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The effects of locally-delivered insulin on equine hoof lamellar tissue

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Abstract

The equine hoof lamellar tissue is a highly specialized structure that plays a critical role in the suspensory apparatus of the distal phalanx (SADP). Failure of this normal attachment between distal phalanx and hoof is a major consequence of laminitis. Several conditions have been associated with laminitis in the past, but those related to insulin resistance (IR) or excess of glucocorticoids appear to be involved in the majority of the reported cases. The common factor in these conditions is hyperinsulinemia. Besides the main role of insulin in regulating glucose metabolism, it is involved in diverse physiologic process such as vascular modulation and cellular growth and proliferation. The role of insulin in laminitis has been assessed by different authors and new experimental model has arisen recently to induce laminitis after systemic administration of high concentrations of the hormone. However, the exact mechanism involved in the pathophysiology of the disease remains unclear.

Utilizing the minimally invasive technique of tissue microdialysis, this work aimed to evaluate the local effects of insulin, delivered in higher than normal concentrations into the lamellar interstitial fluid, by assessing the suggested potential mechanisms involved in this form of the disease: vasodilation, derangements of energy metabolism, and epidermal cellular proliferation. The latter was assessed using 5-ethynyl-2'-deoxyuridine (EdU), a novel marker of mitotic activity.

Insulin was delivered locally to the forefeet of healthy, Standardbred horses using one of the limbs as treatment (n=6) and the contralateral as control (n=6). Although they did not reach significance, changes in lamellar glucose metabolism (decreased interstitial glucose) were observed after delivering an approximated amount of 0.5 µg/ml for 24h at 1 µL/min via microdialysis to the sublamellar tissue. No variations were detected in the other measured metabolites (lactate, pyruvate and urea). The local delivery of insulin did not affect the microcirculation of the lamellae (no changes in urea clearance). These results support the hypothesis of insulin modifying lamellar cellular metabolism by increasing glucose uptake and consumption. This metabolic shift may be relevant in terms of modifying the stability of the cytoskeleton of the epidermal basal cells (EBCs) and the organization of lamellar tissue.

To determine if high concentrations of insulin delivered locally affected the proliferation rate of the EBCs, a second experiment was performed in eight healthy, Standardbred horses. The microdialysis protocol was completed as for the six initial horses. After insulin was delivered for 24h, the proliferation marker EdU (5-ethynil-2'-deoxyuridine) was administered to both forefeet (control and treatment). The EdU technique was developed and standardized as part of this project for the study of the lamellar proliferation. Significative changes in the proliferation rate or total number of cells were found after 24h of local delivery of high concentrations of insulin to the lamellar tissue. These changes were restricted to the tissue sections harvested from close to the microdialysis membrane (L1) of the treatment group. A histomorphometric analysis was added to the experimental protocol to examine changes in the organization of the lamellae. The primary epidermal lamellae (PELs) and the non-keratinized tip of the PELs (NKPELL) measured significantly longer in the tissue sections of the treatment group containing the microdialysis probe. No changes were detected in the length of the secondary epidermal lamellae (SELs) or the keratinized axis of the PELs (KPELL). These results suggest a possible direct effect of insulin on lamellar tissue organization, which could be responsible for weakening of the SADP as was hypothesized by previous authors.

This project has contributed to the understanding of the mechanism involved in the pathophysiology of insulin-induced laminitis. Despite the results being not completely conclusive, this work is the first to assess the direct local effect of insulin in the lamellar tissue and provides new questions and directions for future research. Further work should be focused on the study of the concentration and transport of insulin in the lamellar tissue as well as the interactions between insulin and its receptors in the lamellae. The study of the relationship between hyperinsulinemia, the cytoskeleton of EBCs and the failure of the SADP will contribute to the understanding of the insulin-induced laminitis model.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

Peer-reviewed papers

E.M. Castro-Olivera; C.C. Pollitt; C.E. Medina-Torres; J.I. Al-Alawneh; C. Underwood; A.W. van Eps (2015) The effect of locally delivered exogenous insulin on equine lamellar histomorphometry and epidermal cell proliferation. *Submitted to The Equine Veterinary Journal*.

E.M. Castro-Olivera; C.C. Pollitt; C.E. Medina-Torres; C. Underwood; A.W. van Eps (2015) The effect of locally delivered exogenous insulin on equine lamellar perfusion and glucose metabolism. *Submitted to The Equine Veterinary Journal*.

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Publications included in this thesis

The following paper has been submitted for publication and is incorporated into the thesis as chapter 2:

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Contributor	Statement of contribution
Matias Castro-Olivera (Candidate)	Designed experiments (65%) Performed experiments (100%) Wrote the paper (100%)
Carlos E. Medina-Torres (Candidate)	Assisted with performing experiments (30%) Edited the paper (5%)
John Al-Alawneh	Assisted with statistical analysis (100%)
Claire Underwood	Assisted with performing experiments (15%) Edited the paper (5%)
Christopher C. Pollitt	Assisted with performing experiments (25%) Designed experiments (5%) Edited the paper (20%)
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Contributor	Statement of contribution
Matias Castro-Olivera (Candidate)	Designed experiments (65%) Performed experiments (100%) Wrote the paper (100%)
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Claire Underwood	Assisted with performing experiments (15%) Edited the paper (5%)
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Matias Castro-Olivera (Candidate)	Designed experiments (50%) Performed experiments (100%) Histological analysis (80%) Wrote the paper (100%)
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Christopher C. Pollitt	Assisted with performing experiments (100%) Designed experiments (40%) Histologic analysis (20%) Edited the paper (50%)

Contributions by others to the thesis

The original idea of delivering insulin via microdialysis to the lamellar tissue as well as the experimental design were responsibility of my three supervisors: Dr Andrew van Eps, Prof. Christopher Pollitt and Prof. Martin Sillence, and I. Execution of the experiments and completion of the research plan were primarily my responsibility, always under the supervision of my supervisors. Dr Andrew van Eps was present and actively participated during the conduction of the trials. Prof Pollitt assisted and was my main advisor during the completion of the experiments reported in Chapter 4. Rajesh Gupta and Prof Martin Sillence assisted with the initial insulin determinations at the Central Analytical Research Facility (CARF) – Queensland University of Technology (QUT). Dr Claire Underwood contributed significantly to the project completing the UPLC insulin determinations at the University of Queensland, Gatton campus.

Chapters 2, 3 and 4 have been submitted for publication in international, peer-reviewed journals and subjected accordingly to their editing and proof- reading procedures.

Statement of parts of the thesis submitted to qualify for the award of another degree

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Keywords

Equine laminitis, insulin, microdialysis, cellular metabolism, energy metabolism, cellular proliferation, EdU, urea clearance, histomorphometry, hoof growth.

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List of Abbreviations used in Thesis

ACO – Alimentary carbohydrate overload

ACVIM – American College of Veterinary Internal Medicine

ADAMTS - A disintegrin and metalloproteinase with thrombospondin motif

AEC – Animal ethics committee

AGEs – Advance glycation endproducts

AHF – Animal Health Foundation

AVAs – Arteriovenous anastomoses

BM – Basement membrane

BP 180 – Binding protein 180

BP230 – Binding protein 230

BrdU – Bromodeoxyuridine

BWE – Black walnut extract

CARF – Central analytical research facility

CI – confidence interval

DAPI - 4',6-diamidino-2-phenylindole

DNA - Deoxyribonucleic acid

EBCs – Epidermal basal cells

ECF – Extra cellular fluid

ECS – Equine Cushing's Syndrome

EdU - 5-ethynil-2'-deoxyuridine

EMS – Equine metabolic syndrome

ET-1 – Endothelin 1

FITC – Fluorescent isothiocyanate

GH – Growth hormone

GLUT-1 Glucose transport protein 1

GLUT-12 Glucose transport protein 12

GLUT-3 Glucose transport protein 3

GLUT-4 Glucose transport protein 4

H&E - Hematoxylin and eosin

HPLC – High performance liquid chromatography

HWST – Hoof wall surface temperature

IGF-1 – Insulin growth factor one

IGF-1R – Insulin-like growth factor 1 receptor

IGF-2 – Insulin growth factor two

IGF-2R – Insulin growth factor 2 receptor

IL-1 – Interleukin one

IL-6 – Interleukin six

InsR – Insulin receptor

IR – Insulin resistance

IRR – Insulin receptor-related receptor

IRS – Insulin receptor substrate proteins

KPELL – Keratinized axis of primary epidermal lamellae length

LDH – Lactate de-hydrogenase

LS – Longitudinal section

MAPK – Mitogen-activated protein kinase

Max – Maximum

Min – Minimum

MMP – metalloproteinases

mRNA – messenger Ribonucleic Acid

MWCO – molecular weight cut-off

NKPELL – Non-keratinized tip of primary epidermal lamellae length

NO – Nitric oxide

OF – Oligofructose

PAS - Periodic acid–Schiff

PDLs – Primary dermal lamellae

p-EHC – prolonged Euglycemic hyperinsulinemic clamp

PELL – Primary epidermal lamellae lenght

PELs – Primary epidermal lamellae

PI% - Proliferative index

PI-3K - Phosphatidylinositol 3-kinase

PLMS – Prelaminitic metabolic syndrome

PPID – pituitary pars Intermedia dysfunction

Q1 – First quartile

Q3 – Third quartile

QUT – Queensland University of Technology

RIVI – Retrograde intravenous

RR – Relative recovery

SAA – Serum amyloid A

SADP – Suspensory apparatus of the distal phalanx

SD – Standart deviation

SDLs – Secondary dermal lamellae

SELL – Secondary epidermal lamellae length

SELs – Secondary epidermal lamellae

SLL – Suporting limb laminitis

TLR – Toll like receptor

TNF – Tumor necrosis factor

TNFα – Tumor necrosis factor alfa

TNPC – Total number of positive cells

TPX2 – Targeting protein for Xklp2

TS – Transverse section

UPLC – Ultra high performance liquid chromatography

UQ – The University of Queensland

Part I: General Introduction

Chapter 1: Introduction, literature review and project aims

1.1 Introduction

The equine hoof lamellar tissue is a highly specialized structure that plays a critical role in the suspensory apparatus of the distal phalanx (SADP) (Pollitt, 2010). The failure of this normal attachment between distal phalanx and hoof is a major consequence of laminitis. Several conditions have been associated with laminitis in the past (Heymering, 2010), but those related to insulin resistance (IR) or excess of glucocorticoids appear to be involved in the majority of the reported cases. The common factor in these conditions seems to be hyperinsulinemia (Johnson et al., 2004; McGowan, 2010; Treiber et al., 2006a; Treiber et al., 2006b). Besides the main role of insulin in regulating glucose metabolism, it is involved in diverse physiologic process such as vascular modulation and cellular growth and proliferation. The role of insulin in laminitis has been assessed by different authors (Asplin et al., 2010; Bailey and Harris, 2006; Bailey et al., 2007; de Laat et al., 2011a; du Toit and Trawford, 2010; Jones et al., 1998; Kronfeld, 2005; McGowan, 2008; Walsh et al., 2009) and a new experimental model has arisen recently to induce laminitis after the administration of high concentrations of the hormone (Asplin et al., 2007; de Laat et al., 2010a). However the exact mechanism involved in the pathophysiology of the disease remains unclear. The aim of this work is to evaluate the local effects of insulin delivered in higher than normal concentrations into the lamellar interstitial fluid, assessing the suggested potential mechanisms involved in this form of the disease: vasodilation, derangements of energy metabolism, and epidermal cellular proliferation. This section provides a brief review of the endocrinological aspects of laminitis, including the role of insulin and suggested pathways to disease.

1.2 Literature Review

1.2.1 Structure of the inner hoof wall

The equine hoof is a hard keratinized structure covering the distal end of the digit. In addition to protecting the inner structures of the hoof, it dampens and transmits the impact forces during standing or locomotion of the horse (Goodman and Bonney, 2007). It is constituted by three layers: the *stratum externum, stratum medium* and *stratum internum* (Stump, 1967).

The *stratum externum* is a protective tough layer to inhibit dehydration (Stump, 1967). The *stratum medium* is the thickest of the three layers and is characterized by its tubular and intertubular horn structure**.** It is the result of the proliferative activity of the epidermal basal cells (EBCs) lining the basement membrane of the coronal corium. Numerous papillae project from the coronet corium and fit into the holes of the epidermal coronary groove, nourishing the EBCs which proliferate and differentiate into keratinocytes. These keratinocytes mature forming a keratinized cellular matrix in which the hoof wall tubules are embedded. The hoof wall is apparently reinforced by the tubules but it is the intertubular material that provides mechanical strength and stiffness (Bertram and Gosline, 1986; Kasapi and Gosline, 1998) . The cross-sectional shape of the tubules changes from elliptical in the outer region to circumferential in the inner region; in addition the number of tubules is higher in the outer zones of the stratum medium and declines as it gets close to the *stratum internum.* This special conformation of the *stratum medium* confers the resistance and elastic properties to the hoof wall (Kasapi and Gosline, 1998)

The *stratum internum* is constituted by the epidermal lamellae and the lamellar corium; together they play a fundamental role linking the dorsal surface of the distal phalanx to the stratum medium of the hoof wall. The connection between hoof and bone is termed the suspensory apparatus of the distal phalanx (SADP) (Pollitt, 2010) which includes the dermal and epidermal lamellae, and the connective tissue of the coffin bone (Budras, 2009). The epidermal lamellae are constituted by approximately 600 primary epidermal lamellae (PELs) disposed parallel to each other from the deep edge of the coronary groove to the junction with the sole (Stump, 1967). Along the length of each PEL, 150 to 200 secondary epidermal lamellae (SELs) are found increasing the contact surface between the epidermal and dermal

layers. This particular disposition of the epidermal lamellae is an important specialization of the lamellar tissue to accomplish its suspensory function (Pollitt, 2010).

The corium or dermis, consists of a highly vascularized and innervated dense matrix of connective tissue underlying the hoof wall (Pollitt, 1998). It is comprised by the coronet corium, the lamellar corium and the sole corium. The sole corium, as well as the previously describe coronet corium, has papillae that fit into a socket in the epidermal sole. In contrast, the lamellar corium is interlocked with the epidermal lamellae forming the primary (PDLs) and secondary dermal lamellae (SDLs). The vasculature of the corium is provided mainly by branches from the coronary circumflex artery and the terminal arch; in turn both arteries are branches of the medial and lateral digital arteries (Pollitt, 2010). The lamellar dermal vasculature consists of blood vessel sheets arranged perpendicularly to the hoof wall exhibiting numerous arterioles, capillaries and extensive venous plexus (Marais, 1989; Nasu et al., 1998). The vessels of the dermal papillae of the coronary, sole and frog regions share the same structural organization; a central artery and vein spiralled around each other, surrounded by a sheath of fine capillaries (Pollitt and Molyneux, 1990). The venous circulation is drained from the foot through three different plexuses: the dorsal venous plexus, the palmar/plantar venous plexus and the coronary plexus. The three plexuses are drained by the medial and lateral digital veins (Pollitt, 2010). Arteriovenous anastomoses (AVAs) have been described in both the dermal papillae and the dermal lamellar vasculature in an estimated density of 500 per cm2 (Pollitt and Molyneux, 1990). They are conformed by specialized cells metabolically more active than other endothelial cells of contiguous vessels and play an important role in tissue metabolism, thermoregulation and pressure modulation (Molyneux et al., 1994).

The dense matrix of connective tissue of the corium is responsible for connecting the dorsal surface of the distal phalanx to the basement membrane (BM) at the dermal-epidermal junction. The BM comprises a multiprotein complex that aids the attachment of epidermal keratinocytes to the underlying dermis (McMillan et al., 2003). It is a key structure of the SADP, connecting bands of collagen I from the dorsal surface of the distal phalanx with the EBCs of the SELs (Pollitt, 2010). The BM is constituted by an axial skeleton of collagen IV and several glycoproteins, both together form the electron dense layer termed *lamina densa* (McMillan et al., 2003). At its epidermal side, the BM binds the plasmalemma of EBCs through electron dense adhesion plaques or hemidesmosomes (HD) (Pollitt, 2010). The proteins of the hemidesmosomes plectin and BP230 (intracellular plaque proteins) connect

the keratin cytoskeleton of the EBCs to the transmembrane integrin α6β4 and BP180. These two proteins have domains at both sides of the plasmalemma and bridge the hemidesmosomes to the *lamina densa* through the anchoring filaments composed by Laminin-332 (formerly laminin-5)(Jones et al., 1998). At the dermal side of the BM, long collagen VII filaments bind the *lamina densa* to the connective tissue (collagen I) of the parietal surface of the distal phalanx (Pollitt, 2010).

1.2.2 Laminitis: clinical and histological findings

Laminitis has been defined as the failure of the distal phalanx to maintain its attachment to the lamellae of the inner hoof wall (Pollitt, 2004). The progression of the disease has been divided in three phases: the *developmental phase,* the *acute phase* and the *chronic phase*. The *developmental phase* extends from the exposure of the horse to the inciting cause to the appearance of foot pain. It is in this phase when the destruction of the SADP begins, damaging progressively the inner structures of the hoof capsule. The onset of foot pain and lameness at the trot give rise to the *acute phase,* which last up to the time when radiological evidence of rotation of the distal phalanx is present. This event indicates the start of the *chronic phase,* which can last indefinitely (Pollitt, 2004). The disease severity can vary along the different phases from mild or subclinical manifestation that may go unnoticed to severe manifestation as persistent, mild lameness, continued severe foot pain, further degeneration of lamellar attachments, recumbency, hoof wall deformation and loss of function, leading in many cases to euthanasia (Hunt, 1993).

The different grades of severity described below, correspond to the clinical classification described by Obel (1948**)** which has been universally adopted.

Obel Grade I: At rest the horse will alternately and incessantly lift the feet, lameness is not evident at the walk, but a short stilted gait is noted at the trot.

Obel Grade II: The horse moves willingly at the walk, but the gait is characteristic of laminitis. A hoof can be lifted off the ground without difficulty.

Obel Grade III: The horse moves very reluctantly and vigorously resists attempts to a forefoot.

Obel Grade IV: The horse must be forced to move and may be recumbent.

During the different stages of laminitis several changes occur to the lamellar tissue. The histological alterations occurring in acute laminitis cases are correlated to the clinical progression of the disease and have been classified with light microscopy by (Pollitt, 1996) into three grades:

Grade 1: SELs are decreased in width, elongated and their tips are more pointed than normal. The nuclei shapes are round instead of oval and abnormally orientated with their long axes parallel to the long axis of the SEL. The BM separates from the EBCs, being more noticeable at the SELs' tips where a cytoplasm-free space resembling a teat-shaped bubble is formed (Pollitt, 1996).

Grade 2: SELs are further elongated and separated from the basal cells. PEL tips are stretched and undulated, however basal cells at the tips of PELs are the least abnormal. Mitotic figures may be found as a suggested proliferative response. The change of shape of the EBCs at the SELs nuclei is more evident at this grade, being found closer to the BM rather than their normal apical position. SDL shows a lack of connective tissue and between the bases of the SELs the BM was absent. The major difference with the previous grade is the further separation of the BM from the edge of the PEL axis (Pollitt, 1996).

Grade 3: The normal arrangement of the lamellar anatomy has been lost completely. The PEL tips are tapered and shrunken. SELs are extremely elongated and stretched with no recognisable connective tissue in between of them. Most of the epidermis appeared as an amorphous mass of elongated nuclei and basal cell cytoplasm scattered with the remnants of the keratinised axes of SELs. Some of the epidermal cell nuclei are pyknotic, spindle shaped and elongated in the direction of the lamellae. The PELs tips consist entirely of collapsed tubes of BM lacking of epidermal cells. PAS-positive neutrophils can be found surrounding the PEL tips and within the epidermal compartment between layers of BM (Pollitt, 1996).

The histological classification described by Pollitt using light microscopy, was based on laminitic horses induced utilizing the alimentary carbohydrate overload (ACO) protocol, firstly described by Garner et al. (1975). These lesions have been observed in the different experimental laminitis models with slightly variations. BM damage is more extensive and severe in horses treated with oligofructose (OF) than those treated with insulin. Additionally,

BM derangement has been described as more extensive in horses than ponies subjected to hyperinsulinemia (de Laat et al., 2011b), where only small and occasional areas of BM separated from the tips of SELs were identified (Nourian et al., 2009). The difference of bodyweight between horses and ponies (and therefore the different forces of mechanical distraction acting on the lamellar tissue) has been suggested as a possible explanation for this variability (de Laat et al., 2011b).

The presence of a high number of mitotic figures and apoptotic cells is a characteristic finding in the lamellae of ponies (Asplin et al., 2007; Nourian et al., 2009) and horses (de Laat et al., 2010b) subjected to experimental hyperinsulinemia. Mitotic figures have been also described in other forms of laminitis (Faleiros et al., 2004; Galey et al., 1991; Pollitt, 1996) and associated with reparative processes.

Abnormal proliferative changes have been also described in the lamellar tissue of Kaimanawa feral horses of New Zealand diagnosed with chronic laminitis. The main histopathological lesions found were the presence of excessive cap horn production and abnormal tips of PELs (multi-branched, attenuated and dystrophic). Presumably this chronic presentation of the disease might be associated to consumption of high palatable pastures or IR (Hampson et al., 2012).

1.2.3 Laminitis pathophysiology

The pathophysiology of laminitis has been studied for several years and many hypotheses have been proposed during the last decades trying to elucidate the pathway that leads to lamellar failure. In the last 30 years more than 80 conditions have been associated with laminitis (Heymering, 2010), however most of them are based on observations and only a few conditions have been replicated through experimental models (Asplin et al., 2007; de Laat et al., 2010a; Galey et al., 1991; Garner et al., 1975; van Eps and Pollitt, 2006), with an incomplete understanding of the pathologic process (Heymering, 2010). Due to the diversity of causes for laminitis the idea of a single pathway explaining the disease seems to be very unlikely. Thus the different natural presentations of laminitis have been grouped into three categories: sepsis related laminitis, supporting limb laminitis and endocrinopathic laminitis (Katz and Bailey, 2012).

Sepsis related laminitis is by far the most studied form of the disease. Gastrointestinal injury from various insults, pleuropneumonia and septic metritis are some of the conditions usually related to sepsis and laminitis (Belknap and Black, 2012). In this form of the disease, vascular delivery of "triggering factors" from diverse origins appears to be responsible for activation of enzymatic and/or metabolic events in the lamellar tissue leading to the dermo-epidermal disruption (Pollit 1999). Although the exactly mechanism through which sepsis leads to laminitis remains unclear, the main instigating events appear to be: activation of matrix metalloproteinases (MMP) 2 and 9, increased ADAMTS activity, lamellar leucocyte infiltration and vascular changes (Coyne et al., 2009; Faleiros et al., 2011; Loftus et al., 2009; Mungall and Pollitt, 1999; Roach et al., 2002).

Supporting limb laminitis (SLL) occurs in the healthy limb of horses with severe unilateral lameness when it persists. SLL incidence has been estimated between 11%- 16% of horses at risk (Virgin et al., 2011). Cyclic loading of the horse feet seems to plays an important role in the normal equine foot to avoid ischemia. Reduction of this cycling activity is thought to decrease the lamellar blood flow and may be involved in the pathophysiology of the disease. Ischemia and insufficient glucose delivery to the lamellae appear to be the main factors leading to the SADP failure in SLL (van Eps et al., 2010).

This literature review is focused on the endocrinopathic aspects of laminitis. For detailed discussion about sepsis related laminitis and SLL, the reader is directed elsewhere (Belknap and Black, 2012; Orsini, 2012; van Eps et al., 2010).

1.2.3.1 Endocrinopathic laminitis

Endocrinopathic laminitis arises from hormonal rather than inflammatory conditions (Katz and Bailey, 2012) and has been mainly associated with pathologies involving excess of glucocorticoids (endogenous or exogenous) and/or insulin resistance (IR) (McGowan, 2010). In epidemiological studies from Australia and Finland >80% of horses with a history of laminitis had evidence of an endocrinopathy (McGowan, 2009). Grazing lush pasture, also thought to result in laminitis via insulin imbalances, was the most prevalent form of laminitis in the USA, accounting for approximately 50% of all diagnosed cases (Kane et al., 2000). Similar percentages have been described in the United Kingdom with 61% of laminitis cases occurring in animals kept at pasture (Hinckley and Henderson, 1996).

The pathophysiology of pasture-associated laminitis is still unclear but the most accepted explanation is based on the carbohydrate overload model (Bailey et al., 2004; Eades, 2010). During periods of high risk, mainly spring and early summer or after heavy rains, the grass grows faster and accumulates considerable amounts of non-structural carbohydrates (fructans, simple sugar or starches). The ingestions of this forage generates changes in the hindgut microbiome and damages the intestinal epithelial cells favouring the absorption of laminitis triggering factors into circulation. These events have been replicated by the oligofructose (OF) and the alimentary carbohydrate overdose (ACO) experimental models of laminitis, first described by (Garner et al., 1975) and (van Eps and Pollitt, 2006), respectively. In those experimental models, sepsis is considered as the factor triggering the disease through different mechanisms not yet fully understood (Belknap and Black, 2012). The role of sepsis and endotoxemia in the pathogenesis of pasture-associated laminitis has not been determined (Geor, 2010). Furthermore, only a small proportion of any population develops pasture laminitis during periods of high risk suggesting that consumption of lush pastures during these periods could trigger the expression of an underlying genetic predisposition to laminitis (Bailey et al., 2004). Horses with an insulin-resistant phenotype are prone to the development of pasture-associated laminitis (Frank et al., 2006; Geor and Frank, 2009; Treiber et al., 2006b). A study conducted in ponies showed that intake of feeds rich in carbohydrate constitutes a risk factor to laminitis and may exacerbate hyperinsulinemia in predisposed animals. The term *prelaminitic metabolic syndrome* (PLMS) was used to describe the phenotype of these predisposed animals, including among the risk factors: body condition, plasma triglyceride concentration, insulin sensitivity and insulin secretory response (Treiber et al., 2006b). These characteristics were included in the ACVIM Consensus Statement (Frank et al., 2010) and now this condition is known as Equine Metabolic Syndrome (EMS).

EMS is characterized by the presence of obesity or increased adiposity in specific locations, usually subcutaneous adipose tissues surrounding the nuchal ligament in the neck, fat pads close to the tail or fat accumulated behind the shoulders, prepuce or mammary gland region. Insulin resistance (IR) (and hyperinsulinemia) is another characteristic of EMS and is accompanied by obesity in the majority of cases; although affected horses can have an apparently normal body condition. Obesity might be involved as a cause/consequence of IR through down-regulation of insulin pathways induced by adipokines and cytokines produced in adipose tissue of horses with EMS (Frank et al., 2010). The predisposition to laminitis is the third component of the phenotype for the majority of equids affected by EMS. Other

alterations such as hypertriglyceridemia, hyperleptinemia, arterial hypertension, reproductive alterations and increased systemic markers of inflammation may also be present (Frank et al., 2010).

Pituitary Pars Intermedia Dysfunction (PPID), also known as equine Cushing's syndrome (ECS), is the term used to describe a collection of clinical signs attributed to the loss of dopaminergic inhibition of the pars intermedia of the pituitary (Schott, 2002). As well as other neurodegenerative diseases, the most important risk factor for the development of PPID is age, affeceting 15 to 30% of aged horses (Brosnahan and Paradis, 2003). Clinical signs of PPID include: hirsutism, muscle atrophy, polyuria/polydipsia, hyperhidrosis, abnormal fat distribution and insulin resistance, immunosuppression, behavioural abnormalities, infertility, neurologic and laminitis (McFarlane, 2011). More than 50% of horses affected by PPID suffer chronic insidious-onset laminitis, which has been described as the major clinical complication of the disease (Schott, 2002).

PPID and EMS are both metabolic conditions that predispose horses and ponies to laminitis and may even induce the condition (Tadros and Frank, 2011). The association between laminitis and EMS or PPID has been widely described and the common factor between these conditions seems to be IR and subsequent hyperinsulinemia (Johnson et al., 2004; McGowan, 2010; Treiber et al., 2006a; Treiber et al., 2006b). Horses with PPID usually have IR as a consequence of the inhibiting effect of glucocorticoids on insulin activity, causing hyperinsulinemia (Johnson et al., 2004). This has been detected in a considerable proportion of PPID affected horses (McGowan et al., 2004) and consistently associated with laminitis in these cases (Walsh et al., 2009). In addition, in a field study, horses with EMS and PPID presented with significantly higher plasma insulin concentrations than control horses, suggesting the baseline plasma insulin concentrations as a potential indicator of susceptibility of horses to laminitis (Walsh et al., 2009).

IR is a general term for the inability of a normal concentration of insulin to produce a normal response in the target tissues (Kahn, 1978). It was suggested by (Asplin, 2009) as a possible cause of laminitis due to an impaired glucose uptake by the lamellar tissue. This hypothesis was based on the high metabolic requirements of the lamellar tissue, even higher that of the head (Wattle and Pollitt, 2010) and the need of the lamellae to consume glucose to maintain hemidesmosome integrity between the BM and the EBCs of the lamellae (French and Pollitt, 2004). Later studies characterizing the glucose uptake in the lamellar tissue

showed a strong reaction for glucose transport protein GLUT-1 antibodies in the EBCs of the SELs, whilst the reactions to GLUT-3 and GLUT-4 were weak or undetectable (Wattle and Pollitt, unpublished data). This data was concordant with the work of (Asplin, 2009), describing strong GLUT-1 mRNA expression in the lamellae of normal and laminitic ponies, and very weak expression of GLUT4 mRNA in the same tissues. The protein GLUT-1 is one of the thirteen members of the family of facilitative sugar transporters (GLUT1- GLUT12 and HMIT) (Wood and Trayhurn, 2003) and is characterized by its insulin-independent regulation of glucose uptake in tissues with high metabolic requirements (Ebeling et al., 1998). (Asplin, 2009) utilizing lamellar explants from healthy horses demonstrated that glucose uptake in the lamellar tissue is a process not responsive to insulin and also, glucose is capable of modulating its own transport and metabolism in tissues where insulin-dependent glucose transporters are not present. Thus, the impairment of glucose uptake by a reduced insulin sensitivity or increased IR seems to be an unlikely mechanism explaining the failure of the SADP.

Recently, laminitis was successfully induced in healthy insulin-sensitive ponies and horses through treatment with a prolonged euglycemic hyperinsulinemic clamp (p-EHC) (Asplin et al., 2007; de Laat et al., 2010a)**.** All treated ponies (Asplin et al., 2007) and horses (de Laat et al., 2010a) developed laminitis within 72 and 48 hours, respectively. These experiments give a clear indication that hyperinsulinemia itself is a major factor in the onset of the disease. The exactly mechanisms by which insulin leads to laminitis are still unclear, and different possible pathways has been investigated. Among the main possibilities are: effects on blood flow, inflammatory effects, glucoses excess, MMP activations (Menzies-Gow, 2012) and morphological derangements (de Laat, 2011).

Vasodilation is a known vascular action of insulin exerted through an endothelium-derived nitric oxide mediated effect (Steinberg et al., 1994) modulated through the phosphatidylinositol 3-kinase (PI-3K) pathway (Kim et al., 2006). A previous study, in which hoof wall surface temperature (HWST) was suggested to mirror perfusion within the hoof, concluded that horses treated with insulin show increased HWST and therefore vasodilation (de Laat et al., 2010a). However, vasodilation alone, provoked using persistent digital hyperthermia fails to induce laminitis; therefore it may be involved in the pathogenesis of the disease but is not the sole factor (de Laat et al., 2012c). Insulin also modulates vasoconstriction through the release of endothelin-1 (ET-1) which is controlled by the mitogen-activated protein kinase (MAPK) pathway. In IR subjects the PI-3K activation is

blocked and the imbalance of NO:ET-1 favours vasoconstriction (Kim et al., 2006). Field and Jeffcott (1989) related the vascular theory of laminitis with hyperinsulinemia through a different pathway in ponies. They proposed that hyperinsulinemia and IR increase thromboxane A2 release and platelet aggregation, inducing vasoconstriction in the peripheral vasculature of the horse and laminitis. However, experiments conducted in horses and ponies inducing laminitis utilizing a p-EHC protocol, did not evidence histologic lesions compatible with ischemia or procoagulatory conditions (Asplin et al., 2007; de Laat et al., 2010a). It must be mentioned that these horses were insulin sensitive and the exactly role of IR on the process of laminitis remains unclear.

Inflammation appears to be a minor characteristic of insulin-induced laminitis. Leukocyte infiltration into the dermal tissue of experimental insulin treated horses was milder than that reported in horses subjected to ACO models (de Laat, 2011). Obesity and IR, conditions found in horses with endocrinopathic laminitis, have been associated with inflammation by several authors. Vick et al. (2007) described correlation between elevated tumor necrosis factor (TNF) p, mRNA expression of TNFα and obesity. Also, these factors were associated with increased circulating concentrations of insulin and reduced insulin sensitiveness in mares with high body corporal score and percent of body fat. In addition, mares treated with insulin for 6 h experienced increased concentrations of TNFα and IL-6 (Suagee et al., 2011); supporting the theory that hyperinsulinemia could stimulate inflammation. Suagee et al. (2012) demonstrated that non-obese geldings fed with high glycemic diets showed increased serum TNF concentrations, proposing that high glycemic diets promote inflammation independent of obesity. In another study, peripheral blood cells of obese hyperinsulinemic horses showed decreased gene expression of IL-1 and IL-6, suggesting that obesity and IR are not associated with a generalized pro-inflammatory state in the horse as usually is described in humans (Holbrook et al., 2012). In the horse the acute phase protein serum amyloid A (SAA) appears to be the principal acute phase protein. Body condition and insulin seems to influence SAA more than the commonly measured pro-inflammatory cytokines. Hence, SAA has been proposed as a better marker of obesity-associated inflammation and laminitis risk than are TNF, IL-6 and IL-1β (Suagee et al., 2013). The presence of Toll-like receptor (TLR), a family of pattern recognition receptors crucial in the regulation of non-infectious inflammation, has been confirmed in the lamellar tissue (de Laat et al., 2014). TLR signalling activation modulates the release of inflammatory cytokines among other inflammation mediators (Yu et al., 2010). Horses treated with a p-

EHC showed increased lamellar protein expression of TLR4, TNF-α, but no IL-6 (de Laat et al., 2014). These results are consistent with the minimal cellular inflammatory response observed previously in the insulin-induced model (de Laat et al., 2012b). Although the literature supports a correlation between laminitis, obesity, hyperinsulinaemia and inflammation, the latter appears to be a consequence rather than an initiating factor.

The participation of MMPs in the pathogenesis of laminitis has been described in the ACO, OF and black walnut extract (BWE) laminitic models (Loftus, 2008; Visser and Pollitt, 2012). MMP-2 can be found in the normal lamellar tissue and its expression has been found to be elevated in the lamellae of horses during the developmental phase of laminitis (Mungall et al., 2001). Horses subjected to the OF model had delayed activation of lamellar MMP-2 and no activation of proMMP-9 relative to the initiation of the basement membrane damage, indicating that MMP may not play the initiating role in the lamellar lesion (Visser and Pollitt, 2012). In contrast, horses subjected to p-EHC showed decreased proMMP-2 at the early developmental stage of laminitis (6 h) compared to later time points (24 h) and the acute phase (48 h). MMP-9 levels were observed to gradually increase during the developmental stage with a marked elevation in the acute phase (de Laat et al., 2011b). The increase of MMP-9 is mediated by insulin via the MAPK signalling pathway in monocytes (Fischoeder et al., 2007) and may be the explanation for the increase of lamellar MMP-9 found in lamellar tissue during hyperinsulinemia (de Laat et al., 2011a). Despite the role of MMPs in the developmental stage of insulin-induced laminitis being unclear, MMP activation may be a relevant factor at later stages of the disease.

Exposure of tissues to an excess of glucose over prolonged periods of time produces glycosylation of proteins and formation of advanced glycation endproducts (AGEs). These events play an important role in the progression of diabetic complications in humans (Delafontaine et al., 2004) and may be also involved in the pathogenesis of laminitis. The lamellar tissue could be susceptible to protein glycosylation and oxidative stress during periods of increased glucose metabolism due to its particular glucose uptake regulation independent of insulin (de Laat et al., 2012a). Healthy horses subjected to p-EHC for 48 h showed accumulation of AGEs and increased GLUT-1 transporter in the lamellar tissue. However these changes were not observed at early time-points suggesting that AGEs may be

involved in the pathogenesis of insulin-induced laminitis but not as the main cause of the disease (de Laat et al., 2012a).

The presence of mitotic figures and apoptotic cells was a common finding in the experimental ponies and horses treated with insulin by Asplin et al. (2007) and (de Laat et al., 2010a)**.** These proliferative changes were observed during the early stages of the disease and are likely to be primarily involved in the pathophysiology of insulin-induced laminitis (de Laat et al., 2012b). Insulin via its receptors regulates cellular proliferation and cellular growth through different signalling pathways. The presence of the insulin receptor (InsR) is restricted to the lamellar tissue microvasculature; however the Insulin-like growth factor 1 receptor (IGF-1R), which can be also activated by insulin, is widely distributed in the lamellar vascular elements, epithelial cells and fibroblasts (Wattle and Pollit, unpublished data; Burns et al, 2012) and its activation could be responsible for the epidermal cell growth and proliferative dysregulation observed in the insulin-induced laminitic model (Burns et al., 2012; de Laat et al., 2010a). IGF-1R is known to be mitogenic in vivo and in vitro, it promotes growth and an increase in size of the cell, it sends a powerful anti-apoptotic signal, can induce differentiation in some cell types, and plays a major role in the transformation of cells (Baserga, 2000). Considering these effects and the presence of the IGF-1R in the epidermal lamellar tissue, it has been suggested that changes observed in the p-EHC horses could be the result of insulin stimulation of this receptor (Bailey and Chockalingham, 2010)**.** Furthermore, lamellar epithelial cells exposed *in-vitro* to high concentrations of insulin showed increased cell proliferation and when these cells were co-incubated with a blocking antibody against the IGF-1R, the proliferative effect of insulin significantly decreased (Bailey and Chockalingham, 2010). Hence, the direct proliferative effects of insulin may lead to structural derangements and weakening of the lamellar tissue organization resulting in failure of the SADP and laminitis (de Laat et al., 2011b).

1.2.4 Insulin signalling

Insulin is a polypeptide hormone with a molecular weight of 5747.6 Da in the horse. It consists of two chains, α and β, of 21 and 30 amino acids respectively that are connected by two disulphide bonds (Ho et al., 2011). Insulin structure has been highly conserved throughout evolution and exhibits slight differences among species (Conlon, 2001), mainly at positions 8, 9 and 10 of the α chain (Martin and Crump, 2003). Insulin is synthesised and

secreted in the exocrine pancreas by the β cells mainly in response to increased blood glucose levels, although other factors such as increased concentration of some amino acids, fatty acids (Aronoff et al., 2004), hormones and neuropeptides among others can stimulate insulin secretion (Martin and Crump, 2003).

The main function of insulin is related to the control and regulation of glucose metabolism (Aronoff et al., 2004). However, insulin has an important role in diverse processes such as vascular tone modulation, proliferation, growth and differentiation of cells, and its recently suggested neuromodulatory role in the mammalian central nervous system (Schulingkamp et al., 2000). Whatever the pathway stimulated by insulin, such stimulation must be initiated by interactions with any of its possible receptors: the insulin receptor (InsR) and/or insulin-like growth factor 1 receptor (IGF-1R).

1.2.4.1 Insulin Receptor (InsR)

InsR is widely distributed in different tissues of mammals. The highest expression was found in adipose tissue, whilst in liver, heart and lung tissue only 30% was expressed relative to adipose tissue. Furthermore, muscle, brain, spleen, placenta and kidney tissue expressed only 10% of that found in adipose tissue (Bailyes et al., 1997). Wattle and Pollitt (unpublished data), using antibodies against InsR have described the distribution of the InsR in the lamellar tissue. The receptor was found in the coronet and blood vessels of the lamellar dermis, especially within the small vessels of the secondary dermal lamellae (SDL) adjacent to the glucose dependent lamellar basal cells. However, no InsR has been observed in the epidermal lamellae (Burns et al., 2012).

InsR protein is a disulphide-linked (αβ)2 homodimer. The extracellular portion of each αβ promoter contains six domains (L1, CR, L2, FnIII-1, FnIII-2 and FnIII-3) and an insert domain (ID) within FNIII-2. The α-chain component of the ID is terminated by a segment termed αCT (McKern et al., 2006). These segments are important to understand the insulin-InsR splicing process. Two different sites of the receptor are described interacting with insulin: a low affinity and a high affinity insulin binding site. Insulin binds first to the low affinity site (site 1) composed of the αCT fragment of one α-subunit and then to the high affinity site (site 2) composed of the L1 fragment of the other α-subunit (De Meyts, 2008). The engagement between the insulin molecule and the site 1 of the InsR has been recently

described by (Menting et al., 2013), showing in detail the conformational changes of the receptor as well as of the insulin molecule. The binding of insulin to the InsR brings the two β-subunits into close opposition, activating the InsR tyrosine kinase domain through auto-phosphorylation, initiating a complex cascade of biochemical signals. Two major signalling pathways resulting from the receptor stimulation have been described, phosphoinositide 3-kinase (PI-3K) and Ras (Belfiore et al., 2009).

PI-3PKs belongs to a lipid kinases family that catalyses the phosphorylation of phosphatidylinositol and phosphoinositides generating new intracellular second messengers (Cheng et al., 2012). The protein serine/threonine kinase AKT (also known as PKB) activation is directly facilitated by PI-3PK (Belfiore et al., 2009). In muscle and fat AKT activation regulates glucose uptake, stimulating the translocation of the glucose membrane transporter protein GLUT-4 and the glycogen synthesis (inhibiting the enzyme glycogen synthase 3), whilst in the liver, AKT inhibits gluconeogenesis blocking the transcription of gluconeogenic enzymes (Cheng et al., 2012). AKT also promotes other process such as cell survival and apoptotic modulation. Moreover, through the activation of the regulatory-associated protein mammalian target of rapamycin (mTOR), AKT regulates cellular growth and protein synthesis (Taniguchi et al., 2006).

The Ras pathway, (also called Ras-MAPK pathway) is activated by insulin following the binding of growth factor receptor-bound protein 2 (Grb2) to the insulin receptor substrate proteins (IRS). This binding triggers a complex kinase cascade that modulates cell growth as well as cellular survival and differentiation (Belfiore et al., 2009).

1.2.4.2 Insulin-like growth factor 1 receptor (IGF-1R)

IGF-1R belongs to the same family of transmembrane tyrosine kinases that include the InsR and the orphan insulin receptor-related receptor (IRR). They share the homodimeric structure and domains with slight variations in the amino acid sequences. IGF-1R has a 70% homology to the InsR (Baserga, 2000) and also heterodimers InsR/IGF-1R have been described in cells expressing both receptors (Schaffer et al., 1993). Three ligands are described binding IGF-1R: insulin growth factor 1 (IGF-1), insulin growth factor-2 (IGF-2) and insulin (Baserga, 2000). IGF-1 and IGF-2 are produced by the liver and also by other extrahepatic tissues such as tumor cells and stromal fibroblasts (Adams et al., 2000). IGF-1R

binds IGF-1 with high affinity, whilst insulin (at high concentrations) and IGF-2 bind with lower affinity. IGF-2 is involved in the foetal developmental stages and is mainly regulated by the IGF-2R (Chitnis et al., 2008).

IGF-1 is the main ligand of IGF-1R and its plasma levels have been described in different species. Several factors such as nutritional status, growth hormone (GH) secretion, age, genetic factors, IGF binding proteins, insulin, thyroxine, cortisol, testosterone, estrogen obesity and cytokines regulate IGF-1 secretion in humans (Clemmons, 2007). In the horse, Champion et al. (2002) described that age plays a significant role in IGF-1 plasma levels, with higher concentrations in young animals. In addition, intact males have higher concentrations than mares or geldings suggesting a sex effect. Geographic location and level of training also played a significant role in the IGF-1 plasma levels.

IGF-1R shares some of the signalling pathways with the InsR. The PI3K-AKT and MAPK pathway are common to both receptors. In some tissues IGF signaling regulates cellular differentiation via the PI3K-AKT pathway (Adams et al., 2000), while in others such as myocardium and brain PI3K-AKT and MAPK pathways combined are required (Morisco et al., 2007; Russo et al., 2005). It plays an important role in growth of normal and malignant cells (Chitnis et al., 2008) and is also involved in proliferation, modulation of cell size, transformation of cells and cellular adhesion (Baserga, 2000).

1.2.5 Tissue microdialysis

Tissue microdyalisis is a non-invasive sampling technique developed for studying the extracellular fluid (ECF) (Benveniste, 1989). The first attempts to sample the ECF were performed by Gaddum (1961) using a “push-pull” technique in brain tissue of dogs. That technique used two cannulas, one for pumping liquid directly into the tissue and another to withdrawn the liquid. Years later, Bito et al. (1966) implanted “dialysis sacs” into the subcutis and brain tissue of dogs. Those sacs contained a mixture of colloidal and saline solution and were kept in place for several weeks to then be extracted surgically and analysed. From these studies emerged the concept of a “compartment” which can equilibrate with the surrounding tissue through a semi-permeable membrane (Ungerstedt, 1991). In early 1970s, hollow fibres were used successfully for sampling ECF in neuroscience at the Department of Pharmacology in the Karolinska Institute, (Stockholm, Sweden) (Ungerste.U

and Pycock, 1974) and since this initial design, the "microdialysis probe" evolved into the technique used today.

A microdialysis system is constituted by a microdialysis pump, a probe, a tubing system and a collection system**.** The principle of microdialysis is based on the perfusion of a fluid throughout a microdialysis probe inserted into the desired tissue that equilibrates with the fluid outside of the probe by diffusion through a semipermeable membrane (Ungerstedt, 1991). In ideal conditions, the perfused fluid should be isosmotic with the tissue, the hydrostatic pressure minimal and no net water transfer between the perfused solution and the animal tissue should exist: the concentration gradient of the studied substance should be the sole driving force of the process (Janle and Kissinger, 1996).

Recovery is the term used to quantitate the amount of analyte collected in the dialysate. Absolute recovery corresponds to the total amount of analyte recovered whilst relative recovery (RR) represents the ratio of analyte relative to that of the tissue or solution sampled. The recovered volume in µD is irrespective of the membrane surface area, but depends on the perfusion speed rate (Janle and Kissinger, 1996). Normally, microdialysis pumps allow perfusion speed flow between 0.1 – 5 uL/min. Slow perfusion speeds should be selected to allow the perfusion solution and the ECF to equilibrate. Also a slow flow rate helps to reduce possible disturbances of the normal tissue physiology (Ungerstedt, 1991)**.** Probe outer diameters are usually smaller than 400 µm. This feature permits probes to be inserted virtually in any tissue with minimum disturbances to its physiology and normal parenchymal structure. Several authors have reported its utilization in subcutis (Dostalova et al., 2009; Hagstrom et al., 1987; Jansson et al., 1992; Kaptein et al., 1998; Wientjes et al., 1998), muscle (Deboer et al., 1991; Deguchi et al., 1991; Hallstrom et al., 1989; Miller et al., 2011; Mimura et al., 1996; Olausson et al., 2013; Rosdahl et al., 2000; Stallknecht et al., 1999), liver (Bjornsson et al., 2012; Davies and Lunte, 1995, 1996; Haugaa et al., 2010; Takahashi et al., 1996; Vanbelle et al., 1995), brain (Bjerring et al., 2011a, b; Bossers et al., 2013; Li et al., 2012; Notkina et al., 2012; Sato et al., 2011), adipose tissue (Arner and Bolinder, 1991; Jansson et al., 1995; Kopterides et al., 2012; Lourido et al., 2002; Song and Lunte, 1999; Summers et al., 1998), and recently in the lamellar tissue of the horse hoof (Nourian et al., 2010). Also tissue microdialysis can be used for continuous monitoring for time periods ranging from a few hours to several weeks (Wientjes et al., 1998). A fibrotic reaction has been described around the probe during prolonged microdialysis experiments

and decreased analyte concentrations collected has been associated with this phenomenon (Smith and Kaplitt, 2009).

The recovery of substances from the ECF is dependent on the length (surface area) of the dialysis membrane; the flow rate of the perfusion liquid; the diffusion speed of the substance through the ECF and the properties of the membrane (Amberg and Lindefors, 1989; Benveniste, 1989; Bungay et al., 1990; Lindefors et al., 1989). Relative recovery is inversely related to the perfusion flow rate, the diffusion is limited due to an increased hydrostatic pressure gradient across the membrane (Benveniste, 1989) and also because of reduction in time available for the equilibrium of the substance (Lindefors et al., 1989). The logical thinking is that the membrane is the main barrier during dialysis; however the ability of the substance to diffuse through the ECF plays an important role in both the amount of substance that can be recovered and the speed needed to detect concentration changes (Ungerstedt, 1991).

Microdialysis can be used to measure almost any molecule or metabolite able to pass across the semipermeable membrane. The pore size of the membrane usually ranges between 6 and 300 KDa of molecular weight cut-off (MWCO) depending on the size of the molecule being studied. Most of the energy metabolites (e.g. glucose, lactate, pyruvate, and urea) and small proteins can diffuse across the membrane without problems, however large protein diffusion can be difficult. Rosenbloom et al. (2005), using a 100 kDa MWCO probe described that the diffusion of large proteins such as ovoalbumin (43 kDa), albumin (66 kDa) and transferrin (80 kDa) is considerably affected by the pore size. Published MWCO values may not predict the size of the proteins that can be recovered and also protein recoveries appear to decrease markedly over time. Small proteins were less affected than large ones; but significant differences among proteins similar in size were observed perhaps due to protein aggregation, shape, surface charge or hydrophobicity. During sampling of proteins utilizing probes with large MWCO, loss of fluid can be experienced (Sjogren et al., 2002). In these situations a high molecular weight substance such as dextran should be added to the perfusate to maintain osmotic balance. Rosenbloom et al. (2005) described an improvement of 10% to 20% in the recoveries of protein utilizing this technique.

Diverse methods have been proposed to determine the actual concentration of a substance in the sampled tissue using microdialysis, among them: *difference method* or *no net flux*; *extrapolation to zero flow*; *retrograde dialysis* and *endogenous reference compound*.

The *difference method* or *no net flux* was first proposed by Lonnroth et al. (1987) and is based on the principle that if the concentration of the desired substance is the same in both the perfusate and the sampled solution, there will be no net transfer between membrane and tissue. Using this method several perfusate concentrations have to be tested to find the tissue concentrations, being a time-consuming technique (Janle and Kissinger, 1996). As previously mentioned, the recovery is inversely related to the flow rate perfusion. In the *extrapolation to zero flow* method, the perfusion speed is set up as slow as possible allowing the sampled tissue and the perfused solution to equilibrate. Then concentrations are determined and plotted against flow rate and extrapolated to find zero flow rate (Jacobson et al., 1985). *Retrograde dialysis* or *retrodialysis* is the perfusion of a solution containing a substance identical (or as similar as possible) to the target analyte. Assuming that the diffusion across the membrane will be the same in both directions (from the perfusate to the tissue and vice versa), the loss of the substance perfused can be used to calculate the recovery of the analyte. The drawback of this technique is the difficulty in finding a substance whose behaviour imitates exactly the analyte without interfering with the experiment. The *endogenous compound reference* technique is based in the utilization of an internal substance as a reference to calculate the RR of the desired analyte. The first compound used as an endogenous reference was glucose (Hashimoto et al., 1998). Urea has been proposed as a possible endogenous reference molecule (Brunner et al., 2000; Schwalbe et al., 2006; Strindberg and Lonnroth, 2000) since it is a substance equally distributed in the ECF and its concentrations in the tissue are very similar to that of the plasma. This technique is based on the assumption that the ratio of RR for two substances either *in vivo* or *in vitro* is constant over time (Schwalbe et al., 2006). Utilizing urea RR calculated *in-vivo* and *in-vitro,* and the recovery of the substance of interest *in vitro,* the *in vivo* recoveries of the desired substance can be determined. Strindberg and Lonnroth (2000) and Schwalbe et al. (2006) demonstrated that interstitial concentrations of glucose, lactate and glycerol; and linezolid respectively, determined in muscle and subcutaneous tissue of humans using urea as an *endogenous reference compound* were significantly correlated with those using other calibration techniques such as retrodialysis. In turn, Brunner et al. (2000) could not determine glucose concentration in human muscle ECF using this calibration technique. High variability was observed when the technique was compared to *no net flux*, mainly due to a marked difference in the ratio between urea and glucose recovery *in vivo* and *in vitro*. Also, performing *in vivo* experiments involves tissue dynamic process and conditions that differ from *in vitro* experiences and can subsequently affect the outcome of the dialysis process.

Tissue microdialysis, besides being a sampling technique, also allows delivery of substances to the tissue. Recently microdialysis has been used in neuroscience for delivering drugs to the central nervous system (Boehnke and Rasmusson, 2001; Ludvig et al., 1995; Osman et al., 2005) and insulin to human skeletal muscle . Rosdahl et al. (2000) assessed the changes in the concentration of muscular interstitial glucose, lactate and urea during the perfusion of different concentrations of insulin through a 100kDa MWCO probe. The ultrafiltration often experienced during dialysis when probes of high MWCO (100kDa) are used was successfully solved by the addition of dextran to the perfused solution avoiding the loss of fluid to the tissue and therefore maintaining stable recovery volumes. Insulin perfused in supra-physiological concentrations led to decreasing glucose levels and increasing lactate levels whilst urea remained constant. These changes in the metabolite concentrations were determined simultaneously with insulin delivery, supporting the utilization of tissue microdialysis for the evaluation of the effects of large peptide molecules such as insulin *in vivo*.

1.3 Hypotheses

We hypothesise that insulin, delivered in higher concentrations than normal into the lamellar interstitial fluid, has profound local effects including vasodilation, derangements of energy metabolism and induced epidermal cellular proliferation. It is these local tissue effects of insulin that result in laminitis in experimental models and naturally occurring cases where hyperinsulinemia is a feature.

1. 4 Aims and Objectives

Objectives addressing the hypothesis

- To determine the *in vivo* effects of local (lamellar) delivery of excess insulin on:
    a. lamellar tissue perfusion
    b. lamellar energy metabolism
    c. lamellar histomorphometry
    d. lamellar epidermal cellular proliferation (mitotic activity)

Objectives adding background information to studies of laminitis

- To validate a new technique for determining cellular proliferation rates in the equine lamellar tissue.

- To characterise the rate of epidermal proliferation in normal hooves.

Part II: Experiments

Chapter 2: The effect of locally delivered exogenous insulin on equine lamellar histomorphometry and epidermal cell proliferation.

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Ethical Considerations: The project was approved by The University of Queensland Animal Ethics Committee (AEC) that monitors compliance with the Animal Welfare Act (2001) and the Code of Practice for the care and use of animals for scientific purposes (current edition). All animals were monitored continuously by the investigators.

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2.1 Summary

Reasons for performing study: Increased lamellar epidermal cell proliferation and tissue disorganization have been described in the hyperinsulinaemic laminitis model. However, it is unclear whether these changes are direct effects of insulin.

Objectives: To determine the effects of locally delivered exogenous insulin on the proliferation rate of epidermal basal cells (EBCs) and lamellar histomorphometry.

Study Design: Randomised, controlled (within subject), experimental trial

Methods: Both forefeet of eight Standardbred horses were instrumented for tissue microdialysis: control limbs were perfused with isotonic polyionic solution, whereas recombinant human insulin (100 µg/ml) was added to the perfusate for treated limbs. The tissue was perfused continuously at 1 µl/min for 24h. Prior to euthanasia, 60 mg of the cell proliferation marker EdU, was administered bilaterally via retrograde intravenous injection with a tourniquet placed for 1 h. Lamellar tissue was harvested from 5 different areas around the microdialysis membrane (L1, central; L2, lateral; L3, medial; L4, proximal and L5, distal). The total number of epidermal cells (TNC) and the number of EdU-positive epidermal cells (TNPC) were counted in 250 fields per section. Histomorphometry measurements included length of primary epidermal lamellae (PELL), their keratinized axes (KPELL) and non-keratinized tips (NKPELL).

Results: Histomorphometric differences were found only in L1 sections: means (± SD) of PELL were 8% longer in the treated group (5.67 ± 0.18 mm) compared to control (5.26 ± 0.14 mm) ($p < 0.05$), as a result of an increased NKPELL (940 ± 124 vs 681 ± 64 µm; $p < 0.05$). The total cell number did not differ, but EdU-positive cells (42.50 95% CI 0.78–2325.37) were more numerous in the treated group compared to controls, with a strong trend towards significance ($p = 0.07$). L1 had the highest mean count of EdU-positive cells compared to other areas.

Conclusions: Continuous local delivery of exogenous insulin induced changes in lamellar tissue morphology, suggesting that insulin may act directly at the lamellar level in laminitis.

2.2 Introduction

Insulin deregulation, a key component of several endocrinopathic conditions, has been recently implicated in the development of laminitis [1-3]. Previous research has demonstrated that normal horses and ponies subjected to systemically high levels of exogenous human insulin using a prolonged hyperinsulinemic euglycemic clamp (pHEC) for 24 to 48 h developed laminitis, suggesting that insulin itself (at high concentrations) might be the major triggering factor for laminitis [4; 5]. The histologic alterations observed in p-EHC models differ from those previously described with the black walnut extract [6; 7] and carbohydrate overload models (CHO) [8; 9]. The insulin-induced lamellar lesions are mainly characterized by lengthening and narrowing of the secondary epidermal lamellae (SELs) with increased mitotic activity of the epidermal basal cells (EBCs) leading to considerable tissue disorganization, but with minimal effect on the basement membrane (BM) [4; 10].

Insulin is central to glucose metabolism, participates in the modulation of vascular tone, and has a primary role in general cellular metabolism, proliferation, growth and differentiation [11; 12]. Hyperinsulinaemia may lead to over-stimulation of the intracellular mitogen-activated protein kinase signaling pathway [10] which is responsible for the modulation of cellular proliferation [13], and through this it could affect the EBC proliferation rate. However, mitotic figures and increased proliferative activity of the EBCs have been found in other models of laminitis, and it is still unclear if this is part of the tissue reparation process or a primary direct effect of insulin on the lamellae [10]. It is also possible that systemic states such as obesity, insulin resistance and hyperinsulinemia may ultimately affect the lamellae indirectly, through the induction of other systemic pathways such as inflammation [14; 15]. It is important therefore to establish whether insulin acts directly at the local tissue level, or whether it requires systemic interactions in order to cause lamellar lesions.

Also, it has been hypothesized that weight-bearing and mechanical distractions may contribute to the histological changes observed in horses treated systemically with hyperinsulinaemia. Tissue microdialysis has been used previously as a minimally invasive means of local exogenous insulin delivery into target tissues [16; 17]. Local delivery of insulin via microdialysis permits the assessment of the insulin effects in a circumscribed area of the lamellae preserving the rest of the dermo-epidermal junction intact. This is a major advantage of the present model and a novel approach to the study of laminitis.

The aim of this study was to determine the effects of supranormal insulin levels delivered directly into the lamellar tissue using microdialysis, on lamellar histomorphometry and epidermal cell proliferation.

2.3 Materials and Methods

2.3.1 Animals

Eight Standardbred geldings (aged 6 to 12 years, weight 420 to500 kg) with clinically normal hooves were used in this study. The project was approved by the University of Queensland Animal Ethics Committee (approval number: SVS/338/11/UQ) that monitors compliance with the Animal Welfare Act (2001) and The Code of Practice for the care and use of animals for scientific purposes (current edition).

2.3.2 Tissue microdialysis

In every horse one forelimb was randomly assigned to the treatment group (n = 8) and the contralateral limb to the control group (n = 8). Both fore feet of all horses were instrumented with microdialysis probes using a technique previously described [18]. Briefly, keratinized tissue was resected from the white line region (on the midline, cranial to the point of the frog) to create a hole of 1.5 cm diameter. After bilateral abaxial sesamoid perineural anaesthesia and aseptic preparation, a modified (loose hub) 18G x 15 cm-long spinal needle[a] was introduced through the white line and advanced proximally (at a ~5˚ angle toward the third phalanx) until the tip appeared through the skin above the coronet. A coaxial microdialysis probe (CMA 20[b]) was inserted through the needle in order to position the probe membrane in the mid lamellar region. Once the probe is placed, the needle is projected and removed proximally above the coronet. The resected defect at the toe was filled with dental silicone putty (Ortho-Pak Putty[c]). This introduction technique aimed at positioning the probe in the sublamellar dermis, close to the primary epidermal lamellar (PEL) tips.

Microdialysis solutions were prepared aseptically and stored at 4°C until used. The control solution was a commercial polyionic microdialysis perfusion fluid (T1[d]) with the addition of urea 20mmol/l and dextran 40 mg/ml (Dextran-70[e]) to prevent perfusate loss [19].

In vitro determinations performed previously in our laboratory (Appendix 1) demonstrated that a considerable amount of insulin is lost in the microdialysis system as previously described [20]. The mean (range) insulin recovery outside the membrane was 0.5 (0.14 – 1.1) % of the prepared perfusate concentration.

Microdialysis provides a single point source for drug delivery. Drug diffusion and equilibration are subjected to the drug concentration gradient in the tissue surrounding the probe [21]. As such, the insulin concentration selected aimed to be at least ten times greater than that achieved in plasma during previous p-EHC studies [4; 5]. Thus, the treatment solution was prepared by adding 28 µl of recombinant human insulin (Actrapid[f]) to 972 µl of control solution, achieving a final concentration of 100 µg/ml of human insulin. The predicted concentration delivered to the tissue was 0.5 µg/ml.

In one subject human insulin was replaced by fluorescent FITC-insulin at the same concentration and following the same preparation protocol. FITC-insulin has comparable biological activity to non-labelled human insulin and has been used previously for the study of insulin transport and biological interactions [22; 23]

Following probe placement every foot in each group received control solution during the stabilization period (2h) and during the baseline period (1h). After this, treated and control groups were perfused with their respective solutions for 24 h at a flow rate of 1µl/min using precision microdialysis pumps (CMA 107[g]). Control and treatment perfusates were replaced every 12 h using freshly prepared solutions.

2.3.3 EdU perfusions

After 24 h of microdialysis perfusion, the horses were sedated with intravenous detomidine (Detomo Vet[h]) at 0.01 mg/kg BWT. Both forelimbs were desensitized with abaxial sesamoid perineural anaesthesia and rubber tourniquets were applied at the level of the fetlock. Retrograde intravenous infusion of 60 mg of EdU[i] dissolved in 30 ml of 0.9% sterile saline solution was performed via the lateral palmar digital vein of each foot. The tourniquet was kept in place for 60 minutes and then the horses were killed by I.V. sodium pentobarbitone (Lethabarb[j]) administration.

2.3.4 Tissue sample preparation

Both control and treated feet were disarticulated at the level of the metacarpophalangeal joint and tissue samples (15×10×5 mm) collected from the dorsal hoof wall as follows. A central section (L1) was harvested from the midline at the level of the microdialysis membrane. Four more sections were taken from a distance of 15 mm (to the centre of the section) medial, lateral, proximal and distal (L2, L3, L4 and L5) from the central section. Tissue sections were stained with hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS) for histomorphometry analysis and duplicate sections were processed unstained for click chemistry (Alexa fluor 647 Click iT imaging kit®[k]) and nuclei labelling (Hoechst 33342[l]).

2.3.5 EdU analysis

Fluorescence microscopy analysis was performed on a confocal microscope (N1R confocal, Nikon[m]). Cells were counted in 250 fields per section at 60X magnification using NIS Elements 10.0[m] software for windows. The number of epidermal cells stained with Hoechst 33342 only (TNC) and the number of epidermal cells that stained with EdU and Hoechst (TNPC) was determined for each section.

2.3.6 Histology and histomorphometry

Sections were evaluated by one investigator who was blind to the treatments (AVE), and scored for laminitis histopathology according to the scoring system of Pollitt [24]. Histomorphometry measurements were also performed on sections blinded using software (ImageJ 1.48v[n] and ImagePro Plus[o], for Windows) following a similar protocol to that previously described by de Laat *et al.* [10]. Briefly, the length of the PELs (PELL), their keratinized axis (KPELL) and non-keratinized tip (NKPELL) were measured in the six consecutive PELs closest to the microdialysis membrane. Secondary epidermal lamellar length (SELL) was measured in 10 consecutive SELs in the axial half of previously described PELs.

2.3.7 Data analysis

Data were summarized using mean (Standard Deviation; SD), median (1^{st} and 3^{rd} Quartiles), minimum and maximum. Data normality was tested using the Shapiro-Wilk normality test. Means (± SD) of the normally distributed variables were compared between the group using paired *t*-tests. Statistical significance was declared at $p \leq 0.05$.

For the analysis of TNPC, a zero inflated negative binomial model was used to adjust for the inflated zero counts and any overdispersion in the data. For inclusion in the multivariable analysis, the associations between explanatory variables and cell counts were screened using univariable, mixed effects zero-inflated negative binomial models with horse fitted as a random effect. Explanatory variables with a likelihood ratio p-value ≤ 0.20 were selected for the multivariable model. Multivariable modelling process was done using forward and backward elimination procedure and statistical significance was declared using the likelihood ratio test and an alpha level at 0.05 or less. Two-way interactions were tested and model fit was assessed using *Pearson* goodness-of-fit test. The model was fitted using menbreg procedure in Stata (Stata13.1[p]) with unstructured variance-covariance structure of the random effects and robust standard errors.

2.4 Results

2.4.1 Histology and histomorphometry

The microdialysis membranes were located in the sub-lamellar tissue in the control and treated groups at a median distance of 2.03 mm (range 1.17 - 5.86) and 1.86 mm (range 0.8 - 4.5) respectively from the tip of the closest PEL and therefore did not directly disrupt PEL architecture. There appeared to be some mild laminitis histopathology (grade 1) [21] present in the sections immediately surrounding the probe (L1) in the treated limbs. This consisted of rounding of EBC nuclei, lengthening and attenuation of SELs and some BM separation visible with PAS staining. Apoptotic cells and mitotic figures were rare in both treated and control sections. The median (interquartile range) histological score for the treated sections at this level was 1 (0.25-1.75) compared with 0 (0-0.75) in control limbs (Fig. 2.1), and the difference approached significance ($p = 0.0625$). At all other section levels the median scores for both treated and control limbs were 0 and were not different.

Total number of cells, PELL, KPELL, NKPELL and SELL were normally distributed and differences in the histomorphometry measurements between groups reached significance only at L1. The PELLs were significantly longer in the treated group (5.67 mm±0.18) compared to the control group (5.26 mm ± 0.14; p = 0.034) and NKPELL were longer in the treated group (0.94 mm ± 0.124) compared to control (0.68 mm ± 0.064; p = 0.05) (Fig. 2.2 and 2.3). Comparisons of KPELL and SELL did not reach significance in any of the sections (Fig. 2.2).

In the one treatment limb where FITC-insulin was used in the perfusate, a fluorescent area of 277.27 mm^2 was detected around the microdialysis probe, demonstrating that insulin successfully diffused through the microdialysis membrane to the interstitium.

2.4.2 EdU

The EdU labelled and total cell counts are summarized in tables 2.1. Analysis by a zero inflated negative binomial model (Table 2.2) showed that the mean count of TNPC was 42 (95% CI 0.8 to $2e^3$; p = 0.07) times higher in the treatment group compared with control group. Levels 3 to 5 were grouped for comparison purposes as they were not statistically different from each other. The mean count of TNPC at L1 and L2 was 25 (95% CI 8.6 to 72; p < 0.01) and seven (95% CI 2.2 to 22; p < 0.01) times that of levels 3-5 respectively. No significant differences were found in the TNC between groups at any sample level.

2.5 Discussion

The local delivery of insulin for 24 h affected the histomorphometry of the lamellar tissue in the region immediately surrounding the microdialysis membrane. The PELL and NKPELL were increased significantly (p < 0.05) only in the central sections (L1) of the treated limbs. The combination of longer PELL and conserved KPELL observed exclusively in the sections closest to the membrane (L1) in the treated group supports a direct effect of insulin contributing to changes in the lamellar tissue, affecting mainly the SELs at the axial tips of the PELs (NKPELL). These effects were absent in control limbs and the amount of insulin delivered was too small to affect systemic insulin status, indicating therefore that insulin can cause direct alterations to lamellae without the need for systemic interactions.

Insulin delivered locally to the lamellar tissue via microdialysis exerted a similar effect on the EBCs to that observed when it is administered systemically. Elongation and narrowing of the SELs has been described previously in ponies [25] and horses [4] subjected to the p-EHC model. de Laat *et al.* [10] suggested that elongation of the SELs may be related to an increase in the total number of epidermal cells, supported by the increased number of mitotic figures and the TPX2 immunolocalisation in those tissues after 24h of the p-EHC model. Additionally, it was proposed that the loss of normal orientation and shape of basal cell nuclei observed may reflect an insulin effect on EBC metabolism and cytoskeletal integrity [26]. Karikoski *et al.* [27] attributed the lengthening and narrowing of the SELs of ponies subjected to p-EHC induced laminitis mainly to cell stretching. Considering that no differences were found in the total number of cells between treated and control limbs in the current study (Table 2.1), the lengthening of the PELs cannot be explained by an increase in the number of cells, and therefore the data supports cell stretching as the means by which insulin disrupts lamellar architecture when delivered locally.

Besides the well described effects of insulin on cellular metabolism, growth and proliferation [11], it has been reported that insulin can exert direct effects on the cytoskeleton of different types of cells via the insulin receptor (IR) and the insulin-like growth factor 1 receptor (IGF-1R) [28-30]. Previous studies using cultured rat mesangial cells demonstrated rearrangement of the cytoskeleton after exposure to insulin for one hour. The changes observed in the lamellae in the present work might be explained by a similar pathway. As previously described by Burns *et al.* [31], the distribution of IR in the lamellar tissue is restricted to the vasculature whilst the IGF-1R is ubiquitous. Thus, high local levels of insulin may stimulate the EBCs via IGF-1R, affecting the cytoskeleton and subsequently the shape of the cells, organization of the organelles and stability of the tissue.

It is still likely that cellular proliferation plays an important role in the pathophysiology of insulin-induced laminitis. Lamellar epidermal proliferation has been previously studied by Daradka and Pollitt [32] using the proliferation marker BrdU (Bromohydroxiurudine), and by de Laat *et al.* [26] using TPX2. The novel proliferation marker EdU was used in the current study. EdU is a thymidine analogue which is incorporated into the DNA exclusively during the synthesis stage (S) of mitosis, labelling only the cells that are actually proliferating [33]. EdU was selected for this study over the other markers available because of its good sensitivity and simple protocol for sample analysis [34; 35]. Our group has standardized and used this technique (EdU delivered via

regional perfusion) for the study of proliferation in normal equine lamellar tissue (Chapter 4), with similar results to previous studies [32]. The proliferative response to the local delivery of insulin in the current study was not as substantial as described by de Laat *et al.* [26] for the p-EHC. However the number of EdU positive cells in the treated group was 42 times that of the control group, with a clear trend toward significance ($p = 0.07$). Also, increased proliferation was found at L1 (control and treatment groups together) compared to the other areas. Although the TNPC in the control group is consistent with the proliferation rate previously described for normal horses at the level of the hoof (Chapter 4), the increased cellular proliferation detected in the treated group cannot be attributed as the result of local insulin effects.

The fact that the difference in TNPC between groups did not reach significance ($p = 0.07$) may be related to the small sample size and the variability in the counts between feet within the same group, that could be associated with the labelling technique used in this study. The effect of the sample size on counts of positive cells is difficult to predict (can be bidirectional i.e. away or towards the null) and cannot be evaluated from the current dataset. In the present study, the administered dose of the proliferation marker EdU was lower (60 mg per foot) than that used previously (*Chapter 4)* (75 mg per foot). This small dose difference should not have caused a notable effect in the determination of the cell proliferation rate. However, cell proliferation at the mid-lamellae is rare or absent [32] and the sensitivity of the EdU labelling technique can be modified in a concentration-dependent manner [36; 37]. Thus, a higher EdU concentration may be required to detect significant changes in cell proliferation in the mid-lamellar region. More replicates would be recommended for future experiments.

One of the histological characteristics of the p-EHC laminitis model is minimal disruption of the BM compared to that observed in the oligofructose model [10]. It has been hypothesized that weight-bearing and mechanical distraction plays a role in the disruption of the BM in p-EHC models of laminitis [4], explaining the differences observed between ponies and full sized horses in this model. Interestingly, there was some BM detachment noted with local insulin delivery in the current study, appearing to be confined to the sections immediately surrounding the microdialysis membrane (and presumably with the highest concentration of insulin). This suggests that there were sufficient disruptive forces present in the focal area around the membrane to cause BM separation, even in the absence of widespread failure of the lamellar attachment as would occur with generalized laminitis.

Systemically circulating insulin requires binding to its receptor in the endothelium to be transported from the vasculature to the tissues. This process has been described by Yang *et al.* [38] and Wang *et al.* [39] as the most likely limiting step for insulin delivery. In the case of microdialysis, the limiting factors are the concentration of the analyte in the tissue, the diffusion coefficient and the tortuosity of the tissue [40]. The estimated concentration of insulin delivered using microdialysis to the lamellae in this experiment exceeded the concentrations described previously that achieved a biological effect in rat brain [16] and human muscle [17]. The effect on cellular proliferation in the experiment was only slightly outside the significance level ($p = 0.07$). A concentration-dependent increase in mitotic activity has been reported in cultured lamellar epithelial cells incubated for 3 days with 500 µIU/ml (0.017 µg/ml) of insulin [41]. In that study, EBCs exposed to insulin increased their proliferative activity by more than 50% compared to controls. Examination of concentrations of insulin in the tissue harvested in the current study was not performed, but would be required to confirm the existence of an apparent concentration-response relationship. The substitution of regular human insulin with FITC-insulin in 1 horse in the current study allowed confirmation that insulin was diffusing out of the membrane into the lamellar interstitium, being readily detected by fluorescent microscopy [22].

Time of exposure of the tissue to insulin is a relevant factor to consider when assessing the effects of insulin on cellular proliferation. In previous experiments by de Laat *et al.* [26], horses treated with p-HEC did not show variation in the proliferation rate after 6 h and 12 h of hyperinsulinemia, whilst the expression of the proliferation marker TPX2 increased significantly after 24 h and 48 h. In the current study, the EdU labelling technique only detected cells entering the S phase of mitosis during the 1 h retrograde intravenous infusion period of exposure, and this may explain why the total number of proliferating cells reported here was lower than that described by de Laat *et al.* [10] after 48h of hyperinsulinemia. Despite this, a clear trend of increased proliferative activity was detected in the treatment group. A longer insulin perfusion period, or adjustment of the EdU protocol, might be required to confirm a time-dependent effect of insulin on EBC proliferation.

2.6 Conclusion

Lamellar tissue morphology is affected by continuous local delivery of exogenous insulin, and although EBC proliferation processes are triggered, the morphological changes appear to precede any increase in EBC numbers. Insulin appears to be capable of directly acting on lamellar tissue without the requirement for systemic interactions, and these direct effects may be responsible for the lamellar changes seen in endocrinopathic laminitis.

2.7 Manufacturers

[a] Becton Dickson Infusion Therapy Systems Inc., Sandy, Utah, USA
[b, d, g] CMA Microdialysis, Sweden
[c] Hoof-Life, Baltimore, MD, USA
[e] Sigma-Aldrich, NSW, Australia
[f] NovoNordisk, Denmark
[h] Nature Vet, Glenorie, NSW, Australia
[i, k, l] Innvitrogen, LifeTechnologies, Carlsbad, CA, United States of America
[J] Virbac Animal Health, Milperrin, NSW, Australia
[m] Nikon, Konan, Minato-ku, Tokyo, Japan
[n] National Institute of Mental Health (NIMH), USA
[o] MediaCybernetics, Rockville, MD 20850 USA
[p] StataCop LP, 4905 Lakeway Drive, College Station, TX 77845, USA

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Table 2.1: Mean (SD), Median (Range) values of total number of epidermal cells (TNC) labelled using Hoechst 33342 and total number of proliferating positive cells (TNPC) labelled using EdU**,** counted in five different areas of lamellar tissue (L1 to L5) of control (n=8) and treated limbs (n=8) from the hooves of horses dialysed with recombinant human insulin for 24h.

	Control		Treatment	
	Mean (SD)	Median (Range)	Mean (SD)	Median (Q1, Q3)
TNC*				
L1	21 (6)	20 (16-24)	21 (6)	22 (18-27)
L2	23 (6)	24 (19-25)	21 (7)	22 (19-27)
L3	19 (2)	19 (17-20)	23 (7.0)	231 (19-30)
L4	22 (4)	21 (20-24)	21 (7)	21 (15.-27)
L5	21 (6)	20 (18-25)	24 (7)	27 (18-29)
TNPC				
L1	1.9 (3.6)	0 (0-9)	9.8 (18.3)	0 (0-45)
L2	0.4 (1.1)	0 (0-3)	6.0 (9.3)	1 (0-14)
L3	0.4 (1.1)	0 (0-3)	2.9 (5.0)	0 (0-5)
L4	0.1 (0.4)	0 (0-1)	9.5 (20.1)	0 (0-12)
L5	0.0 (0.0)	0 (0-0)	1.6 (3.0)	0 (0-5)

*total cells count × 1000

Table 2.2: Regression coefficients from a zero inflated negative binomial model of the total number of EdU positive epidermal cells (TNPC)**,** *counted in five different areas of lamellar tissue of control (n=8) and treatment limbs (n=8).*

Explanatory variable	Coefficient (SE)	Mean count ratio (95% CI)	P-value
Group			
Control	Reference	-	-
Treatment	3.8 (2.0)	43 (0.8 – $2e^3$)	0.07
Lamellar area			
3-5	Reference	-	-
1*	3.2 (0.5)	25 (8.6 – 72)	<0.01
2	1.9 (0.6)	7 (2.2 – 22)	<0.01

Key: SE standard error; CI confidence interval.

Overdispersion parameter was 2 (0.2); 95% CI 1.3 to 2.7; p<0.01; Horse-level random effect coefficient was $2.6e^{-29}$ (SE $3.7e^{-29}$) and 95% CI of $1.65e^{-30}$ to $4.13e^{-28}$

**Interpretation: after accounting for the effect of other variables in the model, the mean count ratio of positive cells at level one was 25 (95% CI 8.6 to 72]) times that of areas 3-5 (p < 0.01).*

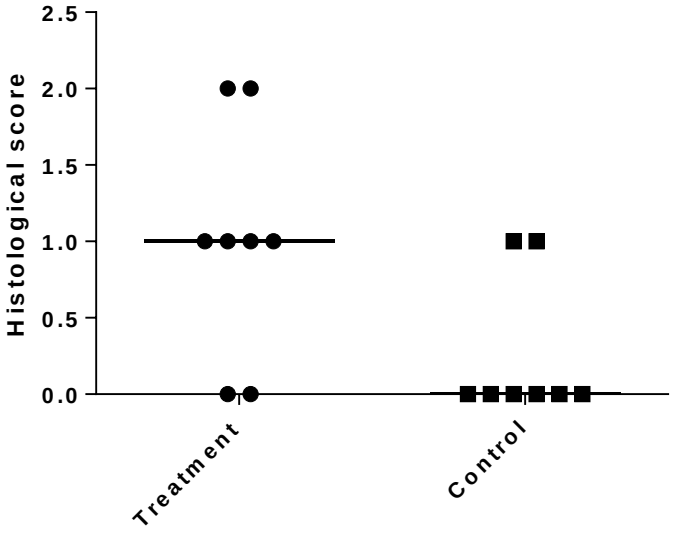

Figure 2.1: Histological scores for L1. The median (Interquartile range) score for the treatment sections at this level was 1 (0.25-1.75) compared with 0 (0-0.75) in controls. However the difference was not significant (p=0.0625). There were no significant differences for the other section sites.

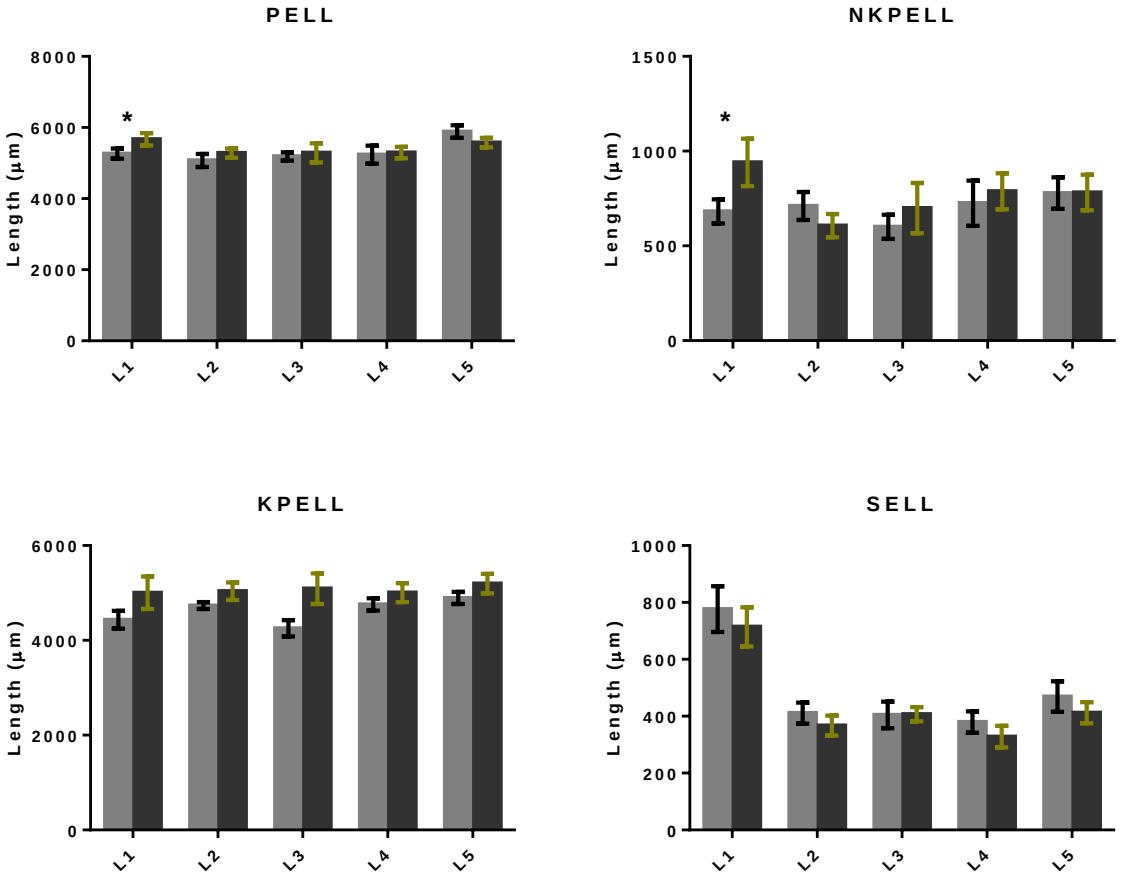

Figure 2.2: Mean ±SEM measurements of histomorphometric parameters of five lamellar tissue areas (L1 to L5) for control (grey bar, n = 8) and treatment (black bar, n = 8) groups. The lengths (µm) of the primary epidermal lamellar (PELL) and the non-keratinized tip of the primary epidermal lamellar (NKPELL) were significantly longer only at L1 in the treated group. The length of the keratinized axis of the primary epidermal (KPELL) did not differ between groups. Significant difference defined as $p < 0.05$. ***** = significant difference between treatment and control group for that area.

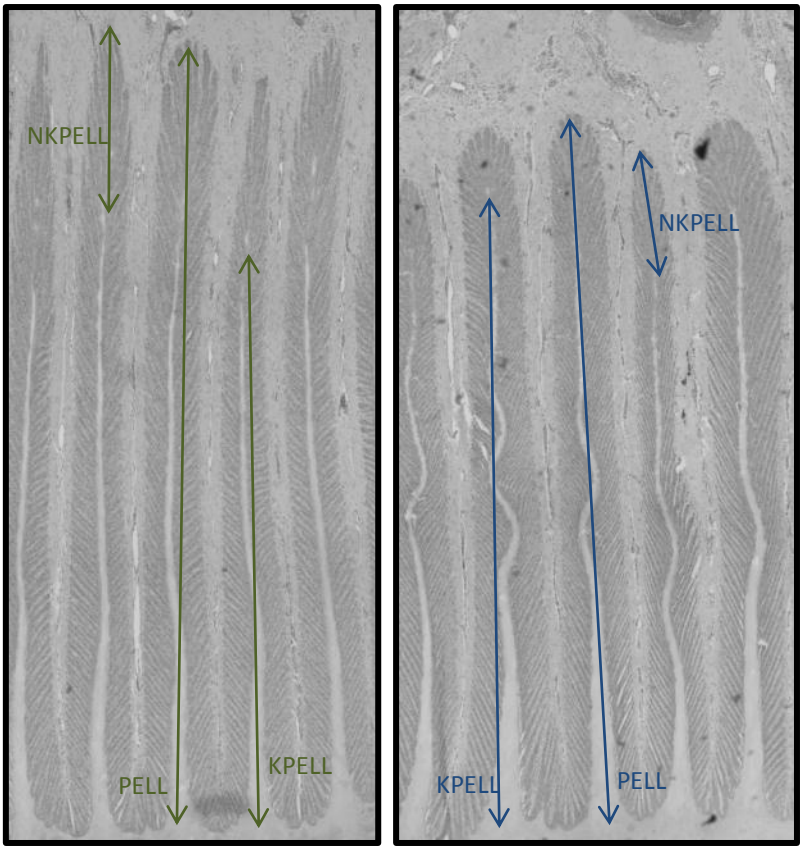

Figure 2.3: Photomicrographs of treated (left) and control (right) sections of area L1 (closest to the microdialysis membrane) from the same horse showing the histomorphometric measurements performed. There is a greater PELL caused by a longer NKPELL in the treated foot compared to the control foot. (PELL: primary epidermal lamellar length; KPELL: keratinized axis of the primary epidermal lamellar length; NKPELL: no keratinized tip of the primary epidermal lamellar)

Chapter 3: The effect of locally delivered exogenous insulin on equine lamellar perfusion and glucose metabolism

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Word Count: 5116

Ethical Considerations: The project was approved by The University of Queensland Animal Ethics Committee (AEC) that monitors compliance with the Animal Welfare Act (2001) and the Code of Practice for the care and use of animals for scientific purposes (current edition). All animals were monitored continuously by the investigators.

Competing Interests: The authors have no competing interests to declare

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3.1 Summary

Reasons for performing study: Hyperinsulinemia appears to be central to the pathophysiology of endocrinopathic laminitis, however the direct effects of insulin on hoof lamellar tissue have not been studied.

Objectives: To determine the effects of locally delivered exogenous insulin *in vivo* on lamellar tissue perfusion and energy metabolism using tissue microdialysis.

Study Design: Controlled (within subject) experimental trial

Methods: Both forefeet of six Standardbred horses were instrumented for tissue microdialysis: control limbs were perfused with isotonic polyionic solution, whereas human insulin (100 µg/ml) was added to the perfusate for contralateral limbs. The tissue was perfused continuously at 1 µl/min for 24 h. Dialysate was collected hourly and metabolite concentrations (glucose, lactate, pyruvate and urea) were measured immediately. At the end of the experiment the horses were euthanized and tissue samples collected for routine histology.

Results: No significant differences were found in the area under the curve (AUC) of metabolite concentrations between groups after the delivery of insulin via microdialysis for 24 h. Although decreased glucose concentrations and smaller AUC were detected in five out of six treatment limbs during the period from 9 to 24h compared to control, the differences between groups were not significant ($p=0.17$). Insulin diffusion into the tissue was confirmed by fluorescent FITC-insulin. No differences were found in urea clearance between groups.

Conclusions: No significant effects were detected in lamellar glucose metabolism or local perfusion after 24 h of insulin delivery via microdialysis; however the apparent effects of insulin on glucose concentration in all but one horse warrant further investigation.

3.2 Introduction

The development of laminitis as a result of endocrine influences, a phenomenon known as endocrinopathic laminitis [1] has received special research interest in recent years. Described as the most common form of the disease [2], endocrinopathic laminitis has been associated with obesity, consumption of lush pastures, pathologies producing high levels of glucocorticoids, and insulin resistance (IR) [3-6]. The common factor in these conditions appears to be the development of hyperinsulinemia [7; 8]. Several authors have assessed the role of insulin in laminitis [9-16], and the disease has been induced experimentally using a prolonged euglycemic hyperinsulinemic clamp (p-EHC) [17] [18]. This supports a direct effect of insulin on lamellar tissue rather than any systemic metabolic dysregulation, although the mechanism by which hyperinsulinemia causes laminitis remains unclear.

Insulin is a polypeptide hormone with a well described role in the control of glucose metabolism [19]. Nonetheless via the insulin receptor (InsR) and insulin like growth factor 1 receptors (IGF1R) it also plays a crucial role in the modulation of vascular tone, cell proliferation, growth and differentiation [20]. Stimulated epidermal basal cell (EBC) division and/or disturbances of cellular energy metabolism may result in weakening or dissolution of hemidesmosomes, the vital connections between EBC and their basement membrane, as previously described in laminitis models [21-23]. In addition, persistent digital hyperthermia occurring during the p-EHC model is suggestive of an effect on digital vascular perfusion, which could play some role in laminitis development [17]. Thus, the study of the direct metabolic and vascular effects of exogenous insulin on the lamellar tissue is required.

Tissue microdialysis is a minimally invasive technique that has been used for the study of the energy metabolism in different tissues including muscle [24], subcutis [25; 26] and equine lamellar tissue [27]. By adding urea to the perfusate, urea clearance can be used as an estimate of local tissue perfusion [27-30]. Although microdialysis is traditionally used as a sampling technique, it can also be used simultaneously for the delivery of substances into the tissue. McNay *et al.* [31] and Rosdahl *et al.* [24] have assessed the effects of locally delivered exogenous insulin on the energy metabolism of human muscle and rat brain, respectively, using tissue microdialysis. Using similar techniques, the aims of the current study were to determine the effects of locally delivered exogenous insulin on lamellar perfusion and glucose metabolism.

3.3 Materials and Methods

3.3.1 Animals

Six Standardbred geldings (aged 6 to12 years, body weights of 420 to 500 kg) with clinically normal hooves were used for this study. The project was approved by the University of Queensland Animal Ethics Committee (approval number: SVS/338/11/UQ) that monitors compliance with the Animal Welfare Act (2001) and The Code of Practice for the care and use of animals for scientific purposes (current edition).

3.3.2 Tissue microdialysis

Both fore feet of all horses were instrumented with microdialysis probes using a previously described technique [27]. Briefly, keratinized tissue was resected from the white line region (on the midline, cranial to the point of the frog) to create a 1.5 cm diameter hole. After bilateral abaxial sesamoid nerve block using 2% mepivacaine[a] and aseptic preparation a modified (loose hub) 18G x 15 cm long spinal needle[b] was introduced through the white line and advanced proximally (at a ~5˚ angle toward the third phalanx) until the tip appeared through the skin above the coronet. A coaxial microdialysis probe (CMA 20[c]) was inserted through the needle in order to position the probe membrane in the mid lamellar region. The resected defect at the toe was filled with dental silicone putty (Ortho-Pak Putty[d]). This introduction technique aims at positioning the probes in the sublamellar dermis, close to the primary epidermal lamellar (PEL) tips.

In every horse one forelimb was randomly assigned to the treatment group (n=6) and the contralateral limb to the control group (n=6). Microdialysis solutions were prepared aseptically and stored at 4°C until used. The control solution consisted of a commercial polyionic microdialysis perfusion fluid (T1[e]) with the addition of 20 mmol/l urea for the calculation of urea clearance, and 40 mg/ml dextran (Dextran-70[f]) to prevent perfusate loss [32]. The treatment solution was prepared by adding recombinant human insulin (Actrapid[g]) to the control solution to obtain a final concentration of 100 µg/ml. Preliminary studies *in vitro* (Appendix 1), evidenced high variation in insulin recoveries depending on the concentration prepared. Considering this, a concentration of 100 µg/ml was selected to ensure

that similar concentrations to previously described (0.035 µg/ml) [18; 33] were achieved. In one of treated limbs, human insulin was replaced by fluorescently-labelled FITC-insulin[h] following the same preparation protocol.

Every foot received control solution during the initial stabilization period (2 h) and during a subsequent baseline period (1 h). After this, treatment and control feet were perfused with their respective solutions for 24 h at a flow rate of 1 µl/min using precision microdialysis pumps (CMA 107[i]). Control and treatment perfusates were replaced every 12 h with freshly prepared solutions.

3.3.3 Sampling

After probe placement, a 2 h stabilisation period was allowed before the collection of a baseline sample (dialysate) in both groups. Dialysate samples were then collected hourly for a further 24 h and metabolite concentrations (glucose, lactate, pyruvate and urea) determined immediately using a commercial microdialysis analyser (Iscus Flex[j]). Standard indices of energy metabolism (lactate:glucose (L:G) and lactate:pyruvate (L:P) ratios) were calculated.

At the end of the perfusion period the horses were euthanized using pentobarbital sodium 20 mg/kg I.V. (Lethabarb[k]). Mid-dorsal lamellar tissue samples were harvested at the level of the membrane, fixed and stained with hematoxylin and eosin (H&E) for confirmation of microdialysis membrane position. The distance (median [IQR]) from the centre of the dialysis probe to the tips of the 5 closest PELs was calculated using NIS Elements 10.0[l] software for windows. The presence of FITC-insulin in the tissue was detected using fluorescence microscopy analysis on a confocal microscope (N1R confocal[m])

3.3.4 Data analysis

The area under the curve (AUC) of dialysate glucose metabolites and their ratios (lactate:glucose (L:G) and lactate:pyruvate (L:P)) as well as urea concentrations was calculated for three periods: baseline to 24 h, baseline to 8 h and 9 to 24 h. 1. The data were partitioned into separate time periods rather than taking an average over the entire infusion period as it was clear from a visual appraisal of the graphs that there was an initial

period of change, followed by a period of stability, which was consistent across all metabolites. Similarities in the variance between groups were tested using F-tests. The distribution of the data was assumed to be normal because the small size of the data set precluded any meaningful normality testing (e.g. Shapiro Wilk test).Then, the AUC of every metabolite was compared between groups using paired t- tests.

Median metabolite concentrations/ratios were calculated and correlated with the distance between the microdialysis membrane and the closest PELs in each foot using Spearman's rank coefficients.

The statistical analysis was performed using GraphPad Prism v6.00 for Windows[n]. Statistical significance was set at $p < 0.05$.

3.4 Results

3.4.1 Histology

The microdialysis probe membranes were confirmed to be located in the sub-lamellar dermis in the control and treated groups at 2.03 mm (1.54 to3.56 mm) and 1.86 mm (1.60 to 1.96 mm) respectively from the tip of the closest PEL, minimally affecting the normal tissue organization. Evaluation of the FITC-insulin[h] perfused limb evidenced an area of 277 mm^2 fluorescent to FITC, confirming that the insulin was diffusing into the lamellar interstitium.

3.4.2 Glucose metabolites

Median metabolite concentrations for the entire 24 h period are summarised in table 3.1 and figure 3.1. The analytes concentrations fluctuated during the first 8 h reaching stable concentrations during the 9 to 24 h period (Fig. 3.1). Differences in the AUC of the analytes between groups failed to reach significance during the baseline to 24 h, baseline to 8 h or 9 to 24 h periods (table 3.2). However, individual AUC and glucose concentration remained considerably lower in horses 3, 4 and 6 over the entire 24 h period. Additionally, five out of six horses showed markedly decreased glucose levels and smaller AUC in the treated group in the period 9 to 24h compared to control. Horse number five showed higher glucose concentrations and AUC in the treated foot compared to the control over the baseline to 24 h and 9 to 24 h periods (table. 3.3 and fig. 3.2).

3.4.3 Urea clearance

No differences were detected in urea AUC between treated and control feet over the 24 h, baseline to 8 h or 9 to 24 h periods.

3.4.4 Correlations

A trend towards a significant positive correlation ($r = 0.9$; $p = 0.083$) was found in the treated group between dialysate glucose concentration and the distance from the microdialysis probe to the 5 closest PEL, whilst a trend towards negative correlation ($r = -0.9$; $p = 0.083$) was found between the L:G ratio and this distance, both outside of the significance limit. No correlations were found between dialysate glucose concentration and the distance from the microdialysis probe ($r = 0.2$; $p = 0.916$) nor the L:G ratio and this distance ($r = -0.2$; $p = 0.916$) in the control group.

3.5 Discussion

After 24 h of insulin delivery via microdialysis, no significant effects on glucose metabolism or local perfusion were detected. Glucose transport in lamellar tissue has been

described as an insulin-independent process (at insulin concentrations up to 300 µIU/ml) mediated mainly by the glucose transporter GLUT-1 [34]. *In vitro* experiments demonstrated that the expression of this transporter seems to be controlled by the amount of glucose available in the medium and that it is also capable of increasing glucose uptake to supra-physiological levels before saturation [34]. However, the presence of other (insulin-sensitive) glucose transporters in the lamellar tissue has not been discounted. The concentration of insulin achieved in the present study appeared to be insufficient to elicit a significant effect on lamellar vascular tone. However, it has been recently demonstrated that lamellar perfusion is highly influenced by limb movement and the lack of load cycling in the standing, static horse may outweigh any vasomodulatory effect of insulin [35].

Tissue concentrations of insulin measured in human skeletal muscle and subcutaneous tissue have been reported to be approximately 50% of the plasma concentration [36; 37]. Insulin determinations have not been performed in the lamellar tissue of healthy nor laminitic horses and insulin transport from or to the lamellar vasculature has not been described. Insulin concentration in the lamellar tissue was not measured in the current study, however based on the *in vitro* calibration of the microdialysis system (Appendix 1), the median (range) concentration of insulin expected outside of the membrane was estimated as 0.5 (0.14 – 1.1) % of the prepared solution. Microdialysis provides a single point source for drug delivery. Drug diffusion and equilibration are subjected to the drug concentration gradient in the tissue surrounding the probe [38]. As such, the insulin concentration selected aimed to be at least ten times greater than that achieved in plasma during previous p-EHC studies. Mathematical models have estimated that the diffusion radius of a neurotransmitter around a microdialysis catheter in the brain could be up to 0.85 ± 0.25 mm depending on the concentration of the analyte in the tissue, the diffusion coefficient and the tortuosity of the tissue [39]. Additionally, histological analysis of lamellar tissue from the horse perfused with FITC-insulin[h] confirmed that insulin was present in the lamellar insterstitium, outside the probe membrane. Although FITC-labelled insulin was observed outside the membrane in one limb, a more accurate determination of the insulin concentration and distribution in the lamellar tissue should be performed. The measurement of the down-regulation of the different insulin receptors and the use of immunohistochemistry techniques for the localization of the insulin molecules in the tissue should be considered for future studies.

Although local insulin delivery did not cause a statistically significant difference in glucose concentrations, the AUC of glucose concentrations in 5 of 6 horses was consistently

lower in the treated group compared to control during the 9 h to 24 h period. In the absence of any change in local perfusion (no change in urea clearance) and the lack of any significant increase in L:P (a marker of ischemic/non-ischemic bioenergetic failure [40-42]) the most likely explanation proposed for the apparent decrease in dialysate glucose concentration is increased cellular glucose uptake and consumption. The relatively high consumption of glucose by the equine digit as a whole has been demonstrated previously [43]. The lamellar dermis is constituted mainly of connective tissue, nerves and blood vessels with limited metabolic activity [43], whereas the EBCs are metabolically active and require a continuous supply of glucose to maintain cellular functions including the dermo-epidermal connection. Pass *et al.* [44], previously demonstrated *in vitro* that glucose is crucial for maintaining hemidesmosomes in order to resist lamellar separation. Thus the fall in the glucose levels with no changes in perfusion (no changes in urea clearance) observed in this experiment might reflect an increased glucose uptake by the EBCs possibly stimulated by insulin.

At normal physiological concentrations insulin exerts its effects via the InsR. In the lamellar tissue InsR distribution is restricted to the vasculature, being absent in the EBCs. However, insulin at high concentrations is capable of stimulating the IGF1R, which is ubiquitous in the lamellar tissue and therefore present in the EBCs [45]. Recently, dysregulation of epidermal cell growth and division through activation of IGF1R has been suggested as a potential mechanism for the laminitis lesion associated with hyperinsulinemia based on the increased proliferative activity and morphological derangements of the EBCs observed during the developmental stage of insulin-induced laminitis [47]. In previous studies, cultured lamellar epidermal epithelial cells incubated with insulin showed a concentration-dependent increase in cell proliferation which was significantly inhibited when the IGF1R was blocked [48]. The insulin-IGF1R interaction can activate multiple signalling pathways, affecting proliferation and cellular metabolism [49]. For example, the pathways PI3K, AKT and mTOR, modulate glucose uptake, glycolysis, fatty acid, protein and nucleotide synthesis and also interact with each other to coordinate the cellular state and metabolism [49].

Additionally, it has been demonstrated that an increase in temperature can lead to increased cellular metabolic rate and therefore energy cellular consumption [50]. Although tissue temperature was not measured in the current study, an increase in the hoof wall temperature during the p-EHC laminitis model, possibly associated with vasodilation, has been previously reported [51]. Vasodilation is a well described effect of insulin, modulated

by the PI-3K pathway [52] and exerted via nitric oxide on the endothelium [53]. Although no vascular changes (no changes in urea clearance) were observed in the present study, insulin could modulate lamellar arterio-venous anastomosis (AVAs) in particular, without a detectable effect on urea clearance. The opening of AVAs could increase tissue temperature and increase cellular metabolic rate indirectly. Thus, the lower concentrations of interstitial glucose observed in the treated group might be the result of a metabolic shift of the EBCs triggered by insulin, modifying the cellular energy requirements indirectly through the stimulation of cellular metabolism, growth and/or proliferation.

Decrease in glucose levels were observed in some of the control feet along time. Interstitial glucose levels depend on local perfusion and glucose uptake by the tissue. Lamellar perfusion is highly influenced by limb load cycling activity and should be considered when the horses are kept in stocks, as reduced limb load cycling frequency has been previously associated with reduced lamellar perfusion [54]. Inflammation can also influence tissue glucose levels via local hyperaemia and migration of inflammatory cells to the target tissue. A mild inflammatory reaction has been observed in the lamellar tissue surrounding the probe with a cellular predominance of neutrophils [27] influencing interstitial glucose levels particularly during the period baseline-8h. Variation in the glucose levels within group were minimal being the differences between control and treatment limb more relevant.

Some of the limitations of the microdialysis technique observed in this study include the variability between horses in the location of the probes placed in the lamellar tissue and the actual sampled/perfused area. The proximity of the membrane to the EBCs or to the bone surface might alter the metabolite concentrations due to a metabolic gradient between the dermis and epidermis. In addition, this distance could also affect the amount of insulin reaching the EBCs and their response to the insulin stimulus. The correlations observed in the treated group between the levels of glucose and the L:G ratio and the distance from the probe to the PELs support the idea that the insulin effect is affected by the proximity of the microdialysis probe to the EBCs and therefore the insulin concentration reaching the epidermis. The proximity of the membrane to the dermal vasculature must be also considered. Wang *et al.* [55] described the transport of the insulin molecule from vasculature to muscle in dogs, demonstrating that the InsR is required by the endothelial cells to transport insulin from the blood stream to the tissues. The lamellar dermis is highly vascularized and the lamellar capillaries are capable of expressing the InsR, which might result in the removal

of the insulin molecule from the tissue, possibly affecting the insulin diffusion and distribution in the lamellar tissue. The high coefficient of variation observed in the AUC of glucose of the treated group (60.88) is likely a consequence of the analyte concentrations of horse five, which showed a larger AUC in the treated foot compared to the control. Concentrations of the other metabolites of control and treatment feet determined in horse five were similar to that of the other horses. As glucose cannot be stored within the hoof [43], the tissue concentrations of glucose will depend on the local perfusion and on the amount of glucose consumed by the tissue.

In conclusion, local delivery of insulin did not cause significant detectable effects on lamellar perfusion or glucose metabolism. However, the apparent decrease in interstitial glucose concentration observed in five out of six treated horses during the 9 to 24 h period, deserves further research. Increased glucose uptake by the EBCs may occur as a consequence of insulin modulation of cellular metabolism via stimulation of IGF1R rather than InsR. Insulin dynamics in lamellar tissue warrant further study.

3.6 Manufacturers

[a] Nature Vet, Glenorie, NSW, Australia
[b] Becton Dickson Infusion Therapy Systems Inc., Sandy, Utah, USA
[c, e, i, j] CMA Microdialysis, Sweden
[d] Hoof-Life, Baltimore, MD, USA
[f, h] Sigma-Aldrich, NSW, Australia
[g] NovoNordisk, Denmark
[k] Virbac Animal Health, Milperrin, NSW, Australia
[l, m] Nikon, Konan, Minato-ku, Tokyo, Japan
[n] Graphpad Software Inc., La Jolla, CA, USA

3.7 References

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Table 3.1: Median [±IQR] of metabolite concentrations in the forelimbs of horses perfused with a control solution or recombinant human insulin over 24 hours.

	Treatment	Control
Glucose	0.57 [0.31 - 0.78]	0.74 [0.6 - 1.01]
Lactate	1.37 [0.97 - 1.49]	1.25 [1.1 - 1.32]
Pyruvate	48.7 [41.55 - 53.80]	47.7 [34.08 – 55.55]
Urea	14.65 [13.37 - 15.70]	14.42 [13.68 - 15.87]
L/G	2.36 [1.34 – 5.23]	1.49 [1.29 – 1.88]

Table 3.2: Mean and Coefficient of Variation (CV%) of the Areas Under the Curve of Glucose, Lactate, Pyruvate, Urea, and Lactate to Glucose and Lactate to Pyruvate ratios, measured in over 24 h in control (n = 6) and treated (n = 6) feet.

	Period					
	Baseline-8h		9-24h		Baseline-24h	
	Treatment	Control	Treatment	Control	Treatment	Control
Glucose						
Mean	5.70	7.19	8.304	12.85	14.61	20.83
CV %	37.1	26.25	60.88	23.04	45.61	21.19
p value	0.3		0.2		0.17	
Lactate						
Mean	10.7	10.89	19.03	17.15	31.06	29.27
CV %	33.05	10.56	18.08	12.18	20.65	9.93
p value	0.9		0.24		0.45	
Pyruvate						
Mean	489.3	498.9	674.4	654.8	1212	1199
CV %	21.14	20.85	19.67	26.10	15.28	23.53
p value	0.89		0.79		0.92	
Urea						
Mean	109.3	107	219.3	220.5	343.5	342.7
CV %	13.99	9.50	8.47	8.78	10.17	8.62
p value	0.71		0.82		0.94	
L:P						
Mean	184.3	198.8	443.2	430.4	655.8	659.7
CV %	26.07	16.83	18.19	25.35	11.92	22.20
p value	0.56		0.78		0.94	
L:G						
Mean	18.73	13.3	118.5	23.51	140.4	38.72
CV %	63.76	27.13	154.99	22.43	137.36	21.69
p value	0.33		0.26		0.25	

Table 3.3 Areas under the curve for glucose concentration versus time in the hooves of individual horses perfused with electrolyte solution (control n=6) or recombinant human insulin (n = 6) for 24 hours. Values are Measn and Coefficient of Variation (CV%).

	Baseline-8h		9-24h		Baseline-24h	
Horse	Treatment	Control	Treatment	Control	Treatment	Control
1	8.105	5.46	7.84	11.45	16.91	17.41
2	7.43	5.275	8.07	9.33	15.97	15.11
3	2.57	6.45	2.925	15.29	5.715	22.4
4	3.85	7.885	4.1	16.37	8.275	25.26
5	6.05	7.76	17.19	10.03	24.18	18.8
6	6.205	10.32	9.7	14.64	16.59	26.01
Mean	5.702	7.192	8.304	12.85	14.61	20.83
CV %	37.1	26.25	60.88	23.04	45.61	21.19
p value	0.3		0.2		0.17	

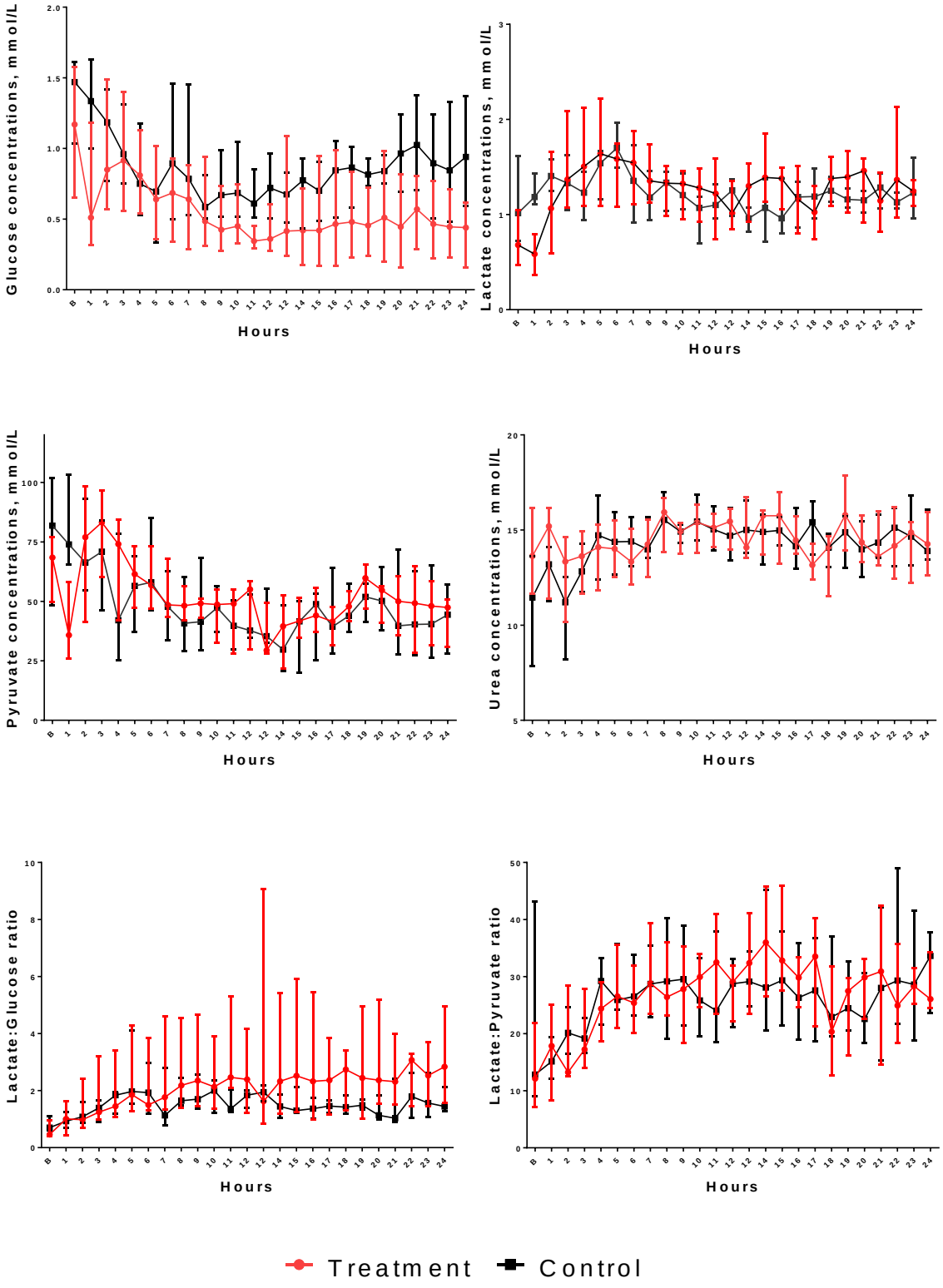

Figure 3.1: Median ±IQR concentrations of glucose, lactate, pyruvate, urea, lactate-glucose ratio and lactate-pyruvate ratio vs. time in the forelimbs of horses perfused with a control solution or insulin for 24 h. Glucose concentration decreased progressively in remaining below baseline until the end of the experiment; however this difference was not statistically significant. No significant differences were found between groups in the other metabolites. different from baseline.

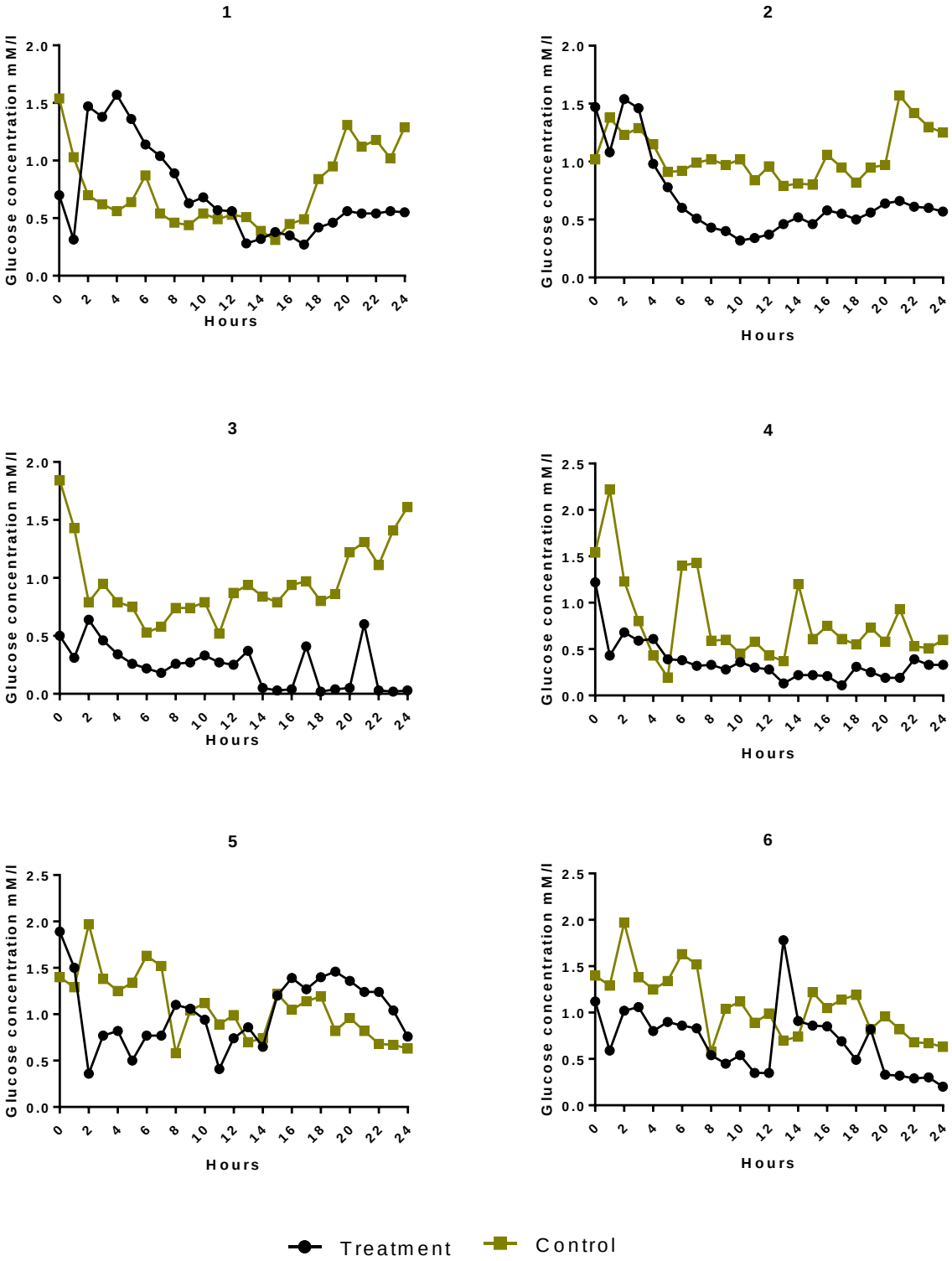

Figure 3.2: Glucose concentration of the horses (1 to 6) perfused with control and human insulin solutions for 24 h via tissue microdialysis. Concentrations in the treatment feet of horses 2, 3, 4 and 6 remained lower than control feet over the 24 h period. In all horses but number 5, glucose concentrations in the treatment feet remained below the control feet between 9 and 24 h. Horse 5 showed higher glucose concentrations in the treatment group.

Chapter 4: Characterization of normal hoof proliferation using 5-ethynil-2'-deoxyuridine (EdU): a novel proliferation marker

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Ethical Considerations: The project was approved by The University of Queensland Animal Ethics Committee (AEC) that monitors compliance with the Animal Welfare Act (2001) and the Code of Practice for the care and use of animals for scientific purposes (current edition). All animals were monitored continuously by the investigators.

Competing Interests: The authors have no competing interests to declare

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4.1 Summary

Reasons for performing study: A simple and sensitive method is required to study cellular proliferation in the equine hoof. Characterization of normal cellular proliferation is crucial to understanding normal growth processes of the hoof and their implications for laminitis.

Objectives: To develop a simple technique for the study of cellular proliferation in the equine hoof and characterize epidermal proliferation rates in the normal equine foot using a regional delivery technique.

Methods: 5-ethynil-2'-deoxyuridine (EdU) was administered to one of the forefeet of 5 Standardbred horses using regional intravenous infusion (RIVI). After 60 min with a tourniquet in place, horses were euthanized, tissue harvested and EdU detected by click chemistry. Tissue sections were analysed using confocal fluorescent microscopy. Total cells were digitally counted and a proliferative index (PI%) was calculated for transverse coronary, terminal and sole tubular epidermis, frontal lamellar sections and ten transverse levels of dorsal hoof wall lamellae.

Results: Proliferative activity was restricted to the coronet (coronary tubules) PI% =23.97 (±1.50); terminal 9.58 (±0.66) and sole 6.02 (±0.29) tubules and proximal lamellae 4.01 (±0.54). Proliferation was rare or absent at middle L5=0.16 (±0.02) and distal lamellae 0.07 (±0.01).

Conclusions: EdU labelling via RIVI is an alternative technique for the study of cellular proliferation in the equine hoof. EBCs actively proliferate at the coronet; proximal lamellae; coronary, terminal and sole epidermis but not in the main lamellar region. This technique may be especially useful for the study of epidermal basal proliferative disorders related to laminitis.

4.2 Introduction

The *stratum lamellatum* consists of interdigitating dermal and epidermal layers closely attached to each other to form the dermo-epidermal junction. The dermal side contains nerves, vasculature and a dense matrix of connective tissue, whilst the epidermal side is lined by a layer of basal cells and deeper layers of parabasal cells attached to a cornified lamellar axis [1].

Understanding of the growth and repair process of the hoof wall is incomplete. Daradka and Pollitt [2], using the proliferation marker bromohydroxiuridine (BrdU), characterized the proliferative activity of the hoof wall, describing it as a process exclusive to epidermal basal cells (EBCs) at the coronet, proximal lamellae, and terminal horn. However, the proliferation rates for coronary, terminal and sole tubular epidermis remain undetermined. Additionally, de Laat *et al.* [3] using TPX2, a nuclear protein expressed during the S, G2 and M phases of mitosis, detected increased proliferative rates in the EBCs of horses undergoing insulin-induced laminitis suggesting that proliferative changes may play a role in the physiopathology. Thus the study of epidermal cellular proliferation of the lamellar tissue is of special significance.

The main drawback of the currently utilized proliferation markers (BrdU and TPX2) for the determination of proliferation rates in the lamellar tissue is the complexity of the post-processing steps required before detection of the actual markers. These steps normally include pre-treatment of the cells (DNA denaturation) and immunohistochemistry which are complex and time consuming techniques [4; 5].

The present study describes the use of the novel proliferation marker EdU (5-ethynil-2'-deoxyuridine) for determining epidermal cellular proliferation in normal equine hooves. EdU, as with BrdU, is a thymidine analogue that is incorporated into DNA during the S phase of mitosis, thus labelling proliferating cells. The main advantage of EdU over its predecessors (BrdU and other proliferation markers) is the simplicity of the detection method in which a fluorescent dye is attached to the EdU molecule through a covalent bond making it detectable with simple fluorescence microscopy. This reaction takes only 30 min and does not require the use of antibodies, the denaturation of DNA or other complex steps.

This study aims to evaluate EdU, administered locally to the foot of the horse using IV regional limb perfusion (RIVI). Regional limb perfusion is well established for regional

delivery of antimicrobials to distal limb synovial structures, bone, and subcutaneous tissues [6-8] and has recently been established as an effective means of drug delivery to lamellar tissue [9]. The present study combines for the first time EdU labelling and RLP techniques, describing a new protocol for the study of cellular proliferation in the equine foot.

4.3 Materials and Methods

4.3.1 Animals

Five Standardbred horses (four geldings and one mare, aged 3-25 years, weighing 380-515 kg) with clinically normal feet were utilized for this study. The project was approved by the University of Queensland Animal Ethics Committee (approval number: SVS/338/11/UQ) that monitors compliance with the Animal Welfare Act (2001) and The Code of Practice for the care and use of animals for scientific purposes (current edition).

4.3.2 EdU perfusions

One of the fore limbs of every horse was randomly assigned to the treatment group and the contralateral limb to the control group. The instrumented digits were desensitized using a bilateral abaxial sesamoid nerve blocking applying 3 ml of Mepivacaine 0.2%[a] per site of injection. Then, the digit was aseptically prepared and a tourniquet applied at the level of the fetlock. Thereafter, a 21G butterfly catheter was inserted into the lateral palmar digital vein and the treatment (EdU[b] 75mg dissolved in 35 ml of 0.9% saline solution) and control (35 ml of 0.9% saline solution) solutions were administered immediately to the respective limbs. The tourniquets were kept in place for sixty minutes and then the horses euthanized by I.V. sodium pentobarbitone (Lethabarb[c]) administration.

4.3.3 Sampling

The forefeet were disarticulated at the fetlock joint. Ten tissue samples (15 x 10 x 5 mm) evenly spaced between the coronet and sole were collected from the midline section of the dorsal hoof wall of the treatment feet. Samples were labelled from TL1 (proximal) to TL10 (distal). Control feet samples were taken only from proximal lamellae (CL1), mid-lamellae (CL5) and distal lamellae (CL10). Longitudinal tissue samples of coronet, periople, skin below and above the tourniquet as well as tongue were also harvested from both groups (Fig. 4.1).

4.3.4 Histological analysis

Tissue blocks were fixed in formalin for 24 h and then kept in 70% ethanol until processed. The blocks were then embedded into paraffin blocks and trimmed, then 7 µm sections were cut and placed on standard microscope slides. The sections were de-waxed, rehydrated and processed for click chemistry (Alexa fluor 647 Click iT imaging kit[d]) according to the manufacturer's instructions. Finally, cell nuclei were stained using Hoechst 33342[e] and sections cover-slipped using a water based mounting medium[f].

Fluorescence microscopy analysis was performed on a confocal microscope N1R[g]. Epidermal cells positive and negative to EdU were counted in 40 fields per section for the coronet (transverse (TS) and longitudinal sections (LS)), terminal papillae (TS), sole papillae (TS) and frontal sections. Cells were counted as well in the ten treatment lamellar sections (TL1-TL10) in 4 PELs randomly selected in every section in 40 fields per section at 60X magnification using NIS Elements 10.0 software. The cells stained with both EdU and DAPI (red + blue fluorescence, respectively) were considered as positive cells, whilst those stained only with DAPI (blue) were considered negative. Using this information a proliferation index (PI%) was calculated

4.3.5 Data analysis

Simple Poisson models were used to quantify the association between positive cells count and tissue sections levels, accounting for the total number of cell counted in each section (used as an offset in the model). Because of the different nature of the sampled sections, three

models were used grouping the sections in: transverse lamellar sections (T1 to T10); coronary, sole and terminal tubular and intertubular hoof; and frontal sections (proximal and distal). All models were adjusted for clustering within horse and coefficient standard errors were generated using the cluster function in Stata 13[h].

4.4 Results

EdU labelling in the foot was restricted to epidermal basal cells (EBCs) with occasional positive cells within the dermis. Positive cells were found in the skin and hair bulbs below the tourniquet. No EDU positive cells were identified in the skin above the tourniquet, the tongue or the control foot.

Total number of epidermal cells, EdU positive epidermal cells and the derived PI% are summarized in Table 4.1. Proliferation was restricted to the coronary, terminal, and solar tubular and intertubular hoof and proximal lamellae. Proliferation at the coronet was significantly higher than in any other area sampled ($p < 0.01$) (Fig. 4.2). The PI% (mean±SEM) determined for LS coronary sections was 17.68 ± 1.23, whilst for the TS coronary section was 23.97±1.50. Differences between these sections were significant ($p < 0.01$). Active proliferation was found in terminal tubular hoof (PI% = 9.58 ± 0.66) and solar tubular hoof (PI% = 6.02 ± 0.29), representing 32% and 15%, respectively (table 4.2), that of the coronet level ($p < 0.01$). Transverse sections of lamellae showed subjectively that there was consistent EBC proliferation located particularly at the axial tips of the primary epidermal lamellae (PELs) which appeared to diminish abaxially (Fig. 4.3A). The PI%± for the proximal lamellae (TL1) (4.01±0.54) was significantly higher than the other nine distal lamellar sections ($p < 0.01$). The PI% decreased distally from TL2 (0.25± 0.09), being almost non-existent in the middle and distal lamellae (PI% at TL10= 0.07 ± 0.01) (Fig. 4.4).

4.5 Discussion

The administration of EdU via RIVI resulted in successful labelling of proliferating epidermal basal cells in the equine hoof. Previous studies used EdU to assess *in vitro* cell cultures [10; 11] chick embryos [12] or live mice utilizing an intra-peritoneal approach [4; 5].

This is the first study describing the administration of EdU intravenously to horses. The regional delivery of the marker via (RIVI) to a single foot allowed use of a reduced total dose, cost and exposure to possible toxic effects. No EdU positive cells were found in the skin above the tourniquet, tongue or contralateral foot. This showed a tourniquet at the level of the fetlock for 60 minutes, in the static standing horse, effectively restricted the distribution of the EdU to just the foot thus avoiding wastage into the systemic circulation. Cells positive to EdU were found in in the bulb and outer sheath of hair and in the *stratum basale* and *spinosus* of skin sampled below the tourniquet (Fig. 4.5). The distribution of proliferating positive cells in hair and skin, after 60 min of exposure to EdU, was similar to that described in previous studies using BrdU in ponies [2] and sheep [13] for the same period of time, however no PI have been previously calculated for equine hair and skin.

Sectioning of the tissue is an important factor for the correct determination of the proliferative index (PI%) at different levels of the hoof wall. As an example, the highest number of proliferating cells was in the epidermis of the coronary inter-tubular and tubular hoof. The distribution of positive cells in tubular horn varied and most were located proximally diminishing distally down the walls of the tubule. Thus, PI% for the LS section was derived from both high and low PI zones and was 17.68(±1.23) whilst TS sections reached 23.97(±1.50). These TS sections were through the proximal high PI zone and skewed the result

Daradka and Pollitt [2] previously described the presence of proliferating cells at the coronary and terminal epidermis using BrdU; however this is the first time a PI% has been calculated for sole papillae (PI%=6.02(±0.29)). Lamellar proliferative activity was studied at ten different levels (TL1 to TL10). The highest PI% was found proximally at TL1 and decreased from TL2 to TL10, similar to previous descriptions [2]. Frontal sections of the proximal and distal lamellae confirmed this proliferation pattern showing the majority of positive EBCs at the most proximal region of the lamellae decreasing to be almost non-existent distally (Fig. 4.6A and 4.6B).

The distribution of these proliferating cells along the primary epidermal lamellae was especially interesting. After 60 minutes of exposure to EdU the majority of positive cells were found only at the axial side of the PELs extending from their tips to the beginning of the keratinized axis of the PELs. The proliferative potential, origin and fate of cells located in the abaxial side of the PELs deserve further research. The analysis of LS sections containing

coronary papillae and proximal lamellae evidenced a continuum of EBC proliferation from coronary horn to the proximal lamellae. The absence of proliferation in the middle and distal lamellae confirms that the main cellular proliferation activity in the hoof wall is provided by the coronet and proximal lamellae, whilst terminal papillae and sole papillae contribute to the proliferation of the distal structures (linea alba and sole). The cells located in the distal keratinised axis of PELs are derived from proximal areas where proliferation actively occurs. Proliferation at other levels of the lamellae appears to be occasional or rare, minimally contributing to the growth of the hoof wall.

Based on the present results, the concept of a "sterile bed" proposed by Leach and Oliphant [14] does not apply to all areas of the lamellar tissue. It does apply to the middle and distal lamellae (TL2 to TL10), where minimal proliferation occurs. The mechanisms that allow displacement of the EBCs distally whilst they maintain their attachments to the underlying basement membrane and to surrounding cells is still unresolved. For a better understanding of these processes further investigation should be focused on the cellular cytoskeleton and junctional components and their interactions during the growth and remodelling process of the hoof wall.

Although the distribution of EdU positive cells appears to be equivalent to that observed previously using systemically administered BrdU, PI%s here calculated were consistently lower than that of Daradka and Pollitt [2]. Several factors related to the technique such as: administered marker concentration, time of exposure of the tissue to marker, mechanism of detection and cell counting protocol may have influenced the PI% in this study. A direct concentration effect has been previously described for the fluorescent activity of EdU [10; 15]. In vivo experiments assessed increasing concentrations of the marker delivered via intraperitoneal injection to rats [5]. The EdU concentration evidencing the highest fluorescent activity was 50mg/kg. Estimating the equine foot weight at approximately 1.5kg, a total dose of 75mg was here selected. Higher and lower concentrations of EdU should be assessed in order to determine the best fluorescent response of the EBCs in the lamellae. Time exposure is a considerable factor when comparing the PI% between systemic and regional delivery models (RIVI). Daradka and Pollitt [2] administered BrdU (35mg/kg) systemically to 5 ponies harvesting the lamellar tissue after 60 min. Although in the present study horses' feet were exposed to the proliferation marker for the same period of time, the total EdU dose to the feet may have differed: BrdU was administered intravenously and diluted in the systemic circulation but recirculation of the marker to the lamellae may have enhanced

delivery of BrdU to the cells. In the RIVI model restricted blood circulation caused by the tourniquet may have affected the distribution of the marker within the tissue and compromised uptake by the EBCs.

The mechanism of detection is one of the main advantages of EdU. Unlike BrdU, EdU does not require the use of complex DNA denaturation or immunohistochemistry techniques for the development process. This avoids damage to other antigens and tissue disruption making EdU labelling easy to combine with other immunofluorescence techniques [5; 10; 15]. To make the EdU molecules fluoresce, only a simple and rapid click chemistry reaction is required, forming a very specific covalent bond with the fluorescent azide probe [5]. Additionally, the molecules involved in the click chemistry reaction are $1/500^{th}$ the size of the antibodies needed for BrdU [11], thus they can effectively diffuse within the tissues and into the double-stranded DNA. Lastly, differences in the PI% between the two studies could be accounted for by the number of cells counted: the total number of cells counted in the present study was considerably higher than those analysed previously by Daradka and Pollitt [2]. Although this fact may increase the sensitivity of this study, comparisons between both methods may not be representative. The use of different counting protocols may cause considerable variations in the PI% calculated. As an example, the present study demonstrated that in the proximal TS sections of the lamellae, cell proliferation was concentrated at the tip of the PELs diminishing toward their base. Considering the number of counted cells at the same level in previous studies and the lack of detail in the counting protocol used [2], the inclusion of these areas with lower proliferation rate in the PI% calculation cannot be assured, possibly skewing the results.

The sensitivity of EdU versus BrdU was previously compared *in vitro* using breast cancer cell cultures [10], neurospheres derived from human olfactory mucosa [5] and *in vivo* using pregnant mice [5]. No substantial differences were observed between the two methods, indicationg that the sensitivity of EdU is equivalent to that of BrdU. Additionally it has been previously reported that EdU has a lower cytotoxicity than BrdU making it a better candidate for *in vivo* experimentation [15].Validation of the present protocol should be performed against BrdU to determine if the presence of the tourniquet and/or different EdU doses played a significant role in the detection of proliferating cells in the lamellar tissue. As EdU can be delivered via RIVI to a single foot, both protocols could be assessed simultaneously in the same horse.

EdU labelling appears to be a viable alternative technique to detect proliferating cells *in vivo* in the hoof wall. Although this technique can be potentially applied to virtually any tissue, the present protocol might be of particular use for the study of cellular proliferation in laminitis, including insulin mediated models where EBC proliferation has been implicated [3; 16; 17] and in chronic laminitis cases where the development of a proliferating epidermal lamellar wedge contributes to distal phalanx pathology and complicates rehabilitation [18]; a better understanding of the dynamics and temporal development of the wedge will inform improved control and therapy.

Due to the simplicity of the presented protocol, the EdU labelling technique would be suitable for field research situations and also could easily be used to study clinical cases subjected to euthanasia, contributing to the understanding of the pathologic process of naturally occurring laminitic cases.

4.6 Conclusion

EdU labelling is a simple technique for the study of cellular proliferation in the equine hoof. The novel protocol here described using regional delivery (RIVI) allowed the reduction of the total dose administered, reasonable cost and decreased exposure to possible toxic effects.

Cellular proliferation was restricted to specific zones of the hoof wall. The coronet and proximal lamellae showed the highest cellular proliferation rates contributing to the formation of the *stratum medium* and *internum* of the hoof wall respectively. Lower but considerable proliferation rates were observed at the terminal and sole horn whilst the middle and distal lamellae show negligible proliferative activity.

4.7 Manufacturers

[a] Nature Vet, Glenorie, NSW, Australia
[b, d, e] Innvitrogen, LifeTechnologies, Carlsbad, CA, United States of America
[c] Virbac Animal Health, Milperrin, NSW, Australia
[f] Dako, North Sydney, NSW, Australia
[g] Nikon, Konan, Minato-ku, Tokyo, Japan
[h] StataCorp LP, Texas, USA.

4.8 References

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Table 4.1: Summary statistics of mean±SEM of the total number of epidermal cells (TNC), total number of positive epidermal cells (TNPC) and proliferation index (PI%) determined in 16 different areas of treatment hooves.

Level	TNC (±SEM)	TNPC (±SEM)	PI% (±SEM)
L1	6675.40 (±1423.00)	233.00 (±32.18)	4.01 (±0.54)
L2	7056.80 (±1295.00)	15.80 (±5.89)	0.25 (±0.09)
L3	7967.40 (±1512.00)	8.20 (±2.40)	0.09 (±0.02)
L4	7449.60 (±1065.00)	5.80 (±1.07)	0.08 (±0.02)
L5	8561.20 (±945.60)	13.60 (±3.06)	0.16 (±0.02)
L6	9597.40 (±918.70)	11.40 (±1.54)	0.12 (±0.02)
L7	8228.20 (±469.40)	7.20 (±1.80)	0.09 (±0.02)
L8	8931.60 (±551.30)	9.40 (±2.54)	0.11 (±0.03)
L9	9607.00 (±794.60)	5.80 (±1.20)	0.06 (±0.01)
L10	7688.60 (±1088.00)	5.80 (±1.66)	0.07 (±0.01)
LS coronary tubular hoof	4981.40 (±2243.00)	831.40 (±339.80)	17.68 (±1.23)
TS coronary tubular hoof	717.40 (±43.33)	171.20 (±12.47)	23.97 (±1.50)
Sole tubular hoof	1319.20 (±69.06)	79.00 (±4.31)	6.02 (±0.29)
Terminal tubular hoof	2115.60 (241.20)	197.40 (±15.14)	9.58 (±0.66)
Frontal proximal	2632.00(±217.8)	164.80 (±52.61)	5.86 (±1.88)
Frontal distal	2146.80 (±577.70)	7.60 (±5.90)	0.39 (±0.33)

Key: ±SEM standard error of the mean

Table 4.2: Regression coefficients from a simple Poisson model, comparing EdU positive cells counted in 16 different sections of lamellar tissue of treated horses (n = 8).

Lamellar area	Mean count ratio	95% CI	P value
L1	Reference	-	-
L2	0.07	0.03 – 0.14	< 0.01
L3	0.03	0.02 – 0.05	< 0.01
L4	0.02	0.02 – 0.03	< 0.01
L5	0.05	0.03 – 0.08	< 0.01
L6	0.04	0.03 – 0.06	< 0.01
L7	0.03	0.02 – 0.04	< 0.01
L8	0.03	0.02 – 0.06	< 0.01
L9	0.02	0.01 – 0.03	< 0.01
L10	0.02	0.01 – 0.04	< 0.01
LS coronary tubular hoof	Reference	-	-
TS coronary tubular hoof	0.44	0.29 – 0.68	< 0.01
Sole tubular hoof	0.15	0.9 – 0.26	< 0.01
Terminal tubular hoof	0.32	0.19 – 0.52	< 0.01
Frontal proximal	Reference	-	-
Frontal distal	0.05	0.01 – 0.31	< 0.01

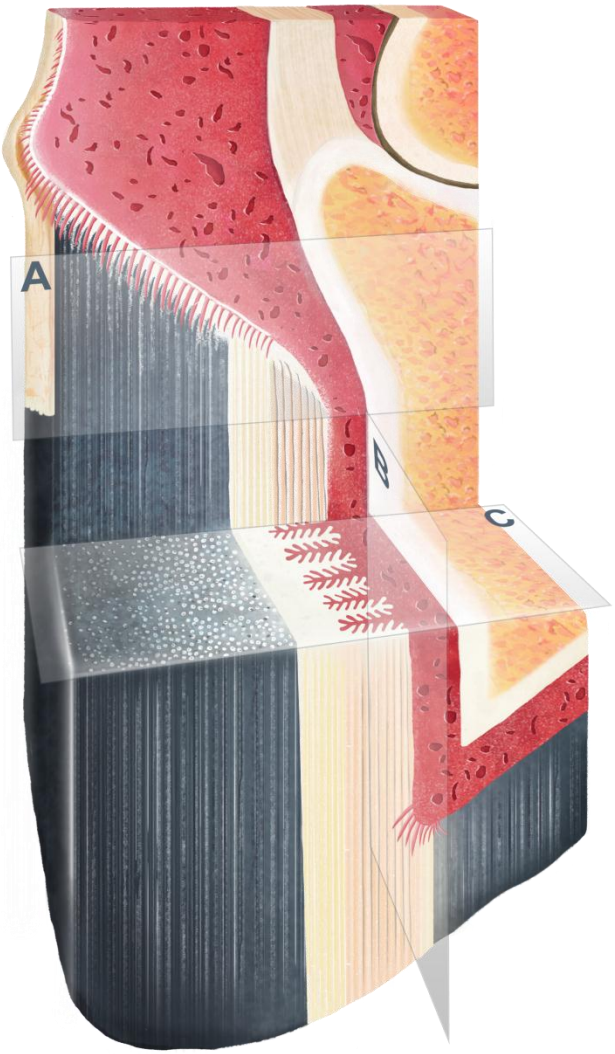

Figure 4.1: Diagram of the hoof's inner structures, showing the different sectioning planes for the acquisition of tissue samples.The orientation of the different planes are: A, longitudinal sections (LS) B, frontal sections and C, transverse sections (TS).

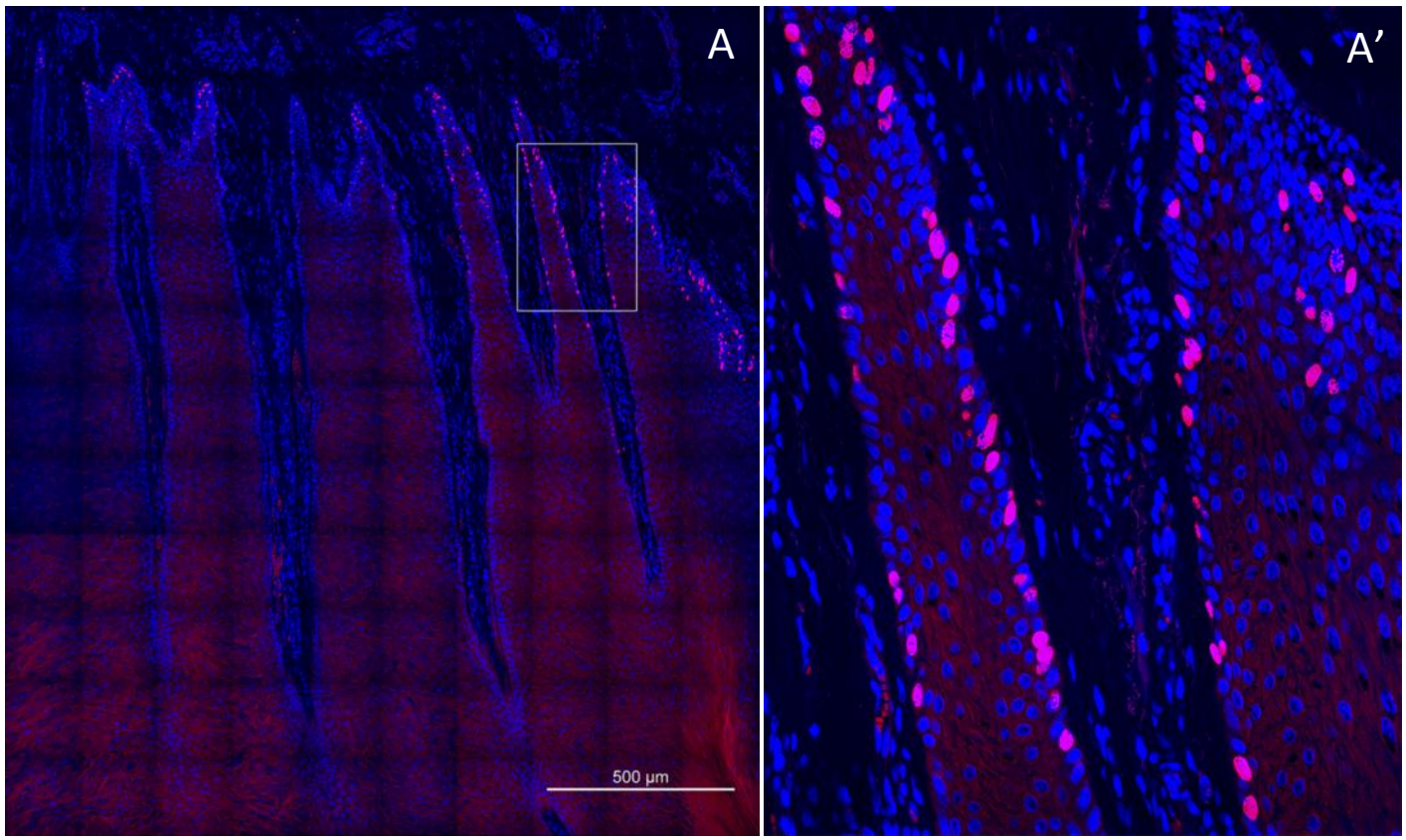

Figure 4.2: Photomicrograph showing the normal proliferative activity in a longitudinal (LS) section of the coronary tubular and intertubular epidermis. A’ is a magnification of the box in A. Nuclei were stained with Hoechst 3342 (DAPI, blue) and proliferating cells labelled with EdU and detected with Alexa fluor 647 Click iT imaging kit (Cy5, red). Proliferation is concentrated in the proximal aspect of the tubular hoof (box) diminishing distally.

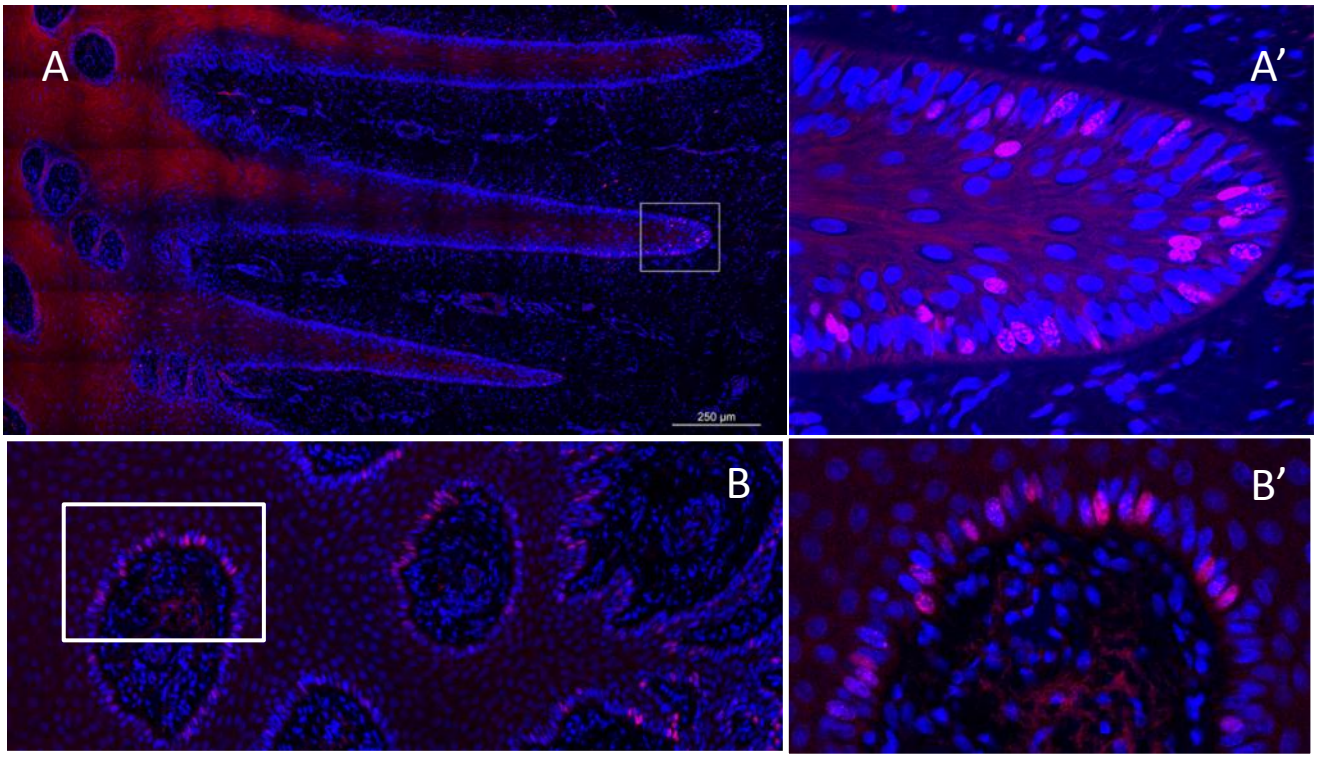

Figure 4.3: Photomicrograph demonstrating the normal proliferative activity in the proximal lamellae (A: transverse section (TS) of area L1) and coronary tubular epidermis TS (B). A’ and B’ are magnifications of respective boxes. Nuclei were stained with Hoechst 3342 (DAPI, blue) and proliferating cells labelled with EdU and detected with Alexa fluor 647 Click iT imaging kit (Cy5, red).

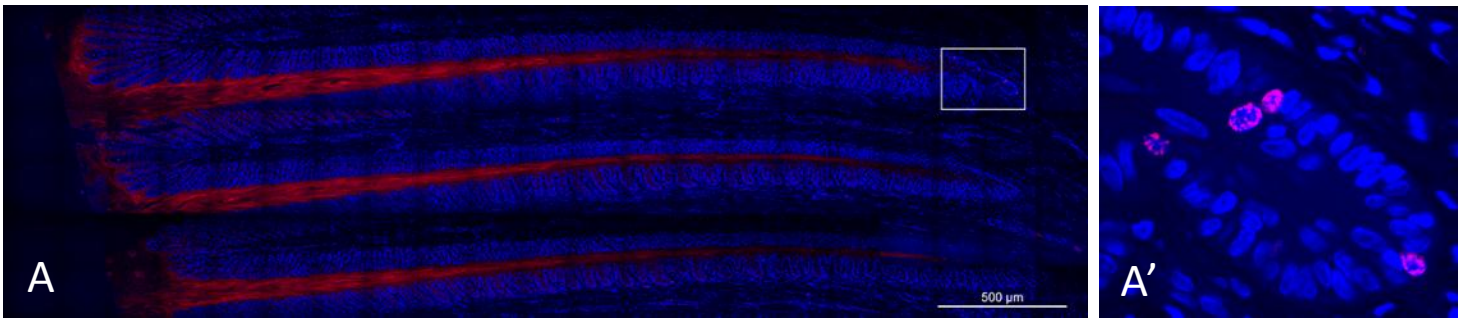

Figure 4.4: Photomicrograph showing the normal epidermal cellular proliferation at the mid-lamellae (TL5). A’ is the magnification of the respective boxe. Nuclei were stained with Hoechst 3342 (DAPI, blue) and proliferating cells labelled with EdU and detected with Alexa fluor 647 Click iT imaging kit (Cy5, red). Proliferation at this level is rare or occasional compared to the proximal levels of the lamellar tissue.

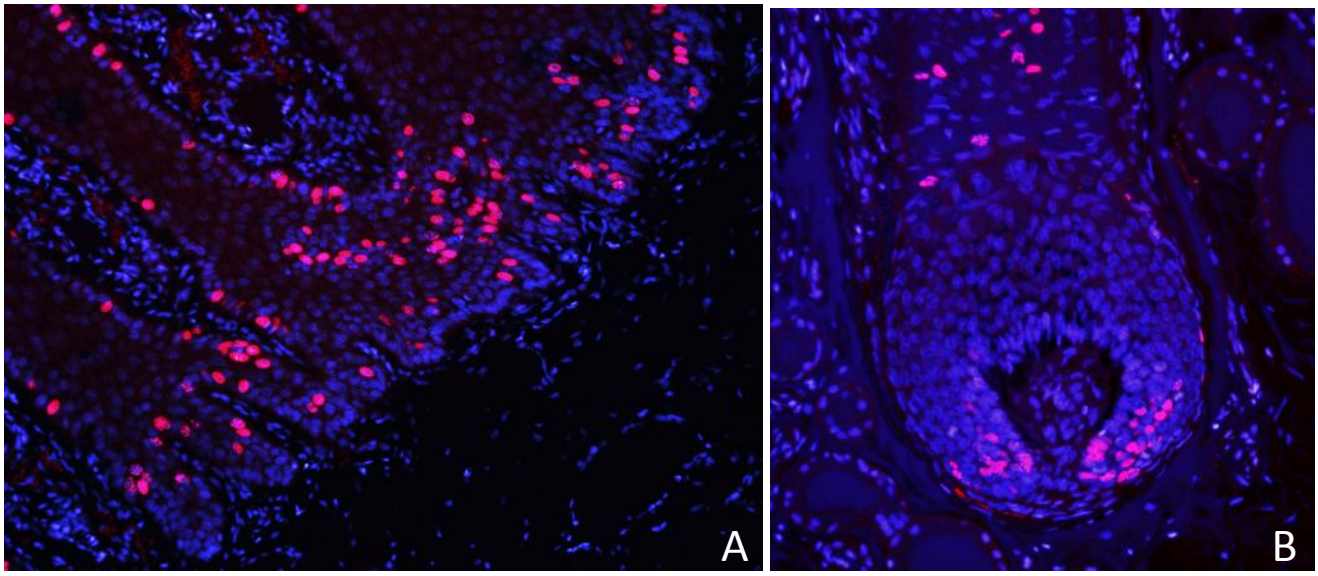

Figure 4.5: Photomicrograph of skin below the tourniquet (A) and a hair follicle from skin below the tourniquet (B). Nuclei were stained with Hoechst 3342 (DAPI, blue) and proliferating cells labelled with EdU and detected with Alexa fluor 647 Click iT imaging kit (Cy5, red). Active cellular proliferation was observed in stratum basale and spinosus of the skin below the tourniquet (A). The majority of proliferating cells in the the hair of skin below the tourniquet were located in the bulb area (B).

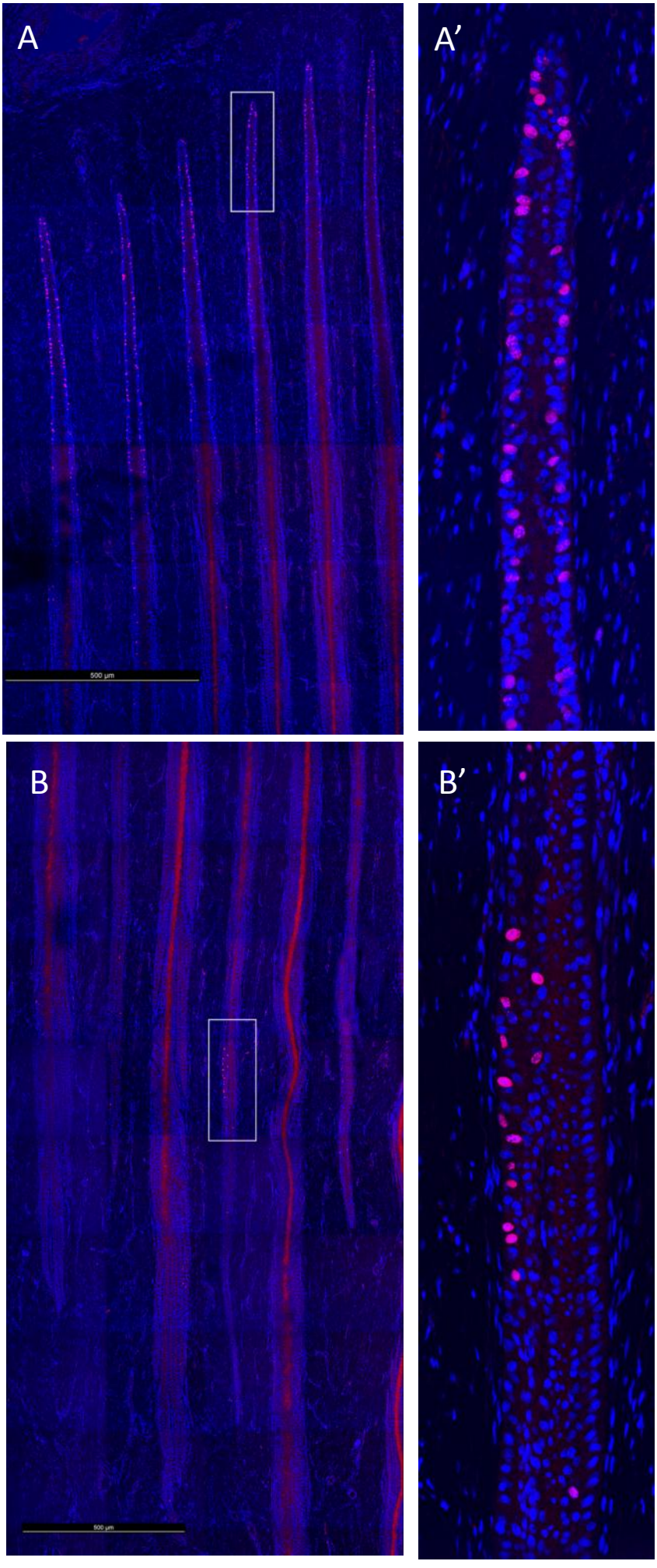

Figure 4.6: Photomicrograph of proximal (A) and distal (B) frontal lamellae sections.A’ and B’ are magnification of respective boxes. Nuclei were stained with Hoechst 3342 (DAPI, blue) and proliferating cells labelled with EdU and detected with Alexa fluor 647 Click iT imaging kit (Cy5, red). Active cellular proliferation was detected at the proximal area of the lamellar tissue (A’). Positive cells were not observed distally in the mid lamellar area (arrows head). The presence of proliferating cells was rare or occasional in the distal lamellae (B’).

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Part III: Conclusion

Chapter 5: General Discussion

The first part (Chapter 2) of the study presented in this thesis describes the local effects of exogenous insulin on cellular proliferation and histomorphometry. The second part (Chapter 3) reports the effects of insulin on equine lamellar perfusion and cellular glucose metabolism. This study is the first to use tissue microdialysis to deliver insulin to the hoof lamellae. This particular model was used because it allowed the researcher to monitor the local effect of a high concentration of insulin delivered into the lamellae in real time in the standing horse.

Several factors were considered when selecting an appropriate concentration of insulin for the perfusion. These included: plasma insulin concentrations achieve in previous studies for the induction of laminitis (Asplin et al., 2007) (de Laat et al., 2010a); the insulin concentrations that elicited a physiological effect in previous microdialysis studies of rat brains (McNay et al., 2013) and human muscle (Rosdahl et al., 2000); the stability of insulin in highly diluted solutions (Blackshear et al., 1983; James et al., 1981; Stickelmeyer et al., 2000); previous evidence of insulin loss in microdialysis systems (Jansson et al., 1993); and *in vitro* determinations performed in our laboratory (Appendix 1). Thus, a concentration of 100 µg/ml of insulin was chosen for the tissue microdialysis perfusate. This delivered adequate insulin to the lamellar tissue to test our hypotheses. Although insulin concentrations in the lamellar tissue of normal or laminitic horses remain undetermined, studies have demonstrated that the insulin concentration in other tissues, including skeletal muscle and adipose tissue, are approximately 50% of circulating concentrations (Jansson et al., 1993; Sjostrand et al., 1999). The estimated concentration delivered into the lamellar tissue in the current studies (0.5 µg/ml) was approximately 14 times greater than the systemic concentration of insulin reached by Asplin et al. (2007) and de Laat et al. (2010a) during the experiments performed on ponies and horses using a prolongued euglycemic hyperinsulinemic clamp (p-EHC). Considering that insulin dynamics in the lamellae are unknown and that the single source point of insulin delivery in our studies was the microdialysis probe, our aim was to deliver a higher concentration of insulin in order to ensure at least a similar biological effect to that previously observed in the systemic insulin-induced model using a p-EHC.

The local delivery of insulin for 24 h affected lamellar histomorphometry and cellular proliferation. In Chapter 2, significant differences ($p < 0.05$) in PELL and NKPEL were observed between treated and control groups. The combination of longer PELL with

conserved KPELL, found exclusively at area L1 (surrounding the microdialysis probe), supports a direct effect of insulin on tissue organization, particularly affecting SELs at the tip of the PELs closest to the microdialysis probe.

Histomorphometric changes have been described previously by Asplin et al. (2010a) and de Laat et al. (2013a) during the induction of laminitis using a pEHC. Elongation and narrowing of the SELs, with minimal compromise of the basement membrane, were the main features of the systemic, insulin-induced laminitis model. Tissue disorganisation was proposed as the probable cause of the weakening of the lamellae, compromising the dermo-epidermal junction (de Laat et al., 2013a; de Laat et al., 2013c). Increased EBC mitotic activity and a concomitant increased number of cells was suggested as the main causes for SEL lengthening after 48 h of exposure to extreme hyperinsulinemia. Using a similar p-EHC technique, Karikoski et al. (2014b) confirmed lengthening of the PELs after exposure of horses to hyperinsulinemia. A combination of more proliferating cells and more apoptotic cells was identified suggesting an accelerated cell apoptotic-proliferation process, with no net change in the total cell number. However cell stretching was suggested as the most likely cause of the lengthening of the PELs rather than increased proliferative activity.

A novel protocol for labelling proliferating EBCs within the lamellar tissue is described in Chapter 4. For the first time, the proliferation marker EdU was administered to the foot of the horse intravenously, using a regional delivery technique. EdU is an analogue of the nucleotide thymidine. It is incorporated in the DNA during the synthesis stage of mitosis, and is detected in fixed samples through a *click chemistry* reaction. The detection process takes 30 min and this is one of the main advantages over its predecessor BrdU, which required DNA denaturation and immunohistochemistry during the developing process. Also, the reagent used during *click chemistry* is $1/500^{th}$ the size of the antibodies needed for BrdU (Salic and Mitchison, 2008), penetrating easily into the sampled tissue and DNA without requiring denaturation.

The EdU labelling technique successfully labelled proliferating EBCs in the equine hoof wall. The results are consistent with previous findings by Daradka and Pollitt (2004b), confirming that the main cellular proliferation activity in the hoof wall is provided by the coronet and proximal lamellae, whilst terminal papillae and sole papillae contribute to the proliferation of the distal structures (*linea alba* and sole). Proliferation at other levels of the

lamellae appears to be occasional or rare, contributing minimally to the growth of the hoof wall.

Although this new technique could be applied to virtually any tissue for the study of cellular proliferation, the present protocol will be of special interest for the study of cellular proliferation in the equine foot and its implications for laminitis models.

In Chapter 2, the EdU labelling technique was used for the study of the cellular proliferation after 24 h of exposure of the lamellae to exogenous insulin *in vivo*. The mean number of TNPC detected in the treatment group was 42 times that of the control, with a clear trend toward statistical significance ($p=0.07$), with sections closest to the microdialysis membrane (L1) having the highest proliferation activity. Considering that no differences were found in the total number of cells between treated and control limbs in the present study, the lengthening detected by increased PELL and NKPEL could not be explained by an increase in proliferative activity as was initially suggested by de Laat et al. (2013a). Therefore, the data support cellular stretching as the cause behind insulin disrupting lamellar architecture when delivered locally.

The proliferation rate of EBCs located in the mid-lamellar region of normal horses is generally negligible (Daradka and Pollitt, 2004a). This corresponds to the area where the microdialysis probes were placed. Control feet showed similar proliferation rates to those described in Chapter 4 for areas sampled at the same level. This demonstrates that the insertion or presence of the microdialysis catheter, or other factors, did not increase EBC proliferation in the lamellar tissue of control horses. Thus, the results support increased proliferation in the treated feet as the likely direct effect of insulin.

Several secondary reactions are triggered once insulin binds to its receptors (InsR or IGF-1R). Insulin modulates glucose metabolism, cell growth, cellular differentiation and cytoskeletal organization (al-Habori, 1993; Berfield et al., 1996; Berfield et al., 1997). The last of these may be of special interest for the understanding of insulin-induced laminitis. The cytoskeleton is a complex network of protein fibres responsible for spatial organization of the cell, cell shape and mechanical resistance to deformation. Additionally, the cytoskeleton is involved in the modulation of other cellular functions such as cell signalling pathways, intracellular transport and cellular division, among others (Schulingkamp et al., 2000b). Previous studies using cultured rat mesangial cells demonstrated the rearrangement of the filamentous polymer of actin (F-actin) from normal patterns to a parallel central aggregated

distribution after exposure to insulin for 1 h. The researchers observed severing of peripheral adhesion points losing the attachment to the nuclei and other organelles, similar to what occurs in cells preparing to move or divide (Berfield et al., 1996). Conversely, the exposure of rat glomerural mesangial cells to insulin-like growth factor I (IGF-I) produced a different response. Cells suffered a progressive elongation, resulting in an extremely stretched bipolar cell (Berfield et al., 1997). A similar process may occur in the lamellae. Research conducted by Burns et al. (2012b) demonstrated that the InsR distribution within the equine hoof was restricted to the lamellar vasculature, whilst IGF-1R were ubiquitous. The main ligand for IGF-1R is IGF, however, in exceptional conditions when insulin concentrations are supraphysiological insulin can bind IGF-1R producing an IGF-like effect. Thus, the increased PELL and NKPEL observed in Chapter 2 may be explained by a direct effect of insulin on the cytoskeletal organization of the EBCs, via the IGF-1R. The histomorphometric changes reported are consistent with the previous histological description by de Laat et al. (2013a). This supports the idea of insulin modifying the EBCs' cytoskeleton, and is also compatible with the loss of the normal histological organization of the lamellar tissue as both de Laat et al. (2013a) and Karikoski et al. (2014b) previously stated.

The occasional compromise of the basement membrane (BM) was one of the characteristics of the insulin-induced model observed by de Laat et al. (2013a)**.** The BM is fundamental for anchoring the parietal surface of the distal phalanx to the cytoskeleton of the EBCs. Hemidesmosomes are the adhesion point between extracellular collagen fibres and the keratin (intermediate filament) cytoskeletal fibres. Cross-talk between insulin and integrins (one of the main proteins constituting the structure of hemidesmosomes) has been identified (Guilherme et al., 1998). Integrins are heterodimer transmembrane receptors that mediate interactions between the cell surface and the extracellular matrix and also initiate signalling events including tyrosine phosphorylation, cytoskeletal reorganization (Burridge et al., 1992; Dedhar and Hannigan, 1996; Schwartz et al., 1995), initiation of mitogen-activated protein kinase (MAPK) cascades, and the modulation of cellular metabolism (Chen et al., 1994). Leipzig et al. (2006) found that IGF-1R collaborate with integrins to regulate focal adhesion proteins, and are also associated with the MAPK signalling pathway. Additionally, an increased amount of F-actin was found in bovine chondrocytes that were treated with IGF-1, causing cellular stiffness and adhesion to the extracellular matrix. The capability of insulin to increase cellular adhesion to extracellular matrix components may explain why BM detachment was minimal in the reports of Asplin et al. (2010a) and de Laat et al. (2011).

Previously, weight bearing was suggested as a possible triggering or contributing factor to the disruption of the dermo-epidermal junction during laminitis. This statement was based on the differences in the level of BM damage present in the lamellar tissue of ponies vs. horses during the systemic insulin-induced model (Asplin et al., 2010a; de Laat et al., 2013a). In the local model presented in this thesis, weight bearing exerted normal tension along all parts of the lamellar tissue. Since the area perfused by the microdialysis membrane was minimal in relation to the total hoof wall surface, mechanical distractions due to increased tension as a consequence of the failure of the SADP were assumed absent However, BM detachment was observed in 30% of the treated sections, varying from grade 1 to 3. Isolated BM separation in control sections was also observed (12.5%), but never exceeding grade 1. The median laminitis histology score for L1 sections in the treatment group was 1 (mild), whilst for all the remaining sections in both groups it was 0 (normal). This difference showed a trend toward significance ($p = 0.062$), and in conjunction with the histomorphometric changes reported, the present work supports the hypothesis of insulin itself affecting the lamellae tissue organization and compromising the BM integrity, as de Laat et al. (2013a) previously reported.

In Chapter 3 the effects of local delivery of insulin on lamellar cellular metabolism and perfusion were studied. Although no significative differences were found in the metabolites concentrations between groups, the AUC of glucose concentrations were lower in five of the six treated horses during the period from 9 to 24 h, with no changes in lamellar perfusion. Glucose is essential to maintain attachment between the dermal and epidermal layers and it is stated that the equine foot consumes more glucose than the head (Wattle and Pollitt, 2010). Previous research by Pass et al. (1998) demonstrated that lamellar tissue explants deprived of glucose are unable to maintain the dermo-epidermal junction, demonstrating that glucose supply is fundamental for the normal function of the lamellar tissue. As glucose cannot be stored within the hoof (Wattle and Pollitt, 2004) , the amount of glucose reaching the lamellae relies on the local perfusion rate. Therefore, the apparent lower concentrations of glucose observed in the treated group might be a consequence of the effects of insulin either reducing the local blood flow to the lamellae, or the result of a higher uptake and metabolism by the EBCs. In this experiment, lamellar perfusion was assessed via tissue microdialysis and urea clearance with no changes in blood flow after 24 h of exposure to insulin. Although lamellar perfusion is influenced by limb load cycling , urea clearance appears to be sensitive technique to assess vascular changes in the lamellar vasculature in the static, standing horse,

as described by Medina-Torres et al. (2014a). The present results do not support changes in lamellar perfusion as a direct consequence of insulin stimulus, and differ from previous results presented by de Laat et al. (2012b) in which increased hoof wall surface temperature (HWST), presumably due to vasodilation, was observed during the developmental stage of insulin-induced laminitis. However, HWST can be influenced, not only by changes in perfusion, but also by ambient temperature and metabolic activity of the submural tissue (Hood et al., 2001). Therefore, it is possible that the observed increases in HWST in previous studies using the p-EHC (de Laat et al., 2012b) were due to increases in metabolic rate, rather than vasodilation. In the current studies, in the absence of changes in urea clearance, the most likely cause for decreased dialysate glucose concentrations in the treated group was increased glucose uptake by the EBCs, possibly driven by increased metabolic rate, indirectly mediated by insulin.

Asplin (2009) previously studied lamellar glucose metabolism and its implications for laminitis. Their work demonstrated that EBCs regulate glucose uptake in an insulin-independent manner, mainly through the transmembrane glucose transporter protein GLUT-1 (Asplin et al., 2011). However, as previously discussed above, the insulin-IGF-1R binding is capable of activating several signalling pathways modulating cellular proliferation and cellular metabolism (Agathocleous and Harris, 2013). The pathways PI3K, AKT and mTOR for example, modulate glucose uptake, glycolysis, fatty acid, protein and nucleotide synthesis, and also interact with each other to coordinate cellular state and metabolism (Agathocleous and Harris, 2013). It has been described that tumours and cells undergoing active proliferation experience a metabolic shift requiring substantially higher amounts of energy in comparison to normal tissues (Fritz and Fajas, 2010; Grabellus et al., 2012). This might be the reason why EBCs respond to the effects of insulin by cytoskeleton disruption and the diverse secondary pathways that can be activated, changing the metabolic requirements of lamellar tissue. Increased metabolic activity is usually an exothermic process (Hood et al., 2001). Future local or systemic insulin delivery models should be performed under temperature-controlled conditions, combining HWST monitoring, energy metabolite and urea clearance in order to confirm this hypothesis.

The limitations of the present work include the microdialysis probe placement protocol, the position of the membrane in relation to the distal phalanx and EBCs, insulin tissue dynamics and the size of the data set. Variability in the location of the microdialysis probes placed in the lamellar tissue was a consequence of the lack of precision in the needle insertion

technique. Individual anatomic differences of the horses' hooves and the angle of insertion, which is approximated using the hoof wall surface as a reference, result in slightly different positioning of the microdialysis probes. The proximity of the membrane to the EBCs or to the bone surface is likely to have influence the actual sampled/perfused area. Metabolite concentrations may fluctuate due to a metabolic gradient between dermis and epidermis. The amount of insulin reaching the EBCs and their response to the insulin stimulus could have been affected by the distance between the microdialysis probe and the tissue. The correlations observed in the treatment group between the concentrations of glucose and L:G, and the distance from the probe to the closest 5 PELs to the membrane, support the idea that insulin effects are affected by the distance from the microdialysis probe to the EBCs and, therefore, the insulin concentration reaching the epidermis. The closeness of the membrane to the dermal vasculature must be also considered. Wang et al. (2006a) described the transport of the insulin molecule from vasculature to skeletal muscle in dogs, demonstrating that the InsR is required by the endothelial cells to transport insulin from the blood stream to the tissues. The lamellar dermis is highly vascularized and the lamellar capillaries express the InsR which might result in the removal of the insulin molecule from the tissue, possibly affecting the insulin diffusion and distribution in the lamellar tissue. Additionally, insulin effects may be time and/or concentration-dependent. In the systemic insulin-induced model (de Laat et al., 2010a) histomorphological changes were evidenced early in disease progression (6 h), whilst in previous proliferation studies *in vitro* (Bailey and Chockalingham, 2010) insulin took up to 48 h to exerts a detectable effect on cell proliferation. Considering the small area perfused here and the unknown behaviour of insulin in the lamellar tissue, longer times of exposure and also different insulin concentrations might cause different or more advanced effects. A bigger data set should be considered in future research to increase statistical power and significance.

The histomorphometric changes observed here support a direct effect of insulin on lamellar tissue, whilst the metabolic effects of insulin warrant further research. As the EBCs lack InsR, an interaction between insulin and IGF-1R is suggested, possibly compromising epidermal cellular metabolism and the EBC cytoskeleton. Future research should be focused on the study of the cellular components of the EBCs in conjunction with local or systemic insulin delivery models. Particular attention should be paid to desmosome and hemidesmosome integrity and stability during laminitis induction. The interaction between insulin and cytoskeletal components may be understood through analysing the lamellar tissue

using electron microscopy. Insulin determinations should be performed in the lamellar tissue of normal and laminitic horses. Understanding how insulin is transported from the systemic circulation to the EBCs and vice versa, as well as the actual insulin tissue concentrations triggering laminitis, is important to comprehend insulin effects on the lamellae and to be able to replicate the disease in experimental models in a better and more realistic way.

Although tissue microdialysis appears to be a good alternative for the delivery of insulin to the lamellae, new possibilities should be assessed. Using the same insertion protocol described here, tissue microdialysis probes may be replaced or used in combination with insulin infusion sets similar to those utilized in human insulin pump therapy. This would allow precise control over the concentration of insulin delivered to the tissue. However, it would necessitate the addition of extra fluid volume to the interstitium and is, therefore, perhaps a less physiologic delivery method than microdialysis. The use of this technique and tissue microdialysis together, in two different areas of the same hoof, could be used for the study of insulin transport and diffusion in the lamellar tissue.

Finally, the study of the InsR and IGF-1R deserve further investigation. The actual role of IGF-1R in the pathophysiology of laminitis remains unclear. However, based on the current results and previous evidence, IGF-1R appears to be fundamental in the interactions between insulin and the EBCs.

5.1 Conclusion

Ttissue microdialysis appears to be suitable for local insulin delivery to the lamellar tissue and may be useful as a model for future studies of the direct effects of insulin on lamellar tissue. However, further work is required in order to determine the local tissue concentrations of insulin achieved with the technique. The present work supports insulin as the main component responsible for the changes in lamellar architecture observed and supports the hypotheses of Asplin et al. (2007) and de Laat et al. (2010a), that insulin itself is a triggering factor for laminitis.

An interaction between insulin, IGF-1R, cellular metabolism and EBCs' cytoskeleton is suggested, according to the lengthening of the PELs and increased proliferative activity described. Bioenergetic failure and ischemia (or hyperaemia) were consequences not

observed during local-delivery of exogenous insulin. The apparent decreased interstitial glucose concentrations, without a detectable change in perfusion (urea clearance), do not support direct vascular effects of insulin on the lamellae, and suggest an increased rate of cellular glucose uptake, possibly secondary to a metabolic shift of the EBCs resulting in increased metabolic rate: however, more research should be carried out to confirm the effects of insulin on the EBCs metabolism.

Further research should be focused on the interactions between insulin, its receptors (InsR and IGF-1R), the secondary signalling pathways and the cytoskeleton. The inspection of the cytoskeletal components, BM and adhesion protein under electron microscopy may aid to understand the actual effects of insulin at a cellular level.

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Appendices

Appendix 1: Preliminary assessment of microdialysis insulin delivery in vitro

A.1 Preliminary assessment of microdialysis insulin delivery in vitro

A considerable amount of insulin adsorption can occur within a microdialysis system (Jansson et al., 1993), therefore an *in vitro* calibration was performed to estimate the actual amount of insulin delivered by the microdialysis system used in the current study.

A.1.1 Microdialysis in vitro

Microdialysis probes (CMA20[a], 0.5 mm, 10 mm long, 100 kDa cut-off membrane) were placed into a 1.5 ml low protein binding polypropylene vial (Lo-bind[b]) containing 1 ml of HPLC grade water[c]. The probe inlet was connected to a microdialysis pump (801 Syringe pump[d]) and the outlet placed into a 0.5 ml polyproplylene vial (Lo-bind[b]). Based on previous studies (McNay et al., 2013; Rosdahl et al., 2000) five insulin preparations (2, 17, 100 and 348 µg/ml) were created by diluting respective amounts of recombinant human insulin (Actrapid[g)] in a commercial polyionic microdialysis perfusion fluid (T1[a]). Each preparation was loaded in a 1 ml syringe[e] and perfused at 1 µL/min for 4 hours, with experiments performed in triplicate. Insulin concentrations were determined in the solution outside the membrane, dialysate and prepared syringe.

A.1.2 Chromatography and mass spectrometric conditions

Analyses were performed on a Shimadzu LCMS-8030 triple quadruple mass spectrometer coupled to Nexera UHPLC system. Chromatographic separation was made using a reverse phase C18 column (Kinetex, 1.7 µm XB-C18, 100A, 50 x 2.1 mm, Phenomenex) column at a temperature of 40˚ C. The mobile phases consisted of UPLC-grade water with 0.1% formic acid (phase A) and 100% acetronile with 0.1% formic acid (phase B). Total acquisition time was 1 min and total flow was 0.2 µl/min, isocratic 70% B. Mass spectrometry parameters were optimized manually with Labsolutions ver. 5 (Table AP-1). Detection was accomplished using ionized recombinant human insulin (Actrapid) $[5803.75 + 5H^-]^{5+}$ precursor 11.61.75 *m/z* and product 226.15 *m/z*. Dwell time was set at 10 msec and

collision energy at -69 V. Calibration and quality control samples were prepared dissolving measured obtained by spiking the blank matrix with known amounts of insulin. The calibration curve (0.01 to 100 µg/ml) was analysed using linear regression with a minimum R^2 of 0.99.

A.2 Results

The insulin concentrations determined for the solution outside the membrane, dialysates and prepared syringe are summarized in the Table AP-2. The mean (range) insulin recovery (concentrations around the microdialysis membrane) was 0.5 (0.14 – 1.1) %, varying markedly depending on the concentration prepared in the syringe. Insulin concentration could not be detected in the solution outside the membrane at 2 µg/ml.

A.3 Conclusion

The mean insulin recovery was 0.5 %, however, high variability in the insulin recovery was found at different insulin concentrations. Insulin adsorption to the plastic surface of the microdialysis system appears to be an important factor. Concentration in the fluid surrounding the microdialysis probe at 2 ug/ml, was probably decreased due to plastic binding. Further work is needed to confirm a concentration-dependent effect on insulin recovery via tissue microdialysis.

Table AP-1: Optimized mass spectrometry parameters for recombinant human insulin (Actrapid) determination on microdialysis dialysate samples using a Shimadzu LCMS-8030 triple quadruple mass spectrometer.

Parameter	**Value**
Nebulizing gas flow	2 l/min
Heating gas flow	10 l/min
Interface temperature	300˚ C
DL temperature	250 ˚ C
HeatBlock temperature	400 ˚ C
Drying gas flow	10 l/min

Table AP-2**:** Concentrations of recombinant human insulin (Actrapid) (µg/ml), determined using UPLC-MS in samples from the prepared syringe, dialysates and solution outside of the microdialysis probe after 4 h of perfusing at 1µl/min an in vitro microdialysis system.

Prepared concentration (ug/ml)	**Prepared syringe (mean ±SD)**	**Dialysate (mean ±SD)**	**Solution outside of the membrane (mean ±SD)**
2	2.00 ±0.47	0.09 ±0.09	-
17	15.90 ±0.07	11.43 ±3.95	0.23 ±0.01
100	78.68 ±3.48	70.95 ±48.95	0.14 ±0.11
348	239.94 ±50.79	308.83 ±5.64	0.32 ±0.17