Equine Laminitis: Comparative Histopathology 48 h after Experimental Induction with Insulin or Alimentary Oligofructose in Standardbred Horses

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Summary

Laminitis has many triggers and comparing the histopathology of lesions induced by different causes may help to establish whether a common mechanism or multiple pathologies are involved. The aim of this study was to describe the microscopical lesions and to quantify morphometric changes in the lamellae of horses with insulin-induced (n = 4) and oligofructose-induced laminitis (n = 4) compared with normal controls (n = 4). Archived lamellar samples collected during two previous studies were used. Laminitis was induced within 48 h in standardbred horses with either a euglycaemic, hyperinsulinaemic clamp (EHC) technique, or in a separate experiment, with an overdose of alimentary oligofructose (OF). Normal tissue was obtained from control horses in the EHC experiment that received a balanced electrolyte solution intravenously for 48 h. Six measurements of lamellar length and width were recorded for each hoof. Leucocyte infiltration was assessed by immunolocalisation of calprotectin. All control horses exhibited normal lamellar architecture, whereas treated horses developed clinical and histopathological changes consistent with laminitis. Laminitic samples displayed lengthening and narrowing of secondary epidermal lamellae (SELs), rounded epidermal basal cell nuclei, mitosis and apoptosis. In the fore feet of laminitic horses, the length from the end of the keratinised axis to the axial tip of the primary epidermal lamellae (PELs) was increased ($P < 0.05$). SELs were significantly longer ($P < 0.05$) and narrower ($P < 0.05$) in the treated horses compared with controls. The two treated groups did not differ from each other in SEL length or width. Calprotectin expression was absent in control horses, moderate in hyperinsulinaemic horses and marked in oligofructose-treated horses. Laminitis induced experimentally with insulin or oligofructose results in comparable

lengthening and narrowing of the SELs and elongation of the axial end of the PELs at 48 h. Immunolocalization of calprotectin indicated that hyperinsulinaemia induces less leucocyte emigration than carbohydrate overload at 48 h. The microscopical lesion of laminitis is similar, but not identical in different forms of the disease.

Keywords: hyperinsulinaemia; histology; epidermal lamellae; calprotectin

Introduction

Risk factors for equine laminitis are diverse, and can be associated with alimentary carbohydrate overload (ACO), sepsis, inflammation and endocrine disease. The clinical outcome of lameness may be the same, but the underlying pathogenesis of the lamellar damage is incompletely understood. Whether different causes of laminitis ultimately trigger a common pathophysiological mechanism, despite resulting from disparate pathways, is also unclear. Scientists have long sought to better describe the pathophysiological processes occurring within lamellar structures in the prodromal and acute stages of this unique disease, but progress has been slow. Experimental models allow analysis of the acute stages of laminitis and relate to the risk factors listed above. Extract of black walnut (BWE) is said to mimic systemic inflammatory response syndrome (SIRS), (Galey *et al.*, 1991; Belknap *et al.*, 2009; Eades, 2010) while ACO with starch (Garner *et al.*, 1975) or oligofructose (OF) (van Eps and Pollitt, 2006) simulates SIRS secondary to colitis. More recently, the use of a prolonged euglycaemic, hyperinsulinaemic clamp (EHC) (Asplin *et al.*, 2007) has provided a way to model hyperinsulinaemic laminitis (the insulin-induction model), which occurs without clinical evidence of systemic illness or inflammation.

Histological descriptions of experimentally-induced laminitis (Pollitt, 1996; Morgan *et al.*, 2003; Faleiros *et al.*, 2004; van Eps and Pollitt, 2009; Asplin *et al.*, 2010) have defined features of the laminitis lesion and have enhanced appreciation of the pathophysiological processes involved. Conversely, histological accounts of naturally-occurring laminitis are scant (Roberts *et al.*, 1980; Ekfalck *et al.*, 1992). An accurate description of various types of experimentally induced and naturally-

occurring cases of laminitis is important in order to understand the disease more fully. Reports include histological descriptions (Pollitt, 1996; Morgan *et al.*, 2003), assessment of the degree of apoptosis (Faleiros *et al.*, 2004) and leucocytic infiltration using calprotectin immunohistochemistry (Visser, 2008; Faleiros *et al.*, 2009). Calprotectin is a calcium binding protein complex that can be found in the cytoplasm of neutrophils and on the membrane of monocytes (Striz and Trebichavsky, 2004). Calprotectin expression by keratinocytes is increased in human inflammatory dermatoses (Kelly *et al.*, 1989).

Endocrinopathic and pasture-associated forms of laminitis occur commonly (USDA, 2000; Geor, 2009). The unifying factor in endocrinopathic (including equine metabolic syndrome) and pasture-associated laminitis is hyperinsulinaemia (Treiber *et al.*, 2006; McGowan, 2008). Understanding how excess insulin damages lamellar integrity may enhance the ability to prevent laminitis. The present study describes the acute laminitis lesions of prolonged hyperinsulinaemia and compares them with those induced by dosing with OF in standardbred horses of a similar bodyweight. This is the first study to describe the lesions of insulin-induced laminitis in detail in horses and the first direct histological comparison between the ACO and EHC models of laminitis. The differences were quantified by primary epidermal lamellar (PEL) and secondary epidermal lamellar (SEL) morphometry and the presence of lamellar leucocytes was confirmed with calprotectin immunohistochemistry (IHC).

Materials and Methods

Horses

Archived lamellar samples from two previous experiments were used in this study (van Eps and Pollitt, 2006; de Laat *et al.*, 2010). In one experiment, laminitis was induced within 48 h in four healthy standardbred horses (427 ± 34 kg), aged 5.8 ± 1.3 years, using a continuous infusion of insulin (Humulin-R™; Eli-Lily Australia, West Ryde, New South Wales, Australia) at a rate of 6 mIU/kg bwt/min, with glucose (50 % dextrose; Baxter Healthcare, New South Wales, Australia) administered concurrently at a variable infusion rate to maintain euglycaemia. Each treated horse was paired with a matched control standardbred horse (432 ± 14 kg; 5.1 ± 0.7 years) that received a balanced electrolyte solution at a rate of 0.57 ml/kg bwt/hr for the same period under identical conditions (de Laat *et al.*, 2010).

Laminitis was induced in a separate experiment in healthy standardbred horses (n = 6) after dosing with OF (Raftilose P-95; Orafti Active Food Ingredients, Tienen, Belgium) at a rate of 10 g/kg bwt (dissolved in 4 l water and administered via nasogastric tube), and four of these horses (451 ± 7.9 kg; 7.0 ± 1.8 years) were selected randomly for this study (van Eps and Pollitt, 2006). Mean ± SE serum insulin concentration at the conclusion of the experiment was higher in the insulin-treated horses (1179 ± 228 µIU/ml) when compared with the OF-treated (16.2 ± 2.3 µIU/ml) and control horses (10.6 ± 1.2 µIU/ml).

All horses had normal radiographic anatomy and no evidence of lameness or hoof abnormalities on clinical inspection, prior to the experiments. Clinical signs of lameness began at 31.5 ± 4.65 h in the insulin-treated horses and 28 ± 1.83 h in OF-treated horses. Horses were humanely destroyed at the onset of grade 2 Obel (Obel, 1948) lameness (insulin group; 46 ± 2.3 h) or at 48 h (OF and control groups). The

Obel grade of laminitis for the OF group was 2.5 ± 0.3 (two horses grade 2 and two horses grade 3). The control horses did not display any clinical signs of lameness.

Ethical approval for this work was granted by the animal ethics committee of The University of Queensland (SVS/108/09/RIRDC) which ensures compliance with the Animal Welfare Act of Queensland (2001) and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition, 2004). The horses were monitored continuously by a registered veterinarian.

Sample Processing

Full thickness lamellar specimens (5 × 5 mm) were placed immediately in 10% neutral buffered formalin for 24 h before being processed routinely. Following embedding in paraffin wax, lamellar specimens were sectioned (5 µm), mounted on Superfrost Plus™ slides (Menzel, Braunschweig, Germany) and either left unstained for IHC or stained with haematoxylin and eosin (H&E) or periodic acid–Schiff (PAS) for light microscopy.

Histological Analysis

H&E and PAS stained sections from all four feet of all horses were randomized and examined via light microscopy (Olympus BX-50; Olympus Corporation Lifesciences, Japan) by one of the authors experienced in describing lamellar histopathology (CCP), who was blinded to the group of origin. Randomization of all sections was then repeated prior to measurement of the primary and secondary epidermal lamellae by a different author (MAD) according to the following protocol. Six sets of measurements were made on each section using image

analysis software (ImagePro; Media Cybernetics Inc, Bethesda, MD): total primary epidermal lamellar length (TPELL), keratinised primary epidermal lamellar length (KPELL), length of the axial tip of the PEL, SEL length of SELs located at the base (abaxial, SELLB) and tip (axial, SELLT) of each selected PEL, and SEL width in the mid-section of the PEL (SELW). Two of these parameters (axial tip length and SEL width) have not, to our knowledge, been measured before. Eight PEL were measured twice for each hoof at ×20 magnification to determine KPELL and TPELL.

Measurement of each PEL (Fig. 1a) was made in a straight line from the tip of the adjacent primary dermal lamella (PDL) to the end of the keratinised axis (KPELL) or the middle of the tip (TPELL). The difference between the TPELL and KPELL was then calculated in order to quantify the length of the axial end of the PELs in each horse (Fig. 2). The length of ten, randomly-selected SELs was measured at ×100 magnification in both the lower (abaxial; 10–30%) and upper (axial; 70–90%) third of each selected PEL (Fig. 1b). The width of twenty SELs was measured at ×200 magnification in the middle section (30–70%) of each PEL (Fig. 1b). In order to ensure the integrity of the measurement protocol, 50% of the sections were selected at random and measured again, according to the above protocol, by a blinded independent party. These measurements were not included in the results, but were used solely to internally validate the measurement protocol.

Immunohistochemistry

IHC was performed using the EnVision+ System-HRP (Dako, Carpinteria, California) according to the manufacturer's instructions. Antigen retrieval was performed using 0.05% proteinase K ready-to-use solution (Dako) for 6 min at room

temperature. Monoclonal mouse anti-human macrophage antibody (MAC-387; Dako) was diluted 1 in 400 in antibody dilution solution with background reducing components (Dako). Mouse IgG was used in place of the primary antibody for a negative control. Positive control tissue was taken from a horse with ACO laminitis (Visser, 2008). Slides were incubated with the chromogen 3, 3'-diaminobenzidine tetrahydrochloride (DAB; Dako) for 8 min and counterstained with Mayer's haematoxylin for 1 min.

Grading of Calprotectin Expression

Immunoreactivity for calprotectin was analysed in samples from all feet, in blinded fashion by one of the authors (CCP). The degree of calprotectin expression in the lamellae of each hoof was graded using the following scale: 0, no labelling or occasional calprotectin-positive cells within vessel walls; 1, mild perivascular infiltration of labelled cells into primary dermal lamellar tissue; 2, moderate, diffuse infiltration of labelled cells throughout the dermal connective tissue; 3, marked tissue infiltration of labelled cells with positive labelling of the SEL basal and parabasal cell cytoplasm.

Statistical Analyses

Within each group, epidermal lamellar measurements for both fore feet were averaged and compared with the average measurement for the hind feet, using a Welch t-test. Epidermal measurements of both fore feet were compared between groups using two-way analysis of variance (ANOVA), with horse number and group used as the two class variables to account for clustering. Pair-wise comparison of means was assessed with Tukey's honestly significant difference test. Within groups,

average fore foot SELL was compared between axial and abaxial locations using a t-test. The level of agreement between observers was assessed with Bland-Altman's limits of agreement (LOA) and Lin's concordance co-efficient (ρ_c) where both co-variation and correspondence are used as a measure of reliability. Pearson's correlation coefficient was used to assess the random variation of inter-rater agreement. Median scores for tissue immunoreactivity to calprotectin for each horse were compared between groups using a Kruskal-Wallis one-way ANOVA on ranks. Median fore and hind foot calprotectin score was compared within each group using a Wilcoxon test. All data are presented as mean ± SE or median (interquartile range). Statistical significance was set at $P < 0.05$. Statistical analyses were performed using R version 7.2.7 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Lamellar Histology

Control Horses: All of the control horses exhibited normal lamellar architecture in sections from all four feet (Figs. 1a–c). Microscopical descriptions of normal equine lamellae have been published previously (Pollitt, 1994; Pollitt, 2004; Asplin *et al.*, 2010) and the lamellae from the control horses in the current study were consistent with these descriptions. Descriptive histology was similar between the fore and hind feet of all control horses.

Insulin-Induced Laminitis: All hyperinsulinaemic horses developed laminitis within the 48 h period, with three out of four horses developing laminitis in all four feet. The

lamellae appeared normal in the right hind foot of the horse with the lowest bodyweight, but laminitis lesions were present in the other three feet (de Laat *et al.*, 2010). The PEL of the hyperinsulinaemic horses showed varying degrees of dyskeratosis (abnormal, premature or imperfect keratinization of EBCs), particularly in the axial regions. Disorganization of SELs was widespread throughout the abaxial, mid and axial regions of the PEL. The SEL appeared lengthened with sharp, pointed tips and a loss of symmetrical angulation to the PEL axis. Many SELs were wider at the base, tapering toward the tip, while some were uniformly narrowed and had become either undulant or crescent shaped (Figs. 1d, e). Clear demarcation of individual SELs at the PEL axis was lost; with secondary epidermal basal cells becoming confluent (Fig. 1e). Many epidermal cell nuclei appeared rounded, with prominent and often multiple nucleoli and many had lost their apical position in the EBC cytoplasm. Apoptotic EBCs and mitotic figures occurred more frequently than in control sections and were seen throughout the axial and mid-sections of the PEL (Fig. 1d inset). Pathological changes tended to be more severe at the axial tips of the PELs, adjacent to the distal phalanx (Fig. 1f). SELs in this region were often severely disorganized and many were devoid of basal cells, yet still outlined by PAS positive BM (Fig. 1f). Empty teat-shaped profiles of PAS positive BM, no longer adherent to EBCs, were present at the tips of attenuated SELs (Fig. 1f inset). This BM dysadhesion from EBCs, and patchy loss of PAS positive BM staining, occurred in the front feet of all horses, but only rarely in hindlimb sections. BM compromise varied in severity between individuals, from showing occasional, mild loss of BM integrity at the tips of axially located SELs in the least affected horse, to BM changes affecting many SELs along the length of the PEL, in the front feet of the two most severely affected horses. The endothelial cells of SDL capillaries were swollen and

protruded into the lumina of vessels. Red blood cells had extravasated into many SDLs and leucocytes were present both within blood vessels and in the connective tissue surrounding them. Infiltrating leucocytes were common in the dermal tissue around the tips of the PELs and some had penetrated SELs empty of EBCs (Fig. 1f). In general, the laminitis lesions were similar in both the fore and hindlimbs of all horses; however, severity was greater in the fore feet.

Oligofructose-Induced Laminitis: Laminitis histopathology of the OF-treated horses showed many similarities to the wheat starch overload model (Pollitt, 1996) and the hyperinsulinaemic horses. Marked attenuation of the SELs along the length of the PEL was accompanied by cellular changes including increased mitosis, apoptosis and rounding of EBC nuclei. There was evidence of BM detachment and disintegration along the length of the SELs, especially at the base of the SEL adjacent to the PEL axis, and of the SEL tips in the axial region of the PELs, in all of the OF-treated horses (Fig. 2b). BM damage appeared to be more widespread and severe in the OF-treated horses than in the hyperinsulinaemic horses, comparable with the wheat starch model (Pollitt, 1996).

Morphometry

Overall, the level of agreement between the two observers for both the PEL and SEL measurements was high (Table 1). Total and keratinized PELL were increased in forelimb hooves compared with the hindlimb hooves ($P < 0.05$), in the insulin-treated group (Table 2). SEL measurements were not different between the fore and hind feet (Table 2). As this is the first detailed morphological study of insulin-induced laminitis to include the hind feet, in order to allow accurate

comparison with previous studies, PEL and SEL measurements were compared between the three groups using the forelimbs only. Neither total nor keratinised PELL differed between any of the groups (Table 2); however, the distance from the end of the keratinised axis to the axial tip of the PELs was increased in laminitic horses compared with control horses (Fig. 2). SEL length was increased ($P < 0.05$) abaxially and axially on the PEL, in both the insulin- and OF-treated groups, when compared with the control group (Table 2). SEL length did not differ between the laminitic groups at either location (Fig. 3). Axial SEL length was not different to abaxial SEL length in any group. Although the SEL were wider ($P < 0.05$) in the control group compared with both laminitis groups (Table 2), the width of the SEL did not differ between the two treated groups.

Calprotectin Expression

Immunolocalization of calprotectin in control horses was limited to an occasional leucocyte confined within venules in the dermal connective tissue (grade 0; Fig. 4a), in both fore and hind feet. In the insulin-treated group there was an increase in the number of calprotectin-positive cells (identified as neutrophils and monocytes on HE-stained sections) located around vessels in the primary dermal lamellae and in the lamellar dermis, around the tips of each PEL (grade 1; range 0.6 - 1.0; Fig. 4b). The lamellar EBC cytoplasm also showed occasional, mild, positive expression of calprotectin at the tips of SELs located at the axial tip of the PEL. Calprotectin-positive cells were also located within the lamellar BM (Fig. 4b). The expression of calprotectin was stronger ($P < 0.05$) in fore foot sections (grade 1.8; range 1–2) when compared with the hind feet (grade 0; range 0–0.3) in the insulin-treated horses, with most of the hindlimb sections showing little to no calprotectin expression. The OF-

induced laminitis group showed diffuse, heavy calprotectin labelling in both the lamellar epidermal basal cells, predominantly in the axial third of the PEL, and cells in the dermis surrounding the tips of the PEL (grade 3; range 2.9–3.0; Fig. 4c), with stronger calprotectin expression than both the control and insulin-treated horses ($P < 0.05$). Strong expression of calprotectin was seen in both the cytoplasm and the nuclei of the lamellar epidermal basal cells (Fig. 4c). Both the fore and hind feet were affected equally in the OF-treated horses (grade 3 [range 3–3] and grade 3 [2.8 – 3], respectively).

Discussion

The histological features of different types and stages of laminitis have been described previously (Pollitt, 1996; Morgan *et al.*, 2003; French and Pollitt, 2004; Visser, 2008; van Eps and Pollitt, 2009; Asplin *et al.*, 2010). The results of the present study add to this field of knowledge by describing lesions resulting from insulin-induced laminitis in standardbred horses for the first time, and by comparing lamellar morphometry and calprotectin immunoreactivity 48 h after the induction of laminitis with hyperinsulinaemia and OF.

The measurement protocol was similar to that used by Asplin *et al.* (2010) who described lesions in ponies with insulin-induced laminitis. Despite the different time points examined (46 versus 55 h), the similarities in experimental design and the Obel grade of laminitis examined should allow reasonable comparisons with the current study. Many histopathological features of insulin-induced laminitis were similar in horses and ponies; however, the degree of BM damage was more extensive

in horses than reported in ponies. Significant elongation of SELs was a consistent finding in both horses and ponies with insulin-induced laminitis and in horses with OF-induced laminitis, when compared with normal horses. Separation of the BM was variable between ponies treated with insulin, and when it did occur, was confined to SELs at the axial tips of the PEL (Asplin *et al.*, 2010). In the current study, BM disintegration and dysadhesion in SELs ranged from mild to extensive, but was present in all insulin-treated horses, appeared more widespread and involved a greater proportion of SELs. This increased degree of BM pathology may be due to the greater bodyweight of horses exerting more strain on the lamellae, compared with ponies. The difference was not associated with the Obel grade of lameness since both horses and ponies were only treated until the onset of Obel grade 2 lameness. However, in insulin-treated horses the degree of clinical severity did differ marginally within this grade. Indeed, the horses that exhibited more frequent shifting of bodyweight in the current study also exhibited more severe BM disruption. The finding of BM separation in this study is consistent with previous descriptions of laminitis in horses associated with ACO and systemic inflammation, albeit to varying degrees (Pollitt, 1996; Morgan *et al.*, 2003; French and Pollitt, 2004; Bailey *et al.*, 2009). Further studies have shown that enzymatic processes, such as unregulated metalloproteinase activity, are unlikely to be an inciting cause of BM disruption in insulin-induced laminitis (de Laat *et al*., 2011), while they have been associated with BM dysadhesion in OF-induced laminitis (Kyaw-Tanner *et al.*, 2008). This may suggest that physical disruption of the BM, rather than enzymatic destruction, is a feature of hyperinsulinaemic laminitis, however further studies to investigate this theory are required. Variably increased numbers of mitotic figures and apoptotic cells in the lamellar epidermis were a feature of insulin-induced laminitis, both in this study

and in ponies (Asplin *et al.*, 2010). Increased mitosis is potentially due to hyperinsulinaemia causing over-stimulation of the intracellular mitogen-activated protein kinase signalling pathway, which is responsible for cell proliferation (Cusi *et al.*, 2000). However, the finding also occurs in other forms of laminitis (Pollitt, 1996; Faleiros *et al.*, 2004) and may reflect general reparative processes.

By including all four feet from each horse, this study allowed direct comparison of fore and hindlimb lamellar pathology, including quantification of SELL and PELL and the degree of leucocyte infiltration during laminitis. Total PELL was greater in the fore feet compared with the hind feet in insulin-treated horses, but not in the OF-dosed or control group, although it is likely that failure to achieve statistical significance in the latter two groups was a consequence of the small sample size, considering the strong trend towards increased fore foot PELL in all groups. Fore and hind foot lamellar histopathological scores have been compared previously in OF-induced laminitis (van Eps and Pollitt, 2006), where it was found that the histological score given to the front feet was always equal to, or more severe than, the hind feet of the same horse. The observation that lesions tended to be more severe in the fore feet in the current study may be related to weight bearing. It has been documented that horses bear up to 60% of their body weight on their front feet compared with 40% on their hind feet (Hood, 1999). A larger sample size would be necessary to determine if this finding is applicable to the wider population. Due to the fact that PELL was not the same for fore and hind foot measurements, and to conform to previous studies, only fore foot data were used when comparing morphometry between the groups.

PELL was not increased at the 48 h time point in insulin-induced or OF-induced laminitis, which is in agreement with insulin-induced laminitis in ponies (Asplin *et al.*, 2010). However, the length of the axial end of the PELs was increased in both the OF and insulin-treated horses compared with the control group, reflecting the severity of the lesions (BM dysadhesion and SEL disorganization) in this location. When TPELL was measured 7 days after OF dosing, a significant difference was recorded between control and OF-treated horses, indicating that an increase in TPELL may occur at later time-points (van Eps and Pollitt, 2009). Cellular proliferation and attempts at tissue repair at the tips of the PEL, especially around areas of detached BM, may account for this overall increase in TPELL in chronic disease.

The finding of significantly elongated SEL at both the base (abaxial) and tip (axial) of the PEL in both groups of treated horses, confirms previous descriptions of laminitis (French and Pollitt, 2004; de Laat *et al.*, 2010) and supports the findings of Asplin *et al.* (2010) in ponies. An important finding here is that SEL length was similar in both the insulin and OF models of laminitis at 48 h, both axially and abaxially, despite quite marked differences in leucocyte recruitment demonstrated using calprotectin. It remains to be determined whether structural or cellular changes are more important in determining the clinical severity of laminitis, but at least structurally, the lesions produced by the two experimental models did not differ greatly in severity from each other at 48 h. Whether this similarity in SEL length would persist at higher grades of laminitis, or at later time-points in the disease, is unknown.

To our knowledge, the current study is the first to measure SEL width in any form of laminitis. The width of the SEL in the middle section of the PEL was substantially reduced in both hyperinsulinaemic (1.9 fold) and OF-induced (1.7 fold) horses compared with normal controls. This finding also confirms previous descriptive reports of insulin-induced (de Laat *et al.*, 2010) and OF-induced (French and Pollitt, 2004) laminitis in horses. This reduction in SEL width may be attributable to changes in shape of the epidermal basal cells and may also result from the general disorganization of SELs in laminitic horses. Temporal information from the developmental stages of insulin-induced laminitis will add to this field of knowledge. Histological analysis of chronic cases of pasture-associated laminitis will also be important in investigating the nature of the hyperinsulinaemic lamellar lesion.

The lack of calprotectin expression in the lamellae of control horses is consistent with findings in other studies where lamellar sections from normal or control horses show little or no calprotectin labelling (Visser, 2008; Faleiros *et al.*, 2009). This finding is also in agreement with studies showing little or no calprotectin expression in the normal, human integument (Kelly *et al.*, 1989). Stronger expression of calprotectin in the fore feet of the insulin-induced horses, when compared with hind feet, is consistent with our earlier observation that lesions tended to be more severe in the front feet. A comparison between the insulin- and OF-induced models shows much stronger and more widespread calprotectin immunolocalization in the OF group. This difference in calprotectin immunoreactivity, up to 48 h, is possibly associated with the severity of the laminitis (insulin; Obel grade 2 versus OF; Obel grade 2.5) or may indicate a difference in the type of lamellar lesion between the experimental models. OF-induced laminitis appears to produce a more inflammatory

lesion, similar to BWE-induced laminitis that has been likened to SIRS in man (Faleiros *et al.*, 2009). Indeed, both the BWE and OF methods of laminitis induction are associated with signs of systemic illness such as depression or anorexia (Galey *et al.*, 1991) and diarrhoea and fever (van Eps and Pollitt, 2006). An inflammatory response has also been detected in affected lamellae (Belknap *et al.*, 2007; Leise *et al.*, 2011). In contrast, laminitis caused by the insulin-induction model does not result in systemic illness (de Laat *et al.*, 2010), indicating that hyperinsulinaemia may be less inflammatory than either OF or BWE, and result in less lamellar inflammation, as suggested by the subdued calprotectin response in the current study. Studies on early and developmental stages of BWE laminitis have shown a significant increase in calprotectin-positive cells (Faleiros *et al.*, 2009) and CD163-positive macrophages (Faleiros *et al.*, 2011) in lamellar tissue as early as 1.5 h post induction. This differs considerably to the findings of a temporal study of OF-induced laminitis, where calprotectin expression was first detected between 18 and 30 h post induction (Visser, 2008). The authors of the BWE studies postulated that leucocyte infiltration played a developmental role in the BWE-induced lamellar lesion. However, findings from the temporal OF study showed that leucocyte infiltration occurred after changes in BM pathology were underway, thus downplaying an early pathogenic role for leucocytes. A study of calprotectin immunolocalization at earlier time-points in hyperinsulinaemic horses would help to demonstrate the temporal pattern of leucocyte invasion in this type of laminitis.

Overall, the findings of this study contribute to the current available data on the lamellar damage sustained by insulin-treated horses, and provide the first direct histological comparison between the insulin- and OF-induced models of laminitis.

While there are major similarities between the lamellar lesions seen in the two groups, such as the extent of lengthening and narrowing of the SEL and the occurrence of BM damage, mitosis and apoptosis, there are also differences. The degree of calprotectin expression is more marked and widespread in the OF-induced model, which may suggest a greater role for inflammation in the lamellae of these horses when compared with the hyperinsulinaemic horses. Examining the histopathology of insulin-induced laminitis and comparing it to other forms of the disease may facilitate improved understanding in this complex field of research and allow the development of appropriate treatment strategies in the future.

Acknowledgments

This study was funded by the Rural Industries Research and Development Corporation, Australia. The authors thank A. Nourian for measuring the subset of tissue sections and R. Greer for statistical advice.

Conflict of Interest Statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Tables

Table 1: Agreement between two observers who each measured all of the parameters

Statistical test	*TPELL*	*KPELL*	*SELLB*	*SELLT*	*SELW*
Concordance (Lin's ρ_c)	0.94	0.89	0.80	0.92	0.84
(Lower 95% CI)	(0.92)	(0.86)	(0.77)	(0.90)	(0.81)
Pearson's r	0.94	0.90	0.81	0.92	0.84
(95% CI)	(0.92, 0.96)	(0.85, 0.93)	(0.76, 0.84)	(0.90, 0.93)	(0.80, 0.87)
Bland Altman (LOA)	15.4 µm	10.4 µm	3.75 µm	2.00 µm	0.27 µm
(lower and upper confidence limit)	(-134,165)	(-174,194)	(-67, 74)	(-70, 74)	(-7.8, 8.4)
% between 95% CI	93%	93.7%	89.5%	91.2%	95.6%

Agreement was determined with Bland-Altman's limits of agreement (LOA) and Lin's concordance co-efficient (ρ_c). Pearson's correlation coefficient was used to assess the random variation of inter-rater agreement (r [95% CI]). The percentage of measurements that fell between the 95% confidence intervals (CI) was also measured for each parameter. Overall, there was excellent agreement between the two observers. TPELL, total primary epidermal lamellar length; KPELL, keratinised primary epidermal lamellar length; SELLB, length of the secondary epidermal lamellae at the abaxial end of the PEL; SELLT, length of the secondary epidermal lamellae at the axial end of the PEL; SELW, secondary epidermal lamellar width at the midpoint of the PEL.

Table 2: Mean epidermal lamellar lengths

Length (µm)	*TPELL*	*KPELL*	*SELLB**	*SELLT**	*SELW**
Control					
fore	3335 ± 194	2965 ± 146	140 ± 19*	116 ± 23*	25 ± 2.5*
hind	2826 ± 195	2654 ± 235	137 ± 14	121 ± 6.5	23 ± 1.7
Insulin					
fore	3367 ± 54 †	2746 ± 59†	234 ± 14	256 ± 45	13 ± 1.5
hind	2708 ± 58	2226 ± 44	201 ± 18	216 ± 11	17 ± 3.3
Carbohydrate					
fore	3334 ± 187	2764 ± 178	237 ± 14	251 ± 27	15 ± 2.2
hind	2829 ± 204	2422 ± 138	229 ± 26	195 ± 18	17 ± 1.5

Mean ± SE epidermal lamellar lengths (µm) of three groups of horses (n = 4) were measured after 48 h of treatment with either a balanced electrolyte solution (control), a combined insulin and glucose infusion (insulin) or oral oligofructose (carbohydrate). Measurements were made in all four feet of each horse. Eight primary epidermal lamellae (PEL) were measured twice and total and keratinized lengths recorded. The length of ten secondary epidermal lamellae (SEL) was measured at both the base and tip of all eight PEL. The width of twenty SEL was measured in the mid-section of each PEL. TPELL, total primary epidermal lamellar length; KPELL, keratinised primary epidermal lamellar length; SELLB, length of the secondary epidermal lamellae at the abaxial end of the PEL; SELLT, length of the secondary epidermal lamellae at the axial end of the PEL; SELW, secondary epidermal lamellar width at the midpoint of the PEL. The asterisk denotes whether the forelimb measurements differ (P < 0.05) between the three groups for each parameter using two-way analysis of variance and Tukey's test. The † indicates if the forelimbs differ (P < 0.05) to the hindlimbs for each parameter within each group using a Welch t-test.

Figures

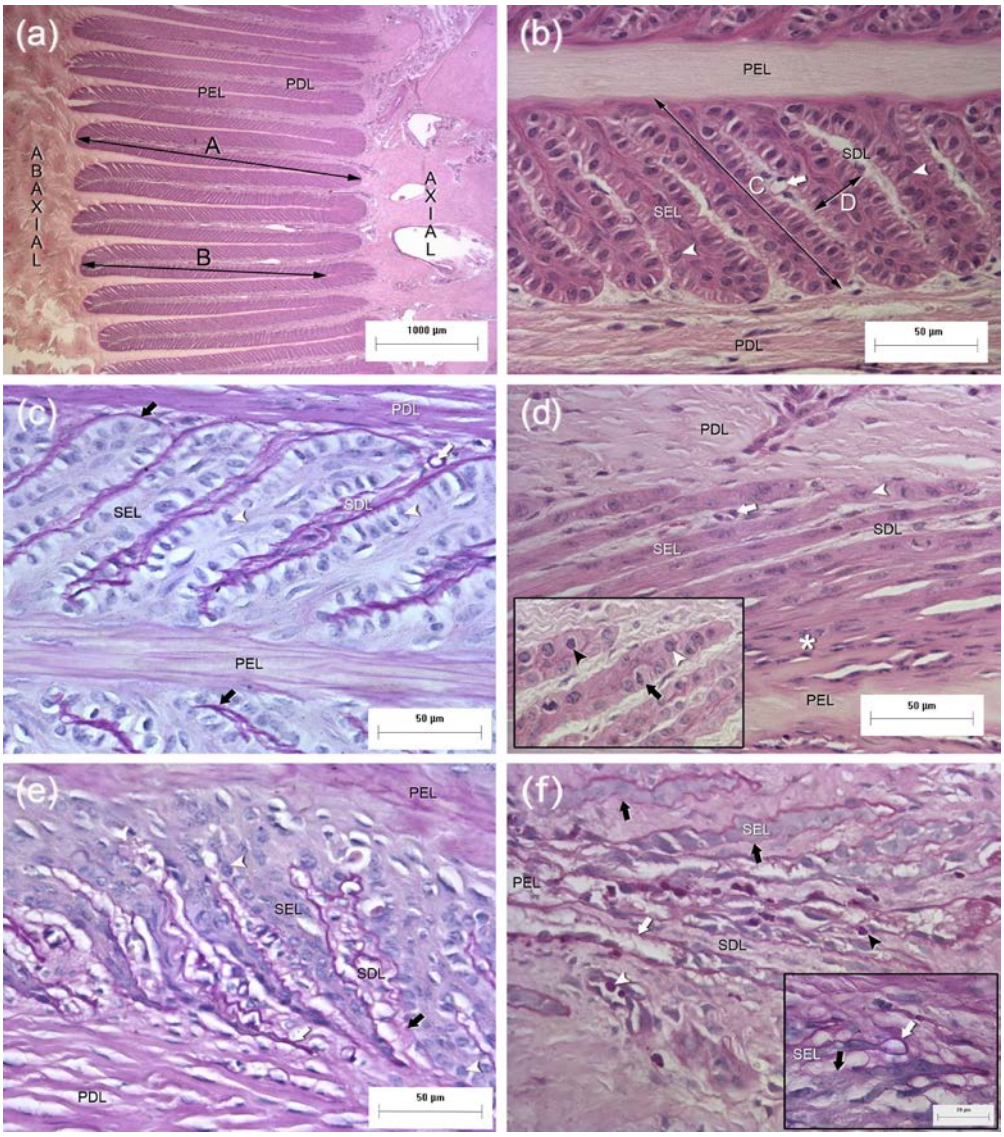

Fig. 1: Photomicrographs of hoof lamellar histology from control (a–c) and insulin-treated (d–f) standardbred horses in transverse section. Lamellar measurements (a, b) included the total (A) and keratinized (B) length of the primary epidermal lamellae (PEL) and the length (C) and width (D) of the secondary epidermal lamellae (SEL). In control horses the PEL were straight and uniform in length, with the keratinized axis finishing before the PEL axis (a). The SEL were uniform in length and clearly separated by each secondary dermal lamella (SDL), which extended almost to the PEL axis (b, c). Epidermal basal cell (EBC) nuclei (white arrowheads in b) were ovoid and located apically in the cell and the PAS stained basement membrane (BM)

was tightly adherent to the rounded tips of each SEL and continued intact to within one or two EBCs of the PEL axis (black arrows in c). The microvasculature surrounding the SEL appeared normal (white arrows in b and c). In insulin-treated horses the SEL appeared lengthened with sharp, pointed tips (d, e) and the EBC nuclei were variable in size and rounded with prominent, and often multiple, nucleoli (white arrowheads in d and e). Clear demarcation of individual SEL at the PEL axis was lost (asterisk in c). Pyknotic (black arrowhead in d inset) and mitotic (black arrow in d inset) figures were present. Loss of PAS positive BM staining was apparent (black arrows in e and f) with empty profiles of BM (white arrows in f) and a lack of visible BM at SEL bases (e). The microvasculature appeared more prominent (white arrow in d and white arrowhead in f) when compared with control sections (white arrows in b and c). H&E (a, b, d) and PAS (c, e, f).

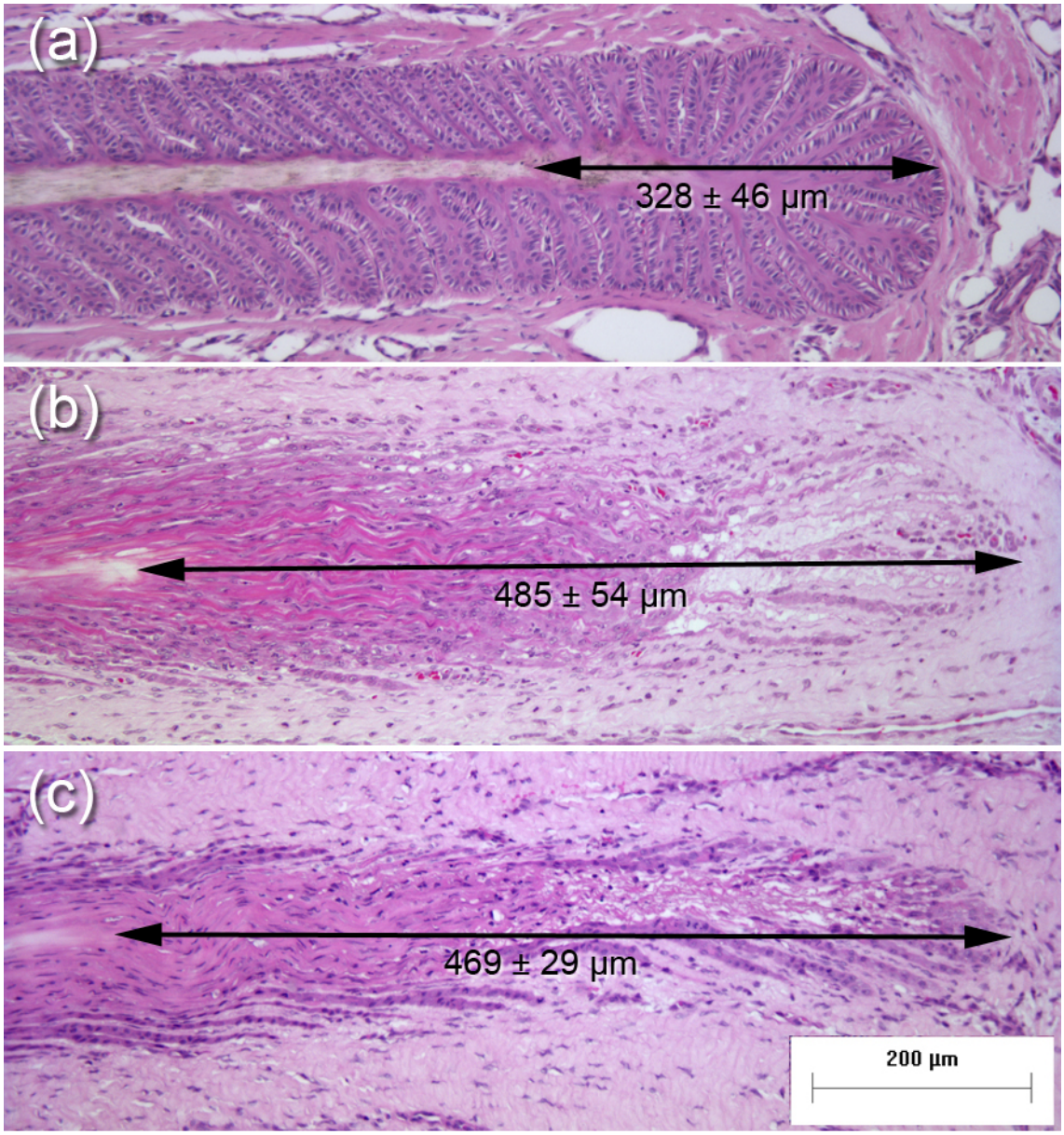

Fig. 2: Photomicrographs of hoof lamellar histology at the axial end of PELs from (a) control, (b) oligofructose and (c) insulin-treated standardbred horses in transverse section. The average length from the end of the keratinized axis of the PEL to the axial tip of the PEL was shorter ($P < 0.05$) in control horses (328 ± 46 µm) when compared with horses with oligofructose (485 ± 54 µm) and insulin-induced (469 ± 29 µm) laminitis. H&E.

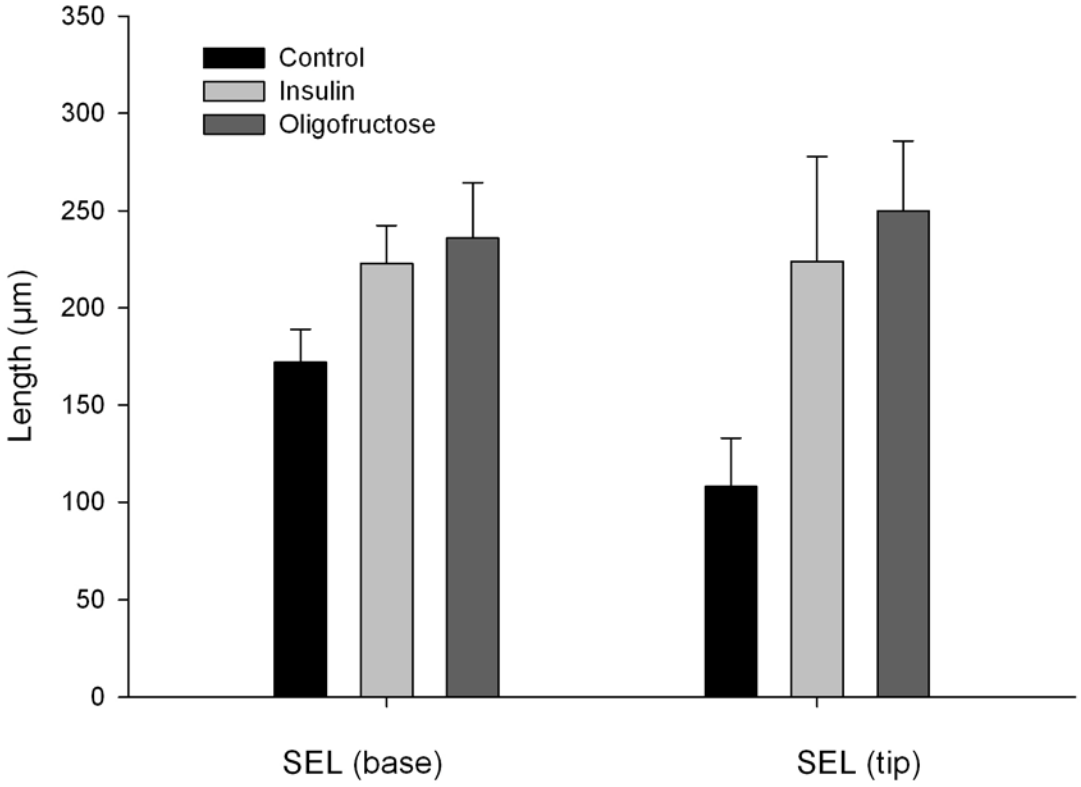

Fig. 3: Mean ± SE secondary epidermal lamellar (SEL) length (µm) was measured at the base and the tip of the primary epidermal lamellae (PEL) in three groups of horses (n = 4) 48 h after treatment with a balanced electrolyte solution (▉), a combined insulin and glucose infusion (▒) or oral oligofructose (▓). SEL length was longer (P < 0.05) at both the base and tip in both insulin and oligofructose treated horses when compared with the control horses. The two treated groups did not differ from each other.

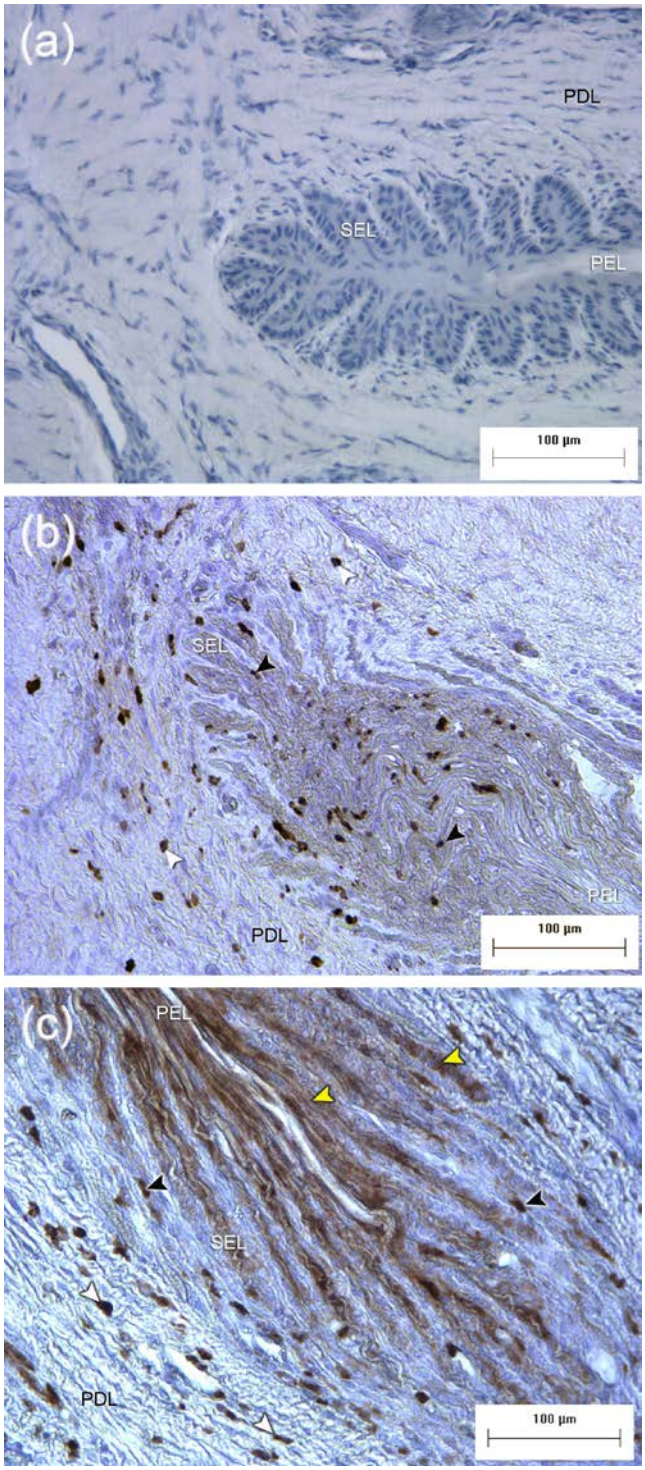

Fig. 4: Photomicrographs of hoof lamellae labelled for calprotectin expression in (a) control, (b) insulin-treated and (c) oligofructose-treated standardbred horses in transverse section. (a) Immunolocalization of calprotectin in control horses was largely non-existent. (b) In insulin-treated horses, there was an increase in the number of calprotectin-positive cells in the lamellar dermis and around the tips of each PEL

(white arrowheads) with a few cells appearing to be within the lamellar BM (black arrowheads). (c) In oligofructose-treated horses, calprotectin immunoreactivity was marked, with labelling of cells in the lamellar dermis (white arrowheads) as well as apparent nuclear (black arrowheads) and cytoplasmic (yellow arrowheads) involvement of epidermal cells. IHC.