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Effect of Continuous Digital Hypothermia on Lamellar Inflammatory Signaling When Applied at a Clinically-Relevant Timepoint in the Oligofructose Laminitis Model

K. Dern, A. van Eps, T. Wittum, M. Watts, C. Pollitt, and J. Belknap

Background: Although continuous digital hypothermia (CDH) protects lamellae from injury in the oligofructose (OF) model of sepsis-related laminitis (SRL), conflicting results exist from these studies regarding effects of CDH on lamellar inflammatory events.

Hypothesis/Objectives: To determine the effect of CDH on lamellar inflammatory events in normal and OF-treated horses when instituted at a clinically relevant time point (onset of clinical signs of sepsis in this model).

Animals: Standardbred geldings (n = 15) aged 3–11 years were used.

Methods: In a randomized, controlled discovery study, animals were administered either OF (OF group, n = 8) or water (CON group, n = 8) by nasogastric tube and CDH was initiated in one forelimb (ICE) 12 hours later. Lamellar tissue samples were collected 24 hours after initiation of CDH (ICE and ambient [AMB] forelimbs). Lamellar mRNA concentrations of inflammatory mediators and lamellar leukocyte numbers were assessed using qPCR and immunohistochemistry, respectively; values from four sample groups (CON AMB, OF AMB, CON ICE, and OF ICE) were analyzed using mixed model linear regression.

Results: Although lamellar mRNA concentrations of multiple inflammatory mediators (IL-1β, IL-6, CXCL1, MCP2, COX-2) were increased after OF administration (OF AMB group versus CON AMB; $P < 0.05$), only 2 inflammatory mediators (IL-6 and COX-2) and lamellar leukocyte numbers were decreased with CDH (OF ICE versus OF AMB; $P < 0.05$).

Conclusions and Clinical Importance: Continuous digital hypothermia initiated at a time point similar to that commonly used clinically (clinical onset of sepsis) resulted in a more focused inhibition of inflammatory signaling.

Key words: Cryotherapy; Digital hypothermia; Equine laminitis; Inflammation; Leukocyte.

Sepsis-related laminitis (SRL) is a common sequela to diseases associated with systemic sepsis in the horse, resulting in extensive lamellar injury and subsequent displacement of the distal phalanx within the hoof capsule.[1] Multiple reports regarding the pathophysiology of SRL-primarily studied in the black walnut extract model or carbohydrate overload models (in which an intragastric bolus of either corn starch gruel or oligofructose is administered),[2–4] have supported a central role of inflammation in the form of lamellar leukocyte influx and markedly increased lamellar concentrations of inflammatory mediators.[5–16] In regards to therapy, SRL is the only type of laminitis in which an effective therapy, continuous digital hypothermia (CDH, also termed "cryotherapy"), has been established.[17] Using the oligofructose model of SRL, CDH has been reported to effectively protect lamellae from structural failure when initiated prior to the onset of systemic sepsis (initiated at the time the OF bolus was administered)[18] and when initiated at the onset of Obel Grade 2 (OG2) laminitis.[19] In a retrospective clinical study in which CDH was administered prophylactically to equine patients exhibiting signs of enterocolitis, it was found to significantly decrease the incidence of SRL.[17] Determining the mechanism by which CDH protects the lamellae is important, as it may provide therapeutic targets for pharmaceutical drugs to supplement or replace CDH, which can be cumbersome and impractical in the clinical setting.

Due to the findings of a previous study which demonstrated profound inhibition of a wide array of inflammatory mediators when CDH was instituted prior to either systemic sepsis or lamellar disease,[20] CDH has been purported to limit lamellar injury due to its anti-inflammatory effects. However, a more recent study in which CDH was initiated at the onset of clinical lameness (OG2) did not find the same degree of inhibition

Abbreviations:

AMB	limbs at ambient temperature
CDH	continuous digital hypothermia
CON	limbs from study subjects in which laminitis was not induced
ICE	limbs treated with continuous digital hypothermia
OG1	Obel grade 1
OG3	Obel grade 3
OG4	Obel grade 4
qPCR	real time quantitative polymerase chain reaction
SRL	sepsis-related laminitis

From the Department of Veterinary Clinical Sciences, Ohio State University, Columbus, OH, USA (Dern, Watts, Belknap); Australian Equine Laminitis Research Unit, School of Veterinary Science, The University of Queensland, Gatton, Qld, Australia (van Eps, Pollitt); and Department of Veterinary Preventive Medicine, Ohio State University, Columbus, OH, USA (Wittum).

Corresponding author: J. Belknap, Department of Veterinary Clinical Sciences, The Ohio State University, 440 VMAB, 1900 Coffey Rd., Columbus, OH 43210; e-mail: belknap.16@osu.edu.

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of lamellar inflammation with CDH despite it still conferring a dramatic protective effect upon the lamellae.[19,21] Similarly, a recent study investigating the effects of CDH (instituted immediately after administration of enteral OF) on leukocyte infiltration in the OF SRL model reported only minor changes in lamellar leukocyte numbers, indicating that leukocyte infiltration may have less of a role in lamellar tissue damage than previously thought.[22]

The current study was performed to assess the effect of CDH on lamellar inflammatory signaling and leukocyte infiltration when CDH was instituted at the time point in which it is commonly instituted in the clinical situation, the onset of clinical signs of systemic disease. In contrast to previous studies of CDH, a non-septic control group was incorporated into the study in order to (1) assess the effect of CDH on lamellar signaling in the normal limb, and (2) to be able to accurately assess whether CDH effectively inhibits inflammatory signaling in SRL to a level comparable to that observed in the normal limb. Additionally, in contrast to the study in which minimal anti-inflammatory effect was detected when CDH was initiated at the onset of OG2 laminitis[19,21] (where lamellar samples were harvested late in the disease process), the objective of the current study was to harvest samples at an earlier time point (prior to the onset of severe lamellar separation/failure) to avoid the effect that extensive structural tissue injury may have on inflammatory signaling.

Materials and Methods

This project was approved by the University of Queensland Animal Ethics Committee (AEC) that monitors compliance with the Animal Welfare Act (2001) and The Code of Practice for the care and use of animals for scientific purposes (current edition). All animals were monitored continuously by the investigators.

Laminitis Induction, Controlled Digital Hypothermia, and Sample Acquisition

Sixteen Standardbred geldings (aged 3–11 years) with no evidence of lameness or gross abnormalities of the feet were randomly assigned to one of two groups: a control group (CON; n = 8) not administered oligofructose (OF), and an OF group (n = 7). All horses were placed in stocks, remaining there for the duration of the experiment with constant access to hay and water. Horses in the OF group were immediately administered a 10 g/kg bolus of OF (up to a maximum dose of 4.2 kg) via nasogastric tube upon being placed in the stocks.

Forelimb hoof temperature was monitored using hoof wall thermistors attached to data –logging devices and human pedometer devices[a] were taped onto the antebrachium of both forelimbs to provide data on frequency of weight shifting as previously described.[19,20] Study subjects were constantly monitored, with 2 hourly recording of vital parameters and other clinical observations (including subjective assessment of forelimb weight shifting). A single dose of 4.4 mg/kg phenylbutazone[b] was administered intravenously at the onset of laminitis signs (Obel grade 1 lameness marked by incessant weight shifting in the forelimbs).[23]

Continuous Digital Hypothermia

Continuous digital hypothermia was initiated 12 hours after OF administration (a timepoint chosen from previous OF studies[4,20] when horses consistently start to show clinical signs of sepsis [tachycardia, hyperemic mucus membranes and pyrexia]) and develop diarrhea, in order to mimic the timing of CDH initiation in clinical patients with colitis.[4,17,20] In the CON group, CDH was applied 12 hours after the beginning of confinement in stocks. CDH was applied to one randomly assigned forelimb (ICE) with the other forelimb remaining at ambient temperature (AMB). CDH was achieved by placing the ICE limb in a rubber boot which contained an ice slurry (approx. 50% water/50% ice mixture) to the level of the proximal metacarpus as previously described.[19] The ice slurry was constantly maintained for the remainder of the protocol.

Sample Acquisition

In order to minimize the influence that lamellar destruction may have on lamellar inflammatory signaling, lamellae were harvested at an earlier time point (36 hours post OF administration) than in a previous study that also evaluated inflammatory signaling in which AMB lamellae had already undergone severe physical destruction.[19] Thus, at 36 hours after the beginning of the experimental period (ie, 36 hours post OF administration in the OF group), each study subject was humanely euthanized with pentobarbital sodium (20 mg/kg bwt IV). The dorsal lamellae were dissected from the hoof and third phalanx in each forelimb (AMB and ICE) from each study subject. The sections were either snap frozen (for real time quantitative PCR [qPCR]) or fixed in formalin and processed for light microscopy.

Histological Evaluation

H&E and PAS were used on formalin fixed sections, which were randomized and coded for histological analysis by 2 blinded observers (AE and CP). Histopathological scores were assigned using the system described by Pollitt[24] with the addition of a fourth category (score of 4), defined as complete physical separation of lamellar epidermis from dermis, with no association between epidermal and dermal tissues on the section, as previously described.[19]

RNA Isolation, cDNA Synthesis, and qPCR

Flash frozen lamellar tissue samples were processed using a commercially available preparation[c] to isolate total RNA. mRNA was then isolated using a separate preparation[d] and reverse transcriptase was used to generate cDNA for each tissue sample. This cDNA was then frozen at −20°C until qPCR could be conducted.

Real-time quantitative PCR (qPCR) was performed using a thermocycler,[e] as previously described. Equine specific primers (specificity confirmed in a previous study) for COX-1, COX-2, CXCL-1, CXCL-6, E-selectin, ICAM-1, IL-1β, IL-6, IL-8, IL-10, MCP1, MCP2, and 3 housekeeping genes were used to amplify cDNA as previously described.[8,20] Previously designed templates were used to generate standard curves for each qPCR reaction; Diethylpyrocarbonate treated (DEPC) water was used as a negative control and each sample was run in duplicate. Amplification was performed in a 20 μL reaction volume consisting of 15 μL of PCR master mix (made on site) and 5 μL of sample cDNA. The master mix included Taq polymerase,[f] SYBR Green stock solution,[f] uracil-N-glycosylase,[g] forward and reverse primers, PCR Nucleotide plus,[g] and PCR buffer (20 mmol/L Tris-HCl, 0.05% Tween20, and a nonionic detergent). The thermal cycler was programmed as previously described with an initial denaturation period followed by 40–45 cycles of amplification: the annealing temp was 1–5°C below melting temperature; 72°C for 5 seconds for extension; and 10 seconds for acquisition. An increase in temperature from 65 to 95°C was used to acquire melting curves for each PCR product. Copy number data were normalized using geNorm[h] software and 3 housekeeping genes (β-Actin, β2-microglobin, and glyceraldehyde-3-phosphate dehydrogenase).[25] Two housekeeping genes were used to make a normalization factor for each

sample, and the amplification data produced by RT-qPCR were divided by the normalization factor, creating a normalized copy number value. The normalized copy numbers of the hypothermic control limb (ICE-CON), laminitic control limb (AMB-OF), and hypothermic laminitic limb (ICE-OF) were compared to the copy numbers of the ambient control limb (AMB-CON) to determine a fold change for each inflammatory mediator of interest.

Leukocyte Assessment

Paraffin embedded, formalin-fixed tissues were sectioned to 4-μm thickness and stained for MAC387/calprotectin[i] (a marker for neutrophils and activated monocytes) and CD163[j] (a marker of tissue macrophages and monocytes in the horse).[9,15,16] Immunohistochemistry detection was conducted using an avidin-biotin complex kit.[k] For detection of MAC387 cells, each section was deparaffinized, treated with proteinase K solution, with concurrent quenching of endogenous peroxidase activity using 3% hydrogen peroxide. The slides were then incubated: (1) for 1 hour at 22°C using a blocking solution (2% serum), (2) overnight at 4°C with a mouse monoclonal anti-human MAC387 antibody, (3) 1 hour at 22°C with biotinylated secondary antibody,[k] and (4) 30 minutes at 22°C with avidin-horseradish peroxidase complex.[k] DAB chromagen[k] was then used to develop the signal, and tissues were counterstained with hematoxylin.

Staining for CD163 positive cells was carried out as above, with the exception that a primary antibody for CD163 was utilized.

An operator blinded to the origin of the tissue manually counted MAC387-positive and CD163-positive leukocytes at 10 random locations using 40× magnification in the lamellar tissue from each study subject.

Data Analysis

The distribution of qPCR data were skewed and were therefore transformed logarithmically in order to fulfill the assumption of Gaussian distribution. A linear mixed model was used to analyze the data. The model was specified with logarithmically transformed mRNA copy number data as the dependent variable and treatment (ICE or AMB), induction (OF or CON), and interaction between treatment and induction as main fixed effects. Horse ID was specified as a random effect. Regression models were created using JMP statistical analysis software.[l] The number of lamellar MAC387(+) and CD163(+) cells was compared between ICE and AMB horses in both OF and CON groups. Graphical analysis of the data revealed non-Gaussian distribution, so the data was logarithmically transformed to fulfill the assumption of Gaussian distribution. The data were then analyzed using a linear mixed model, with cell counts specified as the dependent variable and treatment (ICE or AMB), induction (OF or CON), and interaction between treatment and induction as fixed effects. Horse ID was specified as a random effect. Regression models were created using JMP statistical analysis software.[l] The median histological scores were compared between the treated OF (ICE OF) and untreated OF (AMB OF) lamellar tissue using the Wilcoxon signed ranks tests of Graphpad Prism[m] with differences considered significant at $P < 0.05$.

Results

Induction of Sepsis in Study Subjects

All horses in the OF group developed pyrexia (rectal temperature >38.4°C) within 18 hours of the oligofructose bolus (median/interquartile range, 14/10–14), with the exception of one study subject which was administered 2.0 g phenylbutazone IV at 10 hours due to the onset of incessant weight shifting at this time. In the remaining OF horses the onset of OG1 lameness was 20 hours (n = 1); 22 hours (n = 2) and 24 hours (n = 3), with phenylbutazone administered at these times. All horses in the OF group developed tachycardia (>45 beats/min) within 16 hours of the oligofructose bolus (median/interquartile range, 16/10–16.5). Pedometer data in OF study subjects revealed an increase in limb movement of the ambient limbs that peaked at 18 hours, at which time it was significantly ($P < 0.05$) greater than ICE OF (data not shown).

Hoof wall surface temperature data (median, interquartile range) obtained during the experiment was 6.07 (5.40–6.64)°C for ICE OF limbs, 27.82 (26.71–29.05)°C for the AMB OF limbs, 5.06 (4.83–5.92)°C for the ICE CON limbs, and 24.67 (23.25–26.06)°C for the AMB CON limbs: ICE caused a significant ($P < 0.05$) decrease compared to AMB from 12 to 36 hours within both OF and CON groups, however there was no significant difference between ICE CON and ICE OF limbs, or between AMB CON and AMB OF limbs.

Effect of CDH on Histopathological Scores of Lamellar Tissue

Median histological scores of the middle lamellar sections were significantly lower ($P < 0.05$) in the limbs treated with CDH (ICE OF; 0 [0–0]), compared with the limbs maintained at ambient temperature (AMB OF; 2 [1.5–2.5]). The maximum score recorded was 3.

Effect of CDH on mRNA Concentrations of Lamellar Pro-inflammatory Cytokines

When considering the main effect of the interaction between induction (OF) and treatment (ICE), induction of SRL via administration of oligofructose bolus did not result in a change in the lamellar mRNA concentrations of any of the inflammatory cytokines, nor did treatment with CDH (ICE) result in a change in lamellar mRNA concentrations of the inflammatory cytokines.

When the main effect of induction (OF) was considered, alone, increases in gene expression of IL-1β and IL-6, were observed ($P < 0.0001$; see Table 1, and Figs S1 and S5).

When the main effect of treatment (ICE) was considered, alone, a decrease in mRNA concentration of IL-6 was discovered in the lamellar tissue from the limbs subject to CDH ($P < 0.01$).

Effect of CDH on mRNA Concentrations of Lamellar Chemokines

When considering the main effect of the interaction between induction (OF) and treatment (ICE), induction of SRL via administration of oligofructose bolus resulted in an increase in gene expression of CXCL1 and MCP2 ($P < 0.05$). Treatment (ICE) and induction resulted in an increase in lamellar mRNA concentration

Table 1. The Least Squares (LS) means of the copy number data predicted using each model are presented, along with the *F* test value (*P*-value) for each model when applied to each inflammatory mediator.

	Inflammatory Mediator										
	IL1β	IL6	IL8	IL10	CXCL1	CXCL6	MCP1	MCP2	COX1	COX2	ICAM	e-SEL
Regression model applied												
Treatment	$P = 0.34$	$P < 0.01$	$P = 0.08$	$P = 0.064$	$P = 0.03$	$P = 0.12$	$P = 0.56$	$P = 0.02$	$P < 0.001$	$P < 0.0001$	$P = 0.0001$	$P = 0.06$
ICE	15,245	3,229	12,926	1,784	22,697	2,052	2,090	195,634	879,404	2,460	45,297	20,744
AMB	12,469	12,657	6,063	3,415	15,244	3,415	1,758	124,492	324,811	8,300	16,236	10,583
Induction	$P < 0.0001$	$P < 0.0001$	$P < 0.01$	$P = 0.61$	$P < 0.01$	$P < 0.01$	$P = 0.01$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
OF	37,572	21,844	584,54	2,326	52,891	11,661	11,407	308,045	199,187	19,400	57,354	56,162
CON	5,064	191	1,341	2,620	6,536	602	322	79,063	1,435,466	1,053	12,823	3,909
Interaction	$P = 0.085$	$P = 0.385$	$P = 0.85$	$P = 0.88$	$P < 0.01$	$P = 0.53$	$P = 0.79$	$P < 0.01$	$P < 0.0001$	$P < 0.001$	$P = 0.03$	$P = 0.92$
ICE-OF	50,262	89,500	88,521	1,639	85,135	8,184	11,968	518,658	643,708	5,682	13,053	77,265
ICE-CON	4,624	117	1,888	1,943	6,051	515	365	73,792	1,202,604	1,064	15,741	5,569
AMB-OF	28,057	510,936	38,599	3,301	32,859	16,597	10,873	182,956	61,636	66,171	25,235	40,864
AMB-CON	5,541	313	952	3,533	7,059	703	284	84,711	1,711,704	1,041	10,446	2,741

of MCP2 which was greater than the increase seen with induction alone ($P < 0.01$; see Table 1, and Figs S2 and S5).

When the main effect of induction (OF) was considered, alone, increases in lamellar mRNA concentration of CXCL1, IL-8/CXCL8, MCP1, and MCP2 were observed ($P < 0.05$).

When the main effect of treatment (ICE) was considered alone, an increase in lamellar mRNA concentration of MCP2 and CXCL1 was discovered in the lamellar tissue from the limbs subject to CDH ($P < 0.05$; no decreases in lamellar chemokine mRNA concentrations were detected).

Effect of CDH on mRNA Concentrations of Lamellar Cyclo-oxygenases and Endothelial Adhesion Molecules

When considering the main effect of the interaction between induction (OF) and treatment (ICE), induction resulted in an increase in lamellar mRNA concentration of COX-2 ($P < 0.05$; see Table 1, and Figs S3 and S5). In the case of COX-1, induction in the absence of treatment resulted in a decrease in lamellar mRNA concentration ($P < 0.0001$). Treatment resulted in a decrease in lamellar mRNA concentration of COX-2 ($P < 0.01$). Induction (OF) and treatment (ICE) resulted in an increase in lamellar mRNA concentration of ICAM-1 ($P = 0.03$).

When the main effect of induction (OF) was considered alone, increases in lamellar mRNA concentration of COX-2, ICAM-1, and E-selectin were observed ($P < 0.05$). A decrease in lamellar mRNA concentration of COX-1 was observed ($P < 0.0001$).

When the main effect of treatment (ICE) was considered alone, a decrease in lamellar mRNA concentration of COX-2 was observed ($P < 0.001$). Conversely, an increase in lamellar mRNA concentration of COX1 and ICAM-1was observed in lamellar tissue from limbs subject to CDH ($P < 0.005$).

Summary of Effect of CDH on mRNA Concentrations of Lamellar Inflammatory Proteins

Oligofructose induction resulted in increased lamellar concentrations of cytokines (IL-1β and IL-6), chemokines (CXCL1, IL8/CXCL8, MCP1, and MCP2), COX-2 and endothelial adhesion molecules (ICAM-1 and E-selectin). The treatment of OF-induced horses with CDH resulted in significant decreases in lamellar mRNA concentrations of IL-6 and COX-2 and increased lamellar mRNA concentrations of ICAM-1 and MCP2 (see Table 1, and Figs S1–S3).

Effect of CDH on Lamellar Concentration of MAC387-positive Cells

When considering the main effect of the interaction between induction (OF) and treatment (ICE), induction of SRL via administration of oligofructose bolus resulted in an increase in MAC387(+) cells in lamellar tissue. Although the lamellar tissue from the ICE OF

group had an increase in MAC387(+) cell number over the lamellar tissue from the AMB CON group, the ICE OF lamellar tissue still had significantly less MAC387 (+) cells than the AMB OF group ($P < 0.0001$) (see Fig 1, Table 2, and Fig S4).

Effect of CDH on Lamellar Concentration of CD163-positive Cells

When considering the main effect of the interaction between induction (OF) and treatment (ICE), the lamellar tissue from the digits subject to CDH in the study subjects administered oligofructose (ICE OF) had the least amount of CD163(+) cells. The lamellar tissue from the ambient hoof in the study subjects administered oligofructose (AMB OF) had the greatest amount of CD163(+) cells. The lamellar tissue from the study subjects that were not administered oligofructose (AMB CON, ICE CON) had a greater amount of CD163 (+) cells than the ICE OF tissue, but cell counts were less than that of the AMB OF lamellar tissue ($P = 0.0011$) (Fig 1, Table 2, and Fig S4).

Summary of Effect of CDH on Lamellar Concentrations of Leukocytes

Oligofructose induction resulted in an increase in lamellar concentrations of both MAC387(+) and CD163 (+) cells. The treatment of OF-induced horses with CDH (ICE OF) resulted in significantly less MAC387(+) and CD163(+) cells than the AMB OF study subjects ($P < 0.0007$ and $P < 0.0001$, respectively).

Discussion

This study determines the effect of hypothermia on gene expression in normal lamellae. These data demonstrate no significant effect of hypothermia on inflammatory mediator gene expression (at least at the transcriptional level) in normal lamellar tissue across the broad array of inflammatory mediators including cytokines, chemokines, endothelial adhesion molecules, and isoforms of COX. Although CDH has been suggested to be effective by causing an overall decrease in metabolic rate,[26] this work demonstrates that hypothermia of the lamellar tissue does not cause an overall decrease in activity of all cellular processes (eg, gene expression). Similar to the minimal effect of CDH on inflammatory gene expression in normal lamellae, preliminary findings from these same samples have failed to find an inhibitory effect of CDH on enzyme activity of several kinases in the normal tissue, as the concentrations of phospho-proteins of interest were not different between lamellar samples from ambient or hypothermic limbs in normal horses (Belknap laboratory, unpublished data). The lack of effect of CDH on gene

Table 2. The Least Squares (LS) means of the leukocyte cell count data predicted using each model are presented, along with the *F* test value (*P*-value) for each model when applied to each inflammatory mediator.

	Leukocyte cell numbers	
	MAC387(+)	CD163(+)
Regression model applied		
Treatment	$P = 0.0007$	$P = 0.0001$
ICE	1.7	8.7
AMB	18.5	20.2
Induction	$P < 0.0001$	$P = 0.5405$
OF	19.4	17.1
CON	0.8	11.8
Interaction	$P < 0.0001$	$P = 0.0011$
ICE-OF	2.5	6.7
ICE-CON	0.9	10.7
AMB-OF	36.2	27.5
AMB-CON	0.8	12.9

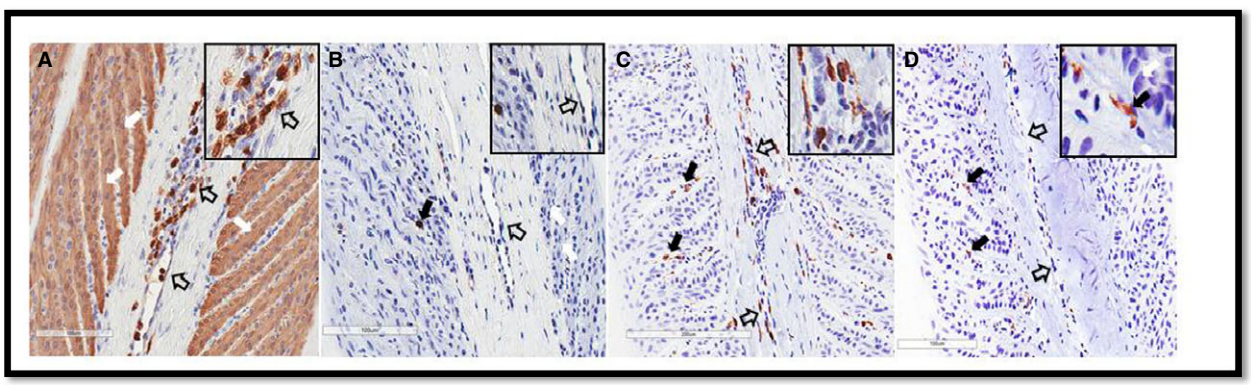

Fig. 1. Note the increased number of calprotectin-positive WBC (**A**) and CD163-positive WBC (**C**) in the perivascular region of the primary dermal lamellae (open black arrows) in the AMB OF lamellae (A) compared to the OF ICE lamellae (**B**, calprotectin; **D**, CD163). Also note that, in the secondary epidermal lamellae (white arrows), the consistent calprotectin staining in the epithelial ells of the OF AMB lamellae (A) compared to the OF ICE lamellae (B). In the secondary dermal lamellae (solid black arrows), CD163-positive cells are also present in both the OF AMB (C) and OF ICE (D) lamellar sections. AMB, limbs at ambient temperature; OF, oligofructose; ICE, limbs treated with continuous digital hypothermia.

expression in normal horses allows us to determine that any effect of hypothermia on lamellar inflammatory gene expression in the OF group is not a general effect of hypothermia on healthy or diseased tissue; rather, the effects of hypothermia on lamellar signaling in the current study are likely distinct effects of CDH on inflammatory gene expression in lamellar tissue from patients with sepsis.

In systemic sepsis, bacterial products termed PAMPs (pathogen-associated molecular pattern molecules; eg, endotoxin) are described to bind to pattern recognition receptors on leukocytes and most other cell types (the classic receptors being Toll-like receptors [TLRs]), resulting in activation of multiple cell types.[27,28] Circulating activated leukocytes reportedly extravasate into the tissue of target organs after being exposed to chemokines and adhesion molecules (eg, ICAM-1, E-selectin) on the surface of the activated endothelium (and chemokines in the tissue).[29] The release of proinflammatory cytokines from these leukocytes, in combination with activated tissue macrophages, are discussed as a major event leading to organ/tissue dysfunction and injury that occurs in sepsis in multiple species and equine laminitis.[30] Similar to previous studies assessing inflammatory events occurring in lamellae in experimental models of SRL,[7–11,13,22,31–33] an increase in lamellar leukocytes (MAC387 [+] and CD163 [+] cells in lamellar dermis) and lamellar concentrations of many inflammatory signaling molecules including cytokines, chemokines, endothelial adhesion molecules, and COX-2 were increased in the untreated limbs of animals administered oligofructose in the current study. However, although CDH in the current study was found to confer the same structural protection to the lamellae as reported in previous SRL studies in which CDH was initiated at different times,[20,34] the treatment's impact on inflammatory events differed. In regards to lamellar leukocyte dynamics, a decrease in both lamellar leukocyte numbers (MAC387 [+] and CD163 [+])-much greater than that reported previously when CDH was initiated prior to onset of systemic disease-occurred. This decrease in leukocyte number occurred despite the CDH-induced increase in two molecules normally described as promoting leukocyte activation and extravasation in sepsis, the chemokine MCP1 and endothelial adhesion molecule ICAM-1. The difference between the leukocyte response to hypothermia in the current study compared to a previous study is likely at least partially accounted for by the effect of the difference in timing of lamellar harvest on the number of leukocytes normally present in the lamellar tissue in the OF model (ie, 36 MAC387[+] cells/40× field in untreated/ambient lamellae in current study [approx. OG4 time point] versus mean: 3.1 cells/40× field in untreated/ambient lamellae in the previous study [approx. OG1 time point]).[22]

The fact that CDH only decreased lamellar mRNA concentrations of two inflammatory molecules assessed, IL-6 and COX-2, while markedly decreasing lamellar leukocyte numbers indicates that lamellar cells other than leukocytes are responsible for expression of many of the inflammatory mediators assessed. The fact that we have previously reported an increase in TLR expression in lamellar basal epithelial cells in SRL,[35] concomitant with an increase in lamellar epithelial expression of cytokines (IL-6, -12),[5] chemokines (CXCL1 & 6, CCL 8 & 13),[5,8,10] and COX-2 in SRL models,[5,13] indicates that the lamellar epithelial cell itself is immunocompetent and likely reacting directly to circulating bacterial products. Although we do not have histologic confirmation that these signaling molecules are inhibited in the epithelium by CDH (due to a paucity of antibodies that detect these equine proteins), MAC387, itself termed a damage-associated molecular pattern protein (DAMP) when expressed by inflamed/injured epithelial cells as occurred in this and previous SRL studies,[9,16] can be observed to undergo a distinct inhibition with CDH on histologic examination of the lamellar epithelium in the current study (see Fig 1). Thus, CDH is likely to be directly inhibiting epithelial inflammatory events (ie, IL-6, COX-2, and MAC387 expression) in animals at risk of SRL.

By instituting CDH at a similar time point (onset of signs of systemic disease) as used in the clinical case of equine sepsis, and harvesting the lamellae at a time point prior to severe destruction of the lamellae, this work likely gives the most accurate picture of lamellar events inhibited by CDH in the septic clinical patient. The combined findings of this study, including that: (1) CDH in normal/non-septic horses does not affect lamellar mRNA concentrations of inflammatory mediators of interest and (2) CDH initiated at the onset of clinical signs of sepsis only inhibits specific inflammatory gene expression (IL-6 and COX-2), indicate that the protective effects of CDH as used in the clinical equine septic patient are likely more focused than a broad effect on either lamellar cellular/metabolic events in general or on lamellar inflammatory events. The inhibition of lamellar leukocyte numbers by CDH support a likely role of the leukocytes in lamellar epithelial dysregulation and injury. The influx of leukocytes in the OF model may also indicate that leukocyte extravasation is a harbinger for epithelial injury (ie, leukocytes are responding to chemotactic products [eg, MAC387/calprotectin] released by an already inflamed injured/lamellar epithelium) and are not the primary event leading to lamellar injury.

In regard to COX-2 as a therapeutic target for SRL, therapies to inhibit COX activity have had conflicting results in the treatment of human sepsis[35] and had minimal efficacy when non-selective NSAIDs were assessed in an equine model of SRL.[36] In light of this apparent lack of efficacy of NSAIDS in laminitis, NSAIDs have been used in models of SRL (including this study) for their analgesic effects, and study animals develop laminitis despite the use of this class of drugs.[4,37] Potential effects of the use of NSAIDS in study subjects include suppression of COX enzymes and other inflammatory pathways, possibly resulting in an overall decrease in the expression of proinflammatory cytokines.[38,39]

The fact that IL-6 signaling has consistently shown the greatest increases in the lamellae in different models of sepsis-related laminitis,[7,11,40] combined with the fact that it has been correlated with organ injury and death in multiple studies of human sepsis,[30,41–43] indicate that signaling induced by this cytokine should be intensively investigated in SRL. IL-6 signaling, which is initiated through binding of the IL-6/IL-6 receptor complex to the gp130 receptor,[44] has recently received a great deal of attention as a therapeutic target in inflammatory disease states ranging from rheumatoid arthritis to sepsis. Whereas a commercial IL-6 receptor inhibitor (monoclonal antibody to IL-6 receptor) is now being used clinically for rheumatoid arthritis,[45] inhibition of IL-6 signaling is still in the experimental stage in sepsis due primarily to the concern that immunosuppression secondary to IL-6 inhibition may exacerbate the septic state.[30] In addition to its role in inflammatory cells, IL-6 related signaling has also been reported to be important in the transformation of epithelial cells to tumor cells (the first stage of which is termed epithelial to mesenchymal transition; EMT) in inflammatory disease states such as inflammatory bowel disease.[46–48] Of interest to laminitis, where loss of structural integrity of the lamellae is purported to occur due to disruption of the lamellar epithelial cell cytoskeleton and adhesion of these cells to the underlying matrix,[49–53] is the fact that two of the first events to occur in EMT are dysregulation of cellular adhesion and disruption of cytoskeletal dynamics.[54] Although the lamellar epithelial cells obviously do not progress past EMT into neoplastic cells, similar epithelial gp130-related signaling driven by IL-6 may lead to the lamellar epithelial cell stretching and separation from the lamellar dermis, the two central histologic findings documented to occur with lamellar failure in both sepsis-related and endocrinopathic laminitis.[31] Importantly, not only has gp130 been reported to work through the same signaling (ie, mTORC1/RPS6) as we have recently reported to occur in the lamellae in the EHC model of endocrinopathic laminitis,[55,56] but we have also recently discovered the same increases in phosphorylated/activated proteins downstream of mTORC1 (eg, p70S6K, RPS6) in the lamellae of ambient/untreated limbs in horses administered OF in the current study (Belknap laboratory, unpublished data) as we reported in the EHC study.[56]

Due to the possibility that IL-6-related signaling leads to lamellar failure due to signaling mechanisms downstream of the gp130 receptor, investigation of these downstream signaling mechanisms might lead to the discovery of a novel therapeutic target and an effective pharmaceutical agent for the treatment of not only sepsis-related laminitis. Additionally, as some of the same signaling mechanisms downstream of the gp130 receptor (eg, mTORC1/p70S6K signaling in endocrinopathic laminitis) are also reported to be important in other types of laminitis,[56] it is possible that further study of these signaling mechanisms might lead to the discovery of a pharmaceutical agent effective for multiple types of laminitis.

Footnotes

[a] Yamasa Tokei Keiki Co, Ltd, Tokyo, Japan
[b] Ausrichter Pty Ltd, Anandale, Australia
[c] Absolutely RNA Miniprep kit, Agilent Technologies, Stratagene Products Division, La Jolla, CA
[d] mRNA extraction kit, Roche Applied Science, Indianapolis, IN
[e] Roche LightCycler 480; Roche Applied Science, Indianapolis, IN
[f] Thermo Fisher Scientific, Waltham, MA
[g] Roche; Roche Applied Science, Indianapolis, IN
[h] Ghent University, Ghent, Belgium
[i] Abcam, Cambridge, MA
[j] Cosmo Bio, Carlsbad, CA
[k] Vector Laboratories, Burlingame, CA
[l] JMP, Version 12. SAS Institute Inc., Cary, NC, 1989–2007
[m] GraphPad Prism 6; GraphPad Software, La Jolla, CA

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Conflict of Interest Declaration: Authors declare no conflict of interest.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Lamellar mRNA concentrations of cytokines: Limbs maintained at ambient temperature in which laminitis was not induced (AMB CON; n = 8), limbs subject to digital hypothermia in which laminitis was not induced (ICE CON; n = 8), limbs maintained at ambient temperature in which laminitis was induced (AMB OF; n = 7), limbs subject to digital hypothermia in which laminitis was induced (ICE OF; n = 7).

Figure S2. Lamellar mRNA concentrations of chemokines: Limbs maintained at ambient temperature in which laminitis was not induced (AMB CON; n = 8), limbs subject to digital hypothermia in which laminitis was not induced (ICE CON; n = 8), limbs maintained at ambient temperature in which laminitis was induced (AMB OF; n = 7), limbs subject to digital hypothermia in which laminitis was induced (ICE OF; n = 7).

Figure S3. Lamellar mRNA concentrations of cyclooxygenases and adhesion molecules: Limbs maintained at ambient temperature in which laminitis was not induced (AMB CON; n = 8), limbs subject to digital hypothermia in which laminitis was not induced (ICE CON; n = 8), limbs maintained at ambient temperature in which laminitis was induced (AMB OF; n = 7), limbs subject to digital hypothermia in which laminitis was induced (ICE OF; n = 7).

Figure S4. Lamellar cell counts of assessed leukocytes: Limbs maintained at ambient temperature in which laminitis was not induced (AMB CON; n = 8), limbs subject to digital hypothermia in which laminitis was not induced (ICE CON; n = 8), limbs maintained at ambient temperature in which laminitis was induced (AMB OF; n = 7), limbs subject to digital hypothermia in which laminitis was induced (ICE OF; n = 7).

Figure S5. The Least Squares (LS) means of the log transformations of the copy number data predicted using each model are presented, along with the *F* test value (*P*-value) for each model when applied to each inflammatory mediator.