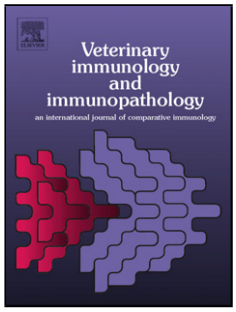

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The effect of hypothermia on influx of leukocytes in the digital lamellae of horses with oligofructose-induced laminitis

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Highlights:

- Digital hypothermia decreased lamellar number of MAC387(+) but not CD163(+) cells
- Digital hypothermia has minor effects on lamellar leukocyte emigration in laminitis
- Anti-inflammatory effect of hypothermia may be due to effect on lamellar host cells

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

(10g/kg, n=14 horses). Lamellae were harvested at 24 hours 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 (40x fields/digit for MAC387 and 20x 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.

Keywords:

Laminitis, sepsis, hypothermia, CD163, MAC387, oligofructose

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;

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 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, Allen et al. 1995, Belknap 2010) and two carbohydrate overload (CHO) models, the more traditional corn starch/wood flour model, (Garner et al. 1978, Faleiro 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, Blikslager 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. 2012) 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.

Material and methods:

1.1 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 colleagues (2006). Each horse was intubated with a nasogastric tube and administered a bolus dose of 10g/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 hours throughout the protocol.

The first group (DEV) of horses (n=7) was subjected to euthanasia with sample collection 24 hours 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 hours post oligofructose administration. At each determined endpoint, lamellar sections were rapidly dissected and either fixed for 48 hours in 10% neutral buffered (Fisher Scientific Pittsburgh, PA USA), followed by immersion in 70% ethanol until embedding or snap-frozen in liquid nitrogen.

1.2 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) 20ug/ml 22^{o}C, 6 minutes). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide (22^{o}C, 5 minutes); the slides were then incubated in blocking solution containing 2% serum (22^{o}C, 1 hour) and then incubated with the mouse monoclonal anti-human MAC387 antibody (1:250, 4^{o}C, overnight). The slides were subsequently incubated with a biotinylated secondary antibody (Vector Laboratories Burlingame, CA USA) (22°C, 30 minutes) and then

finally with avidin-horseradish peroxidase complex (Vector Laboratories Burlingame, CA USA) (22°C, 30 minutes). Signal was developed by incubation with DAB chromagen, (Vector Laboratories Burlingame, CA USA) (22°C, 5 minutes) and counterstained with hematoxylin (30 seconds).

For CD163 staining, the avidin-biotin complex method was again utilized in a similar fashion as for MAC387, but this time an automatic processing system was used, with antigen retrieval of 10mM sodium citrate pH 6.0 under controlled heat and pressure (125°C, 20 minutes using a decloaking chamber) and incubation with CD163 primary antibody (1:40, 37°C, 30 minutes).

1.3 Image Analysis:

All slides were examined by light microscopy to document the presence of positively-stained cells. Whole-slide digital images were randomly acquired with an automated scanning robot with a magnification of 40X HPF for MAC387 at a spatial sampling period of 0.2 µm per pixel. A total of 10 captured images were obtained from each slide. The same spatial sampling period was obtained for CD163 at a 20X HPF magnification again for a total of 10 captured images. A lower magnification was used as the high positive cell stain uptake made the cells indistinguishable further afield. MAC387-positive and CD163-positive leukocytes were manually counted in each individual image. The operator was blinded to the origin of all digital images (i.e. whether the images

were captured from ambient or hypothermic lamellae and from the identity of the horse) during image capture and cell counts. CD163-positive and MAC387-positive cells were counted in different microanatomic locations to assess cell numbers within the vasculature (in the primary dermis), primary dermal lamellae, secondary dermal lamellae, primary epidermal lamellae, secondary epidermal lamellae, and total cell count in each image. The same operator performed both image assessment and cell counts for the entire study.

1.4 Statistics:

Due to the small number of cells observed in each 40X field, statistical analysis was performed on the total number of cells identified in each field (different microanatomic locations were not compared). The number of lamellar MAC387(+) and CD163(+) cells were compared between ICE and AMB conditions at DEV and OG1 time points. Normality of sample data was determined using the D'Agostino and Pearson omnibus normality test and determined to be normally distributed. A one-way analysis of variance (ANOVA) was used to assess the effect of hypothermia on lamellar CD163-positive and MAC387-positive cell numbers per high power field across time points (independent analysis performed for each stain). Significance was set at $p<0.05$. Post hoc comparisons of ANOVA results were performed using the Tukey's test. All analyses were performed using Graphpad Prism software (La Jolla, CA USA).

Results:

Data for this study were normally distributed. On qualitative assessment (Figs 1-4), all sections contained few MAC387(+) cells (Fig. 1) and a larger number of CD163(+) cells (Fig. 2) at each time point examined (the two cell types were not compared statistically due to leukocyte counts being performed on images at different magnifications for the two markers). There were few positive cells for either marker in the epidermal lamellae, with the vast majority of cells observed in the primary or secondary dermal lamellae (see Figs. 1 and 2).

Similar to what has previously been published in a traditional CHO (corn starch gruel) model of SRL (Faleiros et al. 2011a), there was a greater number ($P<0.05$) of MAC387(+) cells in the lamellae from ambient limbs of animals at the OG1 time point compared to the DEV time point. Whereas hypothermia was not associated with any difference in the number of lamellar MAC387(+) cells ($P > 0.9$) at the DEV time point (Fig. 3; AMB [1.2 ± 0.3 cells/HPF; vs ICE [1.3 ± 0.5 cells/HPF] limbs), there were fewer ($P = 0.002$) MAC387(+) cells in the lamellae of the ICE limbs (1.7 $\pm$ 0.2 cells/HPF) compared to AMB limbs [3.1 ± 1.7 cells/HPF]) at the OG1 time point (Fig. 3). The perivascular location (in the primary dermal lamellae) of the majority of MAC387(+) cells in the ambient limb at the OG1 time point (Fig. 1c) suggests that the increase in lamellar MAC387(+) cells is due to an influx of MAC387(+) cells into the lamellae in the OF model.

On qualitative assessment, there were more lamellar CD163(+) cells than MAC387(+) cells at all time points examined (Fig. 2); there was also an increased number ($P<0.05$) of lamellar CD163(+) cells in the ambient limbs at the OG1 time point compared to the DEV time point (Figs. 2 and 4); this increase is similar to what has already been reported in the starch gruel model of CHO (Faleiros et al. 2011a). However, there was no effect of hypothermia observed on the number of lamellar CD163(+) cells at either the DEV (Fig. 4; AMB [90.3 ± 34.2 cells/HPF] vs. ICE [78 ± 33.1cells/HPF]) or OG1 time points (Fig. 4; AMB [111.4 ± 40.5 cells/HPF] vs. ICE [124.1 ± 48.2 cells/HPF]).

Discussion:

Systemic inflammatory response syndrome (SIRS) can be a product of multiple sepsis-related disease states seen in both humans and horses, with uncontrolled SIRS potentially leading to devastating consequences including end-organ dysfunction and failure (Maiers 2000, Belknap et al. 2009). In horses with SRL, lamellar dysfunction and failure can be the result of multiple sepsis-related disease processes, including duodenitis-proximal jejunitis, placental retention, enterocolitis, and pleuropneumonia (Garner, et al. 1975, Parsons, et al. 2007, Belknap et al. 2009, Kullmann et al. 2014). Due to the intense lamellar inflammatory response in SRL and the role leukocytes are reported to play in inflammatory tissue/organ injury in sepsis, investigators have attempted to determine the temporal relationship of leukocyte

influx to the onset of histologic evidence of lamellar injury; this has primarily been performed in an attempt to establish if lamellar leukocyte influx is playing an initiating role in injury or if the leukocyte influx is occurring in response to the primary lamellar injury in SRL. Unfortunately, conflicting results related to this issue are reported in the SRL literature, with two reports using the OF model of SRL stating that histologic changes to the lamellae are present prior to detection of leukocytes (Visser 2008, de Laat et al. 2011) and another report in which the traditional starch gruel model of SRL was used reporting that leukocyte influx occurred prior to histologic evidence of structural lamellar changes (Faleiros, Johnson et al. 2011). Due to the importance of determining the role of leukocyte influx in lamellar injury (as this event could be a potential therapeutic target if central to laminitis pathophysiology), we approached the question in the current study from a different direction, assessing lamellar leukocyte concentrations in lamellar samples from a digital hypothermia study in which local hypothermia was demonstrated to effectively inhibit both inflammatory signaling and lamellar injury in the OF model of SRL (van Eps, Leise et al. 2012).

Based on these previously published results, we have suggested that the increase in lamellar inflammatory mediator observed in SRL is potentially due to the influx of mononuclear cells specifically through leukocyte activation, extravasation into tissues, transendothelial migration, and adhesion to post capillary

venules (Singer et al. 2009, Wang et al. 2013). In SRL, the primary leukocyte present in the lamellae has been the monocyte/ macrophage (Faleiros et al. 2011a). Although neutrophils are commonly a focus of interest in models of human sepsis, macrophages and monocytes are known to function in initiating the acute inflammatory and vascular changes associated with sepsis and leukocyte extravasation into the perivascular tissues of multiple organs (Mills 2012). Macrophages are often classified according to two different phenotypes. Type 1/M1 is known to have a pro-inflammatory phenotype, whereas type 2/M2 demonstrates anti-inflammatory properties (Belknap et al. 2011, Mills 2012). When humans are diagnosed with SIRS, the M1 phenotype dominates the acute inflammatory disease process (Mills, 2012). MAC387 reportedly stains the classically activated M1 phenotype, whereas CD163 is thought to stain for both M1 and M2 phenotype in the horse (Faleiros et al. 2011a). Normal equine lamellar tissue contains very few MAC387-positive leukocytes and a moderate number of CD163-positive cells (which are thought to represent resident tissue macrophages, Faleiros et al. 2011a).

In the current study, we assessed the same two leukocyte markers, MAC387 and CD163, previously used to demonstrate leukocyte influx in both CHO models (corn/wood starch and OF) of SRL (de Laat et al. 2011; Faleiros et al. 2011a, Johnson et al. 2011). Whereas MAC387 appears to be a marker for similar

leukocytes (neutrophils and activated monocytes) in horses as in other species, we, similar to recent reports in inflammatory disease in humans, have found CD163 to be a marker not only for the classically-described anti-inflammatory/M2 phenotype of monocytes/macrophages (Buechler et al. 2000), but also for activated mononuclear cells (M1 phenotype) infiltrating the interstitium from the vasculature in inflammatory diseases (Kim et al. 2006; Faleiros et al. 2011a).

Our findings demonstrated an increase in both MAC387(+) and CD163(+) leukocytes at the OG1 vs the DEV time point (Fig. 3 & 4). This coincides with previous reports of a marked increase in lamellar expression of multiple inflammatory molecules at the onset of OG1 laminitis in the CHO model (Faleiros et al. 2011a, Leise et al. 2011). However, the inability of hypothermia to block the increase in CD163(+) cells at the OG1 time point was unexpected (Fig. 4). Hypothermia decreased the number of MAC387(+) cells at the OG1 time point ($p<0.05$); however, there is an incredibly small number of MAC387(+) cells when compared to the CD163(+) cells (thereby calling the validity of any conclusions drawn from this finding into question). CD163 immunohistochemistry is most likely staining a combination of both resident tissue macrophages and an influx of activated monocytes from the vasculature into the tissues as the numbers are increasing, as previously reported in our other work (Black et al. 2006, Faleiros et al. 2011a). It is possible that CD163

immunohistochemistry in the current study is recognizing an increase in tissue macrophages in the M2 phenotype in the hypothermic limbs, which would possibly indicate an anti-inflammatory role of hypothermia. However, monocytes extravasating into the lamellar tissue in the current model of sepsis-related laminitis are more likely in the classically activated/M1 phenotype as is described for extravasating monocytes in early sepsis in other species (Italiani and Boraschi 2014; Faleiros et al. 2011a); this is consistent with the increase in CD163-positive cells in affected lamellae being monocytes of the inflammatory/M1 phenotype with minimal effect of hypothermia on this event.

In human medicine, the down regulation of inflammatory mediators is thought to play a major role in the protective effect of hypothermia used as an adjunct treatment for sepsis and trauma (Crouser 2012, Rim et al. 2012, Yenari & Han 2012, Coyan et al. 2014). Digital hypothermia in horses has been shown to be very effective in blocking early lamellar inflammatory events likely to play an important role in lamellar injury, including the expression of chemokines, pro-inflammatory cytokines, COX-2 and endothelial adhesion molecules (van Eps et al. 2011, Kullmann et al. 2014). Clinically, hypothermia has been shown to be the only therapy that can attenuate the severity of laminitis at various stages of SRL (van Eps & Pollitt 2004, van Eps 2010, van Eps et al. 2012, van Eps et al. 2014). The cellular origin of the massive

increase of lamellar inflammatory mediators (Leise et al. 2011) that is being blunted with the use of digital hypothermia (van Eps et al. 2011) is unknown. It is likely that much of the inflammatory response is from the host cells of the lamellae including not only resident tissue macrophages but also the lamellar epithelial cells. It is well described that SIRS results from activation of both the innate and adaptive immune responses when stimulated by the presence of both bacterial products and injured host cells (Belknap et al. 2007). The cells of the innate immune system employ pattern recognition receptors (e.g. Toll-like receptors [TLRs]) to recognize circulating proteins both from pathogens and injured host cells including pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPS) (Belknap et al. 2007, McConachie & Hart 2016). This eventually leads to downstream inflammatory signaling, including activation of the transcription factor nuclear factor kappa B (NF-kB); this activation results in activation of pro-inflammatory cascades and production of selectins, chemokines, and adhesion molecules (Wullaert et al. 2011).

Lamellar epidermal epithelial cells, the most abundant cell type in the lamellae, are well characterized as part of the innate immune system capable of expressing PRRs and undergoing NF-κB related signaling (Leise et al. 2010, Muller-Anstett et al. 2010, Leise et al. 2015). Not only have lamellar epithelial cells been documented to express inflammatory mediators (Blikslager et al.

2006, Faleiros et al. 2009a, Leise et al. 2014) bioinformatic analysis of results of next generation sequencing of laser-captured lamellar basal epithelial cells from the OG1 time point in the a CHO model of SRL strongly predicted NF-κB signaling taking place (Leise et al. 2015). These data, combined with data indicating a very permeable microvascular system in the lamellae in SRL (Allen, Clark et al. 1990), highly suggest lamellar epithelial cells and other lamellar host cells (e.g. resident macrophages, fibroblasts) play a central role of role in the marked lamellar inflammatory response in SRL, being activated by circulating PAMPs and DAMPs leaking into the lamellar interstitium in the septic equid.

Given the results of this study, it is likely that hypothermia is causing a marked decrease in lamellar inflammatory signaling through affecting signaling mechanisms in the lamellar host cells themselves-including non-immune cells such as the epithelial cells, and not primarily through the influx of leukocytes. These results also support the results of others that leukocyte influx into the lamellae may be more of a response to lamellar dysregulation/injury than playing a central initiating role in lamellar pathophysiology. Thus, future studies in the search for pharmaceutical agents to mimic the efficacy of hypothermia on lamellar protection in equine sepsis should likely focus on the effect of hypothermia on central inflammatory signaling in the

lamellar keratinocyte and other host cells making up this critical soft tissue structure.

Conclusions:

Although hypothermia induced a decrease in lamellar MAC387-positive leukocytes in the OF model of equine SRL, the lack of effect on lamellar CD163-positive leukocytes, in light of well documented efficacy of digital hypothermia in inhibiting sepsis-induced lamellar inflammatory gene expression, indicates that lamellar injury is not initiated by influx of leukocytes into the lamellar interstitium.

Conflict of Interest:

None of the authors have a financial or personal relationship with people or organizations that could influence or bias this paper.

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Captions for Figures & Graphs:

Figure 1 & 2 need to be printed in color.

Figure 1: Representative images of MAC387-positive cells in both primary dermal (black arrows) and secondary dermal (yellow arrows) lamellae in the limbs maintained at ambient (AMB) or hypothermic (ICE) temperatures at both the DEV and OG1 time points in the OF model of SRL. Note the small number of MAC387-positive cells overall, the increased number of positive cells in the ambient limb at the OG1 time point (c) compared to DEV time point (a), and the decreased number of MAC387-positive cells in the hypothermic limbs at OG1 (d) compared to the ambient limb at OG1 (c).

Figure 2: Representative images of CD163 positive cells in both primary dermal (black arrows) and secondary dermal (yellow arrows) lamellae in the limbs maintained at ambient (AMB) or hypothermic (ICE) temperatures at both the DEV and OG1 time points in the OF model of SRL. Note the similar appearance (both in location and approximate numbers) of CD163 positive cells in the hypothermia-treated lamellae (b and d) compared to the limbs maintained at ambient temperature (a and c) indicating minimal effect of hypothermia on the presence of CD-163 positive leukocytes.

Figure 3: Mean fold changes in laminar MAC387 positive cells in limbs kept at ambient temperature (AMB) or treated with

hypothermia (ICE) at DEV and OG1 time points after OF administration (DEV, n=7 and OG1, n=7). Note the increase (*) in total positive cell numbers in the ambient limbs between the DEV and OG1 time point (AMB-DEV vs AMB-OG1), and the hypothermia-induced decrease (#) in MAC387 positive cells at the OG1 time point (ICE-OG1) compared to AMB-OG1).

Figure 4: Mean fold changes in laminar CD163-positive cells in limbs kept at ambient temperature (AMB) or treated with hypothermia (ICE) at DEV and OG1 time points after OF administration (DEV, n=7 and OG1, n=7). Note the increase (*) in total positive cell numbers in the ambient limbs between the DEV and OG1 time point (AMB-DEV vs AMB-OG1) and no effect of hypothermia (ICE) on lamellar numbers of CD163 positive cells at either the DEV or OG1 time points.