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Equine laminitis: loss of hemidesmosomes in hoof secondary epidermal lamellae correlates to dose in an oligofructose induction model: an ultrastructural study

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Keywords: horse; laminitis; basement membrane lesion; oligofructose; hemidesmosome; transmission electron microscopy

Summary

Reasons for performing study: **Light microscopical studies show that the key lesion of laminitis is separation at the hoof lamellar dermal-epidermal interface. More precise knowledge of the damage occurring in the lamellar basement membrane zone may result if laminitis affected tissue is examined witb the transmission electron microscope. This could lead to better understanding of the pathogenesis of lesions and the means of treatment or prevention.**

Objectives: **To investigate the ultrastructure of acute laminitis as disease of greater severity is induced by increasing oligofructose (OF) dosage.**

Methods: **Three pairs of normal horses, dosed with OF at 7.5, 10 and 12.5 g/kg bwt via nasogastric intubation, developed laminitis 48 h later. Following euthanasia, their forefeet were processed for transmission electron microscopy. Lamellar basal cell hemidesmosome (HD) numbers and the distance between the basal cell plasmalemma and the *lamina densa* of the basement membrane were estimated and compared to control tissue.**

Results: **Increasing OF dosage caused greater HD loss and more severe laminitis. The characteristic separation of the basement membrane, cytoskeleton failure and rounded basal cell nuclei results from combined HD dysassembly and anchoring filament failure.**

Conclusions: **Without properly assembled HDs, dysadhesion between the *lamina densa* of the basement membrane (BM) and epidermal basal cells occurs, emphasising the fundamental importance of HDs in maintaining attachment at the lamellar interface. Medical conditions that trigger lamellar matrix metalloproteinase (MMP) activation and/or compromise entry of glucose into lamellar basal cells appear to promote loss and failure of HDs and, therefore, laminitis development.**

Potential relevance: **A correlation between lameness severity and escalating loss of lamellar HDs now exists. Therapy aimed at protecting the lamellar environment from haematogenous delivery of MMP activators or from glucose deprivation may control laminitis development.**

Introduction

A model of laminitis in which the dermo-epidermal separation characteristic of the disease was artificially induced in hoof explants has been described by French and Pollitt (2004). Ultrastructural examination of this tissue showed lesions in the basement membrane (BM) zone, connecting the dermis and epidermis and, furthermore, that the first elements to fail were hemidesmosomes (HDs) and anchoring filaments (AFs). However, hoof separation occurred by 2 different mechanisms. In one, glucose starvation caused HDs to disappear inducing the collapse of the epidermal basal cell cytoskeleton and, in the other, chemical activation of the matrix metalloproteinases (MMPs) present in the epidermal basal cells (Kyaw-Tanner and Pollitt 2004) destroyed AFs, leaving HDs in the basal cell plasmalemma intact.

Because laminitis is triggered by a variety of medical conditions and its pathogenesis is poorly understood, new knowledge of the events occurring at the lamellar BM zone is required if the lesion of naturally occurring laminitis is to be understood and treated effectively. HDs are of fundamental importance in maintaining attachment at all dermal/epidermal junctions (Burgeson and Christiano 1997; Borradori and Sonnenberg 1999) and in this study transmission electron microscopy (TEM) was used to investigate HD and lamellar BM zone changes occurring in horses with acute laminitis. Laminitis was induced via carbohydrate overload using oligofructose (C. C. Pollitt and A. van Eps, unpublished data). A correlation between the dose of oligofructose (OF) administered and HD and BM zone damage was investigated. The lesions were compared with those induced *in vitro*.

Materials and methods

Laminitis induction with oligofructose

Eight mature Standardbred horses (5 geldings and 3 mares) with normal feet (based on physical and radiological examinations) and no lameness were randomly allocated in pairs. Three pairs received doses of 7.5, 10 or 12.5 g/kg bwt OF

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TABLE 1: The number of hemidesmosomes (HDs) per micrometer of basal cell plasmalemma (Pl) and the surface area of the basal cell Pl occupied by HDs decreased significantly as oligofructose (OF) dose increased. The average distance between the basal cell Pl and the centre of the *lamina densa* of the BM increased significantly as OF dose increased

	HD/µm basal Pl mean ± s.e.	% HD surface area mean ± s.e.	Distance from HD to centre of *lamina densa* (µm) mean ± s.e.	Number of HDs measured per treatment
Control	4.64 ± 0.15	28.57 ± 0.60	0.057 ± 0.002	n = 55
7.5 g/kg bwt	3.62 ± 0.11**	23.38 ± 0.77*	0.061 ± 0.001	n = 62
10 g/kg bwt	2.15 ± 0.01**	12.55 ± 0.82**	0.067 ± 0.002*	n = 70
12.5 g/kg bwt	1.75 ± 0.01**	8.76 ± 0.44**	0.071 ± 0.0022**	n = 64

Dosed horse lamellae vs. control *(P<0.05), **(P<0.001). Each treatment group consists of pooled data from 4 feet.

powder (Raftilose)[1] dissolved in 4 l of tap water, respectively. One pair received sham treatment with 4 l of tap water. All treatments were administered via nasogastric tube. Prior to dosing, 10% of the induction dose was added to the diet every day for 3 days. All horses were recently retired from competition and acclimated to a high-grain diet for at least 4 weeks. Experiments were conducted according to The University of Queensland Animal Ethics Committee guidelines and inspected by the Animal Welfare Officer.

Clinical observations were made and data collected at 4 h intervals over a 48 h study period. The horses were housed in stables on rubber mats with free access to water; and fed lucerne chaff and a pelleted ration. Appetite, water intake, general demeanor, foot behaviour, lameness, oral mucous membrane capillary refill time, heart rate, digital pulse, faecal consistency, faecal pH, gut sounds (left colon and ileo-caecal sounds) and rectal temperature were recorded. Blood was collected for haematological and biochemical analysis.

The horses were monitored continuously and treated pre-emptively with 0.25 mg/kg bwt flunixin meglumine (Flunix)[2] and i.v. fluids at the first signs of discomfort or dehydration. All but the 2 sham-treated control horses were subjected to euthanasia at 48 h by a shot to the head and the fore and hind feet removed. Each foot was cut on a band saw to harvest dorsal hoof wall lamellae (Pollitt 1996).

All horses survived alimentary dosing with OF at the 3 dose rates used and none developed colic. Clinically, OF dosing was characterised by profuse, watery diarrhoea at 12–16 h that ceased by 36–44 h. All horses developed mild to moderate depression and inappetance at 12–16 h that persisted until 28–36 h. Appetite and demeanor steadily improved after this period and were normal at 48 h. One horse, dosed with 7.5 g/kg OF, and moderately dehydrated at 24 h, responded to 5 l of polyionic, i.v. fluid therapy. All but one 7.5 g/kg bwt dosed horse, received flunixin meglumine at 32 h to relieve mild fever, tachycardia, depression and transient, mild colic. Examination of the data indicated there was no effect between treatment groups on appetite, oral mucous membrane capillary refill time, demeanor or gut sounds.

Horses, sample collection and processing

Hoof lamellar tissues from the dorsal hoof wall of the 3 pairs of Standardbred horses that developed laminitis after dosing with OF (Raftilose)[1] were collected and processed for TEM as previously described (Pollitt 1994; French and Pollitt 2004). Control tissue came from 2 normal horses subjected to euthanasia for teaching purposes.

Hemidesmosome and basement membrane analysis

Specimens were examined and photographed using a JEOL 1010 transmission electron microscope. Secondary epidermal lamellae (SELs) from the middle region of primary epidermal lamellae were selected for analysis. An approximate 200 µm length of the *lamina densa* of the BM was photographed at 15,000 times magnification and digital images were generated from the negatives (French and Pollitt 2004). A digital image mosaic of continuous *lamina densa* spanning 3–4 cells (34 fields) was generated from the photographs using digital image editing software (Adobe Photoshop 6.0)[3]. Image analysis software (ImagePro Plus)[4] was used to count the number of HDs per micrometer of basal cell plasmalemma (N_B). The length of each HD was measured and assuming HDs to be thin flat discs, HD length (B_{HD}) was converted to true HD diameter (HD_T) (Madigan and Holden (1992) by the formula: $HD_T = 4/\pi \times B_{HD}$. Using these parameters, the numerical density of HDs per unit surface of basal cell plasmalemma (N_S) was derived and then the approximate percentage area of basal cell plasmalemma occupied by HDs (% $area_{HD}$) (Madigan and Holden 1992). The distance from the basal cell plasmalemma to the centre of the *lamina densa* of the BM was also measured.

The values for N_B, % $area_{HD}$ and the distance from epidermal cell to the centre of the *lamina densa* of the BM were compared between OF treatments and to the controls. Data were analysed using repeated-measures analysis of variance (ANOVA) and Student's *t* test. Statistical analysis was conducted with computer software (Instat GraphPad 2.02)[5].

Results

Morphometric analysis

As the dose of oligofructose increased the number of HDs per micrometer of the basal cell plasmalemma and the mean percentage surface area of the basal plasmalemma occupied by HDs, decreased significantly compared to controls (Table 1). However, the mean distance between the epidermal cell plasmalemma and the centre of the *lamina densa* of the BM increased (Table 1).

Ultrastructural morphology of control lamellar tissue

The BM zone of control SELs was characterised by the dark-staining *lamina densa* that closely followed the contours of the epidermal basal cells (Fig 1a). Between the *lamina densa* and the basal cell plasmalemma was the lightly stained *lamina lucida*. HDs were present in the basal cell plasmalemma each with an electron dense intracytoplasmic plaque and sub-basal dense plate (Fig 2a).

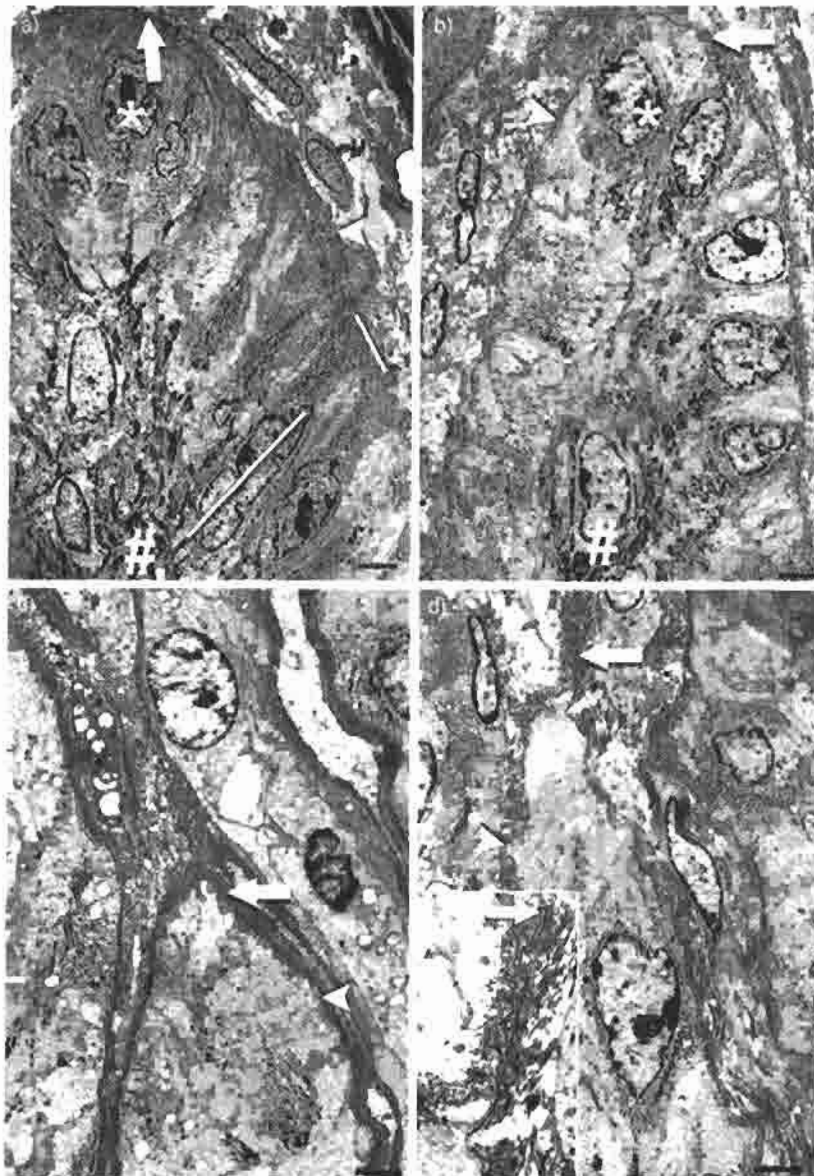

Fig 1: Transmission electron micrograph (TEM) of equine hoof secondary epidermal lamellae (SELs). In control horses (a) the SEL tip (arrowed) was rounded. SEL tips were more pointed (arrows) as the dose of oligofructose increased. b) = 7.5 g/kg, c) = 10 g/kg and d) = 12.5 g/kg bwt. The SEL of a healthy control horse (a) consists of epidermal basal cells (*) and parabasal cells (#). The lamina densa *(arrowhead) closely follows the contours of the epidermal basal cells. Basal cell nuclei are oval shaped, situated at the cell apex and orientated with their long axis (long line) at approximate right angles to the BM zone (short line). In horses dosed at 7.5 g/kg (b) basal cell nuclei are rounded and situated at the cell base close to the BM zone (arrowhead). In horses dosed at 10 g/kg (c) the* lamina densa *is crenellated (arrowhead) and basal cell cytoplasm is diffuse with few dark staining elements of the cytoskeleton. Adjacent to the SEL tip (arrowed) are 3 abnormal, attenuated SELs, barely more than one cell wide. In horses dosed at 12.5 g/kg (d) crenellation of the* lamina densa *is more pronounced (arrowhead) and* lamina densa *has left the SEL tip (arrowed) forming a bi-layered bubble (inset) in the dermal connective tissue. Bars = 2 µm.*

Ultrastructural morphology of lamellar tissue from horses dosed with 7.5 g/kg bwt oligofructose

Hoof SELs from horses dosed with 7.5 g/kg bwt of OF resembled controls, although the SEL tips were more pointed (Fig 1b). Near SEL tips the *lamina densa* was slightly crenellated. Some basal cells showed thinning and loss of tonofilaments and their associated HDs. Basal cell nuclei had lost the oval shape of controls and were rounded, situated abnormally close to the BM zone, in the basal part of the cell (Fig 2b). There were a few areas, small in size, which were lacking in AFs, allowing the *lamina densa* to lift away from the epidermal cell plasmalemma.

Ultrastructural morphology of lamellar tissue from horses dosed with 10 g/kg bwt oligofructose

Hoof SELs from the horses dosed with 10 g/kg bwt of OF had ultrastructural changes compared with controls. SEL tips were markedly pointed instead of rounded (Fig 1c) and the BM zone had a wavy or crenellated appearance (Fig 2c). Epidermal cell nuclei were round and either centrally or basally located within the cells. The *lamina densa* of the BM was less densely stained and indistinct. There was thinning and loss of tonofilaments adjacent to HDs and the cytoplasm in these areas was pale staining and granular (Fig 2c). Tonofilaments, no longer attached to HDs and

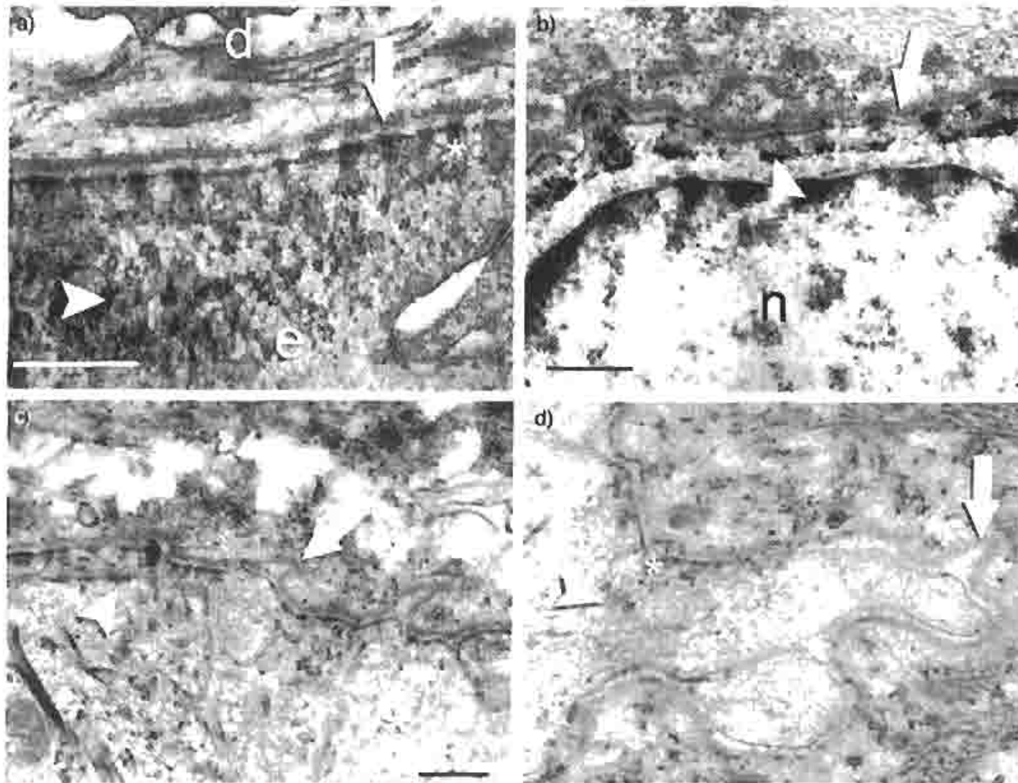

Fig 2: TEMs of lamellar BM zone. In control horses (a) the lamina densa *(arrowed) was a dense unbroken line parallel to the epidermal basal cell (labelled e) plasmalemma that contained numerous, evenly spaced hemidesmosomes (HDs) (*). Anchoring filaments (AFs), especially dense adjacent to HDs, cross the* lamina lucida, *and merge with the* lamina densa. *Dark staining cytoskeleton tonofilaments (arrowhead) criss-cross the cell in all directions and closely associate with HD sub-basal dense plates. Banded collagen fibrils in the dermis (labelled d) merge with* lamina densa. *In horses dosed with 7.5 g/kg (b) the* lamina densa *is unchanged but the number of HDs has decreased. Dark staining tonofilaments (arrowhead) have clumped in the cytoplasm and the now rounded basal cell nucleus (labelled n) is positioned abnormally close to the BM zone. In horses dosed with 10 g/kg (c) portions of the* lamina densa *have separated from the basal cell plasmalemma at sites (arrowed) where HDs have disappeared. HDs are unevenly spaced and smaller and fewer in number. A few clumps of cytoskeleton remain (arrowhead) but most of the cytoplasm is diffuse. These changes are more pronounced at the SEL tips (d). There are only a few HDs, and the* lamina densa *(arrowed) has separated from the basal cell plasmalemma except at sites where HDs still remain (*). Bars = 500 nm.*

therefore the cell plasmalemma, were lying against the cell nucleus in wavy bundles. The intercellular spaces between epidermal cells also seemed to be wider. Areas of the *lamina lucida* lacking AFs, that were observed in the animals dosed with 7.5 g/kg bwt OF, had become more widespread and there were patches of *lamina densa* that had separated from the basal cell plasmalemma (Fig 2c,d).

Ultrastructural morphology of lamellar tissue from horses dosed with 12.5 g/kg bwt oligofructose

Hoof SELs from horses dosed with 12.5 g/kg bwt OF were more severely affected than 10 g/kg bwt OF horses, with ultrastructural changes markedly different to control tissue. Crenellation of a weakly stained, indistinct *lamina densa* was pronounced, and bilayers of separated *lamina densa* were present at SEL tips (Fig 1d). HDs were few in number, small and unevenly distributed in the plasmalemma of basal cells, and HD intracytoplasmic dense plaques were pale and disorganised as if in the process of dysassembling (Fig 3a). *Lamina densa* was detached from the basal cell plasmalemma where HDs were absent or faint but remained attached at zones where HDs survived (Fig 3b). Basal cell cytoplasm was pale and amorphous and contained clumps of dense-staining cytoskeleton. At higher magnification, remnants of AFs lined the inner layers of the empty, separated *lamina densa* (Fig 3c). Some degenerate SELs were reduced to attenuated *lamina densa* enclosed tubes containing the debris of basal cell organelles, clumps of pale amorphous cytoplasm and dense granules of cytoskeleton-like material. Many polymorphonuclear leucocytes (PMNs) were associated with these damaged epidermal compartments, sometimes on the epidermal side of the BM zone (Fig 3d).

Discussion

The ultrastructure of the BM zone of hoof lamellae was affected in a dose-dependent manner after nasogastric administration of OF. Control lamellae, had lamellar ultrastructural features as described by Pollitt (1994) and Leach and Oliphant (1983), and even OF at the lowest dose of 7.5 g/kg bwt significantly decreased both the size and number of lamellar HDs. The magnitude of HD shrinkage and loss increased as OF dose increased. The moderate dose of 10 g/kg bwt OF was required to induce sporadic separation of epidermal basal cells from their underlying *lamina densa*. The highest dose tested was associated with extensive *lamina densa* separation, especially at SEL tips. This is the first time that an objective measure of laminitis severity, based on ultrastructure, has been devised. The relationship between decreasing HD size and number and increasing severity of laminitis has clinical relevance. A correlation between the degree of lameness and grade of

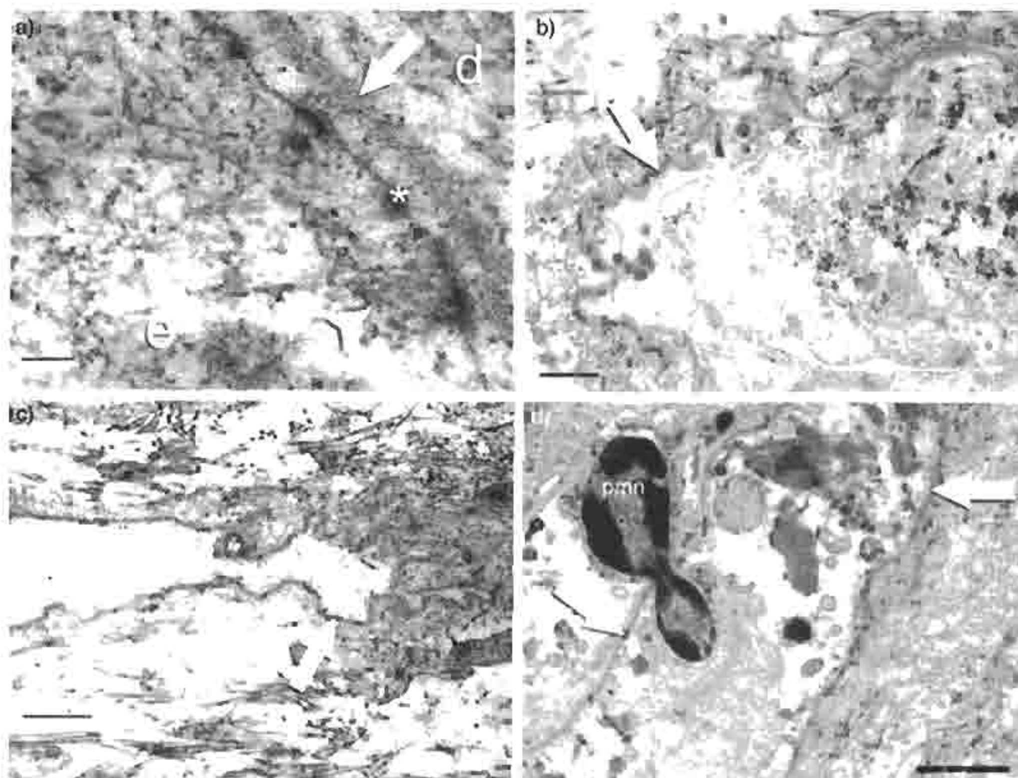

Fig 3: TEMs of lamellar BM zone of horses dosed with 12.5 g/kg. HDs (*) were fewer in number (a), small and unevenly distributed in the plasmalemma of basal cells (labelled e). HD intracytoplasmic dense plaques were pale and disorganised as if in the process of dysassembling (bar = 1 nm). At SEL tips (b) the lamina densa *(arrowed) was detached from the basal cell plasmalemma which was often disrupted and feintly stained. Between the* lamina densa *and the occasional, surviving HD (*) were a few anchoring filaments (AFs). Basal cell cytoplasm was pale and amorphous interspersed with clumps of cytoskeleton (arrowhead). Empty bubbles of* lamina densa *(c) that had completely separated from the basal cells of SEL tips were lined with the remnants of AFs.* Lamina densa *enclosed tubes (arrowed in d) contained debris of basal cell organelles, pale, diffuse cytoplasm and dense granules of cytoskeleton-like material. Polymorphonuclear leucocytes (PMNs) were often within damaged epidermal compartments and one is shown apparently passing through a gap it is has created in the lamellar* lamina densa *(bars in b), c) and d) = 500 nm).*

laminitis severity already exists (Pollitt 1996), therefore it follows that loss of HDs also correlates to lameness. Ultimately the laminitis lesion may be resolved to the molecular level and loss of a key molecule or molecules at the lamellar dermo-epidermal junction may therefore also correlate to lameness.

Pollitt (1996), using light microscopy (LM), developed a laminitis, histopathology grading system. Briefly, SELs with *grade 1* (mild) lesions were narrow, elongated and had pointed instead of rounded tips. Basal cell nuclei were round instead of oval in shape and were situated abnormally close to the BM zone (basally instead of apically located). The BM at SEL tips was not attached and had separated to form teat-shaped bubbles or thin bi-layered tapers. *Grade 2* lesions (moderate) were similar but more extensive with the addition of BM disappearance from between the bases of adjacent SELs. *Grade 3* lesions (severe) were characterised by extensive separation of BM from SEL basal cells. SELs were extremely elongated with spindle-shaped, basal cell nuclei orientated along the long axis of the SEL. BM was recognisable around the tapered, stretched tips of a few SELs but the bulk of the BM occurred as free, wavy strands along the edges of the primary dermal lamellae. The remaining SELs, stripped of their BM, coalesced on either side of the axial core of each primary epidermal lamellae. Blood vessels in the lamellar dermis were surrounded by leucocytes, many of which were within epidermal compartments.

Laminitis tissue examined by low magnification TEM showed morphology consistent with Pollitt's (1996) LM grading system. In lamellar tissues from horses dosed at 7.5 g/kg bwt, SEL tips were pointed, instead of rounded, and many basal cell nuclei were spherical instead of oval and were situated abnormally close to the lamellar *lamina densa*. However, the lesions were not LM *grade 1* as *lamina densa* separation from SEL tips had not occurred. Lamellar tissue from animals dosed with 10 g/kg bwt OF showed structural damage typical of LM *grade 1* (mild) laminitis. TEM showed many rounded basal cell nuclei and SEL tips that were pointed and had shed electron dense *lamina densa*. Therefore, TEM confirmed that the teat-shaped bubbles and thin tapers of periodic acid Schiff positive material at SEL tips of laminitis affected tissue, examined by LM (Pollitt 1996), was in fact the *lamina densa* of the BM. As expected, the ultrastructure of laminitis tissue from horses dosed at 12.5 g/kg bwt paralleled the more severe, *grades 2* and *3*, LM histopathology. SELs were often extremely attenuated, many surrounded and penetrated by PMNs. The rapidly developing, more extensive SEL lesions of *grade 3* laminitis were clearly chemotactic to PMNs, themselves a potent source of MMPs (Mungall *et al.* 1998). The magnitude of PMN influx in early acute laminitis probably entrains a cascade of self-perpetuating lamellar degradation and is, therefore, a harbinger of chronically increasing severity. It is predicted that chronic, severe laminitis tissue will show greater MMP expression than tissue with newly developed, acute laminitis (Kyaw-Tanner and Pollitt 2004).

Studies of laminitis *in vitro* demonstrated that the ultrastructural changes leading to lamellar separation could be initiated in two ways: withdrawal of glucose caused a loss of HDs and their underlying tonofilaments, leaving intact the AFs connecting the basal cell to the *lamina densa*, while activation of MMPs destroyed only the AFs (French and Pollitt 2004).

The ultrastructural changes of OF induced laminitis in the present study were not clear cut; features of both *in vitro* mechanisms (French and Pollitt 2004) were present in the laminitic tissue. The shrinkage and loss of HDs, associated with the collapse of cytoskeleton tonofilaments, leaving a uniform, electron lucent cytoplasm, was a feature of OF induced laminitis tissue. This was also present in lamellar tissues cultured *in vitro* without glucose. However, destruction of the AFs linking the basal cell plasmalemma to the *lamina densa* were also present in the OF induced laminitic tissue and resembled similar ultrastructural changes observed *in vitro* when constituent lamellar MMPs were activated. Thus the pathology of OF induced laminitis seems to result from at least two processes; activation of constituent MMPs and glucose deprivation of SEL basal cells.

There is indirect evidence that this may occur during the developmental phase of laminitis. The molecular components of BMs and AFs are substrates for MMP activity (Giannelli *et al.* 1997). Increased MMP concentrations are found in lamellar homogenates from laminitic horses (Johnson *et al.* 1998; Pollitt *et al.* 1998). In addition, increased transcription of MMP-2 RNA is well under way 48 h after administration of OF and the amounts also correlate to dose.

Lamellar hoof tissue has previously been shown to require glucose to maintain its integrity (Pass *et al.* 1998). OF dosing causes significant hyperglycaemia, especially at high dosage, that does not appear to correlate to changes in insulin concentration. The hyperglycaemia associated with OF overload could result from an abrupt failure of peripheral glucose uptake. Lamellae suddenly unable to transport glucose, may undergo HD disintegration that would contribute to lamellar dermo-epidermal separation, the lesion that characterises laminitis.

This study illustrates the fundamental importance of HDs in maintaining attachment at the lamellar dermal/epidermal interface. Without properly assembled HDs, dysadhesion between the BM *lamina densa* and the basal cell plasmalemma occurs. Medical conditions that trigger lamellar MMP activation and/or compromise entry of glucose into lamellar basal cells appear to promote loss and failure of HDs and, therefore, laminitis development. Laminitis may be prevented by therapies designed to protect the lamellar environment against uncontrolled MMP activation and glucose deprivation.

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Manufacturers' addresses

[1]ORAFTI Active Food Ingredients, Aandorenstraat, Tienen, Belgium.
[2]Parnell Laboratories (Australia) Pty., Ltd., Alexandria, New South Wales, Australia.
[3]Adobe Systems Inc., San Jose, California, USA.
[4]Media Cybernetics, Silver Spring, Maryland, USA.
[5]GraphPad Software Inc., San Diego, California, USA.

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