

## Decreased glucose metabolism causes separation of hoof lamellae *in vitro*: a trigger for laminitis?

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**Keywords:** horse; laminitis; glucose metabolism; 2-deoxyglucose; aminophenylmercuric acetate

### Abbreviations

D-MEM	Dulbecco's modified Eagle medium
APMA	Aminophenylmercuric acetate
2-DG	2-deoxyglucose
H&E	Haematoxylin and eosin
PAS	Periodic acid-Schiff
DTT	Dithiothreitol

### Summary

Explants of horses' hooves remained intact for up to 8 days when incubated in Dulbecco's modified Eagle medium (D-MEM) containing 25 mmol/l glucose but separated within 36 h when incubated in saline. The separation occurred between the basal epidermal cells and their basement membrane which is characteristic of the hoof separation that occurs in laminitis. Separation of hoof explants was prevented by addition of glucose to saline and was induced by adding 2-deoxyglucose or aminophenylmercuric acetate to D-MEM. Glucose consumption by the hoof explants was inhibited by 2-deoxyglucose and aminophenylmercuric acetate. The explants consumed relatively large amounts of glucose during the first 2 days of incubation and then little over the next 6 days. Despite the reduced glucose consumption, the hoof explants did not separate over 8 days of incubation. The results indicated that the integrity of the hoof explants was initially dependent on consumption of glucose and provide a possible explanation for the development of laminitis caused by conditions such as carbohydrate overload, acute inflammatory conditions, corticosteroid therapy and hyperlipidaemia. It would be expected that these conditions would induce a major hormonally-mediated metabolic shift away from glucose consumption by many peripheral tissues. It is suggested, therefore, that if the metabolic change occurred faster than the hoof tissue could adapt to an alternative energy substrate, then hoof separation and laminitis would occur.

### Introduction

A key feature in the development of laminitis in horses is separation of the secondary epidermal and dermal lamellae of the hoof. This separation occurs between the epidermal basal cells and their basement membrane (Pollitt 1996). It has been proposed

that the primary insult leading to this separation is ischaemia resulting from vasoconstriction in the hoof (Hood *et al.* 1993). However, other studies have thrown some doubt on this proposition in that they have shown vasodilation and an increase in hoof blood flow rather than vasoconstriction during the development of laminitis (Trout *et al.* 1990; Pollitt and Davies 1998). It is still unclear if the lamellar tissues are perfused during the development of laminitis (Robinson 1990).

Epithelial cells are attached to the basement membrane by specific adhesion molecules on the plasma membrane. These include integrins which attach to components of the basement membrane such as collagen and laminin (Albelda 1991; Ruoslahti 1991; Quaranta 1993). Although the adhesion molecules and components of the basement membrane in the horse's hoof have not been completely characterised, laminin and collagen have been identified (Pollitt 1996). Therefore, it is reasonable to assume that the attachment system is similar to that described in other species. Detachment of epithelial cells from the basement membrane occurs in both physiological and pathological processes. During tissue growth and repair, cells detach and attach as the tissue enlarges and remodels. In diseases such as asthma and bronchiectasis epithelial cell detachment occurs (Venaille *et al.* 1995). Detachment of epithelial cells from the basement membrane does not necessarily imply injury to the cell (Venaille *et al.* 1995) and these processes of detachment and cell injury and death should be distinguished from each other. Indeed, in cases of laminitis, detached hoof epithelial cells appear viable at least morphologically.

Metalloproteinase (MMP) enzymes degrade elements of the extracellular matrix including collagen (Krane 1994) and this can lead to separation of cells from the basement membrane. MMPs are synthesised as inactive molecules and activation of the enzymes occurs after secretion of the proenzyme from the cell (Murphy *et al.* 1994). Furthermore, activated MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMP) (Murphy *et al.* 1994). Therefore, MMP-induced detachment of cells from the basement membrane depends on the relative concentrations of active MMPs and TIMPs in the tissue. Recently it has been demonstrated that MMPs are active during detachment of lamellar epithelial cells from the basement membrane in cultured explants of horses' hooves (Pollitt *et al.* 1998). This has led to the hypothesis that activation of metalloproteinases in the hoof may be responsible for separation of the secondary epidermal and dermal lamellae in laminitis

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**TABLE 1: Effects of incubating hoof explants for 2 days in a variety of culture media**

Incubation medium	No. tested	No. intact	No. separated
D-MEM	33	33	
Saline	33		33
D-MEM:saline 1:1	8	8	
D-MEM:saline 1:3	2	2	
D-MEM:saline 1:4	11	7	4
D-MEM:saline 1:5	3		3
D-MEM:saline 1:6	5		5
D-MEM:saline 1:8	12	1	11
D-MEM:saline 1:10	2		2
Buffer solution	6		6
Ionic solution	4		4
Saline + 25 mmol/l glucose	8	8	
Saline + 1 mmol/l pyruvate	4		4
Saline + 4 mmol/l glutamine	2		2
Saline + 0.4 mmol/l arginine	2		2
D-MEM + 20 µmol/l lactic acid	4	4	

D-MEM = Dulbecco's modified Eagle medium.

(Pollitt *et al.* 1998).

However, it is still not clear what triggers the separation process and how disparate clinical states, such as carbohydrate overload, septic metritis, corticosteroid therapy and hyperlipaemia, can induce laminitis. Here, we advance the hypothesis that acute alterations in whole body glucose metabolism as a result of these conditions may be a trigger for laminitis.

#### Materials and methods

D-MEM (glucose 25 mmol/l, pH 7.2), gentamycin<sup>1</sup>, APMA, DMSO, DTT, glutamine, arginine•HCl and 2-DG<sup>2</sup> were obtained. APMA was dissolved in DMSO (5 mg/ml) before addition to D-MEM. All other reagents were reagent grade chemicals purchased from chemical manufacturers.

Explants of horses hooves were prepared as described elsewhere (Pollitt *et al.* 1998). They were cultured in 24 well culture plates in one ml of culture medium containing gentamycin (0.1 mg/ml) at 37°C in an atmosphere of 5% CO<sub>2</sub> in air. The explants were such that they contained hoof wall, lamellae and dermal connective tissue. The integrity of the explants was tested after a period of culture by grasping the hoof wall and connective tissue with forceps and pulling them in opposite directions. The result was scored as the tissue being intact if it did not separate between the lamellae or separated if it did (Pollitt *et al.* 1998). Testing of the integrity of the tissue was performed without the operator knowing the treatment given to each explant and the same operator tested all explants. Tissues were fixed in 10% buffered formalin. Histological sections were prepared of selected samples and stained with H&E and PAS. They were examined under a light microscope.

Explants from all hooves were cultured in D-MEM and in normal saline (0.9% sodium chloride) for 2 days in order to test the suitability of that horse's tissue for the experiment. The tissue was judged as suitable if the explants cultured in D-MEM remained intact and those in saline separated. Other explants were cultured concurrently in the media to be tested in that experiment. Duplicate samples were cultured for all experiments. One sample was tested for its integrity and the other left undisturbed for histological examination if required. Culture medium from duplicate wells was pooled for analysis

when required. In all cases, control and test explants were obtained from the same hoof and replicate experiments were performed on separate hooves.

In the first set of experiments, explants were cultured in the following culture media for 2 days and then tested for their integrity: D-MEM; normal saline; D-MEM diluted 1:3, 1:4, 1:5, 1:6, 1:8 or 1:10 with normal saline; glucose (25 mmol/l) in saline; buffer solution (6.4 g/l NaCl; 280 mg/l Na<sub>2</sub>PO<sub>4</sub>•12H<sub>2</sub>O; 3.7 g/l NaHCO<sub>3</sub>); ionic solution (200 mg/l CaCl<sub>2</sub>; 97.67 mg/l MgSO<sub>4</sub>; 400 mg/l KCl dissolved in the buffer solution); sodium pyruvate (110 mg/l) in saline; L-glutamine (584 mg/l) in saline; and arginine•HCl (84 mg/l) in saline. The concentrations of these chemicals were the same as in the D-MEM. Other explants were cultured in 20 µmol/l lactic acid in D-MEM (pH 6.27); 2-DG (0.1, 1, 10, 25, 50 and 100 mmol/l) in D-MEM; APMA (0.7 mmol/l) in D-MEM; and APMA (0.7 mmol/l) and DTT (100 mmol/l) in D-MEM. After 2 days in culture the explants were tested for their integrity and the glucose concentration estimated in some of the culture media. Glucose concentration was estimated using a Cobas Mira analyser<sup>3</sup> and a Unimate 5 gluc hk kit<sup>3</sup>.

In the second set of experiments, sets of explants were cultured in D-MEM or glucose (25 mmol/l) in saline for up to 8 days. In some cases, explants from an individual hoof were tested for their integrity at 2 day intervals and the culture medium assayed for concentration of glucose. In others, the medium was replenished with fresh D-MEM every 2 days for the first 6 days and the removed medium analysed for concentration of glucose. The explants were tested for integrity on Day 8.

Significant differences between groups were tested by Fisher's exact test, the Kruskal-Wallis analysis of variance (ANOVA) or the Wilcoxon-Mann-Whitney U statistic calculated by the STP method (Siegel 1956; Sokal and Rohlf 1969; Motulsky 1995).

#### Results

All explants cultured in D-MEM (n = 33) for 2 days remained intact and all those cultured in saline (n = 33) separated (P < 0.0001, Fisher's exact test) (Table 1). Histological examination of some explants revealed that the separation occurred between the epidermal basal cells and their basement membrane as described elsewhere (Pollitt 1996). Separation was first observed after 36 h in culture in saline (Table 3). Of the components of the D-MEM added to saline, only glucose prevented separation of the hoof tissues (P = 0.0002, Fisher's exact test) (Table 1). Buffers and the other components of the D-MEM tested did not prevent separation (Table 1). Dilution of the D-MEM with saline consistently resulted in separation of the tissue when the dilution was 1:5 or greater (Table 1). This was equivalent to a glucose concentration of 5 mmol/l. Separation sometimes occurred at a dilution of 1:4 (Table 1).

When explants were cultured in D-MEM, the medium became more acid as the incubation progressed, as shown by the phenol red in the medium turning from pink to yellow. However, addition of lactic acid to the D-MEM did not cause separation of the hoof (Table 1).

When 2-DG was added to D-MEM separation occurred when the 2-DG concentration was 50 mmol/l or above (Table 2). Addition of APMA to the D-MEM also induced separation of the hoof tissue and this was prevented by addition of DTT to the culture medium (Table 2).

Analyses of culture medium after the incubation period revealed that 2-DG and APMA inhibited the utilisation of

**TABLE 2: Effects of 2-DG, APMA and APMA + DTT on hoof explants cultured for 2 days**

Incubation medium	No. tested	No. intact	No. separated
D-MEM + 100 mmol/l 2DG	5		5
D-MEM + 50 mmol/l 2DG	5		5
D-MEM + 10 mmol/l 2DG	5	5	
D-MEM + 1 mmol/l 2DG	5	5	
D-MEM + 0.1 mmol/l 2DG	5	5	
D-MEM	5	5	
D-MEM + 0.7 mmol/l APMA	6		6
D-MEM + 0.7 mmol/l APMA + 100 mmol DTT	6	6	

2-DG = 2-deoxyglucose. APMA = Aminophenylmercuric acetate. DTT = Dithiothreitol. D-MEM = Dulbecco's modified Eagle medium.

**TABLE 3: Effects of the time of incubation on hoof explants in different incubation media**

Incubation medium	No. tested	No. intact	No. separated
D-MEM - 0.5 days incubation	2	2	
D-MEM - 1 day incubation	2	2	
D-MEM - 1.5 days incubation	2	2	
D-MEM - 2 days incubation	8	8	
D-MEM - 4 days incubation	8	7	1
D-MEM - 6 days incubation	8	7	1
D-MEM - 8 days incubation	8	8	
D-MEM - replenished every 2 days, 8 days incubation	8	8	
Saline - 0.5 days incubation	2	2	
Saline - 1 day incubation	2	2	
Saline - 1.5 days incubation	2		2
Saline - 2 days incubation	4		4
Saline + glucose 2 days incubation	8	8	
Saline + glucose 4 days incubation	4	3	1
Saline + glucose 6 days incubation	4	2	2
Saline + glucose 8 days incubation	4	3	1

D-MEM = Dulbecco's modified Eagle medium.

glucose by the hoof tissue in that the concentration of glucose was higher in the presence of the inhibitors than in the control cultures at the end of the incubation period ( $P = 0.003$  for 2-DG, Fisher's exact test;  $P < 0.05$  for APMA, STP method) (Figs 1 and 2). DTT reversed the effect of APMA on glucose consumption ( $P < 0.05$ , STP method) (Fig 2).

Separation of hoof explants did not occur consistently when they were incubated in D-MEM for up to 8 days (Table 3). Glucose was consumed by these explants during the first 2 days of incubation but, after this time, less glucose was consumed ( $P = 0.0002$ , Kruskal-Wallis ANOVA) (Fig 3). A similar result was observed when the medium was replenished every 2 days in that consumption was higher for the first 2 days and then decreased substantially ( $P = 0.0011$ , Kruskal-Wallis ANOVA) (Fig 4). Incubation in saline containing glucose prevented separation in some explants for up to 8 days (Table 3).

## Discussion

A variety of clinical conditions including carbohydrate overload, septic metritis, hyperlipaemia and corticosteroid therapy have been recognised as being initiators of laminitis (Jeffcott and Field 1985; Baxter 1994). However, a unifying hypothesis to

explain how these divergent conditions induce laminitis is lacking. The results of the current experiments suggest such an hypothesis.

Adhesion of basal epidermal cells to the basement membrane was maintained for more than one week when hoof explants were cultured in medium containing glucose, amino acids, vitamins, buffers and a variety of ions but for less than 2 days when cultured in physiological saline. The component that appeared to be responsible for maintenance of adhesion was glucose because, when it was added to saline, the explants remained intact for at least 2 days of incubation and in most instances up to 8 days.

The longer term study indicated that glucose consumption from D-MEM diminished considerably as the period of incubation increased, even if the medium was replaced with fresh medium every 2 days. It seemed that the hoof tissue was reliant on glucose for maintenance of adhesion between the epidermal basal cells and the basement membrane for more than 2 days after removal from the horse. However, as time progressed, the cells appeared to adapt to another substrate for energy synthesis. The alternative energy substrate has not been identified but could be an amino acid as these were readily available in the medium and may have been available in the tissue itself.

The experiments with 2-DG and APMA further highlighted the importance of glucose as an energy substrate for the hoof tissue. Both 2-DG and APMA caused separation of the epidermal basal cells from the basement membrane. 2-DG inhibits the glycolytic pathway (Webb 1966). It is believed that 2-DG is converted to 2-DG-6-phosphate which competitively inhibits the metabolism of glucose-6-phosphate by phosphoglucose isomerase (Webb 1966). Glucose-6-phosphate is the first product of glucose metabolism in the glycolytic pathway. The lack of glucose consumption by the tissue explants in the presence of 2-DG in the present study confirmed the inhibitory effect 2DG has on glycolysis.

Aminophenylmercuric acetate (APMA) also induced separation of the hoof; APMA is an organomercurial compound which is recognised as an activator of metalloproteinases which are known to degrade components in the basement membrane (Jones *et al.* 1994). The possible role of these enzymes in the pathogenesis of laminitis, has been discussed elsewhere (Pollitt *et al.* 1998). Organomercurials are also known to inhibit enzymes of the glycolytic pathway; an effect inhibited by DTT (Webb 1966; Kanda *et al.* 1976; Larson and Pate 1976; Thompson 1978). The present results indicate that APMA inhibited glycolysis in the hoof explants because addition of APMA to the culture medium inhibited glucose consumption and this was reversed by DTT. Dithiothreitol (DTT) also prevented the lamellar separation induced by APMA.

The metalloproteinase inhibitor BB-94 also inhibited the separation of hoof tissue treated with APMA and in explants cultured in saline (Pollitt *et al.* 1998). BB-94 does not however inhibit the effect of APMA on glucose metabolism (M. A. Pass, unpublished data). These observations suggest that inhibition of glucose utilisation by cells in the hoof may be a trigger for activation of metalloproteinases that could then cause separation of the epithelial cells from the basement membrane.

Although inhibition of glucose metabolism causes separation of hoof tissue *in vitro*, is there evidence that such a mechanism could account for the development of laminitis *in vivo*? There is circumstantial evidence suggesting that such a mechanism is possible. Laminitis is often a consequence of an acute metabolic stress such as occurs with metritis, carbohydrate overload and

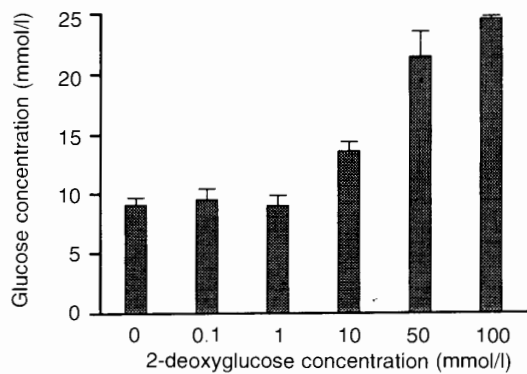


Fig 1: Effect of 2-deoxyglucose (2-DG) on glucose consumption by hoof explants cultured for 2 days in Dulbecco's modified Eagle medium (D-MEM). The results are the mean  $\pm$  s.e. of 5 estimations of the glucose concentration in the medium at the end of the incubation period.

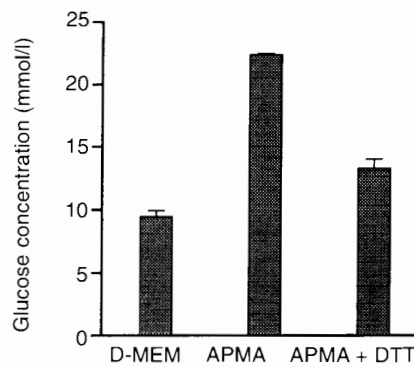


Fig 2: Effect of aminophenylmercuric acetate (APMA) (0.7 mmol/l) and APMA (0.7 mmol/l) + dithiothreitol (DTT) (100 mmol/l) on glucose consumption by hoof explants cultured in Dulbecco's modified Eagle medium (D-MEM) for 2 days. The results are the mean  $\pm$  s.e. of 6 estimations of the glucose concentration in the medium at the end of the incubation period.

hyperlipaemia. Such acute conditions are considered to invoke changes in the pattern of metabolism in the animal similar but probably more pronounced than that occurring during starvation (Moore 1971; Jeffcott and Field 1985; Cunningham 1992). The major feature of these changes are that glucose consumption in many peripheral tissue is reduced and gluconeogenesis is increased. The purpose of this change is to maintain glucose and therefore energy supplies to the injured tissue and the vital organs at the expense of other tissues. The metabolic changes in response to sepsis and other acute diseases are regulated by hormones including insulin, glucagon, cortisol and adrenalin with insulin promoting glucose utilisation and the other hormones promoting the metabolism of other substrates and reducing glucose consumption. It is not known if firstly the hoof tissues are normally reliant on glucose, secondly if they are responsive to hormones regulating glucose metabolism or thirdly if they can change energy substrates. The results of the current experiments suggest that hoof tissue does utilise glucose and that it can change, at least slowly, to an alternate substrate if glucose availability becomes limited. However, rapid withdrawal of glucose *in vitro* causes separation of the hoof. In acute, severe septic diseases and other conditions inducing severe changes in metabolism, changes in glucose metabolism can be rapid and may mimic the conditions established *in vitro*. If the metabolic insult was severe, there may be insufficient time for the hoof to adapt to an alternative

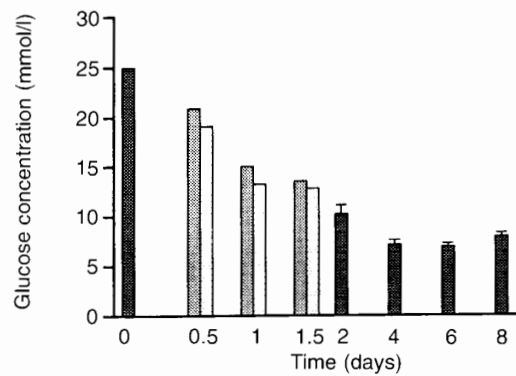


Fig 3: Glucose concentration in Dulbecco's modified Eagle medium (D-MEM) from hoof explants cultured for up to 8 days. The results are the mean  $\pm$  s.e. of 6 or 8 estimates except at 0.5, 1 and 1.5 days where the individual results of 2 estimates are shown.

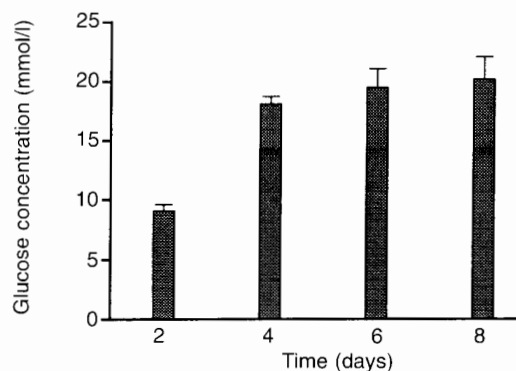


Fig 4: Glucose concentration in Dulbecco's modified Eagle medium (D-MEM) from cultured hoof explants. The D-MEM was replenished every 2 days and the results are the mean  $\pm$  s.e. of 8 estimates on samples collected after each 2 day period of incubation.

substrate, and hoof separation would occur. Other epithelia may be similarly weakened, but gross separation would be manifest most readily in the hoof because of the large mechanical forces generated by weight bearing.

The time course of the metabolic changes in the hoof explants is consistent with the time course of development of laminitis. The explants continued to utilise glucose at a relatively fast rate for 2 or 3 days and separated within 36 h if glucose was unavailable. Clinical signs of laminitis become evident within 24–56 h of induction by carbohydrate overload (Hood 1984) which is shorter than the time the hoof tissue takes to adapt to an energy substrate other than glucose.

There is evidence that the metabolic changes described above do occur as a consequence of carbohydrate overload in horses developing laminitis. Hood (1984) and Clarke *et al.* (1982) demonstrated an increase in blood cortisol during the development of laminitis consistent with a metabolic change to conserve glucose. Furthermore, preliminary experimental data from our laboratory from one horse which developed laminitis after dosing with carbohydrate, showed changes in plasma insulin and glucagon concentrations consistent with a metabolic switch to conserve glucose and increase gluconeogenesis (data not shown).

Further support for a relationship between changes in glucose metabolism and laminitis comes from observations on horses with hyperlipaemia. Hyperlipaemia is a state of negative energy

balance occurring rapidly and often precipitated by some form of stress (Jeffcott and Field 1985). It has been suggested that laminitis related to hyperlipaemia is a result of vasoconstriction in the hoof as a consequence of the altered metabolism in the animal (Field and Jeffcott 1989). An alternative explanation is that the metabolic changes leading to hyperlipaemia result in the hoof tissues being starved of glucose thereby precipitating the chain of events leading to separation of the hoof as occurred in the cultured explants in the current experiments.

Significantly reduced concentrations of 11 $\beta$ -hydroxysteroid dehydrogenase (HSD) have been documented in the skin of horses with laminitis (Johnson *et al.* 1996). If hoof lamellar tissues and skin behave similarly, then an aberration of local metabolism of glucocorticoid, leading to an increased concentration of cortisol in the tissue, could reduce glucose metabolism and cause lamellar separation.

Alpha adrenergic antagonist drugs, such as phenoxybenzamine, appear to prevent laminitis in some circumstances and this has been attributed to the drug inhibiting vasoconstriction in the hoof (Hood *et al.* 1993), presumably by blocking the alpha adrenergic effects of endogenous catecholamines. However, catecholamines also have metabolic effects. In particular, stimulation of alpha adrenoceptors inhibits insulin secretion and stimulation of alpha and beta adrenoceptors increases hepatic glycogenolysis (Robinson 1986; Hoffman and Lefkowitz 1991). Therefore, blockage of alpha adrenoceptors in stressful situations would be expected to increase insulin secretion (Kashiwagi *et al.* 1986) and still maintain increased hepatic glucose production. This could result in increased utilisation of glucose by peripheral tissues and protect the hoof tissues from the effects of glucose starvation.

The results of the present *in vitro* experiments offer support to the hypothesis that changes in glucose metabolism as a result of a primary disease elsewhere in the body may be a trigger for laminitis. As yet, it is not clear if the *in vivo* situation mimics the *in vitro* conditions. For instance, does the hoof tissue in the intact animal normally rely on glucose for most of its energy production; are the cells in the hoof responsive to the hormonal mediators of metabolism; and are the metabolic changes associated with laminitis-inducing diseases severe enough to starve the hoof tissue of glucose rapidly enough to induce separation of epidermal cells from the basement membrane? Answers to these questions are needed to confirm the proposed hypothesis and to develop a framework for manipulating metabolism as a potential approach for the prevention of laminitis.

### Acknowledgements

This project was funded by a grant from the Rural Industries Research and Development Corporation of Australia. The authors are grateful to the Animal Health Trust of Missouri, USA, for funds to purchase the BX-50 Olympus microscope. Bruce Mungall and Mousa Daradka are thanked for collecting knackery specimens.

### Manufacturers' addresses

<sup>1</sup>Gibco BRL, Life Technologies Pty Ltd, Mulgrave, Victoria, Australia.

<sup>2</sup>Sigma Australia, PO Box 970, NSW 2150, Australia.

<sup>3</sup>Roche Diagnostic Systems, Hoffman-La Roche Ltd, CH-4002, Basel, Switzerland.

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