Persistent digital hyperthermia for 48 hours does not induce laminitis in horses

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Abstract

Persistent digital hyperthermia, presumably due to vasodilation, has been shown to occur during the developmental and acute stages of insulin-induced laminitis. This study sought to determine if persistent digital hyperthermia is the principle pathogenic mechanism responsible for the disease. A potent vasodilator, ATP-$MgCl_2$ was infused continuously into the left forefoot of six Standardbred racehorses for 48 h via an intra-osseous infusion into the distal phalanx (0.11mg/kg/min), to promote persistent digital hyperthermia. The right forefoot was infused with saline solution and acted as an internal control. Clinical signs of lameness at the walk were not detected at 0 h, 24 h or 48 h. Mean ± se hoof wall temperature of the left fore (29.4 ± 0.25 ºC) was higher ($P < 0.05$) than the right (27.5 ± 0.38 ºC). Serum insulin (15.0 ± 2.89 µIU/mL) and blood glucose (5.4 ± 0.22 mM) concentrations remained unchanged during the experiment. Histopathological evidence of laminitis was not detected in any horse. The results show that digital vasodilation up to 30°C for a period of 48 h, does not cause laminitis in the absence of hyperinsulinaemia. Thus, although digital hyperthermia may play a role in the pathogenesis of laminitis, it is not the sole mechanism.

Keywords

ATP-$MgCl_2$; Equine; Vasodilation; Intra-osseous; Laminitis

Introduction

Equine laminitis is a serious foot disease of horses (USDA, 2000) characterised by lameness, increased digital pulses and palpably warm hooves. While it is assumed that an increase in perfusion occurs in the acute phase of laminitis, there has been considerable debate on the role of the vasculature during the developmental phase. Some researchers have suggested that a decrease in blood flow to the foot may occur (Adair et al., 2000), or blood may be shunted away from the digital microcirculation through dilated arteriovenous anastomoses (Robinson, 1990) during laminitis development, resulting in lamellar ischaemia. On the other hand, an increase in hoof temperature, and presumably blood flow to the foot, has been recorded during the developmental phase of both carbohydrate- (Pollitt and Davies, 1998) and insulin-induced (de Laat et al., 2010) laminitis. Insulin achieves vasodilation through its interaction with the vascular endothelium and the release of nitric oxide (Baron, 1996) which is the potential mechanism for the digital vasodilation seen during hyperinsulinaemic laminitis in horses (de Laat et al., 2010).

The developmental phase of naturally-occurring laminitis often goes unrecognised and as a result, the opportunity to monitor blood flow during this pre-clinical phase is missed. In particular, endocrinopathic laminitis offers little opportunity to study its developmental phase as the precise time of onset in at-risk individuals can be difficult to predict (Geor, 2008; Menzies-Gow et al., 2010). Experimentally-induced hyperinsulinaemic laminitis is associated with persistently elevated hoof wall temperature during the developmental phase that is consistently higher, and less variable than the hoof temperature recorded in control animals (de Laat et al., 2010).

The increased hoof temperature recorded in this study was presumably a reflection of increased digital perfusion and was attributed to the vasodilatory effects of insulin. The purpose of the current study was to investigate the role of persistent digital vasodilation in the pathogenesis of insulin-induced laminitis. The aim of the study was to induce persistent digital vasodilation over 48 h with ATP-$MgCl_2$, in order to determine whether digital hyperthermia *per se* is sufficient to cause laminitis in the absence of hyperinsulinaemia.

Adenosine 5' triphosphate (ATP) is a powerful vasodilator and acts via purinoceptors located on vascular endothelial cells, to achieve its effects on vascular tone (Burnstock and Kennedy, 1986). A combination of ATP and magnesium chloride ($MgCl_2$) has been investigated as a potential therapy to improve perfusion following sepsis (Chaudry et al., 1980), shock (Chaudry et al., 1976) and ischaemia (Paskitti and Reid, 2002). ATP-$MgCl_2$ has been demonstrated to be safe for intravenous use in horses and has successfully increased blood flow in a dose-dependent manner up to a maximal safe infusion rate (Tetens et al., 1999). ATP-$MgCl_2$ was selected for use in the current study as it was likely to increase digital blood flow following intraosseous infusion into the distal phalanx. Thus, a secondary aim of the study was to determine the suitability of ATP-$MgCl_2$ as a vasodilatory agent following intraosseous perfusion in the horse.

Intraosseous infusion techniques have been used successfully in the metacarpal (Keys et al., 2006; Mattson et al., 2004) and phalangeal (Nourian et al., 2010; Rubio-Martinez et al., 2005) bones of horses. There are numerous medullary sinuses in the body of the distal phalanx of the horse traversed by abundant blood vessels (Nourian,

2009). These blood vessels connect directly to the lamellar vasculature via numerous anastomoses (Nourian, 2009). Intraosseous infusion of the distal phalanx (IOIDP) provides access to the medullary circulation and thus a direct route for administration of substances into the terminal digital circulation and lamellar microenvironment (Nourian et al., 2010). In the current study, continuous intraosseous infusion of the distal phalanx was preferred over intra-arterial or intravenous catheterisation due to the extended nature of the experiment (48 h) and the perceived likelihood of equipment failure during prolonged vascular catheterisation of both forelimbs in a standing, conscious horse. Furthermore, successful infusion of the distal phalanx occurs without the need for a tourniquet (Nourian et al., 2010). The hypothesis of this study was that prolonged infusion of ATP-$MgCl_2$ into one forelimb would result in lamellar vasodilation and persistent hyperthermia, and this would not result in development of laminitis in the treated limb.

Materials and Methods

Subjects

Six clinically healthy (mean bodyweight: 430 ± 17 kg), male Standardbred racehorses in moderate body condition (body condition score: 4-5/9; (Henneke et al., 1983) were randomly selected for use in this study. All of the horses (mean age: 5.2 ± 0.73 years) were unremarkable on physical examination with no evidence of endocrine disease or any phenotypic indicators of insulin resistance (cresty neck score: 0/5; (Carter et al., 2009a). All horses were walked and trotted out for lameness while being videotaped before, during and after the experiment, and any horse that was unsound at

commencement was eliminated from the experiment. Plain, lateral radiographs were taken, and visual inspection of both front feet was performed, before the study to identify horses with pre-existing foot pathology. The horses were then paired randomly and the experiment was conducted in a purpose-built, climate-controlled facility as three replicates over a ten-day period during sub-tropical mid-winter. The horses were accommodated in the facility for 48 h prior to the study to allow environmental acclimatisation. Appetite, demeanour, heart and respiratory rate and rectal temperature were monitored every 4 h throughout the experimental period. Urine analysis was performed prior to, and at the end of the study to assess urine specific gravity and to look for the presence of glucose, ketones, protein and red and white blood cells. Medium quality lucerne chaff, lucerne hay and water were available to the horses *ad libitum* for the 48 h preceding, and throughout the experiment.

Blood sampling

Blood samples (20 mL) were drawn at the beginning and end of the study from a 14 G intravenous, extended-use catheter (MilaCath, Mila) placed in the left jugular vein, to monitor haematologic (vacutainer containing EDTA; Greiner) and biochemical (plain vacutainer; Greiner) variables as an indicator of general health status. Haematologic parameters included haemoglobin, red and white cell count and cell morphology, packed cell volume, platelet count and thrombocytes, while biochemical analysis included electrolytes (sodium, potassium, chloride), aspartate transaminase, alkaline phosphatase, γ-glutamyltransferase, bicarbonate, calcium, anion gap, magnesium, phosphorus, protein (total, albumin, globulin), urea , creatinine, cholesterol, triglycerides, creatinine kinase and total bilirubin. Blood samples (5 ml) were also

drawn to measure serum insulin and blood glucose concentrations at 6 h intervals throughout the study. Blood glucose was measured immediately using a handheld glucometer (Accucheck Go, Roche) previously calibrated against the hexokinase method for equine blood by the authors ($\rho_c = 0.96$). Serum was obtained after allowing the blood sample to clot for 30 min and centrifuging it at 3000 g for 10 min. Aliquots of serum (1 mL) were stored at -80ºC until analysed. Serum insulin concentration was determined for each sample using a radioimmunoassay (Coat-a-count, Siemens) previously validated for use in horses (McGowan et al., 2008).

Formulation and administration of ATP-$MgCl_2$

The ATP-$MgCl_2$ solution was prepared using adenosine 5'-triphosphate disodium salt (A3377, Sigma) and magnesium chloride hexahydrate (M2670, Sigma) according to previous descriptions (Chaudry, 1982). Adenosine 5' triphosphate (0.605 g; 1000 µmol) of was dissolved in 2.5 mL of cold, deionised water and the pH adjusted to 7.0 using 1.0 M sodium hydroxide (NaOH). The final pH of the solution was increased to 7.4 with 0.1M NaOH. The volume was then made up to 5 mL with cold, deionised water and kept on ice. Magnesium chloride (0.2033 g; 1000 µmol) was dissolved in 5 mL of cold, deionised water, mixed with the ATP solution and sterilised with a 0.22 µm filter (Millipore). This solution was then kept on ice until use or stored at -20ºC in 10 mL aliquots for up to 2 weeks.

The safe maximal intravenous infusion rate of ATP-$MgCl_2$ in horses has been shown to be 0.3 mg/kg/min, however a dose rate of 0.2 mg/kg/min was demonstrated as sufficient to achieve peripheral vasodilation without accompanying hypotension

(Tetens et al., 1999). For the current study, a dose rate of 0.2 mg/kg/min was selected for the intra-osseous infusion and was calculated based on the weight of the hoof rather than the horse. The average weight (1.61 ± 0.05 kg) of the distal limb of a Standardbred horse was calculated by weighing fresh cadaver limbs (n = 12) obtained from a local abattoir that were disarticulated at the fetlock. ATP-$MgCl_2$ (7.66 mL; 463.68 mg) of solution was mixed with 22.34 mL of cold 0.9% saline (Baxter) and infused into the left fore foot over 24 h with a 30 mL, re-usable, spring-driven syringe pump (Springfusor, Go Medical) through flow control tubing (Springfusor, Go Medical), to deliver a constant intra-osseous infusion rate of 0.02 ml/min. The springfusor was reloaded with fresh solution and sterile flow control tubing was attached for the second 24 h period. The right fore foot was infused with 30 mL of cold 0.9% normal saline over each 24 h period using an identical Springfusor.

Intra-osseous infusion technique

The horses were administered 0.04 mg/kg bwt of the sedative romifidine hydrochloride (Sedivet, Boehringer Ingleheim) IV prior to bilateral, abaxial, perineural, digital nerve blockade using a short-acting local anaesthetic (2% lignocaine, Troy). An insertion site was marked on the dorsal surface of the front hooves, 25 mm below the hairline and 30 mm lateral to the midline, prior to the hooves being cleaned with a wire brush and disinfected with chlorhexidine. A circular area of 10 mm radius around the insertion point was then cauterised to a depth of 2 mm using a custom-made iron, heated to red-hot in a gas forge. An intra-osseous needle (EZ IO AD 25mm, Vidacare) was inserted at right angles to the hoof wall at the midpoint of the sterilised area using a specialised drill (EZ IO Power Driver,

Vidacare). The needle was passed through the hoof wall and lamellar region into the dorsal cortex of the distal phalanx (Fig 1). Once resistance to drilling decreased, indicating penetration of the dorsal cortex, the drill and needle stylet were removed and a pre-loaded syringe of heparinised saline with 15 cm extension tubing (EZ Connect, Vidacare) was attached to the luer-lock hub of the intra-osseous needle, and the system flushed. Once patent, the Springfusor was attached to the needle via the flow control tubing, and the infusion commenced. The needle was protected with Elastoplast (Beiersdorf) and the Springfusor was secured in a custom-designed neoprene boot (Fig 2). Further local anaesthetic nerve blockade was not required. The horses were fitted with equine nappies (Equisan) to avoid urine or faecal contamination of the intra-osseous needle site.

Hoof wall surface and ambient temperature

A surface thermistor (TinyTag, Gemini) was placed on the midline of the dorsal aspect of the hoof wall, 25 mm below the hairline and 30 mm medial to the midline, on both front feet of each horse (Fig 2). Hoof wall surface temperature (HWST) was measured separately for both front feet every 5 min throughout the experimental period by a data logger connected to the thermistor. Ambient temperature was measured every 5 min by an identical thermistor and data logger placed within 1 m of the horse.

Lamellar histopathology

At the conclusion of the experiment the horses were euthanased and samples of lamellar tissue were immediately obtained from the dorsal mid-section of both front feet. The forefeet were disarticulated at the metacarpo-phalangeal joint, sectioned with a band saw and then dissected to obtain 5 mm x 5 mm samples of lamellar tissue. The tissue was placed in 10% neutral buffered formalin for 24 h before being processed for histology by routine methods. The sections were stained with haematoxylin and eosin (H & E) and periodic acid Schiff (PAS). Samples were randomised then examined via light microscopy (Olympus BX-50) and classified as laminitis positive or negative by one author (C.C.P.) who was blinded to the origin of the samples.

Ethical considerations and statistical analysis

The experimental protocol was approved by the animal ethics committee of The University of Queensland (SVS/108/09/RIRDC) which ensures compliance with the Animal Welfare Act of Queensland (2001) and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th edition 2004). All horses were continuously monitored throughout the experimental period by a registered veterinarian. Repeated measures analysis of variance (ANOVA) was used to measure the change in insulin, glucose, heart and respiratory rates and body temperature for the duration of the experiment. HWST data over time was also analysed with a repeated measures ANOVA, with ambient temperature included as a covariate in the analysis. Overall HWST was compared between the treated and control limbs, or between basal and infusion time-points, using an unpaired and paired t-test respectively. Agreement between the two methods for assessing blood glucose was

analysed using Lin's concordance co-efficient (ρ_c). All data are presented as mean ± se and statistical significance was accepted at $P < 0.05$. Statistical analyses were performed using the R project for statistical computing, version 7.2.7.

Results

Subjects and clinical outcomes

The intraosseous infusion of the ATP-$MgCl_2$ solution was reasonably well tolerated in all horses. Four of the horses showed mild, periodic discomfort associated with the infusion of ATP-$MgCl_2$ as evidenced by occasional pawing with the left forelimb. This discomfort was usually seen after changing the springfusor at the 24 h time-point, but also occurred at a later time-point (40 h) in one horse. Discomfort was treated with a single, intravenous dose of flunixin meglumine (1.1 mg/kg), a non-steroidal anti-inflammatory drug (NSAID) at 24 h in three horses and at 40 h in a fourth horse, and effectively abolished pain within 15 min. None of the horses developed clinical signs of lameness and were sound at the walk at the 24 h (performed before all doses of NSAID were administered) and 48 h time-points.

Appetite, heart rate (37.2 ± 1.97 beats per min), respiratory rate (12.0 ± 1.97 breaths per min) and rectal temperature (37.7 ± 0.22 ºC) did not change throughout the experiment. All of the horses remained clinically normal except for one horse that developed a mild, transient cough. Biochemistry, haematology and urinalysis test results were within accepted diagnostic laboratory reference ranges for the duration of the experiment for all horses (Olson et al., 1993). Serum insulin (15.0 ± 2.89 µIU/ml)

and blood glucose concentrations (5.4 ± 0.22 M) also remained stable, and within previously published reference ranges for basal values (Kronfeld, 2006; Pratt et al., 2005; Treiber et al., 2005), in all horses throughout the experimental period (Fig 3).

Due to the viscous nature and the cool temperature (to minimise ATP degradation) of the ATP-$MgCl_2$ solution, and the cool temperature of the saline solution, the Springfusor system was unable to maintain the desired intra-osseous infusion rate of 0.02 ml/min (optimal solution temperature is 25°C). Instead of 60 mL, the treated limbs received an average of 32.5 ± 2.4 ml while the control limbs received 38.3 ± 2 ml, in the 48 h period. This resulted in an infusion rate of 0.01 mL/min in both the left and right fore. This reduced infusion rate meant that the final dose rate of ATP-$MgCl_2$ was 0.11 mg/kg/min.

Hoof wall surface and ambient temperature

Overall mean (± se) HWST of the foot treated with ATP-$MgCl_2$ (left fore; 29.4 ± 0.25 ºC) was higher ($P < 0.05$) than the foot treated with 0.9% saline (right fore; 27.5 ± 0.38 ºC) during the 48 h treatment period. Basal HWST of the left fore (26.8 ± 0.31 ºC) was similar to the right fore (26.3 ± 0.6 ºC) at the start of the experiment (Table 1). However, the temperature of the treated foot increased ($P < 0.05$) during the first 4 h of the experiment and remained significantly elevated above basal and control foot temperature, at every time-point during the remainder of the experiment (Fig 4). The HWST of the right forefoot (control) during the infusion period did not differ from the basal level at any time-point (Table 1). Ambient temperature remained constant (17.1 ± 0.31 ºC) throughout the experiment for all of the horses.

Lamellar histopathology

All sections from both front feet of all the horses were classified as normal (Fig 5a and b). Lamellar histology of all samples had the appearance of classic lamellar architecture in horses with normal foot health (Obel, 1948). The primary epidermal lamellae were uniform in length and interdigitated with primary dermal lamellae (Fig 5). The secondary epidermal lamellae were uniform in length, symmetrically angled to the primary epidermal lamellar axis and had rounded tips with intact basement membrane (Fig 5). The epidermal basal cell nuclei were ovoid and either apically or centrally located in the cell. Mitotic figures were rare and apoptotic cells infrequent. Dermal vessels were prominent in treated foot sections (Fig 5).

Discussion

Hoof wall surface temperature (HWST) is assumed to be a reliable indicator of underlying perfusion if measured in environmentally-acclimatized horses under conditions of constant, moderate ambient temperature and minimal mechanical activity (Hood et al., 2001), as was done in the current study. A previous study has shown that experimentally-induced hyperinsulinaemic laminitis was associated with increased HWST (de Laat et al., 2010). Elevated HWST has also been recorded in ponies that had previously suffered from naturally-occurring cases of pasture-associated laminitis, when compared to ponies that had never been laminitic (Carter et al., 2009b), although ambient temperature was not controlled for. The HSWT of the treated feet of horses in this current study slightly exceeded the HWST recorded in

laminitic horses subject to prolonged hyperinsulinaemia previously (de Laat et al., 2010). Thus, the failure of the horses to develop clinical and histopathological laminitis within 48 h, while the horses with prolonged hyperinsulinaemia did, suggests that the pathogenesis of insulin-induced laminitis is not entirely due to a dilatory vascular mechanism.

The hyperinsulinaemic, laminitic horses also showed minimal variation in HWST over the 48 h treatment period when compared to the variable HWST of their controls. This HWST variability in the control group was presumed to reflect periods of digital vasodilation and vasoconstriction and may have been important for maintaining normal lamellar health. However, in the current study, the untreated (control) limb showed only marginally more variability in HWST than the treated limb. It is likely that using a contralateral limb from the same horse as a control, limited the ability to compare HWST variability. However, as neither the untreated nor treated limb developed laminitis, the ability to regularly vasoconstrict the digit may not be important in preventing laminitis development in the absence of other factors (systemic inflammation or hyperinsulinaemia).

Mean HWST of the control limbs in the current study also increased marginally above basal levels following commencement of the infusion, although this increase was not significant. This mild elevation in HWST was potentially caused by a local inflammatory response to the intraosseous needle resulting in an increase in blood flow. It is also possible that bandaging the feet may have marginally increased foot temperature through reduced atmospheric heat loss. Regardless, both the treated and control foot were treated in an identical fashion which should account for these

external factors. However, the IOIDP procedure and bandaging may have influenced the decrease in HWST variability seen in the control limbs in the current study when compared to HWST in control horses used in a previous study (de Laat et al., 2010). Another variable of this study was the administration of the NSAID, flunixin, to four of the horses for pain relief. Potentially, flunixin treatment may have altered the degree of digital perfusion by inhibiting inflammation and the attendant vasodilation, hence decreasing HWST in treated horses. However, systemic administration of the drug ensured that both the treated and control limbs were medicated and NSAID treatment did not appear to overtly affect the outcome of significantly increased HWST in the treated limb.

While persistent vasodilation may play a role in the developmental phase of hyperinsulinaemic laminitis (de Laat et al., 2010) the results of the current study would suggest the involvement of other factors, such as insulin or glucose. Insulin is an important vasomediator (Baron, 1994) and its potent vasodilatory effect was probably responsible for the increased HWST seen in horses with insulin-induced laminitis (de Laat et al., 2010). The current study endeavoured to determine whether vasodilation alone (in the absence of hyperinsulinaemia) was laminitogenic. While the current findings do not support this suggestion, they do not rule out the possibility that the vasodilatory effects of insulin are contributory to the development of insulin-induced laminitis.

Intra-osseous infusion of ATP-$MgCl_2$ at the dose rate of 0.11 mg/kg/min significantly raised HWST, and, by implication, increased lamellar perfusion. The slow infusion rate (0.01ml/min) minimised volume overload of the tissues, thus minimising the

degree of foot pain. In four of the horses minor foot discomfort occurred in response to the ATP-$MgCl_2$ infusion. This was assumed to be due to the cool temperature of the solution or an unpleasant, local sensation secondary to the action of ATP-$MgCl_2$. Properties of the solution itself, such as osmolality, may have also been irritant to the tissues. However, lamellar histopathology indicated that no visible detrimental effect on the lamellar tissue occurred. Thus, ATP-$MgCl_2$ would appear to be a safe and effective compound for inducing local digital vasodilation in the horse. The inclusion of a local anaesthetic drug, such as lignocaine, in the infusate may have helped to abolish foot pain and further studies to investigate this are required. Potential clinical applications of the findings of this study may include using ATP-$MgCl_2$ for therapeutic management of ischaemic injuries to tissues within the equine digital environment. For example, if the pathogenesis of laminitis involves lamellar ischaemia-reperfusion injury then a local infusion of ATP-$MgCl_2$ may provide therapeutic benefit. Furthermore, our results suggest that procedures resulting in persistent digital vasodilation for up to 48 h, such as prolonged nerve blocks, are unlikely to induce laminitis *per se*, via a vasodilatory mechanism. On the other hand prolonged low HWST and digital vasoconstriction appeared to confer protection in horses dosed with carbohydrate to induce laminitis (Pollitt and Davies, 1998). The pathogenesis of hyperinsulinaemic and carbohydrate-overload laminitis may be different and vasomodulatory therapies must be considered carefully and tailored individually if genuine benefit is to ensue. Indeed it has been suggested that indiscriminate vasodilation may promote the development of lamellar pathology in the carbohydrate-induction model (Pollitt and Davies, 1998).

Intra-osseous infusion of the distal phalanx is now confirmed as a feasible method for delivering substances to the lamellar circulation over 48 h. The technique does require specialised equipment and skills but these are not beyond the reach of equine veterinary hospitals or research facilities. Catheterisation of the digital artery has been used to deliver medications into the local circulation of the distal limb (Roberts, 1965) and IOIDP is now an additional viable option. While previous studies showed IOIDP effected drug delivery to the lamellar circulation for 6 h (Nourian et al., 2010), further work using other medications and for prolonged periods is required. In the current study no attempt was made to determine the concentration of ATP-$MgCl_2$ in the lamellar tissue, as an increase in HWST was the aim of the study. Before IOIDP can be fully accepted in clinical practice, studies assessing the tissue penetration of infused substances over 48 h need to be performed.

Conclusion

Overall, the findings of the current study indicate that while persistent vasodilation throughout the developmental phase of insulin-induced laminitis may be involved in the pathogenesis of this condition, it is not the sole factor. Persistent digital hyperthermia up to 30°C in the distal forelimb of the horse secondary to a continuous intra-osseous infusion of ATP-$MgCl_2$ at a dose rate of 0.11 mg/kg/min, does not appear to have any detrimental effects on the hoof or the horse, and does not result in laminitis. Prolonged intra-osseous infusion of the distal phalanx may prove to be a safe and effective method of drug delivery for the treatment or prevention of laminitis in the digit.

Conflict of interest statement

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

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Tables

Table 1: Mean ± se HWST was measured in the front feet of Standardbred racehorses (n = 6) treated with an intraosseous infusion into the distal phalanx of both forelimbs. The left fore (LF) was infused with a vasodilator, ATP-$MgCl_2$, to induce digital hyperthermia while the right fore (RF) was infused with saline and acted as a control. HWST data from both forefeet is shown prior to commencement of the experiment (0 h) and for each time-point during the 48 h infusion period. The HWST of the LF was higher (*, $P < 0.05$) than basal and control values throughout the infusion period. The RF HWST did not differ from basal levels during the experiment at any time-point.

Time-point	*HWST °C LF - treated*	*HWST °C RF - control*
0 h	26.9 ± 0.3	26.3 ± 0.6
4 h	29.0 ± 0.15 *	27.0 ± 0.5
8 h	29.1 ± 0.16 *	27.6 ± 0.47
12 h	29.1 ± 0.21 *	27.1 ± 0.56
16 h	29.2 ± 0.4 *	26.9 ± 0.45
20 h	29.4 ± 0.4 *	27.3 ± 0.32
24 h	29.5 ± 0.33 *	27.3 ± 0.41
28 h	30.2 ± 0.28 *	27.8 ± 0.6
32 h	29.5 ± 0.4 *	28.1 ± 0.5
36 h	29.3 ± 0.44 *	27.6 ± 0.61
40 h	29.1 ± 0.27 *	27.5 ± 0.39
44 h	29.1 ± 0.26 *	27.7 ± 0.31
48 h	29.5 ± 0.44 *	27.5 ± 0.37

Figures

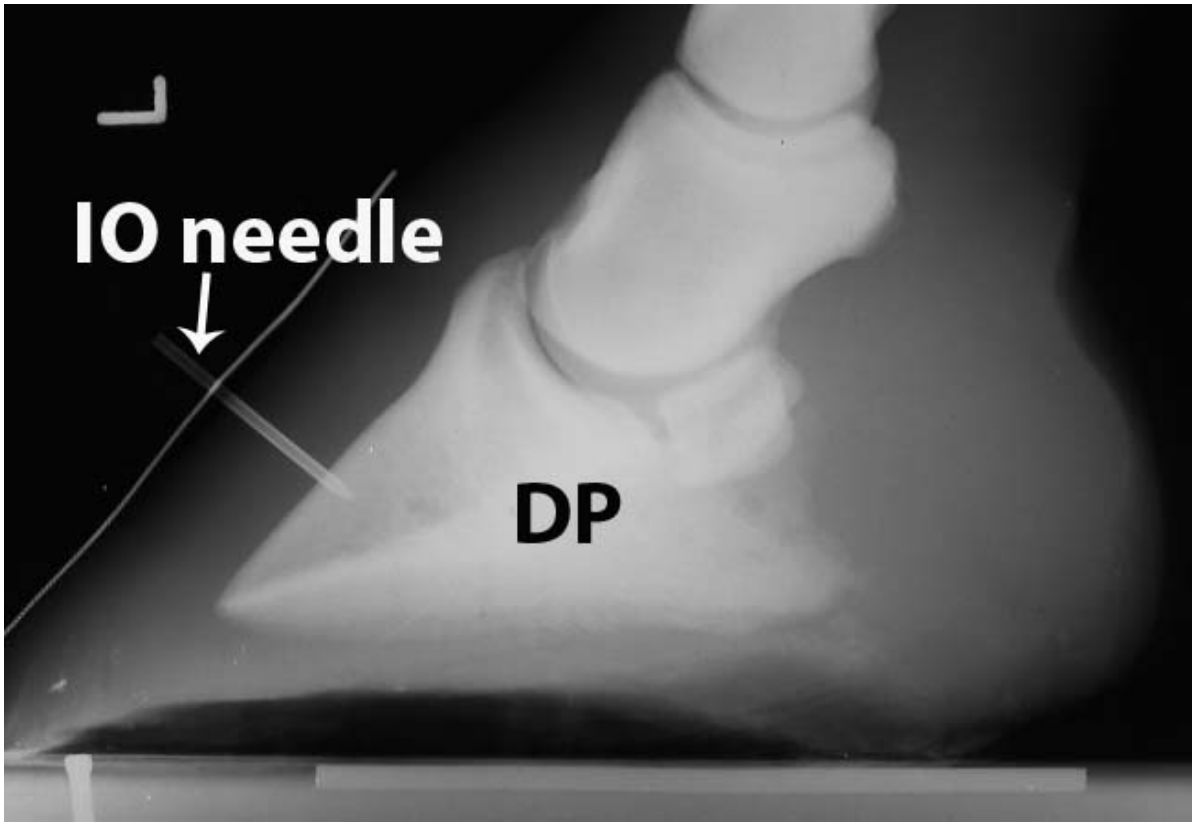

Figure 1: Plain lateral radiograph of the left distal limb of one of the horses after placement of the intra-osseous (IO) needle (EZ IO AD 25 mm, Vidacare). The intra-osseous needle has penetrated the hoof wall and lamellar region and the tip is inserted into the dorsal cortex of the distal phalanx (DP).

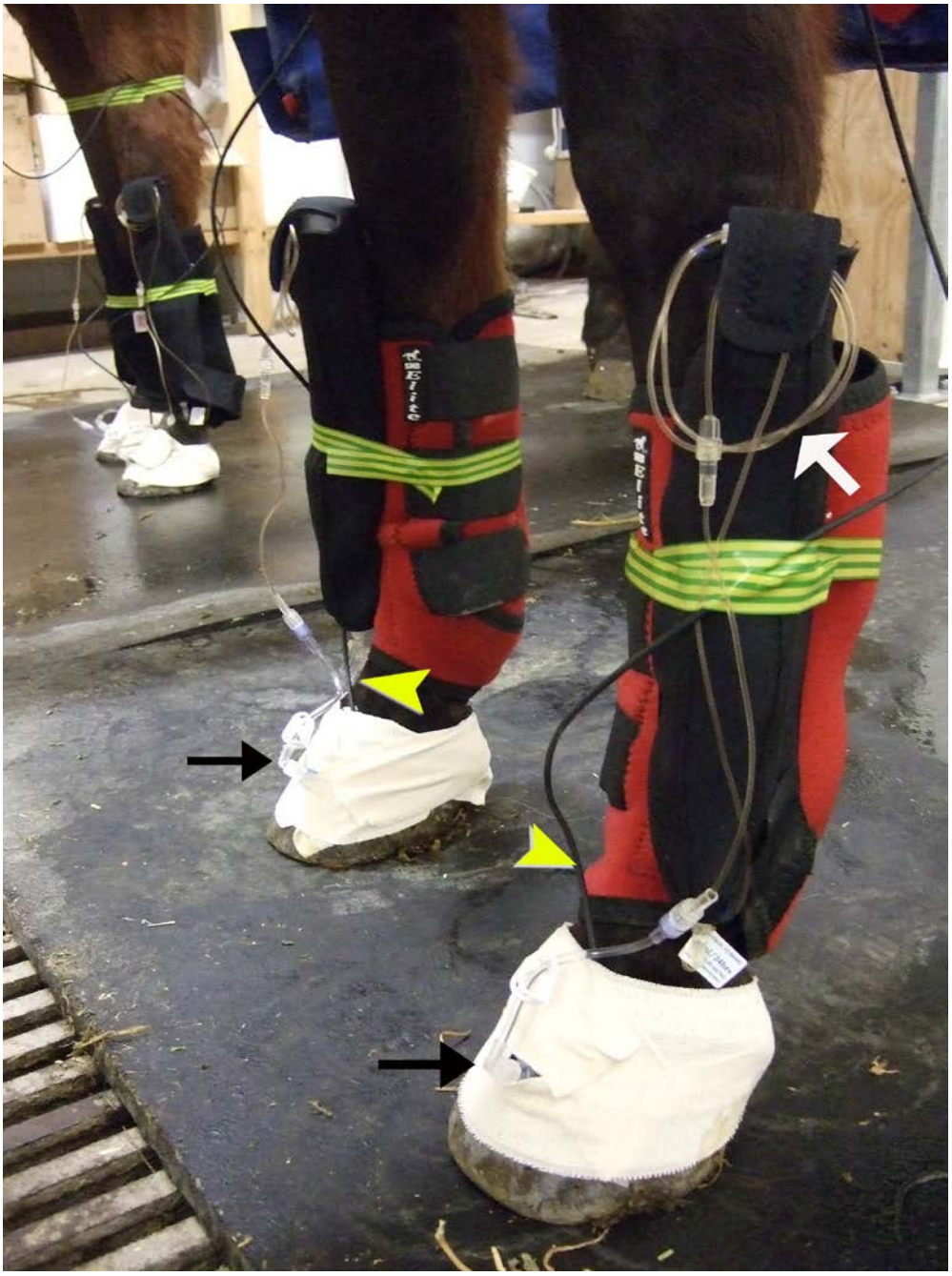

Figure 2: Both forelimbs of all horses were instrumented to allow intra-osseous infusion of a vasodilator, ATP-$MgCl_2$, or 0.9% saline into the distal phalanx. The intra-osseous needle (EZ IO AD 25mm, Vidacare) was inserted through the dorsal surface of the hoof, at right angles to the hoof wall, 25 mm below the hairline and 30 mm lateral to the midline (black arrows). A Springfusor (Go Medical) with pre-calibrated flow control tubing (white arrows) was attached to the luer-lock hub of the intra-osseous needle. The needle was protected with Elastoplast (Beiersdorf) and the Springfusor was secured in a custom-designed neoprene boot. Surface thermistors (TinyTag, Gemini) were placed 25 mm below the hairline and 30 mm medial to the midline on the dorsal surface of both front feet and attached to data loggers worn by the horse that recorded the hoof surface temperature every minute (arrowheads). Blue equine nappies (Equisan) were worn by the horses to prevent waste contamination of the needles.

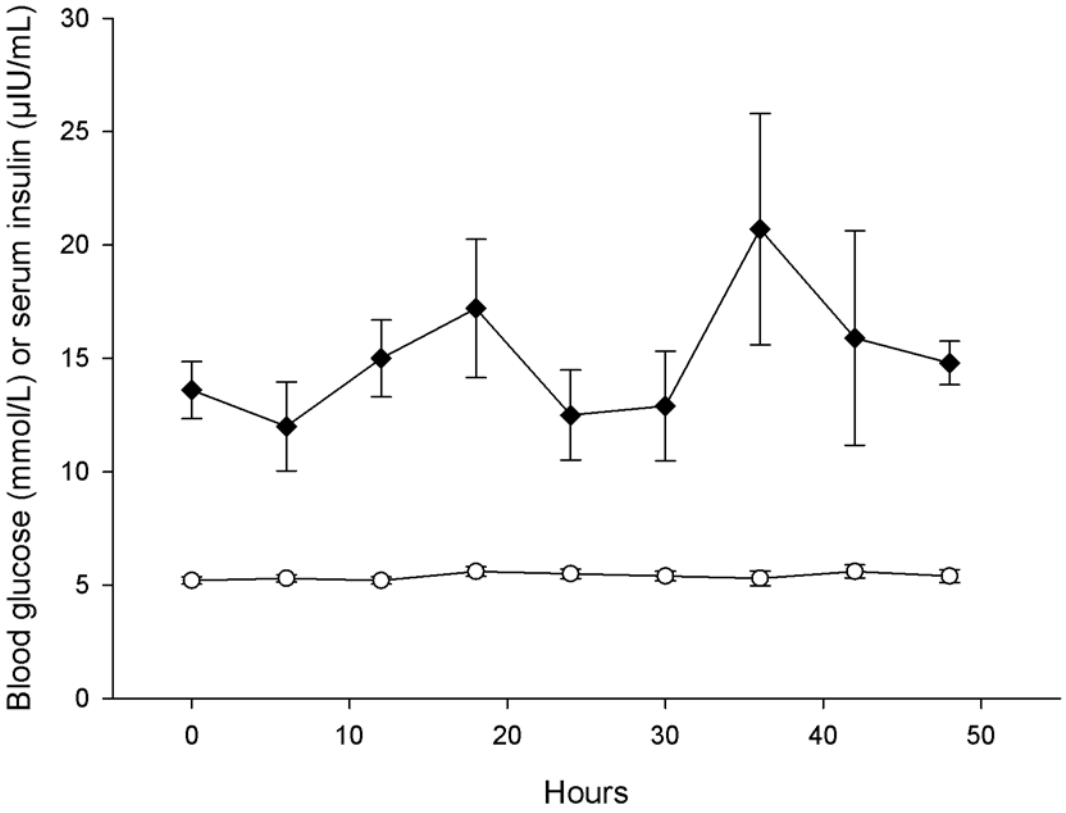

Figure 3: Mean ± se serum insulin (♦) concentration (µIU/mL) and blood glucose (○) concentration (mmol/L) of Standardbred horses (n = 6) treated with an intra-osseous infusion of a vasodilator, ATP-$MgCl_2$ (LF) or normal saline (RF) into the distal phalanx for 48 h. Mean serum insulin and blood glucose concentrations did not change during the experiment.

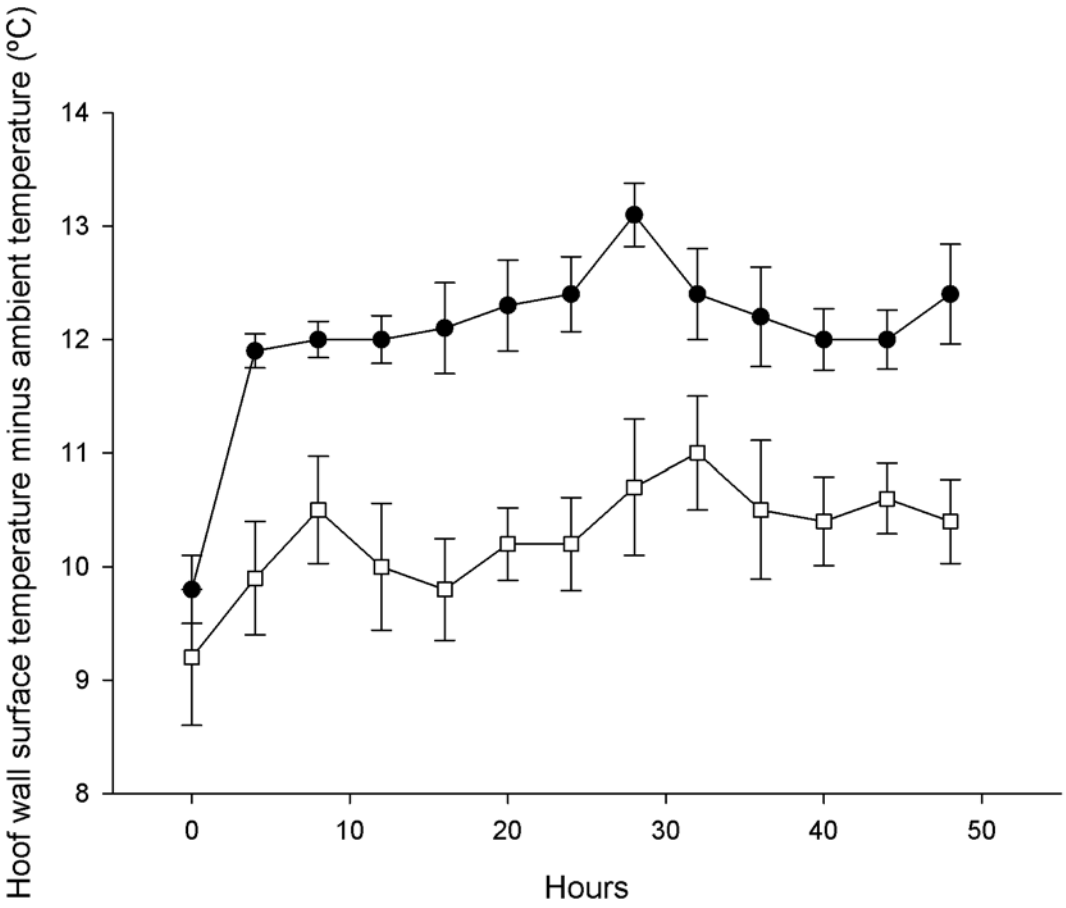

Figure 4: Mean ± se hoof wall surface temperature (°C) was measured in both front feet of Standardbred racehorses (n = 6). The left fore (●) was treated with a vasodilator (ATP-$MgCl_2$) administered by an intra-osseous infusion over 48 h. The right fore (□) was infused intra-osseously with normal saline and acted as a control. Mean (± se) ambient temperature has been subtracted from the mean HWST for each horse. The treated limb was consistently warmer ($P < 0.05$) than the control limb at every time-point after 0 h. Neither foot developed any clinical or histopathological abnormalities.

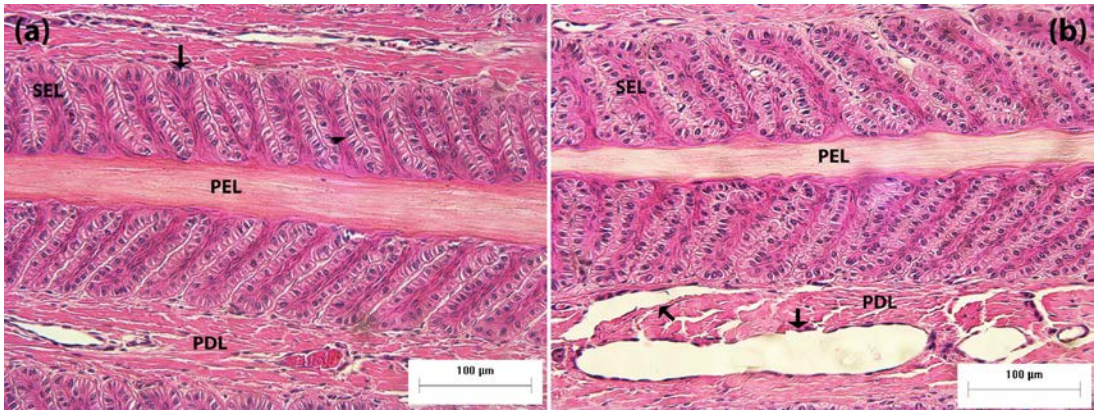

Figure 5: Photomicrographs of hoof lamellar histology from the right (control; a) and left (ATP-$MgCl_2$-treated; b) fore foot of Standardbred horses in transverse section. Hoof lamellar histology was normal in all sections examined. The primary epidermal lamellae (PEL) were uniform in length and interdigitated with primary dermal lamellae (PDL). The secondary epidermal lamellae (SEL) were symmetrically angled to the primary epidermal lamellar axis and had rounded tips with intact basement membrane (black arrow in a). The epidermal basal cell nuclei were ovoid (black arrowhead in a). The microvasculature in the dermis was subjectively noted to be dilated in the sections from the treated limb (black arrows in b) when compared with the untreated limb, in all horses. Stain – haematoxylin and eosin. Bar = 100 µm.