromised, and necrosis will occur before the apoptosis-inducing proteases are activated.[24] Caspase activation and the intracellular ATP content are critical factors that determine whether a cell will undergo necrosis or apoptosis.[24] The ATP is required for activation of cytochrome c, which induces apoptosis.[24] If ATP is decreased, inhibiting caspases that activate the apoptotic process, cells will undergo necrosis.[24] If glycolytic substrates can provide an alternative source of ATP, then apoptosis is likely to occur. Although speculative, intestinal hypoxia during strangulation may cause this intracellular mechanism to induce more cells to undergo necrosis, whereas during simple obstruction, inflammatory mediators may induce apoptosis when there is sufficient ATP. This is not an all-or-nothing event, because renal apoptosis after ischemia is induced by hypoxia[25] and ATP depletion.[26]

Apoptosis is a physiologic mechanism that allows cells to be eliminated without associated tissue inflammation. In the intestine, apoptosis plays a role in the maintenance of homeostasis,[27] and apoptotic cells are ingested by phagocytic cells or shed into the lumen.[28] Although apoptosis that occurs as part of the intestine's normal function should not cause inflammation, excessive apoptosis experimentally causes inflammation. For example, in rats, occasional apoptosis in the intestine does not cause an alteration in the intestinal barrier integrity; however, when doxyrubicin is administered IP, there is an increase in mucosal apoptosis and a concomitant increase in epithelial and endothelial permeability.[12] Apoptosis is considered an important source of inflammation in the kidney.[29] During severe forms of glomerulonephritis, apoptosis of the glomerular endothelial cells may contribute to the development of glomerulosclerosis.[30] In a murine model, administration of antiapoptotic agents at the time of reperfusion of the kidney after ischemia prevents the early onset of apoptosis, inflammation, and tissue injury.[10] Glutamine deprivation-induced apoptosis in rat intestinal epithelial cells and subsequent disruption in homeostatic balance between cell proliferation and cell death is proposed to contribute to intestinal atrophy.[31] The significant increase in numbers of apoptotic cells in the circular muscle of intestine in horses with simple obstruction and in intestine distant to a primary strangulating obstruction lesion suggests that the apoptosis may be associated with intestinal inflammation.

It was interesting that there were significantly higher numbers of apoptotic cells in the circular muscle of the intestine taken distant to the site of a strangulating lesion, compared with that taken distant to a simple obstruction lesion or at the site of either type of lesion. As an example, high numbers of apoptotic cells were in the large colon of horses with a strangulating obstruction of the small intestine. Increases in apoptotic cells at distant sites may be a result of systemic inflammatory mediators resulting from the inflammation during ischemia and reperfusion. Many mediators, such as cytokines, growth factors, chemotactic peptides, hypoxia, and acidosis, can activate leukocytes and initiate or inhibit cell apoptosis.[32] For example, in human patients following elective surgery, **interleukin 6 (IL-6)** was notably increased at 24 hours after surgery and at this concentration inhibited neutrophil apoptosis. This effect was attenuated with the addition of recombinant human IL-10. The authors of that study[33] concluded that imbalances of proinflammatory and anti-inflammatory cytokine release affected the apoptotic capacity of the plasma. Ischemia-reperfusion after occlusion of the superior mesenteric artery in rat small intestine induces apoptosis in the jejunum and ileum.[34] The exact mechanism of ischemia-reperfusion-induced apoptosis in that study was not explained, because the regulation of intestinal cell death and proliferation is highly complex and likely controlled by a variety of factors.[35] Because free radicals cause apoptosis,[36,37] it is reasonable to hypothesize that free radicals produced during reperfusion in the equine intestine may stimulate apoptosis.[34]

Another possible explanation of the significantly increased apoptotic cells distant to a strangulating lesion in intestine from horses undergoing surgery may be the effect of surgical manipulation of the intestine. The intestine is known to be susceptible to stress at surgery, and results of 1 study indicate that surgical trauma induces oxidative stress and damage through enhanced production of reactive oxygen species.[38] Surgical stress induced in rat intestine (induced by opening the abdominal wall and handling the intestine as done during exploratory laparotomy) induces and activates protease activity in the villus and crypt cells.[39] Both the mitochondrial and cytosolic proteases were activated in that study, and free radicals generated by xanthine oxidase were proposed as possible mediators of protease activation after surgical stress in the intestine.[39] The increased protease activity observed in the mitochondria may be responsible for the mitochondrial permeability transition and mitochondrial dysfunction leading to apoptosis.[39] The intestine is highly susceptible to surgical manipulation at remote locations, and mild handling of intestine following laparotomy causes oxidative stress in enterocytes and functional alterations in the intestine.[40]

In certain horses with either a simple obstructive or strangulating lesion, there were high numbers of apoptotic cells in the circular muscle of the intestine from horses. If the apoptosis is associated with an increased inflammatory response in the muscle, it could affect the contractility of the muscle. Contractility of equine intestine is reduced experimentally by inhibition of the circular muscle with nitric oxide in jejunum[41] and ventral colon. Recently, muscle injury (measured by evaluation of creatinine kinase activity) resulting from 90 to 120 minutes of ischemia was reduced by the pancaspase inhibitor z-VAD.[10] This suggests that apoptosis blocked by pancaspase inhibitor is responsible for or associated with muscle inflammation and subsequent injury.[10] A better understanding of the relationship of intestinal muscle cell apoptosis and intestinal inflammation may help development of new therapy to attenuate apoptosis in intestinal muscle.

The locations of the different types of apoptotic cells in the intestine were recorded; however, the number of cells in the specific regions of the 4 intestinal layers was not quantitated. Apoptotic cells were observed in the mucosa, and large numbers were located within the connective tissue of the lamina propria at the tips of the intestinal villi. Apoptosis may be associated with the shedding of the cells from the villus tips in the small intestine[12] and has been induced in the mucosa of the ileum and jejunum of rats after occlusion of the superior mesenteric artery.[34] In those rats, apoptotic cells were most evident at the villus tips.[34] The apoptosis, which increased during the ischemic period in the rat small intestine, was maximal after 1 hour of reperfusion and then significantly decreased after only 6 hours, indicating it was a transient event.[34] Apoptosis was also observed in the muscle fibers of the longitudinal muscle and within the fascial planes between the groups of muscle fibers. Whether this apoptosis in the muscle is detrimental to motility is not yet known.

Apoptosis of Schwann cells, glial cells, or activated satellite cells and neurons in the myenteric plexus also suggests a possible relationship between apoptosis and intestinal function. Because the myenteric plexus is important for functional motility and other gastrointestinal tract functions such as secretion, apoptosis of cells in the enteric nervous system may alter intestinal function.[42]

Few apoptotic cells were observed in the serosa, although ischemia and reperfusion and intraluminal distension and decompression cause severe morphologic changes in the equine jejunal serosa.[7] Mesothelial and endothelial cells were apoptotic in the serosa but appeared to be few in number, compared with other layers. In our study, the musculoskeletal and gastrointestinal tract disease groups had large numbers of apoptotic cells, compared with the control group. Three of the 5 horses in the musculoskeletal group had been treated with phenylbutazone for ≥ 14 days prior to euthanasia. Because phenylbutazone causes an increase in apoptosis in vitro in the right dorsal colon of horses,[43] the apoptosis in the musculoskeletal group may have been related to systemic inflammation or medication. The horses with gastrointestinal tract disease in our study all received flunixin meglumine at various times prior to specimen collection. Although it may be possible for flunixin meglumine to induce apoptosis in the intestine of treated horses, this does not explain the differences in the number of apoptotic cells observed between simple obstructed and strangulated intestine or differences between cells from the primary lesion and those at a distant site. The effect of nonsteroidal anti-inflammatory drugs on apoptosis in intestine of horses is not completely known.

[a]Apoptag In Situ Apoptosis detection kit, Intergen Co, Purchase, NY.
[b]Sigma Chemical Co, St Louis, Mo.
[c]SAS system, version 8.2, SAS Institute Inc, Cary, NC.

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