Insulin-Induced Laminitis

— An investigation of the disease mechanism in horses —

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An investigation of the disease mechanism in horses

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Foreword

Equine laminitis is common and costly. This unique disease has a multitude of causes including overconsumption of lush pasture or grain, hormonal disturbance or sepsis. Despite the diverse array of inciting factors, the clinical end-point of laminitis onset is the same. Failure of the lamellar structures in the horse’s foot results in crippling lameness and in some cases, death. The incidence of laminitis associated with hormonal dysfunction (termed endocrinopathic laminitis) is on the rise. Obesity, sedentary lifestyles and improved pasture species are all contributing to rising serum insulin concentrations in affected horses and ponies, and as a result, more laminitis. The aims of this research project were to investigate the mechanism of laminitis occurring secondary to elevated serum insulin concentrations in insulin-sensitive horses.

This project has demonstrated that insulin-sensitive horses, in addition to ponies, develop laminitis experimentally if high concentrations of insulin circulate in their bloodstream for 48 hours. This discovery shifts the focus of endocrinopathic laminitis pathogenesis away from insulin resistance and hypercortisolaemia towards insulin itself having a direct laminitogenic affect. The research has discounted pathogenic roles for digital vasodilation, lamellar metalloproteinase activity and inflammation in the development of this form of laminitis. The major outcome from this project is that hyperinsulinaemia triggers uncontrolled lamellar cell proliferation and attenuation. This process weakens the lamellar suspensory apparatus of the distal phalanx until the clinical signs of foot pain are manifest. This new, alternative theory for the pathogenesis of insulin-induced laminitis maintains Australia’s leadership in equine laminitis research.

Horse owners, veterinarians and scientists armed with this new knowledge now have better strategies to identify horses at risk of laminitis, to prevent laminitis from occurring, and to treat the condition early in its development. While this new knowledge provides an immediate avenue for better prevention and management of laminitis, the development of new treatments will require further understanding of the laminitogenic potential insulin.

The investment by RIRDC in this project will enable veterinarians to diagnose laminitis earlier than ever before thus potentially preventing an estimated 6% of horses and ponies developing the disease. The financial burden of treating laminitis, often for very long periods, will thus be significantly reduced.

This report is an addition to RIRDC’s diverse range of over 2200 research publications and it forms part of our Horse R&D program, which aims to assist in developing the Australian horse industry and enhancing its export potential.

Most of RIRDC’s publications are available for viewing, free downloading or purchasing online at www.rirdc.gov.au. Purchases can also be made by phoning 1300 634 313.

Craig Burns
Managing Director
Rural Industries Research and Development Corporation
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Abbreviations

Please see Glossary for the list of abbreviations used in this report.
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Executive Summary

What the report is about

Equine laminitis is a debilitating condition affecting the sensitive lamellar structures between the inner hoof wall and distal phalanx (pedal bone) in the horses’ foot. It is a common cause of lameness in horses and ponies and often leads to death in affected animals (USDA, 2000). Hormonal disturbances, such as hyperinsulinaemia, are frequently associated with laminitis and this form of the disease is known as endocrinopathic laminitis (de Laat et al., 2010b). Despite considerable research, the pathogenesis of laminitis is uncertain, and several theories have been suggested. An improved understanding of the mechanism underlying endocrinopathic laminitis will allow the development of targeted treatment strategies and the ability to prevent and more successfully manage the disease. As a result, the costs to the horse industry associated with dealing with the disease will be reduced. This study has investigated several theories on the pathogenesis of laminitis occurring secondary to hyperinsulinaemia, and has significantly improved our understanding of the condition.

Who is the report targeted at?

All facets of the horse industry are likely to be aware of, and potentially deal with, laminitis at some point. The data contained in this report will primarily benefit horse owners, veterinarians, farriers and research scientists. Horse owners can focus on the recommendations provided in this report to ensure the best care and health for their animal. Veterinarians and farriers will be armed with an improved understanding of the condition and made aware of the best ways to ensure accurate assessment and management of their patients. By working together, research scientists can continue to progress our understanding of the condition and strive toward developing specific therapeutic options with the aim of eradicating this insidious disease.

Where are the relevant industries located in Australia?

The horse breeding and sporting industries, equine veterinary industry and farriery associations across all regions of Australia can potentially benefit from the data contained in this report.

Background

The cost of death and disability due to laminitis represents a significant loss to the Australian horse industry. This project builds on the work of a previous RIRDC project, Investigating the role of impaired glucose uptake in laminitis, that led to the ground-breaking discovery that high blood concentrations of insulin precipitated laminitis (Sillence et al., 2007, RIRDC publication no 07-158). Previously, hyperinsulinaemia had been regarded as a sign of glucose intolerance (i.e. intolerance to diets high in soluble carbohydrates) and as a marker for ponies and horses at risk of laminitis, but not as a direct cause. However, ponies are inherently more insulin resistant than horses (Jeffcott et al., 1986) and it was not known whether this new model of laminitis induction would also cause laminitis in the more insulin-sensitive horse.

Conditions associated with endocrinopathic laminitis include equine Cushing’s disease and equine metabolic syndrome, both of which occur in horses. These conditions are increasingly problematic for the horse breeding industry as hyperinsulinaemia can affect fertility, as well as causing laminitis (Vick et al., 2006). Equine Cushing’s disease and equine metabolic syndrome are both characterised by disturbed insulin and glucose metabolism and, more importantly, the development of insulin resistance. Insulin resistance is defined as a failure of the tissues to respond appropriately to insulin and in the horse it is manifest as hyperinsulinaemia, but not hyperglycaemia (Frank, 2006, 2011).

Laminitis is the most critical consequence of insulin resistance in horses. However, the way in which hyperinsulinaemia precipitates lamellar failure is currently poorly understood. There are four major
theories on the mechanism of endocrinopathic laminitis and these have been formulated based on outcomes of research into other forms of laminitis (i.e. inflammatory and alimentary) as well as extrapolations from research on human insulin resistance and metabolic syndromes. These theories are: that glucose uptake into, and metabolism by, lamellar tissue is altered; unrestrained degradative enzyme activity damages lamellar tissue; vascular dysfunction adversely affects the lamellar microcirculation; and that pro-inflammatory cytokines cause tissue damage (Bailey et al., 2004; de Laat et al., 2010b).

Determining whether an infusion of insulin induces laminitis in insulin-sensitive horses was an important first step in investigating the mechanism of hyperinsulinaemic laminitis. The confirmation of the experimental model in horses greatly facilitated our ability to study the disease mechanism more effectively. In particular, the crucial developmental phase of the disease that precedes the onset of clinical lameness is when pathology commences. A thorough investigation of this phase is only possible using a controlled experimental model and is crucial to unlocking the inciting factors of lamellar failure.

Aims/objectives

- investigate the mechanism of insulin-induced laminitis leading to the development of new treatment and improved prevention strategies;
- characterise the lesion caused by hyperinsulinaemia both before and after laminitis onset, and compare this to laminitis triggered by other factors;
- identify the endogenous pancreatic response to glucose overload in insulin-sensitive horses and determine the impact of glucose overload on the lamellae;
- refine laminitis induction techniques to improve animal welfare while decreasing time and labour costs, thus accelerating the rate of future progress;
- develop improved treatment/prevention strategies for laminitis;
- maintain Australia’s leadership in equine laminitis research.

Methods used

The methodology used in this project can be principally divided into two categories: horse studies that model the disease in the live animal and laboratory analyses that further investigate the impact of hyperinsulinaemia in the lamellae by examining lamellar pathology and determining changes in lamellar proteins and gene expression.

Results/key findings

Hyperinsulinaemia induced laminitis in insulin-sensitive Standardbred racehorses treated with a prolonged-euglycaemic, hyperinsulinaemic clamp (p-EHC) (n = 4), thus confirming the characteristics of the p-EHC model in horses. In-depth histological evaluation of the lamellar pathology induced by the insulin infusion found that structural changes to the secondary epidermal lamellae (SEls), such as lamellar lengthening and narrowing, commence early. Nuclear changes, such as rounding of basal cell nuclei, also commence early. Mitosis gradually increased as the lesions progressed and potentially contributed to increases in lamellar length.

Damage to the lamellar basement membrane (BM) was not as pronounced in insulin-induced laminitis as alimentary forms of the disease, but was present in all horses that developed laminitis secondary to hyperinsulinaemia. Loss of structurally important hemidesmosomes and widening of the BM zone was seen in all insulin-treated horses and this may contribute to weakening of the lamellar interface.

Insulin-induced laminitis produced a less inflammatory lesion than other experimental models of the disease (de Laat et al., 2011c; Faleiros et al., 2009) when assessed with calprotectin immunohistochemistry. The objectives of 1) describing lamellar lesions before and during disease onset and comparing them to laminitis triggered by other factors and 2) determining the role of degradative enzymes and inflammation in lamellar pathology, were met.
Persistently elevated hoof wall surface temperature (HWST) occurred in the feet of all horses receiving an insulin infusion and this was in contrast to the control horses that had variable HWST over the infusion period. Thus, a vascular component to the disease was identified. Further investigation of the role of the persistent digital vasodilation using intra-osseous infusion of the distal phalanx (IOIDP) demonstrated that insulin-induced laminitis does not occur purely as a result of a vascular mechanism.

Continuous hyperglycaemia over 48 hours induced endogenous hyperinsulinaemia (~200 micro international unit [µIU]/mL) and subclinical lamellar pathology in treated horses. Hyperinsulinaemia resulted in down-regulation of both the insulin and insulin-like growth factor-1 (IGF-1) receptors. Thus, the IGF system is implicitly involved in the pathogenesis of endocrinopathic laminitis and future research needs to be directed at examining this pathway.

Overall, significant progress in our understanding of hyperinsulinaemic laminitis has been achieved as a result of this project and the objectives have been met. Managing persistent hyperinsulinaemia and reducing it below 200 µIU/0/mL is paramount in managing and preventing episodes of laminitis. Further study of the IGF pathway in laminitis pathophysiology may lead to novel treatment options, such as the use of IGF-1 receptor blockers.

**Implications for relevant stakeholders**

Hyperinsulinaemia is the key pathogenic factor in endocrinopathic laminitis and horse owners and veterinarians now have a target serum insulin concentration which they can use as a benchmark for the management of insulin-resistance and prediction of likely subclinical lamellar pathology (~200 µIU/mL). The reduction of serum insulin concentrations will reduce the incidence of endocrinopathic laminitis in the equine population.

Losses to the horse industry from endocrinopathic laminitis should be lessened if the findings of this project are used to better manage the condition. Fewer laminitis related deaths will lower the emotional impact of the condition and improved management and prevention strategies for the disease should reduce the financial impact on the horse industry.

Future development of targeted treatment protocols for the condition is required. Results from this project indicate that research funding for endocrinopathic laminitis should be directed at an investigation of how the IGF system promotes lamellar failure. Investigations of this nature may lead to therapeutic options for laminitis via IGF-1 receptor blockers.

**Recommendations**

Horse owners need to:

- reduce serum insulin concentrations well below 200 µIU/ml in horses and ponies with hyperinsulinaemia using a modified diet and exercise regimes
- maintain a low bodyweight for their horses and ensure body condition scores are in the optimal range
- monitor pasture species and seasonal variation of pasture growth and to avoid excessive consumption of non-structural carbohydrate-rich pasture.
Veterinarians need to:

- determine serum insulin concentration as a priority in horses and ponies that are overweight or have a phenotype consistent with equine metabolic syndrome (Frank et al., 2010) or are laminitis prone
- ensure serum insulin concentrations are assayed according to a validated protocol.

Research scientists need further funding to:

- further develop a single limb induction technique for the study of laminitis
- investigate the ability of a local IGF-1 infusion to promote the development of lamellar pathology
- investigate the potential of IGF-1 receptor blockers as a therapy for endocrinopathic laminitis.
Introduction

Laminitis is a common cause of lameness in horses and ponies (USDA, 2000) and frequently results in permanent loss of function for an affected animal. Despite continued research into this complex condition the mechanisms leading to lamellar failure have not been completely described and effective treatment strategies do not exist. The costs associated with managing laminitis are high, and have a financial and emotional impact on the horse owning community. Laminitis can be caused by a number of factors but is commonly affiliated with overconsumption of lush pasture or grain, hormonal disturbance or sequelae to sepsis (Heymering, 2010).

Laminitis associated with hormonal disturbances is termed endocrinopathic laminitis (Johnson et al., 2004). Failure of the normal regulation of insulin, glucose and cortisol metabolism is the key factor that predisposes a horse or pony to endocrinopathic laminitis (Johnson et al., 2004; Schott, 2002). Endocrine conditions associated with an increased tendency to develop laminitis include pituitary pars intermedia dysfunction (equine Cushing’s syndrome) and equine metabolic syndrome. Both of these syndromes are characterised by an increase in tissue resistance to insulin and hence, hyperinsulinaemia. Insulin resistance in horses has been defined by Frank (2006) as the failure of the tissues to respond appropriately to insulin. The reduced capacity for glucose uptake by target tissues, such as skeletal muscle, stimulates the pancreas to release more insulin. A direct link between hyperinsulinaemia and laminitis has been documented in naturally-occurring forms of the disease (McGowan et al., 2004; Treiber et al., 2006; Walsh et al., 2009).

Insulin is a hormone synthesised by the beta cells of the pancreas. It is secreted into the bloodstream in response to glucose, proteins and parasympathetic nervous system stimuli. Insulin promotes rapid uptake, storage and use of glucose by the body, in particular, the skeletal muscles, adipose tissue and the liver (Magkos et al., 2010). Insulin is also involved in protein metabolism, fat metabolism, modulation of blood flow (primarily vasodilation) and DNA replication. Insulin mediates its effects by binding with a tyrosine kinase receptor, known as the insulin receptor. Receptor binding by insulin then activates numerous intra-cellular signalling pathways that trigger insulin’s actions.

Research funded by RIRDC has found that exogenously administered insulin induces laminitis in insulin-sensitive ponies within 72 h. The level of hyperinsulinaemia attained in the study was high, with serum insulin concentrations in excess of 1000 µIU/mL. Serum insulin concentrations of this magnitude are infrequently detected in field cases of endocrinopathic laminitis, which suggested that the model was intensifying the developmental phase of laminitis. However, all of the ponies developed Obel grade 2 laminitis (Obel, 1948) in this short timeframe, which confirmed insulin as a cause of laminitis. Previously developed research models of laminitis have focused on alimentary and inflammatory forms of the condition (Galey et al., 1991; Garner et al., 1975). Now, an experimental model using hyperinsulinaemia exists, and will facilitate research specifically focused on the endocrinopathic form of laminitis.

Laminitis researchers have worked hard to determine the pathophysiological events that lead to lamellar failure using the aforementioned experimental models (Bailey et al., 2004). Four principal theories regarding the disease mechanism exist:

- altered glucose delivery to, and uptake by, the hoof
- enhanced degradative enzyme activity
- altered blood flow to the hoof and
- pro-inflammatory cytokine release in the hoof.
Previously, it was considered that a failure of glucose uptake by lamellar tissue would result in weakening and failure of the structure (Pass et al., 1998). However, the finding that glucose uptake in the hoof is largely facilitated by GLUT-1 glucose transporters and is therefore insulin-independent, made this supposition less likely (Asplin et al., 2011). Laminitis occurring in insulin-resistant horses and ponies is often accompanied by the intake of large amount of non-structural carbohydrate rich pasture. Further, the insulin model of laminitis necessitates the administration of large amounts of glucose. It is feasible that the quantity of glucose consumed by, or infused into, these animals contributes to lamellar failure. Horses rarely become hyperglycaemic, so either the relentless insulin production by the pancreas in response to the glucose contributes to lamellar failure, or the glucose itself may be toxic to the tissues. Human diabetic patients experience glucose toxicity which increases the morbidity and mortality associated with the disease, and this is largely associated with basement membrane (BM) pathology (Brownlee, 1992; Dronavalli et al., 2008). It is possible that similar pathophysiology contributes to lamellar failure. This theory requires investigation.

Basement membrane breakdown is a feature of equine laminitis (Pollitt, 1996). Studies have further defined the BM damage as loss of essential structural components of the extra-cellular matrix (ECM) including laminins, collagen and hemidesmosomes (French and Pollitt, 2004a, b; Visser, 2008). This breakdown of the lamellar BM is thought to be largely mediated by local degradative enzymes, known as metalloproteinases (Coyne et al., 2009; Johnson et al., 1998; Mungall et al., 1998; Pollitt, 2004). However, the capacity for degradative enzymes to be responsible for BM breakdown in endocrinopathic laminitis has not been investigated, and preliminary studies have shown that BM dysadhesion may not be a ubiquitous feature of the disease (Asplin et al., 2010; Nourian et al., 2009). BM proteolysis needs to be investigated in horses with hyperinsulinaemia.

Acute laminitis is associated with bounding digital pulses (Asplin et al., 2007) and this has led to considerable conjecture on the potential for laminitis to occur secondary to vascular dysfunction. Potential mechanisms for blood flow related lamellar damage include vasodilation (Pollitt and Davies, 1998), ischaemia due to blood bypassing the lamellar capillary bed (Adair et al., 2000) and vasoconstriction leading to ischaemia (Hood et al., 2001). The developmental stages of insulin-induced laminitis that precede the bounding digital pulses of the acute phase can now be examined in a more controlled manner using the insulin-induction model. An investigation of the changes in blood flow to the foot during disease development will form part of this project.

Lastly, inappropriate release of inflammatory mediators in lamellar tissue has been postulated as a mechanism for laminitis. However, work has primarily been conducted in inflammatory and alimentary models of the disease (Belknap et al., 2007; Fontaine et al., 2001; Leise et al., 2011; Waguespack et al., 2004). Obesity is now recognised as a condition of chronic inflammation with adipose tissue known to actually function as an endocrine organ (Tilg and Moschen, 2006). Ponies and horses with equine metabolic syndrome are frequently obese with an abnormal deposition of fat (Frank et al., 2010). This obesity and enhanced pro-inflammatory cytokine release may contribute to laminitis pathophysiology in laminitis-prone, insulin-resistant animals. A preliminary investigation of the role of inflammation in the endocrinopathic form of laminitis will form part of this research project.

Endocrinopathic laminitis is common, but poorly understood. Previous barriers to research progress can now be overcome with the use of the insulin-induction model of laminitis. Many theories on laminitis pathogenesis that have been described in alimentary and inflammatory disease models are potentially also involved in the endocrinopathic form. Enhanced comprehension of the mechanisms underlying the disease will promote improved management and treatment strategies for laminitis.
Objectives

The original aims of the project were to:

- investigate the mechanism of insulin-induced laminitis leading to the development of new treatment and improved prevention strategies by -
  - determining the role of increased (or decreased) blood flow of the hoof before, during and after laminitis;
  - determining the role of inflammation and degradative enzymes in causing laminitis;
  - investigating the role of IGF-1 in laminitis onset and its potential as a treatment strategy;
- characterise the lesion caused by hyperinsulinaemia before, during and after laminitis onset, and compare this to laminitis triggered by other factors;
- provide insight into corticosteroid-induced laminitis;
- refine the induction technique to improve animal welfare while decreasing time and labour costs, thus accelerating the rate of future progress;
- develop improved treatment/prevention strategies for laminitis;
- maintain Australia’s leadership in equine laminitis research.

Shortly after commencement of the project, another research group published data on the impact of dexamethasone, a corticosteroid drug, on the development of laminitis associated with hyperinsulinaemia (Bailey et al., 2007). Rather than wasting valuable resources by replicating work that had already been published we opted to remove the investigation of corticosteroid-induced laminitis from the project. Instead it was replaced with a new study that would further investigate the role of glucose toxicity in endocrinopathic laminitis. A new objective was added to the original list of aims for the project:

- identify the endogenous pancreatic response to glucose overload in insulin-sensitive horses and determine the impact of glucose overload on the lamellae.
Methodology

The methodology used in this project can be principally divided into two categories: horse studies that model the disease in the live animal and laboratory analyses that further investigate the impact of hyperinsulinaemia in the lamellae by examining lamellar pathology and determining changes in lamellar proteins and gene expression.

Horse studies

Prolonged-euglycaemic, hyperinsulinaemic clamp (p-EHC)

Endocrinopathic laminitis is a difficult disease to study effectively in the field with naturally-occurring cases often precipitated at different times and by different events in prone individuals. Furthermore, investigation of the lamellar pathology is not possible in a live animal due to the inaccessible location of the lamellae between the inner hoof wall and the distal phalanx (pedal bone). The use of a prolonged-euglycaemic, hyperinsulinaemic clamp (p-EHC) to successfully induce laminitis in ponies has confirmed insulin as a primary cause of laminitis in ponies (Asplin et al., 2007). This discovery will enable investigation of this unique disease under controlled laboratory conditions.

The technique uses a constant rate insulin infusion to cause prolonged hyperinsulinaemia in horses. Hypoglycaemia is avoided with a concurrent infusion of glucose given at a variable rate according to regular blood glucose analyses, to maintain normal blood glucose concentrations. The EHC technique is used as a diagnostic test for insulin sensitivity in horses and ponies. However, when used as an experimental model for laminitis induction the EHC is prolonged until the onset of Obel grade two laminitis (Obel, 1948). This occurred within 72 h in ponies. Further details of the clamp are provided in chapter one and have been published (de Laat et al., 2010a).

In the current project, the p-EHC technique was used in horses for the first time in order to study its effects in this more insulin-sensitive group (Jeffcott et al., 1986). In addition to determining if the p-EHC induces laminitis in horses, the technique can be used to study the developmental stage of the disease, which occurs prior to the onset of clinical signs. Originally, temporal investigation of the developmental stages of the disease was to be performed using serial lamellar biopsies. However, inconsistencies with this technique led to an adapted p-EHC being used instead. The p-EHC model of laminitis induction was repeated and ceased at sequential time-points during the developmental, pre-clinical phase of the disease (6 h, 12 h and 24 h) and lamellar tissue harvested. This approach allowed in-depth analysis of the lamellar pathology that precedes the onset of clinical disease and greatly enhanced our understanding of disease progression and pathophysiology.

The role of the vasculature during the developmental and acute stages of the p-EHC was assessed using non-invasive surface thermistor probes taped to the dorsal surface of the hoof wall of all four feet of all horses. The thermistors were attached to a data logger that recorded the temperature of the hoof wall every minute of the infusion period. The HWST of treated horses was compared to control horses.

Intra-osseous infusion of the distal phalanx (IOIDP)

The use of horses to study equine diseases is necessary when studying complicated conditions such as laminitis. However, ways to reduce the number of horses used in these experiments, and lower the impact on the animals, are constantly being sought. By developing a new model of laminitis induction whereby laminitis could be induced in a single limb by a local infusion, fewer horses would be required and the impact on the horses used would be reduced. Each horse would have its own internal control, the contralateral forelimb, thereby negating the need for a separate control group. A technique
designed to infuse the lamellar microcirculation via intra-osseous access to the distal phalanx was developed to complement the p-EHC and had already been shown to be effective for up to 6 h in horses (Nourian et al., 2010). The current project extended this technique to persist for 48 h therefore matching the time to the onset of the acute phase on insulin-induced laminitis in horses.

IOIDP offers a simple and stable way of perfusing the lamellae via the terminal digital circulation, without the need for a tourniquet, which is required during catheterisation and infusion of digital arteries. The intra-osseous needles were inserted into the distal phalanx using a power driven system and infusions administered with a syringe pump and flow control tubing (Springfusor, GoMedical). The use of a single limb infusion technique allowed the infusion of insulin and a vasodilator, ATP-MgCl₂ into the lamellar microcirculation in much smaller quantities than is used during a whole-body technique. In the future, there is also potential for IOIDP to be applied to investigations that aim to test new experimental compounds that are either expensive or available in limited quantities, such as IGF-1. The exact details of the placement and maintenance of the IOIDP needles is detailed in chapter four.

Hyperglycaemic clamp (HC)

The hyperglycaemic clamp (HC) is a diagnostic technique used in the assessment of insulin sensitivity, much like the EHC. The technique induces hyperglycaemia (6.9 mM above normal) with a glucose infusion that is constantly altered to ensure a stable plateau of blood glucose concentration. This in turn enables calculation of the amount of glucose metabolised by the tissues in response to the endogenous insulin produced by the pancreas and hence, insulin sensitivity (DeFronzo et al., 1979). The HC is used less frequently than the EHC as it is reported to be a less reliable measure of insulin sensitivity (Rijnen and van der Kolk, 2003).

An approximated HC was used in this project to investigate the role of glucose in the pathogenesis of the disease. The technique was adapted to suit the objectives of the project and uses a constant rate glucose infusion rather than a variable rate. The HC technique was used instead of a p-EHC as it permitted an examination of the effects of endogenous (equine) insulin rather than exogenous (human) insulin. Further details of the HC for use in studying laminitis pathophysiology are provided in chapter five and have been published (de Laat et al., 2011b).

Laboratory work

Radioimmunoassay

The Coat-a-count solid-phase radioimmunoassay (Siemens) has been proved to be the most accurate method for analysing serum insulin concentration in horses (McGowan et al., 2008) and was thus selected for use in this project. The principal of the assay is that radio-labelled iodine (¹²⁵I) competes with the insulin in the horse’s serum for sites on an insulin-specific antibody. Decanting the supernatant removes the unbound insulin and the radio-labelled antibody remains fixed to the tube wall. The tube is then counted in a gamma counter and the insulin concentration calculated against a standard curve.

Horses treated with a p-EHC have a serum insulin concentration in excess of 1000 µIU/mL. Thus, the serum samples from these horses required dilution to enable accurate results to be obtained. Unfortunately, the insulin-free diluent provided with the radioimmunoassay kit was insufficient for the number of samples to be analysed. Instead, two diluents were validated for use with the assay; phosphate buffered saline and insulin-free serum. The charcoal-stripped insulin-free serum was a superior diluent to phosphate buffered saline and was therefore used with each assay during this project. All assays were performed by the same investigator using the same equipment to ensure consistent results across all experiments. Sample dilutions were determined separately for each assay and varied between 1:10 and 1:5 (sample serum: insulin-free serum).
A standard curve of insulin concentrations was prepared using the calibrators provided with the assay kit as follows: A; 0 µIU/mL, B; 5 µIU/mL, C; 15 µIU/mL, D; 50 µIU/mL, E; 100 µIU/mL, F; 200 µIU/mL and G 350 µIU/mL. Appropriately diluted serum samples from each horse were then pipetted into the insulin antibody-coated tubes and 1.0 mL of 125I-insulin was added prior to vortexing (5 s). The tubes were then incubated for 18 h at room temperature prior to decanting, air drying and counting in a gamma counter for 1 min. The assay was validated for each batch of samples and consistently had inter-assay and intra-assay coefficients of variation below 5% which was deemed acceptable. The results were analysed with AssayZap 3.1 software (Biosoft).

**Pathology**

**Histology**

Lamellar sections from each horse were examined using routine histological stains to identify the presence or absence of lamellar pathology. Haematoxylin and eosin (H&E) was useful for most routine histological examinations, but the periodic acid Schiff (PAS) method was used to examine the lamellar basement membrane (BM). Detailed of sample preparation are included in the individual chapters. The sections were examined with light microscopy (Olympus BX-50) by the authors. Measurements of lamellar morphology were made on each section using image analysis software (ImagePro, Cybernetics). The morphometry measurement technique has been fully described and validated in de Laat et al. (2011c).

**Immunohistochemistry**

The technique of immunohistochemistry uses antigen–antibody binding to visualise the tissue distribution of an antigen and has proved useful for examining specific pathological events occurring in the lamellae during laminitis. The technique allows deeper investigation of pathological processes that are identified during routine histological examination. Antigens are unmasked using proteolytic enzymes before incubation of the tissue with the primary antibody. Incubation with a secondary antibody (directed to the primary antibody) and an enzyme complex follows. The tissue is then stained with a chromagen that is activated by the enzyme complex, resulting in a colour reaction at the antigen site to illustrate the tissue location of the antigen of interest.

In this study, immunohistochemistry was used to illustrate the distribution of infiltrating leucocytes in lamellar tissue during the developmental and acute stages of the disease. The EnVision+ System-HRP system from Dako was used on paraffin-embedded sections from all four feet of the horses examined during this project. Full details of the technique are included in chapter two.

**Transmission electron microscopy**

Ultrastructural analysis of lamellar tissue with transmission electron microscopy (TEM) allows the analysis of the extremely fine intra-cellular detail of diseased and normal lamellar epidermal basal cells (EBC). This allows analysis of cellular pathology that is not visible with normal light microscopy. Blocks of lamellar tissue were embedded in resin to allow ultra-thin sectioning of the tissue. The ultra-thin sections were examined with a trans-electron microscope (Jeol 1010, Tokyo) which used a collimated beam of electrons to illustrate the differing densities of the tissue being examined. TEM analysis of the alimentary model of laminitis induction has greatly enhanced our comprehension of disease pathophysiology at the cellular levels. Analysis of lamellar tissue from horses with insulin-induced laminitis will no doubt allow similar advances to be made in this unique form of the disease. The precise methodological details of the TEM used in this project are included in chapter three.
Zymography

Gelatin zymography has been used extensively to study the activity of a class of metalloproteinases known as gelatinases that participate in degradation of the BM (Troeberg and Nagase, 2004). Zymography uses co-polymerisation of a gel with gelatin to examine the presence or absence of gelatinases in a tissue. Protein from a tissue of interest are loaded into and dragged through the gel with electrophoresis. Following electrophoresis, the gel is stained and the enzymes in the sample can be identified after digestion of the protein substrate in the gel against known standards run with the samples. The breakdown of the lamellar BM in laminitis has been attributed to zealous digestion with metalloproteinases. The activity of metalloproteinases has not been investigated in insulin-induced laminitis. Gelatin zymography provides a way in which the presence of matrix metalloproteinases (MMP) 2 and 9 can be quantified in lamellar tissue in both their active and inactive forms. Specifics of the zymography technique are provided in chapter three and have been published (de Laat et al., 2011a).

qRT-PCR

Quantitative, real time-polymerase chain reaction (qRT-PCR) is a commonly used technique in the field of molecular biology. It allows examination of changes in the gene expression of any gene of interest during disease states with a high degree of accuracy. qRT-PCR has the advantage of being efficient, able to detect only small changes in gene expression and the ability to confirm the product being examined. Amplification of a fragment of DNA of interest was used to determine whether certain genes were up-regulated during insulin-induced laminitis, in comparison to more stable, housekeeping gene that did not change during acute laminitis. Quantitative PCR was used to examine the gene expression of metalloproteinases (MMP-2, MT1-MMP, MMP-9, ADAMTS-4), a metalloproteinase inhibitor (TIMP-3) as well as the insulin receptor and the IGF-1 receptor (IGF-1R) in laminitis and control horses. Background-subtracted, qPCR fluorescence kinetics has been used in this project and the methodology employed is outlined in both chapters three and six.
1. Prolonged-euglycaemic, hyperinsulinaemic clamp

Introduction

Research generated during a previous RIRDC project (UCS-35) indicated that the hyperinsulinaemia attained during a prolonged-euglycaemic, hyperinsulinaemic clamp (p-EHC) induced laminitis in insulin-sensitive ponies within 72 h (Asplin et al., 2007). This previous study confirmed that elevated serum concentrations of insulin are capable of damaging the lamellae in a short period of time. This was an important breakthrough in research into the pathogenesis of endocrinopathic laminitis. However, ponies are more insulin-sensitive than horses, particularly young, Standardbred racehorses, (Jeffcott et al., 1986), and there was some doubt over whether insulin would be similarly laminitogenic in horses. In order to further examine the mechanism by which insulin causes lamellar pathology, it was necessary to determine if it would induce laminitis in Standardbred racehorses.

Data from the pony study showed that lamellar pathology was already substantial by the time that clinical signs (Obel grade 2) of the condition occurred (Asplin et al., 2010; Obel, 1948). Hence, it was important to be able to examine the developmental stages of insulin-induced laminitis, in order to determine when and how lamellar pathology commences. Knowledge of the pathophysiological events that occur during the developmental stages of insulin-induced laminitis may facilitate a deeper understanding of disease pathogenesis. This may result in the development of improved treatment and prevention strategies for the management of naturally-occurring cases of endocrinopathic laminitis.

The use of serial lamellar biopsies taken throughout the developmental phase of the disease was the original method selected to achieve an examination of the lamellae during the developmental phase of the disease. However, work in our laboratory identified some potential problems with the technique that may have led to the inability to detect true pathology. Thus, lamellar biopsies were deemed unsuitable for use in this project. Instead, we opted to cease the p-EHC at sequential time-points during the developmental phase. This approach was also deemed to have minimal impact on the subjects as lameness and repeated invasive procedures (lamellar biopsies) could be avoided.

It has been postulated that laminitis occurs due to a vascular mechanism (Bailey et al., 2004; Hood et al., 1993). Studying the development of laminitis with a model such as the p-EHC allows close analysis of the vascular events that occur during the developmental and acute phases of the disease. Determining the nature of these vascular events may improve our understanding of the role that the vasculature plays in the development of insulin-induced laminitis.

The aims of this first study were to determine whether the p-EHC would induce laminitis in Standardbred horses and if so, to examine sequential time-points during the developmental phase of the induction model. A further aim of the study was to determine the nature of vascular changes in the hooves during the acute and developmental stages of the model, by measuring hoof wall surface temperature (HWST).
Study One: Acute phase

Horses

Eight Standardbred racehorses (7 males, 1 female) purchased in South-East Queensland were used in part one of the study. Their age, weight and body condition score (BCS) parameters are outlined in Table 1.1. No abnormalities were detected on physical examination. All horses were sound on lameness examination and veterinary and radiographic examination of their feet revealed no abnormalities. Radiographs were taken again at the conclusion of the experiment to assess for any changes in pedal bone placement (Figure 1.1). They were fed medium quality lucerne hay and chaff for 48 h preceding, and during the study. The horses were paired randomly with one horse assigned to the treatment group (n = 4) and the other to a control group (n = 4). The experiments were performed in a climate-controlled facility to ensure a constant ambient temperature. Extended-use intravenous (IV) catheters (14 gauge; MilaCath) were aseptically placed and sutured in both jugular veins of each horse and used for the administration of the infusions (right) and blood sampling (left).

Table 1.1  The age, bodyweight and body condition score (mean ± se) of horses treated with a prolonged-euglycaemic, hyperinsulinaemic clamp.

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (years)</th>
<th>Weight (kg)</th>
<th>BCS (1-9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.1 ± 14 0.7</td>
<td>432 ± 14</td>
<td>4.3 ± 0.25</td>
</tr>
<tr>
<td>6 h</td>
<td>9.0 ± 2.05</td>
<td>428 ± 17.6</td>
<td>4.5 ± 0.65</td>
</tr>
<tr>
<td>12 h</td>
<td>5.75 ± 1.75</td>
<td>463 ± 40.1</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>24 h</td>
<td>7.25 ± 2.2</td>
<td>429 ± 16.4</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>48 h</td>
<td>5.8 ± 1.3</td>
<td>427 ± 34</td>
<td>4.4 ± 0.47</td>
</tr>
</tbody>
</table>

Figure 1.1  Lateral radiograph of a p-EHC horse showing the placement of the distal phalanx (DP) within the hoof capsule. A marker is placed along the outer hoof wall (arrow). The surface thermistor is also visible (arrowhead).
Prolonged-euglycaemic, hyperinsulinaemic clamp technique

The p-EHC was adapted from the technique previously described in humans (DeFronzo et al., 1979) and modified to persist beyond 3 h (Asplin et al., 2007). A constant rate insulin infusion was used to cause hyperinsulinaemia, while euglycaemia was maintained with a glucose infusion of variable rate. The p-EHC commenced with an insulin bolus (45 mIU/kg; Humulin-R, Eli Lilly) which was diluted in 50 mL of 0.9% saline (Baxter) and administered over 60 s. A constant rate insulin infusion (6 mIU/kg/min) followed, and was given concurrently with an infusion of glucose (10 µmol/kg/min; 50% w/v, Baxter). The horses in the control group received an infusion of balanced, electrolyte solution (0.57 mL/kg/h; Hartmanns, Baxter) for the same period as their matched pair. The infusions were continued until the onset of Obel grade 2 laminitis, at which point the experiment was ceased and the horses euthanased.

Blood samples were collected from all horses before and at regular intervals throughout the experiment in order to determine blood glucose and serum insulin concentrations (Figure 1.2). Blood was placed in plain vacutainers (Vacuette, Greiner Bio-One), left to clot at room temperature for 30 min and then centrifuged (10 min at 3000 x g). Serum was separated into aliquots (1 mL) and stored at -80 ºC until analysed. Blood glucose was measured using a handheld glucometer (Accucheck Go, Roche), calibrated against the hexokinase method using blood from these horses (data not shown). Samples were taken every 5 min during the first 3.5 h to check blood glucose. Once stable (> 3.5 h), blood glucose was analysed every 30 min until euthanasia to ensure euglycaemia. Serum insulin concentrations were measured in duplicate by a radioimmunoassay kit (Siemens, Coat-a-count) previously validated for use in horses (McGowan et al., 2008). Insulin samples obtained during the clamp were diluted in insulin-free serum to avoid interference problems associated with the assay (Tinworth et al., 2009). Free catch urine samples were collected prior to, and during the experiment in order to detect the presence of glucose in the urine using dipsticks (Multistix, Roche).

Figure 1.2  Mean serum insulin concentration of horses treated with a prolonged-euglycaemic, hyperinsulinaemic clamp for 6 h (□, n = 4), 12 h (Δ, n = 4), 24 h (◇, n = 4), 48 h (◇, n = 4), or a balanced electrolyte solution for 48 h (◇, n = 4).
Insulin sensitivity

The amount of glucose administered during the p-EHC provided a measure of the insulin sensitivity (SI) of the muscle and adipose tissue of the horses to the exogenous insulin (Firshman and Valberg, 2007). Thus, SI was only able to be measured in the horses treated with a p-EHC (Table 1.2). The rate of glucose metabolism (M) was calculated during the steady-state period of the p-EHC. The amount of glucose metabolised per unit of endogenous insulin (M-to-I) was also calculated. Calculations were made following previously published protocols for horses and included space corrections (Rijnen and van der Kolk, 2003).

Table 1.2  Blood glucose and serum insulin values for five groups of horses (n = 4) before and during an insulin infusion. Insulin sensitivity results are given for insulin-treated horses.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>6 h group</th>
<th>12 h group</th>
<th>24 h group</th>
<th>48 h group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal glucose (mM)</td>
<td>5.15 ± 0.34</td>
<td>5.3 ± 0.32</td>
<td>5.5 ± 0.14</td>
<td>5.6 ± 0.21</td>
<td>6.33 ± 0.57</td>
</tr>
<tr>
<td>Clamp glucose (mM)</td>
<td>5.19 ± 0.18</td>
<td>4.3 ± 0.19</td>
<td>4.1 ± 0.35</td>
<td>4.4 ± 0.21</td>
<td>5.53 ± 0.25</td>
</tr>
<tr>
<td>Basal insulin (µIU/mL)</td>
<td>13.4 ± 2.71</td>
<td>10.2 ± 2.95</td>
<td>8.68 ± 1.07</td>
<td>12.0 ± 4.2</td>
<td>22.3 ± 11.2</td>
</tr>
<tr>
<td>Clamp insulin (µIU/mL)</td>
<td>13.6 ± 2.9</td>
<td>811 ± 132</td>
<td>977 ± 86</td>
<td>747 ± 41</td>
<td>1036 ± 129</td>
</tr>
<tr>
<td>M (mM/kg/min)</td>
<td>-</td>
<td>0.02 ± 0.003</td>
<td>0.023 ± 0.004</td>
<td>0.021 ± 0.002</td>
<td>0.024 ± 0.001</td>
</tr>
<tr>
<td>M-to-I ratio (10^-6)</td>
<td>-</td>
<td>3.3 ± 0.4</td>
<td>3.2 ± 0.7</td>
<td>3.9 ± 0.2</td>
<td>3.07 ± 0.17</td>
</tr>
</tbody>
</table>

Histology

Following euthanasia, all four limbs of all horses were disarticulated at the fetlock and sectioned with a bandsaw to produce sagittal blocks of lamellar tissue. These blocks were rinsed in cold water and trimmed to produce 5 mm by 5 mm squares of lamellar tissue with a small sliver of hoof wall included. Lamellar tissue was then placed in 10% neutral buffered formalin for 24 h before being embedded in paraffin by routine methods. Paraffin blocks were sectioned and stained with haematoxylin and eosin (H & E) and periodic acid Schiff (PAS) ready for histological analysis.

Hoof wall surface temperature

Non-invasive surface thermistors (Figure 1.3) were placed on the dorsal aspect of both front feet of all eight horses to measure changes in hoof wall surface temperature (HWST). Temperature readings were logged by data loggers connected to the thermistors every minute throughout the infusion period (Figure 1.3). The ambient temperature was kept constant at all times.

Figure 1.3  Surface thermistor with data logger attached (TinyTag, Gemini).
**Statistical analyses**

The development of laminitis was compared between control and treatment groups using a Fisher’s Exact Probability test. Radiographic measurements, blood glucose and serum insulin were compared between groups or with basal values using a Welch $t$-test. Repeated measures analysis of variance (ANOVA) was used to assess HWST. Ambient temperature was used as a covariate in the analysis. Statistical analysis was performed with R, version 7.2.7 and significance was set at $p < 0.05$. Results are presented as mean ± standard error (se).

**Results**

All of the horses in the treatment group developed laminitis whereas none of the control horses did ($p < 0.05$). The onset and progression of the clinical signs associated with disease in the treated horses is shown in Table 1.3. All of the horses remained healthy with blood haematology, blood biochemistry, heart rate and respiratory rates remaining unchanged from basal values for each group. All of the horses maintained a normal body temperature except for one control horse which was briefly pyretic following transient diarrhoea. Urinalysis results did not change from basal values for any horses and no horse developed glycosuria. Radiographic measurements of distal phalanx placement in both front feet of all horses did not reveal that any significant differences in the distance from the hoof wall to the dorsal aspect of the pedal bone occurred as a consequence of the experiment (Table 1.4).

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bolus administration, commence infusions</td>
<td>0 h</td>
</tr>
<tr>
<td>Increased digital pulses, restlessness episodic shifting of 4 feet</td>
<td>$31.5 ± 4.65$ h</td>
</tr>
<tr>
<td>Consistent shifting of weight, turning to look at feet (Obel grade 1)</td>
<td>$40.5 ± 3.87$ h</td>
</tr>
<tr>
<td>Lameness (Obel grade 2) Euthanasia</td>
<td>$46 ± 2.31$ h</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean difference (mm)</th>
<th>Standard error of difference</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>0.72</td>
<td>0.36</td>
<td>-0.10, 1.53</td>
</tr>
<tr>
<td>Control</td>
<td>0.12</td>
<td>0.30</td>
<td>-0.6, 0.84</td>
</tr>
</tbody>
</table>

Basal serum insulin and blood glucose values did not differ between the groups (Table 1.2). Blood glucose concentration also remained unchanged during the experiment for both groups (Table 1.2). Serum insulin concentration increased ($p < 0.05$) in the treated group, as a consequence of the infusion, as expected. All of the treated horses were calculated to have normal insulin sensitivity (Table 1.2).

Laminitis was confirmed histologically in all four feet of three of the treated horses and three feet in the treated horse with the lowest bodyweight (Figure 1.4). Lamellar changes included mitosis, apoptosis, lamellar lengthening and narrowing and BM disruption. The lamellar pathology of these horses is described in more detail in chapter two.
Figure 1.4 The conditional proportions of laminitis-affected hooves of horses treated with insulin (treatment) or control horses.

The HWST of the control horses (22.0 ± 1.72 °C) was variable throughout the infusion period (CV 42% at 48 h). In the treated horses, the HWST was persistently elevated (Figure 1.5), higher (27.0 ± 0.46 °C) than in the control horses \( (p < 0.05) \) and less variable (CV 4% at 48 h). The ambient temperature was constant at 15.9 ± 0.4 °C.

Figure 1.5 Hoof wall surface temperatures of horses treated with a p-EHC (●, n = 4) and control horses (◆, n = 4) for 48 h.
**Study two: Developmental phase**

**Horses**

Twelve Standardbred racehorses (all male) purchased in South-East Queensland were used in part two of the study. Their age, weight and body condition score (BCS) parameters are outlined in Table 1.1. No abnormalities were detected on physical examination. All horses were sound on lameness examination and veterinary and radiographic examination of their feet revealed no abnormalities. As no radiographic changes in distal phalanx placement were detected in horses treated for up to 48 h, radiographs were not taken at the conclusion of the experiment. The horses were fed medium-quality lucerne hay and chaff for 48 h preceding, and during the study, as before. The horses were paired randomly and both horses were assigned to one of three treatment groups (n = 4). Horses were treated with a p-EHC for 6 h, 12 h or 24 h. The experiments were performed in a climate controlled facility to ensure a constant ambient temperature. Extended-use intravenous (IV) catheters (14 gauge; MilaCath,) were aseptically placed and sutured in both jugular veins of each horse and used for the administration of the infusions (right) and blood sampling (left).

**Prolonged-euglycaemic, hyperinsulinaemic clamp technique**

The p-EHC was performed as described for study one, but was ceased at sequential time-points during the developmental phase (0 h to 48 h). The groups were treated for either 6 h, 12 h or 24 h at which point the experiment was ceased and the horses were euthanased.

Blood samples were collected at the same intervals throughout the experiment, as in study one, up to the end of the infusion period in order to determine blood glucose and serum insulin concentrations (Figure 1.1). Blood was prepared for analysis as per study one. The hand-held glucometer (Accucheck Go, Roche) was re-calibrated against the hexokinase method for these horses (data not shown). Samples were taken every 5 min during the first 3 h to check blood glucose. Once stable (> 3 h), blood glucose was analysed every 30 min until euthanasia, to ensure euglycaemia. Analysis of serum insulin concentrations was performed in an identical manner to study one. Free catch urine samples were collected prior to, and during the experiment, in order to detect the presence of glucose in the urine using dipsticks (Multistix, Roche).

**Insulin sensitivity**

The SI values (M and M-to-I) were calculated and the results are presented in Table 1.2. Calculations were performed as for study one.

**Histology**

Following euthanasia, all four limbs of all horses were disarticulated at the fetlock and prepared for histology using the same protocol as in study one.

**Hoof wall surface temperature**

Hoof wall surface temperature (HWST) was not measured in study two as the non-invasive nature of the thermistors allowed examination of the developmental phase of insulin-induced laminitis in study one.
**Statistical analyses**

Blood glucose and serum insulin were compared with basal values for each group with a Welch $t$-test. Blood glucose and serum insulin values were compared between groups using a one-way analysis of variance (ANOVA). A repeated measures ANOVA was used to assess HWST. Ambient temperature was used as a covariate in the analysis. Statistical analysis was performed with R, version 7.2.7 and significance was set at $p < 0.05$. Results are presented as mean ± se.

**Results**

None of the horses developed clinical signs of laminitis. All of the horses remained healthy with blood haematology, blood biochemistry, heart rate and respiratory rates remaining unchanged from basal values for each group. All of the horses maintained a normal body temperature. Urinalysis results did not change from basal values for any horses and no horse developed glycosuria.

Basal serum insulin and blood glucose values did not differ between the groups (Table 1.2). Blood glucose concentration also remained unchanged during the experiment for all three groups (Table 1.2). Serum insulin concentration did increase ($p < 0.05$) compared to basal values in each group, as a consequence of the infusion, as expected. All of the treated horses were calculated to have normal insulin sensitivity (Table 1.2).

Lamellar pathology was examined histologically in all four feet of all twelve horses. Lamellar changes such as mitosis, apoptosis, lamellar lengthening and narrowing and BM disruption varied in the degree of severity and time of onset of each change but commenced as early as 6 h after the onset of hyperinsulinaemia. The lamellar pathology of these horses is also described in more detail in chapter two.

**Conclusion**

The results of study one confirmed that the p-EHC induces laminitis in young Standardbred racehorses. This result confirms the laminitogenic potential of insulin. With laminitis induced in just 48 h, this study has shown that insulin can produce marked lamellar change in a short period of time. The shorter time to the onset of clinical signs of laminitis seen in this study, when compared to ponies (Asplin et al., 2007), may be related to the fact that horses are more insulin-sensitive than ponies, and/or to bodyweight. The heavier bodyweight of horses, compared to ponies, may have resulted in the faster progression of clinical disease onset in the current study than occurred with ponies.

The degree of hyperinsulinaemia experienced by the horses in study one (> 1000 µIU/mL) was marked and although this degree of hyperinsulinaemia does not occur frequently in field cases of endocrinopathic laminitis or equine metabolic syndrome, concentrations of this magnitude can and do occur. However, in most naturally-occurring cases of endocrinopathic laminitis it is likely that lower and more variable serum insulin concentrations occur over a longer period of time in order to precipitate laminitis. Determination of the threshold for the onset of naturally-occurring endocrinopathic laminitis is likely to be difficult as individual and genetic predisposition to the disease may also be involved. However, a more accurate definition of this threshold than is currently appreciated would be beneficial in allowing more focused prevention strategies to be employed.

Histological analysis of the developmental stages of insulin-induced laminitis has demonstrated that lamellar changes commence as early as 6 h after the onset of hyperinsulinaemia. This lamellar pathology then continues to worsen throughout the developmental phase until BM disruption commences around 24 h and continues to progress up to 48 h. This BM breakdown in all horses is not consistent with lamellar histology findings from ponies with insulin-induced laminitis where BM dysadhesion was not prominent (Nourian et al., 2009). Further examination of the BM lesions in horses with insulin-induced laminitis is covered in chapter three. The predominant lamellar pathology
seen in both studies contained in this chapter are consistent with descriptions of other forms of laminitis (Morgan et al., 2003; Pollitt, 1996, 2004; van Eps and Pollitt, 2009). A more detailed and direct comparison of hyperinsulinaemic laminitis with the alimentary (oligofructose-induced) form of laminitis is required and has been performed in chapter two.

The HWST results indicate that there is a vascular component to the development of insulin-induced laminitis. The persistently elevated HWST seen in the horses that were treated with insulin is in direct contrast to the variable HWST of control horses that were treated with a balanced electrolyte solution. Insulin has vasodilatory properties (Baron, 1994) and is likely to have caused the consistently high HWST values recorded during the insulin infusion. Whether this elevation in HWST contributed to the development of laminitis is yet to be determined. Further investigation of the role of the vasculature in the pathogenesis of insulin-induced laminitis is presented in chapter four.
2. Lamellar pathology

Introduction

Descriptions of the lamellar pathology associated with naturally-occurring endocrinopathic laminitis are few (Johnson et al., 2004). However the lamellar pathology of insulin-induced laminitis in ponies has been well described (Asplin et al., 2010). The lesions described in ponies were not entirely consistent with the histological findings from the p-EHC studies in horses reported in chapter one. Thus, further characterisation of the lamellar lesions associated with hyperinsulinaemia in horses was considered important. The primary aim of the current study was to fully describe the lamellar pathology in both the developmental and acute phases of insulin-induced laminitis in horses.

Laminitis lesions from other experimental models of laminitis have been described (Morgan et al., 2003; Pollitt, 1996; van Eps and Pollitt, 2009). Alimentary forms of laminitis are usually associated with overt clinical signs such as depression, diarrhoea or fever (Garner et al., 1975) whereas hyperinsulinaemic laminitis is not (chapter one). Differences in the lamellar pathology between inciting causes may indicate that not all forms of laminitis follow a common pathophysiological pathway. A direct comparison of different types of laminitis had not been undertaken previously. So, a secondary aim of this study was compare the lamellar pathology of insulin-induced and alimentary carbohydrate overload (ACO) laminitis to determine if differences exist. Any differences could then provide direction for further pathophysiological studies on the disease mechanism.

Horses

Lamellar tissues from all horses treated with a p-EHC in chapter one (6 h, 12 h, 24 h and 48 h) were used in this study. Control horses from the p-EHC experiment that were treated with a balanced electrolyte solution were included to provide a reference point of normal lamellar morphology. Archived lamellar tissue from horses treated with oral oligofructose (10 g/kg) in an ACO model of laminitis induction were used to permit a direct comparison of the two experimental models of laminitis. Each group contained four Standardbred racehorses. The details of the p-EHC horses are outlined in chapter one. The ACO group (451 ± 7.9 kg, 7.0 ± 1.8 years) developed clinical laminitis (Obel grade 2.5 ± 0.3) and were euthanased 48 h after dosing with oligofructose.

Sample preparation

Lamellar sections from all four feet of all horses were obtained from blocks of lamellar tissue removed from the mid-dorsal section of the hoof as described in chapter one. Following fixation in 10% neutral buffered formalin for 24 h they were embedded in paraffin and processed for histology by routine methods. Paraffin blocks were sectioned and stained with H & E and PAS for histological analysis or left unstained for immunohistochemistry.

Immunohistochemistry

Calprotectin is a protein complex that is located in the cytoplasm of neutrophils and on the membrane of monocytes (Striz and Trebichavsky, 2004). Immunolocalisation of calprotectin was used in the current study to identify and locate leucocytes in the lamellar sections.

Paraffin sections were warmed for 10 min at 60 ºC before deparaffinisation in xylene (3 x 2 min) and rehydration through gradients of ethanol (100% 2 x 5 min, 90% 1 x 5 min) using an automated stainer (Shandon Vari-stain 24-2) before being rinsed in tap water (1 x 2 min) and placed in ddH2O. Antigen retrieval was performed using 0.05% Proteinase K ready-to-use solution (Dako) for 6 min at room
temperature before washing (3 x 5 min) in 50 mM Tris-buffered saline with tween (TBST, Dako). Non-specific binding was blocked with 2% normal goat serum (Vector) for 30 min at room temperature. Sections were blocked with peroxidase blocking solution (Dako) for 10 min at room temperature before rinsing with ddH2O and washing with TBST (1 x 5 min). Slides were then incubated for 30 min at room temperature with monoclonal mouse anti-human macrophage antibody (MAC-387, Dako) optimally diluted in antibody dilution solution (1:400) with background reducing components (Dako). Mouse IgG was used in place of the primary antibody for a negative control. Positive control tissue was taken from a horse with ACO laminitis (Visser, 2008). After rinsing with ddH2O and washing with TBST (1 x 5 min) the slides were incubated for 30 min at room temperature with peroxidase-labelled, polymer-HRP, anti-mouse secondary antibody (EnVision+ System-HRP, Dako). Slides were stained with chromagen 3,3’-diaminobenzidine tetrahydrochloride (DAB, Dako) for 8 min and counter-stained with Mayer’s haematoxylin for 1 min. The slides were mounted with an aqueous mounting medium (Faramount, Dako) and examined within 24 h.

The degree of calprotectin immunoreactivity was graded using the following scale: 0 = no staining or occasional calprotectin-positive cell within vessel walls; 1 = mild perivascular infiltration of calprotectin-positive cells into primary dermal lamellar tissue; 2 = moderate, diffuse infiltration of calprotectin-positive cells throughout dermal connective tissue; 3 = marked tissue infiltration of calprotectin-positive cells with positive staining of SEL basal and parabasal cell cytoplasm (de Laat et al., 2011c).

Statistical analyses

Inter-rater agreement was assessed with Lin’s concordance co-efficient ($\rho_c$) and Bland Altman’s limits of agreement. Measurements of epidermal length and width were compared between groups using two-way analysis of variance (ANOVA). Horse identification and group were used as the two class variables to account for clustering of the forelimbs of each horse. Pair-wise comparison of means was assessed with Tukey’s test. Median (interquartile range) scores for calprotectin immunoreactivity were compared between groups using a Kruskal-Wallis one-way ANOVA on ranks. All other data are presented as mean ± se. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using R, version 7.2.7.

Histology

Control horses

Lamellar sections from the control horses had normal lamellar architecture. The primary epidermal lamellae (PELs) of control horses were straight and relatively uniform in length, except for the infrequent appearance of shorter PELs, 50% to 75% of the length of adjacent PELs. The keratinised axis of each PEL did not extend to the axial (distal phalanx) tip of the PEL, finishing just short of the entire PEL. The primary dermal lamellae (PDL) were adjacent to the entire PEL. The histological features of the secondary epidermal lamellae (SELS) in the four control horses included uniformity of length, symmetrical angulation (45 to 90º) of the SEL to the PEL axis and rounded bulbous tips with tightly adhered, PAS stained BM. The secondary dermal lamellae (SDL) interdigitated with adjacent SELS, extended uninterrupted almost to the PEL axis and contributed to the symmetrical architecture that typifies normal lamellae (Figure 2.1). The epidermal basal cells (EBCs) had ovoid, apical or centrally located nuclei principally orientated at right angles to the SEL axis (Figure 2.1). Mitotic figures (indicators of cell proliferation) and apoptotic cells (indicators of cell death) were rare. Microvasculature in the form of blood and lymphatic capillaries were present within the SDL connective tissue surrounding the SEL (Figure 2.1). Descriptive histology was similar between the fore and the hind feet of all control horses.
Figure 2.1 The secondary epidermal lamellae (SELs) of the control horses were uniform in length with rounded bulbous tips and tightly adhered basement membrane (arrow). The secondary dermal lamellae (SDLs) interdigitated with adjacent SELs and epidermal basal cells (EBCs) had ovoid, apical or centrally located nuclei (N) principally orientated at right angles to the SEL axis. Blood vessels (V) were present within the SDL connective tissue surrounding the SELs. Stain = H&E.

Six-hour group

Elongation, narrowing and tapering of the SELs had commenced by the 6 h time-point of hyperinsulinaemia. The EBC nuclei were already disorientated from their apical position in the cell and were more centrally located. Mitotic figures were infrequent and did not appear to be increased compared to control horses. In comparison, apoptosis appeared to be considerably more frequent than in control sections. BM disruption was not evident at this time-point. There did not appear to be an increase in the number of extravasated leucocytes compared to control section.

Twelve-hour group

By 12 h, the SELs were further lengthened and narrowed with more EBC nuclei appearing rounded with enlarged nucleoli. Mitotic figures were slightly increased in number compared with the 6 h time-point and control horses (Figure 2.2). Interestingly, the number of apoptotic bodies had decreased at the 12 h time-point compared with the 6 h horses. BM damage was not evident. More leucocytes were starting to infiltrate in the dermal connective tissue at this time-point but were not extensive.
Figure 2.2 Both apoptotic bodies (arrow) and mitotic figures (arrowhead) were noted in the secondary epidermal lamellae (SEL) of lamellar sections from horses treated with an insulin-infusion for 12 h. Stain = H&E.

Twenty four-hour group

The SELs were longer and narrower again by the 24 h time-point and many had come into contact with adjacent SELs, thus obliterating the intervening SDLs. The EBC nuclei were rounded with multiple and prominent nucleoli. The number of mitotic figures had increased significantly at this time-point compared with the 6 h, 12 h and control groups. Apoptosis was not prevalent. BM dysadhesion had commenced at the axial tips of the PELs in all horses and there were leucocytes in the dermal connective tissue around the PEL tips with BM damage.

Forty eight-hour group

The degree of lamellar pathology varied between the horses and was more severe in the front feet than the hind feet. In particular, pathological changes were more severe at the axial (distal phalanx) end of the PELs (Figure 2.3). The PELs of the 48 h horses were not visibly elongated, but were dyskeratotic, predominantly in the axial regions. The SELs were elongated and disorganised along the whole length of the PEL. The SELs had sharp, pointed tips and were not symmetrically angled to the PEL axis. Many SELs tapered from the base to the tip, while some were uniformly narrowed. Many were either undulant or crescent shaped. Clear demarcation of individual SELs was lost with secondary EBCs becoming confluent.

Many epidermal cell nuclei were no longer located apically in the cell and were rounded. The nuclei were large and usually contained multiple nucleoli. Mitosis was noted more frequently at this time-point than any other and occurred throughout the axial and mid-sections of the PEL. SELs in the axial region were extensively disorganised and pockets of BM often contained no basal cells (Figure 2.3). BM dysadhesion from EBCs occurred in the front feet of all horses, but only rarely in the hind feet. BM damage was variable between individuals. Leucocytes were present both within blood vessels, and in the dermal connective tissue, particularly around the axial tips of the PELs (Figure 2.3).
Figure 2.3  Pathological changes were more severe at the axial (distal phalanx) end of the PELs in sections from horses treated with an insulin infusion for 48 h. The secondary epidermal lamellae (SEL) in the axial region were extensively disorganised and pockets of BM often contained no basal cells (arrows). Leucocytes were present in the dermal connective tissue, particularly around the axial tips of the PELs (arrowhead). Stain = H&E.

Alimentary carbohydrate overload group (48 h)

The lamellar lesions of the horses treated with oral oligofructose were similar to the horses with insulin-induced laminitis. Lamellar lengthening, narrowing, tapering and nuclear changes were accompanied by increases in mitosis and apoptosis. Notably there appeared to be a greater degree of BM separation in the horses with ACO than the hyperinsulinaemic horses at the same time-point (48 h). Furthermore, the numbers of leucocytes infiltrating the dermal connective tissue appeared to be significantly greater in the ACO horses.

Morphometry

Measurements of PEL and SEL length and SEL width were made in the front feet of all groups of horses. The length of eight PELs and ten SELs in the axial region of each of these eight PELs was measured in the dorsal mid-section of each fore foot. The width of twenty SELs was measured on all eight PELs for each horse. The measurement protocol was validated by assessing the inter-rater agreement between two observers in order to ensure the accuracy of the measurements (de Laat et al., 2011c). There was excellent agreement between the two sets of measurements.

PEL length did not increase over time and was not different between any of the groups (Table 2.1). SEL length was significantly increased ($p < 0.05$) in both the 48 h insulin-induction and ACO groups compared with control horses (Figure 2.4). The SELs were narrower by the 12 h time-point in the insulin-induction model and at 48 h in the ACO group (Table 2.1).
Table 2.1  Epidermal length and width of six groups of horses (n = 4) treated with either a p-EHC (insulin), alimentary carbohydrate overload (ACO) or a balanced electrolyte solution (control)

<table>
<thead>
<tr>
<th>Group</th>
<th>PEL length (µm)</th>
<th>SEL length (µm)</th>
<th>SEL width (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3335 ± 194</td>
<td>116 ± 23</td>
<td>25 ± 2.5 *</td>
</tr>
<tr>
<td>Insulin 6 h</td>
<td>3155 ± 203</td>
<td>164 ± 29</td>
<td>24.3 ± 1.9 *</td>
</tr>
<tr>
<td>Insulin 12 h</td>
<td>3303 ± 230</td>
<td>173 ± 33</td>
<td>18.1 ± 2</td>
</tr>
<tr>
<td>Insulin 24 h</td>
<td>3174 ± 82</td>
<td>190 ± 38</td>
<td>17.3 ± 2.4</td>
</tr>
<tr>
<td>Insulin 48 h</td>
<td>3367 ± 54</td>
<td>256 ± 45 *</td>
<td>13 ± 1.5</td>
</tr>
<tr>
<td>ACO 48 h</td>
<td>3334 ± 187</td>
<td>251 ± 27 *</td>
<td>15 ± 2.2</td>
</tr>
</tbody>
</table>

Key: PEL: primary epidermal lamellae, SEL secondary epidermal lamellae, * denotes whether one group differs ($p < 0.05$) to the others for each measurement.

Figure 2.4 The length of the secondary epidermal lamellae (SEL) was increased ($p < 0.05$) in horses treated with insulin for 48 h (a) compared with control horses (c). Stain = H&E.
Calprotectin immunohistochemistry

Calprotectin-positive cells were not present outside of dermal vessels in control horse, 6 h or 12 h time-point sections (Table 2.2). There was a mild infiltration of leucocytes as indicated by increased calprotectin immunoreactivity at the 24 h time-point of insulin-induction although this was only evident in half of the group. Extravasation of calprotectin-positive cells into the dermal connective tissue had occurred in all horses by the 48 h time-point of the p-EHC however this was not as pronounced as the 48 h time-point of the ACO model (Figure 2.5).

Table 2.2  Median (IQR) score for calprotectin immunoreactivity in six groups of horses (n = 4) treated with either a p-EHC (insulin), alimentary carbohydrate overload (ACO) or a balanced electrolyte solution (control).

<table>
<thead>
<tr>
<th>Group</th>
<th>Median score</th>
<th>Inter-quartile range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0 - 0</td>
</tr>
<tr>
<td>Insulin 6 h</td>
<td>0</td>
<td>0 - 0</td>
</tr>
<tr>
<td>Insulin 12 h</td>
<td>0</td>
<td>0 – 0</td>
</tr>
<tr>
<td>Insulin 24 h</td>
<td>0</td>
<td>0 – 0.5</td>
</tr>
<tr>
<td>Insulin 48 h</td>
<td>1.8</td>
<td>1.0 – 2.0</td>
</tr>
<tr>
<td>ACO 48 h</td>
<td>3</td>
<td>3.0 – 3.0</td>
</tr>
</tbody>
</table>

Figure 2.5 Immunolocalisation of calprotectin-positive cells (arrows) in the dermal connective tissue had increased by the 48 h time-point of the p-EHC (a) compared to earlier time-points. However, this was not as pronounced as the 48 h time-point of the ACO model (b).
Conclusion

The current study provides the first detailed histological description and morphometric analysis of the developmental and acute phases of insulin-induced laminitis. It also provides the first direct comparison between the ACO and insulin models of laminitis induction. Lamellar lesions occurring secondary to a p-EHC have been described in ponies and the results of this study are not in complete agreement with the findings in ponies (Asplin et al., 2010). While the morphometry results were largely comparable between the two studies, BM separation at the axial tips of the SELs was only reported in some of the ponies. In contrast, BM dysadhesion was widespread in the current study, occurring in all horses and along greater proportions of the PELs. However, BM separation in the horses treated with the p-EHC for 48 h was still less marked than in horses treated with ACO. The increased degree of BM damage in horses compared to ponies may be a result of their greater bodyweight exerting more stress on the lamellae. BM separation in ACO has been linked to increased metalloproteinase activity (Coyne et al., 2009; Visser, 2008). Factors that initiate BM proteolysis in insulin-induced laminitis are unknown and have been investigated in the next chapter.

Changes to lamellar morphology commenced early in the developmental phase of insulin-induced laminitis. Appreciable increases in length and decreases in width occurred as early as 6 h following the onset of hyperinsulinaemia. These morphological changes continued to progress in their severity throughout the developmental phase. Significant changes in length were not apparent until the onset of clinical lameness at the 48 h time-point. This significant increase in SEL length may be the key factor that initiates lameness and coincide with SEL length surpassing a critical point where it can no longer maintain a strong attachment between the inner hoof wall and distal phalanx. Increasing EBC mitosis may be an indicator of increased cell proliferation contributing to SEL lengthening. Strategies to prevent lengthening and weakening of the lamellae may prove to be a key factor in prevention and treatment of the disease. Investigations into the cause of SEL lengthening, be it secondary to an increase in cell proliferation or changes in cell morphology, will be an important focus for future research into laminitis pathophysiology.

Previous studies have reported strong calprotectin immunoreactivity in other forms of laminitis (Faleiros et al., 2009; Visser, 2008) and this was confirmed in the ACO group in the current study. The direct comparison of the grade of calprotectin immunolocalisation performed between groups in this study showed that insulin-induced laminitis is associated with less leucocyte infiltration than other forms of laminitis. This fits with the clinical presentation of the different forms of laminitis. Horses with hyperinsulinaemia are not clinically unwell (chapter one) whereas horses with ACO develop a fever and diarrhoea (van Eps and Pollitt, 2006). This finding may indicate that not all forms of laminitis have a common pathophysiology and may influence the choice of treatment modality for different forms of the disease.
3. Basement membrane pathology

Introduction

The lamellar basement membrane (BM) separates the epidermal basal cells (EBCs) from the underlying dermal connective tissue in the horse’s foot. BMs are essential for the maintenance of cell structure, proliferation and migration (Timpl, 1996) and they play an important role in maintaining the lamellar structure (Pollitt, 1994). The BM is composed largely of collagen, glycoproteins (laminin and nidogen) and proteoglycans (Yurchenco and Schittny, 1990). The BM is damaged during laminitis, losing its structural integrity (Pollitt, 1994).

Ultrastructural analysis of the lamellar BM has shown that, as with BMs in other locations, it consists of distinct regions (French and Pollitt, 2004b; Nourian et al., 2007). The EBC plasmalemma is populated with hemidesmosomes (HDs) from which anchoring filaments cross the lamina lucida and engage with the lamina densa (Ghadially, 1997). The HD-anchoring filament complex is an important structural component of the BM, forming a strong bond between the EBC and the lamina densa, and enables the structure to withstand mechanical stress (Litjens et al., 2006). HD breakdown and reassembly is normal and allows cells to proliferate and migrate, but also occurs at an accelerated rate in pathological conditions such as cancer (Litjens et al., 2006). The number of HDs in the lamellar BM reduces during ACO laminitis (French and Pollitt, 2004b; Nourian et al., 2007) and hyperinsulinaemic laminitis in ponies (Nourian et al., 2009) and it is likely that this reduction in HD number contributes to weakening of the lamellar interface. Ultrastructural studies on the BM zone have not been performed in horses with insulin-induced laminitis.

Metalloproteinases have been implicated in the BM proteolysis that occurs in ACO laminitis. Studies on metalloproteinase activity have shown that MMP-2, MMP-9 and ADAMTS-4 are all up-regulated in horses with alimentary forms of laminitis (Coyne et al., 2009; Kyaw-Tanner and Pollitt, 2004; Kyaw-Tanner et al., 2008). Metalloproteinases are present in extracellular matrix (ECM) and play an essential role in the breakdown of the BM in both physiological and pathological circumstances. They work in concert with specific inhibitors (TIMPs) to maintain the ECM in a healthy and adaptable state (Woessner, 1993). Up-regulation of metalloproteinases leads to increased BM proteolysis, often with catastrophic consequences. Laminitis is one such condition that may be attributable to inappropriate metalloproteinase activity, but this is yet to be proved in endocrinopathic forms of the disease.

The aim of the current study was to examine BM ultrastructure using transmission electron microscopy (TEM) in horses with acute insulin-induced laminitis to determine if HD disassembly is a feature of the disease. Following this, analysis of metalloproteinase concentrations in the lamellar microenvironment of horses in the developmental and acute stages of insulin-induced laminitis determined the role of specific metalloproteinases in lamellar BM breakdown. Identification of specific metalloproteinases that are potentially involved in disease pathogenesis may allow targeted therapeutic options to be developed.

Horses

Lamellar samples from three horses that developed acute, clinical laminitis secondary to a p-EHC (chapter one) were examined using TEM. Their three matched control horses were also examined to provide a reference point for normal lamellae. Measurements of BM width were made along a length of ~ 200 µm of lamellar BM in all horses. The number of HDs per µm of BM was counted along the same length of BM for each group.

Assessment of metalloproteinase activity was assessed in all horses that underwent a p-EHC (chapter one) including the developmental time-points (6 h, 12 h, and 24 h), as well as horses with acute laminitis (48 h) and the control horses. Concentrations of active and inactive MMP-2 and MMP-9
were quantified using gelatin zymography in all horses. The gene expression of MMP-2, MMP-9, MT1-MMP, ADAMTS-4 and TIMP-3 was analysed with qRT-PCR in the 48 h and control horses.

**Sample preparation**

**Transmission electron microscopy**

Lamellar samples from the left forefoot of three horses were collected from the mid-dorsal section of the hoof as described in chapter one and trimmed to obtain a very small block of tissue (1 mm x 1 mm x 3 mm). This small block was fixed in 4% glutaraldehyde in sodium cacodylate buffer and stored at 4 °C until processed. Samples were then further fixed in 0.1% osmium tetroxide and microwave processed (Pelco biowave, Pelco Int.). The tissue was infiltrated with resin before being embedded, at the correct orientation for sectioning, in epoxy resin (EPON). Resin blocks were trimmed and sectioned on an ultramicrotome (Leica Microsystems) and mounted on formvar-coated copper slot grids. Tissue was stained with uranyl acetate (5% in 50% ethanol, 120 s) and lead citrate (60 s) and examined with a Jeol 1010 electron microscope (Jeol, Japan) at an accelerating voltage of 80 kV.

**Gelatin Zymography**

Blocks (5 mm x 5 mm) of lamellar tissue were collected from the left forefoot of each horse immediately upon euthanasia, rapidly frozen in liquid nitrogen and stored at -80 °C until use. Frozen lamellar tissue (100 mg) from each horse was crushed and homogenised (Omnitip, Omni Int.) in 1mL of cold Triton-X-100 buffer (1.25 mL (0.25%) Triton-X-100, 735 mg (10 mM) CaCl2·2H2O in 500 mL distilled H2O) on ice (5 x 10 s). The homogenate was then centrifuged (10,000 x g, 15 min at 4 °C) and the supernatant was removed. The total protein concentration of each sample was determined in quadruplicate (CV = 4.2%) with the bicinchoninic acid (BCA) protein assay kit (BCA, Pierce). Absorbance at 562 nm was measured on a NanoDrop (Thermo Scientific) spectrophotometer.

Protein (20 µg) was added (1:2) to zymogram sample buffer and incubated at room temperature for 10 min. Samples were run on 10% zymogram ready gels with gelatin (BioRad) for 100 min at 100 V. SDS was removed from the gels with renaturation buffer (2.5% Triton X-100) on a rotary shaker (30 min). Gels were developed in zymography development buffer (50mM Tris-HCl pH 7.5, 5mM CaCl2) for 18 h at 37 °C and then stained for 1 h at room temperature with 0.5% Coomassie R-250 (40% methanol, 10% acetic acid, 0.5% Coomassie blue R-250). Destaining (40% methanol, 10% acetic acid) continued until clear bands were visible against the blue background. Gels were scanned using an image editing software (Adobe Photoshop CS4) and analysed by densitometry using digital image analysing software (ImageJ v. 1.38).

**PCR analysis**

Blocks (5 mm x 5 mm) of lamellar tissue were collected from the left fore foot of each horse immediately upon euthanasia, rapidly frozen in liquid nitrogen and stored at -80 °C until use. One microgram of total RNA was extracted from lamellar tissue (100 mg) with Trizol reagent (Invitrogen) according to the manufacturer’s instructions. RNA concentration was determined by UV spectrophotometry (absorbance; 230 nm). Decontaminated RNA samples were reverse-transcribed to cDNA using the Reverse Transcription System (Promega). cDNA samples from each horse were then analysed with qRT-PCR using 2xSYBR Green Master Mix (Applied Biosystems) and the forward and reverse primers (designed from equine specific sequences using Primer3 v. 0.4.0 software) for the genes of interest (MMP-2, MMP-9, MT1-MMP (MMP-14), ADAMTS-4 and TIMP-3) and a housekeeping gene (β2-microglobulin). The primers were synthesized by Integrated DNA technologies and their specificity checked with a Blast search. The accession number and optimised primer concentrations for each gene are shown in Table 3.1. The exact primer sequences used can be found in de Laat et al. (2011a).
The PCR reactions were run on an ABI 7900 HT Fast Real-Time PCR System (Applied Biosystems) and the results expressed as cycle threshold (CT) values. All PCR reactions were cycled on the same plate at 95 °C for 10 min, followed by 45 cycles of 15 s at 95 °C, and 1 min at 60 °C and included no-template controls. The PCR results were analysed using the 2-ΔCT method (Schmittgen and Livak, 2008).

Table 3.1 The accession number and concentration for the primers used for the quantification of metalloprotease genes in the lamellae of horses with insulin-induced laminitis (n = 4) and control horses (n = 4).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession number</th>
<th>Amplicon size (base pairs)</th>
<th>Primer conc. (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2- microglobulin</td>
<td>X69083</td>
<td>101</td>
<td>200</td>
</tr>
<tr>
<td>MMP-2</td>
<td>AJ24331</td>
<td>95</td>
<td>300</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>EF077282</td>
<td>101</td>
<td>200</td>
</tr>
<tr>
<td>MMP-9</td>
<td>EF581171</td>
<td>101</td>
<td>200</td>
</tr>
<tr>
<td>ADAMTS-4</td>
<td>EU025848</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>IMP-3</td>
<td>AJ243283</td>
<td>101</td>
<td>200</td>
</tr>
</tbody>
</table>

Statistical analyses

Measurements of lamellar width and HDs/µm were compared between treated and control horses with an unpaired t-test. Gelatinase densitometry results were compared between the time-points using one-way ANOVA and Tukey’s test. Gene expression was compared between control and treated groups with Wilcoxon’s t-test. All data are presented as mean ± se. Statistical significance was set at p < 0.05. Statistical analyses were performed using R, version 7.2.7.

Results

Ultrastructure

Control group

The lamellar BM of the control horses matched descriptions of normal BM in other ultrastructural studies (Pollitt, 1994). The plasmalemma, lamina densa and lamina lucida were consistent across all sections examined (Figure 3.1a). The average width of the BM in control horses was 0.14 ± 0.01 µm. The HDs occurred at regular intervals and were uniform in structure. The average number of HDs per µm of plasmalemma was 3.6 ± 0.08.

Insulin-induced laminitis group

The lamellar BM appeared grossly widened in a large number of the ultramicrographs examined, although some sections of the BM appeared almost normal. In some sections it was difficult to determine the location of the BM at all. It appeared to have completely disintegrated with only small fragments of HD-like material in an otherwise amorphous area. The lamina lucida was variably absent or distended and the lamina densa was widened (Figure 3.1b). The width of the BM in treated horses was 0.25 ± 0.03 µm.

Large sections of the lamellar BM did not contain HDs. When they were present they were often structurally indistinct and orientated at an angle to the BM instead of at right angles to it. Fragments of disassembled HDs were frequently seen in the lamina lucida and lamina densa. The number of HDs per µm was 1.88 ± 0.08.
Figure 3.1 Ultramicrographs taken with TEM of the epidermal basal cell (EBC) basement membrane in a control horse (a) and a horse treated with a p-EHC (b). The plasmalemma (white arrowhead), lamina densa (white line) and lamina lucida were uniform in control horses. Hemidesmosomes (arrow) were intact and occurred at regular intervals along the BM. In p-EHC treated horses the lamina lucida was variably absent or distended and the lamina densa (black line) was widened. HDs (arrow) were disorganised or disassembled. The cytoskeleton (black arrowhead) often appeared to be retracting away from the BM zone in p-EHC treated horses. Magnification: 50000 x.

Metalloproteinases

Gelatinase zymography can detect both the active and inactive zymogens of metalloproteinases. Pro MMP-2 (inactive) was present in the lamellar homogenate from all of the horses (Table 3.2). However, activated MMP-2 was generally not present in any horse. In contrast, pro MMP-9 (inactive) was barely detectable in the control, 6 h and 12 h groups. Increased amounts of pro MMP-9 were present at 24 h and again at higher concentration at 48 h. (Table 3.2). However, activated MMP-9 was not detected in any group.

Table 3.2 The mean ± s.e relative intensity units (RIU) for gelatinase analysis of MMP-2 and MMP-9 in four groups of horses (n = 4) treated with a p-EHC for either 6 h, 12 h, 24 h and 48 h and a control group (n = 4) treated with a balanced electrolyte solution.

<table>
<thead>
<tr>
<th>Group</th>
<th>Pro MMP-2 (RIU)</th>
<th>Pro MMP-9 (RIU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2335 ± 510</td>
<td>250 ± 43</td>
</tr>
<tr>
<td>6 h</td>
<td>1842 ± 346</td>
<td>221 ± 63</td>
</tr>
<tr>
<td>12 h</td>
<td>2329 ± 270</td>
<td>256 ± 104</td>
</tr>
<tr>
<td>24 h</td>
<td>3859 ± 473</td>
<td>1506 ± 344</td>
</tr>
<tr>
<td>48 h</td>
<td>4012 ± 589</td>
<td>3586 ± 782</td>
</tr>
</tbody>
</table>

The gene expression of MMP-2, MT1-MMP, ADAMTS-4 and TIMP-3 was not different between the treated and control horses (Table 3.3). MMP-9 gene expression was negligible in control horses but was markedly up-regulated in the treated horses (Table 3.3).
Table 3.3  The mean ± s e gene expression of each target gene is shown relative to the house keeping gene (β2-microglobulin) in horses treated with a p-EHC for 48 h (n = 4) and control horses (n = 4) treated with a balanced electrolyte solution.

<table>
<thead>
<tr>
<th>Gene expression</th>
<th>Control horses</th>
<th>48 h p-EHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>5.49 ± 3.38</td>
<td>3.65 ± 1.87</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>0.06 ± 0.04</td>
<td>0.06 ± 0.04</td>
</tr>
<tr>
<td>MMP-9</td>
<td>0.0006 ± 0.0002</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>ADAMTS-4</td>
<td>0.04 ± 0.02</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>0.06 ± 0.02</td>
<td>0.03 ± 0.01</td>
</tr>
</tbody>
</table>

**Conclusion**

After 48 h of hyperinsulinaemia (> 1000 µIU/mL) a large proportion of the lamellar BM had lost its normal architecture and appeared disorganised and disintegrated. A significant reduction in the number of structurally important HDs had occurred. This presumably contributed to a weakening of the normally strong bond between the EBC and the lamina densa, potentially contributing to lamellar failure.

This disorganisation and break down of the lamellar BM may also have resulted in the significant increase in BM width that occurred in the insulin-treated animals when compared with controls. Alternatively, an increase in the production of the structural components of the BM, such as laminin and collagen, could have contributed to lamellar BM widening. In human diabetic patients persistent hyperglycaemia permits accumulation of advanced glycation end products which in turn leads to overproduction of components of the BM, including laminins and collagen (Brownlee, 1992). This thickening of BMs in various locations in the body is associated with pathologies such as diabetic nephropathy and retinopathy (Dronavalli et al., 2008).

Research on the fate of individual structural components of the lamellar BM in other experimental forms of the disease has demonstrated that they are lost or damaged, rather than increased (French and Pollitt, 2004a; Visser, 2008). However, alimentary forms of laminitis are not associated with endocrinopathic dysfunction, and the pathogenesis of the two forms of the disease may be quite different.

Minimal metalloproteinase activity was found to be occurring in the lamellae of the insulin-treated horses which further supports the notion that the pathogenesis of different forms of the condition may not be the same. Although an increase in pro MMP-9 occurred in the acute phase of the disease compared with control horses, it appeared after other significant events in lamellar pathology were underway and was in the inactive form. MMP-9 levels were positively correlated with neutrophil numbers (Goetzl et al., 1996; Loftus et al., 2006). An increase in leucocyte numbers did occur by the acute phase of the disease in the current study and this is likely to have accounted for the increase in inactive MMP-9.

A lack of up-regulation of metalloproteinases in damaged lamellae during the acute and developmental stages of insulin-induced laminitis is surprising and is not in keeping with research findings in other forms of laminitis (Coyne et al., 2009; Kyaw-Tanner et al., 2008; Visser, 2008). This finding suggests that BM proteolysis by metalloproteinases is unlikely to occur in endocrinopathic forms of the disease. Physical disruption of the lamellar BM as opposed to enzymatic degradation may instead be occurring. Further studies on the causative factors for the lamellar BM damage occurring during hyperinsulinaemic laminitis are required. In addition, assessment of the fate structural components of the BM during the acute and developmental phases of the disease would improve our understanding of the disease mechanism.
4. Intra-osseous infusion of the distal phalanx

Introduction

In-depth examination of the p-EHC model of insulin-induced laminitis has greatly improved our understanding of this unique disease. However, the p-EHC technique causes systemic hyperinsulinaemia and involves the administration of large quantities of glucose. Thus, it is unclear whether insulin induces laminitis by acting directly within the digit or whether a secondary mediator, at the whole body level, is required. Furthermore, the role of the glucose administered during the clamp in uncertain.

Administration of insulin directly into the circulation of the digit, to cause local hyperinsulinaemia, is necessary to establish whether insulin has a direct effect on the tissue. By creating high concentrations of insulin in one front foot only and using the contralateral forelimb as a control, it may be possible to induce laminitis unilaterally, therefore confirming the site of action of insulin. Previous work from our research group has developed a technique using intra-osseous infusion of the distal phalanx (IOIDP) to deliver substances directly into the digital microcirculation (Nourian et al., 2010). This technique was adapted to allow the administration of an infusion for 48 h, thereby enabling the administration of insulin directly into the digit. If laminitis is induced in this way, a new model for the induction of laminitis will have been developed. A model that involves lower costs and more importantly, lower impact on the horses and smaller sample sizes as each animal acts as its own control.

A further advantage of the single limb induction model is the potential to determine the effects of insulin and glucose separately. Local hyperinsulinaemia should not result in any systemic increases in serum insulin concentration. Thus, IOIDP of insulin should not need to be accompanied by a glucose infusion. If local hyperinsulinaemia is laminitogenic in the absence of glucose then a role for glucose in the pathogenesis of the disease can be discounted.

Results from the p-EHC experiments in chapter one have shown that insulin-induced laminitis is accompanied by persistent vasodilation, thus suggesting a role for the vasculature in insulin-induced laminitis pathophysiology. If prolonged vasodilation of the digit is the sole way in which insulin induces laminitis then causing persistent vasodilation in the digit with a vasodilator other than insulin, should also induce laminitis. This hypothesis was suitable to be tested using IOIDP. Thus, a second study was undertaken that aimed to produce persistent vasodilation of a single equine digit with an infusion of a vasodilator that works in a similar way to insulin (i.e. reduce vascular tone) and determine if this vasodilation was laminitogenic.
Study one: Intra-osseous infusion of insulin into a single foot

Horses

Six clinically healthy (2 mares, 4 geldings, mean bodyweight 394 ± 31.8 kg; mean age 5.3 ± 1.7 years) Standardbred racehorses in moderate body condition (BCS: 4.5/9; (Henneke et al., 1983) were used for study one. No abnormalities were detected on physical examination. All horses were sound on lameness examination and veterinary and radiographic examination of their feet revealed no abnormalities. Radiographs were taken again at after insertion of the IOIDP needle to ensure correct placement. The horses were fed medium quality lucerne hay and chaff for 48 h preceding, and during the study. The experiment was performed in a climate controlled facility to ensure a constant ambient temperature.

Extended-use intravenous (IV) catheters (14 gauge; MilaCath) were aseptically placed and sutured in the left jugular vein for blood sampling. Jugular blood samples (10 mL) were drawn at 30 min intervals to measure blood glucose concentration and every 6 h to measure serum insulin concentrations throughout the study. Digital blood samples (2 mL) were drawn from both lateral digital veins at the level of the fetlock at 0 h, 24 h and 48 h. Blood glucose was measured immediately using a handheld glucometer (Accucheck Go, Roche), previously calibrated against the hexokinase method (chapter one). Blood was placed in plain vacutainers (Vacuette, Greiner Bio-One), left to clot at room temperature for 30 min and then centrifuged (10 min at 3000 x g). Serum was separated into aliquots (1 mL) and stored at -80 ºC until analysed. Serum insulin concentrations were measured in duplicate by a radioimmunoassay kit (Siemens, Coat-a-count) previously validated for use in horses.

Intra-osseous infusion of the distal phalanx

The horses were sedated with romifidine hydrochloride (Sedivet, Boehringer Ingleheim; 0.04 mg/kg IV) before perineural, digital nerve blocks of both front feet with a short-acting local anaesthetic (2% lignocaine, Troy). Both front hooves of all horses were rasped and cleaned with dilute chlorhexidine prior to sterilisation of a small area in the mid-section of the dorsal wall of the hooves with cautery. An intra-osseous needle was then inserted aseptically with a specialised drill (Vidacare) through the hoof wall and lamellae before engaging the dorsal cortex of the distal phalanx. Once in place, the needles were flushed before the spring-loaded syringe pumps (Springfusor, Go Medical) were attached (Figure 4.1).

The infusions were commenced at a constant rate of 0.02 mL/min. The dose rate of insulin (Humulin-R, Eli Lilly) was the same as the one used in the p-EHC (6 mIU/kg/min). The volume of insulin required was calculated based on the mean weight (1.61 ± 0.05 kg) of a Standardbred foot. Thus, 0.14 mL of insulin was mixed with 0.86 mL of the horse’s own serum and diluted in 29 mL of 0.9% saline. This was infused over a 24 h period and the process repeated with freshly prepared solution for the second 24 h period. The control limb was infused with 0.9% saline at the same rate. The horses were fitted with equine nappies (Equisan) to avoid urine or faecal contamination of the intra-osseous needle site.
Figure 4.1 The left fore foot showing the extension tubing (black arrow), flow control tubing (white arrowhead) and the spring-driven syringe pump (Springfusor, Go Medical) which was housed in a lateral pouch. The HWST thermistor (white arrow) was placed on the dorsal hoof wall.

Hoof wall surface temperature

Non-invasive surface thermistors were placed on the dorsal aspect of both front feet of all eight horses to measure hoof wall surface temperature (HWST). Temperature readings were logged by the thermistors every minute throughout the infusion period. The ambient temperature was kept constant at all times.

Statistical analyses

A repeated measures ANOVA was used to analyse glucose, heart and respiratory rates, body temperature and HWST data during the experiment. Insulin data were compared between groups using a Kruskal one-way analysis of variance on ranks. All data are presented as mean ± se or median (interquartile range) and statistical significance was accepted at $p < 0.05$. Statistical analyses were performed using R, version 7.2.7.

Results

Clinical signs of lameness and therefore, laminitis, did not occur at any point during or after the experiment. A failure to induce lamellar pathology with intra-osseously administered insulin was confirmed with histological examination of both the treated and control feet. Samples were prepared for histology in the same manner as described in chapter one.

Periodically throughout the infusion (0.02 mL/min) the horses experienced moderate discomfort in both fore-feet, which was evidenced by pawing and weight shifting. The horses were treated with a non-steroidal analgesic injection (flunixin meglumine at 1.1 mg/kg IV). This abolished foot pain in all
horses. The infusion was ceased until pain relief was achieved. All of the horses were sound at the walk at the 24 h and 48 h time-points.

Appetite, heart rate (38 ± 2.7 bpm), respiratory rate (12 ± 1 breaths per min) and rectal temperature (37.8 ± 0.2 ºC) did not change throughout the experiment and all of the horses remained clinically normal. Blood and urine test results were within the accepted reference ranges for the duration of the experiment for all horses (Olson et al., 1993). Blood glucose concentrations from jugular (5.6 ± 0.2 mM) and digital vein samples did not change (left; 5.5 ± 0.5 mM and right; 5.9 ± 0.5 mM), in any horse throughout the experimental period. Supplemental glucose administration was not required at any stage.

Median jugular serum insulin concentrations (13.0 (8.4 - 15.3) µIU/mL) were not increased and did not differ from either the right or left digital vein samples (Figure 4.2). The median digital serum insulin concentration of the insulin-treated foot (left fore) increased ($p < 0.05$) from 6.1 (5.0 - 7.0) µIU/mL at the start of the experiment to 15.3 (12.6 - 31.1) by 48 h. The serum insulin concentration of the control foot (right fore) remained unchanged; 6.8 (5.4 - 9.1) µIU/mL at the start and (5.6 (5.2 - 9.6) µIU/mL) at 48 h. The insulin concentration of the right forefoot was lower ($p < 0.05$) than the left forefoot (Figure 4.2).

The ambient temperature remained constant at 16.7 ± 0.24 ºC throughout the experiment. Treated foot HWST (left fore) was high at 29.2 ± 0.35 ºC but did not differ from the control forefoot (right) which was treated also high (28.9 ± 0.57 ºC).

**Figure 4.2** Serum insulin concentration was measured in blood samples taken from the jugular vein (⧫) and the left (∆) and right (⊘) digital veins of Standardbred racehorses (n = 6) following IOIDP with insulin into the left forefoot.
Study two: Intra-osseous infusion of ATP-MgCl₂ into a single foot

Horses

Six clinically healthy (6 geldings, mean bodyweight 430 ± 17 kg; mean age 5.2 ± 0.7 years) Standardbred racehorses in moderate body condition (BCS: 4.5/9; (Henneke et al., 1983) were used for study two. No abnormalities were detected on physical examination. All horses were sound on lameness examination and veterinary and radiographic examination of their feet revealed no abnormalities. Radiographs were taken again after insertion of the IOIDP needle to ensure correct placement. They were fed medium quality lucerne hay and chaff for 48 h preceding, and during the study. The experiment was performed in a climate controlled facility to ensure a constant ambient temperature. Blood sampling procedures were as outlined for study one.

Intra-osseous infusion of the distal phalanx

The placement of the IOIDP needles proceeded as described in study one. ATP-MgCl₂ acts on the purinoceptors in the cells of the foot vessels to cause vasodilation (Burnstock and Kennedy, 1986). It has been used safely as an IV infusion in horses previously at a dose rate up to 0.3 mg/kg/min (Tetens et al., 1999). A lower dose rate that has been shown to be sufficient to produce peripheral vasodilation was used in the current study (Tetens et al., 1999) to avoid potential unwanted hypotension. The left fore received an infusion of ATP-MgCl₂ and the right fore received an infusion of 0.9% saline. The ATP-MgCl₂ was prepared fresh or stored for up to 2 weeks at -20 °C. The pH was adjusted to 7.4 to prevent tissue reaction to the solution. The solution was sterilised with a 0.22 µm filter (Millipore) prior to use or storage. The infusions were commenced at a constant rate of 0.01 mL/min in both the left and right fore feet. The dose rate of ATP-MgCl₂ was 0.11 mg/kg/min.

Hoof wall surface temperature

As for study one, non-invasive surface thermistors were used to measure hoof wall surface temperature (HWST). Temperature readings were logged by the thermistors every min throughout the infusion period. The ambient temperature was kept constant at all times.

Statistical analyses

Change in insulin, glucose, heart and respiratory rates and body temperature were analysed with repeated measures ANOVA as for study one. HWST data over time was also analysed with a repeated measures ANOVA, with ambient temperature included as a covariate in the analysis. Single time-point HWST was compared between the treated and control limbs, or between basal and infusion time-points, using an unpaired and paired t-test respectively.

Results

Lameness did not occur at any point during the study in any horse. Normal lamellar architecture was found during histological examination of both front feet from all horses. The intra-osseous infusion of ATP-MgCl₂ was better tolerated than the insulin infusion. Pain relieving non-steroidal therapy was only required in four horses. This occurred after changing the springfusor at the 24 h time-point. Appetite, heart rate (37.2 ± 1.97 bpm), respiratory rate (12.0 ± 1.97 breaths per minute) and rectal temperature (37.7 ± 0.22 °C) did not change throughout the experiment and all of the horses remained clinically normal. Blood and urine test results were within the accepted reference ranges for the duration of the experiment for all horses (Olson et al., 1993). Blood glucose (5.4 ± 0.22 mM) and
serum insulin (15.0 ± 2.89 µIU/mL) concentrations from jugular vein samples did not change throughout the experimental period.

The foot treated with ATP-MgCl₂ (left fore) had a mean ± se HWST of 29.4 ± 0.25 ºC. This was higher (p < 0.05) than the foot treated with 0.9% saline (right) which was 27.5 ± 0.38 ºC. The HWST of both feet was similar at the start of the experiment (Figure 4.3). However, during the first 4 h of the experiment the treated foot HWST increased (p < 0.05) and remained high for the remainder of the experiment. In contrast, the HWST of the control foot did not change significantly from basal levels (Figure 4.3). Ambient temperature remained constant (17.1 ± 0.31 ºC).

![Figure 4.3 The mean ± se HWST of the left and right fore feet of Standardbred horses (n = 6). The left fore was treated with ATP-MgCl₂ and the right with 0.9% saline.](image)

**Conclusion**

Although serum insulin concentrations were increased in the left forefoot as a consequence of the insulin IOIDP, this increase was well below the degree of hyperinsulinaemia that is induced during the p-EHC. Furthermore, the left forefoot serum insulin concentration was not higher than the jugular serum insulin concentration. The failure to attain the same level of hyperinsulinaemia as a p-EHC in the left digit resulted in a failed experiment. Laminitis was not produced in the treated limb, but this does not rule out that higher serum insulin concentrations in the digit would be laminitogenic.

The highly vascular nature of the digit may have contributed to the failure to attain and maintain supraphysiological hyperinsulinaemia. Rapid venous drainage coupled with probable loss through lymphatic vessels might have led to an inability of the infused insulin to remain in the lamellar circulation sufficiently long enough to saturate the area. Alternatively, the dose rate of insulin might have been too low. Further work on the insulin dose rate required to achieve serum insulin concentrations of > 1000 µIU/mL in the equine digit are required. A repetition of this study needs to
be performed after pilot studies can ascertain an appropriate dose rate for insulin. This work was beyond the timeframe and financial capacity of this project.

The second IOIDP study aimed to determine if persistent digital vasodilation would induce laminitis in the vasodilated limb in the absence of insulin. Insulin receptors have only been found to be present in the vasculature of the digit (Burns, 2011) so the hypothesis that insulin causes laminitis by a vascular mechanism required investigation. The results of study two showed that persistent vasodilation for 48 h (induced by ATP-MgCl₂) did not cause any lamellar pathology. Thus, this study disproved the hypothesis that endocrinopathic laminitis is entirely due to a vascular mechanism. Persistent vasodilation may play a role in laminitis development by increasing the delivery of other substances such as glucose to the digit, or it may be a benign consequence of the disease.

IOIDP has been confirmed as a feasible technique for the study of laminitis pathophysiology. The technique requires smaller sample sizes as each horse acts as its own control, which reduces the costs associated with equipment and medications and importantly, results in less stock usage. However, some refinement of the technique is still required. Following these studies the contribution of glucose to the development of laminitis still required further investigation.
5. Hyperglycaemic clamp

Introduction

Glucose has been shown to be integral to lamellar health (Pass et al., 1998). However, the insulin-independent nature of the lamellae (Asplin et al., 2011) means that they are unlikely to be susceptible to interruptions in glucose supply and therefore glucose starvation. Alternatively, this insulin-independent status may result in overzealous glucose metabolism during periods of increased supply. Glucotoxicity is a feature of diabetes mellitus in humans (Poitout and Robertson, 2002) and although horses rarely seem to develop diabetes (Johnson et al., 2005) they may still experience periods of excessive glucose uptake in tissues rich in insulin-independent glucose transporters (GLUT-1).

Endocrinopathic laminitis is frequently linked with the consumption of inappropriate quantities of grasses rich in non-structural carbohydrates (Harris et al., 2006; Treiber et al., 2008). This increased dietary intake of glucose may play a role in disease development in cases of pasture-associated laminitis. Furthermore, the p-EHC requires large quantities of glucose to be administered concurrently with the insulin in order to prevent hypoglycaemia (chapter one). If increased glucose uptake leading to glucotoxicity is demonstrated to contribute to laminitis development, then medications that limit glucose uptake in certain tissues may be beneficial in the treating the condition.

The p-EHC produces serum insulin concentrations in excess of 1000 µIU/mL. However, in naturally occurring cases of laminitis serum insulin concentrations are infrequently observed at this upper level. The p-EHC does not allow investigation of the threshold for the onset of lamellar pathology. It is likely that laminitis development in horses and ponies occurring secondary to hyperinsulinaemia is influenced by many factors including breed, genetic variation, bodyweight, concurrent illness and individual susceptibility to the disease. An appreciation of the approximate serum insulin concentrations that precipitate disease would greatly enhance management of laminitis.

Separating the roles glucose and insulin play in laminitis is difficult due to their complementary roles in the integration of fuel metabolism (Guyton, 1991). The aims of the current study were two-fold; to determine the endogenous pancreatic response of insulin-sensitive Standardbred racehorses to a continuous infusion of glucose over 48 h, and to determine the effect of this glucose on the lamellae. The glucose infusion would facilitate an examination of the capacity of the equine pancreas to respond to glucose overload. Further, the effects of this glucose overload on the lamellae could be investigated. By administering the same amount of glucose as is infused during a p-EHC, the effect of that component of the p-EHC could be assessed in the absence of exogenous insulin. If insulin-induced laminitis occurs purely as a result of glucose overload in an insulin-independent tissue then laminitis should also result following dosing with the glucose given during a laminitis-inducing p-EHC.

Horses

Seven Standardbred racehorses (male; 417 ± 16 kg BW; 6.3 ± 0.74 years; BCS 4.3/9) purchased in South-East Queensland were used in this study (Figure 5.1). No abnormalities were detected on physical examination, blood or urine tests prior to the study. All horses were sound on lameness examination and veterinary and radiographic examination of their feet revealed no abnormalities. They were fed medium quality lucerne hay and chaff for 7 days preceding, and during the study. The horses were randomly assigned to the treatment group (n = 4) and a control group (n = 3). Extended-use intravenous (IV) catheters (14 gauge; MilaCath) were aseptically placed and sutured in both jugular veins of each horse and used for the administration of the infusions (right) and blood sampling (left).
Hyperglycaemic Clamp

The hyperglycaemic clamp (HC) was adapted from the technique previously described in humans (DeFronzo et al., 1979) and modified to be administered as a constant-rate infusion rather than a variable rate. A constant-rate glucose infusion of 0.68 mL/kg/h was used to cause hyperglycaemia over 48 h. The horses in the control group received an infusion of balanced electrolyte solution (0.68 mL/kg/h; Hartmanns, Baxter) for 48 h.

Blood samples were collected from all horses before and at regular intervals throughout the experiment in order to determine blood glucose and serum insulin concentrations (Figure 5.2). Blood was placed in plain vacutainers (Vacuette, Greiner Bio-One), left to clot at room temperature for 30 min and then centrifuged (10 min at 3000 x g). Serum was separated into aliquots (1 mL) and stored at -80 °C until analysed. Blood glucose was measured using a hand held glucometer (Accucheck Go, Roche), calibrated against the hexokinase method using blood from these horses (data not shown). Samples were taken every 15 min during the first hour to check blood glucose. Once stable blood glucose was analysed every 30 min until euthanasia to ensure hyperglycaemia was not excessive.

Serum insulin concentrations were measured in duplicate using a radioimmunoassay kit (Siemens, Coat-a-count) previously validated for use in horses (McGowan et al., 2008). Insulin samples obtained during the clamp were diluted in insulin-free serum to avoid intolerance problems associated with the assay (Tinworth et al., 2009). Free catch urine samples were collected prior to, and during the experiment in order to detect the presence of glucose in the urine using dipsticks (Multistix, Roche).

Insulin sensitivity

The insulin sensitivity of the four horses treated with glucose was assessed using an approximated HC. A steady-state period during the first 2 h of the glucose infusion allowed assessment of insulin sensitivity to be made according to previously published equations (Rijnen and van der Kolk, 2003).
Histology

Following euthanasia, all four limbs of all horses were disarticulated at the fetlock and sectioned with a bandsaw to produce sagittal blocks of lamellar tissue. These blocks were rinsed in cold water and trimmed to produce 5 mm by 5 mm squares of lamellar tissue with a small sliver of hoof wall included. Lamellar tissue was then placed in 10% neutral buffered formalin for 24 h before being embedded in paraffin by routine methods. Paraffin blocks were sectioned and stained with H&E and PAS ready for histological analysis.

Measurements of SEL length and width were made in the front feet of all horses. The length of ten SELs in the axial region of each of eight PELs was measured in the dorsal mid-section of each forefoot. The width of twenty SELs was measured on all eight PELs for each horse. The measurement protocol has been validated (de Laat et al., 2011c).

Statistical analyses

Blood glucose and serum insulin concentrations were compared within (paired) and between (unpaired) groups with a $t$-test. The presence or absence of lamellar histopathology was assessed as an outcome using Fisher’s exact probability test. Histometric measurements from each forefoot were averaged to obtain a single value for each horse and compared between groups using a Welch $t$-test. All data are presented as means ± se and statistical significance was accepted at $p < 0.05$. Statistical analyses were performed using R, version 7.2.7.

Results

None of the horses treated with glucose developed clinical signs of laminitis or lameness. The control horses were also not lame at any time. The appetite, heart and respiratory rates remained within normal limits for all horses and did not differ between the groups. Routine haematology and biochemistry blood tests results did not change from initial values as a consequence of either infusion. However, all of the glucose-treated horses developed glycosuria, hyperglycaemia and hyperinsulinaemia (Table 5.1) whereas none of the control horses did. Serum insulin concentrations in the treated horses increased ($p < 0.05$) above the control horses within the first 4 h of the infusion and remained elevated for the infusion period (Figure 5.2). All of the glucose treated horses were insulin sensitive (Table 5.1).

Table 5.1 The blood glucose, serum insulin and insulin sensitivity results for horses treated with a 48 h infusion of glucose (n = 4) or 0.9% saline (control; n = 3).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal blood glucose (mM)</td>
<td>5.7 ± 0.43</td>
<td>5.1 ± 0.13</td>
</tr>
<tr>
<td>Infusion blood glucose (mM) *</td>
<td>10.7 ± 0.78</td>
<td>5.0 ± 0.15</td>
</tr>
<tr>
<td>Basal serum insulin (µIU/mL)</td>
<td>7.84 ± 0.29</td>
<td>8 ± 0.58</td>
</tr>
<tr>
<td>Infusion serum insulin (µIU/mL) *</td>
<td>208 ± 26.1</td>
<td>10.6 ± 1.36</td>
</tr>
<tr>
<td>M (mM/kg/min)</td>
<td>0.039 ± 0.0012</td>
<td>---</td>
</tr>
<tr>
<td>M-to-I ratio (x 100)</td>
<td>0.014 ± 0.002</td>
<td>---</td>
</tr>
</tbody>
</table>

Key: * indicates if measurements differ ($p < 0.05$) between groups
Figure 5.2 Mean serum insulin concentration for horses treated with a glucose infusion (♦, n = 4) and control horses treated with a balanced electrolyte solution (♦, n = 3).

All four glucose-treated horses developed lamellar pathology consistent with the lesions documented in the developmental stages of laminitis (chapter two). The severity of the lamellar lesions varied between horses, with one horse only affected in one foot, two horses in both front feet and one horse in all four feet. The lesions included lengthening and narrowing of the SELs, variably increased numbers of mitotic figures, apoptotic bodies and leucocytes in the dermis, and cellular changes including rounding and disorientation of the EBC nuclei (Figures 5.3). The BM dysadhesion typical of acute laminitis (chapter three) was not apparent. The lamellae of control horses were unaffected. Morphometric measurements showed that significant ($p < 0.05$) increases in length and decreases in width occurred in the glucose-treated group compared with control horses (Table 5.2).

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Treatment group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEL length *</td>
<td>226 ± 23</td>
<td>145 ± 34</td>
</tr>
<tr>
<td>SEL width *</td>
<td>18.7 ± 0.75</td>
<td>31.8 ± 1.85</td>
</tr>
</tbody>
</table>

Key: * indicates if measurements differ ($p < 0.05$) between groups
Conclusion

Moderate endogenous hyperinsulinaemia (~ 200 µIU/mL) secondary to a continuous glucose infusion causes lamellar pathology consistent with the developmental stages of laminitis within 48 h. This result suggests that a large proportion of hyperinsulinaemic horses and ponies may in fact be affected by lamellar pathology that is yet to induce the clinical signs of laminitis. These animals would presumably be at a greater risk of developing acute laminitis than normoinsulinaemic horses and ponies. Thus, the results of this experiment not only confirm insulin as a key factor in endocrinopathic laminitis development, but have identified a potential threshold for laminitis development. The development of subclinical lamellar pathology commences at or below a serum insulin concentration of ~ 200 µIU/mL.

With respect to the second aim of this study, the results do not suggest that glucose is the key pathophysiological factor in triggering endocrinopathic laminitis. Glucose may contribute to laminitis development or complicate the chronic phase of the disease, but does not appear to induce acute laminitis in the absence of marked hyperinsulinaemia. Further studies on the role of glucose in chronic laminitis are warranted. Examination of role of glucose in extra-cellular matrix thickening is also necessary.
6. Insulin-like growth factor-1

Introduction

Insulin-like growth factor-1 (IGF-1) is a hormone that has a similar molecular structure and functional homology to insulin (Chitnis et al., 2008). IGF-1 can bind to its own receptors, the insulin-like growth factor-1 receptors (IGF-1R), and also to insulin receptors. IGF-1 will preferentially bind its own receptor but at high IGF-1 concentrations binding of IGF-1 to the insulin receptor can result. This can impair insulin from binding to its own receptor and cause insulin resistance (Ciaraldi and Sasaoka, 2010). Conversely, although insulin has a higher affinity for its own receptor, it will also bind the IGF-1R during hyperinsulinaemia (Ciaraldi and Sasaoka, 2010).

IGF-1 binding to its receptor causes some different effects in the body to insulin. Both insulin and IGF-1 are capable of stimulating glucose uptake, however, IGF-1 is not known to influence vascular tone like insulin does (Pavelic et al., 2007). A role for IGF-1 in the pathophysiology of endocrinopathic laminitis is possible, particularly if IGF-1 binding of insulin receptors occurs during hyperinsulinaemia. By determining whether IGF-1 is capable of inducing laminitis or not, the mechanism of insulin-induced laminitis could be further explained. More importantly, if IGF-1 is not involved in the pathogenesis of insulin-induced laminitis, it may prove to be useful in disease management or even treatment.

An infusion of IGF-1 into a single digit using IOIDP was planned for this study. However, following the failure of IOIDP with insulin, due to problems with rates of vascular clearance of infused substances, it was considered imprudent to proceed with IOIDP of IGF-1 at this stage. Further work on determining appropriate flow rates for IOIDP infusions is required before this work can be undertaken effectively. Instead it was decided to undertake preliminary laboratory analysis of pre-existing samples from the p-EHC to further determine the role of IGF-1 in insulin-induced laminitis. Thus, the aim of the current study was to investigate engagement of the IGF-1R in lamellar tissue at the mid-developmental and acute stages of insulin-induced laminitis, and compare this insulin receptor engagement.

Samples

Archived, frozen lamellar samples from the left fore foot of horses treated with a p-EHC for 24 h (mid-developmental phase; n = 4) and 48 h (acute phase; n = 4) and control horses treated with a balanced electrolyte solution for 48 h (n = 4) were used in the current study. The details of the p-EHC experiment are given in chapter one. Blocks (5 mm x 5 mm) of lamellar tissue were collected from the left fore of each horse immediately upon euthanasia, snap frozen in liquid nitrogen and stored at -80 °C until use.

PCR analysis

One microgram of total RNA was extracted from lamellar tissue (100 mg) with Trizol reagent (Invitrogen) according to the manufacturer’s instructions. RNA concentration was determined by UV spectrophotometry (absorbance; 230 nm). Decontaminated RNA samples were reverse-transcribed to cDNA using the Reverse Transcription System (Promega). cDNA samples from each horse were then analysed with qRT-PCR using SensiMix 2xSYBR Green Master Mix (Bioline) and the forward and reverse primers (designed using Primer3 v. 0.4.0 software) for the genes of interest (insulin receptor and IGF-1R) and two house-keeping genes (GAPDH and β2-microglobulin). Equine specific sequences for the insulin receptor and IGF-1R were not available on Genbank therefore the primer sets (Table 6.1) for the InsR were designed from the most conserved regions of human (HUMINSR), rat (RATINSAB) and mouse (MUSINSR) sequences, and of cattle (X54980), rat (RATIGFIR) pig
(AB003362) and human (X04434) sequences for IGF-1R. The PCR primer sets for two house-keeping genes (Table 11-1) were designed from equine-specific sequences (GenBank accession no: β2-microglobulin, X69083 and GAPDH, AF157626). The primers were synthesized by Integrated DNA technologies and their specificity checked with a Blast search.

The PCR reactions were run on a Corbett Rotor-Gene 6000 series (Qiagen) and the results expressed as cycle threshold (C_T) values. All PCR reactions were cycled on the same 100-well genediscs at 95 °C for 10 min, followed by 45 cycles of 15 s at 95 °C, 15 s at 58 °C and 30 s at 72 °C and included no-template controls. The PCR results were analysed using the 2^−∆CT method (Schmittgen and Livak, 2008).

Table 6.1 Primer sets and optimised primer concentrations for target genes (Insulin receptor and IGF-1R) and house-keeping genes (β2-microglobulin and GAPDH) for qRT-PCR in lamellar tissue from Standardbred horses.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
<th>Amplicon size (base pairs)</th>
<th>Primer conc. (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2-microglobulin</td>
<td>ACCCAGCAGAGAATGGAAAGC</td>
<td>CATCTTCTCTCCATTTCAGAAATGC</td>
<td>101</td>
<td>600</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAT TGT CAG CAA TGC CTC CT</td>
<td>AAG CAG GGA TGA TGT TCT GG</td>
<td>193</td>
<td>500</td>
</tr>
<tr>
<td>Insulin receptor</td>
<td>CGAGTGGATTATTGCTCTCAAAG</td>
<td>CGTACTCAGTCTGATTGTGCTCTG</td>
<td>101</td>
<td>600</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>TCCAGACAGGAGCTACAGGA</td>
<td>AGAAGACACGGGATCCGCTCA</td>
<td>127</td>
<td>600</td>
</tr>
</tbody>
</table>

**Statistical analyses**

The qRT-PCR data was not normally distributed (Shapiro-Wilk test) and was analysed non-parametrically. Gene expression was compared between groups using a Kruskal-Wallis ANOVA on ranks. Pair-wise comparisons were made with Tukey’s post-hoc test. All data are presented as median (interquartile range) and statistical significance was accepted at \( p < 0.05 \). Statistical analyses were performed using R, version 7.2.7.

**Results**

The horses treated with a p-EHC were hyperinsulinaemic (747 ± 41 µIU/mL for the 24 h group and 1036 ± 129 µIU/mL for the 48 h group) while the control horses were not (13.6 ± 2.9 µIU/mL). The insulin receptor and the IGF-1R were both expressed in the lamellar tissue of all horses. The hyperinsulinaemic horses had decreased \( p < 0.05 \) expression of both the insulin receptor and IGF-1 receptor compared to the control horses (Figure 6.1).
Conclusion

Tyrosine kinase receptors such as the insulin receptor and the IGF-1R become down-regulated (i.e. are endocytosed and degraded) when they are bound (Komada and Kitamura, 2005). Furthermore, the overstimulation of these receptors causes a reduction in gene expression so that fewer receptors are made. Both insulin receptor and IGF-1R expression was significantly decreased in lamellar tissue from the hyperinsulinaemic horses in the current study. This indicates that both of the receptors were overstimulated. It is reasonable to assume that the insulin receptor was being bound by the excessive concentrations of circulating insulin. The serum and lamellar IGF-1 concentration of these horses is unknown, so the IGF-1Rs were potentially being bound by either IGF-1, or insulin, or both. Analysis of IGF-1 concentrations from the p-EHC horses would be useful to further investigate the likely source of IGF-1R binding. However, an accurate assay for determining serum IGF-1 concentrations in horses is not currently available.

Regardless of the binding molecule, once bound the IGF-1R promotes cell survival through an increase in the rate of cell proliferation and reduction in the rate of apoptosis (Pavelic et al., 2007). This is a potential mechanism for the increased length of the SELs documented in chapter two. Lamellar epithelial cells incubated in increasing concentrations of insulin show a dose-dependent increase in cell proliferation (Bailey and Chockalingham, 2009) which supports this theory. Thus, the IGF-1R potentially plays a crucial role in endocrinopathic laminitis pathophysiology.

Further work on the role of the IGF system in insulin-induced laminitis is crucial to furthering our understanding of the disease mechanism. This work has provided a clear direction for future studies. Furthermore, the results of the current study have illustrated that IGF-1 itself would not be useful in disease management or treatment. However, techniques to selectively block the IGF-1R are being trialled in human medical research as an adjunct to cancer therapy (Atzori et al., 2009). Once more is understood about the role of the IGF-1R in the disease, these receptor blockers may prove to be useful in the management of endocrinopathic laminitis in the future.
Results

This project has thoroughly investigated the mechanism of insulin-induced laminitis in Standardbred racehorses and the results have met each of the revised objectives set for the project. The methodical approach of this project to investigating the pathophysiology of laminitis occurring secondary to hyperinsulinaemia in horses has led to a significant bank of data now being available on the subject. The findings from this work have defined a new potential threshold for the onset of subclinical lamellar pathology in the equine population and have considerably narrowed down the likely pathophysiological events that lead to lamellar failure during hyperinsulinaemia. Furthermore, there is an opportunity for horse owners and veterinarians to improve their management and prevention strategies for the disease in a guided manner and potential for new therapeutic options to be developed following further work.

Hyperinsulinaemia induced laminitis in all young Standardbred racehorses treated with a p-EHC \( (n = 4) \). Clinical signs of laminitis commenced at \( 31.5 \pm 4.65 \) h and progressed to Obel grade 2, acute laminitis \( (\text{Obel, 1948}) \) by \( 46 \pm 2.31 \) h. The time to onset of laminitis was shorter in horses \( (48 \text{ h}) \) than ponies \( (~55 \text{ h}) \) and indicated that insulin-sensitive horses of higher bodyweight were just as, or more susceptible to the disease than ponies. All of the horses treated with a p-EHC were insulin-sensitive, as determined by standard insulin sensitivity calculations \( (\text{Rijnen and van der Kolk, 2003}) \), which suggests that tissue resistance to insulin is not required for disease development, just elevated serum insulin concentrations. Significant lamellar pathology consistent with prior descriptions of laminitis \( (\text{Pollitt, 2004}) \) was confirmed histologically in all horses, and affected all feet in the three heavier horses and three feet in the horse with the lowest bodyweight. Thus, the characteristics of the p-EHC model were fully described in Standardbred racehorses fulfilling a key objective of the project.

Histological evaluation of the lamellar pathology induced by the insulin infusion revealed that some subtle differences to other experimental models of the disease exist. A deeper investigation of the pathological events occurring during the developmental (pre-clinical) and acute phases of the disease further described the histopathological appearance of insulin-induced laminitis. Adaptation of the p-EHC allowed temporal analysis of three time-points during the developmental phase of the model when the lamellar pathology was commencing and key steps in disease development were occurring. Structural changes to the SELs commence early. SEL length started to increase only 6 h after the onset of marked hyperinsulinaemia and this lengthening progressed throughout the developmental phase until it became significant at 48 h, when clinical signs of the disease appeared. Likewise, SEL width started to decrease within 6 h and continued to narrow. These changes to lamellar structure are likely to be key factors that weaken the attachment of the inner hoof wall to the distal phalanx, thereby precipitating lameness. Cellular changes also commenced early. Mitosis gradually increased as the lesions progressed and potentially contributed to increases in lamellar length. Nuclear disorientation and rounding may indicate that support for the EBCs was lost and support the notion of weakening of the lamellar structure leading to lamellar failure.

Damage to the lamellar BM was not as pronounced in insulin-induced laminitis as alimentary forms of the disease. Widespread and catastrophic BM demise was a feature of ACO and accompanied marked lameness. Although the horses treated with a p-EHC experienced Obel grade 2 laminitis, their lamellar BM disruption was less widespread than in the ACO model. Further, BM damage during hyperinsulinaemia appeared to occur by other means than enzymatic breakdown, which is also not consistent with ACO models of the disease \( (\text{Coyne et al., 2009; Kyaw-Tanner and Pollitt, 2004}) \). Physical disruption secondary to excessive lengthening of the SELs may have occurred and if reductions in the rate and degree of SEL lengthening can be achieved, BM disruption may be able to be managed or prevented.

Insulin-induced laminitis also produces a less inflammatory lesion that other experimental models of the disease \( (\text{de Laat et al., 2011c; Faleiros et al., 2009}) \). Further corroboration of this finding with lamellar analysis of inflammatory
mediators is required. However, if inflammation is a minor player in insulin-induced laminitis pathophysiology then anti-inflammatory medications may only be useful for their analgesic properties in this form of laminitis, and may not alter progression of the disease. Examination of the lamellar pathology in this project has been thorough and illuminating. The objectives of 1) describing lamellar lesions before and during disease onset and comparing them to laminitis triggered by other factors and 2) determining the role of degradative enzymes and inflammation in lamellar pathology, have been met.

The other major finding from the p-EHC experiments was that the horses that developed laminitis experienced persistently elevated HWST of their feet, which was presumed to reflect digital vasodilation. This was in contrast to the control horses that had variable HWST over the infusion period. Thus, a vascular component to the disease had been described. Further investigation of the role of the persistent digital vasodilation using IOIDP demonstrated that insulin-induced laminitis does not occur purely as a result of a vascular mechanism. However, the vasculature may contribute to disease progression. Thus, the objective of determining the role of blood flow before, during and after laminitis has been fulfilled.

The development of the IOIDP technique for studying laminitis pathophysiology not only enabled the investigation of a possible blood flow mechanism for the disease but will, with further refinement, accelerate the rate of future progress in laminitis research. The technique reduces labour costs, stock usage and improves animal welfare, all of which were aims of the project. In the future, further research into the role of the IGF system in hyperinsulinaemic lamellar pathology will be aided by single limb inductions methods.

This project has found that the IGF system is implicitly involved in the pathogenesis of endocrinopathic laminitis and future research needs to be directed at examining this pathway further. Importantly, the current results have discounted IGF-1 as a potential treatment strategy for endocrinopathic laminitis. Alternatively, blockers of the IGF-1R hold future promise as a potential therapy for treatment and/or prevention of the disease.

Overall, significant progress in our understanding of hyperinsulinaemic laminitis has been achieved as a result of this project. Australia continues to be at the forefront of laminitis research, providing clear directions for future study and new hope for the horse owning community.
Implications

The outcomes of this project should enable significant improvements in the management of cases of endocrinopathic laminitis. Hyperinsulinaemia is the key pathogenic factor of endocrinopathic laminitis and a common denominator linking other conditions that include laminitis amongst their clinical signs; equine Cushing’s disease, equine metabolic syndrome and iatrogenic hypercortisolaemia. Horse owners and veterinarians now have a target serum insulin concentration which they can use as a benchmark for management of insulin-resistance and prediction of likely subclinical lamellar pathology.

With inappropriate foot vasodilation, increased metalloproteinase activity and inflammation largely removed from the therapeutic equation, the veterinary practitioner can concentrate on the core issue of normalising hyperinsulinaemia. All efforts to reduce the incidence of endocrinopathic laminitis in the equine population can now focus of the reduction of serum insulin concentrations. Management of plasma cortisol levels and analysis of the thyroid hormone axis are less crucial than reducing hyperinsulinaemia.

The effects of graded exercise and dietary regimens that reduce adiposity and insulin resistance are already proven. In field cases, the research implies that lamellar damage may be gradual in onset and less catastrophic than the septic/inflammatory forms of laminitis. Thus, there is the reasonable expectation, especially in the acute and early chronic stages, that hyperinsulinaemic laminitis may be more responsive to therapy, with better potential for restoration of functional lamellar anatomy.

The uncontrolled lamellar cell proliferation and attenuation that hyperinsulinaemia triggers via our proposed pathogenic role for IGF-1R resembles the proven role IGF-1R has in human and animal malignant neoplasia. Agents that block IGF-1R activity are already under trial as anticancer therapies and for laminitis, offer a hopeful new line of treatment options worthy of investigation and new funding.

Losses to the horse industry from endocrinopathic laminitis should be lessened if the findings of this project are used to better manage the condition. Fewer laminitis related deaths will lower the emotional impact of the condition and improved management and prevention strategies for the disease should reduce the financial impact on the horse industry.

Future development of targeted treatment protocols for the condition is required. Results from this project indicate that research funding for endocrinopathic laminitis should be directed at an investigation of how the IGF system promotes lamellar failure. Investigations of this nature may lead to therapeutic options for laminitis via IGF-1 receptor blockers.

The investment by RIRDC in this project will enable veterinarians and horse care-givers to diagnose laminitis earlier than ever before. This will potentially prevent an estimated 6% of horses and ponies developing the disease and save the horse industry countless thousands of dollars. The financial burden of treating laminitis, often for very long periods, will be significantly reduced.
Recommendations

Further development of single limb induction techniques for the study of laminitis is required. The IOIDP technique has the potential to greatly enhance our comprehension of key pathophysiological features of the disease, while reducing the impact of laminitis research on horses. Investigation of the ability of a local IGF-1 infusion to promote the development of lamellar pathology is central to progressing the research in this area.

Lamellar IGF research needs future support and funding as it has the greatest potential for the development of novel treatments strategies for laminitis.

In order to reduce the incidence of endocrinopathic laminitis in the equine population of Australia, several recommendations can be made following this study:

- determine serum insulin concentration as a priority in horses and ponies that are overweight or have a phenotype consistent with equine metabolic syndrome (Frank et al., 2010) or are laminitis prone;
- ensure serum insulin concentrations are assayed according to a validated protocol;
- reduce serum insulin concentrations in horses and ponies with hyperinsulinaemia (e.g. with equine metabolic syndrome, insulin resistance or equine Cushing’s disease) well below 200 µIU/ml with modified diet and exercise regimes;
- maintain a low bodyweight and ensure body condition scores are in the optimal range (3-5/9);
- monitor pasture species and seasonal variation of pasture growth and to avoid excessive consumption of non-structural carbohydrate rich pasture;
- closely monitor serum insulin concentrations throughout the year to ensure continued compliance.
Appendix

The publications that have arisen from the research described in this report are listed below.


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>3’</td>
<td>three prime end of primer sequence</td>
</tr>
<tr>
<td>5’</td>
<td>five prime end of primer sequence</td>
</tr>
<tr>
<td>ACO</td>
<td>alimentary carbohydrate overload</td>
</tr>
<tr>
<td>ADAMTS</td>
<td>a disintegrin and metalloproteinase with thrombospondin repeats</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP-MgCl₂</td>
<td>adenosine tri-phosphate magnesium chloride</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BCS</td>
<td>body condition score</td>
</tr>
<tr>
<td>BM</td>
<td>basement membrane</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>C&lt;sub&gt;T&lt;/sub&gt;</td>
<td>cycle threshold</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>distilled, deionised water</td>
</tr>
<tr>
<td>EBC</td>
<td>epidermal basal cell</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3’phosphate dehydrogenase</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HC</td>
<td>hyperglycaemic clamp</td>
</tr>
<tr>
<td>HD</td>
<td>hemidesmosome</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>haematoxylin and eosin</td>
</tr>
<tr>
<td>HWST</td>
<td>hoof wall surface temperature</td>
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<tr>
<td>IGF-1</td>
<td>insulin-like growth factor-1</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>insulin-like growth factor-1 receptor</td>
</tr>
<tr>
<td>IOIDP</td>
<td>intra-osseous infusion of the distal phalanx</td>
</tr>
<tr>
<td>IQR</td>
<td>interquartile range</td>
</tr>
<tr>
<td>IV</td>
<td>intravenous</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>kV</td>
<td>kilovolts</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>M</td>
<td>amount of glucose metabolised</td>
</tr>
<tr>
<td>µIU</td>
<td>micro international unit</td>
</tr>
<tr>
<td>µm</td>
<td>micrometres</td>
</tr>
<tr>
<td>µmol</td>
<td>micro moles</td>
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<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mIU</td>
<td>milli international units</td>
</tr>
<tr>
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<td>milligram</td>
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<td>millilitre</td>
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<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Meaning</td>
</tr>
<tr>
<td>--------------</td>
<td>---------</td>
</tr>
<tr>
<td>M-to-I</td>
<td>amount of glucose metabolised per unit of endogenous insulin</td>
</tr>
<tr>
<td>n</td>
<td>sample size</td>
</tr>
<tr>
<td>nm</td>
<td>nanometres</td>
</tr>
<tr>
<td>nM</td>
<td>nanomolar</td>
</tr>
<tr>
<td>p</td>
<td>probability</td>
</tr>
<tr>
<td>PAS</td>
<td>periodic acid Schiff</td>
</tr>
<tr>
<td>PDLs</td>
<td>primary dermal lamellae</td>
</tr>
<tr>
<td>p-EHC</td>
<td>prolonged-euglycaemic, hyperinsulinaemic clamp</td>
</tr>
<tr>
<td>PELs</td>
<td>primary epidermal lamellae</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real time-polymerase chain reaction</td>
</tr>
<tr>
<td>R</td>
<td>statistical software package</td>
</tr>
<tr>
<td>RIU</td>
<td>relative intensity units</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SDLs</td>
<td>secondary dermal lamellae</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SELs</td>
<td>secondary epidermal lamellae</td>
</tr>
<tr>
<td>SI</td>
<td>insulin sensitivity</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of matrix metalloproteinases</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline with tween</td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
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</table>
References


Equine laminitis is common and costly. The incidence of laminitis associated with hormonal dysfunction (termed endocrinopathic laminitis) is on the rise. Obesity, sedentary lifestyles and improved pasture species are all contributing to rising serum insulin concentrations in affected horses and ponies, and as a result, more laminitis.

This research investigates the mechanism of laminitis occurring secondary to elevated serum insulin concentrations in insulin-sensitive horses.

Horse owners, veterinarians and scientists armed with this new knowledge now have better strategies to identify horses at risk of laminitis, to prevent laminitis from occurring, and to treat the condition early in its development.

While this new knowledge provides an immediate avenue for better prevention and management of laminitis, the development of new treatments will require further understanding of the laminitogenic potential insulin.

The investment by RIRDC in this project will enable veterinarians to diagnose laminitis earlier than ever before thus potentially preventing an estimated 6% of horses and ponies developing the disease. The financial burden of treating laminitis, often for very long periods, will thus be significantly reduced.

This report is an addition to RIRDC’s diverse range of over 2200 research publications and it forms part of our Horse R&D program, which aims to assist in developing the Australian horse industry and enhancing its export potential.

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