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Histopathology of insulin-induced laminitis in ponies

K. E. ASPLIN, **J. C. PATTERSON-KANE**[†], **M. N. SILLENCE**[‡], **C. C. POLLITT** and **C. M. McGOWAN**[*§]

School of Veterinary Science, The University of Queensland, Australia; [‡]Faculty of Science and Technology, Queensland University of Technology, Australia; [†]Faculty of Veterinary Medicine, University of Glasgow, UK; and [§]School of Veterinary Science, Faculty of Health and Life Sciences, University of Liverpool, UK.

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Summary

Reasons for performing study: **Ponies with laminitis associated with insulin resistance and hyperinsulinaemia lack systemic and/or intestinal inflammatory signs, suggesting a different pathogenesis potentially reflected in differing histopathology.**

Objectives: **To describe the histological appearance and quantify morphological changes in primary and secondary epidermal lamellae (PEL and SEL) of laminitis lesions from ponies with insulin-induced laminitis.**

Methods: **Equine hoof lamellar tissue was obtained from 4 control ponies and 5 ponies with laminitis induced following infusion of insulin (1036 ± 55 μU/ml) while maintaining euglycaemia for 55.4 ± 5.5 h. Sections from all 4 hooves were stained and examined by a veterinary pathologist. Measurements of lamellar length (PEL and SEL) were made in mid-dorsal sections of the right forefeet by 2 blinded observers. Immunolabelling for calprotectin was performed using a monoclonal antibody.**

Results: **No lesions were detected in normal ponies. Lesions detected in ponies with laminitis were variable in severity between ponies. Within ponies, SEL lesions were more severe along the axial region of PEL. Lesions included swelling, disorganisation and abnormal keratinisation of epidermal cells, increased mitotic activity and apoptosis. Separation of basement membranes was minimal. Immunostaining revealed inflammatory cells within the lamellar dermis. SEL were significantly elongated in laminitic hooves relative to controls, with the greatest elongation in those attached to abaxial and middle regions of PEL.**

Conclusions: **Laminitis induced by prolonged infusion of insulin lacked widespread basement membrane disintegration, and increases in epidermal cellular proliferation at axial aspects were marked for this acute stage of disease.**

Potential relevance: **Defining equine laminitis entirely in terms of separation of the basement membrane may not be appropriate for laminitis associated with hyperinsulinaemia.**

Introduction

Laminitis can occur following systemic inflammation, usually secondary to inflammation and disruption of the microbial populations in the large intestine. However, laminitis is a disease syndrome with many cases occurring due to hormonal dysfunction; endocrinopathic laminitis (McGowan 2008). Evidence suggests that endocrinopathic laminitis is the most common form of laminitis, and that insulin resistance and associated hyperinsulinaemia is an important aetiological factor (USDA 2000; Treiber *et al*. 2006; McGowan 2008). Clinical and histological signs of laminitis have been triggered in experimental ponies and horses through the induction of prolonged hyperinsulinaemia while maintaining euglycaemia, which may be a model of the latter form of laminitis (Asplin *et al*. 2007; De Laat *et al*. 2010). The lack of any clinical or clinicopathological evidence of systemic or gastrointestinal inflammatory disease in ponies or horses with insulin-induced laminitis (Asplin *et al*. 2007; De Laat *et al*. 2010) implicates a different pathogenesis from previous experimental models of laminitis in horses. Such models have principally been designed to represent severe inflammatory and/or gastrointestinal disease, including carbohydrate (CHO) overload using starch (Garner *et al*. 1975), Black Walnut extract (BWE) (Galey *et al*. 1991) or CHO overload using oligofructose (van Eps and Pollitt 2006). If the pathogenesis of laminitis induced by insulin differs from that induced by CHO overload or BWE this may be reflected as differences in histological lesions, at least in the acute phase.

Despite several descriptive reports of the histological appearance of laminitis in horses occurring as a result of inflammatory or gastrointestinal causes, to date, the lesions in endocrinopathic or insulin-induced laminitis have not been described. There has been insufficient clinical detail from the few naturally occurring cases for which the pathology has been described in the literature to determine the underlying pathogenesis or to correlate it with histological lesions (Roberts *et al*. 1980). Results from insulin-induced experimental laminitis revealed a normal histological appearance of the lamellar tissues obtained from all the control ponies, whereas lamellae obtained from treated ponies showed changes similar to laminitis due to inflammatory causes (Pollitt 1996; Asplin *et al*. 2007). The use of detailed histological measurements is warranted to describe these changes more fully and allow further comparisons of laminitis resulting from different causes and at different time points. Immunohistochemical labelling is also of value to more clearly define lesions, as inflammatory cells can be difficult to identify and quantify in routinely stained histological sections even by experienced observers.

*Corresponding author email: cmcgowan@liv.ac.uk
Dr Asplin's present address is: School of Veterinary Medicine and Science, The University of Nottingham, Sutton Bonington Campus, Leicestershire, UK.

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The aim of this study was to examine and describe the histological appearance of dorsal hoof wall lamellae from ponies with insulin-induced laminitis, with quantification of morphological changes in PEL and SEL, and immunohistochemical labelling to assist in the detection of leucocyte accumulation in the lamellar dermis.

Materials and methods

The protocol was approved by the Animal Ethics Committee of the University of Queensland.

Animals

Five treated ponies (*Ponies 1–5*) and 4 control ponies (*Ponies 6–9*) were included in the study. The ponies weights were 258.4 ± 26 kg, they were 4 ± 1.5 years old (mean ± s.e.) and were clinically normal before the experiment; with no known history of laminitis and no evidence of previous laminitis on inspection of the hooves. The ponies were subjected to euthanasia at the onset of Obel *grade 2* laminitis (treatment) (Obel 1948), or after 72 h of saline infusion (controls). All treated ponies received 4.4 mg/kg bwt phenylbutazone i.v. or *per os* on completion of the infusion and were subjected to euthanasia within 6–12 h after the infusion ceased, except for one treated pony that was subjected to euthanasia 5 days later due to a problem with transport.

Induction of laminitis with insulin

Laminitis was induced in 5 treated ponies via a prolonged euglycaemic–hyperinsulinaemic clamp (pEHC) technique as described previously (Asplin *et al*. 2007). Briefly, 9 ponies were allocated to either a treated (n = 5) or control (n = 4) group, to receive infusions of recombinant human insulin (Humulin R)[1] plus glucose, or an equivalent volume of isotonic saline, respectively. For treated ponies, one jugular catheter was used for the simultaneous infusion of insulin at a fixed rate, and glucose at a variable rate. The opposite jugular catheter was used to collect blood samples, which were used to monitor blood glucose and insulin concentrations (data not shown). After collection of baseline samples, a priming dose of insulin (45 mU/kg bwt in 50 ml of 0.9% saline) was administered i.v. as a bolus injection, followed by insulin infusion at a steady rate of 6 mU/min/kg bwt (DeFronzo *et al*. 1979), which was maintained throughout the experiment. An infusion of glucose solution (50% w/v; Baxter) was initiated at a rate of 24.4 ± 3.0 µmol/min/kg bwt (DeFronzo *et al*. 1979). Euglycaemia was defined as a blood glucose concentration of 5 mmol/l, and the glucose infusion rate was adjusted whenever blood concentrations differed from this value by >1 mmol/l. Control ponies received a single infusion of 0.9% saline at a fixed rate of 14.7 µl/min/kg bwt for 72 h, which represented the average rate of fluid infusion in the treated ponies over the same period.

Sample collection

The distal aspect of all 4 limbs of all ponies was disarticulated at the metacarpophalangeal or metatarsophalangeal joint, within 10 min of death and sectioned with a bandsaw as described previously (Pollitt 1996). A section at the midpoint between the coronary band and the ground-bearing surface on the dorsal aspect of the hoof was obtained using a bandsaw and was then dissected to obtain 5 mm square samples, extending from the inner hoof wall to the dermal connective tissue. All samples were fixed in 10% neutral buffered formalin for 24 h, processed by routine methods, embedded in paraffin wax and sectioned at 5 µm.

Histological description of lamellae

Sections were stained with haematoxylin and eosin or periodic acid Schiff (PAS). All slides were examined by a veterinary pathologist (J.P.K.) blinded to the treatment group.

Measurement of primary and secondary epidermal lamellar lengths

Measurements of lamellar length were made in the mid-dorsal sections of the right forefeet of all 9 ponies by 2 blinded observers, using haematoxylin and eosin sections. The images were randomised (http://www.randomizer.org/form.html) and coded prior to measurement. A continuous line was drawn along the base of the PEL in each section, and the PEL length (PELL) measured from the tip of each PEL to this baseline at 20× magnification (Fig. 1a). Ten PELL measurements were made per hoof. Ten SEL length (SELL) measurements were obtained at 200× magnification from each of 3 regions of each analysed PEL; i.e. 10%, 50% and 90%, corresponding to the tip (axial, adjacent to the distal phalanx), middle and base (abaxial, closest to the stratum medium of the hoof wall) portions, respectively (Morgan *et al*. 2003). The SELL was measured from the tip of each SEL to the base, at the junction with the keratinised axis of the associated PEL (Fig 1b). All measurements were made using image analysis software (ImagePro)[2].

Immunolabelling for calprotection (leucocyte marker)

Immunohistochemical staining was performed on sections from the same wax blocks as described above. The sections were deparaffinised in xylene and hydrated through gradients of ethanol. Antigen retrieval was achieved by heating the sections to boiling temperature in a pressure cooker in 10 mmol/l sodium citrate buffer (pH 6.0) for 5 min, then cooling for 30 min by placing the pressure cooker in ice. Sections were washed in 50% methanol, then incubated in a 3% H_2O_2 solution for 20 min at room temperature to block endogenous peroxidase activity, and washed in phosphate-buffered saline (PBS) containing 0.3% Triton X. Nonspecific binding was blocked using 1% bovine serum albumin (BSA) in PBS for 1 h. A monoclonal mouse antihuman macrophage antibody (MAC387)[3] was diluted at 1 : 200 in BSA and left in contact with the sections for 2 h at room temperature. After being washed 3 times in PBS, sections were incubated with the secondary antibody (peroxidase-labelled goat antimouse antibody 1 : 200)[4] for 30 min at room temperature. Sections were again washed 3 times in PBS before being stained with the chromagen 3,3′-diaminobenzidine tetrahydrochloride (DAB)[5] and H_2O_2 for approximately 5 min, and counterstained with Mayer's haematoxylin and Scott's Bluing Reagent. Nonspecific negative control samples were incubated with PBS only. Equine calprotectin-positive lamellar tissue obtained from a horse with oligofructose-induced laminitis was used as a positive control.

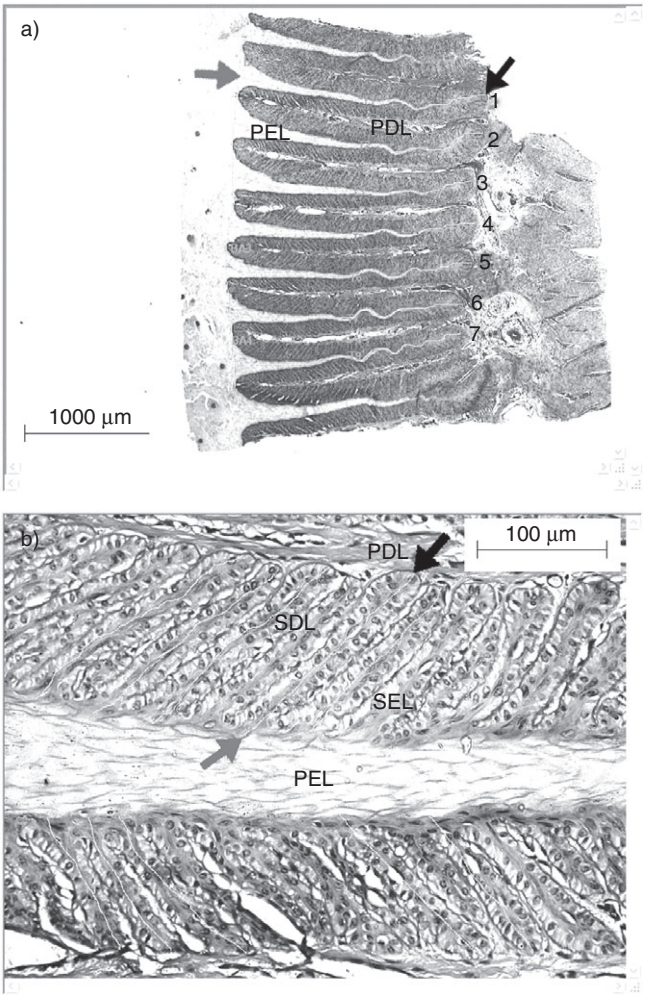

Fig 1: Measurement of epidermal lamellar length, in control ponies. (a) For primary epidermal lamellar length (PELL) measurements, a continuous line was drawn along the bases of the PEL and a measurement taken from the tip of each (black arrow) to the baseline (red arrow). PDL = primary dermal lamellae. Periodic acid-Schiff. Bar = 1000 µm. (b) For secondary epidermal lamellar length (SELL) measurements, lines were drawn from tips of each (black arrow) to the junction with the keratinised axis of the associated PEL (red arrow). Periodic acid-Schiff. Bar = 100 µm.

Presentation of the results and statistical analysis

Results of lamellar measurements were analysed for agreement between observers using Lin's concordance correlation coefficient and Bland-Altman's limits of agreement (LOA) with the Stata vs. 10 for Windows software[6]. Mean PELL from control tissue was compared with laminitic tissue using a Student's *t* test. SELL variables were not normally distributed, thus logarithmic transformation (base e) was performed to achieve normality of distribution. Multivariable linear regression analysis was conducted using the Stata vs. 10 for Windows software[6] to compare SELL of control tissue with SELL of laminitic tissue. Separate models were developed for each operator. Pony was fitted as a random effect, whereas treatment (laminitic or control) and region (10, 50 and 90%) were fitted as fixed effects. Control was the reference level for the coefficient for treatment. As the interaction term for treatment by region was significant for both observers ($P<0.001$) and an interaction was highly plausible a priori, this term was retained in the model and separate estimates are reported for the effect of treatment in each region (10, 50 and 90%). As SELL variables were log transformed before analyses, coefficients estimating the effect of treatment were exponentiated and interpreted as ratios of (geometric) means for laminitic tissue relative to control tissues. PELL and SELL were analysed using the Stata vs. 10 for Windows software.

Results

Histological description of lamellae

In sections from control ponies, the PEL were either straight or slightly curved, with SEL extending out at either right angles or approximately 45°. The SEL had rounded tips and were 10–15 epidermal cells in length. Epidermal cells were plump and columnar with finely vacuolated cytoplasm and apical or central ovoid (or sometimes round) nuclei. The nuclei contained finely granular, partially marginated chromatin and 1–5 small, central or paracentral, deeply eosinophilic nucleoli. Many of the ovoid nuclei were oriented perpendicular to the basement membrane. Basement membranes of SEL extended within 1–2 epidermal cell lengths of the keratinised cores of the PEL. In one control pony (*Pony 7*), 0–2 mitotic figures per high power (400×) field were noted in cells of SEL close to the (axial) PEL tips. Mitotic figures were not seen in these locations in the other control animals. Apoptotic epithelial cells (with a shrunken, rounded cytoplasm containing nuclear fragments) were rarely observed. In the dermis overlying the axial PEL region, small numbers of lymphocytes were scattered around blood vessels, and plasma cells were also noted in a few fields.

In sections from ponies with laminitis (Fig 2), the lesions varied markedly in severity. The PEL were undulant in some sections, and all had irregular and poorly defined PEL-SEL interfaces. PEL keratin was increased in amount in some foci and discontinuous in others. The SEL axes contained increased amounts of keratin that was disorganised and occasionally forming small whorls. SEL towards the PEL tips were swollen, rounded and sometimes club-shaped, and were often touching adjacent SEL; the epidermal cells comprising them were swollen with variably increased cytoplasmic vacuolation, and they were often arranged in 2–4 layers. The nuclei of SEL cells were enlarged and more variable in size that those in control ponies, with enlarged and/or more numerous nucleoli; the nuclei were variable in their orientation within the cell (i.e. not all were apical), and also varied in their orientation to the basement membrane.

Collapsed tubes of PAS-positive basement membrane material separated from SEL tips were noted at axial surfaces (i.e. SEL at PEL tips; Fig 2a); these involved highly variable numbers of PEL in individual ponies. This was severe and extensive in P2 and absent in P1. In some fields/animals there was only slight separation of the basement membrane from some intact SEL tips. Many of these basement membrane tubes were infiltrated by degenerate neutrophils. Over the remaining two-thirds to three-quarters of the PEL, the SEL were markedly elongate rather than thickened, had pointed tips and were difficult to distinguish from adjacent lamellae. Examination of sections using PAS indicated that the basement membranes were not separated in these elongated abaxial and middle regions.

Adjacent to the keratin of both SEL and PEL, small to moderate numbers of epithelial cells contained coarsely granular or homogeneous brightly eosinophilic material (dyskeratosis; Fig 2b). There were up to 15 mitotic figures per high power field

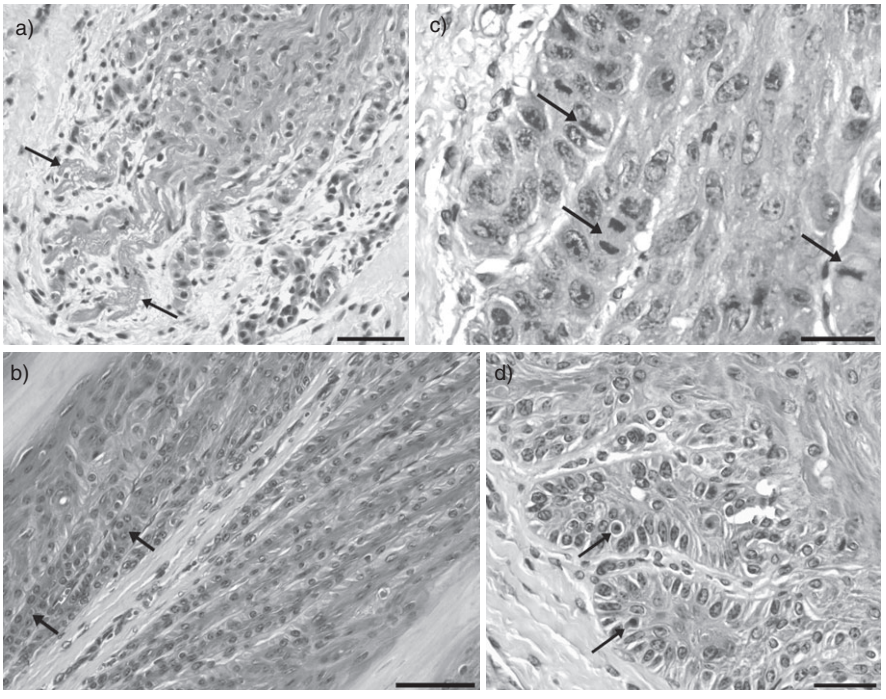

Fig 2: Histological lesions in hoof lamellar tissue obtained from a pony following prolonged infusion of insulin. (a) Collapsed tubes of basement membrane material (arrows) from secondary epidermal lamellae at the axial aspect of a primary epidermal lamella (arrows), with a few neutrophils scattered within. Bar = 100 μm. (b) Epidermal cells containing intracytoplasmic eosinophilic material, indicating inappropriate keratinisation (dyskeratosis; arrows). Bar = 100 μm. (c) Epidermal cells of secondary epidermal lamellae at the axial aspect of a primary epidermal lamella, containing significantly increased numbers of mitotic figures (arrows). Bar = 50 μm. (d) Increased numbers of apoptotic cells in secondary epidermal lamellae (arrows). Bar = 50 μm. Haematoxylin and eosin. Note: This pony had the most severe histological lesions in the treatment group.

(400×) within SEL cells, some of which had a bizarre appearance (Fig 2c). The mitotic figures were most numerous in, but not confined to, SEL at the PEL tips, and their numbers varied significantly between ponies. The number of apoptotic epidermal cells was significantly increased (up to 30 per high power field) and these were noted at SEL-PEL interfaces in addition to within SEL basal layers. The number of apoptotic cells was highly variable between ponies (Fig 2d).

The number of lymphocytes around blood vessels in the dermis was slightly increased, with greater prominence of plasma cells and slight expansion of perivascular stromal tissue by small clear spaces with dilation of lymphatics (oedema). Occasional dermal veins contained small fibrin thrombi.

Measurement of primary and secondary lamellae following induction of laminitis with insulin

The inter-rater agreement for measuring PELL was 0.97 (Lin's concordance correlation coefficient; 95% CI: 0.96–0.98), indicating substantial observer agreement, with negligible random variation (Pearson's r: 0.98) and bias (C_b: 0.99). The LOA measure for PELL (average difference 69.2 μm, 95% LOA: -172.3, 310.7) did not indicate significant average departure from agreement. The inter-rater agreement for measuring SELL was 0.57, 0.53 and 0.61 at 10, 50 and 90% of the associated PEL, respectively (95% CI: 0.53–0.61 at 10%, 0.48–0.58 at 50% and 0.56–0.65 at 90%). The moderate, but not high concordance between observers for measurements of SELL was due mainly to random variation (Pearson's r: 0.64, 0.53, 0.61 for 10, 50 and 90% measurements, respectively), rather than bias (C_b: 0.89, 0.95 and 0.97 for 10, 50 and 90% measurements, respectively). The LOA measures for SELL at 10% (average difference 51.91 μm; 95% limits of agreement: -129.1, 232.9), 50% (average difference 41.8 μm; 95% LOA: -203.1, 286.6) and 90% (average difference 26.05 μm; 95% LOA: -195.8, 247.9) of the associated PEL indicated that the average difference between observers was only 26–50 μm and, for most samples, the results for the 2 operators differed by less than 150–250 μm.

Mean PELL in ponies with laminitis (3558 ± 212 μm) ponies were not significantly higher than in control ponies (2848 ± 245 μm; P = 0.07). In contrast, mean SELL was significantly increased in laminitic tissue when compared with controls at 10% (240 ± 31.4 μm vs. 113 ± 16.5 μm), 50% (323 μm ± 27.2 vs. 190 ± 38.6 μm) and 90% (302 ± 40.2 μm vs. 196 ± 2.7 μm) regions of PEL (P<0.001) (Table 1). The elongation was most prominent in SEL closest to the hoof wall at the abaxial (90%) and middle (50%) regions, where the basement membranes were intact. Secondary epidermal lamellae at PEL tips were stretched to a lesser extent but variable loss of basement membrane was evident as described above (Fig 3).

Immunolabelling for calprotectin

There was positive labelling of leucocytes in equine hoof lamellar dermis obtained from all treated ponies, but not control specimens.

Discussion

This is the first study to describe the histological appearance of laminitis induced by prolonged infusion of insulin, i.e. endocrinopathic laminitis. Our results indicate that while there were some similarities between the histological appearance of

TABLE 1: Ratio of the mean secondary epidermal lamellar length (SELL) for ponies with laminitis induced by prolonged hyperinsulinaemia (n = 5), compared to SELL in control ponies (n = 4). SELL was measured by two blinded observers at three regions of primary epidermal lamellae 10, 50 and 90%, corresponding to the axial, middle and abaxial regions, respectively

	Observer 1		Observer 2	
Region	Ratio of SELL insulin-treated : control	95% Confidence interval	Ratio of SELL insulin-treated : control	95% Confidence interval
Axial	1.39*	1.05–1.84	1.55**	1.25–1.92
Middle	1.73**	1.31–2.31	1.81**	1.46–2.24
Abaxial	1.80**	1.36–2.39	1.55**	1.25–1.92

*P<0.05; **P<0.001.

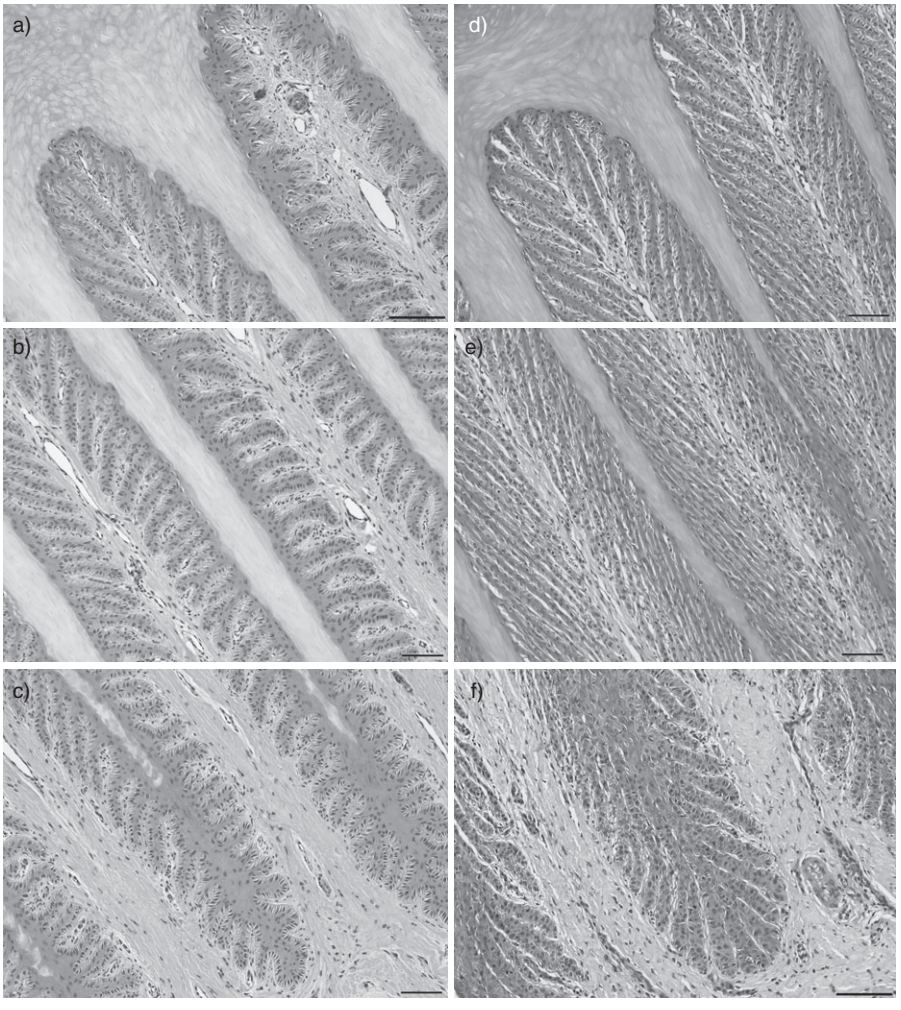

Fig 3: Examples of SEL elongation in a treated pony vs. control pony. (a) Abaxial SEL (90%, at the base of the PEL) of the control pony. (d) Abaxial SEL of the treated pony. (b) SEL in the middle of the PEL (50%) of the control pony. (e) SEL in the middle of the PEL of the treated pony. (c) Axial SEL (10%, tip of the PEL) of the control pony. (f) Axial SEL of the treated pony. Haematoxylin and eosin. Bar = 100 μm. The elongation is most severe in abaxial (90%) and middle regions (50%); however, the basement membranes are intact. PEL = primary epidermal lamellar; SEL = secondary epidermal lamellar.

insulin-induced laminitis and other experimentally induced forms of the disease, there were also considerable differences, reflecting the different initiating cause of this form of laminitis. The histological appearance at acute stages of both the CHO overload (Garner *et al*. 1975; Pollitt 1996; Morgan *et al*. 2003) and the BWE models (Galey *et al*. 1991) of experimental laminitis have been described, with some cases of naturally-occurring disease also described (Roberts *et al*. 1980). However, the different time points examined and small number of horses in each study make direct comparisons difficult. As for lesions reported in CHO overload laminitis (Pollitt 1996; Morgan *et al*. 2003), laminitis induced by prolonged infusion of insulin demonstrated elongated SEL with pointed tips often difficult to distinguish from adjacent lamellae, with irregular, poorly defined PEL/SEL interfaces, cytoplasmic vacuolation in SEL predominantly involving the PEL tips (axial region), neutrophil infiltration around the PEL tips (axial region), variable numbers of mitotic figures and variably sized epidermal nuclei; more rounded in shape and tended to be oriented differently within the cell and in terms of the basement membrane compared with controls. In the current study, cellular and basement

membrane changes were more severe in SEL located along the (axial) PEL tips while elongation was more prominent in SEL located along the middle region of the PEL (closest to the stratum medium of the hoof wall). The increased cellular and basement membrane changes found axially are consistent with the findings of Pollitt (1996) using CHO overload-induced laminitis, but opposite to that observed by Morgan *et al*. (2003) using the same CHO overload model as Pollitt (1996). The reason for the discrepancy is unclear and may be related to the fact that the samples were collected at different time points (48 vs. 72 h, respectively). However, in a more recent paper using oligofructose overload-induced laminitis, samples collected 7 days following induction of laminitis also demonstrated more severe cellular and basement membrane changes axially (van Eps and Pollitt 2009). Morgan *et al*. (2003) propose that the more severe lesions (including pyknosis, i.e. cell death and basal cell 'dissolution') seen abaxially in their model support a vascular pathogenesis, as the most severe changes in their model occur in areas furthest from the primary vascular supply. The results of the current study do not support such a theory. However, in the current study swelling of cells at SEL tips, and mild axial perivascular oedema were also observed indicative of some vascular dysfunction.

This study was also the first to compare both PEL and SEL length in laminitic and healthy hoof tissue. In the present study SELL in laminitic tissue was increased by approximately 1.6-fold compared to that of controls in all three regions, but this difference was more pronounced in the middle and abaxial regions of the PEL. The lack of a significant increase in PELL is in contrast to the findings of a recent study, where a significant increase in total PELL was reported in horses 7 days after the induction of laminitis with oligofructose (van Eps and Pollitt 2009). The latter timeframe was different from the current study where horses were subjected to euthanasia from 6–12 h post induction of laminitis except for one pony where this was delayed for 5 days. Furthermore, the severity of the disease induced in the horses dosed with oligofructose was greater, such that 4 of the 6 horses reached Obel *grade 3* lameness, whereas the ponies in the current study were subjected to euthanasia at the onset of Obel *grade 2* lameness. Alternatively, the increased bodyweight of Standardbred horses compared with the ponies in the current study may have contributed to increased PELL.

Interestingly, the defining lesion of basement membrane disintegration and separation in CHO-induced laminitis (Pollitt 1996) was rarely observed in laminitis induced by prolonged infusion of insulin. Despite the elongation, the basement membrane appeared intact along SEL throughout the majority of the PEL in PAS-stained sections. Only slight separation of the basement membrane was observed along SEL at the most axial regions of the PEL. It is possible that more severe basement membrane pathologies might have been evident had the insulin infusions continued beyond the development of Obel *grade 2* laminitis. Alternatively, as the lamellae function as a suspensory mechanism for the distal phalanx during peak loading of the foot, it is possible that the lower bodyweight of the ponies in the current study might have also been a factor in preventing basement membrane separation, compared to the horses used in other studies. Another explanation may be related to the pathogenesis of laminitis induced by insulin compared to laminitis induced by CHO; with only the latter being associated with severe systemic inflammation, cytokine production (Belknap *et al*. 2007) and clinical illness in the affected horses. Whether this is merely a matter of degree of inflammation or a completely different pathogenesis remains to be determined. Yet the basement membrane of 10 horses subjected to euthanasia 72 h after administration of CHO was also found to be physically intact and attached to basal epithelial cells, despite considerable loss of integrity of the epidermal laminae (Morgan *et al*. 2003). Therefore, the definition of laminitis as separation of the basement membrane may need to be revised, or only applied to certain models.

Previous studies using CHO overload models of laminitis reported pyknotic nuclei, representing cell death (Pollitt 1996; Morgan *et al*. 2003). In the current study, apoptotic epidermal cell numbers were increased significantly at the SEL/PEL interface and within SEL basal layers in treated ponies compared with controls, although this was highly variable between sections and between ponies. Further, apoptotic cells were largely concentrated at PEL tips (axially) in the current study, which is in contrast to findings by Morgan *et al*. (2003) but similar to Pollitt (1996). However, only the end stages of apoptosis are visible morphologically in routinely stained histological sections. In a study using immunohistochemistry (caspase-3 positive) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) to identify apoptotic cells, horses with acute and chronic naturally acquired laminitis, and both BWE and CHO overload models were compared. The number of apoptotic cells was increased in horses with laminitis but most significantly in those with the acute natural disease (Faleiros *et al*. 2004). In that study there were no differences in apoptotic cell numbers between abaxial and axial PEL regions in horses from any group (Faleiros *et al*. 2004). However, the experimental horses were subjected to euthanasia relatively early, approximately 12–18 h following administration of walnut shavings or CHO, and the acute laminitis cases were only noted to be less than 1 week in duration, without information as to the potential cause of disease. This illustrates the point that pathological lesions must be described in the context of clear information regarding both pathogenesis and the time point at which the tissue is sampled.

In the current study, more mitotic figures were consistently observed in treated ponies, as was described in CHO overload-induced *grade 2* laminitis (Pollitt 1996), and they occurred in the same region; the SEL at PEL tips. The relative numbers of mitotic figures were not reported for CHO overload-induced laminitis, precluding a direct comparison. Hyperplasia has also been described as a variation in the lamellae of nonlaminitic Thoroughbred horses, although mitotic figures were not reported (Kawasako *et al*. 2009). Increased and/or abnormal mitotic figures are commonly used as a marker of proliferative activity. In the current study, such accelerated cell activity may have been related to the cellular apoptosis or basement membrane degeneration and is considered to have been an early healing response. In addition to the increased mitotic figures observed in this study, there was also increased keratinisation and dyskeratosis (abnormal and premature keratinisation) of epidermal cells within SEL layers. It might be possible that the proliferation occurred as a result of aberrant insulin signalling via the MAP-kinase pathway, which not only acts independently from the metabolic PI3K pathway, but is possibly overstimulated by hyperinsulinaemia, leading to increased cell proliferation (Kim *et al*. 2006).

Immunolocalisation of calprotectin in the lamellar dermis from treated ponies confirmed the presence of leucocytes identified by routine histology that had migrated into the lamellar dermis. This is consistent with observations in human skin, that calprotectin is either barely detectable or absent in normal skin, whereas

expression is increased significantly during inflammation (Kelly *et al*. 1989). It is also consistent with the recent observation that lamellar epidermal tissues and perivascular leucocytes stain positively following the induction of laminitis with BWE, compared with controls (Faleiros *et al*. 2009), as well as a report that leucocyte infiltration around the dermal lamellar vasculature occurs during the developmental stage of BWE-induced laminitis (Black *et al*. 2006). The authors postulated that the increased calprotectin staining at the development of BWE-induced laminitis provides support for the hypothesis that leucocyte infiltration precedes the lamellae epithelial damage observed in laminitis (Faleiros *et al*. 2009). The BWE model of laminitis has been compared with inflammatory injury observed in human sepsis, and is also associated with a more significant leucocyte influx than that observed in CHO overload-induced laminitis (Pollitt 1996; Belknap *et al*. 2007). Immunostaining for calprotectin was much stronger in the BWE model of laminitis compared to the present study supporting the lack of systemic inflammatory response. Again, this may be related to a lesser degree of inflammation in insulin-induced laminitis where the leucocyte infiltration seen was a secondary event, in response to the initial injury or a completely different pathogenesis. However, the current study only represents a single time point and further studies using earlier time points are warranted to support this. Interestingly, recent research indicates that the MAP-kinase signalling pathway may be involved in neutrophil migration during laminitis (Eckert *et al*. 2009). As discussed above, MAP-kinase is stimulated by insulin and appears to be involved in insulin-stimulated cell proliferation in various tissues, as well as being activated by various cytokines and inflammatory mediators. Thus, the involvement of MAP-kinase in laminitis resulting from prolonged insulin infusion warrants further investigation.

The histological and immunohistological appearance of laminitis induced by prolonged infusion of insulin exhibited many similarities to other forms of experimentally induced laminitis. However, there were some significant differences, in particular the absence of widespread basement membrane disintegration and separation. Defining equine laminitis entirely in terms of separation of the basement membrane may not be appropriate for insulin-induced laminitis, at least in ponies. Further characterisation of the lamellar lesions associated with insulin-induced laminitis at different time points during disease development is warranted, as is comparison with lesions in ponies with naturally occurring laminitis associated with hyperinsulinaemia.

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Manufacturers' addresses

[1]Eli Lilly and Company, Indianapolis, USA.
[2]Cybernetics, Silver Spring, Maryland, USA.
[3]Abcam, Cambridge, UK.
[4]DakoCytomation, Carpinteria, California, USA.
[5]Sigma, St Louis, Missouri, USA.
[6]StataCorp, College Station, Texas, USA.

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