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Systemic leukopenia, evaluation of laminar leukocyte infiltration and laminar lesions in horses with naturally occurring colic syndrome

Luciane Maria Laskoski [a,*], Rosangela Locatelli-Dittrich [a], Carlos Augusto Araújo Valadão [b], Ivan Deconto [c], Kamila Alcala Gonçalves [a], Fabiano Montiani-Ferreira [a], Juliana Sperotto Brum [a], Harald Fernando Vicente de Brito [a], Renato Silva de Sousa [c]

[a] Post-Graduation in Veterinary Sciences, Federal University of Paraná, Campus of Curitiba. Rua dos Funcionários, 1540, 80035-050, Curitiba, Paraná – Brazil
[b] Post Graduation in Veterinary Surgery, Júlio de Mesquita Filho São Paulo State University - UNESP (Jaboticabal campus). Via de Acesso Professor Paulo Donato Castellane, s/n, 14884-900, Jaboticabal, São Paulo – Brazil
[c] Departament of Veterinary Medicine, Federal University of Paraná, Campus of Curitiba. Rua dos Funcionários, 1540, 80035-050, Curitiba, Paraná – Brazil

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ABSTRACT

The present study was aimed at identifying laminar lesions and leukocyte infiltration in hoof laminar tissue of horses with colic syndrome and its correlation with the total leukocyte count before death. Six healthy horses were used as control group (CG), and eighteen horses with lethal gastrointestinal disease were divided into two groups: leukopenic group (LG) with seven leukopenic horses, and non-leukopenic group (NLG) with 11 horses with total leukocyte count within reference range for the species. Leukocyte infiltration was examined by immunohistochemistry. Laminar lesions were observed in both LG and NLG, with no differences in severity between them. LG showed increase of the leukocyte infiltration in the hoof laminar tissue, when compared to CG and NLG. Horses with severe colic syndrome (LG and NLG) developed intense laminar lesions without clinical signs of laminitis, with increased leukocyte infiltration. However, the LG demonstrated an even higher increase of leukocyte infiltration compared to both CG and NLG.

1. Introduction

Laminitis is one of the most serious diseases in horses, and its pathophysiology is currently unknown. The disease affects the laminar tissue of the hoof, responsible for suspending the distal phalanx within the hoof wall. The laminar tissue is formed by interdigitations between the dermal and epidermal tissue, jointed by the basement membrane (Pollitt, 1996).

The study of laminitis is often performed using different experimental induction models, which, sometimes, produce varied results according to the model in question. The insulin administration model is associated with the metabolic laminitis that occurs in obese horses and ponies or those with pituitary pars intermedia dysfunction (Asplin et al., 2007). The carbohydrate overload model (Weiss et al., 1996; Garner et al., 1975) and the black walnut extract (Galey et al., 1991) are associated with inflammatory laminitis that commonly occurs secondary to systemic inflammation such as gastrointestinal disease (Parsons et al., 2007). In the prodromal phase of laminitis, induced by experimental inflammation models, clinical symptoms such as fever, tachycardia, and injected mucous membranes are observed (Maier, 2000). Also, in this phase, elevated concentrations of inflammatory cytokines into the bloodstream (Loftus et al., 2007; Faleiros et al., 2011), leukocyte infiltration in the laminar tissue of the hoof (Black et al., 2006; Faleiros et al., 2011), and leukocyte count change (Galey et al., 1991; Leise et al., 2011) were reported. Therefore, it is suggested that inflammatory laminitis is associated to a process of organ failure, and a failure of many organs can occur in response to inflammatory mediators in sepsis (Belknap et al., 2009). Lesion development in the laminar tissue of the hoof, rather than other organs during systemic inflammatory response, may be related to a decrease in metabolic rates in the area, since under normal conditions it is in a more ischemic environment when compared to other tissues (Pawlak et al., 2004).

The presence of leukocytes in the laminae of the hoof can result in tissue injuries (Black et al., 2006) due to the release of substances such as metalloproteinases (Pollitt, 1996) and reactive oxygen species (Hurley et al., 2006). In the hoof laminar tissue of healthy horses, there are rare leukocytes (Faleiros et al., 2009), and leukocyte infiltration associated with laminitis has been demonstrated experimentally in the developmental phase of laminitis (Galey et al., 1991; Hurley et al., 2006). Leukopenia, in the prodromal phase of laminitis, has been observed after the administration of black walnut extract (Galey et al., 1991). However, there are inconsistencies in the data regarding the role of leukocytes in laminar injury in different experimental models of laminitis (Loftus et al., 2007; Leise et al., 2011).

* Corresponding author. Tel.: +55 41 3350 5740.
E-mail address: luci.laskoski@gmail.com (L.M. Laskoski).

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Gastrointestinal disorders commonly induce acute laminitis in critically ill horses (Slater et al., 1995; Pierce et al., 2010). The strong association of laminitis with colic syndrome occurs because laminitis is not merely a foot disease, but the local manifestation of systemic changes, as it involves multiple organ systems (Hood, 1999). However, it is not possible to state that the same laminar events observed in experimental models of inflammatory laminitis occur secondary to the colic syndrome. The present study was aimed at identifying leukocyte infiltration in the hoof laminar tissue of horses with naturally occurring gastrointestinal disorders, the correlation of infiltration intensity with laminar damage, and the total leukocyte count before death.

2. Materials and methods

2.1. Ethics committee

The experiment was approved by the Research Ethics Committee of the Federal University of Parana (Protocol 038/2012).

2.2. Animals

Twenty-four horses, male and female, of different breeds, aged 2–15 years and weighing between 280 and 560 kg, were used in the experiment. Six of them were from a commercial slaughterhouse[1] considered to be healthy by the local inspection service, and 18 of them were horses suffering from naturally occurring gastrointestinal lethal diseases, treated at veterinary hospitals of higher education institutions.

2.3. Hemogram

Blood samples were collected from horses with gastrointestinal diseases within 6 hours before death or prior to animal euthanasia. The samples were collected by venipuncture of the jugular vein into tubes with EDTA-anticoagulant for later hemogram using a cell counting system.[2] Blood smears were made immediately after blood collection. The results obtained were compared with the reference values ($5.4 – 14.3 \times 10^3/\mu L$) for the equine species (Jain, 1986).

2.4. Experimental groups

2.4.1. Control group (CG)

Six horses from horse commercial slaughterhouses.

2.4.2. Leukopenic group (LG)

Seven horses presenting low white blood cell count before of death.

2.4.3. Non-leukopenic group (NLG)

Eleven horses presenting normal white blood cell count (within normal limit for the species) before of death.

2.5. Gastrointestinal lethal diseases

Data about causes of gastrointestinal disease, duration of illness, presence of fever in the moment of death, anti-inflammatory administration, and presence of clinical signs of laminitis during the clinical evolution of colic were collected.

2.6. Morphology of the hoof laminar tissue

After the death of the animals, hoof laminar tissues of the four limbs were collected with a scalpel blade and forceps after cross-sectioning of the hoof wall (Pollitt, 1996). One horse of the NLG had only the tissue of the forelimbs sampled. The samples were fixed in 10% buffered formalin for 48 hours, then routinely processed and stained with hematoxylin and eosin for histologic evaluation (HE). Paraffin blocks of the hoof laminar tissue were also stained using periodic acid-Schiff (PAS) histochemical methods. Light microscopy was performed by three observers, based in the references described by Pollitt (1996), and the severity of laminar lesions were classified using ordinal categorical scores 0, 1, 2, and 3. Score 0: Normal morphology of interdigitations between secondary epidermal laminae (SEL) and secondary dermal laminae (SDL) with contour of basement membrane (BM) and nuclei of basal cells alongated; Score 1: SEL lengthening, primary epidermal laminae (PEL) undulations, and basal cells (BC) with round-shape nuclei; in Score 2, there was lost BM in some segments and your contour is wavy and undulating, with lesions more pronunciated in SEL and PEL; Score 3: Destruction of interdigitations and morphology of epidermal and dermal laminae, especially in the base of the PEL, with increase distance of keratinizes axis of PEL and basement membrane.

2.7. Immunohistochemistry for white blood cells calprotectin

Leukocyte infiltration in hoof laminar tissue was investigated in horses of both experimental groups using immunohistochemistry of hoof laminar tissue, with 5 μm thick paraffin-embedded tissue sections placed on positively charged slides. After deparaffinization, antigen retrieval was performed in sodium citrate using a slow cooker for 30 minutes. A commercial kit was used to block proteins[3] and endogenous peroxidase.[4] Rabbit anti-calprotectin antibodies were diluted 1:2000 and incubated for 30 minutes at room temperature. Thereafter, they were incubated with universal immunoperoxidase polymer for 30 minutes. The DAB reaction was performed for 4 minutes, then they were counterstained with Harris hematoxylin for 30 seconds, ending with dehydration in alcohol baths and xylene, and the slides were mounted for light microscopy.

The evaluation of leukocyte infiltration into the hoof laminar tissue was performed by three observers using a grade system, as follows: Grade 0, rare presence of leukocytes in blood vessels (less than 10 cells immunostained in laminar section); Grade 1, leukocytes only in the dermal vessels (between 10 and 30 cells immunostained in laminar section); Grade 2, infiltration in the dermal laminae and laminae that interdigitate with epidermal laminae (more than 30 cells immunomarcated in laminar section); Grade 3, immunostaining spreading across epidermal laminae (more than 60 cells immunomarcated in dermal laminae and disseminated immunostaining in epidermal laminae of laminar section).

2.8. Statistical methods

After assessing total white blood cell count, scores of laminar lesions and grades of laminar leukocyte infiltration using Shapiro–Wilk test, the data were analyzed using the Kruskal–Wallis test and the means between groups were compared using Fisher's exact test, with $p < 0.05$. Additionally, the Spearman correlation test was used to determine correlation between scores of laminar lesions and grades of laminar leukocyte infiltration.

3. Results

3.1. Gastrointestinal lethal diseases

The following causes of gastrointestinal disorders were found: intestinal impactation (six animals), gastric overload (two animals), colon displacement/twisted bowel (seven animals), enterolithiasis (two animals), and proximal duodeno-jejunitis (one animal), which caused the death due to ischemia and necrosis of bowel, observed at necropsy.

[1] Slaughterhouse and refrigerator Pomar SA, Araguari – MG, Brasil.
[2] BC 2800, Mindray, Nanshan, Chinaradi.
[3] DPB-125. Spring Bioscience. Pleasanton, CA - EUA.
[4] DHP-125. Spring Bioscience. Pleasanton, CA - EUA.