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Effect of Delayed Digital Hypothermia on Lamellar Inflammatory Signaling in the Oligofructose Laminitis Model

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Background: In the oligofructose (OF) model of sepsis-related laminitis (SRL), digital hypothermia ("cryotherapy") initiated before the onset of clinical signs is reported not only to limit lamellar injury, but also to cause marked inhibition of lamellar inflammatory signaling.

Hypothesis/Objectives: Because hypothermia also has been reported to be protective when not initiated until the onset of lameness in the OF model of SRL, we hypothesized that the lamellar protection conferred by hypothermia is caused by local lamellar inhibition of inflammatory signaling as described when hypothermia was initiated earlier in the disease process.

Animals: Eight Standardbred geldings aged 3–11 years with no lameness and no abnormalities of the feet detectable by gross or radiographic examination.

Methods: Using the OF model of SRL, lamellar mRNA concentrations of proinflammatory cytokines, chemokines, and endothelial adhesion proteins were compared between samples from treated limbs (CRYO, submerged in ice water for 36 hour starting at the onset of lameness), untreated limbs (NON-CRYO, opposite limb from CRYO limbs maintained at ambient temperature), and untreated limbs from normal horses in which laminitis was not induced (CON).

Results: Although OF administration resulted in increases in lamellar mRNA concentrations of several inflammatory mediators in NON-CRYO limbs (vs CON), digital hypothermia had no significant effect on these increases.

Conclusions and Clinical Importance: The lack of inflammatory inhibition in lamellar tissue samples in our study indicates that the protective effects of digital hypothermia instituted at the onset of clinical signs of laminitis do not arise from inhibition of inflammatory pathways.

Key words: Cryotherapy; Equine; Inflammation.

Recent studies specifically investigating sepsis-related laminitis (SRL) have classified the lamellae as a "target organ" of sepsis in the horse, triggering investigation of inflammatory signaling in the early stages of the disease, preceding structural failure of the lamellae.[1–3] In study subjects in which laminitis was induced using various models of SRL, marked increases in a wide range of proinflammatory mediators were detected when compared to nonseptic control horses.[4–8] Similar to the reports from the use of moderate regional hypothermia (approximately 30°C) in human patients to limit damage to vital organs in cases of inflammatory injury, continuous marked digital hypothermia (5–10°C) limited the structural failure of the lamellae both in horses with clinical sepsis and in experimentally induced laminitis.[7,9–12] Initiation of cryotherapy at the time of oligofructose (OF) administration (before the onset of sepsis or lameness) has been linked to a marked decrease in expression of molecules associated with inflammatory signaling when compared to limbs maintained at ambient temperature.[7] In a recent study that more closely simulated the time point at which hypothermia is initiated in many clinically affected horses, investigators did not initiate digital hypothermia until the study subjects exhibited lameness (OG2, Obel Grade 2).[13,14] The first publication from this study established that, whereas the lamellae from the untreated limb had severe histologic changes consistent with Obel Grade 3–4 (OG3–4) lameness,[15] there was minimal histologic evidence of lamellar injury in the limb treated by hypothermia.[13] This work confirms that digital hypothermia still has markedly protective effects even when initiated after the onset of lameness, but the mechanism of this lamellar protection at the later time point has yet to be elucidated.

The aim of our study was to determine whether initiation of hypothermia at the onset of lameness results in a similar inhibition of inflammatory signaling as described when hypothermia was initiated before the

Abbreviations:

CON	limbs from study subjects in which laminitis was not induced
CRYO	cryotherapy
NON-CRYO	limbs at ambient temperature
OF	oligofructose
OG1	Obel Grade 1
OG2	Obel Grade 2
OG3	Obel Grade 3
OG4	Obel Grade 4
RT-qPCR	real-time quantitative polymerase chain reaction
SRL	sepsis-related laminitis

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onset of either sepsis or lameness[7] and to investigate the relationship between gene expression of inflammatory proteins and grade of histologic damage to lamellar tissue. Lamellar samples from the same study demonstrating the protective effect of hypothermia initiated at the onset of lameness were used to determine the gene expression of proinflammatory cytokines, chemokines, endothelial cell adhesion molecules, and COX-2.[13] As a result of finding a minimal effect of hypothermia on inflammatory signaling in this study, lamellar mRNA concentrations from the current study were compared to those of previous SRL studies in which lamellae were harvested at earlier time points to determine whether the lack of an anti-inflammatory effect of hypothermia in this study was due to a lack of hypothermia-induced inhibition of inflammatory mediators or simply the result of a generalized decrease in gene expression of inflammatory mediators in late-stage laminitis.[2,4,7]

Materials and Methods

The 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 and Sample Acquisition

As described in a previous publication by the same study subjects,[13] 8 Standardbred geldings were given an OF bolus (10 g/kg, maximum 4.2 kg) by nasogastric intubation, confined to stocks, and carefully monitored for the onset of lameness by gait evaluation, performed every 4 hours by 2 investigators and by pedometer counts. Pedometer counts were obtained to determine the amount of shifting back and forth between the forelimbs of the study subjects, an early sign of Obel Grade 1 (OG1) laminitis. Lameness of 1 or both forelimbs at the walk (OG2 laminitis) was the time point used for initiation of digital hypothermia in 1 forelimb (other forelimb maintained at ambient temperature) and institution of pain management with perineural anesthesia in both forelimbs. Hoof wall surface temperature was measured with hoof wall thermistors attached to data-logging devices as described previously.[16] At 36 hours after the development of OG2 laminitis (likely OG3 laminitis due to histologic changes observed),[15] horses were euthanized and lamellar tissue samples were collected both from limbs that experienced digital hypothermia and limbs maintained at ambient temperature, as described previously.[13] Tissue intended for gene expression analysis was immediately snap-frozen in liquid nitrogen.

Two other sets of lamellar tissue were analyzed as follows: archived nonseptic control (CON) lamellar tissue from a previous study in which the subjects were not treated with OF[4] and lamellar tissue samples from a previous study in which the tissue samples were harvested at OG1 laminitis after induction using the OF model.[7] In each of these previous studies, the lamellar tissue was harvested in a manner identical to this study, and the OF laminitis induction model (10 g/kg BW) was identical between the OG1 study and this study.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from lamellar tissue samples using a commercially available preparation[a] , and genomic DNA contamination was prevented by use of DNase treatment. mRNA then was isolated using a separate commercially available preparation[b], and cDNA was generated for each tissue sample by reverse transcriptase; cDNA then was frozen at −20°C until real-time quantitative polymerase chain reaction (RT-qPCR) could be performed.

Real-Time Quantitative Polymerase Chain Reaction

A thermocycler[c] was used to perform real-time qPCR, as previously described.[7] To determine the effects of cryotherapy on gene expression of inflammatory mediators in lamellar tissue from the current study, previously optimized equine-specific primers for E-selectin, intracellular adhesion molecule-1 (ICAM-1), CXCL-1, CXCL-8 (IL-8), IL-6, IL-1β, CXCL-6, IL-10, MCP-1, MCP-2, COX-2, and 3 housekeeping genes (β-actin, β2-microglobin, and glyceraldehyde-3-phosphate dehydrogenase) were used to amplify cDNA as described previously.[7] The specificity of each primer for the cDNA fragment of interest was confirmed in a previous study by gel electrophoresis,[7] melt curve analysis, and sequencing of produced cDNA fragments (Table S2). Standard curves for each RT-qPCR were generated using previously designed templates,[7] and water was used as a negative control. All PCRs were performed in 20 μL volumes consisting of 5 μL of sample cDNA and 15 μL of PCR master mix. The master mix included Taq polymerase[d], uracil-*N*-glycosylase[e], SYBR Green stock solution[d], forward and reverse primers, PCR Nucleotide plus[c], and PCR buffer prepared on site (20 mmol/L Tris-HCL, 0.05% Tween-20, and a nonionic detergent). Amplification occurred as previously described for 40–45 cycles; the annealing temperature was 1–5°C below melting temperature; extension temperature was set at 72°C for 5 seconds and fluorescence acquisition for 10 seconds in the SYBR Green format. Melting curves for each PCR product were acquired by an increase in temperature from 65 to 95°C. Each sample was run in duplicate. To determine whether the time of lamellar tissue harvest affected the inflammatory profile in limbs maintained at ambient temperature, RT-qPCR was conducted (as described above) on cDNA obtained from lamellar tissue samples from this study (OG3), from nonseptic archived control lamellar tissue,[4] and from lamellar tissue harvested at OG1[7] (both from previous studies). These RT-qPCRs were run in identical fashion to those described above, with the exception that, because of limited quantity of cDNA from previous studies, these samples were not analyzed for mRNA concentrations of CXCL-6, IL-10, MCP-1, or MCP-2. Copy number data from genes of interest were normalized by geNorm[17] software to analyze copy number data of the 3 housekeeping genes to determine which 2 genes received the best acceptable score and then by the data from those 2 genes to make a normalization factor for each sample.[7] The amplification data obtained by RT-PCR for the respective genes of interest were divided by the normalization factor of the selected housekeeping genes in the same sample, creating a normalized copy number value. The fold changes for each inflammatory mediator of interest were determined by comparing the copy numbers of the hypothermic (CRYO) limb with the ambient control (NON-CRYO) limb from the same horse. For the analysis of the inflammatory profile of the ambient limbs, fold changes were determined by comparing the copy number from the OG1[7] and OG3 limbs (respectively) to the nonseptic archived control (CON).[4]

Histologic Evaluation

Histologic evaluation was performed on formalin-fixed sections (stained by hematoxylin and eosin and periodic acid-Schiff) by 2 blinded observers. A 0–4 grading scale was used as previously described, and a grade of 0 was assigned to normal lamellar tissue and a grade of 4 was assigned to sections in which there was complete physical separation of lamellar epidermis from dermis.[13,15]

Data Analysis

To determine the effects of cryotherapy on the inflammatory profile of laminitic tissue, normalized copy numbers for each inflammatory mediator in CRYO vs NON-CRYO limbs from the current study (OG3) were compared by Wilcoxon signed rank tests. To compare the mRNA concentrations of inflammatory proteins in ambient limbs from the current study (OG3) with those from nonseptic archived control (CON) samples and samples harvested at OG1, RT-qPCR results were compared to each other, respectively, by Mann–Whitney tests. Histopathology scores from the previous publication by the same samples were compared to RT-qPCR results from the lamellar tissue from both CRYO and NON-CRYO limb by Spearman/Pearson correlation tests. All statistics were performed by GraphPad Prism[f]. Significance was set at $P < .05$.

Results

All study subjects developed OG2 lameness between 17 and 21 hours after the OF bolus dose, as previously reported.[13] Hoof wall surface temperature was found to be consistently lower ($P < .05$) in CRYO feet (median 7.1°C) than in NON-CRYO feet (median 30.2°C), as anticipated.

Effect of Digital Hypothermia on mRNA Concentrations of Lamellar Inflammatory Proteins

Digital hypothermia only resulted in a statistically significant ($P < .05$) change in lamellar mRNA concentrations of 2 of the assessed molecules (E-selectin and ICAM-1), both of which were increased in the hypothermic (CRYO) limb (Table 1, Fig S1). No significant changes ($P < .05$) were noted between the CRYO vs NON-CRYO limb for proinflammatory cytokines, chemokines, or COX-2 (Table 1).

Correlation of Lamellar mRNA Concentrations with Histologic Scores

Correlation analysis of lamellar mRNA concentrations and histologic scores of the same subjects/digits

Table 1. Lamellar tissue inflammatory mediator copy number expression data: Forelimbs which were treated with hypothermia (CRYO) compared with untreated (NON-CRYO) limbs.

Inflammatory Mediator	NON-CRYO	CRYO
E-selectin		
Fold increase/NON-RX		3.47
Copy number	1.70×10^4 (7.90×10^3–3.53×10^4)	5.90×10^4 (3.45×10^4–1.03×10^5) ↑
ICAM-1		
Fold increase/NON-RX		28.25
Copy number	1.01×10^4 (5.00×10^3–1.53×10^4)	2.85×10^5 (3.38×10^4–7.04×10^5) ↑
CXCL-1		
Fold increase/NON-RX		1.50
Copy number	3.15×10^4 (8.91×10^3–1.10×10^5)	4.74×10^4 (2.37×10^4–9.55×10^4)
CXCL-8 (IL-8)		
Fold increase/NON-RX		0.51
Copy number	1.70×10^4(3.96×10^3–8.96×10^4)	8.92×10^3 (2.71×10^3–1.42×10^4)
IL-6		
Fold increase/NON-RX		0.37
Copy number	3.22×10^4 (7.02×10^3–8.70×10^4)	1.17×10^4 (9.55×10^2–14.7×10^4)
IL-1β		
Fold increase/NON-RX		0.49
Copy number	5.86×10^4 (1.62×10^4–1.20×10^5)	2.88×10^4 (1.28×10^4–1.09×10^5)
CXCL-6		
Fold increase/NON-RX		0.88
Copy number	7.79×10^3 (4.52×10^3–2.61×10^4)	6.83×10^3 (2.22×10^3–1.51×10^4)
IL-10		
Fold increase/NON-RX		0.96
Copy number	7.79×10^2 (2.89×10^2–1.20×10^3)	7.49×10^2 (4.79×10^2–1.61×10^3)
MCP-1		
Fold increase/NON-RX		2.79
Copy number	4.32×10^3 (1.62×10^3–6.45×10^3)	1.20×10^4 (2.33×10^3–2.70×10^4)
MCP-2		
Fold increase/NON-RX		1.47
Copy number	1.56×10^5 (1.00×10^5–3.48×10^5)	2.35×10^5 (1.46×10^5–5.45×10^5)
COX-2		
Fold increase/NON-RX		0.17
Copy number	2.30×10^4 (4.58×10^3–6.75×10^4)	3.90×10^4 (1.41×10^3–3.12×10^4)

ICAM-1, intracellular adhesion molecule-1.

The copy number data are expressed as cDNA copies per normalization factor (interquartile range) in both CRYO and NON-CRYO columns; median fold increase of CRYO copy number (compared to NON-CRYO) is also presented (top number for each mediator) in the CRYO column.

↑ denotes a statistically significant increase in fold change when compared to the untreated limb.

from a previous study[13] indicated that lamellar mRNA concentrations of ICAM-1, IL-1β, and MCP-1 were inversely correlated ($R > 0.5$, < -0.5; $P < .05$) with the histologic scores in CRYO limbs (ie, higher lamellar mRNA concentrations were seen when the lamellae were intact). In NON-CRYO limbs, the lamellar mRNA concentrations of COX-2, CXCL-6, IL-1-β, and IL-8 were positively correlated ($R > 0.5$, < -0.4; $P < .05$) with the histologic scores (ie, higher lamellar mRNA concentrations were seen when the lamellae were more separated; Table 2).

Comparison of Lamellar mRNA Concentrations of Inflammatory Molecules Between Nontreated/Ambient Limbs Harvested at OG3 (NON-CRYO OG3), OG1 (NON-CRYO OG1) and Nonseptic Control Animals

Lamellar mRNA concentrations of the majority of inflammatory molecules, with the exception of IL-1β, were increased ($P < .05$) in NON-CRYO limbs from the current study when compared with archived lamellar samples from control animals that were not treated with OF (Fig 1, Table S1). When lamellar mRNA concentrations in NON-CRYO limbs of horses in the current study (OG3) were compared with mRNA concentrations in NON-CRYO limbs of horses in which tissue was harvested at onset of OG1 lameness,[7] lamellar concentrations of CXCL-1, E-selectin, and ICAM-1 were lower ($P < .05$) in the OG3 samples (Table S1).

Discussion

We failed to find a decrease in lamellar concentration of molecules associated with inflammatory signaling when digital hypothermia was initiated after the onset of lameness in the OF model of SRL, in contrast to the marked decrease in gene expression of these same molecules observed when hypothermia was initiated at the time of administration of a carbohydrate overload (ie, before signs of sepsis or lameness) in a previous study.[7] Possible explanations for this lack of inflammatory inhibition are as follows: a difference in the severity of lamellar injury between the studies, a difference in inflammatory profile as a consequence of the later time point at which the lamellar tissue was evaluated in the current study (ie, minimal inflammation may be present to inhibit), or the protective effects of digital hypothermia are not mediated through inflammatory pathways.

The possibility of a lack of induction of lamellar injury being responsible for the minimal effect of hypothermia on inflammatory signaling in the present study is contradicted by the histologic evaluation of lamellae from these same animals in a previous report that identified a high degree of lamellar separation consistent with OG3–4 laminitis in limbs maintained at ambient temperature (NON-CRYO).[13] Additionally, evidence of marked inflammation in NON-CRYO limbs in the current study is provided by comparison of lamellar mRNA concentrations of inflammatory mediators in NON-CRYO limbs from the current study with mRNA concentrations of inflammatory mediators in archived lamellar tissue from nonseptic control animals.[4,7] These data indicate that NON-CRYO limbs in the current study exhibited up to 90-fold increases ($P < .05$) in lamellar mRNA concentrations of several classes of inflammatory molecules (E-selectin, CXCL-1, IL-8, IL-6, and Cox-2) when compared to the low mRNA concentrations of these same molecules in nonseptic control samples (Table S1). The increase in gene expression of inflammatory mediators in these limbs, coupled with the histologic evidence of lamellar separation in these samples, confirms that both severe lamellar injury and increased inflammatory signaling were present in the untreated limbs from our study.

Although prominent inflammatory signaling still was present in the current study when compared to nonseptic controls, further evaluation of the

Table 2. Statistical correlation of lamellar mRNA concentrations of specific mediators with the histological scores (from previous publication) of both hypothermic (CRYO) and untreated (NON-CRYO) limbs.

Inflammatory Mediator	CRYO	NON-CRYO
E-selectin		
Spearman/Pearson *R* value	−0.43	−0.10
P-value	ns	ns
ICAM-1		
Spearman/Pearson *R* value	−0.75*	0.44
P-value	.04	ns
CXCL-1		
Spearman/Pearson *R* value	−0.58	0.57
P-value	ns	ns
CXCL-8 (IL-8)		
Spearman/Pearson *R* value	−0.70	0.75*
P-value	ns	.04
IL-6		
Spearman/Pearson *R* value	−0.68	0.45
P-value	ns	ns
IL-1ß		
Spearman/Pearson *R* value	−0.80*	0.91*
P-value	.02	.002
CXCL-6		
Spearman/Pearson *R* value	−0.36	0.86*
P-value	ns	.01
IL-10		
Spearman/Pearson *R* value	−0.60	0.54
P-value	ns	ns
MCP-1		
Spearman/Pearson *R* value	−0.73*	0.49
P-value	.04	ns
MCP-2		
Spearman/Pearson *R* value	−0.55	0.48
P-value	ns	ns
COX-2		
Spearman/Pearson *R* value	−0.44	−0.44*
P-value	ns	.002

ICAM-1, intracellular adhesion molecule-1; Ns indicates that the value was not statistically significant.

*Denotes a statistically significant correlation between the lamellar mRNA concentrations and histologic score of the lamina. A negative value indicates an inverse correlation.

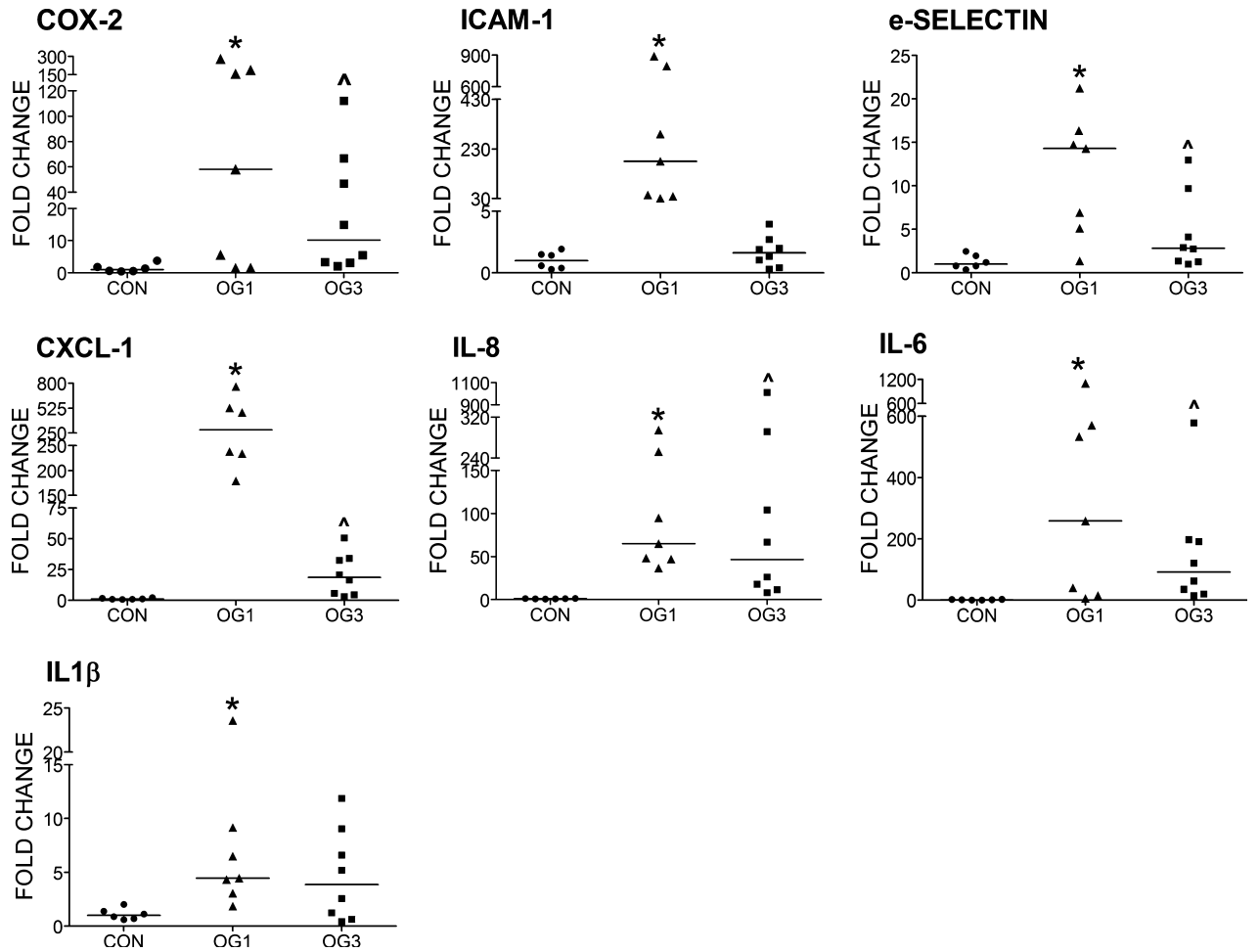

Fig 1. Lamellar mRNA concentrations of inflammatory molecules: Archived nonseptic controls (CON; n = 6) vs OG3 (harvested 36 hours after the onset of lameness; n = 8) vs OG1 (untreated harvested at the onset of OG1 lameness; n = 7). The copy number data are expressed as cDNA copies per normalization factor (interquartile range), median fold increase over CON. *Denotes significant difference between OG1 and CON ($P < .05$); ^Denotes significant difference between OG3 and CON.

inflammatory gene expression data indicates that the severity of lamellar inflammation in the nontreated limbs (NON-CRYO, OG3) in the current study was not as marked as reported in tissue samples harvested from nontreated (NON-CRYO) limbs at an earlier stage of laminitis (OG1) in a previous study.[7] The significantly decreased gene expression of E-selectin, ICAM-1, and CXCL-1 in samples of NON-CRYO limbs from the current study (OG3) when compared with the previous study (OG1) indicates a lower degree of lamellar inflammatory signaling at OG3 in the OF model of SRL (Fig 1, Table S1). This finding is consistent with recent work performed on the OG3 time point in the traditional (starch gruel/wood flour) model of laminitis (J. Belknap and S. Eades, Eades laboratories, unpublished data), in which lower lamellar concentrations of inflammatory mediators were present than previously reported for the OG1 time point with the same model.[7] Although continuous perineural analgesia used in our study subjects precluded assignment of laminitis grades beyond OG2, the degree of lamellar separation evident on histologic evaluation of the samples is indicative of OG3 laminitis.[15] Interestingly, perineural analgesia itself has been reported to cause a decrease in gene expression of inflammatory proteins, and a relationship between cytokines and neuro-hormonal feedback and perineural anesthesia and suppression of inflammation has been observed in both humans and animals.[18–21] Although this relationship has yet to be investigated in laminitis, a recent study of inflammatory signaling in SRL was conducted without the use of perineural anesthesia. This study demonstrated a similar waning of lamellar inflammatory signaling at a later time point, which indicated that lamellar inflammation waned at later time points compared to early/OG1 laminitis with or without perineural anesthesia (J. Belknap and A. van Eps, unpublished data).

The most notable finding from the present study is that, although multiple inflammatory mediators still were markedly increased in untreated limbs when compared to nonseptic controls, digital hypothermia did

not cause a significant decrease in the lamellar mRNA concentrations of any of the cytokines, chemokines, or endothelial adhesion molecules associated with inflammatory signaling (Table 1). Interestingly, ICAM-1 and E-selectin, 2 adhesion molecules important in leukocyte emigration, were the only mediators in the present study to be significantly different in CRYO versus NON-CRYO limbs. However, in contrast to the previous study in which hypothermia initiated earlier induced a significant decrease in lamellar concentrations of these molecules, mRNA concentrations of both were increased in the current study. Although there is a paucity of data in the human literature about this increase in endothelial adhesion molecules under the influence of hypothermia, the marked degree of hypothermia reached in our study (7°C) as compared to the moderate hypothermia (30°C) described in the human literature could be responsible for the lack of similar findings. The apparent disparity between the effect of hypothermia on inflammatory gene expression and the protection against lamellar injury provided by digital hypothermia in the present study is further highlighted by the correlative statistical analysis of the fold changes and histologic scores from lamellar samples. As seen in Table 2, lamellar mRNA concentrations of the inflammatory mediators ICAM-1, IL-1β, and MCP-1 were inversely correlated with histology scores from the same samples, indicating that the highest mRNA concentrations of these inflammatory mediators were obtained from lamellae that had the least histologic signs of injury and separation in the hypothermic limbs. This discrepancy between hypothermic protection and inflammation is not unprecedented in therapeutic hypothermia in other species, which has led to the investigation of other possible links between hypothermia and tissue protection including the effect of hypothermia on energy metabolism, mitochondrial dysfunction, free radical production, and cold-shock proteins.[22]

The lack of inhibition of inflammatory mediators in lamellar tissue samples in the current study, when evaluated in conjunction with the degree of lamellar protection provided by cryotherapy in the same study subject, indicates that the protective effects of digital hypothermia may not be mediated through inflammatory pathways. This finding correlates well with investigations in the human literature in which, although mild hypothermia is the standard of care in the intensive care units of many hospitals due to its protective effect from tissue damage secondary to traumatic brain injury, spinal cord injury, cardiac arrest, and neonatal hypoxic encephalopathy, the specific mechanism of protection has not been elucidated and appears to be more than inhibition of inflammatory signaling.[23–25] Thus, additional work investigating alternate pathways as the protective mechanism of action of hypothermia in SRL is warranted, with the ultimate goal being the discovery of a specific and targeted pharmaceutical intervention that would prevent the development and progression of laminitis in horses with sepsis.

Footnotes

[a] Absolutely RNA Miniprep kit, Agilent Technologies, Stratagene Products Division, La Jolla, CA
[b] mRNA extraction kit, Roche Applied Science, Indianapolis, IN
[c] Roche 480®, Roche Applied Science
[d] Thermo Fisher Scientific, Waltham, MA
[e] Roche, Roche Applied Science
[f] GraphPad Prism 5®, GraphPad Software, La Jolla, CA

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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:

Fig S1. Lamellar mRNA concentrations of inflammatory molecules: Limbs undergoing digital hypothermia (CRYO; n = 8) vs limbs kept at ambient temperature (NON-CRYO; n = 8).

Table S1. Lamellar tissue inflammatory mediator copy number expression data: control (CON) compared with untreated (NON-CRYO; kept at ambient temperature) limbs in subjects in which tissue was harvested at OG1 and 36 hours after onset of OG1 (present study).

Table S2. List of primers' sequences used for real-time qPCR.