

# **The Role of Neutrophil Apoptosis in Horses with Acute Abdominal Disease**

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## **ABSTRACT**

Neutrophils, the chief phagocytic cells in most mammals, are critical in the inflammatory response. Regulation of neutrophil activity occurs through several mechanisms, including apoptosis. Dysfunction of neutrophil apoptosis has been implicated as a cause of organ damage in hyper-inflammatory conditions in human patients. This pilot study investigated apoptosis in circulating neutrophils from horses with surgical lesions in the large and small intestine. We hypothesized that delayed neutrophil apoptosis occurs in peripheral blood of horses undergoing surgery with acute abdominal disease, compared with elective orthopedic cases.

Adult horses undergoing surgery for acute abdominal disease (N=10) and elective orthopedic surgery (control) (N=10) were studied. Peripheral blood was collected preoperatively and postoperatively. Neutrophils were isolated using Percoll gradient. Cells undergoing apoptosis were determined by flow cytometry using a commercially available staining kit (Annexin V-PE Apoptosis Detection Kit I, BD Pharmingen™). The Mann-Whitney *U* test was used to detect significant differences in neutrophil apoptosis between the two groups as well as between lesion types in the abdominal surgery group. Correlations between neutrophils in apoptosis and postoperative parameters were detected using Spearman's rank correlation coefficient.

No significant differences in percentages of apoptotic neutrophils between groups were found; however, a significantly lower percentage of neutrophil apoptosis was present in horses with strangulating intestinal lesions versus nonstrangulating lesions. Current investigations about neutrophil apoptosis in human medicine may result in therapeutic intervention to prevent organ damage in hyper-inflammatory states. Understanding the role of neutrophil apoptosis in equine acute abdominal disease may guide the use of new treatments as they become available.

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## LIST OF ABBREVIATIONS

7-AAD: 7 amino actinomycin  
APAF-1: apoptotic protease activating factor 1  
ARDS: acute respiratory distress syndrome  
ATP: adenosine triphosphate  
ATPase: adenosine triphosphatase  
BAX: B-cell lymphoma- associated X  
BCL2: B-cell lymphoma 2  
BCL2A1: B-cell lymphocyte 2-related protein A1  
DNA: deoxyribonucleic acid  
Fas: TNF receptor superfamily, member 6  
FITC: fluorescein isothiocyanate  
fMLP: formyl-methionyl-leucyl-phenylalanine  
GCSF: granulocyte colony-stimulating factor  
GMCSF: granulocyte-macrophage colony-stimulating factor  
H and E: hematoxylin and eosin  
HIV: human immunodeficiency virus  
IAP: inhibitor of apoptosis protein  
IFN- $\gamma$ : interferon gamma  
IL: interleukin  
LPS: lipopolysaccharide  
LTB<sub>4</sub>: leukotriene B<sub>4</sub>  
MAPK ERK1/2: mitogen activated protein kinase extracellular signal-related kinase 1/2  
MCL1: myeloid cell leukemia 1  
MODS: multiple organ dysfunction syndrome  
MPO: myeloperoxidase  
NADP: nicotinamide adenine dinucleotide phosphate  
NF- $\kappa$ B: nuclear factor kappa beta  
NO: nitric oxide  
p53: protein 53  
PAF: platelet activating factor  
PE: phycoerythrin

PGE2: prostaglandin E2

PI: propidium iodide

PS: phosphatidylserine

ROM: reactive oxygen metabolites

SHIP-1: Src homology 2 domain-containing inositol 5'-phosphatase

SMAC: second mitochondria-derived activator of caspases

TGF- $\beta$ : transforming growth factor beta

TNF- $\alpha$ : tumor necrosis factor alpha

TRAF-1: tumor necrosis factor receptor-associated factor 1

TRAIL: tumor necrosis factor-related apoptosis-inducing ligand

TUNEL: terminal deoxynucleotidyl transferase nick end labeling

## **ATTRIBUTION**

Descriptions of the qualifications of the authors

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## LITERATURE REVIEW

Equine colic is recognized as one of the most important medical problems in horses. It has been reported as the most common cause of death in some horse populations. The annual number of colic cases has generally been reported to be between 4-10 cases per 100 horses.<sup>1,2</sup> While most cases of colic resolve spontaneously or respond to medical management, 2-10% of colic cases have been reported to require surgical intervention.<sup>3,4</sup>

Surgical treatment is required for some forms of intestinal obstruction, which is classified as simple or strangulating. Nonstrangulating infarction of the intestine can also require surgical intervention.<sup>5</sup> All types of obstruction or intestinal ischemia cause some degree of intestinal injury including loss of the mucosal barrier; while a compromised mucosal barrier can lead to endotoxemia and systemic circulation of LPS, intestinal ischemia has also been shown to produce a systemic inflammatory response.<sup>6</sup> Neutrophils are an important component of the systemic response to an infectious or inflammatory insult due to production of ROM and degradative enzymes; however, the potential exists for inadvertent tissue damage when neutrophil homeostasis is not tightly controlled. One primary mechanism for regulating neutrophil homeostasis is apoptosis, or programmed cell death. A myriad of human studies have documented a relationship between systemic infectious or inflammatory conditions and delayed peripheral blood neutrophil apoptosis, and have demonstrated prolonged neutrophil functionality when apoptosis is inhibited.

Systemic inflammatory mediators such as TNF- $\alpha$  and IL-1 have been documented in the human literature to promote an inflammatory response and to delay neutrophil apoptosis.<sup>7,8</sup> These cytokines have been shown to promote an inflammatory response in horses as well.<sup>9</sup> Delayed neutrophil apoptosis in horses following emergency surgery for acute abdominal disease may be the reason for increased intestinal inflammation resulting in postoperative ileus, and may be due to upregulation of systemic inflammatory mediators. Manipulation of neutrophil apoptosis rate may be a key component in more timely resolution of postoperative inflammation and prevention of complications which arise following emergency abdominal surgery in the horse.

## **A. THE NEUTROPHIL**

### **I. Lifespan and functions of the neutrophil**

The neutrophil is the chief phagocytic leukocyte present in horses and other mammalian species, and is a critical component of the host's response to inflammation and infection. It is derived from pluripotent stem cells in bone marrow. Differentiation from myeloblasts to mature neutrophils takes approximately 14 days. Once released into the bloodstream, neutrophil half-life is 6-8 hours. Neutrophil turnover reportedly averages  $10^{11}$  cells per day in an average adult human.<sup>10</sup>

Neutrophils are mobilized from the circulation and bone marrow in response to chemotactic factors produced by the host or by pathogens. Neutrophils roll along the walls of postcapillary venules, surveying tissues for signs of distress and the presence of chemoattractants.<sup>11</sup> One of the most potent neutrophil chemoattractants is the chemokine IL-8.<sup>12, 13</sup> This chemokine is produced by many cell types, including monocytes, macrophages, endothelial cells, and neutrophils during inflammatory states.<sup>14</sup> Bacteria also produce molecules (e.g. *N*-formylated peptides) that directly recruit neutrophils.<sup>15, 16</sup> Neutrophil "rolling" in blood vessels is mediated by selectins, a family of C-type lectin glycoproteins that are expressed on activated endothelial cells (E- and P-selectin), platelets (P-selectin), and activated neutrophils (L-selectin).<sup>17</sup> As part of the inflammatory response, E- and P-selectin become upregulated on endothelial cells and interact with L-selectin expressed on the surface of neutrophils in a process known as "tethering".<sup>17</sup> Tethering facilitates subsequent rolling of neutrophils along endothelial surfaces.

Selectins as well as chemokines presented on the inflamed endothelium may initiate neutrophil signaling pathways that regulate integrin adhesiveness. Binding of activated integrins to their counter-receptors leads either to reduction of rolling velocity or arrest, depending on the conformation of the integrins.<sup>18</sup> Integrins are also able to transfer signals from the extracellular domain into the cell.<sup>19</sup> These signals strengthen adhesion and induce superoxide production, respiratory burst (rapid oxygen consumption and release of ROM), and transmigration.<sup>18</sup>

Once neutrophils are firmly bound to endothelium, several neutrophil surface markers, including CD31,<sup>20</sup> CD54,<sup>21</sup> CD44,<sup>22</sup> and CD47<sup>23</sup> facilitate transmigration through the endothelium into tissues. Transmigration of neutrophils along with direct recognition of bacteria produced molecules, such as peptidoglycan and LPS,<sup>14</sup> permits phagocytosis (binding and

ingestion of invading microorganisms). The efficiency of phagocytosis is enhanced if microbes have been opsonized with serum host proteins, such as complement and/or antibody.<sup>14</sup> Bacterial phagocytosis triggers degranulation as well as neutrophil genes encoding many immunomodulatory agents, including various interleukins and TNF- $\alpha$ . In addition to recruiting more neutrophils and modulating subsequent neutrophil function, these agents potentially coordinate early responses of monocytes, macrophages, dendritic cells, and lymphocytes during inflammatory states.<sup>14</sup> Microorganisms are ultimately destroyed by ROM and cytotoxic granule components produced by neutrophils. Under inflammatory conditions without the presence of microorganisms, a variety of intracellular signaling molecules regulate translocation of granules to the cell membrane for docking and fusion to release their contents.<sup>24</sup>

Inadvertent host tissue damage can occur via neutrophil production of degradative enzymes and ROM. Cell activation and granule exocytosis, prolonged acute inflammatory responses, tissue remodeling, and neutrophil lysis at the site of infection contribute to inflammatory disorders.<sup>14</sup> For example, a relationship has been established between rheumatoid arthritis and production of ROM by neutrophils following activation by immune complexes in synovial fluid. The potential for inadvertent host tissue damage underscores the importance of effective regulation of neutrophil homeostasis and turnover during infection and inflammatory states. One principal way that neutrophil homeostasis is regulated is through apoptosis.

## II. Neutrophil apoptosis

Apoptosis refers to programmed cell death. In Greek, apoptosis translates to “dropping off” of petals or leaves from plants or trees.<sup>25</sup> It is a normal mechanism that occurs in multicellular organisms and effectively clears effete cells. Apoptosis occurs rapidly, leaving no trace, and is likely as important as cell division in producing the correct cell types in the right numbers and locations. Normal apoptosis is considered “suicide” in that the cell itself activates the death program. A significant amount of apoptosis occurs in the vertebrate hemopoietic system; billions of neutrophils die this way each day in an adult human.<sup>26</sup> Apoptotic cells are rapidly phagocytosed by macrophages, preventing leakage of cytosolic contents that could cause an inflammatory response.

Apoptotic cells demonstrate a characteristic morphology (see Figure 1); they shrink and become rounder in shape with increased cytoplasmic density and tight packing of organelles.

Chromatin condenses into compact patches against the nuclear envelope (pyknosis), and discontinuity of the nuclear envelope and fragmentation of DNA occur (karyorrhexis). The cell membrane develops irregular buds, known as blebs. Finally, the cell breaks apart into vesicles known as apoptotic bodies which are phagocytosed.

Apoptosis, also referred to as Type I cell death, is a non-inflammatory form of cell death characterized by orderly destruction of the infected/damaged cell without resultant inflammation in the surrounding tissues.<sup>17</sup> Autophagy, also known as Type II cell death, involves catabolization of damaged components by cells so that essential building blocks, such as amino acids, lipids, and carbohydrates can be used for cellular repair. Autophagy-induced cell death is non-inflammatory programmed cell death, but it is distinct from apoptosis in that it is induced under conditions of cellular starvation or endoplasmic reticulum stress.<sup>27</sup>

Pyroptosis is another programmed cell death mechanism. Unlike apoptosis, it results in cell lysis, tissue inflammation, and recruitment of host professional phagocytes.<sup>17</sup> Oncosis, also known as Type III cell death, is characterized by cell swelling and lysis. Oncosis occurs when membrane integrity is compromised and an efflux of inorganic ions increases intracellular and intraorganelle osmotic pressure.<sup>28, 29</sup> Necrosis, previously defined as cell swelling and lysis, is now widely accepted as the end result of cell death. Necrosis includes lysis of infected cells and the resulting damage that is caused to surrounding tissues.<sup>30</sup>

### *1. REGULATION OF NEUTROPHIL APOPTOSIS*

Balance between neutrophil survival and death is required to resolve infection and control inflammation. Enhanced neutrophil survival, or a delay of apoptosis, may be desirable during the early stages of the inflammatory response, as it increases the window of time during which neutrophils can be recruited to appropriate sites and remain fully functional. Potentially harmful tissue effects can be seen with enhanced neutrophil survival in later stages of the inflammatory response due to production of ROM and cytotoxic granule components.

Spontaneous (constitutive) neutrophil apoptosis is an intrinsic process, meaning that the signal initiating apoptosis originates within the cell.<sup>17</sup> Mitochondria are an essential component of spontaneous neutrophil apoptosis. Mitochondrial proteins known as SMACs are released into the cytosol following increased permeability resulting from mitochondrial interaction with apoptotic proteins.<sup>25</sup> SMACs bind to IAPs and deactivate them, allowing apoptosis to proceed.

The IAPs normally suppress the activity of caspases, cysteine proteases that cleave intracellular substrates and carry out degradation of the cell, ultimately leading to apoptosis.

Depolarization of the mitochondrial membrane, induced as a result of loss of anti-apoptotic BCL2 family proteins such as MCL1<sup>31</sup> and BCL2A1,<sup>17</sup> ROM, or DNA damage results in release of pro-apoptotic factors such as cytochrome c.<sup>32</sup> When cytochrome c is released from mitochondria, it associates with APAF-1 and procaspase 9 to form the apoptosome.<sup>32</sup> This heptameric complex recruits and activates caspases, resulting in cell death by apoptosis.

Many proteins are known to regulate spontaneous neutrophil apoptosis, and several examples are provided here. SHIP-1 is an intracellular protein critical for signal transduction during neutrophil apoptosis.<sup>33</sup> It is recruited to the plasma membrane of neutrophils after ligation of CD18,<sup>34</sup> and this initiates an anti-apoptotic signaling cascade. BAX is a BCL2 family member that promotes neutrophil apoptosis via its interaction with mitochondria.<sup>17</sup> Upon translocation to mitochondria, BAX is cleaved by calpain-1, a nonlysosomal, intracellular cysteine protease, to an 18-kDa fragment unable to interact with BCL2 family members, thereby promoting apoptosis.<sup>35,36</sup> Beyond its interaction with BAX, release of calpain-1 from neutrophil mitochondria can activate other pro-apoptotic factors or degrade anti-apoptotic proteins.<sup>37</sup>

In contrast to spontaneous neutrophil apoptosis, FAS-mediated, TNF- $\alpha$ -mediated and TRAIL-mediated apoptosis occur via extrinsic pathways, as an extracellular signal initiates apoptosis.<sup>17</sup> Interaction of FAS (CD95) with FAS ligand initiates neutrophil apoptosis in a caspase-dependent manner. Because neutrophils express FAS and soluble FAS ligand, neutrophil apoptosis is triggered through both autocrine and paracrine pathways.<sup>17</sup> TNF- $\alpha$  possesses both pro-apoptotic and anti-apoptotic activity towards neutrophils, depending on its concentration.<sup>7</sup> At low concentrations ( $\leq 0.1$  ng/ml), TNF- $\alpha$  delays PMN apoptosis and elicits production of pro-inflammatory cytokines, while at higher concentrations it initiates apoptosis.<sup>7</sup> TNF- $\alpha$  has been shown to induce both “classical” caspase-dependent and “nonclassical” caspase-independent cell death.<sup>38</sup> When caspases are fully inhibited, mitochondria-derived ROM initiate a “nonclassical” death sequence. Extracellular matrix proteins have been shown to accelerate TNF- $\alpha$  mediated apoptosis.<sup>39</sup> Of the 5 TRAIL receptors, only TRAIL-R1 and TRAIL-R2 are able to induce pro-apoptotic, caspase-dependent signaling.<sup>17</sup> TRAIL expressed and secreted by neutrophils acts in an autocrine manner to induce apoptosis<sup>40</sup> and appears important for

senescent neutrophils that preferentially home to bone marrow after circulating in peripheral blood.<sup>41</sup>

Recent studies performed evaluating human, rodent and rabbit neutrophils have elucidated additional pathways, cytokines, and enzymes associated with apoptosis. The majority of these studies have been performed *in vitro*; therefore, there may be involvement of additional factors *in vivo*. Suppression of apoptosis in human neutrophils has been demonstrated by various cytokines, including IL-1,<sup>8</sup> IL-2,<sup>42</sup> IL-6 (both alone and via a mechanism involving PAF),<sup>8, 43, 44</sup> IL-8 (both spontaneous and TNF- $\alpha$ -mediated),<sup>45-48</sup> and TGF- $\beta$ .<sup>49</sup> Suppressed apoptosis in human neutrophils has been observed after incubation with several inflammatory mediators, including GM-CSF, G-CSF, fMLP, complement 5a, IFN- $\gamma$ , and LPS.<sup>8</sup> The pro-inflammatory lipid mediator LTB<sub>4</sub> has been shown to suppress constitutive human neutrophil apoptosis.<sup>49</sup> Thrombin-stimulated platelets have been found to inhibit human neutrophil apoptosis, providing novel evidence to support the central role of platelets in inflammation.<sup>50</sup> NF- $\kappa$ B is a key element in apoptosis regulation in several immune modulating cells and prevents soluble fibrinogen induced apoptosis in human neutrophils with the participation of MAPK ERK 1/2.<sup>51</sup> Inhibition of human neutrophil apoptosis and induction of TRAF-1 by LPS and TNF- $\alpha$  is NF- $\kappa$ B dependent.<sup>52</sup> TRAFs appear to be mediators of apoptosis through signaling pathways for their production in stimulated neutrophils.

Using a peritoneal inflammation model in mice, it has been shown that IFN- $\gamma$  controls neutrophil infiltration and modulates IL-6 signaling through its soluble receptor to promote neutrophil apoptosis and clearance.<sup>53</sup> When evaluating sterile and polymicrobial bacterial peritonitis models in mice, no increase in immediate peripheral leukocyte apoptosis occurred in either model. However, an increase in delayed peripheral leukocyte apoptosis was observed by 18 hours in both models.<sup>54</sup> Coculture of cells from 2- or 5-day-old wounds in rats, or of macrophages purified from such preparations, with neutrophil-rich wound cell populations obtained 1 day after wounding increased neutrophil apoptosis greater than 3-fold.<sup>55</sup> These findings demonstrated that wound macrophages, as opposed to other macrophage populations, can use an integrin- and membrane-bound TNF- $\alpha$ -dependent effector mechanism to actively induce neutrophil apoptosis.<sup>55</sup>

In rabbits, neutrophils in the circulation with little or no L-selectin on their surfaces have features consistent with apoptosis (DNA fragmentation) to a greater degree than those cells

expressing high levels of L-selectin.<sup>56</sup> This suggests that the level of L-selectin expression can serve as a marker for cell age, and that older circulating neutrophils are prone to undergoing apoptosis.<sup>56</sup>

## 2. RELATED DISEASE

Studies have shown that neutrophils undergo apoptosis at sites of inflammation *in vivo*, which helps to resolve inflammation.<sup>57,58</sup> Dysregulation of neutrophil apoptosis is an important factor in various diseases, such as pulmonary inflammation, rheumatoid arthritis, and glomerulonephritis.<sup>59,60</sup> This abnormality is also thought to contribute to an exaggerated inflammatory response in ARDS and MODS in critically ill patients.<sup>47,48</sup> Delayed *in vitro* apoptosis has been reported in circulating neutrophils from human patients with burn injury, systemic inflammatory response syndrome, and sepsis.<sup>61-64</sup> Neutrophils isolated from human patients who sustained moderate to severe blunt trauma injury exhibited delayed apoptosis up to 72 hours post injury.<sup>65</sup> Presumably, the delay in neutrophil apoptosis in these patients allows prolongation of neutrophil function as an important part of a significant systemic response to inflammation and infection. Neutrophils maintain the ability to release clinically significant amounts of chemokines which may help recruit additional neutrophils to the inflammatory site by paracrine mechanisms.<sup>66,67</sup> These chemokines, which are antiapoptotic, help to promote survival of neighboring neutrophils through paracrine mechanisms.<sup>48</sup>

Several recent studies have evaluated the relationship between neutrophil apoptosis and human intestinal injury, with some interesting findings. Transepithelial migration has been shown to induce downregulation of proapoptotic protein expression in transmigrated neutrophils, resulting in increased lifespan.<sup>68</sup> Intestinal epithelial cells have been shown to modulate neutrophil response to bacteria and hypoxia/reoxygenation insults, resulting in production of activated neutrophils with a prolonged lifespan, which may promote remote organ failure.<sup>69</sup> Apoptosis in Crohn's patient neutrophils was delayed in suspension and accelerated in adhesion on fibronectin, which was the opposite of the apoptosis patterns in control cases.<sup>70</sup> These results demonstrated for the first time, by direct experimental evidence, that apoptosis in Crohn's patient neutrophils is regulated differently from that of control neutrophils and potentially reflects the variation between a chronic systemic inflammatory response in Crohn's patients and a non-inflammatory systemic state of healthy patients.

### 3. *EFFECTS OF SURGERY*

Several human studies have evaluated the relationship between neutrophil apoptosis and performance of surgical procedures. In a population of 27 patients undergoing elective surgery under general anesthesia, a significantly increased frequency of apoptotic neutrophils in peripheral blood was present at 12 hours postoperatively compared to preoperatively; however, overall measurements returned to preoperative values 24 hours after surgery.<sup>71</sup> This finding likely reflects an appropriate adaptive response of the host to surgical trauma, which is directed at down-modulating surgical inflammation and limiting the risk of exaggerated tissue injury by neutrophils.<sup>71</sup>

In 8 patients undergoing bilateral sagittal split ramus osteotomy under general anesthesia, the Fas-induced apoptotic response levels in peripheral blood neutrophils 1 day after surgery following exposure to autogenous plasma were significantly suppressed compared to levels at 2 days preoperatively and 5 days postoperatively.<sup>72</sup> This finding points toward the importance of the neutrophil's environment, in this case plasma, as a source of inhibitory factors of apoptosis. In a study of peripheral blood neutrophils after elective orthopedic surgery under epidural anesthesia in 6 patients, apoptosis was significantly inhibited within one hour of surgical incision, and this effect persisted to 24 hours after surgery.<sup>73</sup> IL-6 was notably increased 24 hours postoperatively and at that concentration, inhibited apoptosis of normal neutrophils.<sup>73</sup> Potentially, the reduction in apoptosis may initially be due to release of more immature neutrophils into the circulation due to a surgical stimulus; however, this study demonstrated that by 24 hours post surgery, apoptosis inhibition is at least partly due to circulating plasma factors.

### **B. PERIPHERAL BLOOD NEUTROPHIL CHANGES IN HORSES WITH GASTROINTESTINAL DISEASE**

In contrast to human medical research, where numerous studies have been performed evaluating neutrophil response to systemic inflammatory stimuli, sepsis, and elective surgery, limited studies have been performed evaluating peripheral blood neutrophil changes in horses. To date, these studies have been limited respiratory disease,<sup>74</sup> endometritis,<sup>75</sup> and gastrointestinal disease.<sup>76, 77</sup> Decreased neutrophil deformability has been observed in horses with intestinal lesions compared to control horses.<sup>76</sup> This change corresponded with the severity of illness in these cases.<sup>76</sup> Neutrophil activation by chemoattractants involves cytoskeleton reorganization,

which increases cytoplasmic stiffness. A potential relationship appears to exist between endotoxemia and neutrophil deformability based on greater decreases in neutrophil deformability in non-surviving horses in the group with intestinal lesions.<sup>76</sup>

Neutrophil activation was compared in horses with naturally occurring colic associated with strangulating obstruction, nonstrangulating obstruction, or inflammatory bowel disease.<sup>77</sup> No evidence of activation was observed in horses with nonstrangulating obstructions whereas there was consistent evidence of activation in horses with inflammatory bowel disease. Evidence of neutrophil activation included decreased deformability, increased CD11-CD18 expression, increased size, and decreased granularity.<sup>77</sup> Variable results were obtained in horses with strangulating obstructions, however, changes in neutrophil deformability, size and granularity correlated with an adverse outcome, indicating that activated neutrophils are likely a negative prognostic indicator.<sup>77</sup>

A significantly higher plasma level of MPO, a specific enzyme of neutrophil azurophilic granules with strong oxidative activity, has been observed in horses operated for strangulating obstruction of the large intestine compared to horses with nonstrangulating displacement of the large intestine.<sup>78</sup> The authors of this study stated that the high MPO values in the strangulation cases undoubtedly indicated that neutrophil activation had occurred, and suggested that the difference in MPO between the groups could be partially explained by the release of LPS from ischemic intestine. The high MPO levels in plasma were associated with a decrease in total leukocyte count, indicating that activation can occur even when neutrophils are trapped in organs, and that degranulation products can be released into the general circulation.<sup>78</sup>

Horses with gastrointestinal disease often exhibit signs of sepsis and endotoxemia as a result of the pathologic processes occurring within the gastrointestinal tract. During the initial stages of gram-negative sepsis, neutropenia occurs due to vascular margination. During the latter stages, mononuclear-derived factors (TNF- $\alpha$ , IL-1, GCSF and GMCSF) enhance myeloid proliferation in the bone marrow, causing neutrophilia.<sup>79</sup> Neutrophils often exhibit toxic changes (cytoplasmic vacuolation, toxic granules) and immature (band) neutrophils appear.

During sepsis and endotoxemia, cytokines activate integrins on neutrophils' surfaces, causing firm adhesion to the endothelium and ultimately leading to neutrophil transmigration.<sup>80</sup> The neutrophilic oxidative burst for bacterial killing is activated by the presence of LTB<sub>4</sub>, activated complement components, and antigen-antibody complexes.<sup>81</sup> The oxidants produced

by neutrophils are capable of killing bacteria and degrading endotoxin, but they are also powerful mediators of host endothelial and tissue injury.<sup>82</sup>

## **C. MECHANISMS OF EQUINE INTESTINAL INJURY AND THE ROLE OF THE NEUTROPHIL**

While only a small amount of work has been performed evaluating peripheral blood neutrophil changes in relation to equine gastrointestinal disease, more study has been devoted to exploring the relationship between neutrophils and their behavior within the equine intestine. To comprehend this relationship, there must first be a clear understanding of the mechanisms of equine intestinal injury, which include distention, ischemia, and reperfusion.

### I. Intestinal injury: distention

In the equine small intestine, the degree of intraluminal distention is associated with survival. Horses surviving surgery for small intestinal obstruction have significantly lower intraluminal pressure in the intestinal segment proximal to the primary lesion compared to nonsurvivors.<sup>83</sup> As intraluminal pressure increases, it is transmitted through the interstitium to the veins, resulting in venous outflow obstruction.<sup>84-86</sup> Increased venous pressure increases capillary hydrostatic pressure causing excessive capillary filtration, with subsequent edema and increased interstitial pressure. Eventually, the increased interstitial pressure collapses capillaries and venules resulting in compromised blood flow.<sup>86</sup> More specifically, increased venous pressure reflects back through the capillary bed, causing a myogenic reflex that increases arteriolar tone and causes vasoconstriction which hypothetically results in decreased mural blood flow. This increased vascular resistance results in a reduction in the number of perfused capillaries.<sup>87, 88</sup> Increased capillary permeability during these events occurs due to endothelial cell damage resulting from cellular hypoxia and reperfusion during intestinal distention and decompression.<sup>89</sup> Increased permeability can also persist after decompression due to endothelial cell response to reperfusion. Retraction of endothelial cells and gap formation are caused by various inflammatory mediators (histamine, prostaglandins, leukotrienes, platelet-activating factors, or cytokines).<sup>90</sup> These mediators can originate from leukocytes adhering to vascular endothelium or from stimulated endothelial cells.<sup>89</sup>

The small intestinal seromuscular layer is the most severely affected during luminal distention. The serosa undergoes mesothelial cell loss, neutrophil infiltration, and edema both during distention and after decompression, suggesting an inflammatory reaction as the result of reperfusion injury.<sup>89</sup> Small intestine with luminal distention proximal to strangulated lesions can also undergo seromuscular injury even though it grossly appears normal.<sup>91</sup> Experimentally, seromuscular injury and inflammation can result in adhesion formation. Compared to the small intestine, the large colon is resistant to seromuscular injury resulting from comparable ischemia and reperfusion.<sup>92</sup>

## II. Intestinal injury: ischemia

Ischemic injury to the intestine occurs at the cellular level. The villus tip of the small intestine is most susceptible to mucosal ischemic injury. During low blood flow, capillary architecture in the villus creates a counter current exchange mechanism resulting in ischemia at the villus tip. Oxygen from the central arteriole diffuses across the lamina propria to the villous venous structure, thereby depriving the villus tip of oxygen. Hypoxia of the villus tip results in vascular injury and alterations in epithelial cells. The mucosal epithelium of the small intestine is especially vulnerable to hypoxic injury due to the high energy requirements of Na<sup>+</sup>/K<sup>+</sup> ATPase, an electrogenic and transmembrane enzyme which is required for epithelial ion and nutrient transport. During hypoxia, oxidative phosphorylation is lost, inhibiting the Na<sup>+</sup>/K<sup>+</sup> ATPase transfer system due to diminishing ATP concentration. Cytosolic pH decreases as lactic acid and inorganic phosphates accumulate from anaerobic glycolysis. Fluid leakage from capillaries at the tip of the villus forms a fluid-filled separation of the epithelial cells from the basement membrane known as Gr uenhagen's space.<sup>93</sup> The epithelium sloughs first from the villus tips and advances toward the crypts, which are the last component of the intestinal mucosa to undergo morphologic injury.<sup>94-96</sup> Early morphologic changes occurring in the large colon under ischemic conditions are similar to those described in the small intestine, with initial loss of the more superficially located cells, followed by sloughing within the crypts.<sup>97,98</sup>

## III. Intestinal injury: reperfusion

Reperfusion injury refers to tissue damage caused when blood supply returns to the tissue after a period of ischemia. Inflammation and oxidative damage occur as a result of the condition

created by return of oxygen and nutrients to previously hypoxic tissue during restoration of blood flow.<sup>99</sup> Oxidative stress occurs rather than restoration of normal function. During ischemia, hypoxanthine accumulates due to ATP utilization.<sup>100, 101</sup> During reperfusion, xanthine oxidase catalyzes the conversion of hypoxanthine to xanthine and in the presence of oxygen adds a single additional electron to oxygen which results in production of superoxide.<sup>100</sup> In addition to inflicting oxidative tissue damage, superoxide is important in the generation of neutrophil chemoattractants.<sup>94, 95</sup> Superoxide interacts with lipid membranes, triggering arachidonic acid metabolism. As an example, the arachidonic acid metabolite LTB<sub>4</sub>, produced from the interaction of superoxide and lipid membranes, is a chemoattractant and also activates neutrophils.<sup>102</sup>

As superoxide is created, other ROM such as hydrogen peroxide and hydroxyl radical are generated via the activity of NADPH oxidase in sequestered neutrophils.<sup>103</sup> Reactive oxygen metabolites (mainly hydroxyl radical and hydrogen peroxide) cause mucosal injury directly by damaging cellular membranes via lipid peroxidation which inactivates enzymes and damages nucleic acids leading to DNA strand breakage. ROM cause indirect mucosal injury by disrupting intracellular calcium homeostasis.<sup>104</sup> Injured endothelial cells can elaborate PAF, which attracts and primes neutrophils for the generation of ROM.<sup>105</sup> Reperfusion injury is also characterized by a loss in NO produced by the vascular endothelium. The loss of NO results in upregulation of endothelial adhesion molecules, which promotes neutrophil adherence in the capillaries and venules.<sup>106</sup>

It is believed that neutrophil-derived oxidants mediate the majority of mucosal and microvascular injury associated with reperfusion of ischemic intestine.<sup>107-109</sup> Some evidence has indicated that resident interstitial granulocytes in the intestine contribute more significantly to the increased mucosal permeability associated with reperfusion injury than those granulocytes recruited from the vascular space.<sup>107</sup> However, significant attenuation of damage caused by experimental ischemia-reperfusion in rat small intestine occurred when the intestine was reperfused with a perfluorochemical solution free of leukocytes.<sup>110</sup> The protective effects of this solution were eliminated when neutrophils were added to the perfusate.

The addition of neutrophil adhesion to increased microvascular permeability creates a no-reflow phenomenon with decreased flow after an initial hyperemic phase of reperfusion. This

cyclic event initiates local ischemia and can result in tissue inflammation and subsequent necrosis.

Reperfusion injury after low-flow ischemia creates a similar response to that of total vascular occlusion. Vascular leakage, neutrophil adhesion to the endothelium, neutrophil migration, and no-reflow phenomenon occur.<sup>111</sup> The inflammation created by experimentally induced reperfusion injury is associated with postoperative intestinal adhesions and most likely with ileus in naturally occurring cases of equine acute abdominal disease.<sup>91, 112, 113</sup>

#### IV. Neutrophil response to intestinal injury: serosa and myenteric layers

During experimental low flow ischemia with reperfusion in equine small intestine, neutrophils migrate into the serosa. Additionally, substantial neutrophilic inflammation in the myenteric layers of equine small intestine by 18 hours post laparotomy was present following experimentally induced ischemia and reperfusion, as well as after bowel manipulation only.<sup>91</sup> Because the inflammatory infiltrate into the small intestine can increase for at least 48 hours after surgery, smooth muscle activity may be suppressed, leading to signs of postoperative ileus.<sup>114</sup>

Significantly more neutrophils have been found infiltrating the serosa of resected small intestine compared to control tissues, and a significant increase in MPO has been identified in the middle of strangulating small intestinal lesions.<sup>115</sup> Similar to experimental ischemia with subsequent reperfusion, a significant increase in neutrophil numbers was found in proximal resection margins of all small intestinal tissue layers in horses with naturally occurring strangulating obstruction compared with control horses.<sup>91</sup> This finding demonstrates that reperfusion injury (as indicated by the increased neutrophil numbers) occurs in normal appearing intestine adjacent to a devitalized segment and highlights the difficulty in deciding location of resection margins during surgery.

#### V. Neutrophil response to intestinal injury: mucosa

Neutrophil migration into small intestinal mucosa both oral and aboral to the primary lesion has been observed and indicates a systemic response to the localized ischemia.<sup>6</sup> In the large colon, significantly greater mucosal neutrophil numbers were observed in horses undergoing 6 hours of ischemia or 3 hours of ischemia and 3 hours of reperfusion compared to sham-operated horses.<sup>116</sup> The use of leukocyte scintigraphy in this study revealed a significant

increase in mucosal radioactivity by 1 hour of reperfusion and this activity remained increased throughout the reperfusion period. This increased radioactivity was likely attributable to increased mucosal neutrophil infiltration.<sup>116</sup>

Within equine colonic submucosal venules, calprotectin positive cells and H and E stained neutrophils increased with duration of ischemia and peaked after 30 minutes of reperfusion.<sup>117</sup> Calprotectin is an inflammatory marker originally discovered as an antimicrobial protein in the cytoplasm of neutrophil granulocytes. Based on the results of this study, immunohistochemical staining for calprotectin can be used to demonstrate neutrophil accumulation and migration in the large colon following ischemia and reperfusion. After 2 hours of ischemia, neutrophils began migrating into the colonic mucosa towards the epithelium, with peak infiltration observed after 18 hours of reperfusion.<sup>117</sup> The results of these studies show that neutrophils likely contribute to reperfusion injury of the large colon in addition to the small intestine.

#### VI. Neutrophil response to intestinal injury: peritoneal fluid

Neutrophils increase in the peritoneal fluid of horses with naturally occurring intestinal obstruction and those animals undergoing exploratory abdominal surgery.<sup>118</sup> The ratio of neutrophils to mononuclear cells also increases. Peritoneal fluid parameters have been evaluated in relation to equine gastrointestinal disease in two recent studies.<sup>119, 120</sup> The percentage of apoptotic cells, particularly neutrophils, was found to be increased in a series of horses with gastrointestinal disease. However, only horses with small intestinal strangulating obstruction had a significantly increased percentage of necrotic cells.<sup>119</sup> These findings indicate that neutrophil apoptosis may be increased in response to inflammatory mediators produced in the local environment; for example, increased TNF- $\alpha$  concentrations could initiate apoptosis.

In another case series, horses with strangulating obstruction of the large intestine, strangulating obstruction of the small intestine, or inflammatory bowel disease had significantly higher MPO levels in peritoneal fluid than did horses with nonstrangulating obstruction of the large intestine or healthy horses.<sup>120</sup> In this study, a high level of MPO in the peritoneal fluid in strangulating obstructions was associated with necrotic bowel and a poor prognosis. However, MPO level was not correlated with the neutrophil count.<sup>120</sup> Activated neutrophils may degranulate and disappear rapidly, so that only the proteases remain. Based on these findings, it

is possible that a MPO assay could be useful in predicting the severity of equine gastrointestinal disease.

## **D. LABORATORY TECHNIQUES**

### I. Cell culture and neutrophil apoptosis

Neutrophil isolation from peripheral blood and subsequent *in vitro* study by maintaining the cells in culture medium is commonly performed to learn more about the process of apoptosis and how it is regulated. After 24 hours of culture in medium with autologous serum, 97.3%  $\pm$  1.9% of human neutrophils from 20 healthy donors remained viable as determined by trypan blue dye exclusion; this number decreased to 36.8%  $\pm$  5.3% after 48 hours of culture.<sup>121</sup> Unlike a number of other cell types, neutrophils undergo spontaneous apoptosis when cultured *in vitro*.<sup>122</sup> Work with human neutrophils demonstrated that by 10 hours in culture, up to 40% of cells had undergone apoptosis, and, over 24 hours in culture, an increasing proportion of human neutrophils from peripheral blood underwent morphological changes characteristic of apoptosis.<sup>123</sup> The rate at which a population of neutrophils becomes apoptotic can be influenced by addition of cytokines and bacterial products,<sup>121, 124, 125</sup> by altered levels of second messengers within the cell,<sup>126</sup> and by manipulating the extracellular environment.<sup>125, 127</sup> The rate of constitutive neutrophil apoptosis has been shown to be decreased in a manner that is inversely related to the cell density per cm<sup>2</sup> of the vessel.<sup>127</sup> Neutrophil survival has been enhanced when the cells were cultured in a medium containing protein<sup>127</sup> as well as under hypoxic conditions.<sup>125</sup>

### II. Available assays for measuring apoptosis

A variety of assays that can detect apoptosis in cells and cell cultures has been described. The activity of caspase-3 can be measured via commercially available kits and provides a quantitative measure of apoptosis; however, it is not possible to identify the specific cells undergoing apoptosis.<sup>128</sup> LysoTracker Red is a dye that accumulates in the acidic intracellular compartments and regions where there is a high amount of phagolysosomal activity resulting from engulfment of apoptotic bodies by adjacent cells.<sup>129</sup> This technique is inexpensive and fast, and mild staining conditions allow preservation of antigens required for cell identification.<sup>128</sup> However, the potential for false positives exists for cells with acidic compartments such as macrophages and oocytes.<sup>128</sup> Mitochondrial assays can detect dysfunctions triggering the onset

of apoptosis; although these can be utilized in living cells, it is important to remember that mitochondrial events occur in both apoptosis and necrosis, and distinguishing between the two can be challenging.<sup>128</sup>

The TUNEL technique assays the regularly sized DNA fragments that accumulate and remain concentrated in packages surrounded by intact nuclear membranes.<sup>128</sup> Studies have shown that this technique cannot always distinguish apoptotic and necrotic cells<sup>130, 131</sup> and that cells in the process of active gene transcription can also yield false positive TUNEL results.<sup>132</sup> The TUNEL technique is also quite expensive. Because of the potential for false positive results, the TUNEL technique is often paired with another assay.

Annexin V assays detect apoptotic cells fairly early in the process, when antigens required for cell identification may still be intact, and allow for double and triple labeling of cells when necessary.<sup>128</sup> They are expensive when whole animal studies are undertaken. Apoptosis in peripheral blood neutrophils from horses has been effectively detected by an Annexin V FITC propidium iodide PI assay, and good correlation was found between this assay and light microscopy with an acceptably low mean difference.<sup>133</sup>

### *1. THE PE ANNEXIN V 7-AAD ASSAY*

In apoptotic cells, the membrane phospholipid PS translocates from the inner to the outer leaflet of the plasma membrane, resulting in expression of PS to the extracellular environment. Annexin V is a 35-36 kDa calcium dependent phospholipid-binding protein with high affinity for PS, and binds to cells with exposed PS.<sup>134</sup> Because Annexin can be conjugated to the fluorochrome PE, it serves as a sensitive probe of flow cytometric analysis of cells that are undergoing apoptosis while retaining its high affinity for PS.<sup>134</sup> Since PS externalization occurs in the earlier stages of apoptosis, PE Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes.<sup>134</sup>

PE Annexin V staining precedes the loss of membrane integrity seen with later stages of cell death. Staining with PE Annexin V is usually performed in conjunction with a vital dye such as 7-AAD to allow identification of early apoptotic cells; cells with intact membranes exclude 7-AAD, while membranes of dead and damaged cells are permeable to 7-AAD.<sup>134</sup> From a practical standpoint, this means that viable cells are negative for both PE Annexin V and 7-

AAD; early apoptotic cells are PE Annexin V positive and 7-AAD negative; and late apoptotic or dead cells are positive for both stains.

### III. Flow cytometry

Flow cytometry is a powerful tool allowing measurement of characteristics of single cells suspended in a fluid stream. A basic flow cytometer consists of five main components: a light source (laser), a flow cell, optical components to focus light of different colors on to the detectors; electronics to amplify and process the resulting signals; and a computer. While most scientific procedures obtain average values for an entire population, flow cytometric analysis permits individual measurements on each particle within the suspension in turn.

Flow cytometry allows measurement of several parameters on tens of thousands of individual cells within a few minutes. The properties measured include a particle's relative size, granularity, and fluorescence intensity. These characteristics of flow cytometry demonstrate why it is such a powerful tool in evaluation of neutrophil apoptosis.

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# **The Role of Neutrophil Apoptosis in Horses with Acute Abdominal Disease**

**Reasons for performing study:** Neutrophils, the chief phagocytic cells in mammalian species, are critical in the inflammatory response. Regulation of neutrophil activity occurs through several mechanisms, including apoptosis. Dysfunction of neutrophil apoptosis has been implicated as a cause of organ damage in hyper-inflammatory conditions in human patients. This pilot study investigated circulating neutrophil apoptosis in horses with surgical lesions of the large and small intestine.

**Hypothesis:** Delayed neutrophil apoptosis occurs in peripheral blood of horses undergoing surgery with acute abdominal disease, compared with elective orthopedic cases. Within the group of horses with intestinal injury, delayed peripheral blood neutrophil apoptosis will be more pronounced in horses with potential signs of postoperative complications (tachycardia, leukopenia) compared to horses with no signs of postoperative complications.

**Materials and Methods:** Adult horses undergoing surgery for acute abdominal disease (N=10) and elective orthopedic surgery (control) (N=10) were studied. Peripheral blood was collected preoperatively and 24 hours postoperatively. Neutrophils were counted and were subsequently isolated using a Percoll gradient. Cell populations undergoing apoptosis were determined by flow cytometry using a commercially available staining kit (Annexin V-PE Apoptosis Detection Kit I, BD Pharmingen™).

**Results:** No significant differences in percentages of apoptotic neutrophils between groups were found. Delayed neutrophil apoptosis was not more pronounced in horses with abnormal postoperative parameters. A significantly lower percentage of neutrophil apoptosis was present in horses with strangulating intestinal lesions versus nonstrangulating lesions.

**Conclusion:** Horses with strangulating intestinal disease are susceptible to less apoptosis of circulating neutrophils. Decreased neutrophil apoptosis may be responsible for an increased inflammatory response in horses with injured intestine. Investigating therapeutic interventions to

hasten neutrophil apoptosis in horses with strangulating intestinal lesions may provide valuable new methods to prevent morbidity and mortality in these patients. Future studies designed to increase understanding of the role of neutrophil apoptosis in equine acute abdominal disease may guide the use of new treatments as they become available.

## Introduction

Equine intestinal disease has been reported as one of the most common causes of death in some horse populations.<sup>1,2</sup> The annual number of intestinal disease cases has generally been reported as 4-10 cases per 100 horses,<sup>1,3</sup> although up to 46 cases per 100 horses per year have been reported.<sup>4</sup> While most cases resolve spontaneously or respond to medical management, 2-10% of equine intestinal disease cases have been reported to require surgical intervention.<sup>5,6</sup>

Surgery is required to resolve strangulating intestinal obstruction, some types of nonstrangulating intestinal obstruction, and nonstrangulating intestinal infarction. Horse survival after surgery is related to the amount of intestinal injury as predicted by the length of intestine affected, the degree of intestinal distention and the resulting systemic shock.<sup>7-9</sup>

Morphologic and physiologic abnormalities result from ischemia and reperfusion injury created by the change in blood flow to the intestine, cytokine production in response to ischemia and reperfusion, and alterations in endothelial cell and neutrophil activity. The result is intestinal inflammation, which is associated with loss of motility, systemic shock and ultimately, in horses that survive after relief of obstruction, intestinal adhesions.<sup>10,11</sup>

Neutrophils, the chief phagocytic cells in the horse, play an important role in creating intestinal injury. Neutrophil activation, adhesion and migration occur during reperfusion of previously ischemic tissue, which is caused by vascular obstruction, decreased flow during intestinal distention, or low flow during shock.<sup>12,13</sup> Reactive metabolic oxidants produced by endothelial cells and neutrophils mediate the majority of mucosal and microvascular injury during reperfusion.<sup>14-16</sup> This injury includes neutrophil adhesion to the endothelium, migration through the capillaries and venules into the interstitium, and plugging of capillaries causing a no-reflow phenomenon.<sup>17</sup> Increased capillary permeability increases interstitial fluid resulting in capillary collapse from increased interstitial pressure.<sup>13</sup> Neutrophil activation and migration have been documented in experimental ischemia-reperfusion and in naturally occurring intestinal obstruction.<sup>18-20</sup>

Apoptosis, or programmed cell death, is one of the principal mechanisms by which neutrophil homeostasis is maintained. When neutrophils undergo apoptosis, they are generally functionally quiescent<sup>21</sup> allowing other phagocytes, principally macrophages, to ingest them without subsequent destruction due to high levels of damaging granule contents. Delayed apoptosis may be desirable during the early stages of the inflammatory response for recruitment

to appropriate tissue insults. Neutrophils maintain the ability to release clinically significant amounts of antiapoptotic chemokines which may help recruit additional neutrophils to the inflammatory site and promote survival of neighboring neutrophils by paracrine mechanisms.<sup>22</sup> Inhibition of neutrophil apoptosis by a variety of inflammatory mediators can prolong functional integrity *in vitro*.<sup>21</sup> Enhanced neutrophil survival, or a delay of apoptosis, has been reported *in vitro* from human patients with burn injury, systemic inflammatory response syndrome, sepsis, and moderate to severe blunt trauma injury.<sup>23-27</sup> While prolonged functionality may be beneficial in some instances, it may be detrimental by contributing to an exaggerated inflammatory response in acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS) in critically ill human patients.<sup>22, 28</sup>

Neutrophil isolation from peripheral blood and subsequent *in vitro* study by maintaining the cells in culture medium is commonly performed in human medicine to learn more about the process of apoptosis and how it is regulated. *In vitro* neutrophil apoptosis can be measured by several methods, including Annexin V assays. Apoptosis in peripheral blood neutrophils from horses has been effectively detected by an Annexin V fluorescein isothiocyanate (FITC) propidium iodide (PI) assay, and good correlation was found between this assay and light microscopy with an acceptably low mean difference.<sup>29</sup> Annexin V assays detect apoptotic cells fairly early in the process due to the high affinity of Annexin V for the phosphatidylserine that has translocated from the inner to the outer leaflets of the plasma membrane as the cell initiates apoptosis. Because Annexin can be conjugated to a fluorochrome, it serves as a sensitive probe of flow cytometric analysis of cells that are undergoing apoptosis while retaining its high affinity for phosphatidylserine.<sup>30</sup>

Staining with PE Annexin V is usually performed in conjunction with a vital dye such as PI or 7-amino-actinomycin (7-AAD) to allow identification of early apoptotic cells. Cells with intact membranes exclude the vital dye, while membranes of dead and damaged cells are permeable to it.<sup>30</sup> Using 7-AAD as the vital stain, this means that viable cells are negative for both PE Annexin V and 7-AAD; early apoptotic cells are PE Annexin V positive and 7-AAD negative; and late apoptotic or dead cells are positive for both stains.

Although it has been widely researched in human medicine, reports of neutrophil apoptosis in the horse are limited to research during exercise<sup>31</sup> and in septic joints.<sup>32</sup> Detection of peripheral blood neutrophil apoptosis in response to intestinal injury has not been reported in

the horse. We hypothesized that delayed peripheral blood neutrophil apoptosis occurs postoperatively in horses undergoing emergency surgery for intestinal obstruction compared to horses undergoing elective arthroscopy. Within the group of horses with intestinal injury, we hypothesized that delayed peripheral blood neutrophil apoptosis would be more pronounced in horses with potential signs of postoperative complications (tachycardia, leukopenia) compared to horses without postoperative complications.

## **Materials and Methods**

### ***Animals***

Twenty adult horses greater than 2 years of age presenting to the Marion DuPont Scott Equine Medical Center as clinical cases were used after the study design was approved by the Virginia Tech University Institutional Animal Care and Use Committee. Ten horses undergoing surgery for acute abdominal disease and ten horses undergoing elective arthroscopy were selected as they presented to the hospital. Data collected for each case included age, breed and gender; lesion treated at surgery or surgical procedure performed; anesthesia time; and heart rate 12 hours after surgery.

### ***Neutrophil Collection and Isolation***

Peripheral blood (17 ml in 2 BD Vacutainer® Acid Citrate Dextrose (ACD) Solution A tubes) was collected via jugular venipuncture at 2 time points: preoperatively and 24 hours postoperatively. Complete blood count taken preoperatively was used to calculate neutrophil count/ml by multiplying the total white blood cell count by the percentage of neutrophils in the differential. This same calculation was performed using the postoperative sample, from which only white blood cell count and differential were measured.

Once peripheral blood was collected, the ACD tubes were centrifuged (500 g for 8 minutes at 22 degrees C). Most of the resulting plasma was removed and discarded, leaving a small amount to facilitate buffy coat collection. The buffy coat was collected and mixed with 1X Hanks' solution<sub>a</sub> without calcium or magnesium so that the final concentration was 50% cells in plasma and 50% 1X Hanks' solution. Centrifugation of the ACD tubes was repeated to ensure that as much buffy coat as possible was collected.

Percoll<sub>b</sub> was used to make the gradient by which neutrophils were isolated from the peripheral blood samples. Iso-osmotic Percoll was made by mixing 40 ml of stock Percoll with 3.63 ml of 10X Hanks' solution<sub>c</sub> without calcium or magnesium. The 59% Percoll used as the top layer of the gradient was made by mixing 23.6 ml of iso-osmotic Percoll with 16.4 ml of the 1X Hanks' solution. Using a refractometer<sub>d</sub>, the density of the 59% Percoll was checked; if the density was 115-120 n-n<sub>0</sub> x 10<sup>4</sup>, no adjustments were made. If the density was higher than 120 n-n<sub>0</sub> x 10<sup>4</sup>, 1 ml at a time of 1X Hanks' was added to decrease the density until it was in the appropriate range. If the density was lower than 115 n-n<sub>0</sub> x 10<sup>4</sup>, 1 ml at a time of iso-osmotic Percoll was added to increase the density until it was in the appropriate range. The 75% Percoll used as the bottom layer of the gradient was made by mixing 30 ml of iso-osmotic Percoll with 10 ml of 1X Hanks' solution. Using a refractometer, the density of the 75% Percoll was checked; if the density was 140-145 n-n<sub>0</sub> x 10<sup>4</sup>, no adjustments were made. If the 75% Percoll was slightly denser than 145 n-n<sub>0</sub> x 10<sup>4</sup>, no adjustments were made so as to prevent neutrophils from passing through the bottom layer of the gradient.

To make the Percoll gradient, 5 ml of the 59% Percoll were placed in a 15 ml sterile conical tube. The stylet from a 16 gauge intravenous catheter<sub>e</sub> was primed with 75% Percoll via a 5 ml syringe to remove air and prevent disturbance as the gradient was being made. The catheter was placed on the bottom of the conical tube and held steady as the 75% Percoll was slowly injected into the tube, and an effort was made not to introduce any air into the gradient at completion of the injection.

The mixture of buffy coat and 1X Hanks solution was slowly applied to the Percoll gradient using a serological pipette, with care taken to minimally disturb the gradient. Centrifugation was performed (930 g for 40 minutes at 22 degrees C) to separate the cells on the gradient. The mononuclear cells from the top band in the gradient were removed and discarded. The neutrophils were removed from the middle band in the gradient and were placed in 30 ml Dulbecco's Phosphate Buffered Saline (DPBS)<sub>f</sub> without calcium or magnesium for washing. Care was taken to avoid the red blood cell pellets in the bottoms of the tubes when harvesting the neutrophils. The neutrophils in DPBS were then centrifuged (600g for 8 minutes at 22 degrees C). The resulting supernatant was removed, the neutrophils were resuspended in another 30 ml of DPBS, and centrifugation followed by supernatant removal was repeated.

### ***Cell Culture***

Once washing in DPBS was completed, the isolated neutrophils were resuspended in Roswell Park Memorial Institute (RPMI) complete media<sub>g</sub> containing fetal calf serum<sub>n</sub>. See table 1 for a listing of all components of the cell culture media. Typically, the cells were initially resuspended in 5 ml of the media, and more was added if necessary to ensure a final isolated neutrophil concentration of  $1 \times 10^6$  cells/ml.

A small amount (100  $\mu$ l) of the neutrophil suspension was removed. A slide was prepared and stained; to obtain a manual differential cell count of each sample. Manual cell count of 0.4% trypan blue stained cells (50  $\mu$ l) was performed using a hemocytometer and cell counts were adjusted to  $1 \times 10^6$  cells/ml using the cell culture media. This concentration of neutrophils was required for performance of flow cytometry with the selected apoptosis staining kit<sub>j</sub>. Percentage of recovered neutrophils was calculated using the results of the manual cell count divided by the total neutrophil count from the peripheral blood sample. Neutrophils in the peripheral blood samples were identified as toxic if they contained Döhle bodies, basophilic cytoplasm, and intracytoplasmic vacuoles.

Neutrophils were incubated in the cell culture medium (5% CO<sub>2</sub> at 37 degrees C) for 24 hours,<sup>33</sup> after which they were prepared for flow cytometry.

### ***Flow Cytometry***

After centrifugation (600 g for 8 minutes at 22 degrees C), neutrophils were washed twice in cold PBS (30 ml per wash with resuspension prior to each centrifugation at 600 g for 8 minutes at 22 degrees C). The neutrophils were resuspended in 1X binding buffer, made according to kit instructions, at a concentration of  $1 \times 10^6$  neutrophils/ml in 4 aliquots: one containing unstained cells, one containing cells stained with only PE Annexin V, one containing cells stained with only 7-AAD, and one containing cells stained with both stains. After 100  $\mu$ l ( $1 \times 10^5$  cells) of the cell and buffer solution were transferred to each tube, 5  $\mu$ l each of PE Annexin V and 7-AAD were added to the appropriate tubes. The tubes were gently vortexed and were incubated for 15 minutes at room temperature in the dark. After 400  $\mu$ l of binding buffer were added to each tube, all tubes were analyzed by flow cytometry<sub>k</sub> within 1 hour.

Dot plots of forward and side scatter enabled visualization of voltage adjustments in order to move neutrophils into a gated region (see figure 2). The unstained cells, cells stained

with only PE Annexin V and cells stained only with 7-AAD were used to set the voltages. A second dot plot with FL2 on the x axis and FL3 on the y axis was used to demonstrate results for the stained neutrophils (see figure 3). Ten thousand gated events were counted for each sample. Fluorescent compensation was adjusted using the cells stained only with PE Annexin V and the cells stained only with 7-AAD. After the instrument settings had been adjusted, the aliquot containing cells stained with both stains was used to obtain results for each case.

### ***Statistical Analysis***

Data were entered into a computer spreadsheet and analyzed using commercial statistical analysis software. Data were summarized using means and medians as appropriate. A non-parametric method (Mann-Whitney U test) was used to compare the primary outcome variable (percentage of apoptotic neutrophils) between treatment groups. The percentage of postoperative apoptotic neutrophils in strangulating and nonstrangulating intestinal obstruction was also compared using the Mann-Whitney U test. Additional comparisons including anesthesia time between treatment groups, percentage of apoptotic neutrophils pre and postoperatively, and percentages of recovered neutrophils between groups were analyzed using the Mann-Whitney U test. The Pearson product-moment correlation coefficient was used to evaluate the correlation between percentages of neutrophils obtained on the manual differential and on flow cytometric analysis. Correlation between anesthesia times and percentages of apoptotic neutrophils was evaluated using Spearman's rank correlation coefficient. This test was also used to evaluate correlation between apoptotic neutrophils and heart rate 12 hours after surgery as well as correlation between apoptotic neutrophils and white blood cell count measured 24 hours postoperatively. The P value for significance was pre-set at  $P < 0.05$ .

### **Results**

Mean age of the control (arthroscopy) group was 7.8 years (range, 2 years 3 months – 17 years). Mean age of the colic group was 14 years (range, 5 years – 26 years). Warmbloods and Warmblood crosses (n=7) and Thoroughbreds and Thoroughbred crosses (n=7) were the most prevalent breeds (see table 2). The colic group consisted of horses with strangulating (n=5) and nonstrangulating (n=5) lesions. One case with strangulating adhesions had had previous abdominal surgery approximately 18 months prior to surgery in this study; none of the other

abdominal surgery cases had undergone prior surgery. All horses in both groups survived to discharge from the hospital.

The overall percentage of recovered neutrophils in the colic group was significantly less ( $P=0.0080$ ) than the percentage of neutrophil recovery in the control group. There were significantly fewer ( $P=0.0375$ ) neutrophils recovered from the preoperative colic group samples compared to the preoperative control group samples. No significant differences were present when postoperative control and colic group samples were compared ( $P=0.1733$ ), or when all preoperative samples were compared to all postoperative samples ( $P=0.3575$ ) (see table 3). Three of the preoperative samples and 4 of the postoperative samples in the colic group contained toxic neutrophils. A statistically significant ( $P = 0.0320$ ;  $\rho=0.33967$ ) mildly linear relationship was found between percentages of neutrophils obtained on manual differential and on flow cytometric analysis (see figure 4).

No significant differences in percentages of apoptotic neutrophils were present, when the groups were compared as all preoperative samples vs. all postoperative samples ( $P=0.2559$ ); all control cases vs. all colic cases ( $P=0.4488$ ); preoperative control cases vs. postoperative control cases ( $P=0.1988$ ); or preoperative colic cases vs. postoperative colic cases ( $P=0.7624$ ) (see table 4). There was, however, a significantly lower percentage of postoperative neutrophil apoptosis in horses with strangulating intestinal lesions than in horses with nonstrangulating intestinal lesions ( $P=0.0472$ ) (see table 5). No significant difference in anesthesia time was present between the two groups (see table 6).

No significant correlation between anesthesia times and percentages of apoptotic neutrophils existed in both groups evaluated together ( $P=0.8008$ ;  $\rho=0.06024$ ), in the control group ( $P=0.2335$ ;  $\rho=0.41464$ ), or in the colic group ( $P=0.5903$ ;  $\rho=-0.19447$ ). No significant correlation between postoperative heart rates and percentages of apoptotic neutrophils ( $P=0.9574$ ;  $\rho=0.01356$ ) or between postoperative white blood cell count and percentages of apoptotic neutrophils ( $P=0.2217$ ;  $\rho=0.28593$ ) was detected.

## **Discussion**

No significant differences in percentages of apoptotic neutrophils between or among the control and colic groups were present. This result may have been due in part to the low yields of neutrophils occurring in some cases, as evaluation of missing cell populations could have altered

the results. Within the colic group, however, there was significantly less neutrophil apoptosis in the cases with strangulating lesions compared to the cases with nonstrangulating lesions. These findings suggest that neutrophil apoptosis is delayed in association with intestinal inflammation, which has been documented after experimental ischemia and in seromuscular layers 18 hours after surgery, potentially implicating the involvement of intestinal inflammation in the development of postoperative ileus.<sup>18</sup> Furthermore, delayed neutrophil apoptosis may prolong neutrophil function for this inflammatory process. Production of systemic inflammatory mediators such as TNF- $\alpha$  and IL-1 in the postoperative equine acute abdominal disease patient is likely an important stimulus for delayed neutrophil apoptosis, as these cytokines have been shown to play significant roles in systemic inflammatory response in horses<sup>34</sup> and to delay apoptosis in human neutrophils.<sup>35, 36</sup>

Significantly lower percentages of neutrophils were recovered overall in the colic group and in preoperative samples from the colic group, but these results were not consistently associated with toxic neutrophils, as might have been expected due to increased fragility resulting from labilization of lysosomes and autophagic vacuoles in these cells.<sup>33</sup> It is possible that the neutrophils collected from the abdominal surgery cases, while not toxic in most cases, were more likely to be activated owing to some degree of increased systemic inflammation. Activation of human peripheral blood neutrophils in response to a synthetic chemotactic peptide has been shown to decrease cellular density,<sup>35</sup> suggesting that activation of equine peripheral blood neutrophils may have a similar result. Decreased density of equine neutrophils resulting from cellular activation could result in sedimentation changes and reduced cell recovery. Previous work with human neutrophils has shown that activation does not result from incubation with Percoll,<sup>39</sup> supporting the thought that in our study, equine neutrophils were likely activated prior to use of the Percoll gradient.

No significant correlation between anesthesia times and percent apoptotic neutrophils was present. It was thought that anesthesia time could be a confounding variable affecting percentages of apoptotic neutrophils in this study. This may be similar to the significant increase in apoptotic circulating neutrophils 12 hours postoperatively<sup>40</sup> and to the significant decrease in neutrophil apoptosis 24 hours postoperatively<sup>41</sup> in human patients undergoing elective surgery under general anesthesia. Since surgery and anesthesia were performed in all horses in the current study, it is impossible to differentiate the effect of one procedure versus the other. Our

results may have differed had we measured apoptosis sooner; however, general anesthesia and its duration did not appear to have a significant effect on percentages of apoptotic neutrophils in horses undergoing elective surgery compared to horses undergoing emergency abdominal surgery.

There were several limitations in this study. The statistical power was quite low with only 10 horses in each group. Based on the observed rate of apoptosis, the study would have needed at least 165 horses to obtain 0.80 power. Selection of a uniform lesion type would have increased the statistical power of the study and would have potentially allowed for clearer interpretation of results. The low yield of neutrophils in some cases, particularly cases in the abdominal surgery group, was a significant limitation, as evaluation of these missing populations of cells could have altered the results. Apoptosis in peripheral blood neutrophils from horses has been effectively detected by an Annexin V fluorescein isothiocyanate (FITC) propidium iodide (PI) assay, and good correlation was found between this assay and light microscopy with an acceptably low mean difference.<sup>29</sup> Because this correlation is the conclusion from only one previous equine study, work may be needed to establish the repeatability and accuracy of Annexin V assays for use in detecting equine peripheral blood neutrophil apoptosis.

The decreased neutrophil apoptosis seen in the equine cases with strangulating lesions is potentially linked to the systemic inflammatory reaction known to occur in horses in shock due to the response to the injured intestine.<sup>42</sup> The question remains whether intervening in key signaling pathways, such as those involving nuclear factor- $\kappa$ B, mitogen-activated protein kinase, phosphoinositide-3-kinase, Bcl-2, some lipid mediators, and cyclin-dependent kinase<sup>43</sup> to increase neutrophil apoptosis could prevent postoperative complications in horses. By pharmacologically increasing neutrophil apoptosis, intestinal inflammation could hypothetically be decreased, helping to prevent postoperative ileus.

## **Conclusion**

In conclusion, our hypotheses were not supported. There was no significant difference in neutrophil apoptosis between the arthroscopy and abdominal surgery groups, and there were no significant correlations between percentages of apoptotic neutrophils and measured postoperative values (tachycardia, leukopenia). A significantly lower median percentage of postoperative neutrophil apoptosis was observed in horses with strangulating intestinal lesions compared to

horses with nonstrangulating lesions. This finding has potential implications for development of novel treatments to diminish postoperative complications by manipulating neutrophil apoptosis thereby reducing their systemic and local activity. Future studies evaluating peripheral blood neutrophil apoptosis in horses undergoing emergency abdominal surgery should include a greater number of horses, or should focus on one particular lesion to gain more statistical power than the pilot study presented here. Investigation of neutrophil apoptosis in the intestine could provide additional information on neutrophil behavior as it relates to postoperative intestinal inflammation and its consequences. Investigation of *in vivo* apoptosis detection methods using tracers comprised of Annexin V and its derivatives has been reported in the human literature<sup>44</sup> and could provide information about apoptosis in equine peripheral blood neutrophils without the effects of *ex vivo* manipulation.

<sup>a</sup>Hanks' Balanced Salt Solution, Sigma-Aldrich®, USA

<sup>b</sup>Percoll®, Sigma-Aldrich®, USA

<sup>c</sup>Hanks' Balanced Salt Solution 10X, Sigma-Aldrich®, USA

<sup>d</sup>TS Meter Hand-Held Refractometer, Reichert®, USA

<sup>e</sup>Milacath®, 16 gauge x 13 cm (5.25 in), Mila International, USA

<sup>f</sup>HyClone 1X Dulbecco's Phosphate Buffered Saline, Thermo Scientific, USA

<sup>g</sup>Gibco® RPMI 1640, Life Technologies™, USA

<sup>h</sup>Gibco® Fetal Bovine Serum, Qualified, Heat Inactivated, USDA Approved Regions, Life Technologies™, USA

<sup>i</sup>Harleco Hemacolor® Solutions I, II and III, Fisher Scientific, USA

<sup>j</sup>PE Annexin V Apoptosis Detection Kit I, BD Pharmingen™

<sup>k</sup>BD FACSCalibur™

<sup>l</sup>SAS 9.3, SAS Institute, Cary NC

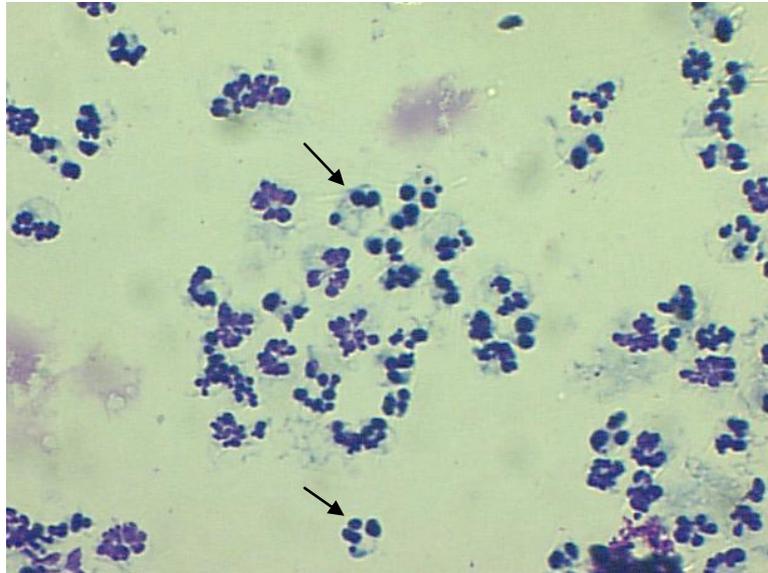
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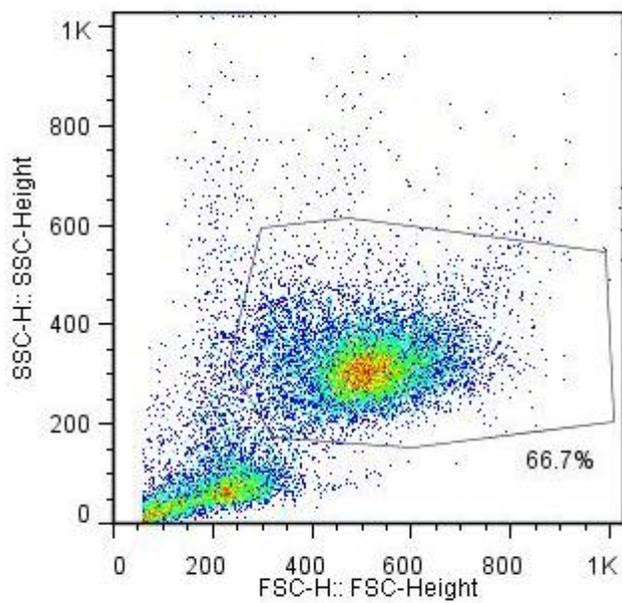
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**Figure 1.** Neutrophils showing apoptotic changes, indicated by arrows. Source: Figure 37C, Wereszka MM, Masters of Science thesis, Virginia Polytechnic Institute and State University, 2007.



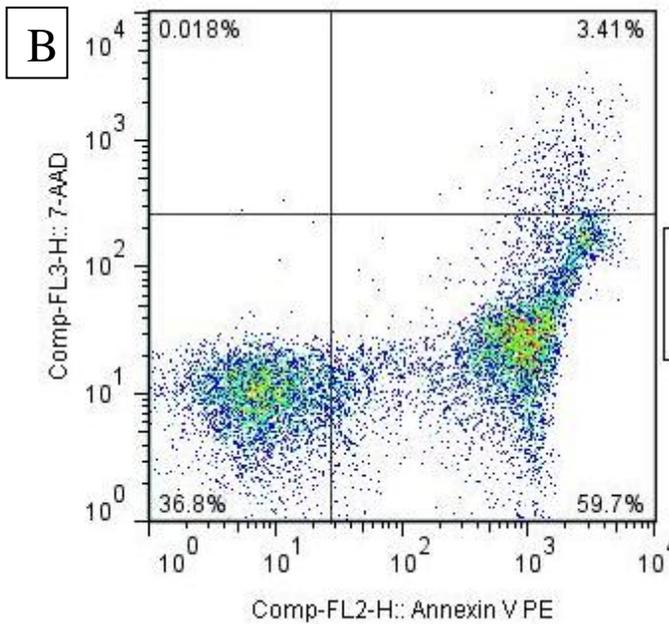
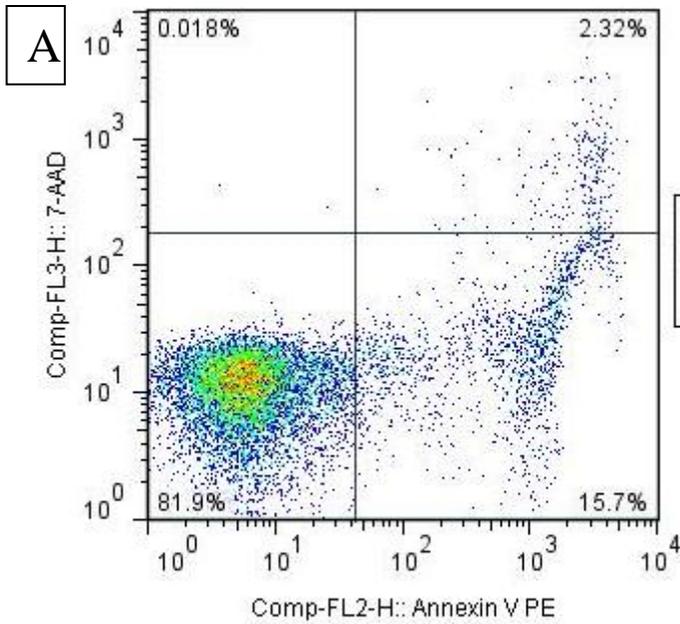
**Figure 2.** Example of a forward scatter and side scatter flow cytometric dot plot used to set voltages and gate neutrophils. Source: K. Krista data



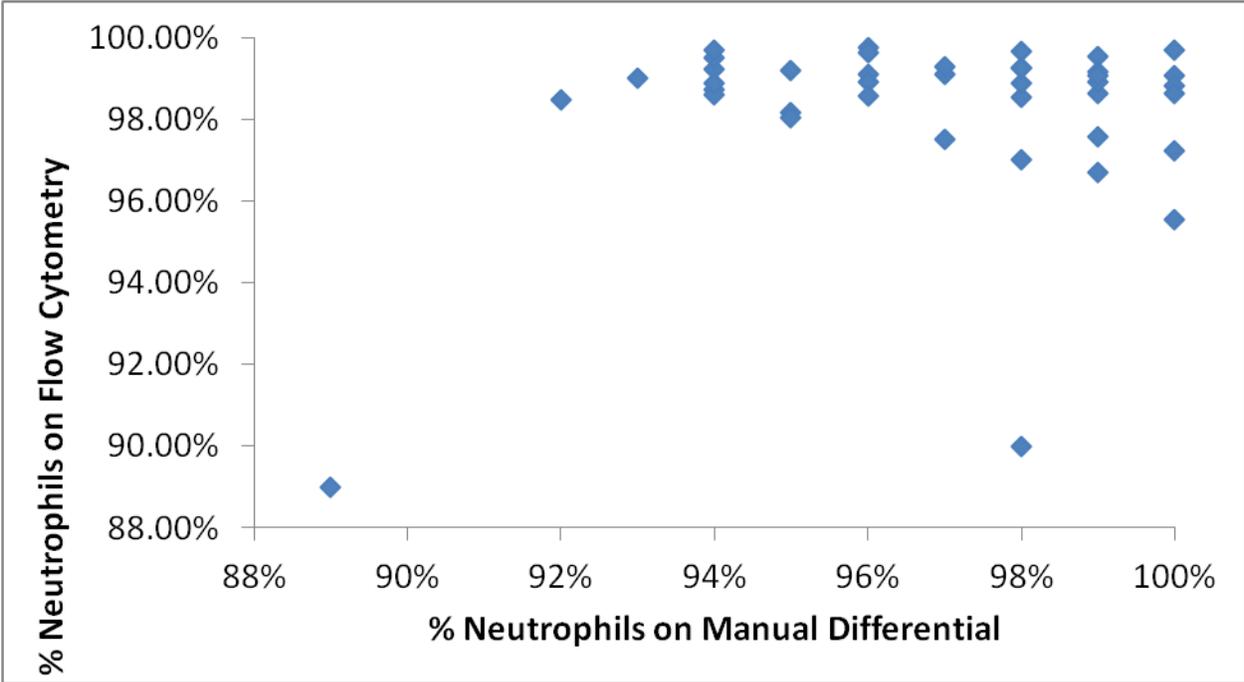
**Figure 3.** Examples of flow cytometric analysis of neutrophils using the Annexin V PE 7-AAD staining kit; apoptotic neutrophils are in the lower right quadrant. Source: K. Krista data

**A)** Lower percentage of apoptotic neutrophils

**B)** Higher percentage of apoptotic neutrophils.



**Figure 4.** Relationship between percentages of neutrophils obtained on manual differential and on flow cytometric analysis ( $\rho=0.33967$ )



**Table 1. Cell Culture Media Components**

| <b><u>Ingredient</u></b>                              | <b><u>Amount</u></b> | <b><u>Manufacturer</u></b> |
|---|----------------------|----------------------------|
| RPMI 1640 + HEPES + glutamine                         | 125 ml               | Life Technologies™         |
| 2-Mercaptoethanol                                     | 0.45 ul              | Sigma-Aldrich®             |
| Penicillin (10,000 IU/ml) and streptomycin (10 mg/ml) | 2.5 ml               | Life Technologies™         |
| L-glutamine (200 mM)                                  | 2.5 ml               | Life Technologies™         |
| Sodium pyruvate (100 mM)                              | 1.25 ml              | Sigma-Aldrich®             |
| Heat inactivated fetal calf serum                     | 12.5 ml              | Life Technologies™         |
| Gentamicin (10 mg/ml)                                 | 2.5 ml               | Life Technologies™         |

**Table 2. Case Information**

| <b><u>Case</u></b> | <b><u>Signalment</u></b>         | <b><u>Surgery/Lesion</u></b>   |
|--------------------|----------------------------------|--|
| 106005             | 3 yo Irish Sport Horse M         | Carpal canal arthroscopy   |
| 106064             | 8 yo Friesian G                  | Right tibiotarsal arthroscopy  |
| 104406             | 7 yo Thoroughbred X G            | Right stifle arthroscopy   |
| 106107             | 2 yrs, 3 months Thoroughbred X G | Right fore fetlock arthroscopy   |
| 102785             | 17 yo Thoroughbred M             | Right stifle arthroscopy   |
| 106632             | 3 yo Westphalian G               | Right hind fetlock arthroscopy   |
| 106663             | 13 yo Oldenburg M                | Left stifle arthroscopy  |
| 103669             | 8 yo Irish Sport Horse G         | Bilateral fore fetlock arthroscopy   |
| 107105             | 12 yo Thoroughbred G             | Right shoulder arthroscopy   |
| 107216             | 4 yo Dutch Warmblood X M         | Bilateral stifle arthroscopy   |
| 105882             | 7 yo Dutch Warmblood G           | Large colon volvulus (360 degrees), right dorsal displacement of the large colon |
| 106007             | 5 yo Warmblood G                 | Right dorsal displacement of the large colon                                     |
| 106208             | 23 yo Thoroughbred G             | Strangulating lipoma   |
| 101251             | 15 yo Thoroughbred G             | Right dorsal displacement of the large colon                                     |
| 105908             | 12 yo Percheron G                | Right dorsal displacement of the large colon                                     |
| 104857             | 9 yo Warmblood M                 | Strangulating adhesions  |
| 107296             | 26 yo Pony X G                   | Strangulating lipoma   |
| 107444             | 7 yo TB M                        | Ileus  |
| 107568             | 18 yo Percheron X M              | Gastric impaction, small intestinal distention                                   |
| 107843             | 19 yo Warmblood G                | Strangulating lipoma   |

**Table 3. Comparisons of Percentages of Recovered Neutrophils**

| <b>Group</b>              | <b>25%</b> | <b>Median</b> | <b>75%</b> |
|---------------------------|------------|---------------|------------|
| All arthroscopy           | 46         | 58*           | 70         |
| All colic                 | 25.5       | 35.5*         | 45         |
| Preoperative arthroscopy  | 46         | 66.5*         | 86         |
| Preoperative colic        | 26         | 32.5*         | 40         |
| Postoperative arthroscopy | 48         | 57.5          | 61         |
| Postoperative colic       | 25         | 39            | 76         |
| All preoperative          | 26.5       | 40.5          | 66.8       |
| All postoperative         | 38         | 49.5          | 64.5       |

\* = statistically significant difference

**Table 4. Comparisons of Percentages of Apoptotic Neutrophils**

| <b>Group</b>              | <b>25%</b> | <b>Median</b> | <b>75%</b> |
|---------------------------|------------|---------------|------------|
| All arthroscopy           | 32.6       | 41.4          | 50.7       |
| All colic                 | 20.1       | 36.7          | 50.9       |
| Preoperative arthroscopy  | 33.5       | 40.4          | 43.5       |
| Preoperative colic        | 23.1       | 36.7          | 40.5       |
| Postoperative arthroscopy | 31.6       | 50.7          | 57.8       |
| Postoperative colic       | 15.7       | 43.6          | 66.3       |
| All preoperative          | 20         | 50.7          | 62.4       |
| All postoperative         | 32         | 39.4          | 42.5       |

\* = statistically significant difference

**Table 5. Comparison of Postoperative Percentages of Apoptotic Neutrophils Within the Colic Group**

| <b>Group</b>             | <b>25%</b> | <b>Median</b> | <b>75%</b> |
|--------------------------|------------|---------------|------------|
| Strangulating lesions    | 14.9       | 18*           | 31.3       |
| Nonstrangulating lesions | 65.1       | 66.3*         | 68.7       |

\* = statistically significant difference

**Table 6. Comparison of Anesthesia Times (minutes)**

| <b>Group</b> | <b>25%</b> | <b>Median</b> | <b>75%</b> |
|--------------|------------|---------------|------------|
| Arthroscopy  | 90         | 112.5         | 135        |
| Colic        | 90         | 135           | 180        |

\* = statistically significant difference

**Appendix A. Complete Protocol for Isolating and Culturing Equine Peripheral Blood Neutrophils and Preparing the Neutrophils for Flow Cytometric Analysis**

**A. Making the Percoll Gradient for Monocyte and Granulocyte Isolation from Equine Blood**

1. Always mix Percoll (by shaking thoroughly) before using.
2. Each gradient will require **5 ml** of final prep of both the 59% and 75% Percoll.
3. Gradient can be made 12-24 hours prior to use.

**4. Iso-osmotic Percoll:**

|                      |               |               |              |              |              |
|----------------------|---------------|---------------|--------------|--------------|--------------|
| <b>Stock Percoll</b> | <b>300 ml</b> | <b>100 ml</b> | <b>50 ml</b> | <b>40 ml</b> | <b>10 ml</b> |
| <b>10X Hanks'</b>    | 27.2 ml       | 9.07 ml       | 4.535 ml     | 3.63 ml      | .907 ml      |

**5. 59% Percoll:**

|                            |               |              |              |              |              |
|----------------------------|---------------|--------------|--------------|--------------|--------------|
|                            | <b>100 ml</b> | <b>50 ml</b> | <b>40 ml</b> | <b>20 ml</b> | <b>10 ml</b> |
| <b>Iso-osmotic Percoll</b> | 59 ml         | 29.5 ml      | 23.6 ml      | 11.8 ml      | 5.9 ml       |
| <b>1X Hanks'</b>           | 41 ml         | 20.5 ml      | 16.4 ml      | 8.2 ml       | 4.1 ml       |

**\*Make sure the Hanks' is calcium and magnesium free**

6. Check density of the 59% Percoll; it should be **115-120** (use refractometer). It can be just below 120; otherwise, it is too dilute. If slightly out of this range, adjust with iso-osmotic Percoll or Hanks, as needed. Should add about 10 drops (1 ml) at a time for a 40 ml volume; adding Hanks' will decrease the density.

**7. 75% Percoll:**

|                            |               |              |              |              |              |
|----------------------------|---------------|--------------|--------------|--------------|--------------|
|                            | <b>100 ml</b> | <b>50 ml</b> | <b>40 ml</b> | <b>20 ml</b> | <b>10 ml</b> |
| <b>Iso-osmotic Percoll</b> | 75 ml         | 37.5 ml      | 30 ml        | 15 ml        | 7.5 ml       |
| <b>1X Hanks'</b>           | 25 ml         | 12.5 ml      | 10 ml        | 5 ml         | 2.5 ml       |

8. Check density of the 75% Percoll; it should be **140-145** (use refractometer). It is OK if it is a little denser so neutrophils do not slip through!!

**Note that the iso-osmotic, 59% and 75% Percoll mixtures should be refrigerated between uses and should be warmed to room temperature prior to use. These should always be used under a laminar flow hood and should be aliquoted into smaller volumes to prevent contamination of larger bottles. These suspensions should be useable for several months once they are made, provided they are only used in a sterile environment. They should always be mixed well immediately prior to use.**

- 9. Making the gradient:** Put 5 ml of 59% Percoll in a 15 ml sterile conical tube. Be careful not to allow the Percoll to splash up onto the side of the tube. Aspirate 5 ml of 75% Percoll into a syringe. Don't use a huge syringe for this. Attach one of the 16

gauge catheters (can either use needle or stylet) to the tip of the syringe and eliminate all air from the syringe; wipe off the outside of the catheter, then expel a small amount onto a Kimwipe so that no air remains in the syringe. It's OK to pull up a little extra of the 75% Percoll so air is not inadvertently injected into the conical tube. Insert the catheter through the 59% Percoll until it is resting on the bottom of the conical tube. **Slowly** inject the 75% Percoll into the conical tube. Be careful not to produce turbulence during injection, since that will disturb the gradient. A ring stand may be helpful for stabilizing the syringe during the injection process, or one's elbows may be rested on the work surface. When the Percoll is injected, **STOP** before reaching the end of the volume in the syringe so that air is not injected after the 75% Percoll is loaded into the gradient. Injection of air will cause turbulence and mixing of the gradients.

**NOTE:** The 16 gauge catheter can be reused; just rinse with distilled water and push air through after each use. Then pull up Hank's and discard prior to pulling up the 75% Percoll.

#### **B. How to Separate Neutrophils from Equine Blood**

1. Collect 10 ml of blood from the standing equine in a yellow top (ACD) tube.
2. Centrifuge blood in ACD tubes at 500-x g for 8 minutes.
3. Remove plasma above blood cells and place plasma in separate tube to be discarded.
4. Add 2 ml of 1X Hanks to a 15 ml conical tube. Collect the buffy coat (can use a plastic pipette) and add it to the Hanks in the 15 ml conical tube. Avoid RBCs as much as possible. Use a 2 ml glass pipette and electric pipettor to collect the buffy coat.
5. Repeat steps 2-4 to ensure as complete a collection of buffy coat as possible.
6. Check the final volume of buffy coat in Hanks. If it exceeds 4 ml, add additional 1X Hanks so that the final solution is no more than 50% cells. For example, if you have a volume of 5 ml after collecting the buffy coat and you started with 2 ml of 1X Hanks, you must have added 3 ml of buffy coat. You will therefore need to add 1 more ml of Hanks to the solution.
7. Load cells and 1X Hanks onto Percoll that has already been prepared and aliquoted as 5 ml volumes into 15 ml conical tubes.
8. Load no more than 5 ml of the buffy coat and 1X Hanks suspension to each 15 ml conical tube containing Percoll gradient.
9. Centrifuge at 2200 rpm (Jouan GR4.11) which is about 930 X G for 40 minutes at 22 degrees C (this temperature may be used for all centrifugation). Make sure that acceleration is **SLOW**, and **DO NOT** use the brake when slowing gradients. Be sure the buckets are loaded and balanced well.
10. Remove **mononuclear** cells from the top band in the gradient and place in a 50 ml conical tube; won't need these.

11. Remove **neutrophils** from the middle band in the gradient and place them in a 50 ml conical tube that contains 30 ml DPBS without Ca or Mg for washing.
12. RBC pellet should be visible in the bottom of the tube; **do not** include these cells when harvesting the neutrophil band.
13. Centrifuge the neutrophils at 600 x g for 8 minutes. Remove the supernatant and resuspend the cells in another 30 ml DPBS. Centrifuge again, and remove supernatant when done.
14. Add RPMI complete media with fetal calf serum (see “C” for how to make media). Add 5 ml of media if the cell pellet covers the bottom of the conical tube; add less with a smaller pellet. The amount will be adjusted more accurately once the manual cell count is complete.
15. Once neutrophils are resuspended in complete RPMI w/o, remove 50 ul for a manual cell count; using the hemacytometer is best. You can estimate 2 million neutrophils per ml of blood.
16. You must determine how much media must be added to give you a final concentration of  $1 \times 10^6$  mononuclear cells per ml which is what the Annexin staining kit used in this protocol requires.
17. Manual cell count: Remove 50 ul of the cell suspension and mix with 50 ul of trypan blue. Agitate and pipette 20 ul of the mixture into a pipette tip. Remove the tip from the pipettor, and load each side of the cytometer by capillary action. Count cells and dilute according to exact calculations.
18. If less than 40% of neutrophils are recovered based on the original count, there is a problem.
19. For cell culture, use the 5% CO<sub>2</sub> incubator set at 37 C and make sure it is humidified (tray of sterile distilled water in the bottom).
20. Cell culture media containing fetal calf serum is good for about 1 month. However, if it becomes cloudy, has particulate debris visible, or starts to smell, it should be discarded immediately.
21. The cell culture media should be refrigerated between uses and should be allowed to warm to room temperature prior to use. It should **always** be used under the laminar flow hood and should be aliquoted into smaller volumes so an entire bottle is not contaminated.

C. How to Make the Media (amounts may be divided by 4 to make a smaller amount of the media)

- 500 ml RPMI 1640 + HEPES + Glutamine
- 1.8 ul 2-Mercaptoethanol,  $14.3 \times 10^4$  M

**Note:** 2-Mercaptoethanol is not  $5.5 \times 10^{-2}$  M; it is 14.3 M/l (1117.259 g/l, MW = 78.13). To get final concentration of 50 uM in 500 ml of media, need to add 0.002 g of

2- Mercaptoethanol to 500 ml. To do this, add **either** 3.7 ml of the media that is 7 mM **or** 1.8 ul of the 14.3 M solution to 500 ml of media.

- 10 ml penicillin (10,000 IU/ml) and streptomycin (10 mg/ml)
- 10 ml L-glutamine in NaCl, 200 mM
- 5 ml Na-pyruvate, 100 mM
- 50 ml fetal calf serum, heat inactivated
- 10 ml gentamicin, 10 mg/ml

**D. Using the apoptosis kit (Annexin-5 PE with 7-AAD)**

1. Reagents:

- a. PE Annexin V: Use 5 ul per test
- b. 7-Amino-Actinomycin (7-AAD): Use 5 ul per test
- c. 10X Annexin V Binding Buffer: For a 1X working solution, dilute 1 part of the 10X Annexin V Binding Buffer to 9 parts of distilled water.

2. Staining:

- a. Wash cells twice with cold PBS (use 30 ml each time and centrifuge at 600 x g for 8 minutes at 22 C) and then resuspend cells in 1X binding buffer at a concentration of  $1 \times 10^6$  cells/ml.
- b. Transfer 100 ul of the solution ( $1 \times 10^5$  cells) to a 5 ml culture tube.
- c. Add 5 ul of PE Annexin V and 5 ul 7-AAD.
- d. Gently vortex the cells (30 seconds) and incubate for 15 minutes at room temperature (25 C) in the dark.
- e. Add 400 ul of 1X binding buffer to each tube. Analyze by flow cytometry within 1 hour.

3. Suggested controls for setting up flow cytometry:

- a. Unstained cells
- b. Cells stained with PE Annexin V (no 7-AAD)
- c. Cells stained with 7-AAD (no PE Annexin V)