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Investigations Into the Pathogenesis of Acute Equine Laminitis.

Linda Elizabeth Cummings
Louisiana State University and Agricultural & Mechanical College

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Investigations into the pathogenesis of acute equine laminitis

Cummings, Linda Elizabeth, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1992

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INVESTIGATIONS INTO THE PATHOGENESIS
OF ACUTE EQUINE LAMINITIS

A Dissertation

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

in

The Interdepartmental Program in Veterinary Medical Sciences

by Linda E. Cummings
D.V.M., Louisiana State University, 1984
August 1992
Dedicated to my children,
Sarah Elizabeth and
Zachary Mark Olcott
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ABSTRACT

A profound decrease in perfusion of the laminar capillaries has been documented during acute equine laminitis, resulting in ischemia of the laminar tissue. Oxygen radicals are believed to mediate ischemia/reperfusion injury. The effects of 1) three hours of surgical ligation of the major blood supply of the equine digit followed by ligature release, 2) resection of the medial and lateral palmar digital arteries, and 3) digital intraarterial infusions of an oxygen radical generating system, were investigated. Laminitis was not induced by any of these manipulations. Clinical signs and alterations in hemograms observed following these procedures are reported. Preliminary identification of xanthine oxidase and superoxide dismutase activities in laminar tissue is described.

Due to similarities in physiologic alterations and histopathologic lesions, laminitis has been described as the manifestation of a Shwartzman reaction. The effects of palmar digital intraarterial infusions of E. coli endotoxin followed in 24 hours by jugular vein infusion of the same endotoxin were investigated. Clinical signs and alterations in hemograms were consistent with the known effects of endotoxin in the horse. Laminitis did not develop.
Exposure to black walnut (*Juglans nigra*) trees or shavings has been associated with the development of laminitis in the horse. Experimentally, laminitis may be consistently induced by the intragastric administration of an aqueous extract of black walnut heartwood. The laminogenic agent in black walnut has not been reported. In the present study the partial characterization of black walnut heartwood extracts was accomplished by gas chromatographic/mass spectrometric methods. Subsequently, identified components of the extracts were individually administered to horses, as were various crude fractions obtained from pH fractionations and precipitations of black walnut heartwood extracts. The clinical effects and laminogenic potentials of each is reported.
Anatomy of the Equine Digit

The equine digit is composed of the epidermal hoof wall and the structures therein encased (Figure 1). These structures include the corium (dermis) which contains the blood and nerve supply of the digit, 11 ligaments, two tendons, the digital cushion which acts as a shock absorber for the foot, the navicular bone and bursa, and the distal or 3rd phalanx (PIII) as well as part of the 2nd phalanx (1).

The hoof is a modified extension of the epidermis of the skin. Histologically the hoof epidermis lacks a stratum granulosum and a stratum lucidum. It consists, therefore, of a thin inner layer of stratum germinativum and a wide outer layer of stratum corneum. The stratum germinativum forms a continuous layer over the corium and proliferates to cause hoof growth. The stratum germinativum is subdivided into the stratum basale and the stratum spinosum. The stratum basale is a single layer of cells adjacent to the corium. As cells migrate outward from the basal layer and begin to keratinize the stratum spinosum is formed. This outer layer can be one to several cell layers thick. That portion of the spinous layer next to the stratum corneum where increased
Figure 1 - The equine digit, sagittal section.

a - periopile, b - perioplic corium,
c - coronary corium, d - laminar corium,
e - solar corium, f - deep digital flexor tendon, g - common digital extensor tendon, 
h - digital cushion, i - hoof wall, 
j - sole, k - frog.
keratinization is occurring is referred to as the keratogenous zone (1). The formation of keratin in epidermal tissue can occur either by "soft keratinization" as in the skin or by "hard keratinization" as seen in the hoof wall. Soft keratinization is characterized by the formation of basophilic keratohyalin granules in the stratum granulosum of the epidermis. The keratin thus formed has a sulfur content of about 1% of the dry substance which is equally represented by methionine and cystine. Hard keratinization, on the other hand, is distinguished by a progressive increase in acidophilic fibrils in the keratogenous zone with the resultant keratin being of greater strength and sulfur content (up to 5% of the dry substance) than its "soft" counterpart. The sulfur content of hard keratin is primarily composed of cystine (2).

The equine hoof wall is comprised of three layers: the stratum externum (stratum tectorium or perioplic epidermis), the stratum medium (coronary epidermis) and the stratum internum (stratum lamellatum) (Figure 2) (1,3). The periople is a narrow band of epidermis at the junction of the skin and hoof wall. The deepest layers of the perioplic epidermis are carried downward as the wall grows. These layers form the thin, shiny coating of the hoof wall. This protective coating, the stratum externum, restricts moisture loss from the hoof. The middle and largest layer of the hoof, the
Figure 2 - Schematic illustration of a histologic section of the dorsal wall of the equine hoof.
stratum medium, is produced by the stratum germinativum of the coronary groove. New hoof wall produced at the coronary band grows at a rate of approximately 8 to 10 mm per month. It thus requires 9 to 12 months to grow a new hoof wall (1,4). Injuries to the coronary band can result in hoof wall defects which migrate distally as new growth occurs. The innermost layer of the hoof wall, the stratum internum, is composed of a series of epidermal laminae aligned almost perpendicular to the ground but parallel to each other and extending from the coronary groove to the solar surface. The primary epidermal laminae are derived from the stratum germinativum of the coronary groove and become keratinized as they are moved distally by hoof growth. The primary epidermal laminae are considered part of the stratum corneum of the hoof wall. The secondary epidermal laminae which are fingerlike projections off the primary laminae are composed of stratum germinativum. The central core of stratum spinosum attaches to the sides of the primary epidermal laminae and is surrounded by the single cell layer of stratum basale which is adjacent to the underlying laminar dermis (Figure 3). The contribution of this stratum germinativum covering the laminar dermis to the thickness of the keratinized hoof wall has been debated over the years. The sterile bed concept (3,5) which states that the equine laminar epidermis is relatively sterile and does not produce horny tissue in the normal hoof has been challenged. Budras, et al (5), did identify progressive keratinization of
Figure 3 - Schematic illustration of A - Cross section of the hoof wall: 1 - primary epidermal lamina, 1a - secondary epidermal lamina, 2 - primary dermal lamina, 2b - secondary dermal lamina; B - Histologic section of the stratum internum: 1 - secondary epidermal lamina, 1a - stratum spinosum, 1b - stratum basale.
the secondary epidermal laminae which ultimately contributed approximately 20% of the thickness of the hoof wall. It has generally been accepted, however, that the laminar epidermis will produce horn in response to hoof wall injury (1,3).

There are approximately 600 keratinized primary epidermal laminae per hoof, each of which has about 100 nonkeratinized secondary laminae, comprising a surface area of about one square meter (1,6). These epidermal laminae interdigitate with complementary dermal laminae. The dermal component along with the stratum germinativum of the epidermis are termed the "sensitive" laminae. The remaining layers of the epidermis are called the "insensitive" laminae. On its innermost surface the dermis is continuous with the periosteum of the distal phalanx (Figure 1). Thus this complex dermoepidermal junction provides an extensive surface which suspends PIII within the hoof wall. In addition, other forces within the hoof serve to counteract the downward thrust of the horse's weight through the bony column of the leg and foot. The deep digital flexor tendon at its insertion on the semilunar crest of PIII and the common digital extensor tendon which attaches on the extensor process of PIII provide an upward pulling force. Further support for PIII is provided distally by the sole and the frog which forms the caudal, wedge-shaped portion of the ground surface of the hoof. As disruption of the deep digital flexor tendon or the common digital extensor tendon
not usually associated with displacement of the distal phalanx, the integrity of the laminar attachments appears to be the essential component for normal anatomic alignment of the distal phalanx within the hoof wall (1,7).

Arterial blood flow to the equine foot is derived from the medial and lateral palmar or plantar digital arteries. These arteries end by anastomosing to form the terminal arch in the semilunar canal of the distal phalanx (Figure 4). Approximately 8 to 10 primary arteries originate from the terminal arch and penetrate PIII distally to form the circumflex artery. The circumflex artery encircles the entire distal circumference of PIII and from its dorsal portion gives origin to the dorsal laminar arteries which perfuse the dorsal laminae. The dorsal laminar arteries are of note in that their blood flow is against gravity, i.e., in a distal to proximal direction, and that angiography has indicated that they are the last arteries in the foot to fill. These vessels may, therefore, be particularly affected by any event which compromises digital blood flow (1,6). The arterial network of the equine foot has been divided into three anatomic regions of independent blood supply: 1) the dorsal coronary corium, 2) the caudal coronary corium and caudal hoof wall laminae, and 3) the dorsal hoof wall laminae and corium of the sole. Several arteries supply the caudal coronary corium and caudal
Figure 4 - Schematic illustration, dorsopalmar view, of the arteries of the left equine digit. a - medial palmar digital artery, b - lateral palmar digital artery, c - terminal arch, d - primary artery of the terminal arch, e - circumflex artery.
hoof wall laminae, rendering this region of the digit less susceptible to perfusion deficits (6).

The hoof has a complex venous drainage network consisting of plexuses of large veins in the corium of the sole and the coronary band. Plexuses of smaller veins are associated with the laminar corium adjacent to the hoof wall. This extensive venous system by nature of its compressible fluid medium acts as part of a shock-absorbing mechanism in the foot. When the foot strikes the ground blood is compressed and forced upward. Expansion and compression of the collateral cartilages of PIII and the digital cushion enhance venous outflow from the plexuses. All of these plexuses converge to form the medial and lateral digital veins (1,8). Within the hoof all veins are valveless thus permitting retrograde flow (8,9). Venous drainage from the dermal laminae is in a proximal direction, i.e. upward against gravity toward the coronary band (10). This, too, may predispose the laminae to perfusion deficits. The capillaries of the dermal laminae have been reported to contain unusual enlargements and focal diverticulae, the function of which is unclear. One proposed function of these structures is to accommodate the retrograde flow of venous blood not redistributed during weightbearing. Electron microscopic studies suggest that these capillary enlargements may have a smooth muscle component allowing them to regulate perfusion of the laminar capillary bed, much as arteriovenous
shunts do (9). The horse's hoof has abundant arteriovenous anastomoses (AVA) with a reported density of 500/cm². This density is surpassed only by that found in the flippers of marine animals (8). AVAs have been documented in the coronary, sole and frog corium, the digital cushion, and the dermal laminae (11). These AVAs, when open, provide a direct low-resistance channel between the arterial and venous systems. This channel bypasses the capillaries.

Equine digital veins are thick-walled with mural dimensions similar to that of the arteries. This thickness renders these vessels relatively inelastic resulting in a low compliance. Equine digital vascular compliance is approximately one-fifth to one-tenth that in the canine limb. It is proposed that this low compliance may be enhanced by the encasement of the vasculature between the hoof wall and PIII (12). Low compliance aids the veins in returning blood to the general circulation from a dependent area and enables them to withstand high intravascular pressures present in the equine hoof. The horse at rest has a digital venous pressure ranging from 59 to 100 mm Hg. Walking or trotting increases this pressure dramatically with mean peak ranges of 232 to 352 mm Hg and 320 to 643 mm Hg, respectively (13).
Starling Forces in the Equine Digit

The Starling forces (the hydrostatic, oncotic, and permeability factors which regulate the rate and direction of transvascular fluid movement) are unusual in the equine digit as compared to similar structures in other animals. Capillary pressure is much higher in the digit of the horse (36.7 mm Hg) than in the canine forelimb (12.0 mm Hg) and analogous tissues in other species (12,14). The osmotic reflection coefficient, a measure of vascular permeability, is 0.67 in the normal equine digit as compared to 0.95 in connective tissue structures of other animals (15). This relatively permeable vasculature does not produce oncotic pressure gradients sufficient to limit the edemogenic effect of high capillary pressures. Rather, efflux of fluid from the vascular to the extravascular space is opposed by a very high interstitial fluid pressure (>25 mm Hg) in the healthy horse. Interestingly, this value approaches the 30 mm Hg associated with the production of compartment syndromes in other species, i.e. syndromes characterized by ischemic necrosis. The horse may, therefore, be at risk for the development of digital ischemia with only small aberrations in the Starling equilibrium (12).
Equine Laminitis

As mentioned above, several anatomic and physiologic factors would seem to render the equine digit susceptible to perfusion deficits resulting in tissue ischemia. And indeed a common disease of the equine foot, laminitis, is believed to be due to a peripheral vascular insult. The term, laminitis, implies an inflammation of the laminae of the hoof. However, there seems to be no active inflammation during the initial 72 hours of the disease process (16). Laminitis is characterized by lameness which can affect all four feet but which most often is seen in the forelimbs bilaterally. In the latter case, the horse assumes a stance characteristic of the disease, i.e. with forelimbs extended anteriorly and hindlimbs brought under the body to assume most of the weightbearing. Lameness may range from mild to severe. In the most severe cases downward deviation of the third phalanx occurs due to loss of support from the structurally disrupted laminae. PIII, in some cases, will prolapse through the sole of the hoof. Prognosis in these instances is grave. Laminitis may result in permanent loss of function in the performance horse and the more complicated cases may necessitate euthanasia. Approximately 100,000 horses are afflicted annually in the United States (17). Estimates based on insurance claims attribute up to 4.7% of annual equine losses to laminitis (18). There is a 4-fold higher incidence in ponies than in
other breeds. In horses, a lower incidence has been reported in geldings versus mares and stallions (19,20). While laminitis affects horses of all ages, the disease occurs most commonly in mares 4 to 7 years old and in male horses between the ages of 7 and ten years.

Laminitis may be classified as acute, subacute, and chronic. In the acute disease the horse is depressed, anorexic, and stands with obvious discomfort. Increased hoof temperature and bounding digital pulses are hallmarks of the disease. Generalized sensitivity over the sole of the foot is detected with hoof testing. Pyrexia is common as are elevated pulse and respiratory rates which can be manifestations of intense pain and/or other systemic alterations. Lameness severity is graded according to the criteria of Obel (20):

Grade 1 - When standing the horse lifts its feet incessantly. There is no lameness apparent at the walk, however, the gait is short and choppy at the trot.

Grade 2 - The horse walks willingly but with a gait characteristic of laminitis and does not object to the lifting of a forefoot.

Grade 3 - The horse moves very reluctantly and strenuously resists the raising of a forelimb.
Grade 4 - The horse refuses to move without the use of force.

The subacute case of laminitis may exhibit any of the clinical signs of the acute disease but the symptoms are much less severe. A change in stance, some reluctance to walk, and a mild increase in hoof sensitivity to pressure may be noted. Horses experiencing subacute and acute laminitis seem to be predisposed to recurrence of the disease (19). Laminitis is considered chronic when the horse remains symptomatic more than 48 hours or when deviation of PIII occurs. PIII may deviate as early as 3 hours following the initial onset of lameness. Chronic disease is manifested as intermittent or persistent lameness, a dropped or convex sole, and an abnormal pattern of hoof growth, i.e. the hoof wall at the heel having a faster rate of growth than that at the toe.

**Etiologies of Equine Laminitis**

Laminitis may be induced by a number of seemingly unrelated diseases and circumstances. It occurs frequently as a sequelae to grain overload and to infectious diseases, especially those with an associated endotoxemia. Laminitis can also be induced by corticosteroid drug therapy, imbibition of a large volume of cold water, and grazing lush, new pastures. The development of lameness in these cases is
usually preceded or accompanied by systemic derangements. Thus it is widely held that laminitis is a local manifestation of a systemic syndrome (19,21). However, there are cases of laminitis due to excessive concussion (road founder) and to excessive weightbearing by one foot due to contralateral limb breakdown (support leg laminitis). Laminitis in these situations is unaccompanied by metabolic disturbances and is believed to be secondary to physical factors, i.e. local trauma to the laminae.

Histopathologic Changes in Equine Laminitis

In spite of the various inciting factors, the histopathologic changes associated with cases of laminitis are similar. Endothelial cell swelling and mild edema are apparent within four hours of the onset of lameness (16). Rupture of endothelial cells and increased numbers of pseudopodia on the luminal side of the cells have been reported (17). Acute laminar changes are correlated with the severity of clinical signs. In the mild case, pathology is confined to the epidermis and consists of vacuolization, pyknosis and disorganization of the tissue. In the severely afflicted horse, epidermal coagulation necrosis is apparent and is accompanied by widespread dermal congestion and edema of the epidermal-dermal interface. Extensive disruption of the normal laminar architecture may occur. Other pathologic
changes reported include microvascular thrombosis, hemorrhage and epidermal hyperplasia (16,22).

The Carbohydrate Overload Model of Laminitis and Endotoxin

Laminitis has been extensively studied using the carbohydrate (CHO) overload model. In this model laminitis is induced by administering a commercially prepared diet (Theracon, Inc., Topeka, Kansas) of 85% cornstarch and 15% wood flour at a dose of 17.6 g/kg bodyweight. The gruel is administered via a nasogastric tube (23). Within 56 hours Obel grade 3 lameness occurs in approximately 60 to 70% of the horses fed the experimental ration (23,24,25). This method is believed to be a valid model of naturally occurring alimentary laminitis as similar systemic alterations are seen in both. These alterations include increases in body temperature, cardiac output, arterial pressure, heart rate, total leukocyte count, packed cell volume, and total serum protein (23). The mechanism by which this model induces laminitis is unknown. Up to 15% of horses administered the experimental ration die of circulatory collapse. In these cases, the progression of clinical signs resembles that seen in endotoxemia and consequently, gram-negative endotoxin has long been implicated in the pathogenesis of CHO-induced laminitis. Evidence in support of a role for endotoxin includes the documentation by Garner et. al. of a change in the bacterial flora of the cecum.
following CHO overload. An increase in lactic acid-producing microorganisms i.e., lactobacillus and streptococcal spp., occurs coincident with a decrease in cecal pH and the number of gram-negative bacteria (26). The increased acidity of the cecum causes lysis of the gram-negative organisms with a resultant increase in cecal endotoxin levels (27). Compromise of the cecal wall occurs due to the low pH and the presence of cytotoxic endotoxin. Systemic absorption of endotoxin is thus facilitated. Rises in plasma gram-negative endotoxin levels are associated with the occurrence of Obel grade 3 lameness in 85% of affected horses (28).

The Shwartzman Reaction

Due to histopathologic similarities Sprouse et al (29) described equine laminitis as a local Shwartzman reaction. The local Shwartzman reaction is a two-stage phenomenon classically produced by sequential injections of endotoxin. The preparatory or sensitizing intradermal injection is followed in 18-24 hours by an intravenous provoking injection. Granulocytes infiltrate the preparatory site following the intradermal injection. Within 24 hours of provocation dermal hemorrhagic necrosis occurs at this site accompanied by microvascular thrombi (30). Sprouse et al (29,31) successfully induced the local Shwartzman reaction in the skin of ponies and horses, thus establishing the susceptibility of
this species to the phenomenon. As the equine hoof is of epidermal/dermal origin the authors also proposed that laminitis could be a local Shwartzman reaction. Subsequent documentation of biphasic increases in plasma endotoxin levels in 45% of horses exhibiting CHO-induced laminitis substantiated their hypothesis (28). The peaks in circulating endotoxin were separated by 16 hours, approximating the 18-24 hour interval between injections described for induction of the Shwartzman reaction. However, the CHO-induced exposure to endotoxin more closely simulated the generalized Shwartzman reaction in which both preparatory and provoking injections are intravenous and in which subsequent pathology is disseminated throughout the body (30). Occurrence of the generalized reaction has been reported in the horse (32).

Laminitis and the Shwartzman reaction have much in common, giving credence to the possibility that they are one in the same. It is known that the sensitized state required for manifestation of the Shwartzman reaction can be elicited by means other than injection of endotoxin. Pregnancy, renal hypertension, and diabetes are known to substitute for the preparatory injection (33). Large doses of glucocorticoids are a classic means of sensitization, an effect believed to be mediated by an increased sensitivity of alpha-adrenergic receptors to catecholamine stimulation (34). Interestingly, Eyre et. al. (35) have demonstrated corticosteroid-mediated
potentiation of the vasoconstrictor responses of equine digital vasculature to sympathomimetic catecholamines. They have proposed this finding as the mechanism of corticosteroid-induced laminitis. Amelioration of both the Shwartzman reaction and laminitis has been reported following pretreatment with the alpha-adrenergic blocker, phenoxybenzamine (34,36). A large release of catecholamines is believed to be essential for the manifestation of the generalized Shwartzman reaction. Administration of catechol-O-methyl transferase inhibitors can produce the prepared state in male rats (37). It has been suggested that increased sympathetic discharge secondary to digital pain is responsible for the hypertension seen in association with acute laminitis. Nerve blocks of affected digits have resulted in reduction of arterial pressure within 10 minutes of the blockade (38). Adrenal catecholamines are released in response to pain and significant increases in their plasma levels have been documented after the onset of lameness (39). Further evidence of adrenal gland hyperactivity in the acutely laminitic horse include the findings of a stress leukogram and hyperglycemia (16).

Both the Shwartzman phenomenon and CHO-induced laminitis have been proposed to involve a coagulation dysfunction. The generalized Shwartzman reaction in animals is considered to be a model of human disseminated intravascular coagulopathy.
Hemorrhagic necrosis secondary to microvascular thrombi in the small veins and venules is characteristic of the reaction. Activation of the Hageman factor (Factor XII) in the intrinsic clotting pathway ultimately results in the production of fibrin which is believed to be primarily responsible for the thrombi formation (30). Likewise intravascular thrombi have been identified in the dermal laminae of the acutely laminitic horse (22,29). Pretreatment with large doses of heparin reportedly inhibits both the Shwartzman reaction in experimental animals and the development of CHO-induced laminitis in the horse (30,40). Heparin inhibits coagulation by preventing the conversion of prothrombin to thrombin. Thrombin is necessary for the formation of fibrin and the subsequent microthrombi (41). Conversely, a retrospective study of clinical laminitis cases demonstrated no significant difference in the incidence of the disease in untreated versus heparin-treated horses (42). Likewise, fluctuations in coagulation parameters during the development of laminitis have been inconsistent between studies. Alterations in prothrombin time (PT), activated partial thromboplastin time (APTT), and in levels of fibrin degradation products and platelets have been observed (43,44,45). However, a lack of significant changes in the above parameters has also been reported (46,47).
Despite evidence suggesting that laminitis is a Shwartzman phenomenon, definitive proof is lacking. Laminitis is not reported as a manifestation of the generalized Shwartzman reaction in the horse (29,32). Furthermore, sublethal doses of endotoxin have consistently failed to induce laminitis in horses and ponies (16,21,48). Duncan et al. (49) did, however, report mild signs of hoof discomfort in horses receiving 24-hour portal vein infusions of endotoxin. While there seems to be an association between gram-negative endotoxemia and laminitis, a cause-effect relationship remains to be established.

The Carbohydrate Overload Model and Systemic Derangements

CHO overload induces a multitude of systemic derangements with laminitis being a local manifestation of the syndrome. Severe diarrhea accompanies CHO overload and results in marked compartmental fluid shifts. Fluid loss due to diarrhea and sequestration in the large intestine is not readily compensated for because of anorexia and adipsia (23,45,50). Hypokalemia resulting from potassium loss in the feces is commonly found (38,50). Resultant stimulation of the renin-aldosterone-angiotensin system is reflected in elevated plasma aldosterone levels and plasma renin activity (39,50). Other systemic alterations variably reported in conjunction with alimentary laminitis include metabolic acidosis (38),
hyponatremia (50), hypochloremia (50), hypophosphatemia (50), elevated plasma cortisol and testosterone levels (51), and decreased plasma thyroid hormone levels (21).

**Vascular and Physiologic Alterations in Equine Laminitis**

Major research efforts have been directed at determining the local digital vascular perturbations that occur during the development of CHO-induced laminitis. Nuclear medical and angiographic techniques have demonstrated a profound decrease in perfusion of the laminar capillaries (25,52). In the angiographic study this reduction is attributable to a marked decrease in the diameter of the terminal arteries with occlusion of the terminal arch and circumflex arteries. Both studies show evidence of increased blood flow just proximal to the digit in agreement with Robinson et. al., who made quantitative blood flow measurements in the midmetacarpal region (53). This seeming paradox of increased total blood flow to the distal limb accompanied by decreased perfusion of the laminae is explained by evidence suggesting that a significant amount of arteriovenous shunting was present (25).

Evaluation of hemodynamic changes and Starling forces during the prodromal stages of laminitis have indicated an increased digital capillary pressure (mean=55.13 mm Hg) caused by elevated postcapillary vascular resistance (54). The total
vascular resistance to blood flow is increased 3.5 times over normal. However, there is not a proportional decrease in digital blood flow. Perfusion is reduced to approximately 50% of normal, indicating a greater volume of blood is reaching the digital vasculature than would be expected based on the resistance to flow. The concurrent opening of arteriovenous anastomoses would allow maintenance of higher than predicted digital blood flows and may explain the discrepancy seen. High capillary pressure causes a hydrostatic-mediated movement of fluid into the interstitium with a resultant increase (mean=44.5 mm Hg) in interstitial fluid pressure over normal values. This elevation in interstitial pressure is sufficient to exceed the critical closing pressure of the capillary beds and thus cause laminar ischemia. This sequence of events is analogous to the development of compartmental syndromes described in other species. It has been proposed that this high interstitial pressure may be the impetus for arteriovenous shunting (16).

Whether caused by arterial or venous constriction, there seems little doubt that a perfusion deficit in the digital laminae occurs during the development of equine laminitis. Due to its ischemic nature, it is possible that reperfusion (post-ischemic) injury plays a role in the pathogenesis of this disease. Clinical observations support this possibility. Laminitis is often a sequelae to severe systemic illness,
e.g., acute diarrhea and endotoxemia. Dehydration accompanies many of these systemic diseases and results in poor peripheral perfusion characterized by prolonged capillary refill time and cooling of the extremities. It is often after fluid replacement therapy and reestablishment of peripheral perfusion that the bounding digital pulses and increased hoof temperature characteristic of laminitis onset are noted. Twenty-four hours following CHO overload, the limbs of experimental horses are noted to be "cold" and remain so until the development of lameness. At lameness onset hoof temperature is elevated and thermography reveals increased thermal patterns (23,55).

Ischemia/Reperfusion Injury

Oxygen-derived free radicals (molecules derived from molecular oxygen which have unpaired electrons, such as the superoxide and hydroxyl radicals) have been shown to mediate reperfusion injury in the skin, intestines, kidney, and other tissues of numerous species (56,57,58). Sources of these oxygen radicals in vivo are numerous. During ischemia the cytosolic enzyme xanthine dehydrogenase (XD) is converted via activation of a calcium-dependent protease to xanthine oxidase (XO) which uses oxygen, rather than NAD, as an oxidant. Concomitantly, ATP is broken down with a resultant accumulation of the substrate hypoxanthine (HX) in the tissue.
Upon reperfusion of the tissue, xanthine oxidase produces the superoxide radical during its reaction with hypoxanthine. The presence of xanthine oxidase in equine laminar tissue remains to be established, although enzyme activity has been measured in equine serum (59). Neutrophils are known to accumulate in ischemic tissue and accumulation increases rapidly upon reperfusion, probably due to activation by superoxide of a latent extracellular neutrophil chemotactic factor. These cells are an additional potent source of superoxide due to their membrane-associated NADPH oxidase activity (56). In addition, neutrophils secrete the lysosomal enzyme myeloperoxidase (MPO) which enhances the toxicity of hydrogen peroxide, a free radical precursor (60). Generation of oxygen radicals also occurs during the metabolism of arachidonate, a cell membrane fatty acid. Arachidonic acid breakdown involves the production of free radical intermediates which may contribute to tissue injury (61). A role for products of arachidonic acid metabolism in laminitis is speculative at this time. However, phenylbutazone and other cyclooxygenase inhibitors which prevent the formation of prostaglandins from arachidonic acid ameliorate the clinical signs of laminitis and are a first line treatment for the disease (21,62).

Univalent reduction of molecular oxygen to superoxide radical may also occur nonenzymatically. Numerous soluble cell components are capable of undergoing electron transfer
reactions (e.g., hydroquinone + oxygen → semiquinone + superoxide radical + hydrogen) or auto-oxidation reactions (an uncatalyzed reaction needing only the parent compound) (63,64). The auto-oxidation of catecholamines has been proposed to mediate ischemia-reperfusion injury of the myocardium. This proposal is based on the indirect evidence that nonphysiologically high blood levels of catecholamines are associated with functional and ultrastructural damage to the heart. Also, the beta-antagonists which block the binding of catecholamines to the receptor provide varying degrees of protection against ischemia-reperfusion injury. The occurrence of catecholamine auto-oxidation has been disputed based on data suggesting the involvement of various catalysts in the reaction. It remains to be determined if oxidation of catecholamines by any mechanism is associated with ischemia-reperfusion injury (65).

Antioxidant enzymes provide the primary defense against oxygen radicals thus formed (66). Superoxide dismutase (SOD) catalyzes the dismutation of superoxide radicals to form hydrogen peroxide. Removal of hydrogen peroxide is accomplished by catalase and glutathione peroxidase. There are no specific enzymatic activities for scavenging hydroxyl radical, a very reactive species formed by the interaction of superoxide and hydrogen peroxide via a metal-catalyzed Haber-Weiss reaction. During oxidant stress these enzymatic
defenses may be overwhelmed or the enzymes may be altered or functionally impaired (67). Reduction in the levels of the antioxidant enzymes has been suggested to be responsible for tissue damage seen during liver ischemia (68) and during reperfusion of the ischemic rat heart (69). The detection and quantitation of antioxidant enzymes has not been reported in equine laminar tissue. However, superoxide dismutase and dimethylsulfoxide, a purported hydroxyl radical scavenger, have been used with some success in the treatment of various equine lamenesses.

Virtually all cellular components can be targets of oxygen radical-mediated damage. Particularly reactive targets are the unsaturated bonds of lipid membranes and sulfur-containing molecules. Lipid peroxidation of membranes can alter crucial membrane properties such as deformability, ion transport, and enzyme activity. Proteins containing the sulfur amino acids, methionine and cysteine, can undergo free radical-mediated modifications with resultant loss of activity (70). Larsson et. al., reported aberrant metabolism of sulfur-containing amino acids in the keratogenous zone of the equine hoof during acute laminitis (71). Methionine supplementation has long been recommended in the treatment of laminitis to provide a disulfide bond substrate for maintenance of the laminar-pedal bone interface (72).
Black Walnut-Induced Laminitis

As in the CHO-overload model evidence of a perfusion deficit has been reported for black walnut-induced laminitis. The first known cases of equine laminitis associated with horses being bedded on black walnut (Juglans nigra) shavings were reported in the late 1970s by True and Lowe (73). They documented a 37-100% incidence of acute laminitis on six farms. The laminitis developed within 24 hours of exposure. Subsequent case reports indicated that shavings consisting of as little as 20% black walnut by weight could cause toxicosis and that the development of laminitis generally occurred within 12 hours of exposure (74,75,76). Other clinical signs reported in association with these cases of black walnut toxicosis were mild colic, depression/sedation, anorexia, pyrexia, tachycardia, tachypnea, increased hoof temperature, increased strength of digital pulses, and pitting edema of the distal limbs and ventral midline. The occurrence and severity of these clinical signs varied among horses and were not necessarily accompanied by lameness. Removal of horses from the shavings and treatment with phenylbutazone resulted in cessation of clinical signs and resolution of lameness within 48 hours to one week. Aeration of the shavings for variable periods (24 hours to 3 months) reportedly resulted in loss of toxicity with subsequent use as bedding possible. This suggested a volatile or unstable toxic principle.
Preliminary investigations of the toxic principle of black walnut focused on the substance juglone (5-hydroxy-1,4-naphthoquinone) which is found in members of the walnut family (Figure 5). Juglone was known to be toxic when injected into certain plants. Consequently, it was believed to be responsible for the naturally occurring allelopathic effect of the black walnut tree on plant species growing within the boundaries of its root system (77,78). In addition, juglone was reported to cause sedation in goldfish, rats, and rabbits (79) and to be lethal to a number of animals (80). However, controlled studies using synthetic juglone failed to consistently produce laminitis in the horse. Topical administration to the distal forelimbs of horses resulted in local skin irritation only. Large oral doses of juglone resulted in increased hoof temperatures and digital pulses in 25% of ponies exposed, but no lameness occurred. Prior oral or intravenous exposure to juglone followed by a second intravenous dose elicited an apparent anaphylactic response characterized by acute pulmonary edema in 2 out of 2 ponies. No lameness was seen, however (81). Allergic syndromes in humans have been suggested to be caused by exposure to black walnut and to juglone (82).
5-hydroxy-1,4-naphthoquinone

Figure 5 - Chemical structure of juglone.
In 1987 Minnick et al. (80) induced laminitis in 80% of experimental horses by intragastric administration of aqueous extract of black walnut heartwood. Laminitis was seen within 12 hours of administration. No juglone was detected in the extract by gas chromatographic/mass spectrometric analysis. This finding essentially ruled out juglone as the laminogenic agent in Juglans nigra. In addition, Minnick et al. had developed an experimental model of laminitis which had an induction rate similar to CHO-overload with the advantage of few side effects. Only laminitis, dependent edema, and tranquilization were seen following extract administration. A subsequent study reported a 100% induction of laminitis within 8 to 12 hours of administration with depression and anorexia being the only other clinical signs. Hematological and chemical changes reported in this study included a mild decrease in the erythron (hematocrit, red blood cell count, and hemoglobin) at postdosing hour (PDH) 12. A transient neutropenia was detected at PDH 4 followed by a neutrophilia by PDH 8. Additionally, prolongation of the diurnal cortisol peak was seen in severely affected horses. Histopathological lesions paralleled the severity of clinical signs and were similar to lesions seen in laminitis due to other causes (83).

Gamma-scintigraphic analysis of perfusion of the equine digit during black walnut-induced laminitis has demonstrated
decreased blood flow to the forefoot as compared to the entire distal forelimb (84). Additionally, the deficit in perfusion to the dorsal laminae is greater than the deficit seen elsewhere in the forefoot. At PDH 84 perfusion to the dorsal laminae is increased relative to the distal forelimb, suggesting that reperfusion has occurred. These findings are consistent with changes in perfusion seen with CHO-overload laminitis. Interestingly, black walnut aqueous heartwood extract has been shown to reversibly enhance vasoconstriction induced in isolated equine digital arteries and veins by epinephrine and by epinephrine potentiated with hydrocortisone (85). The extract does not have a direct contractile effect, however. Therefore, similar to the mechanism proposed for the corticosteroids, the extract may induce laminitis by potentiating the action of vasoactive amines.

The toxic principle and the mechanism of toxicity of black walnut-induced laminitis have yet to be determined. Analyses of components of the heartwood extract and the physiologic effects of these components are not reported in the literature. Elucidation of the toxic principle and its mechanism of action may provide a crucial key to the understanding not only of black walnut-induced laminitis but of laminitis in general.
Chapter 2

THE ROLE OF OXYGEN RADICALS IN THE PATHOGENESIS OF ACUTE EQUINE LAMINITIS: CLINICAL TRIALS

Introduction

Major research efforts have been directed at determining the local digital vascular perturbations that occur during the development of equine laminitis. Studies using nuclear medical and angiographic techniques have demonstrated a profound decrease in perfusion of the laminar capillaries during acute CHO-induced laminitis (25,52). In the angiographic study this reduction is attributable to near occlusion of the terminal arch and circumflex arteries. Gamma scintigraphic analysis of perfusion of the equine digit during black walnut-induced laminitis has demonstrated decreased blood flow to the forefoot with the deficit in perfusion being greatest at the dorsal laminae (84). Evaluation of hemodynamic changes and Starling forces during the prodromal stages of CHO-induced laminitis have indicated an increased digital capillary pressure caused by elevated postcapillary vascular resistance (54). High capillary pressure causes a hydrostatic-mediated movement of fluid into the interstitium with a resultant increase in interstitial fluid pressures over
normal values. This elevation in interstitial pressure is sufficient to exceed the critical closing pressure of the capillary beds and thus cause laminar ischemia.

Alterations in perfusion of the dorsal laminae may be due to microthrombosis. Intravascular thrombi have been identified in the dermal laminae of the acutely laminitic horse (22,29). Due to histopathologic similarities, i.e., microvascular thrombi with secondary hemorrhagic necrosis, Sprouse et. al. (29) described equine laminitis as a local Shwartzman reaction. The local Shwartzman reaction is a two-stage phenomenon classically produced by sequential injections of endotoxin. The preparatory or sensitizing intradermal injection is followed in 18-24 hours by an intravenous provoking injection. Within 24 hours of provocation dermal hemorrhagic necrosis occurs at this site accompanied by microvascular thrombi (30).

Whether caused by arterial or venous constriction or microthrombi, there seems little doubt that a perfusion deficit in the digital laminae occurs during the development of equine laminitis. Due to its ischemic nature, it is possible that reperfusion (post-ischemic) injury plays a role in the pathogenesis of this disease. Clinical observations support this possibility. Laminitis is often a sequelae to severe systemic illnesses, e.g., acute diarrhea and
endotoxemia. Dehydration accompanies many of these systemic diseases and results in poor peripheral perfusion characterized by prolonged capillary refill time and cooling of the extremities. It is often after fluid replacement therapy and reestablishment of peripheral perfusion that the bounding digital pulses and increased hoof temperature characteristic of laminitis onset are noted. Twenty-four hours following CHO overload, the limbs of experimental horses are noted to be "cold" and remain so until the development of lameness. At lameness onset hoof temperature is elevated and thermography reveals increased thermal patterns (23,55).

Oxygen-derived free radicals (molecules derived from molecular oxygen which have unpaired electrons, such as the superoxide and hydroxyl radicals) have been shown to mediate reperfusion injury in the skin, intestine, kidney, and other tissues of numerous species (56,57,58). The major source of these oxygen radicals in postischemic tissue is the cytosolic enzyme, xanthine oxidase (XO), which produces both the superoxide radical and hydrogen peroxide via reduction of molecular oxygen (56,59). The cellular membrane- associated NADPH oxidase of neutrophils can also generate oxygen radicals (56). Adherence of neutrophils to endothelium has been reported during ischemia with subsequent streaming of the cells to the infarcted site upon reperfusion. Univalent reduction of molecular oxygen to superoxide radical may also
occur nonenzymatically. Numerous cellular components are capable of undergoing electron transfer reactions or auto-oxidation reactions (an uncatalyzed reaction needing only the parent compound) \((63,64)\). In addition, arachidonic acid metabolism involves the production of free radical intermediates which may contribute to tissue injury \((61)\).

Virtually all cellular components can be targets of oxygen radical-mediated damage. Particularly reactive targets are the unsaturated bonds of lipid membranes and sulfur-containing molecules. Lipid peroxidation of membranes can alter crucial membrane properties such as deformability, ion transport, and enzyme activity. Proteins containing the sulfur amino acids, methionine and cysteine, can undergo free radical-mediated modifications with resultant loss of activity \((70)\). Larsson et. al., reported aberrant metabolism of sulfur-containing amino acids in the keratogenous zone of the equine hoof during acute laminitis \((71)\). Methionine supplementation has long been recommended in the treatment of laminitis to provide a disulfide bond substrate for maintenance of the laminar-pedal bone interface \((72)\). Some protection against oxidative injury in tissue is afforded by the presence of the antioxidant enzymes, superoxide dismutase, catalase, and glutathione peroxidase. Levels of these enzymes in equine laminar tissue have not been reported.
Detection of oxygen radicals in vivo is difficult due to their high reactivity, short half-lives, and low tissue concentrations. Implication of oxyradicals in a disease process is usually based on indirect methods. The use of mechanisms and/or chemicals known to produce free radicals can be employed in an attempt to reproduce the lesions of the disease in question. Other indirect methods include the detection of products of oxygen radical-mediated reactions e.g., lipid peroxidation, or the demonstration of protection from injury with the use of agents that remove radicals.

The purpose of the study reported here was to attempt the induction of equine laminitis by 1) ischemia/reperfusion modeling, 2) palmar digital arterial infusion of the oxygen radical generating system, hypoxanthine/xanthine oxidase (HX/XO), and 3) Shwartzman reaction modeling. Preliminary investigations to determine the presence of XO and SOD in equine laminar tissue were also performed.

Materials and Methods

Animals - Six horses and two ponies (geldings and mares aged 2 1/2 to 12 years) were acclimated for at least one week prior to use. A physical examination and complete blood count and chemical profile were done on all animals to ensure the absence of significant medical problems. A lameness
examination was likewise performed to rule out the presence of hoof-related conditions. All animals were free of clinical signs of previous laminitis. Animals were housed in individual box stalls bedded with wood shavings. Hay and water were available on a free choice basis. A pelleted ration was fed twice daily.

Surgical procedures - Feed was withheld for 24 hours prior to surgery. All surgical procedures were conducted with animals under general anesthesia. Prior to anesthetic induction animals were tranquilized with 0.04 mg/kg acetylpromazine\(^1\) intravenously. General anesthesia was induced with 5\% guaifenesin\(^2\) and 4.5 mg/kg thiamyl sodium\(^3\) administered intravenously to effect. Following intubation, general anesthesia was maintained with halothane.\(^4\) Horses were monitored for 2 weeks until suture removal, except in those instances where animals were euthanized.

Horses were divided into three experimental groups. Group 1 horses underwent ischemia/reperfusion modeling, i.e., surgical isolation and ligation or resection of the digital blood supply. Group 2 horses received digital infusions of an

\(^1\)PromAce®, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa

\(^2\)Ruger Chemical Co., Irvington, New Jersey

\(^3\)Surital®, Parke-Davis Co., Morris Plains, New Jersey

\(^4\)Fluothane®, Pitman-Moore, Mundelein, Illinois
oxygen radical generating system. Group 3 horses underwent Shwartzman reaction modeling, i.e., two sequential infusions of endotoxin.

**Group 1: Ischemia/reperfusion modeling** - Horse 1 - The horse was placed in right lateral recumbency. Medial and lateral palmar digital arteries of the right and left forelimbs were isolated in the midpastern region through 5 cm. incisions in the skin and fascia. One cm. pieces of silastic tubing split lengthwise on one side were placed around the medial and lateral palmar digital arteries of both forelimbs. Braided dacron ligatures (#5) were hand-tied over the silastic tubing on the arteries of the left forelimb. To ensure that adequate occlusion had been attained, arteries were checked distal to the ligatures for blanching and the absence of pulses. No ligation was performed on the right control forelimb. Ligatures were removed after 3 hours. Palmar digital venous blood collection was performed on both forelimbs at 0 and 180 minutes following ligation and at 5 and 60 minutes following reperfusion or removal of the ligatures. Skin incisions were closed in a single layer using 2-0 nylon in a continuous pattern. Support bandages were applied.

Pony 2 - The procedure was identical to Horse 1 with the following exceptions. The medial and lateral palmar digital arteries were isolated at the level of the sesamoids.
Indwelling catheters (20 g) were placed in the right medial and left lateral palmar digital veins for purposes of blood collection. Catheters were flushed with heparinized saline following each sample collection.

Horse 3 - No silastic tubing was used beneath the ligatures. Digital venous blood samples were collected at 0 and 180 minutes following ligation and at 5 and 30 minutes after removal of the ligatures. The remainder of the procedure was as for Pony 2.

Horse 4 - A 12 cm. skin incision was made caudal to the radius approximately midway between the carpus and the elbow on the medial aspect of the right forelimb. The right median artery was isolated using blunt dissection. The right medial and left lateral palmar digital veins were isolated at the level of the sesamoids and catheters (18 g) were inserted. The median artery was then ligated with a ligature of #2 vicryl. One hour later an additional vicryl ligature and an umbilical tape ligature were placed on the artery. Ligation was maintained for 3 hours after placement of the initial ligature. Simple interrupted sutures of 2-0 vicryl were used to close the fascial and subcutaneous layers of the forearm incision. The skin was closed with 2-0 nylon in a simple interrupted pattern. Distal skin incisions were closed with
2-0 nylon in a simple continuous pattern. Digital venous blood samples were collected as for Horse 3.

Horse 5 - The left medial and lateral palmar digital arteries were isolated at the level of the sesamoids as previously described. A 1-1 1/2 cm. segment of each artery was resected between previously placed proximal and distal ligatures of #1 braided dacron. Proximal arterial stumps were assessed for leakage prior to closure of the incision. A catheter was placed in the lateral palmar digital vein for blood collection and was left indwelling at the completion of the procedure for postoperative sampling. Digital venous blood samples were collected at 0 minutes and at 5 minutes following completion of the resection. Postoperative samples were collected at 24 and 48 hours after resection. The skin incision was closed with interrupted sutures of 2-0 nylon. On the third postoperative day Horse 5 was euthanized using T-61® euthanasia solution.'

Group 2: Digital infusion of an oxygen radical generating system - Horse 1 - Following a two week recovery from the previously described arterial ligation, Horse 1 was given an intraarterial (palmar digital artery) infusion of the oxygen generating system, xanthine oxidase/hypoxanthine (86,87). The medial and lateral palmar digital arteries of

'Hoechst-Roussell, Somerville, New Jersey
the left forelimb were isolated at the level of the sesamoids. A nonocclusive indwelling catheter (18 g) was placed in each artery. Hypoxanthine\textsuperscript{6}, 28\% in 0.9\% saline, and xanthine oxidase\textsuperscript{7}, 0.05 U/ml 0.9\% saline, were infused from separate reservoirs via Y-type solution administration sets. The bags of solution were placed in pressurized cuffs to overcome arterial pressure. The hypoxanthine and xanthine oxidase solutions were allowed to mix in the drip chambers of the infusion sets before entering the arterial catheters. Infusion rate was maintained at 1.11 ml/min. using Dial-a-Flo Regulators\textsuperscript{8}. Infusion duration was 90 minutes. Venous blood samples were collected at 0, 30, 60 and 90 minutes following infusion onset via indwelling catheters in the left lateral and right medial palmar digital veins. Following the procedure all catheters were removed. On the second postoperative day Horse 1 was euthanized with T-61 euthanasia solution.

Horse 6 - Infusion of hypoxanthine/xanthine oxidase with venous occlusion. Infusion setups were modified in that the drip chambers were removed. The hypoxanthine and xanthine oxidase solutions were thus not allowed to mix except in the 11.5 cm. of tubing immediately proximal to the arterial

\textsuperscript{6}Sigma Chemical Co., St. Louis, Missouri
\textsuperscript{7}Sigma Chemical Co., St. Louis, Missouri
\textsuperscript{8}Abbott Laboratories, North Chicago, Illinois
catheters. Hypoxanthine and xanthine oxidase, 0.084 U/ml 0.9% saline, were infused into the right forefoot at a rate of 2.5 ml/min. for 2 hours. Venous catheters were placed in the right medial and lateral and left medial palmar digital veins. The veins of the right foot were occluded with catgut ligatures placed around the venous catheters. Ligatures were left in place for 3 hours. Blood samples were collected at 0, 60 and 180 minutes post-occlusion and at 5 and 60 minutes following removal of the ligatures. All catheters were removed at the end of the procedure.

Group 3 - Shwartzman reaction modeling - Pony 7 - Under general anesthesia catheters were placed in the left medial and lateral palmar arteries and in the left lateral and right medial digital veins at the level of the sesamoids. Escherichia coli 055:B5 endotoxin⁹, 10 ug in 10 ml. of 0.9% saline, was injected via catheters simultaneously into each left palmar digital artery. Venous occlusion via tenting of the veins was performed during the injection and for 5 minutes following in an attempt to temporarily localize the endotoxin. Venous blood samples were taken at 0, 5, 15 and 45 minutes following endotoxin administration. All catheters were then removed and the pony was allowed to recover from anesthesia. Twenty-four hours following this procedure, E. coli endotoxin, 10 ug/kg bodyweight in 100 ml 0.9% saline, was administered

⁹Sigma Chemical Co., St. Louis, Missouri
intravenously over a 15 minute period via a catheter placed in the right jugular vein.

Horse 8 - Catheterizations were as described for Pony 7. *Escherichia coli* 055:B5 endotoxin, 5 ug in 10 ml. 0.9% saline was injected into the palmar digital arteries of the right forefoot. Occlusion of the right medial and lateral palmar digital veins was accomplished for one hour with catgut ligatures tied around the venous catheters. Venous blood samples were taken at 0, 5, 15, and 45 minutes following endotoxin administration. Twenty-four hours following the preparatory injection, 1 ug/kg bodyweight of *E. coli* endotoxin in 150 ml. 0.9% saline was administered by slow drip via a jugular vein catheter.

**Hematology** - Neutrophil counts and complete blood counts were obtained on whole blood collected in EDTA using the Serano-Baker, Model 9000, Automated Cell Counter.

**Histopathology** - The hoof was disarticulated at the fetlock and then sectioned with a band saw. A single vertical cut was made through the dorsal midline of the hoof followed by repeated full-thickness horizontal cuts. Tissue was fixed in 10% formalin and then processed by standard paraffin techniques and hematoxylin and eosin stains were used.
Laminar tissue supernatant preparation - Tissue was harvested as for histopathology from euthanized clinical cases with and without laminitis. The epidermal and dermal laminae were then dissected free of the hoof wall and underlying tissue and immediately frozen in liquid N\_2. Each sample was weighed and then homogenized using a Tekmar Tissuemizer (speed setting = 50) in 50 mM cold potassium phosphate buffer (4:1 volume/weight) containing 1 mM EDTA, pH 7.8. The homogenates were centrifuged at 14,000 g for 15 minutes. The resultant supernatant volumes were measured and then were centrifuged at 105,000 g for 30 minutes. Final supernatant volumes were measured. The supernatants were stored at -70° C until assays were performed.

Xanthine dehydrogenase/xanthine oxidase (XD/XO) assay - XD/XO in laminar tissue was measured by the fluorometric method of Beckman et al. (92) using a Perkin-Elmer LS-5 fluorometer set to 345 nm excitation and 390 nm emission with 5 nm bandwidth slits. The XO catalyzed conversion of pterin (2-amino-4-hydroxypteridine) to isoxanthopterin is the basis of this assay. In the presence of pterin only XO activity is assayed. Combined XD and XO activity is determined with the addition of methylene blue (MB) which acts as an electron acceptor for XD in lieu of NAD+. NAD+ is not used because of its overlapping fluorescence with that of isoxanthopterin. Briefly, 100 ul of laminar tissue supernatant were added to
2.8 ml of 37°C 50 mM potassium phosphate buffer containing 0.1 mM EDTA, pH 7.8, and a baseline fluorescence was obtained. The rate of pterin oxidation was determined following the addition of 30 ul of 1 mM pterin. Once a linear rate was established 30 ul of 1 mM MB were added and the combined activities of XD/XO were measured. The reaction was then stopped by the addition of 30 ul of 1 mM allopurinol which inhibits both XD and XO. Following inhibition of the reaction 30 ul of 10 uM isoxanthopterin were added to the cuvette as an internal standard and to correct for fluorescence quenching. Preliminary Michaelis-Menten kinetics of XO, i.e., $K_m$ and $V_{max}$, were determined using the above procedure but varying the pterin concentration in the reaction mixture as follows: 1.25 uM, 1.6 uM, 2.5 uM, 3.3 uM, 5 uM, 10 uM, and 20 uM. Values for $K_m$ and $V_{max}$ were obtained from a Lineweaver-Burke plot.

**Superoxide dismutase activity** - Equine laminar SOD activity was measured by inhibition of the rate of cytochrome c reduction by superoxide, with one unit of SOD defined as that amount which produces a 50% inhibition (93). The reaction of pure bovine milk xanthine oxidase and xanthine supplied a constant source of the superoxide radical. The 3.0 ml reaction volume contained 50 mM potassium phosphate buffer with 0.1 mM EDTA, pH 7.8, 50 uM xanthine, and 10 uM horse heart cytochrome c. Sufficient xanthine oxidase (~6 nM) was
added to give a $\Delta A_{340}/\text{min.} = 0.025$ ($^{1.2}$ uM superoxide/min.).

Laminar tissue supernatant was then added until 50% inhibition was obtained.

Results

**Group 1 - Horse 1 -** Difficulty obtaining venous blood was encountered with repeated sticks of the digital veins. Cooling of the experimental forefoot was apparent during the period of ischemia. Following recovery the temperature of the forefoot was similar to touch as that of the control limb. No lameness developed. Following 180 minutes of ligation the experimental hoof exhibited a 13.3% decrease in the neutrophil count (Table 1). At 5 minutes post-reperfusion (PR) the neutrophil number was further depressed by 24.4% of baseline. By 60 minutes PR the cell count had rebounded to 91.1% of the original value. Conversely, in the control limb only a 9.1% decrease in neutrophil number was seen at 5 minutes PR. However, a further decline of 22.7% of baseline value occurred at 60 minutes PR.

**Pony 2 -** Placement of catheters in the digital veins greatly facilitated blood collection. Venous blood samples were easily obtained throughout the surgical period. Although a faint pulse was occasionally palpable distal to the arterial
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C = Control limb, E = Experimental limb
PR = post-reperfusion; PRs = post-resection
* tube clotted, no count obtained
ligature on the medial side, the experimental hoof was palpably cooler than the control limb. Contact thermograms of the experimental and control forefeet during ischemia revealed profound cooling of the ligated hoof. Thermograms taken 40 minutes following ligature removal demonstrated warming of the experimental hoof suggestive of increased blood flow. However, the experimental hoof remained cooler than the control hoof for 3 days post-operatively. No lameness developed. A 66.6% increase in neutrophils occurred in the experimental hoof after 180 minutes of ligation. Subsequently, cell counts began declining to a low at 60 minutes PR of 41.0% above baseline. In the control limb an increase in neutrophil numbers was also seen following 180 minutes of ligation. However, a peak count of 18.7% over baseline occurred at 5 minutes PR with a subsequent decline to 12.5% above the initial count seen at 60 minutes PR.

Horse 3 - Cooling of the experimental hoof occurred during the period of ligation. Following removal of the ligatures the experimental limb warmed although it remained somewhat cooler than the control limb. This discrepancy in forefeet temperature remained for several days postoperatively. No lameness developed. No baseline neutrophil count was obtained for the experimental limb due to clotting of the blood sample. However, neutrophil counts for
latter time points paralleled those of the control limb closely.

Horse 4 - At one hour following ligation of the median artery no cooling of the experimental hoof was noted. Placement of two additional ligatures did result in subsequent cooling of the hoof but to a lesser degree than had been noted in previous procedures. Lameness did not occur. Neutrophil count in the experimental hoof dipped to a low of 17.4% of baseline at 180 minutes of ligation. The cell count approximated the initial value at 5 minutes PR and rose to 28.1% above baseline by 30 minutes PR. The control hoof exhibited a decrease in neutrophils at 180 minutes of ligation. However, the nidus of 25.9% of baseline was reached at 5 minutes PR and remained essentially unchanged at 30 minutes PR.

Horse 5 - Post-operative recovery was complicated by slippage of the proximal medial arterial ligature resulting in significant hemorrhage. Hemorrhage was successfully controlled with pressure bandaging. On the first postoperative day a grade 2 lameness was noted in the experimental limb. However, the horse was unresponsive to hoof testing and did not have an unusual amount of incisional pain. The experimental hoof was palpably cooler than the control hoof. On the second postoperative day grade 2
lameness was still apparent. Hoof testing elicited no response and incisional pain was not remarkable. The temperature of the experimental hoof approximated the control hoof. By the third postoperative day, lameness had decreased to a grade 1 and experimental and control limbs remained similar in temperature. A 13.0% decrease in neutrophil number was seen at 5 minutes post-resection (PRs) in the experimental hoof. Twenty-four hours following resection an 89.1% increase over baseline neutrophil count occurred. This was followed at 48 hours PRs by a decline to 32.6% above the initial count. Although a baseline control limb neutrophil count was not obtained, the values obtained at subsequent time points were similar to the experimental samples. Histopathology of laminar tissue from the experimental hoof showed no signs of laminitis or ischemic necrosis.

Group 2 - Horse 1 - Postoperatively no lameness developed and no discrepancy in the temperature of the experimental versus the control limb was noted. Neutrophils in the experimental limb virtually doubled following 30 minutes of infusion and then began falling (Table 2). After 90 minutes of infusion the neutrophil count was 73.3% over
<table>
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<th>90</th>
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</tr>
<tr>
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<tr>
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</tr>
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<td>3.6</td>
<td>2.9</td>
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VLR = Following venous ligature removal
baseline. The neutrophil numbers in the control limb remained essentially unchanged. Histopathology of laminar tissue from both hooves failed to show any lesions compatible with laminitis.

Horse 6 - A mild radial paralysis of the control left forelimb was apparent upon recovery from surgery. The paralysis was felt to be secondary to positioning of the horse in left lateral recumbency during the surgical procedure. Paralysis resolved within 24 hours. No lameness or change in hoof temperature was noted postoperatively. Neutrophil count decreased by 27.5% following 60 minutes of infusion and venous ligation. Neutrophil numbers then began increasing to reach a high of 24.1% over baseline at 5 minutes following the removal of the venous ligatures. Sixty minutes after ligature removal (VLR) a decline to baseline neutrophil count occurred. Likewise in the control hoof the neutrophil number decreased by 3.7% after 60 minutes of infusion and then began increasing. A peak count of 44.4% above baseline was reached at 5 minutes VLR. A fall to 33.3% over the initial value was seen at 60 minutes VLR.

Group 3 - Pony 7 - Within 15-20 minutes of intraarterial endotoxin administration, the pony experienced respiratory arrest. Resuscitative measures were successful and included cessation of halothane, administration of oxygen with
bagging, intravenous doxapram, and increased rate of administration of lactated Ringer's solution. Upon recovery the pony was febrile and exhibited a grade 2 experimental limb lameness. The pony was unresponsive to hoof testing but did exhibit marked sensitivity to palpation of the incision sites. The experimental hoof was cooler to touch than the control hoof. By 24 hours postoperative lameness had resolved and the pony was afebrile. The experimental hoof remained cooler than the control hoof. Examination of digital venous blood obtained intraoperatively revealed a 9.4% decrease in total white blood cells, an 11.1% decrease in neutrophils, and a 26.2% decrease in thrombocytes in the experimental hoof by 5 minutes postendotoxin (Table 3). At 45 minutes postendotoxin the total white cell count rose 17% and the thrombocyte count fell 60% as compared to the baseline values. The neutrophil count was essentially unchanged. The hematocrit was elevated 76.2%. In the control hoof at 5 minutes postendotoxin total white cell count was essentially unchanged but a 16.2% elevation in neutrophil numbers was seen. Platelets were decreased but to a lesser degree than seen in the experimental hoof (9.4% versus 26.2%). At 45 minutes postendotoxin total white count was increased 34% while the neutrophil count was essentially unchanged. Platelet numbers decreased 11.4% while hematocrit rose 73.1% from baseline. Within 30 minutes of the systemic injection of endotoxin the pony exhibited profound hypovolemia and became recumbent. Following intravenous
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<td></td>
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<tr>
<td><strong>PMN</strong></td>
<td>10^3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TC</strong></td>
<td>10^3</td>
<td></td>
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</tbody>
</table>

**Pony**
- C: 5.5 28.7 3.7 202
- E: 5.3 27.4 3.6 183

**Horse**
- C: 6.1 19.4 4.2 121
- E: 5.4 15.4 3.7 102

*15 minute samples not obtained.
WBC = white blood cells, HCT = hematocrit, PMN = neutrophils, TC = thrombocytes
administration of 5 1/2 liters of lactated Ringer's solution, the pony was able to rise. Additional fluids over the course of the day were required to stabilize the pony's condition. The pony was severely depressed, febrile, and anorectic. On the following day complete blood count revealed a neutrophilia with a left shift, lymphopenia, and thrombocytopenia. On the third day the pony became afebrile and began eating some hay and grass. On the fifth day the previously catheterized jugular vein was noted to be thrombosed. Treatment with phenylbutazone, trimethoprim - sulfa, and hydrotherapy was instigated. Over the following two weeks the pony recovered uneventfully. No hoof-related lameness or sensitivity was noted at any time over the course of the recovery.

Horse 8 - No untoward effects of the intraarterial injection of endotoxin were encountered. At 5 minutes postendotoxin a 33.3% decrease in total white cells accompanied by a 21.6% decline in neutrophils occurred in the experimental limb. Platelets fell 21.5% while the hematocrit rose 50.6%. White blood cell and neutrophil counts rebounded by 45 minutes postendotoxin with an 11.1% and 9.75% increase over baseline, respectively. Likewise the platelet count rose to 3.92% above its initial value. Hematocrit decreased to 22.7% over baseline. The control limb exhibited a 4.91% increase in total white blood cells but a 9.52% decrease in neutrophils at 5 minutes postendotoxin. Platelets declined by
2.5%. Hematocrit remained essentially unchanged. At 45 minutes postendotoxin increases of 14.7% and 23.8% in the white cell and neutrophil counts, respectively, were seen over the baseline measurements. Platelets decreased to 13.2% of the initial count while hematocrit approximated its original value. Following intravenous infusion of approximately half of the provoking dose of endotoxin the horse became ataxic, developed an elevated heart rate, and exhibited congested mucous membranes with a capillary refill time of 3 seconds. The unsteadiness of the horse worsened with continued administration of the endotoxin and, therefore, the infusion was ceased. The approximate dose of endotoxin administered was 0.5 ug/kg bodyweight. Due to the prolonged capillary refill time, lactated Ringer's solution was administered intravenously. The horse became febrile and the experimental hoof was markedly warmer to touch than the control hoof. By the next morning the horse was afebrile and no discrepancy between the temperatures of the forefeet could be detected. No lameness or hoof sensitivity ever developed.

**XD/XO activity** - Xanthine oxidase activity was measured in all 11 of the laminar supernatants assayed. In 3 of the 11 samples (27.2%) an increase in slope was seen following the addition of MB indicative of the presence of unconverted XD. Interestingly, XD activity was detected only in control
samples, i.e., those obtained from laminitis-free horses. Equine laminar tissue XO exhibited a $K_m$ of 2.56 uM pterin and a $V_{max}$ of 5 uM/min.

**SOD activity** - Preliminary results indicate the presence of SOD-like activity in laminar tissue. In general, 100 ul of supernatant resulted in 50% inhibition of cytochrome c reduction, i.e., 100 ul of supernatant contained 1 unit of SOD activity. An increase in the reduction of cytochrome c in the presence of potassium cyanide occurred and is evidence that the inhibition observed is likely due to CuZnSOD and not cytochrome c reductases or other inhibitors of the reactions. However, repetition of the assays with more stringent controls including the use of succinylated cytochrome c is necessary for conclusive determination of SOD activity.

Discussion

Several peculiarities of the equine dorsal laminar tissue would seem to render it particularly susceptible to perfusion deficits. The blood supply of the dorsal hoof wall laminae is from a single origin, the circumflex artery, from which the dorsal laminar arteries are derived. The dorsal laminar arteries are of note in that their blood flow is against gravity, i.e., in a distal to proximal direction, and that angiography has indicated that they are the last arteries in
the foot to fill (1,6). Venous drainage from the dorsal laminae is upward against gravity toward the coronary band (10). Within the hoof, however, the veins are valveless thus permitting retrograde flow (8,9). Additionally, the healthy horse has a very high digital capillary pressure (36.7 mm Hg) which is opposed by an interstitial fluid pressure (>25 mm Hg) that approaches the 30 mm Hg associated with the production of compartment syndromes in other species. (12,14,15) The horse would seem, therefore, to be at risk for the development of laminar ischemia with any event which would compromise digital blood flow or produce small aberrations in the Starling equilibrium.

The failure of ligation or resection of the main blood supply of the digit to produce any clinical or histopathological effects referable to the dorsal laminar tissue in the present study is, at first, surprising. However, this is in agreement with the findings of Scott, et al. (88) who reported no lameness or change in tissue viability following ligation of either the medial palmar artery or the medial palmar digital artery in ponies. Angiographic studies of the limbs of these ponies did reveal decreased blood vessel filling time in the case of medial palmar artery ligation; however, adequate perfusion of the distal limb was maintained by the lateral palmar, dorsal metacarpal and palmar metacarpal arteries. No change was seen
in the vascular pattern or the vessel filling time following medial palmar digital artery ligation. The authors attributed this to the presence of vascular anastomoses in the coronary corium and first phalanx. A more proximal ligation of the median artery, which would seemingly negate the effects of distal collateral vessels, failed to produce any untoward effects in the present study. Likewise, in their study of the clinicopathological effects of neurectomy, Said et al. (89) reported that ligation of the median artery, median vein, median artery and vein, the palmar digital arteries, or the palmar vein of donkeys did not result in any ischemic changes in the hoof. Following resection of the medial and lateral palmar digital arteries in ponies, Rijkenhuizen et al. (90) reported that arteriograms on the seventh postoperative day revealed no filling defects distal to the site of arterial disruption. However, severe lameness did occur immediately post-resection. Lameness was believed to be due to the pain associated with acute ischemia. An initial cooling of the experimental hoof suggestive of a decreased blood supply was reported in 45.5% of these ponies. A subsequent increase in temperature greater than the control hoof occurred on the second postoperative day in 80% of the originally cooler hooves. The remaining ponies exhibited no differences in hoof temperatures. In agreement with these findings, the resection performed in the present study resulted in postoperative lameness, though it was of moderate severity. The lameness
was not attributable to pain elicited by palpation of the incision sites or by hoof testing. The experimental hoof was palpably cooler than the control on the first postoperative day with no detectable temperature difference on the following day. It would seem, therefore, that sudden interruption of the peripheral arterial supply is well tolerated in the horse. Preexisting collateral circulation is able to compensate for loss of the normal circulatory pathways and is sufficient to maintain tissue viability (88-91).

While ischemia/reperfusion modeling was not successful in inducing laminitis in this study, a role for this mechanism in the pathogenesis of the disease cannot be ruled out. The severity of an ischemic injury depends on both the intensity of the ischemic insult and the duration of arterial occlusion (93). It is likely that some degree of ischemia, albeit temporary, was produced by the arterial ligations and resection performed, given the marked cooling of the experimental hooves. However, the occurrence of a significant ischemic insult is questionable given the ease with which digital venous blood samples were obtained following all ligations and the resection. Prolongation of the period of ischemia in all probability would not have affected the outcome of the procedures due to the extensive collateral circulation. Long-term ligations of the medial palmar and medial palmar digital arteries (1 month), the median artery (4
months), and both palmar digital arteries (3 weeks to 4 months) have not resulted in any pathologic consequences (88-91). Minimal, transient ischemia would likely not elicit the biochemical changes associated with ischemia/reperfusion injury, e.g., the conversion of xanthine dehydrogenase to xanthine oxidase. Thus, the production of tissue-damaging oxygen radicals would not likely occur. It may be that in the naturally-occurring case of laminitis some circulating chemical or hormone induces a widespread constriction of the peripheral arteries, including the potential collateral vessels, thus producing a profound ischemia.

No consistent pattern of change in neutrophil numbers was apparent during ischemia/reperfusion modeling. Although in two horses a decrease in neutrophil numbers was seen during ligation consistent with the reported ischemia-induced adherence of these cells to the endothelium, no subsequent increase in numbers was seen upon reperfusion (56). Interestingly, a lower peripheral neutrophil count as compared to the systemic count was consistently seen in these animals even prior to any procedure. Neutrophil counts in digital venous blood samples were up to 50% less than in systemic samples. A similar discrepancy has been reported for systemic vs. peripheral hematocrit in other species. The lower peripheral hematocrit is believed to be due to the presence of parallel circuits between the arteriolar and venular vessels.
which are more accessible to red cells than to plasma (94). In addition, it has been proposed that the reduced peripheral hematocrit is due in part to a cell-free plasma layer which exists adjacent to the walls of the microvasculature. Capillary transit times and the relative distribution of red cells to plasma can also be altered by the degree of constriction of the arterioles, in particularly the terminal arterioles. This can explain heterogeneity in peripheral hematocrits drawn concurrently from different sites. These factors may also explain the reduced peripheral neutrophil counts as well as the heterogeneity seen in 0 hour samples taken from control and experimental limbs.

Studies indicate that the major source of superoxide during ischemia/reperfusion is xanthine oxidase (95). The horse seems likely to be susceptible to ischemia/reperfusion injury given that xanthine oxidase activity is relatively high in equine serum (59) and now has been detected in laminar tissue. However, many variables may affect the relative sensitivity of a tissue to this type of injury. The minimum amount of xanthine oxidase required to produce biologically significant amounts of superoxide radical is unknown. While XO is widely distributed, enzyme content and the kinetics of the XD-to-XO conversion vary among tissues and may be determinants of tissue susceptibility to ischemia/reperfusion injury. The intestine and liver are particularly rich in XO
activity (95). However, where only one minute of ischemia results in complete rat intestinal D-to-O conversion, half-time of conversion in the rat liver is 3.6 hours (95). Significant hepatic XD-to-XO conversion occurs only following 2 hours of complete ischemia. During oxidant stress SOD and other enzymatic defenses may be overwhelmed or the enzymes may be altered or functionally impaired (67). A reduction in the level of the antioxidant enzymes has been suggested to be responsible for tissue injury seen during liver ischemia (68) and during reperfusion of the ischemic rat heart (69). The identification of superoxide dismutase-like activity in equine laminae suggests that the balance between oxygen radical production and the relative amount of SOD present may be important in determining laminar tissue damage as well.

Enzymatically generated oxygen radicals have been shown to increase vascular permeability when topically applied to the hamster cheek pouch and when introduced intraluminally or intraarterially into the small intestine of the dog and cat, respectively (86,87). In addition, topical application of XO/xanthine to the cerebral cortex of cats causes arteriolar dilatation and morphologic lesions of the endothelium and vascular smooth muscle (95). However, in this study the intraarterial infusions of the oxygen radical generating system, HX/XO, did not result in any clinical or histopathologic signs of laminitis. These findings coupled
with the reported lack of increase in vascular permeability during the prodromal phases of CHO-induced laminitis (54) suggest that oxygen radicals may not be instrumental in inducing the disease. However, other possibilities must be considered. In the previously cited studies, the exposure of tissue to oxyradical generating systems was controlled either by topical administration or by isolation of intestinal segments and their respective segmental vascular supply. The present study did not deal with an isolated system. Given the extensive collateral circulation of the equine hoof it is difficult to estimate actual exposure of digital vasculature to generated oxygen radicals. The reaction of hypoxanthine with XO is very rapid and the oxygen radicals thus produced are reactive and short-lived (87). It is conceivable that following palmar digital artery infusion no circulating oxygen radicals are presented to the dorsal laminar arteries when they are finally filled. These variables could be circumvented with HX/XO infusions done in isolated, perfused limbs or with topical administration to the dorsal laminae via hoof wall windows.

Interestingly, Horse 1 did show a marked increase in neutrophil numbers in the experimental but not the control limb following 60 minutes of HX/XO intraarterial infusion. This suggests that the enzyme system was generating superoxide as this oxygen radical is capable of activating an albumin-
associated neutrophil chemoattractant (56). While Horse 6 exhibited a decline in neutrophil count in the experimental limb following 60 minutes of infusion, this may have been due to the concurrent venous ligation in this animal.

Sprouse et. al. (29,31) successfully induced the local Shwartzman reaction in the skin of ponies and horses, thus establishing the susceptibility of this species to the phenomenon. As the equine hoof is of epidermal/dermal origin these authors proposed that laminitis could be a local Shwartzman reaction. Subsequent documentation of biphasic increases in plasma endotoxin levels in 45% of horses exhibiting CHO-induced laminitis substantiated their hypothesis (28). The peaks in circulating endotoxin were separated by 16 hours, approximating the 18-24 hour interval between injections described for induction of the Shwartzman reaction. In the present study, Shwartzman modeling did not induce any clinical signs of laminitis. It is well-documented that the dose of endotoxin required to produce the prepared state for the Shwartzman phenomenon can be minute, with only 5 ng necessary in the dermal or local reaction (29). The ug doses of preparative endotoxin used here would therefore seem adequate. In addition, the immediate decreases seen in the neutrophil and thrombocyte numbers of the experimental limbs suggests that endotoxin-associated changes did occur with the doses used. Endotoxin is known to induce an abrupt
neutropenia secondary to margination and sequestration of the cells (30). This is followed by a neutrophilia with a left shift which is maximal by 12-24 hours after endotoxin (96). Thrombocytopenia also occurs rapidly due to platelet aggregation and the removal of platelet-bound endotoxin from the circulation (30). In addition, a febrile response was seen following the preparatory injection. Endotoxin is pyrogenic with the minimal pyrogenic dose in the horse estimated to be 0.001 ug/kg (97). Clinical signs and changes in hematologic parameters following the provoking dose of endotoxin were similar to those reported following a single sublethal injection of endotoxin in the horse and pony (98,99).

Thus it would appear that despite similarities between the two, laminitis is not a manifestation of the Shwartzman phenomenon. Another possibility to consider is that it may be difficult to elicit the Shwartzman reaction in the horse with simply two spaced injections of endotoxin, as is the case in the rat (30). The generalized reaction can, however, be elicited in the pregnant rat and likewise has been reported in the pregnant mare (32). This refractoriness seems unlikely in the horse given its sensitivity to endotoxin and the successful induction of the local Shwartzman reaction in equine skin reported by Sprouse and Garner (31).
Chapter 3

INVESTIGATIONS INTO THE CHARACTERIZATION OF AN EXTRACT OF BLACK WALNUT (JUGLANS NIGRA) HEARTWOOD AND ITS LAMINOGENIC POTENTIAL IN THE HORSE

Introduction

The first known cases of equine laminitis associated with horses being bedded on black walnut (Juglans nigra) shavings were reported in the late 1970's by True and Lowe (73). Subsequent case reports indicated that shavings consisting of as little as 20% black walnut by weight could cause toxicosis and that the development of laminitis generally occurred within 12 hours of exposure (74,75,76). Other clinical signs associated with these cases of black walnut toxicosis included mild colic, anorexia, pyrexia, depression/sedation, tachycardia, tachypnea, increased hoof temperature, increased strength of digital pulses, and pitting edema of the distal limbs and ventral midline. The occurrence and severity of these clinical signs varied among horses and were not necessarily accompanied by lameness. Aeration of the shavings for variable periods (24 hours to 3 months) reportedly resulted in loss of toxicity with subsequent use as bedding possible. This suggested a volatile or unstable toxic principle.
Preliminary investigations of the toxic principle of black walnut focused on the substance juglone (5-hydroxy-1,4-naphthaquinone) which is found in members of Juglandaceae, e.g., walnut, pecan. Juglone was believed to be responsible for the allelopathic effect of the black walnut tree on plant species growing within the boundaries of its root system (77,78) In addition, juglone was reported to cause sedation in goldfish, rats, and rabbits (79) and to be lethal to a number of animals (80). However, controlled studies using synthetic juglone failed to consistently produce laminitis in the horse (81).

In 1987 Minnick et. al. (80) induced Obel grade 3-4 laminitis in 80% of experimental horses by intragastric administration of an aqueous extract of black walnut heartwood. Laminitis was seen within 12 hours of administration. Gas chromatographic/mass spectrometric analysis, however, detected no juglone in the heartwood extract. This finding essentially ruled out juglone as the laminogenic agent in Juglans nigra.

The toxic principle and the mechanism of toxicity of black walnut-induced laminitis have yet to be determined. Analyses of components of the heartwood extract and the physiologic effects of these components are not reported in the literature. The goals of the present study were to (1)
characterize the components of an extract of black walnut heartwood and (2) fractionate the extract by various means in an attempt to isolate the laminogenic principle.

Materials and Methods

Animals - Ten horses (geldings and mares aged 3 to 16 years) were acclimated for a minimum of 1 week prior to use. A physical examination and complete blood count and chemical profile were done on all animals to ensure the absence of significant medical problems. A lameness examination was likewise performed to rule out the presence of hoof-related conditions. All animals were free of clinical signs of previous laminitis. Animals were housed in individual box stalls bedded with wood shavings. Bedding was examined and determined to be free of black walnut wood. Hay and water were available on a free choice basis. A pelleted ration was fed twice daily.

Black walnut wood - Black walnut logs were obtained from West Virginia and Mississippi and were cut from living trees in January and October, respectively. Bark and sapwood were removed from the logs with a band saw. Heartwood was then planed into shavings. The shavings from the West Virginia logs were frozen at -20° C with aliquots defrosted as needed to make individual doses of extract. Shavings derived from the
Mississippi wood were also frozen at -20° C. However, all but approximately 1/4 of this wood was used to make extract ahead of time. Therefore, the shavings were frozen only as long as was required to prepare all of the extract. The extract was then frozen in individual aliquots at -20° C until needed for dosing.

**Aqueous heartwood extract** - Aqueous heartwood extract was prepared in a manner similar to the procedure described by Minnick et. al. (80). Shavings were defrosted and weighed into aliquots of 2 g/kg or 3 g/kg of bodyweight. Extract made in advance was prepared based on the average-sized horse, i.e., 454 kg bodyweight. The shavings were then divided approximately in half and placed into two plastic bags. Distilled water (3.5 liters/bag) was added and the bags were then put into stainless steel buckets. The buckets were placed in agitating waterbaths at room temperature for approximately 16 hours. The resultant deep brown fluid (approximately 5 L) was filtered through a triple layer of cheesecloth to remove particulate matter. The filtrate was then administered via nasogastric intubation to the horse. Aliquots of all extracts were frozen at -20 °C for gas chromatographic/mass spectrometric (GC/MS) analysis.

**pH fractionation of aqueous heartwood extract** - Aqueous heartwood extract (1000 ml, pH 4.38) was placed in a 2 liter
separatory funnel and extracted with 200 ml of ethyl acetate (EtOAc). Ten (10) ml isopropanol were added to reduce emulsion formation. The aqueous fraction was reserved. This procedure was repeated on an additional 4 liters of extract resulting in a total EtOAc acidic fraction volume of 550 ml. The reserved aqueous fractions were pooled (5700 ml) and the pH was adjusted to 10 with 45% NaOH. EtOAc extraction was performed as previously described. The resultant total EtOAc basic fraction volume was 1200 ml. The neutral aqueous fraction (5760 ml) was reserved and frozen at -20°C. Aliquots of both the EtOAc acidic and basic fractions were frozen at -20°C prior to evaporation to dryness of the fractions with a rotary evaporator. Residues were subsequently frozen overnight.

**Administration of acidic and basic EtOAc and neutral aqueous fractions** - The acid residue was dissolved in 100 ml distilled water with heating to 37°C. The basic residue would not dissolve in 100 ml distilled water. Neither the addition of acetic acid (2 ml) nor water (1 liter) and heating to 37°C enhanced dissolution. However, the residue did dissolve in the presence of 0.2 N NaOH. The acidic and basic fractions were brought to final volumes of 1200 ml with distilled water. The pH of the acidic and basic fractions following reconstitution were 5.0 and 6.2, respectively. The neutral aqueous fraction (5760 ml, pH 7.66) was thawed and
was noted to smell strongly of EtOAc. A rotary evaporator was used to remove as much residual EtOAc as possible. The fraction volume following evaporation was 4300 ml, pH 6.1. A very faint vinegary odor remained.

The three fractions, acidic, basic and aqueous, were administered intragastrically to three horses via nasogastric intubation. The horses were monitored closely over a 20 minute period for untoward effects. Temperature, pulse, respiration, hoof temperature, strength of digital pulses, gait, and any other significant observations were recorded approximately every 2 hours for the first 10-12 hours and then again the following morning.

**Ethanolic heartwood extract** - Ethanolic heartwood extract was prepared similarly to the aqueous extract. Shavings (Mississippi wood), however, were placed directly into stainless steel buckets rather than in plastic bags. In the case of the first horse to which it was administered extract was prepared at a dose of 2 g shavings/kg bodyweight. Subsequent extract was made from 3 g shavings/kg bodyweight and 8 liters of 190 proof, undenatured ethanol. Following filtration of the extract, a rotary evaporator was used to evaporate the extract to dryness. The residue was then weighed. Immediately prior to administration the residue was
reconstituted in approximately 1 liter of 5% ethanol. Aliquots of all extracts were frozen at -20° C for GC/MS analysis.

**Fractionation of ethanolic heartwood extract: Precipitate and Supernatant** - Ethanolic extract was prepared as previously described and then evaporated to dryness via a rotary evaporator. Residue weight was 38.0 gm. The residue was reconstituted in 250 ml of 25% ethanol. Sequential filtrations under vacuum were then performed using #4 Whatman, #5 Whatman, 0.45 u, and 0.22 u filter paper. Final filtrate volume was approximately 600 ml. Molecular weight ultrafiltration was then attempted at 80 psi using a 180 ml stir cell (Amicon) containing a 3000 MW limit filter (Filtron Omega Series OM003062, 62 mm diameter, NMWL 3K). However, due to an extremely slow filtration rate and the large volume to be filtered, the ultrafiltration procedure was abandoned. Because of the observation that precipitate formed upon addition of water to the ethanolic mixture, precipitation and ultracentrifugation of the filtrate was performed. Briefly, 400 ml of deionized, distilled water was added to the 600 ml of filtrate and the mixture refrigerated overnight. Using a Sorvall OTD-65 Ultracentrifuge (Dupont Industries) the filtrate was centrifuged at 105,000 g X 20 minutes at 5 °C. The supernatant was decanted and the precipitate was dissolved in a minimal amount of ethanol. Both the supernatant and the
precipitate were then frozen at -20°C. The following morning the supernatant was defrosted and centrifuged at 10,000 g for 60 minutes at 5°C (Beckman Model J-21B). The supernatant was decanted and the precipitate was dissolved in ethanol and added to the previously isolated precipitate. Ultrafiltration as described above was again attempted. An extremely slow filtration rate was again encountered despite removal of large amounts of precipitate. All filters used in this fractionation procedure were covered with ethanol and sonicated for 25 minutes. Filters were then rinsed with additional small volumes of ethanol. The ethanol mixture was then pooled with the precipitate. Final precipitate volume was approximately 500 ml. Final supernatant volume was approximately 1000 ml. Aliquots of precipitate and supernatant were reserved for GC/MS analysis.

**Administration of ethanolic supernatant and precipitate**

The supernatant and precipitate were defrosted. Using a rotary evaporator, the supernatant was evaporated down to approximately a 50 ml volume. The precipitate was evaporated to dryness and the residue weighed. Precipitate residue weight was 8.65 g, 23% of the original whole extract residue (38.0 g). Immediately prior to administration, the precipitate was reconstituted in 50 ml of ethanol and 1450 ml of distilled water. Likewise the supernatant volume was increased to 1500 ml by the addition of distilled water. The
fractions were individually administered to two horses by means of nasogastric intubation. The horses were subsequently monitored as described for the pH fractionation.

**Hydrolysis of ethanolic precipitate** - Three 1 ml aliquots of precipitate were evaporated to dryness under nitrogen stream and frozen overnight at -20°C. The following morning the precipitates were defrosted. To the first precipitate sample was added 10 ml of 6 N HCl, to the second sample 10 ml of 6 N NaOH, and to the third sample 10 ml of distilled water (pH 7). The mixtures were stirred overnight at 37°C. The samples were then each extracted with 2 ml of EtOAc. The EtOAc fraction of the NaOH mixture remained colorless unlike that of the HCl and H2O mixtures which were dark orangy-brown. In an attempt to enhance the EtOAc extraction from the aqueous fraction, the NaOH aqueous fraction was adjusted to pH 7.3 and again extracted with EtOAc. For comparison purposes the aqueous fraction of the HCl mixture was treated likewise. The subsequent NaOH and HCl aqueous fractions were adjusted to pH 2 and pH 12, respectively, and then each extracted with 2 ml of EtOAc. The EtOAc samples were evaporated under nitrogen stream to approximately 0.5 ml and then GC/MS analyses were performed. Derivatization of samples evaporated to dryness and subsequent GC/MS analyses were also done.
Chemicals - 1-naphthol, 1,4-naphthoquinone (1,4-NQ), 1,4-naphthalenediol (1,4-ND), and gallic acid (GA) were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. Pyrogallol (PG) was purchased from Kodak Chemical CO., Rochester, NY.

Sample preparation for gas chromatography/mass spectrometry - Aqueous extract samples were evaporated to dryness under a nitrogen stream and then reconstituted with 100 ul ethyl acetate (EtOAc) prior to injection. Ethanolic extract samples were injected without manipulation except for dilution with additional ethanol when necessary. When possible, spectra were verified with commercially available standards at a concentration of 100 ppm in ethanol. Sample derivatization - Following GC/MS analysis underivatized samples were evaporated to dryness under a nitrogen stream. Fifty (50) ul of N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) were added to each vial and the samples incubated at 65 °C for 20 minutes.

Gas chromatography/mass spectrometry - A Hewlett-Packard 5890A/5970 MSD was used for analyses. The HP5890A gas chromatograph was equipped with a 30 m X 250 um I.D. DB-5 5% phenyl and 95% methyl capillary column of 0.25 um film thickness\textsuperscript{10}. The injector was operated in the splitless mode.

\textsuperscript{10} J & W Scientific, Folsom, California
at 250 °C with a helium head pressure of 10 psi. For underivatized samples the oven temperature was held at 50 °C for one minute and then heated at a rate of 30 °C/min. to 300 °C and there maintained for 10 minutes. For silylated samples the oven temperature was maintained at 100 °C for one minute and then heated at a rate of 30 °C/min. to 300 °C where it was held for 10 minutes. The GC/MS transfer line was maintained at 300 °C. The mass spectrometer was operated with the temperature of the ion source at 150 °C and an electron energy of 2600 eV. Mass range scanned was 40 - 650 AMU.

**EtOAc extraction of basic aqueous heartwood extract** - It had been noted that adjustment of heartwood extract to a basic pH (pH = 12) resulted in a preponderance of an unidentified compound of MW 162 in the EtOAc fraction upon extraction. In order to perform NMR analysis which requires a somewhat purified sample, the following extraction procedure was done to isolate a fraction containing a high percentage of the unknown. Approximately 1 liter of aqueous black walnut heartwood extract was adjusted to pH 12 with 6 N NaOH. Extraction of this aqueous extract was done with 200 ml EtOAc + 10 ml isopropanol in a separatory funnel and then repeated. The final EtOAc volume was 275 ml. GC/MS analysis of the EtOAc fraction was performed. The EtOAc fraction was then evaporated to dryness with a resultant residue weight of 100 mg. The basic heartwood residue (BHR) was dissolved in a
minimal amount of ethanol and frozen at -20 °C. GC/MS analysis of the BHR was done before and after NMR analysis described below.

**NMR analysis of the basic heartwood residue (BHR)** - The BHR was defrosted and evaporated to relative dryness under a nitrogen stream. The residue was then placed in a vacuum dessicator for approximately 60 hours for removal of residual water or ethanol. The dried residue was removed and dissolved in deuterated chloroform (CDCl₃) for NMR analysis. NMR spectra were taken on a Bruker AM-400 spectrometer. Proton magnetic resonance spectra were recorded at 400.13 MHz with a repetition time of 4.638 sec and 16,384 data points collected over a sweep width of 5000 cycles. The ¹³C magnetic resonance spectra were recorded at 100.61 MHz with a repetition time of 3.655 sec and 32,768 data points collected over a sweep width of 25,000 cycles. A distortionless enhancement polarization transfer (DEPT) sequence was used to determine the multiplicity of the carbons.

**Direct exposure probe MS (DEPMS)** - The mass spectrum of BHR following NMR analysis was determined using a direct exposure probe on a Finnigan MAT TSQ-4500 mass spectrometer at an ionization potential of 70 eV and the electron multiplier at 1500 eV. The exposure probe was ramped from 0.0 to 1.2 A at 20 mA/sec.
Synthesis of 4-hydroxytetralone - Based on the method of Boyland and Manson (100) reduction of 1,4-naphthoquinone with LiAlH₄ was performed. Two grams of 1,4-naphthoquinone and 0.2 g LiAlH₄ were placed in an Erlenmeyer flask. Fifty ml of ether were added to the flask and the mixture was then stirred for 2 hours until the quinone was dissolved. The mixture was cooled to 0°C and then 30 ml of ether, 100 ml of distilled water, and 50 ml of 2 N H₂SO₄ were added to decompose both excess LiAlH₄ and the reaction complex. The mixture was then filtered under vacuum using #4 Whatman filter paper and an ether wash. The ethereal layer was then separated and washed with 100 ml of distilled water and then with 100 ml of 2 N NaOH. The resultant emulsion was allowed to settle and then the NaOH layer was removed and the ethereal layer washed once again with 100 ml distilled water. The ethereal layer was then separated and an aliquot submitted for GC/MS analysis.

Results

Aqueous heartwood extract - Three horses were administered aqueous heartwood extract derived from the West Virginia black walnut wood. The first horse exhibited an Obel grade 2 lameness at postdosing hour (PDH) 6. At PDH 12 lameness was accompanied by fever, elevated hoof temperature, and an increase in the strength of the palmar digital pulses. Marked
edema in all of the distal limbs was apparent by PDH 24. Approximately 3 1/2 months later a second horse was administered extract freshly prepared from frozen shavings. A mild increase in digital pulse strength was noted from PDH 4-15. However, no other clinical signs of laminitis were observed. As black walnut-induced laminitis is reported to occur in approximately 80-100% of horses exposed (80,83,84), an additional horse was administered the extract to rule out refractoriness in the second animal. This third horse developed no clinical signs referable to laminitis. Because of the apparent loss of toxicity the West Virginia shavings were no longer used for extract preparation.

Black walnut shavings were subsequently obtained from Mississippi-derived wood. Because of the apparent instability of the toxic principle in frozen wood shavings, batches of aqueous extract were made as quickly as possible from the shavings and frozen at -20 °C. Subsequent administration of extract prepared with 2 g shavings/kg bodyweight and 3 g shavings/kg bodyweight to two horses resulted in Obel grade 1 laminitis at PDH 30 and PDH 22, respectively. Although present, the laminogenic activity in these extracts was weak resulting in prolonged onset of mild lameness.
**pH fractionation of aqueous heartwood extract** - In an attempt to partially characterize the toxic principle and possibly concentrate it in a volume feasible to administer intragastrically, a pH fractionation of aqueous heartwood extract was performed. Extraction of weak acids and neutrals from the acidic heartwood extract resulted in a sherry-colored EtOAc fraction. Administration of this fraction after removal of EtOAc and dissolution in 5% ethanol produced a two-fold elevation in respiratory rate which returned to baseline by PDH 2. Anorexia and a transient increase in the digital pulses also occurred. Extraction of weak bases and neutrals from basic heartwood extract produced a rose-colored EtOAc fraction. Respiratory rate increased 2.5 times over baseline immediately after dosing but had resolved by PDH 2. However, an increased inspiratory effort remained apparent and had not resolved at the study's finish. Likewise a pulse rate 1.3 times greater than the initial value was observed immediately after dosing and was sustained throughout the remainder of the experiment. A mild elevation in hoof temperature accompanied by increased digital pulses and depression were noted at PDH 2 but resolved by the following morning. A temperature spike of 101.6°F occurred at PDH 4.5. Body temperature had returned to normal by PDH 9.5. Administration of the aqueous fraction resulted in a respiratory rate 1.3 times greater than baseline. A return to the baseline rate was seen at PDH 3. A fever spike of 101.4°F and a questionable increase in
digital pulse strength were noted at PDH 11.5. Also at this time the horse was observed to be weight-shifting on the rear limbs and to have an edematous right rear leg from the tarsus distally. By PDH 22.5 marked distal edema was apparent in all four limbs and pulse rate was elevated. However, no lameness occurred and the edema resolved over the next 24 hours.

**Ethanolic heartwood extract** - Enhancement of the laminogenic activity demonstrated in the aqueous extracts was attempted by extraction of the Mississippi shavings with ethanol, a less polar solvent than water. One horse was administered reconstituted residue from ethanol-extracted heartwood. At PDH 3 hoof temperature seemed elevated. By PDH 4.5 body temperature although still within normal limits had risen a degree, hoof temperature was elevated, and digital pulses were increased. Obel grade 2 lameness was apparent by PDH 10. At PDH 32.5 distal edema of all four limbs and an increased pulse rate were noted.

**MW fractionation of ethanolic heartwood extract** - Administration of the supernatant produced mild sedation (lowered head, drooping ears) within 10 minutes of dosing. At PDH 2 the horse was in sternal recumbency but rose quickly when approached. At PDH 4 the horse exhibited a low grade fever (101.2°F) and was reluctant to rise. By PDH 8 a mild elevation in hoof temperature and an increase in the strength
of the digital pulses were noted and the mare exhibited an equivocal lameness on turning to the right or left. Obel grade 1 lameness was apparent at PDH 12 and progressed to a grade 2 accompanied by mild distal edema by PDH 24. Comparison of complete blood counts (CBC) of digital venous blood samples at PDH 0 and 24 revealed a stress leukogram and hyperfibrinogenemia. Administration of the precipitate produced no sedation in the horse dosed. An increase in strength of the digital pulses was noted at PDH 4. The gelding exhibited a febrile response (101.6°F) at PDH 8. By PDH 12 hoof temperature was elevated and an Obel grade 2 lameness was apparent. Progression of the lameness to an Obel grade 3 and the development of mild distal edema occurred by PDH 24. PDH 24 digital blood sample revealed a stress leukogram and hyperfibrinogenemia.

Analytical Data - Gas chromatographic analyses of aqueous whole black walnut heartwood extracts consistently revealed one major and two minor peaks in underivatized samples (Figure 6). Mass spectra of these peaks showed compounds with molecular weights of 144, 162, and 178 in order of elution from the GC. Relative abundance of these compounds was MW 162 >> MW 144 > 178. These compounds were also found in underivatized samples of ethanolic whole heartwood extracts. In addition, compounds of MW 158 and MW 160 were identified in the ethanolic samples (Figure 6). Relative abundance of these
Figure 6 - Gas chromatograms of underivatized black walnut heartwood extracts. A - Aqueous; B - ethanolic. 1 = 1-naphthol, 2 = 4-hydroxytetralone, 3 = regiolone (4,8-dihydroxytetralone), 4 = 1,4-naphthoquinone, 5 = 4-oxotetralone.
compounds was MW 162 > 158 > 144 > 160 > 178. Data system library spectra based on the National Bureau of Standards, identified compounds of MW 144, MW 158, and MW 160 as 1-naphthol, 1,4-naphthoquinone (1,4-NQ), and 1,4-naphthalenediol (1,4-ND), respectively. Spectra of standards of 1-naphthol and 1,4-NQ confirmed their identification (Figures 7 and 8). However, GC/MS analysis of a standard of 1,4-ND resulted in a mass spectrum identical to that of 1,4-NQ rather than that of the compound of MW 160 in the extract (Figure 9). It was suspected that the MW 160 substance was 4-oxotetralone (4-OT) rather than 1,4-ND. The compound of MW 178 (Figure 10) had a mass spectrum consistent with that reported in the literature for regiolone, 4,8-dihydroxy-tetralone (101). The compound of MW 162 was suspected to be 4-hydroxytetralone (4-HT) based on its fragmentation pattern (Figure 10) and on the naphthalene-derived structures of the other compounds. Additional evidence came from the observation that acidification of heartwood extract to pH 1-2 resulted in a relative abundance of 1-naphthol greater than that of the unknown compound. Adjustment of extract to neutrality or an alkaline pH caused an inversion in the relative abundance ratio. Conversion of 4-HT to 1-naphthol under acidic conditions is reported in the literature (102). However, the mass spectrum of the unknown compound exhibited an M+ -15 (m/e 147) suggestive of the loss of a methyl group which could not be resolved with the
Figure 7 - Mass spectra of underivatized 1-naphthol.
A - In heartwood extract; B - 100 ppm standard.
Figure 8 - Mass spectra of underivatized 1,4-naphthoquinone. A - In heartwood extract; B - 100 ppm standard.
Figure 9 - Mass spectra of underivatized compounds of molecular weight 160. A - In heartwood extract; B - 100 ppm standard of 1,4-naphthalenediol.
Figure 10 - Mass spectra of underivatized compounds found in heartwood extract. A - Regiolone (4,8-dihydroxytetralone); B - 4-hydroxytetralone.
suspected structure. Structures of the above compounds are given in Figure 11.

Derivatized samples of whole heartwood extracts revealed two additional compounds with molecular weights of 126 and 170 (Figure 12). These were identified as pyrogallol (1,2,3-benzenetriol) and gallic acid (3,4,5-trihydroxybenzoic acid) and were confirmed with purified standards (Figures 13 and 14). Structures of these compounds are given in Figure 15. Mass spectra of the trimethylsilyl (TMS) derivatives of 1-naphthol (Figure 16) and 1,4-NQ (Figure 17) in the extracts were identical to those seen with derivatized standards. The TMS derivative of the standard of 1,4-ND (Figure 18) was indistinguishable from that of 1,4-NQ, i.e., m/e 304. The TMS derivative of the compound of MW 162 exhibited a molecular ion, m/e 234, consistent with derivatization of the lone hydroxyl group of 4-HT (Figure 19). The TMS derivative of regiolone could not be identified.

Proton NMR analysis of a BHR composed of approximately 59% of the unknown substance revealed bands at 2.00 to 3.90 (4 H), 3.59 (1 H), 4.89 (1 H), 7.27 to 7.70 (3 H), and 7.92 (1 H) (Figure 20). These bands correspond to the four methylenic protons, the hydroxyl proton, the geminal proton of the hydroxyl group, the benzylic protons, and the benzylic proton vicinal to the ketone moiety, respectively. This proton
Figure 11 - Chemical structures of naphthalene derivatives in black walnut heartwood extracts.
Figure 12 - Gas chromatograms of derivatized heartwood extracts. A - Aqueous; B - Ethanolic.
1 = pyrogallol, 2 = 4 - hydroxytetralone, 
2a = 4-hydroxytetralone (underivatized), 
3 = m/e 304, 4 = gallic acid, 5 = 1,4- naphthoquinone.
Figure 13 - Mass spectra of derivatized pyrogallol. A - In heartwood extract; B - 100 ppm standard.
Figure 14 - Mass spectra of derivatized gallic acid.
A - In heartwood extract; B - 100 ppm standard.
Figure 15 - Chemical structures of benzene derivatives in black walnut heartwood extracts.
Figure 16 - Mass spectra of derivatized 1-naphthol.
A - In heartwood extract; B - 100 ppm standard.
Figure 17 - Mass spectra of derivatized compound, m/e 304. A - In heartwood extract; B - 100 ppm standard of 1,4-naphthoquinone.
Figure 18 - Mass spectra of derivatized compound.
A - 100 ppm standard of 1,4-naphthalenediol;
B - 100 ppm standard of 1,4-naphthoquinone.
Figure 19 - Mass spectrum of derivatized 4-hydroxy-tetralone in heartwood extract.
Figure 20 - Proton NMR spectrum of 4-hydroxytetralone.
magnetic resonance spectrum established the structure of the unknown substance as 4-HT and was consistent with published NMR spectra of this compound (102, 103). The $^1$H NMR analysis confirmed the absence of a methyl group. GC/MS analysis of the BHR before and after NMR analysis, however, revealed a M+ -15 (m/e 147). This ion was not reported in the mass spectrum of 4-HT produced during bacterial metabolism of naphthol (103).

Further confirmation of the identity of the unknown compound was obtained by GC/MS analysis of synthesized 4-HT (Figure 21). The synthetic 4-HT had a mass spectrum (Figure 22) identical to that of the naturally-occurring unknown, including the M+ -15 (m/e 147). A DEPMS-derived spectrum also contained this ion. Synthesis of 4-HT also resulted in the production of a compound of MW 160 the mass spectrum of which (Figure 23) was identical to that seen for the naturally-occurring compound of MW 160 seen inconsistently in underivatized samples of heartwood extract. Based on possible products of the employed synthetic procedure and the knowledge that 1,4-ND does not show up as such in an underivatized sample, this compound was presumed to be 4-oxotetralone (4-OT), a less reduced precursor formed in the stepwise reduction of 1,4-NQ to 4-HT. Although no standard was available for verification, presumably 4-OT could undergo electron transfers and subsequent bond rearrangements to exhibit a TMS derivative.
Figure 21 - Gas chromatograms of synthetic 4-hydroxytetralone. A - Underivatized sample; B - Derivatized sample. 1 = 1,4-naphthoquinone, 2 = 4-oxotetralone, 3 = lawsone, 2-hydroxy-1,4-naphthoquinone, 4 = 4-hydroxytetralone, 4a = 4-hydroxytetralone (underivatized), 5 = 1-naphthol.
Figure 22 - Mass spectra of synthetic 4-hydroxytetralone.  
A - Underivatized sample; B - Derivatized sample.
Figure 23 - Mass spectra of synthetic 4-oxotetralone. A - underivatized sample; B - Derivatized sample.
with a molecular ion, m/e 304, indistinguishable from that seen with 1,4-NQ and 1,4-ND (Figure 24). Additional synthetic products were identified as 1-naphthol, a minor constituent, and lawsone (2-hydroxy-1,4-naphthalenedione, m/e 174) which constituted a major peak. Several other minor peaks were evident but were not identified.

Presence of the low molecular weight compounds identified in the various aqueous and acid/base heartwood extract fractions administered to horses is listed in Table 4. Absolute quantitation was not possible as no internal standard was used. Normalized abundances measured as peak height X/peak height 4-HT, however, are listed for underivatized and derivatized samples. Derivatization of samples introduces many variables into the calculation of relative abundances e.g., are structural changes induced by derivatization, is all of the compound present in the sample derivatized, etc. However, because some compounds showed up only with derivatization while others were not identified after derivatization it was necessary to present normalized abundances for both sample types. It is of note that 1,4-NQ was identified in a trace amount in the whole aqueous extract when a specific ion search was performed. However, its associated peak was recognized only as background on the gas chromatograph and therefore was not integrated. Thus no peak
Figure 24 - Formation of trimethylsilylated derivatives.
Table 4. GC/MS analysis of whole aqueous heartwood extract and acid/base fractions; normalized abundances based on peak height X/peak height 4-HT

<table>
<thead>
<tr>
<th>Compound</th>
<th>1-naphthol</th>
<th>1,4-NQ</th>
<th>4-MT *</th>
<th>4-HT</th>
<th>Regiolone</th>
<th>Gallic acid</th>
<th>Pyrogallol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whole Aqueous</strong></td>
<td>U</td>
<td>0.6</td>
<td>0**</td>
<td>0</td>
<td>1</td>
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<td>D</td>
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<td>0</td>
<td>0.07</td>
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<td>.37</td>
</tr>
<tr>
<td><strong>Acid Fraction</strong></td>
<td>U</td>
<td>0.06</td>
<td>0.55</td>
<td>0</td>
<td>1</td>
<td>.06</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>D</td>
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<td>0</td>
<td>.39</td>
<td>0.40</td>
<td>0</td>
<td>.59</td>
</tr>
<tr>
<td><strong>Basic Fraction</strong></td>
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<td>0</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>0</td>
<td>0</td>
<td>.09</td>
<td>0.1</td>
<td>0</td>
<td>.07</td>
</tr>
<tr>
<td><strong>Aqueous Fraction</strong></td>
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<td>--</td>
<td>--</td>
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<td>D</td>
<td>0</td>
<td>0</td>
<td>.08</td>
<td>.21**</td>
<td>1**</td>
<td>0</td>
</tr>
</tbody>
</table>

* Underivatized, D = derivatized
* * Mie 104 in derivatized samples may reflect contributions from 1,4-NQ and 1,4-ND
* ** Trace amount
height ratio could be reported for this compound in whole aqueous extract in Table 4.

GC/MS analysis of the supernatant derived from MW fractionation revealed the presence of most of the previously detected compounds (Table 5). Similar analysis of the precipitate showed no major peaks but trace amounts of 1-naphthol, 1,4-NQ, 4-HT, 4-OT, and gallic acid were detected. The presence of these compounds in trace quantities was felt to be secondary to either contamination from the supernatant, as the precipitate was not washed prior to analysis, or breakdown of a high molecular weight species. The appearance of underivatized 1-naphthol, 1,4-NQ, 4-HT, and 4-OT in the derivatized precipitate sample suggested that breakdown of a HMW compound had occurred after the derivatization procedure was completed, i.e., breakdown had occurred in the injection port of the GC. The presence of derivatized 1-naphthol and gallic acid was probably due to supernatant contamination of the precipitate sample. Aside from these trace levels essentially no other low molecular weight compounds were detectable upon analysis of the precipitate. Hydrolysis of the precipitate was accomplished in the presence of distilled water and 6 N HCl. The precipitate dissolved in but was not hydrolyzed by 6 N NaOH. GC/MS analysis of the hydrolysates revealed the presence of all of the previously identified low molecular weight compounds. In the water hydrolysate (pH = 7)
Table 5. GC/MS analysis of whole ethanolic heartwood extract and molecular weight fractions: normalized abundances based on peak height X/peak height 4-HT

<table>
<thead>
<tr>
<th>Compound</th>
<th>1-naphthol</th>
<th>1,4-NQ</th>
<th>4-CP</th>
<th>4-HT</th>
<th>Regiolone</th>
<th>Gallic acid</th>
<th>Pyrogallol</th>
</tr>
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<tbody>
<tr>
<td>Whole Ethanol</td>
<td>U</td>
<td>0.04</td>
<td>0.18</td>
<td>0.06</td>
<td>1</td>
<td>0.02</td>
<td>0</td>
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<tr>
<td></td>
<td>D</td>
<td>0</td>
<td>0.18</td>
<td>0.37</td>
<td>1</td>
<td>0</td>
<td>0.12</td>
</tr>
<tr>
<td>Supernatant (LMW)</td>
<td>U</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>0</td>
<td>0.16</td>
<td>0.61</td>
<td>1</td>
<td>0</td>
<td>0.58</td>
</tr>
<tr>
<td>Precipitate (HMW)</td>
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* = underivitized, D = derivitized
* * m/e 304 in derivitized samples may reflect contributions from 1,4-NQ and 1,4-ND
** = trace amount
4-HT was in greater abundance than 1-naphthol while in the HCl hydrolysate (pH = 1) and inverse ratio existed. However, on adjustment of the HCl hydrolysate to pH = 7 and pH = 12 4-HT became more prevalent.

Discussion

Heartwood is defined as the nonliving, usually dark-colored, inner layers of wood in which the reserve materials such as starch, which are present in the overlying sapwood, are removed or converted into heartwood substances (104,105). Heartwood contains a variety of extractives which include polyphenols (e.g., tannins), resins, fats, oils, etc. The polyphenols are one of the most common extractives, being present in trace to large quantities in all heartwoods. The composition of extractives from a given heartwood can be used for purposes of chemotaxonomic classification as the mixture of components is qualitatively the same within a species. The site of formation of heartwood extractives is controversial with evidence to support both in situ production in sapwood cells, thus changing them to heartwood, and synthesis in the leaves, needles, or cambium with translocation via phloem and rays to the heartwood. In Juglans nigra a causal relationship has not been found between the number and relative volumes of sapwood parenchymal cells and the quantity of phenolic heartwood extractives (106). However, between-tree
differences in the amount of total extractives in black walnut heartwood are greatly influenced by physiological conditions near the heartwood periphery. In addition, a relationship between quantity of extractives and soil properties seems to exist and explains to some extent within-species variation (105). Theories of heartwood formation abound. It has been viewed as an aging process and as a translocation process for biochemical waste. It has been proposed that heartwood is formed due to lowered water content and in response to fungal infection. The mechanism of heartwood formation has not as yet been conclusively determined.

In the present study characterization of the low molecular weight components of black walnut heartwood extract revealed the presence of 1-naphthol, 4-HT, regiolone (4,8-dihydroxytetralone), 4-OT, gallic acid and pyrogallol. To this author's knowledge 1-naphthol and 4-HT have not been previously reported in *Juglans nigra*. While 1,4-NQ was also identified, the behavior of the standard sample of 1,4-ND upon GC/MS analysis raises some questions. Is 1,4-NQ present in native heartwood extract or is its presence an artifact of the conditions under which analysis was performed, i.e., is 1,4-ND simply oxidized to 1,4-NQ in the injection port of the gas chromatograph? Spectra of derivatized standards of 1,4-NQ and 1,4-ND are not helpful in answering these questions as they are identical with both revealing underivatized 1,4-NQ and its
TMS derivative, m/e 304. However, given that 1,4-NQ has been previously identified in extracts of *Juglans regia* plants (107) and fruits (108) by thin layer and gas chromatographic methods and in *Juglans cordiformis* Maxim leaves by GC analysis (107), it seems likely that this naphthaquinone is not artifactual. Total 1,4-NQ content of black walnut extract determined by GC/MS analysis is likely, however, to include a contribution from the conversion of 1,4-ND. It has been shown that radiolabelled 1,4-NQ applied to *Juglans regia* plant material is incorporated into 4-hydroxy-1-naphthalenyl-glucoside (109). An O-glycosylation process occurs in Juglans and would require prior hydroxylation of 1,4-NQ to 1,4-ND. In addition, 4-OT, an isomer of 1,4-ND, has been speculatively identified in this study and evidence for its presence in *Juglans regia* has been documented elsewhere (109). An equilibrium may exist between 4-OT and 1,4-ND.

Most studies of *Juglans* species extractives have been done using bark, nut hulls, or the nut itself. Regiolone has been isolated from the stem bark of *Juglans regia* along with juglone, betulinic acid, and sitosterol (101). Regiolone has not been previously identified in heartwood. A study of polyphenols in *Juglans nigra* reported hydrojuglone-4-glucoside, myricetin, myricitrin, sakuranetin, sakuranin and neosakuranin in stem bark but not in heartwood or sapwood (110). However, in agreement with the present study gallic
acid was found in appreciable quantity in an alcohol extract of the heartwood. A related substance, ellagic acid, has been identified in the nut hulls of *Juglans nigra* (111). Although ellagic acid can be obtained from sodium persulfate oxidation of gallic acid or from acid hydrolysis of crude gallotannins, it was not identified in the heartwood extracts used in the present study. Interestingly, both ellagic acid and a strong acid fraction of the hulls have been shown to depress the growth rate of BW-10232 mammary adenocarcinoma in mice (111).

Acid/base extractions of whole aqueous heartwood extract in the present study yielded not only fractionation of low molecular weight compounds but separation of some clinical symptoms. The acid fraction which contained most of the compounds of interest resulted in a transient tachypnea and increase in the strength of the digital pulses. The basic fraction, which was essentially 4-HT although trace amounts of several other compounds were detected, resulted in depression and fever in addition to those symptoms described for the acid fraction. The aqueous fraction, despite being devoid of all LMW compounds except in trace amounts, proved the most active fraction. Transient tachypnea, increased strength of digital pulses, evidence of mild hoof discomfort (weight-shifting), fever, and distal edema ensued following aqueous fraction administration. Symptoms induced by the acid fraction could be ascribed to 1,4-NQ as quinones are known to increase
respiration and dilate blood vessels (112). However, the lack of 1,4-NQ in the basic fraction would seem to implicate 4-HT in the induction of the clinical signs associated with both fractions. Given the relative absence of these compounds in the more active aqueous fraction, a more plausible explanation would seem to be that an amphoteric substance not detectable by gas chromatographic methods is responsible for the clinical symptoms seen. A polymeric substance is likely as greater than 80% of the polyphenolic extractives of heartwood are reportedly polymeric materials (104). A dark violet-colored polymeric substance has been reported in an alcohol extract of black walnut heartwood (110).

Extraction of black walnut heartwood shavings with ethanol resulted in enhancement of laminogenic activity over that seen with the aqueous extract, thus possibly ruling out highly polar compounds e.g., proteins, as the toxic principle. In addition, fractionation of ethanolic heartwood extract provided further evidence for the involvement of a high molecular weight or polymeric substance in the pathogenesis of black walnut toxicosis. Administration of the HMW fraction which contained only trace amounts of the LMW compounds of interest resulted in the induction of laminitis. While the LMW fraction also induced laminitis, contamination of this fraction with the HMW substance(s) seems likely and cannot be ruled out with the GC/MS analyses performed. The possibility
that the precipitate was composed of the LMW compounds which were released upon hydrolysis of the precipitate in the stomach was investigated. Hydrolysis of the precipitate was accomplished with 6 N HCl and yielded 1-naphthol, 1,4-NQ, regiolone, and gallic acid. The TMS derivative, m/e 304, associated with 1,4-NQ, 1,4-ND, and 4-OT was also seen. Given that these LMW compounds were present in the acid fraction of the aqueous heartwood extract but induced no laminitis raises several possibilities. One or a combination of these LMW compounds may be the laminogenic agent but an insufficient concentration or the improper ratio of compounds was present in the acid fraction and thus no laminitis occurred. The combined action of several chemicals has been documented in some cases of allelopathic inhibition (113). Alternatively, the hydrolysis of the precipitate may yet yield a laminogenic substance of higher molecular weight than is detectable by GC methodology.

Several possibilities exist for a high molecular weight substance in black walnut heartwood. Quinones can readily polymerize during oxidation in air (112). Juglone (5-hydroxy-1,4-naphthaquinone) has been isolated from the bark of Juglans species in the oligomeric forms of 3,3'-bijuglone (8,8'-dihydroxy[2,2'-binaphthalene]-1,1',4,4'-tetrone) and unsymmetrical cyclotrijuglone (1,7,16-trihydroxy-5,6,11,12,17,18-trinaphthalenehexone) (114,115). The related
compounds isolated here in heartwood could likewise polymerize to produce a homogeneous or heterogeneous polymer. Alternatively, the dimeric lignans and polymeric gallotannins of heartwood must be considered. Interestingly, one of the principal groups of lignans are tetrahydronaphthalene (tetralin) derivatives (116) as are some of the LMW compounds identified in the present study. Lignans are of taxonomic interest as they can be characteristic of a certain botanical group. While the hydrolyzable gallotannins cannot be ruled out, they would seem a less likely candidate for toxicity. Their occurrence is widespread in plants, however, laminitis has not been reported in association with stall bedding derived from trees other than black walnut. In addition, oak poisoning which is attributed to oak gallotannins is not associated with laminitis in the horse or cow (117). Neither is the toxicity significantly decreased by freezing or drying. This is in contrast to the apparently unstable toxin in *Juglans nigra*. In the present study black walnut shavings lost all appreciable toxicity after being frozen for several months. Shavings reputedly lose their toxicity after variable periods of aeration (74-76). However, frozen aqueous heartwood extract has remained active for up to 3 months (84). The numerous evaporative procedures used in the preparation of various doses and fractions of heartwood extract in the present study would seem to rule out a volatile toxic
principle. Oxidative or microbial/fungal degradation of the toxic substance seems more likely.

Although evidence suggests the possibility of a laminogenic polymeric substance, the role of the identified low molecular weight compounds in the toxicity of black walnut heartwood extract warrants further investigation. Administration of purified compounds to horses may elucidate the contribution, if any, of these substances to the toxicity associated with black walnut. Enhanced activity has been reported for purified antitumor compounds versus crude fractions (111). Perhaps this may be the case for the laminogenic activity of black walnut as well.
Introduction

The horse is susceptible to a number of plant-related toxicoses including red maple and oak poisoning which result usually from ingestion of leaves. Red maple contains an unidentified oxidant which produces a hemolytic anemia in exposed horses (118). Oak poisoning has been attributed to the gallic acid moiety of oak gallotannin with the degree of toxicity reflecting the amount of tannin content in the leaves (117). As a group, tannins are classified as hepatotoxins although nephrotoxic properties are also associated with oak toxicosis. The mechanism of oak poisoning has not been elucidated; however, the characteristic precipitation of proteins by tannins (i.e., tanning of leather) may be involved.

Another Angiospermae (hardwood) poisoning seen in the horse is black walnut toxicosis. Toxicosis is manifested primarily as acute laminitis accompanied by dependent edema. Most cases of black walnut-induced laminitis result from
horses being bedded on black walnut shavings. However, the occurrence on two farms of toxicosis in multiple horses on pasture containing black walnut trees has been reported (82). Removal of all black walnut trees from the pastures prevented further occurrence of laminitis. While ingestion of the toxicant would seem the likely route of exposure, no correlation has been noted between horses known to ingest shavings and the severity of clinical signs (75). Experimentally, black walnut toxicosis may be induced by intragastric administration of an extract derived from Juglans nigra heartwood. Another possible route of exposure which has been considered is percutaneous absorption. However, the packing of black walnut shavings on the lower limbs of ponies for several days was reported to be without effect (73).

Gas chromatographic/mass spectrometric characterization of black walnut heartwood extracts has revealed the presence of numerous low molecular weight compounds. These include the naphthalene derivatives 1-naphthol, 1,4-naphthoquinone, 1,4-naphthalenediol, and 4-hydroxytetralone. The benzene derivatives, 1,2,3-benzenetriol (pyrogallol) and 3,4,5-trihydroxybenzoic acid (gallic acid) have also been identified. The purpose of the present study was 1) to administer these compounds individually to horses and assess the physiologic effects and 2) to perform gas chromatographic/mass spectrometric analyses of serum and urine.
samples from these animals in order to obtain preliminary information concerning the absorption and metabolism/excretion of the administered compounds.

Materials and Methods

**Animals** - Fourteen horses (mares and geldings aged 2 to 16 years) were acclimated for a minimum of 1 week prior to use. A physical examination and complete blood count and chemical profile were done on all animals to ensure the absence of significant medical problems. A lameness examination was likewise performed to rule out the presence of hoof-related conditions. All animals were free of clinical signs of previous laminitis. Animals were housed in individual box stalls bedded with wood shavings. Bedding was examined and determined to be free of black walnut wood. Hay and water were available on a free choice basis. A pelleted ration was fed twice daily.

**Chemicals** - 1-naphthol, 1,4-naphthoquinone (1,4-NQ), 1,4-naphthalenediol (1,4-ND), and gallic acid (GA) were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. Pyrogallol (PG) was purchased from Kodak Chemical Co., Rochester, NY.
Isolation of 4-HT by continuous ethyl acetate (EtOAc) extraction of aqueous black walnut heartwood extract - Because 4-HT was not readily available in purified form, isolation of the compound from an aqueous heartwood extract was performed. Three continuous extraction apparatus were arranged in series to allow a relatively large volume of aqueous heartwood extract to be processed at one time. Each unit consisted of a one liter erlenmeyer flask to which was attached, via a 45 degree angle 3-way ground glass adapter, a condenser and a 1 liter round bottom flask. The three units were placed in series by attaching the water outlet tubing from one condenser to the water inlet of the next. Approximately 900 ml of aqueous heartwood extract adjusted with 6 N NaOH to pH 12 was placed in each erlenmeyer flask. EtOAc (150 ml) was added to the heartwood extract and also to the round bottom flasks (400 ml/flask). Glass tubing of adequate length to reach through the adapter to the condenser tip was then positioned in the erlenmeyer flask. To allow condensing EtOAc to bubble up through the extract the end of the tubing inserted into the erlenmeyer flask was cut on a diagonal. The round bottom flasks were placed in heating mantles set at 65% of power. The aqueous extract was magnetically stirred and the extraction was allowed to proceed for 22-24 hours. At this time the EtOAc in the round bottom flasks was pooled and evaporated to dryness using a rotary evaporator. The EtOAc fraction was noted to be violet-brown
in color. The extraction procedure was repeated until the desired amount of residue weight was obtained. Gas chromatographic/mass spectrometric (GC/MS) analyses were performed on residue samples prior to administration of reconstituted residue to horses.

**Administration of naphthalene and benzene derivatives** - All compounds were administered to two horses, one receiving a low dose and the second a high dose. In all cases the compounds were reconstituted in 1 liter of 5% ethanol and administered intragastrically via nasogastric intubation. The initial low dose was approximated based on the relative percentage of the major component, 4-HT, in a black walnut heartwood residue of known weight and laminogenic activity. This relative calculated weight of 1.38 g was then approximately doubled to give a minimum low dose of 3 g. The following compounds were administered at a low dose of approximately 3 g: 1,4-NQ, GA, PG, and 4-HT. In the case of 4-HT 6.59 g of residue was actually administered. Although the residue contained 4-HT in great excess as compared to other individual components, it remained a somewhat crude fraction. Therefore the administered weight of residue was that calculated to contain approximately 3 g of 4-HT. This was calculated based on the relative percentage of 4-HT (~50%) determined by GC/MS analysis of a sample of the residue. The administered low doses of 1-naphthol and 1,4-ND were 3.5
g and 5 g, respectively. High dose administration was as follows: 6 g - 4-HT, 1,4-NQ, GA, PG; 7 g - 1-naphthol; and 10 g - 1,4-ND. Following administration of compounds the horses were monitored closely over a 20 minute period for untoward effects. Temperature, pulse, respiration, hoof temperature, strength of digital pulses, gait, and any other significant observations were recorded approximately every 2 hours for the first 10-12 hours and then again the following morning. Venous blood samples were collected at postdosing hours (PDH) 0, 2, 4, 8, 12, and 24 hours.

Preparation/administration of ethanolic heartwood extract
Ethanolic heartwood extract was prepared at a dose of 3 g shavings/kg of bodyweight. Shavings were weighed and divided approximately in half and placed into two 5 gallon stainless steel buckets. Four liters of 190 proof, undenatured alcohol was added to each bucket and the shavings were allowed to soak overnight at room temperature. The ethanolic extract was then filtered through a triple layer of cheesecloth and evaporated to dryness using a rotary evaporator. Residue weight was recorded prior to reconstitution with 1 liter of 5% ethanol.

Ethanolic heartwood extract was administered intragastrically to two mares via nasogastric intubation. Parameters monitored and venous blood collection were similar to that conducted following the administration of individual
compounds. Urine samples were collected via indwelling #28 foley catheters (35 cc balloon) at PDH 0, 2, 4, 8, and 12. After collection of the 12 hour sample the catheters were removed. Urine samples were obtained at 24 hours by introduction of mare catheters into the urinary bladders.

**Extraction of serum samples** - Two ml of serum were adjusted to a pH of 1-2 with 0.5 ml 1 N HCl and additional aliquots of 6 N HCl. Centrifugation was performed, however, no protein precipitate was detected. Following centrifugation 4 ml of EtOAc were added to the serum and the mixture was vortexed resulting in a slight emulsion formation. Centrifugation was again performed. The top EtOAc layer containing acids and neutrals (acid/neutral fraction) was subsequently removed. The remaining aqueous layer was adjusted to a pH = 10 with 6 N NaOH and then extracted with 4 ml of EtOAc. The EtOAc fraction containing compounds of a basic nature was reserved (basic fraction) for GC/MS analysis. The acid/neutral fraction was adjusted to a pH of about 10 with 6 N NaOH. The resulting neutral compound-containing EtOAc layer (neutral fraction) was removed and reserved. The aqueous layer was then adjusted to a pH of approximately 3 and extracted with 4 ml of EtOAc. The EtOAc layer containing acidic compounds (acidic fraction) was then removed and reserved. Extraction procedure controls included blank equine serum samples spiked with 10 ppm 1,4-NQ and distilled,
deionized water (ddH₂O) samples spiked with 100 ppm 1,4-NQ or 100 ppm 1-naphthol. In the case of spiked serum, paired samples of unincubated versus incubated (at 37 °C X 20 minutes) sera were prepared to evaluate possible protein binding. Spiked serum was extracted as previously described. Spiked ddH₂O samples were adjusted to pH = 1-2 with 6 N HCl and extracted with 4 ml of EtOAc. The acid/neutral EtOAc fraction was reserved for GC/MS analysis.

Extraction of urine samples - Urine samples were paired, untreated (UT) versus glucuronidase-treated (GT), to evaluate if glucuronide conjugation of the low molecular weight compounds found in black walnut heartwood extract occurred in vivo in the horse. UT urine sample - Five ml of an untreated urine sample were adjusted to pH = 1-2 with 6 N HCl and then mixed with 5 ml of EtOAc. The sample was then centrifuged and the resultant acid/neutral EtOAc fraction was reserved for GC/MS analysis. GT urine sample - To five ml of urine was added 2 ml of 1.0 M phosphate buffer (pH = 5) and 1 ml of B-glucuronidase". The mixture was then vortexed and incubated at 65 °C for 2 hours. The mixture was allowed to cool to room temperature and then was extracted as described for the UT urine samples.

"Sigma Chemical Co., St. Louis, Missouri"
Aeration of black walnut heartwood shavings - Twenty-five grams of black walnut heartwood shavings were spread out in weigh boats and placed in a ventilating hood. The shavings were left exposed to light and air for one week. They were then placed in 150 ml of 95% undenatured ethanol overnight. Also extracted in this manner were shavings from the same wood which had not been aerated. The ethanolic extracts were then filtered through a triple layer of cheesecloth and analyzed by GC/MS.

Gas chromatographic/Mass spectrometric analyses - A Hewlett-Packard 5890A/5970 MSD was used for analyses. The HP5890A gas chromatograph was equipped with a 30 m X 250 um I.D. DB-5 5% phenyl and 95% methyl capillary column of 0.25 um film thickness17. The injector was operated in the splitless mode at 250 °C with a helium head pressure of 10 psi. For underivatized samples the oven temperature was held at 50 °C for one minute and then heated at a rate of 30 °C/min. to 300 °C and there maintained for 10 minutes. For silylated samples the oven temperature was maintained at 100 °C for one minute and then heated at a rate of 30 °C/min. to 300 °C where it was held for 10 minutes. The GC/MS transfer line was maintained at 300 °C. The mass spectrometer was operated with

17J & W Scientific, Folsom, California
the temperature of the ion source at 150 °C and an electron energy of 2600 eV. Mass range scanned was 40 - 650 AMU.

Samples in ethanol or EtOAc were injected directly into the GC/MS for analyses of underivatized spectra. Subsequent derivatization of all samples was done as follows. Samples were evaporated to dryness under a nitrogen stream. Fifty (50) ul of N,O-bis-trimethylsilyl)trifluoroacetamide (BSTFA) were then added to the dry tube under nitrogen. The tube was immediately capped and then vortexed. The sample was then incubated at 65 °C for 20 minutes using a heating block. GC/MS analysis was then performed.

Results

Administration of 4-hydroxytetralone - Prior to administration of the residues of the continuous EtOAc extractions, GC/MS analyses were performed to determine the relative percentages of 4-HT in the residues. In the case of both the low dose residue and the high dose residue 4-HT accounted for approximately 45-50% of the residue. Administration of the low dose of 4-HT (3 g) resulted in an immediate decrease in pulse rate (from 48 to 30 beats/min.) and in respiratory rate (from 24-16 breaths/min.). The horse, however, was not depressed but rather appeared agitated. By postdosing hour (PDH) 4 pulse rate had returned to its
baseline value but respiratory rate was unchanged. At PDH 8 respiratory rate was elevated to 30 breaths/min. and inspiration was accompanied by nostril flaring. The strength of the digital pulses were increased at this time and remained so through PDH 24. No increase in hoof temperature was detected. Respiratory rate had returned to its previous level by PDH 12. No signs referable to hoof-related pain were seen at any time during the 24 hours of observation. No lameness or dependent edema developed.

Within 10 minutes of the administration of the high dose of 4-HT (6g) pulse rate was noted to be 24 beats/min., decreased from a baseline value of 36. By PDH 1, however, the pulse rate had rebounded to 42 beats/min. and remained between 42-48 beats/min. for the remainder of the experiment. At PDH 1 urticaria was apparent over the neck and shoulder areas bilaterally. An increased strength of the digital pulses was also noted at this time. Facial sweating was observed at PDH 2. By PDH 3 the urticaria was beginning to resolve; however, respiratory rate was increased to 30 breaths/min. from a baseline rate of 12. Respiratory rate remained between 30-42 breaths/min. through PDH 24. At PDH 8 hoof temperature was elevated and a further increase in the strength of the digital pulses was noted. By PDH 24 hoof temperature was normal, however, the digital pulses remained
increased in strength. No lameness or dependent edema developed.

**Administration of 1,4-naphthoquinone** - Immediately following administration of 3 g of 1,4-NQ an increase in pulse rate from 66 to 90 beats/min. was recorded. Twenty minutes after dosing the pulse rate had fallen to 48 and the respiratory rate had decreased to 30 from a baseline value of 36 breaths/min. By PDH 1 respiratory rate had jumped to 60 breaths/min. and was accompanied by a greater inspiratory effort than previously and nostril flaring. The horse appeared somewhat depressed. At PDH 3 pulse rate had increased to 60 beats/min. and respiratory rate had fallen to 48 breaths/min. The strength of the digital pulses was increased at this time and remained so through PDH 24. By PDH 4 pulse rate had decreased to 42 beats/min. and respiratory rate was 54 breaths/min. Pulse rate remained stable but respiratory rate fluctuated between 48-60 breaths/min. through PDH 12. By PDH 24 pulse rate was 36 beats/min. and respiratory rate was 36 breaths/min. Other than the increased strength of the digital pulses no hoof-related symptoms of laminitis were observed. No dependent edema developed.

The high dose (6 g) of 1,4-NQ resulted in a marked increase in respiratory rate (from 36 to 66 breaths/min.) at PDH 1. Respiration was rapid and shallow and accompanied by
nostril flaring. At PDH 2 respiratory rate remained elevated. The horse was noted to have shavings on its left side indicative that it had been lying down in the stall. The horse was depressed, hung its head, and appeared uneasy at this time. At PDH 4 the pulse rate was decreased to 36 from a baseline rate of 42 beats/min. Respiratory rate was also decreased to 42 breaths/min.. At PDH 12 the pulse rate had increased to 54 beats/min., however, the respiratory rate had decreased further to 36 breaths/min. Hoof temperature and the strength of the digital pulses were increased at this checkpoint. At PDH 24 vital signs had returned to baseline levels and hoof parameters were normal. No lameness or dependent edema developed.

Administration of 1-naphthol - Within 20 minutes of administration of 3.5 g of 1-naphthol a decrease in both the pulse rate (from 42 to 36 beats/min.) and the respiratory rate (from 30 to 16 breaths/min.) occurred. At PDH 4 pulse rate was elevated to 60 beats/min. and the respiratory rate had returned to 30 breaths/min.. At this time the strength of the digital pulses were noted to be increased over baseline and remained so through PDH 24. By PDH 8 pulse rate had decreased to 48/min. and remained at this rate through PDH 24. At PDH 12 the respiratory rate had fallen to 20 and fluctuated between 18-20 breaths/min. for the remainder of the
experiment. The only other clinical symptom exhibited by this animal during the experimental period was partial anorexia.

Following administration of 7 g of 1-naphthol a transient increase in pulse (from 48 to 60 beats/min.) and respiratory rates (from 18 to 24 breaths/min.) were seen. However, by 15 minutes after dosing the pulse rate had fallen to 36 beats/min. and the respiratory rate was likewise depressed to 12 breaths/min.. Although respiratory rate was decreased, inspiratory effort seemed greater than previously and was accompanied by an abdominal component which had not been noted prior to this time. At PDH 1 the strength of the digital pulses had increased and remained so until PDH 24 at which time normal pulsations were present. At PDH 4 the horse seemed depressed. Pulse rate and respiratory rate were 42 and 20, respectively, and remained stable through PDH 24. At PDH 24 the horse was still depressed and nostril flaring accompanied inspiration. No lameness or dependent edema developed during the experimental period.

Administration of 1,4-naphthalenediol - Immediately after the administration of 5 g of 1,4-ND a decrease in the pulse rate from 36 to 30 beats/min. and an increase in respiratory rate from 12 to 8 breaths/min. occurred. At PDH 1 a five-fold increase in respiratory rate (60 breaths/min.) was noted and was accompanied by an increased abdominal effort
and nostril flaring. The horse was in sternal recumbency at this time, however, it rose readily when approached. At PDH 2 the strength of the digital pulses and the hoof temperature of the right forefoot only were increased. By PDH 4 respiratory rate had decreased to 30 breaths/min. and pulse rate had increased to 42 beats/min. Although the digital pulses of the right forefoot remained increased in strength, no palpable difference in hoof temperature could be discerned between it and the left forefoot at this time. At PDH 6 pulse rate returned to its baseline value and respiratory rate dipped to 24 breaths/min. The digital pulses of both the right and left forefoot were now increased and remained so through PDH 24. At PDH 24 the respiratory rate remained elevated at 30 breaths/min. The only additional symptom exhibited by this animal during the 24 hours of observation was anorexia. No lameness or edema was noted at any time.

A decrease in pulse rate (from 48 to 36 beats/min.) was noted immediately following administration of 10 g of 1,4-ND. The pulse rate fluctuated between 36-42 beats/min. through PDH 24. By PDH 1 a 1.8-fold increase in respiratory rate (from 20 to 36 breaths/min.) had occurred. At PDH 2 the horse was found lying down in the stall and was noted to be occasionally looking at its abdominal region. Increased gut sounds were audible without a stethoscope. Respiration was shallow and accompanied by nostril flaring. Hoof temperature of both the
right and left forefeet was elevated, the right more than the left, at PDH 4. The respiratory rate had decreased to near baseline level (24 breaths/min.). Hoof temperature had returned to normal and the respiratory rate was 12 breaths/min. by PDH 8. No further changes were seen in these parameters through PDH 24. In addition to the above described changes, anorexia was exhibited until PDH 24. Lameness and distal edema did not occur.

**Administration of gallic acid** - Following administration of 3 g of gallic acid pulse and respiratory rates remained essentially unchanged throughout the 24 hours of observation. This horse exhibited a marked temperature difference between the two front hooves, with the right hoof being much cooler than the left, prior to dosing. The warmer left foot was similar in temperature to the back feet. This discrepancy was detectable until PDH 2 at which time the temperature of the right front hoof was not noticeably different from the left. An increase in hoof temperature above normal or in the strength of the digital pulses was not noted at any time. No anorexia, lameness or edema was evident through PDH 24.

An initial drop in both pulse (from 54 to 36) and respiratory (from 18 to 12) rates were seen immediately following the administration of 6 g of gallic acid. After this time pulse rate fluctuated between 36-48 beats/min. and
respiratory rate varied from 12-18 breaths/min. The horse remained asymptomatic through PDH 24.

Administration of pyrogallol - At PDH 1 following the administration of 3 g of pyrogallol pulse and respiratory rates showed a decrease from 48 to 36 beats/min. and from 36 to 20 breaths/min., respectively. From this time through PDH 24 the pulse rate varied between 36-48 beats/min. and the respiratory rate fluctuated from 30-36 breaths/min. At PDH 4 the horse, though standing quietly in the stall, was noted to be sweating over the back and neck regions. The sweating resolved within an hour. At PDH 8 the strength of the digital pulses was increased and remained so until PDH 24. The horse exhibited anorexia during the 24 hours of observation; however, no other clinical symptoms were discerned.

Administration of 6 g of pyrogallol elicited minimal fluctuations in pulse and respiratory rates. At PDH 2 an increase in the temperature and strength of digital pulses of the left front hoof were detected. By PDH 4 these parameters in the two hind hooves approximated those found in the left front. However, the right front hoof remained icy cold and digital pulses were not increased. A definite line of demarcation between the cold foot and the warmer upper leg could be palpated immediately above the right front fetlock. By PDH 8 all hooves exhibited similar increases in temperature
and strength of digital pulses. Lameness and dependent edema were not observed through PDH 24.

Administration of ethanolic heartwood extract - Two mares (BW #1 and BW #2) received reconstituted residue derived from ethanolic heartwood extract. Administered residue weight was approximately 40 g in both cases. Following administration of the extract BW #1 exhibited a decrease in pulse rate from 60 to 42 beats/min. and a rise in respiratory rate from 24 to 36 breaths/min. At PDH 2 collected urine was a dark greenish-brown. Blood collected at this time showed no evidence of hemolysis. At PDH 4 pulse rate was 30 beats/min. and respiratory rate was 16 breaths/min. From this time through PDH 24 the pulse rate ranged from 30-48 beats/min. and the respiratory rate varied between 12-18 breaths/min. The mare was anorectic and depressed throughout the experiment. An increase in hoof temperature and digital pulses of the forefeet were noted at PDH 12. Hoof temperature remained elevated at PDH 24 but the strength of the digital pulses had returned to normal. All urine samples collected were dark greenish-brown although some lessening of color intensity was apparent at PDH 24. No lameness or edema developed in this mare.

Pulse rate fluctuated between 42-54 beats/min. and respiratory rate ranged from 12-18 breaths/min. during the 24
hours following administration of extract to BW #2. At PDH 4 a febrile response occurred (102 F) and the mare was noted to be shivering. The fever increased to 103 F by PDH 6. At PDH 12 increased digital pulses were noted in the forefeet bilaterally, however, only the left forefoot exhibited an increased hoof temperature. At PDH 24 hoof temperature was elevated in the right forefoot as well. Extensive dependent edema was apparent from the carpi and hocks distally. All urine samples collected after PDH 0 were a dark greenish-brown in color. A decreasing intensity of urine color was noted from PDH 8 through PDH 24.

**GC/MS analyses of serum samples** - Analyses of serum samples, underivatized and derivatized, from horses to which individual compounds or extract had been administered revealed the absence of the naphthalene and benzene derivatives of interest. This was the case as well for serum samples spiked with 1,4-NQ. However, GC/MS analyses of the acid/neutral fractions of ddH₂O samples spiked with either 1,4-NQ or 1-naphthol did reveal the presence of these compounds. Therefore, failure of detection of the compounds in serum samples was not due to an ineffective extraction procedure. Possible explanations include serum levels of these compounds below the level of detection, a lack of systemic absorption, or a high degree of protein binding in the serum.
**GC/MS analyses of urine samples** - Analyses of untreated/underivatized urine samples revealed the presence of 1-naphthol, 4-HT, and 4-oxotetralone (4-OT). 1,4-NQ was not identified as such in these samples. Identification of additional metabolites from data system library spectra based on the National Bureau of Standards included naphthalene and naphthalenamine. Analyses of glucuronidase-treated/underivatized samples revealed the aforementioned compounds and, in addition, 4,5-dihydroxytetralone (regiolone) and 1-methoxynaphthalene. 1,4-NQ was again not identified. Calculation of the average ratio of peak height in glucuronidase-treated (GT) versus untreated (UT) urine samples (i.e., peak height $X_{GT}$/peak height $X_{UT}$) gave the following: 1-naphthol = 0.99, 4-OT = 1.12, 4-HT = 1.52, naphthalene = 0.88, and naphthalenamine = 1.05. Both regiolone and 1-methoxynaphthalene were found only in the glucuronidase-treated samples and therefore no ratio could be calculated. While gallic acid was detected in derivatized untreated urine, it could not be identified in the glucuronidase-treated derivatized sample. This situation was reversed for pyrogallol i.e., it was not identified in the derivatized untreated urine but was detected in glucuronidase-treated/derivatized samples.

**GC/MS analyses of aerated versus unaerated shavings** - Comparison of the normalized abundance (peak height $X$/peak height $X_{GT}$/peak height $X_{UT}$)
height 4-HT) of each compound can be seen in Table 6. The normalized abundances of 1,4-NQ and regiolone in unaerated shavings were 2-fold and 1.75-fold greater, respectively, than that seen in the aerated sample. Normalized abundances of 1-naphthol were similar between samples, however, the aerated shavings were completely devoid of 4-OT. Conversely, both pyrogallol and gallic acid were in greater abundance in the aerated shavings as compared to the unaerated.

Discussion

Gamma-scintigraphic analysis of perfusion of the equine digit during experimentally induced black walnut laminitis has demonstrated decreased blood flow to the forefoot as compared to the entire distal forelimb, resulting in ischemia (84). Additionally, the deficit in perfusion to the dorsal laminae is greater than the deficit seen elsewhere in the forefoot. At 84 hours following administration of the heartwood extract perfusion to the dorsal laminae is increased relative to the distal forelimb, suggesting reperfusion has occurred. Oxygen-derived free radicals have been shown to mediate ischemia/reperfusion injury in numerous tissues of varied species (56,57,58). One of the primary sources of these
Table 6. Normalized abundances of compounds in aerated vs. unaerated black walnut heartwood shavings (peak height X/peak height 4-HT)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Aerated</th>
<th>Unaerated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-naphthol</td>
<td>0.13</td>
<td>0.14</td>
</tr>
<tr>
<td>1,4-NQ</td>
<td>0.22</td>
<td>0.44</td>
</tr>
<tr>
<td>4-OT</td>
<td>0</td>
<td>0.18</td>
</tr>
<tr>
<td>Regiolone</td>
<td>0.04</td>
<td>0.07</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>0.77</td>
<td>0.67</td>
</tr>
<tr>
<td>Gallic Acid</td>
<td>0.69</td>
<td>0.54</td>
</tr>
</tbody>
</table>
oxygen radicals in vivo is the cytosolic enzyme xanthine oxidase (XO). During ischemia ATP is broken down with a resultant accumulation of hypoxanthine, a substrate of XO. Upon reperfusion of the tissue, the superoxide radical is generated during the reaction of XO with hypoxanthine, a reaction which uses oxygen as an oxidant.

Quinones, such as 1,4-NQ identified in black walnut heartwood extract in the present study, have been shown to function in electron transport in association with certain enzymes including xanthine oxidase (112). Most of the quinones are fairly potent oxidants, e.g., the reduction potential of 1,4-NQ, $E'_o$, is 470 mV (119). Unlike substituted naphthoquinones, 1,4-NQ undergoes a two-proton, two-electron reduction in the whole range of pH. Oxidation-reduction reactions of quinones generally involve the formation of an intermediate free radical semiquinone which can undergo redox cycling with oxygen to yield active oxygen species (120). Alternatively, the semiquinone radical can directly react with cellular targets, e.g., the sulfhydryl groups of proteins (112). Quinones may alter protein structure by the formation of disulfide interchain and interprotein links. Such cross-linking by quinones has been demonstrated in keratin. It has been proposed that quinones can be potent allergenic agents because they combine rapidly with proteins. Quinones are also known to have significant effects on respiration. An increase
in respiration is a characteristic response to quinones. Quinones inhibit oxidative phosphorylation by a number of mechanisms and 1,4-NQ has been shown to cause a rapid oxidation of hemoglobin to methemoglobin (112).

The toxic dose of 1,4-NQ has been reported as 10-15 mg/kg with an LD₉₀ of 25-30 mg/kg (112). The 3 g and 6 g amounts administered in the present study were calculated to be approximately 6 mg/kg and 12 mg/kg doses, respectively. The two horses which received 1,4-NQ did exhibit markedly increased respiratory rates and dyspnea consistent with quinone toxicity. The cyanosis and "chocolate"-colored blood associated with methemoglobinemia was not seen. However, this is in agreement with other in vivo animal studies in which only trace amounts of methemoglobin were found in the blood, even at the time of death (112). The increased strength of the digital pulses seen in these horses likely reflects the fact that toxic doses of quinones generally cause vasodilation with a resultant increase in peripheral blood flow and a temperature rise in the extremities (112). The relatively quick recovery of these animals is undoubtedly due to the rapid metabolism of quinones in the body. Indeed, near-toxic daily doses can be given to some animals without marked effects due to this rapid metabolism and the fact that quinones are not believed to be cumulative poisons. An additional consideration in the toxicity of 1,4-NQ is that
1,4-naphthoquinone has been reported to be much less reactive toward oxygen than naphthoquinones derived from other black walnut extractives i.e, juglone and naphthazarin (5,8-dihydroxy-1,4-naphthoquinone) (121).

Similarities do exist between the potential pathogenesis of quinone toxicosis and laminitis. Larsson et. al., reported aberrant metabolism of the sulfur-containing amino acids in the keratogenous zone of the equine hoof during acute laminitis (71). Laminitis has been proposed to involve a coagulation dysfunction with at least one report documenting a prolonged prothrombin time (44). Because vitamin K is a naphthoquinone necessary for prothrombin synthesis in the liver, the possibility of competitive inhibition of prothrombin synthesis by other naphthoquinones exists and has been documented in the case of 2-chloro- and 3-hydroxy-2-chloro-1,4-naphthoquinone (112). Despite these similarities, however, no laminitis was induced by 1,4-NQ administration. Given that the administered doses of 1,4-NQ approximated, in the case of the low dose, or exceeded the estimated amount present in previously administered laminogenic heartwood residues, it seems probable that this compound alone is not responsible for black walnut induced laminitis in the horse.

Ingestion of large amounts of 1-naphthol reportedly may cause nephritis, lens opacity, abdominal pain, vomiting and
diarrhea, convulsions, hemolytic anemia, circulatory collapse, and death in humans (122). The LD₉₀ of l-naphthol in rats is 2.59 g/kg. In the present study horses were administered 7.5 mg/kg and 13.1 mg/kg of l-naphthol. In both cases a change in the character of respiration, but not necessarily the rate, was noted following administration. A greater abdominal effort, deeper inspiration, and nostril flaring were observed. Other symptoms seen included depression, partial anorexia, and increased strength of the digital pulses. Although some differences did exist in the time of onset, severity, and duration, the clinical signs seen following administration of 1,4-ND (10 mg/kg and 21.6 mg/kg) and 4-HT (7.6 mg/kg and 12.6 mg/kg) were similar to those associated with l-naphthol.

It may be that the common naphthalene ring structure is responsible for the similar clinical signs associated with the above compounds. The presence of the naphthalene nucleus as well as the 1,4 position in various quinone and dihydroxyquinone compounds has been shown to be responsible for the potency of some inhibitory effects (123). Alternatively, the similar effects of these compounds may be due in part to their metabolism. Fungal species are known to metabolize aromatic hydrocarbons to metabolites identical to those reported for mammalian metabolism (124). The fungi Cunninghamella elegans metabolizes l-naphthol to 4-HT via 1,4-ND and 1,4-NQ intermediates (102). In addition, the
metabolism of tetralin to 4-HT in the rat is believed to occur via oxidation of 1,4-ND (125). This may be the case in the horse as well.

The plant phenolic, gallic acid, has been shown to promote the reductive release of iron from horse spleen ferritin (126). Iron is important in oxygen radical-mediated tissue damage because it catalyzes the formation of the highly reactive hydroxyl radical from hydrogen peroxide (63). Despite this gallic acid is reported to have very low systemic toxicity in humans (122). In rabbits the LD50 is 5.0 g/kg (127). In the present study horses administered 7.5 mg/kg and 12.8 mg/kg of gallic acid remained asymptomatic.

Pyrogallol ingestion has been associated with severe gastrointestinal irritation, renal and hepatic damage, methemoglobinemia, hemolysis, and death (122). However, the administration of 6.9 mg/kg and 14.5 mg/kg of pyrogallol produced minimal symptomatology in the horse. The increased strength of the digital pulses and elevation of hoof temperature seen is ironic given the catechol-O-methyltransferase inhibition exhibited by pyrogallol (37).

Given that the administered doses of the above compounds approximated or exceeded the amounts calculated (% of total weight) in a laminogenic residue of black walnut heartwood, it
seems probable that none of these low molecular weight compounds are the laminitogenic agent. Although the compounds were not detected in serum, their presence in urine confirms absorption. Apparently these compounds are highly protein bound. This is consistent with the known reactivity of quinones (112) and gallic acid (127) with proteins. Glucuronidation of 1-naphthol (124) and 4-HT (124,125) has been reported. In the present study glucuronidase treatment yielded higher relative amounts of 4-OT, 4-HT, regiolone, and pyrogallol than detected in untreated urine. However, no increase in 1-naphthol was seen. The failure to identify 1,4-NQ suggests that it is metabolized prior to conjugation and/or excretion.

Comparison of aerated versus unaerated shavings revealed that the relative distribution of compounds was similar except for the absence of 4-OT in the aerated sample. However, 4-OT has been inconsistently seen even in laminogenic extracts. The decrease in the relative abundances of 1,4-NQ and regiolone reflect their instability in light and air and their susceptibility to oxidation/reduction reactions (112). In light of the failure of these compounds to individually induce laminitis, the decrease in relative abundances seen with aeration is probably unimportant in terms of loss of laminitogenic activity.
Chapter 5
GENERAL CONCLUSIONS

Ischemia/Reperfusion Modeling

While ischemia/reperfusion modeling was not successful in inducing laminitis in the present study, a role for this mechanism in the pathogenesis of the disease cannot be ruled out. It is likely that some degree of ischemia, albeit temporary, was produced by the arterial ligations and resection performed, given the marked cooling of the experimental hooves. However, the occurrence of a significant ischemic insult is questionable given the ease with which digital venous blood samples were obtained following all ligations and the resection. Minimal, transient ischemia would likely not elicit the biochemical changes associated with ischemia/reperfusion injury, e.g., the conversion of xanthine dehydrogenase to xanthine oxidase. Thus the production of tissue-damaging oxygen radicals would not likely occur. The sudden interruption of the peripheral arterial supply is well-tolerated in the horse due to preexisting collateral circulation. It may be that in the naturally-occurring case of laminitis some circulating chemical or hormone induces widespread constriction of the peripheral arteries, including the potential collateral vessels, thus producing a profound ischemia.
Digital Infusion of Hypoxanthine/Xanthine Oxidase

The identification of xanthine oxidase activity in laminar tissue in the present study suggests that this tissue is likely to be susceptible to ischemia/reperfusion injury. The intraarterial infusions of the oxygen radical generating system, hypoxanthine/xanthine oxidase, did not result in any clinical or histopathologic signs of laminitis. However, the present study did not deal with an isolated system. Given the extensive collateral circulation of the equine hoof it is difficult to estimate actual exposure of the digital vasculature to generated oxygen radicals. The reaction of hypoxanthine with xanthine oxidase is very rapid and the oxygen radicals thus produced are reactive and short-lived (87). It is conceivable that following palmar digital artery infusion no circulating oxygen radicals are presented to the dorsal laminar arteries when they are finally filled. These variables could be circumvented with infusions done in isolated, perfused limbs or with topical administration to the dorsal laminae via hoof wall windows.

Many variables including the endogenous levels of antioxidant enzymes may affect the relative sensitivity of the laminae to ischemia/reperfusion injury. Further characterization of the antioxidant enzymes in laminar tissue, including the superoxide dismutase-like activity identified in
the present study, would help to elucidate the susceptibility of the laminae to oxidative stress.

**Shwartzman Reaction Modeling**

In the present study, Shwartzman modeling did not induce any clinical signs of laminitis. It is well-documented that the dose of endotoxin required to produce the prepared state for the Shwartzman phenomenon can be minute, with only 5 ng necessary in the dermal or local reaction (29). The ug doses of preparative endotoxin used here would therefore seem adequate. In addition, the febrile response and the immediate decreases seen in the neutrophil and thrombocyte numbers of the experimental limbs suggests that endotoxin-associated changes did occur with the doses used. Thus it would appear that despite similarities between the two, laminitis is not a manifestation of the Shwartzman phenomenon.

**Characterization of a Black Walnut Heartwood Extract**

The characterization of the low molecular weight components of black walnut heartwood extract revealed the presence of 1-naphthol, 4-hydroxytetralone, regiolone (4,8-dihydroxytetralone), 4-oxotetralone, gallic acid and pyrogallol. To this author's knowledge 1-naphthol and 4-hydroxytetralone have not been previously reported in *Juglans*
nigra and regiolone has been identified only in the stem bark of *Juglans regia*.

Comparison of the laminogenic potential of aqueous versus ethanolic heartwood extracts revealed greater activity following ethanolic extraction. Ethanolic extraction would likely rule out highly polar substances, such as proteins, as the toxic principle. The results of administration of acidic, basic, aqueous, precipitate and supernatant fractions suggest that an amphoteric substance not detectable by gas chromatography is responsible for the induction of laminitis. A polymeric substance is likely as greater than 80% of the polyphenolic extractives of heartwood are reportedly polymeric materials (104).

**Administration of Individual Black Walnut Extractives**

The administration of l-naphthol, 1,4-naphthoquinone, 1,4-naphthalenediol, 4-hydroxytetralone, gallic acid, and pyrogallol did not induce laminitis in the horse. However, increases in respiratory rate and effort, strength of the digital pulses, and hoof temperature were frequently observed. These symptoms are associated with laminitis in the horse regardless of the etiology i.e., in the absence of black walnut-related extractives. The presence of these extractives may contribute to the overall clinical picture seen with black
walnut-induced laminitis. However, it seems probable that these physiologic changes are induced by some other mechanism common to all cases of laminitis, thus resulting in the similar symptomatology seen.

Although the above compounds were not detected in serum, most likely due to a high degree of protein binding, their presence in urine confirms their systemic absorption. Glucuronidation of 4-hydroxytetralone, regiolone, and pyrogallol occurred in the horse.

The failure of the low molecular weight compounds to induce laminitis in the horse is further evidence for the involvement of a high molecular weight substance in the pathogenesis of the black walnut induced disease. Several possibilities exist for a high molecular weight substance in black walnut heartwood. Quinones can readily polymerize during oxidation in air (112). Oligomeric forms of juglone have been isolated (114,115) and the related compounds identified in the present study could likewise polymerize to produce a homogeneous or heterogeneous polymer. Alternatively, the dimeric lignans and polymeric gallotannins of heartwood must be considered. One of the principal groups of lignans are tetrahydronaphthalene (tetralin) derivatives (116) as are some of the low molecular weight compounds identified in the present study. If, indeed, lignans proved
to induced laminitis in the horse an oxygen radical mediated mechanism might be ruled out as a direct cause of the disease, as many lignans possess antioxidant properties. However, even if laminitis is not induced per se by an oxidative mechanism, oxygen radicals might still play a role in the ultimate tissue injury seen.

While the hydrolyzable gallotannins cannot be ruled out, they would seem less likely candidates for toxicity. Their occurrence is widespread in plants; however, laminitis has not been reported in association with stall bedding derived from trees other than black walnut. In addition, oak poisoning which is attributed to oak gallotannins is not associated with laminitis in the horse or cow (117). The lack of symptomatology following gallic acid administration in the present study is further evidence that the gallotannins are not involved in the pathogenesis of laminitis. Also, the toxicity of oak is not significantly decreased by freezing or drying. This is in contrast to the unstable toxic principle of *Juglans nigra*. In the present study black walnut shavings lost all appreciable toxicity after being frozen for several months. Shavings reputedly lose their toxicity after variable periods of aeration (74-76). However, the numerous evaporative procedures used in the preparation of various doses and fractions of heartwood extract in the present study would seem to rule out a volatile toxic principle.
Further studies to characterize the lignans, gallotannins, and other high molecular weight components of black walnut heartwood would be of interest. Subsequent evaluation of the physiologic effects of these components in the horse might elucidate the toxic principle of black walnut induced laminitis. Effective treatments might then be defined.
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VITA

Linda Elizabeth Cummings was born in New Orleans, Louisiana on December 20, 1957. She attended Mercy Academy of New Orleans from which she graduated in May, 1975.

Following the fulfillment of preveterinary requirements at Louisiana State University and University of New Orleans, she gained admission to the School of Veterinary Medicine at Louisiana State University in 1980. She received the Doctor of Veterinary Medicine degree in 1984. After a year of private practice she was accepted into a large animal internship at the School of Veterinary Medicine at Louisiana State University.

In August of 1986 she was admitted to the PhD graduate program in the Department of Veterinary Physiology, Pharmacology, and Toxicology at Louisiana State University. She completed the PhD degree with a major in Veterinary Toxicology in August, 1992.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Linda F. Cummings

Major Field: Veterinary Medical Sciences

Title of Dissertation: Investigations into the Pathogenesis of Acute Equine Laminitis

Approved:

[Signature]
Major Professor and Chairman

[Signature]
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signature]

Date of Examination:

7/14/92