Morphological and cellular changes in secondary epidermal laminae of horses with insulin-induced laminitis

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Objective—To determine cellular changes associated with secondary epidermal laminae (SEL) in forefeet and hind feet of ponies with insulin-induced laminitis.

Animals—8 ponies.

Procedures—Laminitis was induced in 4 ponies by IV administration of insulin and glucose; 4 control ponies received saline (0.9% NaCl) solution IV. Laminar tissue samples obtained from the dorsal aspects of the hooves were histologically evaluated. Primary epidermal lamina (PEL) length and width and SEL length, width, and angle were determined. Numbers of epidermal cell nuclei per micrometer and per total length of SEL and numbers of apoptotic and proliferative cells in axial, middle, and abaxial laminar regions were determined.

Results—SEL in treatment group ponies were significantly longer, were significantly narrower, and had a smaller angle relative to PEL in all laminar regions versus control ponies. In treatment group ponies, the number of epidermal cell nuclei per SEL was typically higher and the number of cells per micrometer of SEL was lower in laminar regions, apoptotic cell numbers were higher in abaxial and middle regions in forefeet and hind feet, and proliferating cell numbers were higher in axial laminar regions in forefeet and all laminar regions in hind feet, versus control ponies.

Conclusions and Clinical Relevance—Results indicated SEL elongation, narrowing, and alteration in orientation developed in all feet of ponies with insulin-induced laminitis. This was primarily attributable to cell stretching that developed at the same time as an accelerated cell death–proliferation cycle; differences in cell cycle responses among laminar regions between forefeet and hind feet may have been attributable to differences in load bearing.

Received April 18, 2013.
Accepted September 3, 2013.
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Laminitis is a debilitating disease of equidae that has major welfare and economic consequences for the equine industry worldwide. The frequency of this disease in equids is estimated to be 1.5% to 34%. Laminitis has typically been considered to develop secondary to systemic inflammation or gastrointestinal tract disease or uneven weight distribution in limbs. More recently, results of other studies have indicated that endocrine disturbances (ie, endocrinopathic laminitis) account for most cases of laminitis evaluated at first-opinion practices in Europe and the United States. Such endocrinopathies include pituitary pars intermedia dysfunction and hyperinsulinemia attributable to equine metabolic syndrome. Recently, laminitis has been induced in horses and ponies with a Prolonged euglycemic-hyperinsulinemic clamp technique. Hyperinsulinemia in the absence of systemic inflammation and hyperglycemia consistently occurs in animals with laminitis that is associated with pitu-
The laminar region of the hoof in equids holds the distal phalanx and the hoof wall together; it consists of epidermis connected to underlying dermis by a basement membrane. The surface area of this dermal-epidermal junction is greatly increased by complex primary and secondary infoldings, called laminae. In an anatomically normal hoof, there are 550 to 600 PEL, each with 150 to 200 SEL. Regardless of disease etiology, the laminar structure in horses with laminitis weakens, which leads to stretching of laminae because of ground reaction forces, pull of the deep digital flexor tendon on the palmar or plantar aspect of the distal phalanx, and pressure that develops along the dorsal aspect of the hoof wall during breakover (ie, the period between the point at which the heel leaves the ground and the toe leaves the ground at the end of the stance phase of the stride). Studies of laminitis have typically involved histologic examination of hoof lamina samples obtained from horses that received an overload of carbohydrate (starch or oligofructose). However, results of studies in which a pEHC technique was used indicate substantial differences between endocrinopathic and nonendocrinopathic forms of laminitis. In all animals with laminitis, considerable elongation of SEL develops; however, severe basement membrane damage may be the most prominent pathological finding for horses with laminitis induced with carbohydrate overload. For horses that undergo a pEHC technique, basement membrane damage is minimal and frequently localized to SEL of the axial aspects of PEL. Additionally, in ponies and horses with insulin-induced laminitis, increased apoptotic cell death and mitotic and proliferative activity have been detected, but these factors have only been quantified for horses.

Insulin is a peptide hormone secreted by pancreatic \( \beta \) cells. Secretion of insulin is mainly regulated in response to plasma glucose concentration, but compensatory hyperinsulinemia develops when target cells have insulin resistance. After binding to its receptor, insulin may have metabolic effects via the phosphatidylinositol 3-kinase (PI-3K) pathway or mitogenic and differentiation. Insulin resistance in human keratinocytes alters patterns of proliferation and differentiation. Proliferative effects of insulin may cause inappropriate laminar keratinocyte proliferation, resulting in the elongation of SEL detected for equids with laminitis induced by means of a pEHC technique; however, the relative contributions of cell proliferation and stretching to elongation of SEL have not been determined. We hypothesized that elongation of SEL is attributable to cell stretching rather than simple addition of cells of the same size. Our objective was to measure morphological features of the SEL of ponies with insulin-induced laminitis and to quantify and localize changes in cell death and proliferation. Given that stretching of cells would indirectly indicate a reduction in resistance to mechanical forces of weight bearing, another objective was to determine differences in histomorphometric variables of SEL between forefeet and hind feet; in all previous studies of laminitis in ponies and horses, only forefeet have been evaluated, to the authors’ knowledge.

Materials and Methods

Animals—All experimental procedures were performed in another study, and archived samples collected during that study were used in the present study. The experimental protocol was approved by the Animal Ethics Committee of the University of Queensland, which monitors compliance with the Animal Welfare Act (2001) and The Code of Practice for the care and use of animals for scientific purposes. Eight healthy ponies (mean ± SE age, 6.3 ± 1.7 years; body weight, 244.1 ± 32 kg) with no known history of laminitis and no evidence of previous laminitis detected during examination of the hooves were included in the study. Ponies included 6 geldings, 1 sexually intact male, and 1 mare. By means of a randomization procedure, 4 of the ponies were allocated to a treatment group and 4 to a control group. Laminitis was induced with a pEHC technique. Treatment group ponies received recombinant human insulin and glucose IV. Control group ponies received an equivalent volume of saline (0.9% NaCl) solution IV. For treatment group ponies, 1 jugular venous catheter was used for the simultaneous administration of insulin at a fixed rate and glucose at a variable rate to maintain euglycemia (blood glucose concentration, 5 mmol/L). A catheter in the other jugular vein was used to collect blood samples for monitoring of blood glucose and insulin concentrations. After collection of baseline blood samples, insulin (45 mU/kg in 50 mL of saline solution as a bolus followed by 6 mU/min/kg for the duration of the experiment) was administered IV. Intravenous administration of 50% glucose solution was initiated at a mean ± SE rate of 24.4 ± 3.0 mmol/min/kg. The glucose administration rate was adjusted when blood glucose concentrations differed from the target concentration (5 mmol/L) by > 1 mmol/L. Control ponies received saline solution (14.7 mL/min/kg for 72 hours, which was the mean rate of fluid administration for treatment group ponies during the same period).

Obel grade 2 laminitis occurred 50.3 ± 5.3 hours (mean ± SE) after initiation of insulin and glucose administration in treatment group ponies, at which time insulin and glucose administration was stopped. All treatment group ponies received phenylbutazone (4.4 mg/kg, IV or PO) at the time of completion of insulin and glucose administration and were euthanized within 6 to 12 hours. Control group ponies were euthanized after 72 hours of saline solution administration. All ponies were euthanized with an overdose of barbiturate.

Hoof tissue sample collection and histologic examination—The distal aspect of all 4 limbs of each pony was disarticulated at the metacarpophalangeal or metatarsophalangeal joint within 10 minutes after death and sectioned with a band saw, as previously described. A sagittal section of hoof at the midpoint between the coronary band and the ground-bearing surface on the dorsal aspect was obtained. This section was then dissected to
obtain 5-mm² tissue samples, extending from the stratum medium to the dermal connective tissue. All hoof tissue samples were fixed in neutral-buffered 10% formalin for 24 hours, processed with routine histologic methods, embedded in paraffin wax, sectioned (thickness, 5 µm), and stained with H&E or by means of the PAS method.

**Histomorphometry**—Sections of hoof tissue samples were histologically examined by 2 of the authors (NPK and JPK) with a light microscope. Digital images were obtained with image capture software and a digital microscope camera. Images of PAS-stained sections from each of the 4 feet of all 8 ponies were obtained at 40X magnification and then merged (for each section) with photo-editing software for measurements of PEL. Then, images from each examined region (axial [adjacent to the distal phalanx], middle, and abaxial [adjacent to the stratum medium of the hoof wall]) of each of 10 randomly selected PEL were obtained at 200X magnification for measurements of SEL (Figures 1 and 2). All the measurements were performed with the aid of image analysis software.

The length of each PEL was measured with a drawing tool from the tip (axial) to the base (abaxial; Figure 1). The width of a PEL was measured at a location 50% along the length of the measurement line for that lamina. This measurement was determined from the tips of SEL on one side to the tips of SEL on the other side. The SEL length, width, and angle were measured for 10 randomly selected SEL (5 from the left side and 5 from the right side) at 200X magnification from each of the 3 examined regions of PEL (Figure 2). The regions were located 10%, 50%, and 90% along the PEL length, corresponding to the axial, middle, and abaxial regions, respectively. The lengths of SEL were measured from the tip to the base of such lamina at the junction with the keratinized axis of associated PEL. The widths of SEL were measured from basement membrane to basement membrane at the longitudinal midpoint of each SEL (identified along a straight line). The angle between axes of PEL and those of SEL was measured with the angle tool in the image analysis software. The number of epidermal cell nuclei per micrometer of an SEL was calculated by dividing the number of cell nuclei by the SEL length (for an individual SEL). Percentage differences between

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Figure 1—Photomicrograph indicating measurement of PEL length (a) and width (b) and the locations of abaxial (c), middle (d), and axial (e) PEL regions. For PEL length measurements, a continuous line was drawn from the axial tip of the PEL to the level of the base (where it joined the adjacent PEL). The PEL width was measured at the middle of each PEL by drawing a straight line that extended to the tips of SEL on each side. PDL = Primary dermal lamina. PAS stain; bar = 500 µm.

Figure 2—Photomicrograph indicating measurement of SEL length (a), width (b), and angle (c) in the abaxial region of a PEL. For SEL length measurements, a continuous line was drawn from the tip of each SEL to the base at the junction with the keratinized axis of the associated PEL. The SEL width measurements were performed at the midpoint of each SEL by drawing a straight line. The angle between the PEL axis and the SEL axis was measured with an angle tool in computer image analysis software. PAS stain; bar = 200 µm.

Figure 3—Photomicrograph of a representative SEL of a pony with insulin-induced laminitis. Notice the numerous rounded, hypereosinophilic apoptotic epithelial cells and large numbers of apoptotic bodies, some of which contain nuclear fragments. Some of the apoptotic bodies have been phagocytosed by adjacent, viable epithelial cells (arrow). H&E stain; bar = 50 µm.
the control and treatment group ponies were calculated for each variable (PEL length and width; SEL length, width, and angle; cell number per SEL; and cell number per micrometer of SEL).

**Quantification of apoptotic and proliferative cells in SEL—**Apoptosis of SEL cells was confirmed on the basis of morphological criteria, including cytoplasmic rounding, nuclear shrinkage, chromatin condensation and fragmentation, and formation of apoptotic bodies. Some of the apoptotic bodies had been phagocytosed by neighboring epithelial cells (as expected). The number of apoptotic cells per 200X field was counted for each of the 3 regions (axial, middle, and abaxial) of evaluated PEL (n = 10/hoof) in H&E-stained sections (Figure 3). Immunolocalization of proliferating cells was performed with a mouse monoclonal antibody (Ki-S2) to the TPX2 protein; TPX2 is a nuclear protein (repp86/p100) associated with cell proliferation that is specific for S, G2, and M phases of the cell cycle. Unlike the more commonly used immunohistochemical proliferation markers Ki-67 and topoisomerase-IIα antigens, TPX2 does not label cells in the G1 phase of the cell cycle, thereby avoiding inclusion of cells in damage-induced G1 arrest. In addition, because TPX2 is required for mitotic spindle formation, the antibody to that protein can label dividing cells that do not contain mitotic figures, which makes it superior to conventional staining techniques for detection of mitosis. The TPX2 immunolabeling was performed with an automated stainer and a horseradish peroxidase system. Antigen retrieval was achieved by heating sections in a digital electric pressure cooker in sodium citrate buffer (pH, 6; 125°C for 1 minute and 40 seconds). This antibody to the TPX2 protein has been validated for use with equine tissue samples by means of Western blot analysis and immunolabeling of positive control (epidermal) tissues. Primary antibodies were diluted at an optimal ratio (1:800) in antibody-dilution solution. Positive control tissue samples were human and equine skin samples. For negative reagent control samples (to exclude nonspecific binding identified by the detection system), mouse IgG was used instead of the primary antibody. Sections were stained with chromagen 3,3′-diaminobenzidine tetrahydrochloride twice for 5 minutes (with an intervening Tris-buffered saline with Tween wash) followed by counterstaining with Mayer hematoxylin (30 seconds). Five PEL on each slide were randomly selected, and the total number of cells with positive results for TPX2 in each region (axial, middle, and abaxial) was counted by one of the authors (MMN) with the assistance of image analysis software.

**Statistical analysis—**Comparisons of variables between ponies in treatment and control groups were performed for PEL length and width; SEL length, width, and angle; cell number per SEL; cell number per micrometer of SEL; and number of apoptotic and proliferative cells per 200X field. Results are reported as median and IQR values. Statistical analysis was performed with computer software. Values of all variables, except PEL width, were nonnormally distributed; therefore, a nonparametric Kruskal-Wallis test with Bonferroni adjustment was used for pairwise comparisons. Values of P < 0.05 were considered significant.

**Results**

**Histomorphometry—**No significant differences were detected in PEL length or width between right and left hooves for forefeet or hind feet; therefore, measurements for right and left hooves were pooled. Significant differences were detected in PEL length between forefeet and hind feet for control group ponies; therefore, all variables were analyzed separately for forefeet and hind feet. For treatment and control group ponies, PEL length was significantly (P = 0.015 for both groups) greater in forefeet than hind feet. For control group ponies, the median PEL length and width were 3,566 μm (IQR, 2,940 to 4,060 μm) and 295 μm (IQR, 263 to 341 μm) in hind feet, respectively. For treatment group ponies, the median PEL length and width were 4,105 μm (IQR, 3,754 to 4,504 μm) and 294 μm (IQR, 249 to 330 μm) in hind feet, respectively. The PEL length for forefeet and hind feet were significantly (P < 0.001) longer (mean percentage difference, 15%) in treatment group ponies than they were in control group ponies (Figure 4). Median SEL length, width, and angle and percentage differences between groups for each evaluated region in forefeet and hind feet were summarized (Table 1). For control group ponies, abaxial region SEL were significantly longer (P < 0.05).
0.003) and narrower (P < 0.001) and had a smaller angle toward the PEL axis (P = 0.002) in forefeet than they did in hind feet. In treatment group ponies with experimentally induced laminitis, SEL were longer, were narrower, and had a smaller angle in all 3 examined laminar regions in all feet, compared with control ponies; this change was most prominent in the middle region (P < 0.001).

For control group ponies, there were significantly (P < 0.001) more epidermal cell nuclei per SEL in forefeet (abaxial and middle laminar regions) than in hind feet. For treatment group ponies, there were no significant differences between forefoot and hind feet regarding the number of epidermal cell nuclei per SEL. Significantly greater numbers of cell nuclei per SEL were detected in axial (P < 0.001) and middle regions (P = 0.003) of forefoot laminae and axial and middle (P < 0.001) and abaxial (P = 0.001) regions of hind feet than in control pony SEL (Figure 5). The number of epidermal cell nuclei per SEL in treatment group ponies was a median percentage of 6% (forefeet) and 17% (hind feet) higher than values for control ponies.

Table 1—Median (IQR) SEL length, width, and angle and percentage difference between control ponies (n = 4) and ponies with insulin-induced laminitis (treatment; 4) in axial, middle, and abaxial laminar regions in forefeet and hind feet.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Treatment</th>
<th>Difference (%)</th>
<th>Control</th>
<th>Treatment</th>
<th>Difference (%)</th>
<th>Control</th>
<th>Treatment</th>
<th>Difference (%)</th>
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<tr>
<td>Forefeet</td>
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<td></td>
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<tr>
<td>Length (µm)</td>
<td>115.0 (115.5-193.0)</td>
<td>284.6 (183.5-405.8)*</td>
<td>158</td>
<td>21.0 (15.0-29.0)*</td>
<td>209.5 (154.4-288.6)</td>
<td>32.5 (20.5-39.0)*</td>
<td>159</td>
<td>21.0 (15.0-29.0)*</td>
<td>209.5 (154.4-288.6)</td>
</tr>
<tr>
<td>Width (µm)</td>
<td>39.3 (28.0-42.1)</td>
<td>22.6 (118.0-30.6)</td>
<td>50</td>
<td>8.1 (6.0-9.0)</td>
<td>25.5 (20.9-30.6)</td>
<td>17.2 (14.7-21.2)*</td>
<td>47</td>
<td>8.1 (6.0-9.0)</td>
<td>25.5 (20.9-30.6)</td>
</tr>
<tr>
<td>Angle (°)</td>
<td>19.5 (19.5-103.0)</td>
<td>21.0 (115.0-29.0)</td>
<td>200</td>
<td>0.5 (0.5-0.5)</td>
<td>37.1 (34.8-55.3)</td>
<td>18.0 (14.7-23.2)*</td>
<td>106</td>
<td>0.5 (0.5-0.5)</td>
<td>37.1 (34.8-55.3)</td>
</tr>
<tr>
<td>Hind feet</td>
<td></td>
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<tr>
<td>Length (µm)</td>
<td>198.2 (111.6-199.0)</td>
<td>360.2 (250.0-539.0)*</td>
<td>88</td>
<td>18.9 (18.9-29.0)*</td>
<td>198.2 (146.0-297.8)</td>
<td>401.3 (346.4-569.9)*</td>
<td>133</td>
<td>18.9 (18.9-29.0)*</td>
<td>198.2 (146.0-297.8)</td>
</tr>
<tr>
<td>Width (µm)</td>
<td>21.0 (25.3-38.0)</td>
<td>21.4 (116.7-27.9)*</td>
<td>42</td>
<td>0.5 (0.5-0.5)</td>
<td>25.4 (21.9-29.0)</td>
<td>16.4 (14.3-19.6)*</td>
<td>55</td>
<td>0.5 (0.5-0.5)</td>
<td>25.4 (21.9-29.0)</td>
</tr>
<tr>
<td>Angle (°)</td>
<td>76.5 (49.9-103.0)</td>
<td>21.0 (115.0-29.0)</td>
<td>270</td>
<td>0.5 (0.5-0.5)</td>
<td>39.1 (28.8-54.2)</td>
<td>19.4 (15.4-24.2)*</td>
<td>102</td>
<td>0.5 (0.5-0.5)</td>
<td>39.1 (28.8-54.2)</td>
</tr>
</tbody>
</table>

One hundred SEL length, width, and angle measurements were determined for each laminar region in each foot of the ponies. *Values are significantly (P < 0.05) different between treatment and control group ponies.
Apoptosis and cellular proliferation—Apoptotic cells and cells with positive results for TPX2 were rarely detected in hoof tissue samples obtained from control group ponies, with no significant differences detected among regions (Figure 6). Numbers of apoptotic cells were significantly \( (P < 0.001) \) higher in treatment group ponies versus control ponies in abaxial and middle regions of laminae in forefeet and hind feet. Numbers of cell nuclei with positive results for TPX2 were significantly higher for treatment group ponies in forefeet for axial regions \( (P = 0.001) \) and in hind feet for abaxial \( (P = 0.049) \), middle \( (P = 0.017) \), and axial \( (P = 0.043) \) regions.

Discussion

The results of the present study indicated a significantly lower number of cell nuclei per micrometer of SEL in all laminar regions in forefeet and hind feet for treatment group ponies versus control group ponies, without evidence of laminar disruption. These findings suggested that such cells in treatment group ponies were longer than those in control group ponies. The percentage increase in total SEL cell number (approx 12%) was considerably smaller than the percentage decrease in cell number per micrometer (approx 75%) in treatment group ponies versus control ponies. These findings suggested that cell elongation was likely the primary mechanism for laminar stretching during laminitis. The relationship between such lesions and hyperinsulinemia in equids with laminitis was not determined. The finding that numbers of both apoptotic and proliferating SEL cells were higher in treatment group ponies than they were in control group ponies suggested a complex relationship between cell stretching and alteration in cell cycles. The temporal relationship between these processes cannot be determined by means of histologic examination of tissue samples obtained at 1 time; however, the spatial localization of such changes may suggest potential relationships. For example, in the hind feet, compared with the forefeet, the extent of cellular elongation was the same, but the increase in total numbers of cells forming the SEL, compared with control ponies, was greater, and proliferating cells were detected in all SEL regions (abaxial, middle, axial). Conversely, total SEL cell number was not significantly higher in the abaxial region of the forefeet of treatment group ponies versus control ponies, and apoptosis was more prominent than proliferation. Differences in biomechanics between hind feet and forefeet attributable to differences in weight-bearing loads may have caused such variation in results among anatomic locations. Our finding that the greatest numbers of apoptotic cells were located in abaxial and middle laminar regions in all feet of treatment group ponies might also have been attributable to mechanical factors. Mechanical stress induces apoptosis of multiple cell types, including epithelial cells. The abaxial region SEL were longer than those in other regions in control ponies. Therefore, if long SEL have a lower tolerance for mechanical forces versus short SEL, the small amount of elongation of SEL in the abaxial region may have been sufficient to cause cell death. Additionally, the thick keratin cores of PEL attached to and between rows of SEL in abaxial regions might have limited elongation.
and contributed to mechanical stress. The micromechanics of PEL and SEL have not been determined at this level of anatomic detail, to the authors’ knowledge.

The SEL cell proliferation detected in treatment group ponies in this study could have been a response to cell death, as develops during epithelial homeostasis and wound-healing processes, rather than aberrant proliferation of laminar epidermal cells. Changes in cell shape in response to cell death are exposed to different cyclic mechanical strains in fibroblasts obtained from equine digital tendons that can be maintained) among regions. For example, internal cytoskeletal tension and external tension range of tissue tensions within which the balance of apoptosis and proliferation of laminar epidermal cells to various amounts of stretching among laminar regions is likely greater in forefeet than they are in hind feet and lesions may be more severe in forefoot. The potential complexity of the accelerated cell death–proliferation cycle during laminitis is also suggested by comparison of results between horses and ponies. In the forefeet of Standardbreds, for which a higher mechanical load would be expected versus that of ponies, increased numbers of proliferating cells are detected in axial and abaxial laminar regions and increased proliferation in axial regions. In horses, approximately 58% of body weight is supported on forefeet; therefore, mechanical forces on the laminar region of feet are likely greater in forefeet than they are in hind feet and lesions may be more severe in forefoot. The potential complexity of the accelerated cell death–proliferation cycle during laminitis is also suggested by comparison of results between horses and ponies. In the forefeet of Standardbreds, for which a higher mechanical load would be expected versus that of ponies, increased numbers of proliferating cells are detected in axial and abaxial laminar regions at 24 and 48 hours after induction of laminitis (middle laminar regions were not analyzed in that study), whereas numbers of apoptotic cells were lower at those times versus earlier times. The proliferative response of laminar cells may continue after the rate of apoptotic cell death has decreased, and that rate of apoptotic cell death may decrease beginning in axial laminar regions. Evaluation of equids with other types of experimentally induced laminitis and those with naturally developing disease at various times (acute and chronic) may provide further information regarding how the 3 evaluated cellular mechanisms by which hyperinsulinemia induced or facilitated such processes are not known, to the authors’ knowledge.

**References**

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