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

THESIS

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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, 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 hypothermia 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 (20x fields/digit for MAC387 and 40x fields/digit
for CD163)] obtained using Aperio microscopy imaging analysis software (Leica
Biosystems Inc. Buffalo Grove, IL USA). Data were assessed for normality and analyzed
with a paired t-test and one-way ANOVA with significance set at p<0.05.

MAC387(+) 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(+) 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(+) 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(+) mononuclear cells.
Dedication:

For my mother, for without her, I would not be the person I am today. Her endless support, encouragement, and constant love have sustained me throughout my life.
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Publications


Fields of Study

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Chapter 1: Introduction

1.1 Laminitis: A Historical Perspective

Laminitis in the horse has been described throughout history for approximately 2000 years. Varying accounts first started with Aristotle (330 BC) and Xenophon (380 BC) who each described a type of barley disease culminating in a crippling foot disease. In 55AD, Collumella, went on to describe this as a “crippling disease due to blood descending to the feet” (Heymering 2010). He noted that the feet were hot and recommended bleeding the middle of the leg as the first historical recorded treatment. It was not until 1576 that the first publication on laminitis was written by Malbie although at this time the cure was thought to be through exercise. In 1683, Snape, a farrier to Charles II, had the forethought to begin vertical hoof grooving which is still used in present day treatment. In 1886, Zundel was the first to describe the lamellar wedge with a description of the third phalanx perforating the sole. He recommended trimming/shoeing techniques in conjunction with the first recorded use of cryotherapy by cooling the hooves with snow or crushed ice. It was Martin in 1916 who was the first person to suggest that microbes potentially cause laminitis, and, in 1934, Akerblom was the first to experimentally induce laminitis with the use of carbohydrates in conjunction with repeated doses of histamine. Multiple suggestions on shoeing and various treatments continued throughout the early 20th century. In 1948, Obel endeavored to
establish a grading system of lameness (Grade I-IV) to further categorize laminitis to which we still use today.

In 1993, RJ Hunt performed a retrospective evaluation of laminitis in 202 horses to determine their long term outcome. Horses with laminitis had an extremely poor prognosis with 25% of horses becoming sound, 25% remaining somewhat lame, and approximately 50% not surviving long term. This study determined that the presence or absence of distal phalangeal displacement and degree of rotation cannot predict the eventual outcome (Hunt 1993). In 1996, a study of over 100,000 horses was performed in the United Kingdom which found an incidence of laminitis to be 7.1% (Hinckley & Henderson 1996). In 2000, the USDA via the National Health Monitoring System performed a similar study looking at the incidence of laminitis in 1200 operations with 28,000 horses (USDA 2000). This review estimated that at least 13% of these operations had encountered at least one horse with laminitis in the previous year with an overall incidence of 2.1% (USDA 2000).

Experimental designs and treatment options have continued into present day with no concrete determination as to why some horses develop laminitis while others with the same clinical signs and disease processes do not. Risk factors for developing acute laminitis in hospitalized horses are endotoxemia, colitis, surgical acute abdomen, pneumonia, and vascular anomalies (Parsons 2007). There are many speculations as to why laminitis develops in these sick hospitalized patients, but there are only four truly reliable research models that will create a model of laminitis for study. These are the corn starch/wood flour, black walnut extract, oligofructose, and insulin models. To date, the
carbohydrate overload models (corn starch/wood flour and oligofructose) are the only models to consistently give researchers the form of laminitis that specifically represents the systemic inflammatory response seen in hospitalized septic patients leading to sepsis related laminitis.

1.2 Structure and Anatomy of the Equine Foot

The equine foot is a complex structure which supports the 3rd phalanx of the horse’s appendicular skeleton. Impact/force in conjunction with stress/strain on the foot have resulting implications on the external load and mechanical behavior of the foot. Extrinsic modifiers to include gait, speed, and shoeing can have a direct effect on the external load of the foot, whereas intrinsic modifiers such as the actual shape of the foot, internal anatomy and biological responses/remodeling can have a direct effect on mechanical behavior. All of the aforementioned variables have a direct effect on the internal structure of the foot itself.

The hoof is made up of the wall and lamellar corium which suspend and protect the 3rd phalanx of the horse. Simply put, the interior lamellae attach to the corium of the pedal bone which consists of a complex network of blood vessels, lymph vessels, and nerves. This intricate interface allows the hoof to remain stable but also be flexible when the horse is standing or in motion and, these soft tissue structures effectively bear the entire weight of the horse.
The wall of the hoof can be broken down into three distinct layers. From the outside working inward they are the stratum tectorium, stratum medium, and stratum lamellatum. The outermost layer, the stratum tectorium, is composed of cornified epithelial cells which attach the hoof to the epidermis of the skin of the foot. The stratum medium is comprised of prominent tubular and intertubular horn. This layer makes up the majority of the hoof wall (Ownby 2002). This leads to the stratum lamellatum known as the “layer of leaves” which is the epidermis and dermis in the lamellar region of the foot. This innermost layer is critical in the suspension of the 3rd phalanx within the hoof wall. It is made up of dermal papillae and epidermal pegs which form elongated ridges which are perpendicular to the ground. The ridges are formed from primary and secondary lamellae. The secondary lamellae are formed at right angles to the primary lamellae. There are approximately 600 primary lamellae and approximately 100-200 secondary lamellae for each individual primary lamellae (Ownby 2002). The construction of this internal system comprised of interdigitating primary and secondary lamellae provide the tight bond that holds the hoof in place with the underlying dermis through all mechanical forces in the normal foot.

When looking grossly at the lamellae, it is clear to see the intertwining epidermal and dermal components that fit like a closed zipper. On a microscopic level, the secondary epidermal and dermal lamellae can be seen to interdigitate as well. These secondary epidermal lamellae are made up of a single layer of basal epithelial cells which attach to the basement membrane by the type 1 hemidesmosome structure similar to that present in skin (Litjens et al. 2006). The secondary dermal lamellae attach to the
secondary epidermal lamellae at the basement membrane. The secondary dermal lamellae are similar in structure to connective tissue and extend from the corium of the third phalanx. Laminitis can be defined as the separation of the secondary epidermal and secondary dermal lamellae. The key histological feature is often a degradation of the basement membrane and type 1 hemidesmosome attachment (French & Pollitt 2004; Nourian et al. 2007). This destruction of this delicate network can be a potentially devastating and ultimately a life ending complication in many equids.

1.3 Sepsis and the Systemic Inflammatory Response Syndrome Defined

In 1992, the American College of Chest Physicians and the Society of Critical Care Medicine published a consensus report of definitions for systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis, and multiple organ dysfunction (MODS) which has been subsequently adapted to veterinary medicine for better consistency with diagnosis (Bone et al. 1992). The systemic inflammatory response syndrome is used to describe systemic inflammation caused by infectious agents (bacteria, viral, or fungal) or noninfectious causes (trauma, burns, toxins, and acidosis). The current criteria of SIRS for veterinary species are the presence of two of the following: hyperthermia or hypothermia; tachycardia; tachypnoea or hyperventilation; leucopenia, leukocytosis, or > 10% band neutrophils (Smith 2014).
Sepsis by definition is a systemic, deleterious host response to infection that can lead to severe sepsis (acute organ dysfunction secondary to documented or suspected infection) and septic shock (severe sepsis plus hypotension not reversed with fluid resuscitation) (Dellinger et. al 2012). Simply put, SIRS in addition to infection equals sepsis. The most common cause of sepsis in human medicine is bacterial in origin with the most frequent complication being multiple organ dysfunction (MODS) (Dellinger et. al 2012). In humans, the most common manifestation of organ dysfunction is respiratory failure with up to 75% of cases requiring mechanical ventilation. Other common manifestations of organ dysfunction include sepsis-related myocardial depression with circulatory failure and renal dysfunction in 56% of patients (Martin & Wheeler 2009). Unlike humans who attain mostly visceral organ failure, the most common focus of organ/tissue dysfunction in the septic equine patient manifests as the dysfunction and destruction of the lamellar tissue of the hoof leading to crippling and life threatening lameness (Belknap 2009). In human medicine, sepsis is known to occur in 2% of all hospitalizations and can occur in 6-30% of ICU patients. Sepsis accounts for 10% of deaths and approximately 17 million dollars per year (Martin & Wheeler 2009). In horses, sepsis is the leading cause of illness and death in both neonates and adults (Cohen et al. 1994, Hoffman et al. 1992, Peek et al. 2006, Werners et al. 2005).
1.4 DAMPs, PAMPS, & Alarmins

In order for cells to distinguish between invading pathogens and cell damage or death, an intricate system of alarms has evolved to aid in surveillance, defense, and repair mechanisms for the body. Pathogen-associated molecular pattern molecules (PAMPs) are molecules that are recognized as being part of microbial pathogens. These PAMPs can be identified as entire molecules or parts of the structure of molecules. Exogenous PAMPs are recognized by the immune system (both innate and acquired) by Toll-like receptors (TLRs) which are part of the pattern recognition receptors (PRRs) leading to activation of downstream cell signaling (Bianchi 2007). There are currently 10 TLRs that have been identified with specificity towards binding ligands to bacteria, fungi, and yeast proteins (Cohen 2002). TLR2 identifies Gram-positive cell wall structures (peptidoglycans) whereas TLR4 recognizes the lipopolysaccharide (LPS) from Gram-negative cell lysis. Flagellin is a virulence factor recognized by TLR5 from both Gram-positive and Gram-negative bacteria. TLR9 recognizes CpG elements of bacterial DNA (Cohen 2002). All known TLRs signal through the adaptor protein MyD88 in the cytoplasm of neutrophils, macrophages, and monocytes, thus leading to downstream activation of NF-κβ. This ultimately leads to either destruction of the pathogen itself or pathogen-infected cells, and a positive feedback response of the immune system by activation of cytokine gene expression and receptor expression along with antibodies of the adaptive immune system (Bianchi 2007).
Pathogens are not the only forces causing inflammation and a response from the 
immune system. Trauma, burns, chemical insults, radiation, drugs, toxins, and oxygen 
withdrawal can cause a similar chain of events from the immune system. Once a 
traumatic injury occurs, it is very likely that infection can and will follow if able. When 
the body undergoes non-pathogen associated cell injury, danger signals are released 
which are termed “alarmins.” Alarmins in conjunction with PAMPs constitute the family 
of damage-associated-molecular patterns (DAMPs). Alarmins are very similar to PAMPs 
but are endogenous molecules that have been released into the body after non-
programmed cell death from non-apoptotic cells and alternatively can be released by 
specialized secretion pathways from cells including those of the immune system (Bianchi 
2007). Alarmins can recruit cells to activate their receptor expression to promote 
adaptive immunity, and they can also promote homeostasis by reconstruction of tissue. 
Examples of alarmins are high mobility group box 1 (HMGB-1), S100 proteins, heat 
shock proteins, IL-1alpha, and uric acid among others. HMGB1 is a nuclear protein that 
binds to nucleosomes and promotes DNA bending (Bianchi 2007). When cells 
unexpectedly die and are not associated with programmed apoptosis, HMGB1 is released. 
Myeloid cells and natural killer cells of the immune system can secrete this alarmin when 
activated through secretory lysosomes. Neurons, enterocytes, smooth muscle, endothelial 
cells and macrophages that have engulfed and phagocytosed apoptotic cells can also 
secrete HMGB-1 thru secretory pathways. The secretion of this alarmin allows for 
chemotactic activity on neutrophils, monocytes, macrophages, and dendritic cells of the 
immune system. S100 proteins, which are also known as calgranulins, are expressed by
phagocytes and secreted at sites of inflammation in the body. There are more than 20 of these calcium binding proteins which produce increased vascular permeability along with prothrombotic effects (Bianchi 2007). One of the S100 alarmins is recognized by MAC387 immunohistochemical staining. Both HMGB1 and S100 proteins interact with the RAGE ligand although S100A8/9 may react with TLRs (Bianchi 2007).

1.5 Ischemia-Reperfusion Injury

In normal tissue, blood flow and tissue perfusion are necessary to maintain normal cellular homeostasis. When blood supply at the capillary level becomes decreased adequate oxygen delivery is compromised and the resultant event is localized tissue ischemia and likely if ongoing cellular death (Moore et al. 1995). The degree of injury and duration of obstruction or constriction of the tissue’s blood supply will ultimately determine viability. Most tissues and cells are programmed to withstand ischemia to a degree as they have cellular energy reserves and can increase the rate of oxygen extraction from the blood. When blood flow is restored to tissues, deleterious effects can occur which is known as reperfusion injury. In horses, typically the most common association with reperfusion injury is compromised intestine in the surgical acute abdomen.

Oxygen is required for normal cellular metabolism with the majority of consumption occurring intracellularly in the mitochondria. Both the electron transport chain and synthesis of ATP make up the cell’s energy source. Oxygen deprivation,
decreases in ATP, or when oxygen demand exceeds availability cause the cell extreme injury an potentially death. When cells are functioning normally, oxygen is decreased to water through oxidative phosphorylation. This reaction occurs in a series of steps reducing the oxygen species by one electron per step. The first reduction step results in the formation of superoxide anion; superoxide ion is then reduced by another electron to yield hydrogen peroxide. Further reduction of hydrogen peroxide results in production of hydroxyl radical; and finally with reduction of oxygen by four electrons forms water (Flaherty & Weisfeldt 1988). During this multi-step reaction from oxygen to water, free radical formation occurs at each step. Free radicals are partially reduced oxygen molecules that contain an unpaired electron and are important mediators in the processes of bacteriocidal mechanisms of leukocytes, inflammatory disease, organ damage, ischemia-reperfusion injury, among others in both humans and veterinary species (Moore et al. 1995). These molecules are electrically unstable because they can either donate or accept electrons and can react with hydroxyl radical and superoxide anion noted to be the most intense form of oxygen radicals that can result in significant tissue damage. In order to counteract free radical formation, the body built in antioxidant defense mechanisms. These include the removal of superoxide anion and hydrogen peroxide by enzymatic antioxidants (superoxide dismutase, catalase, and glutathione peroxidase) and nonenzymatic α tocopherol, ascorbic acid, and β carotene (Moore et al. 1995).

There are many pathways which can produce oxygen-derived free radicals. Xanthine oxidase (XO) is a cytosolic enzyme present in most human and animal cells. XO interacts with oxygen leading to the formation of xanthine and uric acid from
hypoxanthine thereby generating a free oxygen radical. XO is mostly present as xanthine dehydrogenase (XDH) in tissues, and is connected with nicotinamide dinucleotide (NAD+). Neutrophils can produce superoxide radicals through the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system (Moore et al. 1995) also known as the “respiratory burst,” an important and necessary reaction involved in cellular phagocytosis. Superoxides can also occur during the activation of arachidonic acid/eicosanoids leading to conversion of prostaglandin G2 to Prostaglandin H2 through oxidation of catecholamines (Moore et al. 1995). Specifically, intestinal ischemia-reperfusion injury is highly associated with superoxide formation through the XO pathway in both the endothelium and mucosal epithelium in conjunction with neutrophil respiratory burst.

A massive inflammatory response begins to occur when reperfusion is initiated and can lead to further tissue injury. The inflammatory response includes activation of endothelial cells, leukocytes, platelets, parenchymal cells, complement, cytokines, reactive oxygen species, nitric oxide, among many other mediators triggered by cellular debris and signaling of PAMPs, DAMPS, and alarmins. The reactive oxygen intermediates formed lead to endothelial cell damage by cellular swelling, local increased capillary permeability, increased intracellular calcium concentration in damaged cells and proinflammatory cellular mediator synthesis (Kerrigan & Stotland 1993). Multiple organ dysfunction can also be a complication of reperfusion injury that can occur as a result of systemic disease. Reactive oxygen species formation initiates cell signaling due to activation of platelet activating factor and histamine in conjunction with neutrophil
Neutrophil activation can stimulate mucosal and submucosal injury along the gastrointestinal tract, leading to translocation of gastrointestinal bacteria in conjunction with vascular leakage (Cerqueira et al. 2005). This intricate sequence of inflammatory events can further potentiate the risk for endotoxemia to develop.

1.6 Syndrome of Endotoxemia

Endotoxemia is a clinical syndrome defined as free endotoxin in the blood stream and is a common cause of gram negative induced sepsis. In humans, endotoxin is noted to be the “most notorious exogenous microbial component of gram-negative bacteria” (Martin & Wheeler 2009). The presence of Gram-negative bacterial lipopolysaccharide (LPS) within the blood, is a common finding in bacteraemic neonatal foals and is a common sequela of equine gastrointestinal disease, pleuropneumonia and metritis among others. The most notable complications of endotoxemia are laminitis, coagulopathies, and mortality (King and Gerring 1988, Steverink et al. 1994, Ryu et al. 2004, Vinogradov et al. 2008, Hilton and Pusterla 2009, Lopes et al. 2010, Senior et al. 2011).

The pathophysiology of gram negative bacterial sepsis begins with the lysis of the bacteria itself which releases the lipopolysaccharide (LPS)/endotoxin from the outer membrane of the cell wall. Free LPS is first sensed by lipid binding protein (LPB). LPB is an acute phase protein that binds to LPS in order to create an immune response by presenting the LPS/LBP combination to cell surface pattern recognition receptors (PRRs) in order to potentiate cell signalling. The first is by complexing onto CD14/TLR4/MD-2
receptor on the neutrophil, macrophage, or monocyte cell surface. This complex then causes intracellular signaling through binding of the TLR domain, TIR (Toll/IL-1 receptor), to IRAK (IL-1 receptor associated kinase) (Cohen 2002). This process is facilitated by either MyD88 (myeloid differentiation protein 88) or TIRAP/MAL (TIR domain-containing adaptor protein/MyD88 adaptor-like protein) (Cohen 2002) and can be inhibited by Tollip (Toll-interacting protein). Both the dependent MyD88 pathway and independent MyD88 pathway trigger downstream reactions intracellularly that activate NF-κB. NF-κB enters the nucleus to facilitate transcription in order to produce cytokines and effector molecules including: interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α), interleukin-8 (IL-8 also designated CXC ligand 8), and interleukin-6 (IL-6). Both IL-1 and TNF-α promote inflammatory responses in Type 2 immune responses, while upregulation of IL-8/CXCL-8 on the cell surface encourages signaling of tight adhesion formation prior to leukocytes undergoing endothelial extravasation.

Transcription of these aforementioned cytokines and effector molecules can also occur thru the dependent MyD88 pathway through MAPK and independently through IRF 3 (interferon regulatory factor 3) that do not involve NF-κB. NOD1 and NOD2 intracellular proteins may also be able to respond to LPS independently but the mechanism in the cytosol is unknown. Other pathways that are stimulated on the cell surface receptors that sense the LPS/LPB complex are the macrophage scavenger receptor (MSR), CD11b/CD18 receptor, and ion channels (Cohen 2002).

Twenty five percent of horses admitted to hospitals with acute abdominal disease will have circulating endotoxin in their blood (Moore et al. 2012). The half-life is less
than 2 minutes which immensely decreases the likelihood of diagnosing every patient from plasma sampling. Due to this short half-life, most horses that are admitted to hospital with suspect endotoxemia are diagnosed by clinical examination findings (fever, tachycardia, tachypnea, discolored mucous membranes, increased capillary refill time, among others), complete blood count (often leukopenia, neutropenia, and left shift), and arterial blood gas with evidence of hypoxemia and metabolic acidosis (Moore et al. 2012). Diagnosis of endotoxemia has been found to be the only factor significantly associated with laminitis (Parsons et al. 2007). Several attempts to induce laminitis by experimental infusion of LPS have been tried and have been unsuccessful; therefore, other sepsis models have been studied in order to replicate clinical sepsis and outcome (MacKay et al. 1991, MacKay & Lester 1992, Turek et al. 1985, Ward et al. 1987, Tadros & Frank 2012).

1.7 Inflammation and the Role of Leukocytes

In order for the body to respond to infection or inflammation, the role of leukocytes and their ability to cross blood vessels in both human and veterinary species is essential. The release of Danger-Associated Molecular Patterns (DAMPs), Pathogen-Associated Molecular Patterns (PAMPs), alarmins, cytokines/interleukins, and chemoattractants all come from the presence of invading bacteria or damaged cells. DAMPs bind to toll like receptors and RAGE receptors (both pattern recognition
receptors or PRRs) and thus begins the process of activating the acute inflammatory cascade to signal leukocyte extravasation to sites of inflammation. This initial step leads to vasoactive changes in the tissue and changes in hemodynamics at the site of inflammation specifically in the microvasculature of arterioles, capillary beds, and post capillary venules. A reduced blood flow rate in post capillary venules in conjunction with presence of histamine and acute inflammatory mediators (TNF-α & IL-1β) activate P-selectin (a cell adhesion molecule) initially stored as granules from intracellular Weibel-Palade bodies to translocate to the cell membrane. This allows for leukocytes’ ligands to adhere to endothelial cells and slow down while rolling along endothelial cells. Endothelial cells that have been activated by pro-inflammatory cytokines also engage the action of E-selectin which encourage further slowing and rolling of the leukocytes along the endothelial border. This is followed by activation of integrins which favor binding of specific ligands. Leukocytes carry CD18 integrins which have a compatible ligand on endothelial cells. CD18 as part of the beta 2 family of integrins binds to both ICAM-1 and ICAM-2 which are intercellular adhesion molecules (Martin 2012, Muller 2013). Lymphocytes and monocytes also contain integrins of the beta 1 family which include CD49d and CD29 which bind to VCAM-1, a vascular cell adhesion molecule (Martin 2012). These two processes allow for adhesion of the leukocyte and the beginning of extravasation into tissues through tight junctions between the endothelial cells. This is mediated by PECAM-1 a platelet and endothelial cell adhesion molecule which then allows perivascular migration through the extracellular matrix via integrins.
1.8 Models of Sepsis-related Laminitis

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, Martin & Wheeler 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, Faleiros et al. 2011a) and the more recent oligofructose model (van Eps and Pollitt 2006). The BWE model is a short-term transient model which rarely leads to severe lamellar injury (Belknap 2010). The carbohydrate overload models more closely resemble what occurs in clinical cases of laminitis in which enterocolitis develops before the onset of clinical signs of laminitis. This is ultimately 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 forelimbs in these experimental models of SRL have documented a marked increase in pro-inflammatory cytokines, selectins, 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). Hind limb lamellar tissue has also been assessed and found to have a similar pro-inflammatory profile with increased mRNA concentrations of IL-6, COX-2, CXCL1, CXCL8, in conjunction with MAC387 positive leukocytes in lamellar tissue (Leise et al. 2011). Signaling events related to inflammation in lamellar tissue have also
been documented to lead to dysadhesion of lamellar basal epithelial cells. This is a central event demonstrating that the basal epithelial cells are actively participating in cell signaling and extracellular matrix regulation further resulting in structural failure (Leise et. al 2014).

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 model (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). Later work using the same immunohistochemical techniques in a CHO model of SRL documented an increase primarily in mononuclear cells with CD163 and MAC387 stains in the lamellae at the onset of clinical lameness (Faleiros et al. 2011a).

Most of the literature on inflammation has been focused on the lamellar tissue of the foot. However, Tadros et al. in 2012, looked at a wider perspective by documenting the inflammatory mediators in whole blood in the CHO model. The results showed that inflammatory mediators-specifically interleukins IL-6, IL-8, and IL-10-increased and preceded the onset of lameness. In 2015, Laskoski et al. examined lamellar tissue of horses with colic and total leukocyte count before death in hospitalized patients. The
results of this study showed that leukocyte infiltration was present with MAC387 positive staining cells present within the lamellar tissue. Lamellar lesions were observed in non-leukopenic horses as well as leukopenic horses with no differences in the disease severity between either of the groups. The leukopenic group also demonstrated an increase in leukocyte infiltration in the lamellar tissue when compared to the control group (Laskoski et al. 2015). The combination of global inflammation and documentation of pro-inflammatory mediators in conjunction with leukocyte infiltration within the lamellar tissue demonstrate a further need to investigate the leukocyte role in the lamellar tissue.

1.9 Digital Hypothermia

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 (hypoxic ischemic encephalopathy in neonates, cardiac by-pass, trauma, among others) and animal models of human disease with a focus on ischemic reperfusion injury, inflammation in the microvasculature, and apoptosis (Martin & Wheeler 2009, Crouser
In post-cardiac arrest, therapeutic hypothermia counteracts neuroexcitation, suppressing the inflammatory response, and reducing cerebral edema. Cerebral metabolism decreases 6-10% for each degree Celsius the body temperature decreases. Side effects of induced hypothermia have been associated with changes in ECG and cardiac rhythm, diuresis, systemic immune suppression, and electrolyte imbalances (Soleimanpour et al. 2014). Diuresis and electrolyte imbalance occurs due to a decrease in solute absorption at the loop of Henle and an increase in intracellular movements of potassium, magnesium, and phosphate thereby decreasing serum ionized concentrations (Soleimanpour et al. 2014). A decreased magnesium concentration may be most detrimental as this ion is especially thought to potentially be neuroprotective and is required both directly and indirectly in ion traffic and neurotransmitter release. Magnesium also plays a role in reperfusion and is associated with decreased infarct size and improved myocardial function after myocardial infarction (Soleimanpour et al. 2014). Unlike humans who require three distinct phases of cooling, only focal continuous digital hypothermia in the equid at <10°C has been used both 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). Cryotherapy has been documented in the OF model of SRL to result in a remarkable decrease (up to 100-fold) in lamellar expression of inflammatory mediators including cytokines, chemokines, COX-2, and endothelial adhesion molecules (van Eps
et al. 2012). To date, it is unknown whether this effect of hypothermia also results in (and is possibly due to) an inhibition of influx of leukocytes into the lamellar tissue.

2.1 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 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, 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 approximates 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). 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.

2.2 Materials & Methods

Animals and Sample Collection:

Previously obtained paraffin-embedded archived lamellar samples from an OF model were used for this study (Faleiros et al. 2011). The University of Queensland Animal Care and Use Committee approved and oversaw all live 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 hoof wall 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.

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 (Obel 1948). 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.

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°C, 6 minutes). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide (22°C, 5 minutes); the slides were then incubated in blocking solution containing 2% serum (22°C, 1 hour) and then incubated with the mouse monoclonal anti-human MAC387 antibody (1:250, 4°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 chromogen, (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).

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 20X 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 40X HPF magnification again for a total of 10 captured images. A higher magnification was used for CD163 to allow for the identification of each positive cell as many positive cells were in close proximity to each other (i.e. were indistinguishable as separate cells on 20X). MAC387(+) and CD163(+) 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(+) and MAC387(+) 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.

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 was 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(+) and MAC387(+) cell numbers per high power field (HPF) 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 V.6 software (La Jolla, CA USA).

2.3 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 (+) 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 ± 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 ± 33.1 cells/HPF]) or OG1 time points (Fig. 4; AMB [111.4 ± 40.5 cells/HPF] vs. ICE [124.1 ± 48.2 cells/HPF]).
Figure 1: Images of MAC387(+) cells

Representative images of MAC387(+) 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(+) 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(+) cells in the hypothermic limbs at OG1 (d) compared to the ambient limb at OG1 (c).
Figure 2: Images of CD163(+) cells

Representative images of CD163(+) 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(+) 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 CD163(+) leukocytes.
Figure 3: MAC387(+) Graph

Mean fold changes in lamellar MAC387(+) 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(+) cells at the OG1 time point (ICE-OG1) compared to AMB-OG1).
Figure 4: CD163(+) Graph

Mean fold changes in lamellar CD163(+) 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(+) cells at either the DEV or OG1 time points.
Chapter 3: Discussion

3.1 Conclusions

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 2009, 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 et al. 2011a). 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 from a different direction. In this study we evaluated 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 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.
Normal equine lamellar tissue contains very few MAC387(+) leukocytes and a moderate number of CD163(+) 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).

The 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 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).

In conclusion, although hypothermia induced a decrease in lamellar MAC387(+) leukocytes in the OF model of equine SRL, the lack of effect on lamellar CD163(+) 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.

3.2 Future Directions

In human medicine and experimental rodent models, the down regulation of inflammatory mediators can be provided by controlled local hypothermia and is used as an adjunct therapy for treatment of sepsis and trauma (Crouser 2012, Rim et al. 2012, Yenari and Han 2012, Coyan et al. 2014). Currently, positive results are seen in human neonates with hypoxic ischemic encephalopathy, adults undergoing cardiac by-pass surgery, trauma patients, and microvascular inflammation in rat models (Coyan et al. 2014, Crouser 2012, Rim et al. 2012, Yenari and Han 2012, Jenkins et al. 2013). Digital hypothermia in horses has been shown to be 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 and 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 activity of both the innate and adaptive immune responses in stimulated by the presence of both bacterial products and damaged cells (Belknap et al. 2007). The cells of the innate immune system employ pattern recognition receptors (i.e. 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 and 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 pattern recognition receptors (Toll-like receptors and NOD-like receptors) and undergoing NF-kB 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 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 et. al. 1990), highly suggest lamellar epithelial cells and other lamellar host cells (i.e. 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 by 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.
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