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Role of endothelin in the pathogenesis of acute laminitis in horses

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ROLE OF ENDOTHELIN IN THE PATHOGENESIS OF ACUTE LAMINITIS IN HORSES

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To all Veterinarians.

May we gain knowledge regarding this devastating disease

to better guard the health and well-being of the graceful, honest, and most beautiful of our companions – the Horse.

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ABSTRACT

Acute laminitis is a severely debilitating disease of the laminae of the equine digit; however, the mechanism(s) of pathogenesis have yet to be fully elucidated. In physiologic states, the endothelium synthesizes substances, such as nitric oxide (NO; vasodilator) and endothelin-1 (ET-1; profound vasoconstrictor), which play a crucial role in vasomotor regulation. The overall hypothesis is that the initiating factor in the onset of acute laminitis is a disruption in the balance between NO and ET-1, which leads to digital vasoconstriction and subsequent laminar ischemic necrosis.

In vitro studies with digital vessels from healthy horses and horses with naturally-acquired laminitis determined that ET-1 caused concentration-dependent, sustained contraction of arteries and more profound contraction of veins, and incubation with the nonselective ET receptor antagonist (PD145065) at a 10^{-5} M concentration abolished these contractile effects. ET-1 was then administered into the digit of healthy conscious horses, which resulted in reduced blood flow and the ET antagonist, especially in combination with a NO donor, reversed these reductions.

Naturally-acquired laminitic horses had a trend for increased jugular and cephalic venous plasma ET-like immunoreactivity, and horses during the development of black walnut extract (BWE)-induced laminitis developed increased digital venous plasma ET-like immunoreactivity. After validation for equine tissues, ET-1 immunohistochemical staining was conducted on digital vascular and laminar tissues, but no notable differences were found between healthy and naturally-acquired or experimentally-induced laminitic horses.

During the developmental stages of BWE-induced laminitis, digital blood flow initially decreased followed by hyperemia, corresponding with demonstration of clinical signs of

laminitis. Administration of the ET antagonist, and the antagonist combined with a NO donor, improved Starling force alterations by improving digital vascular resistances and blood flow. Utilizing digital vessel rings from BWE-treated horses, ET-1 caused a concentration-dependent contraction in vitro that was abolished by the ET antagonist. Endothelium-dependent vasodilation was decreased in these vessels, demonstrating possible altered endothelial function due to BWE administration.

Based on the results of these studies, ET-1 appears to play a role in the pathophysiology of acute laminitis in horses and continued investigations evaluating ET antagonists as preventative and therapeutic agents for this devastating disease are warranted.

CHAPTER 1. REVIEW OF LAMINITIS AND ENDOTHELIN-1 LITERATURE

1.1 Laminitis Introduction

Laminitis is a disease that has devastated horses, and their owners and caretakers, for centuries. The earliest record of laminitis dates back to around 350 BC when it was referred to as "Barley Disease" by Aristotle.[1] During the fourth century, the Greek Apsyrtus wrote a book Hippiatrika (Equine Medicine) by order of Emperor Constantine and described treatment of barley disease incorporating mild bleeding, light exercise, and diet restriction. Since that time, various names and etiologies have been linked to laminitis and treatments from "bleeding of the bad humors" to the development of special ointments applied to the digit have been employed. The term "founder" was derived during the 1500s from the word *morfounde* that was used by sailors to describe a ship that had been driven under water by a succession of waves, much like the sinking of the distal phalanx within the hoof capsule.[2] The term "laminitis" evolved as the precise location of the disease within the foot was better understood during the early 1700s. [3]

Acute laminitis is a severely debilitating, excruciatingly painful, and potentially career-ending and life-threatening disease of the sensitive and insensitive laminae of the equine digit. Laminitis is important to all horse owners/ trainers and horse enthusiasts because it can occur in adult horses and ponies of any breed or use. Laminitis usually occurs secondary to other diseases such as acute gastrointestinal tract disease, particularly strangulating obstruction and inflammatory bowel disease; grain overload; retained fetal membranes and subsequent metritis; pleuropneumonia; and other diseases accompanied by endotoxemia.[4] Additionally, support limb laminitis occurs commonly in the contra lateral limb owing to overload or excessive weight bearing in horses that have a severe non-weight bearing lameness.[5]

Laminitis causes profound emotional stress and economic loss to horse owners and trainers because of the pain experienced by these horses; this disease often leads to poor body

condition and prolonged periods of recumbency with secondary pressure sores. Approximately 75% of laminitic horses treated at a university hospital did not return to athletic soundness; the majority of these horses were ultimately euthanized owing to severe pain associated with separation of the sensitive and insensitive laminae, resulting in rotation and/or distal displacement of the third phalanx.[6] It is estimated that 15% of horses in the United States are afflicted with laminitis over the course of their lifetime, and 75% of these horses develop severe or chronic lameness and debilitation that necessitates euthanasia. This represents a substantial number of horses in the US and worldwide that suffer from this devastating disease that are ultimately destroyed. From an economic perspective, the diagnosis and treatment of laminitis is estimated to cost approximately $8 million annually and the monetary loss of animals euthanized each year subsequent to complications of laminitis also ranks in the millions of dollars annually.

1.2 Anatomy of the Foot

The ability of the equine athlete to walk depends on the integrity of the interdigitating primary and secondary laminae, which structurally unite the hoof wall, distal phalanx, and the sole of the foot into a single unit.[7] The bulk of the hoof is comprised of the stratum medium composed of avascular highly keratinized stratified squamous epithelium.[8] This layer blends with the stratum internum that is comprised of the primary and secondary epidermal laminae. There are approximately 600 primary laminae that form longitudinal grooves for interdigitation with the vascular laminae of the laminar dermis (corium). The laminar corium unite with the subcutis and periosteum of the distal phalanx.[8]

The bones of the digit are the proximal, middle, and distal phalanx and the distal sesamoid bone (navicular bone) (Fig 1.1). The primary joint of the digit is the distal interphalangeal joint comprised of the middle and distal phalanx and the distal sesamoid bone.

The short collateral ligaments join the distal end of the middle phalanx and the proximal edges of the distal phalanx. The collateral sesamoidean ligaments extend from the distal aspect of the proximal phalanx and insert on the edges of the distal sesamoid bone. A branch of this ligament also inserts on the palmar process of the distal phalanx. The distal sesamoid impar ligament arises from the distal aspect of the distal sesamoid bone and extends to the palmar surface of the distal phalanx. A T-ligament is formed by a fibrous connection between the palmar surface of the middle phalanx and the deep digital flexor tendon. The deep digital flexor tendon inserts on the palmar aspect of the distal phalanx and the common digital extensor tendon inserts on the extensor process of the distal phalanx.[8,9]

The distal interphalangeal joint capsule joins with the common digital extensor tendon, the collateral ligaments of the distal interphalangeal joint, the distal sesamoid impar ligament, and the T-ligament. There are two main pouches of the joint capsule, the dorsal pouch and the palmar pouch and the palmar pouch is further divided into proximal and distal pouches. [8]

There are two cartilaginous structures located palmar to the collateral ligaments composed of hyaline cartilage. As the animal ages, these cartilages progress to become predominately fibrocartilage. The cartilages provide structure and support to the heel of the foot. The digital cushion is a large soft tissue structure located between the base of the cartilages. The digital cushion is composed of fibroelastic tissue, adipose tissue, and a small percentage of fibrocartilage. A venous plexus is also located within the digital cushion.[8] As the horse has fluctuations in weight bearing of the digit, as in walking or exercise, the digital cushion is compressed leading to forcing of blood from the venous plexus up the digit for return to the heart. The ability of the digital cushion to compress is thought to act as a shock absorber for the foot. It is also possible that this pumping action aids in venous return from the digit.

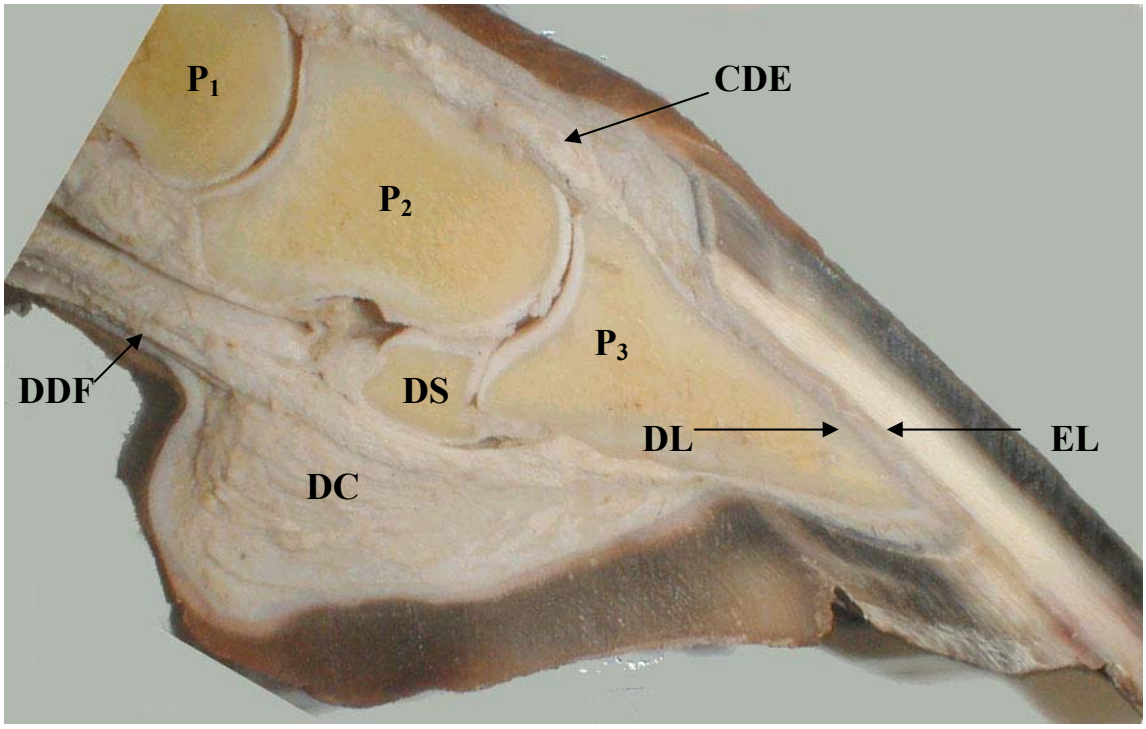

Figure 1.1 – Cross section of the equine digit. P_1 = proximal phalanx, P_2 = middle phalanx, P_3 = distal phalanx, DS = distal sesamoid bone, DC = digital cushion, DDF = deep digital flexor tendon, CDE = common digital extensor tendon, DL = dermal laminae, and EL = epidermal laminae.

The arterial supply to the forefoot is mainly derived from the medial and lateral palmar digital arteries. The first branch at the level of the proximal interphalangeal joint is the bulbar artery, which supplies the digital cushion and gives off the axial branch that supplies the laminae of the heel and bar.[8] The coronal artery is the next branch of the palmar digital artery and supplies the perioplic and coronary coria. The next branch is the dorsal artery of the middle phalanx, which forms the coronary arterial circle with the contralateral vessel. The coronal artery and coronary arterial circle eventually supply the skin, extensor tendon insertion, distal interphalangeal joint, and coronary corium. A collateral arch at the level of the middle phalanx is formed by the next branch of the palmar digital artery, the palmar artery of the middle phalanx. This arch supplies the distal sesamoid bone, distal interphalangeal joint, digital cushion, and the cuneate corium. At the level of the distal phalanx, the palmar digital artery gives off the dorsal artery of the distal phalanx, which supplies the digital cushion and gives off branches to the circumflex artery and the terminal arch. These structures supply the distal sesamoid bone, laminar corium, and the solar and cuneate coria. Blood flow within the laminae is from distal to proximal.[8] Arteriovenous anastomoses occur in the dermis of the coronary band, in neurovascular structures within the dermal laminae, and at the entrance to and along the length of the dermal laminae.[10,11] One study reported density of arteriovenous shunts within the laminae at 500 shunts/cm^2.[11] There are several hypotheses for the function(s) of these shunts. One hypothesis is that during long periods of cold exposure (i.e. standing in snow), the shunts open to allow for warming of the feet by increasing blood flow. Another hypothesis is that high pressure fluctuations (i.e. pressure increases in the digit due to galloping or jumping) cause opening of the shunts to diffuse the tremendous increases in pressure and may act as a "safety valve" for the vasculature.[11]

The deep structures of the foot and the distal sesamoid bone are drained first by the axial and abaxial parallel veins within the solar canal of the distal phalanx.[8] The parallel veins join to form the terminal vein and is joined by the inner venous plexus branches to form the palmar digital vein. Located at the middle palmar surface of the middle phalanx, an anastomosis between the palmar digital veins drains the distal sesamoid bone and the digital cushion. A majority of the veins of the foot are valveless and the direction of their flow is dependent upon weight bearing forces.[8] Most likely due to the anatomy of the distal limb and the forces required for venous return to the heart, the digital veins are quite muscular compared with veins from other tissues and from other species.[12]

The nerve supply to the foot is principally derived from the medial and lateral palmar digital nerves. A dorsal branch, and in approximately 30% of horses an intermediate branch, supply sensory and vasomotor innervation to the dorsal aspect of the distal interphalangeal joint and the perioplic and laminar coria.[8] The palmar digital nerve continues distal to supply the laminar and solar coria.[8,10,13-18]

1.3 Forces Transferred Through and Within the Foot

The normal horse bears approximately 28% of its weight on each forefoot and 22% on each hindfoot.[19] There are five primary loads placed on the digit while standing: (1) compressive loads due to the weight of the horse; (2) tensile force from the pull of the deep digital flexor tendon; (3) tensile force from the pull of the interdigitated laminae from the hoof wall; (4) tensile force from the pull of the common digital extensor tendon; and (5) the compressive force of the sole on the ground.[7] The three predominant forces in ascending order of importance are the compressive load due to body mass, the tensile force from the pull of the deep digital flexor tendon, and the tensile force of the laminar interface.[7] The digital venous pressure increases as

the load applied to the digit increases and decreases as the load decreases, possibly acting in a shock-absorbing fashion.[20] Additionally, the digital cushion has been hypothesized to act as a shock-absorber during locomotion to decrease the compressive loads placed on the digit.[21]

Overall, the forces applied to the interdigitations of the dermal (sensitive) and epidermal (insensitive) laminae are substantial and disruption or interference of the attachment of these tissues can lead to catastrophic failure and collapse of the distal phalanx within the hoof capsule.

1.4 Physiology of the Normal Digital Vasculature

In the standing horse, digital blood flow is relatively stable and minor shifts in weight do not result in significant alterations in flow.[22] As the horse shifts weight onto the digit, the flow decreases and vice versa.[22] In relation to vasoactive properties, the digital arteries and veins supplying the hoof have unique characteristics. The digital veins are highly muscular, have a relatively inelastic vascular wall, and are located in a noncompliant compartment (course between the hoof wall and distal phalanx), resulting in vasculature of low compliance.[12] The equine digital arteries and veins are highly sensitive to vasoconstrictive substances, most notably norepinephrine and endothelin.[23] Furthermore, the digital veins are more sensitive than arteries in vitro to the vasoconstrictive substances angiotensin, thromboxane, norepinephrine, serotonin, and endothelin. [23] Baxter et al also examined vasodilation of palmar digital arteries and veins in vitro and found that veins dilated more than arteries to acepromazine and isoxsuprine.[24] Administration of acetylcholine, prostaglandin E_2, and prostaglandin I_2 (prostacyclin) resulted in vasodilation through the endothelium-dependent actions of nitric oxide and dilation through this mechanism was abolished by incubation of tissues with the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester.[24-26] Comparison of palmar digital vessels to other vessels within the horse found that 5-hydroxytryptamine (serotonin) was more potent as a vasoconstrictor of

digital arteries than facial or tail arteries.[27] Overall, the culminating effects of low compliance and high sensitivity to venoconstrictive substances predispose the equine digit to high venous pressures, thereby increasing hydrostatic pressure and thus the likelihood of laminar edema formation.

The microcirculation of the equine foot is poorly adapted to handling edema. In normal tissues, the three main safety factors that counteract edema formation are capillary permeability, pre-to-post capillary resistance, and lymphatic drainage. An impermeability of the capillary endothelium serves as a barrier to fluid and protein transudation. This results in a higher gradient between the capillary and tissue oncotic pressure, favoring movement of fluid into the capillary lumen. Evaluated by use of the pump-perfused extracorporeal digital preparation to measure Starling forces within the equine digit, the normal equine digital capillary bed is highly permeable to fluid and macromolecules (Fig. 1.2).[12] The capillary bed only retains 67% of the macromolecules within the vasculature and is more permeable than the vasculature of the dog and rat paw.[28] This results in a greater concentration of interstitial protein, favoring edema formation. A high precapillary and low postcapillary resistance (92 % and 8%, respectively) reduces the capillary pressure, thereby reducing the hydrostatic pressure for transcapillary fluid filtration. The pre-to-postcapillary resistance ratio in healthy horses is comparable to that in other musculoskeletal beds in other species.[12] The third edema safety factor is provided by lymphatic drainage. The lymphatics of the digit are few in number and small in diameter; therefore, it is unlikely that lymphatic circulation can effectively protect the foot against edema when the hydrostatic forces in the capillary favor edema formation.[29]

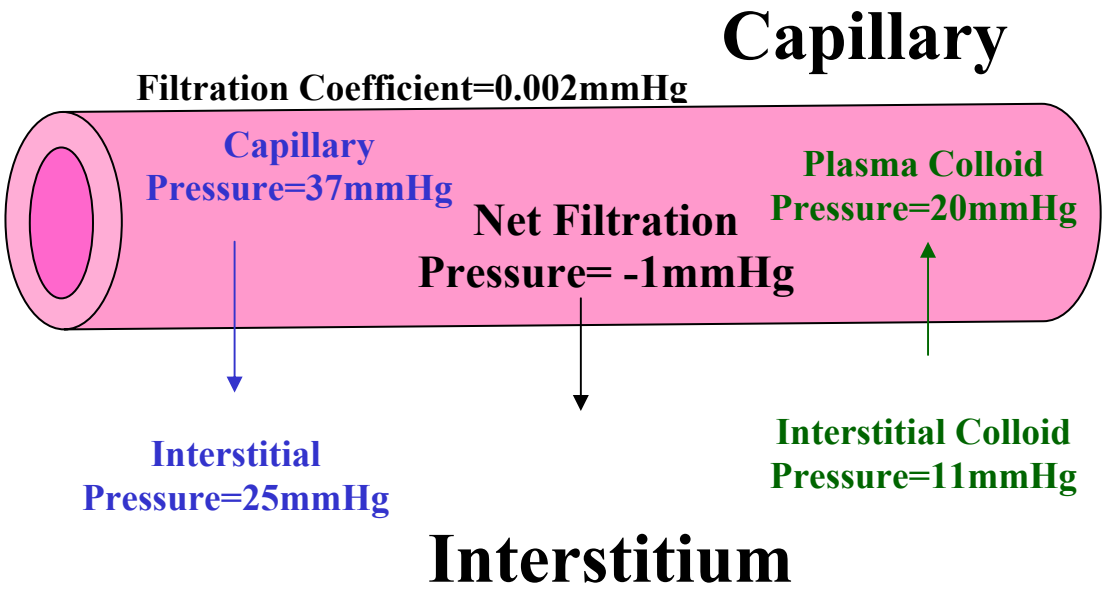

Figure 1.2 – Diagram of Starling force measurements from the digital vasculature of normal horses using the pump-perfused extracorporeal digital preparation. Note the net filtration pressure is -1mmHg in normal tissues due to balances of capillary and interstitial pressures with plasma and interstitial colloid pressures. The result is a net efflux of fluid from the interstitium. Data is from Allen et al, 1988.[12]

1.5 Naturally-Acquired Laminitis

1.5.1 Risk Factors - Several risk factor studies have been completed to improve clinicians' ability to identify horses at high risk for the development of laminitis. Dorn et al determined that in the laminitic horses studied, there were fewer castrated males than intact males indicating that hormonal factors may play a role in the pathogenesis of the disease.[30] Dorn and coworkers also found a seasonal association with the development of laminitis in their population. Another study found that mares were more likely than geldings and intact males to develop acute laminitis.[31] This same study found that there was an increased risk for horses to develop acute laminitis from 5 to 7 years of age and from 13 to 31 years of age. In contrast, age, breed, sex, and seasonality were not statistically associated with the diagnosis of acute laminitis in other studies.[32,33] Laminitis is often associated with other diseases such as colic, particularly strangulating obstruction and inflammatory bowel disease, grain overload, retained fetal membranes and subsequent metritis, pleuropneumonia, and other diseases associated with endotoxemia.[34,35] Gastrointestinal tract disease was the most common primary disease in 54% of horses that developed acute laminitis in a study conducted in seven private practices and at a university veterinary hospital.[33] Additionally, laminitis occurs commonly in the contralateral limb of horses that have a severe non-weight bearing lameness in the opposite limb.[5]

1.5.2 Stages of Laminitis - The progression of laminitis has been divided into four phases, namely the developmental, acute, subacute, and chronic phases. The developmental stage (also called the prodromal stage) encompasses the period between the initial insult and the first appearance of clinical signs associated with laminitis.[36] The actual duration of this phase is dependent upon the inciting factor leading to the development of the disease. Since this phase

concludes with the development of clinical signs of laminitis, studies of pathogenesis and development of therapeutics for the prevention of this disease are best focused during this stage. The acute phase is defined as beginning once clinical signs are observed and extends to either a duration of disease over 72 hours, or to evidence of mechanical collapse of the distal phalanx within the hoof capsule.[37] If the horse maintains clinical signs over 72 hours, but does not have structural failure of the foot, the laminitis is classified as subacute. If at any time the horse develops structural failure, rotation or sinking of the distal phalanx, then the laminitis would be considered to be in the chronic phase. The classifications of subacute and chronic are highly associated with prognosis since horses with chronic laminitis are likely to be affected by the disease for the remainder of their life.[37]

1.5.3 Clinical Signs - Laminitis is a disease that can affect all four feet; however, it most commonly affects the forelimbs since they bear approximately 60% of the horse's mass.[7] The increased load of the forelimbs compared to the hindlimbs is thought to account for the increased occurrence of laminitis in the forelimbs. To better define the severity of clinical signs exhibited by horses, a grading system was established by Obel in 1948.[38] Grade 1 is the least severe and states that the horse alternately and incessantly lifts the feet, lameness is not evident at a walk, but at a trot a short stilted gait is noted. Horses that walk with a stilted gait but can still have a foot lifted are classified as grade 2. Horses with grade 3 move very reluctantly and vigorously resist lifting of a foot. The most severe classification is grade 4 noted by the horse refusing to move unless forced.[38] Other clinical signs characteristic of laminitis are heat present over the dorsal surface of the hoof wall, bounding of the digital pulse (increase in the difference between the systolic and diastolic digital arterial pressure), sensitivity to hoof testers, swelling of the coronary band, and alteration of stance to redistribute weight to the hind limbs ("sawhorse

stance" or rocking of weight to the hind limbs) if laminitis is principally affecting the front limbs. More severe signs are a dropped sole or palpation of a depression located at the level of the coronary band, both indications of rotation or sinking of the distal phalanx within the hoof wall.[9,39]

1.6 Models of Acute Laminitis

1.6.1 The Endotoxemia Model - A publication by Backus in 1937 stated that laminitis was due to the effects of a toxic substance entering the blood stream affecting digital vasoconstriction and hemodynamics.[40] This report stated that high body temperature, increased pulse, increased respiration, muscle tremors, and injection of mucous membranes were precursors to the development of laminitis. The developmental phase of laminitis was difficult to distinguish from enteritis, pneumonia and peritonitis. Over the past 20 years, the leading hypothesis regarding the initiating factor in the cascade of events leading to necrosis and structural failure of the interdigitating sensitive and insensitive laminae has focused on digital hemodynamic alterations; however, the initiating event that triggers these vascular alterations has yet to be determined. Laminitis usually occurs secondary to other diseases, such as those described by Backus, primarily diseases accompanied by endotoxemia. In vitro and in vivo studies have shown that following experimentally-induced laminitis, the mucosal barrier was substantially damaged, potentially allowing entry of endotoxin into the circulation.[41-43] Administration of 0.03 g/kg Escherichia coli endotoxin 055:B5 did not induce changes in equine digital hemodynamics or Starling forces associated with the developmental stages of acute laminitis found using other models of induction such as the black walnut extract (BWE) and carbohydrate overload (CHO) models. However, use of the endotoxin model in this study resulted in arterial vasoconstriction and digital hypoperfusion.[44] It is important to note that acute

laminitis was not consistently induced after administration of 0.03 g/kg Escherichia coli endotoxin 055:B5 in this study. Circulating levels of endotoxin are increased in horses following CHO-induced laminitis and in horses with naturally-occurring gastrointestinal tract disease.[45,46] Administration of the 0.03 g/kg dose of endotoxin in another study found low dosages of endotoxin cause pulmonary hypertension without causing systemic hypotensive, hypodynamic shock.[47] Systemic hypotension and shock are associated with many of the primary diseases that are accompanied by the development of acute laminitis in horses, and horses in other studies administered greater doses of endotoxin developed profound reductions in peripheral perfusion.[48] The cascades of cellular interactions that occur following endotoxemia in horses is extremely diverse including activation of inflammatory mediators (tumor necrosis factor, interleukins –1 and -6, and numerous eicosanoids), the coagulation cascade (microthrombi formation), and endothelial cell disturbances (increased permeability, structural and metabolic alterations, and altered release of endothelial derived substances).[49-57]

Disturbances of endothelial cell function following exposure to endotoxin have been studied using in vitro preparations and have demonstrated that low dose endotoxin decreased endothelium-dependent relaxation and increased adrenergic contraction of palmar digital arteries from horses.[58] Incubation of palmar digital blood vessels in cell culture media containing endotoxin resulted in no release of nitric oxide from endothelial cells, a further sign of endothelial damage due to endotoxin.[59] Endothelial damage has been confirmed after a single infusion of endotoxin and finding endothelial cell retraction and pyknosis, a loss of barrier function, and eventual cell lysis.[60]

Overall, the endotoxin model did not consistently induce acute laminitis in horses when administered as 0.03 g/kg Escherichia coli endotoxin 055:B5. However, substantial evidence

exists supporting a role of endotoxin in the pathophysiology of acute laminitis secondary to diseases commonly associated with endotoxemia, such as gastrointestinal tract disease, metritis, and pleuropneumonia. Continued research investigating the involvement of endotoxin in the pathophysiology of laminitis is warranted.

1.6.2 The Carbohydrate Overload Model - A consistent and clinically relevant method for induction of acute laminitis in horses was needed to thoroughly evaluate the mechanisms of pathogenesis and to develop potential preventions and therapeutics of the disease. Although experimentally-induced laminitis was reported previously, Garner et al in 1975 are recognized as developing the commonly used model of CHO for study of this disease.[61] The laminitis-inducing ration, a combination of 85% corn starch and 15% wood cellulose flour, was administered via a stomach tube at a dose of 17.6 g/kg. The exact cascade of events linking the administration of CHO to the development of acute laminitis is unclear. Alterations in cecal flora, lactic acidosis, and endotoxemia have been associated with CHO administration, but the direct stimulant of the systemic and digital alterations that occur has yet to be determined.[41-43,45,62,63] Horses developed clinical signs of laminitis and progressed to Obel grade 3 by approximately 40 hours post-CHO administration. Horses experienced alterations similar to horses developing naturally-acquired laminitis such as increased heart rate, rectal temperature, increased packed cell volume, leukocytosis, and hyperproteinemia.[61,64-67] The consistent alterations preceding development of clinical signs using the CHO model were arterial and venous hypotension followed by arterial hypertension, increased packed cell volume, leukocytosis, and hyperproteinemia.[61] Garner also noted that the limbs of horses from 24 hours post-CHO administration until demonstration of clinical signs of laminitis were cold, but as clinical signs developed the coronary bands were warm to the touch. The marked systemic hemodynamic changes included a decline in right atrial

pressure, diastolic systemic arterial pressure, and systolic systemic arterial pressure, which reached a maximum about 16 hours after starch was administered.[68] This pressure drop was followed by a steady increase in right atrial pressure, diastolic arterial pressure, and systolic arterial pressure. These results suggest that appreciable systemic cardiovascular and local digital vascular changes occur in horses with laminitis and increased release or activation of vasoactive mediators occurs.

In subsequent studies using the isolated perfused digit, the specific hemodynamic forces acting on the laminar microcirculation in experimentally-induced laminitic horses have been extensively defined.[29,69,70] Garner et al introduced the hypothesis that the predominant cause of laminitis after CHO was a disturbance in digital blood flow, which occurred during the onset of the syndrome after carbohydrate overload of the gastrointestinal tract.[61] Measurements of Starling forces by Allen et al found increased venous resistance with the development of laminitis.[29,70-73] Of particular importance is the finding that the pre-to-post capillary resistance ratio is decreased in the prodromal stages of laminitis. This finding supports the hypothesis that increased venomotor tone initiates laminitis. Increased venoconstriction results in increased vascular resistance and capillary hydrostatic pressure. This imbalance increases the hydrostatic force in the capillary promoting the flux of fluid across the capillary bed within the foot, resulting in laminar edema while capillary permeability remains normal (Fig. 1.3). [29,69,70] The increased laminar interstitial pressure, due to edema formation, exceeds the critical closing pressure of equine digital capillaries because they are located between the rigid hoof capsule and the hard bony surface of the third phalanx, thereby leading to a "compartment-like syndrome".[29] When tissue pressure increases above the capillary critical closing pressure, the capillaries

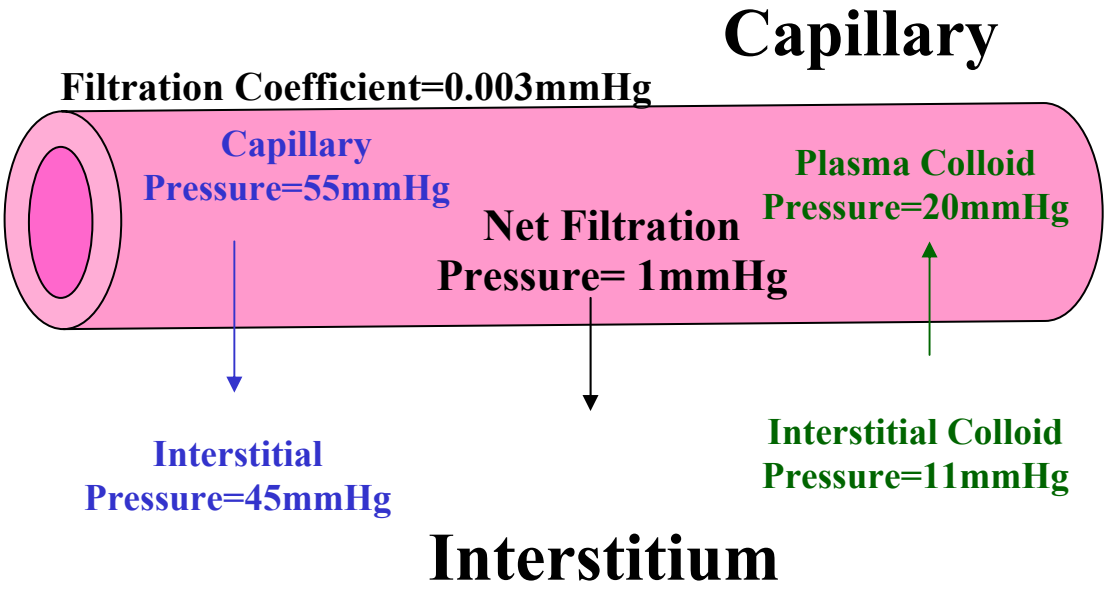

Figure 1.3 – Diagram of Starling force measurements from the digital vasculature of horses after carbohydrate overload-induced laminitis using the pump-perfused extracorporeal digital preparation. Note the net filtration pressure is 1mmHg in these tissues due to an imbalance of capillary and interstitial pressures with plasma and interstitial colloid pressures. The imbalance is from increased capillary pressure which results from venoconstriction. The result is a net efflux of fluid from the capillary (interstitial edema formation). Data is from Allen et al, 1990.[29]

collapse leading to tissue ischemia. Many researchers have hypothesized that blood flow is further reduced by formation of arteriovenous shunts at the level of the coronary band.[4,29,74] Researchers have indirectly demonstrated reduced perfusion in the vasculature of the foot using contrast radiography, and hoof wall surface temperature.[75-77] Using contrast radiography, researchers demonstrated reduced perfusion in the terminal vasculature of the foot after CHO-induced laminitis.[75] During the developmental stages of laminitis using the CHO model, Hood et al used hoof wall surface temperature as an indication of laminar perfusion and found decreases 8 to 12 hours before the onset of lameness, an indication of decreased laminar perfusion or decreased metabolic activity.[76] Additional findings of this study were that hoof wall surface temperature significantly increased above baseline as clinical signs of laminitis became evident. Pollitt and co-workers recently demonstrated increases in hoof temperature (indicator of increased lamellar blood flow) 16- 40 h after CHO overload.[29,61,76,78]

Although numerous mediators are likely to contribute to the previously mentioned vascular alterations, the principal mediators have yet to be determined. Katwa et al recently demonstrated that the expression of ET-1 in laminar connective tissues obtained from CHO-induced acutely laminitic horses, and naturally-occurring chronically laminitic horses were increased compared with a control group.[71]

Following CHO-induced laminitis, microvascular thrombi formation has been documented in the laminae, in addition to the previously mentioned hemodynamic alterations, and likely contribute to the decreases in laminar perfusion due to microvascular obstruction.[79,80] Blood platelets were significantly decreased 8 h after the onset of severe lameness in another study examining the hematological alterations associated with CHO-induced laminitis.[67] In

contrast, Prasse et al did not find significant differences in coagulation and fibrinolysis in 15 horses after CHO administration suggesting an imbalance in these pathways are not a significant factor in the pathogenesis of laminitis.[81]

Histological study of laminar changes during laminitis has been performed 48 to 96 hours after induction of laminitis with the CHO model. Following the onset of lameness, the initial histological alteration occurs in the digital vasculature, including swelling of endothelial cells and mild edema formation.[36,82,83] Laminar capillaries become congested with erythrocytes within 8 hours. Within 6 to 12 hours, a perivascular leukocyte infiltration occurs that then dissipates as the inflammatory cells migrate into the epidermal layer. Arteriolar endothelial cells become deformed as a result of cytoplasmic processes that extend into the lumen. Microvascular thrombi and accompanying severe edema formation occur within 24 hours, and hemorrhage occurs within the primary dermal laminae by 72 hours. Histological evaluation of the laminae within 8 hours after lameness develops reveals thinning and lengthening of the lamellar structures accompanied by reduction, flattening and displacement of epithelial cells.[82,83] The secondary lamina become redirected such that lamina nearer the base of the dermal lamina are directed toward the coffin bone, and those nearer the laminar tips are directed toward the hoof wall. Morphologic alterations secondary to epithelial cell damage include swelling, vacuolization, nuclear swelling and/or pyknosis, and leukocytic infiltration of the secondary epidermal lamina, which can be observed as early as 24 hours after the onset of lameness.[82] Additionally, Pollitt found that in samples from horses 48 hours after CHO administration, the lamellar basement membrane had disintegrated and the attachment of the basement membrane to the basal cells of the epidermis had failed.[84]

Alterations in endothelial cell function due to acute laminitis may affect endothelium-dependent actions and may alter the responsiveness of the digital vasculature to vasoactive agents. The results of recent studies indicate that normal equine digital vessels have a substantial capacity for endothelial-dependent relaxation by nitric oxide in vitro, accounting for approximately 70% to 85% of the maximal relaxation induced by acetylcholine.[23,85] In horses with CHO-induced laminitis, acetylcholine-mediated relaxations of digital vessels in vitro are reduced, suggesting that the NO producing capacity of the digital vascular endothelium is reduced, thereby rendering the vessels more sensitive or vulnerable to vasoconstrictive agents.[26]

1.6.3 The Black Walnut (Juglans nigra) Extract Model - The black walnut (Juglans nigra) extract (BWE) model for induction of acute laminitis was first described by Minnick et al in 1987 as an improved model over the CHO model described by Garner et al in 1975.[61,86] Horses were known to develop acute laminitis after exposure to fresh shavings from the black walnut tree and investigators were interested if the extract given via nasogastric tube would consistently induce acute laminitis and also wanted to test the extract to identify the laminogenic agent.[86] The methods call for selection of a branch approximately 25 cm in diameter cut in the fall of the year from a live tree, removal of the bark and sapwood, and passing of the wood through a planer to produce shavings of the heartwood.[86] Soaking of the shavings (2 g/kg body weight) followed by straining results in a dark tea-colored extract that is then given via nasogastric tube. Unfortunately, analysis of the extract has yet to yield the laminogenic factor.[86] Horses developed edema of the coronary band, occasional depression, and 8 out of 10 horses demonstrated clinical signs of laminitis within 12 hours of BWE administration. Hematologic measurements revealed an initial decrease in white blood cell counts (nadir of at least a 30% decrease from baseline at 4 hours post-BWE), an increase in white blood cell counts above

baseline starting 8 hours post-BWE, increased packed cell volume, and hyperglycemia.[87]The alterations in white cell count are very similar to the findings after CHO administration.[61] Galey et al suggested that these alterations in white blood cells are characteristic of horses with endotoxemia, but Eaton et al examined blood samples for endotoxin up to 3 hours post-BWE and did not detect endotoxin in any of the samples.[70,87]The decrease in central venous pressure and initial decrease in systemic arterial pressure were not as pronounced using the BWE model as compared with the CHO model of induction.[70] The BWE model was considered to be an improvement since diarrhea and signs of endotoxemia were not associated with this model and the time to the development of signs associated with acute laminitis is shorter compared with the CHO model. [70,77,86-88]

Using the same pump-perfused extracorporeal digital preparation used after CHO administration, researchers have evaluated digital Starling forces after BWE administration.[70] Starling force measurements were obtained 2 – 4 hours after BWE administration (once there was a 30% decrease in WBC count) and the predominant finding was an increase in postcapillary (venous) resistance.[70] Increased venous resistance and high capillary hydrostatic pressures occurred as consistent findings using BWE models of induction, similar to the findings after CHO overload.[29,70] In previous studies using the BWE model and Starling force evaluation, precapillary resistance did not change, compared with healthy horses, but using the CHO model precapillary resistance significantly increased.[29,70] Differences between these models may be due to differences in timing of events in the development of laminitis. The severity of the venoconstriction accompanying laminitis induced with BWE was less than that associated with CHO overload. These less severe changes with BWE may be due to a difference in the pathophysiology of the disease, or more likely because the Starling forces accompanying

laminitis due to BWE were evaluated at a different stage (2 to 4 hours versus 16 hours) of the disease.

Using laser Doppler flow probes to measure laminar capillary perfusion, Adair et al recently determined that laminar microvascular blood flow decreases in the first 1 to 2 hours after BWE administration.[77] This initial decrease is then followed by a return of laminar microvascular blood flow to near baseline values. Then, at approximately 8 hours into the disease, laminar blood flow again decreases, which temporally corresponds with development of clinical signs of laminitis. Although a different method for examining laminar perfusion was utilized, these findings are similar to those of Hood et al where they used hoof wall surface temperature as an indication of laminar perfusion after CHO administration.[75-77] Another study used gamma scintigraphy of regionally infused 99mTc- labeled macroaggregated albumin, before and 12 hours after BWE administration and when compared with baseline images, perfusion to the forefoot of horses after the development of acute laminitis was decreased. Also with the onset of laminitis, perfusion was decreased to the dorsal laminar and coronary dermis compared to the remainder of the forelimb.[89] These studies are in agreement with the studies examining perfusion alterations after CHO-induced laminitis and together demonstrate strong evidence of an ischemic event occurring within the digit during the early phases of the development of laminitis.

The role of the inflammatory response in the development of laminitis has been questioned. A recent report by Fontaine et al examined expression of interleukin-1 beta in the laminae of horses after the characteristic 30% decrease in white cell count (approximately 3 hours post-BWE).[88] They found an increase in interleukin-1 beta expression thereby providing evidence of an inflammatory response occurring early in the developmental stages of the disease

after BWE administration. The authors state that increased expression of inflammatory cytokines, such as interleukin-1 beta, alter multiple systems including the release of vasoactive, procoagulant, and other proinflammatory mediators that result in the measured hemodynamic and hematologic alterations known to occur with the development of the disease.[88]

Histological sections from horses 12 hours post-BWE revealed mild vacuolization of secondary dermal laminae.[87] The vasculature within these sections were mildly congested. The tips of the primary epidermal laminae lacked cellular definition and were necrotic in some areas. Tissues from horses euthanized 84 hours post-BWE revealed severe changes including necrosis of the tips of the primary epidermal laminae, loss of normal tissue architecture, and the presence of cellular debris. Regeneration of epithelial cells were found within areas of necrosis and these areas were also marked by a high number of mitotic figures.[87] In contrast to the findings of Weiss et al, thrombi were not found within these sections from BWE-induced horses.

Use of the in vitro preparation for the investigation of BWE-induced alterations in equine digital vessel constriction and dilation has not been extensive to date. One study examined the direct effects of BWE on the contractile effects of epinephrine in digital vessels and found that BWE led to increased vasoconstriction compared with vessel rings not exposed to BWE.[90] Vessel rings exposed to other extracts (the eastern white pine, eastern red cedar, and pin oak) did not contract differently to epinephrine compared with contraction with epinephrine alone.[90] Further investigations into the effects of BWE administration on the vasoactive properties of digital vessels are warranted.

1.6.4 The Fructan Model - The CHO model of laminitis has been associated with high morbidity. Nevertheless, this model has become the standard for laminitis studies partially due to its similarity to grain overload, a common cause of naturally-occurring laminitis cases. In an

effort to retain the clinical ties of the model but decrease morbidity, Pollitt and van Eps have developed the fructan model of laminitis induction.[91] Fructan is a form of oligofructose, a component of the CHO ration, derived from chicory roots that is administered via a nasogastric tube. Six of 6 horses developed clinical signs of laminitis, an improvement from the CHO and BWE models. Horses developed diarrhea, pyrexia, elevated heart rate, and the hematological alterations were similar to the CHO model.[67,91] Overall, Pollitt and van Eps suggest that this model is more humane and is more effective than the CHO model and future investigations using this new model are warranted.

1.7 Theories of Pathogenesis

There are three principal theories regarding the mechanisms responsible for the development of laminitis: the ischemic/vascular, mechanical/traumatic, and metabolic/enzymatic theories.[39]

1.7.1 The Ischemic/Vascular Theory - The ischemic/vascular theory involves altered digital perfusion as the initiating factor in the cascade of events that leads to metabolic dysfunction and structural failure of the laminae.[36] Although the pathogenesis of laminitis is not fully understood, the initial vascular mechanisms are characterized by hypoperfusion due to venoconstriction, laminar edema formation, opening of arteriovenous shunts, allowing blood to bypass laminar tissues, leading to tissue ischemia, necrosis of the interdigitating laminae, and ultimately mechanical failure with rotation or sinking of the distal phalanx away from the hoof wall.[29,29,61,69,92] Venoconstriction is considered the initiating factor causing decreased laminar perfusion.[4] Increased venoconstriction results in increased vascular resistance and capillary hydrostatic pressure. Increased capillary hydrostatic pressure forces fluid out of the capillaries and into the interstitium thereby increasing laminar interstitial pressure. When tissue pressure

increases above the capillary critical closing pressure, the capillaries collapse leading to tissue ischemia. Increased pressure in a confined anatomical space can affect blood flow of those tissues and can lead to ischemia of those tissues; this condition is referred to as "compartment syndrome". Allen et al hypothesized that horses develop compartment syndrome within the digit during the developmental stages of laminitis, leading to laminar ischemia.[29] It is hypothesized that blood flow is further reduced by formation of arteriovenous shunts at the level of the coronary band.[4,29,74] The digital laminae undergo necrosis after prolonged ischemia. Separation of the interdigitating sensitive and insensitive lamina develops and distal phalanx rotation, distal displacement, or both subsequently occur.[93]

Raynaud's syndrome in humans has many pathologic similarities with equine laminitis such as early ischemia due to decreased digital microcirculatory perfusion followed by reperfusion leading to painful, throbbing digits. Raynaud's syndrome and laminitis have been proposed to be the same disease but in different species.[71,94,95] Raynaud's disease is defined as idiopathic paroxysmal bilateral cyanosis of the digits due to arterial and arteriolar contraction. It is often precipitated by cold and results in blanching and numbness or pain of the fingers.[96] Endothelin-1 gene expression is increased and cutaneous vascular plasma ET-1 levels are increased in patients with Raynaud's disease.[71,94,95,97] It is hypothesized that the vasospasm associated with this disease is due to endothelial dysfunction with excessive production of ET-1 and decreased production of NO.[98]

The ischemic hypothesis has focused on digital hemodynamic alterations; however, the initiating mediator(s) that trigger these vascular alterations have yet to be determined. Katwa et al recently demonstrated that the concentration of ET-1, a potent endothelial-derived vasoconstrictor, in laminar connective tissues obtained from experimentally-induced acutely

laminitic horses, and naturally-occurring chronically laminitic horses were increased compared with a control group.[71] Another study examining the potent vasoconstrictor ET found that in vitro, contractile responses of equine palmar digital veins were over 3 times greater than arteries to ET-1 administration.[23,72,73,99]Previous research has demonstrated that NO donor administration improves digital perfusion and reduces the bounding digital pulses associated with acute laminitis in ponies.[100,101] Nitric oxide is an endothelial-derived vasodilator that plays a role in regulation of ET-1 release.[102] Based on these studies, it is possible that an imbalance in endogenous endothelial-derived substances, such as ET-1 and NO, may play a role in the vascular alterations that occur during the development of laminitis in horses.

1.7.2 The Mechanical/Traumatic Theory - The mechanical or traumatic theory is based on causes of laminitis that result from direct trauma to the digit and not a primary systemic disease leading to the development of laminitis.[39] Common examples of traumatically-linked laminitis are road founder, laminitis secondary to unilateral lameness of the opposite foot (support limb laminitis), and development of laminitis after long trailer rides.[5,39] The exact mechanisms that lead to structural failure of the laminae are unknown, but several hypotheses have been suggested. Excessive force applied to the dermal and epidermal laminar interdigitations may initiate an inflammatory response with vasospasm, thereby increasing capillary hydrostatic pressure, leading to edema formation ultimately resulting in a compartment-like syndrome much like that of the ischemic/vascular theory.[39] Another hypothesis is that application of excessive force results in tearing of the dermal and epidermal laminar interdigitations, then the inflammatory response and/or vasospasm ensues leading to ischemic damage of the laminar interdigitations.[39]

1.7.3 The Toxic/Enzymatic Theory - The toxic, or enzymatic theory, states that the fundamental event leading to the failure of the laminar interdigitations is delivery of blood-borne toxins to the epidermal laminae resulting in weakening and loss of cellular attachments.[103] Based on this theory, the loss of these cellular attachments are precursors to the vascular and inflammatory alterations described within the ischemic theory. Pollitt states that instead of the hypoperfusion demonstrated by ischemic theory proponents, hyperperfusion of the digit is responsible for delivery of these toxins to the laminar tissues.[78] Pollitt states that the targets of the blood-borne toxins are the mediators of enzymatic remodeling that are a part of the normal processes involved in the movement of the continually proliferating hoof wall past the distal phalanx. Laminin and type IV and type VII collagen are components of the laminar basement membrane and the enzymes metalloproteinase-2 and metalloproteinase-9 are believed to dissolve these substances, and under normal physiological states, controlled dissolution allows the movement of epidermal laminae past the dermal laminae as growth occurs.[103,104] Excessive activation of these enzymes leads to uncontrolled dissolution of the basement membrane components resulting in separation of the epidermal laminae from the dermal laminae. Laminar samples from horses 48 hours after induction of laminitis using the CHO model demonstrated loss of basement membrane attachments.[84] In horses with naturally-acquired acute and chronic laminitis, zymography of laminar connective tissues found increased activation of extracellular metalloproteinases compared with non-laminitic horses.[105] Activation of the metalloproteinase enzymes is hypothesized to be induced by the exotoxin(s) from Streptococcus species, especially Streptococcus bovis, a Gram-positive bacteria found as part of the normal cecal flora.[63,103,106] Using the CHO model, researchers have identified changes in the bacterial population of the cecum with fermentation of the CHO, resulting in excessive lactate production, rapid decline in

intracecal pH, and death of cecal bacteria including Streptococcus species.[63,107] Based on this theory and its supportive data, prevention of laminitis should be aimed toward abolishing activation of the enzymes responsible for the dissolution of the basement membrane.

Development of acute laminitis is often secondary to other primary diseases; therefore, the mechanisms involved in the pathogenesis of laminitis are most likely numerous and interrelated. Currently, there are three main theories regarding the pathogenesis of acute laminitis in horses; but, there are probably multiple factors from each theory that participate in the pathogenesis of the disease.

1.8 Current Treatments

Clinicians currently employ numerous and varied therapies in the prevention and treatment of laminitis, which reflects the lack of a complete understanding of the pathophysiology of this disease. The cornerstones of treatment of horses with acute laminitis are directed at different components of the pathophysiologic process. Acute laminitis should be considered a medical emergency and treatment should be instituted ideally before the onset of clinical signs. The goals of treatment should be to minimize any predisposing factors, reduce pain, reduce the severity of permanent laminar damage, improve digital hemodynamics, normalize digital Starling forces, and prevent further movement of the distal phalanx within the hoof capsule. Considerable controversy exists regarding the treatment of laminitis because of our lack of understanding of the pathophysiology of this disease.

In order to institute preventive treatment for laminitis, those horses at risk must be identified. Once identified, preventatives may include administration of mineral oil, intravenous fluids, parenteral antibiotics, nonsteroidal antiinflammatory drugs (NSAIDs), hyperimmune serum or plasma, and polymyxin B. Other preventive treatments include heparin, aspirin,

vasodilators, rheologic agents, corrective hoof trimming and shoeing, placement of the horse in a deeply bedded stall, and frog support. Many of these preventive measures are also instituted therapeutically.

Based on the extensive research conducted measuring Starling forces and the formation of laminar edema, one of the most important considerations in developing a preventive and therapeutic plan is to attempt to normalize digital Starling forces. The best approach to do this is to make sure plasma oncotic pressure is sufficient by supplementing with either plasma or another colloidal solution such as hydroxyethyl starch.[108] Care should be taken when administering intravenous fluid therapy to horses with acute laminitis because excessive intravascular volume could perpetuate the development of laminar edema in horses with abnormal digital hemodynamics.

A majority of the scientific data supports the theory that vascular alterations, most notably hypoperfusion, are key factors leading to the failure of laminar interdigitations. Based on this data, treatments directed at improving digital blood flow and laminar perfusion are often suggested. The drugs most commonly used to improve digital blood flow are acepromazine (0.03-0.06 mg/kg intramuscularly every 6-8 hours for a minimum of 3 days), isoxsuprine hydrochloride (1.2 mg/kg per os every 12 hours), and topically applied glyceryl trinitrate (2-4 mg/hour).[108] A study conducted in normal horses found that administration of acepromazine caused an increased blood flow to the digit; but isoxsuprine and pentoxifylline did not improve flow to the digit.[109] Since isoxsuprine is suggested to induce vasodilation, decrease blood viscosity and platelet aggregation, it is still used by some clinicians.[110] Experimentally, nitric oxide donors, such as glyceryl trinitrate, reduced the lameness and 'bounding pulses' of ponies with grass-induced laminitis and improved digital perfusion.[100,111] In another study, glyceryl

trinitrate was applied topically over the palmar digital vasculature of normal horses and digital perfusion did not significantly increase as noted by no significant changes in hoof wall surface temperature.[112] There remains a question regarding the effectiveness of nitric oxide donors as treatment for equine laminitis since the pathogenesis of the disease is still not fully understood. Additionally, many of the studies evaluating these agents were conducted in normal horses; the vasculature from horses during the developmental stages of laminitis may respond differently to these agents.

With the recent suggestion that vasoconstriction early in the onset of laminitis may have a protective effect by limiting the delivery of gut-derived blood-borne toxic substances that have direct laminar cellular damaging effects, some investigators and clinicians suggest that vasodilation may not be an appropriate therapeutic in the developmental stages.[103] Soaking the feet in crushed ice or cold water may be preferred to prevent the vasodilatory phase. This is a time-honored treatment; however, it would need to be performed continuously and during the developmental phase prior to the onset of lamellar damage. It is likely that once the feet were removed from the ice, vasodilation and a rebound hyperemia would occur. As many times is the case, the developmental phase goes unnoticed and lamellar damage has already occurred by the time treatment is initiated.

Anti-inflammatory medications are indicated to decrease inflammation, edema and pain associated with laminitis. Phenylbutazone appears to have the best anti-inflammatory and analgesic effect of any of the NSAIDs commonly used in horses and one study found that this particular NSAID is utilized in approximately 86% of laminitis cases.[33] A dose of 2.2-4.4 mg/kg of phenylbutazone can be administered either intravenously or per os every 12 hours. Alternatively, flunixin meglumine can be administered at 0.5 -1.1 mg/kg intravenously or per os

every 8-12 hours. A dose of 0.25 mg/kg flunixin meglumine can be administered intravenously every 8 hours to interrupt eicosanoid production associated with endotoxemia.[108] Since flunixin meglumine has been associated with decreased eicosanoid production due to endotoxemia, this particular NSAID is utilized more with acute cases of laminitis compared to chronic cases which tend to be administered phenylbutazone.[113] Ketoprofen can be administered at 2.2 mg/kg intravenously every 12 hours. Dimethylsulfoxide (DMSO) is an anti-inflammatory drug that scavenges hydroxyl radicals, decreases edema, and is therefore used to counteract the effects of ischemia-reperfusion injury. DMSO should be administered at a dose of 0.1-1.0 gram/kg intravenously diluted in a polyionic fluid with dextrose to a concentration of 10-20%. It can be administered every 8-12 hours.[108] DMSO has been used in the treatment of muscle trauma, tendonitis, laminitis, and arthritis and may potentate the effects of other drugs.[113] Some clinicians prefer to place DMSO topically on the coronary bands.

Because microthrombi have been shown in the laminar vasculature from horses during laminitis, some clinicians prefer to administer heparin and/or aspirin to horses as a preventive or therapeutic agent. Heparin is often given subcutaneously at a dose of approximately 20,000-40,000 units per 450-kg horse. There is no evidence that administration of heparin will prevent the onset of laminitis. One study did not find a difference in the incidence of laminitis between horses treated prophylactically with heparin and those not treated with heparin.[114] Aspirin is often administered at a dose of 10-20 mg/kg per os once every 48 hours.[108] It irreversibly inhibits platelet cyclooxygenase and therefore production of thromboxane, which should decrease platelet aggregation and vasoconstriction. Although not an established treatment, Weiss examined ponies treated with a synthetic analogue of the platelet fibrinogen receptor antagonist peptide and found a significant decrease in the development of CHO-induced laminitis,

demonstrating a role of platelets aggregation in the pathogenesis of the disease.[115] Future developments may lead to the use of an agent similar to this in treatment protocols.

Efforts to reduce mechanical forces and stabilize the distal phalanx are imperative to treatment of acute laminitis. Exercise can exacerbate tearing of already compromised laminar interdigitations and should be avoided. The stall should be bedded deeply with sand or other material that provides support to the frog. Pressure sores are a common complication due to long hours of recumbency; therefore, bedding the stall deeply and frequent repositioning are necessary. Providing early and effective mechanical support of the distal phalanx can spare weakened, separating laminae and improve the outcome.[116] This mechanical support should ideally be instituted prior to or at the onset of foot pain. Frog support is one of the more effective methods of providing support to the distal phalanx.

The preventative and therapeutic protocol decided upon by clinicians will be based on the primary events leading to the development of laminitis and stage of disease each horse is experiencing. The information presented above represents some of the currently used methods, but with further research, more effective treatments will be developed.

1.9 Prognosis

Many horses that demonstrate clinical signs of acute laminitis that receive prompt, appropriate medical treatment of the predisposing condition and mechanical foot support may recover completely. If radiographs demonstrate signs of distal phalanx rotation, the prognosis for soundness and even survival must be guarded. Horses with distal displacement, or sinking, of the distal phalanx with the hoof capsule are more likely to be euthanized than those without distal displacement.[6,117] Horses with less than 5.5 degrees of rotation evident on lateral radiographs returned to athletic performance; whereas, horses with greater than 11.5 degrees of

rotation did not return to athletic performance.[118] Horses with laminitis often require extensive, long-term treatment and the prognosis is grave because of the recurrent, crippling pain and recumbency, which often require euthanasia for humane reasons. In one study of horses with acute laminitis admitted to a university veterinary hospital, 75% did not return to athletic function, and a majority were humanely destroyed within one year because of a lack of response to therapy or development of severe complications.

1.10 Laminitis Summary

Acute laminitis is a severely debilitating, potentially career-ending and often life-threatening disease of the sensitive and insensitive laminae of the equine digit. Laminitis can occur in adult horses of any breed or use and is often associated with primary diseases associated with endotoxemia. The ability of the equine athlete to walk depends on the integrity of the interdigitating primary and secondary laminae, which structurally unite the hoof wall, distal phalanx, and the sole of the foot into a single unit. Clinicians employ numerous and varied therapies in the prevention and treatment of laminitis, which reflects the current lack of a complete understanding of the pathophysiology of this disease. Through future research, the mechanisms responsible for the development of acute laminitis may be elucidated.

1.11 Endothelin-1 Introduction

In physiologic states, the endothelium synthesizes vasoactive substances, such as nitric oxide (NO) and endothelin-1 (ET-1), which regulate vasomotor tone.[119,120] Under normal physiological conditions, it is hypothesized that a balance exists between endothelial-derived vasoconstrictors (i.e. ET-1) and vasodilators (i.e. NO) and that this balance is important for maintaining vasomotor tone, blood flow and tissue perfusion.[121]

Endothelins are a family of peptides synthesized by various cells that exert numerous biologic and pathophysiologic effects.[102] The principal endothelin of importance in vascular diseases or ischemic conditions is ET-1. Endothelin-1 is a potent vasoconstrictor peptide synthesized by endothelial cells, vascular smooth muscle cells, and macrophages. It not only induces prolonged vasoconstriction in arteries and arterioles, but also causes intense profound venoconstriction in both the systemic and pulmonary circulation.[102,122] Although the endothelin family encompasses three important isoforms, this review will focus on ET-1 since it is the predominant endothelin produced by vascular endothelial cells.

1.12 Discovery

In 1982, Hickey et al were examining the dilatory effects of aortic endothelial cell culture media on isolated pig coronary arteries when an unexpected discovery occurred.[123] They had expected vasodilation of the coronary arteries due to endothelial derived relaxing factor (NO) release from the endothelial cells in culture. Instead, the vessels developed a slow and prolonged contraction.[123] Several years later, endothelin, a 21 amino acid peptide, was first isolated, purified, sequenced and cloned by Yanagisawa and colleagues.[120] Since that time, endothelin has been extensively studied and linked to many diseases characterized by smooth muscle alterations.

1.13 Structure

Endothelin has three isoforms, namely ET-1, ET-2, and ET-3.[124] The ET family is structurally similar to the rare snake venom sarafotoxin derived from Atractaspis engaddensis. The ETs are all 21 amino acids in length with complete identity at 10 positions (Table 1.1). [102,120] The structure of ET is highly conserved across species.[125] There are two pairs of disulfide bonds that join cystine residues 1 with 15 and 3 with 11. These disulfide bonds are required for binding

Table 1.1 – Primary amino acid structure of the three endothelin isoforms endothelin-1 (ET-1), endothelin-2 (ET-2), and endothelin-3 (ET-3) with the differences in residues noted for ET-2 and ET-3 in bold. Modified from Rubanyi and Polokoff, 1994.[98]

	Amino Acid Position																			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
ET-1	C	S	C	S	S	L	M	D	K	E	C	V	Y	F	C	H	L	D	I	I	W
ET-2	C	S	C	S	S	**W**	**L**	D	K	E	C	V	Y	F	C	H	L	D	I	I	W
ET-3	C	**T**	C	**F**	**T**	**Y**	**K**	D	K	E	C	V	Y	**Y**	C	H	L	D	I	I	W

of ET to the ET_A receptor.[102] The presence of these two pairs of disulfide bonds within a short peptide chain was a new configuration of mammalian origin.[120] Residues 4 – 7 are the most variable for substitution of alternate amino acids in comparison to residues 8 – 10 which are the most highly conserved across the ET family. The hydrophobic COOH terminus, composed of residues 16 – 21, is also highly conserved and is responsible for binding of ET with the receptors. The COOH terminus has been studied extensively for the development of ET receptor antagonists.[126]

1.14 Genes

There are three separate genes for ET-1, ET-2, and ET-3. They have been mapped to chromosome 6 (ET-1), chromosome 1 (ET-2), and chromosome 20 (ET-3).[127-129] Although multiple cell types are capable of ET-1 mRNA expression, the predominant cells are endothelial cells, macrophages, cardiomyocytes, and vascular smooth muscle cells.[124,130-132] Many factors increase the expression of ET-1 including proinflammatory cytokines, coagulation mediators, other vasoactive substances such as angiotensin and bradykinin, and hypoxia.[102] Sheer stress across endothelial cells decreases expression of ET-1, most likely to allow for flow-induced vasodilation.[120] Researchers have hypothesized that ET production is regulated at the level of mRNA and that ET is not stored in secretory granules for later release.[120,133]

1.15 Biosynthesis and Endothelin Converting Enzyme

Gene translation results in synthesis of the 212-amino acid preproendothelin which is then cleaved from the COOH terminus by peptidases to form the 38-amino acid proendothelin-1, also referred to as big ET-1. Endothelin converting enzyme cleaves this shortened COOH terminus to form the 21-amino acid structure of ET-1.[124] Endothelin converting enzyme is present in the cytosol and in the membrane of endothelial cells and in the membrane of vascular

smooth muscle cells.[134,135] Therefore, unconverted big ET-1 released from endothelial cells can still be cleaved to the more active form, ET-1, in the region of the ET_A receptor on the surface of smooth muscle cells. Since endothelin-1 is over 100 times more potent as a vasoconstrictor than big ET-1, complete cleavage to this most active form is essential to reach the full effects of the peptide.[136]

Vascular endothelium and smooth muscle cells only synthesize the ET-1 isoform.[124,130] In blood vessels, biosynthesis of ET-1 occurs primarily in the endothelium, approximately 80% is released abluminally toward the vascular smooth muscle, and receptors for ET-1 are located on both endothelial and vascular smooth muscle cells (Fig. 1.4).[122,137] The actions of basal ET-1 produced by the endothelium are predominantly paracrine in effect, but endothelium-derived ET-1 can also act in an autocrine manner to induce nitric oxide and prostacyclin release.[138] Endothelin-1 produced in smaller amounts by vascular smooth muscle may function in an autocrine manner to regulate both tone and structural modeling of vasculature.[130]

Endothelin synthesis in multiple species is stimulated by endotoxin, epinephrine, transforming growth factor (produced during platelet aggregation), platelet activating factor (which stimulates platelet aggregation and neutrophil chemotaxis), tumor necrosis factor, tissue hypoxia, and altered shear stress, which are associated with many diseases characterized by an inflammatory response and/or by vascular alterations. [34,49,102,121,139-148] Endothelin-1 synthesis is downregulated by many factors including NO, high sheer stress, and anti-inflammatory cytokines such as interleukin-4.[149-151]

Degradation of ET-1 is by the zinc membrane metalloendopeptidase I located on the cell surface of many tissue types, particularly pulmonary and renal tissues.[152] One study reports that the pulmonary circulation accounts for approximately 47% of ET-1 clearance, demonstrating the

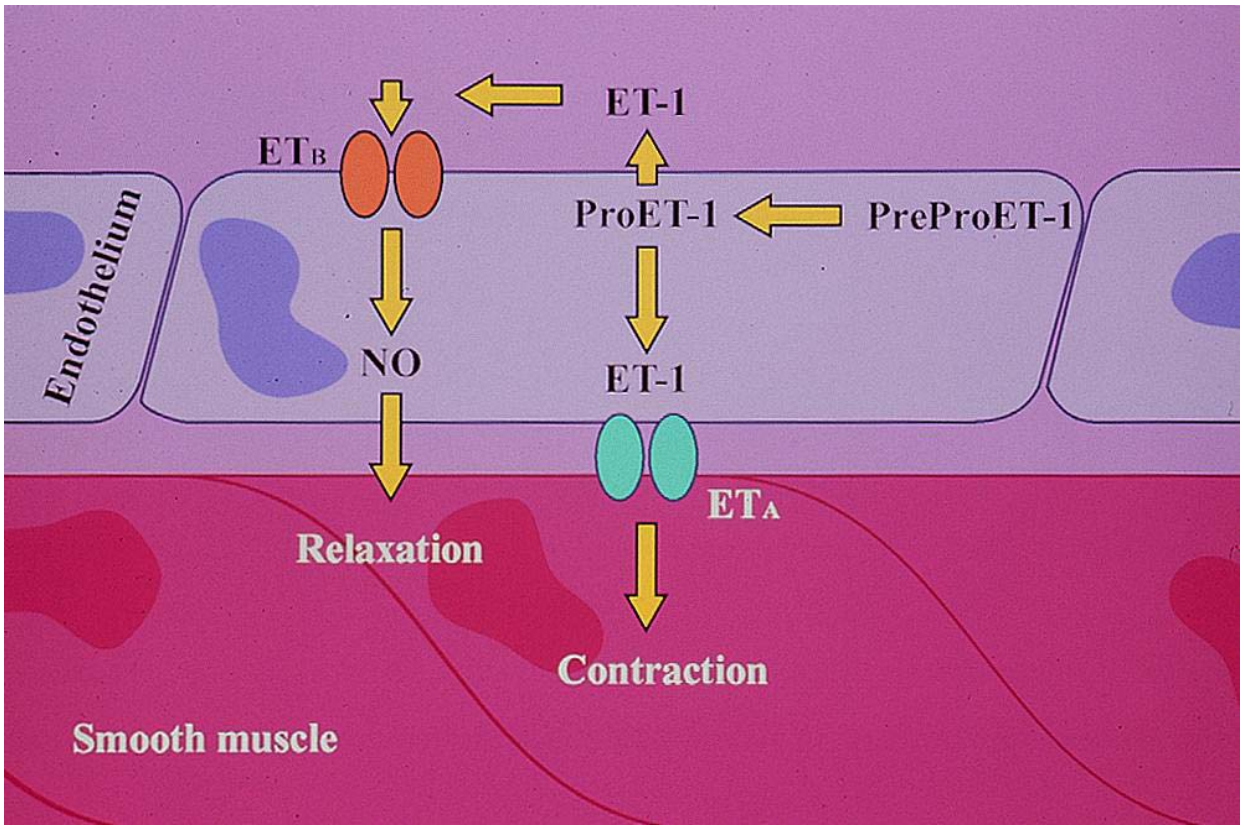

Figure 1.4 – Diagram illustrating the synthesis, release, and actions of endothelin-1 (ET-1) through the two main receptor types, ET_A and ET_B. Stimulation of the ET_A receptor causes profound contraction of the vascular smooth muscle. Stimulation of the ET_B receptor causes release of nitric oxide (NO) that leads to transient smooth muscle relaxation. The predominant effects of ET-1 are through the ET_A receptor.

potential for substantially increased circulating levels of ET-1 with cardiovascular or pulmonary disease.[153]

1.16 Receptors

There are two principal receptor types that respond to ET-1, the ET_A and ET_B receptors. The ET_A receptors are predominantly located on the surface of the vascular smooth muscle to elicit an increase in intracellular calcium that results in slowly developing, but sustained vasoconstriction.[102,154] The ET_B receptors are located principally on endothelial cells and trigger the vascular smooth muscle relaxing factors, such as NO and prostacyclin. Nitric oxide diffuses into the smooth muscle where it stimulates guanylate cyclase and the subsequent generation of cGMP, which leads to vascular smooth muscle relaxation.[121] Nitric oxide released through this mechanism is also believed to regulate release of ET-1, possibly through inhibition of the precursors of ET-1.[102] Recent research has determined that the subtypes ET_{B1} and ET_{B2} may exist and have differing actions when stimulated by ET_B agonists.[155] The subtype ET_{B1} is thought to initiate the synthesis and release of relaxing factors, and ET_{B2} may have contractile properties similar to the ET_A receptor type. The present theory is that under normal physiologic states in vascular smooth muscle, ET-1 has predominately contractile effects through the ET_A receptor type, minor dilatory effects through the ET_{B1} receptor subtype, and few effects through the ET_{B2} subtype.[154] However, during pathologic states, such as hypertension and Raynaud's disease, the contractile effects through the ET_{B2} receptor may become significant.[154]

The genes for the ET_A and ET_B receptors are located on chromosome 4 and chromosome 13, respectively.[156,157] Endothelin receptors are heptahelical G-protein coupled receptors identified as members of the rhodopsin superfamily.[158,159] The ET_A and ET_B receptors were first expressed and cloned by Arai et al and Sakurai et al, respectively, in 1990.[160,161] Binding

specificity of the ET_A receptor is ET-1 ≥ ET-2 > ET-3 and the ET_B receptor is ET-1 = ET-2 = ET-3.[102] The seven amphipathic transmembrane helices are highly conserved whereas the NH_2 and COOH terminals are regions of high diversity. G-protein coupling occurs at the third transmembrane helix and the receptor is anchored to the membrane just past the seventh helix.[162] This anchor is required in the ET_A receptor for binding of ET. The NH_2 terminus is also required for ET binding to the ET_A receptor, but is not required for ET binding to the ET_B receptor.

1.17 Signal Transduction

Stimulation of the ET_A receptor on the surface of vascular smooth muscle results in activation of several pathways. The associated G-protein is activated leading to conversion of phosphatidylinositol to phosphatidylcholine by phospholipase C and to arachadonic acid release through phospholipase A in the cell membrane (leads to increased prostaglandin production). Phosphatidylcholine increases *sn*-1,2-diacylglycerol (DAG) and 1,4,5-inositol triphosphate (IP_3). Increases in DAG stimulate increases in protein kinase C that lead to cellular alkalinization and to long-term alterations of cellular function, such as increased DNA synthesis and increased mitogenesis. Increased IP_3 leads to opening of voltage gated calcium channels and sarcoplasmic reticular release of calcium to increase intracellular concentrations of calcium. Through the actions of calmodulin, myosin light chain kinase is activated to hydrolyze adenine triphosphate to adenine diphosphate to allow for cross-bridge formation leading to smooth muscle contraction. Stimulation of the ET_B receptor located on the endothelial cell also results in increased intracellular calcium, but leads to NO production through the action of NO synthase.[102,138,158,163,164]

1.18 Effects of Endothelin-1

1.18.1 Cardiovascular Effects - Endothelin-1 has been studied extensively in order to characterize its potential role in pathological states such as atherosclerosis, congestive heart failure, pulmonary hypertension, and numerous other cardiovascular diseases.[165,166] During normal physiological conditions, ET-1 is found in low concentrations in circulating plasma.[167] Release of ET-1 by the endothelium increases systemic arterial pressure and vascular resistance; therefore, the primary effect of ET-1 on the cardiovascular system is a pressor effect.[120] Initially, however, intravenous administration of ET-1 decreases peripheral resistance and blood pressure, most likely due to the release of vasodilatory compounds such as NO, prostacyclin, and atrial natriuretic peptide.[168]

In vitro studies show that veins are more sensitive and contract to a greater magnitude to ET-1, compared with arteries. [169] Increased venous constriction induced by ET-1 may be the most important factor regulating increases in vascular resistance and may play an important role in regulation of venous return.[169] Within the cardiovascular system, ET-1 has very specific effects depending on the target organ.

1.18.2 Cardiac Effects - Endothelin-1 administration has positive inotropic and chronotropic effects, prolongation of the action potential in cardiac myocyte and papillary muscle preparations.[168] Yet, in the intact animal, ET-1 administration results in decreased cardiac output, most likely due to decreased myocardial perfusion (coronary vasoconstriction) and to increased afterload (increased arterial resistance).[170] Endothelin-1 has also been demonstrated to induce mitogenesis of cardiac myocytes and may play an important role in ventricular processes that lead to chronic cardiac failure.[168]

1.18.3 Vascular Effects - Endothelin-1 is the most potent endogenous vasoconstrictor known to date. Endogenous generation of ET-1 plays a fundamental physiological role in the maintenance of basal vascular tone.[164] Stimulation of the endothelium of large vessels and microvessels results in stimulation of the ET_B receptor and subsequent release of NO and prostaglandin I_2. The predominant actions of ET-1 are through the ET_A receptor located on the vascular smooth muscle cells of the large vessels and microvessels, causing pronounced vasoconstriction and mitogenesis.[102] Of particular interest is that exogenous ET-1 administration results in a 3 to 10-fold greater constriction of veins than arteries and this finding has been demonstrated in many species and various vascular beds.[99,169,171-173] Takai et al measured the effects of ET-1 administration on dogs and determined that the vasoconstrictive effects of ET-1 were stronger in the venous circulation and the dilatory effects of ET-1, through stimulation of the ET_B receptor and subsequent NO release, were present initially followed by vasoconstrictive effects in the arterial circulation. [171] Overall, Takai et al found that the vasoconstrictive effects of ET-1 dominated as measured by increased total peripheral resistance and mean circulatory pressure. Increased venous constriction induced by ET-1 may be the most important factor regulating increases in vascular resistance and increases in venous return.[99,169] Increases in ET-1 synthesis and release may be associated with diseases characterized by vasoconstriction and increases in vascular resistance, such as hypertension and Raynaud's disease.

1.18.4 Platelet and Neutrophil Effects - Endothelin-1 has been shown to cause rolling and adherence of leukocytes in rat postcapillary venules, and it induces shape changes and activation of human platelets.[143,174] Creating a positive feedback loop, activated neutrophils stimulate additional ET-1 production.[175] To balance the additional ET-1 production and to decrease neutrophil activation, ET-1 stimulates NO production from neutrophils. It is an

imbalance in production of ET-1 and NO that lead to overall altered neutrophil function.[102] One study found that both ET_A and ET_B receptors likely play a role in platelet activation, but another study found that through activation of the ET_B receptor, NO inhibits platelet and neutrophil aggregation and adhesion.[149,174] As with neutrophils, an imbalance in ET-1 and NO are considered to contribute to altered platelet function.

1.19 Development of Endothelin Antagonists

Several ET antagonists acting at the receptor level or influencing endothelin converting enzyme are under investigation and have great potential as agents for use in the treatment of a number of diseases characterized by alterations in ET, and these antagonists are also important components for understanding the fundamental biologic mechanisms of ET. The structure/activity relationship of ET-1 is well characterized and the conformational criteria required for each of the receptors, ET_A and ET_B, has been established.[176] The hydrophobic COOH terminal hexapeptide of ET is the region known to be highly important for receptor recognition.[126] Non-peptide ET antagonists have been developed from a lead compound found during a compound screening program, but selectivity for a receptor type has not been possible using this type of antagonist.[176] Peptidic receptor antagonists have been developed, but the clinical potential of these compounds has been questioned since they are considered more fragile in nature.[176]

1.19.1 PD145065 - Doherty et al of Parke-Davis developed the ET nonselective receptor antagonist PD145065 (Ac-D-5H-dibenzyl[a,d]cycloheptene-10,11-dihydroglycine-L-Leu-L-Asp-L-Lle-L-Lle-Trp).[177] PD145065 is an inhibitor of both the ET_A and ET_B receptors.[178]The effectiveness of PD145065 in preventing vasoconstriction has been confirmed by demonstrating a lack of increased vascular smooth muscle intracellular calcium in the presence of ET-1 after

incubation with the antagonist.[179] PD145065 has proven to be beneficial in models examining the role of ET-1 in ischemia/reperfusion, the role of ET-1 in the systemic inflammatory response, and in ET-induced constriction of vascular and airway smooth muscle.[180-182] In particular, use of PD145065 has been beneficial for identification of the physiologic and pathophysiologic roles of ET-1 in cardiovascular diseases such as hypertension, heart failure, atherosclerosis, coronary heart disease, restenosis after angioplasty, and primary pulmonary hypertension.[138] Therapeutically, administration of the combination of PD145065 and a NO donor has been shown to significantly decrease vasoconstriction, typically associated with bronchoconstriction in adult respiratory distress syndrome and acute lung injury.[183] Short-term administration of PD145065 does not alter ET-1 synthesis; previous studies have demonstrated no effects on resting vascular tone in the cat femoral artery and vein and no alterations in systemic hemodynamics (i.e. mean arterial pressure, cardiac index, and mean pulmonary pressure) in dogs when administered on its own in healthy animals.[178,184] Significant effects of PD145065 administration are seen when ET-1 concentrations are elevated, such as in hypertension and atherosclerosis, as compared to the lack of effect during normal physiological states.[138] Various concentrations of PD145065 have been evaluated in vitro and in vivo. The 10^{-5} M concentration was most effective in blocking ET-1-induced increases in perfusion pressure in pigs in vivo and in blocking ET-1-induced vasoconstriction in equine colonic vessels in vitro.[185,186] Information regarding the use of PD145065 in the prevention or reversal of ET-1-induced smooth muscle contraction supports exploration of its use in additional pathologic states.

1.20 Role of Endothelin in Selected Vascular Diseases

Many pathologic states in humans and other species, such as atherosclerosis, hypertension, tissue hypoxia, Raynaud's syndrome, and asthma, are associated with increased

ET-1-induced smooth muscle contraction, increased plasma ET-1 concentrations, and increased ET-1 immunohistochemical staining.[94,147,165,166,187,188]

1.20.1 Vasospasm (Raynaud's Disease) - Endothelin-1 has been hypothesized to be involved in various forms of vasospasm including coronary vasospasm, cerebral vasospasm after subarachnoid hemorrhage, and Raynaud's disease.[102] Raynaud's disease is defined as idiopathic paroxysmal bilateral cyanosis of the digits due to arterial and arteriolar contraction. It is often precipitated by cold and results in blanching and numbness or pain of the fingers.[96] In particular, Raynaud's syndrome in humans is characterized by early ischemia due to decreased digital microcirculatory perfusion followed by reperfusion leading to painful, throbbing digits. Endothein-1 gene expression is increased and cutaneous vascular plasma ET-1 levels are increased in patients with Raynaud's disease.[71,94,95,97] It is hypothesized that the vasospasm associated with this disease is due to endothelial dysfunction with excessive production of ET-1 and decreased production of NO.[98]

1.20.2 Hypertension - Hypertension is associated with increased peripheral vascular resistance, increased vascular tone, and hypertrophy of vascular smooth muscle. Increased plasma ET-1 concentrations are consistently found with hypertension in a number of species and ET-1 has been shown to cause all of the above mentioned alterations associated with hypertension.[68,140,149,166,184] General consensus of the literature is that an imbalance between ET-1 and NO systems underlie the mechanisms involved in the pathogenesis of systemic and pulmonary hypertension.[189-191] As an example, a special type of hypertension, preeclampsia, has been linked to endothelial dysfunction within the maternal circulation with overproduction of ET-1 and reduced production of NO and prostaglandin I_2.[192,193] Therefore, much like Raynaud's disease, alteration of endothelial function appears to contribute to hypertension.

1.20.3 Ischemia/Reperfusion - Numerous vascular disease states have been associated with elevations in the production and/or release of ET-1 and ET-1 has been implicated in the deleterious changes associated with ischemia-reperfusion injury.[149] Endothelin-1 is the most potent endogenous vasoconstrictor known and increased ET-1 production can lead to tissue ischemia.[194,195] After periods of ischemia, ET-1 concentrations are increased in plasma, affected blood vessels have enhanced reactivity to vasoconstrictive substances, and binding of ET-1 is increased in affected tissues (possibly due to upregulation of receptors).[102] One study demonstrated that after reducing blood flow by 70% to an isolated segment of ileum in pigs, ET-1 plasma concentrations remained unchanged in control segments and in the systemic circulation, slightly increased in the intestinal arterial blood supply, but increased four-fold in the intestinal venous circulation.[173] Venous pressure was also increased during this study although the blood flow had been manually reduced for the duration of the study. Another recent study examining microvascular dysregulation associated with ischemia-reperfusion injury found that after a short period of low flow (60 minutes) and within a few hours of restoration of flow, the major receptor upregulated in the ischemic and nearby tissue was the ET_B receptor.[196] The purpose of this preferential upregulation may be to balance the increased presence of ET-1 in the venous circulation after a period of ischemia with increased NO availability for smooth muscle relaxation. The severity of tissue damage after ischemic conditions has been reduced by administration of ET antibodies, endothelin converting enzyme inhibitors, and ET_A receptor antagonists, further supporting the role of ET-1 in ischemia/reperfusion injury.

1.21 Role of Endothelin in Equine Disease

Endothelin-1 synthesis is stimulated by numerous mediators including epinephrine, transforming growth factor (produced during platelet aggregation), platelet activating factor

(which stimulates platelet aggregation and neutrophil chemotaxis), and tumor necrosis factor, which are increased during many diseases in horses characterized by an inflammatory response, endotoxemia, and/or vascular alterations (i.e. pleuropneumonia, endometritis, intestinal ischemia, enterocolitis, and anterior enteritis).[34,49,102,142-146] Reported concentrations of jugular venous plasma ET in healthy horses are from 0.18 pg/ml to 1.80 pg/ml using radioimmunoassay procedures.[197-199] Similar to values reported in horses, plasma ET-1 like immunoreactivity values in healthy dogs and humans were determined to be 1.83 and 1.7 pg/ml, respectively.[94,139] Systemic administration of ET-1 significantly decreased cardiac output and significantly increased systemic vascular resistance.[200] Infusion of a selective ET_A receptor antagonist completely inhibited these responses to ET-1.[200]

Other studies have examined the potential role of ET-1 in equine disease. Ischemia/reperfusion injury has been associated with various forms of colic and vascular alterations are hypothesized to be the initiating events in the pathogenesis of laminitis. Jugular venous plasma ET-1 like immunoreactivity were significantly greater in horses with colic compared with clinically healthy horses (3.29-10.02 pg/ml; healthy controls 1.8 pg/ml). Horses with strangulating large colon volvulus, enterocolitis, and peritonitis had the greatest plasma concentrations of ET-1.[197] Examining expression of ET-1, Katwa et al found increased laminar connective tissue ET-1 expression from acutely and chronically affected laminitic horses (1.7 pg/mg of tissue) compared with non-laminitic horses (0.4 pg/mg of tissue).[71] Endothelin-1 has also been evaluated in vitro with colonic and digital vessel rings. As found in other species, ET-1 administration induces greater venoconstriction than arterioconstriction.[23,99,169]

Lethal white foal syndrome is a disease associated with breeding of particular spotted horses, generally described as “frame overo”. Breeding can produce foals that are all white or

nearly all white, and die shortly after birth of severe intestinal blockage due to aganglionosis from the distal small intestine to the large intestine.[201] This syndrome has been found to be due to mutations in the ET_B receptor gene. There is a 2-basepair nucleotide change leading to mutation in the first transmembrane helix of the receptor.[201] The ET_B receptor is thought to be involved in the development of neural crest cells that become enteric ganglia and melanocytes.[202]

By far, a majority of our knowledge of ET-1 and its role in disease is based on literature regarding human medicine. The application of this information, plus that gained through veterinary research, will better define the roles of ET-1 in equine health and disease.

1.22 Endothelin Summary

The discovery of ET-1 has greatly advanced our understanding of endothelium-mediated regulation of vasomotor tone in health and vascular alterations that occur in many diseases. The identification of pathogenic mechanisms in disease, an in particular equine disease, that involve ET-1 has just begun. As further research is conducted, the role of ET-1 in diseases characterized by vascular alterations will become clearer and therapeutic tools for prevention and treatment will become more numerous.

1.23 Overall Summary

Acute laminitis in horses is associated with numerous vascular alterations including reductions in digital perfusion. The mediator of these vascular changes has not been identified. Since ET-1 has been implicated in many diseases characterized by vascular alterations, investigation of the potential role of ET-1 in acute laminitis in horses is warranted.

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CHAPTER 2. COMPARISON OF TWO ENDOTHELIN ANTAGONISTS ON IN VITRO RESPONSES OF EQUINE PALMAR DIGITAL ARTERIAL AND VENOUS RINGS TO ENDOTHELIN-1

2.1 Introduction

Endothelin (ET), a potent vasoconstrictor, is a 21 amino acid peptide and was first isolated by Yanagisawa and colleagues in 1988.[1] Endothelin has three isoforms, namely ET-1, ET-2, and ET-3.[2] Vascular endothelium and smooth muscle cells only synthesize the ET-1 isoform.[2,3] In blood vessels, biosynthesis of ET-1 occurs in the endothelium and receptors for ET-1 are located on both endothelial and vascular smooth muscle cells.[4] There are two principal receptor types that respond to ET-1 in these cells, ET_A and ET_B receptors. The ET_A receptors are predominantly located on vascular smooth muscle cells and, through several signal transduction mechanisms, ET-1 binding results in slowly developing but sustained vasoconstriction.[5] The ET_B receptors are located principally on endothelial cells and trigger the release of the endothelial-derived relaxing factor, nitric oxide (NO).[5] Through these mechanisms, ET-1 and NO contribute to maintenance and homeostasis of vasomotor tone.[4]

During normal physiological conditions, ET-1 is found in low concentrations in circulating plasma.[6] Release of ET-1 by the endothelium increases systemic vascular resistance. Increased venous constriction induced by ET-1 may be the most important factor regulating increases in vascular resistance and increases in venous return; in vitro studies show that veins are more sensitive and contract to a greater magnitude to ET-1, compared with arteries.[7,8]

Endothelin-1 has been studied extensively in order to characterize its potential role in pathological states such as atherosclerosis, congestive heart failure, pulmonary hypertension, and numerous other cardiovascular diseases.[9,10] Circulating concentrations of ET-1 were significantly increased 4.5 fold during endotoxemia in dogs.[11] Increases in ET-1 synthesis and

release may be associated with diseases characterized by vasoconstriction and increases in vascular resistance. Jugular venous plasma ET-1 concentrations were significantly greater in horses with colic compared with clinically healthy horses. Horses with strangulating large colon volvulus, enterocolitis, and peritonitis had the greatest plasma concentrations of ET-1.[12]

Acute laminitis is a commonly encountered disease in horses and is characterized by decreased blood flow to the digital lamina, resulting in laminar ischemia, necrosis and subsequent separation of the distal phalanx (P_3) from the hoof wall.[13,14] Venoconstriction is considered the initiating factor causing decreased laminar perfusion.[13] Increased venoconstriction results in increased vascular resistance and capillary hydrostatic pressure. Increased capillary hydrostatic pressure forces fluid out of the capillaries and into the interstitium thereby increasing laminar interstitial pressure. When tissue pressure increases above the capillary critical closing pressure, the capillaries collapse leading to tissue ischemia. Blood flow is further reduced by formation of arteriovenous shunts at the level of the coronary band.[13,14,15] The digital laminae undergo necrosis after prolonged ischemia. Separation of the interdigitating sensitive and insensitive lamina develops and P_3 rotation, distal displacement, or both subsequently occur.[16]

Acute laminitis owing to carbohydrate overload is also associated with systemic vascular alterations.[15] The disease is characterized by an initial decrease in systemic arterial blood pressure with a concurrent increase in central venous pressure.[17,18] Since the onset of laminitis is characterized by digital venoconstriction and changes in systemic arterial and venous

pressures, determining the role of ET-1 in the pathogenesis of this disease may provide further important knowledge for the prevention and treatment of laminitis in horses.

An in vitro study examining the vascular effects of intravenous infusion of low-dose endotoxin found ET-1 is a potent vasoconstrictor of control and endotoxin-treated equine palmar digital vessels.[19] Availability of ET receptor antagonists has made it possible to study the individual effects of ET-1 on equine palmar digital vessels. We hypothesize that ET-1 will cause profound contraction of equine digital vascular smooth muscle in vitro and that the addition of ET antagonists will attenuate these contractile effects. The purpose of the study reported here was to compare the effectiveness of two ET receptor antagonists and to determine the concentrations that effectively block the in vitro vasomotor effects of ET-1 in equine palmar digital arterial and venous rings. Additionally, the effects of ET-1 were compared to those of commonly studied potent vasoconstrictors in equine medial and lateral palmar digital vessel rings in vitro. We also used both medial and lateral vessel rings to validate that both sides respond in a similar manner to these vasoconstrictors in vitro.

2.2 Materials and Methods

2.2.1 Tissue Sources - This study was approved by the Institutional Animal Care and Use Committee of the Louisiana State University. Palmar digital arteries and veins were collected from 8 horses immediately after euthanasia with sodium pentobarbital[a] (90mg/kg, IV). The horses were of various breeds, age ranging from 1 to 25 years (mean ± SEM, 11 ± 3.30) and body weight from 247 to 516 kg (381.2 ± 33.01). Horses were determined to be free of laminitis based on history, thorough physical and lameness examinations, and lateral radiographs of both front feet. No pharmacological agents were administered for at least 72 hours prior to the study.

2.2.2 Vessel Preparation - The medial and lateral palmar digital arteries and veins from one forelimb were collected and placed in chilled, oxygenated (95% O_2 and 5% CO_2) Tyrode's solution (136.87 mM NaCl; 2.68 mM KCl; 11.90 mM $NaHCO_3$; 5.55 mM dextrose; 1.81 mM $CaCl_2$; 1.07 mM $MgCl_2$; 0.36 mM NaH_2PO_4). Vessels were then gently cleansed of excess connective tissue and cut into 4mm wide rings.[8,20,21] Isometric tension was monitored by attaching (using 5-0 silk suture) one side of the vessel ring to the stationary floor of an organ bath containing oxygenated Tyrode's solution at 37 C, and the other side to a force-displacement transducer[b] interfaced with a polygraph.[22, c] Based on preliminary studies in our laboratory and on previously published studies by Baxter et al utilizing palmar digital arteries and veins in vitro, an initial tension of two grams was determined to be the optimal resting tension and was applied to each vessel ring to mimic in vivo diastolic vascular tone.[20,21,23] The rings were allowed to equilibrate for 45 minutes. During this period, the bath solution was gently replenished with fresh Tyrode's solution at 15-minute intervals and the tension was readjusted to two grams.[20,23] Tension was not reapplied after the last bath solution change.

2.2.3 Pharmacological Agents - Two ET receptor antagonists, PD 142893[d] and PD 145065[e], designated in this study as B-1 and B-2, respectively, were selected based on their ability to inhibit ET binding to both ET_A and ET_B receptors. Based on manufacturer's recommendations, methanol was used to dissolve B-1 whereas distilled water was used to dissolve B-2. Tyrode's solution was used to dilute each solution to the desired concentrations (10^{-7} to 10^{-5} M). ET-1[f] was dissolved in distilled water and frozen in aliquots at -80 C. Aliquots were thawed immediately prior to use and diluted with Tyrode's solution to the desired concentrations (10^{-10} to 10^{-6} M). Due to the current expense of ET-1, we were limited to 10^{-6} M

concentration as the strongest concentration of ET-1 for the concentration-response (C-R) curve. Both NE[g] and HST[h] were dissolved in distilled water and were further diluted with Tyrode's solution to the desired concentrations (10^{-10} to 10^{-4} M).

2.2.4 Experimental Design - Two separate studies were conducted with the palmar digital vessels. The first study was conducted to compare the two ET receptor antagonists in their ability to alter C-R relationships to ET-1 on medial palmar digital arteries and veins. The ET-1 C-R relationships were determined with and without incubation with the ET receptor antagonist. Afterward, norepinephrine (NE) or histamine (HST) was added to test for tissue fatigue and viability. The purpose of the second study was to determine C-R relationships for NE and HST in order to compare responses of lateral and medial digital arteries and veins, and to compare the potency of ET-1 to the other vasoconstrictor agents.

2.2.5 Study I - ET receptor antagonists - Each organ bath contained one vessel ring prepared as previously mentioned. A total of eight organ baths were used containing four arterial and four venous rings. The first bath of each vessel type was a control bath and did not receive an ET antagonist. The second, third, and fourth baths of each vessel type were incubated with a 10^{-7}M, 10^{-6}M, or 10^{-5}M concentration of B-1, respectively. Incubation occurred during the last 30 minutes of equilibration by adding the selected antagonist to the bath at each of the three times the bath solution was replenished. This 8-bath design was repeated using the ET receptor antagonist B-2 on additional vessel rings from the same horse. The order of B-1 vs. B-2 was randomly determined so that each antagonist was evaluated an equal number of times in each run.

ET-1 concentration-response relationships - After equilibration and incubation, cumulative C-R relationships were determined for ET-1 for all vessel groups (10^{-10} to 10^{-6} M). Each consecutive concentration of ET-1 was added to the baths at 5-minute intervals. At the end of each ET-1 C-R curve, either NE or HST was added to the baths in increasing concentrations (10^{-6} to 10^{-4} M) to further contract tissues.

Concentration-response curves were recorded for each vessel ring, apparent maximum responses to ET-1 and NE or HST were measured, and relative ET-1 EC_{50} values were calculated using nonlinear regression fitting a sigmoid curve to the C-R data.[i] It should be noted that due to the expense of ET-1, the C-R curves were limited to the concentrations of 10^{-10} to 10^{-6} M. The contraction of vessel rings following this concentration were considered apparent maximum contractions for relative EC_{50} calculations and throughout this manuscript maximum contractions and EC_{50} values are in context of these limitations. The EC_{50} values higher than -6.00 log M concentration were extrapolated based on the corresponding antagonist's C-R curve.[i] The dry tissue weight was determined afterward by allowing the rings to dry at room temperature (20-22 C) and measuring their weight on an analytical balance until weight loss was no longer observed.

2.2.6 Study II - NE and HST concentration-response relationships - NE and HST C-R relationships were determined to compare the potency of ET-1 to other known vasoconstrictors, and to study the response of the medial and lateral digital vessels. Study II was conducted concurrently with Study I using additional vessel rings from the same horse. Each organ bath contained one vessel ring prepared as described previously. A total of 4 organ baths contained one ring of each vessel type (medial and lateral/ artery and vein). After the 45-minute

equilibration period, cumulative C-R relations were determined for NE (10^{-10} to 10^{-4} M) in the absence of ET receptor antagonists. Each consecutive concentration of NE was added to the baths at 2-minute intervals. This 4-bath set-up was simultaneously repeated in adjacent organ baths using HST in place of NE. The dry tissue weights of the vessels were determined and apparent maximum contraction and EC_{50} values were calculated as described in study I.

2.2.7 Determination of Drug Antagonism Values - The pA_2 value, a measurement of drug antagonism, was determined for each ET-1 antagonist based on the analysis of the Schild regression.[24] Briefly, using calculated mean C-R curves for each group, the concentration required to produce 50% of the maximal response to ET-1 (EC_{50}) was determined for arterial and venous rings. For the higher concentrations of the ET antagonists, the EC_{50} values required extrapolation, using nonlinear regression.[i] The negative log of the antagonist concentration was plotted against the corresponding log of the concentration ratio minus 1. The concentration ratio is the ratio of the EC_{50} of ET-1 after addition of an antagonist over the EC_{50} of the control curve. The results from the linear regression of these points yields a line such that the x-intercept equals the pA_2 value.[24] Coefficient of determination (R^2) correlation was determined for each fitted line to its respective data points and the slopes of these fitted lines were compared.[25,i]

2.2.8 Statistical Analyses - The continuous data (relative EC_{50}, apparent maximum contraction) were evaluated for normality, using the Shapiro-Wilk statistic and were considered to follow a normal distribution with failure to reject the null hypothesis of normality at $p \leq 0.05$. Data that did not follow a normal distribution were transformed (log, square) to establish a normal distribution of the data. The data were summarized and graphed as mean ± SEM**.**

Study I - The continuous data (EC_{50}, maximum contraction) were evaluated separately for arteries and veins using the following model:

$$y = \mu + Horse + Antagonist + Concentration + Antagonist*Concentration + Horse*Antagonist*Concentration + \varepsilon$$

where the effect of Horse was considered random and the effect of Antagonist and Concentration was tested using the Horse interaction term. Only the responses of arteries or veins to control conditions, 10^{-5}, 10^{-6}, and 10^{-7} M concentrations of B-1 and B-2 were used in the analysis. Comparisons between arteries and veins were determined using a mixed effect linear model that accounted for the random variance of horse and the repeated measurements of each horse. Responses to HST or NE added after the ET-1 C-R curve were evaluated by descriptive comparison.

Study II - For comparison of the ET-1 C-R of medial control vessels with vessels treated with NE or HST C-R curves, the apparent maximum contraction was evaluated for arteries and veins separately using the following model:

$$y = \mu + Horse + Drug + Horse*Drug + \varepsilon$$

where the effect of horse was considered random and the effect of Drug was tested using the Horse interaction term.

The continuous data (relative EC_{50}, apparent maximum contraction) comparing medial and lateral vessels were evaluated separately for arteries and veins using the following model:

$$y = \mu + Horse + Drug + Side + Drug*Side + Horse*Drug*Side + \varepsilon$$

where the effect of Horse was considered random and the effect of Drug and Concentrations was tested using the Horse interaction term.

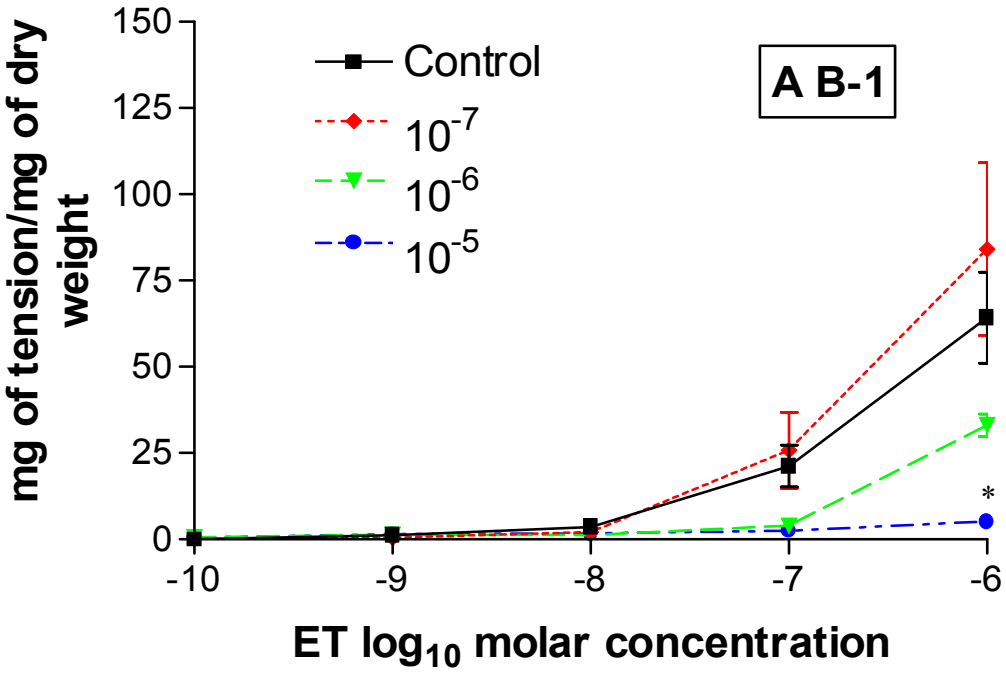
For all analyses, a two-sided hypothesis with α=0.05 was used to determine significance of the main and interaction effects. The p-value for significant interaction effects was reported. Proc mixed[j] was used for the analysis. Where there was significant main or interaction effects, multiple comparisons, using adjusted least squares means, were made among and between drug combinations maintaining an experiment-wise error of $\alpha = 0.05$. Thus, where a difference is noted, unless specified, the p-value was ≤ 0.05.

2.3 Results

2.3.1 Study I – Apparent maximum contraction values – Although 100% maximum contraction of tissues was not obtained due to our limitation of ET-1 at 10^{-6} M being the highest concentration utilized, ET-1 administration in the absence of ET antagonists (control vessel rings) resulted in a concentration-dependent profound contraction of palmar digital arteries and veins. (Figures 2.1-2.2) Responses of control vessel rings to ET-1 between runs were equivalent. For both ET receptor antagonists (B-1 and B-2), the 10^{-5} M concentration was most effective in attenuating the contraction caused by ET-1 in a concentration-dependent manner in palmar digital arteries and veins. At the 10^{-5} M concentration, B-2 was most effective and completely abolished the contractile effect of ET-1 in most vessel rings. Vessel rings treated with either the 10^{-6} or the 10^{-5} M concentration of B-2 had significantly lower maximum contractions compared with control vessels (p=0.0001). Overall, arteries treated with B-2 had significantly lower mean maximum contractions than those treated with B-1.

Veins treated with B-1 or B-2 at the 10^{-5} M concentration had significantly reduced maximum contractions compared with the control vessels and vessels treated with antagonists at

Figure 2.1- Mean ±SEM concentration-response (C-R) curves for arteries with endothelin -1 (ET-1) (10^{-10} to 10^{-6} M) expressed in mg of tension/mg of dry weight. The ET-1 C-R for arteries (A) after incubation with PD 142893 (B-1) (10^{-7} to 10^{-5} M) or Tyrode's (control) (top panel); ET-1 C-R for arteries after incubation with PD145065 (B-2) (10^{-7} to 10^{-5} M) or Tyrode's (control) (bottom panel). *Apparent maximum contraction values differ significantly ($P < 0.05$) from control for these M concentrations of each ET-1 antagonist. Note the difference in the y-axis scale for arteries incubated with B-1 vs. B-2.



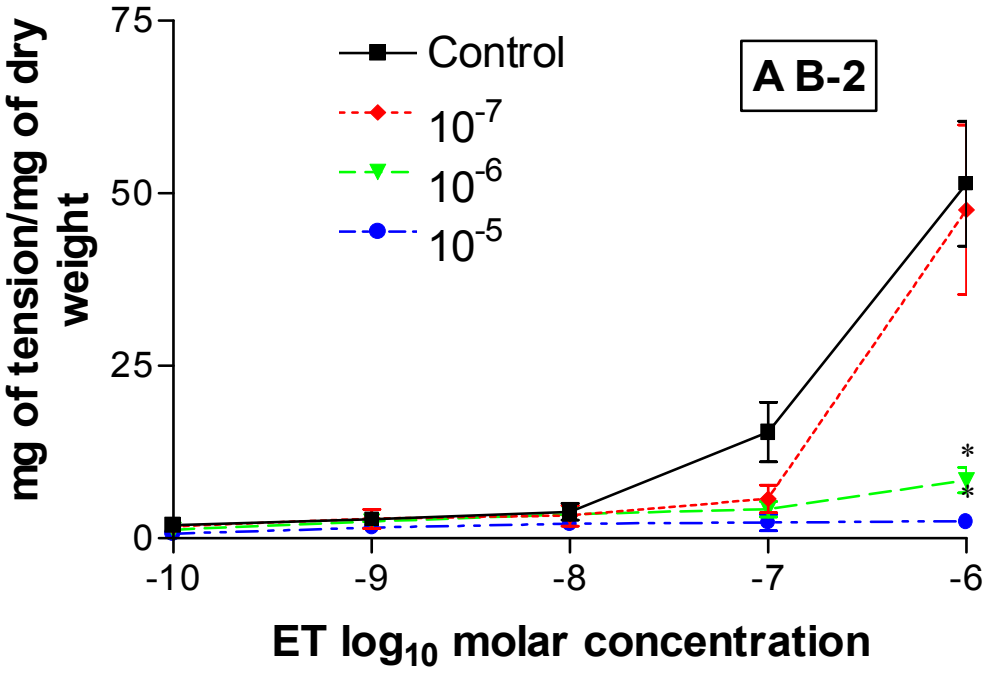
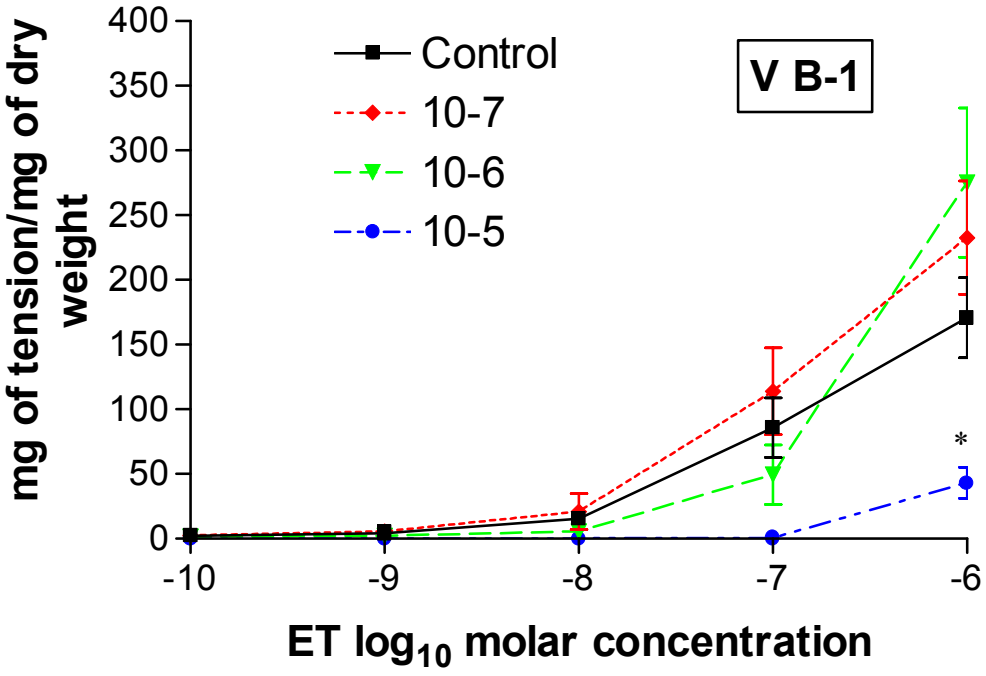
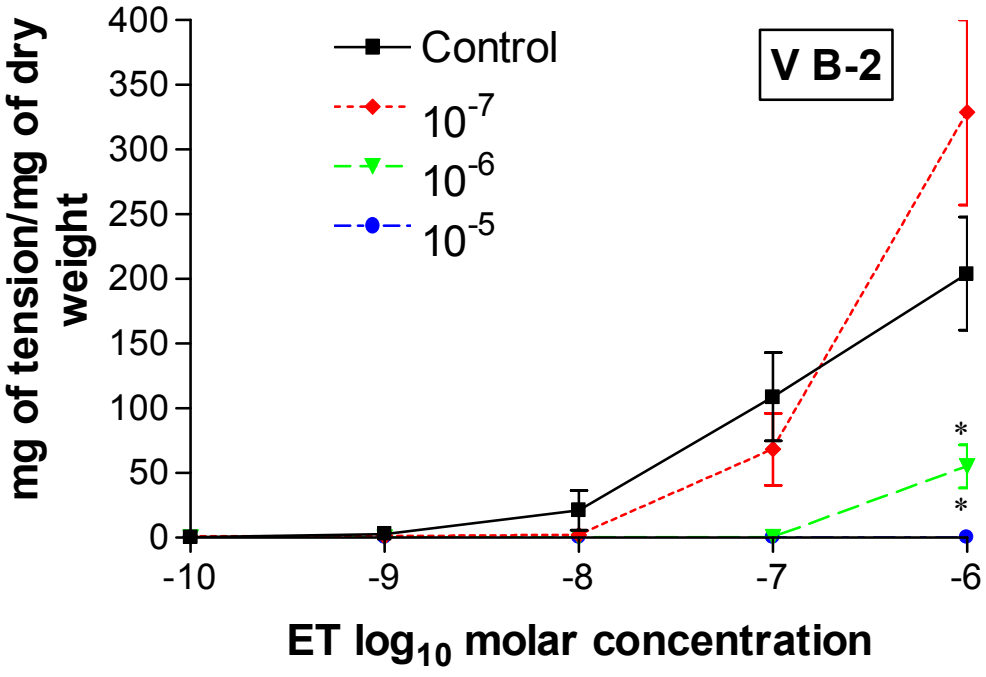


Figure 2.2 - Mean ±SEM concentration-response (C-R) curves for veins with endothelin-1 (ET-1) (10^{-10} to 10^{-6} M) expressed in mg of tension/mg of dry weight. ET-1 C-R for veins (V) following incubation with PD 142893 (B-1) (10^{-7} to 10^{-5} M) or Tyrode's (control) (top panel); ET-1 C-R for veins following incubation with PD145065 (B-2) (10^{-7} to 10^{-5} M) or Tyrode's (control) (bottom panel). *Apparent maximum contraction values differ significantly ($P < 0.05$) from control for these M concentrations of each ET-1 antagonist. Note the difference in the y-axis scale for veins vs. arteries incubated with B-1 or B-2.





the 10^{-7} M and the 10^{-6} M concentrations. The 10^{-6} M concentration for B-1 and B-2 also significantly reduced the maximum contraction of veins compared with the 10^{-7} M concentrations of the respective antagonist. Maximum contractions for veins treated with B-1 at the 10^{-6} M concentration were significantly greater than veins treated with B-2 at the same concentration. In control vessels and those incubated with B-1 or B-2, veins had greater maximal contractions than arteries to ET-1. Maximum contractions to the 10^{-6} M concentration of ET-1 of control veins were over 3 times greater than maximum contractions for arteries.

Relative EC_{50} values – Utilizing C-R curves of vessel rings based on maximum contraction of tissues to ET-1 10^{-6} M concentration, relative EC_{50} values were calculated. A significant difference was found between arterial and venous rings incubated with one of the two ET antagonists for ET-1; C-R EC_{50} values with B-2 having a significantly greater value than B-1 (Table 2.1). The EC_{50} values for arterial rings incubated with B-2 were significantly higher at the 10^{-5} M concentration compared with arterial rings incubated with B-1 at the same molar concentrations. Arteries incubated with 10^{-5} M concentration of either ET antagonist had significantly greater EC_{50} values than arteries in control conditions.

For veins incubated with ET antagonists, the EC_{50} values for ET-1 C-R were significantly greater for B-2 than B-1 at 10^{-6} M and 10^{-5} M concentrations. Veins incubated with 10^{-5} M concentration of either ET antagonist had significantly greater EC_{50} values than veins in control conditions. Veins had a significantly greater EC_{50} value compared with arteries after incubation with B-2 at 10^{-5} M concentration.

Table 2.1 - Mean ±SEM relative EC_{50} and apparent maximum contraction values for arteries and veins after concentration-response (C-R) curves with endothelin-1 (ET-1) for control vessel rings and those incubated with one of the concentrations of the ET receptor antagonist [B-1 (PD142893) or B-2 (PD145065) at 10^{-7}, 10^{-6}, or 10^{-5} M].

	Relative EC_{50} Values (log M concentration)		**Apparent Maximum Contraction (mg tension/mg tissue weight)**	
ET receptor antagonist concentration	**Arteries**	**Veins**	**Arteries**	**Veins**
Control	-6.80 (±0.039)	-7.00 (±0.089)	57.82 (±7.91)	187.49 (±26.24) *
B-1 10^{-7} M	-6.82 (±0.280)	-7.04 (±0.298)	84.12 (±25.07)	232.64 (±43.91)
B-1 10^{-6} M	-6.08(±0.080)	-6.79 (±0.206) †	33.03 (± 3.24)	275.15 (± 57.77)
B-1 10^{-5} M	-4.03 (±0.669)$^{\ddagger, \dagger}$	-4.87 (±0.574)$^{\ddagger, \dagger}$	5.19 (±1.98)$^{\ddagger, \S}$	43.14 (±11.88) ‡
B-2 10^{-7} M	-6.31 (±0.263)	-6.91 (±0.223)	47.62 (±12.26)	328.70 (±71.39)
B-2 10^{-6} M	-4.78 (±0.540)‡	-4.92 (±0.606)‡	8.62 (±1.84)‡	55.06 (±16.64) ‡,§
B-2 10^{-5} M	-2.53 (±0.738)‡	-1.02 (±0.000)‡,*	2.66 (±1.12)$^{\ddagger, \S}$	0 (±0) ‡,§

Control groups, n=16; all remaining groups, n=8. EC_{50} values lower than -6.00 log M concentration were extrapolated based on corresponding antagonist=s C-R curve. *Value significantly different from corresponding arterial value. †Relative EC_{50} values significantly different from B-2 vessel group at the same M concentration. ‡Significantly different from control values within column. §Maximum contraction significantly different from 10^{-7} M concentration within antagonist group. Statistical significance was set at $P < 0.05$ for all comparisons.

All vessel rings contracted following administration of NE or HST. Norepinephrine caused a greater maximal contraction in arteries (mean = 335.7 ± 45.5 SEM mg tension/mg dry weight) and veins (mean 559.4 ± 55.3 SEM mg tension/mg dry weight) after the ET-1 C-R curve compared with HST in arteries (mean 187.4 ± 21.8 SEM mg tension/mg dry weight) and veins (mean 393.8 ± 50.3 SEM mg tension/mg dry weight).

2.3.2 Study II - Comparisons of independent C-R curves for each vasoconstrictor agent revealed that the relative sensitivities (relative EC_{50} values and apparent maximal contractions) of digital vessels were NE = ET-1 > HST (10^{-10} to 10^{-6} M concentrations for ET-1, NE, and HST), and the differences between NE and ET-1 to HST were statistically significant (Table 2.2).

These results were consistent in both arteries and veins with veins being more responsive to all agents compared with arteries (Figure 2.3). At the 10^{-4} M concentration, HST had a significantly lower maximum contraction than NE for both medial and lateral vessels (Figure 2.4; Table 2.3).

No significant differences were observed for medial vs. lateral vessel rings for relative EC_{50} values or apparent maximal contractions for NE or HST.

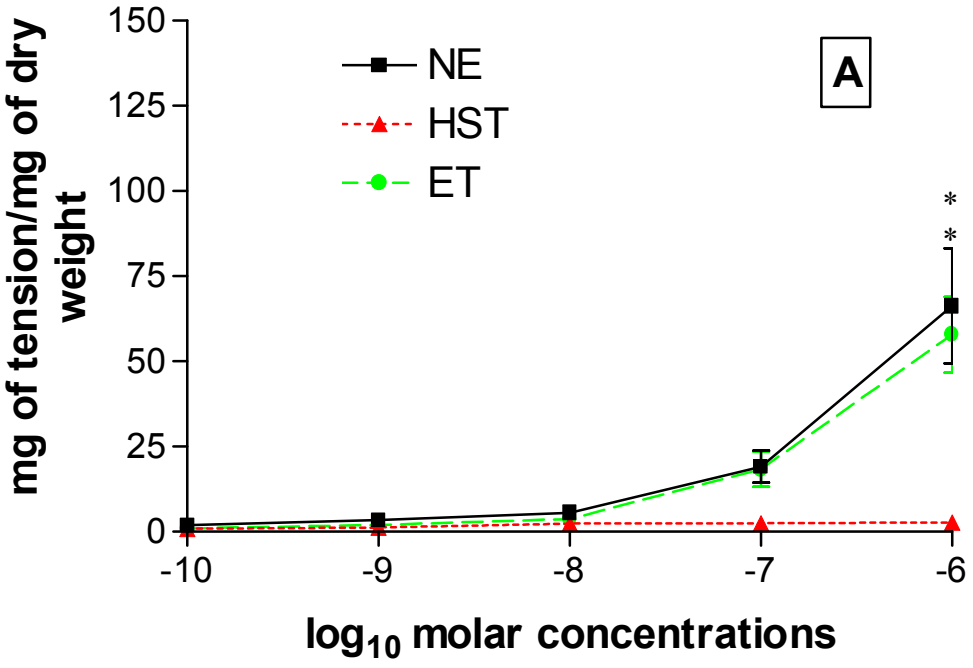
2.3.3 Drug Antagonism Values - The calculated pA_2 values for arteries and veins with B-2 were $10^{-7.121}$ and $10^{-6.747}$ M concentrations, respectively. The correlations (R^2) of the fitted lines with data points were 0.9931 and 0.9893 for B-2 in arteries and veins, respectively. The slopes of the fitted lines for arteries and veins with B-2 were not significantly different. The pA_2 values for B-1 could not be accurately calculated (Figure 2.5).

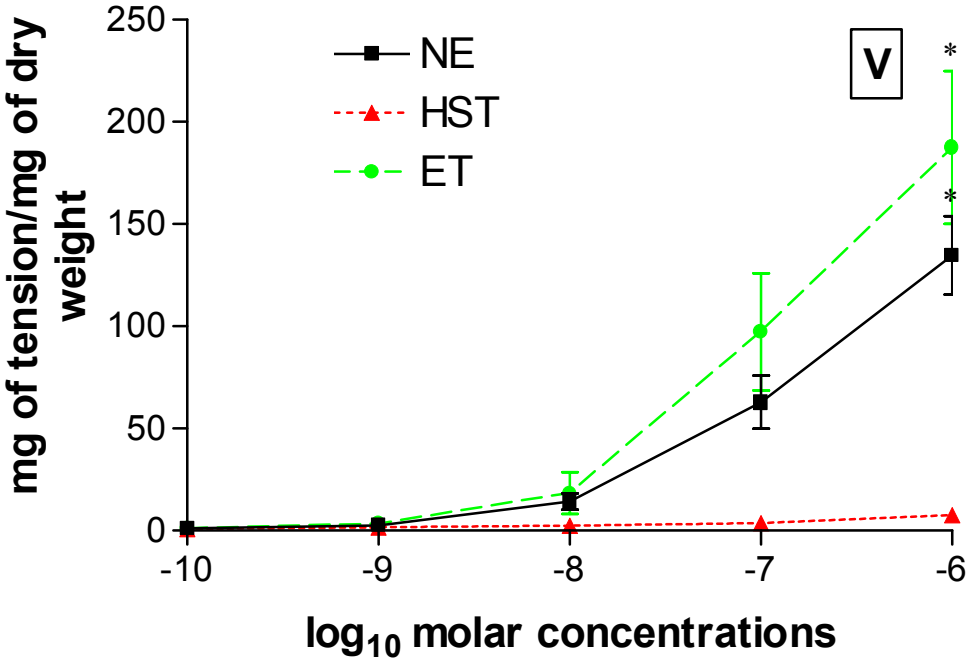
Table 2.2 - Mean ±SEM apparent maximum contraction values for arteries and veins after concentration-response curves with endothelin-1 (ET-1), norepinephrine (NE), or histamine (HST). For this table, only values for the 10^{-6} M concentration were compared for the three vasoconstrictors.

	Apparent Maximum Contraction (mg tension/mg tissue weight)	
Drug	**Arteries**	**Veins**
ET-1 (n=16)	57.82 (±7.91)[a]	187.49 (±26.24)[a]
NE (n=7)	66.31 (±16.85)[a]	134.67 (±19.25)[a]
HST (n=7)	2.75 (±0.84)[b]	7.68 (±2.40)[b]

[a,b]Within each column, different superscripts indicate values that are significantly ($P < 0.05$) different.

Figure 2.3 - Mean ±SEM concentration-response curves of medial arteries (A, top panel) and veins (V, bottom panel) to vasoconstrictors norepinephrine (NE), histamine (HST), and ET (10^{-10} - 10^{-6} M concentrations) expressed in mg of tension/mg of dry weight. *Apparent maximum contraction values differ significantly ($P < 0.05$) from HST.





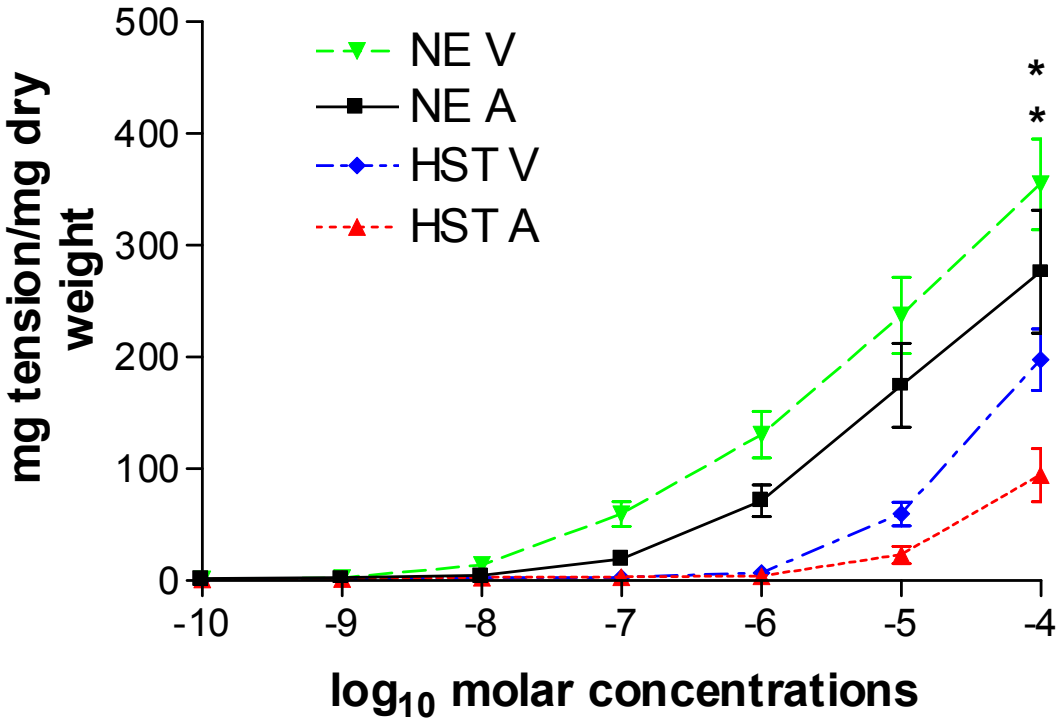


Figure 2.4- Mean ±SEM concentration-response curves of combined medial and lateral arteries (A) and veins (V) to vasoconstrictors norepinephrine (NE) and histamine (HST) (10^{-10} - 10^{-4} M concentrations) expressed in mg of tension/mg of dry weight. *Apparent maximum contraction values differ significantly ($P < 0.05$) from HST.

Table 2.3 - Mean ±SEM relative EC_{50} and apparent maximum contraction values for medial and lateral arteries and veins after concentration-response (C-R) curves with either histamine (HST) or norepinephrine (NE), 10^{-10} - 10^{-4} M concentrations.

	Relative EC_{50} Values				**Apparent Maximum Contraction (mg tension/mg tissue weight)**			
Drug	**Arteries**		**Veins**		**Arteries**		**Veins**	
	Medial	**lateral**	**Medial**	**lateral**	**Medial**	**lateral**	**medial**	**lateral**
HST (n=7)	-4.72 (±0.063)	-4.68 (±0.031)	-4.75 (±0.045)	-4.76 (±0.049)	76.51* (±27.51)	112.35* (±39.59)	231.22* (±30.91)	164.41* (±44.20)
NE (n=7)	-5.37 (±0.101)	-5.26 (±0.093)	-5.65 (±0.078)	-5.50 (±0.164)	240.43 (±49.95)	312.40 (±100.99)	352.58 (±51.35)	356.62 (±67.05)

*Significantly ($P < 0.05$) different from corresponding value for NE

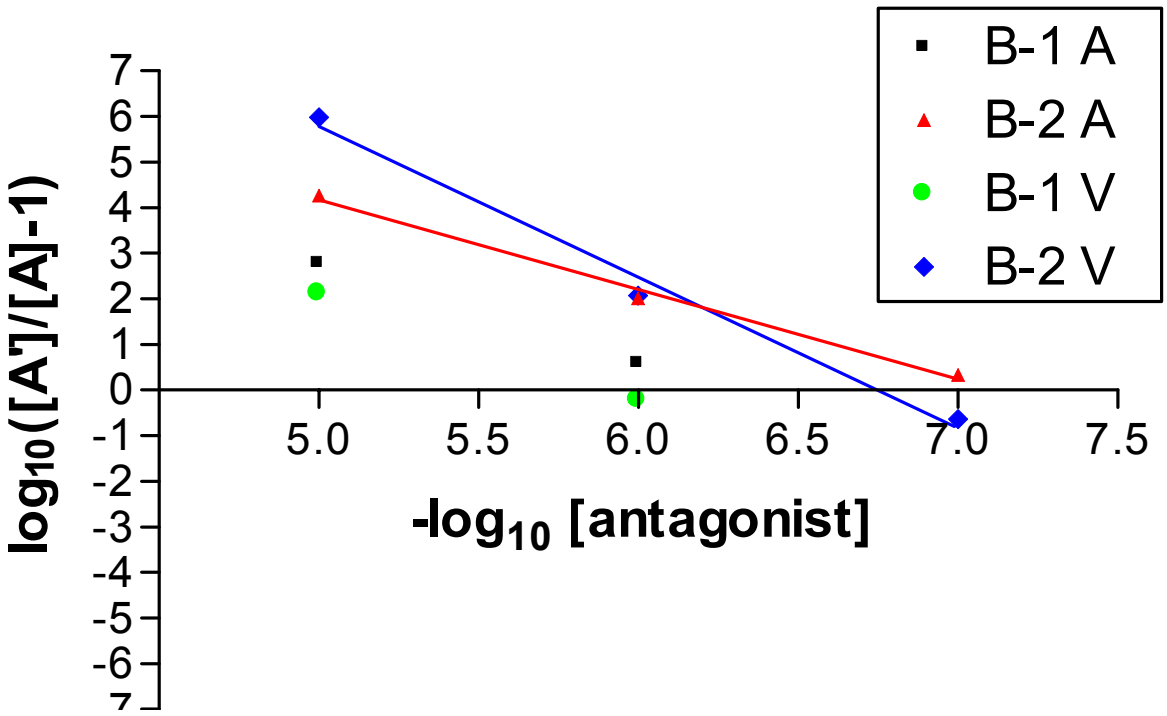


Figure 2.5- Schild regressions comparing antagonist potency between PD142893 (B-1) and PD145065 (B-2) for arteries (A) and veins (V). Regression lines could not be accurately determined for the 10^{-7} M concentration of B-1 for arteries or veins due to potentiation of ET-1-induced vasoconstriction; therefore, pA_2 values could not be calculated for B-1 since this value is equal to the x-intercept of this calculated line.

2.4 Discussion

This study resulted in several important findings. First, ET-1 caused a concentration-dependent contraction of palmar digital arterial and venous rings from clinically healthy horses in vitro. Veins were more sensitive and responded to a greater apparent maximum contraction, compared with arteries. Second, the ET antagonists evaluated in this study caused a concentration-dependent inhibition of the ET-1 induced contraction of arteries and veins except for the 10^{-7} M concentration incubation with B-1. This concentration of B-1 consistently shifted the entire C-R curve to the left and potentiated the effects of ET-1 in both arterial and venous rings. However, B-2 was most effective and at a concentration of 10^{-5} M inhibited the contractile effects of ET-1 in palmar digital veins and significantly reduced the effects in arteries. These findings are supported by studies utilizing rabbit femoral and pulmonary arteries which found that B-2 was 10-fold more potent than B-1 in binding at ET_A and ET_B receptors.[26] Third, the contraction caused by ET-1 was similar to that caused by the previously studied vasoconstrictor, NE.[20] Palmar digital vessels responded with similar sensitivity and to similar maximum contraction between ET-1 and NE, both of which were greater than responses to HST. Finally, the responses of the medial vs. lateral palmar digital vessels to NE and HST were not different.

This study demonstrates the presence of ET-1 receptors in equine palmar digital vessels that may play a role in the vascular constriction that occurs in the early stages of laminitis. Preparation and application of ET-1 using our in vitro organ-bath design produced results that correlated well with the data of Baxter using ET-1 in equine palmar digital vessel rings using the

same in vitro organ-bath design.[19] A previously published study in our laboratory using equine colonic vessels also produced very similar results regarding the in vitro contractility of vessel rings to ET-1.[8] The increased sensitivity and responsiveness of veins to ET-1 is interesting when considering the increased venoconstriction and imbalance of venous and arterial resistances previously documented in the early stages of experimentally-induced laminitis in horses.[14]

The apparent maximum contractile effects and relative EC_{50} values of ET-1, and the inhibitory effects of B-2, are consistent with similar studies using equine colonic vessel rings.[8] A higher EC_{50} value indicates that a greater concentration of ET-1 was required to reach 50% of the maximal contraction for that vessel. The B-2 antagonist was more effective in blocking the receptors and resulted in the vessels requiring a stronger concentration of ET-1 to cause contraction. Due to the current expense of ET-1, this study was limited to the use of a maximum concentration of 10^{-6} M; consequently, maximum contraction and EC_{50} values are based on the maximal response of tissues to this 10^{-6} M concentration of ET-1. Values were not based on an ideal 100% maximum contraction of tissues to concentrations greater than 10^{-6} M of ET-1, but our data correlates well with maximal contractions and EC_{50} values reported by Baxter examining ET-1 in palmar digital arterial and venous rings in vitro, although the maximum concentration of ET-1 in this previous study was 10^{-7} M.[19] In addition, the observed apparent maximum contractions of these tissues support the efficacy of B-2 at the 10^{-5} M concentration in attenuating the contractile effects of ET-1. Since the greatest concentration used in this study for ET-1 was 10^{-6} M, it is possible that with higher concentrations ET-1 may be able to overcome the antagonistic affects of B-2. However, it is unlikely that circulating concentrations of ET-1

would exceed this concentration in normal horses or those with pathological conditions such as laminitis. Katwa et al found increased laminar connective tissue ET-1 expression from laminitic horses (1.7 pg/mg of tissue) compared with non-laminitic horses (0.4 pg/mg of tissue).[27] Other studies have compared plasma ET-1 levels in horses with recurrent airway obstruction (6.53 pg/ml; healthy controls 3.74 pg/ml) and horses with various gastrointestinal diseases (3.29-10.02 pg/ml; healthy controls 1.8 pg/ml); although, care must be taken when comparing plasma ET-1 levels with that available to underlying smooth muscle since approximately 80% of ET-1 release is abluminal.[28,12,29] The lower concentrations of ET-1 used in this study better approximate likely physiological and pathophysiologic plasma levels of circulating ET-1 and these lower concentrations were effectively blocked by B-2 at the 10^{-5} M concentration in our study.

In previous in vitro studies evaluating effects of ET-1, NE, and HST on equine colonic vessels, each consecutive concentration of the agent was added at 2-minute intervals.[30,8,31] For this study, consecutive concentrations of ET-1 were added at 5-minute intervals since during preliminary studies we found the response to ET-1 in digital vessels to be slower, to plateau later, and to be longer lasting than the response to other agents. Since longer intervals were selected for this study, tissue viability and fatigue were a potential concern. Also, following incubation with the 10^{-5} M concentration of the antagonists, the response to ET-1 was minimal to absent. The addition of known potent vasoconstrictors, NE or HST, at the end of the ET-1 C-R curve demonstrated that the tissues were still viable and retained the ability to contract substantially.

Veins were more sensitive to the contractile effects of ET-1, resulting in greater EC_{50} values and maximum contractions, compared with arteries. Previous studies with equine colonic

vessels have suggested that modulation of relaxation may be of more importance for arteries (ex. tissue oxygen demand) and modulation of contraction more important for veins (ex. increases in venous return).[30] Although the primary effect of ET-1 in equine digital vasculature appears to be vasoconstriction through the ET_A receptors located on vascular smooth muscle cells, stimulation of ET_B receptors located on the endothelial cells may lead to vasodilation through the NO pathway, as found in other species.[5] This endothelium-dependent vasodilation may be of more importance in arteries than in veins.[30] This may explain why arteries had lower maximum contractions to ET-1 than veins, and may further suggest a role for ET-1 in the initial venoconstriction observed in the early stages of laminitis.[14]

The ability of an antagonist to reduce the maximum C-R for a given agent is expressed in terms of the pA_2 value; lower pA_2 concentrations indicate the antagonist has more affinity for the receptors in that tissue. Since the ET-1 antagonists used for this study are classified as competitive non-selective inhibitors for both the ET_A and ET_B receptor types, an equation of competitive antagonism can be applied to the data based on parallel log concentration–effect curves and relative EC_{50} values.[24] The B-2 antagonist fit this parallel design for each of the three concentrations allowing for accurate pA_2 calculation. Our calculated pA_2 values for B-2 (arteries $10^{-7.121}$ and veins $10^{-6.747}$ M concentrations) correlate well with previous studies in rats examining structure-activity relationships of various ET antagonists (femoral artery $10^{-6.9}$ and pulmonary artery $10^{-7.1}$ M concentrations).[26] Based on the R^2 correlation values, the linear regression lines applied to the data points demonstrated good fit. Although the pA_2 concentration for arteries was lower than veins, the difference was not statistically different. The B-1 antagonist fit the parallel design for the 10^{-5} M and 10^{-6} M concentrations, but the 10^{-7} M

concentration did not follow this parallel design for arteries or veins. Therefore, the B-1 pA_2 values could not be calculated and pA_2 and slope comparisons to B-2 could not be made. At the 10^{-7} M concentration, B-1 attenuated the ET-1 contractile effects resulting in both a shift of the C-R curve to the left and an overall decrease in relative EC_{50} values, compared with control vessels. Competitive receptor antagonism, as a rule, shifts the C-R curve to the right and increases the EC_{50} values due to a linear decrease in receptor availability. Further studies examining lower concentrations of these antagonists may elucidate the mechanism of this observed response.

Previous studies have not examined whether differences exist between medial and lateral palmar digital vessel responses to vasoconstrictor agents. There were no significant differences between the responses of medial vs. lateral vessels utilizing the vasoconstrictors NE and HST; therefore, as expected the medial and lateral vessels can be used interchangeably in future in vitro studies. Response of vessel rings to NE (10^{-10} to 10^{-4} M concentrations) and HST (10^{-10} to 10^{-4} M concentrations) were similar to those measured in previous studies using equine palmar digital vessel rings.[20,32]

Following experimentally-induced laminitis, microvascular thrombi formation has been documented in the laminae in addition to the previously mentioned hemodynamic alterations.[33,34] Endothelin-1 has been shown to cause rolling and adherence of leukocytes in rat postcapillary venules and induces shape changes and activation of human platelets.[35,36] Previous studies have shown that both ET_A and ET_B receptors likely play a role in platelet activation, thus demonstrating another benefit to the use of a non-selective ET receptor antagonist.[36] Further studies are needed to further define the role of ET-1 in equine platelet-

neutrophil interactions in normal horses and those with acute laminitis. The ability to competitively inhibit local ET receptors may prove to be beneficial in preventing and treating microthrombi formation as well as the hemodynamic alterations found in acute laminitis in horses.

In summary, this study demonstrated that ET-1 is a potent and sustained vasoconstrictor of the equine digital vasculature and ET receptor antagonists are effective in reducing ET-1 contractile effects. Endothelial release of ET-1 in horses with early laminitis may lead to appreciable increases in digital vascular resistance owing principally to venoconstriction. Since the onset of laminitis is believed to be related to early venoconstriction, an ET receptor antagonist may be potentially useful in prevention and treatment of susceptible horses. The receptor antagonist B-2 has the most potential as a therapeutic agent in this capacity, but needs to be investigated further to determine its in vivo effectiveness in reducing digital vasoconstriction and improving blood flow in clinically healthy horses administered ET-1 and in horses with experimentally-induced laminitis.

2.5 Product Information

[a] Sodium pentobarbital, The Butler Co, Columbus, Ohio.

[b] Model FT03 force-displacement transducer, Grass Medical Instruments, Quincy, Mass.

[c] Model 7D polygraph, Grass Medical Instruments, Quincy, Mass.

[d] PD 142893, Sigma Chemical Co, St Louis, MO.

[e] PD 145065, American Peptide Co, Sunnyvale, Calif.

[f] Endothelin-1, Research Biochemicals International, Natick, Mass.

[g] Arterenol, Sigma Chemical Co, St Louis, MO.

[h] Histamine (diphosphate), Sigma Chemical Co, St Louis, MO.

[i] GraphPad Prism, Version 2.0, GraphPad Software, Inc., San Diego, Calif.

[j] Proc mixed, SAS version 6.12, SAS Institute, Cary, NC.

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CHAPTER 3. IN VITRO RESPONSES OF PALMAR DIGITAL VESSEL RINGS FROM HORSES WITH NATURALLY-ACQUIRED LAMINITIS TO ENDOTHELIN-1

3.1 Introduction

Acute laminitis (founder) is a severely debilitating, potentially career-ending and often life-threatening disease of the sensitive and insensitive laminae of the equine digit. Laminitis can occur in adult horses and ponies of any breed or use and is often associated with other diseases such as colic, particularly strangulating obstruction and inflammatory bowel disease, grain overload, retained fetal membranes and subsequent metritis, pleuropneumonia, and other diseases associated with endotoxemia.[1,2] Additionally, laminitis occurs commonly in the contralateral limb of horses that have a severe non-weight bearing lameness in the opposite limb.

The ability of the equine athlete to walk depends on the integrity of the interdigitating primary and secondary laminae, which structurally unite the hoof wall, distal phalanx, and the sole of the foot into a single unit.[3] Clinicians currently employ numerous and varied therapies in the prevention and treatment of laminitis, which reflects the lack of a complete understanding of the pathophysiology of this disease. There are three principal theories regarding the mechanisms responsible for the development of laminitis: the ischemic/vascular, metabolic/enzymatic, and mechanical/traumatic theories.[4] The ischemic/vascular theory involves altered digital perfusion as the initiating factor in the cascade of events that leads to metabolic dysfunction and structural failure of the laminae.[5] The fundamental pathophysiologic mechanisms are believed to be venoconstriction followed by edema, ischemia, and necrosis of the interdigitating laminae ultimately leading to mechanical failure with rotation or sinking of the distal phalanx.[6-8] Measurement of digital perfusion in horses with acute laminitis has resulted in conflicting findings.[9,10] Differences may be due to timing of blood flow measurements since recent studies have findings supporting both decreased (before the onset of lameness) and increased (at the

onset of lameness) digital blood flow occurring during the developmental stages of the disease.[11,12]

In physiologic states, the endothelium synthesizes vasoactive substances, such as nitric oxide (vasodilator) and endothelin-1 (ET-1; profound vasoconstrictor), which play a substantial role in the regulation of vasomotor tone.[13] Endothelin is a 21 amino acid peptide first isolated by Yanagisawa and colleagues in 1988.[14] Endothelin has three isoforms, namely ET-1, ET-2, and ET-3; however, the vascular endothelium and smooth muscle cells only synthesize the ET-1 isoform.[15] Biosynthesis of ET-1 occurs in the endothelium and the two main receptor types for ET-1 are located on vascular smooth muscle cells and endothelium, namely ET_A and ET_B receptors, respectively.[16] Many conditions in humans such as atherosclerosis, hypertension, Raynaud's syndrome, and asthma, are associated with increased ET-1-induced smooth muscle contraction and increased plasma ET-1 concentrations.[17-21] In particular, Raynaud's syndrome in humans has many pathologic similarities with equine laminitis such as early ischemia due to decreased digital microcirculatory perfusion followed by reperfusion leading to painful, throbbing digits, and increased ET-1 expression. Raynaud's syndrome and laminitis have been proposed to be the same disease but in different species.[19,20,22] Therefore, studies examining the effects of ET-1 in the digital vasculature of horses with laminitis may further our understanding of the pathogenesis of this devastating disease.

Our hypothesis was that vessel rings from laminitic horses would respond in a similar manner to ET-1 as vessel rings from non-laminitic horses. The purpose of the study reported here was to determine the in vitro effects of ET-1, an ET receptor antagonist, norepinephrine, and histamine on palmar digital vessels from horses with naturally acquired laminitis and to compare

these results with those from a similar study with non-laminitic horses previously conducted in our laboratory.

3.2 Materials and Methods

3.2.1 Tissue Sources - This study was approved by the Institutional Animal Care and Use Committee of Louisiana State University. Palmar digital arteries and veins were collected from 9 horses with naturally-acquired active laminitis immediately after euthanasia with sodium pentobarbital[a] (90mg/kg, IV). The age of the horses ranged from 1 to 15 years (median, 11.5) and body weight from 373 to 591 kg (mean ± SEM, 448.7 ± 23.39). Horses were diagnosed with active laminitis based on their history, thorough physical and lameness examinations, and lateral radiographs of both front feet. Horses with acute and chronic active laminitis were included in this study.

3.2.2 Vessel Preparation - The medial palmar digital arteries and veins from one forelimb were collected and placed in chilled, oxygenated (95% O_2 and 5% CO_2) Tyrode's solution (136.87 mM NaCl; 2.68 mM KCl; 11.90 mM $NaHCO_3$; 5.55 mM dextrose; 1.81 mM $CaCl_2$; 1.07 mM $MgCl_2$; 0.36 mM NaH_2PO_4). Vessels were then gently cleansed of excess connective tissue and cut into 4 mm wide rings.[23-25] Isometric tension was monitored by attaching one side of the vessel ring to the stationary floor of an organ bath containing oxygenated Tyrode's solution at 37 C, and the other side to a force-displacement transducer[b] interfaced with a polygraph.[26, c] Based on preliminary studies in our laboratory and on previously published studies by Baxter et al utilizing palmar digital arteries and veins in vitro, an initial tension of two grams was determined to be the optimal resting tension and was applied to each vessel ring to mimic in vivo diastolic vascular tone.[24,25,27] The rings were allowed to equilibrate for 45 minutes. During this period, the bath solution was gently replenished with fresh Tyrode's

solution at 15-minute intervals and the tension was readjusted to two grams.[24,27] Tension was not reapplied after the last bath solution change.

3.2.3 Pharmacological Agents - An ET receptor antagonist, PD145065[d] was selected based on the ability to inhibit the contractile effects of ET-1 in palmar digital arteries and veins in a previous study.[28] The antagonist was dissolved with distilled water following manufacturer's recommendations and diluted with Tyrode's solution to the desired concentration (10^{-5} M). The ET-1[e] was dissolved in distilled water and frozen in aliquots at -80 C. Aliquots were thawed immediately prior to use and diluted with Tyrode's solution to the desired concentrations (10^{-10} to 10^{-6} M). Due to the expense of ET-1, we were limited to 10^{-6} M concentration as the strongest concentration of ET-1 for the concentration-response (C-R) curves. Both norepinephrine (NE)[f] and histamine (HST)[g] were dissolved in distilled water and were further diluted with Tyrode's solution to the desired concentrations (10^{-10} to 10^{-4} M).

3.2.4 Experimental Design - This study was conducted to test the contractile effects of ET-1 on equine palmar digital vessel rings from horses with active laminitis. The effectiveness of the ET receptor antagonist in its ability to inhibit ET-1-induced contraction was also examined. Therefore, relative ET-1 C-R relationships were determined with and without incubation with the ET receptor antagonist. Afterward, NE or HST was added to test for tissue fatigue and viability. Using separate vessel rings, C-R relationships for NE and HST were determined in order to examine the relative response of palmar digital vessels from laminitic horses to known potent vasoconstrictors.

Each organ bath contained one vessel ring prepared as previously mentioned. A total of eight organ baths were used simultaneously containing four arterial and four venous rings. The first arterial bath was a control and did not receive the ET antagonist. The arterial ring in the

second organ bath was incubated with a 10^{-5}M concentration of the ET antagonist. Incubation occurred during the last 30 minutes of equilibration by adding the antagonist to the bath at each of the three times the bath solution was replenished. Cumulative C-R relationships were determined for ET-1 for the first and second baths (10^{-10} to 10^{-6} M). Each consecutive concentration of ET-1 was added to the baths at 5-minute intervals. At the end of each ET-1 C-R curve, either NE or HST was added to the baths in increasing concentrations (10^{-6} to 10^{-4} M) to test for tissue fatigue and viability. Tissue baths three and four received increasing cumulative concentrations of NE and HST (10^{-10} to 10^{-4} M), respectively, in the absence of the ET antagonist. Each consecutive concentration of NE or HST was added to the baths at 2-minute intervals. This 4-bath design was repeated simultaneously with venous rings from the same horse in adjacent organ baths. The order of the bath design was randomly determined so that each treatment group was evaluated an equal number of times in each run. The dry tissue weight was determined afterward by allowing the rings to dry at room temperature (20-22 C) and measuring their weight on an analytical balance until weight loss was no longer observed.

Concentration-response curves were recorded for each vessel ring, apparent maximum responses to ET-1, NE, or HST were measured, and relative EC_{50} values were calculated using nonlinear regression fitting a sigmoid curve to the C-R data.[h] It should be noted that due to the expense of ET-1, the C-R curves were limited to the concentrations of 10^{-10} to 10^{-6} M. The contractions of vessel rings following ET-1 10^{-6} M concentration were considered apparent maximum contractions for relative EC_{50} calculations and throughout this manuscript maximum contractions and EC_{50} values are in context of these limitations. Relative EC_{50} values greater than -6.00 log M concentration were extrapolated based on the corresponding C-R curve.[h] Area under the curve was calculated based on each C-R curve.[h]

Responses for these 9 laminitic horses were compared to responses from 8 clinically healthy non-laminitic (control) horses in a previous study where the same experimental procedures were utilized using palmar digital arterial and venous rings.[28]

3.2.5 Statistical Analyses – Data was considered continuous and found to follow a normal distribution using the Shapiro-Wilk test with failure to reject the null hypothesis of normality at $p \leq 0.05$. The data was summarized and presented as mean ± SEM. The data was analyzed using a mixed effect linear model that accounted for the random variance of horse and the repeated measurements on each horse. Where there were significant interaction effects at $p \leq 0.05$, predetermined least squares means comparisons were made to determine where differences were occurring within each group at various concentrations, and between groups at specified concentrations. Type I error was maintained at 0.05. SAS PROC MEANS, UNIVARIATE, and MIXED were used for the analysis.[i]

3.3 Results

3.3.1 Clinical Data – Seven Quarter horses, 1 Quarter horse cross, and 1 Palamino comprised the 9 laminitic horses in this study. Five were female, 2 intact males, and 2 geldings; Obel grade ranged from 2 to 4 with a median of 3. Horses were admitted to the Veterinary Teaching Hospital and Clinic with gastrointestinal disease (3), acute laminitis (1), and chronic active laminitis (5) as primary presenting diseases.

3.3.2 Apparent Maximum Contraction Values – Arterial and venous vessel rings not incubated with the ET antagonist contracted in a concentration-dependent manner to ET-1. Venous rings had greater apparent maximum contractions to ET-1 with control tissues compared with tissues incubated with the ET antagonist PD145065 (Fig. 3.1 and Table 3.1). After administration of the 10^{-6} M concentration of ET-1 in the absence of the antagonist, veins also

had significantly greater maximal contractions compared with arteries. Within the arterial vessel rings, there was no significant difference between antagonist-treated and untreated groups. After completion of ET-1 C-R curves, vessel rings contracted further following administration of NE or HST (10^{-6} to 10^{-4} M concentrations). Comparisons of ET-1 C-R curves to NE and HST C-R curves (10^{-10} to 10^{-6} M concentrations) found ET-1 had significantly greater apparent maximum contractions at the 10^{-6} M concentration for venous rings (Fig. 3.2 and Table 3.2).

3.3.3 Relative EC_{50} Values - For both arteries and veins, vessel rings incubated with the ET antagonist required a significantly stronger concentration to reach 50% of the apparent maximal contraction to ET-1 (relative EC_{50}) compared with vessel rings without the antagonist.

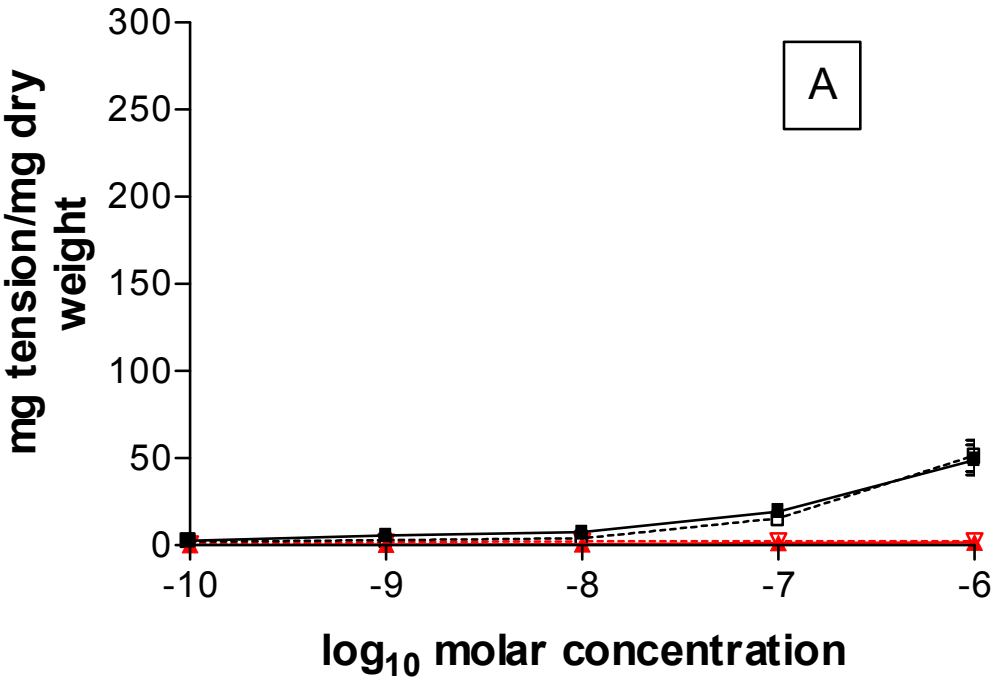
3.3.4 Area Under the Curve – Values for venous rings were significantly lower for antagonist-treated vessels, compared with control vessels. Comparison between arterial and venous rings revealed control veins had greater area under the curve values compared with arterial control rings.

3.3.5 Comparison to Normal Horses – There were no significant differences between laminitic and non-laminitic horses for relative EC_{50}, apparent maximum contraction, or area under the curve values.[28]

3.4 Discussion

The key findings of this study are that (1) arterial and venous rings from laminitic horses contracted in a concentration-dependent manner to ET-1; (2) venous rings from laminitic horses had greater apparent maximal contractions and greater area under the curve values compared to arterial rings; (3) antagonist-treated vessel rings from laminitic horses had greater EC_{50} concentrations than control rings from laminitic horses; (4) antagonist-treated venous rings from laminitic horses had lower apparent maximum contraction and lower area under the curve values

Figure 3.1 – Mean ± SEM concentration-response (C-R) curves for arteries (A) and veins (V) to endothelin-1 (ET-1) (10^{-10} to 10^{-6} M) [laminitic horses (square) and non-laminitic horses (open square)] and to ET-1 after incubation with PD145065 (ET antagonist) [laminitic horses (triangle) and non-laminitic horses (open triangle)] expressed in mg of tension/mg of dry weight. *Values differ significantly ($P \leq 0.05$) from control vessel rings at this equimolar concentration.



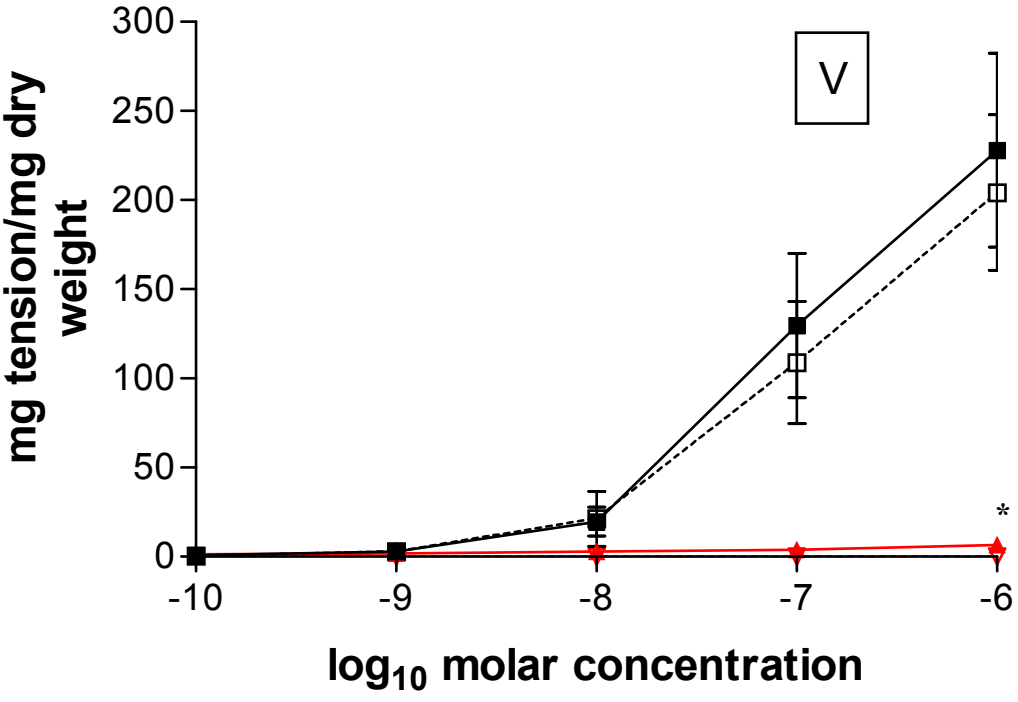
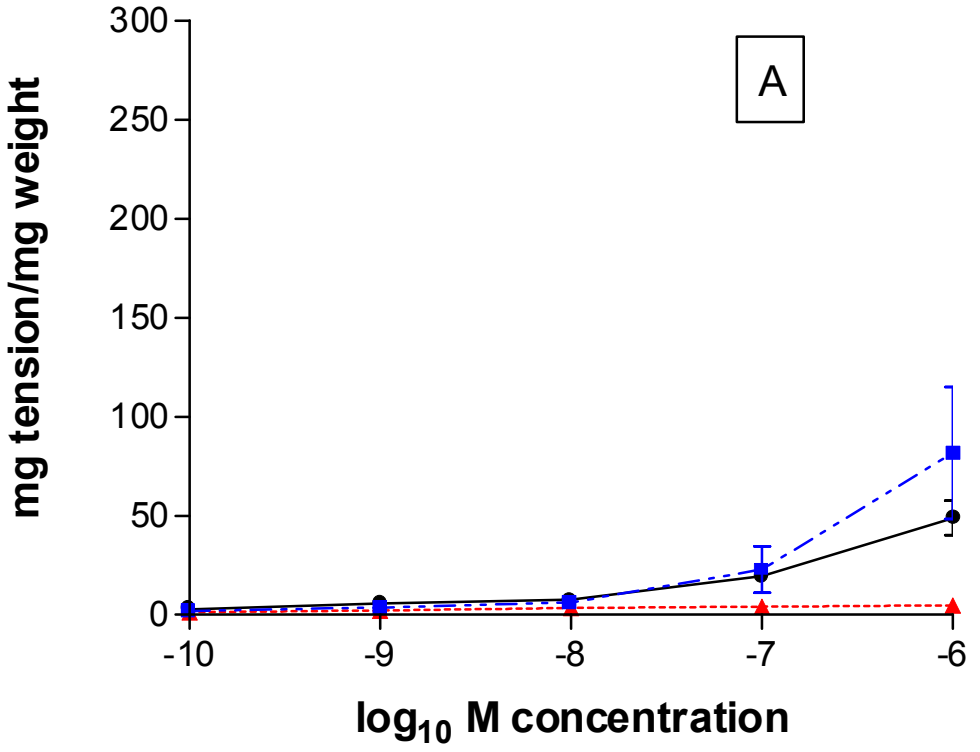


Table 3.1 - Mean (SEM) relative EC_{50}, area under the curve, and apparent maximum contraction values for arterial and venous rings after concentration-response (C-R) relationships to endothelin-1 (ET-1) for untreated vessels and those treated with the 10^{-5} M concentration of the ET receptor antagonist PD145065. Data from a previous study utilizing the same methods with vessel rings from non-laminitic horses are also presented for comparison.[28]

Concentration-Response Agents	Relative EC_{50} Values (log M concentration)		Area Under Curve		Apparent Maximum Contraction (mg tension/mg dry weight)	
Laminitic Horses	**Arteries**	**Veins**	**Arteries**	**Veins**	**Arteries**	**Veins**
ET-1 Control (n=9)	-7.08 (0.120)	-7.02 (0.071)	58.45 (8.508)	266.56 (74.306) *	48.92 (8.641)	227.93 (54.432) *
PD145065 + ET-1 (n=9)	-1.61 (1.082)†	-2.15 (1.046) †	2.58 (1.356)†	12.47 (5.837)†	1.57 (0.729)	6.51 (2.457) †
Non-laminitic Horses	**Arteries**	**Veins**	**Arteries**	**Veins**	**Arteries**	**Veins**
ET-1 Control (n=16)	-6.80 (0.039)	-7.00 (0.089)	48.70 (9.096)	235.19 (72.785) *	57.82 (7.91)	187.49 (26.24) *
PD145065 + ET-1 (n=8)	-2.53 (0.738) †	-1.02 (0.000) †	5.55 (3.365)	0 (0) †	2.66 (1.12) †	0 (0) †

* Value statistically different from corresponding arterial value within a row.
† Value is significantly different from ET-1 control value within laminitic and non-laminitic group. There were no significant differences between laminitic and non-laminitic groups. Statistical significance was set at $P \leq 0.05$ for all comparisons.

Figure 3.2 - Mean ± SEM concentration-response (C-R) curves for arteries (A) and veins (V) collected from laminitic horses to endothelin-1 (ET-1; circle), norepinephrine (NE; square), and histamine (HST; triangle) (10^{-10} to 10^{-6} M) expressed in mg of tension/mg of dry weight. *Values differ significantly ($P \leq 0.05$) from ET-1-treated vessel rings at this equimolar concentration.



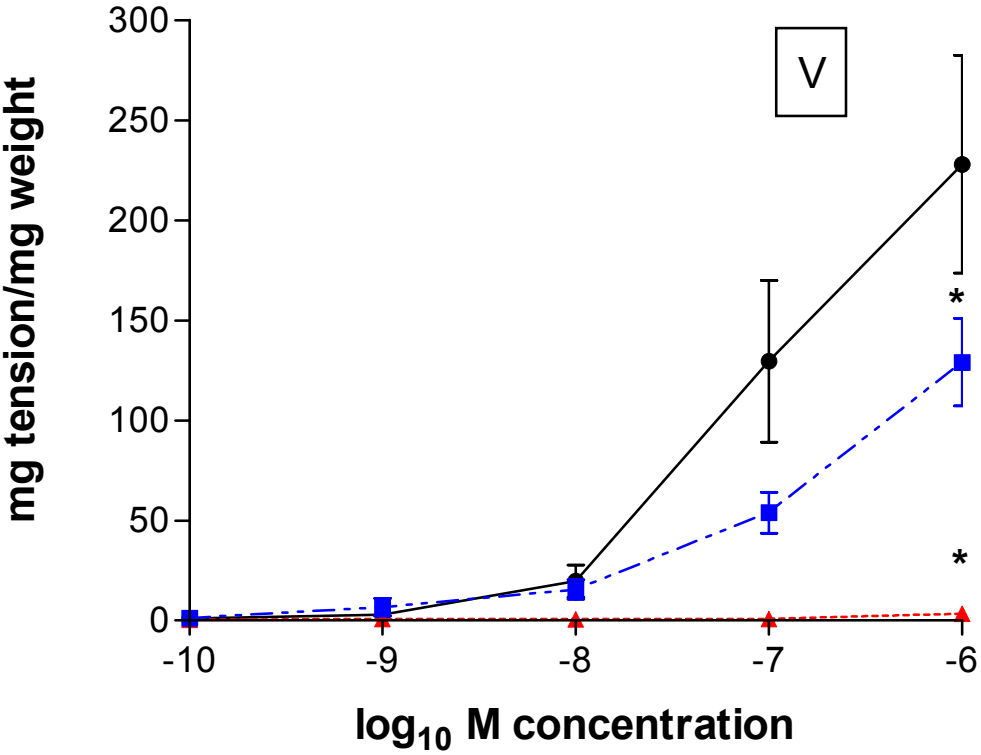


Table 3.2 - Mean (SEM) apparent maximum contraction values for arterial and venous rings from laminitic horses after concentration-response relationships to endothelin-1 (ET-1), norepinephrine (NE), or histamine (HST). For this table, only values for the 10^{-6} M concentration were compared for the three vasoconstrictors.

	Apparent Maximum Contraction (mg tension/mg dry tissue weight)	
Drug	**Arteries**	**Veins**
ET-1 Control (n=9)	48.92 (8.64)*	227.93 (54.43)[a]
NE (n=9)	81.63 (33.27)	129.05 (21.88)[b]
HST (n=9)	4.72 (2.56)	3.34 (0.84)[b]

[a,b]Within each column, different superscripts indicate values that are significantly ($P \leq 0.05$) different. * Values are significantly ($P \leq 0.05$) different between arteries and veins within each row.

than non-treated venous rings; and (5) there were no significant differences between laminitic and non-laminitic horses in response to ET-1 or the ET antagonist.

Our global hypothesis is that the vascular alterations during the developmental stages of acute laminitis are due to an imbalance between vasoconstrictors (ET-1; increased synthesis) and vasodilators (NO; decreased synthesis). The effectiveness of the ET receptor antagonist and the similarity of responses of vessel rings from laminitic, compared with non-laminitic, horses support our hypothesis that increased ET-1 synthesis likely account for the actions of ET-1 in the digital vessels. This hypothesis is further supported by findings in our laboratory in which palmar digital venous plasma ET-1 concentrations increased in horses after black walnut extract (BWE) induced acute laminitis and was also increased in horses with naturally-occurring colic.[29,j]

A limitation of our study is that due to the expense of ET-1, the 10^{-6} M concentration is the highest concentration utilized in this study. Although this is not an ideal experimental design for pharmacological studies with attainment of a true maximal contraction, our findings are consistent with those published by Baxter et al and with findings from studies within our laboratory.[23,24,28] We believe that our findings demonstrate a concentration-dependent contraction of vessels from laminitic horses to ET-1 and that administration of the ET receptor antagonist to vessel rings from these horses results in attenuation of ET-1-induced contraction.

The actions of ET-1 are mediated through two main receptor types, the ET_A and ET_B receptors.[16] The ET_A receptors are predominantly located on the surface of the vascular smooth muscle to elicit an increase in intracellular calcium and results in slowly developing, but sustained vasoconstriction.[16,30] The ET_B receptors are located principally on endothelial cells and trigger the vascular smooth muscle relaxing factors, such as NO and prostacyclin. Nitric

oxide released through this mechanism is believed to regulate release of ET-1, possibly through inhibition of the precursors of ET-1.[16] Recent research has determined that the subtypes ET_{B1} and ET_{B2} may exist and may have differing actions when stimulated by ET_B agonists.[31] The subtype ET_{B1} is thought to initiate relaxing factors, and ET_{B2} may have contractile properties similar to the ET_A receptor type. The present theory is that under normal physiologic states in vascular smooth muscle, ET-1 has predominately contractile effects through the ET_A receptor type and minor dilatory effects through the ET_{B1} receptor subtype, and few effects through the ET_{B2} subtype.[30] Although during pathologic states, such as hypertension and Raynaud's disease, the contractile effects through the ETB2 receptor may become significant.[30]

The ET antagonist selected for these studies is a non-selective, competitive inhibitor of both the ET_A and ET_B receptors.[32] This ET antagonist was selected based on previous in vitro studies within our laboratory that demonstrated it significantly reduced the contractile effects of ET-1 in equine colonic arterial and venous rings and digital vessels from normal horses.[23,28] The authors believe that until the roles of the ET_A and ET_B receptor types and subtypes are further defined, the use of a non-selective antagonist is warranted.

Increased venous resistance and high capillary hydrostatic pressures are consistent findings during the developmental stages of acute laminitis using both the carbohydrate overload (CHO) and BWE models of induction.[8,33] Endothelin-1 has been shown in other species and in other vascular beds in the horse to induce greater veno- than arterioconstriction.[23,24,34] The study presented here demonstrates consistency of greater venoconstriction of equine palmar digital vessel rings with these previous studies and has shown that with the onset of naturally-occurring laminitis, these vessels react in a similar manner compared with normal horses.[28] Current research has not identified the mediator responsible for observed increases in venous resistance

during the developmental stages of the disease, but based on the findings of our study and previous studies, increased ET-1 synthesis could account for these vascular alterations. Additional studies are necessary to determine the in vivo effects of ET-1, the ET receptor antagonist, and to further define the role of ET-1 in the pathophysiology of acute laminitis in horses.

3.5 Product Information

[a] Sodium pentobarbital, The Butler Co, Columbus, OH

[b] Model FT03 force-displacement transducer, Grass Medical Instruments, Quincy, MA

[c] Model 7D polygraph, Grass Medical Instruments, Quincy, MA

[d] PD 145065, American Peptide Co, Sunnyvale, CA

[e] Endothelin-1, Research Biochemicals International, Natick, MA

[f] Arterenol, Sigma Chemical Co, St Louis, MO

[g] Histamine (diphosphate), Sigma Chemical Co, St Louis, MO

[h] GraphPad Prism, Version 2.0, GraphPad Software, Inc., San Diego, CA

[i] Proc mixed, Univariate, and Means; SAS version 6.12, SAS Institute, Cary, NC

[j] Holm AS, Eades SC, Moore RM. Alterations in jugular and digital venous plasma endothlein-1 concentrations in horses administered black walnut extract. *Vet Surg* 31 (5) 491 2002.

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CHAPTER 4. JUGULAR AND CEPHALIC VENOUS PLASMA ENDOTHELIN-LIKE IMMUNOREACTIVITY AND DIGITAL VASCULAR AND LAMINAR IMMUNOHISTOCHEMICAL STAINING FROM HORSES WITH NATURALLY-ACQUIRED LAMINITIS

4.1 Introduction

In physiologic states, the endothelium synthesizes vasoactive substances, such as nitric oxide (NO; vasodilator) and endothelin-1 (ET-1; profound vasoconstrictor), which regulate vasomotor tone.[1,2] Endothelin is a 21 amino acid peptide and was first isolated by Yanagisawa and colleagues in 1988.[2] Endothelin has three isoforms, namely ET-1, ET-2, and ET-3, although vascular endothelium and smooth muscle cells only synthesize the ET-1 isoform.[3] In normal vasculature, the actions of basal ET-1 produced by the endothelium are predominantly paracrine in effect, but ET-1 can also be produced by vascular smooth muscle and may function in an autocrine manner to regulate both tone and structural modeling of vasculature.[4] In blood vessels, biosynthesis of ET-1 primarily occurs in the endothelium. Approximately 80% is released abluminally toward the vascular smooth muscle, and the two main receptor types for ET-1, ET_A and ET_B, are located on vascular smooth muscle cells and endothelium, respectively.[3,5] Many pathologic states in humans and other species, such as atherosclerosis, hypertension, tissue hypoxia, Raynaud's syndrome, and asthma, are associated with increased ET-1-induced smooth muscle contraction, increased plasma ET-1 concentrations, and increased tissue ET-1 (IHC) immunohistochemical staining.[6-11]

The ability of the equine athlete to walk depends on the integrity of the interdigitations of the primary and secondary laminae, which structurally unite the hoof wall, bone (distal phalanx), and the sole of the foot into a single unit.[12] Although the pathogenesis of laminitis is not fully understood, the fundamental mechanisms are believed to be initially vascular in nature, characterized by hypoperfusion due to venoconstriction, laminar edema formation, opening of arteriovenous (AV) shunts, which allow blood to bypass laminar tissues, leading to tissue ischemia, necrosis of the interdigitating laminae, and ultimately mechanical failure with rotation

or sinking of the distal phalanx away from the hoof wall.[13] The leading hypothesis regarding the initiating factor in the cascade of events leading to necrosis and structural failure of the interdigitating sensitive and insensitive laminae has focused on digital hemodynamic alterations; however, the initiating event that triggers these vascular alterations has yet to be determined.[13] Laminitis often occurs secondary to other diseases such as intestinal strangulating obstruction, inflammatory bowel disease, grain overload, metritis, and pleuropneumonia, and ET-1 has been implicated in the mechanisms of pathogenesis for these diseases in horses and other species.[14-17] In vitro studies conducted in our laboratory and those conducted by Baxter et al have determined that the vasoconstrictive effects of ET-1 are especially pronounced in equine palmar digital veins,[3,18,19] which in the development of equine laminitis may lead to increases in venous pressure, capillary hydrostatic pressure, laminar edema formation, and opening of AV shunts, allowing blood to bypass the laminar tissue. Differences between the contractile responses of arteries and veins to ET-1 have been documented in dogs and other species as well, such as in dogs, yet the reason for the increase in reactivity to ET-1 in veins compared with arteries remains uncertain.[20] Investigators have hypothesized that this difference may play a role in maintenance of venous tone during normal physiological states, or during pathologic states, such as hypotensive shock, this may aid in correction of altered vascular function.[20]

Our hypotheses are that plasma concentrations of ET-like immunoreactivity will be greater in horses with naturally-acquired laminitis, compared with the concentrations found in healthy horses, vascular and laminar tissues from naturally-acquired laminitic horses will stain for ET-1 with greater intensity than tissues from healthy horses, and samples of palmar digital veins will stain for ET-1 with greater intensity than tissues of palmar digital arteries. The purposes of these studies were to quantify jugular venous (JV) and cephalic venous (CV) plasma

ET-like immunoreactivity and examine ET-1 IHC staining of vascular and laminar tissues from horses with and without naturally-acquired laminitis.

4.2 Materials and Methods

4.2.1 Selection and Preparation of Horses - This study was approved by the Clinical Animal Care and Use Committee of Louisiana State University.

Forty-six light breed horses [21 females, 14 castrated males, and 11 males; ages ranging 1 – 23 years (mean = 10.9 years); weighing 365 to 548 kg (mean = 458 kg)] were selected for plasma collection for ET-like immunoreactivity quantification. Of these horses, 34 were diagnosed with clinical laminitis based on their history and thorough physical and lameness examinations. The remaining 12 horses were determined to be free of laminitis based on their history as well as physical and lameness examinations. Lateral radiographs of both front feet were available for 24 of the laminitic horses and 5 of the normal horses in order to further confirm the presence or absence of laminitis. Obel grade and complete blood count (CBC) data for packed cell volume (PCV) were recorded at the time of plasma collection.

Tissues were collected for ET-1 IHC staining from 21 light breed horses (16 females, 4 castrated males, and 1 male; ages ranging 1 – 21 years (mean = 9.9 years); weighing 400 to 516 kg (mean = 438 kg)). Of these horses, 12 were diagnosed with clinical laminitis based on their history and thorough physical and lameness examinations. The remaining 9 horses were determined to be free of laminitis based on their history as well as physical and lameness examinations. Lateral radiographs of both front feet were only available for 3 of the laminitic horses and none of the normal horses to further confirm the presence or absence of laminitis. Obel grade was recorded at the time of tissue collection.

4.2.2 Plasma Endothelin-Like Immunoreactivity – Jugular venous and CV blood samples (8 ml) were collected via venipuncture and placed in chilled tubes containing EDTA[a] (500 KIU/ml) and aprotinin[a] (1 mg/ml), an anticoagulant and a protease inhibitor. Samples were centrifuged immediately at 1,500 X g for 10 minutes. The plasma was then transferred into polypropylene tubes and stored at –70 C until analyzed for ET-like immunoreactivity.

Plasma ET-like immunoreactivity was quantified using a commercial human enzyme-linked immunosorbent assay (ELISA) kit.[b] One milliliter of plasma was thawed and mixed with 1.5 ml of precipitating agent (80 mls HPLC grade acetone + 12 mls precipitating agent) provided by the manufacturer. The samples were cooled to 4 C and centrifuged for 20 min at 3,000 x g. The supernatant was transferred into polypropylene tubes in a 37 C water bath and dried under a flow of nitrogen gas. The dried samples were reconstituted in 500 µl of assay buffer. Serial dilutions of the ET stock solution were prepared according to the manufacturer's guidelines to serve as standards. The buffer was used as the zero standard. The antibody used in this assay principally recognized ET-1 with 100% cross-reactivity, however, there was reportedly also 100% and <5% cross-reactivity with ET-2 and ET-3, respectively.

Two hundred microliters of standards, controls, and samples in duplicate were pipetted into wells coated with a polyclonal rabbit anti-ET antibody. Detection antibody (monoclonal mouse anti-endothelin antibody lyophilized with green dye, 50 µl) was added to all wells, except the blank, and then thoroughly mixed. The wells were covered with plastic film and incubated 16-24 hrs at room temperature (20 - 22 C). The contents of the wells were discarded and the wells washed 5 times with washing buffer. Conjugate (anti-mouse IgG antibody conjugated to horseradish peroxidase, 200 µl) was then added to all wells. The wells were covered again with plastic film and incubated for 3 hrs at 37 C. The contents of the wells were discarded and the

wells washed 5 times with washing buffer. Substrate (tetramethylbenzidine, 200 µl) was added and the wells incubated for 30 min at 20 C in the absence of light. Then, 50 µl of stop solution was added to all wells and mixed thoroughly. Absorption was determined immediately with an ELISA reader[c] at 405 nm against 620 nm as reference. All samples were analyzed in duplicate. The sensitivity of the assay was 1.5 pg/ml. The mean percentage recovery of a known quantity of ET-1 standard added to pooled plasma was 102%; the inter- and intra-assay variability for equine plasma was determined to be 15.4% and 6.4%, respectively, in our laboratory. The detection range was 1.59 – 25.65 pg/ml and sample dilution or concentration accounts for reported concentrations above or below the detection range, respectively.

4.2.3 ET-1 Immunohistochemical Staining – Tissue samples from the PDA, PDV, and laminae were collected from horses immediately after euthanasia with sodium pentobarbital[d] (90mg/kg, IV) and placed in zinc formalin for fixation for 24 hours. Tissues were paraffin-embedded, sectioned at 4 µm, attached to silanized slides, and dried overnight in a 37 C oven. Immunostaining was performed using a modified three-step Avidin-Biotin complex (ABC) method with a Vector Elite ABC Rabbit IgG kit[e]. Tissue sections were heated for 10 min at 60 C and were then deparaffinized and hydrated through graded alcohol solutions to distilled water. The remaining steps were completed at room temperature. Slides were placed in 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity, rinsed in distilled water, and equilibrated in 10 mM phosphate buffered saline with 0.25% tween 20 (PBS-Tween, pH 7.6). Slides were incubated in normal goat serum (1:50) for 45 min to block non-specific antibody binding. Excess serum was removed and slides were incubated with the primary antibody rabbit anti-endothelin-1 antiserum[f] for 60 min (1:700; diluted in goat serum). After incubation, slides were rinsed with PBS-Tween and incubated with the biotinylated secondary

antibody goat anti-rabbit IgG antiserum (1:200) for 30 min. Slides were rinsed with PBS-Tween and were incubated with the ABC (prepared according to manufacturer's directions) for 45 min. After rinsing with PBS-Tween, the horse-radish-peroxidase substrates were developed with the chromogen (0.06% diaminobenzidine (DAB) in 50 mM Tris-Cl buffer and 0.003% hydrogen peroxide) for 3 – 5 min while monitoring positive controls under a standard light microscope. The chromogen reaction was stopped by rinsing slides with distilled water. The slides were counterstained with Mayer's hematoxylin for 30 seconds, rinsed in tap water, and allowed to sit in tap water for 5 min. The final steps were dehydration through graded ethanol, clearing with xylene, and mounting in resinous mounting medium.

Validation of the specificity of the primary antibody for binding to ET-1 was completed using the antibody neutralization technique.[21] Briefly, the primary antibody, rabbit anti-ET-1 antiserum, was incubated (1:2) with the ET-1 peptide[g] overnight at 4 C. The IHC procedure described above was conducted on two identical subsets of study tissues with the second subset receiving the neutralized primary antibody substituted for the normal unneutralized primary antibody (subset one).

Proper IHC technique was further confirmed during each staining session of all study samples by inclusion of positive controls (equine heart, lung, and kidney) and negative controls (omission of primary antibody on equine heart, lung, and kidney sections).

Scoring of ET-1 staining intensity was completed using a standard light microscope in three fields of each slide three times by one investigator (AMS) and the modal value was determined. For PDA and PDV samples, the endothelium and the vascular smooth muscle were scored separately. For laminar samples, the epithelial cells and stroma of the epidermal laminae, and the endothelium and vascular smooth muscle of the adjacent parietal arteries and collecting

veins of the dermal laminae were evaluated. A value from 0 to 3 was assigned to each variable based on the relative amount of staining compared with slides using the antibody neutralization technique. A score of 0 was assigned if there was no staining present; 1 for mild brown staining; 2 for moderate brown staining; and 3 if intense brown staining occurred. Increased staining intensity would be associated with increased levels of ET-1 in the tissues.

4.2.4 Statistical Analyses – Plasma endothelin-1 like immunoreactivity - The data was summarized and presented as mean ± SEM and SAS[h] was used for all analysis. Data was considered continuous and found to follow a normal distribution using the Shapiro-Wilk test with failure to reject the null hypothesis of normality at $P \leq 0.05$. The data was analyzed using the following model:

$$y = \mu + \text{Horse} + \text{Disease State} + \text{Horse} * \text{Disease State} + \varepsilon$$

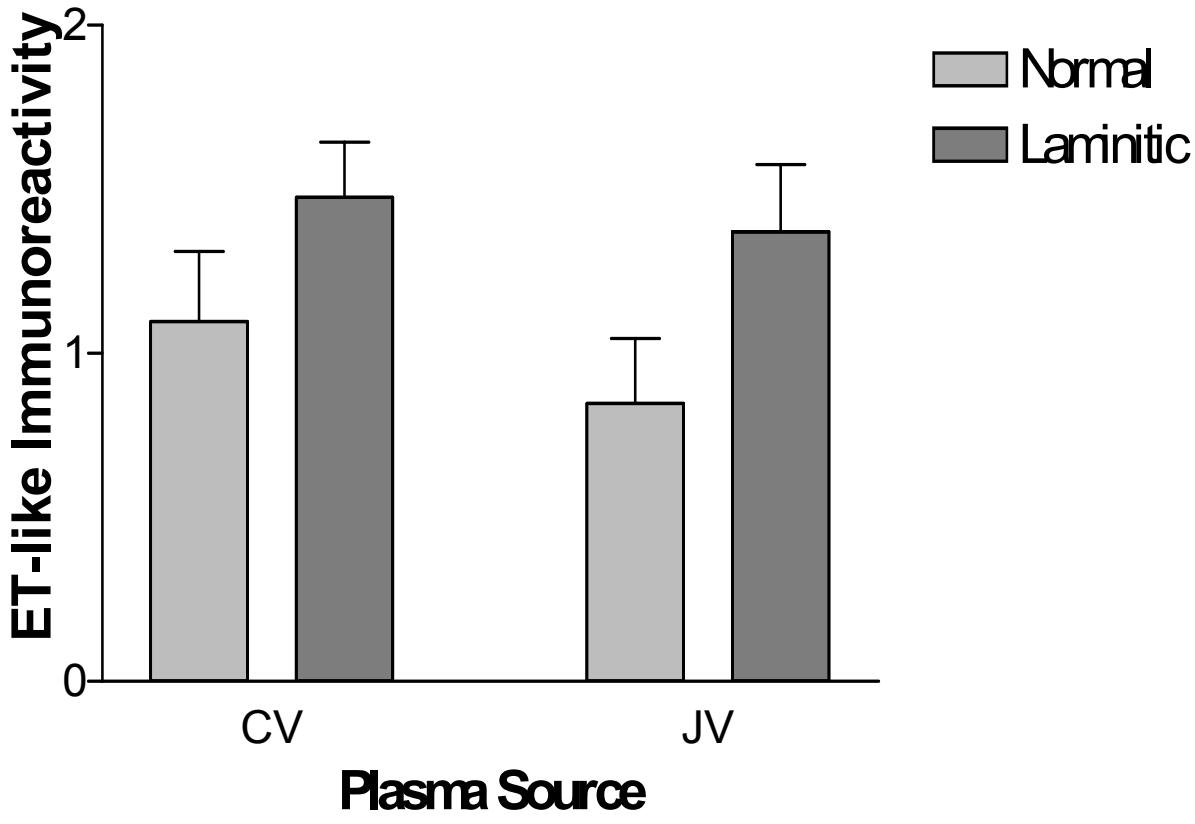
where the effect of horse was considered random and the effect of Disease State (Normal or Laminitic) was tested using the Horse interaction term. Type I error was maintained at 0.05. PROC MEANS, UNIVARIATE, and MIXED were used for the analysis. Further multiple comparisons were not necessary.

ET-1 immunohistochemical staining – Since the data was based on an ordinal/categorical scale of measurement, staining frequency counts were determined for each variable by disease state and data was presented descriptively and graphically.

4.3 Results

4.3.1 Plasma Endothelin-Like Immunoreactivity – Cephalic venous plasma ET-like immunoreactivity was not significantly different but had a trend ($P = 0.10$) for higher values in horses with laminitis (1.475 pg/ml) compared with normal horses (1.097 pg/ml) (Fig. 4.1). Jugular venous plasma ET-like immunoreactivity followed a similar trend ($P = 0.17$) between laminitic (1.370 pg/ml) and normal horses (0.848 pg/ml), but was also not significant. There was

Figure 4.1 – Mean ± SEM endothelin (ET)-like immunoreactivity (pg/ml) in normal, healthy control horses and those with naturally-acquired laminitis in cephalic venous (CV) and jugular venous (JV) plasma.



no significant difference in plasma ET-like immunoreactivity between CV and JV samples. Obel grade ranged from 1 to 4 for the laminitic group (mean = 2.823 +/- 0.171). There were no significant differences between PCV for these groups.

4.3.2 ET-1 Immunohistochemical Staining - Validation of the specificity of the primary antibody for binding to ET-1 was evident using the antibody neutralization technique. The tissue subset incubated with the neutralized primary antibody did not stain brown as evident in the subset incubated with the normal primary antibody (Fig. 4.2). During each IHC staining session, the positive and negative controls stained properly as noted by staining and no staining, respectively.

No differences were found for PDA or PDV samples, however, some parameters within the epidermal and dermal laminae had increased frequency of intense (grade 3) staining for normal horses compared with laminitic horses (Fig. 4.3 – 4.5). Within the epidermal laminae, the epithelial cells and stroma from normal horses were graded as mild to intense (grades 1 - 3), whereas these structures in tissues from laminitic horses were only graded as mild to moderate in staining (grades 1 – 2). Staining was also heavier in normal horses than laminitic horses within the parietal arterial endothelium (normal horses graded 1 – 3; laminitic horses graded 1 – 2) of the dermal laminae. Notable frequency differences were not found for the other variables evaluated within the laminae. Comparisons between parietal arteries and collecting veins of the dermal laminae did not reveal any differences between these vessel types (Fig. 4.6 and 4.7). Obel grade ranged from 1 to 4 for the laminitic group (mean = 2.667+/- 0.309).

4.4 Discussion

The important findings of these studies are that systemic and local cephalic venous plasma concentrations of ET-like immunoreactivity followed a trend to increase in horses with

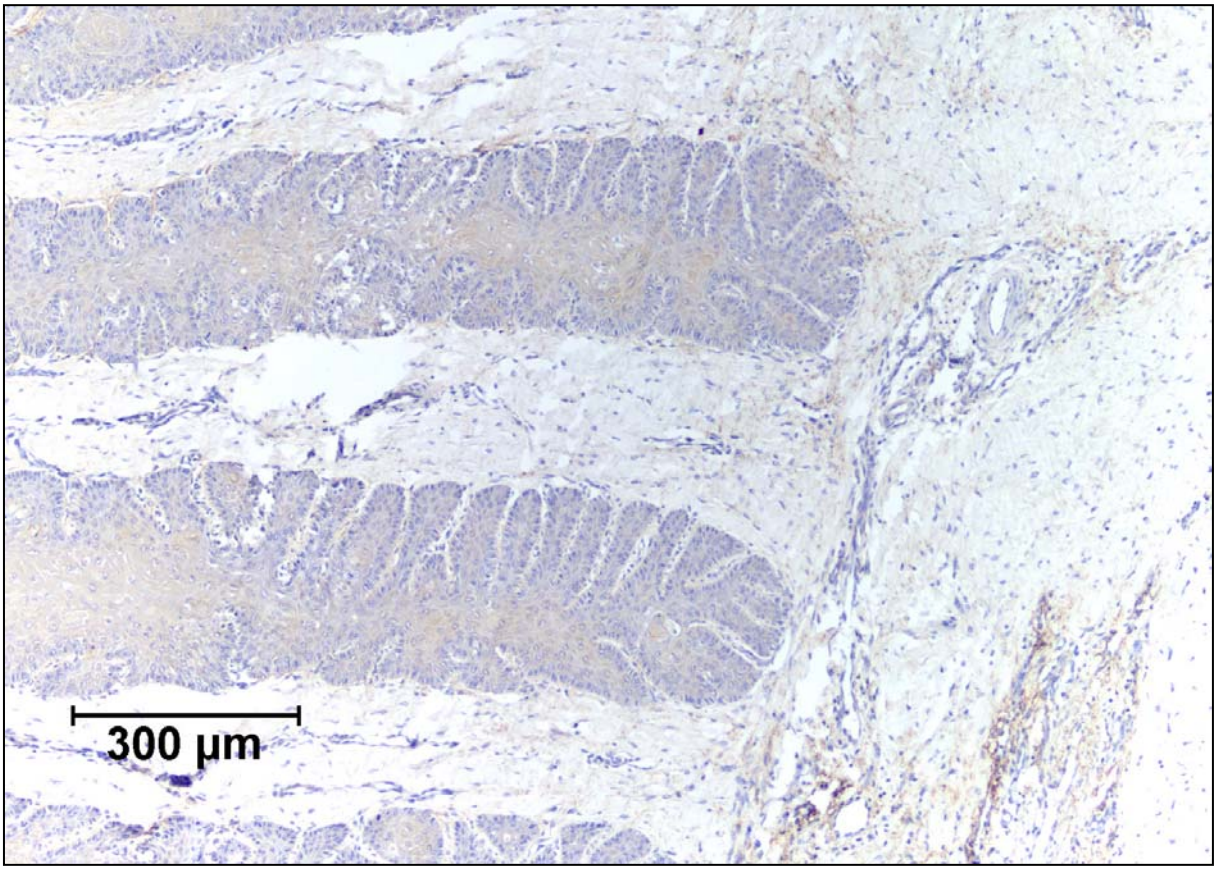
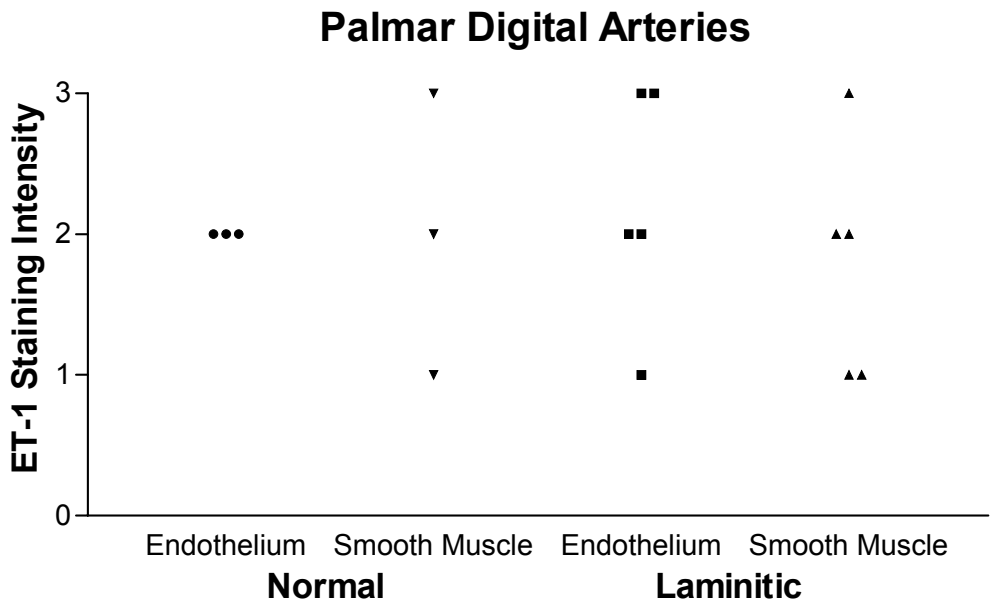


Figure 4.2 – Photomicrograph of equine laminae after immunohistochemistry using the antibody neutralization technique to validate the endothelin-1 antibody in equine tissues. Note the lack of staining in the epithelial cells and stroma of the epidermal laminae and the vascular structures of the dermal laminae.

Figure 4.3 – Scatter plots of modal values for ET-1 immunohistochemical staining intensity of palmar digital arterial and venous samples from normal and laminitic horses. Vascular endothelium and vascular smooth muscle were evaluated in both vessel types. A 0 score was assigned if there was no staining present; 1 if mild brown staining; 2 if moderate brown staining; and 3 if intense brown staining. Increased staining would be associated with increased presence of ET-1.



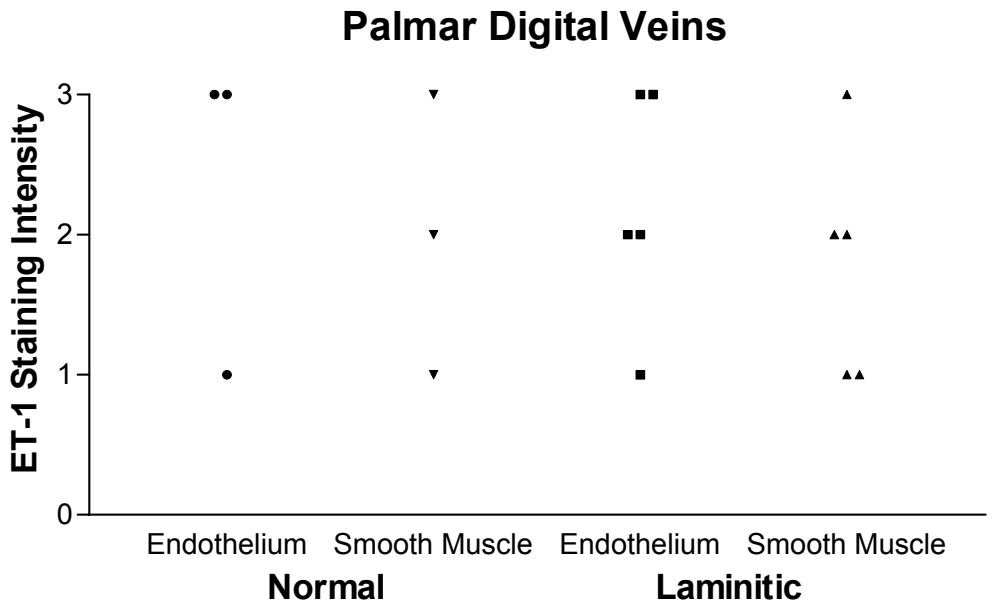
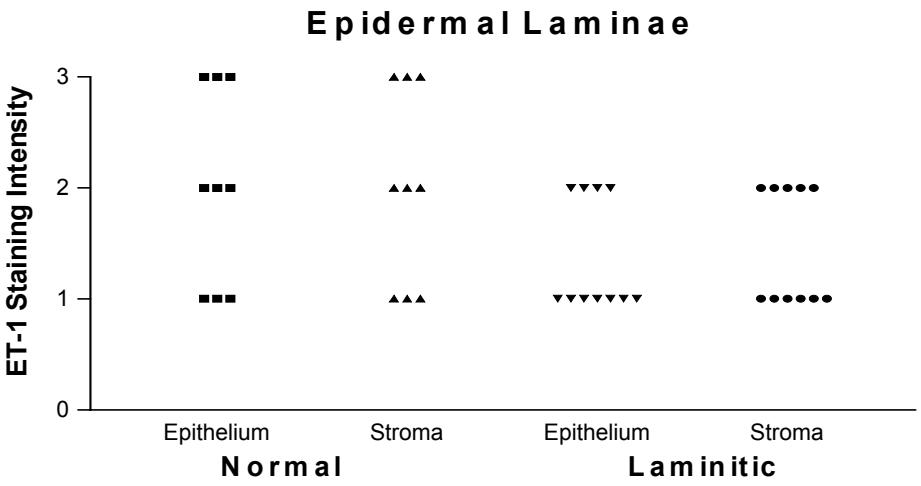
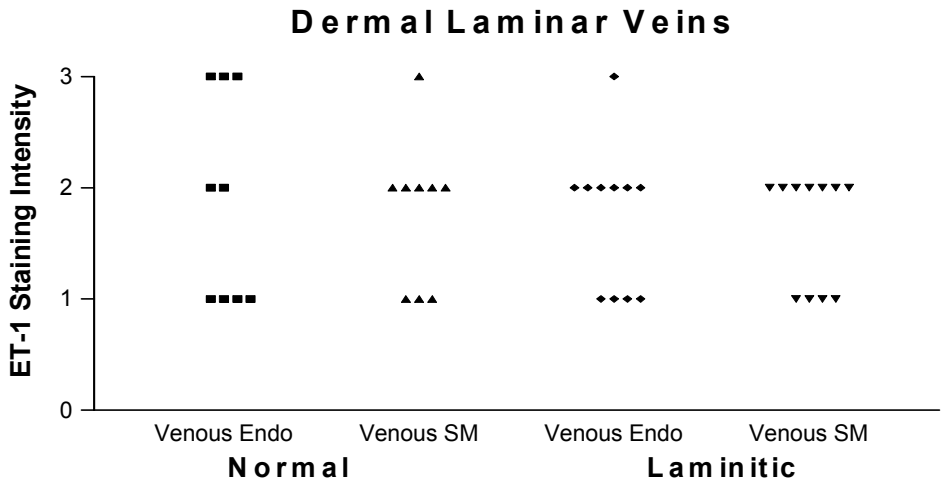
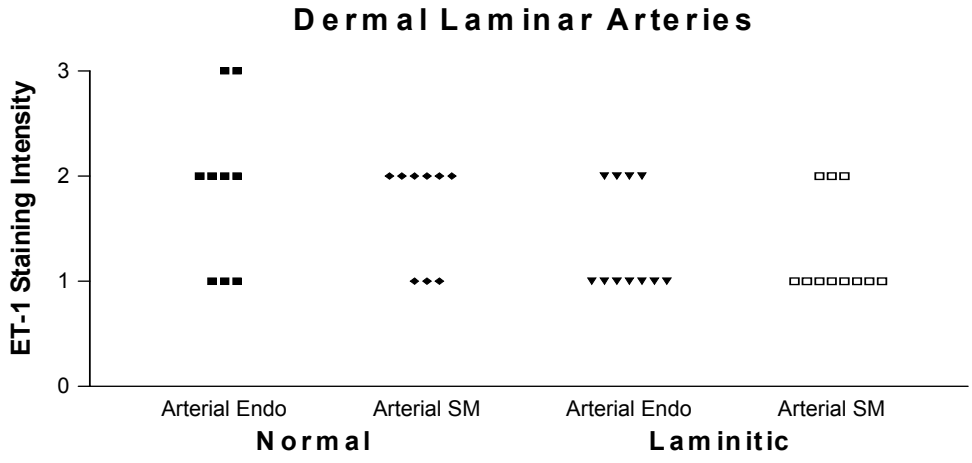


Figure 4.4 – Scatter plots of modal values for ET-1 immunohistochemical staining intensity of laminar samples from normal and laminitic horses. Epithelial cells and stroma of the epidermal laminae and vascular endothelium (Endo) and smooth muscle (SM) of the dermal laminae were evaluated. A 0 score was assigned if there was no staining present; 1 if mild brown staining; 2 if moderate brown staining; and 3 if intense brown staining. Increased staining would be associated with increased presence of ET-1.







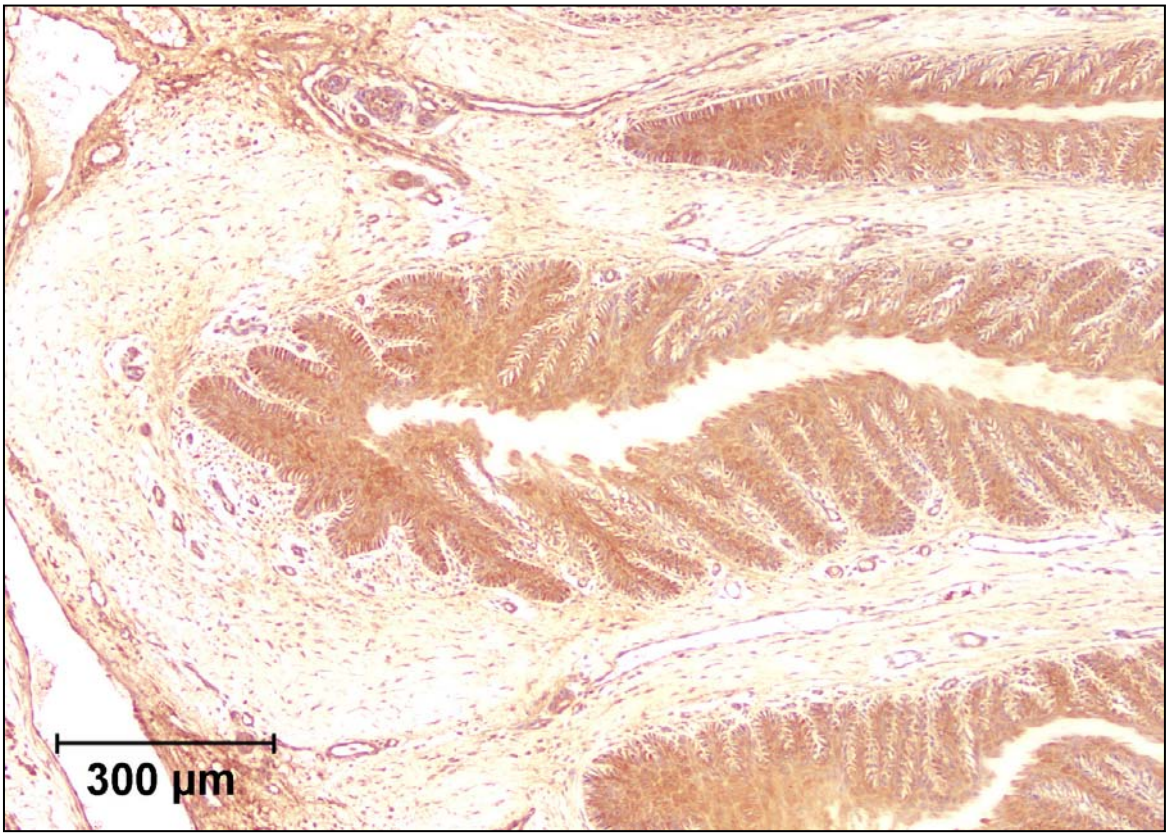
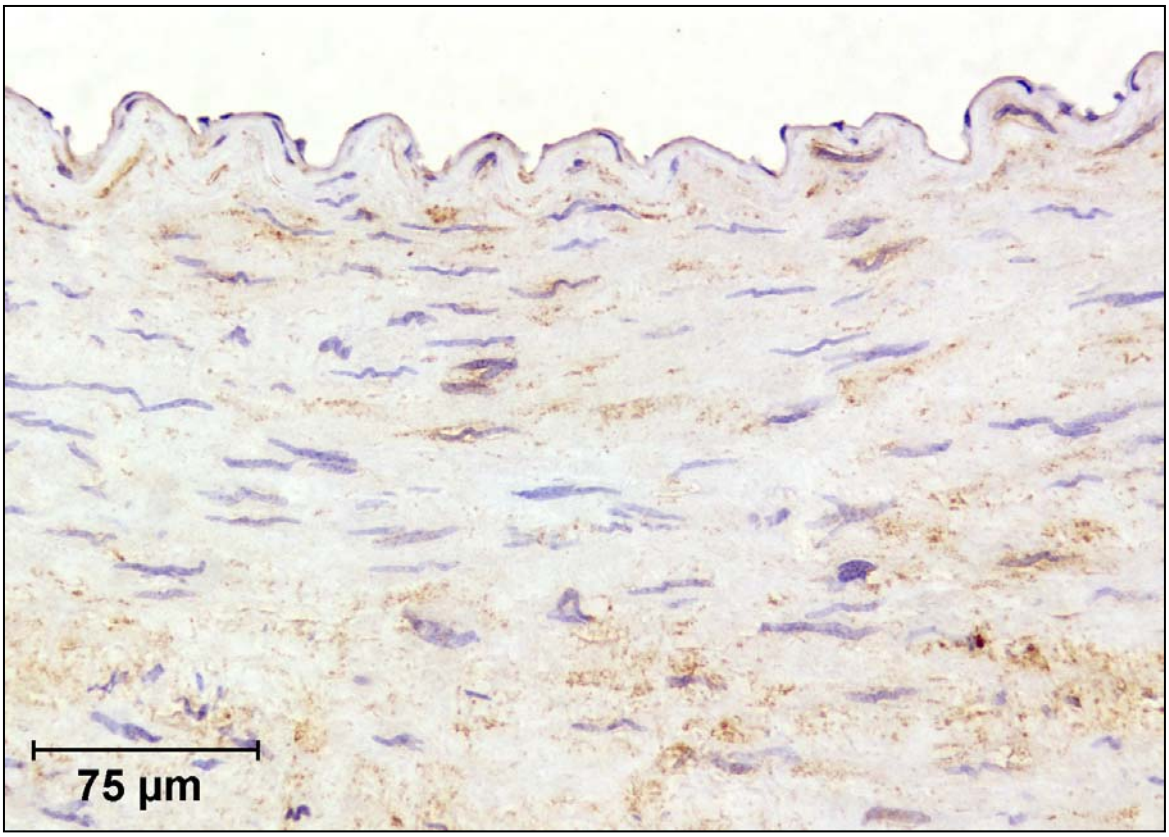


Figure 4.5 – Photomicrograph of laminae from a healthy horse, demonstrating intense endothelin-1 immunohistochemical staining.

Figure 4.6 – Photomicrographs of palmar digital arterial cross-sections from two different horses with naturally-acquired chronic laminitis. Note the difference in intensity of endothelin-1 immunohistochemical staining of the endothelial cells and of the underlying vascular smooth muscle.



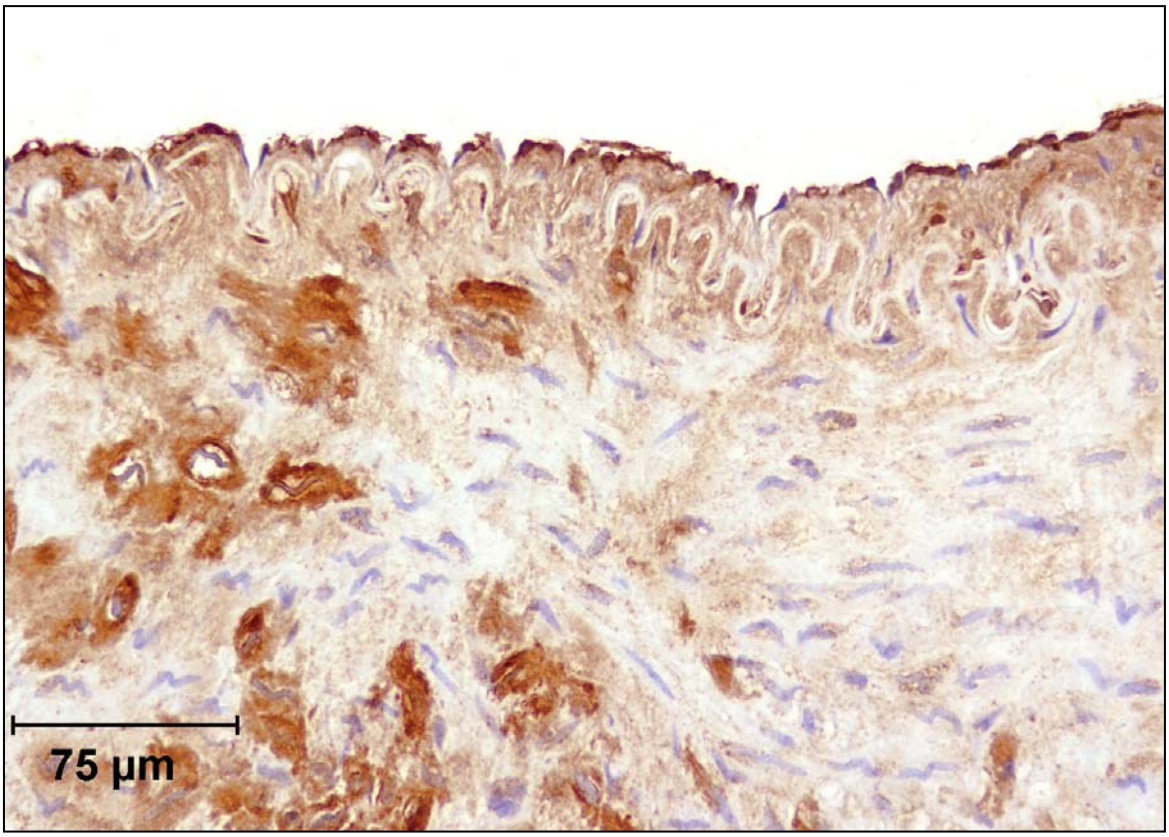
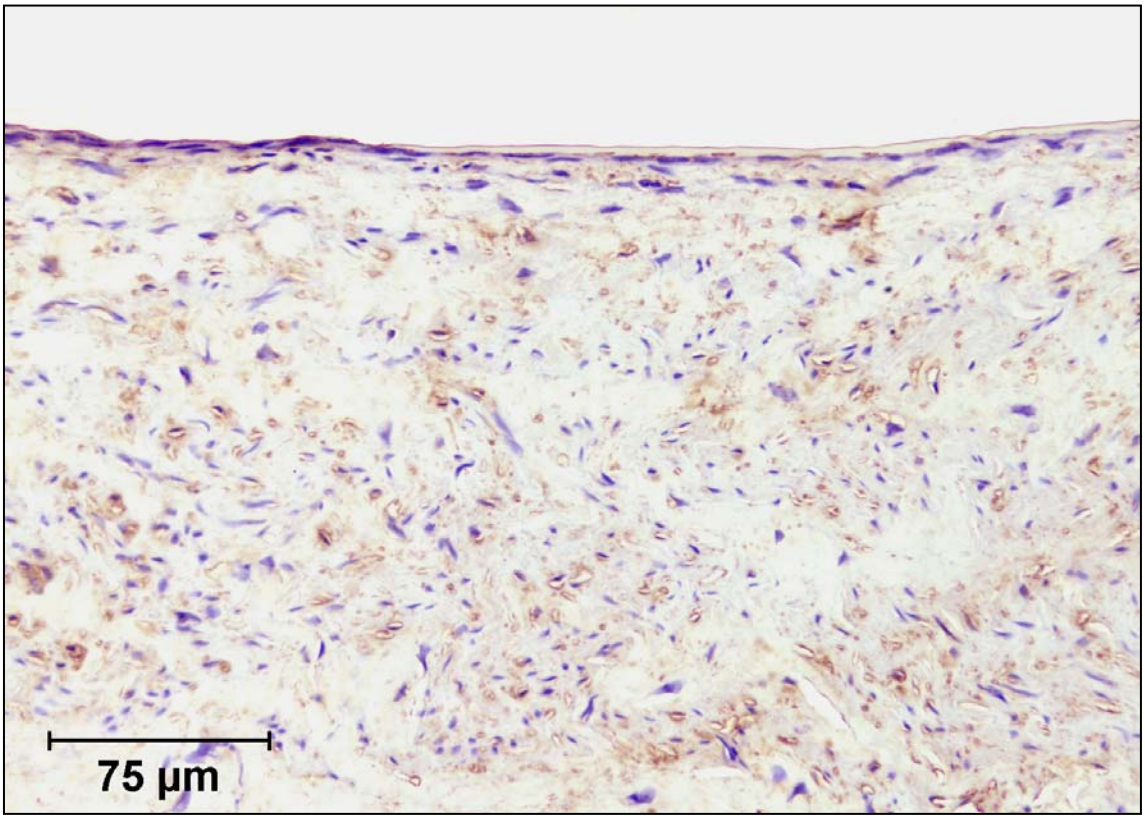
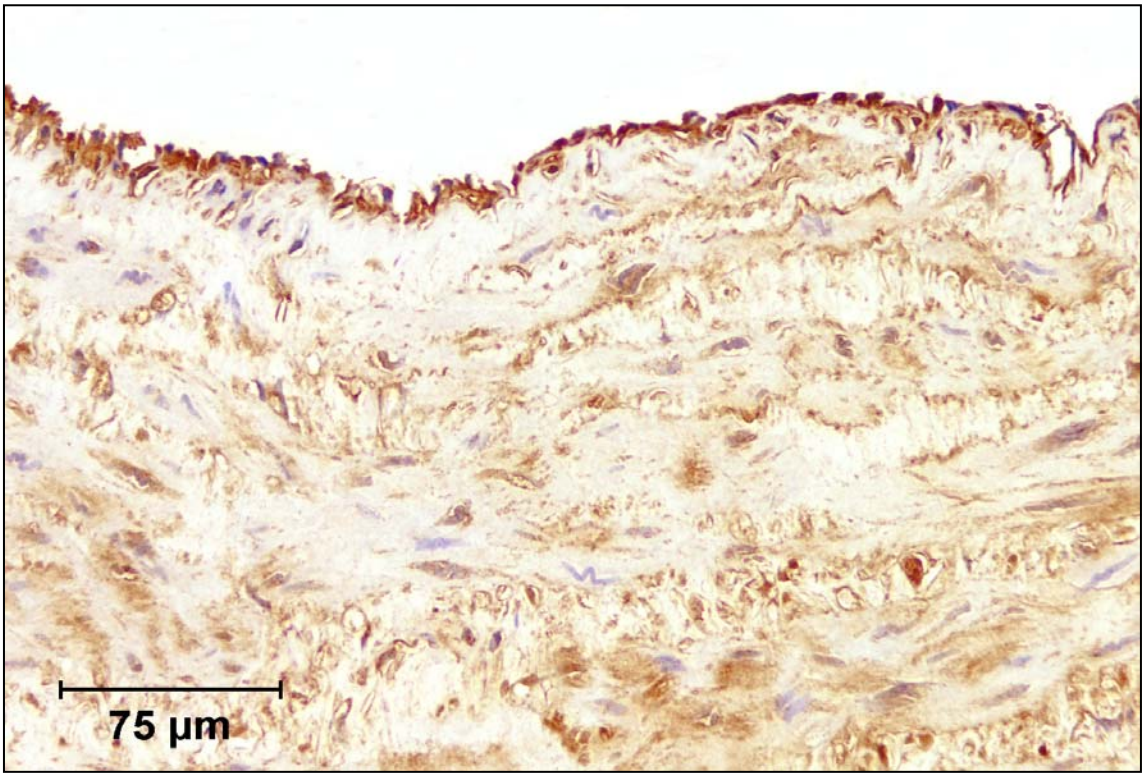


Figure 4.7 – Photomicrographs of palmar digital venous cross-sections from a healthy horse (top) and a horse with naturally-acquired chronic laminitis (bottom). Note the difference in intensity of endothelin-1 immunohistochemical staining of the endothelial cells and of the underlying vascular smooth muscle. The endothelial cells from the horse with naturally-acquired chronic laminitis appear swollen, irregular, and contain multiple mitotic figures.





laminitis compared with healthy horses. In addition, tissue IHC staining for ET-1 resulted in trends for increased ET-1 levels within regions of the epidermal and dermal laminae of healthy horses compared with laminitic horses. A third important finding was that ET-1 IHC staining was not increased in dermal laminar parietal collecting veins compared to parietal arteries in either group of horses.

Cephalic venous plasma ET-like immunoreactivity increased with a similar increase in systemic venous immunoreactivity; therefore, the data supports our hypothesis that horses with laminitis have increased concentrations of plasma ET-1.

Using black walnut extract to induce acute laminitis in a study conducted in our laboratory, we found increased palmar digital venous plasma ET-like immunoreactivity without a concomitant increase in systemic venous immunoreactivity.[22] This lack of increased systemic ET-1 differs from the horses with naturally-acquired laminitis in the study presented here. Systemic hypertension is associated with equine laminitis, and increased plasma ET-1 concentrations are consistently found with hypertension in a number of species.[7,23-25] Systemic hypertension was not a finding during our study using black walnut extract to induce laminitis, which corresponds to the finding that JV plasma ET-1 concentrations did not increase. Blood pressure was not evaluated in the laminitic horses in this study, although it is possible that these horses had laminitis-associated hypertension, which could be related to increased systemic ET-1 plasma concentrations. Of particular interest, 5 of 6 horses with substantial increases in JV plasma ET-1 like immunoreactivity in this study (> 2.95 pg/ml) were presented with laminitis as the primary presenting problem and were of chronic duration and progression of the disease. It is also of interest that in these same 5 horses, JV plasma concentrations of ET-like immunoreactivity were nearly double that of the CV values. Formal comparisons between acutely and chronically

affected horses could not be accurately conducted. To better understand the relationship between these observed increases in JV plasma ET-like immunoreactivity in chronically affected horses and increased CV plasma ET-like immunoreactivity in acutely affected horses would require sampling of more horses and the presence or absence of hypertension would need to be evaluated.

Although there is cross-reactivity between ET types (esp. ET-1 and ET-2) using this ELISA, the predominant isoform expected in the plasma is ET-1 since this is the principal isoform synthesized and released by vascular endothelial cells.[3] The structure of ET-1 is highly conserved across species and the ELISA kit used for sample analysis was validated for equine plasma in our laboratory. Since the PCV from laminitic horses was not significantly different from normal horses, increased or decreased values obtained during our study are not likely to be due to hemoconcentration or hemodilution.

Approximately 80% of ET-1 is released abluminally; therefore, the actual concentration of ET-like immunoreactivity released from the endothelial cells is likely substantially greater than that measured in plasma.[5] The authors believe the increased CV plasma ET-like immunoreactivity found in laminitic horses most likely represents a much greater release of ET-1 abluminally toward smooth muscle, potentially leading to digital vasoconstriction associated laminitis; however, the ET-1 IHC staining results of this study do not support increased tissue ET-1 levels. Other research within our laboratory and other laboratories support the role of ET-1 in this disease and Katwa et al found increased ET-1 expression within the laminae during the developmental and chronic stages of laminitis.[26]

Our laboratory and a study by Baxter et al have demonstrated the in vitro contractile effects of ET-1 in non-laminitic and laminitic horses, verifying the effectiveness of ET-1 as a

potent constrictor of equine digital vessels.[18,19,27] Measurements of Starling forces by Allen et al and Eaton et al found increased venous resistance with the development of laminitis, and during in vitro studies ET-1 induced greater contraction of veins than arteries.[13,18,19,26,28] Based on these findings, we hypothesized that PDV samples would have increased IHC staining compared with PDA samples. However, the IHC finding that PDA and PDV staining was not significantly different did not support our hypothesis. Possible reasons for these contradictory findings in our data are that alterations in ET-1 concentrations associated with the pathophysiology of laminitis were below the detection level of IHC techniques; the IHC staining technique was not specific for ET-1 and masked true ET-1 presence within the tissues; ET-1 levels are not affected by laminitis within these tissues. Although we used the antibody neutralization technique to examine primary antibody specificity for ET-1 in equine tissues, a study examining 7 primary antibodies used to detect ET-1 found 5 of these antibodies to bind nonspecifically to tissue proteins.[29] Multiple studies have successfully stained tissues using the same primary antibody utilized in the study presented here and steps were taken within our assay to minimize nonspecific binding to tissue proteins (incubation with goat serum and dilution of primary antibody with goat serum). Although it is possible that tissue ET-1 levels are not increased in horses with laminitis, more studies, and possibly more sensitive and specific techniques to examine these tissues, are warranted to clarify the regulation and localization of ET-1 in digital vascular and laminar tissues of horses with acute and chronic laminitis.

In summary and based on the findings of the studies presented here, increased plasma concentrations of ET-like immunoreactivity, but not digital vascular or laminar ET-1 IHC staining, may be associated with naturally-acquired laminitis in horses.

4.5 Product Information

[a] Sigma Chemical Co, St. Louis, MO

[b] Biomedica, American Research Products, Inc., Belmont, MA

[c] Bio-tek, Winooski, VT

[d] Sodium pentobarbital, The Butler Co, Columbus, OH

[e]Vectastain ABC Elite Kit – Rabbit IgG, Vector Laboratories, Inc., Burlingame, CA

[f] Rabbit anti-Endothelin-1, Peninsula Laboratories, Inc., Belmont, CA

[g] Endothelin-1, Peninsula Laboratories, Inc., Belmont, CA

[h] Proc Mixed, Univariate, Freq, and Means; SAS version 8, SAS Institute, Cary, NC

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CHAPTER 5. EFFECT OF ENDOTHELIN-1, AN ENDOTHELIN ANTAGONIST AND NITROGLYCERINE ON DIGITAL HEMODYNAMICS IN CLINICALLY HEALTHY CONSCIOUS HORSES

5.1 Introduction

Acute laminitis is a severely debilitating and painful disease of the sensitive and insensitive laminae of the equine digit characterized by decreased pre-to-post capillary sphincter resistance ratio, increased capillary pressure, increased interstitial pressure and edema, microvascular thrombosis, ischemia, and laminar necrosis.[1-4] Garner et al introduced the hypothesis that the predominant cause of laminitis after carbohydrate (CHO) overload was a disturbance in digital blood flow, which occurred during the onset of the syndrome after carbohydrate overload of the gastrointestinal tract.[5] Researchers have indirectly demonstrated reduced digital vascular perfusion using contrast radiography, hoof wall surface temperature, and laser Doppler flowmeters.[6-8] Garner also determined that the changes in digital perfusion are associated with marked systemic hemodynamic changes including a decline in right atrial pressure, diastolic systemic arterial pressure, and systolic systemic arterial pressure, which reaches a maximum about 16 hours after starch is administered via nasogastric tube.[9] This pressure drop is followed by a steady increase in right atrial pressure, diastolic arterial pressure, and systolic arterial pressure. These results suggest that appreciable systemic cardiovascular and local digital vascular changes likely occur in horses with laminitis, and increased release or activation of vasoactive mediators occurs. Although numerous mediators are likely to contribute to these vascular alterations, the primary mediators have yet to be determined.

Endothelins are a family of peptides (ET-1, ET-2, ET-3) synthesized by various cells that exert numerous biologic and pathophysiologic effects.[10] The principal endothelin of importance in vascular diseases or ischemic conditions is ET-1. Endothelin-1 is a potent vasoconstrictor peptide synthesized by endothelial cells, vascular smooth muscle cells, and macrophages. It not only induces prolonged vasoconstriction in arteries and arterioles, but also causes intense

profound venoconstriction in both the systemic and pulmonary circulation.[10,11] Endothelin synthesis is stimulated by epinephrine, transforming growth factor (produced during platelet aggregation), platelet activating factor (which stimulates platelet aggregation and neutrophil chemotaxis), and tumor necrosis factor, which are increased during many diseases in horses characterized by an inflammatory response (pleuropneumonia, endometritis, intestinal ischemia, enterocolitis, anterior enteritis, etc), and which are empirically linked to the development of laminitis.[10,12-18]

Katwa et al recently demonstrated that the concentration of ET-1 in laminar connective tissues obtained from experimentally-induced acutely laminitic horses, and naturally-occurring chronically laminitic horses were increased compared with a control group.[19] Previous in vitro studies in our laboratory have found that ET-1 causes a concentration-dependent contraction of equine palmar digital vessel rings from normal horses and those with naturally-acquired laminitis, and that administration of the ET antagonist (PD145065; 10^{-5} M concentration) significantly attenuates these contractile responses to ET-1.[20,21] Palmar digital venous rings contracted over 3 times greater than arterial rings to ET-1 indicating the potential importance of ET-1 in leading to a decreased pre-to-post capillary resistance ratio found during the developmental stages of acute laminitis.[1,20,21]

The purpose of this study was to evaluate the effect of ET-1, a potent vasoconstrictor, on digital hemodynamics in conscious horses to investigate the link between ET-1 and laminitis. Our study hypotheses were that ET-1 administration would result in dose-dependent reductions in blood flow and capillary perfusion, and increases in palmar digital arterial and venous blood pressures, and the administration of an ET antagonist into the digital arterial circulation would reverse the alterations caused by ET-1 infusion. In addition, we hypothesized that administration

of a NO donor (nitroglycerine) would further improve digital hemodynamics. The objectives were to evaluate the effects of ET-1, an ET antagonist (PD145065), and a NO donor (nitroglycerine, NG) on digital hemodynamic variables in clinically healthy conscious horses.

5.2 Materials and Methods

5.2.1 Selection and Preparation of Horses - This study was approved by the Institutional Animal Care and Use Committee of Louisiana State University. Nine adult light breed horses (5 to 15 years of age and body weight 370 to 530 kg) were determined to be free of laminitis and gastrointestinal tract disease based on their history, thorough physical and lameness examinations, and lateral radiographs of both front feet. Horses were housed in stalls, fed grass hay, and acclimated to the study area for two weeks prior to the study.

5.2.2 Instrumentation – As previously described, an ultrasonic Doppler flow probe[a] was surgically placed around the lateral palmar digital artery two weeks prior to the study for measurement of palmar digital blood flow (BF).[22] On the day of the study, horses were placed in stocks, instrumented, and allowed a 1-hour period of equilibration prior to the start of the study. All catheters were placed percutaneously after aseptic preparation of the skin and desensitization by subcutaneous infiltration of lidocaine solution. A 14-gauge, 13.3-cm Teflon catheter[b] was inserted into the left jugular vein (JV) for collection of blood samples. A 20-gauge, 4.45-cm polyurethane catheter[c] was placed in the right transverse facial artery for measurement of mean systemic arterial pressure (MAP). A 20-gauge, 4.45-cm polyurethane catheter was placed in the right lateral palmar digital artery for measurement of mean digital arterial pressure (MDAP) and administration of ET-1[d], the ET antagonist PD145065[e], and the NO donor NG[f]. A 20-gauge, 4.45-cm polyurethane catheter was placed in the right lateral palmar digital vein for collection of blood samples and measurement of mean digital venous pressure (MDVP). The pressure

transducers were placed at the level of the point of the shoulder. Correct catheter placement was confirmed by characteristic pressure waveforms. A 6-mm hole was drilled 3 cm distal to the coronary band in the dorsal aspect of the hoof wall on the same limb containing the ultrasonic flow probe and other instrumentation, using a hand-held, multiple-use electric burr.[23] The hole was drilled through the hoof capsule to the junction of the epidermal and dermal laminae, but the dermal laminae were not entered. The appropriate depth was confirmed by placing the calibrated laser Doppler flow probe[g] in the hole, occluding the digital arteries, and observing a decrease in the number of capillary perfusion units (CPU). Blood flow, pressure, and CPU measurements were interfaced with a physiograph[h] and recorded continuously.

5.2.3 Study Design – Horses were allocated to each of 3 treatment groups with each horse receiving all 3 treatments with studies separated by a minimum of 7 days. All doses were administered over 2 minutes in the palmar digital arterial catheter and doses were calculated based on measured palmar digital arterial blood flow to achieve appropriate drug concentrations. During study I, horses received a dose of 0.9% NaCl followed by doses of ET-1 (to achieve approximate plasma concentrations equivalent to 10^{-12} to 10^{-7} M in palmar digital blood flow) to establish ET-1 dose-response curves. Each successive dose was infused at least 10 minutes apart or once the blood flow response stabilized. During studies II and III, horses received ET-1 at a dose to reduce blood flow by 75% (ET_{75}, based on individual responses to ET-1 during study I) followed by 3 doses of either the ET antagonist PD145065 (10^{-7}, 10^{-6}, and 10^{-5} M concentrations in palmar digital blood flow) or 3 doses of 0.9% NaCl (equivalent volume), respectively. Variables were recorded 1 hour after initiation of the study to determine the duration of treatment effects of the ET-1 and the antagonist. Then, NG (equivalent to a 10^{-5} M plasma concentration) was administered as a continuous infusion over 6 minutes during studies II and III. Variables

(BF, pressures, and CPU) were measured for 5 minutes after completion of each drug administration and averages were calculated.

5.2.4 Statistical Analyses – Data were considered to be continuous and were summarized and presented as mean ± SD. Data from studies II and III are presented as percentage change from values after the ET_{75} dose and were analyzed using the arcsin transformation. The data were analyzed, using a repeated measures mixed effect linear model that accounted for the random variance of horse. Where there were significant interaction effects at $p \leq 0.05$, predetermined least squares means comparisons were made to determine where differences were occurring between groups. Type I error was maintained at 0.05. PROC MEANS, UNIVARIATE, and MIXED were used for the analysis.[i]

5.3 Results

5.3.1 Study I – Administration of ET-1 resulted in a dose-dependent decrease of blood flow with significant reduction after infusion of the 10^{-7} M concentration and remained significantly decreased at the 1-hour time point (Fig. 5.1). There were no significant differences over time for CPU (Fig. 5.2), MDVP, or MAP. Mean digital arterial pressure was significantly increased after the 10^{-7} M concentration of ET-1 and remained increased at the 1-hour time point (Fig. 5.3). Concurrent with the ET-1 dose-response curve in these horses, changes in clinical signs were noted with the higher doses of ET-1. Most horses demonstrated clinical signs associated with marked digital pain by repeatedly lifting the treated foot and exhibiting anxiety. These horses were administered xylazine (150 mg, IV) at the conclusion of the study and no further signs of discomfort were noted.

5.3.2 Studies II and III – There were significant differences in percentage change of blood flow (increased flow) with ET antagonist administration and there were significant

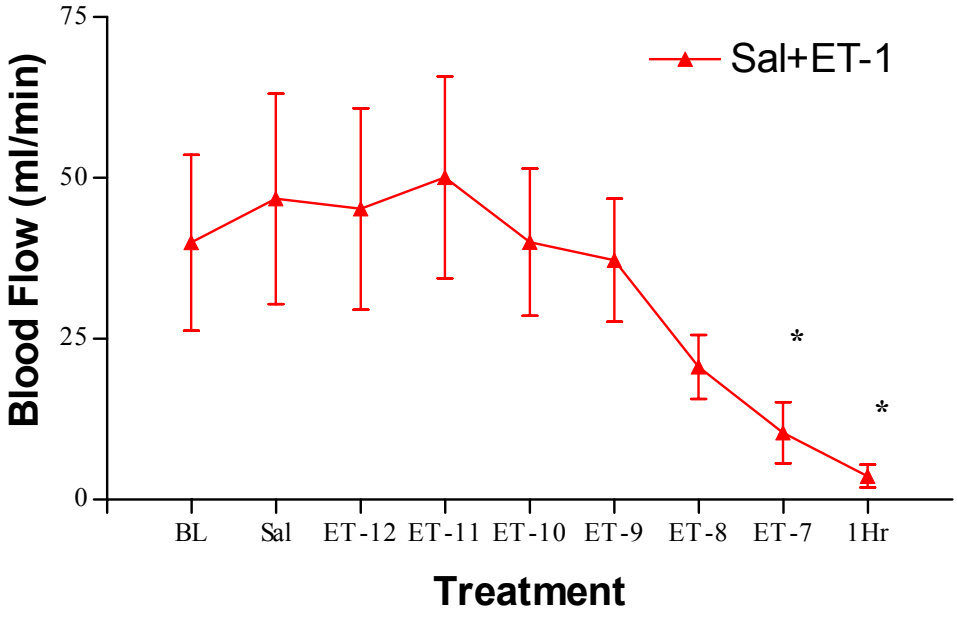

Figure 5.1 – Mean +/- SD palmar digital blood flow values from baseline (BL), infusion of 0.9% NaCl (Sal), and infusion of endothelin-1 (ET-1; 10^{-12} to 10^{-7} M concentrations) into the palmar digital artery. * Indicates significantly ($p < 0.05$) different from the BL value.

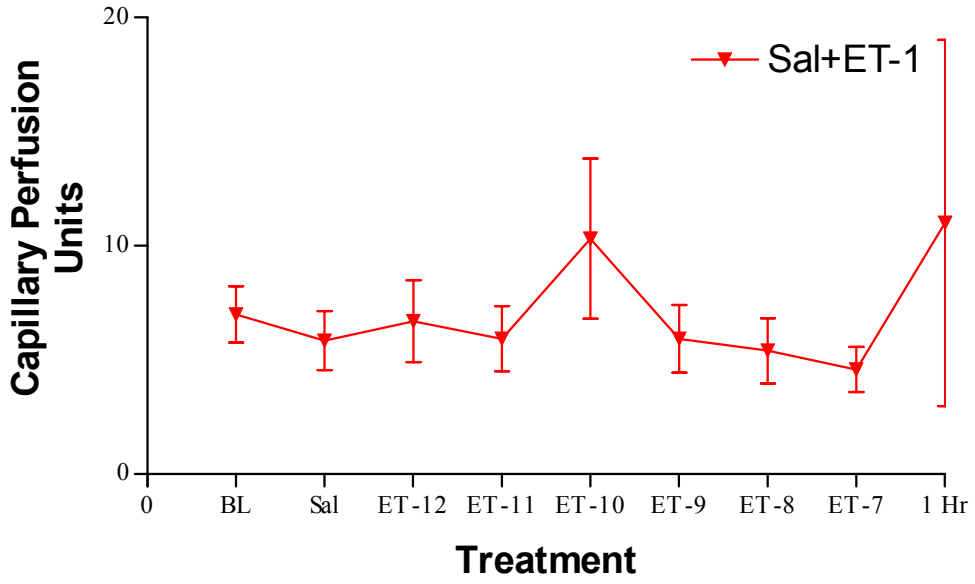

Figure 5.2 – Mean +/- SD laminar capillary perfusion unit (CPU) values from baseline (BL), infusion of 0.9% NaCl (Sal), and infusion of endothelin-1 (ET-1; 10^{-12} to 10^{-7} M concentrations) into the palmar digital artery. There were no significant differences between time points.

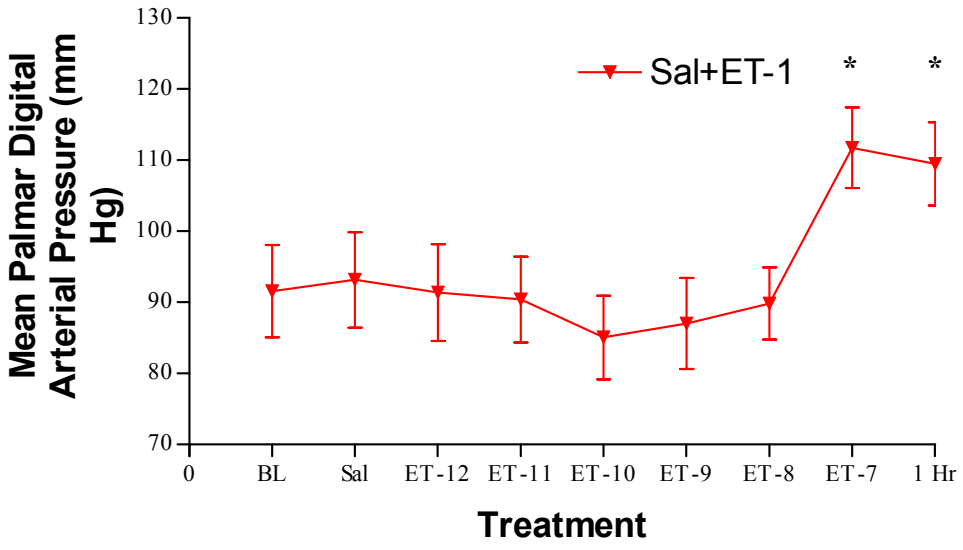

Figure 5.3 – Study I mean +/- SD palmar digital arterial pressure values from baseline (BL), infusion of 0.9% NaCl (Sal), and infusion of endothelin-1 (ET-1; 10^{-12} to 10^{-7} M concentrations) into the palmar digital artery. *Indicates significantly ($p < 0.05$) different from the values from BL to ET 10^{-8} M.

differences between saline- and ET antagonist-treated studies (Fig. 5.4). There were no significant differences between CPU values within or between studies II and III. There were significant differences for MDAP, MDVP, and MAP from ET_{75} administration with ET antagonist administration and MDVP was significantly different between saline- and ET antagonist-treated studies (Table 5.1). Administration of NG significantly improved blood flow in both studies (Fig. 5.4 and Table 5.1).

5.4 Discussion

The important findings of these studies are that administration of ET-1 into the medial palmar digital artery induced a dose-dependent sustained decrease in digital blood flow and a significant increase in digital arterial pressure at higher doses in clinically healthy conscious horses. Administration of the ET antagonist reversed ET-1-induced decreases in blood flow and NG infusion further improved digital hemodynamics. Additionally, higher doses of ET-1 led to temporary clinical signs associated with marked digital pain, characterized by repeated lifting of the treated limb.

Under normal physiological conditions, it is hypothesized that a balance exists between endothelial-derived vasoconstrictors (i.e. ET-1) and vasodilators (i.e. NO) and that this balance is important for maintaining vasomotor tone, blood flow and tissue perfusion.[24] Our global hypothesis is that during the developmental stages of acute laminitis, an imbalance exists (increased ET-1 and decreased NO) resulting in the hemodynamic alterations known to occur during experimentally-induced laminitis (i.e. decreased digital blood flow and decreased pre-to-post capillary resistance ratio). In support of our study hypothesis, ET-1 administration into the digital arterial circulation resulted in a dose-dependent decrease in digital blood flow and an increase in digital arterial pressure. This finding demonstrates the presence of ET receptors

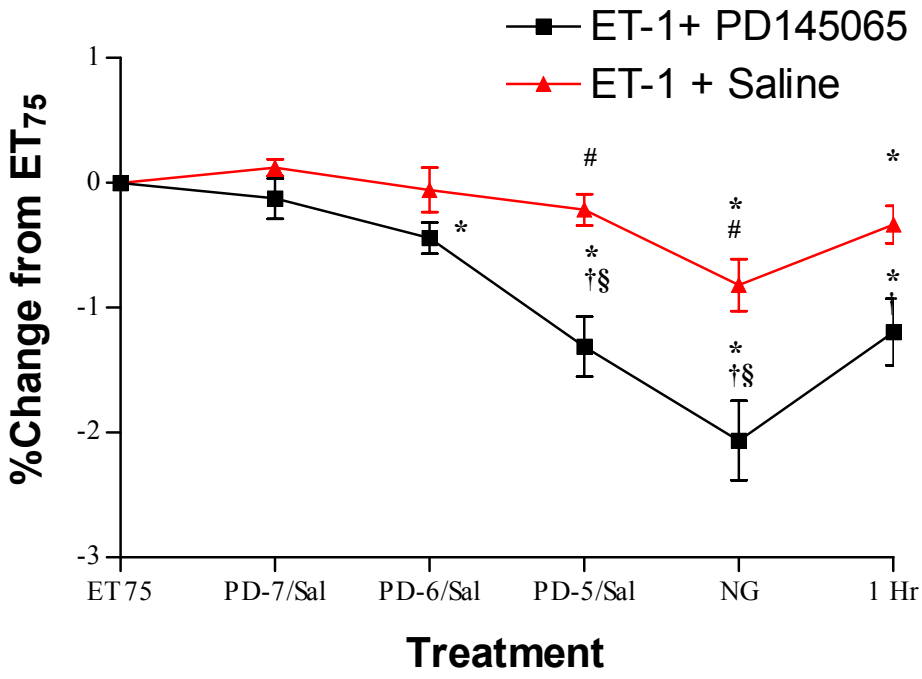

Figure 5.4 – Mean +/- SD percentage change in palmar digital blood flow values from the dose of endothelin-1 (ET-1) required to reduce blood flow by 75% (ET_{75}) followed by either infusion of PD145065 (PD; 10^{-7} to 10^{-5}; Study II) or an equivalent volume of 0.9% NaCl (Sal; Study III) into the palmar digital artery. Nitroglycerine (NG; 10^{-5} M concentration) was then infused followed by a 1-hour measurement. Negative values indicate increases in blood flow. * Indicates significantly ($p < 0.05$) different from ET_{75} values. # Indicates significantly different ($p < 0.05$) between PD145065 and 0.9% NaCl treatments at these time points. † Indicates significantly ($p < 0.05$) different from PD145065 at the 10^{-7} M concentration. § Indicates significantly ($p < 0.05$) different from PD145065 at the 10^{-6} M concentration.

Table 5.1 – Mean percentage change for palmar digital arterial pressure (top), palmar digital venous pressure (middle) and systemic arterial pressure (bottom) from the value after reduction of flow by ET-1 to 75% baseline value (ET_{75}) for horses treated with ET antagonist PD145065 (PD; [10^{-5} M], Study II) or saline (Sal; Study III), after nitroglycerine (NG; [10^{-5} M]) administration, and a final measurement taken 1 hour (1 Hr) after administration of the ET_{75} dose.

Palmar digital arterial pressure

	ET_{75}	**PD^{-7}/Sal**	**PD^{-6}/Sal**	**$PD10^{-5}$/Sal**	**NG**	**1Hr**
ET_{75} + PD145065	0^{a}	-1.1^{b}	2.7^{b}	2.7^{b}	-2.5^{b}	2^{b}
ET_{75} + Saline	0^{a}	0.3^{b}	2.1^{b}	-1.6^{b}	-2.5^{b}	1.4^{b}

Means in a row with a different letter are significantly ($P < 0.05$) different from ET_{75}.

Palmar digital venous pressure

	ET_{75}	**PD^{-7}/Sal**	**PD^{-6}/Sal**	**$PD10^{-5}$/Sal**	**NG**	**1Hr**
ET_{75} + PD145065	0^{a}	$-24.1^{a,b}$	34.3^{b}	-95.9^{b}	33.8^{b}	151^{b*}
ET_{75} + Saline	0^{a}	144^{b}	-4.9^{b}	36^{b}	$201^{a,b}$	$6^{a,b}$

Means in a row with a different letter are significantly ($P < 0.05$) different from ET_{75}.
*Mean is significantly ($P < 0.05$) different from that for saline-treated horses.

Systemic arterial pressure

	ET_{75}	**PD^{-7}/Sal**	**PD^{-6}/Sal**	**$PD10^{-5}$/Sal**	**NG**	**1Hr**
ET_{75} + PD145065	0^{a}	0.9^{b}	0.5^{b}	2.1^{b}	7.8^{b}	6.1^{b}
ET_{75} + Saline	0^{a}	3.4^{b}	0.8^{b}	3.7^{b}	7^{b}	3.2^{b}

Means in a row with a different letter are significantly ($P < 0.05$) different from ET_{75}.

within the digital vasculature since saline administration immediately prior to ET-1 infusion did not induce any blood flow or pressure alterations, compared with ET-1. The increase in digital arterial pressure confirms that the decrease in blood flow is due to an increase in digital arterial vascular resistance.

The actions of ET-1 are mediated through two main receptor types, the ET_A and ET_B receptors.[10] The ET_A receptors are predominantly located on vascular smooth muscle cells and, through several signal transduction mechanisms, ET-1 binding results in slowly developing, but sustained vasoconstriction.[10] The ET_B receptors are located principally on endothelial cells and trigger the release of the endothelial-derived relaxing factor, NO. Nitric oxide released through this mechanism is believed to regulate release of ET-1, possibly through inhibition of the precursors of ET-1.[10]

Because 80% of ET-1 synthesized by the endothelium is released abluminally toward the smooth muscle where the contractile ET_A receptors are located, it is possible that during laminitis, the amount of ET-1 reaching and binding to these receptors is greater than was achieved by infusion of the ET-1 into the medial palmar artery in the horses of this study.[25] Infusion of ET-1 into the digital circulation was performed over two minutes, and it is possible that the blood flow carried at least some of the ET-1 out of the digital bed limiting the time for its diffusion through the vessel wall to the smooth muscle receptors. Therefore, the magnitude of vascular and microvascular alterations caused by ET-1 may be greater in horses with experimentally-induced or naturally acquired laminitis.

Increased laminar ET-1 gene expression was demonstrated in horses with acute CHO-induced laminitis and with chronic laminitis by Katwa et al, which further supports the presence of ET-1 synthesis and or binding to ET receptors within the digit.[19] Additionally, digital plasma

ET-like immunoreactivity was significantly increased in horses after black walnut extract administration.[j] Together, these findings demonstrate that if ET-1 increases within the digit during acute laminitis, the vasculature is likely to respond by a reduction in blood flow and subsequent laminar perfusion.

We did not find significant differences for laminar CPU using the laser Doppler flow probe during any of the presented studies. The authors believe that because of the complexity of laminar anatomy and blood flow physiology, the use of a single laser Doppler flow probe provided inconsistent results; therefore, we do not have confidence that our laminar perfusion results are indicative of the true alterations resulting secondary to ET-1-induced vasoconstriction. Adair, et al have reported on an improved method for measurement of laminar perfusion by using multiple custom-designed laser Doppler probes simultaneously and demonstrated significant biphasic alterations in perfusion after BWE administration.[8]

The ET antagonist selected for these studies is a non-selective, competitive inhibitor of both the ET_A and ET_B receptors.[26] This ET antagonist was selected based on previous in vitro studies conducted in our laboratory that demonstrated it significantly reduced the contractile effects of ET-1 in equine colonic arterial and venous rings and digital vessels from normal and laminitic horses.[20,21,27] During this study, infusion of the ET antagonist reversed ET-1-induced decreases in blood flow, further demonstrating the activity of ET-1 within the digital vasculature and demonstrating the effectiveness of this ET antagonist to attenuate the effects of ET-1 on digital blood flow. Since the action and presence of ET-1 within the digit of the horse has been demonstrated in our laboratory and by other researchers, future investigations into the use of this ET antagonist in the prevention and treatment of laminitis are warranted.

Nitroglycerine alone, but especially in combination with the ET antagonist, significantly improved digital hemodynamics in the studies presented here. Previous research has demonstrated that NO donor administration improves digital perfusion and reduces the bounding digital pulses associated with acute laminitis in ponies.[28,29] In other species, administration of the combination of PD145065 and a NO donor has been shown to significantly decrease vasoconstriction, typically associated with bronchoconstriction in adult respiratory distress syndrome and acute lung injury.[30]

The studies presented here document ET-1 as a potent vasoconstrictor of the digital vasculature in clinically healthy conscious horses resulting in altered hemodynamics. Additionally, our studies demonstrate the effectiveness of the ET antagonist PD145065, especially in combination with the NO donor, NG, in reducing these vascular alterations. Further studies are warranted to investigate the use of these agents in the prevention and treatment of acute laminitis in horses.

5.5 Product Information

[a] Transonics Systems, Inc., Ithaca, NY

[b] Angiocath Vascular Access, Becton Dickinson & Co, Sandy, UT

[c] Arrow Catheters, Arrow International, Reading, PA

[d] Endothelin-1, Research Biochemicals International, Natick, MA

[e] PD 145065, American Peptide Co, Sunnyvale, CA

[f] Hoesch Marion Roussel, Kansas City, MO

[g] Transonic Systems, Inc., Ithaca, NY

[h] Grass Medical Instruments, Quincy, MA

[i] Proc Mixed, Univariate, and Means; SAS version 8, SAS Institute, Cary, NC

[j] Holm AS, Eades SC, Moore RM. Alterations in jugular and digital venous plasma endothlein-1 concentrations in horses administered black walnut extract. *Vet Surg* 31 (5) 491 2002.

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CHAPTER 6. SYSTEMIC AND LOCAL DIGITAL HEMODYNAMIC AND HEMATOLOGIC ALTERATIONS IN HORSES ASSOCIATED WITH ACUTE LAMINITIS INDUCED USING THE BLACK WALNUT EXTRACT MODEL

6.1 Introduction

Garner et al introduced the hypothesis that the predominant cause of laminitis after carbohydrate (CHO) overload was a disturbance in digital blood flow, which occurred during the onset of the syndrome after CHO overload of the gastrointestinal tract.[1] Using contrast radiography, researchers demonstrated reduced perfusion in the terminal vasculature of the foot.[2] In subsequent studies using the isolated perfused digit, the specific hemodynamic forces acting on the laminar microcirculation in healthy and experimentally-induced laminitic horses have been extensively defined.[3-5] Several alterations in the digital vascular system of horses with experimentally-induced Obel grade I laminitis [both CHO overload and black walnut extract (BWE) models] have been identified.[3] Of particular importance is the finding that the pre-to-post capillary resistance ratio is decreased in the prodromal stages of laminitis. This imbalance increases the hydrostatic force in the capillary promoting the flux of fluid across the capillary bed within the foot, resulting in laminar edema while capillary permeability remains normal. These findings support the hypothesis that increased venomotor tone initiates laminitis.

In physiologic states, the endothelium synthesizes vasoactive substances, such as nitric oxide (vasodilator) and endothelin-1 (ET-1; profound vasoconstrictor), which regulate vasomotor tone and have anticoagulant properties.[6-9] Many pathological states characterized by vascular or smooth muscle alterations, such as endotoxemia, atherosclerosis, hypertension, Raynaud's syndrome, and asthma, are associated with increased plasma concentrations and tissue levels of ET-1.[10-15] Endothelin is a 21 amino acid peptide and was first isolated by Yanagisawa and colleagues in 1988.[7] Endothelin has three isoforms, namely ET-1, ET-2, and ET-3; however, vascular endothelium and smooth muscle cells primarily synthesize the ET-1 isoform. In blood vessels, biosynthesis of ET-1 occurs in the endothelium, approximately 80% is released

abluminally toward the vascular smooth muscle, and the two main receptor types for ET-1 (ET_A and ET_B) are located on vascular smooth muscle cells and endothelium, respectively.[9,16]

The ET antagonist PD145065 selected for these studies is a non-selective, competitive inhibitor of both the ET_A and ET_B receptors.[17] It has proven to be beneficial in models examining the role of ET-1 in ischemia/reperfusion, the systemic inflammatory response, and in ET-induced constriction of vascular and airway smooth muscle.[18-20] Since laminitis is characterized by alterations in vascular resistance and blood flow, the potential use of this agent to ameliorate these hemodynamic changes would be beneficial.

Our hypotheses are that with the onset of experimentally-induced acute laminitis using the BWE model, horses administered saline will demonstrate an initial decrease in digital blood flow followed by a period of increased flow corresponding to demonstration of clinical signs. Administration of the ET antagonist PD145065 will prevent decreased blood flow and will minimize the hyperemic period. Additionally, the antagonist will prevent digital pressure alterations and will decrease the formation of platelet neutrophil aggregates within the digital circulation.

6.2 Materials and Methods

6.2.1 Selection and Preparation of Horses - This study was approved by the Institutional Animal Care and Use Committee of Louisiana State University. Fourteen light breed horses (4 mares and 10 geldings; ages ranging 5 – 20 years; weighing 411 to 591 kg) were determined to be free of laminitis and gastrointestinal tract disease based on their history, thorough physical and lameness examinations, and lateral radiographs of both front feet. Two weeks prior to the study, an ultrasonic Doppler flow probe[a] was surgically placed around the medial palmar digital artery in the mid-pastern region for blood flow (BF) measurements and the

medial palmar artery was surgically elevated to a subcutaneous location in the proximal to middle one-third of the metacarpus of the same limb to facilitate catheter placement.[21] Horses were housed in stalls, fed grass hay, and acclimated to the study area for two weeks prior to the study. On the day of the study, horses were placed in stocks, instrumented, and then moved to the study area where they were allowed a 1-hour period of equilibration prior to the start of the study. All catheters were placed percutaneously after aseptic preparation of the skin and desensitization by subcutaneous infiltration of lidocaine solution. A 14-gauge, 13.3-cm Teflon catheter[b] was inserted into the left jugular vein (JV) for collection of blood samples. Palmar digital venous (PDV) blood was collected and palmar digital venous blood pressure (MDVP) was measured via a 20-gauge, 4.45-cm polyurethane catheter[c] placed in the palmar digital vein. A 20-gauge, 4.45-cm polyurethane catheter was placed in the medial palmar artery (PA) for administration of the ET receptor antagonist PD145065[d] in 7 horses and saline solution (0.9% NaCl) in the remaining 7 horses, and for measurement of palmar arterial pressure (MPAP) in all horses. Systemic arterial pressure (MAP) was measured by placement of a 20-gauge, 4.45-cm polyurethane catheter in the transverse facial artery. Pressure transducers were placed at the level of the point of the shoulder. Blood flow and pressure measurements were interfaced with a physiograph[e] and recorded continuously. Correct positioning of all catheters was confirmed by observation of characteristic pressure wave forms.

6.2.2 Preparation of Extract – Preparation of the BWE was as previously described.[5,22] Briefly, shavings were made from the heartwood of a black walnut tree cut in the fall of the year and were stored at –20 C until use. Two grams of black walnut shavings/kg body weight were combined with 8 liters of distilled water and mixed in a shaker bath[f] for 14 hours at room

temperature (20 – 22 C). The mixture was filtered, refrigerated, and the fluid was administered within 24 hours of preparation.

6.2.3 Experimental Design – Fourteen horses were instrumented for measurement of hemodynamic variables, collection of blood samples for hematologic evaluation, and measurement of physical examination variables. Hemodynamic variables measured were palmar digital BF, MDVP, MPAP, and systemic MAP. Jugular venous blood samples were collected for CBC determination including packed cell volume (PCV), total plasma protein (TPP), white blood cell (WBC) count, segmented neutrophil count, platelet count, fibrinogen, and for evaluation of jugular venous platelet/neutrophil (P/N) aggregate formation. Palmar digital venous blood samples were collected for P/N aggregate determination. Physical examination parameters evaluated were heart rate, respiratory rate, rectal temperature, Obel grade, attitude, subjective hoof temperature, digital pulse, response to hoof testers, capillary refill time/mucous membrane color, and gastrointestinal motility.[23] Baseline measurements and samples were acquired 8, 4, and 1 hour before BWE administration. Laminitis was induced in all horses by administration of the BWE via a nasogastric tube. Samples were collected and horses were monitored hourly after BWE administration until horses demonstrated Obel grade 1.[23] Efficacy of the extract was confirmed by at least a 30% decrease in WBC count.[5]

Horses were randomly assigned to one of the two treatment groups. Seven horses received local digital administration of the ET receptor antagonist PD145065 (10^{-5} M concentration) at two designated time points and seven horses received an equivalent volume of saline solution (0.9% NaCl) to serve as control. Doses were delivered over two minutes into the medial palmar arterial catheter at 1.5 and 7.5 hours post-BWE administration. These times were selected based on the timing of known decreases in laminar perfusion after BWE

administration.[24] Endothelin antagonist doses were calculated based on measured palmar digital blood flow to achieve the appropriate drug concentration. The study was terminated once horses demonstrated Obel grade 1 laminitis.

6.2.4 Hematologic Variables - Jugular venous blood (3 ml) was collected into tubes containing EDTA for CBC determination and jugular and PDV blood (3 ml) was collected into tubes containing sodium citrate for P/N aggregate determination.[25] Within 30 min of blood collection, 1 ml of citrate-anticoagulated blood was placed in a 10-ml plastic tube, and RBCs were allowed to settle for 20 min. Thereafter, the platelet and leukocyte-rich plasma layer was removed, combined with 2 ml of autologous platelet-poor plasma, and centrifuged at 57 x g for 5 min. The platelet-rich plasma layer was removed, and leukocyte-rich sediment was resuspended in 150 µl of autologous plasma. Wedge-type smears were prepared and stained with modified Wright's stain. Two hundred cells were counted on each of two smears and the percent of neutrophils with platelets attached were enumerated.

6.2.5 Statistical Analyses – At each time point, hemodynamic variables were continuously recorded for 5 minutes with a sampling rate of 150 times/minute using Workbench 3 for Windows[g], and the average of these values was calculated for each time point for use in the analyses. Data was considered continuous and found to follow a normal distribution using the Shapiro-Wilk test with failure to reject the null hypothesis of normality at $p \leq 0.05$. Data for the 3 baseline measurements, before BWE administration, were averaged and used as comparisons for data after BWE administration. The data was summarized and presented as mean ± SEM. The data was analyzed using the following mixed effect linear model that accounted for the random variance of horse and the repeated measurements on each horse:

$$y = \mu + \text{horse} + \text{treatment group} + \text{time} + \text{group*time} + \text{horse*time} + \varepsilon$$

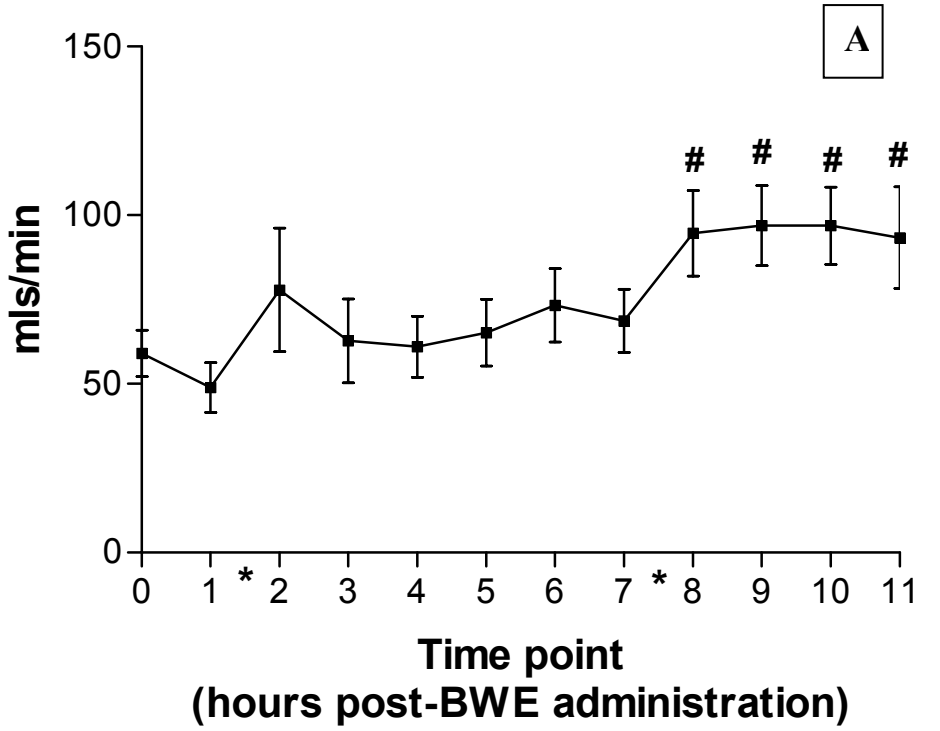
Select hemodynamic, all hematologic variables, and all physical exam variables were analyzed using a mixed effect model not examining for treatment group effects. Where there were significant interaction effects at $p \leq 0.05$, predetermined adjusted least squares means comparisons were made to determine where differences were occurring. Type I error was maintained at 0.05. PROC MEANS, UNIVARIATE, and MIXED were used for the analysis.[h] The physical examination parameters attitude, hoof temperature, digital pulse, response to hoof testers, capillary refill time/mucous membrane color, and gastrointestinal motility are presented descriptively.

6.3 Results

Eleven horses (six PD145065-treated and 5 saline controls) developed Obel grade 1 laminitis between 5 and 11 hours after BWE with a median value of 9 hours. These 11 horses had over a 30% decrease in WBC count. The remaining three horses did not develop Obel grade 1 laminitis, did not have at least a 30% decrease in WBC count, and data from these horses were not included in the analysis.

6.3.1 Hemodynamic Variables – From a baseline (BL) value of 59.1 ± 6.89 mls/min, palmar digital BF initially decreased 1 hour post-BWE administration (48.9 ± 7.35 mls/min), followed by an increase in blood flow that was significantly greater than BL 8 – 11 hours post-BWE with the greatest flow occurring with the onset of Obel grade 1 laminitis (96.9 ± 11.90 mls/min) (Fig. 6.1a and Table 6.1). There were no significant differences between PD145065-treated and saline-treated horses for palmar digital BF (Fig. 6.1b). PD145065 administration at the 1.5-hour time point resulted in an increase in BF within the treated group from 46.64 ± 4.47 mls/min at 1-hour post-BWE to 85.11 ± 16.13 mls/min at 2 hours post-BWE. Administration of PD145065 at the 7.5 hour time point also increased flow from 67.42 ± 13.00 mls/min at 7 hours

Figure 6.1 – Mean ± SEM palmar digital arterial blood flow measured using an ultrasonic Doppler flow probe in all 11 horses (A) and in horses divided by treatment group (B) before and after black walnut extract (BWE) administration. Horses demonstrated Obel grade 1 laminitis an average of 9 hours post-BWE administration. *Indicates timing of local digital treatment with either saline (n = 5) or PD145065 (10^{-5} M concentration; n=6). # Indicates values significantly ($p < 0.05$) different from baseline (0) values. There were no significant differences between saline and PD145065 treated horses.



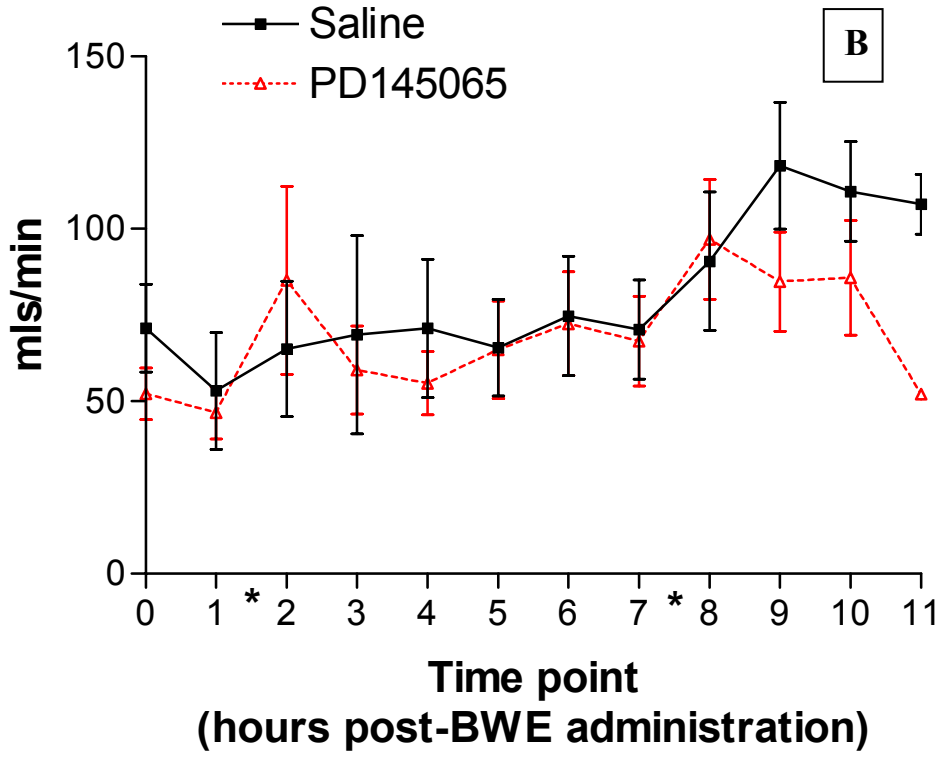


Table 6.1 - Mean (+/-) SEM values for hemodynamic and physical examination variables for all horses (n = 11) at baseline (before black walnut extract (BWE) administration) and at 9 hours after BWE administration corresponding to demonstration of Obel grade 1 laminitis. *Value is significantly ($P < 0.05$) different from baseline value.

Variable	Baseline	Obel Grade 1
Digital Blood Flow (mls/min)	59.10 (6.88)	96.93 (11.90)*
Digital Venous Pressure (mm Hg)	8.95 (0.99)	13.63 (3.08)
Digital Arterial Pressure (mm Hg)	96.58 (8.49)	81.50 (9.67)
Systemic Arterial Pressure (mm Hg)	101.43 (3.13)	107.90 (6.87)
Heart Rate (beats/min)	36.45 (1.14)	45.82 (1.36)*
Respiratory Rate (breaths/min)	13.89 (1.01)	18.00 (2.17)
Rectal Temperature (F)	100.46 (0.10)	102.48 (0.31)*

to 96.95 ± 17.38 mls/min at 8 hours post-BWE administration. After the 8-hour time point, the PD145065-treated group did not demonstrate as high levels of BF as the treated group, however, this difference was not significant. A significant overall effect of treatment was found ($p = 0.02$) for MDVP and PD145065-treated horses had a significantly greater ($p = 0.03$) value at 5 hours post-BWE administration (12.68 ± 1.83 mmHg for PD145065-treated horses compared with 6.43 ± 1.01 mmHg for saline-treated horses) (Fig. 6.2) Analysis of MDVP over time with all horses combined did not reveal any significant differences. There was a significant increase ($p = 0.02$) in MPAP over time with the greatest pressure occurring 11 hours post-BWE (106.7 ± 8.88 mmHg compared with the BL value of 96.6 ± 8.49 mmHg) (Fig. 6.3a). Systemic MAP did not significantly change across time (Fig. 6.3b).

6.3.2 Hematologic Variables – A significant 41.5% and 46.1% decrease ($p < 0.0001$) in WBC and segmented neutrophil counts, respectively, were noted 2 to 3 hours after BWE administration. White blood cell counts then significantly increased ($p < 0.003$) from 7 to 10 hours post-BWE (Fig. 6.4a) and segmented neutrophils significantly increased ($p < 0.004$) from 6 to 10 hours post-BWE (Fig. 6.4b and Table 6.2). Packed cell volume, TPP, platelet count, and HPP did not significantly change across time (Fig. 6.5 – 6.6). Jugular venous P/N aggregates did not significantly change over time and were not significantly different from palmar digital venous P/N aggregate counts (Fig. 6.7a). Palmar digital venous P/N aggregate counts significantly changed over time ($p = 0.02$) and were significantly lower ($p < 0.006$) at 4, 7, and 9 hours post-BWE compared with BL values (Fig. 6.7b). ET antagonist administration did not alter palmar digital venous P/N aggregate counts.

6.3.3 Physical Examination Variables – Heart rate significantly increased across time ($p < 0.0001$) from a BL value of 36.45 ± 1.14 beats/min at 4, 5, and 8 – 11 hours with the

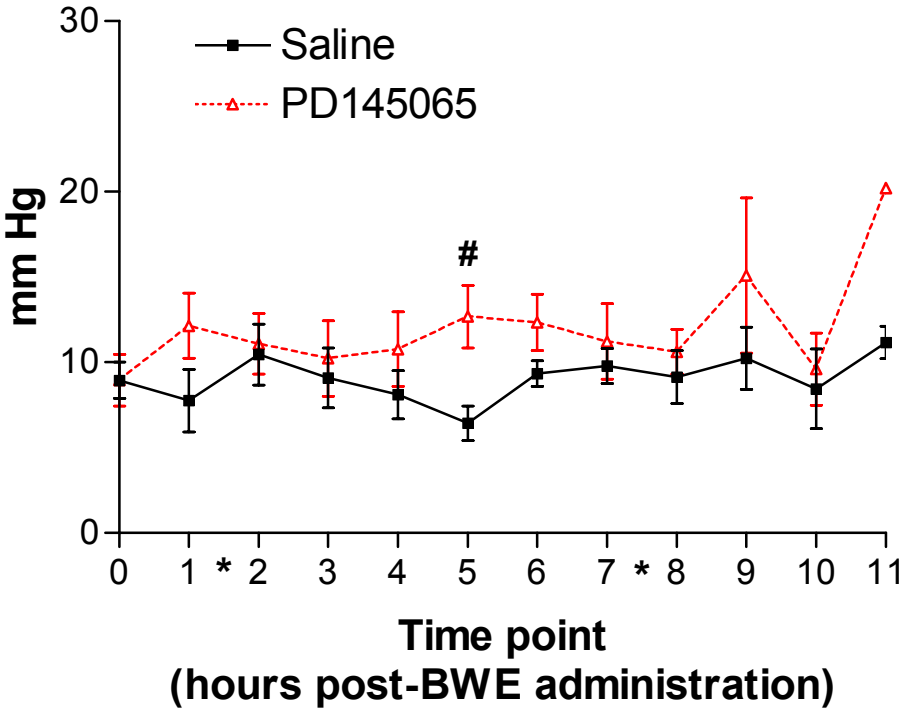
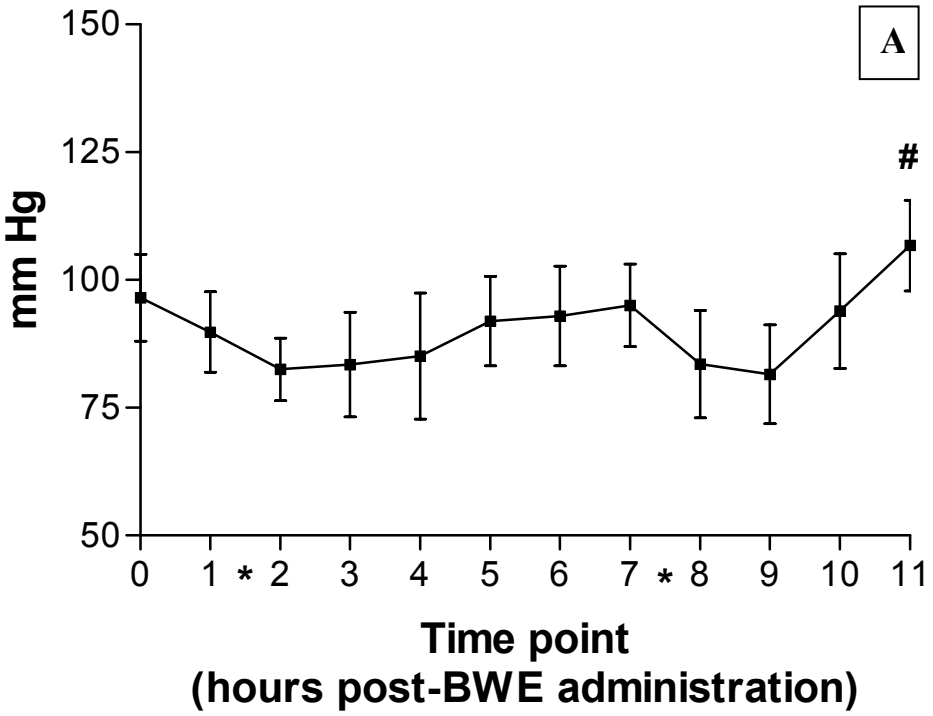


Figure 6.2 – Mean ± SEM mean palmar digital venous pressure measured in horses before and after black walnut extract (BWE) administration and locally treated with either saline (n = 5) or PD145065 (10^{-5} M concentration; n = 6) infused into the palmar artery. Only one horse was measured at the time point 11 for the PD145065 treated group for this variable. 0 = baseline. *Indicates timing of treatment with either saline or PD145065. # Indicates values significantly ($p < 0.05$) different from saline-treated group.

Figure 6.3 – Mean ± SEM mean palmar arterial pressure (A) and mean systemic arterial pressure (B) measured in 11 horses before and after black walnut extract (BWE) administration. *Indicates timing of local digital arterial infusion with either saline (n = 5) or PD145065 (10^{-5} M concentration; n=6). There were no significant differences between saline- and PD145065-treated horses, therefore horses were combined for analysis. # Indicates values significantly ($p < 0.05$) different from baseline (0) values.



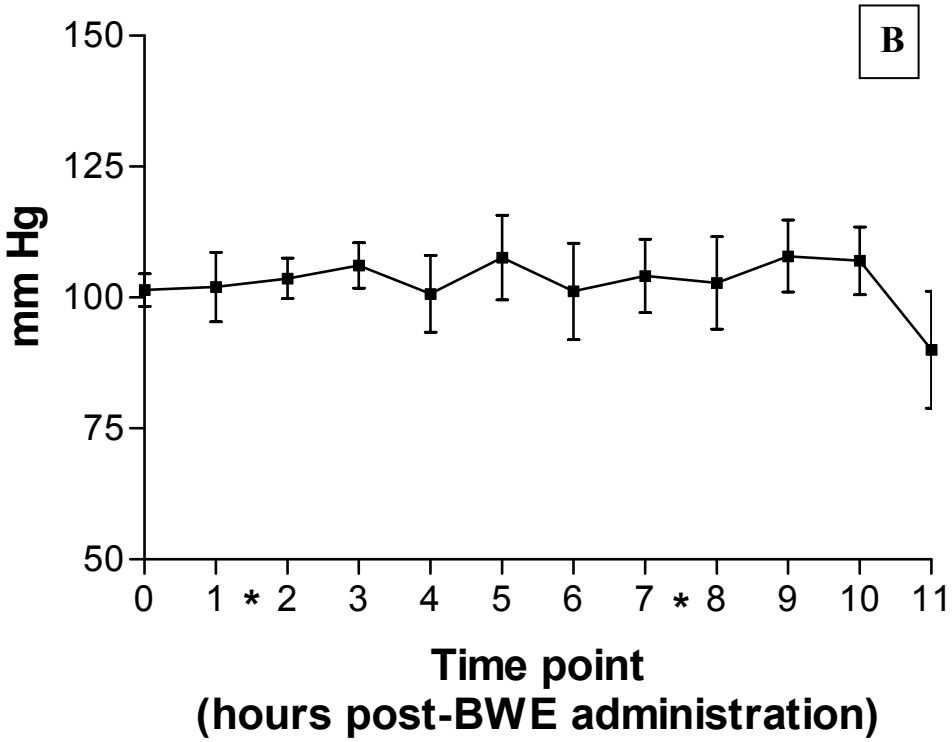


Figure 6.4 – Mean ± SEM white blood cell counts (A) and segmented neutrophil counts (B) measured in 11 horses before and after black walnut extract (BWE) administration. *Indicates timing of local digital arterial infusion with either saline (n = 5) or PD145065 (10^{-5} M concentration; n=6). There were no significant differences between saline- and PD145065-treated horses, therefore horses were combined for analysis. # Indicates values significantly ($p < 0.05$) different from baseline (0) values.

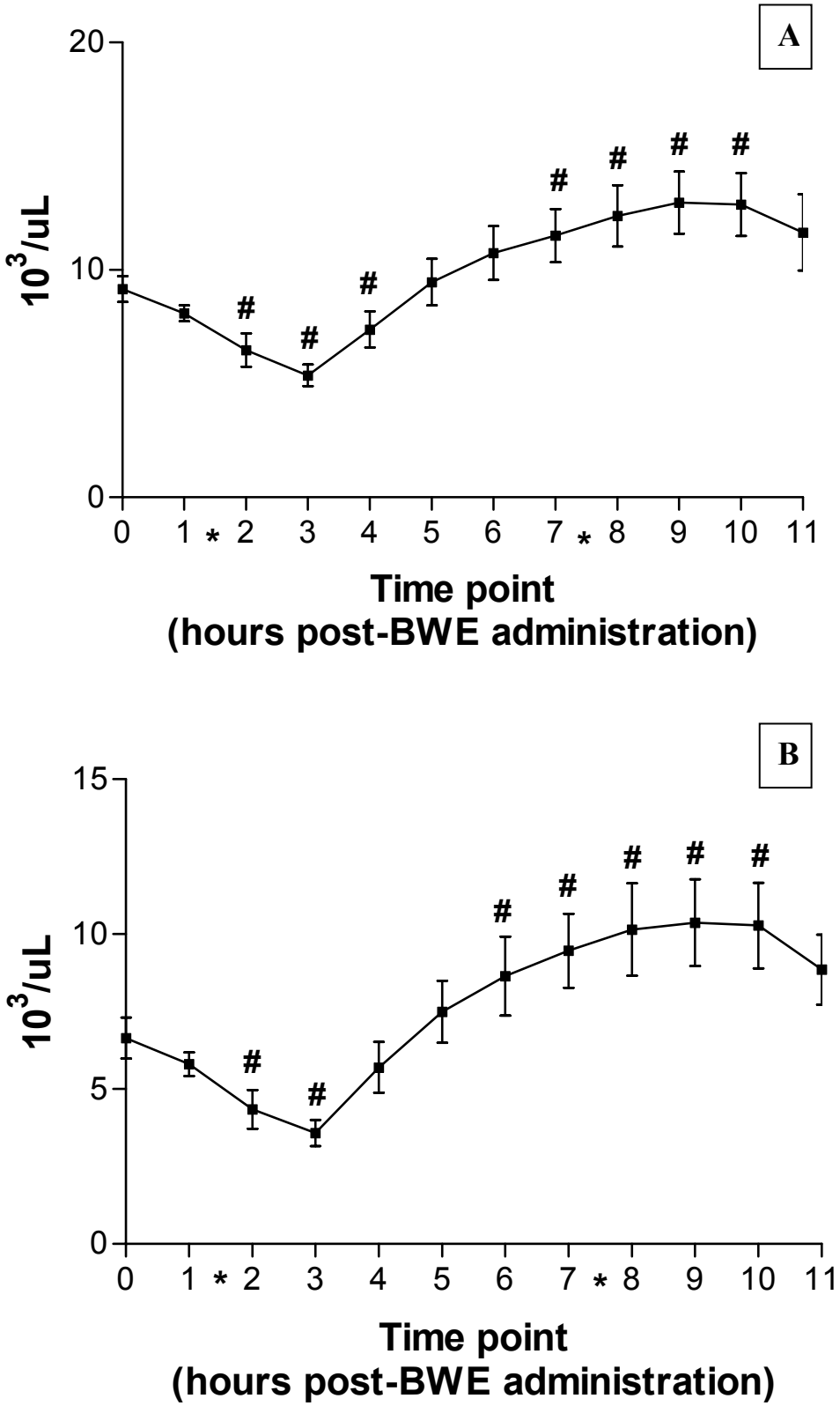
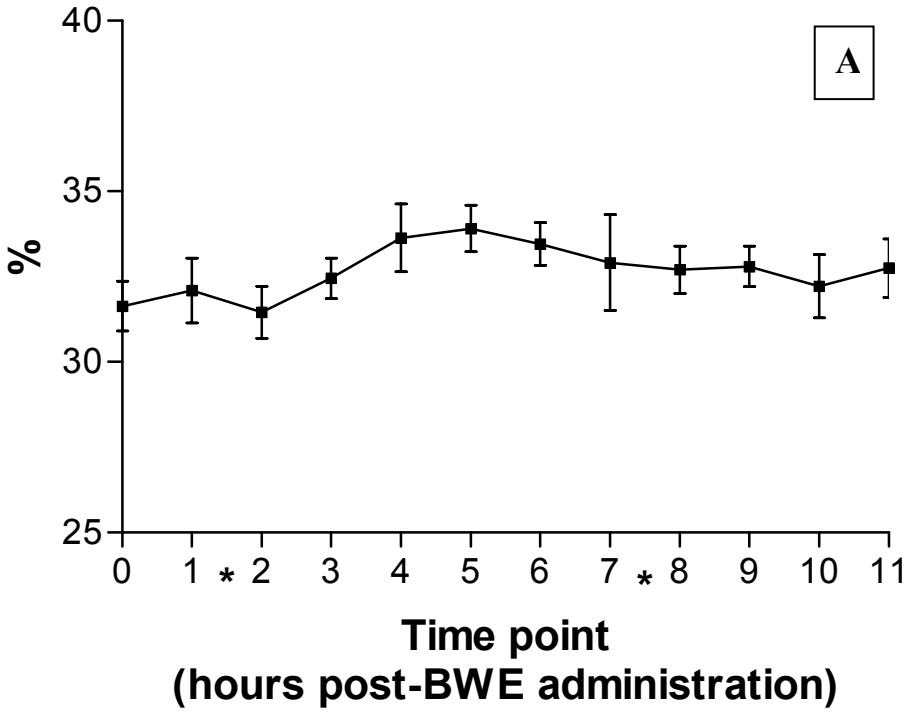


Table 6.2 - Mean (+/-) SEM hematologic values for all horses (n = 11) at baseline (before black walnut extract (BWE) administration) and at 9 hours after BWE administration corresponding to demonstration of Obel grade 1 laminitis. *Value is significantly ($P < 0.05$) different from baseline value. # Over a 40% drop was noted 3 hours post-BWE administration.

Variable	Baseline	Obel Grade 1
Packed Cell Volume (%)	31.64 (0.73)	32.80 (0.60)
Total Plasma Protein (g/dL)	6.77 (0.13)	6.66 (0.17)
White Blood Cell Count (10^3/uL)	9.16 (0.57)	12.96 (1.36)*#
Segmented Neutrophils (10^3/uL)	6.64 (0.66)	10.37 (1.40)*#
Platelet Count (10^3/uL)	145.07 (10.84)	157.33 (8.53)
Jugular Venous Platelet/Neutrophil Aggregate Ratio	0.037 (0.01)	0.020 (0.006)
Digital Venous Platelet/Neutrophil Aggregate Ratio	0.033 (0.004)	0.017 (0.002)*
Fibrinogen (mg/dL)	286.36 (18.91)	270.00 (28.60)

Figure 6.5 – Mean ± SEM packed cell volume (A) and total plasma protein concentration (B) measured in 11 horses before and after black walnut extract (BWE) administration. *Indicates timing of local digital arterial infusion with either saline (n = 5) or PD145065 (10^{-5} M concentration; n=6). There were no significant differences between saline- and PD145065-treated horses, therefore horses were combined for analysis. There were no significant changes in these variables from baseline (0) values.



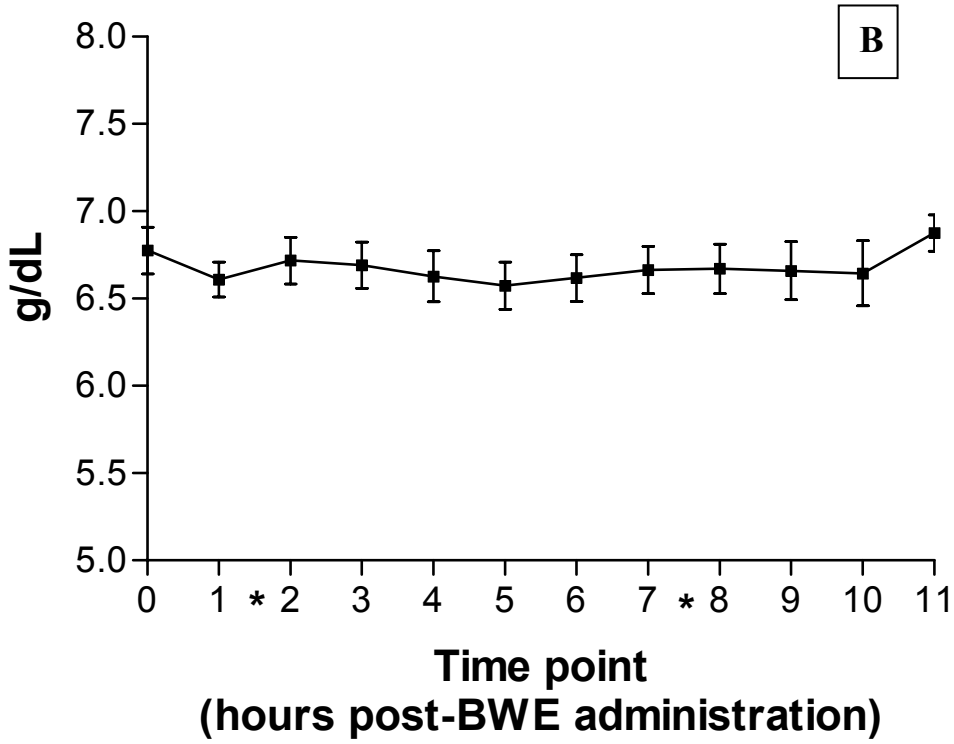
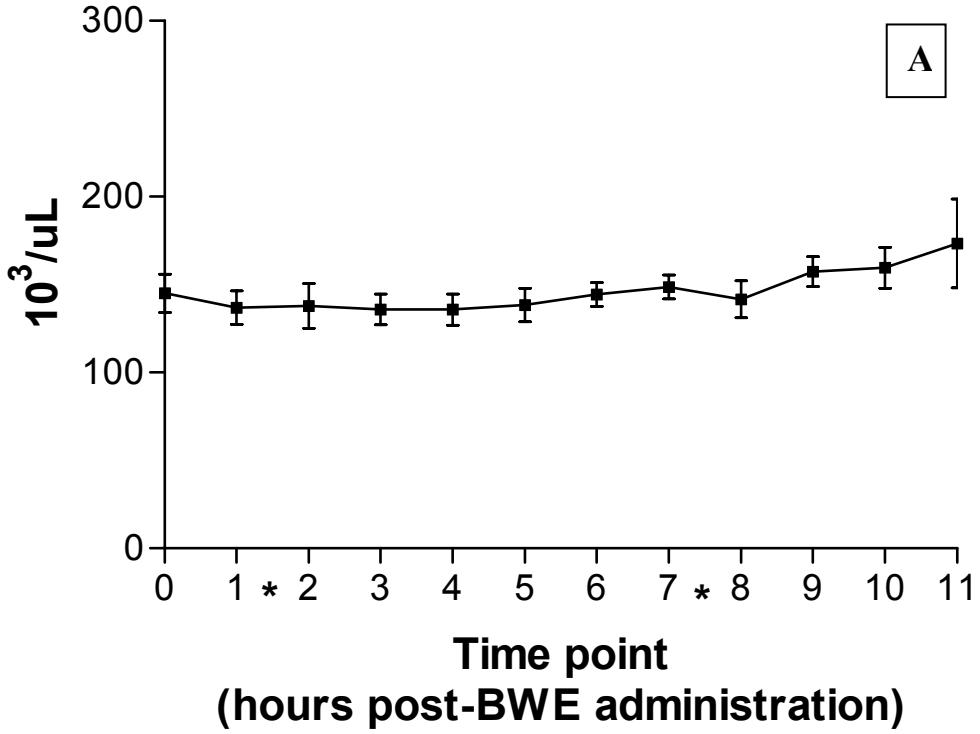


Figure 6.6 - Mean ± SEM platelet count (A) and fibrinogen concentration (B) measured in 11 horses before and after black walnut extract (BWE) administration. *Indicates timing of local digital arterial infusion with either saline (n = 5) or PD145065 (10^{-5} M concentration; n=6). There were no significant differences between saline- and PD145065-treated horses, therefore horses were combined for analysis. There were no significant changes in these variables from baseline (0) values.



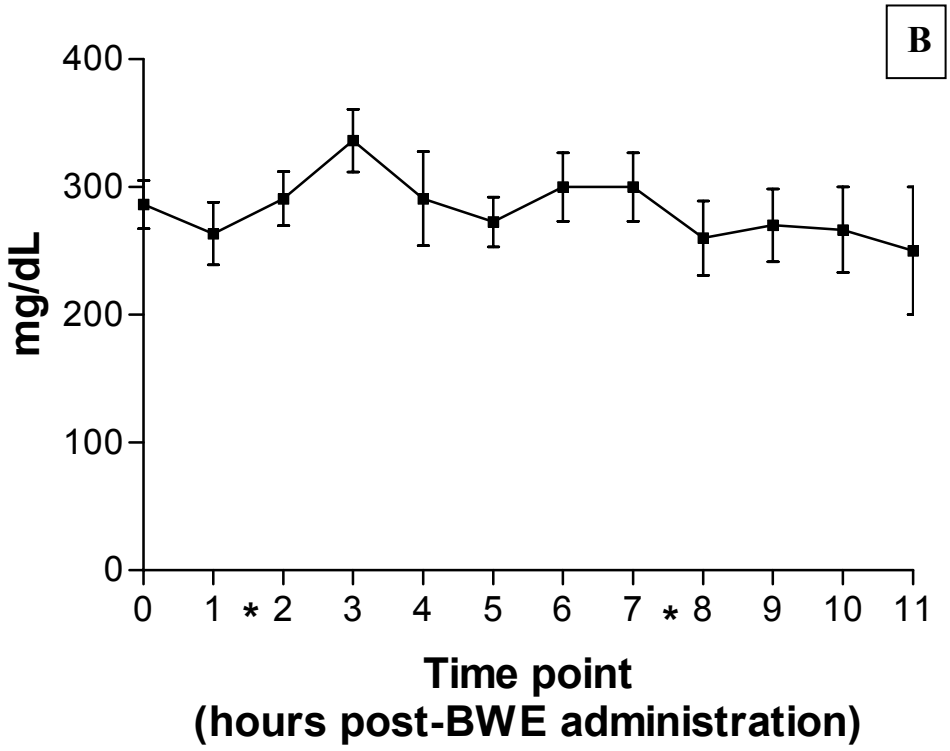
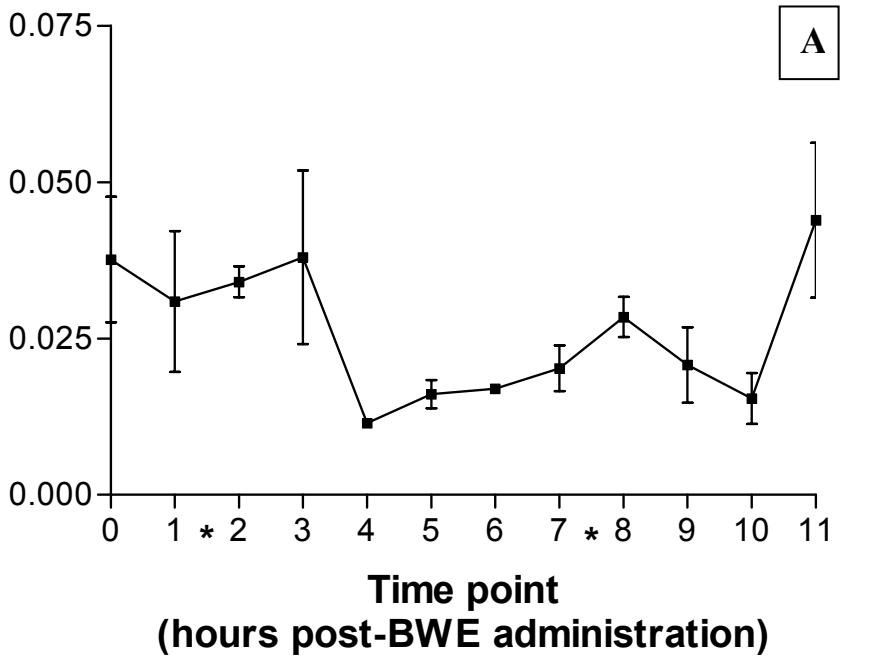
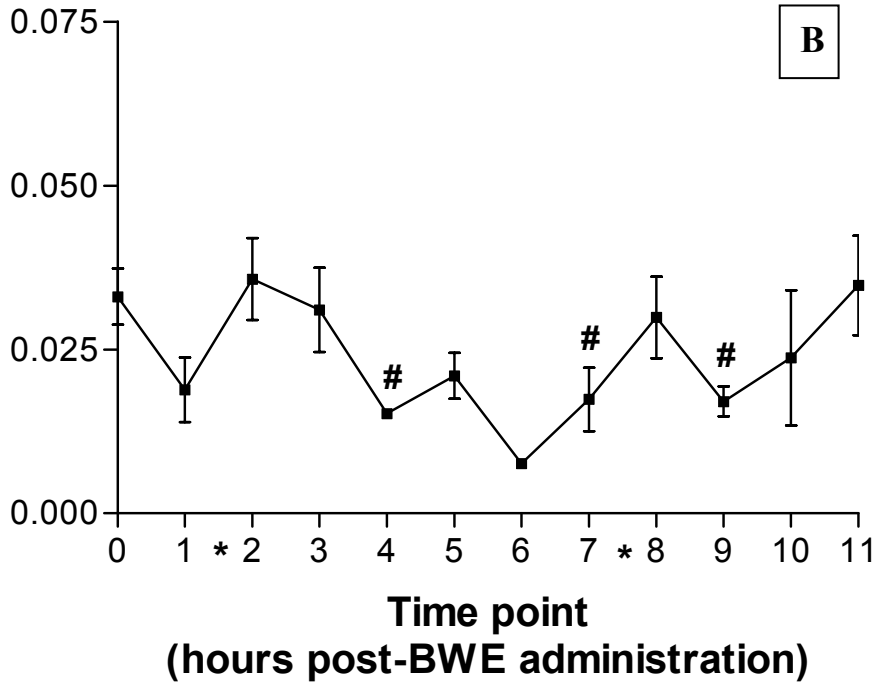


Figure 6.7 - Mean ± SEM jugular venous (A) and palmar digital venous (B) platelet/neutrophil aggregates (number of aggregates (platelets adhered to neutrophils) per 200 neutrophils evaluated) measured in 11 horses before and after black walnut extract (BWE) administration. *Indicates timing of local digital arterial infusion with either saline (n = 5) or PD145065 (10^{-5} M concentration; n=6). There were no significant differences between saline- and PD145065-treated horses, therefore horses were combined for analysis. # Indicates values significantly ($p < 0.05$) different from baseline (0) values.



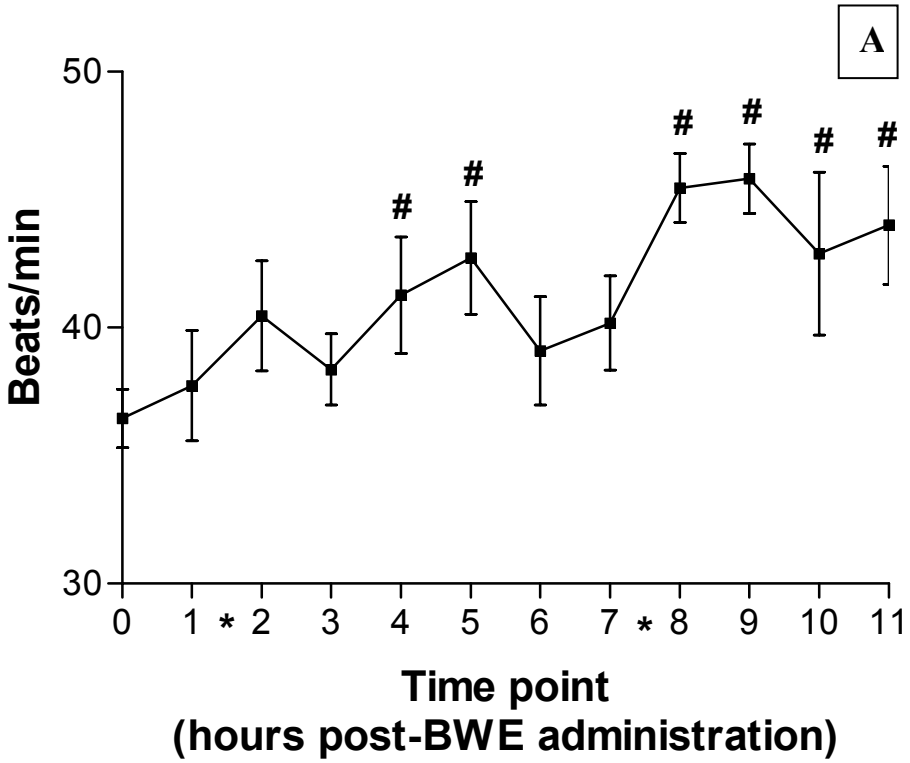


greatest value occurring 9 hours post-BWE (45.82 ± 1.36 beats/min) (Fig. 6.8a and Table 6.1). Respiratory rate also significantly increased ($p = 0.004$) across time with the highest rate 6 hours post-BWE (23.18 ± 5.09 breaths/min compared to BL of 13.89 ± 1.01 breaths/min) (Fig. 6.8b). Rectal temperature significantly increased over time ($p < 0.0001$) with significant differences from 3 to 11 hours post-BWE (Fig. 6.9a). The rectal temperature at BL was 100.4 ± 0.09 F and increased to the greatest temperature of 102.6 ± 0.33 F at 10 hours post-BWE. Obel grade significantly increased ($p < 0.0001$) across time and was significantly greater from 8 to 11 hours post-BWE (Fig 6.9b). The average time required for horses to demonstrate Obel grade 1 laminitis was 9 hours post-BWE. The remaining physical examination findings are presented in Table 6.3.

6.4 Discussion

Use of BWE for induction of laminitis in this study resulted in several key findings. First, use of the BWE model resulted in induction of acute laminitis in 11 of 14 horses with demonstration of Obel grade 1 laminitis after an average of 9 hours post-BWE administration. Palmar digital blood flow initially decreased 1 hour post-BWE, and then increased above BL corresponding with the demonstration of clinical signs of laminitis. Local digital administration of the ET antagonist PD145065 did not alter blood flow, mean palmar arterial pressure, systemic arterial pressure, or palmar digital venous P/N aggregate counts. PD145065 did result in an increased palmar digital venous pressure, especially 5 hours post-BWE administration, and since alterations in flow were not noted during this time, the increase in venous pressure is most likely related to increased post-capillary resistance. The alterations in resistance were not of sufficient magnitude to decrease flow. Mean palmar arterial pressure increased after the demonstration of clinical signs of laminitis; increases in blood flow were also occurring during this time but may

Figure 6.8 - Mean ± SEM heart rate (A) and respiratory rate (B) measured in 11 horses before and after black walnut extract (BWE) administration. *Indicates timing of local digital arterial infusion with either saline (n = 5) or PD145065 (10^{-5} M concentration; n=6). There were no significant differences between saline- and PD145065-treated horses, therefore horses were combined for analysis. # Indicates values significantly ($p < 0.05$) different from baseline (0) values.



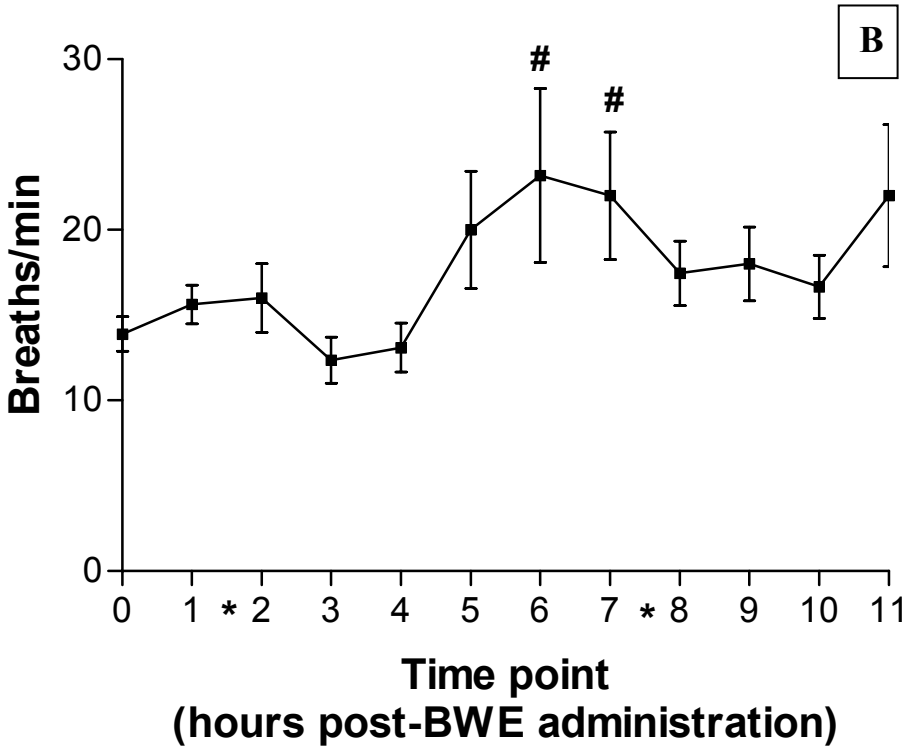
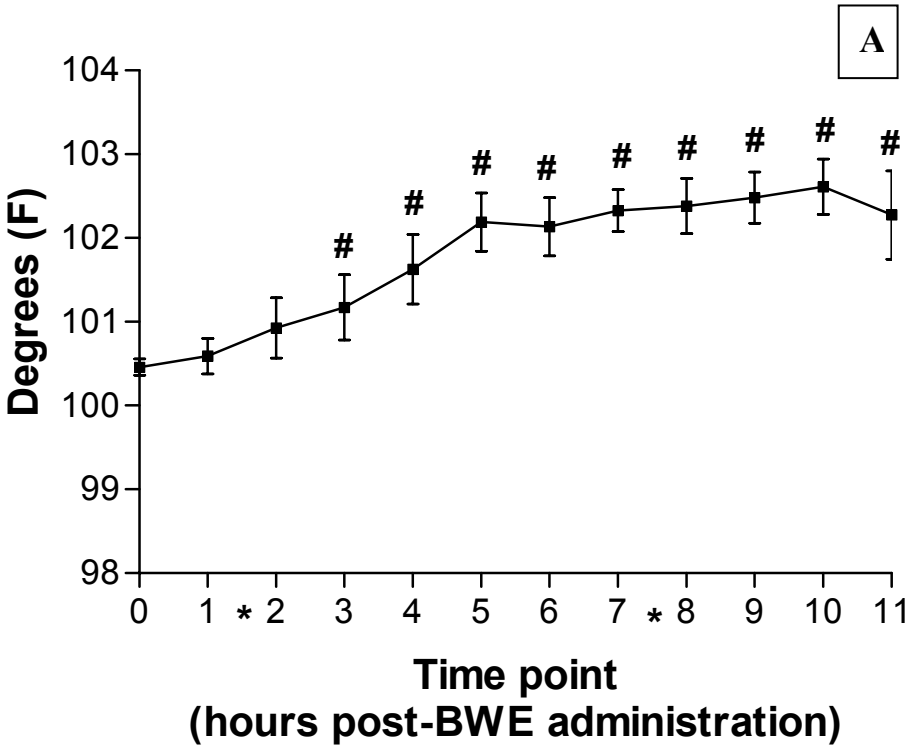


Figure 6.9 - Mean ± SEM rectal temperature (A) and Obel grade (B) measured in 11 horses before and after black walnut extract (BWE) administration. *Indicates timing of local digital arterial infusion with either saline (n = 5) or PD145065 (10^{-5} M concentration; n=6). There were no significant differences between saline- and PD145065-treated horses, therefore horses were combined for analysis. # Indicates values significantly ($p < 0.05$) different from baseline (0) values.



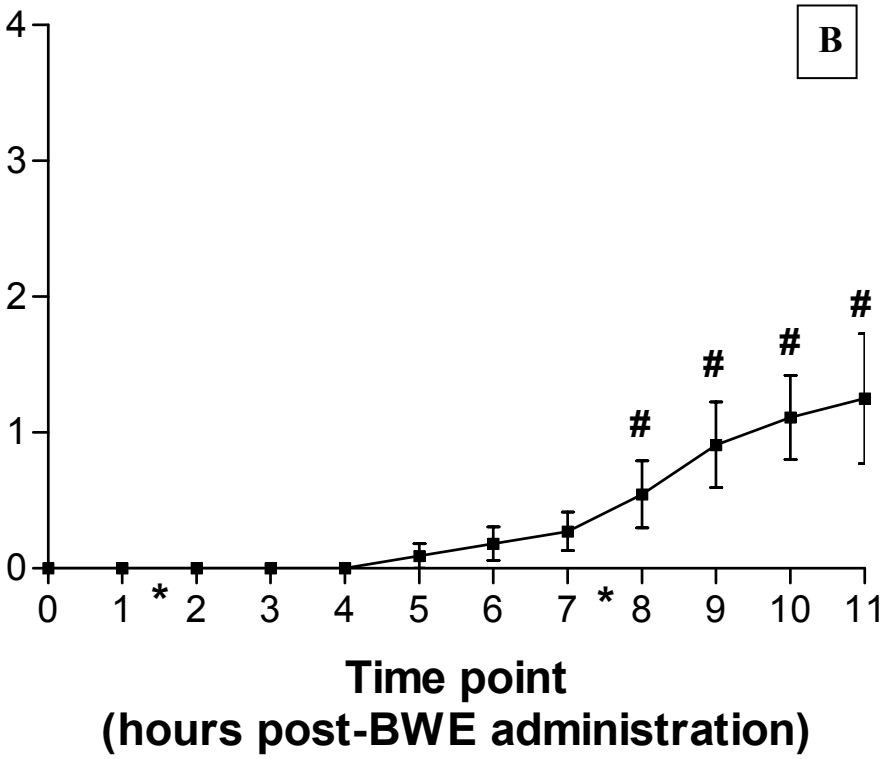


Table 6.3 – Number of horses (n = 11) demonstrating certain physical examination findings during each of three time intervals during the developmental stages of acute laminitis induced by black walnut extract (BWE). The mean time for horses to demonstrate Obel grade 1 laminitis was 9 hours post-BWE administration.

Physical Examination Variable	1 – 3 Hours Post-BWE	4 - 6 Hours Post-BWE	7 - 11 Hours Post-BWE
Depression	4	3	6
Signs of Abdominal Discomfort	9	5	4
Increase in Hoof Wall Temperature	1	9	11
Increase in Digital Pulse	1	5	11
Positive Response to Hoof Testers	0	3	11
Prolonged Capillary Refill Time	3	5	9
Decreased Gastrointestinal Motility	6	2	4

have been bypassing laminar tissues due to opening of the numerous arteriovenous shunts found within the digital vasculature. Finally, alterations in hematologic and physical examination variables were similar to findings of other studies using this model for induction of acute laminitis demonstrating the consistency and usefulness of this model.[5,24,26]

The BWE model for induction of acute laminitis was first described by Minnick et al in 1987 as an improved model over the CHO overload model described by Garner et al in 1975.[1,22] The BWE model was considered to be superior to the CHO overload model since diarrhea and signs of endotoxemia were not associated with it. The use of BWE in our laboratory resulted in findings comparable to those of Minnick et al, Galey et al, Eaton et al, Adair et al, and Fontaine et al, all of which used the BWE model for induction of acute laminitis in horses.[5,22,24,26,27] The focus of each of these studies has determined the duration of sample collection and monitoring of the horses before induction of anesthesia or euthanasia; Eaton et al and Fontaine et al anesthetized the horses once a 30% decrease in WBC count occurred; whereas Minnick et al, Galey et al, and Adair et al followed the horses beyond the demonstration of clinical signs of laminitis until recovery in horses without severe laminitis. For the report presented here, the study was terminated once horses demonstrated Obel grade 1 laminitis (9 – 11 hours post-BWE). The initial decrease in WBC and segmented neutrophil counts followed by a substantial increase in both indices are consistent with these other studies.[26] Galey et al suggested that these leukocyte alterations are characteristic of horses with endotoxemia, but Eaton et al examined blood samples for endotoxin up to 3 hours post-BWE and did not detect endotoxin in any of the samples.[5,26]

Although the use of an ultrasonic Doppler blood flow probe has been successful in measuring digital blood flow in horses, this study is the first to report its implementation using

the BWE model.[21,28,29] Our findings support the findings of Adair et al regarding laminar capillary perfusion measurements whereby perfusion initially decreased 1 hour post-BWE followed by substantial increases in flow just before horses demonstrated clinical signs of laminitis.[24] Using the CHO model, decreased hoof wall surface temperature was noted during the developmental stages of laminitis and was considered an indication of decreased laminar perfusion or decreased metabolic activity.[30] Based on these studies, a period of altered digital blood flow and laminar perfusion occurs during the first 3 hours of BWE-induced laminitis and is followed by a substantial period of reperfusion as the clinical signs of laminitis develop.

Contrary to our hypothesis, administration of the ET receptor antagonist PD145065 did not significantly improve digital blood flow compared with saline control horses; however, PD145065 administration at the 1.5 and 7.5 hour time points did result in a considerable increase in blood flow within the treated group and this group did not appear to experience as great a hyperemic episode as the saline-treated group. Also contrary to our expected findings, the group treated with the ET antagonist had significantly greater MDVP 5 hours post-BWE administration than the saline-treated horses. Previous studies have not examined digital venous pressure using the BWE model for comparison to our findings. Digital blood flow was not increased at 5 hours post-BWE, therefore the difference in pressure is most likely due to increased venous resistance. Digital venoconstriction has been considered an important component early in the developmental stages of laminitis, but administration of the ET antagonist was expected to improve the venous pressure compared with saline-treated horses.[5] A study demonstrated that after reducing blood flow by 70% to an isolated segment of ileum in pigs, ET-1 plasma concentrations remained unchanged in control segments and in the systemic circulation, slightly increased in the intestinal arterial blood supply, but increased four-fold in the intestinal venous circulation.[31] Venous

pressure was also increased during this study although the blood flow had been manually reduced for the duration of the study. Although the findings are not a part of this report, we measured jugular and digital venous ET-like immunoreactivity in the horses before and after BWE administration and found significant increases in digital venous blood 3 to 10 hours post-BWE.[32] Another recent study examining microvascular dysregulation associated with ischemia-reperfusion injury found that after a short period of low flow (60 minutes) and within a few hours of restoration of flow, the major receptor upregulated in the ischemic and nearby tissue was the ET_B receptor.[33] The purpose of this preferential upregulation may be to balance the increased presence of ET-1 in the venous circulation after a period of ischemia with increased NO availability for smooth muscle relaxation. It is possible that after the decrease in blood flow to the digit during our study, administration of the non-selective (ET_A and ET_B) receptor antagonist PD145065 resulted in blockade of the upregulated ET_B receptor to a greater extent than the ET_A receptor. Greater blockade of the ET_B receptor would cause less NO release leading to greater contractile effects of ET-1 through the ET_A receptor, resulting in increased venous pressure. The overall significance of the change in digital venous pressure is questionable. A significant difference occurred at only a single time point and the change in pressure was not of sufficient magnitude to alter blood flow.

Since we did not find significant differences between saline- and PD145065-treated horses for blood flow or palmar arterial pressure, potentially the dose of the antagonist (10^{-5} M concentration delivered for a 2-minute duration into the palmar artery) was not sufficient to effectively block the contractile effects of ET-1. Previous in vitro and in vivo studies in our laboratory have identified the 10^{-5} M concentration of PD145065 as the most effective dose resulting in significant blockade of the contractile effects of ET-1. Consequently, the duration of

administration would presumably be the target of future studies examining the usefulness of this agent in the prevention and treatment of laminitis.

Measured increases in digital arterial pressure occurred after demonstration of Obel grade 1 laminitis in this study. Previous studies have not evaluated digital arterial pressure, but have examined precapillary resistance.[5] Previous studies using the BWE model and Starling force evaluation, precapillary resistance did not change compared with healthy horses, but using the CHO model precapillary resistance significantly increased.[3,5] Differences between these models may be due to differences in timing of events in the development of laminitis. The ischemic theory of acute laminitis states that venoconstriction is the initiating factor causing decreased laminar perfusion.[34] Increased venoconstriction results in increased venous resistance and capillary hydrostatic pressure. Increased capillary hydrostatic pressure forces fluid out of the capillaries and into the interstitium thereby increasing laminar interstitial pressure. When tissue pressure increases above the capillary critical closing pressure, the capillaries collapse leading to tissue ischemia. Blood flow is further reduced by formation of arteriovenous shunts at the level of the coronary band.[3,34,35] It is probable that digital arterial pressure increases as the capillaries collapse due to increased arterial resistance; therefore, the digital arterial pressure would increase later than the venous pressure as found during our study. Also, increases in blood flow corresponded with the increase in digital arterial pressure, possibly contributing to the increase in pressure.

The hematologic findings were similar to those reported by Galey et al and Eaton et al.[5,26] A variable examined as part of the study reported here was the presence of P/N aggregates in jugular and palmar digital venous blood. Weiss et al found microvascular thrombi in the laminae of ponies after induction of laminitis using the CHO overload model.[25] We detected a low

number of platelets adhered to neutrophils per 200 neutrophils examined. The only significant differences noted were for digital venous aggregate counts with fewer aggregates noted 4, 7, and 9 hours post-BWE. A possible explanation for this finding is that during these time points the aggregated cells were lodged in the microvasculature of the digit or other organs. Aggregate counts from jugular samples were also decreased at these time points but were not statistically decreased. The platelet count determined from jugular venous samples at the corresponding time points were not changed from BL measurements, therefore platelet consumption does not appear to account for these observed P/N aggregate decreases. Other causes of the decreased P/N aggregate formation are unclear.

In summary, the findings of this study in conjunction with other studies using the BWE model for induction of laminitis demonstrate that an early ischemic event occurs and may precede the other hemodynamic events known to occur during the prodromal stages of the disease, such as alterations of digital pressures, resistances, and hydrostatic-mediated movement of fluid into the laminar interstitium. The stimulus of the decreased blood flow remains unclear; however, with further investigation, the role of ET-1 in the pathophysiology of digital ischemia may be further defined.

6.5 Product Information

[a] Transonics Systems, Inc., Ithaca, NY

[b] Angiocath Vascular Access, Becton Dickinson & Co, Sandy, UT

[c] Arrow Catheters, Arrow International, Reading, PA

[d] PD 145065, American Peptide Co, Sunnyvale, CA

[e] Grass Medical Instruments, Quincy, MA

[f] Eberbach Corp, Ann Arbor, MI

[g] Workbench 3 for Windows, IOtech, Inc., Cleveland, OH

[h] Proc Mixed, Univariate, and Means; SAS version 8, SAS Institute, Cary, NC

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CHAPTER 7. JUGULAR AND PALMAR DIGITAL VENOUS PLASMA ENDOTHELIN-LIKE IMMUNOREACTIVITY AND DIGITAL VASCULAR AND LAMINAR IMMUNOHISTOCHEMICAL STAINING IN HORSES WITH BLACK WALNUT EXTRACT-INDUCED ACUTE LAMINITIS

7.1 Introduction

Over the past 20 years, the leading hypothesis regarding the initiating factor in the cascade of events leading to necrosis and structural failure of the interdigitating sensitive and insensitive laminae has focused on digital hemodynamic alterations; however, the initiating event that triggers these vascular alterations has yet to be determined.[1] Laminitis usually occurs secondary to other diseases such as intestinal strangulating obstruction, inflammatory bowel disease, grain overload, metritis, pleuropneumonia, and other diseases, which are often accompanied by endotoxemia. Administration of 0.03 g/kg *Escherichia coli* endotoxin 055:B5 did not induce consistent changes in equine digital hemodynamics or Starling forces associated with the developmental stages of acute laminitis found using other models of induction such as the black walnut extract (BWE) and carbohydrate overload (CHO) models.[2] However, circulating levels of endotoxin are increased in horses following CHO-induced laminitis and in horses with naturally-occurring gastrointestinal tract disease.[3,4] The cascades of cellular interactions that occur following endotoxemia in horses is extremely diverse including activation of inflammatory mediators (tumor necrosis factor, interleukins -1 and -6, and numerous eicosanoids), the coagulation cascade (microthrombi formation), and endothelial cell disturbances (increased permeability, structural and metabolic alterations, and altered release of endothelial-derived substances).[5-13]

Although the association between endotoxemia and laminitis has never been solidly linked by research, many studies have reported the cascade of events, led by alterations in vascular function, which occur during the developmental stages of laminitis in horses.[1,14-17] Garner et al introduced the hypothesis that the predominant cause of laminitis after CHO was a disturbance in digital blood flow, which occurred during the onset of the syndrome after CHO

overload of the gastrointestinal tract.[18] Using contrast radiography, researchers demonstrated reduced perfusion in the terminal vasculature of the foot.[19] In subsequent studies using the isolated perfused digit, the specific hemodynamic forces acting on the laminar microcirculation in healthy and experimentally-induced laminitic horses have been extensively defined.[1,14,20] Several alterations in the digital vascular system of horses with experimentally-induced Obel grade I laminitis (both CHO overload and black walnut extract (BWE) models) have been identified.[1] Of particular importance is the finding that the pre-to-post capillary resistance ratio is decreased in the prodromal stages of laminitis. This imbalance increases the hydrostatic force in the capillary promoting the flux of fluid across the capillary bed within the foot, resulting in laminar edema while capillary permeability remains normal. These findings support the hypothesis that increased venomotor tone initiates laminitis.

In physiologic states, the endothelium synthesizes vasoactive substances, such as nitric oxide (vasodilator) and endothelin-1 (ET-1; profound vasoconstrictor), which regulate vasomotor tone and have anticoagulant properties.[21-24] Many pathological states characterized by vascular or smooth muscle alterations, such as endotoxemia, atherosclerosis, hypertension, Raynaud's syndrome, and asthma, are associated with increased plasma concentrations of ET-1 and increased tissue ET-1 immunohistochemical staining.[25-30] Endothelin is a 21 amino acid peptide and was first isolated by Yanagisawa and colleagues in 1988.[22] Endothelin has three isoforms, namely ET-1, ET-2, and ET-3 although vascular endothelium and smooth muscle cells principally synthesize the ET-1 isoform. In blood vessels, biosynthesis of ET-1 occurs in the endothelium, approximately 80% is released abluminally toward the vascular smooth muscle, and the two main receptor types for ET-1 (ET_A and ET_B) are located on vascular smooth muscle cells and endothelium, respectively.[24,31]

The ET antagonist PD145065 selected for these studies is a non-selective, competitive inhibitor of both the ET_A and ET_B receptors.[32] It has proven to be beneficial in models examining the role of ET-1 in ischemia/reperfusion, the role of ET-1 in the systemic inflammatory response, and in ET-induced constriction of vascular and airway smooth muscle.[33-35] Since laminitis is characterized by alterations in vascular resistance and blood flow, the potential use of this agent to ameliorate these hemodynamic changes would be beneficial.

Our hypotheses are that with the onset of experimentally-induced acute laminitis using the BWE model, digital plasma concentrations of ET-like immunoreactivity and digital vascular and laminar tissue ET-1 immunohistochemical staining will increase, which likely contribute to the vascular alterations (venoconstriction) characteristic of acute laminitis. Administration of the ET antagonist is not expected to alter ET-1 plasma immunoreactivity or IHC staining since it acts as a competitive receptor antagonist. The purposes of these studies were to quantify systemic and digital venous plasma ET- like immunoreactivity in horses before and after administration of BWE and evaluate palmar digital arterial (PDA), PDV, and laminar IHC staining for ET-1 after induction of acute laminitis using BWE.

7.2 Materials and Methods

7.2.1 Selection and Preparation of Horses - This study was approved by the Institutional Animal Care and Use Committee of Louisiana State University. Fourteen light breed horses (4 mares and 10 geldings; ages ranging 5 – 20 years; weighing 411 to 591 kg) were determined to be free of laminitis and gastrointestinal disease based on their history, thorough physical and lameness examinations, and lateral radiographs of both front feet. Horses were housed in stalls, fed grass hay, and acclimated to the study area for two weeks prior to the study. On the day of the study, horses were placed in stocks, instrumented, and then moved to the study

area where they were allowed a 1-hour period of equilibration prior to the start of the study. A 14-gauge, 13.3-cm Teflon catheter[a] was inserted into the left jugular vein (JV) for collection of blood samples. Palmar digital venous (PDV) blood was collected via a 20-gauge, 4.45-cm polyurethane catheter[b] placed in the palmar digital vein. A 20-gauge, 4.45-cm polyurethane catheter was placed in the medial palmar artery for administration of the ET receptor antagonist PD145065[c] in 6 horses. Tissues for ET-1 IHC staining were collected from these horses after termination of the study. Tissues were also collected for ET-1 IHC staining from 9 light breed horses (6 females and 3 castrated males; ages ranging 4 - 17 years; weighing 409 to 455 kg) determined to be free of laminitis based on their history and physical and lameness examinations.

7.2.2 Preparation of Extract – Preparation of the BWE was as previously described.[14,36] Briefly, shavings were made from the heartwood of a black walnut tree cut in the fall of the year and were stored at –20 C until use. Two grams of black walnut shavings/kg body weight were combined with 8 liters of distilled water and mixed in a shaker bath[d] for 14 hours at room temperature (20 – 22 C). The mixture was filtered, refrigerated, and the fluid was administered within 24 hours of preparation.

7.2.3 Experimental Design – Baseline JV and PDV blood samples (8 ml) were drawn 8, 4, and 1 hour before BWE administration and placed in chilled tubes containing EDTA[e] (500 KIU/ml) and aprotinin[e] (1 mg/ml), an anticoagulant and a protease inhibitor, respectively. Samples were centrifuged immediately at 1,500 X g for 10 minutes and the plasma transferred into polypropylene tubes and stored at –70 C until analyzed for endothelin-like immunoreactivity using a commercial human enzyme-linked immunosorbent assay (ELISA) kit.[f] Jugular venous blood samples (3 ml) were also collected into tubes containing EDTA for CBC determination. Laminitis was induced in all horses by administration of the BWE via a nasogastric tube. Blood

samples (JV and PDV) were collected and horses were monitored hourly after BWE administration until horses demonstrated Obel grade 1.[37] Efficacy of the extract was confirmed by at least a 30% decrease in WBC count.[14] Seven horses were administered a 10^{-5} M concentration of PD145065 (ET receptor antagonist) and 7 horses received infusion of an equivalent volume of 0.9% NaCl over two minutes into the medial palmar arterial catheter at 1.5 and 7.5 hours post-BWE administration. These times were selected based on the timing of known decreases in laminar perfusion after BWE administration.[15] The dose required to maintain a 10^{-5} M concentration of the ET antagonist in the blood for two minutes was based on the measured palmar digital blood flow using an ultrasonic Doppler blood flow probe[g] surgically placed around the palmar digital artery 10 days before the start of the study. The study was terminated once horses demonstrated Obel grade 1 laminitis and tissues were collected for IHC staining.

7.2.4 Plasma Endothelin-Like Immunoreactivity – Plasma ET-like immunoreactivity was quantified using a commercial human ELISA kit.[f] One milliliter of plasma was thawed and mixed with 1.5 ml of precipitating agent (80 mls HPLC grade acetone + 12 mls precipitating agent) provided by the manufacturer. The samples were cooled to 4 C and centrifuged for 20 min at 3,000 x g. The supernatant was transferred into polypropylene tubes in a 37 C water bath and dried under a flow of nitrogen gas. The dried samples were reconstituted in 500 µl of assay buffer. Serial dilutions of the endothelin stock solution were prepared according to the manufacturer's guidelines to serve as standards. The buffer was used as the zero standard. The antibody used in this assay principally recognized ET-1 with 100% cross-reactivity, however, there was reportedly also 100% and <5% cross-reactivity with ET-2 and ET-3, respectively. Two hundred microliters of standards, controls, and samples in duplicate were pipetted into wells

coated with a polyclonal rabbit anti-ET antibody. Detection antibody (monoclonal mouse anti-endothelin antibody lyophilized with green dye, 50 µl) was added to all wells, except the blank, and then thoroughly mixed. The wells were covered with plastic film and incubated 16-24 hrs at room temperature. The contents of the wells were discarded and the wells washed 5 times with washing buffer. Conjugate (anti-mouse IgG antibody conjugated to horseradish peroxidase, 200 µl) was then added to all wells. The wells were covered again with plastic film and incubated for 3 hr at 37 C. The contents of the wells were discarded and the wells washed 5 times with washing buffer. Substrate (tetramethylbenzidine, 200 µl) was added and the wells incubated for 30 min at 20 C in the dark. Then, 50 µl of stop solution was added to all wells and mixed thoroughly. Absorption was determined immediately with an ELISA reader[h] at 405 nm against 620 nm as reference. All samples were analyzed in duplicate. The sensitivity of the assay was 1.5 pg/ml. The mean percentage recovery of a known quantity of ET standard added to pooled plasma was 102%. The inter- and intra-assay variability for equine plasma was determined to be 15.4% and 6.4%, respectively, in our laboratory. The detection range was 1.59 – 25.65 pg/ml and sample dilution or concentration accounts for reported concentrations above or below the detection range, respectively.

7.2.5 ET-1 Immunohistochemical Staining – Tissue samples from the PDA, PDV, and laminae were collected from horses immediately after euthanasia with sodium pentobarbital[i] (90mg/kg, IV) and placed in zinc formalin for fixation for 24 hours. Tissues were paraffin-embedded, sectioned at 4 µm, attached to silanized slides, and dried overnight in a 37 C oven. Immunostaining was performed using a modified three-step Avidin-Biotin complex (ABC) method with a Vector Elite ABC Rabbit IgG kit.[j] Tissue sections were heated for 10 min at 60 C and were then deparaffinized and hydrated through graded alcohol solutions to distilled water.

The remaining steps were completed at room temperature. Slides were placed in 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity, were rinsed in distilled water, and equilibrated in 10 mM phosphate buffered saline with 0.25% tween 20 (PBS-Tween, pH 7.6). Slides were incubated in normal goat serum (1:50) for 45 min to block non-specific antibody binding. Excess serum was removed and slides were incubated with the primary antibody rabbit anti-ET-1 antiserum[k] for 60 min (1:700; diluted in goat serum). After incubation, slides were rinsed with PBS-Tween and incubated with the biotinylated secondary antibody goat anti-rabbit IgG antiserum (1:200) for 30 min. Slides were rinsed with PBS-Tween and were incubated with the ABC (prepared according to manufacturer's directions) for 45 min. After rinsing with PBS-Tween, the horse-radish-peroxidase substrates were developed with the chromogen (0.06% diaminobenzidine (DAB) in 50 mM Tris-Cl buffer and 0.003% hydrogen peroxide) for 3 – 5 min while monitoring positive controls under a standard light microscope. The chromogen reaction was stopped by rinsing slides with distilled water. The slides were counterstained with Mayer's hematoxylin for 30 seconds, rinsed in tap water, and allowed to sit in tap water for 5 min. The final steps were dehydration through graded ethanol, clearing with xylene, and mounting in resinous mounting medium.

Validation of the specificity of the primary antibody for binding to ET-1 was completed using the antibody neutralization technique.[38] Briefly, the primary antibody rabbit anti-ET-1 antiserum was incubated (1:2) with the ET-1 peptide[l] overnight at 4 C. The IHC procedure described above was conducted on two identical subsets of study tissues with the second subset receiving the neutralized primary antibody substituted for the normal unneutralized primary antibody (subset one).

Proper IHC technique was further confirmed during each staining session of all study samples by inclusion of positive controls (equine heart, lung, and kidney) and negative controls (omission of primary antibody on equine heart, lung, and kidney sections).

Scoring of ET-1 staining intensity was completed using a standard light microscope in three fields of each slide three times by one investigator (AMS) and the modal value was determined. For PDA and PDV samples, the endothelium and the vascular smooth muscle were evaluated. For laminar samples, the epithelial cells and stroma of the epidermal laminae, and the endothelium and vascular smooth muscle of the adjacent parietal arteries and collecting veins of the dermal laminae were evaluated. A value from 0 to 3 was assigned to each parameter based on the relative amount of staining compared with slides using the antibody neutralization technique. A 0 score was assigned if there was no staining present; 1 if mild brown staining; 2 if moderate brown staining; and 3 if intense brown staining. Increased staining intensity would be associated with increased levels of ET-1 in the tissues.

7.2.6 Statistical Analyses – Plasma endothelin-1 like immunoreactivity - Data was considered continuous and found to follow a normal distribution using the Shapiro-Wilk test with failure to reject the null hypothesis of normality at $p \leq 0.05$. Data for the 3 baseline measurements, before BWE administration, were averaged and used as comparisons for data after BWE administration. Since horses developed Obel grade 1 laminitis at different time points, the time points for each horse corresponding to the development of Obel grade 1 laminitis were selected and labeled "Obel 1" in the results and graphs. The data was summarized and presented as mean ± SD. The data was analyzed using a mixed effect linear model that accounted for the random variance of horse and the repeated measurements on each horse. Where there were significant interaction effects at $p \leq 0.05$, predetermined least squares means

comparisons were made to determine where differences were occurring. Type I error was maintained at 0.05. PROC MEANS, UNIVARIATE, and MIXED were used for the analysis.[m]

ET-1 immunohistochemical staining – Since the data was based on an ordinal/categorical scale of measurement, staining frequency counts were determined for each variable by treatment (saline or PD145065) or by disease state (normal or BWE-induced laminitic horses) and data was presented descriptively and graphically.

7.3 Results

7.3.1 Plasma Endothelin-Like Immunoreactivity – Eleven horses (six PD145065 treated and 5 saline controls) developed Obel grade 1 laminitis between 5 and 11 hours after BWE with a median value of 9 hours. The remaining three horses did not develop Obel grade 1 laminitis, did not have at least a 30% drop in WBC count, and plasma samples from these horses were not included in the analysis. There were no statistical differences between ET antagonist-treated horses and saline-treated horses at any time point for ET-like immunoreactivity; therefore, all horses were pooled for analysis. Mean baseline JV values (0.802 pg/ml) were not significantly different from mean baseline PDV values (0.803 pg/ml) (Fig. 7.1). Overall, plasma ET-like immunoreactivity (pg/ml) was significantly greater over time, compared with baseline, after BWE administration ($p < 0.0001$) in PDV samples. Values in PDV samples were significantly greater than JV samples ($p < 0.0001$) after BWE administration. Palmar digital venous samples had significantly greater ET-like immunoreactivity than JV samples from 3 hours to 8 hours post-BWE. Palmar digital venous samples after BWE administration were significantly greater than baseline samples 3 to 10 hours post-BWE and upon reaching Obel grade 1 laminitis. Endothelin-like immunoreactivity peaked and remained at 2.59 pg/ml at 4, 5,

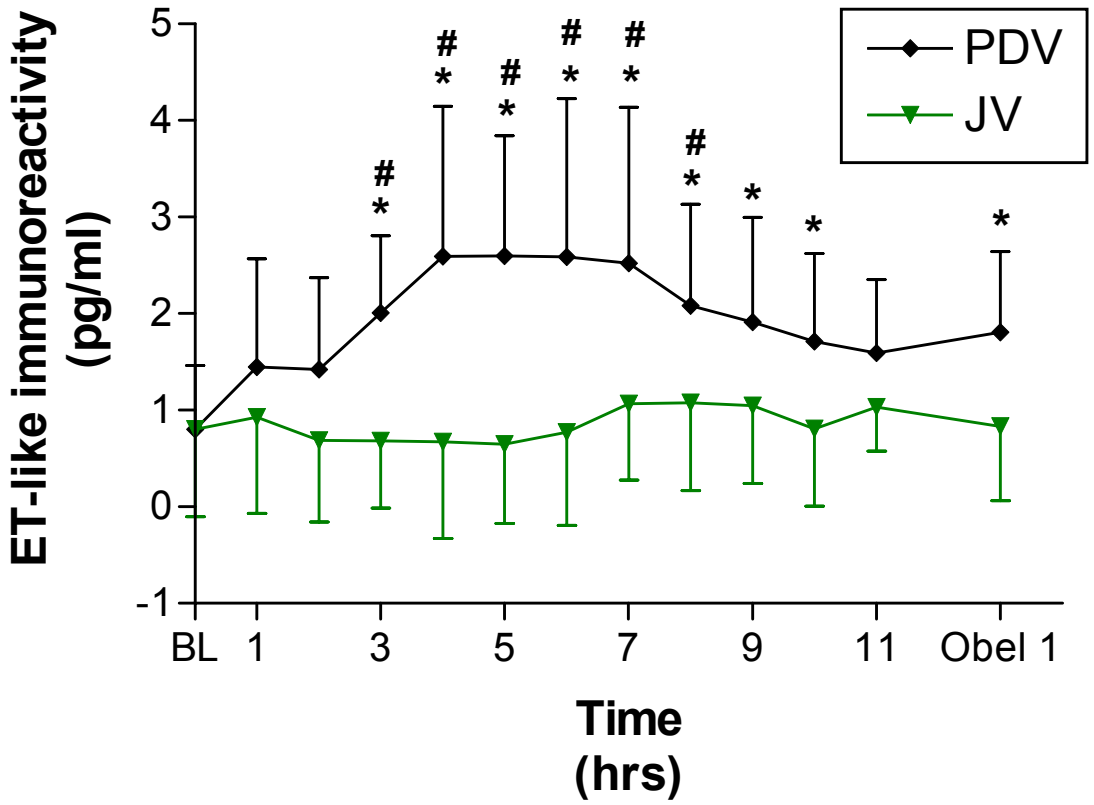


Figure 7.1 – Mean ± SD endothelin (ET)-like immunoreactivity (pg/ml) pre-black walnut extract (BWE) administration (baseline [BL]) and hourly after BWE administration for palmar digital venous (PDV) and jugular venous (JV) plasma. The time point "Obel 1" corresponds to the development of Obel grade 1 laminitis at 5 – 11 (median = 9) hours post-BWE. *Indicates values significantly ($p < 0.05$) different from baseline values. # Indicates values significantly ($p < 0.05$) different from JV samples at the same collection time point. N = 11 except at 10 hours post-BWE (N = 9) and 11 hours post-BWE (N = 3).

and 6 hours post-BWE in the PDV samples, compared with JV samples at these same time points with 0.673, 0.648, and 0.774 pg/ml, respectively. Although not statistically significant, the greatest concentration of JV ET-1 like immunoreactivity (1.08 pg/ml) occurred 8 hours post-BWE administration and by Obel stage 1 laminitis, the mean value had returned to the baseline value of 0.80 pg/ml. There were no significant differences for PCV or total plasma protein concentrations over time in either group of horses.

7.3.2 ET-1 Immunohistochemical Staining - Validation of the specificity of the primary antibody for binding to ET-1 was evident using the antibody neutralization technique. The subset of tissues incubated with the neutralized primary antibody did not stain brown as occurred in the subset incubated with the normal primary antibody (Fig. 7.2). During each IHC staining session, the positive and negative controls stained properly as noted by staining and no staining, in that order.

Only tissues from the 11 horses that developed laminitis and the normal horses were included for ET-1 IHC staining. No trends or differences were found for PDA, PDV, or laminar samples (Fig 7.3 – 7.9) between saline- and PD145065-treated horses or between normal and BWE horses. Within the vessel samples (PDA and PDV), epidermal laminae, and dermal laminae, structures were more frequently graded as moderate staining (grade 2) than mild or intense staining (grades 1 & 3).

7.4 Discussion

Palmar digital venous plasma ET-1 like immunoreactivity increased without a concomitant increase in systemic venous immunoreactivity; therefore, the data supports our hypothesis that with the development of BWE-induced acute laminitis, local digital plasma ET-1

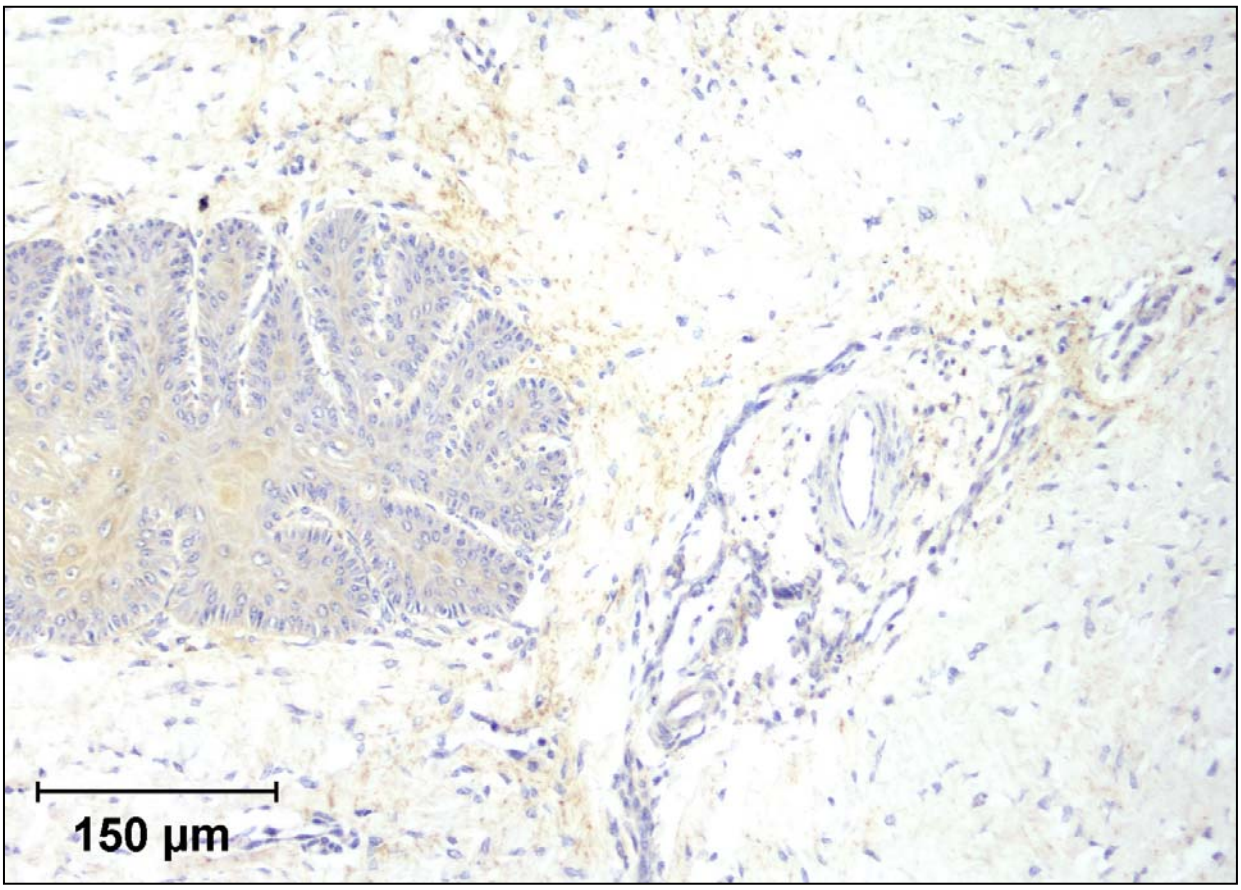
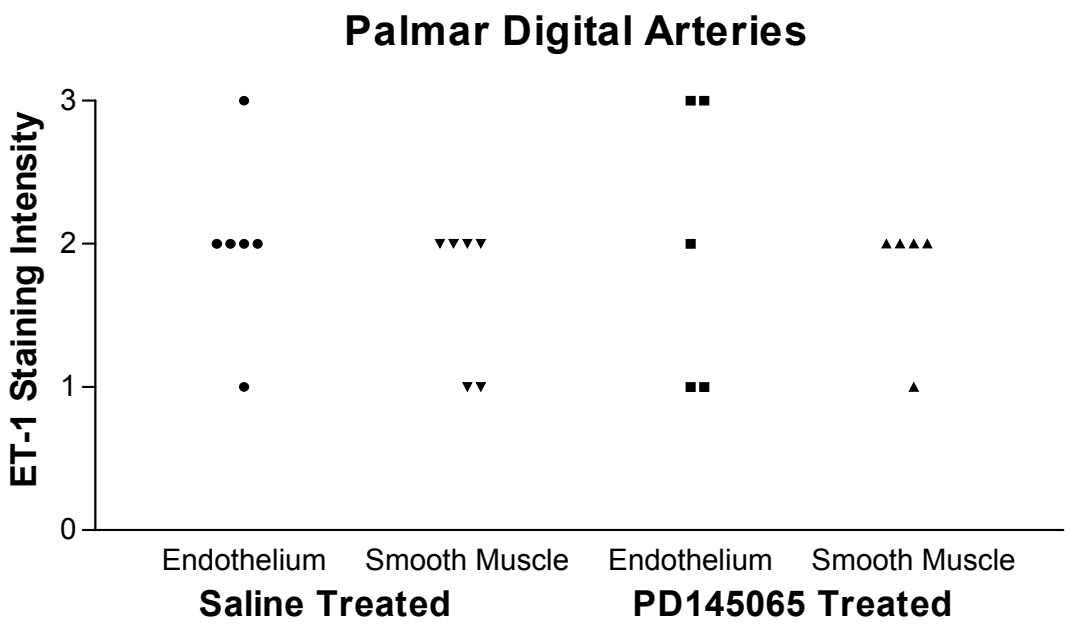


Figure 7.2 – Photomicrograph of equine laminae after immunohistochemistry using the antibody neutralization technique to validate the endothelin-1 antibody in equine tissues. Note the lack of brown staining in the epithelial cells and stroma of the epidermal laminae and the vascular structures of the dermal laminae.

Figure 7.3 – Scatter plots of modal values of ET-1 immunohistochemical staining intensity of palmar digital arterial and venous samples from saline- and PD145065 (ET antagonist)-treated horses after induction of laminitis using black walnut extract (BWE). Vascular endothelium and vascular smooth muscle were evaluated in both vessel types. A 0 score was assigned if there was no staining present; 1 if mild brown staining; 2 if moderate brown staining; and 3 if intense brown staining. Increased staining would be associated with increased presence of ET-1.



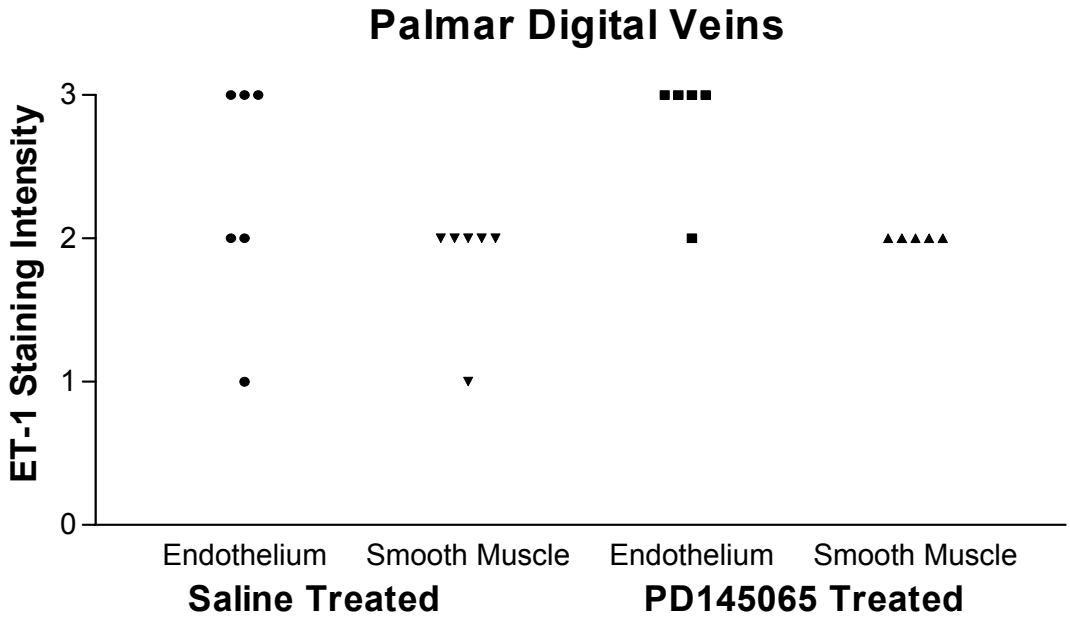
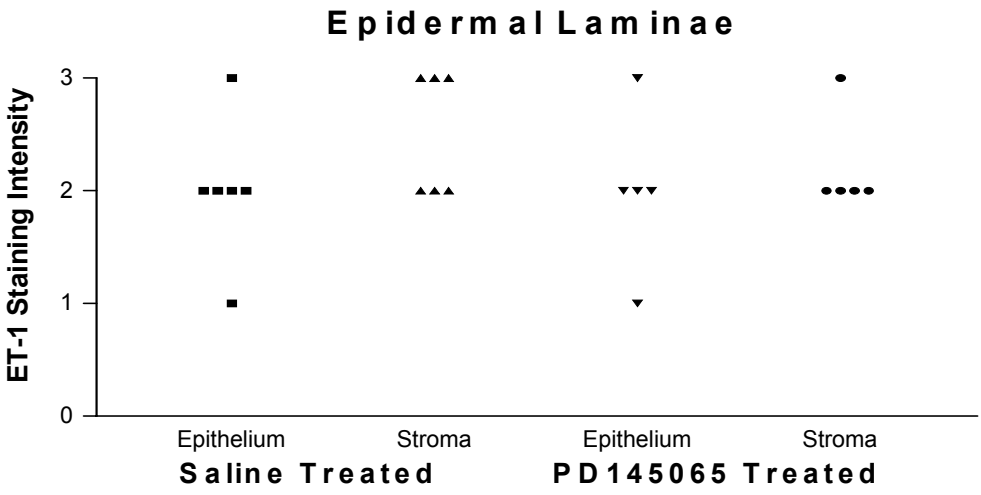
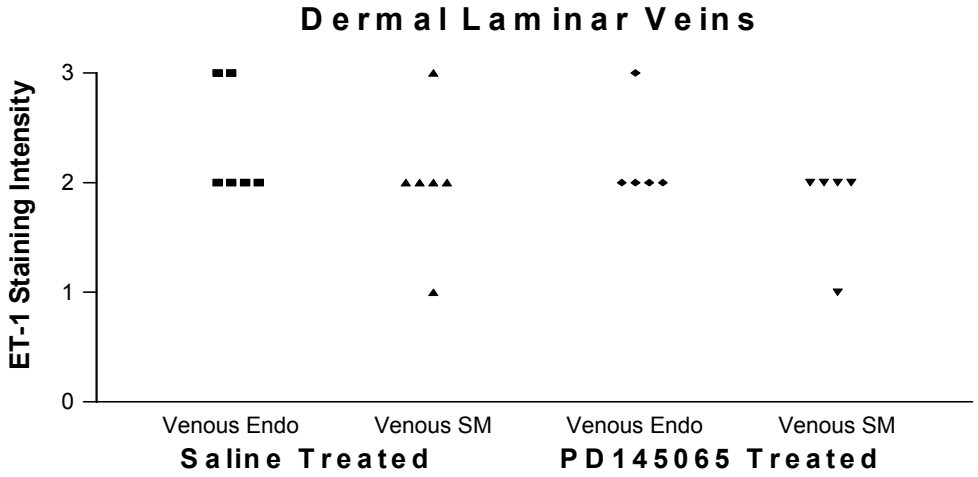


Figure 7.4 – Scatter plots of modal values of ET-1 immunohistochemical staining intensity of laminar samples from saline- and PD145065 (ET antagonist)-treated horses after induction of laminitis using black walnut extract (BWE). Epithelial cells and stroma of the epidermal laminae and vascular endothelium (Endo) and smooth muscle (SM) of the dermal laminae were evaluated. A 0 score was assigned if there was no staining present; 1 if mild brown staining; 2 if moderate brown staining; and 3 if intense brown staining. Increased staining would be associated with increased presence of ET-1.





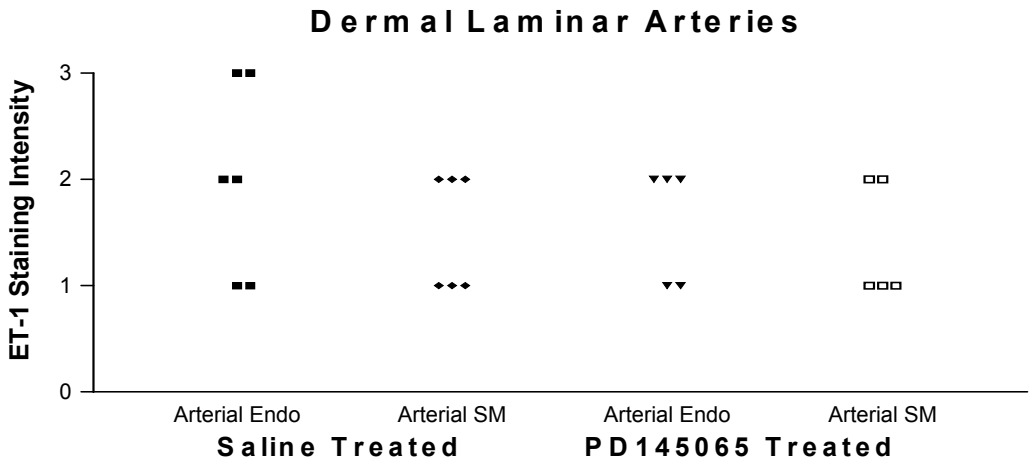
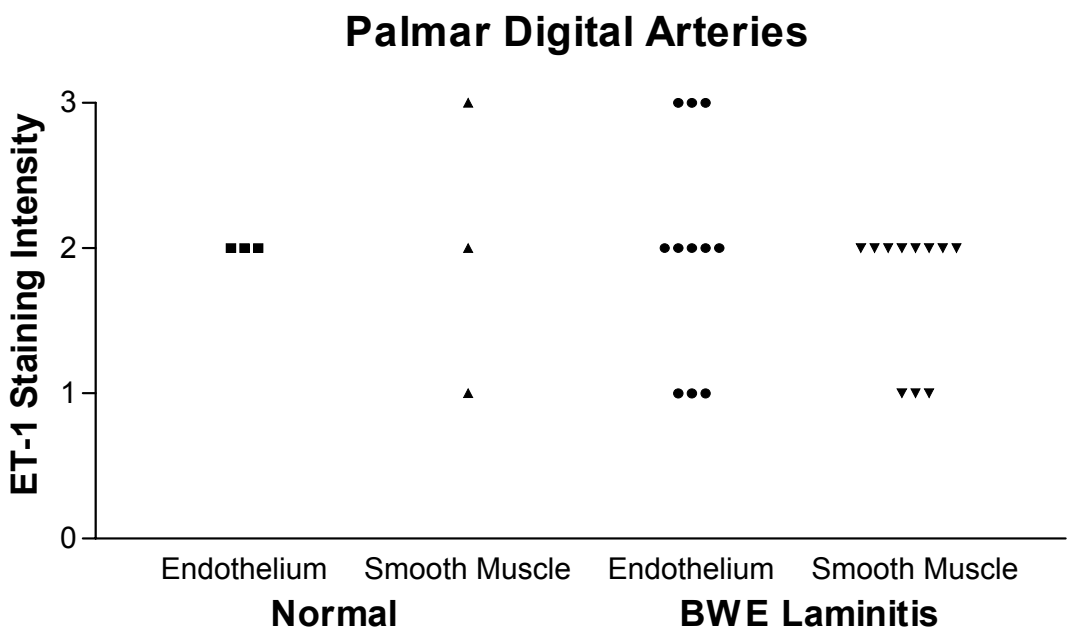


Figure 7.5 – Scatter plots of modal values of ET-1 immunohistochemical staining intensity of palmar digital arterial and venous samples from normal horses and those after induction of laminitis using black walnut extract (BWE). Vascular endothelium and vascular smooth muscle were evaluated in both vessel types. A 0 score was assigned if there was no staining present; 1 if mild brown staining; 2 if moderate brown staining; and 3 if intense brown staining. Increased staining would be associated with increased presence of ET-1.



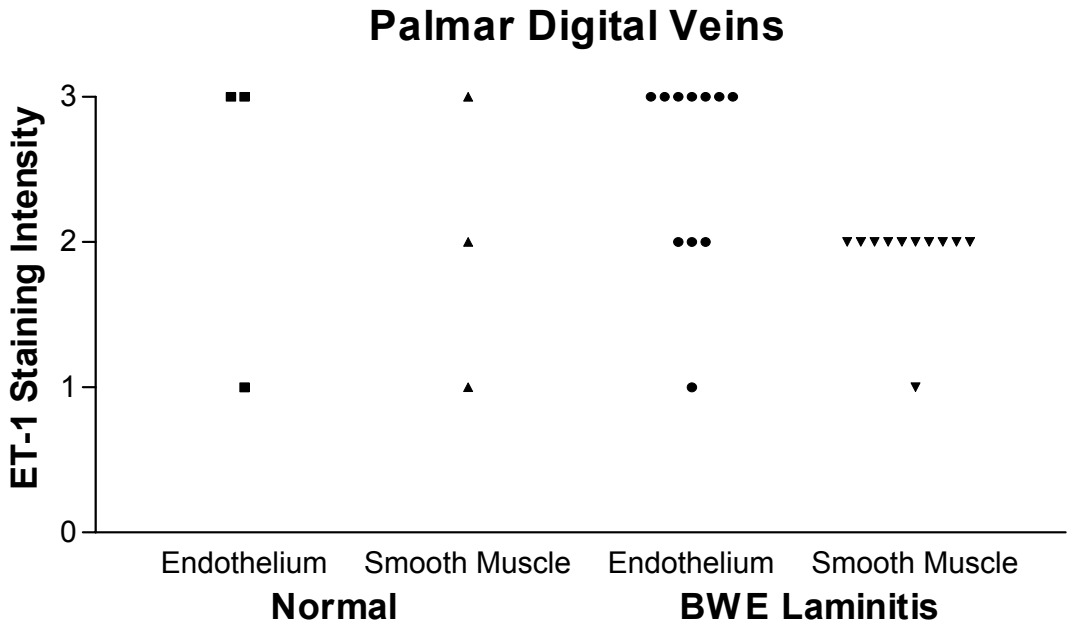
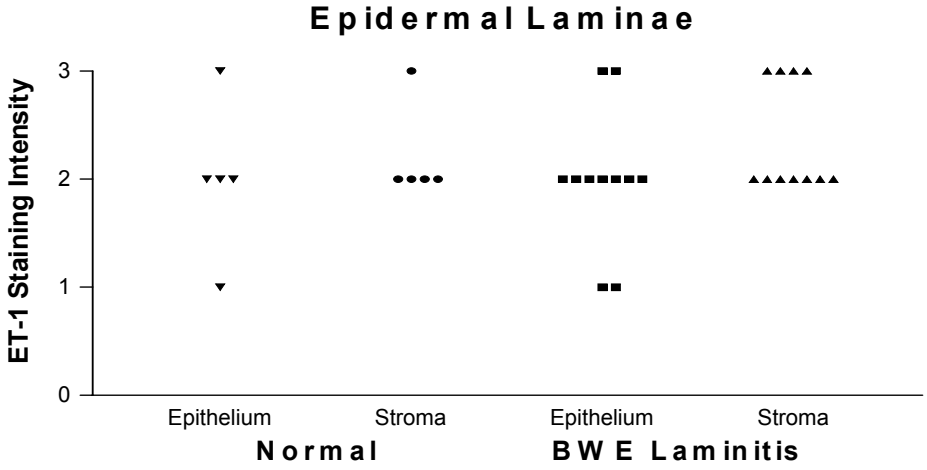
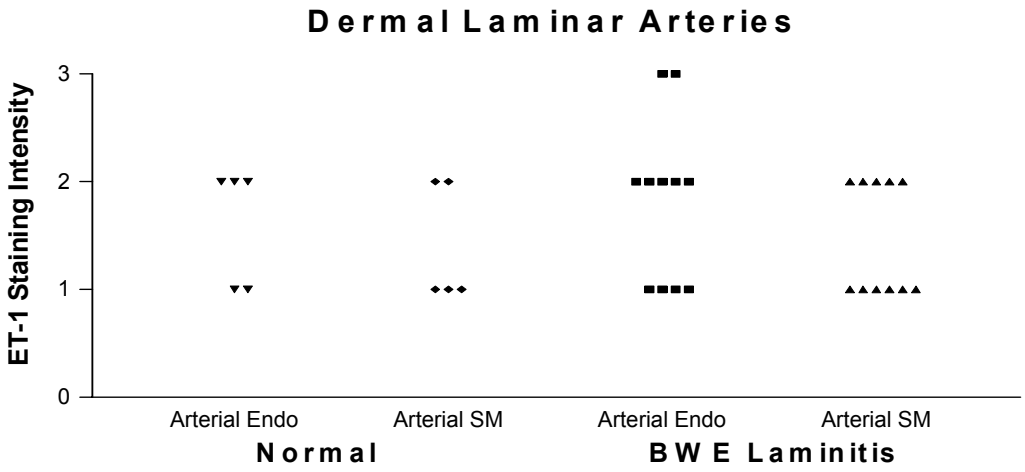
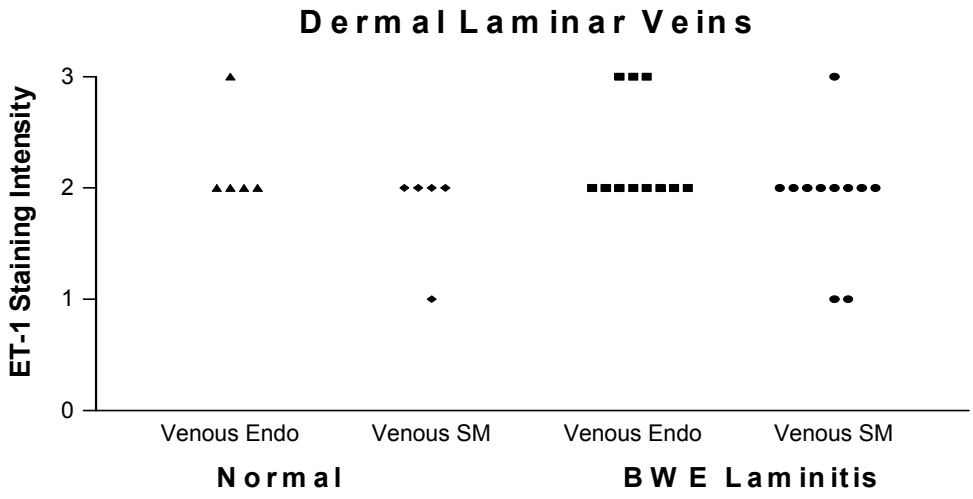


Figure 7.6 – Scatter plots of modal values of ET-1 immunohistochemical staining intensity of laminar samples from normal horses and those after induction of laminitis using black walnut extract (BWE). Epithelial cells and stroma of the epidermal laminae and vascular endothelium (Endo) and smooth muscle (SM) of the dermal laminae were evaluated. A 0 score was assigned if there was no staining present; 1 if mild brown staining; 2 if moderate brown staining; and 3 if intense brown staining. Increased staining would be associated with increased presence of ET-1.







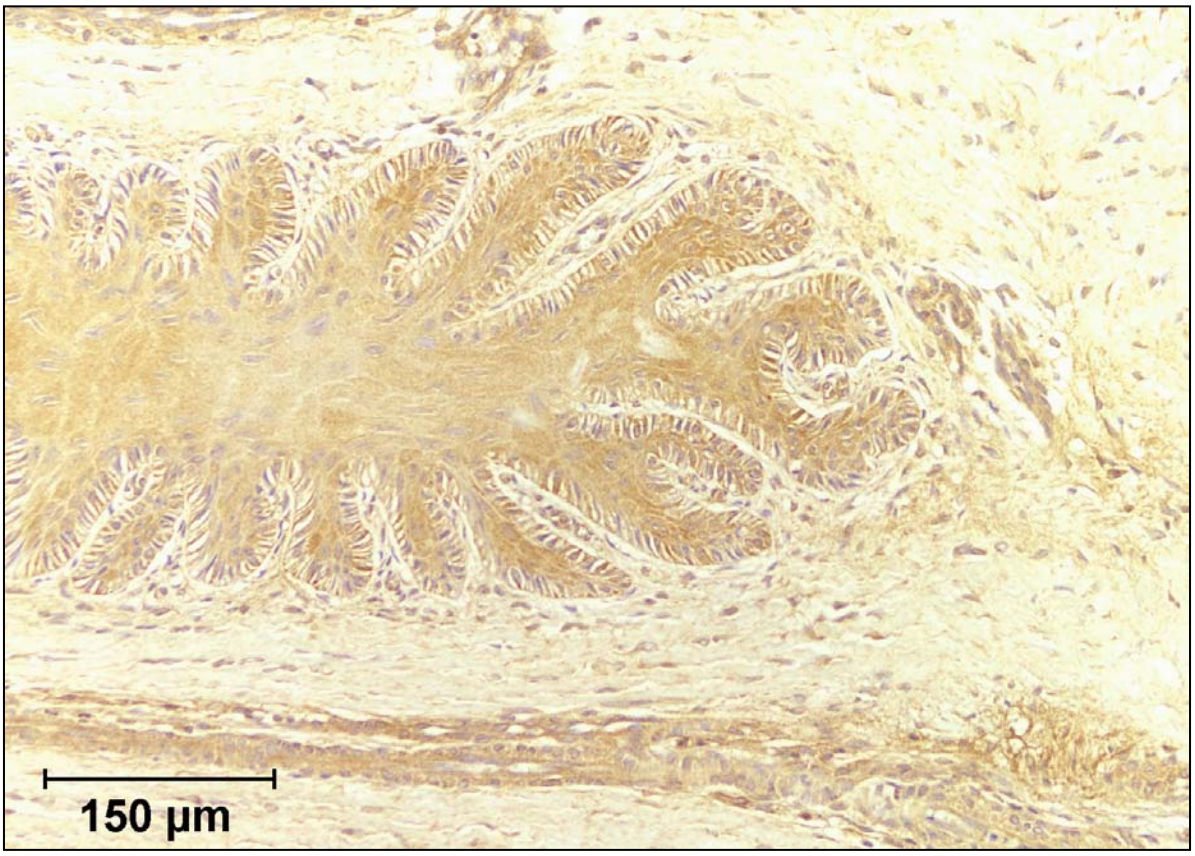
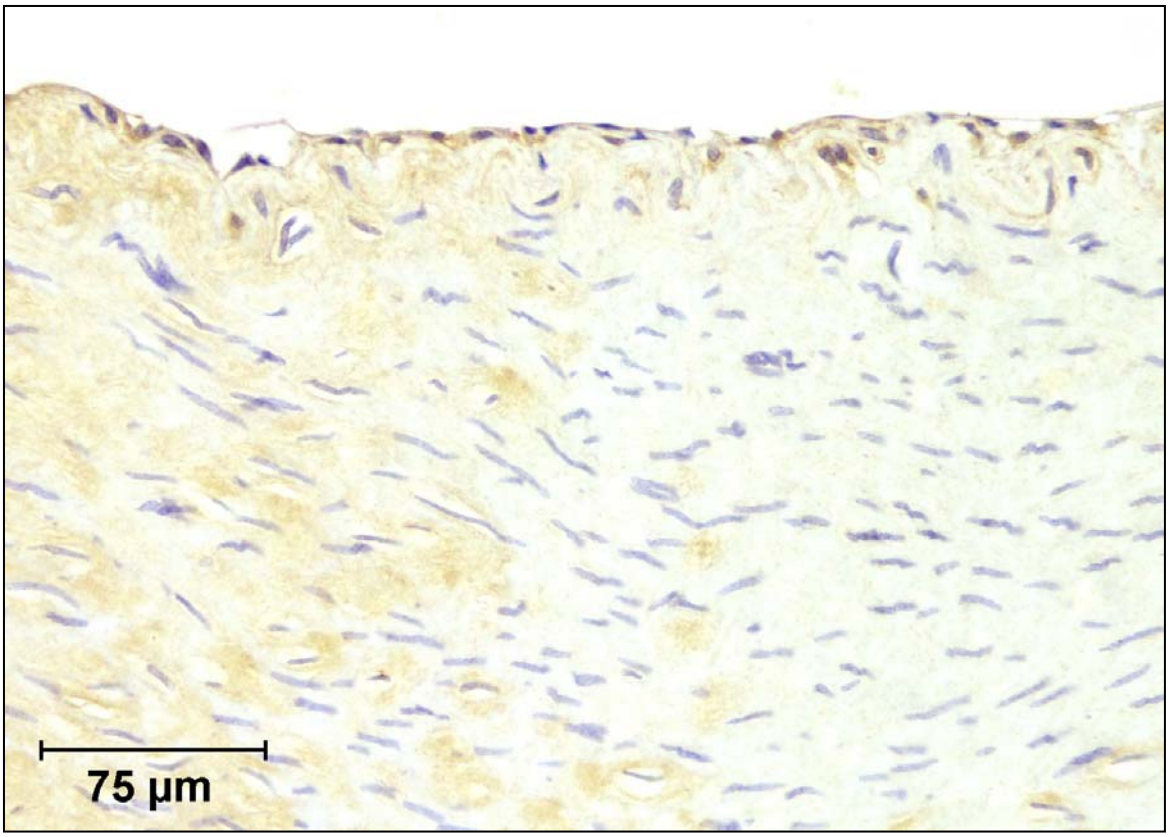


Figure 7.7 – Photomicrograph of laminae from a horse after black walnut extract-induced acute laminitis demonstrating intense brown endothelin-1 immunohistochemical staining of the epithelial cells and stroma of the epidermal laminae.

Figure 7.8 – Photomicrographs of palmar digital arterial cross-sections from horses after black walnut extract-induced acute laminitis demonstrating mild (top) and moderate (bottom) brown endothelin-1 immunohistochemical staining of the endothelium and vascular smooth muscle.



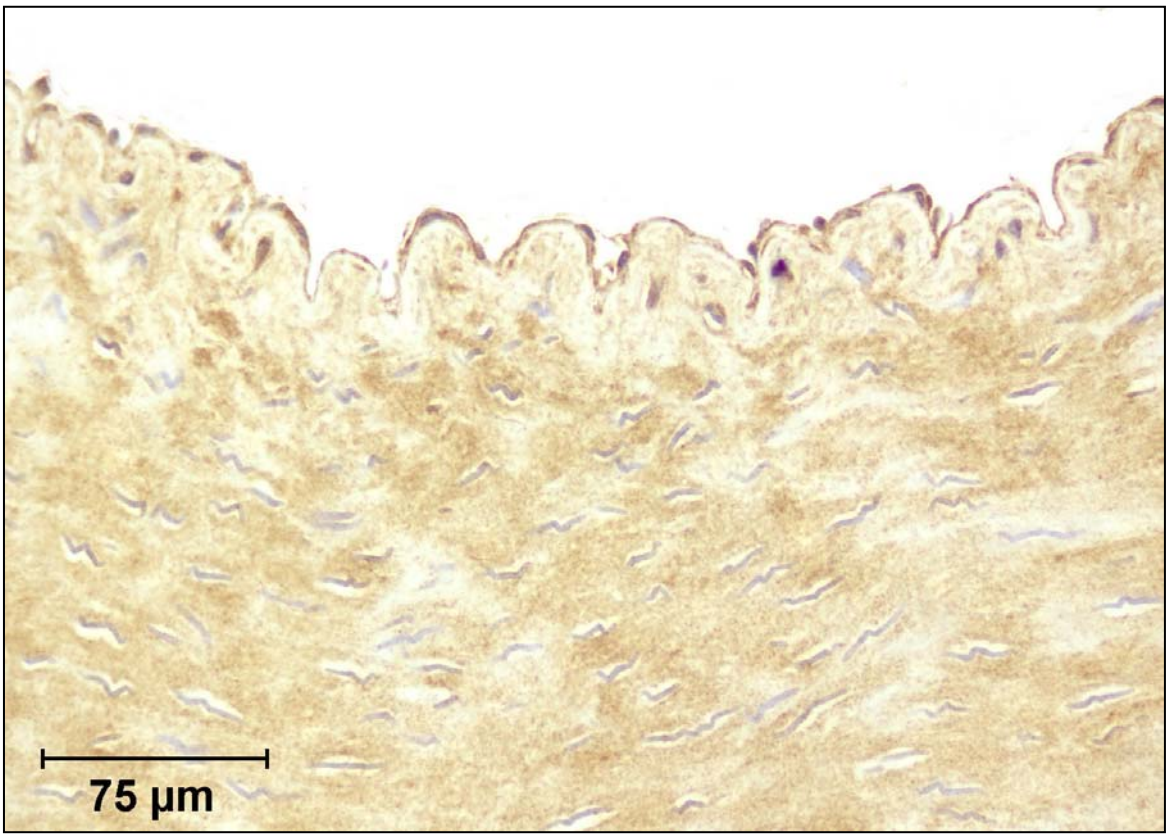
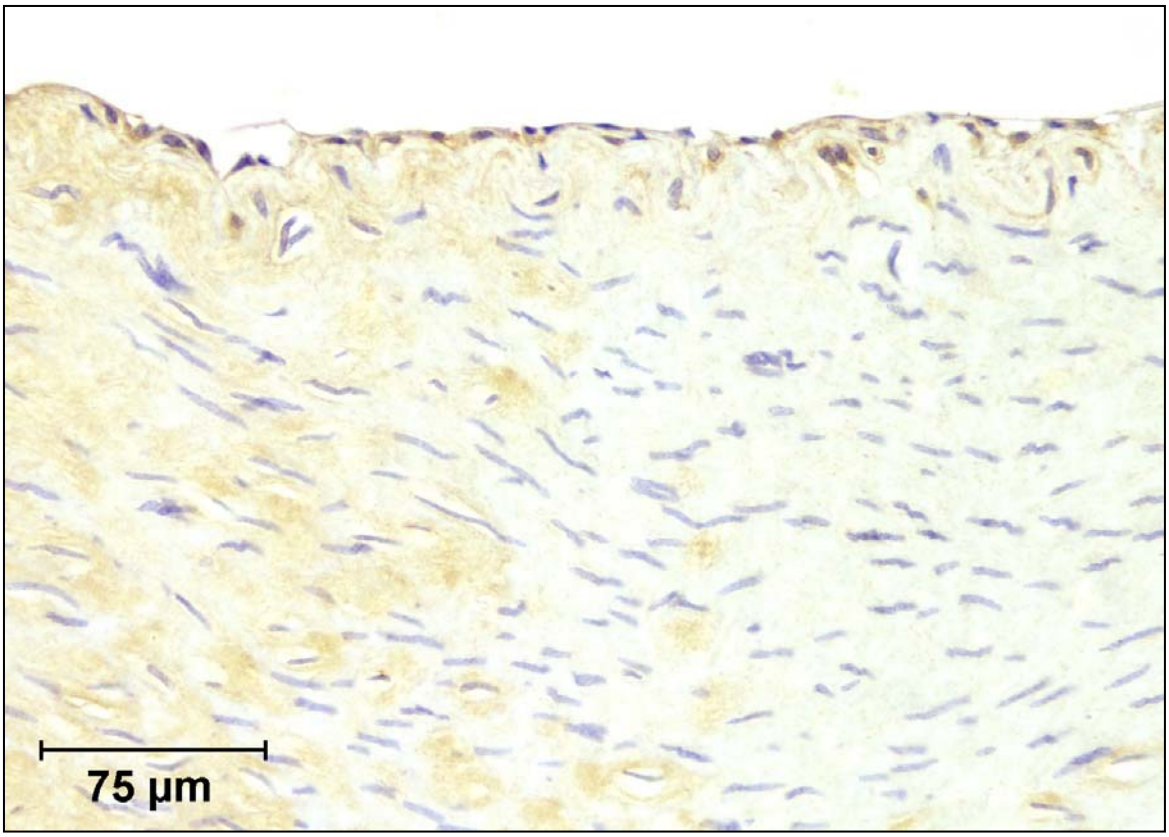
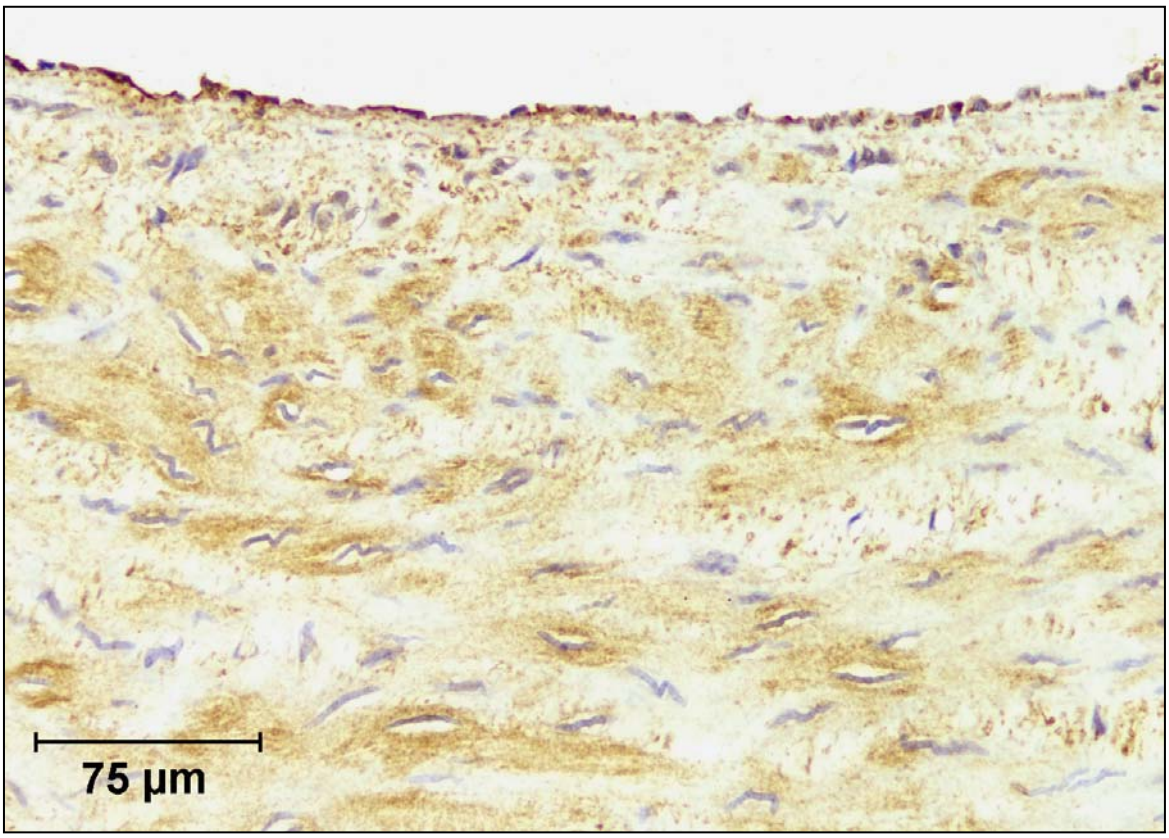


Figure 7.9 – Photomicrographs of palmar digital venous cross-sections from horses after black walnut extract-induced acute laminitis demonstrating mild (top) and moderate (bottom) brown endothelin-1 immunohistochemical staining of the endothelium and vascular smooth muscle.





concentrations of ET-1 increase. Although there is cross-reactivity between ET isoforms (esp. ET-1 and ET-2) using this ELISA, the predominant isoform expected in the plasma is ET-1 since this is the principal isoform synthesized and released by vascular endothelial cells.[24] The structure of ET-1 is highly conserved across species and the ELISA kit used for sample analysis was validated for equine plasma in our laboratory. It is not expected that short-term administration of the ET antagonist would alter ET-1 synthesis or plasma concentrations since previous studies have demonstrated no effects of PD145065 on resting vascular tone in the cat femoral artery and vein and did not alter hemodynamics (i.e. mean arterial pressure, cardiac index, and mean pulmonary pressure) in dogs when administered alone.[32,39] Since the PCV and total plasma protein concentrations were not significantly different over time, values obtained during our study are not likely affected by hemoconcentration or hemodilution.

Approximately 80% of ET-1 is released abluminally; therefore, the actual concentration of ET-1 released from the endothelial cells is likely substantially greater than that measured in the plasma.[31] Reported concentrations of jugular venous plasma ET in healthy horses are from 0.18 pg/ml to 1.80 pg/ml using radioimmunoassay procedures.[40-42] Similar to values reported in horses, plasma ET-like immunoreactivity values in healthy dogs and humans were determined to be 1.83 and 1.7 pg/ml, respectively.[25,28] Our baseline value of 0.80 pg/ml is similar to these previous studies and similar to values obtained from normal horses in another study in our laboratory.[43] The authors believe the increased palmar digital venous plasma ET-like immunoreactivity found after BWE administration most likely represents a much greater release of ET abluminally toward smooth muscle, potentially leading to digital vasoconstriction associated with the developmental stages of acute laminitis. Although determination of the hemodynamic effects of the measured increases in plasma ET-like immunoreactivity was not

part of the data presented here, the findings of this study support future investigation into the potential role of ET-1 in causing digital vasoconstriction associated with the developmental stages of laminitis.

The finding of no differences in ET-1 IHC between normal and BWE-induced horses does not support our hypothesis that ET-1 tissue concentrations would increase during the developmental stages of the disease. Possible reasons for these contradictory findings in our data are 1) that alterations in ET-1 concentrations associated with the pathophysiology of laminitis were below the detection level of IHC techniques; 2) the IHC staining technique was not specific for ET-1 and masked true ET-1 presence within the tissues; and 3) that ET-1 levels are not affected by the development of laminitis within these tissues. These findings were similar to our findings comparing IHC ET-1 staining of the same structures in horses with naturally-acquired laminitis.[43]

The finding that digital plasma ET-1 concentrations increase with induction of laminitis provides evidence that ET-1 may be involved in the pathogenesis of acute laminitis in horses. Research within our laboratory and other laboratories also support the role of ET-1 in this disease. Katwa et al found increased ET-1 expression within the laminae during the developmental stages of laminitis.[44] Our laboratory and a study by Baxter et al have demonstrated the in vitro contractile effects of ET-1 in non-laminitic and laminitic horses. Measurements of Starling forces by Allen et al and Eaton et al found increased venous resistance with the development of laminitis, and during in vitro studies ET-1 induced greater contraction of veins than arteries.[1,14,44-46] Substantial data exists supporting the association of ET-1 and equine laminitis even though our IHC techniques did not find increased ET-1 staining in digital tissues with the development of the disease. It is possible that techniques such as *in situ*

hybridization will allow for more accurate assessment and localization of ET-1 expression within these tissues.

Although we have not definitively identified the stimulus of increased ET-1 release within the horse, potential mediators are LPS, cytokines, and altered shear stress across vascular endothelium all of which have been found to increase ET-1 concentrations in other species.[25,27,47,48] Systemic plasma concentrations of ET-like immunoreactivity did not significantly increase in the horses after BWE administration, however, increases occured within the digital circulation. Possible reasons for this difference are that systemic circulation results in clearance of ET-1 or that local digital concentrations are increased. Since the JV plasma concentrations of ET-like immunoreactivity found during this study are in agreement with previous work in our laboratory, it is most likely that local digital increases in ET-1 account for this difference.

Increased digital plasma ET-1 concentrations may lead to venoconstriction of the digital venous circulation resulting in altered Starling forces, in particular increased venous resistance, during the developmental stages of acute laminitis.[1] The findings of this study support our hypothesis that with the onset of BWE-induced acute laminitis, digital plasma ET-1 concentrations would increase, likely contributing to the vascular alterations (venoconstriction) characteristic of acute laminitis.

7.5 Product Information

[a] Angiocath Vascular Access, Becton Dickinson & Co, Sandy, UT

[b] Arrow Catheters, Arrow International, Reading, PA

[c] PD 145065, American Peptide Co, Sunnyvale, CA

[d] Eberbach Corp, Ann Arbor, MI

[e] Sigma Chemical Co, St. Louis, MO

[f] Biomedica, American Research Products, Inc., Belmont, MA

[g] Transonics Systems, Inc., Ithaca, NY

[h] Bio-tek, Winooski, VT

[I] Sodium pentobarbital, The Butler Co, Columbus, OH

[j]Vectastain ABC Elite Kit – Rabbit IgG, Vector Laboratories, Inc., Burlingame, CA

[k] Rabbit anti-Endothelin-1, Peninsula Laboratories, Inc., Belmont, CA

[l] Endothelin-1, Peninsula Laboratories, Inc., Belmont, CA

[m] Proc Mixed, Univariate, and Means; SAS version 8, SAS Institute, Cary, NC

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CHAPTER 8. EFFECT OF AN ENDOTHELIN ANTAGONIST AND NITROGLYCERINE ON DIGITAL STARLING FORCES IN HORSES WITH BLACK WALNUT EXTRACT-INDUCED ACUTE LAMINITIS

8.1 Introduction

Although the pathogenesis of acute laminitis is not fully understood, there are three main theories regarding the initiating factor in the cascade of events that lead to separation of the sensitive and insensitive laminae, ultimately resulting in rotation and/or sinking of the distal phalanx within the hoof, namely the vascular, enzymatic, and mechanical theories.[1] Experiments examining the vascular theory have resulted in conflicting data. Although studies have demonstrated a reduction in hoof wall surface temperature (indicator of decreased lamellar blood flow) and decreases in digital blood flow and perfusion during the developmental stages of experimental acute laminitis, Pollitt recently demonstrated increases in hoof temperature (indicator of increased lamellar blood flow) associated with its development.[2-5] Despite these differences, systemic and digital hemodynamic alterations characterize the developmental stages of laminitis; however, the exact mechanisms and factors responsible for these changes remain unknown.

Previous Starling force studies in horses with experimentally-induced laminitis using the black walnut extract (BWE) and carbohydrate overload (CHO) models have demonstrated alterations in digital hemodynamics.[4,6,7] Of particular importance is the finding that pre-to-post capillary resistance ratio is decreased and capillary pressure and laminar interstitial pressure are increased in the prodromal stages of laminitis. The imbalance with the pre-to-post capillary resistance ratio increases the hydrostatic force in the capillary bed promoting the flux of fluid across the capillary wall within the foot, resulting in laminar edema. The increased laminar interstitial pressure, due to edema formation, exceeds the critical closing pressure of equine digital capillaries because they are located between the rigid hoof capsule and the hard bony surface of the third phalanx, thereby leading to a "compartment-like syndrome".[4] One of the

predominant hemodynamic alterations in experimentally-induced laminitis is development of increased venous pressure subsequent to increased venomotor tone.[4] Venous constriction reduces the pre-to-post capillary resistance ratio, which likely is the initiating factor in the development of increased laminar edema and interstitial pressure, and subsequent devastating laminar ischemia and necrosis. These findings support the hypothesis that increased venomotor tone initiates laminitis.

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide synthesized by endothelial cells, vascular smooth muscle cells, and macrophages.[8] Endothelin synthesis is stimulated by numerous factors, which are increased during many diseases in horses characterized by an inflammatory response (pleuropneumonia, endometritis, intestinal ischemia, enterocolitis, anterior enteritis, etc.), which are empirically linked to the development of laminitis. Katwa et al recently demonstrated that the expression of ET-1 in laminar connective tissues obtained from experimentally-induced acutely laminitic horses, and naturally-acquired chronically laminitic horses was increased.[9] In vitro studies conducted in our laboratory have demonstrated concentration-dependent contraction of digital arterial and venous rings from normal and laminitic horses to ET-1.[10,11] Additionally, we found that administration of an ET antagonist (PD145065; 10^{-5} M concentration) significantly decreased the contractile effects of ET-1, thereby, demonstrating the potential usefulness of this antagonist for the in vivo attenuation of ET-induced vasoconstriction. Of particular importance was the finding that veins contracted over 3 times greater than arteries with ET-1 administration. This finding has also been published by other authors in equine digital and colonic vessels.[10-13] These findings support the potential role of ET-1 in the pathogenesis of acute laminitis, especially the likely role of ET-1 in the venoconstriction observed during the developmental stages of the disease.

Nitric oxide (NO) is released from endothelial cells and results in transient vasodilation and has an inhibitory effect on platelet aggregation and neutrophil adhesion, which helps ensure vascular patency and tissue perfusion.[14] The results of recent studies indicate that normal equine digital and colonic vessels have a substantial capacity for endothelial-dependent relaxation by NO in vitro, accounting for approximately 70% to 85% of the maximal relaxation induced by acetylcholine.[12,15] Furthermore, intravenous infusion of endotoxin to horses for 60 minutes appears to alter the sensitivity of the digital vascular segments to various endothelium-dependent compounds and reduces the maximal relaxation induced by these compounds.[16] In horses with CHO-induced laminitis, acetylcholine-mediated relaxations of digital vessels in vitro are reduced, suggesting that the NO producing capacity of the digital vascular endothelium is reduced, thereby rendering the vessels more sensitive or vulnerable to vasoconstrictive agents.[17] Experimentally, NO donors reduced the lameness and 'bounding pulses' of ponies with grass-induced laminitis and improved digital perfusion.[18,19] However, there remains a question regarding the effectiveness of NO donors as treatment for equine laminitis since the pathogenesis of the disease is still not completely understood.

Our hypothesis was that the use of an ET antagonist and a NO donor will improve Starling forces, compared with saline-treated horses, following BWE administration.
The objectives of the study were to administer either an ET antagonist or saline during the developmental stages of acute laminitis followed by measurement of digital Starling forces using a pump-perfused extracorporeal digital preparation. After the first set of Starling force measurements were recorded, a NO donor (NG) was administered to both groups (ET antagonist-treated and saline-treated) and Starling force measurements were repeated.

8.2 Materials and Methods

8.2.1 Selection and Preparation of Horses - This study was approved by the Institutional Animal Care and Use Committee of Louisiana State University. Fourteen light breed horses (4 mares and 10 geldings; ages ranging 5 – 20 years; weighing 411 to 591 kg) were determined to be free of laminitis and gastrointestinal tract disease based on their history, thorough physical and lameness examinations, and lateral radiographs of both front feet. Horses were housed in stalls, fed grass hay, and acclimated to the study area for two weeks prior to the study. Ten days before the study, the medial palmar artery was surgically elevated to a subcutaneous location in the proximal to middle one-third of the metacarpus and an ultrasonic Doppler flow probe[a] was placed around the medial palmar digital artery in the pastern region of the same limb. On the day of the study, horses were placed in stocks, instrumented, and moved to the study area where they were allowed a 1-hour period of equilibration prior to the start of the study. All catheters were placed percutaneously after aseptic preparation of the skin and desensitization by subcutaneous infiltration of lidocaine solution. A 14-gauge, 13.3-cm Teflon catheter[b] was inserted into the left jugular vein (JV) for collection of blood samples. A 20-gauge, 4.45-cm polyurethane catheter[c] was placed in the right medial palmar artery. The catheter was used for administration of the ET receptor antagonist PD145065[d] or 0.9% NaCl.

8.2.2 Preparation of Extract – Preparation of the BWE was as previously described.[7,20] Briefly, shavings were made from the heartwood of a black walnut tree cut in the fall of the year and were stored at –20 C until use. Two grams of black walnut shavings/kg body weight were combined with 8 liters of distilled water and mixed in a shaker bath[e] for 14 hours at room temperature (20 – 22 C). The mixture was filtered, refrigerated, and the fluid was administered within 24 hours of preparation.

8.2.3 Induction of Laminitis – Jugular venous samples were collected before BWE administration and baseline WBC counts were determined. Laminitis was induced in all horses by administration of the BWE via a nasogastric tube. Horses were monitored hourly after BWE administration until reaching Obel grade 1.[21] Efficacy of the extract was confirmed by at least a 30% decrease in WBC count from samples collected hourly after BWE administration.[7] Seven horses were administered a 10^{-5} M concentration of PD145065 (ET receptor antagonist) and 7 horses received an equivalent volume of 0.9% NaCl for two minutes into the medial palmar arterial catheter at 1.5 and 7.5 hours post-BWE administration. The dose required to maintain a 10^{-5} M concentration of the ET antagonist in the blood for two minutes was based on the measured palmar digital blood flow, using an ultrasonic Doppler blood flow probe surgically placed around the lateral palmar digital artery 10 days before the day of the study.[22,23]

8.2.4 Measurement of Digital Starling Forces – Once Obel grade 1 was reached, general anesthesia was induced with xylazine hydrochloride (0.51.0 mg/kg), sodium thiamylal (10 mg/kg) and sodium pentobarbital (7.5 mg/kg), IV, and maintained with pentobarbital (5 to 15 mg/kg/hr, IV). Horses were positioned in right lateral recumbency and ventilated with positive pressure using 100% oxygen. The treatment of either PD145065 or 0.9% NaCl was repeated into the medial palmar arterial catheter. Sodium heparin was administered via the jugular venous catheter (500 IU/kg). A 20-gauge, 4.45-cm polyurethane catheter was placed in the transverse facial artery for measurement of systemic blood pressure. The instrumented forelimb was used for the pump-perfused extracorporeal digital preparation as described.[24] Briefly, the medial and lateral palmar artery and vein were cannulated with polyethylene tubing (PE 320 to 240; the largest diameter that each vessel could accommodate was utilized) at the level of the fetlock and a pump-perfused circuit was formed (Fig. 8.1). The arterial circuit arose from the median artery

and the venous effluent was collected into a reservoir and then returned to the horse via the cephalic vein. The circuit was interfaced with pressure transducers and connected to a physiograph[f] for measurement of arterial (Pa) and venous (Pv) pressures (mmHg). Ligation and electrocautery were used to disarticulate the digit at the level of the fetlock. The digit was placed in a wire basket suspended from a force transducer[g] interfaced with the physiograph to measure weight changes of the digit. Vascular pressures were adjusted by regulating the arterial pump speed and height of the venous reservoir to maintain the digit in an isogravimetric state. Systemic and digital vascular pressures and digital weight changes were continuously recorded. An initial set of Starling forces were measured. Three of the horses treated with 0.9% NaCl and 5 horses treated with PD145065 received NG (10^{-5} M concentration) into the arterial circuit and Starling force measurements were repeated. At the end of the study, the isolated digit was weighed, the soft tissues completely removed via boiling, and the remaining bone, hoof, and sole were reweighed. Subtraction of these values from the total weight approximates the perfused soft tissues, which minimizes variability due to foot size.[24] The soft tissue weight was used to calculate blood flow per 100 g of soft tissue of the isolated digit.

8.2.5 Hemodynamic Measurements – Based on pressures of the anesthetized laterally recumbent horse, arterial pressure was set at approximately 100 mmHg by regulation of flow, using the arterial perfusion pump and venous pressure was set at approximately 30 mmHg by regulating the height of the extracorporeal venous reservoir.[24] Capillary pressure (Pc; mmHg) was determined using the previously described venous occlusion technique.[25] Isolated digital blood flow (Qb; ml/min/100g of tissue) was measured by timed collection into a graduated cylinder.

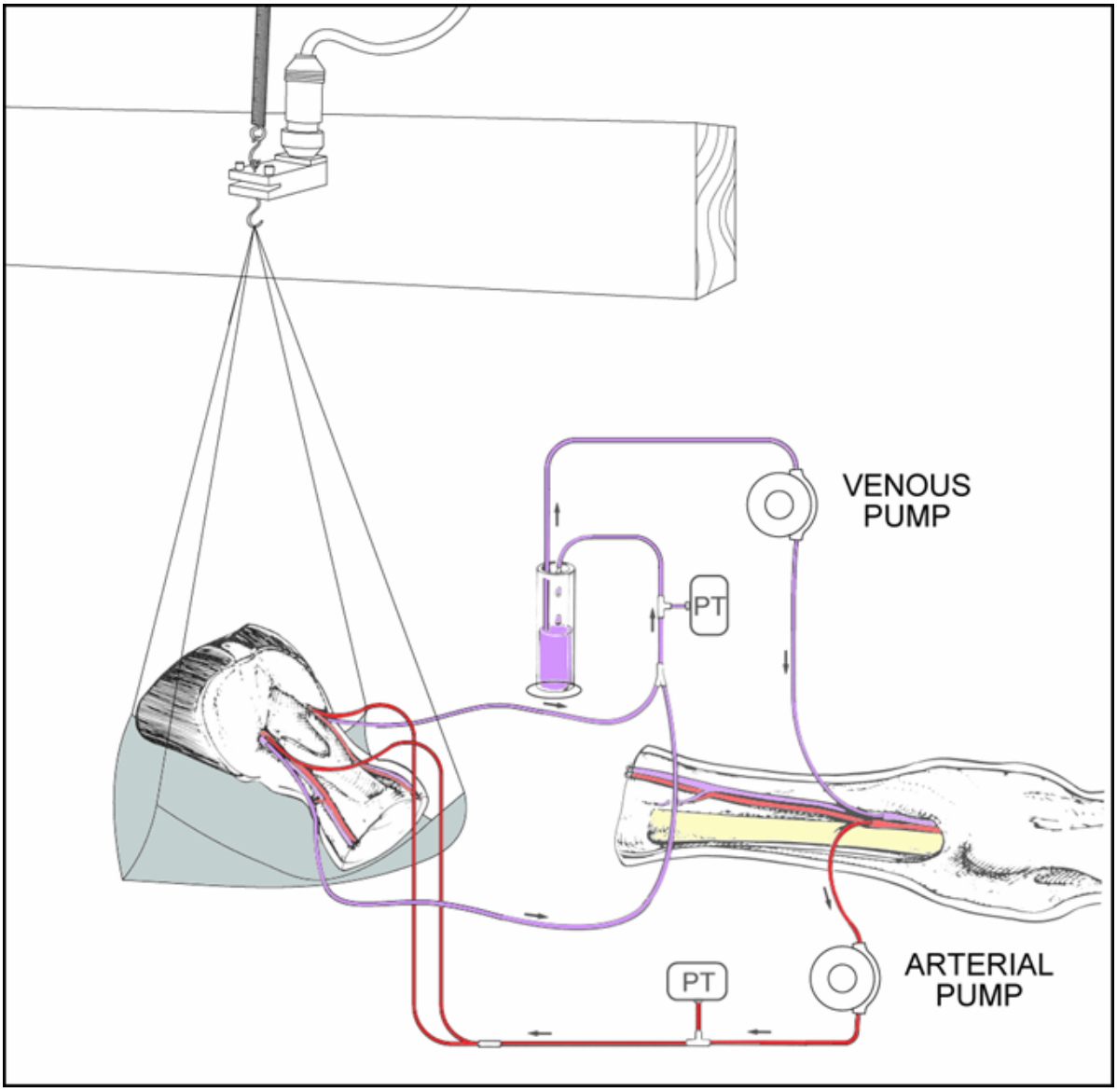

Figure 8.1 – The pump-perfused extracorporeal digital preparation for measurement of Starling forces in the equine digit. The arrows indicate direction of blood flow and the location of the arterial and venous pumps are shown. Circuits were interfaced with pressure transducers (PT), and the venous outflow was collected into a graduated cylinder for blood flow measurements. The digit was disarticulated at the level of the fetlock, and the isolated digit was placed in the wire basket suspended from a force transducer interfaced with a polygraph to measure weight changes.

8.2.6 Calculations – After an equilibration period, Pa, Pv, Pc, and Qb were recorded. Digital vascular resistances were calculated using the following formulas (mmHg/ml): total vascular resistance = Pa – Pv / Qb; precapillary resistance = Pa – Pc / Qb; postcapillary resistance = Pc – Pv / Qb. The pre- to postcapillary resistance ratio was calculated.[24]

The filtration coefficient (Kfc; ml/min/mmHg/100 g tissue) was calculated using the formula: $Kfc = Jv_2 – Jv_1 / Pc_2 – Pc_1$ with Jv_1 equal to the initial filtration rate, Pc_1 equal to the initial capillary pressure, Jv_2 and Pc_2 equal to the filtration rate and capillary pressure, respectively, after the venous pressure was increased by 7 - 10 mmHg.[24,26] After the venous pressure was increased by raising the height of the extracorporeal blood reservoir, there was a two phase weight gain within the isolated digit; first, a rapid increase in weight due to vascular volume increase, and then a slower increase in weight due to capillary filtration. The Jv_2 was measured during the second slower phase followed by measurement of Pc_2 using the venous occlusion technique. Using the initial phase of the weight gain curve, vascular compliance (ml/mmHg) was calculated using the formula: compliance = $\Delta W / \Delta Pc$ with ΔW equal to the change in vascular volume in the isolated digit and ΔPc equal to the change in capillary pressure.[24,27]

8.2.7 Statistical Analyses – Data was considered to follow a normal distribution using the Shapiro-Wilk test with failure to reject the null hypothesis of normality at $p \leq 0.05$. The data was summarized and presented as mean ± SEM. The data was analyzed using a mixed effect linear model that accounted for the random variance of horse. Where there were significant interaction effects at $p \leq 0.05$, predetermined comparisons were made using least squares means to determine where differences were occurring between groups. Type I error was maintained at 0.05. PROC MEANS, UNIVARIATE, and MIXED were used for the analysis.[h]

8.3 Results

Eleven of the 14 horses (6-PD145065 treated horses and 5 saline control horses) developed at least a 30% decrease in WBC count and progressed to Obel grade 1 laminitis between 5 and 11 hours after BWE with a median value of 9 hours. The remaining three horses did not develop Obel grade 1 laminitis, did not have at least a 30% drop in WBC count, and were not anesthetized for Starling force measurements. Starling force data is summarized in Table 8.1. Treatment with the ET receptor antagonist significantly lowered digital arterial pressure ($p = 0.037$), and caused a trend ($p = 0.1$) for lower total and precapillary resistances than saline-treated horses. ET antagonist-treated horses receiving NG infusion had a further reduction in vascular resistance and caused a significant increase in digital blood flow ($p = 0.046$) compared with the ET antagonist alone. In this group, there was a trend ($p < 0.1$) for lower digital arterial pressure, higher digital capillary pressure, and a higher capillary filtration coefficient. Additionally, those treated with the ET antagonist and NG had trends ($p < 0.1$) for lower total vascular resistance, precapillary resistance, postcapillary resistance, and higher digital blood flow compared to saline-treated horses. There were no significant differences between saline-treated horses and those receiving saline followed by NG for any variables.

8.4 Discussion

This study provides indirect evidence to support our global hypothesis that the initiating event of acute laminitis involves an imbalance in endothelium-derived vasodilators (decreased nitric oxide) and vasoconstrictors (increased endothelin-1). This study also provides direct evidence to support our study hypothesis that decreasing ET receptor availability by using an ET antagonist and increasing NO concentrations by administration of a NO donor improves digital Starling forces in horses with BWE-induced laminitis.

Table 8.1 – Mean +/- SEM values for Starling measurements and calculations for horses treated with saline or an ET antagonist (PD145065; [10^{-5} M]) during the developmental stages of BWE-induced laminitis (Set 1) and another set (Set 2) of Starling measurements and calculations after nitroglycerine (NG; [10^{-5} M]) administration into the arterial circuit.

Variable	Set 1		Set 2	
	Saline	PD145065	Saline+NG	PD145065+NG
Digital Blood Flow (ml/min/100 g tissue)	2.929 ± 0.8911	4.021 ± 1.4255	2.112 ± 0.9668	6.364 ± 1.3708*
Digital Arterial Pressure (mmHG)	196.4 ± 44.0063	133.705 ± 21.1537#	66.288 ± 34.3477	105.34 ± 5.2168
Digital Venous Pressure (mmHg)	30.3 ± 0.5612	30 ± 0	10.187 ± 9.9077	30.3 ± 0.3
Digital Capillary Pressure (mmHg)	38.1 ± 2.4806	36.058 ± 1.7146	13.417 ± 11.3223	40.52 ± 2.9917
Total Vascular Resistance (mmHg/ml)	122.476 ± 63.1839	55.263 ± 22.7175	47.054 ± 12.3816	15.12 ± 4.0694
Precapillary Resistance (mmHg/ml)	119.248 ± 62.5273	52.863 ± 22.4891	45.959 ± 12.0624	13.486 ± 4.1783
Postcapillary Resistance (mmHg/ml)	3.3 ± 0.7118	2.33 ± 0.6546	1.232 ± 0.5491	1.625 ± 0.4588
Precapillary-to-Postcapillary Resistance Ratio	28.03 ± 10.5558	25.746 ± 8.5924	14.965 ± 5.4205	10.598 ± 4.0083
Capillary Filtration Coefficient (ml/min/mmHg/100 g tissue)	0.0093 ± 0.002	0.0057 ± 0.0012	0.0030 ± 0.0014	0.0130 ± 0.0025
Vascular Compliance (ml/mmHg)	0.1564 ± 0.0199	0.136 ± 0.0275	0.0611 ± 0.0374	0.1678 ± 0.0262

* Significantly different ($p < 0.05$) from ET antagonist-treated group.
# Significantly different ($p < 0.05$) from saline-treated group.

Although laminitis induced by BWE administration and CHO overload are both accompanied by increases in capillary pressure and tissue pressure, there are differences between the two models.[4,7] The severity of the venoconstriction accompanying laminitis induced with BWE was less than that associated with CHO overload. These less severe changes with BWE may be due to a difference in the pathophysiology of the disease, or more likely because the Starling forces accompanying laminitis due to BWE were evaluated at a different stage (2 to 4 hours versus 16 hours) of the disease. Using laser Doppler flow probes to measure laminar capillary perfusion, Adair et al recently determined that laminar microvascular blood flow decreases in the first 1 to 2 hours after BWE administration.[28] This initial decrease was then followed by a return of laminar microvascular blood flow to near baseline values. Then, at approximately 8 hours after administration of BWE, laminar blood flow again decreased, which temporally corresponded with development of clinical signs of laminitis. Administration of a CHO ration results in hemodynamic and clinical alterations that take longer to develop compared to the BWE model. During the developmental stages of laminitis using the CHO model, Hood et al used hoof wall surface temperature as an indication of laminar perfusion and found decreases 8 to 10 hours before the onset of lameness (mean onset of lameness was 33 hours from carbohydrate administration).[2]

Starling force measurements during the study presented here were made after the development of Obel grade 1 laminitis (mean of 9 hours post BWE administration), later than a study published by Eaton et al in which Starling force measurements were obtained 2 – 4 hours after BWE administration (once there was a 30% decrease in WBC count).[7] Our objective was to determine the effectiveness of the ET antagonist in preventing Starling force alterations during early stages of laminitis (Obel grade 1), compared with saline treatment, thus, requiring

progression of laminitis beyond that of Eaton et al. The temporal differences could account for the observed differences between the horses in our study and those of the study by Eaton et al.

The increase in precapillary resistance in the horses administered BWE in this study is in contrast to the findings of Eaton et al where they reported predominantly an increase in postcapillary resistance.[7] Our finding is, however, in agreement with the hypothesis of Allen, et al that the initiating vascular event is venoconstriction, which causes an increase in postcapillary resistance.[4] The authors hypothesize this increase in venous resistance causes an increase in the capillary hydrostatic pressure, which forces fluid out of the capillary bed into the laminar interstitial space, leading to laminar edema.[4] The laminar edema causes an increase in the interstitial pressure because the fluid is essentially trapped between the rigid hoof capsule and the hard bony surface of the third phalanx; this leads to compression and collapse of the capillary beds ultimately resulting in an increase in precapillary resistance. Therefore, the timing after BWE with which we evaluated Starling forces in this study lends support to the theory that increased postcapillary resistance eventually progresses to increased precapillary resistance due to a "compartment-like" syndrome. A compartment-like syndrome is defined by increased pressure in a confined anatomical space, affecting blood flow of the soft tissues within the confined space leading to tissue ischemia.

Previous studies in our laboratory have shown that ET-1 causes a dose-dependent, sustained contraction of palmar digital arteries and veins in vitro with venous ring contraction being more pronounced than contraction of arterial rings.[10,11] In a preliminary study (unpublished data) in our laboratory, infusion of ET-1 (at a dose to yield a 10^{-6} M concentration in digital blood) into the arterial side of the circuit in three clinically healthy horses instrumented for Starling force evaluation revealed ET-1 caused a decrease in digital blood flow due to a 30%

increase in postcapillary resistance (i.e. venoconstriction). Capillary pressure increased from 36 to 52 mmHg, a value similar to the capillary pressure measured in horses after BWE administration.[7] These findings further support the role of ET-1 in at least contributing to the digital hemodynamic and Starling force alterations characteristic of the early phases of acute laminitis.

Horses administered BWE have been shown to have a significant increase in digital venous, but not jugular venous, plasma concentrations of ET-like immunoreactivity that develops within 3 hours and persists for up to 10 hours post-BWE administration.[29] This increase in digital plasma concentrations of ET-like immunoreactivity likely represents only a small fraction of the total ET-1 synthesized and released by the digital vasculature in horses administered BWE considering that approximately 80% of ET-1 normally synthesized by the vascular endothelium is released abluminally toward the smooth muscle.[30] Horses in the developmental stages of laminitis may have substantially increased ET-1 synthesis with a resultant saturation of ET receptors on the digital vascular (venous) smooth muscle that leads to increases in postcapillary resistance and initiates the cascade of events that progresses to increased precapillary resistance and laminar ischemia.

The two main receptor types for ET-1 in vasculature are the ET_A and ET_B receptors located on smooth muscle cells and endothelial cells, respectively.[31] The vasoconstrictive effects of ET-1 are principally through the ET_A receptor, although the receptor antagonist we selected (PD145065) based on previous studies is nonselective for both ET_A and ET_B receptor types.[10,32] In the study reported here, the antagonist was infused into the digital arterial circulation over two minutes at a dose that approximated a 10^{-5} M concentration based upon the digital blood flow measurements. Although the antagonist was not injected as a bolus, the blood flow likely carried

some of the antagonist out of the digital circulation before it had time to diffuse from the lumen to the smooth muscle where the ET receptors responsible for contraction are located, resulting in less antagonist reaching the smooth muscle ET receptors. There were several variables in this study with trends ($p < 0.1$) identified where the ET antagonist appeared to improve Starling forces. These trends may have become statistically significant if we had been able to increase the number of horses in each group. Additionally, these trends may have become significant if either the dose or duration of administration of the ET antagonist had been increased. Although increasing the duration of infusion of the antagonist may have helped to overcome this potential limitation, it was cost prohibitive.

Administration of NG, a NO donor, into the arterial side of the circuit in the horses of this study significantly improved blood flow above that observed with administration of the ET antagonist. There were also trends for a reduction in total and precapillary resistance in horses administered NG compared with administration of the ET antagonist alone. These findings demonstrate that NO is important in regulating vasomotor tone and blood flow in the digital circulation and that administration of NO may improve digital hemodynamics and Starling forces in horses with BWE-induced laminitis. Nitric oxide, normally released from the vascular endothelium, diffuses into the smooth muscle where it stimulates guanylate cyclase and the subsequent generation of cGMP, which leads to vascular smooth muscle relaxation.[33] This effect helps to reduce vasomotor tone, leading to improved blood flow and perfusion.

The results of this study provide additional supportive evidence that ET-1 may be involved in the pathogenesis of the vascular alterations characteristic of acute laminitis in horses and that administration of an ET antagonist alone or in combination with NG, may help improve digital Starling forces in horses with BWE-induced laminitis. Further studies regarding the role

of ET-1 and NO in the pathogenesis of acute laminitis as well as the potential for ET antagonists and NO donors to prevent or reverse the digital vascular events are warranted.

8.5 Product Information

[a] Transonics Systems, Inc., Ithaca, NY

[b] Angiocath Vascular Access, Becton Dickinson & Co, Sandy, UT

[c] Arrow Catheters, Arrow International, Reading, PA

[d] PD 145065, American Peptide Co, Sunnyvale, CA

[e] Eberbach Corp, Ann Arbor, MI

[f] Grass Medical Instruments, Quincy, MA

[g] FT03, Grass Medical Instruments, Quincy, MA

[h] Proc Mixed, Univariate, and Means; SAS version 8, SAS Institute, Cary, NC

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CHAPTER 9. EFFECTS OF BLACK WALNUT EXTRACT ADMINISTRATION ON IN VITRO RESPONSES OF EQUINE PALMAR DIGITAL ARTERIAL AND VENOUS RINGS TO ENDOTHELIN-1, ACETYLCHOLINE, AND NITROGLYCERINE

9.1 Introduction

Acute laminitis is a commonly encountered disease in horses and is characterized by decreased blood flow to the digital lamina, resulting in laminar ischemia, necrosis and subsequent separation of the distal phalanx (P_3) from the hoof wall.[1,2] Venoconstriction is considered the initiating factor causing decreased laminar perfusion.[1] Increased venoconstriction results in increased vascular resistance and capillary hydrostatic pressure. Increased capillary hydrostatic pressure forces fluid out of the capillaries and into the interstitium thereby increasing laminar interstitial pressure. When tissue pressure increases above the capillary critical closing pressure, the capillaries collapse leading to tissue ischemia. Blood flow is further reduced by formation of arteriovenous shunts at the level of the coronary band.[1-3] The digital laminae undergo necrosis after prolonged ischemia. Separation of the interdigitating sensitive and insensitive lamina develops and P_3 rotation, distal displacement, or both subsequently occur.[4]

Endothelin-1 (ET-1) is a potent vasoconstrictor released by the endothelium that has been associated with various diseases characterized by tissue hypoxia and has been found to have increased expression in the laminae of horses with experimentally-induced and naturally-acquired laminitis.[5-8] There are two types of ET receptors, ET_A receptors on vascular smooth muscle cells (results in sustained vasoconstriction) and ET_B receptors on endothelial cells (triggers the release of nitric oxide).[9] Nitric oxide (NO) is also released by the endothelial cells, independently of the ET_B receptor, and causes vasodilation.[5] Many vasoactive agents, such as acetylcholine (ACh), require intact endothelium to cause vasodilation by facilitating endothelial release of NO.[10] Alterations in endothelial cell function due to acute laminitis may affect endothelium-dependent functions and may alter the responsiveness of the digital vasculature to

vasoactive agents.[11,12] Since the onset of laminitis is characterized by digital vasoconstriction, and in particular venoconstriction,[2] determining the roles of ET-1 and NO in the pathogenesis of this disease may provide further important knowledge for the prevention and treatment of laminitis in horses.

Our hypotheses are that after BWE-induced laminitis, in vitro ET-1 administration will cause concentration-dependent vasoconstriction and the ET antagonist PD145065 will attenuate these contractile effects. These findings are expected to be similar to results of a similar study conducted in our laboratory of normal horses. Vessel rings will demonstrate a biphasic response to ACh, however, this response may be altered due to endothelial dysfunction. The endothelium-independent dilatory effects of nitroglycerine (NG) will be concentration-dependent. The purpose of this study was to examine the effects of BWE administration on the in vitro vasomotor effects of ET-1, ACh, and a NO donor in equine palmar digital arterial and venous rings.

9.2 Materials and Methods

9.2.1 Tissue Sources - These studies were approved by the Institutional Animal Care and Use Committee of the Louisiana State University. These studies presented here were conducted using tissues collected from horses that were part of a larger study examining acute laminitis using the BWE model of induction. Eleven light breed horses (2 mares and 9 geldings; ages ranging 5 – 19 years; weighing 430 to 591 kg) were determined to be free of laminitis and gastrointestinal tract disease based on their history, thorough physical and lameness examinations, and lateral radiographs of both front feet.

9.2.2 Preparation of Extract – Preparation of the BWE was as previously described.[13,14] Briefly, shavings were made from the heartwood of a black walnut tree cut in the fall of the year and were stored at –20 C until used. Two grams of black walnut shavings/kg body weight were combined with 8 liters of distilled water and mixed in a shaker bath[a] for 14 hours at room temperature (20 – 22 C). The mixture was filtered, refrigerated, and the fluid was administered within 24 hours of preparation.

9.2.3 Induction of Laminitis – Laminitis was induced in all horses by administration of the BWE via a nasogastric tube. Horses were monitored hourly after BWE administration until horses demonstrated Obel grade 1.[15] Efficacy of the extract was confirmed by at least a 30% decrease in WBC count.[14] After horses demonstrated Obel grade 1 laminitis, they were anesthetized (general anesthesia was induced with xylazine hydrochloride (0.51.0 mg/kg), sodium thiamylal (10 mg/kg) and sodium pentobarbital (7.5 mg/kg), IV, and maintained with pentobarbital (5 to 15 mg/kg/hr, IV) and vessel segments were surgically removed.

9.2.4 Vessel Preparation - The lateral palmar digital arteries and veins from one forelimb were collected and placed in chilled, oxygenated (95% O_2 and 5% CO_2) Tyrode's solution (136.87 mM NaCl; 2.68 mM KCl; 11.90 mM $NaHCO_3$; 5.55 mM dextrose; 1.81 mM $CaCl_2$; 1.07 mM $MgCl_2$; 0.36 mM NaH_2PO_4). Vessels were then gently cleansed of excess connective tissue and cut into 4mm wide rings.[16,11,17] Isometric tension was monitored by attaching (using 5-0 silk suture) one side of the vessel ring to the stationary floor of an organ bath containing oxygenated Tyrode's solution at 37 C, and the other side to a force-displacement transducer[b] interfaced with a polygraph.[18, c] Based on preliminary studies in our laboratory and on previously published studies by Baxter et al utilizing palmar digital arteries and veins in vitro,

an initial tension of two grams was determined to be the optimal resting tension and was applied to each vessel ring to mimic in vivo diastolic vascular tone.[11,17,19] The rings were allowed to equilibrate for 45 minutes. During this period, the bath solution was gently replenished with fresh Tyrode's solution at 15-minute intervals and the tension was readjusted to two grams.[11,19] Tension was not reapplied after the last bath solution change.

9.2.5 Pharmacological Agents – Two incubation agents were selected for these studies. The ET receptor antagonist PD145065[d] was selected based on its ability to inhibit ET binding to both ET_A and ET_B receptor types. The other incubation agent was the NO synthase inhibitor Lω-nitro-L-arginine methyl ester (L-NAME)[e]. Based on manufacturer's recommendations, distilled water was used to dissolve both of these agents and Tyrode's solution was used to dilute PD145065 and L-NAME to their desired concentrations, 10^{-5} and 10^{-4} M, respectively. Endothelin-1 was selected for use as a preconstrictor and for concentration-response (C-R) curves. ET-1[f] was dissolved in distilled water and frozen in aliquots at -80 C. Aliquots were thawed immediately prior to use and diluted with Tyrode's solution to the desired concentrations (10^{-10} to 10^{-6} M). Due to the current expense of ET-1, we were limited to 10^{-6} M concentration as the strongest concentration of ET-1 for the C-R curve. Concentration-response curves were also conducted using ACh and NG. The ACh[g] was dissolved in distilled water and was further diluted with Tyrode's solution to the desired concentrations (10^{-10} to 10^{-4} M). The NG[h] was initially diluted with distilled water and then further diluted with Tyrode's solution to the desired concentrations (10^{-10} to 10^{-4} M).

9.2.6 Experimental Designs - Two separate studies were conducted with the palmar digital vessels and a comparison was made between these findings and those of a previous study

using palmar digital vessel rings from normal horses.[20] The first study was conducted to compare ET-1 C-R curves with and without incubation with the ET antagonist PD145065 (10^{-5} M concentration) or the NO synthase inhibitor Lω-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M concentration). The purposes of the second study were to examine: (1) the responsiveness of vessels from laminitic horses to the endothelium-dependent actions of ACh; and (2) the ability of these vessel rings to respond to direct vasodilation via NG. This was accomplished by pre-constricting vessel rings with the EC75 concentration of ET-1 (10^{-7} M concentration) and then determining C-R relationships for ACh and NG separately (10^{-10} to 10^{-4} M concentrations). Vessels serving as controls received the ET-1 EC75 pre-contraction, but did not receive ACh or NG and were monitored for the duration of the study; therefore, a declining response over time to ET-1 could be differentiated from ACh/NG-induced relaxation. A third component to this report is a comparison of results of the ET-1 C-R curves from this study to the ET-1 C-R curves of a previous study of normal horses with and without incubation with the ET antagonist PD145065.[20]

9.2.7 Study I – PD145065 and L-NAME incubation - Each organ bath contained one vessel ring prepared as previously mentioned. A total of six organ baths were used containing three arterial and three venous rings and were randomly assigned to organ baths (Table 9.1). The first bath of each vessel type was a control bath and did not receive an incubation agent. The second and third baths of each vessel type were incubated with a 10^{-5}M concentration of PD145065 or a 10^{-4}M concentration of L-NAME, respectively. Incubation occurred during the

Table 9.1 – Study I organ bath design.

Bath #	Vessel Type	Incubation Agent	Concentration-Response Agent
1	Artery	None	[Endothelin-1 10^{-10} to 10^{-6} M]
2	Artery	[PD145065 10^{-5} M]	[Endothelin-1 10^{-10} to 10^{-6} M]
3	Artery	[Lω-nitro-L-arginine methyl ester 10^{-4} M]	[Endothelin-1 10^{-10} to 10^{-6} M]
4	Vein	None	[Endothelin-1 10^{-10} to 10^{-6} M]
5	Vein	[PD145065 10^{-5} M]	[Endothelin-1 10^{-10} to 10^{-6} M]
6	Vein	[Lω-nitro-L-arginine methyl ester 10^{-4} M]	[Endothelin-1 10^{-10} to 10^{-6} M]

last 30 minutes of equilibration by adding the selected agent to the bath at each of the three times the bath solution was replenished.

ET-1 concentration-response relationships - After equilibration and incubation, cumulative C-R relationships (10^{-10} to 10^{-6} M concentrations) were determined for ET-1 for all vessel groups. Each consecutive concentration of ET-1 was added to the baths at 5-minute intervals. Concentration-response curves were recorded for each vessel ring and apparent maximum responses to ET-1 were measured. It should be noted that due to the expense of ET-1, the C-R curves were limited to the concentrations of 10^{-10} to 10^{-6} M. The contraction of vessel rings following this concentration was considered the apparent maximum contractions and throughout this manuscript maximum contractions are in context of these limitations. The dry tissue weight was determined afterward by allowing the rings to dry at room temperature (20-22 C) and measuring their weight on an analytical balance until weight loss was no longer observed.

9.2.8 Study II – L-NAME incubation and ET-1 precontraction – Study II was conducted concurrently with Study I using additional vessel rings from the same horse. Each organ bath contained one vessel ring prepared as previously mentioned. A total of 10 organ baths were used containing five arterial and 5 venous rings and were randomly assigned to organ baths (Table 9.2). Arterial rings in organ baths two and four and venous rings in organ baths seven and nine were incubated with a 10^{-4} M concentration of L-NAME during the last 30 minutes of equilibration as described above. After the 45-minute equilibration period, each of the 10 organ baths received ET-1 10^{-7} M concentration that was determined from previous studies to be the

Table 9.2 – Study II organ bath design.

Bath #	Vessel Type	Incubation Agent	Precontraction Agent	Concentration-Response Agent
1	Artery	None	[Endothelin-1 10^{-7} M]	[Acetylcholine 10^{-10} to 10^{-4} M]
2	Artery	[Lω-nitro-L-arginine methyl ester 10^{-4} M]	[Endothelin-1 10^{-7} M]	[Acetylcholine 10^{-10} to 10^{-4} M]
3	Artery	None	[Endothelin-1 10^{-7} M]	[Nitroglycerine 10^{-10} to 10^{-4} M]
4	Artery	[Lω-nitro-L-arginine methyl ester 10^{-4} M]	[Endothelin-1 10^{-7} M]	[Nitroglycerine 10^{-10} to 10^{-4} M]
5	Artery	None	[Endothelin-1 10^{-7} M]	None
6	Vein	None	[Endothelin-1 10^{-7} M]	[Acetylcholine 10^{-10} to 10^{-4} M]
7	Vein	[Lω-nitro-L-arginine methyl ester 10^{-4} M]	[Endothelin-1 10^{-7} M]	[Acetylcholine 10^{-10} to 10^{-4} M]
8	Vein	None	[Endothelin-1 10^{-7} M]	[Nitroglycerine 10^{-10} to 10^{-4} M]
9	Vein	[Lω-nitro-L-arginine methyl ester 10^{-4} M]	[Endothelin-1 10^{-7} M]	[Nitroglycerine 10^{-10} to 10^{-4} M]
10	Vein	None	[Endothelin-1 10^{-7} M]	None

EC_{75} concentration in equine palmar digital vessel rings.[20] The ET-1 EC_{75} was added to the organ baths and tension was monitored continuously until the contractile response plateaued. Once the contractile response plateaued, a new baseline was established for determination of vessel relaxation or contraction due to the C-R agents ACh or NG.

ACh and NG concentration-response relationships - Cumulative C-R relations (10^{-10} to 10^{-4} M concentrations) were determined for ACh or NG. Each consecutive concentration was added to the baths at 2-minute intervals. The dry tissue weights of the vessels were determined and apparent maximum relaxation and contraction were calculated.

9.2.9 Comparison of ET-1 C-R Relationships to Previous Study – Endothlein-1 C-R data (no incubation agent and PD145065 incubated vessel only) from Study I were compared to data from a previous study of 8 normal horses that utilized the design as described in Table 9.3 and followed the same methodology as described above for Study I.[20]

9.2.10 Statistical Analyses - The continuous data (apparent maximum contraction) were evaluated for normality, using the Shapiro-Wilk statistic and were considered to follow a normal distribution with failure to reject the null hypothesis of normality at $p \leq 0.05$. The data were summarized and graphed as mean ± SEM**.**

Study I - The continuous data (maximum contraction) were evaluated separately for arteries and veins using the following model:

$$y = \mu + \text{Horse} + \text{Incubation Agent} + \text{Time} + \text{Horse*Incubation Agent*Time} + \varepsilon$$

where the effect of Horse was considered random and the effect of Incubation agent was tested using the Horse interaction term. Comparisons between arteries and veins were determined using

Table 9.3 – Organ bath study design of previous study in normal horses for comparison to Study I.

Bath #	Vessel Type	Incubation Agent	Concentration-Response Agent
1	Artery	None	[Endothelin-1 10^{-10} to 10^{-6} M]
2	Artery	[PD145065 10^{-5} M]	[Endothelin-1 10^{-10} to 10^{-6} M]
3	Vein	None	[Endothelin-1 10^{-10} to 10^{-6} M]
4	Vein	[PD145065 10^{-5} M]	[Endothelin-1 10^{-10} to 10^{-6} M]

a mixed effect linear model that accounted for the random variance of horse and the repeated measurements of each horse.

Study II - For comparison of the ACh, NG, and no C-R agent vessel ring responses, the apparent maximum contraction was evaluated for arteries and veins separately using the following model:

$$y = \mu + Horse + Drug + Time + Horse*Drug*Time + \varepsilon$$

where the effect of horse was considered random and the effect of Drug was tested using the Horse interaction term. Comparisons between arteries and veins were determined using a mixed effect linear model that accounted for the random variance of horse and the repeated measurements of each horse.

Comparison of ET-1 C-R relationships to previous study - The continuous data (maximum contraction) were evaluated separately for arteries and veins between horses with BWE-induced laminitis and normal horses using the following model:

$$y = \mu + Horse + Disease\ State + Time + Horse*Disease\ State*Time + \varepsilon$$

where the effect of Horse was considered random and the effect of Disease State was tested using the Horse interaction term.

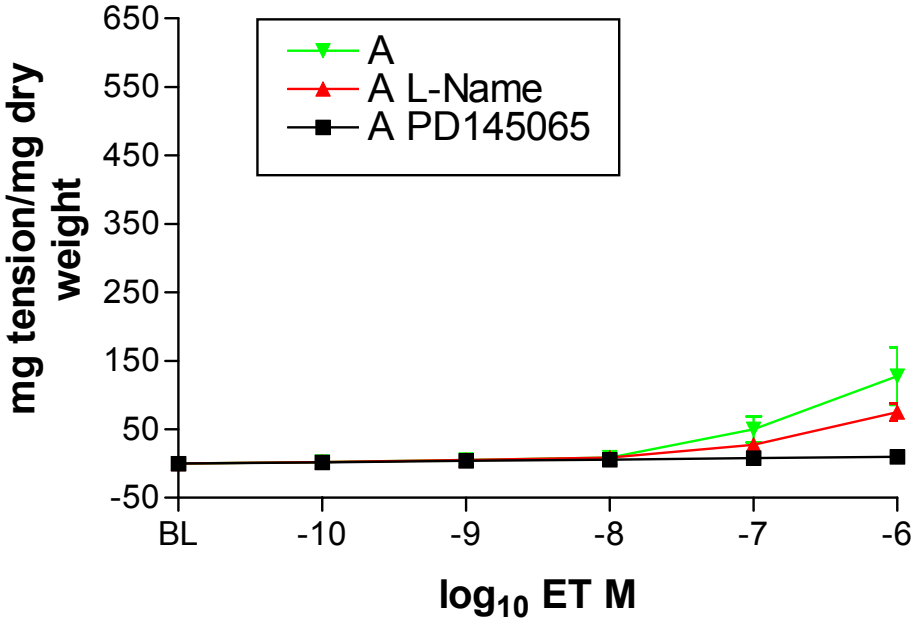
For all analyses, a two-sided hypothesis with $\alpha=0.05$ was used to determine significance of the main and interaction effects. The p-value for significant interaction effects was reported. Proc means, univariate, and mixed were used for the analysis.[i] Where there was significant main or interaction effects, multiple comparisons, using adjusted least squares means, were made among and between drug combinations maintaining an experiment-wise error of $\alpha = 0.05$. Thus, where a difference is noted, unless specified, the p-value was ≤ 0.05.

9.3 Results

9.3.1 Study I - Although 100% maximum contractions of tissues were not obtained due to our limitation of ET-1 at 10^{-6} M being the highest concentration utilized, ET-1 administration in the absence of the ET antagonist (control vessel rings) resulted in a concentration-dependent, profound contraction of palmar digital arteries and veins (Fig. 9.1). Venous rings responded significantly different from arterial rings by contracting 5 times greater to ET-1 than arterial rings. These contractile responses were effectively inhibited by the ET antagonist PD145065 at the 10^{-5} M concentration for both arterial and venous rings. The ET-1 C-R curve was not statistically different between vessel rings incubated with or without L-NAME.

9.3.2 Study II – Once vessel rings reached a plateau after precontraction with ET-1, there was no significant change in mg tension/mg dry weight over the length of the study for arterial or venous control rings not receiving ACh or NG C-R curves. Therefore, the vessels maintained a steady-state once they reached the plateau induced by administration of the EC_{75} for ET-1. After the plateau was reached and ACh was administered (10^{-10} to 10^{-4} M concentrations), a biphasic response was present to ACh (Fig. 9.2). Venous rings had greater dilation to ACh 10^{-10} to 10^{-6} M concentrations than arterial rings; however, arterial rings contracted over two times more to ACh at 10^{-5} to 10^{-4} M concentrations than venous rings. The contraction of arterial rings following the 10^{-4} M concentration of ACh was statistically different from baseline values. Incubation with L-NAME did not significantly alter the response to ACh for arterial or venous rings (Fig. 9.3). Following the NG C-R curve, venous rings dilated over three times greater than arterial rings with significant differences at the 10^{-5} and 10^{-4} M concentrations of NG. Venous rings dilated significantly from baseline values after 10^{-7}

Figure 9.1 - Results from Study I demonstrating mean +/- SEM endothelin-1 (ET-1) concentration-response curves [10^{-10} to 10^{-6} M] for arterial (A; top panel) and venous (V; bottom panel) rings pretreated with either Lω-nitro-L-arginine methyl ester (L-NAME, [10^{-4} M]), PD145065 ([10^{-5} M]), or no pretreatment. Vessel rings were collected from horses after induction of acute laminitis using the black walnut extract model. Note difference in y-axis scale between arterial and venous responses. BL=baseline *Values for control and L-NAME treated venous rings that differ significantly ($p<0.05$) from venous rings pretreated with PD145065.



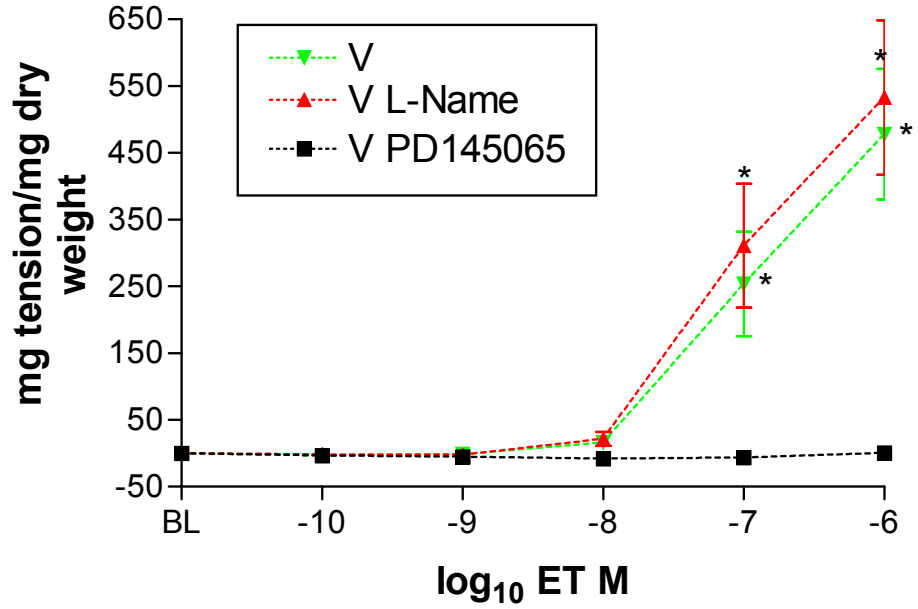
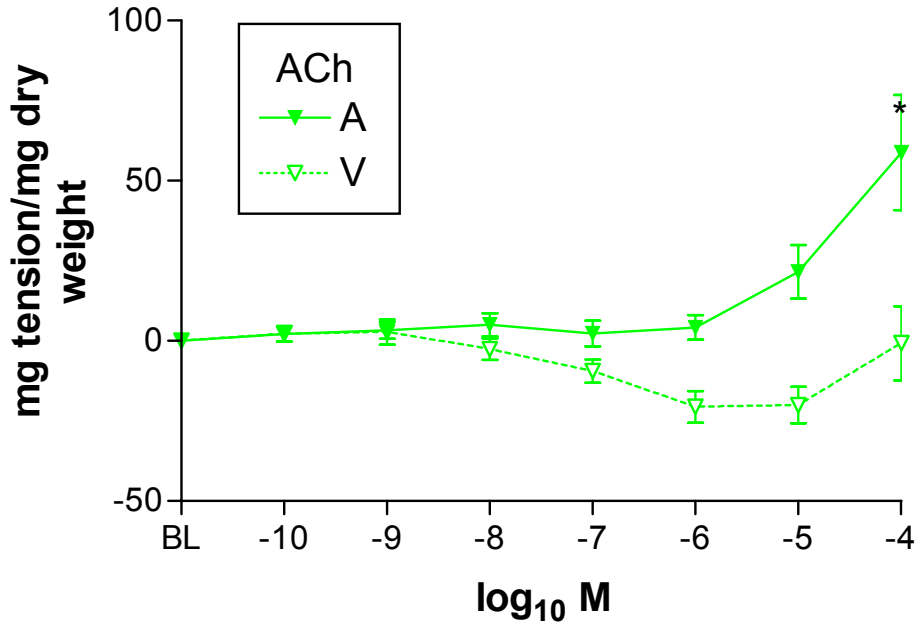


Figure 9. 2 - Results from Study II demonstrating mean +/- SEM acetylcholine (ACh; top panel) and nitroglycerine (NG; bottom panel) concentration-response curves [10^{-10} to 10^{-4} M] for arterial (A) and venous (V) rings with no pretreatment. Vessel rings were collected from horses after induction of acute laminitis using the black walnut extract model. BL=baseline *Values differ significantly ($p<0.05$) from venous rings. Note the change in the y-axis between the two agents.



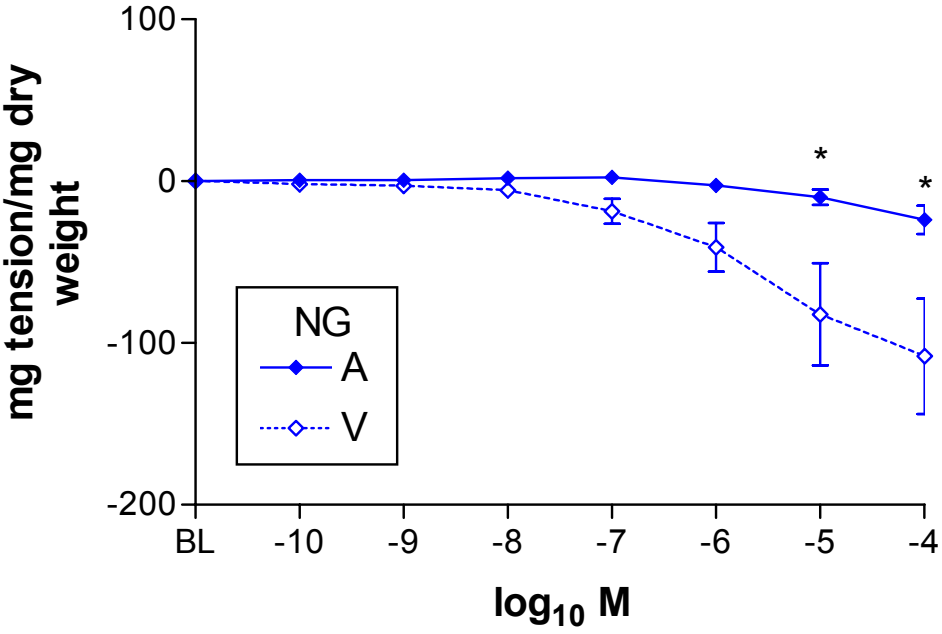
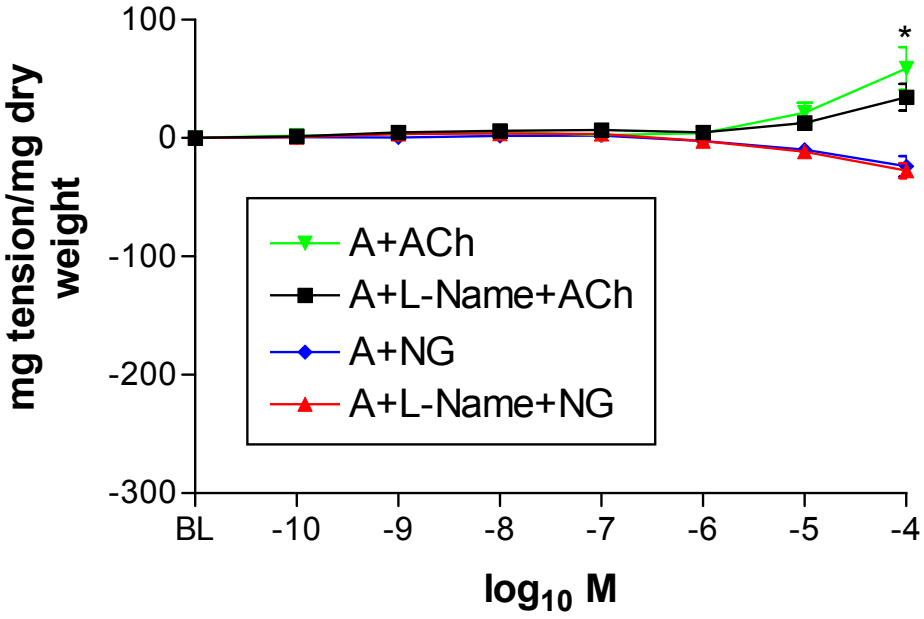
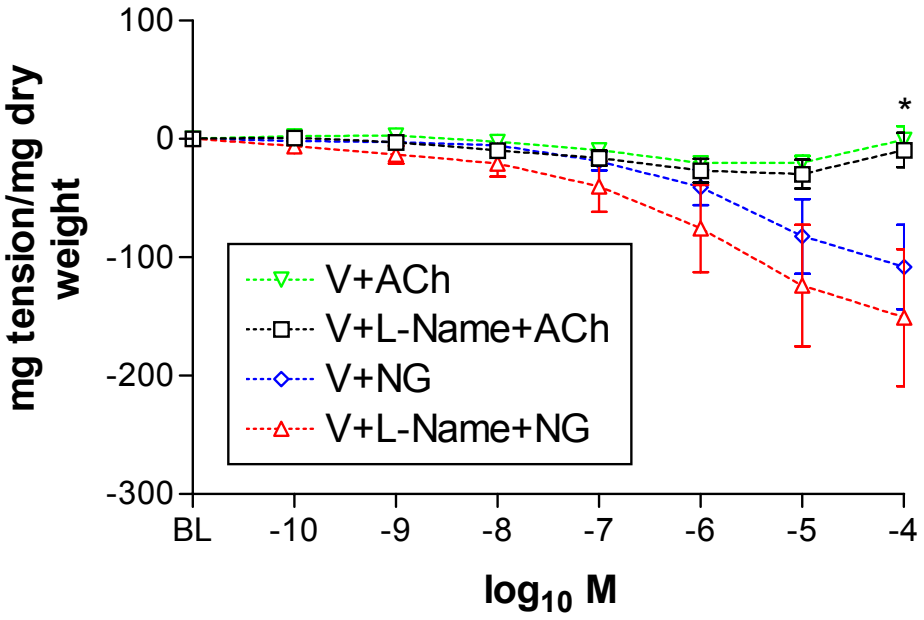


Figure 9.3 - Results from Study II demonstrating mean +/- SEM acetylcholine (ACh) and nitroglycerine (NG) concentration-response curves [10^{-10} to 10^{-4} M] for arterial (A; top panel) and venous (V; bottom panel) rings pretreated with Lω-nitro-L-arginine methyl ester (L-NAME, [10^{-4} M]) or with no pretreatment. Vessel rings were collected from horses after induction of acute laminitis using the black walnut extract model. BL=baseline *Values differ significantly ($p<0.05$) from NG treated rings.





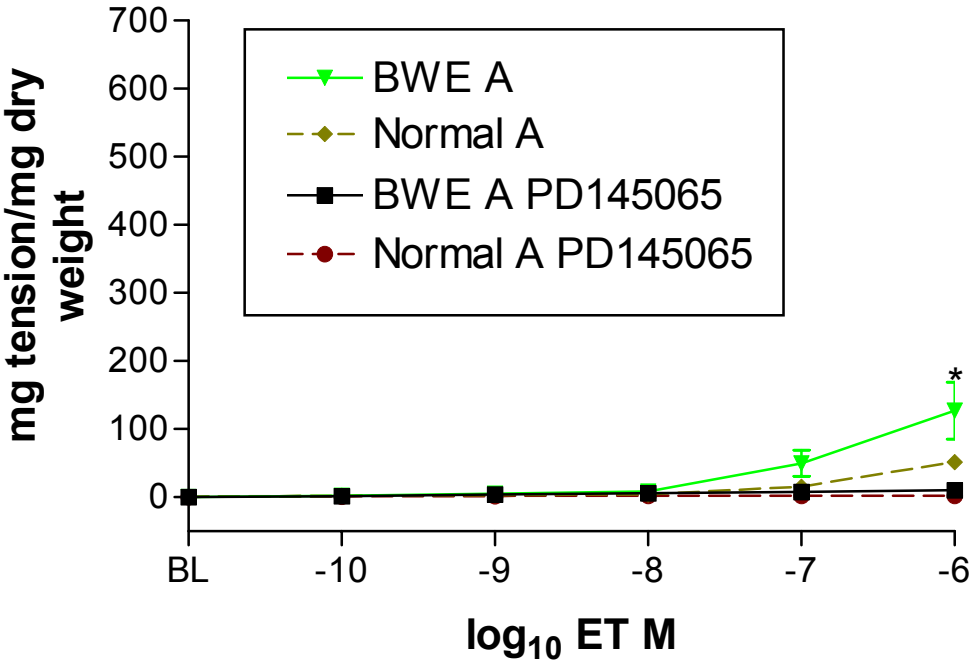
to10^{-4} M concentrations of NG and venous rings incubated with L-NAME also dilated significantly from baseline at 10^{-6} to 10^{-4} M concentrations of NG. There were no significant differences between or within arterial responses to NG with or without L-NAME.

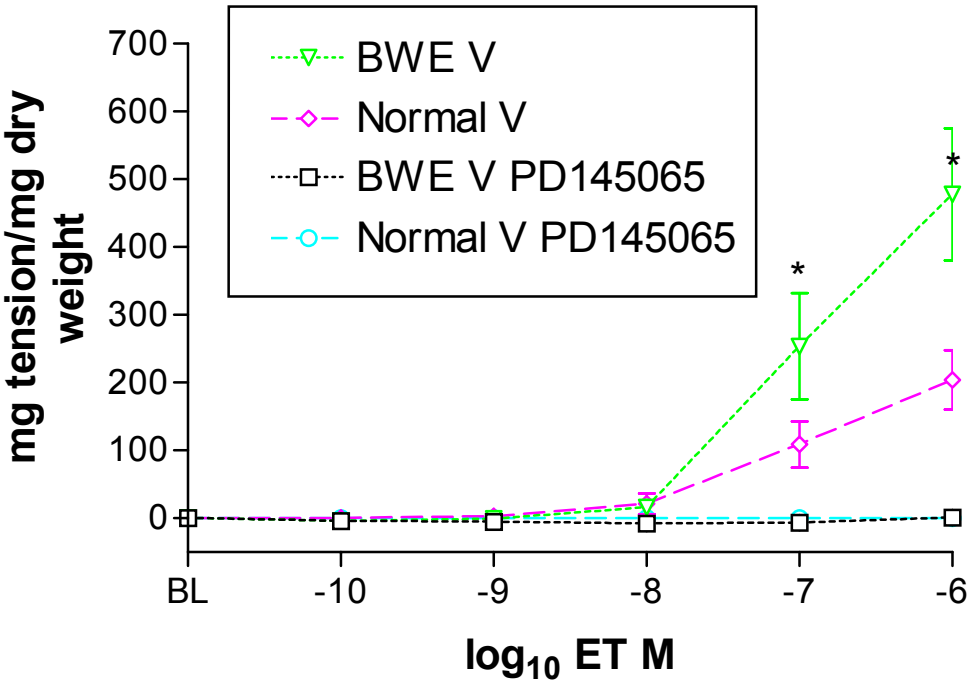
9.3.3 Comparison of ET-1 C-R Relationships to Previous Study - Arterial rings from horses after BWE-induced laminitis contracted significantly greater than corresponding vessel rings from normal horses at the 10^{-6} M concentration (Fig. 9.4). Venous rings from horses after BWE-induced laminitis also contracted significantly greater than corresponding vessel rings from normal horses at the 10^{-7} and 10^{-6} M concentrations. There were no significant differences between PD145065-treated vessel rings for BWE-induced horses, compared with normal horses, since this ET antagonist was effective in attenuating the contractile effects of ET-1 in both groups of horses.

9.4 Discussion

Study I demonstrated four important findings. First, ET-1 administration resulted in a concentration-dependent contraction of vessels from horses with acute BWE-induced laminitis. Second, veins were more sensitive and responded with a greater maximal contraction than arteries to ET-1. Third, in both arteries and veins, the ET receptor antagonist PD145065 at the 10^{-5} M concentration inhibited the contractile effects of ET-1, suggesting the effectiveness of this antagonist in laminitic horses. Lastly, inhibition of NO synthase by the actions of L-NAME did not change ET-1 function; thus, ET-1 activity through the ET_B receptor (NO release) did not

Figure 9.4- Mean +/- SEM arterial (A; top panel) and venous (V; bottom panel) endothelin-1 (ET-1) concentration-response curves [10^{-10} to 10^{-6} M] in normal horses and horses after induction of acute laminitis with black walnut extract (BWE) for vessel rings without pretreatment and those pretreated with the ET antagonist PD145065 [10^{-5} M]. * Values differ significantly ($p<0.05$) from corresponding vessel rings from normal horses.





appear to be an appreciable part of the actions of ET-1 in these vessels from horses with BWE-induced laminitis.

This study demonstrates the presence of ET-1 receptors in equine palmar digital vessels that may play a role in the vascular constriction that occurs in the early stages of laminitis. Preparation and application of ET-1 using our in vitro organ-bath design produced results that correlated well with the publication by Baxter using ET-1 in equine palmar digital vessel rings using the same in vitro organ-bath design.[12] A previously published study in our laboratory using equine colonic vessels also produced very similar results regarding the in vitro contractility of vessel rings to ET-1.[16] Studies in our laboratory with normal horses and those with naturally-acquired laminitis resulted in similar findings; however, comparison of the ET-1-induced contractile response of vessels from Study I to those of normal horses demonstrated greater maximal contraction in horses after BWE-induced laminitis.[20,21] It is possible that experimentally-induced laminitis induced by BWE causes damage to the endothelium resulting in either decreased activation of the ET_B receptor (located on endothelial cells; activation results in release of NO) or decreased release of endogenous endothelial-derived relaxing factors (i.e. NO) independent of ET_B receptor stimulation. Previous studies using the carbohydrate model of acute laminitis have identified swelling of the endothelial cells as early as four hours after the onset of lameness, thus supporting endothelial dysfunction. [22-24] These previous studies support the findings of the study presented here that vessels from BWE-induced laminitic horses have increased reactivity to ET-1 and that veins have increased sensitivity and responsiveness to ET-1 compared with arteries. These findings are interesting considering that venoconstriction and an

imbalance of venous and arterial resistances have been previously documented in the early stages of experimentally-induced laminitis in horses.[2]

The inhibitory effects of PD145065 are consistent with similar studies using equine colonic vessel rings and equine palmar digital vessel rings in normal and naturally-acquired laminitic horses.[16,20,21] The PD145065 antagonist was effective in blocking the receptors and resulted in almost complete attenuation of the ET-1 induced contractile response in arterial and venous rings. One of the goals of these studies was to determine if the ET receptor antagonist would be as effective in attenuating ET-induced contractile effects in horses with experimentally-induced laminitis. The antagonist PD145065 was effective in both normal horses and those with naturally-acquired laminitis.[20,21] Our results were as expected that the antagonist would be effective in horses with experimentally-induced disease. It is important therapeutically that the antagonist is effective in horses after the cascade of events that occurs during the development of the disease has been initiated.

Due to the current expense of ET-1, this study was limited to the use of a maximum concentration of 10^{-6} M; consequently, maximum contraction values are based on the maximal response of tissues to this 10^{-6} M concentration of ET-1. Our data correlates well with maximal contractions reported by Baxter examining ET-1 in palmar digital arterial and venous rings in vitro; although the maximum concentration of ET-1 in this previous study was 10^{-7} M.[12] In addition, the observed apparent maximum contractions of these tissues support the efficacy of PD145065 at the 10^{-5} M concentration in attenuating the contractile effects of ET-1. Since the greatest concentration used in this study for ET-1 was 10^{-6} M, it is possible that with higher concentrations ET-1 may be able to overcome the antagonistic effects of PD145065. However, it

is unlikely circulating concentrations of ET-1 would exceed this concentration in normal horses or those with pathological conditions such as laminitis. Katwa et al found increased laminar connective tissue ET-1 concentrations from laminitic horses (1.7 pg/mg of tissue) compared with non-laminitic horses (0.4 pg/mg of tissue).[8] Other studies have compared plasma ET-1 concentrations in horses with recurrent airway obstruction (6.53 pg/ml; healthy controls 3.74 pg/ml) and horses with various gastrointestinal tract diseases (3.29-10.02 pg/ml; healthy controls 1.8 pg/ml); however, caution should be used when comparing plasma ET-1 concentrations with that available to underlying smooth muscle since approximately 80% of ET-1 release is abluminaly.[25,26,27] The lower concentrations of ET-1 used in this study better approximate likely physiological and pathophysiologic plasma levels of circulating ET-1 and these lower concentrations were effectively blocked by PD145065 at the 10^{-5} M concentration in our study.

Veins were more sensitive to the contractile effects of ET-1 resulting in greater maximum contractions, compared with arteries. Previous studies with equine colonic vessels have suggested that modulation of relaxation may be of more importance for arteries (ex. tissue oxygen demand) and modulation of contraction more important for veins (ex. increases in venous return).[28] Although the primary effect of ET-1 in the equine digital vasculature appears to be vasoconstriction through the ET_A receptors located on vascular smooth muscle cells, stimulation of ET_B receptors located on the endothelial cells may lead to vasodilation through the NO pathway as reported in other species.[9] Based on our previous studies of palmar digital vessels and colonic vessels from normal horses, we suggested that this endothelium-dependent vasodilation through the ET_B receptor may be of more importance in arteries than in veins.[20,28] However, during Study I, incubation of vessel rings with L-NAME did not alter the contractile effects of

ET-1 in either arteries or veins. Therefore, we must question the mechanism(s) by which veins constrict to a greater degree than arteries. Arteries and arterioles are considered to regulate the delivery of blood to tissues and respond directly to the tissue's needs. The response and role of veins in the maintenance of tissue homeostasis is not fully understood. It may be that veins play much larger roles than just blood reservoirs and conduits for return of blood to the right side of the heart. It is possible that veins may respond to alterations in capillary pressure and constrict or dilate to maintain adequate capillary pressure in the face of altered arterial flow or pressure. The use of a selective ET receptor antagonist, such as the ET_B receptor antagonist BQ-788, may further help define the roles of veins and their responses to ET-1.

There are several key findings for Study II: (1) precontraction with the EC_{75} of ET-1 resulted in a steady sustained contraction of digital vessels; (2) vessel rings demonstrated a biphasic response to ACh; (3) venous rings dilated to a greater degree at the lower concentrations of ACh than arteries, whereas arteries contracted to a greater degree at the higher concentrations; (4) incubation with L-NAME did not alter the biphasic response of vessel rings to ACh or to the dilatory effects of NG; and (5) venous rings dilated significantly more than arterial rings to NG.

Since control vessel rings precontracted with ET-1 did not increase or decrease contraction once a steady state was reached using the EC75 concentration, precontraction with ET-1 appears to be an acceptable method for studying these vasodilators. The vessel rings with the ACh C-R curve had a biphasic response with dilation at lower concentrations and contraction at higher concentrations. Arterial and venous rings behaved differently with arteries demonstrating less dilation than venous rings, but greater contraction at the end of the curve. Alternatively, venous rings dilated to a greater extent at the lower concentrations of ACh,

demonstrating their responsiveness to endothelial NO release. L-NAME is an L-arginine analog that acts as a NO synthase inhibitor, and it is of interest that the incubation of vessel rings with L-NAME did not significantly alter the biphasic response to ACh. Baxter et al demonstrated a reduction of the dilatory response to ACh after incubation of palmar digital vessels with L-NAME.[12] One hypothesis is that following BWE-induced laminitis the vascular endothelium becomes damaged resulting in a decreased endothelium-dependent response (decreased release of NO from endothelial cells). This could explain the lack of significant arterial dilation and the lack of changes observed by incubation with L-NAME. Arterial and venous rings also had differing responses following the NG C-R curve. As with ACh, arterial rings did not significantly dilate in response to NG, compared with venous rings, which dilated significantly at the higher concentrations of the C-R curve. As expected, L-NAME did not alter the responses to NG since NG is a direct NO donor and does not rely on the actions of NO synthase.

Together, the findings of Study I and Study II demonstrate the differences between the palmar digital arterial and venous vasculature in response to the studied vasoconstrictors and vasodilators. Takai et al measured the effects of ET-1 administration on dogs and determined that the vasoconstrictive effects of ET-1 were stronger in the venous circulation and the dilatory effects of ET-1, through stimulation of the ET_B receptor and subsequent NO release, were present initially followed by vasoconstrictive effects in the arterial circulation. [29] Overall, Takai et al found that the vasoconstrictive effects of ET-1 dominated as measured by increased total peripheral resistance and mean circulatory pressure; this study was one of the earliest to demonstrate the difference between arteries and veins in their response to ET-1.

Overall, vessel segments from BWE-induced laminitic horses were responsive to the vasoconstrictor ET-1 (especially veins) and this response could be attenuated by the addition of the ET receptor antagonist PD145065 (10^{-5} M concentration). Arterial rings were not appreciably responsive to the endothelium-dependent dilatory effects of ACh or to the direct dilatory effects of NG, whereas venous rings had greater dilation in response to increased NO due to (1) the endothelium-dependent release of NO (ACh), and (2) direct increases in NO (NG). In summary, it appears that an imbalance in ET-1 and NO could be involved in the vascular and hemodynamic alterations observed in acute laminitis. Administration of NO donors and/or ET receptor antagonists may be useful in the prevention and treatment of laminitis in horses and warrants further investigation.

9.5 Product Information

[a] Eberbach Corp, Ann Arbor, MI

[b] Model FT03 force-displacement transducer, Grass Medical Instruments, Quincy, MA.

[c] Model 7D polygraph, Grass Medical Instruments, Quincy, MA.

[d] PD145065, American Peptide Co, Sunnyvale, CA.

[e] Lω-nitro-L-arginine methyl ester (L-NAME), Sigma-Aldrich Inc, St Louis, MO.

[f] Endothelin-1, Research Biochemicals International, Natick, MA.

[g] Acetylcholine, Sigma Chemical Co, St Louis, MO.

[h] Hoesch Marion Roussel, Kansas City, MO

[i] SAS version 8, SAS Institute, Cary, NC.

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SUMMARY

A combination of in vitro and in vivo studies in normal horses, horses with naturally-acquired laminitis, and horses after induction of laminitis using the black walnut extract model were conducted to evaluate the potential role of ET-1 in the pathogenesis of acute laminitis in horses. These studies were designed to test the global hypothesis that an imbalance between the endothelium-derived vasoconstrictor ET-1 (increased production) and the endothelium-derived vasodilator nitric oxide (decreased production) is the initiating factor in the development of acute laminitis in horses.

In vitro ET-1 administration caused pronounced and sustained concentration-dependent contraction of equine palmar digital vessel rings from normal horses and these contractile responses were effectively reduced by the 10^{-5} M concentration of the ET antagonist PD142893 and were completely blocked by the 10^{-5} M of the ET antagonist PD145065. Venous rings had greater apparent maximum contractions to ET-1 than arterial rings. In arteries and veins, the relative sensitivity for vasoconstrictors at the concentrations of 10^{-10} – 10^{-6} M was norepinephrine = ET-1 > histamine and no significant differences were observed between responses of medial vs. lateral palmar digital vessel rings. These studies demonstrated that ET-1 is a potent vasoconstrictor of equine palmar digital arteries and veins and the ET receptor antagonist PD145065 was effective in inhibiting these contractile effects in vitro.

Palmar digital vessel rings from horses with naturally acquired laminitis contracted in a concentration-dependent manner to ET-1 and veins had significantly greater apparent maximal contractions and area under the curve values than arteries. Venous rings incubated with PD145065 had significantly lower apparent maximum contractions and area under the curve values than those not incubated with the antagonist in response to ET-1. At the 10^{-6} M

concentration, the relative potency of agents was ET-1 > norepinephrine = histamine for arteries and veins. Comparison of vessel ring responses between horses with naturally acquired laminitis to responses of normal horses resulted in no significant differences. In horses with clinically active laminitis, ET-1 caused pronounced contraction of vessels (especially veins) and the ET antagonist was effective in attenuating vascular contraction.

Jugular venous and cephalic venous plasma ET-1 concentrations followed a trend to increase in horses with naturally-acquired laminitis compared with normal healthy horses. Immunohistochemical ET-1 staining was not different from samples of the palmar digital artery, palmar digital vein, and dermal laminae between normal and naturally-acquired laminitic horses. Epidermal laminae from normal horses were graded as intense more frequently than samples from laminitic horses. Based on the findings of these studies, increased plasma ET-1 concentrations but not laminar ET-1 IHC staining may be associated with naturally acquired laminitis in horses.

Evaluation of the effect of ET-1, PD145065, and nitroglycerine (a nitric oxide donor) on digital hemodynamics in conscious horses demonstrated that local ET-1 infusion resulted in a significant dose-dependent decrease of blood flow and at the highest concentration caused marked digital pain. Mean digital arterial pressure significantly increased after ET-1 administration. There were no significant differences over time for laminar capillary perfusion units, mean digital venous pressure, or mean systemic arterial pressure. There were significant differences in percent change of blood flow with PD145065 administration and there were significant differences between saline- and PD145065-treated horses for % change blood flow. There were significant differences for mean digital arterial pressure, mean digital venous pressure, and mean systemic arterial pressure with PD145065 administration and mean digital

venous pressure was significantly different between saline- and PD145065-treated studies. Nitroglycerine administration resulted in further improvements in digital hemodynamics and further improved the digital blood flow reduction caused by ET-1. Therefore, ET-1 infused locally into the digit of normal standing horses resulted in a concentration-dependent reduction in blood flow, which could be reversed with administration of the ET antagonist. These results support a role for ET-1 as a vasoconstrictor of the equine digit.

Examination of digital hemodynamics, hematologic parameters, and physical examination parameters before and after induction of acute laminitis using black walnut extract (BWE) and evaluation of the usefulness of the endothelin antagonist PD145065 in attenuating hemodynamic alterations were evaluated in 14 horses. Eleven horses developed Obel grade 1 laminitis. An initial decrease in blood flow occurred 1-hour post-BWE and then increased above baseline 8 – 10 hours post-BWE. PD145065 treated horses had higher mean digital venous pressure compared to saline treated horses 5 hours post-BWE. PD145065 treatment did not alter any other parameters. Hematologic and physical examination variables followed similar patterns to previously published studies using this model. Conclusions of this study are that an initial period of reduced blood flow occurs early in the development of laminitis followed by a period of hyperemia that corresponds with the demonstration of clinical signs of laminitis. The use of the endothelin antagonist PD145065 still remains a possibility for treatment of acute laminitis, although further investigations using this agent are warranted. Additionally, results of this study suggest that alterations in digital blood flow are the initial events in the development of laminitis and should remain the target of therapeutic investigations.

Jugular and palmar digital venous plasma endothelin-like immunoreactivity was determined for samples drawn before and hourly after induction of acute laminitis using BWE.

After the development of acute laminitis, palmar digital arterial, palmar digital venous and laminar samples were collected, evaluated for ET-1 immunohistochemical staining, and compared to tissues from normal horses. ET-like immunoreactivity increased in palmar digital venous samples 4, 5, and 6 hours post-BWE administration and remained increased above baseline with the onset of Obel stage 1 laminitis. This finding supports our hypothesis that digital imbalances in ET-1 concentrations are associated with BWE-induced laminitis. Jugular venous plasma ET-like immunoreactivity did not significantly increase with the development of laminitis using the BWE model. Endothelin-1 immunohistochemical staining was not different between saline and PD145065 treated horses or between BWE-induced laminitic horses and normal horses. The significance of these studies suggest that plasma ET- like immunoreactivity may be increased with the onset of acute laminitis and that use of an ET receptor antagonist may be useful for treatment and prevention of laminitis.

The effects of the ET antagonist PD145065 and nitroglycerine (a nitric oxide donor) on Starling forces in horses after BWE-induced acute laminitis were determined. Treatment with PD145065 reduced digital vascular resistance to blood flow as evidenced by a lower digital arterial pressure, and lower total and precapillary resistances than those with saline-treated horses. Nitroglycerine infusion further reduced vascular resistance and caused an increase in digital blood flow. These findings support our hypothesis that decreasing ET receptor availability and increasing nitric oxide concentrations improves digital hemodynamics after BWE administration. Results of this study suggest that both the ET antagonist and nitroglycerine, a nitric oxide donor, may prevent or limit local digital vascular alterations and Starling force alterations in horses with laminitis and may be potentially useful for treatment and prevention of naturally acquired laminitis in horses.

Finally, ET-1 induced sustained in vitro contraction of palmar digital arteries and veins from BWE-induced laminitic horses. Similar to normal horses and those with naturally-acquired laminitis, venous rings contracted 5 times higher to ET-1 than arterial rings and PD145065 effectively inhibited these contractile responses. Endothein-1 contractile effects were not different between vessel rings incubated with or without the nitric oxide synthase inhibitor Lω-nitro-L-arginine methyl ester (L-NAME); therefore, ET-1 activity through the ET_B receptor (nitric oxide release) does not appear to be an appreciable part of the actions of ET-1 in equine palmar digital vessel rings after BWE-induced laminitis. A biphasic response was present for vessel rings after acetylcholine administration. Venous rings had greater dilation to lower concentrations whereas arterial rings had greater contraction to the higher concentrations of acetylcholine. Venous rings dilated over three times greater than arterial rings to nitroglycerine. Arterial and venous vessel rings from horses after BWE-induced laminitis contracted significantly greater to ET-1 than corresponding vessel rings from normal horses. This suggests a lack of an opposing vasodilatory response, most likely a lack of endothelium-derived nitric oxide due to endothelial damage from BWE administration and the development of acute laminitis. Further studies will better define the source of this observed increase in vascular contractility.

Overall, the findings of all of these studies support the global hypothesis that increased ET-1 and decreased nitric oxide are involved in the pathophysiology of acute laminitis in horses. These findings also support continued investigation into the use of an ET antagonist and a nitric oxide donor for the prevention and treatment of this devastating disease.

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