THESIS

INFLAMMATION AND APOPTOSIS WITHIN THE COLON FROM HORSES WITH BLACK WALNUT EXTRACT-INDUCED LAMINITIS - PROGNOSTIC FACTORS AFTER ESOPHAGEAL OBSTRUCTION IN HORSES

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY LUDOVICA CHIAVACCINI ENTITLED INFLAMMATION AND APOPTOSIS WITHIN THE COLON FROM HORSES WITH BLACK WALNUT EXTRACT-INDUCED LAMINITIS - PROGNOSTIC FACTORS AFTER ESOPHAGEAL OBSTRUCTION IN HORSES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

INFLAMMATION AND APOPTOSIS WITHIN THE COLON FROM HORSES WITH BLACK WALNUT EXTRACT-INDUCED LAMINITIS - PROGNOSTIC FACTORS AFTER ESOPHAGEAL OBSTRUCTION IN HORSES

Laminitis has been defined as a progressive disease in which digital pain may be caused by laminar inflammation or destruction, or may be caused by pressure either from the displacement of the third phalanx into the sole, or laminar pressure secondary to edema, or a combination of these. Numerous studies have been conducted in the last twenty years on the vascular, mechanical and biochemical events occurring in the equine foot, yet the pathogenesis of this life-threatening disease still is unclear. Due to the multifactorial origin of laminitis and the lack of consistency in its manifestation in nature, understanding the pathogenesis depends mostly on reliable experimental models, such as carbohydrate overload and black walnut extract (BWE) toxicosis.

The link between black walnut toxicosis and the mechanism initiating laminitis is not completely understood. One study demonstrated that at the onset of laminitis, horses treated with BWE developed eosinophilic colitis, edema, and hemorrhage associated with concomitant reduction of intestinal transmucosal resistance in all segments of colon *ex vivo*. These findings support the hypothesis that the gastrointestinal tract may be the

first focus of a systemic inflammatory response and that the disruption of the mucosal barrier may allow the release of intestinal toxins and vasoactive amines into the systemic circulation which results in triggering the development of laminitis. However, the cellular processes and events leading to colonic mucosal impairment during the prodromic phase of BWE-induced laminitis need further investigation.

The objective of the present study was to investigate calprotectin expression, epithelial and endothelial apoptosis and their correlation in the colon of horses undergoing BWE toxicosis.

We used paraffin blocks of full sections of colon from 19 horses: 6 horses at the developmental time-point of leukopenia (DTP), 6 at the onset of Obel grade 1 laminitis (LAM) after BWE-administration, and 7 controls.

Specimens were stained with H&E for histopathologic examination. In addition, tissues underwent immunohistochemical evaluation of calprotectin expression with MAC 387 antibody and epithelial and endothelial apoptosis with caspase-3 active antibody. Differences in calprotectin scoring and percentage of apoptotic cells were statistically analyzed. Correlation between calprotectin expression and apoptosis was evaluated.

Histologically, tissues from BWE-treated horses showed increased eosinophil and lymphocyte epitheliotropism, superficial inflammatory cell karyorrhexis and neutrophilic infiltration (LAM group). The DTP group had a higher ($p<0.01$) calprotectin score compared to the control group, while there was no significant difference in percentage of epithelial and endothelial apoptotic cells between groups ($p=0.08$, $p=0.48$), nor was there a significant correlation between calprotectin score and epithelial or endothelial apoptosis ($p=0.69$ and $p=0.29$ respectively).

There is preliminary evidence that exposure of horses to BWE results in an early inflammatory response in the colon, which may contribute to compromise the intestinal barrier. No differences in epithelial and endothelial apoptosis were observed between groups. Different mechanisms, such as disruption of intercellular junctions in non-apoptotic cells may result in increased intestinal permeability and should be further investigated.

* * *

Esophageal obstruction or "choke" is a common clinical disorder and the most common esophageal affliction in the horse. It can be a primary disorder or secondary to pre-existing pathologic conditions, such as squamous cell carcinoma, cysts within the esophageal wall, para-esophageal abscesses, strictures, diverticula, esophageal motility disorders, neurologic and neuromuscular abnormalities, guttural pouch mycosis, pharyngeal trauma, botulism and grass sickness. Complications after an episode of esophageal obstruction are common and may include aspiration pneumonia, pleuritis, mucosal esophageal ulceration and stricture formation, esophageal perforation, chronic recurrent obstruction, postoperative infection, laminitis, laryngeal paralysis and Horner's syndrome.

Previous studies have described clinical findings in horses with esophageal obstruction, but there are no reports that attempt to make correlations of clinical findings with outcome. The objective of our study was to find specific clinical features of horses with esophageal obstruction that are associated with increased likelihood of complications.

The clinical records of 109 horses admitted with esophageal obstruction between April 1992 and February 2009 for esophageal obstruction were reviewed. Variables included breed, sex, age, temperature, heart rate and respiratory rate at admission, whether or not thoracic or esophageal radiographs were performed, and radiographic findings. When endoscopy was performed, tracheal contamination with food material was subjectively graded as "absent" when no visible particles were detected, "mild" when there were single food particles visualized in the trachea, "moderate" when small amounts of aspirated fluid were detected on the tracheal floor, or "severe" when large amounts of aspirated fluid were detected on the tracheal floor and/or food particles were present diffusely within the trachea. Visible esophageal lesions were classified as absence of irritation or swelling, mild to moderate mucosal lesions, or severe ulceration or morphological abnormalities. The location of the obstruction was classified as proximal, middle or distal one-third of the esophagus as determined endoscopically. Hematologic and biochemical variables at the time of admission included packed cell volume (PCV), total protein (TP), blood glucose, bicarbonate, and anion gap. Recorded treatments included whether antibiotics were administered and the route of administration, whether non-steroidal anti-inflammatory drugs (NSAIDs), alpha-2 agonists, opioids, acepromazine and oxytocin were used, and whether general anesthesia was required to resolve the obstruction. The duration of the esophageal obstruction prior to admission (less than 3 hours, 3 to 6.3 hours, 6.4 to 12.3 hours, 12.4 to 48 hours or greater than 48 hours), and the time between admission and resolution of the obstruction (relieved spontaneously at the time of admission, during initial treatment, within 24 hours, greater than 24 hours, or never resolved) were recorded. The association between

clinical, hematological, biochemical, and therapeutic variables with the likelihood of developing complications was investigated by a univariable logistic regression model, followed by multivariable analysis.

Age less than one year or greater than 15 years, radiographic evidence of pulmonary disease, grade of esophageal lesion, TP > 70 g/L, the use of both oral and parenteral antibiotics, the use of general anesthesia, and duration of obstruction longer than 48 hours prior to admission were significantly associated with increased likelihood of complications via univariable analysis. Since aspiration pneumonia alone represented almost 70% of the complications, its association with each variable of interest was further investigated. In addition to age, total protein and the use of general anesthesia, intact male, respiratory rate at admission greater than 22 breaths per minute, and moderate to severe tracheal contamination were significantly associated with the likelihood of aspiration pneumonia via univariable analysis. Multivariable logistic regression analysis revealed a significant association between intact males, age greater than 15 years, and the use of general anesthesia with the likelihood of developing complications. This study gives insight into the understanding of factors that may affect prognosis in horses with esophageal obstruction and could give rise to a prospective study in the future.

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PART I

INFLAMMATION AND APOPTOSIS WITHIN THE COLON FROM HORSES WITH BLACK WALNUT EXTRACT-INDUCED LAMINITIS

Chapter 1: Literature review on laminitis, calprotectin expression and apoptosis in the equine gastrointestinal tract

General Overview on Equine Laminitis with Emphasis on its Pathogenesis

Laminitis has been defined as a progressive disease, in which digital pain may be caused by laminar inflammation or destruction, or may be due to pressure either from the displacement of the third phalanx into the sole or laminar pressure secondary to edema, or a combination of these [1]. Despite numerous studies on the vascular, mechanical and biochemical events occurring in the equine foot, the pathogenesis of this life-threatening disease still is unclear [2,3]. In general, two primary clinical forms of laminitis are recognized: mechanical and inflammatory.

Mechanical laminitis is caused by weight bearing on the supportive forelimb or hind limb, when the contralateral is severely injured, or may occur as a bilateral forelimb disease in horses trained on excessively hard surfaces. The hemorrhagic nature of this laminitis suggests that its pathophysiology resides in the tearing and shearing forces transmitted through the bony column to the third phalanx and ultimately to the dermal-epidermal laminar interface, more than in the compression of the solar vessels or circumflex arteries of the distal aspect of P3 (Fig. 1.1) [1].

Inflammatory laminitis is often secondary to septic disease such as gastro-intestinal tract disorders (e.g. infectious enterocolitis, enteritis secondary to carbohydrate ingestion or

microflora deregulation), pleuropneumonia, retained placenta or acute metritis, endotoxemia or sepsis from any cause [4,5]. Even though the pathogenesis of sepsis-related laminitis remains unclear, there is convincing evidence coming from experimental models (such as oligofructose or black walnut extract models) for systemic inflammation as the main etiopathological event [3,6]. The changes observed in the laminae seem to be a local response to systemic inflammation and share many similarities with the changes observed during multiple organ dysfunction syndrome (MODS) in human sepsis [7,8]. The septic horse often exhibits clinical signs similar to the systemic inflammatory response syndrome (SIRS), a precursor of MODS in humans, such as fever, tachycardia and injected mucous membranes. Second, a systemic activation of the endothelium followed by activation of leukocytes, leads to migration of the latter from the blood stream into the perivascular tissue of the laminae and other organs, as it occurs in human organs [8-10]. As extensively described later in this chapter, the local inflammation, culminating in production and release of reactive oxygen species (ROS) by the neutrophil population, may interfere with cytoskeleton dynamics and trigger the dysadhesion between epidermal and dermal lamellae (Fig. 1.1) [11]. Previous studies suggested that one of the main events leading to the disruption of lamellar bonds is the breakdown of type IV and type V collagen and the protein laminin 5, by the activated gelatinases MMP-2 and MMP-9 [12-14]. Laminin attaches on one side to the collagen in basal lamina, on the other to the transmembrane protein α6β4 integrin, which, through the protein plectin, is linked to keratin intermediate filaments in the cell. This guarantees the connection between the epithelial cell and the basal lamina [15]. MMP-2 is constitutively expressed by several cells, such as keratinocytes and seems to be part of the normal extracellular matrix turnover. Its

expression is normally down-regulated by the tissue inhibitor of metalloproteinase (TIMP) and alpha2-macroglobulin [12,13]. In contrast, MMP-9 is often transiently expressed, except in neutrophils, which store the enzyme in granules and liberate it under various stimuli [14,16]. MMP-2 and MMP-9 are released as zymogens and then activated through the cleavage of the regulatory N-terminus. Even though the trigger factors of MMPs activation remain unclear, possible causes are ischemia, inflammation, oxidative stress [12], and, for MMP-2, the membrane-type MMP1 (MT1-MMP), which is proteolytically activated by the protein convertase PACE4 [14]. Once the bonds between the epidermal and dermal lamellae have been disrupted, the tearing and shearing forces due to weight bearing induce the rotation or the displacement of the third phalanx into the sole, or both, depending on the localization and extension of the dysadhesion [7]. These observations together suggest that laminitis may be the final result of both the local response of systemic inflammation and imbalance between activation and inhibition of the normal laminar healing processes in the foot [7,12,14]. As in human sepsis, not only endotoxins, but multiple types of toxins, such as gram-positive and gram-negative bacterial toxins, fungal toxins and viral components are absorbed systemically and may induce a downstream inflammatory reaction leading to organ failure [7].

Although there are similarities between the pathogenesis of human multiple organ failure and laminitis, the structural and physiological peculiarities of laminae make the foot the "target organ" in the horse, unlike other sepsis models in which lungs, kidney and liver are usually primarily affected [7]. First, the more severe inflammatory reaction in the laminae (as suggested by a higher cytokine expression with respect to other tissue) or the innate lack of superoxide dismutase (SOD), or both, may result in insufficient

antioxidant capacity for dealing with the ROS produced by leukocytes infiltrating during the prodromic phase of systemic inflammation [8,11,17,18]. Second, in contrast to lungs, kidney and liver, the laminae seem to lack a regional monocyte population. This may affect the regional inflammatory response and in the end the activation of MMPs [1]. Third, the foot is subjected to distracting forces due to weight bearing and this makes it more susceptible to the disruption of connections between epidermal cells and basal lamina [1,12]. In addition, the specific vascular anatomic features in this region, such as high interstitial pressure increasing with weight bearing, high capillary pressure, and opening of vascular shunts, make the laminar tissue more sensitive to the hemodynamic changes induced by sepsis [1].

Under the umbrella of inflammatory laminitis we may also include equine metabolic syndrome-associated laminitis and pasture-associated laminitis. The term metabolic syndrome was first proposed by Johnson (2002) for indicating obese horses at risk of laminitis. Later Treiber *et al.* introduced the term prelaminitic metabolic syndrome (PLMS) to specifically address those ponies at higher risk of pasture-associated laminitis, indicating that pasture-associated laminitis can be considered a "subtype" of metabolic syndrome-associated laminitis [19,20]. The pathogenesis of metabolic syndrome and its relationship with increased susceptibility to laminitis is only partially understood [19]. Presently two interrelated mechanisms have been suggested. First, obesity has been associated with low grade chronic inflammation [20,21]. Adipose tissue, including adipocytes and macrophages, once considered only a storage tissue, has been recognized as the largest endocrine organ of the body, which can produce a large array of adipokines such as leptin, resistin, TNFα and TNFp, interferon-γ and a variety of interleukins as

IL1β, IL6, IL8, IL10, monocyte chemotactin protein-1 and complement proteins [21-23]. This chronic inflammatory status per se can increase the risk of a systemic inflammatory response and consequently all the local vascular and inflammatory changes described above leading to laminitis. Second, obesity and chronic inflammation have been demonstrated to be almost always associated with increase insulin resistance (IR) [21,24,25]. Insensitivity to insulin results in hyperinsulinemia, hyperglycemia, and mobilization of fatty acids from the liver (hypertriglyceridemia). Free fatty acids appear to activate toll-like receptor 4 and to aggravate the subclinical inflammatory condition [22]. In addition, IR seems to lead to a vascular impairment in the laminae due to a deregulation between vasodilation and vasoconstriction. In fact, insulin receptors in the laminae activate both vasodilation through the synthesis of nitric oxide (NO) via the phosphatidylinositol 3-phosphate (PIP3) pathway and vasoconstriction through the release of endothelin-1 (ET-1) via the MAPK pathway. For causes that remain unclear, during insulin resistance, the PIP3 pathway is blocked, while the MAPK pathway remains active, leading to vasoconstriction, platelet activation and leukocyte adhesion and extravasation [19,20,25-27]. In agreement with these findings, the concentration of immunoreactive ET-1 is increased in the connective tissue of the foot of horses with laminitis [26,28]. Moreover, it has been recognized that the adipose tissue itself is one of the major sources of angiotensinogen and that it can locally regulate the conversion to angiotensin II, which may contribute to the vascular deregulation [22]. IR seems also to directly activate the production of MMP2 and MMP9 within the laminar tissue [27]. In conclusion obesity creates a state of chronic inflammation, hyperinsulinemia and IR, leading to vascular impairment and initiating the cascade of events that converges into laminitis.

Pasture-associated laminitis alone embodies more than 50% of all cases of laminitis and can be summarized as a combination of genetic, dietetic factors and obesity [19,20,29]. Certain horses and ponies seem to be more affected than others, even when kept in the same environment, and predisposed animals tend to have recurrent laminitis episodes. In general pony breeds seem to be more inclined to develop pasture-laminitis than horses due to inherent lower insulin sensitivity [19]. In an inbred population of Welsh and Dartmoor ponies, a PLMS phenotype seemed to have a dominant mode of inheritance, suggesting a genetic predisposition to laminitis in these breeds. There are few studies addressing the same type of association in horses, but Morgans, Paso Finos, Arabians and Norwegian Fjords seem to have a higher predisposition to EMS. This fact may be an adaptive response in those native breeds coming from poor nutritional environments [19]. In another study conducted by Bailey *et al.* (2008) in a mixed-breed population of ponies in the United Kingdom, laminitis-prone animals showed the prelaminitic phenotype only during the summer, suggesting that the summer pasture may exacerbate the hypertension, IR, hyperinsulinemia and hypertriglyceremia in predisposed animals. The content in non structural carbohydrates (NSCs) such as fructans, starch and simple sugar tends to increase during spring, is intermediate during fall and low in winter and at the end of the summer, with variations between different forages. Moreover NSCs tend to increase during the morning, reaching the maximal peak in the afternoon, and lowering again during the night. These sugars are easily absorbable in the gastrointestinal tract and rapidly increase the glycemia, with consequential exacerbation of IR and hyperinsulinemia in predisposed animals, triggering the laminitic episode [19]. The second possible mechanism is extrapolated from the carbohydrate overload model in which

laminitis is experimentally induced administering a large amount of starch or oligofructose (OF) as a single bolus. The procedure will be explained in more detail later in this chapter. However it has been demonstrated that ingestion of a large quantity of rapidly fermentable carbohydrates into the cecum and the colon causes disequilibrium of the intestinal microflora, with proliferation of lactic-acid producing *Streptococcus* spp. and *Lactobacillus* spp. and subsequent lowering of the intraluminal pH and increasing intestinal permeability [19,30]. These modifications result in production and absorption of endotoxins, exotoxins and vasoactive amines (in particular tryptamine, tyramine and phenylethylamine) by the hindgut environment, and may lead to the onset of laminitis [19,30-33]. In some periods of the year, the quantity of NSCs ingested daily by grazing horses may approach experimental doses [34]. It is also possible that lower doses of NSCs are sufficient to induce laminitis in predisposed animals, due to impaired intestinal barrier function, reduced detoxifying capacity of the liver, or higher sensitivity of the vasculature or the lamellar tissue to trigger factors [19,20,30,32]. Finally, most ponies and horses kept on pasture tend to be obese [1]. The association between obesity and laminitis has been previously discussed.

Although the cascade of events triggering laminitis may differ, it has been suggested that, in the end, lamellar disruption is secondary to dysoxia of the foot [1,35]. Several studies demonstrated that in the prodromal phase of laminitis there is an alternation of hypoperfusion and hyperperfusion of the foot leading to vascular impairment [26,36,37]. Studies on the Starling's forces within the equine laminar vasculature, in both carbohydrate overload and black walnut extract models, demonstrated an early venocostriction, followed by increased capillary pressure, extravasation of fluids and

consequent edema of the digital interstitium [35,37,38]. These findings are corroborated by the fact that laminar veins have been demonstrated more sensitive than arteries to ET-1, serotonin (5-HT), catecholamines, prostaglandins and hindgut-originated vasoactive monoamines *in vitro* [28,33,39], and administration of an ET-1 antagonist significantly reduces the post-capillary resistance in an isolated foot after carbohydrate overload-induced laminitis [35,37]. The role of these molecules during the development of laminitis has been described earlier and will be discussed later in this chapter.

Experimental Models for Inducing Equine laminitis

Due to the multifactorial origin of laminitis and the lack of consistency in its manifestation in nature, understanding its pathogenesis depends mostly on reliable experimental models. There are currently two main models for inducing laminitis of alimentary origin: carbohydrate overload and black walnut extract toxicosis.

1. *Carbohydrate overload*: The carbohydrate overload model draws from the observation that accidental access to grain starch induces laminitis in the horse [40]. The original protocol consisted of the administration of 17.5 g/kg of wheat flour in 8L of tap water through a nasogastric tube [41]. Due to the variable results and the high mortality of this model, the protocol was substituted with the administration of 10 g/Kg of commercial OF (Raftilose P95 [a]) in 4L of water [40]. In a recent study, Kalck *et al.* (2009) obtained the same effect with 5 and 7.5 g/Kg respectively. Both starch and OF are polymers of glucose, the first constituted by a chain of glucose molecules, the second by

[a] Orafti Active Food Ingredients, Tienen, Belgium

one molecule of glucose linked to one or more molecules of fructose, and both are highly fermentable in equine cecum and colon [40]. The starch and OF overload models produce similar clinical and metabolic effects. The onset of laminitis is observed between 20-44 hours and 24-26 hours post ingestion respectively. This is accompanied by profuse diarrhea with electrolyte loss, dehydration, hypovolemic shock and endotoxemia [40-43]. As previously anticipated, feeding high quantities of starch or OF induces a shift in the hindgut bacterial population [44]. The predominant OF fermenting bacteria isolated as early as 2H post-administration (POA) is the equine hindgut streptococcal species (EHSS) *Streptococcus lutetiensis*, a member of the *S. bovis/equinus* complex. The shifts of *Lactobacillus* and *E. coli* subpopulations seem to occur secondarily and later in time with respect to the EHSS shift [29]. Consequentially, *S. lutetiensis* seems to be the main bacteria responsible for the lowering of intraluminal pH, accompanied by increasing intestinal permeability, resorption of bacterial toxins and lactic acidemia [29,40]. A recent study demonstrated that a massive EHSS death and cell lysis is observed around 16H POA, and the consequential release and absorption of bacterial components may trigger laminitis [29]. Previous studies demonstrated that supernatant of cultures of *S. bovis* activates MMP-2 and is able to induce lamellar separation in vitro [45]. More contradictory is the role of endotoxins in the pathogenesis of laminitis. In fact, while different grade endotoxemia accompanies the carbohydrate ingestion [42,43], endotoxin infusions failed to induce laminitis [5,38,46]. A recent study suggested that endotoxins, once

absorbed through the intestine, primarily activate platelets, releasing 5-HT and thromboxane, two potent vasoconstrictors, and lead indirectly to the activation of leukocytes and the cascade of events culminating with the onset of clinical laminitis [42]. Moreover, it has been demonstrated that tryptamine, tyramine, phenylethylamine and other monoamines produced by decarboxylation of amino acids by EHSS and Lactobacilli, once released into the circulation, have digital vasoconstrictor effects. Though the mechanism has not completely been elucidated, two are hypothesized. First, these amines, structurally similar to 5-HT and catecholamines, may directly activate 5-HT receptors (tryptamine) and cause the release of norepinephrine from neuronal vesicles (tyramine) in the digital vessels. Second, they may displace endogenous 5-HT from platelets and/or inhibit 5-HT uptake from the endothelium [3,33].

2. *Black walnut extract (BWE) toxicosis*: The aqueous extract of black walnut (*Junglas nigra*) is obtained incubating 2 g black walnut shavings/kg body weight of in 8L of deionized water overnight at room temperature and filtering it. Six liters of the BWE is then administered to the horse through a nasogastric tube [9,47,48]. The onset of laminitis is observed between 10-12 H post administration up to 90% of the animals [47,49]. The only significant clinical signs observed are mild depression, increase in temperature, heart rate and respiratory rate starting at 4 H POA and limb edema at the time of the onset of laminitis. Increase of epinephrine may be secondary to stress and pain due to laminitis. One of the advantages is that, unlike the carbohydrate overload

model, the BWE model does not cause overt intestinal disturbance, endotoxemia or other secondary severe metabolic effects requiring the euthanasia of the animal [38,47]. The most distinctive finding of this protocol is a 30% reduction in white blood cell (WBC) count at about 3 hours post administration [1,7,38,48,50]. Combined with the leukopenia, massive extravasation of activated leukocytes, mostly neutrophils, occurs in the perivascular regions of the dermal vasculature of the laminae, around skin venules [9,10], and in the hepatic and pulmonary parenchyma [8]. Immediately after administration, the early peak of IL-1β and IL-8 and proinflammatory chemokines as MCP-1 and MCP-3, leading to the expression of E-selectin and ICAM-1, may induce primary endothelial activation and initiation of leukocyte extravasation [6,10,50]. An intense activation of leukocytes in the prodromal stages of BWE induced laminitis is also confirmed by significant increases in gene expression of IL-1β, IL-6, and IL-8 in the laminar tissue and, to a lesser extent, in lungs and liver, and COX-2, MAIL/IkBξ[b], G-CSF and SAA, in the laminae [8,10,17,50-53]. The surprising lack of TNF-α expression in the laminae, unlike pulmonary and hepatic tissue, may be due to the expression of nuclear protein MAIL/IkBξ, which suppresses TNF-α, or due to lack of a resident monocyte population in the foot [10,17]. The hypothesis that BWE administration may induce systemic leukocyte activation is further corroborated by recent studies showing an increase in concentration and activity of myeloperoxidase (MPO)

[b] Molecule possessing Ankyrin-repeats Induced by Lipopolysaccharide: a protein interacting with the transcription factor NF-kβ.

in plasma, laminar tissue and skin, concurrent with the development of leukopenia and the increased production of ROS, in those horses that develop laminitis [48,54]. MPO is not only a marker of leukocyte activation, but a bioactive molecule that can generate ROS and RNS. In addition, the activation of cytokine receptors and toll-like receptors can induce the release of ROS inside the cell [11]. Free radicals such as ROS react with proteins, DNA and especially lipids. The peroxidation of lipids leads to the creation of isoprostanes and aldehydes such as 4-hydroxy-2-nonenal (4-HNE), which are active molecules able to induce the oxidation of other fatty acids, activation of the MAPK cascade with consequential induction of COX-2 expression, apoptosis and interference with cytoskeleton dynamics, and may play a role in the dysadhesion between epidermal and dermal lamellae [11]. Although the origin of BWE induced laminitis seemed to lie in the gut, the link between black walnut toxicosis and the mechanism initiating the laminitis is not completely understood. One study demonstrated that at the onset of laminitis, horses treated with BWE showed significant eosinophilic colitis, edema and hemorrhage, associated with concomitant reduction of intestinal transmucosal resistance in all segments of colon *ex vivo* [49]. These findings support the hypothesis that the intestine may be the first focus of a systemic inflammatory response [1] and that the disruption of the mucosal barrier may allow the release of intestinal toxins and vasoactive amines into the systemic circulation that may directly trigger laminitis [3].

Calprotectin and Its Role in Inflammation

Calprotectin belongs to the S100 proteins, a new group of molecules with an important role in innate immunity modulation, classified as damage associated molecular patterns or DAMPs. To date, more than 20 different S100 proteins have been recognized. They are calcium binding proteins, with two calcium binding helix-loop-helix regions (EF-hands) connected by a central hinge region [55-58]. The C-terminal EF-hand is formed by 12 amino acids and has a higher affinity for calcium, while the N-terminal is formed by 14 amino acids. In addition to binding calcium, they bind zinc in different and independent domains [57,59]. Calprotectin is not an individual protein, but a combination of two S100 proteins: S100A8 (8 kDa, also called Calgranulin A or myeloid related protein 8, MRP8) and S100A9 (14 kDa, also called Calgranulin B or myeloid related protein 14, MRP14), which are found in granulocytes, monocytes and early differentiation stages of macrophages. They represent 40% of cytosolic and 5% of total proteins of neutrophils [58,60,61]. Expression of S100A8 and S100A9 is also reported in epithelial cells, keratinocytes and several tissues and body fluids [55-58,60]. S100A8 and S100A9 exist in different isoforms and they can be found as monomers or may form active heterodimers or tetramers. Computer-based homology studies suggested that the residues Ile_{13}, Tyr_{16}, Phe_{68}, Val_{80} and Ser_{86} of the S100A8 and Ile_{16}, Phe_{19}, Phe_{76}, Trp_{88} and Met_{94} within the amino acid sequences of S100A8 and S100A9 are involved in heterodimer formation [57]. The heterodimer is calcium independent, while the tetramer forms only in the presence of calcium [55,56].

Calprotectin has both intra and extra-cellular activities. As an intracellular calcium-binding molecule, calprotectin has signal transduction functions [60]; it binds

arachidonic acid in a calcium-dependent manner, regulating its storage and mobilization inside the cells [57], and calcium-dependent S100A8/A9 tetramers promote tubulin polymerization and stabilization of microtubules (MTs), modulating cytoskeleton rearrangement during phagocyte migration [55,56]. Upon elevation of calcium levels, the heterodimers are translocated to the plasma membrane, but the mechanism through which they penetrate and are attached to the membrane is not completely understood since both proteins lack a transmembrane region [57]. Noncovalently associated S100A8/A9 complexes are secreted by activated phagocytes and epithelial cells during inflammation. However, neither endosomes nor vesicles containing S100A8 or S100A9 have been identified in the cytosolic fraction of monocytes [55-57]. Secretion involves two independent pathways. First different stimuli (cytokines, LPS…) activate protein kinase C; second, the contact of phagocytes with extracellular matrix protein (ECM) or activated endothelium elevates intracellular calcium concentration, necessary for triggering S100 protein release. The secretion follows an "alternative" secretory pathway, based on active transport via MTs and release through an unknown transmembrane transporter [55-57]. Once secreted, S100A8/A9 induces activation of adhesion molecules such as vascular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and various chemokines. Furthermore it promotes leukocyte adhesion and extravasation. It increases the binding capacity of integrin receptors CD11b-CD18 on the leukocyte surface to ICAM-1 on the endothelial cells, it induces prothrombotic and proinflammatory responses, and increases vascular permeability through disruption of cell junctions and promotion of endothelial necrosis and apoptosis [55-57,60,61]. Calprotectin is a toll-like-receptor endogenous activator, promoting lethal endotoxin-induced shock [55,62], and shows

zinc-dependent and independent inhibition of growth and induction of apoptosis against myeloid cells, mitogen-activated lymphocytes, human fibroblasts and several tumor cell lines *in vitro* [58,63]. Calprotectin seems to have zinc-reversible antimicrobial and candidastatic action [57,58,60]. Recent studies suggested that S100A8 and S100A9 proteins may have biological intracellular activity also as monomers. In addition, murine S100A8 has chemotactic activity in vitro [56-58]. Calprotectin is up-regulated in plasma and exudates in several diseases such as rheumatoid arthritis, Crohn's disease, ulcerative colitis, colorectal cancer, cystic fibrosis, multiple sclerosis, and HIV-infection [56,58,60,61]. Fecal calprotectin is commonly used as a biomarker of intestinal inflammation [55]. Lately, calprotectin has been validated for assessing neutrophil activation, adhesion and migration in the equine small and large intestine during experimental ischemia and reperfusion (I/R) [64-66]. Previous direct and indirect methods used in both human and animal models, such as intravital microscopy, flow cytometry, scintigraphy, measure of MPO activity, histological and biochemical procedures, were cumbersome or they failed to localize the neutrophils within the tissue [60,64]. Moreover, Faileros et al (2009) reported increased epidermal calprotectin signal at the onset of laminitis in the laminae of horses treated with BWE, indicating that activation of epithelial cells follows leukocyte migration into the laminae. This is the first evidence of induction of a proinflammatory molecule, which may play a role in dysadhesion between epidermal and dermal lamellae, in laminar epithelium of horses undergoing BWE-induced laminitis.

Intestinal Apoptosis and Its Role in The Regulation of Barrier Function

Cellular death occurs in two different but complimentary ways: cellular necrosis and apoptosis. While cellular necrosis is a passive phenomenon induced by several pathological factors, apoptosis is an active process that occurs in response to environmental or developmental cues or as a response to cell damage. Necrosis leads to lack of cellular homeostasis, swelling, plasma and organelle burst, and culminates in autodigestion and dissolution of the cell with a local inflammatory reaction [67]. Apoptosis is characterized by loss of cellular contacts and cellular shrinkage, chromatin condensation, DNA fragmentation and cellular budding [68-70]. Apoptosis is programmed by the cell with minimal tissue inflammation and plays an important role in embryogenesis, tissue homeostasis, lymphocyte development, and tumor regression. In the gastrointestinal tract, in addition to its physiological cellular turnover function, apoptosis has been associated with intestinal adenocarcinoma, ulcerative colitis, Crohn's disease, non-steroidal anti-inflammatory drug-related colitis, active celiac disease, burn shock and ischemia-reperfusion injury [69-72]. It has also been demonstrated that horses with gastrointestinal simple obstruction have a significantly higher number of apoptotic cells in all the layers of the intestinal wall with respect to horses with musculoskeletal disease or controls [68].

Although increased epithelial apoptosis has been associated with both small and large intestinal injury, the role of apoptosis in loss of the intestinal barrier function is not completely understood. Epithelial barrier function is controlled by trans and para-cellular permeability, the latter of which is restricted by tight junctions (TJ) [72]. Although disruption of TJs in non-apoptotic cells may result in increased intestinal permeability,

studies both *in vivo* and *in vitro* showed a significant association between apoptotic rate and intestinal bidirectional permeability in both the small and large intestine [70-73]. Moreover, Schultze *et al.* (2006) reported a 5% increase in apoptotic rate, associated with a reduction in intestinal barrier function, in mild to moderately inflamed colon specimens collected from patients with ulcerative colitis and Crohn's disease. Several cytokines, such as TNFα and IL-3, involved in chronic inflammatory diseases, have been reported to increase the apoptotic rate and colonic epithelial conductivity [72]. The reduction of intestinal barrier function may allow bacteria, toxins and other molecules to reach the systemic circulation, resulting in septic morbidity and potentially initiating multiple organ dysfunction syndrome [70,72,74].

As we previously described, there is a strong relationship between gastrointestinal disease and laminitis. Transmission electron microscopy analysis of the cecum of horses with CHO-induced laminitis reported progressive epithelial surface sloughing, disruption of microvilli, pyknotic nuclei, epithelial vacuoles and dilation of the intercellular spaces starting at 24 hours after starch administration [75]. A recent study reported increased intestinal barrier permeability 4 hours after administration of the carbohydrate overload [76]. Colonic impairment at the onset of laminitis has been previously described also for the BWE-toxicosis model [49]. Although the author indicates a significantly decreased intestinal transmucosal resistance in all segments of colon, the cellular processes and events leading to mucosal impairment have not been fully characterized.

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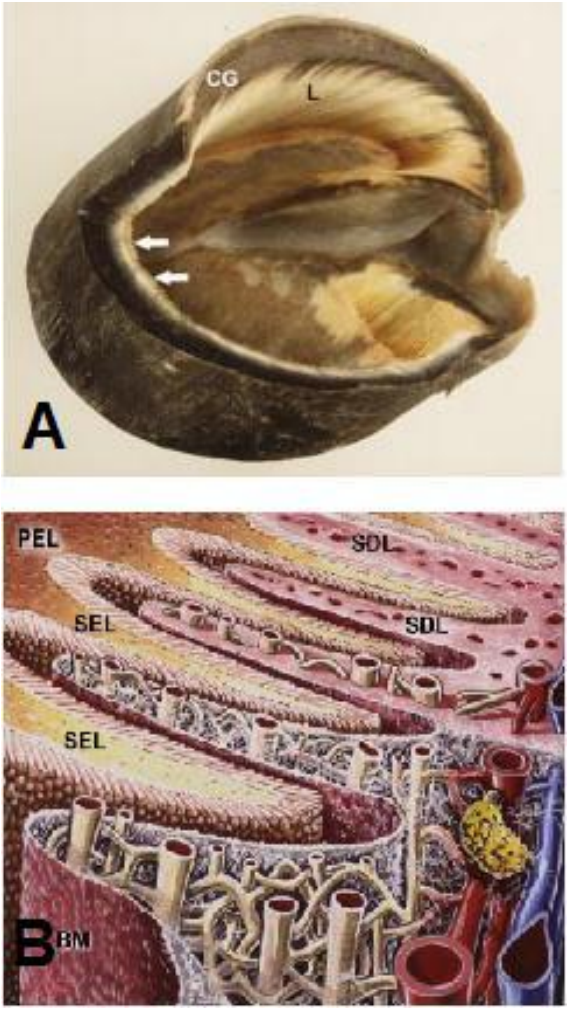
Tables and figures

Figure 1.1: Hoof with its content removed (A) and magnification of the epidermal-dermal junction (B). (A) In this hoof capsule a portion of the wall has been removed to show the inner structures. Notice the curve of the coronary groove (CG), the pigmented *stratum medium* of the wall and the non-pigmented *stratum internum* with the lamellae (L and arrows). (B) Primary epidermal lamellae (PEL) extend their surface into secondary epidermal lamellae (SEL). Notice the basal membrane (BM) which connects the epithelium of the secondary epidermal lamellae to the connective tissue of the secondary dermal lamellae (SDL). [2]



Chapter 2: Detection of calprotectin and its correlation to apoptosis within the equine colon from horses with black walnut extract-induced laminitis

This chapter has been formatted based on the instruction for authors in order to submit it to the American Journal of Veterinary Research. Some information included in Chapter 1 is repeated here in the introduction and discussion in order to have this chapter as a standalone document for subsequent submission and consideration for publication.

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Abbreviations: CHO, carbohydrate overload; BWE, black walnut extract; DTP, developmental time-point; LAM, onset of laminitis; TTBS, tris-tween buffered saline.

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The objective of the present study was to investigate calprotectin expression, epithelial and endothelial apoptosis and their correlation in the colon of horses exposed to orally administered black walnut extract (BWE). Paraffin blocks of full sections of colon from 19 horses including 7 controls not exposed to BWE, 6 horses at the developmental time-point of leukopenia (DTP) and 6 at the onset of Obel grade 1 laminitis (LAM) after BWE-administration. Specimens were stained with H&E for histopathologic examination. Immunohistochemical evaluation for calprotectin expression with MAC 387 antibody was performed along with assessment of epithelial and endothelial apoptosis with caspase-3 active antibody. Calprotectin expression and percentage of apoptotic cells were compared between controls and the two treatment groups and presence of a correlation between calprotectin expression and apoptosis was evaluated. Histological findings from BWE-treated horses included eosinophil and lymphocyte epitheliotropism. The DTP group had a higher ($p<0.01$) calprotectin score with respect to the control group, while there was no significant difference in percentage of epithelial and endothelial apoptotic cells between groups ($p=0.08$ and $p=0.48$ respectively). No significant correlation was found between calprotectin score and epithelial or endothelial apoptosis ($p=0.69$ and $p=0.29$ respectively). There is preliminary evidence that exposure of horses to BWE results in an early inflammatory response in the colon that may contribute to compromise of the intestinal barrier. Further studies are needed to characterize the nature of the colonic injury in BWE-exposed horses and the link to the development of laminitis.

Introduction

Laminitis has been described as a progressive disease, in which digital pain may be caused by laminar inflammation or destruction, pressure either from displacement of the third phalanx into the sole or secondary to edema, or a combination of these.[1] Inflammatory laminitis is often secondary to septic disease such as gastrointestinal tract disorders, pleuropneumonia, retained placenta or acute metritis, endotoxemia or sepsis from any cause.[2,3] Although numerous studies have been conducted on the vascular, mechanical and biochemical events occurring in the equine foot during laminitis, the pathogenesis of this life-threatening disease is still unclear.[4]

Due to the multifactorial origin of laminitis and the lack of consistency in its manifestation, understanding its pathogenesis depends mostly on reliable experimental models. Currently, there are two main models for inducing alimentary laminitis: CHO and BWE toxicosis. In contrast to the CHO model, BWE induces laminitis in up to 90% of horses within 12 hours post-administration without apparent intestinal disturbance, endotoxemia or other secondary severe metabolic effects requiring euthanasia of the animal.[5-7] A consistent clinical finding is a 30% reduction in white blood cell count 3-4 hours post BWE administration,[1,7-10] as is often seen in adult horses with acute gastrointestinal disease undergoing surgery.[a] Combined with the leukopenia, a massive extravasation of activated leukocytes, mostly neutrophils, is observed in the perivascular regions of the dermal laminae, around skin venules,[11,12] and in the hepatic and pulmonary parenchyma.[13] This is further corroborated by demonstration of neutrophil myeloperoxidase in plasma, skin and laminae after BWE administration.[14] These findings, along with significant increases in IL-1β, IL-6 and IL-8 gene expression in

laminar tissue, in lungs and liver, and immunohistochemical evidence of cyclooxygenase-2 (COX-2), molecule possessing ankyrin-repeats induced by lipopolysaccharide (MAIL/IkBξ), granulocyte-colony stimulating factor and serum amyloid A expression in the laminae,[10,12,13,15-18] suggest that laminitis is a local manifestation of a systemic disease,[3,14,16,19] comparable to multiple organ failure in human sepsis.[8,13] Studies based on CHO models hypothesize that the early overgrowth of equine hindgut streptococcal species, followed by *Lactobacillus sp.* and other fermenting bacteria, decreases luminal pH and increases intestinal permeability, allowing the absorption of exotoxins and endotoxins into the systemic circulation.[20,21] Previous studies demonstrated that supernatant of cultures of *S. bovis* activates the matrix-metalloproteinase MMP-2 and is able to induce lamellar separation in vitro,[22] while endotoxins may activate platelets and indirectly lead to the activation of leukocytes and the cascade of events culminating with the onset of clinical laminitis.[23] Other authors focus their attention on the absorption of vasoactive amines (such as tryptamine, tyramine and phenylethylamine) by the hindgut environment as a major cause of laminitis through direct and indirect digital vasoconstriction effects.[4,23-29]

Calprotectin is a combination of two S100 proteins (the 8 kDa S100A8, also called Calgranulin A or myeloid related protein 8, MRP8, and the 14 kDa S100A9, also called Calgranulin B or myeloid related protein 14, MRP14), classified as damage associated molecular patterns or DAMPs, a new group of calcium binding molecules with an important role in innate immunity modulation. Calprotectin is found in granulocytes, monocytes, and early differentiation stages of macrophages and represents 40% of cytosolic and 5% of total proteins of neutrophils.[30-32] Expression of calprotectin is also

reported in epithelial cells, keratinocytes and several tissues and body fluids.[30,31,33-35] Calprotectin is upregulated in plasma and exudates in several human diseases[30-32,34] and today is commonly used as a fecal biomarker of intestinal inflammation.[33] Immunohistochemical detection of calprotectin with the murine anti-human monoclonal antibody MAC387 has been validated for assessing neutrophil activation, adhesion and migration in the equine small and large intestine during experimental ischemia-reperfusion.[36-38] Faileros et al[39] reported increased epidermal calprotectin at the onset of laminitis in the laminae of horses treated with BWE. Calprotectin has both intra- and extracellular activities, including signal transduction[30] and modulation of cytoskeleton rearrangement during phagocyte migration.[33,34] Calprotectin is a toll-like-receptor endogenous activator;[33,39] it induces various chemokines, promotes leukocyte adhesion and extravasation, and increases vascular permeability through disruption of cell junctions and promotion of endothelial necrosis and apoptosis.[30,32-35]

Apoptosis is an active process that occurs in response to environmental or developmental events or as a consequence of cell damage leading to cellular death with minimal tissue inflammation. It plays an important role in embryogenesis, tissue homeostasis, lymphocyte development, and tumor regression.[40] In the gastrointestinal tract, in addition to its physiological cellular turnover function, apoptosis has been associated with intestinal adenocarcinoma, ulcerative colitis, Crohn's disease, non-steroidal anti-inflammatory drug-related colitis, active celiac disease, burn shock and ischemia-reperfusion injury in humans.[41-44] Studies both *in vivo* and *in vitro* showed a significant association between apoptotic rate and disruption of the barrier function in both the small and large intestine.[41-43,45] Studies by Krueger *et al.*[46] and Weiss *et al.*[47]

describe progressive cecal mucosal damage and increased intestinal barrier permeability in horses after CHO administration. McConnico et *al.*[6] demonstrated that at the onset of laminitis, horses treated with BWE showed significant eosinophilic colitis associated with reduction of intestinal transmucosal resistance *ex vivo*. These findings support the hypothesis that the intestine may play a significant role in the BWE-induced systemic inflammatory response[1], with the disruption of the mucosal barrier may allow release of intestinal toxins and vasoactive amines into the systemic circulation and directly trigger laminitis.[4] The cellular events leading to colonic mucosal impairment during the prodromic phase of BWE-induced laminitis need further investigation.

The aim of the present study was to investigate calprotectin expression and apoptosis in colonic mucosa and submucosa in horses after BWE administration. Because of the pivotal role of leukocyte activation at the onset of laminitis,[48] the flux and migration of these cells through the colonic wall was evaluated. Since calprotectin showed inhibition of growth and induction of apoptosis in several cell types *in vitro*,[31,49] we also wanted to investigate the correlation between calprotectin expression and the percentage of epithelial and endothelial apoptotic cells. We hypothesized that there would be an overexpression of calprotectin-positive cells and of epithelial and endothelial apoptosis in the colon of horses undergoing BWE administration compared with controls. We further hypothesized that there would be a direct correlation between calprotectin expression and apoptosis.

Materials and Methods

Archived colon specimens from previous experiments approved by the Institutional Animal Care and Use Committee of The Ohio State University were used. Colon sections from 3 groups were used: the DTP group (onset of leukopenia,[16] approximately 3–4 hours post BWE exposure; n=6), the LAM group (onset of laminitis, approximately 10–12 hours post BWE; n=6) and the control group (n=7).

BWE-induced laminitis and sample collection

Laminitis was induced as previously described.[11,12,39] Briefly, BWE (*Junglas nigra*) was obtained by incubating 2 grams of black walnut shavings/kg body weight in 8L of deionized water overnight at room temperature and filtering it. Six liters of the BWE or water (controls) was administered via nasogastric tube. Horses were anesthetized either 3-4 hours after BWE administration (DTP group), at the onset of Obel grade 1 lameness (LAM group), or 3 to 12 hours post-water administration (control group). After BWE administration, horses were sedated with xylazine (1.1 mg/kg, IV) 5 minutes before anesthetic induction with a combination of diazepam (0.1 mg/kg) and ketamine (2.2 mg/kg), intubated, and maintained on isoflurane. Full thickness samples of the antimesenteric colon were withdrawn through a standard ventral midline approach. All samples obtained were placed in 10% neutral buffered formalin for 24 h and then transferred to 70% ethanol until paraffin embedding. Each horse was euthanized with 20 mg/kg (IV) of a solution containing pentobarbital sodium (390 mg/mL) and phenytoin sodium (50 mg/mL). Paraffin embedded samples were cut in 5 μm sections parallel to the crypt axis.

Histopathologic evaluation

All slices were stained with H&E for examination. Sections of colonic mucosa and submucosa from each specimen were examined via light microscopy by two investigators (E.J.E. and L.C.) using a 20x objective. Tissue samples were subjectively described with respect to edema, focal hemorrhage, karyorrhexis and leukocyte cell infiltration. All photographs were taken with a Q-fire digital camera and analyzed with QCapture[b] software for Windows®.

Immunohistochemistry

Two serial 5 μm sections were deparaffinized with 100% xylene 2 times, followed by 50% xylene in alcohol for 3 min, and rehydrated with 100%, 95%, 70% and 50% ethanol in deionized water for 1 minute each. For antigen retrieval, each tissue underwent heat treatment in citrate buffer pH 6.0[c] with a pressure cooker (125°C for 1 min). After cooling in distilled water, specimens were incubated in 0.3% hydrogen peroxide for 15 minutes at room temperature to quench endogenous peroxidase. After rinsing with TTBS 1 time, slides were incubated with a commercial background blocking reagent [d] for an additional 10 minutes to block nonspecific binding. Each colon section was then incubated with 1:100 mouse anti-human calprotectin monoclonal antibody (MAC387)[e] for 30 minutes at room temperature,[36,37] or with 1:500 affinity purified rabbit polyclonal antibody anti human/mouse caspase 3 active[f] overnight at 4^{o} C. The second section was incubated with TTBS alone as negative control. For each set of study slides subjected to calprotectin or caspase 3 immunohistochemistry staining, two adjacent 5 μm sections of normal equine lung and rat jejunum, from rats treated with 3mg/kg of lipopolysaccharide

intraperitoneally in a previous study, [g] were used as positive and negative controls, respectively.[36,37] After rinsing with TTBS 3 times, the slides were incubated with a commercial horseradish-peroxidase conjugated anti-rabbit and anti-mouse secondary antibody [h] for 30 minutes at room temperature, and rinsed again in TTBS for 3 times. To demonstrate the antigen, the staining was detected with a colorimetric reaction induced with 3,3'-Diaminobenzidine (DAB)[i] for 15 minutes at room temperature. Sections were counterstained with Mayer's Haematoxylin for 1 minute and, after rinsing once in distilled water, they were dehydrated with 50%, 70%, 95% and 100% ethanol for 1 minute, and then passed 1 time in 50% xylene in alcohol and 2 times in 100% xylene for 3 minutes. Sections were mounted in **Cytoseal XYL** mounting medium[j] and covered with glass cover slips. Tissues were observed by light microscopy using 20x (calprotectin) or 40x (caspase 3-active) objectives by two blinded independent investigators (L.C. and M.S.), and photographs were taken using 10x and 20x objectives.

Calprotectin staining evaluation

A scoring system (0–5) was used to score the distribution of positively stained cells: 0 indicated no neutrophils detected; 1, neutrophils limited to vasculature; 2, moderate to marked neutrophils showing margination within the vasculature; 3, marginating neutrophils in addition to perivascular neutrophils; 4, extravascular neutrophils and 5, severe extravascular neutrophilic infiltration in deep layers of the colonic wall.

Epithelial and endothelial apoptosis evaluation

Prior to the evaluation, two independent investigators (L.C. and M.S.) blinded to treatment groups agreed on a threshold for positive staining. Only epithelial and endothelial cells were considered in our study and were independently evaluated. For epithelial cells, the number of positively stained cells in two adjacent crypts in longitudinal section was counted (40x objective), and the count was repeated for 10 fields for each slide. The percentage of positively stained cells was calculated out of the total number of cells counted. For endothelial cells, the percentage of positively stained cells was calculated out of the total number of endothelial cells identified in mucosa and superficial submucosa for each slide.

Statistical analysis

The data are presented as median and inter-quartile range. Significance was set at $p<0.05$. Differences in calprotectin scoring and percentage of epithelial and endothelial caspase-3 active positive cells between the 3 groups were evaluated using the Kruskal-Wallis test. Post hoc pairwise multiple comparisons were performed using a Wilcoxon rank sum test, with Bonferroni-corrected alphas. To assess the correlation between calprotectin expression and apoptosis, calprotectin scores and percentage of epithelial and endothelial caspase 3-active positive cells were compared using Kendall's tau rank correlation coefficient. All statistical analyses were performed with Stata version 10[k].

Results

Histopathologic evaluation

All slides showed a high percentage of eosinophils and lymphocytes in both the lamina propria of the mucosa and in the submucosa. Compared with tissue from control horses, colon specimens from the DTP group had increased eosinophil and lymphocyte epitheliotropism, and increased superficial lamina propria inflammatory cell karyorrhexis (Fig. 2.1A). Colon specimens from the LAM group showed less severe epitheliotropism (with predominance of lymphocytes over eosinophils) and karyorrhexis, with increased free neutrophils in the lamina propria of the mucosa (Fig. 2.1B).

Calprotectin staining evaluation

The staining protocol was reliable since lung control sections showed positive staining in alveolar macrophages and randomly distributed neutrophils with no evidence of spurious binding of the primary or secondary antibody (Fig. 2.2). In colonic tissue specimens, the cells that stained positively for calprotectin were readily differentiated from surrounding and adjacent cells (Fig. 2.3). The leukocytes were consistently intensely stained, which obscured the nuclear morphology thereby impeding differentiation of neutrophils from monocytes or macrophages in most cases. However, the distribution of positive cells closely matched the distribution of neutrophils. Calprotectin scores within groups are shown in Table 2.1. There was a significant difference in calprotectin scores between groups (p=0.04). Colon specimens from the DTP group showed a significantly higher (p<0.01) score with respect to controls (Fig. 2.4).

Epithelial and endothelial apoptosis evaluation

The staining protocol was reliable since rat control sections showed positive staining in all layers of the intestinal wall with no evidence of spurious binding of the primary antibody or nonspecific binding of the secondary antibody. In colonic tissue specimens, the cells staining positive for caspase 3-active demonstrated variability in intensity of the staining. However, the staining distribution was uniform along the entire slide. The percentages of positively stained cells in the epithelium and the endothelium among the groups is summarized in Table 2.1. There was no statistically significant difference in percentage of epithelial (Fig. 2.5) and endothelial (Fig. 2.6) apoptotic cells between groups (p=0.08 and p=0.48 respectively). Images of typical epithelial and endothelial staining among groups are shown in Fig. 2.7 and 2.8. There was no correlation between calprotectin score and epithelial or endothelial percentage of caspase 3-active positive cells (p=0.69 and p=0.29 respectively).

Discussion

Insight into the effect of BWE toxicosis on equine colonic tissue was revealed in this study. McConnico *et al.* showed a significant increase in inflammation, edema, and hemorrhage, with increased eosinophilic infiltration and increased intestinal permeability *ex vivo* in all segments of colonic mucosa from BWE-treated horses with respect to controls, at the onset of Obel grade 1 laminitis.[6] Although our histologic analysis was only qualitative, all colonic samples had high eosinophilic infiltration in both mucosa and submucosa. Specimens from the DTP group, and to a lesser degree from the LAM group,

showed increased eosinophil and lymphocyte epitheliotropism with respect to the control group in addition to inflammatory cells karyorrhexis in the superficial lamina propria. Our findings are in agreement with a study conducted by Rotting *et al.* on normal horses,[50] in which eosinophils were found higher in number and closer to the mucosal surface in the cecum and ascending colon. The function of eosinophils under physiological or inflammatory conditions is not completely understood.[6,50] They have been reported to increase in response to various stimuli including experimentally induced colitis, ischemia-reperfusion injury, parasite infection and food allergies.[50] In a previous study, an association between large intestinal eosinophilic infiltration and cyathostome parasitism was found.[51] Although all horses in our study had been on deworming programs prior to the onset of the study, one horse had histopathologic evidence of parasitic larvae. However, a recent study found that the presence and distribution of eosinophils within different tracts of intestinal mucosa in horses is independent of *S. vulgaris* infestation.[52] The epitheliotropism of eosinophils and lymphocytes in BWE treated horses may be a response to toxicosis and should be further investigated.

Calprotectin immunohistochemical staining resulted in a reliable method for following neutrophilic migration through the colonic mucosa, as previously reported.[36] The dark staining of nuclei sometimes made it difficult to differentiate between neutrophils and monocytes or macrophages. Moreover, some cells were distorted during migration from the vessels to the lamina propria. Therefore, some of the positively stained cells that were identified as neutrophils may have been either infiltrated monocytes or activated local macrophages. At the same time, not all the neutrophils stained positively for calprotectin. These neutrophils may have not been activated or may

have already exhausted cytoplasmic calprotectin following activation.[37] The aim of our study was not to specifically identify neutrophilic infiltration, but to have an aid for scoring the colonic inflammatory response after BWE ingestion. Previous studies compared the accumulation of neutrophils counting the number of calprotectin positive cells per square millimeter of mucosa[38] or dividing the mucosa into five equal zones.[36] Due to the multifocal-segmental aspect of the inflammation, we adopted a scoring system that took into account both cell quantity and degree of infiltration. Horses in the DTP group showed a higher calprotectin score compared with controls. These findings are in agreement with previous studies showing increased activated neutrophil infiltration in the perivascular regions of the dermal vasculature of the laminae, around skin venules,[11,12] and in the hepatic and pulmonary parenchyma[13] at 3-4 hours post BWE administration. Although the LAM group had a higher median score than the DTP group, the difference was not statistically significant with respect to the control group. The small sample size, the high variability between horses, and the use of Bonferroni's adjusted alphas lowered the power of our estimates and with it, the ability to correctly identify a difference.

Calprotectin was associated with zinc-dependent and independent inhibition of growth and induction of apoptosis in endothelial cells, myeloid cells, mitogen-activated lymphocytes, human fibroblasts and several tumor cell lines *in vitro.* [31,32,35,49] Studies both *in vivo* and *in vitro* showed a significant association between apoptotic rate and intestinal bidirectional permeability in both the small and large intestine.[41-43,45] Although a significant correlation between calprotectin and apoptosis was not observed in this study, McConnico *et al.* demonstrated increased colonic mucosal permeability in horses

treated with BWE.[6] This may suggest apoptosis as a component in the pathogenesis of experimental BWE-induced laminitis.

There are limited studies investigating apoptosis in the horse and most are limited to reproductive physiology, exercise physiology and viral infection.[53] One study attempting to quantify apoptosis in the gastrointestinal tract from horses with and without gastrointestinal disease[54] used terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique for detection of fragmented DNA associated with apoptosis. Although largely used, the TUNEL assay may sometimes provide spurious results, since necrotic cells or cells not undergoing apoptosis may result in positive staining.[53,55] Immunohistochemical detection of caspase 3-active, one of the main components of the mitochondrial apoptotic pathway, is a valuable alternative method for apoptosis detection in the horse[53,56]and it was used in our study after optimization by variation of primary antibody incubation times and concentration.

For assessing the role of apoptosis in the BWE model, we limited our attention to epithelial and endothelial cells in the mucosa and superficial submucosa. There was no statistically significant difference in epithelial and endothelial apoptosis between groups although a trend toward a higher percentage of epithelial cell apoptosis was identified in the DTP group compared with controls (p=0.08). Apoptosis was not correlated with calprotectin expression. Rowe *et al.* reported a high variability in intestinal apoptosis in horses with disease not related to the gastrointestinal tract.[54] Calprotectin apoptotic effects have been reported to appear after 20 hours of exposure,[31,39] so our samples may have been collected too early to detect a possible relationship between calprotectin expression and apoptosis.

BWE induced laminitis represents a reliable method for studying the link between gastro-intestinal disease and laminitis in the horse.[6,19] The present study demonstrated that there is increased activated leukocyte infiltration in the colonic mucosa of horses exposed to orally administered BWE as early as 3 hours post-administration. Even though it is difficult to differentiate whether the colon is the first focus of the inflammatory reaction or if this is part of the systemic response observed with BWE-induced laminitis, the epitheliotropism of eosinophils and lymphocytes suggests a local inflammatory reaction in response to BWE toxicosis.[50] Statistically significant differences in epithelial and endothelial apoptosis were not identified at either time point. Different mechanisms, such as disruption of tight junctions in non-apoptotic cells may result in the increased intestinal permeability observed by other authors[6] and should be further investigated.

1

Endnotes

[a] Chiavaccini L. *Prognostic value of white blood cell drop in horses undergoing post-colic surgery intensive care.* DEC Thesis. Faculte' de Medicine Veterinaire, Université de Liège, Sart Tilman-Liège, Belgique, 2006.

[b] Olympus America, Inc., Melville, NY.

[c] Target retrieval solution pH6.0 10x, DakoCytomation, Glostrup, DK-2600 Denmark.

[d] Background Sniper, Biocare Medical, Concord, CA.

[e] MAC387, Abcam, Cambridge, MA.

[f] R&D Systems, Minneapolis, MN.

[g] Chiavaccini, L. and Boscan, P. Unpublished data.

[h] EnVision™+ Dual Link System-HRP, Dako, Glostrup, Denmark.

[I] DAB Peroxidase Substrate Kit, Vector Laboratories, Burlingame, CA.

[j] Richard-Allan **Cytoseal XYL** Mounting Medium, Richard-Allan Scientific, Kalamazoo, MI.

[k] Stata/IC 10.1 for Windows, StataCorp LP, College Station, TX.

Tables and figures

Table 2.1: Median calprotectin score and percentage of epithelial and endothelial apoptotic cells for colonic mucosa and submucosa obtained from horses treated with BWE at 3 hours post-treatment (DTP), at onset of Obel1 lameness (LAM) and controls.

	DTP (median, IQ) (n)	LAM (median, IQ) (n)	Control (median, IQ) (n)
Calprotectin score	3, 2-4 (6)	3.5, 1-4 (6)	1, 1-2 (7)
Apoptosis epithelium	1%, 9-12% (6)	4.5%, 2-6% (6)	4%, 3-27% (7)
Apoptosis endothelium	59.5%, 57-63% (6)	52%, 47-59% (6)	52%, 41-69% (7)

IQ, interquartile range; n, number.

Figure 2.2: Representative photomicrographs of sections of colonic mucosa obtained from horses treated with BWE at 3 hours post-treatment (DTP) and at the onset of Obel1 lameness (LAM). (A) Mucosa obtained at DTP. Notice eosinophil and lymphocyte epitheliotropism (arrow heads) and superficial lamina propria inflammatory cell karyorrhexis (arrows). (B) Mucosa obtained at LAM. Notice predominance of lymphocyte migration in the epithelium (arrow heads) and presence of neutrophils (arrows). H&E (x200).

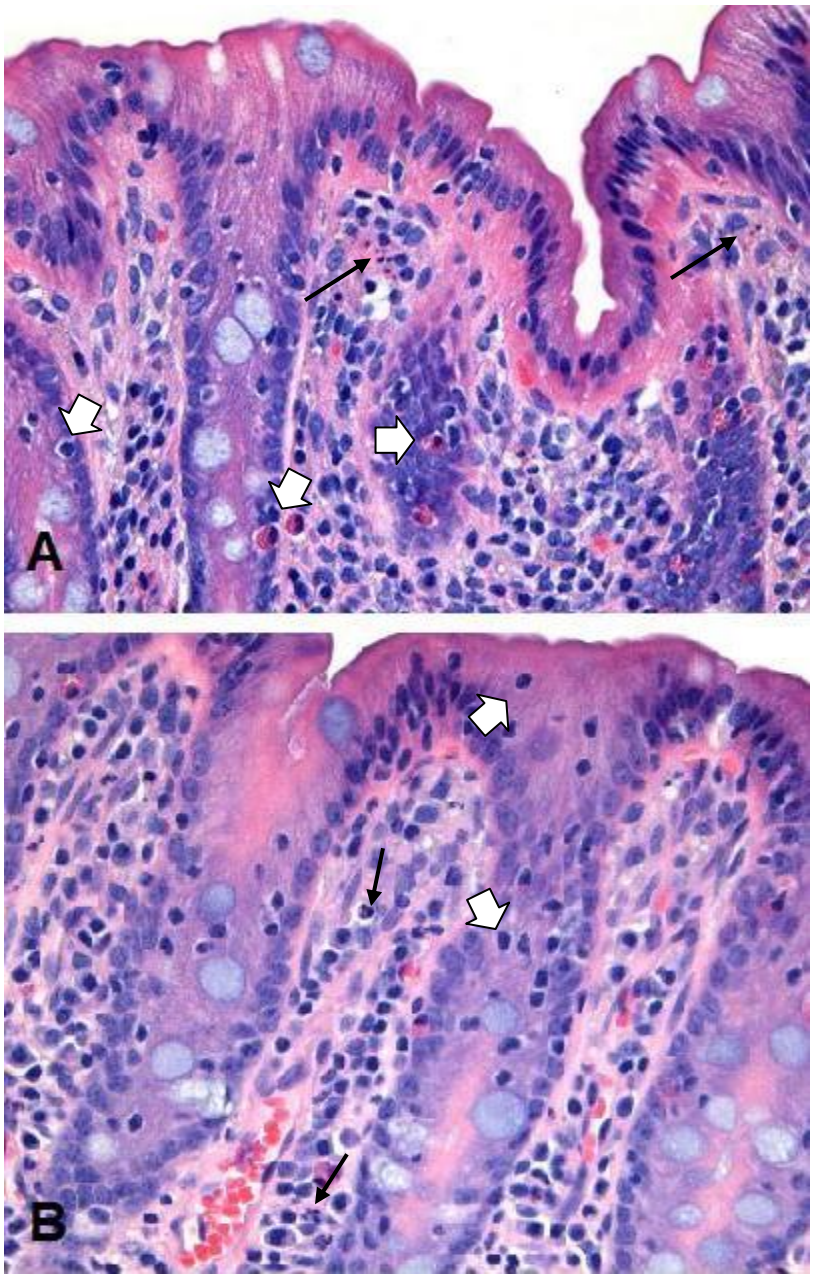


Figure 3.2: Calprotectin immunohistochemistry to detect equine alveolar macrophages (brown-stained cells): Negative (A) and positive controls (B) of equine lung tissue using 1:100 MAC 387 antibody (x100).

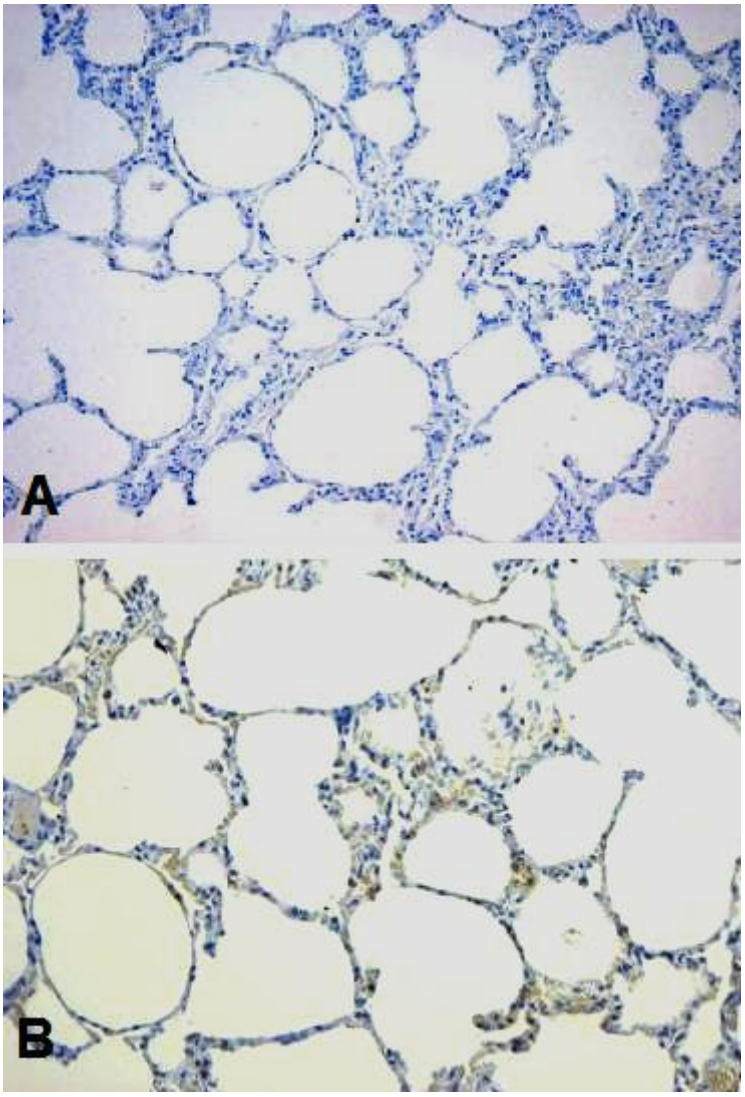


Figure 2.4: Calprotectin scoring. (A) 0, no neutrophils detected; (B) 1, neutrophils limited to vasculature; (C) 2, moderate to marked neutrophils showing margination within the vasculature; (D) 3, marginating neutrophils in addition to perivascular neutrophils; (E) 4, extravascular neutrophils; (6) 5, severe extravascular neutrophilic infiltration in deep layers of the colonic wall. 1:100 MAC 387 antibody (x200).

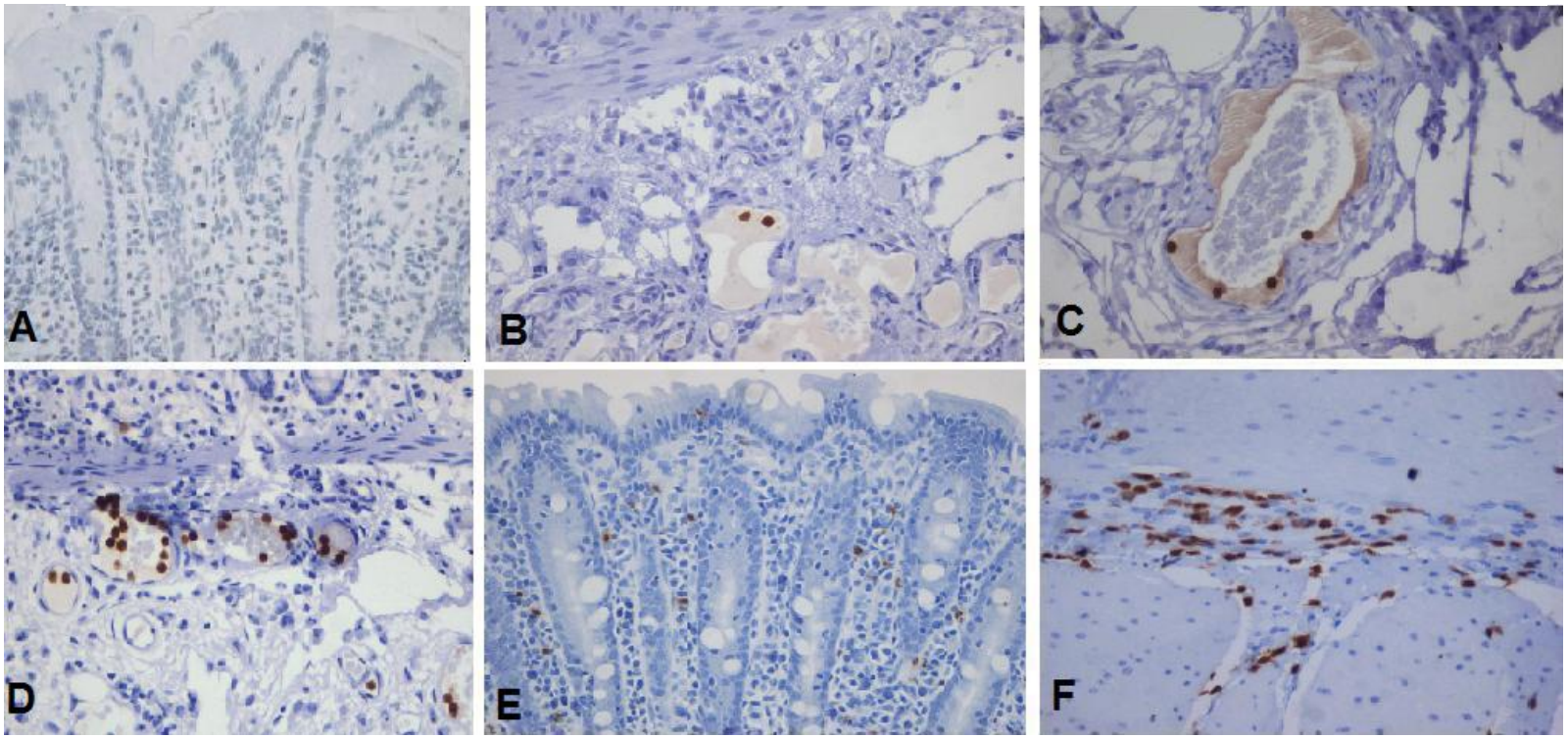


Figure 2.5: Score of calprotectin immunohistochemistry based on frequency of positively stained cells and migratory activity of neutrophils, for colonic mucosa and submucosa obtained from horses treated with BWE at 3 hours post-treatment (DTP), at the onset of Obel1 lameness (LAM) and controls. Median and inter-quartile range.*p< 0.05

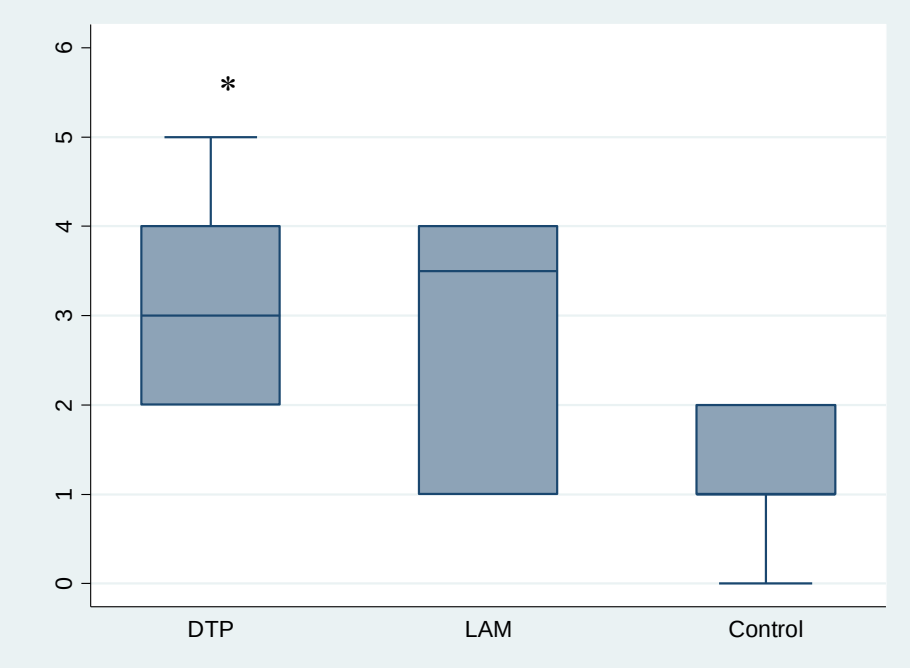


Figure 2.6: Percentage of epithelial apoptotic cells based on positive caspase 3-active staining, for colonic mucosa obtained from horses treated with BWE at 3 hours post-treatment (DTP), at the onset of Obel1 lameness (LAM) and controls. Median and inter-quartile range. *p< 0.05

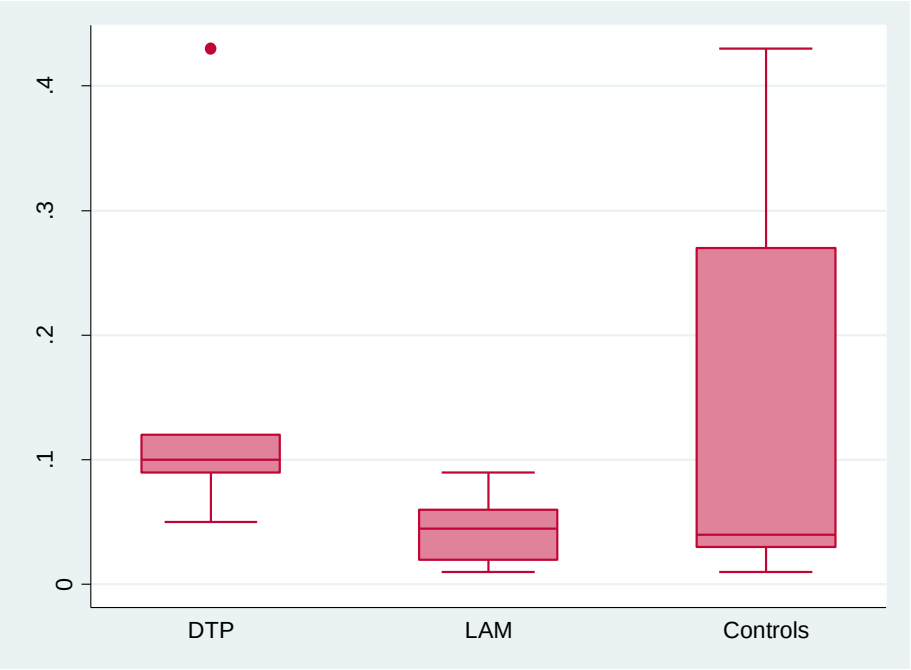


Figure 2.7: Percentage of endothelial apoptotic cells based on positive caspase 3-active staining, for colonic mucosa and superficial submucosa obtained from horses treated with BWE at 3 hours post-treatment (DTP), at the onset of Obel1 lameness (LAM) and controls. Median and inter-quartile range.*$p < 0.05$

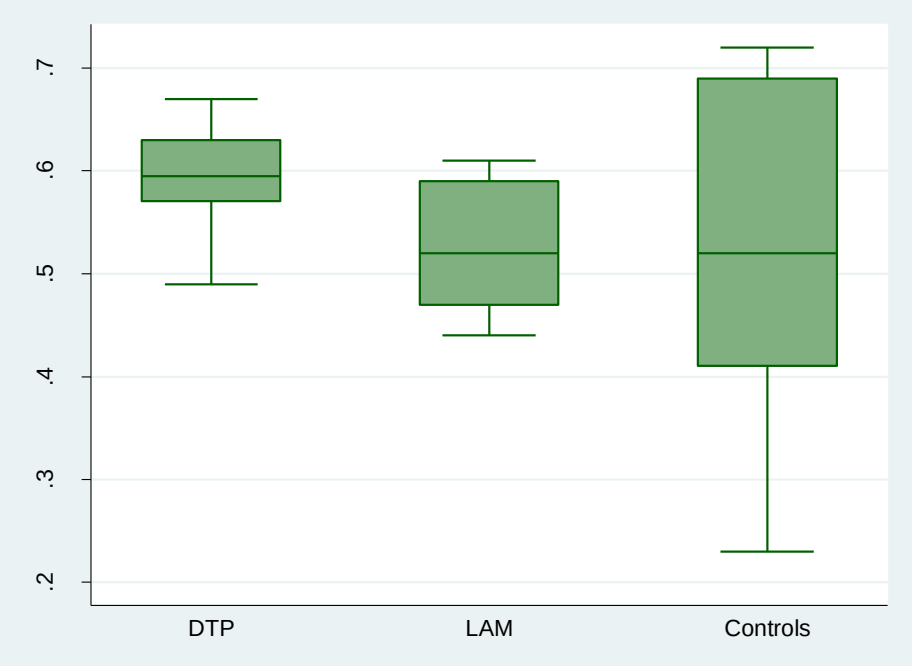


Figure 2.8: Representative photomicrographs of colonic epithelium obtained from controls, horses treated with BWE at 3 hours post-treatment (DTP), and at the onset of Obel1 lameness (LAM). (A) Mucosa from control section. (B) Mucosa obtained at DTP. (C) Mucosa obtained at LAM. 1:500 Caspase 3-active antibody (x100).

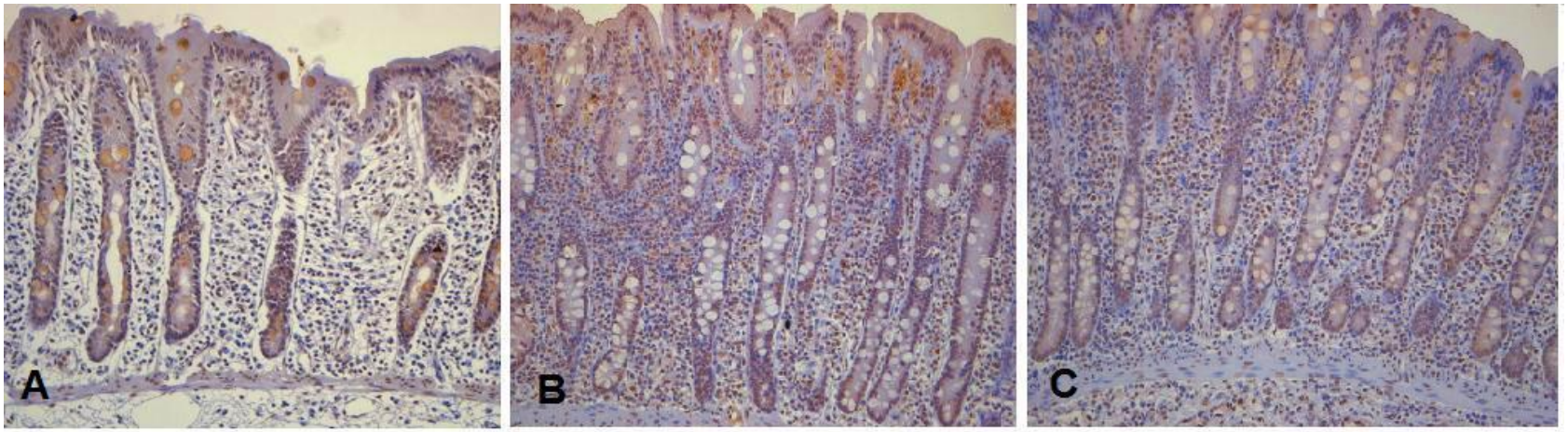
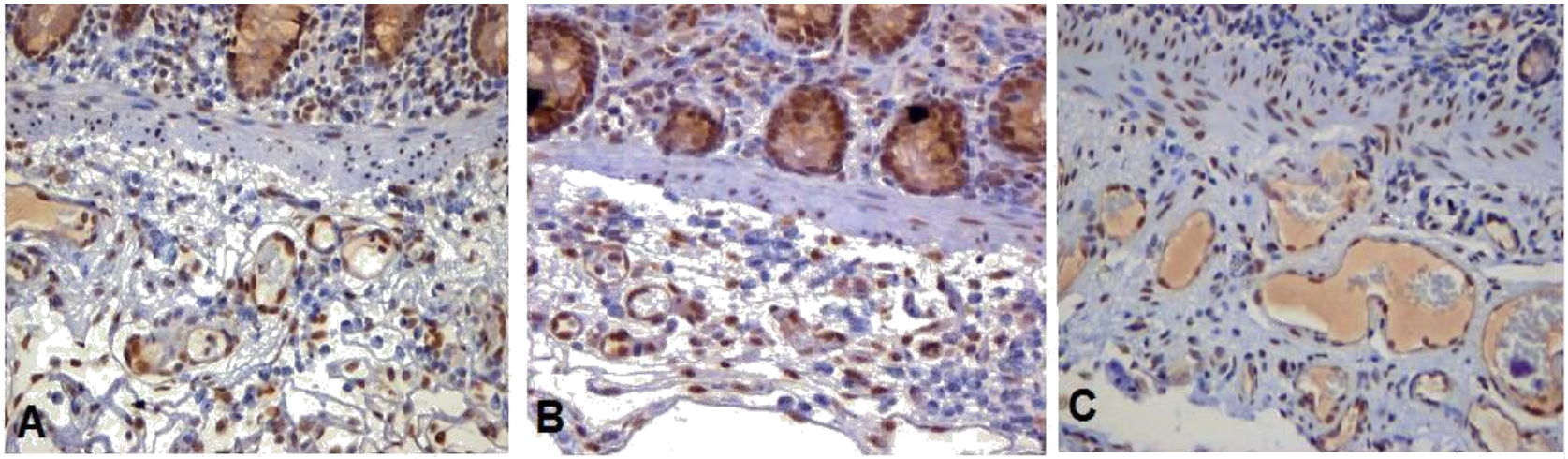


Figure 2.9: Representative photomicrographs of colonic submucosal endothelium obtained from controls, horses treated with BWE at 3 hours post-treatment (DTP), and at the onset of Obel1 lameness (LAM). (A) Mucosa and submucosa from control section. (B) Mucosa and submucosa obtained at DTP. (C) Mucosa and submucosa obtained at LAM. 1:500 Caspase 3-active antibody (x200).



PART II

PROGNOSTIC FACTORS AFTER ESOPHAGEAL OBSTRUCTION IN HORSES

Chapter 3: Literature Review on Esophageal Obstruction (Choke) in Horses

Esophageal obstruction or "choke" is a common clinical disorder and the most common esophageal condition in the horse [1-4]. It can be a primary disorder or due to pre-existing pathological conditions leading to a partial or complete obstruction of the esophageal lumen.

Esophageal Anatomy

The esophagus is a musculomembranous tubular structure extending from the pharynx to the stomach and can be divided into proximal, thoracic and abdominal parts. Its wall is comprised of four layers: mucosa, submucosa, muscularis and adventitia or serosa (depending of the portion). The mucosa consists of stratified squamous epithelium, while the submucosa mostly contains elastic fibers. The *muscularis mucosa* of the lamina propria is thin in the cranial esophagus and becomes thicker approaching the cardia. The *muscularis externa* extends along the entire length of the esophagus. The orientation of fibers is circular toward the luminal surface and longitudinal toward the outer surface. The oral two-thirds of the muscular layers consists of skeletal muscle and is innervated by the pharyngeal and esophageal branches of the vagus nerve, while the aboral one-third consists of smooth muscle and is innervated by the parasympathetic fibers of the vagus nerve. The role of sympathetic fibers in innervation of the esophagus is irrelevant. [1,3,5]

Etiopathogenesis and Symptoms of Esophageal Obstruction

Esophageal obstruction can occur in every portion of the esophagus, but it is more commonly found in the proximal portion aboral to the larynx and at the thoracic inlet [1,3,5,6]. Esophageal obstruction can be primary or secondary [3]. Primary obstruction is the most common cause of "choke" and it is usually caused by feed impaction, such as hay, grain, pelleted feed, beet pulp, carrots, apples or other vegetables, coarse food, corncobs and, more rarely, antibiotic boluses, wood or wood shavings or phytobezoar [1,2,7-9]. Predisposing factors include poor dentition, inadequate water intake, eating when heavily sedated or during the recovery from general anesthesia [1,3,4,10,11]. Primary esophageal obstructions are usually presented as isolated cases and, if promptly treated, typically do not recur [11]. Esophageal obstruction can be secondary to local or systemic disorders, such as squamous cell carcinoma or cysts within the esophageal wall [1,12-15], para-esophageal abscesses [10], strictures and diverticula [1,2,7,16,17], esophageal motility disorders and neurologic and neuromuscular abnormalities. It also may occur secondary to guttural punch mycosis, pharyngeal trauma, botulism and grass sickness [1,4,18-20]. A case of esophageal obstruction associated with esophageal ectasia was described in a thoroughbred foal [3].

Strictures and diverticula can be primary or secondary to a previous episode of obstruction. Strictures have been classified into three types: Type I involves only the mucosa and submucosa, type II involves also the *tunica muscularis* and type III involves the entire esophageal wall. Type II and III strictures have a poor prognosis [21]. Primary strictures are more common in foals. Usually they are idiopathic in nature and symptoms appear within the first two weeks of life [2,18]. An unusual condition is idiopathic muscular

hypertrophy of the esophagus. It consists of the hypertrophy of the circular layer of the *tunica muscularis* in the distal one-third of the esophagus, and sometimes other portions of the gastrointestinal tract. Although it is mostly considered a coincidental finding during necropsy, it has been sometimes implicated in cases of recurrent esophageal obstruction [22]. More common are strictures secondary to previous cases of esophageal obstruction or esophageal reflux, as a result of circumferential ulceration of the mucosa [2,21,23]. Other causes of secondary stricture include trauma from a kick or from nasogastric tube passage and persistent right aortic arch or other vascular ring anomalies [2,24].

Diverticula are differentiated as pulsion or traction diverticula. Pulsion diverticula are formed by herniation of the esophageal mucosa through the muscular layer due to increased intraluminal pressure. They form as a consequence of a kick [2,25] or secondary to esophageal stenosis/stricture. Traction diverticula are formed by pulling forces on the esophageal wall due to periesophageal bands of adhesion [1,17].

Symptoms of esophageal obstruction are pathognomonic and include ptyalism, profuse salivation, foam or alimentary discharge from the nostrils, coughing, frequent and ineffective attempts to swallow, retching, extension and contraction of the neck. If the obstruction is in the cervical part of the esophagus, a local swelling may be seen and palpated on the left side of the neck [1,3,4,7,18,19,24].

Diagnosis

Diagnosis of esophageal obstruction is usually simple based on clinical symptoms. The passage of a nasogastric tube allows the confirmation of an obstruction and its localization [1-4,7].

Determining the primary cause of an obstruction may be challenging. Especially in recurrent or chronic cases, the use of instrumental diagnostic tools may be required. Esophageal endoscopy allows direct visualization and localization of the obstruction [1-4,7], the visualization of intraluminal masses, strictures or diverticula [4,7,18,21] and should be performed on obstructions longer than 24 hours duration for assessing the integrity of the mucosa [1,4]. Motility disorders are harder to diagnose, especially if the horse is sedated. However they may be assessed as luminal dilation and absence of peristalsis, when the endoscope is pulled in a retrograde fashion from the gastric lumen [1,15]. Before the esophagus, the trachea should be examined for evidence of aspiration [7]. Endoscopy of the upper airways may eliminate other differential diagnoses such as guttural punch mycosis or neurological problems [4,19].

Esophagography can give important information regarding the nature of the obstruction. The normal esophagus should not be visible [1], however in cases with distention proximal to an obstruction or due to peristaltic impairment, large amounts of intraluminal air may be detected. Air in the periesophageal region may indicate esophageal rupture [1,3,4]. Plain radiography allows evaluation for the presence of radiopaque foreign bodies. A more accurate evaluation of the nature of the obstruction can be made with contrast radiography or computed tomography (CT scan). Contrast radiography allows recognition of the profile of intraluminal radiolucent foreign bodies and outlining of diverticula or dilations. Pulsion diverticula appear rounded, with a narrow neck, while traction diverticula are usually smaller and have a triangular appearance [1,17,25,26]. Sequential contrast radiographs allow differentiation between anatomical and functional strictures due to esophageal spasms [1,18]. Using double contrast

enables detection of mucosal ulceration. If an irregular intraluminal or periesophageal mass is seen, a squamous cell carcinoma may be suspected, while a smooth regularly marginated appearance is more consistent with an esophageal or periesophageal abscess [1,10,12,13,15]. CT has been used in foals suspected of having vascular ring anomalies [18] and for evaluating the involvement of the central nervous system [19]. In addition, electromyography, manometry and muscle or nerve biopsies may be required when a neuromuscular dysfunction is suspected [3,18].

Ultrasound can be used as a complimentary aid for helping in the diagnosis of feed impaction, stricture or diverticula in the cervical part of the esophagus [1].

Nonsurgical vs. Surgical Management

Some esophageal obstructions may resolve spontaneously, simply via withdrawal of access to feed and water for a few hours in combination with sedation with xylazine, detomidine hydrochloride, or acepromazine to improve esophageal muscle relaxation [1,11]. However, many obstructions require the passage of a nasogastric tube, lavage by gravity or with a stomach pump and gentle pressure on the impaction to push it into the stomach [1-4]. Sometimes removal or disruption of the impaction with endoscopic forceps is required [3]. The use of mineral oil for helping the passage of the bolus is controversial [1,4]. Campbell (2003) suggests using 50-100 ml of 27% lidocaine solution directly on the site of the obstruction, in order to relax the esophageal musculature. Oxytocin (0.11-0.22 IU/kg) has been reported to induce transient relaxation of esophageal striated musculature 5-15 min after IV administration in healthy horses [5]. However, other investigators have not been able to corroborate these findings using manometric evaluations of the

esophagus [27], confirming the results of an *in vitro* study in which oxytocin showed relaxation of only the esophageal smooth muscle [6]. In a previous study, detomidine, acepromazine, and a combination of xylazine and butorphanol have been reported to have potential detrimental effects on the resolution of the esophageal obstruction because of the reduction in swallowing and the change in normal peristaltic activity with increases in high pressure events at the thoracic inlet [27]. General anesthesia may be necessary in some refractory cases [1,3]. Due to the high risk of aspiration pneumonia, all authors agree that many horses with esophageal obstruction should be placed on prophylactic broad-spectrum parenteral antibiotic therapy [1-4,7,11,18,21,28]. Non-steroidal anti-inflammatory drugs (NSAIDs) may help with preventing stricture formation in cases of severe inflammation of the esophageal mucosa [1,2,4,21]. Feed should be withheld for 12-72 hours, depending on the duration of the obstruction and the grade of the mucosal lesion. The initial diet should be very soft and fed in small quantities several times daily [1,4]. In cases of severe mucosal lesions or irresolvable choke, a nasogastric tube can be temporarily placed distal to the obstruction through an esophagotomy [1]. Rarely an esophagotomy is required in order to resolve the obstruction [2,3]. In a study on 61 horses, the open esophagotomy healed with second intention with no complications, while the sutured esophagotomy lead to postoperative infection and laminitis [2]. In one study, a gastrotomy was used for solving an esophageal obstruction [29].

Surgical intervention is more often required in case of structural modification of the esophagus, such as strictures or diverticula. The results of nonsurgical management of esophageal strictures are controversial. If modification of the diet is not strictly enforced, horses managed medically for esophageal strictures tend to have recurrent episodes of

obstruction and a poor survival rate [23]. In a retrospective study on 61 cases of esophageal obstruction, surgical management of esophageal stricture had a better long term survival than medical management [2]. However, studies conducted by Clabough *et al.* (1991) and Knottenbelt *et al.* (1992) on foals less than six months of age refuted these findings, since all horses had a positive response to medical management. Surgical management of stricture includes esophageal myectomy and esophagomyoplasty, partial resection and anastomosis, patch grafting of the sternocephalicus muscle, and mucosal scar fenestration [2,26]. Two successful cases of esophagomyotomy and esophagopexy, and one case of removal of intraluminal exuberant granulation tissue with Nd:YAG laser have been described [16,30].

Surgical management of esophageal diverticula includes diverticulectomy and inversion of the redundant mucosa into the esophageal lumen, followed by the repair of the defect in the *tunica muscularis* [2,3]. Harrison and Cartee (1990) had good results simply by performing an esophagomyotomy extending proximally and distally to the diverticulum and dissecting the *tunica muscularis* for 180^{o} from the submucosal layer.

Complications

Complications are common and may include aspiration pneumonia, pleuritis, mucosal esophageal ulceration and stricture formation, esophageal perforation, chronic recurrent obstruction, postoperative infection, laminitis, laryngeal paralysis and Horner's syndrome [1-3].

Aspiration pneumonia is the most common life threatening complication after an episode of esophageal obstruction [1-3]. In a retrospective study on 34 horses with

esophageal obstruction, the duration of the obstruction prior to admission, but not the grade of tracheal contamination, was a good predictor of whether aspiration pneumonia would develop [7]. The other main complication is stricture formation as a consequence of circumferential mucosal ulceration [1-3,18]. Experimental stricture of the esophagus has been reproduced 15 days after mucosal resection and anastomosis, while naturally strictures may form in only a few days. For this reason, horses with a history of esophageal obstruction should be examined 24-48 hours after resolution for the presence of ulceration, and should undergo anti-inflammatory therapy to reduce the risk of stricture [21]. Esophageal rupture, dehiscence, repeat stricture, mediastinitis, pleuritis, laminitis, laryngeal paralysis, and Horner's syndrome are reported after esophageal surgery [2,3].

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Chapter 4: Retrospective review of clinical features and prognostic variables in 109 horses with esophageal obstruction (1992-2009)

This chapter has been formatted based on the instruction for authors in order to submit it to the Journal of Veterinary Internal Medicine. Some information included in Chapter 3 is repeated here in the introduction and discussion in order to have this chapter as a standalone document for subsequent submission and consideration for publication.

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Short Title: Esophageal obstruction in horses

Keywords: Equine; Esophagus; Obstruction; Esophagitis

Abbreviations: T, Temperature; HR, Heart rate; RR, Respiratory rate; PCV, Packed cell volume; TP, total protein; NSAID, Non-steroidal anti-inflammatory drugs; OR, Odds ratio.

All data collection was obtained from medical records at the Equine Hospital at Colorado State University, Fort Collins, CO.

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Esophageal obstruction is a commonly observed emergency in the horse and can result in life-threatening complications. Previous studies have described clinical findings in horses with esophageal obstruction, but there are no reports that attempt to make correlations of clinical findings with outcome. We hypothesized that specific clinical features of horses with esophageal obstruction are associated with increased likelihood of complications. This retrospective cross-sectional study included 109 horses with esophageal obstruction. All clinical records of horses admitted between April 1992 and February 2009 for esophageal obstruction were reviewed. The association between 24 clinical, hematological, biochemical, therapeutic variables and the likelihood of complications was investigated by a univariable logistic regression model, followed by multivariable analysis. Multiple logistic regression analysis revealed a significant association between sex and age of the horse and general anesthesia with the outcome. Breed, heart rate, respiratory rate, whether the obstruction was in the proximal, middle or distal third of the esophagus, PCV, glucose, bicarbonate, anion gap, and the time elapsed between admission and resolution were not associated with the outcome. Intact males, age greater than 15 years and a need for general anesthesia were associated with increased likelihood of complications after an episode of esophageal obstruction. This study gives insight into the understanding of other factors that may affect prognosis in horses with esophageal obstruction and could give rise to a prospective study in the future.

Introduction

Esophageal obstruction or "choke" is a common clinical presentation in the horse; most frequently it is caused by feed impaction. Suggested causes for simple obstruction include ingestion of inadequately soaked sugarbeet pulp, ingestion of apples or carrots, excessively rapid ingestion of dry fibrous, pelleted or cubed feedstuffs, inadequate mastication from poor dentition, or the swallowing of a foreign body.[1,2] Other reported causes include inadequate water intake, eating when heavily sedated, and esophageal disease including functional neuromuscular disorders, stenosis/strictures, diverticula, neoplasia, abscesses and cysts.[3,4]

Horses with persistent esophageal obstruction often become compromised as a consequence of dehydration, acid-base and electrolyte imbalances, and aspiration pneumonia.[5] Complications are common and may include esophageal mucosal ulceration, stricture formation, esophageal perforation, aspiration pneumonia, chronic recurrent obstruction, postoperative infection, pleuritis, laminitis, laryngeal paralysis, and Horner's syndrome.[6]

There are few published studies describing the clinical findings in horses with esophageal obstruction and these mainly consist of smaller numbers of horses. They also fail to make correlations of clinical findings with outcome. The purpose of the present retrospective cross-sectional study was to describe clinical features of a population of horses presented to a referral institution with esophageal obstruction, and to assess the association between analyzed variables and the likelihood of complications.

Material and Methods

The clinical records of horses admitted for esophageal obstruction to the Equine Hospital at Colorado State University between April 1992 and February 2009 were analyzed. Variables included breed, sex, age, temperature (T), heart rate (HR) and respiratory rate (RR) at admission, whether or not thoracic or esophageal radiographs were performed and radiographic findings. Radiographic evidence of alveolar or bronchoalveolar densities in the caudoventral region of the lungs was considered a sign compatible with aspiration pneumonia secondary to esophageal obstruction. When endoscopy was performed, tracheal contamination with food material was subjectively graded, as previously described,[2] as absent, when no visible particles were detected, mild, when there were single food particles in the trachea, moderate, when small amounts of aspirated fluid were detected on the tracheal floor, or severe, when large amounts of aspirated fluid were detected on the tracheal floor and/or food particles were detected in the dorsal aspect along the trachea. Visible esophageal lesions were classified as absence of irritation or swelling (normal), mild to moderate mucosal lesions, or severe lesions, ulcers or morphological abnormalities. The location of the obstruction was identified as proximal (≤ 60 cm), middle (61-90 cm), or distal (> 90 cm) third of the esophagus as determined endoscopically, measured as centimeters from the nares, and via clinical examination, taking as a reference an adult Warmblood and proportionally adapting the measurement to smaller horses or foals.

Hematologic and biochemical variables at the time of admission included packed cell volume (PCV), total protein (TP), blood glucose, bicarbonate, and anion gap.

Recorded treatments included whether antibiotics were administered and the route of administration (oral, parenteral or both). Additional drug therapies documented included the use of non-steroidal anti-inflammatory drugs (NSAIDs), alpha-2 agonists, opioids, acepromazine and oxytocin. Whether or not general anesthesia was required for resolving the obstruction was recorded.

The duration of the esophageal obstruction prior to admission (less than 3 hours, between 3-6.3 hours, between 6.4-12.3 hours, between 12.4-48 hours or more than 48 hours in duration) was established. The time between admission and resolution of the obstruction was classified as a) relieved spontaneously by admission, b) during initial treatment, c) within 24 hours, d) more than 24 hours or e) never resolved.

For the outcome variable, an animal showing any complication related directly to the esophageal obstruction was considered as "having complications" and these were specifically recorded.

A descriptive analysis of all the recorded variables and their association with the outcome was undertaken. The association between these variables and the outcome was assessed by logistic regression analysis. Continuous variables were evaluated for linear relation with the log odds of the outcome using the Lowess smoothing function and whenever the assumption was not met they were categorized based upon biological reasoning and distribution of the population. Variables with a univariable $p \leq 0.25$ were included into the multivariable model, which was constructed manually. Significance was set at $p \leq 0.05$. If a variable affected more than 20% the coefficient of another variable, it was considered a confounder and was forced into the model. We checked all biologically plausible 2-way interactions. The Hosmer-Lemshow statistic was used to

assess the overall fit of the model. All the analyses were conducted with Stata version 10[a].

Results

The dataset consisted of 109 records from horses admitted to the Equine Hospital at Colorado State University for esophageal obstruction between April 1992 and February 2009. Only 5 records contained a complete collection of all variables of interest. Of these 109 horses, 56 (51.4%) developed complications including aspiration pneumonia (39), esophageal stenosis/stricture (8), fever (4), esophageal diverticula (4) or rupture (2), kidney failure (2), diarrhea (2), mild esophagitis (1), laryngeal hemiplegia (1), esophageal necrosis (1), laminitis (1) or a combination of these, or the obstruction was not resolvable due to undetermined causes (7). Thirteen horses (11.9%) died or were euthanized at the hospital as a consequence of the obstruction. Descriptive statistics for variables significantly associated with the outcome as determined via univariable analysis are summarized in Table 4.1. Since pneumonia alone represented almost 70% of the overall complications, its association with each variable of interest was further investigated and results are summarized in Table 4.2.

Animal level variables

The cases ranged between 2 months and 35 years of age (median 13 years) and included 45 Quarter Horses (41.3%), 17 Arabians (15.6%), 5 Thoroughbreds (4.6%), 8 ponies (7.3%) and 34 horses of other breeds (31.2%). There were 36 females (33.1%), 56 geldings (51.4%), and 17 intact males (15.6%).

Clinical variables

Body temperature (T) on admission ranged between 35.6°C and 40.6°C (median 37.9°C). The heart rate (HR) and respiratory rate (RR) ranged between 30 -100 beats per minute and 8-66 breaths per minute respectively (median 56 beats/min and 24 breaths/min respectively). Signs consistent with aspiration pneumonia were found in 25 of the 38 horses (65.8%) that underwent thoracic radiography at admission. Tracheoscopy was performed on 45 horses (41.3%). Esophagoscopy was performed on 70 horses (64.2%). Anatomical abnormalities included eight cases of stricture. Two strictures were located in the proximal one-third of the esophagus, two in the middle third, two in the distal third and two had an undocumented location. Two of these were associated with post-stenotic diverticula, two with esophageal scars, and one with a para-esophageal abscess in the caudal retropharyngeal region. One half of all cases of stricture were foals (4/8) less than 1 year of age. Other esophageal disorders associated with the esophageal obstruction ranged from moderate circumferential erosion (15) to esophageal ulcers (8), ranging in length from 5 cm to the entire esophagus. Two cases of distal esophageal hyperplasia were diagnosed via subjective assessment at endoscopy or during postmortem examination. Fifteen out of 59 obstructions (25.4%) were located in the middle third of the esophagus, 22 (37.3%) in the proximal and distal thirds respectively. For the other 11 horses undergoing endoscopy, the location of the obstruction was unrecorded.

Hematological and biochemical variables

On admission, the packed cell volume (PCV) ranged between 23% and 59% (median 34%). The mean total protein was 73 g/L ± 10.4 (mean±SD, range 34-99 g/L). Glucose measured at admission ranged between 3.3 and 16.3 mmol/L (median 7.8 mmol/L). Bicarbonate ranged between 13.7 and 30.6 mmol/L (median 25.6 mmol/L) and the anion gap between 9 and 35 mmol/L (median 15 mmol/L).

Treatment-related variables

Of the 71 horses for which antibiotic therapy was recorded, thirteen (18.3%) received oral antibiotics only, 20 (28.2%) received parenteral antibiotics only, and 38 (53.5%) received both oral and parenteral antibiotics. Sixty-one out of 107 horses (57%), for which the medical therapy was recorded, were treated with NSAIDs, 87 (81.3%) received alpha-2-agonists and 59 (55.1%) received opioids. All but one horse received opioids in association with an alpha-2 agonist. Twenty-six (24.3%) were treated with acepromazine and 14 (13.1%) horses received one or more doses of oxytocin (0.11-0.22 IU/kg IV).

Other variables

Out of 78 horses for which the duration of the choke was recorded, 11 (14.1%) were referred to the hospital within three hours from recognition of the choke episode, nine (11.5%) between 3.1 and 6.3 hours, 18 (23.1%) between 6.4 and 12.3 hours and 18 (23.1%) within 24 hours. Of the 22 horses (28.2%) that suffered chronic obstruction (more than 48 hours in duration), 11 (50%) showed severe esophageal lesions or

anatomical abnormalities endoscopically. The majority (32/61) of obstructions were resolved during initial attendance. Thirteen cases (21.3%) were not resolvable and were euthanized. Since euthanasia was inevitably performed on all horses with irresolvable obstructive lesions, these horses were categorized as "never resolved" and removed from further analysis since it was lacking in statistical variability.

Univariable and multivariable logistic regression analysis

Age, presence of radiographic signs of aspiration pneumonia, grade of esophageal lesion as detected endoscopically, total plasma protein, type of antibiotics used, whether general anesthesia was required for resolution of the obstruction, and duration of the obstruction prior to referral had significant associations with the development of complications ($p \leq 0.05$) via univariable analyses (Table 4.1). Breed, temperature, heart rate, whether the obstruction was in the proximal, middle or distal third of the esophagus, PCV, glucose, bicarbonate, anion gap and the time elapsed between admission and resolution were not associated with outcome. Respiratory rate and grade of tracheal contamination, although not associated with complications as a whole, were significantly associated with the specific risk of developing aspiration pneumonia (Table 4.2). Sex, age and whether or not general anesthesia was needed in order to solve the obstruction had complete observations and were thus included in the multivariable model.

The final model was significant ($p<0.01$) and findings are shown in Table 4.3. When considering all the variables in the model, intact males were almost seven times more likely to develop complications than females (OR = 6.6, 95% CI 1.4-31.6, p = 0.02), senior horses (>15 years) were more than six times more likely to develop

complications than adult horses (OR= 6.2, 95%CI 2.2-17.6, $p<0.01$), and the requirement of general anesthesia for solving the obstruction increased the likelihood of developing complications five times (OR= 5.1, 95%CI 1.9-13.4, $p<0.01$). Overall, the model fits the data well based on the Hosmer-Lemeshow statistic (Chi^2 3.2, df=6, $p = 0.78$) and had a good predictive value (AUC ROC 0.79). The specificity and sensitivity of the model were 71.7% and 66.1% respectively.

Discussion

The short term survival rate and the complications associated with esophageal obstruction were in accordance with earlier findings.[6] Previous studies reported that aspiration pneumonia is one of the most frequent complications after esophageal obstruction and a cause of mortality.[6,7] This was confirmed by the present study, as aspiration pneumonia represented nearly 70% (39/56) of overall complications. Other complications were sporadic and often associated with pneumonia. According to our study, the risk of developing aspiration pneumonia was positively associated with respiratory rate at admission. Although an elevated respiratory rate can occur due to several reasons such as pain or stress, this could also be an indicator of early pulmonary impairment, as suggested by our results, in which horses with a respiratory rate greater than 22 breaths per min at admission were nearly six-fold more likely to develop aspiration pneumonia after the episode of esophageal obstruction, with respect to those with a rate of 12 breaths per min or less. Feige *et al.*[2] suggested that clinical and radiographic evaluation of the lungs is of value whenever respiratory impairment is suspected. In our study we demonstrated a significant association between the detection

of radiographic signs compatible with aspiration pneumonia and the subsequent development of complications. Interestingly, radiographic signs were not directly associated with pneumonia. However, this may have been a spurious finding due to the smaller number of horses when only aspiration pneumonia was considered as an outcome. Unlike Feige *et al.*,[2] we found that the extent of tracheal contamination was proportionally associated with the subsequent development of pneumonia (Table 4.2).

In our study, the grade of the esophageal lesion was strongly associated with the subsequent development of complications. This confirms the endoscopic exam as a valuable diagnostic aid for the diagnosis of mucosal impairment. Endoscopy and contrast radiography of the esophagus should be strongly considered in cases of chronic or recurrent obstruction,[2,6-8] as anatomic abnormalities are more likely to be present. The three main anatomic abnormalities encountered were ulcers, strictures and esophageal diverticula. Half of all cases of stricture were foals, in accordance with earlier findings.[6] The etiology of stricture in neonatal foals is not completely clear; it may be a congenital or developmental disorder, reflect a neurological or muscular developmental problem, or be the consequence of circumferential ulceration from a previous obstruction.[9-11] While some of these stenoses documented in equine neonates have been reported to resolve after medical management, the survival rate is higher when treated surgically.[6,9]

Unlike a previous report,[7] we did not find any significant difference in the prevalence of the location of the obstruction, nor could we identify any association between the location and the complication rate.

Among the hematological and biochemical variables, only total protein >70 g/L was associated with an increased likelihood of complications and pneumonia in

particular. Perhaps this is related to either dehydration or hyperglobulinemia at presentation, possibly reflecting a more severe disease process. However, due to the large numbers of missing observations, it is difficult to make statistical inferences on the clinical significance of this finding.

Since our study population included cases collected over 17 years, the treatments differed widely and made it difficult to make inferences about the protocols used. Initial therapy for primary esophageal obstruction is often conservative, as many will resolve spontaneously or with medical management consisting of sedation, smooth muscle relaxants, and analgesics or anti-inflammatory drugs.[1,4,12] Tranquilization and gentle pressure on the obstruction with a nasogastric tube is often necessary to promote passage of the obstruction into the stomach. General anesthesia may be necessary in some refractory cases, and surgical intervention is very rarely required.[5] Oxytocin (0.11-0.22 IU/kg IV) has been reported to induce transient relaxation of esophageal musculature in healthy horses and resolution of choke in 8 of 10 horses.[13] However, other investigators have not been able to corroborate these findings using manometric evaluations of the esophagus in the absence of balloon distention.[14] In a previous study, detomidine, acepromazine, and a combination of xylazine and butorphanol have been reported to have potential detrimental effects on the resolution of the esophageal obstruction because of the reduction in swallowing and the change in normal peristaltic activity with increases in high pressure events at the thoracic inlet.[14] However, the combination of xylazine and butorphanol was commonly used in this population of horses. *In vitro* studies suggest oxytocin induces relaxation of only the smooth muscle portion of the esophagus.[15] In our

study the number of horses treated with acepromazine or oxytocin was too low and the number of those treated with alpha-2 agonists too high to make any statistical inferences.

Only 13 out of 71 horses for which antibiotic therapy was recorded received oral antibiotics exclusively. Horses receiving antibiotic therapy seemed to have a trend toward developing pneumonia (p=0.08) and those receiving both oral and parenteral antibiotics had a higher risk (odds) of developing complications (p=0.02). However, this may have been related to the fact that those horses were considered at greater risk during clinical examination at admission and consequently received more aggressive and more prolonged antimicrobial therapy.

Feige *et al.* (2000) reported that the duration of esophageal obstruction prior to admission was a significant risk factor for aspiration pneumonia, since the longer the obstruction the longer the dysphagia and thus the risk of tracheal contamination and aspiration of saliva and food. Unlike earlier findings, we did not find any association between the duration of the obstruction and the development of aspiration pneumonia; however, horses with a history of chronic obstruction had 9 times higher odds (OR 9.1, 95% CI 1.7-47.7 p <0.01) of developing complications compared to horses with an episode of obstruction less than 3 hours in duration.

The sex and the age of the animals, and the use of general anesthesia in order to resolve the obstruction were significant in the final multivariable logistic regression model. Although we found that intact males were more likely to develop complications compared to females, their number was considerably lower than the number of females or castrated males, so this may represent a spurious statistical finding. We found a strong association between whether the horse underwent general anesthesia and the development

of complications (specifically aspiration pneumonia) in both the univariable and multivariable models. General anesthesia increases the risk of post-operative fatalities.[16,17] However this association may have been affected by the fact that probably only the most refractory cases, already at a higher risk of complications, needed general anesthesia for resolution of the obstruction.

Our findings were likely affected by changes in protocols over time and by the high number of variables excluded from the final model because of missing values. The model is not very accurate at predicting the future onset of complications in a horse experiencing an episode of esophageal obstruction, because of the low sensitivity (66.1%) and specificity (71.7%) of the final model. To our knowledge, this is the first study using a multivariable model for assessing the association between several risk factors and the likelihood of complications following an episode of esophageal obstruction. Only sex, age and whether or not the horse underwent general anesthesia fit the criteria for inclusion in the multivariable model. However, clinical variables along with radiographic and endoscopic results were confirmed as important tools in assessing the severity of the esophageal lesion and pulmonary involvement. These procedures should be applied in cases with recurrent esophageal obstruction because of the likelihood of pre-existing anatomic or functional esophageal disorders. Two weaknesses in our study were the high number of missing values and the variability in procedures and treatments between different clinicians over the years. This is meant to be a preliminary study. A prospective study including all variables of interest, including clinical variables along with complete radiographic and endoscopic findings and the methods used to provide relief of the obstruction (e.g. single tube, pressure lavage, gravity lavage, etc),

not recorded in our population, may provide a real benefit to practitioners and be desirable in the future.

Endnotes

[a] Stata/IC 10.1 for Windows, StataCorp LP, College Station, TX.

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Tables

Table 4.1: Descriptive statistics and univariable analysis of variables showing an association ($p \leq 0.25$) with the likelihood of developing complications after esophageal obstruction.

Variable	*N. (%)*	*N. outcomes (%)*	*OR*	*95% CI*	*LRchi2*	*P-value*
Sex	109	56 (51.4)			5.3	0.07
Female	36 (33.1)	17 (47.2)	Ref.	-	-	-
Gelding	56 (51.4)	26 (46.4)	1	0.4-2.2		0.90
Male	17 (15.6)	13 (76.5)	3.6	1-13.3		0.05
Age (years)	109	56 (51.4)			13.5	**<0.01**
<1	17 (15.6)	12 (70.6)	5.8	1.7-20.1		<0.01
1-15	41 (37.6)	12 (29.3)	Ref.	-	-	-
>15	51 (46.8)	32 (62.7)	4.1	1.7-9.8		<0.01
Temperature (C^o)	91	52 (57.1)			2.9	0.23
≤ 37	14 (15.4)	7 (50.0)	Ref.	-	-	-
37.1-38	35 (38.5)	17 (48.6)	0.9	0.3-3.3		0.90
>38	42 (46.1)	28 (66.7)	2	0.6-6.8		0.30
RR (bpm)	91	52 (57.1)			3.9	0.14
≤12	34 (37.4)	15 (44.1)	Ref.	-	-	-
13-22	25 (27.5)	17 (68.0)	2.7	0.9-7.9		0.07
>22	32 (35.2)	20 (62.5)	2.1	0.8-5.6		0.13
X-rays AP	38	28 (73.7)			3.9	**0.05**
No	13 (34.2)	7 (53.8)	Ref.	-	-	-
Yes	25 (65.8)	21 (84.0)	4.5	1-20.7		
Tracheal contamination	45	28 (62.22)			6.9	0.07
None	17 (37.8)	7 (41.2)	Ref.	-	-	-
Mild	5 (11.1)	3 (60)	2.1	0.3-16.4		0.46
Moderate	18 (40)	15 (83.3)	7.1	1.4-34.4		0.01
Severe	5 (11.1)	3 (60)	2.1	0.3-16.4		0.46
Grade esophageal lesion	70	40 (57.14)			12.7	**<0.01**
Absent	16 (22.9)	4 (25.0)	Ref.	-	-	-
Moderate	29 (41.4)	16 (55.2)	3.7	0.9-14.2		0.06
Severe	25 (35.7)	20 (80.0)	12	2.7-53.6		<0.01
TP (g/L)	64	41 (64.06)			5.5	**<0.01**
≤70	24 (37.5)	11(45.8)	Ref.	-	-	-
>70	40 (62.5)	30 (75.0)	1.2	1-1.4		
Antibiotics	71	37 (52.11)			6.5	**0.04**
Oral	13 (18.3)	3 (23.1)	Ref.	-	-	-
Parenteral	20 (28.2)	10 (50.0)	3.3	0.7-15.8		0.13
Both	38 (53.5)	24 (63.2)	5.7	1.3-24.3		0.02
General Anesthesia	109	56 (51.4)			14.6	**<0.01**
No	69 (63.3)	26 (37.7)	Ref.	-	-	-
Yes	40 (36.7)	30 (75.0)	5.0	2.1-11.8		
Duration (hours)	78	43 (55.13)			9.7	**0.05**
<3	11 (14.1)	3 (27.3)	Ref.	-	-	-
3-6.3	9 (11.5)	4 (44.4)	2.1	0.3-13.8		0.43
6.4-12.3	18 (23.1)	8 (44.4)	2.1	0.4-10.8		0.36
12.4-48	18 (23.1)	11 (61.1)	4.2	0.8-21.4		0.08
>48	22 (28.2)	17 (77.3)	9.1	1.7-47.7		<0.01

N, number of horses; OR, odd ratio; CI, confidence interval; LRchi2, likelihood ratio chi-squared; Ref., reference value; RR, respiratory rate; X-rays AP, radiological signs of aspiration pneumonia; TP, total protein.

Table 4.2: Results of descriptive statistics and univariable analysis of variables showing an association ($p \leq 0.05$) with the likelihood of developing pneumonia after esophageal obstruction.

Variable	*N. (%)*	*N. outcomes (%)*	*OR*	*95% CI*	*LRchi2*	*P-value*
Sex	109	39 (35.8)			7.9	**0.02**
Female	36 (33.1)	9 (25.0)	Ref	-	-	-
Gelding	56 (51.4)	19 (33.9)	1.5	0.6-3.9		0.36
Male	17 (15.6)	11 (64.7)	5.5	1.6-19.2		<0.01
Age (years)	109	39 (35.8)			8.4	**0.02**
<1	17 (15.6)	9 (23.1)	4.6	1.4-15.8		0.01
1-15	41 (37.6)	8 (20.5)	Ref	-	-	-
>15	51 (46.8)	22 (56.4)	3.1	1.2-8.1		0.02
RR (bpm)	91	36 (39.6)			10.7	**<0.01**
≤12	34 (37.4)	7 (20.6)	Ref	-	-	-
13-22	25 (27.5)	10(40)	2.6	0.8-8.1		0.12
>22	32 (35.2)	19 (59.4)	5.6	1.9-16.8		<0.01
Tracheal contamination	45	21 (46.7)			10.7	**0.01**
None	17 (37.8)	4 (23.5)	Ref	-	-	-
Mild	5 (11.1)	1 (20.0)	0.8	0.1-9.5		0.80
Moderate	18 (40)	12 (66.7)	6.5	1.5-28.8		0.01
Severe	5 (11.1)	4 (80.0)	13	1.1-152.3		0.04
TP (g/L)	64	31 (48.4)			8.7	**<0.01**
≤70	24 (37.5)	6 (25.0)	Ref	-	-	-
>70	40 (62.5)	25 (62.5)	5.0	1.6-15.4		
General Anesthesia	109	39 (35.8)			10.1	**<0.01**
No	69 (63.3)	17 (24.6)	Ref	-	-	-
Yes	40 (36.7)	22 (55.0)	3.7	1.6-8.6		<0.01

N, total number of horses; OR, odd ratio; CI, confidence interval; LRchi2, likelihood ratio chi-squared; Ref., reference value; RR, respiratory rate; TP, total protein.

Table 4.3: Results of multivariable analysis for the identification of risk factors for complications or death after esophageal obstruction.

Variable	*Coeff.*	*OR*	*SE*	*95% CI*	*Partial LRchi2*	*P-value*
Intercept	-2.0	-	-	-		-
Sex					6.3	0.04
Female	Ref.	-	-	-		-
Gelding	0.3	1.4	0.7	0.5-3.7		0.52
Males	1.9	6.6	5.3	1.4-31.6		**0.02**
Age (years)					14.2	<0.01
≤1	1.4	4.0	3.0	0.9-17.6		0.07
1-15	Ref.	-	-	-		-
>15	1.8	6.2	3.3	2.2-17.6		**<0.01**
General Anesthesia	1.6	5.1	2.5	1.9-13.4	12.1	**<0.01**

Coeff., coefficient; OR, odd ratio; SE, standard error; CI, confidence interval; LRchi2, likelihood ratio chi-squared; Ref., reference value.