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Continuous digital hypothermia prevents lamellar failure in the euglycaemic hyperinsulinaemic clamp model of equine laminitis

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Summary

Background: Continuous digital hypothermia can prevent the development and progression of laminitis associated with sepsis but its effects on laminitis due to hyperinsulinaemia are unknown.

Objectives: To determine the effects of continuous digital hypothermia on laminitis development in the euglycaemic hyperinsulinaemic clamp model.

Study design: Randomised, controlled (within subject), blinded, experiment.

Methods: Eight clinically normal Standardbred horses underwent laminitis induction using the euglycaemic hyperinsulinaemic clamp model (EHC). At initiation of the EHC, one forelimb was continuously cooled (ICE), with the other maintained at ambient temperature (AMB). Dorsal lamellar sections (proximal, middle, distal) were harvested 48 h after initiation of the EHC and were analysed using histological scoring (0-3) and histomorphometry. Cellular proliferation was quantified by counting epidermal cell nuclei staining positive with an immunohistochemical proliferation marker (TPX2).

Results: Severe elongation and disruption of SEL with dermo-epidermal separation (score of 3) was observed in all AMB feet at one or more section locations, but was not observed in any ICE sections. Overall 92% of AMB sections received the most severe histological score (grade 3) and 8% were grade 2, whereas ICE sections were classified as either grade 1 (50%) or grade 2 (50%). Relative to AMB feet, ICE sections were 98% less likely to exhibit grades 2 or 3 (OR: 0.02, 95% CI 0.001, 0.365; $p<0.01$). Histomorphometry measurements of total and non-keratinised primary epidermal lamellar length were significantly increased ($p<0.01$) in AMB limbs compared to ICE. TPX2 positive cell counts were significantly increased ($p<0.01$) in AMB limbs compared to ICE.

Main limitations: Continuous digital hypothermia was initiated before recognition of laminitis and therefore the clinical applicability requires further investigation.

Conclusions: Continuous digital hypothermia reduced the severity of laminitis in the EHC model and prevented histological lesions compatible with lamellar structural failure.

Introduction

Insulin dysregulation, particularly hyperinsulinaemia, is a key contributor to the development of endocrinopathic laminitis in horses and ponies with equine metabolic syndrome and/or pituitary *pars intermedia* dysfunction [1-3]. This endocrinopathic form of laminitis is typically chronic, slowly progressive and often subclinical [1] however recurrent acute episodes are also common, particularly after access to pasture high in non-structural carbohydrate [4,5]. Hyperinsulinaemia causes acute laminitis experimentally, which is the basis of the EHC model [6-10]. However, despite identification of some key events in recent studies [11,12], our understanding of how excess insulin damages lamellae remains incomplete and there is currently no evidence to support a particular therapeutic intervention to limit lesion progression in acute cases.

Continuous digital hypothermia prevents lamellar structural failure when applied prophylactically [13] and during the acute phase [14] in the oligofructose experimental model of sepsis-associated laminitis. A prophylactic effect has also been demonstrated in clinical cases of colitis [15]. Importantly, the effectiveness of continuous digital hypothermia as an intervention in the oligofructose model has facilitated studies examining the importance of different pathophysiological events in the development of acute laminitis [16,17]. However, the pathophysiology of endocrinopathic laminitis may have little in common with that of the sepsis-associated form, with inflammation in particular demonstrated to play a greater role in the latter [8,18]. Recent studies investigating how excessive insulin causes laminitis have focused on the activation of lamellar

insulin-like growth factor-1 receptor (IGF-1R) by insulin, triggering processes leading to epidermal cellular proliferation, increased cell-death proliferation cycle and cytoskeletal disruption [11,12,19].

In light of the key differences in the pathophysiology of this form of the disease, a therapeutic effect of continuous digital hypothermia cannot be extrapolated from existing studies of sepsis-associated laminitis. Therefore, the objective of the current study was to determine the effect of continuous digital hypothermia on the development of acute laminitis caused by hyperinsulinaemia. We hypothesised that continuous digital hypothermia would reduce the severity of laminitis in the EHC model: we used histopathology, histomorphometry and immunohistochemistry to evaluate its effects.

Material and Methods

Subjects

Eight clinically normal Standardbred geldings recently (<4 weeks) retired from racing in Queensland, Australia, were selected for use in this study. The horses were sound and had no gross or radiographic abnormalities of the feet, and were housed in a stable for one week prior to the experiment and fed lucerne (alfalfa) hay. During the 72 hour experimental period all horses were confined to stocks (a crush) and received *ad libitum* access to lucerne hay and water.

Instrumentation and monitoring

Pedometers (Yamax digiwalker sw700)[a] were taped to the antebrachium of both forelimbs and forelimb hoof temperature was monitored using hoof wall thermistors attached to data-logging devices as previously described [17]. Immediately following instrumentation, a 24 h control period was commenced after which the EHC was performed for 48 h with one forelimb randomly selected to be continuously cooled (ICE) and the other maintained at ambient temperature (AMB). Vital signs

were measured every 2 h. Following 48 h of EHC, each horse was walked in a straight line for 20 metres and video recorded for blinded analysis by a veterinarian experienced in the evaluation of lameness due to laminitis (A.v.E.) who reported the presence or absence of lameness at the walk and noted which limb appeared to be most affected.

Euglycaemic hyperinsulinaemic clamp

The EHC was performed as previously described [7]; briefly, a continuous intravenous infusion of recombinant human insulin (Humulin-R)[b] was administered via a 14-gauge jugular catheter at a rate of 6 mIU/kg bwt/min, after administering an intravenous insulin bolus (45 mIU/kg bwt) diluted in 50 ml of saline (0.9% NaCl). A continuous intravenous infusion of 50% glucose (Baxter)[c] was administered via a 14-gauge catheter (in the opposite jugular vein) with the administration rate adjusted to maintain euglycaemia (5 ± 1 mmol/l). Blood glucose was measured using a portable glucometer (Accu-Chek Performa)[d] every 5 min until euglycaemia was maintained for 30 min without the need to adjust the glucose infusion rate. Blood glucose was then measured every 30 min.

Continuous digital hypothermia

Thirty min prior to starting the EHC, one forelimb was randomly selected (coin toss) to be cooled for the remainder of the experiment in a 50% ice, 50% water mixture to a level just distal to the carpus using a rubber boot (Bigfoot Ice boots)[e].

Histology

After 48 h of EHC the horses were euthanased with pentobarbital sodium (20 mg/kg bwt i.v.). Dorsal lamellae were dissected from the hoof and distal phalanx of both forelimbs and sections were fixed in formalin and processed for light microscopy as previously described [20]. H&E (haematoxylin and eosin) and PAS (periodic acid-Schiff) stained sections from proximal, middle and distal regions of the forefeet were randomised and examined via light microscopy by a blinded veterinary pathologist (J.B.E.). The histological severity of each section was graded on a 0-3 scale as previously described [20].

Histomorphometry

Sections were randomised and examined by a blinded observer (S.M.S.) for morphometry. Five consecutive PEL (primary epidermal lamellae) were measured per section from proximal, middle and distal regions, with the mean of these 5 measurements then used for statistical analyses. Total PEL length was measured from the abaxial to axial margin of each selected PEL as previously described [8]. Non-keratinised PEL length was measured from the axial tip of the keratinised axis of the PEL to the axial margin of the same PEL (Fig 1). All measurements were made using digital slide software (Aperio ImageScope v12.1.0.5029)[6].

Immunohistochemistry

Immunohistochemical staining for cellular proliferation marker TPX2 (targeting protein for xenopus kinesin-like protein 2) was performed on middle formalin-fixed sections as previously validated in horse lamellae [6]. Sections were routinely deparaffinised, hydrated, and treated with 3% hydrogen peroxide in TBS for 10 min. Antigen retrieval was achieved by treating the sections for 5 min in 0.01

M citrate buffer (pH 6.0) at 125°C. Subsequently, the sections were incubated with TPX2 primary antibody (TPX2/ab32795; Abcam[7]) diluted 1:100, for 60 min. The sections were then incubated with MACH1 mouse probe (15 min) and polymer (30 min) (Biocare Medical[8]) at room temperature. Staining signalling was developed with diaminobenzidine chromogen substrate, followed by counterstaining with haematoxylin.

The TPX2-positive cell counts were performed by a blinded observer (S.M.S.) as previously described [6]. Briefly, five consecutive PEL were measured per section and divided in half into axial and abaxial regions. The number of TPX2 positive epidermal cells (intense nuclear staining) was counted in each region across 5 PEL. For each section, a mean count (per PEL) was then calculated for each region for use in the statistical analyses.

Data analysis

All analyses were conducted using Stata 15/MP[9], with two-sided tests of hypotheses and a p-value <0.05 as the criterion for statistical significance. Age and weight data were analysed for normality using Shapiro-Wilk tests. Descriptive statistics are expressed as mean with standard error of the mean (S.E.M.) or 95% confidence intervals (CI) for normally distributed variables and median (interquartile range [IQR]) for other continuous variables. Categorical variables were tabulated. Inference statistical analysis was conducted using a mixed effects multi-level linear regression where the random effects were assigned at the level of the animal and the fixed effects were specified as statistical interaction between treatment (AMB vs. ICE) and either time (for pedometer and hoof temperature data) or section location (TPX2 count and histomorphometry). We utilised an independent covariance structure with one unique variance per random effect. All covariances were set to zero. In order to assess model fit, the distribution of residuals were checked using quantile normal plots. Due to small departures from normality of the residuals for hoof temperature, we decided to use a robust estimation of the variance that provides adjustment for small departures

from normality. The categorical outcome, histological grade (grades 1, 2 or 3), was recategorised into a binary categorical variable where grade 1 was assigned a 0 value and histological grade 2 and 3 were combined and assigned a value of 1 (0 value = mild histological lesions and value of 1 = moderate to severe histological lesions). Univariate analysis was conducted to establish association between histological grade and location and treatment using a chi-squared test. Due to issues with complete separation, final inference statistical analysis was performed using penalised logistic regression (firth logistic regression). In the inference model location and treatment were considered to be independent fixed effects. Regression model estimates are included in the text where appropriate as the coefficient (Coeff.) and 95% CI are also presented for all comparisons in Supplementary Item 1.

Results

All 8 horses (mean age: 6.4 ± 1.8 years; mean bodyweight: 447.8 ± 39.5 kg) developed laminitis within 48 h. There was no significant change in pedometer count frequency over the first 24 h in either the AMB or ICE limbs, however during the EHC (24-72 h) there was a significant increase in AMB limb pedometer count frequency (Coeff. 4.91, 95% CI 3.57, 6.27; $p<0.01$), that was not present in ICE limbs (Supplementary Item 2). Incessant weight shifting (and an increase in AMB limb pedometer count frequency) became clinically apparent (Obel grade 1 laminitis) from 52-58 h (28-34 h after initiating the EHC), at which time each horse was administered phenylbutazone (Nabudone P)[10] 4 mg/kg bwt i.v.. Five of the horses received a second dose 8-10 h later. Video analysis at 72 h showed six horses were lame at the walk in the ambient limb (Obel grade 2 laminitis) and two were not detectably lame at the walk.

Hoof wall surface temperature decreased (Coeff. -0.35, 95% CI -0.37, -0.33; $p<0.01$) in the ICE limbs after ice boot application (24-72 h): the median [IQR] temperature for this period was 6.4 [6-6.9] °C for ICE compared with 29.7 [28.6-31.1] °C for AMB (Supplementary Item 3). There was a gradual

increase in AMB hoof wall surface temperature over the experimental period (Coeff. 0.26, 95% CI 0.19, 0.33; p<0.01).

Histology

Severe SEL elongation and disruption with dermo-epidermal separation (Grade 3) was observed in all AMB feet at one or more section levels (Fig 1a), and in none of the ICE sections (Fig 1b). Representative histopathology from AMB and ICE feet is presented in Figure 2 and Figure 3. Relative to AMB feet, ICE sections were 98% less likely to exhibit grades 2 or 3 (OR: 0.02, 95% CI 0.001-0.365; p<0.01; Table 1). There was no effect of section location on grade. Overall, 92% of AMB sections received a grade of 3 and 8% a grade of 2, compared with ICE sections which were 50% grade 1 and 50% grade 2.

Histomorphometry

Histomorphometry results are presented in Table 2. There was a significant increase (p<0.01) in total PEL length and non-keratinised PEL length in AMB feet compared to ICE. There was no significant effect of section location on histomorphometry variables.

TPX2 Expression

Intense nuclear TPX2 expression was frequent in epidermal basal cells in AMB feet, and occasional in ICE feet (Fig 4). Median [IQR] TPX2 positive cell counts in AMB feet were 118.3 [100.5-159.4] (abaxial) and 159.6 [109.6-172] (axial), whereas in ICE feet they were 0.9 [0.2-2.15] (abaxial), and 8.8 [3.35-12.85] (axial). TPX2 counts were significantly lower in the ICE feet (Coeff. -141, 95% CI -182, -99; p<0.01) compared to AMB and also higher in the axial location (Coeff. 24.5, 95% CI 4, 44.9; p =

0.02). When the interaction between treatment and location was accounted for, this difference was significant only for the ICE feet (Coeff. 7.5, 95% CI 3.6, 11.2; p<0.01).

Discussion

Hyperinsulinaemia has been implicated as the key event in endocrinopathic laminitis [1,2,7,10,21]. In the current study, a protective effect of continuous digital hypothermia was observed in a model of endocrinopathic laminitis when it was initiated at the time of commencement of the EHC. Severe histologic lesions present in AMB feet, including dermo-epidermal separation and marked neutrophilic infiltration, were not observed in ICE feet. Dermo-epidermal separation of AMB lamellae was reflected in the increased PEL length, due largely to the severe separation at the axial tips and consequent retraction of the keratinised axis reflected by increased non-keratinised PEL length. In contrast, ICE feet total PEL length measurements (mid lamellar: 3586 ± 224 µm) were similar to those of control subjects from the same population in a previous study [22] (mid lamellar: 3411 ± 116 µm). Continuous digital hypothermia also mitigated the clinical signs of laminitis that manifested in the AMB limbs as progressive increases in weight shifting (increased pedometer count frequency) and lameness at the walk (6/8 horses). Overall the protective effects of continuous digital hypothermia on the severity of clinical and histological laminitis were similar to those noted in previous studies of sepsis-associated laminitis that utilised the oligofructose model [13,14,22].

The association between insulin dysregulation and laminitis has been well established but the exact pathophysiology remains unclear. Proposed mechanisms include lamellar ischaemia [5,23,24], energy failure at the level of the lamellar epithelial cell [25], and activation of growth factor signalling pathways via IGF-1R in lamellar epithelial cells [12,26]. Following the discovery that hyperinsulinaemia can cause laminitis in the absence of insulin resistance in the EHC model [7,10], there has been growing recognition of the role of hyperinsulinaemia in naturally-occurring cases [1,2,21]. This, together with evidence from *in vitro* studies [27,28], supports the presence of a direct

effect of excess insulin on the lamellae in this form of laminitis. One such key effect of insulin in experimental models appears to be activation of lamellar epithelial IGF-1R [11,12,28] triggering lamellar epithelial cell proliferation [28]. However, a recent in vitro study found that insulin does not readily bind to equine IGF-1R, even at similar increased insulin concentrations to that achieved in serum during the EHC [29]. Although increased cell proliferation may be evidenced by suprabasilar acanthosis in the current study, cell stretch (without necessarily an increase in cell number) may be the main contributor to lamellar lengthening in the acute phase of the EHC model [19]. In terms of lamellar failure, dissolution of epithelial cell hemidesmosomes and cytoskeletal re-organisation may be key early events as noted in cancer cells undergoing epithelial-mesenchymal transition [30,31], which could result in epithelial cell stretch and dysadhesion. In the current study, there was histological evidence of both epithelial cell stretch and cell adhesion loss, as indicated by severe SEL elongation, haphazard basal and suprabasilar cell organisation, and BM dysadhesion in AMB feet, which was mitigated by hypothermia. At what level this mitigation occurred warrants further investigation: hypothermia could have directly affected insulin-receptor binding and internalisation, which is known to be influenced by temperature in other cell types [32,33], or may have affected events downstream from IGF-1R.

The increase in TPX2 positive epithelial cell counts in AMB feet was similar to that reported previously at the 48 h time point of the EHC model [6]. In contrast, TPX2 positive cell counts in ICE limbs of the current study were comparable to those previously reported in normal control feet [6]. TPX2 is a microtubule associated protein that participates in mitotic spindle formation and is utilised as a proliferation marker to provide prognostic value in a number of cancers [34-37]. Overexpression of TPX2 is associated with apoptosis and epithelial-mesenchymal transition in hepatocellular carcinoma [36] and there is evidence that TPX2 can (itself) promote cellular proliferation through activation of the PI3K/Akt signalling pathway in human colon cancer [37]. Lamellar concentrations of phosphorylated (activated) Akt are increased in the EHC model and cellular changes similar to epithelial-mesenchymal transition could play a role in destabilising the lamellae [11]. Furthermore,

an increased apoptosis-proliferation cycle within SEL was identified in ponies undergoing the EHC [19]. It is possible that continuous digital hypothermia prevented lamellar failure in the current study by stabilising the apoptosis-proliferation cycle and by inhibiting signalling events associated with epithelial-mesenchymal transition including PI3K/Akt signalling, however it is unclear whether hypothermia has a direct effect on TPX2 expression or just the events preceding TPX2 expression. There is limited published information on the effects of hypothermia on growth factor signalling pathways in other tissues, however there is evidence that the cardioprotective effects of hypothermia in cardiac arrest models are attributable in part to enhanced Akt phosphorylation [38] and mTOR inhibition (directly and/or via increasing the activity of AMPK) [39]. Nevertheless, in the current study the TPX2 cell counts (together with histomorphometry) provided an objective, quantitative means of evaluating the therapeutic effect of hypothermia in the EHC model.

Histological changes in AMB feet appeared to be more severe than those reported in previous EHC studies, with more marked dermo-epidermal separation and detachment of the BM. There was also more inflammatory cell infiltration than that reported previously with the EHC model [7,10]. The extent and severity of epithelial cell-BM adhesion loss was similar to that seen in sepsis-associated laminitis models, but with less evidence of BM disintegration/dissolution seen in those models [20,40]. In a temporal study of laminitis development in the oligofructose model, BM lesions preceded leukocyte infiltration and perhaps induced it [41]. It is likely that the relatively severe inflammation noted in the current study occurred because of the increased severity of BM detachment or SEL necrosis. Regardless of cause, inflammation was clearly ameliorated by continuous digital hypothermia in the current study, consistent with its anti-inflammatory effects in the oligofructose model [17].

It is unclear why the lamellar pathology was more severe in the AMB limbs of the current study compared with a previous study also in Standardbred horses [7]. The duration of the EHC in the current study was longer (48 h) than that of a previous EHC study (46 ± 2.31 h) in which the

Standardbred horses used also had lower body weight (447 ± 13 kg), with the forces of weight bearing considered to play an important role in lesion development [7]. The early administration of phenylbutazone in the current study may have also contributed to the increased severity of lesions, particularly through alteration of weight bearing patterns during laminitis development. Furthermore, some horses received one dose whilst others received 2; however since the current study compared the effect of ICE treatment between limbs (rather than between horses) it is unlikely that this difference affected the outcome of the ICE vs. AMB evaluation.

Although this study establishes a therapeutic effect of hypothermia against laminitis caused by hyperinsulinaemia in principle, the results have limited clinical applicability since hypothermia was applied immediately at the onset of hyperinsulinaemia (and continuously for the study period). In contrast, the development of naturally occurring endocrinopathic laminitis is insidious, episodic and even advanced chronic lesions may be subclinical [1], therefore identification of a defined developmental period in which to apply hypothermia prophylactically would be impossible in almost all natural cases. Continuous digital hypothermia has a therapeutic effect even when initiated after the development of lameness in the oligofructose model of sepsis-associated laminitis, and a similar study would need to be performed using the EHC model to confirm a similar effect; however, the results of the current study add support for the clinical use of continuous digital hypothermia where insulin dysregulation is a suspected contributor (including episodes of acute pasture-associated laminitis [42]). More importantly, the efficacy of continuous digital hypothermia established in the current study allows its use as a research tool to investigate which mechanistic pathways contribute to lamellar failure in hyperinsulinaemia, with the ultimate aim being development of therapeutic targets for pharmacological intervention in endocrinopathic laminitis.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

The project was approved by The University of Queensland Animal Ethics Committee (AEC). All animals were monitored continuously by the investigators.

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Authorship

S. M. Stokes, J.K. Belknap and A.W. Van Eps contributed to study design, data analysis, interpretation, and preparation of the manuscript. All authors contributed to the study execution and approved the final manuscript.

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Figure legends

Fig 1: H&E stained transverse sections of lamellar tissue from the middle lamellar level of AMB (a) and ICE (b) feet from the same horse. Severe dermo-epidermal separation of AMB lamellae (a) characterised by wavy PEL contours, axial-abaxial PEL elongation with abaxial displacement of the keratinised axis and medial-lateral collapse of SEL, as compared to ICE lamellae (b). Lines demonstrate technique used for histomorphometry measurements: total primary epidermal lamellar length (**A**) and non-keratinised primary epidermal lamellar length (**B**). Bar = 1000µm

Fig 2: PAS stained transverse sections of lamellar tissue from the middle lamellar level of AMB (a, c) and ICE (b, d) feet of the same horse. AMB limbs at abaxial (a), middle, and axial (c) regions demonstrate marked SEL elongation (a, **A**) with collapse of SDL, and SEL basement membrane

detachment (c, **arrows**) with the formation of irregularly-shaped epithelial islands composed of dysplastic keratinocytes (c, **asterisks**). In contrast, ICE limbs at abaxial (b), middle and axial (d) regions the SEL remain attached to the keratinised axis with predominantly round tips, closely adhered basement membrane, and distinct SDL. Occasional mild elongation of SEL (b, **B**) with occasional pointed basement membrane tips (d, **arrows**) and mild disorganisation of basal and suprabasal cell nuclei (d, **asterisks**) are identified within ICE limbs. Bar=300µm

Fig 3: H&E (a, b) and PAS (c, d) stained transverse sections of lamellar tissue from the middle lamellar level of AMB (a, c) and ICE (b, d) feet. In AMB lamellae, entire regions of axial SEL demonstrate necrosis, characterised by hypereosinophilic, compressed keratinocytes with pyknotic nuclei (a, **arrows**), and fibrinous exudate with haemorrhage (a, **asterisk**). In contrast, ICE axial lamellae retain their normal morphology and demonstrate scattered individual keratinocyte necrosis (b, **arrows**). Severe neutrophilic inflammation is associated with necrotic SEL of AMB feet (c, **arrow**). Grade 2 ICE section with suprabasilar acanthosis within regions of most severe lamellar distortion (d, **arrows**). Bar = 100µm

Fig 4: Immunohistochemically stained (TPX2) transverse sections of lamellar tissue from the middle lamellar level of ICE (a) and AMB (b) feet of the same horse demonstrating frequent nuclear TPX2 expression in epidermal basal cells in the AMB limb. The median TPX2 positive cell counts in both the abaxial (b) and axial (c) regions is greater (*$p<0.01$) in AMB feet when compared with ICE. Bar = 100µm

\lementary Information

Supplementary Item 1: Mixed effects regression model estimates.

Supplementary Item 2: Median (± IQR) pedometer count frequency.

Supplementary Item 3: Median (± IQR) hoof wall surface temperature.

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Table 1: Histological scores in forelimb hoof lamellar sections following a 48 h euglycaemic hyperinsulinaemic clamp during which one forelimb was cooled (ICE) and the other maintained at ambient temperature (AMB).

	Proximal		*Middle*		*Distal*	
Score (0-3)	**AMB**	**ICE**	**AMB**	**ICE**	**AMB**	**ICE**
0	0	0	0	0	0	0
1	0	2	0	5	0	5
2	0	6	1	3	1	3
3	8	0	7	0	7	0

Table 1: Blinded histological scoring of forelimb dorsal hoof lamellar sections was used to determine the effect of continuous digital hypothermia on the development of laminitis in the EHC model in 8 horses. Relative to AMB feet, ICE sections were 98% less likely to exhibit grades 2 or 3 (OR: 0.02; $p<0.01$). There was no significant effect of section location (proximal, middle or distal) on histological score.

Table 2: Histomorphometry measurements in forelimb hoof lamellar sections following a 48 h euglycaemic hyperinsulinaemic clamp during which one forelimb was cooled (ICE) and the other maintained at ambient temperature (AMB).

Length (µm)	AMB	ICE
Proximal		
TPELL	4118 (3779, 4457)	3613 (3193, 4032)
NKPELL	1436 (1103, 1768)	760 (655, 865)
Middle		
TPELL	4061 (3604, 4519)	3586 (3056, 4116)
NKPELL	1369 (1111, 1627)	620 (369, 871)
Distal		
TPELL	4176 (3911, 4441)	3596 (3052, 4140)
NKPELL	1320 (1143, 1497)	690 (450, 929)

Table 2: Mean (95% CI) total primary epidermal lamellar length (TPELL) and non-keratinised primary epidermal lamellar length (NKPELL) of dorsal hoof wall lamellae at three section levels. Histomorphometry of forelimb dorsal hoof lamellar sections was used to determine the effect of continuous digital hypothermia on the development of laminitis in the EHC model in 8 horses. Based on the mixed effects linear regression model, both TPELL (Coeff. -520, 95% CI -739, -302) and NKPELL (Coeff. -685, 95% CI -878, -492) were significantly decreased in ICE limbs compared with AMB ($p<0.01$). There was no effect of section location on the measurements. (µm).