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Equine lamellar energy metabolism studied using tissue microdialysis

C.E. Medina-Torres a, C.C. Pollitt a, C. Underwood a, E.M. Castro-Olivera a, S.N. Collins a, R.E. Allavena a, D.W. Richardson b, A.W. van Eps a,*

a Australian Equine Laminitis Research Unit, School of Veterinary Science, Faculty of Science, University of Queensland, Gatton Campus, Gatton, QLD, 4343, Australia
b New Bolton Center, School of Veterinary Medicine, University of Pennsylvania, 382 West Street Road, Kennett Square, PA 19348-1692, USA

* Corresponding author. Tel.: +61 7 5460 1799
E-mail address: a.vaneps@uq.edu.au (A.W. van Eps).
Abstract

Failure of lamellar energy metabolism may contribute to the pathophysiology of equine laminitis. Tissue microdialysis has the potential to dynamically monitor lamellar energy balance over time. The objectives of this study were to develop a minimally invasive lamellar microdialysis technique and use it to measure normal lamellar energy metabolite concentrations over 24 h. Microdialysis probes were placed (through the white line) into either the lamellar dermis (LAM) \((n = 6)\) or the sublamellar dermis (SUBLAM) \((n = 6)\) and perfused continuously over a 24 h study period. Probes were placed in the skin dermis (SKIN) for simultaneous comparison to LAM \((n = 6)\). Samples were collected every 2 h and analysed for glucose, lactate, pyruvate, urea and glycerol concentrations. LAM was further compared with SUBLAM by simultaneous placement and sampling in four feet from two horses over 4 h. Horses were monitored for lameness, and either clinically evaluated for 1 month after probe removal \((n = 4)\) or subjected to histological evaluation of the probe site \((n = 10)\).

There were no deleterious clinical effects of probe placement and the histological response was mild. Sample fluid recovery and metabolite concentrations were stable for 24 h. Glucose was lower (and lactate:glucose ratio higher) in LAM compared with SUBLAM and SKIN \((P < 0.05)\). Pyruvate was lower in SUBLAM than SKIN and urea was lower in LAM than SKIN \((P < 0.05)\). These differences suggest lower perfusion and increased glucose consumption in LAM compared with SUBLAM and SKIN. In conclusion, lamellar tissue microdialysis was well tolerated and may be useful for determining the contribution of energy failure in laminitis pathogenesis.

Keywords: Horse; Laminitis; Lamellae; Bioenergetics; Urea clearance.
Introduction

Failure of lamellar energy metabolism may be an important contributor to laminitis development (Belknap et al., 2009; French and Pollitt, 2004; Pass et al., 1998; van Eps et al., 2010). In vitro lamellar glucose starvation causes hemidesmosome loss with resultant weakening of the dermo-epidermal connection and lamellar separation (French and Pollitt, 2004; Pass et al., 1998). Despite the potential importance of bioenergetic disturbances in the pathogenesis of laminitis, very little is known about normal lamellar energy metabolism.

Tissue microdialysis is a minimally invasive technique used to assess local energy metabolism through measurement of energy metabolites such as glucose, pyruvate and lactate. A probe with a semi-permeable membrane, introduced into the tissue of interest, is slowly and continuously perfused allowing molecules to diffuse across the membrane down their concentration gradient (de Lange, 2013). Therefore, metabolite concentrations in the microdialysis sample (dialysate) are reflective of the metabolite concentration in the interstitial fluid of the studied tissue (Klaus et al., 2004). Microdialysis is used in clinical and research settings to study tissue bioenergetic disturbances (ischaemic and non-ischaemic) particularly in the brain (Benveniste et al., 1989; Hillered et al., 2006; Klaus et al., 2004; Oddo et al., 2012a,b; Schulz et al., 2000). The addition of a known (high) concentration of urea to the perfusate allows for determination of urea clearance, an estimate of local perfusion (Farnebo et al., 2010).

In horses, microdialysis has been used in pharmacokinetic studies and for assessment of muscle energy metabolism (Chou et al., 2001; Edner et al., 2005, 2009; Ingvastlarsson et al., 1992; Murchie et al., 2006; Vickroy et al., 2008). Lamellar microdialysis has been described for pharmacokinetic studies (Nourian et al., 2010a, b). However, the previously reported
technique necessitated removal of a 2 cm diameter portion of hoof wall over the insertion site, potentially introducing heat artefact and interfering with normal mechanical forces acting on the sampled area.

The primary objectives of the current study were: (1) to develop and evaluate a lamellar microdialysis technique without hoof wall disruption at the probe membrane site, and (2) to measure and compare energy metabolites in microdialysate from the lamellar, sublamellar and skin dermis of normal horses over 24 h.

**Materials and methods**

*Animals and experimental design*

Fourteen clinically normal Standardbred horses (12 geldings and 2 mares; mean age, 6.5 ± 3.03 years; mean bodyweight, 461.2 ± 49.3 kg) with radiographically normal forefeet were used. The project was approved by the University of Queensland Animal Ethics Committee (approval number: SVS/257/11/GJRF) that monitors compliance with the Animal Welfare Act (2001) and The Code of Practice for the care and use of animals for scientific purposes (current edition).

Three experiments were performed. In experiment 1, linear microdialysis probes were inserted in the lamellar dermis (LAM) of one forelimb (Figs. 1A and B) and the skin dermis (SKIN) over the tail base in six horses. Simultaneous samples were obtained every 2 h for comparison of metabolites over a 24 h period with horses confined to stocks. Four of these horses then had the probes removed and were monitored for 1 month afterwards to assess recovery. In experiment 2, dialysate metabolite concentrations in the sublamellar dermis and lamellar dermis were compared by inserting two coaxial microdialysis probes into each
forelimb of two horses: one directed into the sublamellar dermis (SUBLAM) and the other into the LAM (Figs. 1C and D). Simultaneous hourly sampling was performed for 4h in stocks. Experiment 3 was performed to further assess dialysate metabolite concentrations in the SUBLAM: coaxial microdialysis probes were placed in the SUBLAM of one foot (Fig. 1C) in six horses and sampling was performed every 2 h for 24 h with horses confined to stocks. In two of these horses, sampling was continued every 6 h for an additional 96 h in a stall.

Microdialysis probe placement and perfusion

For lamellar (LAM and SUBLAM) placement, keratinized tissue was resected from the white line region (on midline, dorsal to the point of the frog) with an electric rotating burr (Die-Grinder, Makita) to create an ovoid 1.5 × 1.0 cm hole, leaving the majority of the stratum medium of the distal hoof wall intact. The depth was slowly increased until the white line was easily depressed with forceps. Bilateral abaxial sesamoid perineural anaesthesia was performed with 2% lignocaine (Ilium Lignocaine 20, Troy Laboratories).

After aseptic preparation of the hoof and pastern, the hoof was covered with a sterile adhesive drape (Ioban, 3M). Spinal needles (15 cm) were used as introducers for lamellar probe placement. A 20 G needle (Becton-Dickinson) was used for linear probes (CMA66, CMA-Microdialysis) and an 18 G needle (Lochimed) for coaxial probes (CMA20, CMA-Microdialysis). The needles were inserted at the white line and advanced proximally (parallel with the dorsal hoof wall for LAM placement and at a ~5° angle toward the third phalanx for SUBLAM placement) until the tip appeared through the skin above the coronet. The stylet was removed and a microdialysis probe with a 10 × 0.5 mm, 100 kDa cut-off membrane was
inserted to position the probe membrane in the mid lamellar region. The needle was removed leaving the probe in place (Figs. 1A and C).

The inlet/outlet tubing was passed through a 2 mm hole drilled in the distal hoof wall defect to emerge on the dorsal surface of the hoof. The outlet tubing was connected to a custom made microdialysis vial holder (Fig. 1B) secured to the dorsal hoof wall, and the inlet tubing was connected to a precision pump (CMA107, CMA-Microdialysis) housed in a custom limb boot over the metacarpus. The defect at the toe was filled with silicone putty (Hoof-Life).

For SKIN probe placement, a linear microdialysis probe with a $10 \times 0.26$ mm, 55 kDa cut-off membrane (CMA31, CMA-Microdialysis) was inserted into the dermis over the tail base using a 21 G, 38 mm needle (Becton-Dickinson), after aseptic preparation and subcutaneous infiltration with local anaesthetic (2% lignocaine). The inlet tubing was connected to a precision pump (CMA107) and the outlet tubing to a microdialysis vial (Microvial, CMA-Microdialysis), both secured to the tail.

All probes were perfused with an isotonic, polyionic sterile perfusion fluid (T1, CMA-Microdialysis) containing 40 g/L dextran-70 (Sigma-Aldrich) to prevent perfusate loss (Rosdahl et al., 1997). The perfusate in experiments 2 and 3 contained urea (20 mmol/L) for determination of urea clearance (Farnebo et al., 2010). All probes were perfused for 30 min prior to implantation, then continuously at a rate of 1 µL/min throughout the experiments. The perfusion rate was decreased to 0.3 µL/min during the additional 96 h sampling period in stalls for two horses in experiment 3.
Sample collection and analysis

After probe insertion, a 2 h stabilization period was allowed before commencement of sample collection. Dialysate samples were simultaneously collected every 2 h from the LAM and SKIN probes (experiment 1), hourly from SUBLAM and LAM probes (experiment 2) and every 2 h from SUBLAM probes in experiment 3. The collected dialysate samples were weighed and fluid recovery calculated as a percentage of the perfused volume during the sampling period (120 µL). Glucose, lactate, pyruvate, urea and glycerol concentrations were determined immediately (analysed within 3 min of sample collection) using a commercially available, dedicated microdialysis analyser (IscusFlex, CMA-Microdialysis). Standard indices of energy metabolism (lactate:glucose [L:G] and lactate:pyruvate [L:P]) were calculated.

Lameness evaluation

Digital pedometers (Digiwalker sw700, Yamax) were taped to the distal antebrachium of both forelimbs in all horses to record weight shifting frequency across the entire sampling period (counts recorded and reset every 2 h). Lameness evaluations were performed immediately prior to and at the conclusion of the entire sampling period in each experiment. Each horse was walked and trotted towards and away from the observer and circled to the right and left. Lameness was graded on a 0 – 5 scale following the AAEP guidelines. In the four horses from experiment 1 where the probes were removed, lameness evaluations and visual inspection of the hooves were performed weekly for 4 weeks.

Histology

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1 American Association of Equine Practitioners; see: www.aaep.org
Two horses from experiment 1, and all horses in experiments 2 \((n = 2)\) and 3 \((n = 6)\) were euthanased with pentobarbital sodium \((20 \text{ mg/kg IV})\) (Lethabarb, Virbac) and lamellar tissue harvested and prepared for light microscopy as previously described (Pollitt, 1996). The area of skin containing the SKIN probe was also harvested. The tissue sections were blindly assessed by a specialist veterinary pathologist (REA) and scored using a semi-quantitative method (Table 1).

**Statistical analysis**

The data failed normality testing therefore non-parametric tests were used. The concentrations of dialysate analytes, ratios \((L:G \text{ and } L:P)\), fluid recovery, and pedometer counts were compared over time using Friedman analysis with Dunn’s post-tests, and also between LAM and SKIN at each time point in experiment 1 using Wilcoxon signed-rank tests. The median concentrations of analytes and calculated ratios were compared between LAM and SKIN (experiment 1), and SUBLAM (experiment 3) using Mann-Whitney tests. Wilcoxon signed-rank tests were also used to compare pedometer count frequencies between limbs (experiments 1 and 3). Data from experiment 2 were analysed descriptively. Statistics were performed using GraphPad Prism v6.00 for Windows (GraphPad Software) with significance set at \(P < 0.05\). Data are expressed as median with interquartile range (IQR).

**Results**

**Probe performance and clinical effects**

In each horse the microdialysis probes remained in position and were functional throughout the 24-h study periods whilst confined to stocks. Pedometer count frequency did not differ significantly between limbs and was not significantly different over time in any experiment (data not shown). The lameness score was 0 at each observation interval for all
horses, including over the 1-month follow-up period \((n = 4\); experiment 1) and there were no visible changes in external hoof wall and sole morphology during this period.

Dialysate analysis

Fluid recovery did not vary significantly over time (Fig. 2), nor was there a difference between LAM \((100.1\%, [97.0-102.4\%])\), SKIN \((95.7\%, [90.7-99.3\%])\) and SUBLAM \((101.7\%, [99.6-103.9\%])\) recovery. When considering the median dialysate analyte values from all time-points during experiments 1 and 3 (Table 2), LAM glucose concentrations were lower and LAM L:G higher than both SKIN and SUBLAM, LAM endogenous urea concentrations were lower than SKIN, and SUBLAM pyruvate concentrations were higher than SKIN \((P < 0.05)\). SUBLAM and LAM urea concentrations were not compared due to the addition of urea to the dialysate for urea clearance studies in experiment 3. In all three experiments, median concentrations of glucose, lactate, pyruvate and urea, as well as L:G and L:P did not differ significantly over time in LAM, SKIN and SUBLAM dialysate, whereas glycerol concentration decreased significantly \((P < 0.05)\) in each (Fig. 2).

In experiment 2, glucose and pyruvate concentrations appeared to be lower, and L:G higher in LAM vs. SUBLAM dialysate; however the data were not statistically analysed (Fig. 3). Urea (mmol/L) also appeared to be higher in LAM \((13.53, [13.2-13.6])\) vs. SUBLAM \((11.18, [10.11–12.03])\). The perfusate urea concentration was not measured in experiment 2. The urea concentration (mmol/L) in SUBLAM dialysate in experiment 3 was 14.83 \([13.95-14.92]\) with a measured perfusate concentration of 17.92 \([17.54-18.36]\).

Extended sampling (96 h in stalls) in two horses (H5 and H6) from experiment 3 yielded an overall fluid recovery of 98.84\% [91.32-100.8]; however breakage of the vial holders (H5:
18 h and H6: 60 h) and disruption of tubing connections (H5: 36 h and H6: 12 h; 24 h) occurred. Metabolite concentrations were similar to the initial 24-h period in stocks and appeared stable until time point 48 h (H5) and 66 h (H6), after which a rapid decrease in glucose and increase in lactate concentrations was observed (data not shown). Glucose concentrations (mmol/L) were 0.06 (H5) and 0.0 (H6) at the last sampling time point. Other metabolite values remained stable.

Probe location and host reaction

Histological examination confirmed that probe membrane placement was in the desired LAM, SKIN and SUBLAM position in all tissue sections (Fig. 4). With LAM placement, damage to adjacent primary epidermal lamellae (PEL) was observed (Fig. 4A), whereas no evidence of damage to PEL was apparent with SUBLAM probes (Fig. 4B). There was evidence of mild to moderate inflammation and cellular debris, mild endothelial reactivity, oedema and haemorrhage around each probe. Neutrophils were the predominant cell type with rare macrophages and lymphocytes. The histological scores for each location are presented in Table 3. Overall, the host response to microdialysis probe implantation was mild, regardless of probe location, and consistent with tissue damage due to probe placement and wound healing responses relative to the time the probe was in situ (24 h vs. 120 h).

Discussion

Lamellar microdialysis allowed for consistent recovery of dialysate samples and was well tolerated. Fluid recovery from LAM, SUBLAM and SKIN probes was similar and consistent throughout the study. The minor disruption of the distal hoof wall and sole that was required for probe insertion was remote from the sampling site (microdialysis membrane). Maintaining tissue integrity around the membrane is likely to be important when studying the
effects of weight bearing and limb load cycling on lamellar perfusion and bioenergetics, as mechanical forces operating on the soft tissue between hoof wall and distal phalanx may affect perfusion (Redden, 2003; van Eps et al., 2010).

Metabolite concentrations were stable for 24 h and there was minimal histological response to the probes, suggesting that analyte recovery was unlikely to be affected by fibrous capsule formation or host response to the probe. These findings suggest that microdialysis is suitable for evaluating disturbances in lamellar energy metabolism over time, particularly during laminitis development; however, it should be noted that sampling in the current study was confined to a single region (dorsal lamellae), which may not be representative of lamellar tissue in other regions of the foot. Sampling in two horses moving freely in a stall for 96 h was achieved, but a decrease in glucose followed breakage of the microdialysis system in both horses. The decrease was likely due to bacterial contamination of the microdialysis system itself (a recognised cause of low glucose concentration artefact) (Heller and Feldman, 2013), since there was minimal histological evidence of a foreign body reaction (the other major cause of low glucose artefact in microdialysis) (Nichols et al., 2011). A more robust collection system is required for ambulatory studies and studies of longer duration.

The lower glucose and endogenous urea concentrations in LAM compared with SKIN may be due to the presence of lower blood flow in lamellar tissue, increased glucose uptake by surrounding cells, or a combination of both. Interstitial glucose concentrations may also be influenced by blood glucose concentration, but this would be expected to equally affect simultaneous LAM and SKIN samples. Both glucose and urea must be delivered to the lamellae and skin haematogenously (Hall, 2011; Sorg et al., 2005), but only glucose is
consumed. Therefore, the lower LAM endogenous urea concentration compared with SKIN supports the existence of comparatively lower perfusion in the lamellar dermis. The lower LAM glucose concentration compared to SUBLAM and SKIN supports the existence of higher glucose consumption at the site of LAM probe placement (between avascular, but glucose-consuming epidermal lamellae). A high rate of glucose consumption has been demonstrated in the equine digit and this probably reflects the rate of glycolysis, since there is no local means of glycogen storage in lamellar tissue (Wattle and Pollitt, 2004).

Lamellar perfusion was assessed further in experiments 2 and 3 using urea clearance (Farnebo et al., 2010). In experiment 3 (SUBLAM), urea concentrations remained stable over time, indicating the method may be useful for documenting changes in perfusion over time. In experiment 2 there appeared to be lower urea concentrations (higher urea clearance) in SUBLAM compared to simultaneous LAM samples, which may suggest lower local perfusion in the LAM region. Urea clearance was not used in experiment 1 because an objective was to determine the endogenous dialysate concentrations of all analytes (including urea). Further evaluation of the urea clearance technique is required to determine whether changes in lamellar perfusion can be detected.

Increased dialysate L:G has been observed with hypoxia and ischaemia in brain tissue, where it is the consequence of a combination of increased extracellular lactate and reduced glucose (Goodman et al., 1999; Meierhans et al., 2010). The high LAM L:G in this study was due to the significantly lower LAM dialysate glucose concentrations (rather than higher lactate concentrations), since LAM dialysate lactate concentrations were similar to those of the SKIN, SUBLAM and previously reported tissues such as brain (Langemann et al., 2001) and muscle (Edner et al., 2005). Therefore, the high L:G in normal LAM (more than twice
that of SKIN, SUBLAM and other previously studied tissues) suggests the presence of a high
glycolytic rate in the lamellae (glucose consumption with production of lactate), which is
typical of epidermal tissues (Ronquist et al., 2003; Wattle and Pollitt, 2004). The lack of
lactate accumulation suggests that lamellar tissue is adapted to the high rate of glycolysis,
with the lactate produced being either utilised as an energy source via lactate dehydrogenase
(LDH; richly present in lamellar epidermis) (Wattle and Pollitt, 2004) or removed efficiently
via a lactate shuttle (Gladden, 2004).

Other metabolite values (lactate, pyruvate, glycerol) were similar in all sampled tissues
and also similar to values reported for the healthy human brain under similar microdialysis
conditions (Reinstrup et al., 2000). In the current study, the perfusion rate of 1 µL/min was
chosen as it allows for collection of adequate volumes of dialysate over short sampling
intervals (≤15 min), a requirement for ongoing studies in the authors’ laboratory. This
relatively high flow rate reduces metabolite recovery (Benveniste and Huttemeier, 1990);
therefore measured dialysate metabolite concentrations were assumed to be much lower than
the absolute values in the interstitial fluid. The pattern of initially high glycerol
concentrations, decreasing over 24 h in the current study was also observed in equine muscle
(Edner et al., 2005), and is likely the result of cell membrane damage caused by probe
insertion. Measurement of other markers of cell membrane damage such as LDH (Glick,
1969) is also possible in microdialysis fluid and should be considered in future studies.

The L:P measured at all sites in the current study resembled values previously reported
for brain (Zhang and Natowicz, 2013) and muscle (Edner et al., 2005). The L:P is indicative
of tissue redox status (which is dependent on mitochondrial function and tissue oxygenation),
and an increase in L:P is the most reliable indicator of energy metabolism dysfunction
(ischaemic and non-ischaemic) in well-studied tissues (Hillered et al., 2006; Setala et al., 2004; Waelgaard et al., 2012). In fact L:P is considered a sensitive marker of cellular metabolic dysfunction (Vespa et al., 2005) and is used in the diagnostic evaluation of disorders of energy metabolism in a range of tissues (Marcoux et al., 2008; Zhang and Natowicz, 2013). Therefore, lamellar dialysate L:P may be useful for detecting the presence of bioenergetic failure during the development of different forms of equine laminitis. The L:P in lamellar dialysate in the present study was similar to that of SKIN and also brain and muscle in previous studies, suggesting a similar resting redox state. This appears to be at odds with the findings of Pawlak et al. (2014), who concluded that the equine epidermal lamellar tissue is comparatively hypoxic.

In the current study, the contribution of epidermal cell metabolism (basal cells and deeper epidermal cell layers) to the dialysate composition is not clear since the probes were placed in the dermis (either between PEL (LAM) or adjacent to the PEL tips (SUBLAM)). The sampling area of a microdialysis probe may depend on the tissue and the analyte. However, it has been estimated to be 50 times the membrane thickness (Benveniste et al., 1989) or calculated by mathematical models to be 0.85 ± 0.25 mm for neuroactive substances in the brain (Diczfalusy et al., 2011), suggesting that in the present study, both epidermal and dermal lamellar tissues should have influenced the dialysate regardless of probe position. However, immediately adjacent tissues are likely to have more influence on dialysate composition, which may account for the differences observed between LAM and SUBLAM.

The limitations of the present study include the use of different microdialysis probes and the lack of ambulation (having the horses restrained in stocks). Membrane characteristics were however similar for all probes, with CMA20 and CMA66 probes having identical
membrane characteristics, and the CMA31 differing only in pore size (55 kDa). Net transport of metabolites across the microdialysis membrane (recovery) depends mainly on perfusate flow rate, membrane size (area) and how diffusible each metabolite is in the sampled tissue (Hamrin et al., 2002; Rosdahl et al., 1998). Therefore, the difference in membrane pore sizes is likely negligible, particularly considering the molecular weight of the largest metabolite measured in the present study (glucose) was 0.18 kDa. Although it is possible that the lamellar bioenergetic profile in stationary horses differs from that of ambulating horses, restraining the study subjects in stocks was necessary for frequent sampling and protection of the equipment. Sampling was only performed in two freely moving horses to assess if long-term lamellar microdialysis was achievable, and a different perfusion rate was used for convenience (6-h sampling interval), preventing comparisons between ambulating and standing static horses from being performed.

This study is the first to evaluate with histology the tissue response to microdialysis probe placement in horses. The scoring system was designed to represent the range of pathology present in the samples scored, rather than to act as a comparison with other foreign body responses. Overall the histological changes were benign and not suggestive of a foreign body response driven by the microdialysis membrane.

**Conclusions**

Tissue microdialysis is a suitable method for energy metabolite sampling in the equine lamellar and skin dermis. Recovery of dialysate was consistent and the concentrations of measured metabolites were stable for 24 h. There were no adverse clinical effects of probe implantation and histological reactions were mild. In the standing, static horse, the lamellar dialysate had lower glucose and urea concentrations and a higher L:G compared to skin
consistent with lower perfusion and/or higher glucose consumption), but lamellar L:P, the most useful marker of energy homeostasis, was similar to skin and previously studied tissues. Probe position (lamellar vs. sublamellar dermis) affected microdialysate metabolite concentrations, which is likely to be a factor of proximity to the avascular, but metabolically active epidermis.

Conflict of interest statement

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

Acknowledgements

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References


Table 1: Semi-quantitative histological scoring system applied to the lamellar and skin dermis tissue slides. The scoring system was designed to represent the range of pathology present in the samples scored and is not a comparison to other foreign body responses. HPF, high power field.

<table>
<thead>
<tr>
<th>Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Epidermal basal cell and parabasal cell hyperplasia</strong> (fold increase in thickness of epidermal cell layer)</td>
<td>Absent</td>
<td>2 fold</td>
<td>3 fold</td>
<td>≥ 3 fold</td>
</tr>
<tr>
<td><strong>Flattening of secondary epidermal lamellae (SEL):</strong> expressed as % of length of unaffected SEL remaining</td>
<td>100%</td>
<td>66-99%</td>
<td>50-66%</td>
<td>&lt;50%</td>
</tr>
<tr>
<td><strong>Mitotic figures:</strong> per HPF (200X)</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>≥3</td>
</tr>
<tr>
<td><strong>Inflammatory cell count</strong> around the probe per HPF at 200X</td>
<td>0</td>
<td>1-50</td>
<td>50-100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><strong>Oedema</strong></td>
<td>Absent</td>
<td>Mild</td>
<td>Moderate</td>
<td>Marked</td>
</tr>
<tr>
<td><strong>Haemorrhage</strong></td>
<td>Absent</td>
<td>Mild</td>
<td>Moderate</td>
<td>Marked</td>
</tr>
<tr>
<td><strong>Fibroplasia:</strong> thickness of the fibrous tissue around the probe</td>
<td>Absent</td>
<td>Mild</td>
<td>Moderate</td>
<td>Marked</td>
</tr>
<tr>
<td><strong>Collagen bundle formation</strong></td>
<td>Absent</td>
<td>Mild</td>
<td>Moderate</td>
<td>Marked</td>
</tr>
<tr>
<td><strong>Cellular debris</strong></td>
<td>Absent</td>
<td>Mild</td>
<td>Moderate</td>
<td>Marked</td>
</tr>
<tr>
<td><strong>Endothelial reactivity:</strong> number of vessels with reactive endothelium per HPF (200X)</td>
<td>0</td>
<td>1-20</td>
<td>21-50</td>
<td>&gt;50</td>
</tr>
<tr>
<td><strong>Tissue necrosis</strong></td>
<td>Absent</td>
<td>Mild</td>
<td>Moderate</td>
<td>Marked</td>
</tr>
</tbody>
</table>
**Table 2:** Median and interquartile range (IQR) concentrations of energy metabolites, urea, glycerol and standard calculated ratios in lamellar dermis (LAM) and skin dermis (SKIN) dialysate in experiment 1, and sublamellar dermis (SUBLAM) dialysate in experiment 3 for the entire 24 h sampling period.

<table>
<thead>
<tr>
<th></th>
<th>LAM</th>
<th>SKIN</th>
<th>SUBLAM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mmol/L)</td>
<td>(mmol/L)</td>
<td>(mmol/L)</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.45&lt;sup&gt;1,2&lt;/sup&gt;</td>
<td>1.23&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.83&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(0.38-0.50)</td>
<td>(1.08-1.39)</td>
<td>(0.73-1.13)</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.32</td>
<td>1.45</td>
<td>1.22</td>
</tr>
<tr>
<td></td>
<td>(1.09-1.52)</td>
<td>(1.34-1.50)</td>
<td>(1.11-1.31)</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>65.45&lt;sup&gt;3&lt;/sup&gt;</td>
<td>72.10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>54.04&lt;sup&gt;3&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>(50.90-76.64)</td>
<td>(69.35-74.88)</td>
<td>(43.51-59.65)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>5.04</td>
<td>3.10</td>
<td>7.38</td>
</tr>
<tr>
<td></td>
<td>(3.79-8.05)</td>
<td>(2.37-4.38)</td>
<td>(4.61-12.06)</td>
</tr>
<tr>
<td>Urea</td>
<td>2.63&lt;sup&gt;1&lt;/sup&gt;</td>
<td>3.72&lt;sup&gt;1&lt;/sup&gt;</td>
<td>14.83&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(2.30-2.81)</td>
<td>(3.55-4.06)</td>
<td>(13.95-14.92)</td>
</tr>
<tr>
<td>Lactate:Glucose</td>
<td>2.84&lt;sup&gt;1,2&lt;/sup&gt;</td>
<td>1.21&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1.44&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(2.64-3.63)</td>
<td>(1.04-1.34)</td>
<td>(1.12-1.77)</td>
</tr>
<tr>
<td>Lactate:Pyruvate</td>
<td>20.33</td>
<td>21.34</td>
<td>25.57</td>
</tr>
<tr>
<td></td>
<td>(18.07-22.82)</td>
<td>(19.36-23.15)</td>
<td>(23.41-28.59)</td>
</tr>
</tbody>
</table>

<sup>1</sup> Statistically significant ($P < 0.05$) difference between LAM and SKIN.  
<sup>2</sup> Statistically significant ($P < 0.05$) difference between LAM and SUBLAM.  
<sup>3</sup> Statistically significant ($P < 0.05$) difference between SKIN and SUBLAM.  
<sup>4</sup> Perfusate contained 20 mmol/L of urea.
Table 3: Median and interquartile range (IQR) histological score in the tissue surrounding the probe for LAM, SKIN, SUBLAM, and for different implantation duration (24 h vs. 120 h). Medians are ≤ 1 for all subcolumns except after 120 h in situ, indicative of a mild host response to microdialysis probe implantation. The scoring system represents the range of pathology present in the samples scored. The histological changes were not suggestive of a foreign body response driven by the microdialysis membrane. EBC, epidermal cell hyperplasia; SEL, secondary epidermal lamellae; PMN, polymorphonuclear neutrophils.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LAM</td>
<td>SKIN</td>
<td>LAM</td>
</tr>
<tr>
<td>EBC hyperplasia</td>
<td>0 (0-0)</td>
<td>N/A</td>
<td>0 (0-0)</td>
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<td>Flattening of SEL</td>
<td>0 (0-0)</td>
<td>N/A</td>
<td>0 (0-0)</td>
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<tr>
<td>Mitotic figures</td>
<td>0.5 (0-1)</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
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<tr>
<td>Inflammatory cell count</td>
<td>1 (1-1)</td>
<td>1.5 (1-2)</td>
<td>3 (1.5-3)</td>
</tr>
<tr>
<td>- cell type</td>
<td>PMN</td>
<td>PMN</td>
<td>PMN</td>
</tr>
<tr>
<td>Oedema</td>
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<td>1 (1-1)</td>
<td>1 (1-2.5)</td>
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<tr>
<td>Haemorrhage</td>
<td>1 (1-1)</td>
<td>1 (1-1)</td>
<td>1 (1-2.5)</td>
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<tr>
<td>Fibroplasia</td>
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<td>0 (0-0)</td>
<td>0 (0-0)</td>
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<td>Collagen bundles</td>
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<td>0 (0-0)</td>
<td>0 (0-0)</td>
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<tr>
<td>Cell debris</td>
<td>2 (2-2)</td>
<td>1.5 (1-2)</td>
<td>2.5 (1.25-3)</td>
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<td>Endothelial reactivity</td>
<td>1.5 (1-2)</td>
<td>1 (1-1)</td>
<td>1 (0.25-2.5)</td>
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<tr>
<td>Tissue necrosis</td>
<td>1.25 (0-5)</td>
<td>0.5 (0-0)</td>
<td>1.5 (0.5-2)</td>
</tr>
<tr>
<td><strong>Total median (IQR)</strong></td>
<td>1 (0-1.5)</td>
<td>1 (0-1.25)</td>
<td>0.5 (0-1)</td>
</tr>
</tbody>
</table>
Figure legends

Fig. 1. Schematic representation of the tissue microdialysis linear probe system used in experiment 1 (A): the microdialysis membrane is located within the mid-dorsal lamellar tissue, the outlet tubing is connected to a collection vial within a custom-made vial holder on the dorsal hoof wall (B). Schematic representation of a tissue microdialysis coaxial probe system used in experiments 2 and 3 (C): the microdialysis membrane is located within the mid-dorsal lamellar tissue: the inlet and outlet tubing emerge through the white line and via a hole in the hoof wall are connected to a microdialysis pump and a collection vial on the dorsal hoof wall, respectively. For Experiment 2, two tissue microdialysis coaxial probes were placed 1 cm lateral and medial of midline for simultaneous comparison of dialysate from LAM (probe introducer inserted parallel to hoof wall) and SUBLAM (probe introducer inserted at a ~5º angle towards the third phalanx) (D). The dotted lines depict the internal location of the probes.

Fig. 2. Median ± interquartile range (IQR) concentrations of energy metabolites, urea, glycerol, L:G, L:P and dialysate recovery in LAM, SKIN (experiment 1), and SUBLAM (experiment 3) vs. time. Glycerol was the only analyte that varied significantly over time, decreasing compared to baseline (2 h) in the dialysate from each tissue. The concentrations of glucose, and urea were significantly lower in LAM compared to SKIN (P<0.05). The L:G was significantly higher in LAM compared SKIN (P < 0.05). Fluid recovery is expressed as a percentage of the infused volume for each sampling interval (120 µL in 2 h). Fluid recovery did not vary significantly over time.

¹, significant difference (P < 0.05) between LAM and SKIN in experiment 1 at this time point; ², significant difference (P < 0.05) vs. 2 h for LAM dialysate in experiment 1 at this
time point; \(^3\), significant difference \((P < 0.05)\) vs. 2 h for SKIN dialysate in experiment 1 at this time point; \(^4\), significant difference \((P < 0.05)\) vs. 2 h for SUBLAM dialysate in experiment 3 at this time point. \(^5\), perfusate contained 20 mmol/L of urea.

Fig. 3. Median and interquartile range (IQR) concentrations of energy metabolites, urea, glycerol and standard calculated ratios in simultaneous LAM and SUBLAM dialysate samples in four feet from two horses (experiment 2) over a 4-h period. A lower concentration of glucose and pyruvate, lower urea clearance, and a higher lactate:glucose (L:G) was apparent in LAM vs. SUBLAM dialysate (descriptive analysis only; \(n = 2\)).

Fig. 4: Haematoxylin and eosin-stained light microscopy at 4x magnification showing the position of the microdialysis probe membranes (P) in representative sections from LAM (A); SUBLAM (B); and SKIN (C). There was damage to primary epidermal lamellae (white asterisks) neighbouring the probe in all of the LAM sections, but not in SUBLAM sections. White arrow heads, skin hair follicles.