Veterinary Immunology and Immunopathology 135 (2010) 181–187

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

# Equine neutrophil elastase in plasma, laminar tissue, and skin of horses administered black walnut heartwood extract

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ARTICLE INFO

*Article history:*
Received 29 March 2009
Received in revised form 17 September 2009
Accepted 17 October 2009

*Keywords:*
Laminitis
Neutrophil elastase
ELISA
Inflammation
Horse

ABSTRACT

Laminitis is a local manifestation of a systemic inflammatory response that is characterized by neutrophil activation and movement of neutrophils into the laminar tissues. Given the evidence for the involvement of neutrophils in the development of laminitis, we measured concentrations of neutrophil elastase, a serine protease released from the azurophilic granules of neutrophils, in plasma, skin and laminar tissues obtained from control horses and horses given black walnut heartwood extract (BWHE) to induce laminitis. Healthy horses (5–15 years old) were randomly assigned to 4 groups: 3 experimental groups given BWHE via nasogastric tube, and a control group given an equal volume of water. The experimental groups consisted of horses euthanized 1.5 h ($n = 5$), 3 h ($n = 6$) or 12 h ($n = 10$) after BWHE administration. Control horses ($n = 7$) were euthanized 12 h after intragastric administration of water. Plasma samples were collected in all horses of the control and 12 h BWHE groups at 0, 1, 2, 3, 4, 6, 8, 10, and 12 h after treatment, and laminar tissue and skin from the middle region of the neck were harvested at the time of euthanasia in all 1.5 and 3 h BWHE horses, in 6 of the 12 h BWHE horses and in 5 of the control horses. Plasma and tissue concentrations of neutrophil elastase were determined using an equine specific ELISA, and statistical significance was set at $p < 0.05$. Plasma concentrations of neutrophil elastase in the BWHE group were significantly higher at 6 and 8 h compared to the control group and at 8 and 10 h compared to time 0. Concentrations of neutrophil elastase in skin and laminar tissue were significantly higher in the 3 and 12 h BWHE groups compared to the control group. Concentrations of neutrophil elastase were significantly higher in the skin than in the lamina in the 12 h BWHE horses. The administration of BWHE thus results in significant increases in the concentration of neutrophil elastase in the circulation, skin and laminar tissue. These results confirm a role for neutrophils in the developmental phase of laminitis, and the systemic nature of the inflammatory process. Furthermore, neutrophil elastase may play a key role in the disintegration of the hoof basal membrane and be a target for the development of new treatments for laminitis.



Abbreviations: BWHE, black walnut heartwood extract; MPO, myeloperoxidase; MMP, matrix metalloproteinase; PBS, phosphate buffer saline.

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doi:10.1016/j.vetimm.2009.10.024

## 1. Introduction

Acute laminitis in the horse is considered to be the local manifestation of a systemic inflammatory disease and commonly develops secondary to conditions such as gastrointestinal diseases, septicaemia, pleuritis, retained placenta and metritis. In a recent review, the development of laminitis was significantly associated with the clinical signs commonly associated with endotoxaemia (Parsons et al., 2007). Laminitis also develops secondary to endocrine disorders including Cushing's disease, equine metabolic syndrome or obesity (Johnson et al., 2004; Geor and Frank, 2009).

A commonly used experimental model of acute laminitis involves the intragastric administration of the aqueous extract of the heartwood of black walnut trees (BWHE). Use of this model has improved our understanding of the mechanisms involved in the prodromal stage of the disease. For example, administration of BWHE results in a pronounced decrease in the number of circulating leukocytes with evidence of activation of polymorphonuclear neutrophils during the development stage of laminitis (Fontaine et al., 2001; Hurley et al., 2006). In additional studies, administration of BWHE resulted in perivenous infiltration of neutrophils in laminar tissues (Black et al., 2006), and local up-regulation of inflammatory cytokines, including interleukin-1 beta, interleukin-6, cyclo-oxygenase-2, and MAIL (Fontaine et al., 2001; Waguespack et al., 2004a,b). These findings are consistent with histological changes observed in laminitis, which include interstitial edema, swelling of endothelial cells and keratinocytes, disintegration of the basement membrane and degeneration of secondary lamellae (Hood et al., 1993; Pollitt, 1996; Morgan et al., 2003).

The laminar tissue is a highly specialized component of the common integument. Due to the systemic nature of the processes that lead to the development of acute laminitis, similar, but sub-clinical, inflammatory processes may affect the entire integument including the skin. The results of a recent study in which significant increases in total and active myeloperoxidase (MPO) were detected in blood, skin, and laminar tissues of horses that were administered BWHE lend credence to the proposed early role of neutrophils in the pathogenesis of laminitis (Riggs et al., 2007).

Neutrophil elastase, a serine protease contained in the azurophilic granules of the neutrophil, is widely recognized as a component and marker of inflammatory disorders (Gross et al., 1993; Jochum et al., 1994; Zorn et al., 2003; Langhorst et al., 2008). This serine protease plays a role in the oxygen-independent microbicidal pathway, and contributes to the tissue remodelling that occurs after injuries (Shapiro, 2002; Chua and Laurent, 2006). The concentrations of neutrophil elastase were increased in the bronchoalveolar lavage fluid of horses with recurrent airway obstruction (Brazil et al., 2005), and we also recently found neutrophil elastase increases in the plasma of horses with colic (de la Rebière de Pouyade et al., 2010). Moreover, a study performed using isolated equine neutrophils revealed kinetic differences between oxidative burst and release of neutrophil elastase by stimulated neutrophils (Dagleish et al., 1999). Because of the potential role for neutrophil elastase in the development of laminitis, the current study was performed to compare concentrations of this enzyme in plasma, skin and laminar tissues from control horses and horses administered BWHE.

## 2. Materials and methods

### 2.1. Animals

Healthy horses ranging in age from 5 to 15 years were included in this study. All horses were free of existing lameness and lacked clinical evidence of systemic inflammatory disease. No radiographic evidence of preexisting laminitis was present on survey lateral and dorsopalmar radiographic views of the forelimb digits. A 12-gauge catheter was placed in the left jugular vein for serial blood sample collection, and the middle region of the neck was clipped prior to the start of the study. The University of Georgia and Ohio State University Animal Care and Use Committees approved the study.

### 2.2. BWHE preparation

The BWHE was prepared as previously described (Minnick et al., 1987). Briefly, 1 kg of black walnut heartwood shavings was agitated in 7 L of water at room temperature (approx. 22 °C) for 24 h. An aqueous filtrate was then obtained by filtering the solution through cheesecloth. Six liters of the resulting BWHE were administered by nasogastric tube to horses in the experimental groups.

### 2.3. Experimental protocol

Experimental protocol: 28 horses were randomly assigned to 1 of the 4 following groups: control ($n = 7$), 1.5-h BWHE ($n = 5$), 3-h BWHE ($n = 6$), and 12-h BWHE ($n = 10$). Horses in the control group received 6 L of water via nasogastric intubation, and were euthanized after 12 h; blood was collected in the 7 animals and tissues in 5 of them. Horses in the 1.5-h BWHE group were euthanized at 1.5 h after intubation. Horses in the 3-h BWHE group were euthanized at the onset of leukopenia (approximately 3 h after administration of BWHE and defined as a $\geq$30% decrease in peripheral white blood cell count from the time 0 value). Horses in the 12-h BWHE group were euthanized at the onset of Obel grade 1 laminitis (clinical signs consisting of weight shifting and bounding digital pulses without evidence of lameness at a walk), or at 12 h after intubation, if signs of Obel grade 1 laminitis had not developed by that time. Tissue samples were obtained in all horses of 1.5 and 3 h BWHE groups and in 6 horses of the 12 h BWHE group, and blood samples were obtained in the 10 horses of the 12 h BWHE group. Each horse was evaluated prior to intubation and every hour thereafter for attitude, heart rate, respiratory rate, capillary refill time, hoof temperature, digital pulses, and evidence of lameness consistent with Obel grade 1 laminitis. The horses were euthanized with a penetrating captive bolt, in compliance with guidelines outlined in the 2000 Report of the AVMA Panel on Euthanasia.