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Equine Laminitis (21-Nov-2003)

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1. Introduction

The mission statement of the AELRU is to elucidate the mechanism of laminitis to make it a preventable disease. This review, which describes some of our research over the last five years, seems to contain seemingly unrelated detail, but, nevertheless, it reveals a pattern that is leading to an understanding of the pathogenesis of laminitis. Laminitis is a dynamic, molecular process superimposed on normal biology. Many of its features include normal processes appearing at the wrong time and place. Although knowing the anatomy of hooves and bones is important, we will understand laminitis better when we learn more about the genes and proteins of the lamellar region. Thus, in addition to ongoing research on the pathogenesis of laminitis, our team has undertaken studies on normal feet, particularly the inner hoof wall lamellar zone, to better understand some key activities and processes that may shed light on how laminitis occurs.

2. How the Hoof Wall Grows

Because the hoof wall of a mature horse "grows" continuously to replace hoof lost to wear and tear at the ground surface, continuous cell proliferation in the coronet must occur. Mitosis of epidermal basal cells in the coronet produces new generations of cells that mature and cornify, thus, adding incrementally to the length of the hoof wall [1]. Similarly, mitosis in the PELs also occurs. Although MFs among the basal cells of the proximal lamellar zone are easily observed, there is no convincing evidence that the more distal lamellae proliferate at all. The fundamental problem of how the inner hoof wall lamellae remain attached to the connective tissue embedded on the surface of the stationary distal phalanx while one moves over the other is unresolved. Is it by continuous proliferation of the lamellar epidermis (laminar flow) or by some other remodeling process (that may also be involved in laminitis pathogenesis)? Cells in mitosis are rarely, if ever, found in normal lamellae below the proximal proliferative zone. This has led to the proposal of the "sterile bed concept," [1] named because the equine lamellar epidermis was non-proliferative, and thus, sterile. However, the "sterile bed concept" has since been described [2] as "no longer tenable" on the basis of MFs located in cap horn arcades at the bases of the lamellae. Therefore, to determine precisely where the cell proliferation occurs within the hoof wall epidermis, we calculated a PI for basal cells of the coronet, lamellae, and toe of the dorsal hoof wall.

An analogue of the DNA nucleotide thymidine BrdU [a], which is incorporated into all cells in the prolonged, synthesis stage of cell division, was infused intravenously into five normal ponies with at least one white foot. After tissue harvesting, BRdU (and thus, basal cell proliferation) was detected immunohistochemically in formalin fixed tissue using mouse BRdU antibody [b]. PI values were calculated for the coronet and 10 levels of the dorsal hoof wall lamellae.

As expected, the highest PI values (mean $\pm$ SE) were in the coronet (12.04 $\pm$ 1.59%) and proximal lamellae (7.13 $\pm$ 1.92%). Thus, these are growth zones of the proximal hoof wall. Distal to this, the PI values of more distal lamellae were very much lower. They ranged from 0.11 $\pm$ 0.04% to 0.97 $\pm$ 0.29%, which is significantly lower ($P < 0.05$) than the lamellar growth zone.

Evidence for a constant supply of new cells in the lamellar region, generating a downward laminar flow, was not provided by this study. The few proliferating cells detected in the main lamellar region had a patchy distribution and were usually located at the PELs tips, not in cap-horn arcades. A 20-fold PI decrease between proximal and more distal lamellae suggests that the

majority of the normal lamellae are non-proliferative and that their main function is to suspend the distal phalanx within the hoof capsule. Remodeling within the hoof wall epidermal lamellae, which must occur as the hoof wall moves past the stationary distal phalanx, is a process not requiring epidermal cell proliferation. Since Leach and Oliphant's 1983 publication [1], remodeling of the epidermis and the ECM is now known to involve the controlled release of activated MMPs and their subsequent inhibition by TIMPs. MMPs have been shown to exist in lamellar hoof, and their uncontrolled activation has been proposed as a mechanism for the pathogenesis of laminitis [3]. The molecular components of desmosomes, HDs, and BMs are substrates for MMP activity [4]; therefore, the mechanistic concept [1] of "formation and destruction of desmosomes in a staggered ratchet-like manner" now has a well referenced, biological explanation [5]. Lamellar epidermal cells and their adjacent BMs are constantly responding to the stresses and strains of growth and locomotion; they release MMPs and TIMPS to accomplish the cellular reorganization required. Because this involves enzymes capable of destroying key components of the attachment apparatus between distal phalanx and inner hoof wall, it is clear that triggering this "loaded gun" will have dire consequences for the future health of the foot. Inadvertent or uncontrolled lamellar MMP activation makes horses, with their generic reliance on a single digit per limb, uniquely susceptible to the destructive effects of laminitis. Indeed, if laminitis can be shown to involve increased transcription of constituent lamellar MMP, it would be put into the disease category of a normal process gone wrong.

3. Hoof Wall Wound Repair

Strips of hoof wall that are removed surgically leave behind a deficit that heals remarkably well. Within a few days, the surface of exposed lamellar corium dries and hardens, and a new hoof wall, generated at the coronet, grows slowly downwards over the superficially keratinized lamellar deficit. There have been no reports on how this is achieved. After wall stripping, we documented the temporal changes in the lamellar BM and epidermis to better understand the biology of hoof lamellae, and thus, the pathophysiology of laminitis.

The gross appearance and histology of wall strips made at the midline in the dorsal hoof wall of six adult Standardbred horses were studied. A standard, side-clipped horseshoe and a custom-made metal plate, shaped to conform to the dorsal hoof wall, were fitted to support the dorsal hoof wall postoperatively. All horses were briefly anesthetized with a combination of xylazine HCl [c] (1.1 mg/kg, IV) and ketamine [d] (2.2 mg/kg, IV) during the wall strip procedure. Wall stripped tissue 1 - 10 days post-surgery came from five euthanized horses, whereas 4- to 6-mo tissue came from one horse. Hoof wall specimens taken at the time of wall stripping were also harvested. Parenteral, post-operative procaine penicillin [e] (12 mg/kg, IM) and a phenylbutazone, sodium salicylate mixture [f] (4.5 and 1.2 mg/kg, IV, respectively) were administered daily for 5 days. The wounds were gently irrigated with normal saline, dressings were changed every second day (without removing the steel hoof plate), and no medicaments were applied to the wounds. One horse, with 3 and 5-day wall strips, was injected with the thymidine analogue BRdU to detect epidermal cell proliferation. The specimens were fixed, processed, and stained [1] for routine histology. In addition, immunohistochemistry [2] was used to show changes in the lamellar BM and proliferation in lamellar epidermal cells.

All wall strip surgery wounds healed without sepsis or other complications. The metal hoof wall plate and the sideclipped shoe kept the edges of the wall strip parallel, resulting in only slight, post-operative lameness. Wall stripping caused the tips of the lamellae to snap at the same point and remain behind in the dermis along with the majority of the lamellar BM and some lamellar basal cells. Three days later, the repaired BM was intact, and new lamellae had been reconstructed by proliferation of surviving basal cells. By 5 days, the surface of the stripped zone was covered with yellow epidermis that subsequently thickened and hardened. Eventually, the hoof wall deficit was replaced by new wall growing down from the coronet.

Analysis of wall strips, performed in the midline of the dorsal hoof wall, showed the sequence of wound healing in the hooves of horses. Immunohistochemistry with antibody directed against BM proteins enabled mapping of subtle changes in structure as the BM was repaired. BRdU, injected into one horse and detected in its lamellar tissues with anti-BRdU, showed that intense epidermal cell proliferation was switched on after lamellar tissues were wounded.

Stripping the equine hoof wall causes the tips of the epidermal lamellae to snap and remain embedded in the dermis. Also, most of the lamellar BM peels from the epidermis and remains in the dermis. Small gaps in the BM are quickly repaired. New epidermal cells, proliferating from the stumps of snapped-off PELs tips, migrate into the empty, collapsed BM shells, filling them with new keratinocytes. Remarkably, new lamellae are formed with near normal anatomy. Small numbers of SELs basal cells, not stripped away during surgery, also contribute to the rapid reconstruction of lamellae. They separate from their underlying BM and, proliferating as they go, repopulate the new lamella. This intense proliferation after wounding is in contrast to normal non-injured lamellae that are quiescent. The repaired lamellar BM acts as a template over which keratinocytes migrate to reconstruct the lamellae. Parallel saw cuts made in the hoof wall to facilitate wall stripping caused the greatest amount of damage; they led to faulty reconstruction of BM, and resembling lamellae was affected by chronic laminitis (enclosed islands of epidermal cells not connected to the PELs). Deep penetration of the saw blade and overheating of the blade should be avoided when performing wall strips. The conforming steel plate, which was screwed to the hoof wall

surface immediately after surgery, successfully stabilized the toe and prevented significant post-operative lameness. The application of dressings, without medicament, protected the wound from contamination, absorbed wound exudates, and provided an optimal environment for healing [6].

There is a key difference between laminitis-affected lamellae and wall stripped lamellae. Lamellae affected by laminitis lose contact with a BM that is damaged, often severely [7,8] and the lamellar epithelium repairs with many anatomical defects [9]. In contrast, the BM survives wall stripping virtually intact, and the lamellae are quickly and effectively repaired. The act of wall stripping, traumatic as it may seem, invokes a built-in mechanism that provides a source of cells (the snapped-off PELs tip) and a BM template over which new epidermal cells can rapidly migrate. In laminitis-affected tissue, this option is not available; the pathology is reversed. Lamellar tips and a BM template are far removed from each other, and anatomical reconstruction is forever compromised. Perhaps this explains the irreversible nature of chronic laminitis and the reason why cases of genuine recovery are so rare.

All experiments on horses, conducted by AELRU, were approved by The University of Queensland Animal Ethics Committee (in concordance with the National Health and Medical Research Councils "Australian code of practice for the care and use of animals for scientific purposes," which is embedded in the Queensland Animal Care and Protection Act 2001), and all horses under experimentation were inspected by an Animal Welfare Officer.

4. A New Laminitis Induction Model Based on Alimentary Overload with OF

Although alimentary carbohydrate overload with grain starch is the standard model for induction of acute laminitis, it has a high rate of morbidity and mortality and a poor success rate. This behooves laminitis researchers to develop a more efficacious and humane model for inducing the disease.

Although accidental access to grain starch is undoubtedly a cause of field cases of laminitis, consumption of pasture rich in fructan, the non-structural, storage carbohydrate, has also been implicated [10]. Under certain conditions of climate, fructan in the stems of grass may reach concentrations (<50% DM) high enough to trigger laminitis. Starch is a carbohydrate polymer consisting entirely of linked glucose molecules, and fructan consists of a single glucose linked to varying numbers of fructose molecules. Most temperate, pasture grasses produce mainly phlein or levan-type fructans (β2,6-linked), but they also produce the inulin (β2,1-linked) type.

We tested the hypothesis that alimentary overload with fructan, in the form of the insulin-like (β2,1-linked) commercial OF (Raftilose P95 [g]) extracted from the roots of chicory (*Cichorium intybus*), would cause laminitis. We used a range of doses that mimicked the amounts of pasture fructan that could be consumed by a horse in 1 day and analyzed the resultant data for a dose response relationship.

Eight mature, clinically normal Standardbred horses were randomly allocated in pairs to three OF treatment groups (7.5, 10, and 12.5 g/kg) and one sham-treated control group. The OF readily dissolved in 4 L of tap water and was administered to each horse as a single bolus dose with a nasogastric tube.

After bolus dosing, clinical observations and blood sampling for hematological and biochemical analyses were made at 4-h intervals over a 48-h study period. All horses were euthanized at 48 h, stained sections of the hoof wall lamellae were examined with a light microscope, and the severity of the laminitis was graded using the scoring system of Pollitt [7]. The effects of OF dosing were statistically analyzed for treatment, time, and treatment by time interactions.

All horses survived the 48-h period after alimentary dosing with OF at the three dose rates used. None developed colic. All horses developed clinical and histological laminitis in at least one foot. Higher doses of OF were associated with significantly more severe (grade 3) histological laminitis ($P < 0.05$). Only minimal supportive treatment was required. All but one horse received 0.5 mg/kg flunixin meglumine [h] at 32 h to alleviate clinical signs of endotoxaemia; this did not affect laminitis outcome.

All the horses developed profuse diarrhea at around 18 h that ceased by 36 - 44 h. Pyrexia and elevated heart rate peaked between 16 and 20 h and returned to near normal by 48 h. Heart rates rose above 60 beats/min in only two of six horses. Fecal pH began to fall within 4 h of dosing, reaching its lowest value (3.7) at around 18 h. For all groups, there were significant losses of bicarbonate after 12 h, which remained low for 12 h and then recovered, although at different rates. Blood D-lactate, of bacterial origin, peaked at 24 h at a concentration (2.87 mmol/l) far in excess of that reported in a similar study using wheat starch [11]. Hematological and serum electrolyte perturbations during the laminitis induction period were similar to those reported for laminitis induced with corn starch [12]. Plasma glucose concentrations in all OF dosed horses peaked at 24 - 28 h, and the transformed glucose data showed significant time by treatment interactions. Serum cortisol concentrations in OF dosed horses peaked at 24 h, but they did not differ from sham-treated control horses and therefore, were not significant. Plasma insulin concentrations did not show significant effects of time or significant time by treatment interactions, although horses in the 12.5 g/kg group consistently had the highest values.

Because all horses developed laminitis, the results establish that a storage carbohydrate, other than starch, can induce the disease. OF is a principal non-structural carbohydrate of pasture grasses, suggesting it is grass OF that causes laminitis and not other substance(s), such as dietary amines [13]. Mammals have no enzyme to metabolize OF; therefore, when consumed,

it passes undigested into the caecum, where it undergoes rapid microbial fermentation [14]. In this situation, Gram positive organisms, notably *Streptococcus bovis* and *S. equinus*, proliferate preferentially and, temporarily, become the dominant microflora [15]. Collectively, during OF fermentation, Gram positive microflora produce lactic acid, causing the low hindgut pH and the peak of blood lactic acid at 24 h, typical of alimentary carbohydrate overload [16]. Mammals produce only the L-isomer of lactate, whereas hindgut bacteria produce both the D- and L-isomers. Thus, when D-lactate appeared in the blood of the OF-dosed horses, it indicated that hindgut fermentation of soluble carbohydrate was occurring [17]. Interestingly, D-lactate disappeared from the blood by 40 h, suggesting that there is a sharp decline in D-lactate producing organisms after an early, rapid population explosion.

In the context of alimentary overload, we have used our *in vitro* laminitis model to test a range of potential "laminitis trigger factors" (cytokines, eicosanoids, gram negative bacterial endotoxins), but only the supernatants of cultured Gram positive hindgut bacteria readily induced *in vitro* laminitis using the MMP activation pathway [17]. We suggest that a link exists between pasture fructan, hindgut gram positive microbial proliferation, BM dysadhesion, and laminitis. Although we now have evidence that disruption of lamellar glucose metabolism also contributes to BM dysadhesion, laminitis may nevertheless result directly from products elaborated by a suite of gram positive organisms proliferating, dose dependently, on a single, pure substrate, OF. The large but temporary gram positive population could liberate exotoxins and endotoxins when they die off at the end of their growth phase or when OF substrate is exhausted. These could cause laminitis when they penetrate the leaky, mucosal barrier of the large bowel during the developmental phase of carbohydrate-induced laminitis [18]. Studies to enumerate and identify the microbes responsible for OF hindgut fermentation are underway in the AELRU.

Compared with traditional alimentary overload with starch, OF dosing resulted in reduced morbidity and no deaths, indicating that alimentary overload with OF (at 7.5 g/kg by weight) is an efficient and more humane experimental model for the induction of acute laminitis. Laminitis seems to be the result of fermentation of pasture OF by gram positive hindgut microbes, reducing fructan production by pasture; therefore, managing horses to reduce pasture fructan consumption and reducing the numbers of fructan fermenting bacteria in the hindgut seem to be realistic, preventive strategies.

5. Increased Transcription of MMP-2 Occurs During the Developmental Phase of Laminitis

Laminitis histopathology shows a characteristic loss and disorganization of the lamellar BM [7,8]. Both zymogen and activated MMP-2 are increased in homogenates of laminitis affected tissue, implying that it is MMP-2 activity that causes the BM degradation of laminitis [3,19]. MMPs are zinc-dependent enzymes that, when activated, degrade ECM, BM components, and the molecules that attach the BM to epidermal basal cells [4,5]. To better understand the involvement of MMPs in the pathogenesis of laminitis, we cloned, sequenced, and quantitated the cDNA encoding equine MMP-2 in normal and laminitis-affected hoof tissue. In addition, using immunohistochemistry and *in situ* hybridization, we showed the location of MMP-2 protein and the location of its mRNA in lamellar tissue.

Lamellar tissues were harvested from the feet of normal horses (n = 4) and from the feet of horses with laminitis induced by alimentary OF overload (n = 18). The mid-dorsal hoof wall lamellae were dissected and formalin fixed or frozen in liquid nitrogen; they were stored at -70ºC until required. Total RNA was isolated, RT-PCR was performed, and major PCR products were gel-purified and cloned. Randomly selected clones were screened for the presence of the correct insert, and DNA was sequenced. Real-time PCR analysis was used to accurately quantitate the MMP-2 gene expression in the DS cDNA synthesized from each tissue sample. Each value was adjusted against a "house-keeping" gene (in this case, GAPDH was used). Rabbit polyclonal anti-equine MMP-2 was produced [i] against a synthetic peptide derived from the amino acid sequences of our equine MMP-2 clones and tested for specificity by Western blot analysis. Hoof wall lamellae were sectioned at 5 μm and processed for immunohistochemistry with the primary anti-MMP-2 diluted to 1/100 parts. *In situ* hybridization with a non-radioactive, indirect DIG labeling system was used to detect MMP-2 RNA in formalin fixed, paraffin-embedded hoof tissues. For each assay, a DIG labeled sense probe as well as "no probe" reactions were used as negative controls.

The cDNA encoding equine MMP-2 was successfully cloned by the PCR method. The open reading frame of equine MMP-2 encoded a 662 amino acid protein. In common with all MMPs, equine MMP-2 had the conserved "cysteine switch" and "catalytic zinc binding site" essential for protease activity [20,21]. DS cDNA (converted from RNA), extracted from each of 4 normal and 18 laminitis foot samples and subjected to the real-time PCR analysis, provided accurate quantitation of MMP-2 expression. Western blot analysis, using anti-equine MMP-2 raised in rabbits from deduced amino acid sequences of the cDNA clones, confirmed both the presence of the MMP-2 proteins in hoof tissue homogenates and the specificity of our antibody. The MMP-2 expression level of laminitic horses was compared with that of normal horses. Mean $\pm$ SE of MMP-2 mRNA expression in four normal horses was 1.03 ± 0.02. In 18 laminitic hooves, it was 2.26 ± 0.20, a significant ($P < 0.01$) increase.

Immunostaining, using our equine specific MMP-2 antibody, showed that MMP-2 was located in the cytoplasm of lamellar basal and parabasal cells, and concentrated in the basal cytoplasm adjacent to the BM. The keratinized axis of PELs and the connective tissue of the dermal lamellae did not immunostain. ISH of formalin fixed, paraffin-embedded tissue (using a DIG-labeled, equine-specific MMP-2 anti-sense probe) showed a pattern of MMP-2 mRNA similar to MMP-2 immunostaining.

ISH with an MMP-2 sense probe and no probe showed no signal.
Our quantitative real-time PCR results support, at the molecular level, the concept that increased MMP-2 transcription, in hoof tissues developing laminitis, promotes increased proteolytic degradation and structural failure of the hoof lamellae at the dermo-epidermal junction [7,8]. Type IV collagen and laminin, key structural components of the lamellar BM, are known targets of activated MMP-2 [20-23] and the molecular up-regulation of lamellar MMP-2 seems to be an important, early event in the pathogenesis of laminitis. Real-time PCR analysis of lamellar MMP-2 accurately monitors the molecular development of laminitis, and it can be used diagnostically and for testing preventive strategies. Targeted inhibition of MMP-2 transcription has the potential to prevent laminitis. The increase in MMP-2 expression occurs in the 48 h between the administration of a carbohydrate alimentary overload and the development of the first clinical signs of lameness. Clinicians should be aware that the enzymatic separation of BM from lamellar epidermal cells is well underway before clinical signs are apparent, and preventive strategies must be in place early if horses are to experience the developmental phase of laminitis without significant lamellar damage.

6. Equine Laminitis *In Vitro*: An Ultrastructural Study of Lamellar Dermo-Epidermal Separation

Partitioning the lamellar epidermal cells from the lamellar dermis is a thin layer of specialized material called the BM [24]. On one side of the BM, epidermal basal cells are firmly attached, whereas on the other, dermal side, tendon-like connective tissue is tightly woven into the mat-like structure of the BM [1]. The signature lesion of laminitis, failure of the attachment between lamellar dermis and epidermis, occurs at the lamellar dermo-epidermal junction and involves the lamellar BM [7,8]. When viewed with the TEM, the BM is dominated by the electron-dense lamina densa, which appears as a dark line along the contours of the epidermal basal cells [24]. The base of each basal cell is attached to the BM by numerous electron dense adhesion plaques or HDs. The various proteins of each HD occur on both sides of the basal cell P1, and form a bridge linking the interior of the basal cell to the exterior connective tissue. The intracytoplasmic HD proteins that attach basal cells to the BM are named plectin, BP230, BP180, and integrin $\alpha_6\beta_4$. Importantly, HDs are maintained and assembled by glucose-consuming phosphorylation reactions [25,26]. Integrin $\alpha_6\beta_4$ and BP180 have domains on both sides of the P1 and form part of the extracytoplasmic sub-basal dense plaque of the HD. Two proteins bridge the gap between the HD and the lamina densa BP180 and laminin 5. TEM resolves these proteins as innumerable fine AFs spanning the lamina lucida, the space between the basal cell and the lamina densa.
We used small samples of inner hoof wall lamellae (lamellar hoof explants), maintained in tissue culture fluid, to study two cellular mechanisms of laminitis [3,27]. The first laminitis mechanism relates to MMP activation. Activated MMPs are found in hoof lamellar tissue taken from horses with laminitis [3,19] and laminitis is associated with increased transcription of lamellar MMP-2. The *in vitro* model for this laminitis mechanism is to culture explants with a chemical MMP activator, APMA. The second laminitis mechanism relates to the essential need for glucose in the hoof lamellae. Insulin resistance and an attendant hyperglycemia is associated with both acute and chronic laminitis. It is a sign that entry of glucose into peripheral tissues (we presume this includes hoof lamellae) is compromised. The profound insulin resistance that occurs in peripheral tissues during stress, injury, or infection [28] could contribute to laminitis through a glucose deprivation mechanism. The *in vitro* model for this second mechanism is to culture lamellar explants without glucose. Our light microscope studies showed that both *in vitro* models of laminitis (MMP activation and glucose deprivation) develop a laminitis-like separation at the dermo-epidermal junction [27]. To gain further insight into the lesion of laminitis, we used TEM to study the separation process of the two *in vitro* models.
Explants, cultured with the MMP activator APMA or without glucose, were subjected to tension and processed for TEM. Explants were sampled at intervals over a 48-h period to study the time course of the ultrastructural events leading to dermoepidermal separation. Digital mosaics of TEM images were constructed using digital photographic software [j], and the number of HDs/μm of basal cell Pl in BM contact was counted using morphometric analysis software [k]. In addition, the numeric density of HDs/unit surface of basal cell Pl was derived as well as the approximate percentage area of BM surface in contact with HDs. The distance from the epidermal cell Pl to the lamina densa of the BM was also measured. The results were analyzed using ANOVA and Student's T test.
Without glucose, or with APMA, explants under tension separated along the dermo-epidermal junction. However, the results showed that this *in vitro* laminitis occurred through two different ultrastructural processes. Lack of glucose reduced HD numbers until they disappeared, and the basal cell cytoskeleton collapsed. The AFs connecting the basal cell Pl to the BM were unaffected by glucose deprivation, although they failed under tension. In contrast, APMA activation of constituent MMPs did not affect HD number or size but instead, caused AFs to disappear; this also led to dermo-epidermal separation under tension.
Glucose is clearly important in the maintenance of lamellar integrity [27]. Glucose deprivation could cause HD denucleation directly by disrupting energy-dependent phosphorylation reactions vital for HD assembly and maintenance [25,26]. The BM remained in fairly close apposition to the epidermal cell Pl throughout the culture period; however, under tension, it separated, presumably because of weak anchoring filament insertions into disintegrating HDs. Defective expression of

intracytoplasmic HD proteins in human blistering skin disorders and their targeted removal in experimental mice also results in loss of HDs and widespread dermo-epidermal separation [29].
MMPs are present in normal hoof tissue, and controlled MMP activity has the physiological role of remodeling the various cells of the secondary and primary lamellae. Explants cultured with APMA caused the surface area of HDs in BM contact to decrease, but HDs were always present in the Pl, despite separation from the BM. Thus, in contrast to HDs without glucose, HDs resist MMP activation and do not disappear. On the other hand, the AF connection between basal cell Pl and the lamina densa of the BM does disappear. In the inherited skin blistering disorder JEB of Belgian horses [30] there is AFs failure, and skin and hooves separate at the BM like in the hoof explant APMA model.
The results support the proposal that the lamina densa of the lamellar BM can be separated from lamellar basal cells by two different mechanisms. Glucose starvation weakens HDs, leads to their disappearance, and causes the cytoskeleton of basal cells to collapse. A similar mechanism may operate in natural cases of laminitis where basal cell uptake of glucose may be compromised. APMA activation of the MMPs resident in lamellar basal cells destroys AFs and sets the lamina densa adrift without significantly altering HD structure. Histopathology of carbohydrate overload laminitis [7,8] shows wholesale dislocation of lamellar BM from basal cells, suggesting that AFs destruction by activated MMPs occurs during the developmental process. Basal cell nuclear rounding, cytoskeleton disruption, and BM dysadhesion, all features of natural laminitis, were inducible *in vitro*, suggesting that both glucose unavailability and MMP activation are involved in the development of the dermo-epidermal separation that characterizes the disease.

7. Loss of Hemidesmosome Ultrastructure Correlates to Dose in an OF Laminitis Induction Model
In this study, we used TEM to investigate the ultrastructural changes occurring in the lamellar BM zone of horses with acute laminitis. Laminitis was induced using alimentary overload with OF; OF was administered at three doses to seek a correlation between the severity of the ultrastructural lesions and the dose of OF administered. The lesions of naturally occurring laminitis were compared with the two *in vitro* models.
The feet of the six normal Standardbred horses involved in the previously described study and the feet of the two normal untreated horses were dissected to produce lamellar samples, which were processed for TEM. As before digital images were generated and from the mosaic of continuous BM, the following parameters were calculated: the number of HDs/μm of basal cell Pl, the numeric density of HDs per unit surface of basal cell Pl, the approximate percentage area of basal cell Pl occupied by HDs, and the distance from the basal cell Pl to the lamina densa of the BM were quantitated. The results from the three OF treatments and the untreated normal horses were analyzed using ANOVA and Student's T test. Statistical analysis was performed using computer software [l].
Hoof tissue from control horses showed a normal arrangement of SELs basal cells with the key features of the lamellar ultrastructure present [1,24]. The average density of HDs/μm of the basal cell Pl was (mean $\pm$ SE) 4.64 $\pm$ 0.15 HDs/μm. The average percentage surface area of the basal cell Pl occupied by HDs was 28.57 $\pm$ 0.60%. The average distance between the epidermal cell Pl and the center of the lamina densa of the BM was 0.057 $\pm$ 0.002 μm.
Hoof SELs from horses dosed with 7.5 g/kg by weight of OF resembled hoof SELs from control horses, although the SEL tips were more pointed. There were a few areas, small in size, that were lacking in AFs, and at these points, the BM had lifted away from the epidermal cell Pl. The average density of HDs/μm of the basal cell Pl was 3.62 $\pm$ 0.11 HD/μm, and the average percentage surface area of the basal Pl occupied by HDs was 23.38 $\pm$ 0.77%. Both parameters had decreased significantly from the control horses. Although the average distance between the epidermal cell Pl and the center of the lamina densa had increased slightly (0.061 $\pm$ 0.001 μm), this was not significant.
Hoof SELs from the horses dosed with 10 g/kg by weight of OF showed ultrastructure dissimilar to hoof SELs from control horses. SEL tips were pointed instead of rounded, and the BM had a wavy or crenelated appearance. Areas of the lamina lucida lacking AFs, which were observed in the animals dosed with 7.5 k/kg by weight of OF, had become more widespread, and there were patches of BM that had separated from the basal cell Pl. The average density of HDs/μm of the basal cell Pl was 2.14 $\pm$ 0.01 HD/μm, and the average percentage surface area of the basal cell Pl occupied by HDs was 12.55 $\pm$ 0.82%, both significantly less than the measurements taken on the control horses. The average distance between the epidermal cell Pl and the center of the BM had increased to 0.067 $\pm$ 0.002 ìm, which significantly different than the measurements taken on the control horses.
Hoof SELs from horses dosed with 12.5 g/kg by weight of OF were more severely affected than hoof SELs from horses dosed with 10 g/kg by weight, with ultrastructure markedly different from the hoof SELs of control tissue. The BM was weakly stained and blurred, and bilayers of separated BM were present at SEL tips. HDs were few in number, small and unevenly distributed in the Pl of basal cells. HD intracytoplasmic dense plaques were pale and disorganized, apparently in the process of denucleating. BM was detached from the basal cell Pl, where HDs were absent or feint but remained attached at zones where HDs survived. Some degenerate SELs were reduced to attenuated, BM-enclosed tubes containing the debris of basal cell organelles, clumps of pale amorphous cytoplasm, and dense granules of cytoskeleton-like material. Many PMNs were associated with these damaged epidermal compartments, sometimes on the epidermal side of the BM. The average

density of HDs/μm of the basal cell Pl was 1.75 ± 0.09 HD/μm, and the average percentage surface area of the basal cell Pl occupied by HDs was 8.76 ± 0.44%, a significant decrease over the measurements taken from the control horses. The average distance between the epidermal cell Pl and the center of the BM had increased significantly (0.071 ± 0.02).

The ultrastructure of the BM zone of hoof lamellae was affected in a dose-dependent manner by alimentary OF overload. Compared with control lamellae, even OF at the lowest dose of 7.5 g/kg by weight significantly decreased both the size and number of lamellar HDs. The magnitude of HD shrinkage and loss increased as the OF dose increased. The highest dose tested was associated with wholesale BM separation, especially at SEL tips. Without properly assembled HDs, dysadhesion between the BM and the basal cell Pl occurred, emphasizing the fundamental importance of HDs in maintaining attachment at the matrix/epidermal basal cell interface. Thus, medical conditions contributing to the loss and failure of HDs must also contribute to laminitis development. The rapidly developing, more extensive SEL lesions in horses dosed with 12.5 g/kg of OF were markedly chemotactic to PMNs, themselves a potent source of MMP [31]. The magnitude of PMN influx in early acute laminitis probably entrains a cascade of self perpetuating lamellar degradation and therefore, is a harbinger of chronically increasing severity. We predict that chronic, severe laminitis tissue will show greater MMP expression than the tissue with newly developed, acute laminitis.

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 degree of lameness and grade of laminitis severity already exists [7]; it follows that the loss of HDs also correlates to lameness. Ultimately, the laminitis lesion will be resolved to the molecular level, and it may be a loss of a molecule at the lamellar dermo-epidermal junction that correlates to lameness.

Studies of laminitis *in vitro* demonstrated that the ultrastructural changes leading to separation can be initiated by (1) withdrawing glucose, causing the loss of HDs but leaving intact the AFs connecting the basal cell to the BM, and (2) activating constituent MMPs-spared HDs but destroyed AFs. The ultrastructural changes of OF-induced laminitis were not clear cut; features of both *in vitro* models were present in the laminitic tissue. The shrinkage and loss of HDs was a notable feature of OF-induced laminitis tissue. This was also the principal lesion of the zero glucose *in vitro* model. However, destruction of the AFs linking the basal cell Pl to the BM were also present in the OF-induced laminitic tissue and resembled similar ultrastructural changes observed in the MMP activation *in vitro* model. Thus, the pathology of OF-induced laminitis seems to result from at least two processes activation of constituent MMPs and failure of glucose to enter SEL basal cells. There is indirect evidence that these two processes occur during the developmental phase of laminitis. The molecular components of BM and AFs are substrates for MMP activity [32] and increased concentrations of MMP-2 are known to be present in both pro-enzyme and active forms in lamellar homogenates from laminitic horses [3,19]. In addition, increased transcription of MMP-2 RNA is well underway 48 h after administration of a laminitis inducing alimentary carbohydrate overload.

Hyperglycemia, particularly in the 10- and 12.5 g/kg groups, was a feature of OF-induced laminitis, and a cause of this could be an abrupt increase in insulin resistance. Stress and sepsis are known causes of insulin resistance [28] and the metabolic reaction associated with alimentary OF overload could well be sufficient to trigger an episode of profound insulin resistance. Lamellae suddenly insensitive to insulin, with glucose uptake blocked, may fail because of damaged hemidesmosome ultrastructure as occurs in lamellar explants developing *in vitro* laminitis when glucose is withheld [27]. A correlation between lameness severity and escalating loss of lamellar HDs now exists. Therapy aimed at protecting the lamellar environment from hematogenous delivery of MMP activators or from glucose deprivation may control laminitis development.

8. Prolonged, Continuous Distal Limb Cryotherapy in Horses

The use of cryotherapy for the treatment of athletic injuries in the horse is well documented [33-35] and cryotherapy remains popular because of its low cost, ease of application, lack of side-effects, and perceived clinical benefits [36]. Continuous cryotherapy at controlled temperatures (5 - 10ºC), for protracted periods (up to 72 h), is superior to traditional cryotherapy strategies used post-operatively in human knee surgery patients [37]. Continuous cryotherapy during the developmental and acute stages of equine laminitis has been suggested as a potential preventative strategy [38]. The aim of this study was to evaluate the clinical effects of ice and water applied continuously to the distal limbs of horses for 48 h.

A boot [m] containing a slurry of ice and water was applied to the right forelimb of four clinically normal horses for 48 h. Forelimb HT, ambient temperature, and internal ice boot temperature were logged continuously using data logging devices [n] attached to thermistors by 2-m cables. The HT thermistors were housed in stainless steel probes that were inserted into holes drilled into the midline dorsal hoof wall 20 mm distal to the coronet of both forelimbs.

Appetite, demeanor, oral mucous membrane capillary refill time, fecal output, rectal temperature, and heart rate were monitored at 2-h intervals. At the conclusion of the 48-h period, the ice boot was removed. Two hours after removal of the ice boot, the horses were examined at the walk and trot for lameness. The horses were then placed into a paddock, and examinations for lameness were repeated at 1 wk, 6 mo, and 1 yr after treatment. Heart rate, respiratory rate, rectal temperature, and HT data were examined over time using repeated-measures ANOVA. HTs of the treated and untreated limbs

were compared at specific time points using one-way ANOVA. All results are expressed as the mean ± SE. Statistical analysis was performed using computer software [l].

With the boot applied and containing the ice and water mixture, the horses were able to use the treated limb for normal weight bearing and limited ambulation. Appetite, oral mucous membrane capillary refill time, and demeanor were not affected by application of the ice boot for the 48-h period. The HT of the untreated limbs ranged between 27.7ºC and 34.8ºC (32.5 ± 0.1ºC). Treated limb HT decreased rapidly after cryotherapy began at 0 h. Mean HT of the treated limbs at 2 h (11.9 ± 1.0ºC) was significantly ($P < 0.05$) less than that of the untreated limbs (33.7 ± 0.5ºC). After 2 h, the HT of the treated limbs (5.3 ± 0.3ºC) remained significantly less than that of the untreated limbs for the remainder of the experimental period ($P < 0.05$). Between 2 and 48 h, the mean difference between the untreated and treated limb HT was 27.1 ± 0.3ºC. At 50 h, 2 h after removal of the ice from the boot, the HT of the treated limbs (24.0 ± 2.7ºC) was still significantly less ($P < 0.05$) than that of the untreated limbs (31.5 ± 0.7ºC).

Rectal temperature (37.7 ± 0.01ºC) did not vary significantly during the 50-h experimental period. Lameness was not detected in any horse before or after experimentation. No edema of the treated limbs was present at any of the examination periods. Treated and untreated hooves appeared normal, and there were no visible "rings" in the treated hooves up to 1 yr after removal of the ice boot.

The application of ice and water to the distal limb of horses for 48 h was well tolerated and resulted in no clinical ill effect. The slurry of crushed ice and water applied to the level of the fetlock was effective in cooling the feet. Most horses in the trial remained fairly still throughout the experimental period. Confinement in a stock, with one or more limbs in unattached boots or a cold water tank, is suggested for routine cryotherapy. The use of HT, measured under appropriate conditions, is considered a valid indicator of digital perfusion [39]. HT measurement using thermistors placed in holes in the hoof wall has been performed previously [40] and relative increases in digital perfusion were readily detected using HT measurement when the ambient temperature was low. The absence of any marked variation in the HT of the treated limbs suggests that sublamellar perfusion and metabolic rate remained fairly constant.

A significant decrease in soft tissue perfusion of the equine digit has been demonstrated scintigraphically in feet subjected to cryotherapy for 30 min [41]. Contrary to what is documented in other species [42] the phenomenon of reflex intermittent vasodilation (hunting reaction) was not observed. This phenomenon is thought to be largely caused by dilatation of blood vessels in muscle tissue [43,44] and the lack of skeletal muscle tissue in the equine distal limb may explain the absence of "hunting" in response to local cryotherapy.

The ability to safely achieve extremely low tissue temperatures in the distal limb of the horse for extended continuous periods provides the unique opportunity to modify the course of pathological processes that rely on up-regulated enzymatic activity and/or increased vascular perfusion. Metabolic enzymatic activity decreases by approximately 50% when tissue temperature is lowered by 10ºC [45]. In this study, the average reduction in treated HT (27.1 ± 0.3ºC) over a 46-h period suggests that a marked and prolonged hypometabolic effect was achieved. The activity of MMP enzymes has been implicated in the pathogenesis of acute laminitis [3,19]. Continuous distal limb cryotherapy during the developmental stage of laminitis could limit MMP activity until the initiating systemic disease state has abated. Furthermore, cold-induced vasoconstriction during this period may limit the delivery of hematogenous "laminitis trigger factors" [38].

Cold-induced pain was observed in human patients when low temperatures (5ºC) were applied to knees continuously for 48 h [37]. Cryotherapy at 10ºC was tolerated better but was less therapeutically effective. Continuous cryotherapy at 5ºC is superior, but humans require increased analgesic medication to tolerate this. Fortunately, cold-induced pain is not a problem in horses; they seem to lack cold nociception in their distal limbs. Horses in the current study showed no cold-induced injury or any clinical signs attributable to cold-induced pain, despite extremely low ice boot and tissue temperatures. Continuous application of ice and water to the equine distal limb for 48 h seems safe, effective, and well tolerated by horses.

9. Cryotherapy Prevents Development of Acute Laminitis

There are two broad pathophysiological hypotheses for the basic mechanisms that ultimately result in failure of the attachment apparatus between the hoof wall and distal phalanx [46]. The first proposes that digital hypoperfusion during the developmental stage of laminitis leads to ischaemia of lamellar tissue. Excessive uncontrolled enzymatic degradation of lamellar attachments caused by hematogenous "laminitis trigger factors" form the basis of the second hypothesis [47]. Delivery of hematogenous factors responsible for triggering lamellar MMP enzyme activity would require adequate digital blood flow during the developmental stage of laminitis. Three studies [40,46,48] report increased digital blood flow before and during acute laminitis induced by alimentary carbohydrate overload. Indeed, digital vasoconstriction during the developmental stage seemed to protect horses against laminitis [40] and led to the suggestion that promoting digital vasoconstriction using cryotherapy may be an effective laminitis preventive strategy [38].

The therapeutic application of cold results in local analgesia, tissue hypometabolism, and a vascular response [42]. A marked reduction in metabolic enzymatic activity [45] and a profound local vasoconstriction [43] occur locally. The application of cryotherapy in humans is limited to 30 45 min to avoid frost-bite and nerve palsy [49] and this time limit is usually applied to

horses. Horses, however, show no adverse effects when their distal limbs are continuously exposed to sub-freezing ambient temperatures in sub-polar climates.

Scalp cryotherapy prevents alopecia in human cancer patients undergoing chemotherapy [49]. Cryotherapy apparently reduces delivery of the chemotherapeutic agent to the scalp as well as reducing cellular uptake and metabolism when the drug reaches the hair follicles. Similarly, cryotherapy could be used during the developmental stage of laminitis to reduce delivery of hematogenous "laminitis trigger factors" to the digit as well as reduce the activity of MMP enzymes at the lamellae. We evaluated the efficacy of cryotherapy continuously applied to one limb in preventing laminitis induced by alimentary carbohydrate overload.

Six mature, Standardbred horses (three geldings and three mares) with normal feet and no lameness received alimentary overload with OF (10 g/kg) to induce laminitis. Each horse was confined to a stock, and one front limb (left) was placed in a rubber boot [o] containing a mixture of 50% cubed ice and 50% water for the duration of the 48-h experimental period. The boot was continually replenished with ice to maintain a level just below the carpus. Forelimb HT, ambient temperature, and internal ice boot temperature were logged continuously using data logging devices. The information stored in each of the four data loggers was downloaded to a computer at the end of the 48-h experimental period and analyzed. Appetite, demeanor, oral mucous membrane capillary refill time, fecal output, rectal temperature, and heart rate were also monitored.

At the end of the 48-h experimental period, the ice boot was removed from the forelimb, and the horses were evaluated at the walk and trot for lameness. After euthanasia, the dorsal hoof wall lamellae were sampled [7] and randomized, stained sections of the dorsal hoof wall lamellae from each foot were coded and submitted for light microscopic examination by four blinded evaluators. The evaluators graded the severity of laminitis using an established scoring system [7].

Samples of lamellar tissue from each hoof were also rapidly frozen by immersion in liquid nitrogen, stored at 70ºC, and later subjected to real-time PCR analysis for MMP-2 mRNA. Results were expressed as the magnitude of the MMP-2 gene relative to that of tissue from four normal hooves.

Temperature, real-time PCR, and histological data were analyzed statistically using a significance level of $P < 0.05$, and an inter-evaluator agreement on histological scores was tested using a weighted κ test. Lameness was evaluated by one observer only at the completion of the 48-h experimental period. Results are expressed as the mean $\pm$ SE.

The six horses maintained the treated limbs within the ice boot voluntarily, only rarely attempting to remove them. All horses developed mild to severe lameness consistent with clinical laminitis in one or more feet. However, the horses appeared to be lame only in the untreated forelimb. Shifting of weight in the fore and hind limbs was noted in all horses beginning between 28 and 46 h. One horse held the untreated forelimb off the ground for prolonged periods between 40 and 48 h.

Mean ice boot temperature was 0.5 ± 1.7ºC. The HT of the treated limbs decreased rapidly within the first hour and remained below 5ºC for the remainder of the experimental period (3.5 ± 0.9ºC). The mean HT of the untreated forelimbs was not significantly different from that of the mean ambient temperature between 12 and 28 h, but then a sharp HT increase to above 30ºC occurred in the untreated forelimb between 28 and 46 h.

Median laminitis histology scores for the treated feet were either 0 (normal) or 0.5. The untreated forefeet had median scores ranging between 1 and 3 (severe). Wilcoxon signed ranks analysis revealed significantly increased histological scores in the untreated forefeet compared with that of the treated forefeet ($P < 0.05$). Mann-Whitney analysis revealed significantly increased ($P < 0.05$) histological scores in the untreated limbs as a group ($n = 18$) compared with the treated limbs ($n = 6$). Detachment of the BM from the SELs never occurred in the treated feet, although elongation of the SELs as well as changes in basal cell nuclear morphology (round instead of oval) and position (more centrally located within the cytoplasm) were present. Inter-evaluator agreement, expressed as a weighted kappa statistic (*Kw*), ranged from fair to moderate.

In all instances (except the right hindfoot of one horse), MMP-2 mRNA expression magnitude was greater in each of the untreated feet than the corresponding treated foot of each horse. MMP-2 mRNA expression magnitude in the treated feet was significantly less ($P < 0.05$) than that of the corresponding untreated forefeet. Mean MMP-2 mRNA expression magnitude in the treated feet ($n = 6$) was significantly less ($P < 0.05$) than that of the untreated feet as a group ($n = 18$), but it was significantly greater ($P < 0.05$) than that of the four normal control feet (1.04 ± 0.02).

Cryotherapy, when applied to one foot, was effective in preventing the development of acute laminitis in the face of a challenge that caused laminitis in the remaining three untreated feet. A significant reduction in the severity of laminitis histology ($P < 0.05$) occurred in the treated limbs. Genetic up-regulation of MMP-2 enzymes, implicated in the pathogenesis of laminitis [3,19] was also significantly reduced in the treated limbs ($P < 0.05$). Subjectively, cryotherapy also prevented the development of clinical laminitis. Thus, the vascular and hypometabolic effects of cryotherapy seem to intervene beneficially in the pathophysiology of acute laminitis.

Substances delivered through the circulation to the digit, such as cytokines [7] and bacterial products of hindgut origin [17] proposed initiators of MMP enzyme production and activation, and subsequently, lamellar separation. Profound, continuous vasoconstriction in the treated limbs may have prevented the delivery of such hematogenous "laminitis trigger factors" to the treated digits in this study. Such profound vasoconstriction would seem contra-indicated if digital hypoperfusion [50] was the primary mechanism involved in the development of laminitis. Directly or indirectly, cryotherapy also reduced the expression of MMP-2 mRNA in the lamellar tissue of the treated feet in this study.

We propose that cryotherapy, applied during the developmental stage of acute laminitis, prevents delivery of hematogenous "laminitis trigger factors" to the lamellar tissue through vasoconstriction of the digital circulation. The low temperature achieved by the application of iced water to the equine distal limb acts to inhibit MMP enzyme production and activity, even if triggering factors are present. We suggest cryotherapy as a potentially effective prophylactic strategy in horses with conditions placing them at risk of developing acute laminitis.

10. Conclusions

Just as it is impossible to understand bleeding without first understanding coagulation, cancer without understanding cell growth and differentiation [51] so it is that the puzzle of laminitis will be deciphered when the biology of normal lamellae is thoroughly investigated.

Our lamellar proliferation studies suggest that the equine hoof has evolved a dependency on controlled enzymatic activity (MMPs) to remodel the non-proliferative lamellae of the "evergrowing" hoof as it moves downwards over the connective tissue of the distal phalanx. Although this mechanism serves the genus well in its natural environment, a problem arises when MMP control and balance is lost (as seems to be the case in laminitis). Interestingly, the hoof lamellar cells, which we take for granted to be permanently and well-attached at the dermo-epidermal junction, can loosen their attachments and migrate (proliferating as they go) when the need arises. Our wall stripping experiments show that this is the situation when the lamellae are wounded. The rapid restoration of near normal anatomy only occurs when the lamellar BM survives wall stripping virtually intact and serves as a scaffold (something that does not occur after laminitis). This shows the fundamental importance of an intact and functional BM. Strategies to preserve the lamellar BM and maintain lamellar basal cell to BM proximity during laminitis should improve the outcome. New biochemical and surgical approaches will be required to achieve this.

We used molecular biological techniques to document the status of the MMPs enzymes in hoof lamellae before and after laminitis, and we found that the transcription of MMP-2 is significantly increased after laminitis induction. We learned that lamellar MMP-2 and its RNA are positioned perfectly to mediate lamellar basal cell movement in relation to the adjacent BM. This happens when lamellar basal cells detach from the BM to migrate during wound healing, during normal growth of the hoof wall, and catastrophically, during the prelude to acute laminitis. Increased transcription and activation of lamellar MMP-2 (in addition to histopathology) should be the gold standard by which laminitis researchers prove that laminitis has or has not occurred.

We have developed a cleaner laminitis induction model using a pure carbohydrate (OF) instead of the usual carbohydrate protein mixture of milled grain. This enables us to induce laminitis of greater or lesser severity depending on the dose of OF administered. Because OF is closely related to the storage carbohydrate of pasture (fructan), the model takes us closer to the natural disease. We have continued our *in vitro* studies of hoof lamellae, and we have discovered that the dermo-epidermal separation that occurs when either glucose is absent or lamellar constituent MMPs are activated occurs because of damage to lamellar HDs. The effect on HDs is treatment specific; lack of glucose causes one type of lesion, and MMP activation causes another type of lesion. Both lesions are present in OF-induced laminitis, suggesting that at least two pathophysiological mechanisms may be operative. Our ultrastructural studies of laminitis show a correlation between laminitis severity and HD survival, which emphasizes the fundamental importance of these adhesion plaques in maintaining lamellar integrity. Our new knowledge of laminitis pathophysiology has enabled us to predict and verify that laminitis is preventable if the delivery of blood-borne "laminitis trigger factors" and activity of lamellar enzymes are controlled. This has been achieved using distal limb cryotherapy.

One-half of laminitis science is incorrect but which half [51]? Of the nebulous laminitis data of the last 60 yr, patterns and trends come about that crystallize into principles in veterinarian's consciousness. Although we have made some progress toward achieving the goals of the AELRU, there are still gaps in our knowledge. The biological basis of laminitis has become molecular, and the discipline of molecular biology has laminitis in its crosshairs. These are exciting times to be involved in equine research we now have tools our forefathers would not have thought possible. A coherent body of knowledge will soon emerge that will demystify laminitis.

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Footnotes

[a] Sigma Chemical Co., St Louis, MO 63178.

[b] Amersham (Australia), Castle Hill, NSW 2154, Australia.

[c] xylazil-100, Ilium Laboratories Pty. Ltd., Smithfield, NSW, Australia.
[d] ketamine, Parnell Laboratories (Aust) Pty. Ltd., Alexandria, NSW 2015 Australia.
[e] Depocillin, Intervet Australia Pty., Ltd., Bendigo East, VIC 3550, Australia.
[f] Butasyl, Fort Dodge, Novartis Animal Health Australasia Pty., Ltd., Pendle Hill, NSW, Australia.
[g] Raftilose, Orafti Active Food Ingredients, Aandorenstraat 1, B-3300 Tienen, Belgium.
[h] Flunix, Parnell Laboratories (Australia) Pty., Ltd., Alexandria, NSW 2015, Australia.
[i] Mimotopes Pty. Ltd. Clayton VIC. 3168 Australia.
[j] Adobe Photoshop 6, Adobe Systems Inc., San Jose, CA 95110-2704.
[k] ImagePro, MediaCybernetics, Silver Spring, MD 20910.
[l] Jacks Whirlpool Boot, Jack's Mfg. Inc., Washington C.H. Ohio 43160.
[m] Tinyview Plus Data Loggers, Gemini Inc., Reno, Nevada 89509.
[n] Analyse-it Software Ltd., Leeds, LS27 7WZ, England, UK.
[o] Bigfoot Ice Boots, Esk, Queensland, 4312 Australia.

Abbreviations
AELRU - Australian Equine Laminitis Research Unit
AFs - anchoring filaments
ANOVA - analysis of variance
APMA - p-aminophenyl-mercuric acetate
BM - basement membrane
BP180 - bullous pemphigoid 180 (antigen)
BP230 - bullous pemphigoid 230
BRdU - 5-bromo-2'-deoxyuridine
cDNA - complementary DNA
DIG - digoxigenin
DM - dry matter; DS cDNA, double strand cDNA
ECM - extracellular matrix
GAPDH - glyceraldehyde-3-phosphate dehydrogenase
HDs - hemidesmosomes
HT - hoof temperature
ISH - *in situ* hybridization
JEB - junctional epidermolysis bullosa
MFs - mitotic figures
MMPs - matrix metalloproteinases
MMP-2 - matrix metalloproteinase-2
mRNA - messenger RNA
OF - oligofructose
PCR - polymerase chain reaction
PELs - primary epidermal lamellae
PI - proliferative index
Pl - plasmalemma
PMNs - polymorphonuclear leukocytes
RT-PCR - reverse transcription polymerase chain reaction
SELs - secondary epidermal lamellae
TEM - transmission electron microscope
TIMPs - tissue inhibitors of metalloproteinases

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