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Oxidative stress in hoof laminar tissue of horses with lethal gastrointestinal diseases

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ABSTRACT

Tissue damage caused by oxidative stress is involved in the pathogenesis of several diseases in animals and man, and is believed to play a role in the development of laminitis in horses. The aim of this study was to investigate the oxidative stress associated with laminar lesions in horses with lethal gastrointestinal disorders. Laminar tissue samples of the hoof of 30 horses were used. Tissue samples were divided as follows: six healthy horses (control group—CG), and 24 horses that died after complications of gastrointestinal diseases (group suffering from gastrointestinal disorders—GDG). Superoxide dismutase (SOD2) and nitrotyrosine immunostaining and the severity of laminar lesions were evaluated. Presence of laminar lesions and immunostaining for nitrotyrosine and SOD2 were only evident in horses from the GDG group. Thus, oxidative stress may play a role in the pathogenesis of laminar lesions secondary to gastrointestinal disorders.

1. Introduction

Laminitis is a serious condition, commonly seen in horses after severe episodes of gastrointestinal diseases (Parsons et al., 2007). Lesions occurs in the hoof laminar tissue located between the hoof wall and the distal phalanx, responsible for joining and supporting these structures (Pollitt, 1996; Karikoski et al., 2014).

The study of laminitis is usually carried out experimentally induced inflammatory models such as by BWE administration or carbohydrate-overload (Loftus et al., 2006; Faleiros et al., 2009; Lima et al., 2013; Steelman et al., 2014) or inducing endocrinopathic laminitis such as by insulin administration (de Laat et al., 2012). However, the presence of morphological lesions in the hoof laminar tissue can also be observed by inducing gastrointestinal disorders (Laskoski et al., 2010) and in naturally occurring colic syndrome even without clinical signs of laminitis (Laskoski et al., 2015).

Distinct theories to explain laminitis secondary to inflammatory diseases exist. One of the theories proposed to explain the physiopathology laminitis suggests the occurrence of laminar ischemia secondary to vasoconstriction or due to vascular obstruction by a thrombus (Weiss et al., 1994; Noschka et al., 2009a). Degradation of basement membrane-type collagen by metalloproteinases also was identified as a potential cause for this injury (Pollitt, 1996). Another theory is the metabolic laminitis, which has many similarities with the disease induced by inflammatory models, such as insulin resistance and resulting hyperglycemia during the prodromic phase of laminitis (Toth et al., 2009). Recently, associations between systemic inflammation and laminitis have been observed (Loftus et al., 2007; Faleiros et al., 2009). However, theories for the development of laminitis and consequent findings seem to vary

Abbreviations: BM, basement membrane; BC, basal cells; BWE, black walnut extract; CG, control group; CS, clinical signs (horse with clinical signs of laminitis before death); DNA, deoxyribonucleic acid; GDG, gastrointestinal disorders group; PEL, primary epidermal laminae; SDL, secondary dermal laminae; SEL, secondary epidermal laminae; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α.

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according to the type of experimentally-induced laminitis model investigated.

Oxidative stress is associated with the pathogenesis of many diseases, causing cellular injury by reaction of free radicals with cellular proteins, lipids and DNA (Berg et al., 2004). Peroxynitrite is derived from the reaction of nitric oxide with superoxide anions (Carreras et al., 1994). It is a potent bactericidal and parasiticidal agent, whose products can lead to the formation of substances that cause oxidative damage (Zhu et al., 1992 Alvarez et al., 2004), inducing cytotoxicity by oxidation of tryptophan and cysteine, tyrosine nitration, formation of dityrosine, and protein fragmentation (Ischiropoulos and Al-Mehdi, 1995). While superoxide anions and nitric oxide are generated almost exclusively by endothelial cells, peroxynitrite can be generated by the reaction of these two substances in the extracellular environment (Frei et al., 1988; Halliwell and Gutteridge, 1990). Nitrotyrosine is a product from tyrosine residues reacting with peroxynitrite, and has been used as a marker of oxidative stress (Ischiropoulos and Al-Mehdi, 1995).

Oxidative stress occurs when the amount of substances responsible for oxidative damage exceeds the capacity of neutralization by the antioxidant system (Macdonald et al., 2003). The SOD is an important cellular antioxidant enzyme, which is also present in the extracellular milieu, plays an important role in preventing the decomposition of nitric oxide, and promotes vasodilatation, besides catalyzing the decomposition of superoxide, leading to the protection of the endothelium and other tissues by inhibiting the subsequent formation of peroxynitrite (Beckman et al., 1990).

Some authors have suggested laminar tissue lesions caused by oxidative stress. Yin et al. (2009) observed an increase of a lipid aldehyde, which causes lipid peroxidation during the developmental phase of laminitis induced by administration of BWE. Antioxidant substances such as catalase and xanthine oxidase are present in the hoof laminar tissue of healthy horses, but SOD is not found in healthy animals (Loftus et al., 2006).

The aim of this study was to evaluate the immunolocalization of SOD and nitrotyrosine in the hoof laminar tissue of healthy horses and horses suffering from serious colic syndrome, and the association of these findings with the development of laminar injury.

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

Samples of hoof laminar tissue from the four limbs were collected immediately after the death of thirty male and female horses from different breeds, weighing between 280–560 kg. The animals were divided in two experimental groups: Control group (CG), with six horses from a commercial slaughterhouse (aged 6–10 years), and gastrointestinal disorders group (GDG), with 24 horses that died or were submitted to euthanasia after complications caused by natural gastrointestinal complications (aged 2–15 years).

2.3. Experimental design

Control Group (CG): with six crossbreed horses, considered to be healthy by the local inspection service of a commercial slaughterhouse (Slaughterhouse and refrigerator Pomar SA, Araguari–MG, Brazil).

Gastrointestinal Disorders Group (GDG): with 24 horses suffering from naturally occurring gastrointestinal lethal diseases, treated at Veterinary Hospitals of Higher Education Institutions. The causes of gastrointestinal disorders were proximal duodenal–jejunitis ($n = 1$), grain overload ($n = 2$), enterolithiasis ($n = 4$), intestinal impactation ($n = 6$) and colon displacement/twisted bowel ($n = 11$), which caused death or led to the indication of euthanasia due to intestinal ischemia and necrosis. All horses were evaluated, during disease evolution period, for the presence of lameness associated to laminitis.

2.4. Morphology of the hoof laminar tissue

After the death of the animals, hoof laminar tissue of the four limbs was collected with a scalpel blade and forceps after cross-sectioning the hoof wall (Pollitt, 1996). Samples were fixed in 10% buffered formalin for 48 h, and routinely processed and stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) for histologic evaluation. Light microscopy was performed by three observers, following the method described by Pollitt (1996) with adaptation, and the severity of laminar lesions were graded as 0, 1, 2 and 3 (Table 1).

2.5. Immunohistochemistry for nitrotyrosine and SOD2

Nitrotyrosine and SOD2 in hoof laminar tissue were investigated in horses of both CG and GAG groups using immunohistochemistry, Paraffin-embedded tissue sections (5 μm) placed on positively charged slides were deparaffinized and antigen retrieval was performed in sodium citrate using a slow cooker for 30 min (92–95 °C/197–203 °F). A commercial kit was used to block proteins (DPB-125, Spring Bioscience. Pleasanton, CA, EUA), and endogenous peroxidase (DHP-125, Spring Bioscience. Pleasanton, CA, EUA). Mouse anti-nitrotyrosine (AB 61392, ABCAM, San Francisco, USA) antibody was diluted 1:6000, and rabbit anti-SOD2 (AB 13534, ABCAM, San Francisco, USA) antibody was diluted 1:4000. Both antibodies were incubated for 60 min at room temperature, following incubation with universal immunoperoxidase polymer (Nichi-414152-F, NICHIREI BIOSCIENCE INC, Tokyo, Japan) for 30 min. The DAB reaction was performed for four minutes and counterstained with Harris hematoxylin for 30 s, dehydrated with ethanol, cleared with xylene and mount with a resinous medium. Slides were evaluated by three independent observers.

Evaluation of nitrotyrosine and SOD2 staining of hoof laminar tissue was graded according to the immunostaining intensity observed in GAG horses (Tables 2 and 3).

2.6. Statistical methods

The Kruskal–Wallis test was used to compare grades of laminar lesions and scores of nitrotyrosine and SOD2 between the groups CG and GDG. Additionally, the Spearman correlation test was used to determine correlation between the grades of laminar lesions and scores of nitrotyrosine and SOD2 immunostaining. In all tests $P < 0.05$ was considered significant.

3. Results

Laminar lesions and immunostaining for nitrotyrosine and SOD2 were not detected in horses of the CG. All laminar lesion grades (Fig. 1) and all scores of nitrotyrosine (Fig. 2) and SOD2 (Fig. 3) immunostaining were observed in horses from the GDG, with medians higher than the CG (Table 4).

One horse from the GDG developed clinical signs of laminitis, such as increase of digital pulse, lameness and hoof tester positivity, Obel grade IV lameness and forelimb pain. Colic signs were caused by impaction of the transverse colon, treated surgically. In the postoperative period, this horse showed serious lameness and