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The effect of hypothermia on influx of leukocytes in the digital lamellae of horses with oligofructose-induced laminitis

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ABSTRACT

Sepsis-related laminitis (SRL) is a common complication in the septic/endotoxemic critically-ill equine patient, in which lamellar injury and failure commonly lead to crippling distal displacement of the distal phalanx. Similar to organ injury in human sepsis, lamellar injury in SRL has been associated with inflammatory events, including the influx of leukocytes into the lamellar tissue and markedly increased expression of a wide array of inflammatory mediators at the onset of Obel grade 1 (OG1) laminitis. The only treatment reported both clinically and experimentally to protect the lamellae in SRL, local hypothermia ("cryotherapy"), has been demonstrated to effectively inhibit lamellar expression of multiple inflammatory mediators when initiated at the time of administration of a carbohydrate overload in experimental models of SRL. However, the effect of hypothermia on leukocyte influx into affected tissue has not been assessed. We hypothesized that cryotherapy inhibits leukocyte emigration into the digital lamellae in SRL.

Immunohistochemical staining using leukocyte markers MAC387 (marker of neutrophils, activated monocytes) and CD163 (monocyte/macrophage-specific marker) was performed on archived lamellar tissue samples from an experimental model of SRL in which one forelimb was maintained at ambient temperature (AMB) and one forelimb was immersed in ice water (ICE) immediately following enteral oligofructose administration (10 g/kg, n = 14 horses). Lamellae were harvested at 24 h post-oligofructose administration (DEV, n = 7) or at the onset of OG1 laminitis (OG1, n = 7). Both MAC387-positive and CD163-positive cells were counted by a single blinded investigator on images [n = 10 (40× fields/digit for MAC387 and 20 x fields/digit for CD163)] obtained using Aperio microscopy imaging analysis software. Data were assessed for normality and analyzed with a paired *t*-test and one-way ANOVA with significance set at $p < 0.05$.

MAC387-positive cells were present in low numbers in the lamellar tissue and were decreased in the hypothermic limbs (vs. AMB limbs, $p < 0.05$) in the OG1 group; no change in CD163-positive cell numbers was noted across the conditions of the model. This study demonstrated that hypothermia of the distal limbs instituted early in the disease process in the horse at risk of SRL significantly attenuates the increase of MAC387-positive leukocytes in the digital lamellae, but has minimal effect on increases in lamellar concentrations of the major leukocyte cell type present in that tissue, CD163-positive mononuclear cells.

1. Introduction

In horses, a variety of illnesses leading to systemic sepsis have been associated with the onset of a severe, crippling form of equine laminitis termed sepsis-related laminitis (SRL) (Garner et al., 1975; Parsons et al., 2007). Many of the same pathophysiologic events reported in organ failure in septic humans in response to systemic inflammatory response syndrome have also been documented to

Abbreviations: AMB, ambient; BWE, black walnut extract; CHO, carbohydrate overload; COX-2, cyclooxygenase-2; DAMPs, damage-associated molecular associated molecular patterns; DEV, developmental; HPF, high power field; NF-kβ, nuclear factor kappa B; OF, oligofructose; OG1, Obel Grade 1; PAMPs, pathogen-associated molecular patterns; PMN, polymorphonuclear; (+), positive; PRRs, pattern recognition receptors; SRL, sepsis–related laminitis; TLRs, Toll-like receptors.

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occur in the lamellae in SRL (Maier, 2000; Belknap et al., 2009). In both septic humans and animal models of sepsis, leukocyte extravasation into tissues due to leukocyte activation, adhesion to post capillary venules, and transendothelial migration is reported to be a primary event in organ dysregulation/injury (Singer et al., 2009; Wang et al., 2013; Heemskerk et al., 2014). This leukocyte emigration, which reportedly occurs due to the systemic activation of leukocytes and the local expression of multiple selectins, integrins, and chemokines from activated vascular wall and surrounding tissues; purportedly leads to inflammatory injury to tissues/organs in human and equine sepsis (Leise et al., 2011; Chaudhry et al., 2013; Maier, 2000; Black et al., 2006) and has thus become a focus of therapeutics in human medicine. (Crouser, 2012; Rim et al., 2012; Yenari and Han, 2012; Coyan et al., 2014; Yuan et al., 2014).

In sepsis in adult horses, the major "target organ" is the digital lamellae of the hoof; whereas human sepsis commonly results in multiple organ dysfunction/failure observed in visceral organs (Stewart et al., 2009). The most common experimental models of SRL have included the black walnut extract (BWE) model (Eaton et al., 1995; Belknap, 2010) and two carbohydrate overload (CHO) models, the more traditional corn starch/wood flour model, (Garner et al., 1978; Faleiros et al., 2011a) and the more recent oligofructose model (van Eps and Pollitt, 2006). Whereas the BWE model is a short-term transient model which rarely leads to severe lamellar injury (Belknap, 2010) carbohydrate overload models more closely approximate what occurs in clinical cases of laminitis in which enterocolitis develops before the onset of clinical signs of laminitis, followed by a similar degree of lamellar injury as observed in clinical cases of SRL (van Eps and Pollitt, 2009). Multiple studies of lamellar tissues in these experimental models of SRL have documented a marked increase in pro-inflammatory cytokines, chemokines, cyclooxygenase-2 (COX-2), and endothelial adhesion molecules in the early stages of laminitis (Waguespack et al., 2004; Bliksłager et al., 2006; Belknap et al., 2007; Leise et al., 2011; van Eps et al., 2012). Due to the role leukocytes reportedly play in sepsis-related end-organ injury, several studies have characterized lamellar leukocyte populations at different stages of laminitis in the SRL models (Black et al., 2006; Faleiros et al., 2009a; Faleiros et al., 2011a). In the BWE model, CD13-positive (+) polymorphonuclear (PMN) cells (Black et al., 2006) were identified entering lamellar tissue, as were a combination of PMNs and mononuclear cells using MAC387/calprotectin (identifies neutrophils, activated monocytes/macrophages, and damaged/stressed/activated epithelial cells) and CD163 (identifies monocytes/macrophages) immunohistochemical stains (Faleiros et al., 2009b; Faleiros et al., 2011b). Whereas CD163 was first described as a marker for the M2 (alternatively activated/anti-inflammatory) macrophage phenotype characteristic of resident tissue macrophages (Mills et al., 2012), CD163 immunohistochemistry has also been reported to recognize mononuclear cells in the M1 (classically activated/pro-inflammatory) phenotype in the horse and other species. (Kim et al., 2006; Faleiros et al., 2011a). Later work using the same immunohistochemical techniques in a CHO model of SRL documented an increase primarily in mononuclear cells in the lamellae at the onset of clinical lameness (Faleiros et al., 2011a).

Due to the complex nature of systemic inflammation leading to increases in tissue leukocyte infiltration (Wang et al., 2013) and pro-inflammatory cytokine expression in multiple disease processes, targeted therapy through hypothermia for these individual processes has been the focus in both human (Crouser, 2012; Coyan et al., 2014; Yuan et al., 2014) and now veterinary medicine (van Eps and Pollitt, 2004; van Eps, 2010; Kullmann et al., 2014; van Eps et al., 2014; van Eps and Orsini, 2016). Hypothermia has been documented to decrease leukocyte infiltration and cytokine expression, leading to decreased end organ inflammation and injury in multiple disease states in humans and animal models of human disease (Crouser, 2012; Rim et al., 2012; Yenari and Han, 2012; Coyan et al., 2014; Yuan et al., 2014). Continuous digital hypothermia in the equid, which has been documented histologically in SRL models to inhibit lamellar injury (van Eps et al., 2004; van Eps et al., 2014) and clinically to protect septic equine patients from the development of laminitis (Kullmann et al., 2014), has been documented in the OF model of SRL to result in remarkable decreases (up to 100-fold) in lamellar expression of a broad spectrum of inflammatory molecules including cytokines, chemokines, and endothelial adhesion molecules (van Eps et al., 2012). To date, it is unknown whether this anti-inflammatory effect of hypothermia also results in (and is possibly due to) an inhibition of influx of leukocytes into the lamellar tissue. The goal of this study was to determine the effect of digital hypothermia on lamellar leukocyte numbers in the OF model of equine SRL.

2. Material and methods

2.1. Animals and sample collection

Previously obtained paraffin-embedded archived lamellar samples from an OF model were used for this study (van Eps et al., 2012). The University of Queensland Animal Care and Use Committee approved and oversaw all animal protocols. Fourteen Standardbred horses, all determined to be healthy with no evidence of lameness or radiographic abnormalities of the feet, were divided into two equal groups. Laminitis was induced by enteral oligofructose overload as previously described by van Eps and Pollitt (2006). Each horse was intubated with a nasogastric tube and administered a bolus dose of 10 g/kg oligofructose. Each horse then had one of the randomly-assigned forelimbs continuously cooled (ICE) by placing the foot in an equal mixture of ice and water to a level immediately below the carpus with continuous hoof temperature monitoring with thermistors as previously described (van Eps et al., 2004). The opposite hoof was maintained at ambient temperature for the duration of the protocol thus allowing each horse to serve as their own control. As previously described (van Eps et al., 2012), horses were constantly monitored during the protocol; clinical parameters (rectal temperature, capillary refill time, fecal output and consistency, heart rate, frequency of weight shifting of forelimbs [primarily limb at ambient temperature]) were recorded every 2 h throughout the protocol.

The first group (DEV) of horses (n = 7) was subjected to euthanasia with sample collection 24 h after administration of the bolus of oligofructose. The second group of (OG1) horses (n = 7) was subjected to euthanasia with sample collection immediately on recognition of Obel Grade 1 lameness (onset of weight shifting of forelimbs; Obel, 1948), which occurred 20–28 h post oligofructose administration. At each determined endpoint, lamellar sections were rapidly dissected and either fixed for 48 h in 10% neutral buffered (Fisher Scientific Pittsburgh, PA, USA), followed by immersion in 70% ethanol until embedding or snap-frozen in liquid nitrogen.

2.2. Immunohistochemistry

Formalin-fixed tissues were embedded in paraffin and sectioned to 4-μm thickness and then stained separately for both MAC387/calprotectin (Abcam Cambridge, MA, USA) and CD163 (Cosmo Bio Carlsbad, CA, USA). Immunohistochemistry utilized the universal avidin-biotin complex detection technique for all samples. To detect MAC387, each individual section was deparaffinized and treated with protease solution (Proteinase-K (Fisher Scientific Pittsburgh, PA, USA) 20 μg/ml 22 °C, 6 min). Endoge-