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An Evaluation of Pathophysiology and Biomechanics of Selected Lamenesses in the Horse

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AN EVALUATION OF PATHOPHYSIOLOGY AND BIOMECHANICS OF SELECTED LAMENESSES IN THE HORSE

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College
in partial fulfillment of the requirements for the degree of Master of Science

in

The Department of Veterinary Clinical Sciences

by
Cole Barrett Sandow
DVM, Louisiana State University, 2013
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The author would like to dedicate this work to his parents, Kathy and Allan Sandow. Without their constant love and support this would not have been possible.
The author would like to thank Dr. Laura Riggs, graduate committee chair, mentor, teacher, but most of all, friend. Her constant support, guidance, and friendship were vital to succeed in this challenging program. She helped the author realize his potential and further develop skills to progress in his career. Simply, the author could have not dreamed for a better mentor and friend than Dr. Laura Riggs.

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ABSTRACT

INTRODUCTION-Laminitis and synovitis are two common causes of equine lameness. Laminitis is most often associated with hyperinsulinemia and osteoarthritis is most commonly the result of synovitis. The objective is to evaluate biomechanical and pathophysiologic events regarding laminitis and synovitis.

MATERIALS AND METHODS-The first study evaluated biomechanical effects of insulin on lamellar strength. For this experiment, lamellar explants were harvested, incubated in media only (serving as a control) or media with a high concentration of insulin for 8 hours. After incubation, structural integrity was evaluated with a mechanical testing device. Data included load to failure (N), stress to failure (MPa), elongation to failure (mm), and Young’s modulus (MPa) to be evaluated with a mixed linear model (P<0.05). The second study evaluating synovitis in horses, used a cross-over design using lipopolysaccharide (LPS) to induce synovitis. Horses were administered either saline (as a control) or PRP as treatment for LPS-induced synovitis. Following a wash-out period the opposite treatment was performed in the contralateral joint.

Serial lameness evaluations and assessments of the synovial fluid for concentrations of prostaglandin-E$_2$ (PGE$_2$), interleukin-1 receptor antagonist (IL-1ra), and collagenase-cleavage neoepitope (C2C) were performed. Statistical significance was assessed using a two-way repeated measures ANOVA with a Bonferroni correction (P<0.05).

RESULTS- Insulin significantly weakened the structural integrity of lamellar explants, but did not make them less stiff. A single injection of PRP improved subjective lameness scores, but did not improve the synovial environment.

DISCUSSION- The use of a mechanical testing device to study hyperinsulinemia-induced lamellar failure through the exposure of lamellar explants to high concentrations of insulin.
provides a novel model to study that form of laminitis and evaluate potential therapies.

Additional injections of PRP as recommended in human literature or a different composition of PRP may have improved results because the PRP used in the study did not improve the synovial environment using that model of synovitis.
CHAPTER 1. INTRODUCTION

1.1 Lameness in the Horse

Lameness can be defined as any gait abnormality in the horse and may be the result of painful or mechanical manifestation of disease (McIlwraith 2011). It is most commonly associated with a musculoskeletal disorder such as injury to a joint or soft tissue structure but may also originate from the feet as well. There are few things more frustrating to an equestrian than a lame performance horse unable to compete to its potential due to osteoarthritis or the emotional roller-coaster ride of having a horse with laminitis such as Barbaro, the 2006 Kentucky Derby winner, who unfortunately succumbed to complications of the disease.

Laminitis is an extremely complex disease affecting the attachments between the hoof wall and the bone encased within it that causes debilitating pain and lameness with no known cure despite millions of research dollars and years of investigations. The importance of the disease which has affected horses for centuries are summed up well by William Percival “Were a veterinary surgeon asked the question from what disease a horse experienced the most suffering, he would, methinks, require little reflection before he determined in favor, or rather disfavor, of the one I am about to describe. There may be, and no doubt are, other morbid conditions, from which the animal suffers more acutely for the time; but there is no one in which pain, while poignant to the extreme, is apt to be so protracted “(Percival 1871). Synovitis due to cyclic trauma within the joint is the most common reason for performance horses to develop osteoarthritis (McIlwraith 2011). In competitions where success or failure is less than a fraction of a second, an effective treatment for synovitis in the face of stricter medication regulations would be ideal to keep horses performing at the maximum potential. This document addresses the biomechanical integrity of the attachments affected by laminitis when exposed to insulin and evaluate the
effects of an autologous therapy for the treatment of synovitis, two common causes of lameness in the horse, with hopes that information may be applied to existing knowledge base so we may better understand and treat equine patients with those disorders.

1.2 Laminitis

There are few, if any, diseases of horses that strike so much fear, concern, or emotional derangement in people as the horse with laminitis. While this condition has affected horses for centuries, there is still no cure for the disease. This is due to the incomplete understanding of the complex pathophysiology of laminitis. However, great strides have been made over the last several years providing more information about how laminitis develops and ways to identify at risk horses. This has allowed for significant improvement in prevention of the disease in some cases. The laminitis portion of this chapter will describe the lamellar tissue that is involved with the disease, different types of laminitis, mechanisms of the disease, and biomechanical evaluation of the dermo-epidermal junction.

1.2.1 Functional Anatomy of Lamellae

Incased within the hoof wall is an intricate network of tissue attachments between the hoof capsule and the distal phalanx that functions to suspend the entire weight of the horse (Thomason 2001; van Eps 2006). The hoof wall can be divided into 3 sections from superficial to deep: stratum externum, stratum medium, and stratum internum. The interdigitating folds of tissue between the hoof wall and distal phalanx are known as lamellae. They are composed of approximately 550-600 primary lamellae and 150-200 folds of tissue on the surface of each primary lamellae known as secondary lamellae (Pollitt 2010). The secondary epidermal lamellae from the hoof wall side interdigitate with the secondary dermal lamellae from the distal phalanx side at the interface of the lamellar basal epithelial cells with that of the basement membrane of
the dermis (Figure 1.1). The suspension of the axial skeleton within the hoof wall and weight of
the horse is dependent on those attachments. Failure of that

Figure 1.1  A 5 mm x 5 mm section of tissue comprised of hoof wall (A) and distal phalanx (B)
showing inter-digitation of epidermal tissue from the hoof wall and distal phalanx as highlighted
by the white bracket.
interface results in displacement of the coffin bone within the hoof capsule resulting in debilitating lameness which is known as laminitis (Pollitt 1998, 2004). The effects of biomechanical forces applied to the lamellae will be explained later but the forces applied by the deep digital flexor tendon on the dorsal lamellae cannot be overlooked. Due to its attachment on the flexor cortex of the distal phalanx and coursing proximally around the distal sesamoid bone incriminates this structure as playing a role in rotation of the distal phalanx in cases of dorsal lamellar failure (Merritt 2008).

The lamellar basal epithelial cells (LBECs) are closely adhered to the underlying basement membrane through attachments called hemidesmosomes while desmosomes provide adjacent attachment of LBECs to each other. The basement membrane has important functions in cell signaling, growth, migration, and attachment (Abrahamson 1986). It is comprised of type IV collagen, laminin, nidogen/entacin, and perlecan. Its glycoprotein components allow for strong uptake of periodic acid-Schiff (PAS) stain (Pollitt 1998). While the basement membrane is important, the function of the lamellar junction to support the weight of the horse hinges on the integrity of LBEC making it a focus of laminitis research. Precise mechanisms of lamellar failure have yet to be elucidated. However, whether failure results from cytoskeletal dysregulation or dysadhesion of the LBEC from the basement membrane is unknown. However, the importance of the LBEC in maintaining the structural integrity of dermo-epidermal junction cannot be overstated (Belknap 2012).

1.2.2 Pathophysiology of Laminitis

Laminitis was traditionally described as a sudden-onset of lameness with varying degrees of pain accompanied with characteristic signs such as stretched-out forelimbs, weight-shifting, increased digital pulses, alterations in hoof wall temperature, and pain when pressure is applied
to the sole (Hood 1999). The varying degrees of lameness due to laminitis have been quantified by Obel as follows: Obel grade 1 - the horse shifts weight between affected limb, no lameness observed at the walk, lameness at the trot is readily apparent; Obel grade 2 - the horse moves willingly at walk but lameness is observed, a foot may be lifted with ease; Obel grade 3 - the horse is reluctant to move, resist attempts to lift contralateral limb to affected foot; Obel grade 4 - the horse does not move without being forced, prefers to be recumbent (Obel 1948). The Obel lameness grades are routinely used in evaluation of horses with laminitis.

Evaluation of the condition has identified 3 phases associated with the disease: developmental, acute, and chronic (Hood 1999). The developmental phase does not result in clinical signs of laminitis; however, this is the beginning of the process involved with lamellar failure (Hood 1999). The duration varies and does not end until first appearance of lameness beginning the acute phase of the disease. The acute phase ends after 72 hours or unless radiographic or physical evidence of displacement of the coffin bone is observed. The severity of the observed lameness during varies during this time and horses often display an altered stance, weight-shifting, and increased digital pulses (Baxter 1994). The chronic phase has varying degrees of lameness and anatomical derangement but is best assessed by the Obel grade of lameness (Hunt 1993). Since the degree of lameness does not always correlate with morphologic changes of the lamellae, the source of lameness during the acute and chronic phase remains unknown. For example, horses during the acute phase may be more uncomfortable than horses in the chronic phase begging the question is inflammation or basement separation or a combination of both to blame for the observed lameness.

Laminitis has also been categorized into 3 distinct categories: sepsis-related, support limb, and endocrinopathic. The different predisposing causes of laminitis have been studied
experimentally. The development of experimental models that mimic clinical sepsis-related and endocrinopathic laminitis and has greatly improved the understanding of this devastating disease. Due to humane reasons, a model that causes support limb laminitis has not been developed, but a period a unilateral weight-bearing using an unstable shoe has been used (Belknap 2013). While all three predisposing causes can result in lamellar failure, the sequence of events and mechanisms involved appears to be unique for each type. However, all types have some contributions from vascular, inflammatory, biomechanical, and metabolic derangements with varying implications in the disease dependent on the type of laminitis. The different types of laminitis and their respective models will be discussed and be related to the mechanisms involved with the development and progression of the disease.

SEPSIS-RELATED LAMINITIS

This category of laminitis is a complication of diseases causing systemic inflammation such as: gastrointestinal strangulation, colitis, pleuropneumonia, duodenitis/proximal jejunitis, and septic metritis (Belknap 2009; Cohen 1999; Hunt 1986; Slater 1995). Laminitis can occur as a complication of those disorders allowing thereby being labeled as sepsis-related laminitis and allows for identification of at risk animals. Clinical experience has shown displacement of the distal phalanx may occur as rotation or distal displacement with forelimbs more commonly affected than hindlimbs. The exact mechanisms of lamellar damage due to this form of laminitis have been studied using experimental models including starch overload, fructan overload, and black walnut extract which mimic the inflammatory response noted in clinical cases. The starch overload involves a week-long pellet only ration followed by nasogastric intubation of wood flour and corn starch at an induction dose of 17.6 g/kg of bodyweight (Garner 1975). The mean time to obel grade 3 lameness is 40 hours with lameness usually most severe in the fore feet.
Obel grade 1 lameness is observed as early as 12 hours. The drawback is that it does not consistently result in laminitis in all subjects. The oligofructose model is another model of carbohydrate overload that is based on the premise of ingestion lush pasture rich in fructans (long-chain polymers of fructose) resulting in laminitis. The protocol involves nasogastric intubation with fructan from roots of chicory and administered to horses at 7.5, 10, and 12.5 g/kg of body weight (van Eps 2006). This reliably induced laminitis with lameness occurring at 24-36 hours post administration. The black walnut extract model (BWE) was established after reports of horses developing laminitis on wood shavings from black walnut (Juglans nigra) trees. Two grams of black walnut shavings per kilogram of body weight is added to 6-7 liters of water at room temperature and agitated for 12-24 hours. The dark red solution is filtered off using a cheesecloth and administered to study subjects via nasogastric tube (Minnick 1987). Subjects develop Obel grade 1 lameness within 8-10 hours of administration (Hurley 2006; Peroni 2005). The drawback of this model is that it does not result in lamellar failure and may not be as clinically relevant as the CHO model.

SUPPORT LIMB LAMINITIS

Support limb laminitis results due to unilateral weight bearing of the so-called “good-leg” due to a primary condition in the contralateral limb making identification of at-risk horses straightforward (Baxter 2008). It is unique in that it occurs in only one foot lacking the usual systemic signs seen in other forms of laminitis and the coffin bone usually has distal displacement instead of rotation (Baxter 2008). The risk of development is believed to be related to the duration and severity of lameness (Redden 2006). A higher incidence of this laminitis is thought to be related to duration of casting and higher body weights (Virgin 2011). It has also been reported to have a 16% incidence in horses with fracture repair using locking compression
plates (Levine 2007). There is little research regarding support limb laminitis due to the lack of a humane consistent model, however the use of a special shoe has led to the development of a short-term, non-weight bearing model (Belknap 2013). While further research is needed to study the pathophysiology of this type of laminitis, it is believed unilateral weight bearing on the unaffected contralateral limb leads to decreased perfusion with a concurrent increase in mechanical force predisposing the lamellae of that digit to failure. Additionally, the inflammatory response due to the primary condition may further prime the lamellar tissue to failure when persistent increase in mechanical force is applied.

VASCULAR MECHANISMS IN LAMINITIS DEVELOPMENT

Hemodynamic changes occur during the development and progression of laminitis and was initially thought to be the major mechanism in the pathophysiology of the disease (Hood 1999). Several techniques have been used to evaluate lamellar blood flow including laser Doppler flowmetry, arteriography, nuclear scintigraphy, thermography, and infrared spectroscopy (Adair 2000; Hinckley 1995; Hood 2001; Hunt 1994; Pollitt 1998; Rosenstein 2000). The pitfall of those techniques is the inability to differentiate capillary flow and flow through arterio-venous anastomosis. For example, identification of reduced capillary flow would be most beneficial to discern if decreased perfusion results in lamellar hypoxia. However, total digital blood flow may be maintained due to AVA leaving the status of capillary perfusion unknown. Despite those drawbacks hemodynamic changes have been documented using models for sepsis-related laminitis. The observed hemodynamic changes using those models include increased post-capillary resistance with resulting interstitial edema due to alterations in Starling forces and morphologic changes to the lamellae (Eaton 1995; Hood 1999). The pre-capillary resistance is greater than post-capillary resistance (92% and 8%, respectively), but the post-
capillary resistance increases with sepsis-related models of laminitis (Allen 1990; Allen 1988). Edema formation is also due to the ‘leaky’ nature of lamellar capillaries with a similar permeability to capillaries of the lungs (Allen 1988; Bailey 1998). The lack of xanthine oxidase in a model of sepsis-related laminitis suggests ischemia/reperfusion is unlikely to play a role in the disease (Loftus 2007).

Further evidence for hemodynamic dysfunction is demonstrated by platelet activation and microthrombi formation (Weiss 1994). The effects of weight-bearing lamellar on perfusion has been evaluated using microdialysis of lamellar interstitium concluding prolonged unilateral weight-bearing creates a hypoxic environment and may implications in support limb laminitis (Medina-Torres 2016). The contribution of vascular disturbance to lamellar damage appears to vary with the type of laminitis and may be most significant in support limb laminitis, followed by sepsis-related laminitis, and lastly endocrinopathic laminitis. Furthermore, the degree of lamellar damage due to blood flow alterations is unknown. However, it is suspected that vascular alterations do have some role in the development and also the progression of laminitis in certain cases.

INFLAMMATORY MECHANISMS IN LAMINITIS DEVELOPMENT

Inflammation has been reported to occur in early the development of laminitis in models of sepsis-related laminitis before clinical signs of digital pathology or lameness, but is less marked and more variable when evaluated in models of endocrinopathic laminitis. Therefore, the majority of information on inflammation has come from research using sepsis-related models of laminitis. The purported sequence of events of with inflammation includes invasion of leukocytes due to inflammatory signaling, increased cytokine production and protease
production initially at the lamellar basal epidermal cells (LBEC) (Faleiros 2009; Leise 2011; Riggs 2007).

Despite the name suggesting an inflammatory response, inflammation specifically was not evaluated early in laminitis research. However, a study found the expression of interleukin-1b (a pro-inflammatory cytokine) in lamellar tissue prior to the development of lameness suggesting this response was important to the developmental phase of sepsis-related laminitis (Fontaine 2001). This sparked further research marking the presence of inflammation in lamellar tissue with CD-13 (poor marker for monocytes) and calprotectin (good marker for neutrophils and monocytes) using similar models of laminitis (Black 2006; Visser 2011). Infiltration of leukocytes demonstrated in the lamellar tissue form these experiments was thought to result in increases in local MMP production (Belknap 2007).

Knowing the earliest location of the of leukocyte signaling would be beneficial to further understand the disease. Tissue participating in inflammatory signaling can be marked for the expression of toll-like receptors (TLR), specifically TLR-2 (Gram-positive ligand) and TLR-4 (Gram-negative ligand) (Werners 2012). A novel study showed LBEC had significant expression of TLR-2 and TLR-4 showing they play an active role in the lamellar inflammatory signaling (Leise 2015). Some of the inflammatory mediators produced by the lamellar tissue include proinflammatory cytokines (interleukin-1b, interleukin-6, interleukin-12), COX-2, adhesion molecules (E-selectin), neutrophilic chemokines (CXCL1, CXCL6, and CXCL18), and monocytic chemokines (CCL2 and CCL8) (Faleiros 2009; Fontaine 2001; Leise 2011; Loftus 2007). Lamellar tissue also has high concentrations of hypoxic-inducible-factor 1a (HIF-1a), which is increased with low tissue concentration of oxygen, in both normal horse and those
undergoing sepsis-related laminitis models (Rius 2008). The presence of high concentration of HIF-1a may prime the lamellae for intensified inflammatory response in sepsis (Pawlak 2014).

Degredation of the extracellular matrix and separation of the basement membrane and from lamellar epithelial basal cells in horses with laminitis lead to research to evaluate the contributions of matrix metalloproteinases (MMPs) and ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs-4) in the early stages of the disease. MMP-2 and MMP-9 are capable of degrading several components of the basement membrane and lamellar dermis while ADAMTS-4 degrades large polysulfated proteoglycans (Porter 2005; Stanton 2011). MMP-2 and MMP-9 were originally thought to cause lamellar damage in the development of laminitis (Mungall 1999). However, they were not present in their active form during the development of laminitis in an experimental model suggesting they are not as vital in the initial stages as first described (Loftus 2009). It now appears that MMP-1, MMP-13, as well as ADAMATS-4 are the proteases responsible for lamellar damage in sepsis-related models of laminitis (Coyne 2009; Leise 2015; Visser 2012). The expression of genes encoding for ADAMTS-4, MMP-1, and MMP-13 were not increased in horses undergoing a model for endocrinopathic laminitis compared to healthy horses suggesting the role of those proteases is not as great in endocrinopathic laminitis.

METABOLIC MECHANISMS IN LAMINITIS DEVELOPMENT

This area has become a major research focus because of its proposed role in the development of endocrinopathic laminitis. It has been shown that glucose deprivation results in detachment of lamellar basal epithelial cells (LBEC) from their basement membrane in-vitro; however, it was later shown that glucose uptake is insulin-independent suggesting glucose deprivation was not a major mechanism in endocrinopathic laminitis (Burns 2013; French 2004).
Review of endocrinopathic laminitis cases suggest insulin is central to causing lamellar dysfunction as the majority of horses have an underlying hyperinsulinemia (Karikoski 2015). The exact cause of lamellar dysfunction as result of high serum concentrations of insulin is purported due to the activation of insulin-like growth factor-1 (IGF-1) signaling. This has been demonstrated through a study IGF-1 signaling in lamellar tissue. Presence of phosphorylated ribosomal protein (RPS6), an end-product of the IGF-1 signaling pathway, has been found in lamellar tissue of horse administered a high concentration of insulin to induce experimental endocrinopathic laminitis (Lane 2017). This signaling pathway has been shown to cause dysregulation of epithelial tissue, disruption of cytoskeletal organization, increased mitotic rate, and cause dissolution of hemidesmosomes (Laval 2014; Magnuson 2012; Mezi 2012; Shahbazian 2006; Yu 2012). Therefore, activation of IGF-1 signaling due to hyperinsulinemia may be responsible for lamellar dysfunction.

BIOMECHANICAL MECHANISMS IN LAMINITIS DEVELOPMENT

The role of biomechanics in the progression of laminitis cannot be overlooked, however there is little research in this area with current theories relying more on biomechanical principles and anecdotal evidence. After lamellar injury due to vascular, inflammatory, or metabolic derangements, it appears the lamellar junction may be susceptible to further injury and eventually separation due to mechanical forces acting on the lamellae. Once the lamellar attachments fail, the coffin bone is displaced in the hoof wall in 3 possible directions: rotation, symmetrical distal displacement, or asymmetrical distal displacement. The role of weight-bearing and therefore a biomechanical component is readily apparent due to the fact the same lamellar signaling events occur within hind feet as they do the fore feet, despite a higher incidence of clinical laminitis in the fore feet (Leise 2012). This is supported by the fact horses at
rest bear 28% of their body weight through a forelimb compared to 66% of their body weight through a forelimb when walking (Merkens 1988). Furthermore, the lower body weight of ponies was thought to be the reason for mild loss of structural integrity at the epidermal-dermal lamellar interface following hyperinsulinemia suggesting a mechanical factor in the progression of laminitis (de Laat 2010; Nourian 2009). During acute stages of disease there is equal weight bearing through the forelimbs despite constant weight shifting between forelimbs; however, weight bearing through the forelimbs is decreased in the chronic stages (Hood 2001). While biomechanical forces play a role in all types of laminitis, it appears to be central in the development of support limb laminitis due to decreased perfusion with a concurrent increase in mechanical forces of the lamellae in the support digit due to unilateral weight bearing.

1.2.3 Endocrinopathic Laminitis and Insulin

Endocrinopathic laminitis (EL) is the most common type of laminitis affecting horses worldwide and is associated with hormonal influences rather than pro-inflammatory or intestinal conditions (de Laat 2010; Karikoski 2011). It is currently a major focus of laminitis research. This form of laminitis is linked with pituitary pars intermedia dysfunction, Equine Metabolic Syndrome, corticosteroid administration, pasture-associated reasons, and insulin dysregulation (either insulin resistance or insulin sensitivity). Since the causes of endocrinopathic laminitis are different than sepsis-related, the mechanisms involved with lamellar dysfunction as well as morphologic changes are different for each when comparing their respective experimental models (de Laat 2011). Furthermore, horses clinically affected with EL appear to have several bouts of lamellar damage prior to presenting with lameness when compared to sepsis-related or support limb laminitis horses that often have a sudden onset of lameness suggesting the initial lamellar damage in the latter two is more severe (Karikoski 2015).
While there are several predisposing causes associated with EL, the common factor amongst horses affected with that disease is hyperinsulinemia suggesting insulin in the cause for lamellar dysfunction (Karikoski 2015). Horses admitted with very high serum insulin concentrations at presentation (>188 µ IU/ml) were more likely to develop laminitis and survive less than two years after diagnosis than those with only moderate elevations or normal insulin levels (<62 µ IU/ml) (McGowan 2004). This lead to the development of a modified euglycemic-hyperinsulinemic clamp technique (EHC) as model for endocrinopathic laminitis (Asplin 2007). It was initially performed in ponies with Obel grade 2 lameness observed at 55 hours, much slower than sepsis-related models (Asplin 2007). This was repeated in Standardbred horses with quicker onset of Obel grade 2 lameness at 46 hours, presumably due to a high body weight (de Laat 2010). Histologic comparison of horses undergoing the EHC and CHO models, show horses in the EHC have more lamellar stretching due to observed elongation of basal epithelial cells compared to more of a wide-spread basement membrane separation (de Laat 2011; Karikoski 2015). Also, the inflammatory response is greater in CHO compared to EL models, but cellular infiltration follows the development of basement membrane lesions and morphologic changes of the secondary epidermal lamellae (de Laat 2011; Visser 2011). The exact mechanisms have yet to be determined but may include glucose deprivation, vascular effect, or inflammatory effects.

Recently, effects of lamellar signaling have proposed that insulin-like growth factor-1 (IGF-1) signaling may be involved with lamellar dysfunction (de Laat 2013; Kullmann 2016; Lane 2017). It has been purported that alterations in lamellar epithelial basal cells is one of the initial events in the development of sepsis-related laminitis (Leise 2011). Cellular alterations at the same level are thought to occur through insulin binding to IGF-1 present on epidermal cells (Kullmann 2016). Additional work performed by Lane et al 2017 confirmed activation of IGF-1
signaling in horses undergoing a model of endocrinopathic laminitis by immunofluorescence staining of lamellar tissue for phosphorylated ribosomal protein S6 (RPS6) an end-product of the IGF-1 pathway. It has been shown that activation of that pathway may lead to cytoskeletal dysregulation, increased mitotic rate, and dissolution of hemidesmosomes (Laval 2014; Mezi 2012; Yu 2012). Although it has not been confirmed, it is speculated similar mechanisms may occur following insulin binding to IGF-1 receptors in the lamellae leading to lamellar dysfunction. Potentially, a threshold of lamellar damage is reached and once mechanical factors (such as weight-bearing) are applied lamellar failure occurs.

1.2.4 Biomechanical Evaluation of Lamellae

Biomechanics evaluates how a force effects tissue or how much displacement occurs when a force is applied to a tissue. This may be plotted on a load-displacement curve or stress-strain curve with the slope of the line representative of the stiffness of a tissue also known as Young’s modulus. Stress is defined as a force divided by the area of the tissue and is often reported in Pascals. Strain refers to the original shape minus the final shape divided the original shape and is reported as a percentage. Forces can be applied in various directions relative to the tissue being tested. The most relevant forces in relation to the lamellae include tension and shear in both a mediolateral and proximodistal direction.

The unique structure of the hoof provides challenges when evaluating biomechanical stress on the equine digit. Stress at the lamellar dermal-epidermal junction has been studied in normal horses through biomechanical testing of lamellar explants with interesting results (Douglas 1998; Kochova 2013). Doulgas et al 1998 evaluated lamellar explants which are block of tissue comprised of hoof wall, dermal tissue, epidermal tissue, and distal phalanx. Explants were harvested from different areas of the foot (dorsal, medial, and lateral) and different forces
applied (tension, mediolateral shear, proximodistal shear). The explants were strongest in tension, weaker in proximodistal shear, and weakest in mediolateral shear. The angle of force application in relation to the primary epidermal lamellae did not have a significant effect. Another interesting finding was that hoof was significant factor but horse was not suggesting two feet from the same horse have varying stiffness (Douglas 1998). Kochova et al 2013 also evaluated lamellar explants and concluded the relative concentration of secondary lamellae correlated with increased stiffness presumably due to more surface area attachment of the basement membrane (Kochova 2013). In conclusion, there is location dependent variation amongst lamellar explants depending on the location from the foot where they are harvested.

An interesting application of biomechanical testing of lamellar explants is the addition of substances that may reduce the structural integrity of the lamellar junction to identify potential therapeutic targets. A primitive application of this concept was applied by exposing lamellar explants to matrix metalloproteinase activators and testing relative structural integrity with by pulling them apart by hand using forceps (Pollitt 1998). However, specific biomechanical testing has not been performed on lamellar explants exposed to an induction agent such as insulin with a mechanical testing device. Application of such a model is described in a later chapter.

1.3 Synovitis

Synovitis due to cyclic trauma is the most common cause for the development of osteoarthritis in athletic horses and osteoarthritis accounts for 60% of lameness problems in horses (Caron 2003). There is no cure for osteoarthritis but rather a multimodal approach to its management. The estimated direct and indirect cost for treatment of a horse with osteoarthritis has been estimated at $3000 and $15,000/year, respectively (Oke 2010). Understanding this prevalent and costly equine disease as well as evaluating new therapies is a major focus of
equine research. This section will describe the pathophysiology of synovitis and the develop of osteoarthritis, synovial fluid biomarkers, medical treatments for osteoarthritis, and platelet-rich plasma.

1.3.1 Functional Anatomy of a Synovial Joint

A joint is now considered a complex organ, such as the kidney, heart, or liver, and is comprised of several components that must perform in unison to maintain function. Joints can be categorized in categories: fibrous joints with dense connective tissue, cartilaginous joints with a cartilage interface, and synovial joints with fluid-filled cavity (Dyce 2010). The basic components of a synovial joint are articular cartilage, subchondral bone, synovial fluid, and a synovial capsule. Just as bone has been known to respond to mechanical loading described by Wolff’s law stating that bone will adapt to the loading to which it is subjected, the same principle can be applied to a synovial joint (Wolff 1892). The responses to loading is important to develop the form and functions of the synovial joint of an equine athlete.

The major components of articular cartilage are collagen, proteoglycans, and water. Water content varies from 70-80%. The dry weight basis is 50% collagen and 35% proteoglycans with the remaining 15% glycoproteins (Todhunter 1996). Articular cartilage has 4 stratified layers including the superficial zone, intermediate zone, and deep zone forming the hyaline cartilage and the distinct calcified cartilage just superficial to the subchondral bone (Aydelotte 1988). Articular cartilage is made up of primarily type II collagen which is unique to the rest of the musculoskeletal system made of primarily type I collagen. The collagen fibers originate from the subchondral bone in a perpendicular direction toward the superficial zone where they become parallel to the joint surface then descend to the subchondral bone forming arches known as the arches of Benninghoff (Benninghoff 1925). This orientation of collagen has
profound effect on the elasticity and absorptive properties of articular cartilage. Mechanical loading in the juvenile period has influence on the orientation of the collagen component, therefore having consequences of the biomechanical behavior of the cartilage (Van Turnholdt 2008). Proteoglycans are made of a protein and sugar as the name implies and linked to collagen fibril either directly or through a connection with hyaluronan. Hyaluronan is a major component of the extracellular matrix but is also found in the synovial fluid. The proteoglycans also have sulfate side chains including aggrecan, versican, and lectican (Ruoslahti 1996). The sulfate side groups are hydrophilic and negatively charged, repelling each other, adding to the elastic properties and stiffness of articular cartilage. The only cell type present within articular cartilage is the chondrocyte with different shapes and densities of chondrocytes dependent on its location in articular cartilage. This structure is also unique due to the fact it is avascular and aneural which explains why initially minor cartilage damage may progress for long time before becoming clinically evident osteoarthritis. Some growth factors have a known anabolic effect on articular cartilage including transforming growth factor-β (TGF-β) and insulin-like growth factor-1 (IGF-1). TGF-β can stimulate proteoglycan synthesis by chondrocytes and expression of collagen type II and may down regulate matrix metalloproteases (Edwards 1987).

Subchondral bone consists of a layer of compact bone directly between layer of calcified cartilage and trabecular bone. It has been shown that there are differences in thickness and therefore deformability of subchondral bone in certain areas of a joint predisposed to increased mechanical forces in response to Wolff’s law (Branch 2005). This has implications in the development of joint disease placing more stress in articular cartilage in areas of denser subchondral bone and this may be initiating factor for osteoarthritis (Radin 1986).
The synovium of the joint capsule has two distinct inner layers: the intimal and subintimal layers. The subintimal layer is very well vascularized and the intimal layer which directly lines the joint capsule is few cell layers thick without a basement membrane. The synoviocytes of the intima have been divided in two categories, Type A and Type B. Type A are macrophage-like synoviocytes mostly involved in phagocytic actions. Type B are fibroblastic synoviocytes mainly responsible for production and excretion into synovial fluid making them responsible for the viscosity joint fluid viscosity (Henderson 1985). They can also produce various cytokines, growth factors, and inflammatory mediators making them crucial in maintenance of joint homeostasis. Synovial fluid is sometimes called an ultrafiltrate of blood plasma due to the lack of basement membrane of the synovial lining. It has a limited cellular component including lymphocytes and a limited number of macrophages with a total cell count usually less than five-hundred cells per microliter.

The frictionless movement of diarthrodial joints is due in part to the organization and engineering of articular cartilage and a fluid interface. Two types of lubrication involved are boundary lubrication and fluid-film lubrication and both play a role in synovial joints. Boundary involves direct contact between gliding surfaces specific substances or molecules adhere to these surfaces and form a protective layer to prevent excess wear and tear (Walker 1968). Hyaluronan is the principle boundary lubricant (Radin 1971). Fluid-film lubrication in regard to synovial joints provides elastohydrodynamic lubrication. In this case, the elastic nature of the articular cartilage deforms under load squeezing fluid that was trapped within creating a leading edge of fluid to allow for frictionless movement. The trapped fluid results from the hydrophilic nature of the extracellular matrix, where compounds within the cartilage such as aggrecan, and repels like-
charges. This principle provides high compressive stiffness and resilience making articular cartilage very much a viscoelastic tissue (Brommer 2005).

1.3.2 Synovitis and Pathophysiology of Osteoarthritis

Synovitis plays a key role in the development osteoarthritis. Articular cartilage depends on functioning synovium for physiologic homeostasis and an inflamed synovium can be a source of deleterious mediators that incite cartilage breakdown. The significance of which was shown in early experimental model of synovitis (McIlwraith 1981). It has since been shown that synovitis not only causes pain for the horse, but increased production of matrix metalloproteases (MMPs), a disintegrin and metalloprotease with thrombospondin motif-4 (ADAMTS-4), prostaglandins E\(_2\) (PGE\(_2\)), as well as cytokines such as interleukin-1 and tumor necrosis alpha (Balkman 1998; Clegg 1997; Frisbie 2002; Trumble 2001). Tumor necrosis factor alpha was abundantly expressed in synovial membrane and cartilage; while interleukin-1beta (IL-1\(\beta\)) expressed from cartilage but not significantly from synovium (Kamm 2010). Production of these various factors from cartilage and synovium resulting in synovitis is the most common problem in high-motion joints of the equine athlete. The factors produced during synovitis contribute to degradative process in articular cartilage by release of enzymes, inflammatory mediators, and cytokines.

It is well accepted that athletic activity carries an increased risk of osteoarthritis. Cartilage development is dependent on increased weight-bearing and it has been shown to increase the volume and thickness of articular cartilage in young horses (Jones 1993). However, studies have shown there is a dose-response curve where increased weight-bearing can reach a threshold leading to cartilage injury (Kiviranta 1992). Central to this process is the development of synovitis and the release of degenerative mediators and cytokines. Interleukin-1 and TNF\(\alpha\)
modulate the synthesis of metalloproteases by both chondrocytes and synovial cells (Wood 1985). Interleukin-1 also deplete proteoglycans in articular cartilage in association with release of MMPs, aggrecanases, and PGE2 from chondrocytes (Morris 1990). MMP-13 has been associated with degradation of type II collagen of articular cartilage (Caron 1996; Moldovan 1997). ADAMTS-4 is known to cause loss of aggrecan from articular cartilage. Prostaglandin E2 is considered to be one of the main mediators of inflammation and pain in osteoarthritis as well as decrease the proteoglycan content of the cartilage matrix (Takafuji 2002). PGE2 is also used as an objective index of the level of synovitis in horses (Frisbie 1998).

The release of inflammatory mediators and enzymes begins with acute synovitis causing a biochemical change of the articular cartilage. Morphologic changes ensue, softening of the articular cartilage, and swelling due to loss of glycosaminoglycan and absorption of water. Fibrillation of the superficial zone of cartilage ensues and progresses through the remaining zones until full thickness erosion occurs. The progressive loss of articular cartilage coincides with further development of synovitis.

1.3.3 Synovial Fluid Biomarkers

Biomarkers provide interesting research applications to measure levels of molecular signals and products of tissue turnover to assess the presence and/or progression of disease states such as synovitis by serial evaluations of synovial fluid over time. They are defined by the Biomarker Definitions Working Group as a “characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Downing 1999). Biomarkers have been classified as “dry” and/or “wet” by Kraus et al 2011. Dry biomarkers include diagnostic imaging findings or clinical pain scores. Wet biomarkers include the presence of mediators in fluid such as blood,
urine, or synovial fluid or specific tissues. Currently biomarkers serve primarily as research tool and have not been accepted for use in clinical cases of joint disease in the horses (Kraus 2011).

Evaluation of biomarkers within synovial fluid of both normal horses and horses with joint disease has been evaluated (de Grauw 2009; de Grauw 2009). They can be divided into different categories such as catabolic or anabolic processes, inflammatory mediators, or growth factors. One example of a biomarker for catabolic process is collagenase-cleavage neoepitope (C2C) which increases with cleavage of type II collagen (the predominant collagen of articular cartilage) by collagenase (Dejica 2008). The breakdown of articular cartilage due to collagenase activity which cleaves type II collagen fibrils exposes an otherwise hidden neoepitope present on type II collagen. The C2C antibody has high affinity for that site once it is exposed making the C2C ELISA a useful diagnostic test for articular cartilage degradation. Anabolic processes can be evaluated by the use of carboxy propeptide of type II collagen for a marker of type II collagen synthesis (Shinmei 1993). Inflammatory mediators can also be evaluated in synovial fluid including substance P and prostaglandin E2 (PGE2). Both of them have been shown to be elevated in equine joints with osteoarthritis compared to controls. PGE2 is released to a greater extent due to an inflamed synovial membrane and a lesser from articular cartilage (de Grauw 2011). As such it is a sensitive marker of active synovitis and commonly used to evaluate the anti-inflammatory and/or analgesic capabilities of joint therapies. The presence of growth factors within in synovial fluid can also be evaluated including transforming growth factor 1 beta, platelet-derived growth factor, and vascular endothelial growth factor (Textor 2011).

Interpretation of results when using biomarkers presents some obstacles in both clinical cases and experimental models. Synovial joints are comprised of a synovial membrane, subchondral bone, and articular cartilage which all must interact with each other to maintain
homeostasis of the joint environment. However, disease involving any of those components can result in the same clinical signs of joint disease including pain, effusion, and/or decreased range of motion despite different structures being affected. For example, two different horses may present with lameness in the same joint but have a different diagnosis such as synovitis in one horse and maladaptive remodeling of subchondral bone in the other joint while both share the clinical signs of joint disease. Further complicating interpretation in clinical cases is variation with regard to age, inciting causes, duration of disease, and synovial inflammation at the time of sampling (Fuller 2001). Therefore, biomarkers are currently best suited for research application using synovitis or osteoarthritis models under controlled conditions decreased potential variations amongst study subjects. An additional factor is that most commercial kits are not validated for use with synovial fluid but rather serum or urine. Despite those drawbacks experimental models of synovitis and osteoarthritis display significant alterations of biomarkers in synovial fluid compared to control subjects. Overall, the use of biomarkers to evaluate synovial fluid is a useful tool in a research setting and multiple biomarkers should be used to obtain a broader perspective on alterations of the synovial environment to determine treatment effects.

1.3.4 Medical Treatment of Osteoarthritis in the Horse

Osteoarthritis is a progressive disease making early intervention important to preserving articular cartilage and slowing the progression of the disease with the hope to maintain athletic function. While surgical options, physical therapy protocols, and complimentary treatment devices exist, this document will highlight medical therapies for osteoarthritis which can be divided into symptom-modifying osteoarthritis drugs (SMOADs), and disease-modifying osteoarthritis drugs (DMOADs), or be a combination of both. SMOADs improve the signs of
lameness and pain. DMOADs may not improve lameness but have chondroprotective properties as well as increase anabolic effects and decrease catabolic effects in the joint. Medical treatments may be systemic or intra-articular, but some treatments may be administered via either route. Systemic therapies include non-steroidal anti-inflammatory drugs (NSAIDs), polysulfated glycosaminoglycans (PSGAGs), hyaluronan, and sodium pentosane polysulfate. Intra-articular treatments include corticosteroids and hyaluronan as well as biologic therapies including interleukin-1 receptor antagonist protein (IRAP), mesenchymal stem cells (bone marrow or fat derived), and platelet-rich plasma (PRP) (Goodrich 2013). The use of corticosteroids in joints has been increasingly scrutinized other the last several years prompting the use and investigation of the aforementioned biological therapies. While the use of IRAP and stem cells has been investigated extensively, controlled experiments using PRP are lacking and is the premise for chapter 3 of this document.

NSAIDs method of action is inhibition cyclooxygenase (COX) that converts arachidonic acid to prostaglandins and thromboxanes thereby reducing inflammation and has been a mainstay for treatment of joint disease (Vane 1971). The two primary isoenzymes are COX-1 (constitutive form) which regulates normal cellular processes and COX-2 (inducible form) which is thought to be responsible for inflammatory responses. Different NSAIDs have different effects on COX-1 and COX-2 or may act on both. The main drawback of COX-1 inhibition is potential for damage to gastrointestinal mucosa or kidney function. Phenylbutazone is the most commonly used NSAID in the horse with oral and intravenously formulations. It has dose of 2.2 mg/kg twice daily or 4.4 mg/kg once daily. The 4.4 mg/kg dose has a half-life of 5.5 hours in plasma but reduced PGE2 and PGI2 in inflammatory exudate up to 24 hours (Lees 1987). Firocoxib is a COX-2 inhibitory drug for OA horses and may be give as an oral formulation or injectable. A
loading dose of 0.27 mg/kg is recommended, and is followed by 0.1 mg/kg every 24 hours. A canine formulation (Previcox) was used to prior licensing of the equine product, Equioxx, but no studies for safety or bioavailability of Previcox have been used in horses.

Polysulfated glycosaminoglycans (PSGAGs) are a mixture of glycosaminoglycans which is a component of the extracellular matrix of articular cartilage made from an extract of bovine lung and trachea. The exact mechanism of action is unknown but it is a disease-modifying osteoarthritis medication used when there is cartilage damage rather synovitis (Trotter 1996). The benefit appears to be anti-inflammatory and inhibition of degradative enzymes that contribute to OA. This medication has also been shown to promote stimulation of hyaluronan and collagen synthesis. It was initially administered in the joint, but a study showed it could potentiate joint infection appeared to slow its use even though the addition of amikacin obviated any risk of infection (Gustafson 1989). There is currently a trend toward its intramuscular use amongst equine veterinarians (Ferris 2011). The IM dose has is capable of inhibiting some cartilage-degrading enzymes, but the duration of effect is unclear (Burba 1993).

Hyaluronan is an important component of synovial fluid and articular cartilage with lubricating properties. Exogenous HA lasts in the joint only hours following IA administration while IV administration lasts only minutes (Fraser 1993; Fraser 1981). Its function is to minimize mediators of joint disease such as white blood cells, free radicals, and pro-inflammatory cytokines. The critical weight for hyaluronan to be effective is 500,000 Daltons or greater (Smith 1987). For IA use, at least 3 injections 1 week apart are needed for clinical improvement. It is commonly added with a corticosteroid for joint injections and shown to have a synergistic effect on proteoglycan matrix metabolism in the presence of IL-1 stimulation compared to both treatments alone (Schaefer 2009). It is recommended to use 20-22 mg of mid-
molecular weight HA with 3 to 5 mg of triamcinolone in a 10-15 ml as a single injection followed by 2 weekly injection of hyaluronic acid. Using the IV administration with a model of equine OA, improved clinical lameness and synovial fluid parameters were noticed 42 days following the last of three weekly 40 mg IV injections (Kawcak 1997).

Sodium pentosane polysulfate has been used in Europe for decades as an antithrombotic agent but its use an OA medication has recently become apparent. It is believed to function by promoting the synthesis proteoglycans, inhibiting enzymes responsible for proteoglycan and collagen degradation, and increase the synthesis of metalloprotease-3 (TIMP-3) (Ghosh 1999). Anecdotally, the drug is administered at a dose of 3 mg/kg IM once a week for 4 weeks and it is recommended to rest the horse for 24 hours following administration due to increase in partial prothrombin time (Dart 2001). It decreased articular cartilage fibrillation in an OA fragment model but clinical lameness scores were not different between groups making this a disease-modifying OA drug (McIlwraith 2012).

Corticosteroids have been under much scrutiny due to the catastrophic fracture of Thoroughbred horses at high profile racing events. They are used due to their anti-inflammatory effects and a prolonged effect is achieved because the bind to cytoplasmic receptors causing changes in protein expression. The most commonly used steroids are triamcinolone, methylprednisolone, and betamethasone. It was shown that triamcinolone is actually chondroprotective, betamethasone had no deleterious effects, and methylprednisolone had deleterious effects on articular cartilage using osteochondral fragment OA model (Foland 1994; Frisbie 1998; Frisbie 1997). There is fear of laminitis using triamcinolone and the earliest report had no reported case of laminitis using less than 18 mg in 1200 horses (Genovese 1983). A follow up study showed there was no association between the occurrence of laminitis and the IA
use of triamcinolone (McCluskey 2004). However, the occurrence of laminitis occurred in 3/2000 cases when 20-45 mg of triamcinolone was used (Bathe 2007). The stricter regulations of corticosteroids in competing equine athletes has caused a shift for alternative biologic therapies.

Interleukin-1 receptor antagonist protein (IRAP) or autologous conditioned serum (ACS) works on the premise there is increased anti-inflammatory cytokines in the absence of the production of pro-inflammatory cytokines. There is more than just IRAP in the product and actually contains insulin-like growth factor, transforming growth factor-β, tumor necrosis factor, and interleukin-1β (Hraha 2011). Reports in human medicine have shown clinical evidence for the use of ACS in human joint disease administered twice a week for 6 treatments (Baltzer 2009). In horses, it is most commonly used in joints not responsive to corticosteroid administration suggesting it is useful in progressive joint disease (Ferris 2011). It was used in an equine osteochondral OA model and showed improved lameness scores, improved synovial membrane, and decreased articular cartilage fibrillation when administered as 6 ml treatment once a week for 4 weeks (Frisbie 2007). Interestingly, there was continued endogenous production of IRAP in synovial fluid 3 weeks after the first treatment and this remained elevated through 35 days which was the end of the study (Frisbie 2007).

Stem cells are those that are able to self-replicate and differentiate into different tissue types. When considering stem cells, the first is tissue source and it appears bone marrow-derived cells are the best source for joint-related tissue (Frisbie 2010). The dose for a joint has been a range of 10-50 million cells in a 10-15 ml joint (Frisbie 2010). It has different goals regarding treatment joint disease including cartilage resurfacing to treat focal defects, diffuse OA, and treatment of intra-articular soft tissue structures. Stem cells appear to populate the articular cartilage and synovial membrane when injected directly into the joint (Agung 2006). However,
the most beneficial application of stem cells appears to be when the goal is treat damaged intra-articular tissue such as the cruciate ligaments or meniscus. The hallmark publication was the transection of the cranial cruciate ligament in goats to destabilize the stifle and then treat the joint with bone marrow-derived stem cells. The remarkable finding was the regeneration of a meniscus-like tissue in seven of the nine stem cell treated joints (Murphy 2003). A multi-center study evaluating horses with meniscal injury treated arthroscopically followed by an injection of 15-20 million bone marrow-derived stem cells along with HA 4 weeks after surgery did better compared to a report where horses with meniscal injury had surgery alone (Ferris 2014; Walmsley 2003).

1.3.5 Platelet-Rich Plasma

Platelet-rich plasma (PRP) works on the premise that growth factors contained within alpha granules of platelets are released when activated. Therefore, whether through a commercial kit or laboratory protocol to obtain a product with a high concentration of platelets, one could deliver growth factors to the site of injury to improve healing. PRP was first used in people for the healing of mandibular defects in humans (Marx 1998). It has since been used for many applications including wound healing, soft tissue injuries, and most recently intra-articular use. Reviews and meta-analysis of PRP use in humans with OA has promising results (Chang 2014; Khoshbin 2013). They did suggest it is best suited for mild cases of OA and at least three injections were recommended. It was first used in horses with OA in 2007 with beneficial results (Carmona 2007). However, its clinical use far outpaced controlled research.

While there are ways to collect the biologic product known as “PRP” the composition amongst those products can vary greatly with regard to platelet concentration relative to baseline, activation method, and white blood cell count relative to baseline (DeLong 2012). These factors
have direct results on the action of PRP and therefore must be specified to be optimal for its intended use. The ideal composition for the intra-articular use of PRP is a hotly debated topic.

Increased platelet concentrations have a direct correlation to the amount of growth factor in PRP primarily transforming growth factor-beta 1 (TGF-β1) and platelet derived growth factor (PDGF) which have been shown to exhibit anabolic effects on articular cartilage through increased expression of type II collagen and aggrecan (Sundman 2011). However, too high of a platelet concentration (6x baseline) has been reported to have negative effects on bone and has been associated with pain on injection (Gruber 2002). Platelet activation has been shown to occur in vitro through exposure to synovial fluid, however is not as great when an activator is used (Textor 2013). It is important to note that activation of PRP with thrombin was associated with pain on intra-articular injection and calcium chloride activated PRP is painful when injected in human joints (Textor 2013). The white blood cell concentration relative to baseline is perhaps the most controversial topic when discussing PRP composition. There does appear to be more reports of negative effects with a high leukocyte concentration compared to low leukocyte concentrations. For example, high leukocyte concentrations had negative effects on cartilage and meniscal explants (Kisiday 2012). Sundman et al 2011 showed a correlation between high leukocyte concentrations and inflammatory cytokines (Sundman 2011). This information would suggest an endogenously activated leukocyte-poor platelet rich plasma (LP-PRP) would be best suited for intra-articular use. This suggestion is confounded by a report by Bertone et al 2014 using an autologous product in clinical cases of OA in horses (Bertone 2014). The product concentrated leukocytes twelve times baseline and platelets one and half times baseline. One injection of this product improved lameness in two weeks and was sustained. They did suggest it
was best for milder cases of OA. Those points highlight the need for further investigation of the optimal PRP composition for intra-articular use.
CHAPTER 2. EX VIVO EFFECTS OF INSULIN ON THE STRUCTURAL INTEGRITY OF EQUINE DIGITAL LAMELLAE

2.1 Introduction

Equine laminitis associated with hyperinsulinemia due to soluble carbohydrate ingestion and endocrine dysfunction is a current focus of laminitis research. The majority of laminitis cases treated by equine veterinarians have an underlying endocrinopathy associated with insulin dysregulation and hyperinsulinemia (Karikoski 2011). An *in vivo* model of endocrinopathic associated laminitis has been developed using a prolonged euglycaemic hyperinsulinemia technique in horses (Asplin 2007; de Laat 2010). Histological evaluations of lamellar tissue from these horses has revealed stretching of the epidermal lamellae, most notably observed by elongation of the secondary epidermal lamellae, changes to the basal epidermal nuclei, increased numbers of mitotic figures, and dysadherence of the lamellar basal epithelial cell layer from the underlying basement membrane presumably due to hyperinsulinemia (de Laat 2013; de Laat 2011; Karikoski 2014; Nourian 2009). Interestingly, loss of structural integrity at the epidermal-dermal lamellar interface was mild in ponies following hyperinsulinemia perhaps due to their lower body weight suggesting a mechanical factor in the development of laminitis (de Laat 2010; Nourian 2009).

Biomechanical stress within the hoof capsule is believed to be a critical pathophysiological event in the development of lamellar failure due to a higher incidence of laminitis in fore limbs despite similar signaling in the hind limbs (Leise 2012). Increased weight bearing is known to play a crucial role in support limb laminitis; thus, decreased activity is frequently recommended in acute cases of laminitis to decrease further injury to the lamellae (Orsini 2012; van Eps 2010). However, the unique structure of the hoof provides challenges when evaluating biomechanical stress on the equine digit. Stress at the lamellar dermal-
epidermal junction has been studied in normal horses through biomechanical testing of lamellar explants (Douglas 1998; Kochova 2013). Additionally, effects of matrix metalloproteinase activators on the structural integrity of the equine lamellar tissue have been evaluated describing the location of lamellar failure at the basement membrane between the dermal-epidermal junction (Pollitt 1998). However, specific biomechanical testing has not been performed on lamellar explants exposed to an induction agent such as insulin.

2.2 Objectives and Hypothesis

The purpose of this study was to determine if exposure of lamellar explants to insulin would affect the structural integrity; thereby, developing an ex vivo model to study mechanisms leading to hyperinsulinemia-induced lamellar failure. We hypothesized that explants incubated in high concentrations of insulin would be less stiff, failing at lesser stress, and lower loads as well as exhibit greater elongation to failure compared to controls.

2.3 Materials and Methods

The experimental protocol was approved by the Institutional Animal Care and Use Committee. Lamellar explants were harvested from four horses undergoing euthanasia for reasons unrelated to this study. Three Thoroughbreds and one Arabian including two geldings, one stallion, and one mare between 2-12 years old were included. All horses were lean with a body condition score (BCS) <5/9 which is a phenotype not associated with insulin dysregulation; however, this was not ruled out specifically. Horses were determined to be free of medical problems related to inflammatory diseases, endotoxemia, and diseases of the digit through complete physical examination as well as lameness examination including hoof testing, and radiographic evaluation of the digit.
HARVESTING AND INCUBATION OF LAMELLAR EXPLANTS

Immediately following euthanasia with sodium pentobarbital (100 mg/kg IV), both forefeet were removed just distal to the metacarpophalangeal joint. Lamellar explants consisting of hoof wall, epidermal lamellae, dermal lamellae, and distal phalanx were harvested as follows from the dorsal aspect of each fore foot (Figure 2.1). After removing the distal aspect of the limb, the foot was trimmed and the solar surface pared with a hoof knife. The feet were then scrubbed with a disinfectant solution (Decon Bacdown, King of Prussia, PA). Using a table saw cleaned with disinfectant between each use, the proximal portion of the digit was removed at the distal interphalangeal joint followed by additional cuts to remove the abaxial and palmar portions of the foot at the level of the digital cushion. Four to five sagittal cuts were made to obtain 5mm wide sagittal sections of the axial portion of the digit. Excess bone of the distal phalanx was removed using a band saw in a plane parallel to the hoof wall so that approximately 5 mm of distal phalanx remained attached to lamellar tissue. This was followed by four to five 5 mm transverse cuts made perpendicular to the hoof wall using a band saw sterilized with disinfectant to obtain 5 mm thick x 5 mm wide lamellar explants.

After harvest, lamellar explants were incubated in Dulbecco’s Modified Eagle’s medium (DMEM, Sigma; St. Louis, MO) for 10 hours at 37 °C. DMEM containing 4.5 g/L glucose, L-glutamine, and sodium pyruvate, with piperacillin (64 µg/ml), tazobactam (8 µg/ml), amikacin (5 µg/ml) and nystatin (100 U/ml) was used to equilibrate the explants to culture conditions prior to the start of the experiment. After equilibration, explants from those horses were randomly assigned to a control group (50 explants) or inulin group (39 explants). Control lamellar explants were incubated in medium alone. Explants in the insulin treatment group were incubated in medium supplemented with 2.5 µg/ml insulin (Humulin-R, Eli-Lilly). Both groups of explants
from each horse were incubated concurrently in humidified air containing 5% CO$_2$ and maintained at 37 °C for 8 hours.

BIOMECHANICAL TESTING

Biomechanical testing of the explants was performed immediately following 8 hours of incubation with insulin (or with medium only for the control explants). Structural integrity of lamellar explants was evaluated using a mechanical testing system (Instron Model 5960, Norwood, MA). A clamp fixture (Model G-227, Test Resources, Shakopee, MN) was applied to both the hoof wall and distal phalanx of each explant in the testing device (Figure 2.2). Explants were placed in the clamp device to apply tension parallel to the long axis of the distal phalanx for consistency in testing. Care was taken to ensure the clamps were applied as close to the lamellar attachments on the distal phalanx and hoof wall as possible. Mechanical load was applied with a 2 kN load cell using a constant elongation rate of 25 mm/min until explant failure. Load to failure was recorded for each lamellar explant. Only explants failing at the dermal-epidermal junction, defined as an “un-zipping” of the explant exposing epidermal leaflets, were used for analysis (Figure 2.3). Prior to testing, the width and thickness of each explant was measured using a digital caliper to determine stress to failure (MPa). Also, once fixed in the mechanical testing device the distance between each clamp was measured using the same electronic caliper to calculate elongation to failure (mm). Elongation to failure was divided by original length to find strain which was then used to obtain Young’s modulus (MPa) by dividing stress and strain. After testing, selected explants were fixed in 10% buffered formalin for histological evaluation. Histological sections (5 µm) were stained with H&E and PAS and the site of failure was evaluated.
**Figure 2.1** Lamellar explant measuring 5 mm x 5 mm with hoof wall (A), epidermal tissue (B), dermal tissue (C), and distal phalanx (D).
Figure 2.2 Photograph of a lamellar explant mounted in the mechanical testing device. One clamp is fixed on the hoof wall (A) and the other clamp is fixed to the distal phalanx (B). After the explant was mounted in the mechanical testing device, the distance between both clamps was measured to obtain elongation to failure (C).
Figure 2.3 Photograph of a lamellar explant following mechanical testing to failure showing exposed epidermal leaflets (A) separated from the dermal tissue (B) demonstrating failure at the dermo-epidermal junction.
STATISTICAL ANALYSIS

Recorded data included load to failure (N), stress to failure (MPa), elongation to failure (mm), and Young’s modulus (MPa) were evaluated separately using the Shaprio-Wilk test, skewness, kurtosis, and q-q plots. Normal data was reported by the mean, standard deviation (SD), and minimum/maximum (min-max). For non-normally distributed values, the median, 10-90% quartiles, and min-max were calculated. Any non-normal data was log transformed for parametric testing. A mixed linear model was used to compare the control and insulin-treated groups. Horses served as the random factor and group as a fixed factor. A p-value less than 0.05 was considered statistically significant. Statistical analysis was performed using SPSS 24.0 (IBM Inc., Armonk, NY, USA).

2.4 Results

A total of 89 explants from 4 horses were tested following 8 hours of incubation in medium alone (50 explants) or medium supplemented with insulin (39 explants). Explant failure at the dermal-epidermal junction, demonstrated by “unzipping” of the epidermis from the dermis occurred more frequently in explants incubated in insulin 35/39 (90%) compared with controls 36/50 (72%). Furthermore, explants incubated with 2.5 μg/ml of insulin failed at a significantly (F-test=14.356, p=0.0001) lesser load (49.8 ± 12.3 N) compared with controls (62.39 ± 14.74 N). Stress to failure was significantly lower (F-test=11.87, p=0.001) with explants incubated with insulin (1.92 ± 0.51 MPa) compared with controls (2.38 ± 0.61 MPa). Elongation at failure was significantly greater (F-test=6.06, p=0.016) in explants incubated in insulin (median: 3.31 mm [2.59-3.97 mm]) compared with controls (median: 3.06 mm [2.46-3.65 mm]). Explants incubated in insulin had a lower Young’s modulus (6.01 ± 2.51 MPa) compared to controls (7.06 ± 2.46 MPa), but this was not significantly different (F-test=1.253, p=0.317) (Table 2.1). Histological
evaluation of selected explants incubated with insulin demonstrated structural failure via separation of the epidermal-dermal attachments at the basement membrane (Figure 2.4), and examined control explants showed separation at the middle of the laminar junction halfway between the epidermal and dermal lamellae.

**Table 2.1** Results of biomechanical testing of lamellar explants (mean ± SD)

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<th>Control Group</th>
<th>Insulin Group</th>
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<tr>
<td>Load to Failure (N)</td>
<td>62.39 ± 17.74</td>
<td>49.8 ± 12.3</td>
<td>0.0001</td>
</tr>
<tr>
<td>Stress to Failure (MPa)</td>
<td>2.38 ± 0.61</td>
<td>1.92 ± 0.51</td>
<td>0.001</td>
</tr>
<tr>
<td>Elongation to Failure (mm)</td>
<td>3.04 ± 0.07</td>
<td>3.33 ± 0.09</td>
<td>0.016</td>
</tr>
<tr>
<td>Young’s Modulus (MPa)</td>
<td>7.06 ± 2.46</td>
<td>6.01 ± 2.51</td>
<td>0.317</td>
</tr>
</tbody>
</table>

**Figure 2.4** Histologic section (5 mm) of a lamellar explant following biomechanical testing to failure that was incubated with media supplemented with insulin (PAS stain, 40x magnification). The blue arrow indicates separation of the dermo-epidermal junction at the basement membrane. Black bar is 100 mm.
2.5 Discussion

The addition of insulin to equine lamellar explants weakened the structural integrity of lamellar attachments. Explants incubated for 8 hours in 2.5 µg/ml of insulin failed with lesser stress and lower load when compared to control explants. Greater elongation to failure demonstrated by increased distance between the epidermal and dermal segments when failure occurred, was also present in the insulin-treated group compared with controls. The greater elongation to failure, suggests that the lamellar explants incubated in insulin exhibit more stretch than controls prior to failure. Furthermore, the failure rate was higher in the insulin group compared to the control group (90% and 72%, respectively) showing that the addition of insulin weakens the structural integrity of lamellar explants.

While the specific sequence of events leading to endocrinopathic laminitis have yet to be elucidated, hyperinsulinaemia has been associated to lamellar dysfunction in both experimental models and clinical cases (Asplin 2007; de Laat 2010; Karikoski 2015; Walsh 2009). Cellular changes within the epidermal lamellae are purported to occur through binding of insulin to insulin-like growth factor-1 receptor (IGF-1Rc) present on the epidermal cells (Kullmann 2016). A recent study by Lane and co-workers described activation of the IGF-1Rc signalling through the upregulation of RPS6 protein, an end-product of that pathway, by the use of immunofluorescence staining for RPS6 on lamellar tissue from horses undergoing the euglycaemic hyperinsulinaemic clamp technique (Lane 2017). Activation of the IGF-1 signaling pathway results in downstream events such as PI3K/Akt, ERK1/2, and mTORC1 activation, all of which have been shown to cause dysregulation of epithelial tissue, disruption of cytoskeletal organization, increased mitotic rate, and cause dissolution of hemidesmosomes (Laval 2014;
Magnuson 2012; Mezi 2012; Shahbazian 2006; Yu 2012). Therefore, IGF-1Rc signaling has been proposed as a potential factor of lamellar dysfunction due to hyperinsulinemia (Lane 2017).

The exact mechanisms leading to decreased structural integrity of lamellar explants in the \textit{ex-vivo} model described in this study are not known and warrant further investigation. While post-mortem autolysis could not be completely ruled out, a similar study showed that there was no significant decrease in structural integrity of lamellar explants incubated at 24 hours compared to 48 hours (Reisinger 2016). The use of an \textit{ex-vivo} model with a control group consisting of medium-alone and a treatment group with the addition of only insulin removed multiple other factors that may be in circulation \textit{in-vivo}. This demonstrated that insulin alone weakened the structural integrity of lamellar explants. Therefore, similar activation of the IGF-1Rc signaling pathway may have occurred in our study and potentially weakened the structural integrity of lamellar explants. However, this would have to be confirmed by immunofluorescence staining for RPS6 of explants and could be performed in future studies using this model to further investigate mechanisms leading to hyperinsulinaemia-induced lamellar failure.

Unpublished data by an author of this manuscript showed increased expression of RPS6 following the incubation of epithelial tissue with insulin at a concentration of 2.5 $\mu$g/ml. Those results were used to begin preliminary biomechanical testing in our laboratory and observed the same 2.5 $\mu$g/ml concentration had the lowest variance. The insulin concentration in media was roughly 70x greater than serum insulin concentrations of horses and ponies in the \textit{in-vivo} model of endocrinopathic laminitis (1000 $\mu$IU/ml) (Asplin 2007; de Laat 2010). The presence of lamellar damage in horses with much lower serum insulin concentrations (200 $\mu$IU/ml) in the same 48-hour time as the period as the \textit{in-vivo} model suggests lamellar damage is based on both
the magnitude of hyperinsulinaemia as well as the time-frame which it occurs (de Laat 2012). Those findings may explain why such high insulin concentration were able to weaken the structural integrity of the lamellae in a shorter time-frame compared to the in-vivo model. The interstitial concentrations of insulin are 10-50% of serum levels when evaluated in human muscle and adipose tissue due to a saturation effect dependent on a capillary delivery system that is not rapid enough to balance consumption in the measured tissue (Bodenlenz 2005; Sjostrand 1999). Metabolism of glucose in lamellar tissue is unique and insulin has been shown to constrict digital vessels which may confound delivery of insulin to lamellar tissue (de Laat 2015; Venugopal 2011). While the ex-vivo nature of this study relied on diffusion rather than perfusion of insulin and penetration of insulin was not confirmed, it was presumed to occur by the weakened structural integrity of lamellar explants incubated in medium with insulin compared to medium alone. Future studies could also evaluate different concentrations of insulin to determine if insulin exhibits a dose-dependent effect on the structural integrity of lamellar explants.

Studies using normal horses have been performed to quantify the biomechanical behaviour of lamellar explants (Douglas 1998; Kochova 2013). It has been concluded that location-dependent variation exists in the density of secondary lamellae leading to a greater surface area for basement membrane attachment resulting in greater structural integrity of the explant (Kochova 2013). The experimental design used in this study attempted to circumvent that potential variation in explant structural integrity by randomly assigning explants to a control and treatment group independent of location within the digit. Also, the forces applied through the equine digital lamellae during normal locomotion is multifactorial depending on hoof shape, surface and speed of the gait (Douglas 1998). However, in this study the tensile force was applied perpendicular to the distal phalanx for consistency with biomechanical testing. Previous
studies examining lamellar tissue free of disease chose an elongation rate (500 mm/min) comparable to what a horse experiences at a gallop (Douglas 1998; Kochova 2013). However, the goal this study was to develop an *ex vivo* model to study hyperinsulinaemia-induced lamellar failure. Horses with clinical laminitis rarely gallop due to lameness, therefore a much slower elongation rate (25 mm/min) to failure was used.

The *ex vivo* design of this study is not without limitations. Diffusion of nutrients, oxygen, and insulin can be variable throughout the explant and could be dependent on the overall size of the explant. This may have been controlled for by using a microcirculation device for each explant. However, to minimize this effect explants were precision cut to minimize variation, and each explant was measured prior to mechanical testing when determining stress to failure to account for minor discrepancies in explant size. Although horses used in this study were not tested for endocrinopathies prior to inclusion, they did not have a body condition score or phenotype suggestive of endocrine dysfunction.

Overall, it is well accepted that hyperinsulinaemia is a risk factor for the development of laminitis and appears to the unifying factor for lamellar dysfunction in both clinical cases as well as the euglycaemic hyperinsulinaemic clamp technique for reasons that are not fully understood (Asplin 2007; de Laat 2010; Karikoski 2011; McGowan 2004; Menzies-Gow 2017). The results of this study demonstrate that an *ex vivo* model using explants incubated in insulin can be used to investigate potential mechanisms of hyperinsulinaemia-induced lamellar failure such as signal transduction events. Evaluation of the phosphorylation events occurring during IGF-1 receptor signaling can be assessed using this experimental model by the use of immunofluorescence staining for RPS6, and blocking various components of the IGF-1 signaling pathway would allow for determination of their significance. For example, rapamycin (an mTORC inhibitor) or
wortmannin (a PI3K inhibitor) may be added after the induction agent insulin to determine if the structural integrity of lamellar explants can be maintained by inhibiting growth factor signaling (Huang 2014; Molinolo 2012). Positive results from that type of study could warrant further evaluation in clinical models to identify therapeutic options for endocrinopathic laminitis.
CHAPTER 3. INTRA-ARTICULAR ADMINISTRATION OF PLATELET-RICH PLASMA IN HORSES USING A SYNOVITIS MODEL

3.1 Introduction

Platelet-rich plasma (PRP) has been used with success in human patients with osteoarthritis based on the premise that growth factors contained within alpha granules of platelets are released when platelets are activated to improve healing (Chang 2014). The main growth factors contained within alpha granules are TGF-1β, VEGF, and PDGF which improve the synovial environment through anabolic effects increasing type II collagen and aggrecan production which are key components of articular cartilage (Edwards 1987).

While there are several commercial kits and laboratory protocols to obtain “platelet-rich plasma”, it is important to consider there are vast differences in composition with regard to platelet and leukocyte concentration relative to baseline values as well as the method of platelet activation (Dohan Ehrenfest 2009). Those parameters have direct correlation to the anabolic as well as anti-inflammatory and catabolic properties of PRP suggesting the composition is dependent on the intended use (Sundman 2011). The ideal composition for intra-articular use is a hotly debated topic. However, there is more evidence to support the use of a leukocyte-poor PRP (LP-PRP) since it is suggested leukocyte-rich PRP is associated with negative effects to the synovial environment when evaluated using in vitro models while (Kisiday 2012; Sundman 2011). While the addition of platelet activators may increase the activity and release of growth factors there use has been associated with discomfort when administered into the joint of horses (Textor 2013). Therefore, synovial fluid may be used as an endogenous activator of platelets limiting the unwanted inflammatory flare in horses treated intra-synovially with PRP.

Osteoarthritis in athletic horses is most often the result of acute or cyclic trauma causing a synovitis resulting in pain and production of inflammatory cytokines and enzymes that cause
articular cartilage degradation leading to the development of osteoarthritis (McIlwraith 2011). Clinical use of biologic therapies in horses outpaced controlled research as a pilot study showed promising results in horses with osteoarthritis (Carmona 2007). Information regarding the use of intra-articular PRP in horses with osteoarthritis is limited. Additionally, the PRP composition used in these reports is sometimes poorly defined making it difficult to compare results from various studies. Compared to widespread clinical use in all facets of equine joint disease, there is a paucity of peer-reviewed literature reporting the intra-articular use of PRP in diseased equine joints either experimentally-induced or naturally-occurring.

3.2 Objectives and Hypothesis

To our knowledge there is no peer-reviewed literature evaluating the intra-articular use of PRP in equine joints in a model of aseptic synovitis and is the purpose of this study. We hypothesize that the intra-articular use of leukocyte-poor platelet rich plasma (LP-PRP) in an experimental synovitis model will 1) improve lameness scores, 2) improve the synovial environment, and 3) stabilize cartilage metabolism by serial lameness and synovial fluid evaluations.

3.3 Materials and Methods

INCLUSION CRITERIA

The experimental protocol was approved by the Institutional Animal Care and Use Committee. This study used six horses ages 6-12 (mean of 8 years old) years old with no appreciable forelimb lameness and no to minimal osteoarthritis on radiographs of the metacarpophalangeal joints. There were five Thoroughbreds and one Quarter Horse and all of them were geldings. Physical exams consisting of temperature, heart rate, and respiratory rate were performed twice daily during data collection.
STUDY DESIGN

A crossover design was employed so that each metacarpophalangeal joint acted as either the control (saline only, no LP-PRP) or treatment (LP-PRP) within each horse. Synovitis was randomly induced in either the right or left metacarpophalangeal joint by the intra-articular administration of a 0.5 ng dose of lipopolysaccharide (LPS) and called post-injection hour (PIH) 0. Eight hours post injection of LPS (PIH 8), horses randomly received intra-articular treatment (into the same joint that was administered LPS) of either 4 ml saline (to serve as control) or 4 ml of LP-PRP. Synovial fluid was collected using a collateral sesamoidian approach to metacarpophalangeal joint at the following times: PIH 0, 8, 24, 48, 72, and 96 for total cell count and total protein as well as later analysis of selected biomarkers. Subjective lameness evaluations were also performed at PIH 0, 8, 24, 48, 72, and 96 using an 8-point scale (0= sound, 2=mild, 4=moderate, 6=severe, 8= nonweightbearing) (Dyson 2011). Following a two-week washout period, synovitis was induced in the opposite metacarpophalangeal joint and the other treatment (either saline or LP-PRP) was administered.

INDUCTION OF SYNOVITIS

Lipopolysaccharide from Escherichia coli O55:B5 (Sigma-Aldrich, St. Louis, Missouri, USA) was thawed from stock, diluted to a concentration of 0.5ng/ml using sterilized phosphate buffered saline (PBS, pH 7.2) and stored in glass vials to minimize degradation and adherence of LPS to the vial. Synovitis was induced at PIH 0 by the intra-articular injection of 0.5 ng of LPS with a 20-guage needle using the lateral collateral sesamoidian ligament approach to the metacarpophalangeal joint.
PLATELET-RICH PLASMA PREPARATION

This study used a leukocyte-poor platelet rich plasma obtained via a commercially available kit (Arthex ACP). At least 15 ml of blood was aseptically obtained using the double syringe provided within the kit and centrifuged at 1,500 g for 5 minutes. At least 6 ml of LP-PRP was produced and 4 ml was injected in the metacarpophalangeal joint using aseptic technique. The remaining 2 ml of LP-PRP was aliquoted and stored at -80°C for evaluation of PGE₂, C2C, TGFβ-1, and IL-1Ra.

SYNOVIOCENTESIS

A volume of at least 2 ml of synovial fluid was collected using aseptic technique with a 20-gauge, 1-inch needle and immediately transferred into plain tubes (Vacutainer, Tyco Healthcare, Mansfield, Mass). A collateral sesamoidian approach was used and alternated between medial and lateral aspects of the joint for each time point. A portion of the fluid was transferred to EDTA tubes for white blood cell count and total protein measurement (Vacutainer, Tyco Healthcare, Mansfield, Mass.). The remainder was centrifuged at 10,000g for 15 min, aliquoted, and stored at -80°C until further analysis. Prostaglandin E₂ (PGE₂) was measured as a marker for inflammation and synovitis. Articular cartilage breakdown was measured using collagenase-cleavage neoepitope (C2C). Interleukin-1 receptor antagonist protein (IL-1ra) was used to measure anti-inflammatory effects of treatment.

STATISTICAL ANALYSIS

Results were expressed as the mean ± standard deviation (SD). Significant differences of normally distributed data were assessed using a two-way ANOVA with repeated measures. A Bonferroni correction was applied. A p-value less than 0.05 was considered statistically significant. Statistical analysis was performed using SPSS 24.0 (IBM Inc., Armonk, NY, USA).
3.4 Results

Intra-articular injection of LP-PRP had no adverse reactions. Collection of at least 2 ml synovial fluid was successful in 71/72 (98%) attempts. Injection of 0.5 ng of LPS resulted in a transient synovitis and lameness. Lameness was significantly increased at PIH 8, 24, and 48 (P<0.05). Horses treated with LP-PRP were less lame compared to controls at PIH 24 (0.67 ± 52 and 2.83± .41, respectively; p=0.03) and PIH 48 (.33 ± .52 and 1.5 ± .54, respectively; p=0.04) (Figure 3.1). There was a significant increase in total protein (Figure 3.2) and total cell count (Figure 3.3) at PIH 8 and 24 but no significant difference between groups (P<0.05). PGE₂ had a significant increase at PIH 8, but no significant differences between groups (Figure 3.4) (P<0.05). IL-1ra was significantly increased at PIH 8 and PIH 24, but there were no significant differences between groups (Figure 3.5) (P<0.05). C2C was significantly elevated at PIH 24, but there were no significant differences between groups (Figure 3.6) (P<0.05). Platelets in LP-PRP were increased 1.5x compared to baseline (233,000 ± 30,113.12/µl and 151, 166 ± 29,498.6/µl, respectively) and had a 6-fold reduction in leukocytes (1,300 ± 931.7/µl and 8,250 ± 882.6/µl, respectively) (Table 3.1). Evaluation of biomarkers in LP-PRP revealed the following mean concentrations: \( \text{PGE}_2 = 1223.5 \pm 1440.3 \text{ pg/ml} \), \( \text{IL-1Ra} = 0.4 \pm .22 \text{ pg/µl} \), \( \text{C2C} = 233.22 \pm 32.85 \text{ ng/ml} \), and transforming growth factor 1-beta was found in LP-PRP = 16.3 ± 5.1 pg/ml. TGF-1β was not found in synovial fluid at PIH 8 or PIH 24 and 48 following administration of LP-PRP.
Figure 3.1 Bar graph representing mean subjective lameness scores for PRP and saline treatments. Synovitis was induced at PIH 0 and saline or LP-PRP administered PIH 24.
Figure 3.2 Bar graph representing mean of total protein in synovial fluid for PRP and saline treatments. Synovitis was induced at PIH 0 and saline or LP-PRP administered PIH 24.
Figure 3.3 Bar graph representing mean total cell count in synovial fluid for PRP and saline treatments. Synovitis was induced at PIH 0 and saline or LP-PRP administered PIH 24.
Figure 3.4 Bar graph representing mean of PGE$_2$ in synovial fluid of PRP and saline treatments. Synovitis was induced at PIH 0 and saline or LP-PRP administered PIH 24.
Figure 3.5 Bar graph representing mean of IL-1ra in synovial fluid for PRP and saline treatments. Synovitis was induced at PIH 0 and saline or LP-PRP administered PIH 24.
Figure 3.6 Bar graph representing mean C2C in synovial fluid of PRP and saline treatments. Synovitis was induced at PIH 0 and saline or LP-PRP administered PIH 24.

Table 3.1 Comparing the composition of LP-PRP to that of baseline blood values using the mean ± SD of platelets/µl and mean ± of white blood cells (WBC)/µl.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>LP-PRP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Platelets (mean + SD)</strong></td>
<td>151,166 ± 29.498.6/µl</td>
<td>233,000 ± 30,113.12/µl</td>
</tr>
<tr>
<td><strong>WBC (mean + SD)</strong></td>
<td>8,250 ± 882.6/µl</td>
<td>1,300 ± 931.7/µl</td>
</tr>
</tbody>
</table>
3.5 Discussion

The use of platelet-rich plasma for the treatment of equine inflammatory and degenerative joint diseases has become increasingly common due to largely anecdotal evidence of decreased pain and return to athletic function. The intra-articular administration LP-PRP was well tolerated by horses in this acute synovitis model as no increase in effusion, heat or worsening of lameness was noted. Leukocyte-poor platelet rich plasma administration did reduce lameness scores compared to saline controls. Following LPS-induced synovitis there were immediate yet transient increases in synovial fluid concentrations of the inflammatory biomarker PGE$_2$ and C2C which is a biomarker for cartilage breakdown; however, concentrations of these biomarkers were not affected by administration of LP-PRP. There was at the same time a greater than 40-fold increase in interleukin-1 receptor antagonist protein beginning prior to administration of the treatment or placebo at PIH 8, remained elevated at PIH 24, and then returned to baseline levels. With the dramatic IL-1ra response, it appears the synovial environment is protected by the animal’s own physiologic response rather than the introduction of LP-PRP. The lack of effect of LP-PRP administration with this model was interesting and perhaps a different model of synovitis such as the interleukin-1β model would yield different results. Limitations of the study include a sex bias since this study had all geldings. Also, there was no objective lameness assessment and biomarkers of the serum were not assessed.

The small numbers in treatment groups may have led to a lack of difference noted. Alternatively, the LPS synovitis model may not be dramatic enough in effect to detect differences. It was also noted that the changes in leukocyte-poor PRP had no deleterious effects on synovial environment and may be an effective therapy in acute synovitis when corticosteroids are unable to be administered. Serial administration or a leukocyte-rich PRP may have afforded
different results. Injection of leukocyte-poor platelet-rich plasma did not appear to significantly alter the synovial environment with regard to those synovial fluid components tested.
4.1 Summary

In summary, we have concluded that exposure of lamellar explants to high concentrations of insulin weakened their structural integrity. This has implications in the evaluation of hyperinsulinemia-induced lamellar failure as this model could be used to investigate mechanisms of that process. It is presumed that insulin-like growth factor-1 signaling is up-regulated due to high concentrations of insulin as noted by increased immunofluorescence staining for ribosomal protein-S6 (an end-product of that pathway) in horses undergoing the euglycemic-hyperinsulinemic clamp technique. The model will also allow for evaluation of potential therapies, such as rapamycin and wortmanin, that would block phosphorylation events of that pathway. Potentially, when rapamycin or wortmanin is added to media along with insulin, the addition of those compounds could potentially maintain the structural integrity of the lamellar explants which would warrant evaluation with *in vivo* models.

A single injection of leukocyte-poor platelet rich plasma in an LPS model of synovitis did not improve the synovial environment, but did improve subjective lameness scores. It is possible that a different model of synovitis such as injection of recombinant equine interleukin-1 beta, a different composition platelet rich plasma, or different injection protocol as used in human medicine would have improved results. It is important to note reports suggest it is best suited for early or mild cases of osteoarthritis and clinical cost-benefit analysis must be discussed as less expensive albeit proven treatments exist. Further work to determine the ideal composition for intra-articular use in horses is warranted.


VITA

Cole Barrett Sadow was born in Baton Rouge, LA in 1989. A passion for horses was cultivated in high school and the desire to be an equine veterinarian was realized. He attended Louisiana State University for two years beginning in 2007 and graduated from the School of Veterinary Medicine in 2013. This was followed by a year-long surgery internship at Hagyard Equine Medical Center in Lexington, KY, followed by a 6-month racetrack and sales internship with EQUI-VET, LLC based in New Orleans, LA, and then a 6-month internal medicine fellowship at Hagyard Equine Medical Institute in Lexington, KY. A 3-year equine surgery residency began in July of 2015 at Louisiana State University School of Veterinary Medicine. Following the completion of the residency, he will return to Hagyard Equine Medical Institute in Lexington, KY to work as an equine surgeon in the Davidson Surgery Center.