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## Induction of laminitis by prolonged hyperinsulinaemia in clinically normal ponies

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### Abstract

The purpose of this study was to determine the effects of prolonged administration of insulin, whilst maintaining normal glucose concentrations, on hoof lamellar integrity in vivo on healthy ponies with no known history of laminitis or insulin resistance. Nine clinically healthy, unrelated ponies were randomly allocated to either a treatment group ( $n = 5$ ;  $5.9 \pm 1.7$  years) or control group ( $n = 4$ ;  $7.0 \pm 2.8$  years). The treatment group received insulin via a euglycaemic hyperinsulinaemic clamp technique modified and prolonged for up to 72 h. Control ponies were infused with an equivalent volume of 0.9% saline. Ponies were euthanased at the Obel grade 2 stage of clinical laminitis and hoof lamellar tissues were harvested and examined for histopathological evidence of laminitis.

Basal serum insulin and blood glucose concentrations were  $15.7 \pm 1.8 \mu\text{U/mL}$  and  $5.2 \text{ mmol/L}$ , respectively (mean  $\pm$  SE) and were not significantly different between groups. Mean serum insulin concentration in treatment ponies was  $1036 \pm 55 \mu\text{U/mL}$  vs.  $14.6 \mu\text{U/mL}$  in controls. All ponies in the treatment group developed clinical and histological laminitis (Obel grade 2) in all four feet within 72 h ( $55.4 \pm 5.5$  h), whereas none of the control ponies developed laminitis. There was no clinical evidence of gastrointestinal involvement and the ponies showed no signs of systemic illness throughout the experiment. The data show that laminitis can be induced in healthy young ponies, with no prior history of laminitis, by maintaining prolonged hyperinsulinaemia with euglycaemia. This suggests a role for insulin in the pathogenesis of laminitis, independent of hyperglycaemia, or alterations in hind-gut fermentation. For the clinician, early detection and control of hyperinsulinaemia may facilitate management of endocrinopathic laminitis.

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**Keywords:** Insulin; Equine; Euglycaemic-hyperinsulinaemic clamp; Glucose; Laminitis

### Introduction

The term endocrinopathic laminitis describes laminitis occurring from putative hormonal dysfunction, rather than occurring in association with pro-inflammatory and intestinal conditions. Endocrinopathic laminitis is most commonly seen in association with equine Cushing's syndrome (ECS), corticosteroid treatment and insulin-resistance syndrome (otherwise known as equine metabolic syndrome) (Johnson et al., 2004c).

In ECS, laminitis is thought to be triggered by hypercortisolaemia, resulting from excessive ACTH production (Love,

1993; Schott, 2002), and under certain conditions, the direct administration of corticosteroids can also trigger laminitis (Bailey and Elliott, 2007). There is a well-established link between insulin and cortisol, which have opposing actions on glucose metabolism, such that hyperinsulinaemia and insulin resistance are potential consequences of ECS or corticosteroid treatment (Reynolds and Walker, 2003; Bailey and Elliott, 2007).

Other workers have also made a connection between insulin and laminitis with the recognition that laminitis is a major clinical sign in animals that display a syndrome of insulin resistance, often associated with pony breeds and (or) obesity (Johnson et al., 2004a; Treiber et al., 2005). Similarities between insulin resistance syndrome in horses and metabolic syndrome in humans have led to

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speculation about the pathogenesis of laminitis (Johnson et al., 2004a). However, an important clue to understanding this disease may lie in the observation that insulin status is a powerful prognostic indicator in horses with ECS, such that animals are much more likely to develop laminitis and survive <2 years after diagnosis if they are also insulin-resistant, as judged by a basal serum insulin concentration >188  $\mu\text{U}/\text{mL}$  (McGowan et al., 2004).

Surprisingly, the most recent evidence in support of a role for insulin in laminitis has emerged from dietary studies, which sought to explain why some horses and ponies that graze on lush pastures can develop laminitis repeatedly. Until now, most investigators have focussed on dietary-induced changes in hind-gut fermentation, and the search for plant or microbial toxins (Galey et al., 1991). However, a 'prelaminitic metabolic syndrome' (PLMS) has now been described, in which elevated serum insulin concentrations served to distinguish ponies that were known to be susceptible to dietary laminitis, from others in the same herd that were not susceptible (Treiber et al., 2006b). Interestingly, among the susceptible group, plasma insulin concentrations were markedly elevated in the ponies that developed laminitis while grazing lush pasture, while glucose, free fatty acid and cortisol concentrations remained normal (Treiber et al., 2006a).

Exploration of the link between cortisol, insulin resistance and laminitis has focussed on the control of glucose uptake in the hoof lamellae. There is evidence that the equine foot has an unusually high glucose demand (Wattle and Pollitt, 2004), and that lamellar explants become separated if incubated in the absence of glucose, or in the presence of a glucose uptake inhibitor (Pass et al., 1998; French and Pollitt, 2004). As insulin resistance is defined as an impairment in the ability of tissues to take up glucose via insulin-stimulated glucose transporters, glucose uptake impairment was an hypothesised mechanism leading to laminitis. However, the recent discovery that glucose uptake in the hoof occurs independently of insulin (Asplin et al., 2007), has cast doubt on this mechanism necessitating further investigation.

Specifically, whilst much has been written about laminitis in the context of insulin resistance, little is known about the effects of hyperinsulinaemia per se in horses or ponies. In other species, insulin has been shown to have important effects on variables such as blood flow (Anderson et al., 1991) and protein turnover (Millward et al., 1983), that are probably independent of glucose transporters and hence 'insulin sensitivity' in the classical sense. Furthermore, in contrast to humans, insulin-resistant horses rarely develop pancreatic exhaustion, and are capable of producing exceptionally high serum insulin concentrations (Reeves et al., 2001; McGowan et al., 2004).

The purpose of the present study was to determine the effects of hyperinsulinaemia, in the absence of cortisol manipulation, dietary modification, or hyperglycaemia on lamellar integrity in the hooves of healthy ponies. It is

not insulin resistance but insulin toxicity that is the causative factor in endocrinopathic or pasture-associated laminitis is the hypothesis of this study.

## Materials and methods

### Ponies

Nine clinically normal, untrained ponies were used for this study. The ponies were not related and all came from different properties. The seven geldings, one colt and one mare had a median age of 4 years (range 2–14 years), a mean bodyweight (BW) of  $258 \pm 25$  kg and were in moderate body condition (scored 3–4, based on a scale of 1–5; Carroll and Huntington, 1988).

None of the ponies had a known history of laminitis, or showed any evidence of this based on a visual inspection of their hooves. The ponies grazed on tropical grass pasture supplemented with lucerne and/or grass hay before the study and were allowed ad libitum access to lucerne hay and fresh water throughout the experimental infusion period. Routine haematology, serum biochemistry and plasma basal ACTH (immulite 1000) analyses were performed on all ponies and any pony with abnormalities was excluded from the study.

### Prolonged euglycaemic-hyperinsulinaemic clamp (pEHC) technique

The ponies were allocated at random to either a treated or control group, to receive infusions of recombinant human insulin (Humulin R, Eli Lilly) plus glucose, or an equivalent volume of isotonic saline, respectively. To perform the infusions, each pony was loosely confined in a space of  $1 \text{ m} \times 2.5 \text{ m}$  in the corner of a stable. Catheters (14 G, MILA, CPP) were then inserted into both jugular veins using a local anaesthetic (2% lignocaine) blockade. For treated ponies, the first catheter was used for simultaneous infusion of insulin at a fixed rate and glucose at a variable rate. The second catheter was used to collect blood samples.

Prolonged insulin infusions were based on the euglycaemic-hyperinsulinaemic clamp (EHC) technique established by DeFronzo et al. (1979), but modified to last for up to 72 h. At the start of the experiment, three blood samples (10 mL) were obtained 10 min apart to determine basal glucose and insulin concentrations. Within the next 10 min, a priming dose of insulin (45 mU/kg BW in 50 mL of 0.9% saline) was administered intravenously (IV) as a bolus injection (DeFronzo et al., 1979). The insulin infusion was then started at a steady rate of 6 mU/min/kg BW, which was maintained throughout the experiment. An infusion of glucose solution (50% w/v; Baxter) was also started, at a rate of  $24.4 \pm 3.0$   $\mu\text{mol}/\text{min}/\text{kg}$  BW (DeFronzo et al., 1979).

Glucose concentrations were measured in 1 mL blood samples collected every 5 min for the first 3 h of the experiment, and every 30 min thereafter, using a portable glucometer calibrated for equine blood (Advantage, Roche Diagnostics). Euglycaemia was defined as a blood glucose concentration of 5 mM, and the glucose infusion rate was adjusted whenever blood concentrations differed from this value by >1 mM.

A steady state was presumed to exist once euglycaemia was maintained for a period of at least 30 min without the need to adjust the glucose infusion rate. During this steady state, three blood samples (10 mL) were taken at 10 min intervals to determine insulin concentrations. The samples were allowed to clot at room temperature then centrifuged for 15 min at 3000 g to obtain serum. Additional blood samples for insulin analysis were taken hourly until 12 h after the initial insulin bolus, and at 8 h intervals thereafter in treated ponies. Serum samples were stored at  $-80$  °C until analysed for insulin concentration using a radioimmunoassay kit validated for use with horse serum (DSL). Control ponies received a single infusion of 0.9% saline at a fixed rate of 14.7  $\mu\text{L}/\text{min}/\text{kg}$  BW for 72 h, which represented the average rate of fluid infusion in the treated ponies over the same period. Blood samples for insulin analysis were taken hourly until 12 h, then every 4 h until 24 h, and at 8 h intervals until 72 h, and blood glucose was measured every 6 h in control ponies.

Infusions continued for a period of 72 h, or stopped following detection of clinical signs of laminitis. These signs were increased digital pulses, palpably increased hoof heat over the dorsal hoof wall, weight shifting and lameness (Obel grade 2). Heart rate, respiratory rate, rectal temperature and demeanour were also monitored at regular intervals throughout the experiment. Once the insulin infusions were stopped, the rate of glucose infusion was decreased steadily over 2–3 h, until the animals were able to maintain euglycaemia unaided. Any pony showing signs of laminitis was given phenylbutazone (4.4 mg/kg BW orally or IV).

All ponies were euthanased with pentobarbitone sodium (162.5 mg/kg BW IV) within 6–12 h after the infusion ceased, except for one pony that was euthanased five days later.

### Insulin sensitivity

The relative insulin sensitivity was determined for each treated pony by calculating the ratio of insulin infused to the amount of glucose metabolised during the 30 min steady state period (*M*-to-*I* ratio). The insulin infusion rate was known and the glucose metabolism rate (*M*) was estimated, based on the glucose infusion rate and the use of a space correction factor to allow for any glucose added or removed from the system by means other than metabolism, as described by DeFronzo et al. (1979).

### Lamellar histopathology

All four hooves were disarticulated at the metacarpophalangeal joint within 10 min of euthanasia, then dissected to obtain 5 mm square explants, extending from the inner hoof wall to the dermal connective tissue, as described by Pollitt (1996). The explants were placed in 4% paraformaldehyde for a minimum of 24 h, then embedded in paraffin and sectioned at 5  $\mu$ m. The sections were then stained with haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS), for the evaluation of lamellar histomorphology and basement membrane abnormalities, respectively. Histological analysis was performed with the investigator (CCP) blinded to the identity of the pony and group.

### Statistical analysis

Analysis of histopathological diagnoses of laminitis for comparison between groups was performed using an exact Chi Square test (Fisher's exact test, Epi Info version 3.4, 2007). Insulin and glucose concentrations were compared between groups using the Student's *t*-test. Significance was accepted as  $P < 0.05$ . All results are presented as mean  $\pm$  standard error of the mean (SE).

The 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 (current edition). All animals were continuously monitored by the investigators and a registered veterinarian throughout the experimental procedure.

## Results

### Ponies

All ponies ( $n = 5$ ) in the treatment group developed laminitis in all four hooves, whereas none developed laminitis in the control group ( $n = 4$ ) ( $P < 0.01$ ). In the treated group, Obel grade 1 laminitis (prominent, palpable digital pulses, heat over the dorsal hoof wall and weight shifting) (Obel, 1948) was evident in all treated ponies at  $32.6 \pm 5.4$  h after infusions started. The time to onset of Obel grade 2 (Obel, 1948) laminitis and the end of infusion in treated ponies was  $55.4 \pm 5.5$  h. Mean respiratory rate increased from  $18.7 \pm 1.0$  to  $36 \pm 3.1$  breaths/min, and heart rate increased from  $49 \pm 2.2 \times$  to  $70 \pm 3.7$  bpm at the onset of Obel grade 2 laminitis.

The ponies all remained bright and alert and were eating and drinking normally throughout the experiment. However, 3/5 ponies showed signs of agitation associated with the onset of laminitis. Rectal temperature remained within the normal range in all ponies. In the treated ponies, clinical signs were ameliorated by one Obel grade by routine doses of non-steroidal anti-inflammatory medication until the animals were euthanased.

### Prolonged euglycaemic-hyperinsulinaemic clamp technique

Basal serum insulin and blood glucose concentrations were  $15.7 \pm 1.8$   $\mu$ U/mL and  $5.2 \pm 0.1$  mM, respectively, and were not significantly different between treated and control groups. Blood glucose concentration ( $5.2 \pm 0.1$  mM)

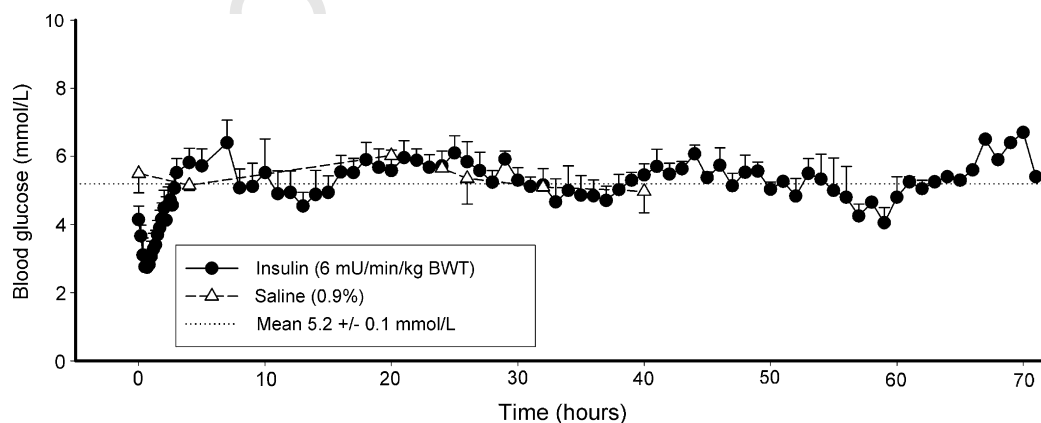


Fig. 1. Blood glucose concentrations observed in ponies infused for up to 72 h with recombinant human insulin (6 mU/min/kg BW), plus glucose at a variable rate designed to maintain euglycaemia at 5 mmol/L (treatment group ●;  $n = 5$ ), or 0.9% saline only at a fixed rate (control group ○;  $n = 4$ ). Samples were obtained every 5 min for the first 180 min, and at 30 min intervals thereafter for treated ponies and every 6 h for 40 h for control ponies. Values are presented as mean  $\pm$  SE. For clarity, only positive error bars are shown for treated ponies and negative error bars for control ponies; and only samples obtained every 10 min for the first 180 min, and hourly thereafter for treated ponies are presented.

was maintained within euglycaemic values for both treated and control ponies and was not significantly different between groups (Fig. 1). Serum insulin concentrations in treated ponies during the infusion period averaged  $1036 \pm 55.0 \mu\text{U/mL}$  vs.  $14.6 \pm 2.6 \mu\text{U/mL}$  in control ponies and was significantly different between groups (Fig. 2).

The glucose metabolism rate ( $M$ ), and the amount of glucose metabolised per unit of exogenous insulin ( $M$ -to- $I$  ratio), were calculated during the first 3 h of the pEHC procedure for each treated pony (Table 1). The first 90 min of the EHC is considered an equilibration period. Therefore, only steady states achieved between 90 min and 180 min after starting the insulin infusion were used for calculations. The  $M$  value and  $M$ -to- $I$  ratio, relative to three of the ponies was high for Pony 2 and low for Pony 3.

### Lamellar histopathology

The histological appearance of the lamellar tissues obtained from the control ponies was judged normal in all cases (Pollitt, 1996). The tissues obtained from treated ponies, which all showed clinical signs of laminitis at the time of euthanasia, was typical of laminitis: the tips of secondary epidermal lamellar (SEL) were elongated and tapered, the basement membrane (BM) was disintegrated, and basal cell nuclei were rounded (Pollitt, 1996).

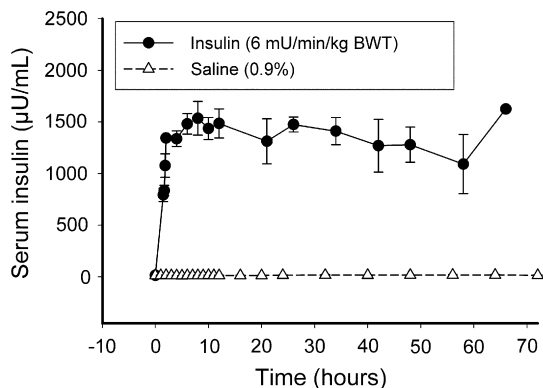


Fig. 2. Serum insulin concentrations observed in ponies infused with insulin (6 mU/min/kg BW) (treatment group ●;  $n = 5$ ) or 0.9% saline (control group ○;  $n = 4$ ) for up to 72 h. Euglycaemia was maintained in all ponies throughout the procedure. Values are mean  $\pm$  SE at all time points for control ponies ( $n = 4$ ), and for treated ponies up to 42 h ( $n = 5$ ), 58 h ( $n = 3$ ), and 66 h ( $n = 1$ ).

Table 1  
Measures of glucose metabolism and insulin sensitivity obtained during a euglycaemic-hyperinsulinaemic clamp in five healthy ponies

Variable	P1	P2	P3	P4	P5	Mean $\pm$ S.E.
$I$ basal (pmol/L)	75.3	128.0	144.1	59.9	117.7	105.0 $\pm$ 16.0
$I$ steady state (pmol/L)	6957.5	5908.0	5143.4	5681.0	7434.8	6224.9 $\pm$ 422.3
$M$ (mmol/kg/min)	0.0138	0.0435	0.0054	0.0220	0.0235	0.0216 $\pm$ 0.006
$M$ -to- $I$ ratio ( $\times 10^{-6}$ )	2.0	7.4	1.0	3.9	3.2	3.5 $\pm$ 1.1

Mean  $\pm$  SE are for five ponies. P1–5 = Pony 1–5.  $I_{\text{basal}}$ : baseline serum insulin;  $I_{\text{steady state}}$ : serum insulin concentration ( $I$ ) at steady state;  $M$ : amount of metabolised glucose;  $M$ -to- $I$  ratio: metabolised glucose per unit of insulin.

### Discussion

The results of this study are consistent with the hypothesis that insulin toxicity is a key factor in triggering equine laminitis. Thus, laminitis was induced in healthy young ponies with no prior history of the condition, by maintaining prolonged hyperinsulinaemia and euglycaemia. The EHC technique, which suppresses endogenous glucose and insulin production (DeFronzo et al., 1979) was used to demonstrate that laminitis could be induced by insulin with no marked change in blood glucose concentration and that this was not dependent on whether the animal was insulin resistant or insulin-sensitive, with respect to glucose metabolism. Of the ponies used in the present study, all treated animals had basal insulin concentrations in the normal range, whereas the  $M$ -to- $I$  ratio suggested three were normal, one was slightly insulin resistant, and one was particularly insulin-sensitive. It may or may not be significant that this insulin-sensitive pony was the first to develop Obel grade 2 laminitis, which occurred after 37 h.

As well as highlighting the importance of insulin in the pathogenesis of endocrinopathic laminitis, it is likely that insulin toxicity plays a key role in the development of certain dietary forms of laminitis, particularly where high carbohydrate intakes drive insulin levels beyond a certain tolerance threshold in the insulin-resistant horse or pony. For example, diets rich in non-structural carbohydrates (NSC) are reported to decrease insulin sensitivity and impair glucose tolerance in horses (Pratt et al., 2006), whereas insulin-resistant ponies that graze lush pasture are those most likely to develop laminitis (Treiber et al., 2006a).

The practical implications of this research are that methods should be developed to identify horses at risk of laminitis, via the early detection of hyperinsulinaemia, although more work needs to be done to identify the insulin toxicity threshold. Techniques should be employed to lower insulin concentrations and restore insulin sensitivity. These include substituting dietary NSC with low glycaemic index (GI) feeds (Johnson et al., 2004b) and progressive weight loss in overweight horses. It has already been shown that physical exercise training can help to prevent insulin resistance in horses (Pratt et al., 2006) and improve glucose tolerance in ponies (Freestone et al., 1992). To our knowledge, the use of insulin-sensitising drugs of the type given to human

patients given to human patients with Type 2 diabetes has not been thoroughly explored in horses.

Elucidation of the mechanism of insulin toxicity might yield other useful treatments or preventative strategies for endocrinopathic laminitis. It has been proposed that the mechanism involved in hyperinsulinaemic-associated laminitis is similar to that of glucotoxic endotheliopathy and microvascular dysfunction commonly seen in human diabetics (Johnson et al., 2004c; Keen et al., 2004). However, glucotoxicity is not supported in the current study where laminitis occurred with no elevation in blood glucose concentrations and many horses and ponies with IR or hyperinsulinaemia that develop laminitis are not overtly hyperglycaemic (Treiber et al., 2005).

Insulin increases blood flow to muscles in humans via endothelium-derived nitric oxide synthesis or release (Steinberg et al., 1994), as well as open arteriovenous anastomoses (AVAs) (Kihara et al., 1994). In fact, up to 25% of the stimulatory effects of insulin on muscle glucose uptake appears to be the result of vasodilation and capillary recruitment (Baron et al., 1995). Increased blood flow to the foot post-prandially has been linked to post-prandial elevation in insulin in horses (Hoffmann et al., 2001). In the present study, the increased digital pulse amplitude that followed injection of the initial insulin bolus may indicate an increase in blood flow to the foot. This occurred as early as 3.5 h in 2/5 treated ponies, and by 14 h in one treated pony. The increased pulse amplitude subsequently returned to normal until the development of laminitis, where it again increased. Prolonged increased blood flow to the foot may increase delivery of glucose to the lamellar basal cells and/or capillary endothelial cells, even during periods of euglycaemia. Since glucose uptake is insulin-independent (Asplin et al., 2007) and GLUT-1 transport proteins are well below saturation in physiological conditions (Gruetter et al., 1998), a marked increase in glucose uptake could occur thus supporting the hypothesis of glucotoxic endotheliopathy and microvascular dysfunction (Johnson et al., 2004c; Keen et al., 2004).

In rats, insulin administration under euglycaemic conditions causes hypoxia in peripheral nerves despite increased total blood flow (Kihara et al., 1994). The hypoxia was ascribed to perfusion failure due to open AVA shunts. Similarly, in the human neuropathic diabetic foot, increased limb blood flow is shunted out of the foot by dilated AVAs, leading to increased venous pressure and foot ulceration (Boulton et al., 1982). Laminitis may develop because blood is shunted away from nutritive lamellar capillaries via dilated AVAs. This results in pathological hypoperfusion of the lamellae, despite increased blood flow to the foot (Hood et al., 1978). In this study, the bounding digital pulse and increased heat observed in the hoof wall during laminitis could be the result of insulin-induced vasodilation. If AVAs were preferentially dilated, then localised lamellar hypoxia and ischaemia could result in degradation of the basement membrane lamellar junction and laminitis.

Endothelin-1 (ET-1) expression in the lamellae is increased during both acute and chronic laminitis and has been implicated in the pathophysiology of laminitis via its vasoconstrictive effects (Katwa et al., 1999). Increased ET-1 production can be induced by hyperinsulinaemia (Juan et al., 2004) and the laminitis developed by the ponies of this study could have resulted from lamellar hypoperfusion and hypoxia associated with ET-1 induced vasoconstriction.

Whether due to glucotoxicity, hypoperfusion, or another mechanism, it is likely that the haemodynamic changes associated with prolonged hyperinsulinaemia ultimately result in matrix metalloproteinase (MMP) upregulation. It has been established that MMPs are present in equine lamellar tissue, with increased expression of the active forms of MMP-2 and MMP-9 in laminitic tissue (Johnson et al., 1998; Pollitt et al., 1998). These enzymes are capable of degrading the BM (Pollitt and Daradka, 1998; French and Pollitt, 2004) and are activated in response to oxidative stress, peripheral hypoperfusion (Woessner, 1991) and increased circulating and local tissue levels of pro-inflammatory cytokines (Salo et al., 1994) and could be involved in the laminitis observed here.

## Conclusions

Prolonged hyperinsulinaemia induces laminitis in healthy, young, lean ponies, independent of changes in blood glucose concentration or insulin sensitivity. This has important implications for the management of horses with insulin resistance due to equine Cushing's syndrome, corticosteroid treatment or insulin resistance syndrome induced by a long-term high intake of non-structural carbohydrates, obesity, and/or or a genetic predisposition. Our previous study established that glucose uptake to hoof lamellae is insulin independent (Asplin et al., 2007). Lamellar epidermal basal cells are rich in GLUT-1 (Asplin et al., 2007), but lack insulin receptors (Wattle and Pollitt, unpublished data). The only lamellar tissue with insulin receptors is the vasculature (Wattle and Pollitt, unpublished data) and this seems a likely target for the hyperinsulinaemia induced by this experiment. The recorded increased digital pulses and hoof wall heat in the insulin treated ponies suggests that hyperinsulinaemia caused a prolonged vascular response. Whether this affected glucose delivery and perturbed lamellar epidermal basal cell metabolism, sufficient to cause laminitis, awaits further investigation. Meanwhile, strategies to lower blood insulin concentration and restore insulin-sensitivity in horses or ponies known to be at risk of laminitis are recommended.

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