Acute Laminitis: Descriptive Evaluation of Serial Hoof Biopsies

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This study describes the progression of histological changes in horses developing laminitis and shows that basement membrane pathology is the earliest and most significant histological lesion. These changes are present as early as 12 h after induction, long before clinical signs of lameness are observed. Clinicians need to be proactive if prevention and treatment are to be successful. Authors’ address: Australian Equine Laminitis Research Unit, School of Veterinary Science, Faculty of Natural Resources Agriculture and Veterinary Science, The University of Queensland, Brisbane, Queensland 4072, Australia; e-mail: ecroser@uq.edu.au (Croser). © 2006 AAEP.

1. Introduction
The histopathology of laminitis-affected hoof lamellae has long been studied to better elucidate the pathogenesis of this debilitating disease.1–13 The histopathologic findings at various stages of disease development have been described, and most reports describe samples taken once clinical signs of lameness have occurred. This study reports the collection and descriptive evaluation of serial biopsies of lamellar tissue taken before and at the prodromal, developmental, and acute phases of laminitis. Six biopsies were taken from each of five horses over a period of 48 h after administration of an alimentary carbohydrate overload.

2. Materials and Methods
This series of experiments was approved by the University of Queensland Animal Experimentation Ethics committee. Laminitis was induced by administration of oligofructosea (OF).14 Briefly, five mature, standardbred horses without lameness or radiological evidence of hoof disease were stabled and acclimated to a high-grain diet for 3 wk. Three days before laminitis induction, 10% of the OF induction dose was added to the diet for 3 consecutive days. On the fourth day, an induction dose of 10 g OF/kg body weight was dissolved in 6 l of tap water and administered by nasogastric tube. Horses were assessed for lameness when walking on concrete flooring and when circled to the right and left.

Six hoof biopsies were taken at 6-h intervals (a control sample taken before induction and 12, 18, 24, 30, and 36 h after induction). There were three biopsy sites on each front hoof (medial, dorsal, and lateral). The dorsal biopsy site was 5 cm distal to the hairline of the coronet, and the medial and lateral sites were 4 cm on either side of the dorsal site. Palmar nerve blocks were performed bilaterally at the abaxial sesamoid site to provide analgesia for the central biopsy and unilaterally for medial and lateral biopsy sites. A hole, ~15 mm in diameter, was created in the outer hoof wall using a rotating burrab until the remaining hoof wall could be depressed by finger pressure and the lamellae were visible. To prevent heat damage to the underlying lamellar tissues, the site was constantly irrigated.
with cold tap water during the procedure. An Esmarch's rubber bandage tourniquet was applied to the fetlock, and the biopsy site was disinfected. A sterile, size 11 scalpel blade was used to incise a 10 × 10-mm square through the epidermal lamellae and sublamellar dermis to the depth of distal phalanx. A sterile, size 15 blade with a curved tip (made by heating the blade and bending with forceps) was used to release the biopsy from the surface of the distal phalanx. The defect in the hoof wall was packed with sterile swabs and covered with methylmethacrylate resin\textsuperscript{c} and a fiberglass cloth patch.\textsuperscript{c} After the resin had polymerized, the tourniquet was removed. The biopsy was fixed in a 4% buffered formaldehyde solution for 6 h and transferred into a 70% ethanol solution until processing. Five-millimeter sections stained with hematoxylin and eosin (H&E), phosphotungstic acid hematoxylin (PTAH), Mason's trichrome, and periodic acid-Schiff (PAS) stains were prepared for histological assessment. Forty-eight hours after OF administration, the horses were euthanized by barbiturate overdose, and the limbs were disarticulated at the fetlock. The hooves were sectioned with a band saw,\textsuperscript{11} and hoof wall strips that included the biopsy sites and adjoining tissue were collected and fixed as before.

3. Results

Clinical Findings

The biopsy technique seemed to be well tolerated by all the horses. Occasionally mild lameness was observed in one fore foot when walking and particularly when circling to the affected side. Mildly increased digital pulses were first detected from between 13 and 29 h (horses 1 and 2, respectively) and became progressively more palpable from this time. The typical constant weight shifting of laminitis was evident in all horses. Time of onset varied between 20 (horse 1) and 43 h (horse 2) after induction. Lameness in four of the five horses progressively worsened with signs of severe laminitis evident in all four feet. The remaining horse (horse 2) exhibited a mild lameness in all four feet that remained constant once detected. All horses developed moderate to marked transient (6- to 8-h duration) episcleritis, blepharospasm, and corneal opacity, which was clinically diagnosed as corneal edema, between 30 and 40 h.

Histological Findings

The tissues obtained by the biopsy method contained adequate hoof lamellae and underlying dermal structures for evaluation. The control samples from each horse conformed to descriptions of normal lamellar histology.\textsuperscript{5,11,15–17} Histological changes were detected as early as 12 h (horses 1 and 4) and as late as 30 h (horse 2). H&E sections allowed evaluation of cell morphology and structure. PAS-stained sections highlighted the glycoprotein components of the basement membrane (BM) a distinct magenta color. Mason's trichrome highlighted the relationship of dermal to epidermal components by staining connective tissue a bright green color and epidermal tissue a brown color. PTAH stain was used to examine the presence of fibrin thrombi within vessels.

Twelve-Hour Biopsy

Two horses (horses 1 and 4) had noticeable changes at this time. The changes were subtle and consisted of a mild degree of change in the orientation of ovoid epithelial nuclei, which were no longer oriented perpendicular to the long axis of the secondary epidermal lamellae (SEL). Some nuclei were rounded and were more centrally located within the epithelial cell cytoplasm. The tips of SELs were mildly pointed. Secondary dermal lamellae (SDLs) appeared mildly thinner and the BM stained intermittently with less intensity around the bases of the SDLs. The majority of these changes occurred on the same side of the primary epidermal lamella (PELs). Vascular endothelial cells had rounded nuclei protruding into vessel lumens.

Eighteen-Hour Biopsy

The biopsies from horses 1 and 4 had SELs that were progressively more elongated and tips that tapered to finer points. Occasionally, short projections of BM extended past the pointed tips of the SELs. Some of these projections formed teardrop-shaped, apparently empty vesicles. The number of ovoid nuclei in the basal epithelial cells decreased, and those remaining were of disorderly alignment. The proportion of rounded, centrally located nuclei increased. The PAS-stained BM between adjacent SELs had decreased intensity and in some cases disappeared altogether, especially at the SEL bases. Remaining BM appeared crenate. The SDL also became thinner and connective tissues no longer extended between SEL bases. Small vesicles lined by BM were visible in the areas vacated by connective tissue. These changes occurred predominately only on one side of the PELs. Vascular endothelial cells bulged more prominently within vessel lumens. Pyknotic nuclei were visible in a small number of epithelial cells, most frequently within the tips of the PELs.

The sample from horse 5 exhibited the mild changes seen in the 12-h sample from horse 4. No significant changes were detected in the samples from horses 2 and 3.

Twenty-Four-Hour Biopsy

The lesions in the biopsies from horses 1 and 4 had worsened; the changes were more generalized and no longer confined to one side of the PELs. Pyknotic epithelial cell nuclei slightly increased in number. Vascular endothelium was prominent. The biopsy from horse 5 showed changes similar to, but slightly milder than, those described in the 18-h sample from horse 4. No significant changes...
were detected within the samples from horses 2 and 3.

Thirty-Hour Biopsy

Laminitis lesions in the biopsies from horses 1, 4, and 5 had increased in severity and had more generalized changes. SELs were thinner and projections of BM continued past the pointed tips of the SELs and often formed small teat-shaped vesicles. The BM had disappeared between SEL bases and the SELs were confluent with each other.

The biopsy of horse 3 at 30 h resembled the biopsy of horse 4 at 12 h. In the biopsy from horse 2, a small, rounded, deeply eosinophilic staining object that contained shrunken white blood cell nuclei and stained PTAH staining positive for fibrin was present in the lumen of a large dermal vessel. Very mild changes similar to those described for the 12-h biopsy from horse 4 were also present.

Thirty-Six-Hour Biopsy

Elongation of horses 1, 4, and 5 SELs had increased and the teat-shaped vesicular BM projections were larger and more numerous. The long axes of the SEL were more acutely angled to the PEL. The apparently empty spaces where SDL were previously were further enlarged. Mason’s trichrome staining confirmed the absence of connective tissue between the SELs.

Changes had progressed within horse 3 SELs similar to those reported in the 18-h biopsy from horse 4. Two small serocellular proteinaceous slightly irregularly shaped objects were present in the center of two separate large dermal vessels. They did not appear attached to the vessel walls; the lumen diameter was much greater than the diameter of the objects themselves. They stained positive for fibrin with PTAH. The biopsy from horse 2 revealed changes had progressed mildly and had become generalized. They were equivalent in severity to those seen within the 18-h sample from horse 4.

Forty-Eight-Hour Post-Mortem Samples

The lesions within the sections from horse 1 were severe and evident grossly and histologically. There was generalized dermo-epidermal separation with large spaces between PEL and PDL. PELs were thinly tapered, and occasional remnant wavy SELs projected into the space. PDLs had often retracted to a level near the tips of the PEL.

The lesions in the biopsies from horses 4 and 5 were similar in appearance. Sections from horse 3 were of similar but less severe appearance. SELs were severely thinned, appeared to be shorter, and contained few epithelial cells within the projecting remaining, BM-lined tips, which were finely tapered to points. These changes were most disorganized at the tips of the PELs. The long axes of the SELs were at a much more acute angle to the long axes of the PEL. Long wavy projections of BM extended past the SEL tips; many of these projections contained large teat-shaped vesicles. The BM became obscure to absent from the proximal third of the SELs and more of the SELs became confluent (Fig. 1).

Collagen fibers did not stain within the tips of remaining BM-demarcated SDLs, and in these areas, this BM formed very large apparently empty vesicles between SELs (Fig. 1). Lymphatics within the PDL appeared to subjectively increase in diameter. Occasional perivascular polymorphonuclear cells were observed. Post-mortem sections revealed many of the PDLs had completely separated from the PELs (Fig. 2).

The biopsies from horse 3 had changes that were not present in samples from horses 4 and 5. Toward the tips of most of the PDLs, numerous large diam-
eter lymphatic vessels were dilated with amorphous moderately eosinophilic staining proteinaceous material. In some lamellae, this material extended into the large spaces previously vacated by SDL.

The histology from horse 2 did not progress past the 36-h biopsy findings with the exception of the 48-h samples having very large tear drop-shaped BM-lined vesicles at the tips of the SEL.

Mild to moderate extravasation of erythrocytes was observed in both control and later sections from all horses.

4. Discussion

This study confirms previously reported findings that the initial changes in the development of laminitis are the rounding of epithelial basal cell nuclei, loss of SDL structure, and elongation and tapering of SELs. Correlation between duration of the clinical signs and severity of the changes can also be made from these sections. There is a temporal progression of changes that correlate well with progression through the grading system previously described for laminitis histopathology. PAS-stained sections revealed BM changes as early as 12 h after induction. All horses had histological changes by 30 h, which occurred before the clinical onset of laminitis in each case. Interestingly, the onset of increased digital pulses was closely related to the time-point when initial histological changes were first observed.

Only three thrombi were detected, but at time-points well after the appearance of early laminitic changes. The diameters of the thrombi were smaller than the large dermal vessels in which they were located, and there was no association with the vessel walls. The cells at the base of the PELs were no more severely affected than any other area in comparison with previous findings that greater ischemic damage was present in regions furthest from the sublamellar blood supply.

There was no evidence of degenerative changes such as intercellular edema within the epithelial basal cells. The density of both the loose connective tissue surrounding vessels in the deep dermis and PDLs, as well as the dense core of connective tissue in the deep dermis comprising the core of the PDLs, remained unchanged. Proteinaceous edema fluid within PDL lymphatics and spaces vacated by SDLs was only observed in three 48-h sections from horse 3. The non-staining empty spaces between SELs and within the BM projections from the SEL tips would most likely have contained interstitial fluid, which does not contain enough macromolecules to stain. They are most likely a result of, not the cause of, the separation. Low-protein edema fluid would also not stain, but if edema was the cause of the apparently empty vesicles, we hypothesize that increasing eosinophilia should have ensued. This did not occur, the vesicles remaining clear for at least 12 h from first detection.

The number of pyknotic epithelial nuclei was mildly to moderately elevated in the early stages of laminitis development. Pyknosis may indicate apoptosis, supporting previous reports of up to 17-fold increases in naturally occurring laminitis. Numbers of pyknotic nuclei became harder to identify once the SELs were elongated and arrangements became haphazard. Special staining techniques that allow greater accuracy and confirm apoptosis have been validated in the horse. Further evaluation of the numbers of cells undergoing apoptosis using a similar staining technique is proposed in subsequent studies of these sections.

The enlarging of vascular endothelial cells and protrusion of their rounded nuclei into vessel lumens is a common response to inflammation. There were no other changes consistent with inflammation within these samples except mild numbers of perivascular neutrophils, which were seen in 48-h samples once laminitis lesions were advanced.

The mild unilateral intermittent lameness seen at early time-points in some horses was most likely caused by biopsy technique. A series of experiments to further evaluate the biopsy procedure is underway. Interestingly the epiphora, blepharospasm, and corneal edema generally occurred before the onset of laminitis and had completely resolved by 48 h. We hypothesize the corneal BM is affected in a similar manner to the lamellae. Unfortunately, no ocular samples could be taken during the affected period.

The detection of similar numbers of extravasated erythrocytes in both control sections and throughout the serial biopsies implies their presence is most likely caused by artifact.

This study has followed the progression of histological changes in horses developing laminitis and shows that BM pathology is the earliest and most significant histological lesion. These changes are present long before clinical signs of lameness are observed. Failure of the BM results initially in the stretching of dermal–epidermal attachments, leading ultimately to a complete failure of this attachment under load.

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