BIOCHEMICAL CHANGES IN THE INTEGUMENT OF HORSES WITH EXPERIMENTALLY-INDUCED LAMINITIS

by

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(Under the Direction of John F. Peroni)

ABSTRACT

The pathogenesis of acute laminitis in horses is complex and poorly understood. As a component of the integumentary system, the hoof, like the skin, is composed of epidermis, dermis, and subcutaneous tissue. For this reason, it may be possible to identify alterations occurring in the skin that correlate with derangements in the laminae. Thus, examination of these two tissues may allow identification of pathologic mechanisms affecting the integument during the onset of laminitis. The equine hoof is a specialized modification of the integumentary system and includes interdigitations of the dermis and epidermis. The purpose of this study was to further define the presence of leukocytes and their enzymatic products in the skin and laminar tissues of horses administered black walnut heartwood extract and compare those findings to horses administered purified lipopolysaccharide (LPS). Data obtained in the present study indicate that CD13-positive leukocytes and MMP-9 expression and activity, increased in laminar tissue, are also increased in skin from horses with experimentally induced laminitis. Additionally, these increases were not evident in horses treated with LPS. Results of the LPS group were not significantly different from Control tissues. The results of this study show that the pattern so leukocyte emigration are similar in skin and laminar tissue collected from the black walnute

extract model. Furthermore, these biochemical events are not occurring in experimental endotoxemia. The ease with which skin specimens can be obtained, compared with the collection of laminar tissue, may facilitate identification of horses at increased risk of developing acute laminitis or for assessing the efficacy of new treatment modalities designed to minimize activation and extravasation of WBCs.

INDEX WORDS: laminitis, inflammation, equine, lameness, neutrophil, myeloperoxidase, matrix metalloproteinase, CD13, LPS, endotoxin, integument

BIOCHEMICAL CHANGES IN THE SKIN AND LAMINAR TISSUE FROM HORSES WITH EXPERIMENTALLY-INDUCED LAMINITIS

by

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DEDICATION

To my parents, Rene and Tom Riggs, for your endless support. Mom, you have given me "roots and wings" and for that I am eternally grateful. Dad, your life is an example of what can be accomplished with dedication and perseverance. To my sister, Aimee, and brother, David, for your love, friendship and support throughout our lives.

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CHAPTER ONE

INTRODUCTION

This submitted work was undertaken to contribute to the understanding of acute laminitis etiology and pathophysiology. Specifically, the integumentary system was evaluated in regard to similarities in cellular expression between skin and laminar tissue in the development phase of laminitis. The dissertation is divided into five chapters and 3 appendices. Chapter 2 is a literature review which encompasses several aspects of acute laminitis research and is divided into three sections. Section I includes an overview of acute laminitis pathophysiology and includes several theories which involve the etiology of the disease. Section II reviews the equine integument and the subsequent association with laminitis. Section III presents the experimental models of acute laminitis and the information produced with each of them. Section IV is a detailed discussion of equine leukocytes and their role in laminitis. Section V details matrix metalloproteinases 2 and 9 and the involvement with developmental laminitis. Section VI reviews endotoxemia in the equine and the association with laminitis. Chapter 3 contains information on neutrophil myeloperoxidase levels in plasma, laminar tissue and skin of horses administered black walnut extract. Chapter 4 explores biochemical activity associated with leukocyte emigration in both the black walnut heartwood extract model of laminitis and in horses given lipopolysaccharide. Chapter 5 further evaluates biochemical activity in the carbohydrate overload model of laminitis. Chapter 6 is a discussion of the results of research presented in the previous three chapters and relates this information to other published material. Appendix A evaluates matrix metalloproteinase 2 and 9 in skin and laminar tissue samples collected from

horses with chronic laminitis. Appendix B reviews a protocol involving nitrotyrosine immunohistochemical expression in laminar tissue and skin in the black walnut heartwood extract experimental model of acute laminitis.

CHAPTER TWO

LITERATURE REVIEW

SECTION I. OVERVIEW OF LAMINITIS PATHOPHYSIOLOGY

The pathogenesis of laminitis is one of the most fundamentally complicated and challenging topics in the area of equine medical research. It is a severely debilitating condition and is cause of huge financial loss and loss of life in the equine industry. As well, the prodromal stage of the disease is clinically silent and, to date, preclinical diagnostic tests are nonexistent. Preclinical diagnosis, prevention and treatment options are essential in order to preclude destruction of essential anatomical structures. The available treatments for laminitis are largely ineffectual because they do little to prevent the processes that cause edema, ischemia, and separation of dermal and epidermal laminae. The clinical signs of laminitis occur from the acute inflammatory process affecting the sensitive laminae of the hoof.[1] When clinical signs develop, the structural anatomy of the hoof and the biomechanical conditions associated with weight bearing hinder the successful implementation of treatment. The inexpansive nature of the hoof does not accommodate for the inflammatory changes and subsequent edema formation leading to progressive laminar necrosis. Moreover, the forces of weight bearing further compromise the dorsal laminae leading to disruption of the normal functional relationship between the distal phalanx and the hoof.[2]

The cascade of events involved in the development of acute laminitis affects multiple organ systems and tissues resulting in the local manifestation of a systemic inflammatory

process. The gross physical changes in the hoof are well documented as are the inciting diseases (colitis, metritis, carbohydrate overload, etc.). It is the cascade of events that links the two is the focus of research and the subject of much debate. Because the developmental phase is clinically silent, naturally occurring laminitis is difficult to scientifically evaluate. Therefore, most of the data describing the pathophysiology of the disease has been collected from experimental induction with unrefined carbohydrate, fructan or black walnut heartwood extract. The histological evaluation of normal and experimentally-induced laminitic tissues have identified pronounced, but varying, morphologic changes in the laminar tissues, including interstitial edema, perivascular inflammation, epidermal cell necrosis, leukocyte infiltration, and separation and loss of the basement membrane attachment between the dermis and epidermis.[1,3-6] Taking into account this gross and histologic evidence, research has always been directed at understanding the critical etiologic steps which produce the clinical disease. From this research two theories evolved, the vascular theory and the metabolic theory. The vascular theory proposed that the vasculature was the primary source of dysfunction in laminitis and that subsequent damage could be traced back to an ischemic event within the laminar tissues.[7,8] The metabolic theory offered that laminar dysfunction was the result of a toxic agent or metabolite which directly altered metabolism in laminar tissues and that any vascular changes were secondary.[9-12]

Each theory is supported by compelling evidence but neither completely clarifies the pathophysiology of the disease. Therefore, it has become widely accepted that the actual mechanisms are likely a combination of the two theories and as yet neither surpasses the other in explaining the etiology of laminitis. The activation and recruitment of leukocytes from the circulation and their immigration into the laminae support the theory of a locally

altered metabolism[13-15] by exogenous factors. Additionally, laminae in the developmental phase contain both leukocyte-derived[16] and locally-derived[10,17,18] proteolytic enzymes capable of extensive tissue damage.

Similarly, cardiovascular dysfunction is likely a fundamental participant in the pathophysiology of the disease. The predisposition of laminar veins to vasoconstriction and established increased digital postcapillary resistance after experimental induction are strongly suggestive of the contribution of venous disturbances to the disease process.[19,20] Support for arterial dysfunction in the digit has not been elucidated. Similarly, studies have shown the uniqueness of the laminar vasculature in its response to applied vasoconstrictors in comparison to the larger digital arteries and veins. Constrictors which have been shown to have a profound effect on the laminar veins include prostaglandin $F_{2\alpha}$, 5-hydroxytryptamine, and endothelin-1. [21]

SECTION II: INTEGUMENT

Laminitis research has traditionally focused on the uniqueness of laminar tissue for an explanation of the localized clinical signs observed at the onset of the disease. In actuality, the specialized function of the hoof may allow the manifestation of a dysfunction occurring in other areas of the integument. The integument is a complex, active organ system involved in a wide range of tasks essential to normal physiologic activity. This system includes the epidermis and dermis always intimately associated with one another in both location and function.[22] The epidermis is the outermost layer of the integument and is embryologically derived from ectoderm. It is composed of keratinized stratified squamous epithelium and includes the skin as well as specialized structures such as hair, hoof, chestnut, ergot and certain glandular components. The dermis is of mesodermal origin and lies just deep to the

epidermis. It consists primarily of a dense connective tissue and contains the blood vessels and nerves supplying the integument.[23] The equine hoof is a specialized modification of the skin and includes epidermis and dermis (also known as corium or laminae). The epidermis or hoof capsule provides support and structure to the foot and is avascular and aneural. The epidermis and dermis of the hoof, like other types of integument are intimately associated via the basement membrane. The basement membrane is a complex network composed of collagen, particularly collagen IV, and glycoprotein molecules, such as laminin, fibronectin, amyloid P, entactin and heparin sulfate.[24,25] In the hoof, specialized interdigitations connect the dermis and epidermis and are termed primary and secondary laminae.[2,26] These interdigitations exponentially increase the surface area between dermis and epidermis allowing for the specialized weightbearing function of the hoof in the equine.[27,28]

The similarities between skin and hoof exist, not only at the gross and microscopic level but equally at the cellular level. The main cellular components of the integument are the keratinocytes. These cells proliferate in the integument in the process known as keratinization, producing cytokeratins. Over 30 different cytokeratins have been recognized in human integument [29], each contributing to the mechanical properties of their respective tissues. The equine laminae, chestnut and skin of normal horses were evaluated, using gel electrophoresis and immunohistochemical staining, for cytokeratin differentiation and content [30]. The distribution patterns of cytokeratins were consistent between hoof and chestnut representing the similarities of maturation patterns between the tissues. Subsequent to this finding, the patterns of expression of cytokeratin subtypes in both normal horses and those affected by laminitis were characterized for chestnut and periople.[31] A significant difference was found between cytokeratin expression in chestnut and periople from horses

with acute laminitis. The information obtained by these earlier studies indicates that the laminae and peripheral skin do function and mature in similar ways and that changes in cellular expression occur in both tissues subsequent to laminitis.

One study has recently addressed the specific integumentary effects of laminitis in horses suffering from chronic laminitis via hypersensitivity skin testing.[32] Both laminitic and control horses were subjected to intradermal skin testing as well as subsequent full thickness skin biopsy of reactive areas. The results of these studies showed a significant increase in clinical hyperreactivity to intradermal antigen challenge in horses affected by laminitis.

SECTION III: MODELS

In an effort to elucidate the pathophysiology of laminitis, researchers have developed experimental models to induce the disease. These include the intragastric administration of an overload of unrefined carbohydrate, of fructan or nonstructural storage carbohydrate and of black walnut heartwood extract (BWHE).[13,33,34] The models vary in the time from administration to clinical signs, what clinical parameters change after administration and how closely the model resembles naturally-occurring laminitis conditions. Acute laminitis develops in approximately 80% of horses administered either carbohydrate or BWHE.[13,33,34] There has been a great deal of debate on the best model for laminitis research and it is unknown how much, if any, these models differ in the etiology of the disease. Each of the experimental models of acute laminitis is based on ingestion of a substance capable of inducing the disease and the gastrointestinal tract is commonly the location of primary disease. Experimentally, once ingested these substances are either themselves absorbed[13,14] or may induce alterations in the normal microflora[35-42] which then cause laminitis. Fructan sugars are recognized as an important carbohydrate source in clinical cases of laminitis caused by grazing

on lush pastures and purified fructan will experimentally induce laminitis.[34] Likewise, monoamines have been found in large quantity in the cecum of ponies[39] and, in a separate study, the production of some of the monoamines were found to be increased *in vitro* when incubated with carbohydrate[37]. The infusion of purified monoamines, tryptamine and phenylethylamine, into normal horses was shown to decrease blood flow to the foot[43]. Captivating is the fact that tryptamine and phenylethylamine are structurally similar to 5-hydroxytryptamine (5-HT), the previously mentioned established constrictor of laminar arteries and veins with veins showing significantly greater sensitivity and a greater maximal response[19,21,37].

Well known to equine veterinarians and researchers are the primary disease processes of which laminitis is a common sequela. The clinical syndrome and the gross pathologic changes are also well documented. A unified theory for the cascade of events that occurs in between remains elusive. The models using both overload of unrefined carbohydrate and fructan are similar to the clinical condition of grain overload, all of which can produce profuse watery diarrhea, mild to moderate depression and inappetance, tachycardia and increased rectal temperature.[34] Clinical signs of laminitis are apparent approximately 24 hours after administration. The BWHE model has the distinct advantage that it does not induce secondary clinical problems, such as colic and diarrhea. It has been established that the development of acute laminitis induced by BWHE is accompanied by a rapid decrease in total white blood cell count that usually occurs within 4 hours of administration of BWHE.[13,14]

Comparisons, as seen above, of the laminitis models have not found one to be superior to the others. When evaluated, the vascular[19,44-46] and biochemical changes[16,18,47] appear to be similar with a slightly different time frame. As a result, it has yet to be determined if one should be used preferentially in laminitis research.

SECTION IV: LEUKOCYTES IN DEVELOPMENTAL LAMINITIS

The neutrophil is the central player in equine innate immunity and comprise close to 80% of the peripheral leukocyte population in the horse.[48] The rate of biosynthesis of mature neutrophils is affected by G-CSF and GM-CSF while release of the neutrophil from bone marrow is mediated by IL-1 and tumor necrosis factor[49]. Once the mature neutrophil has entered the circulation it remains there until it migrates into the tissues or undergoes apoptotic cell death with a half-life of 10.5 hours in the horse[48]. Non-activated peripheral neurophils are equally divided between circulating and marginated pools. Activation of the neutrophil is regulated by binding to and subsequent activation of cell surface receptors[49]. These receptors are activated by factors secreted by bacteria, factors generated during the host organisms' response to bacteria or factors generated by the coagulation cascade. Neutrophil activation enhances the functional activity of the cell and allows its participation in the innate immune response, immune regulation, and inflammation[50]. Activation also prolongs the life of the cell by removing it from the peripheral blood. For these reasons, neutrophil activation is under complex control. If neutrophil activation were more simply regulated, minimal dysfunction of neutrophil activation would result in rapid overwhelming bacterial infection while excessive activation could result in severe and unnecessary tissue damage. The specific activating factor regulates what action will occur from the neutrophil. Activation causes diverse reactions such as changes in receptor expression, adherence to endothelial cells, chemotaxis, phagocytosis, degranulation or production of reactive oxygen species[49,50]. Some of these diverse functions can be activated by the same agonists binding to different receptors or agonist binding to the same receptor with different affinity-states. For example, fMet-Leu-Phe, complement fragment C5a, and leukotriene B_4 can each stimulate

adherence and chemotaxis at low concentrations but can activate degranulation of granules and ROS production at higher concentration[49,50]. Most of the signal transduction cascades appear to involve G-protein coupled receptors. The concept of neutrophil priming is also important to neutrophil activation. In short, some agonists can activate receptors alone at low concentrations, some require higher concentrations to act alone while others require priming by activation of other receptors with a priming agonist[49,50]. For example, some receptors are only functional if the cell has been activated by strong agonists such as LPS or fMLP[49,51].

The neutrophil is the first line of defense from infection in the horse. Migration of neutrophils from the vasculature is a key element of activation and is essential for effective microbe killing[52]. The neutrophil is chemotactically activated to penetrate the vessel wall and enter the interstitium and there are several interrelated chemoattractants with induce extravavasation of neutrophils including complement factor 5a, leukotriene B4, platelet activating factor, and various chemokines[49].

Neutrophils circulate in the vasculature until activated at a site of infection or inflammation. Once activated, the leukocyte appears to roll along the endothelium mediated by selectin proteins present on both the activated neutrophil and the damaged endothelial cells. These selectin receptors are low-affinity receptors allow the slow rolling to take place. Once the neutrophils have slowed they are more tightly adhered to the endothelial cell by integrins with receptors present on the leukocyte cell surface. ICAM-1 is expressed on endothelial cells to adhere neutrophils[49]. Following firm adhesion to the plasma membrane of endothelial cells, neutrophils undergo extravasation via diapedesis. Neutrophils may either migrate by paracellular or transcellular pathways. The paracellular pathway by disruption of interceullular tight junctions appears to overwhelmingly predominate. Also, it appears that

the extravasation of neutrophils generally increases local vascular permeability although the exact mechanism is not clear[49,50].

The neutrophil is usually the first line of defense and as such is the first cell to arrive at the site of inflammation[52]. The cell brings with it a wide variety of microbicidal mechanisms to fight "invasion" and depends on opsonization for effective phagocytosis of whole bacteria and particles. Some organisms and particles can be phagocytized in the absence of recognition markers on their surfaces but the majority require opsonization for recognition by receptors on neutrophil cell membranes[49]. The majority of opsonin proteins are IgG and complement. Once the particle or organism has been phagocytized it is subjected to the "array of armaments" available to the neutrophil[49].

These cytotoxic defenses can basically be classified as either oxidative or non-oxidative. Oxidative defenses involve the production of reactive oxygen species. These are produced by a multi-protein enzyme complex call the NADPH oxidase. This pathway results in the transfer of electrons from NADPH to oxygen which results in the formation of superoxide anion ($O_2\cdot^-$). Superoxide anion is relatively unstable and its direct role in microbicidal activity is likely limited. It is exceptionally important, however, as it is the precursor to much more bacteriocidal oxidant species.[53,54] $O_2\cdot^-$ reacts with H+ to form hydrogen peroxide most often by superoxide dismutase[53]. Simultaneously, primary granules fuse with the plasma membrane and release myeloperoxidase. MPO catalyzes the production of hypochlorous acid (HOCl) from Cl^- and H_2O_2. HOCl is quite a potent oxidant, causing cytotoxicity to bacteria as well as viruses and sometimes fungi[55].

H_2O_2 is not the only product of reactions with $O_2\cdot^-$. Likewise, it may be converted to hydroxyl radicals (HO·), another potent oxidant. The importance of HO· *in vivo* remains

unknown. Singlet oxygen, ozone, nitric oxide and peroxynitrite are all possibly important free radicals in bacteriocidal actions but definitive research is lacking. The chemical reactions that produce them are well known but their importance, particularly in the equine neutrophil is, to date, unknown[53].

Non-oxidative defenses include the ability to degranulate and release toxic enzymes for microbe killing. These enzymes include defensins, lactoferrin, and proteases (MMP-9)[49].

The neutrophil also has the ability to produce cytokines. One group of cytokines, the chemokines act to recruit monocytes, eosinophils, basophils, T-lymphocytes, and additional neutrophils. Probably the most important of the chemokines is IL-8 which predominately recruits more neutrophils[49]. Proinflammatory cytokines such as TNFα and IL-1 are also produced and quite possibly anti-inflammatory cytokines[49].

Interleukin-1β is a pro-inflammatory cytokine that is expressed in laminar tissues of horses administered BWHE[56,57]. This increased local expression of interluekin-1β occurred coincident with a 30% decrease in circulating leukocytes, causing the authors to speculate that the diffuse pattern of interleukin-1β expression in the laminar tissues may have been caused by the entry of activated leukocytes from the adjacent microvasculature[56]. Routine histological staining of laminar tissues has failed to consistently identify increases in leukocyte number in the acute phases of the disease[1,6].

While the cells have not been consistently evident, activated neutrophils express biochemically active markers which can be identified. One of those markers is myeloperoxidase (MPO). MPO is stored in the intracellular granules of neutrophils and is released upon activation of these cells as seen during inflammation.[58] The enzymatic function of MPO is to convert superoxide anion to hydrogen peroxide which is the precursor for the

generation of more potent oxidizing radicals such as hypochlorous acid (HOCl). HOCl contributes to phagolysosome activity and microbial destruction but can also cause significant cellular damage[55,58]. Additionally, myeloperoxidase, oxidizes nitrite and tyrosine to nitrogen dioxide radical and tyrosyl radical, respectively with the end result being the formation of 3-nitrotyrosine[53].

MPO activity can be measured by enzymatic assays or immunological techniques to demonstrate neutrophil activation[58,59]. Importantly, MPO activity can be found in tissue following neutrophil degranulation and is, therefore, a sensitive marker of previous location and activity of activated neutrophils even after these cells have disappeared from the tissues. Therefore, assays performed on tissues which normally contain these cells in high numbers will indicate a high baseline MPO activity[60]. The skin, under normal conditions, contains very few neutrophils making the assay useful for detection of neutrophil activity in laminitic conditions[61].

CD13, like myeloperoxidase, can be used as a marker of leukocyte presence. CD13 is a cell surface protein, also known as aminopeptidase N,[62] which is present on myeloid cells. It has been used as marker for those cells but is not unique to those cells. In humans it has been identified on the surface of fibroblasts, intestinal epithelium, renal tubular epithelium, synapses of the central nervous system and sebaceous glands[62,63]. Like myeloperoxidase, CD13 not only acts as a marker for leukocyte presence but is also an enzymatically active protein. It is a zinc-dependent metalloproteinase with an extracellular domain capable of cleaving N-terminal amino acid residues from oligopeptides[62,64] In humans, it has been associated with acne and malignant neoplasms of epithelial and lymphoid origin.[63,65] While

the enzymatic activity of CD13 has not been evaluated in equine disease its has been used as a marker of equine leukocytes in a model of laminitis.[47]

Evidence obtained using both the carbohydrate-overload and BWHE models of laminitis indicate that profound effects on circulating leukocytes precede the development of symptoms in the hooves, and represent a systemic response that may affect many body systems. This generalized response includes the development of neutrophil-platelet aggregates in horses after carbohydrate overload[66,67], and an abrupt 30-40% decrease in circulating leukocytes in horses administered BWHE[14]. These facts support the hypothesis that the initial cascade of events of laminitis may have systemic effects and form the basis for the desire to investigate the effects of BWHE administration on the integumentary system as a whole. As previously stated, aggregates of platelets and/or platelets and neutrophils have been observed in carbohydrate overload induced laminitis in ponies[67]. These findings have been accompanied by a significant reduction in circulating platelet counts, shortened survival time of ^{111}indium-labeled autologous platelets, and diminution of blood flow in the feet of laminitis affected horses, in the absence of activation of the systemic coagulation system.[68,69]

The leukopenia which occurs after induction of experimental laminitis is not due to the extravasation of neutrophils, alone. In a study specifically evaluating these changes in circulating leukocytes[14], a significant decrease in circulating monocytes was found as well as a shift in neutrophil population from a smaller, more mature population to a larger, immature one. Additionally, reactive oxygen species production by leukocytes increased in the horses showing lameness and the occurrence coincided with the decrease in circulating leukocytes.[14]

SECTION V: MATRIX METALLOPROTEINASES

Matrix metalloproteinases (MMP's) have been studied extensively in experimental models of laminitis.[9,10,17,47,70] Like CD13 and myeloperoxidase, the matrix metalloproteinases are enzymatically active proteins.[71] MMPs are zinc-associated calcium dependent enzymes with considerable sequence homology which retain a cysteine switch and zinc-binding motif. MMP-2 and MMP-9 are a subgroup of MMPs termed gelatinases. These two proteins have enzymatic activity capable of degrading the basement membrane, specifically, able to degrade Type IV, V and VII collagen as well as laminin, fibronectin and elastin key components of laminar tissue.[24] Under normal physiologic conditions, the activities of MMPs are regulated by transcription, activation of the precursor zymogens, interaction with specific extracellular matrix components and inhibition by endogenous inhibitors.[72] A loss of control of these processes results in tissue damage. Tissue inhibitors of metalloproteinases (TIMPs) are specific proteins involved in controlling the local activities of MMPs in tissues. TIMPs prevent activation of the proenzyme form of the enzymes. In studies of mice and humans, TIMP-1 is particularly important in inhibiting MMP-2 and MMP-9[72].

MMP-2 and MMP-9 in zymogen and active forms are present in extremely low levels in normal equine skin and laminar tissues.[16,61] These enzymes, with TIMPs, are thought to be involved in the normal growth and remodeling of the tissues. In lamina, they may participate in growth and elongation of the hoof by controlled "unlocking" of the dermal epidermal junction.[1,25] If the enzymes are not closely regulated, as in some inflammatory pathologic conditions, the basement membrane may detach from the epidermis.[1,25] In the hoof, this is defined as laminitis.

From studies with other species, some cytokines induce the upregulation of MMPs directly and as well as inhibit TIMPs.[72] In particular, TNF-B induces Pro-MMP-9 formation and TNF-α inhibits TIMP-1.[72] Many inflammatory mediators are increased in experimentally induced laminitis, IL-1B, MPO, MAIL and and COX-2[57,73,74] and similarly upregulated mediators are likely in the equine skin.

In the most recent studies of acute laminitis, the presence of MMP-2 and 9 in laminar tissue has varied depending on the origin of the disease (naturally-occurring cases, carbohydrate overload-induced or black walnut extract-induced). Using SDS-PAGE gelatin zymography, increased levels of MMP 2 and 9 were demonstrated in laminar tissue obtained from several horses with naturally occurring acute and chronic laminitis [70] and in two horses with experimentally-induced carbohydrate overload laminitis [18]. Tissues obtained from the horses with carbohydrate overload induced laminitis also had increased expression of MMP-2, although the authors did not indicate the timepoint at which the samples were collected.[75] In contrast, laminar tissue obtained from horses after administration of black walnut extract had increased expression and activity of MMP-9 but not MMP-2, as determined by RT-PCR and gelatin zymography. [16]

SECTION VI: ENDOTOXEMIA AND LIPOPOLYSACCHARIDE

Horses are extremely sensitive to endotoxin (lypopolysaccharide, LPS) and the lethal doses of LPS range between 200-400 μg/kg[76,77] while other species (rats, mice, rabbits or guinea pigs) succumb to much higher systemic doses (3.0-10.0mg/kg).[78] The association between primary diseases which cause endotoxemia (strangulating colic lesions, colitis, bacterial pleuropneumonia, retained placenta) and the development of laminitis is well established.[39,79] Intuitively, researchers questioned whether gram-negative endotoxin and the subsequent

overwhelming reaction of the horse to it was the direct causative factor in the development of laminitis. Regrettably but not surprisingly the pathogenesis of the disease is not that simple. Administration of 20-30 ng/kg of LPS results in hyperthermia, tachypnea, tachycardia and leukopenia, all associated with septis in horses and systemic inflammatory response syndrome (SIRS), described in humans. It does not, however, induce laminitis.[76,77,80] The link is clearly present but is not a directly causative one.

The effects of endotoxin on the equine immune and cardiovascular systems have been studied extensively.[51,81-90] Not only is endotoxemia associated with laminitis but it has a direct, profound and negative effect on prognosis in the equine patient[87-90]. Likewise, experimental administration of LPS, like the experimental induction of laminitis by CHO or BWHE, causes significant leukopenia in the horse.[51,80] LPS, in vitro, binds to and activates neutrophils when other blood factors are present, causing an increased expression of integrin adhesion molecules, changes in neutrophil size, and degranulation.[86] It is interesting to note that both LPS and laminitis cause vascular derangements, but the specific digital abnormalities appear occur by different mechanisms. As stated previously, experimental induction of laminitis causes an increase in post-capillary resistance[19,21] while endotoxin appears to cause pre-capillary resistance.[46,83] Because of the association between endotoxemia and laminitis, any data collected on laminitis should answer the following question: Is the event studied a critical etiologic step in the development of the disease, a noncontributing consequence or a coincidence.[91] Comparisons between data from experimental induction of endotoxemia and laminitis will help to answer this question.

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CHAPTER THREE

NEUTROPHIL MYELOPEROXIDASE LEVELS IN PLASMA, LAMINAR TISSUE AND SKIN OF HORSES ADMINISTERED BLACK WALNUT EXTRACT [1]

[1] Riggs LM, Franck T, Moore JN, Krunkosky TM, Hurley DJ, Peroni JF de la Rebiere G, and Serteyn DA. 2007. *American Journal of Veterinary Research*. 68(1):81-6

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ABSTRACT

Objective—To compare measurements of myeloperoxidase (MPO) in plasma, laminar tissues, and skin obtained from control horses and horses given black walnut heartwood extract (BWHE).

Animals—22 healthy horses ranging in age from 5 to 15 years.

Procedure—Horses were placed in 4 groups, a control group given water (n = 5) and 3 experimental groups given BWHE (17) via nasogastric intubation. Each horse was monitored for signs of laminitis. Plasma samples were obtained hourly until euthanasia of BWHE-treated horses at 1.5 (n = 5), 3 (6), and 12 hours (6) and of control horses at 12 hours (5). Laminar tissue and skin from the middle region of the neck were harvested at the time of euthanasia. Plasma and tissue MPO concentrations were determined via an ELISA; tissue MPO activity was measured by use of specific immunologic extraction followed by enzymatic detection.

Results—The tissues and plasma of horses receiving BWHE contained significantly higher concentrations of MPO beginning at hour 3. Laminar tissue and skin from horses in all 3 treatment groups contained significantly higher MPO activity than tissues from control horses. Concentrations and activities of MPO in skin and laminar tissues were similar over time.

Conclusions and Clinical Relevance—In horses, BWHE administration causes increases in MPO concentration and activity in laminar tissue and skin, and the time of increased MPO concentration correlates well with emigration of WBCs from the vasculature. These findings provide additional support for the hypothesis that activation of peripheral WBCs is an early step in the pathogenesis of acute laminitis.

INTRODUCTION

Acute laminitis occurs secondary to a variety of insults, including gastrointestinal diseases, pleuritis, retained placenta, carbohydrate overload, and exposure to shavings from black walnut trees.[1] Results of recent studies indicate that peripheral blood WBCs become activated during the prodromal stage of acute laminitis induced by administration of an aqueous BWHE and leave the circulation.[2,3] Concomitant with this leukopenic response are increases in leukocyte production of radical oxygen species, numbers of WBCs in laminar soft tissues, and expression of inflammatory mediators in the same tissues.[3-5] These early inflammatory events precede the development of morphologic changes in the digit, including interstitial edema, epidermal cell necrosis, and separation and loss of the basement membrane that characterize laminitis [6-10]. Although the laminar tissue is a highly specialized tissue able to withstand large biomechanical forces, it remains part of the common integument, which includes the skin, ergots, and the chestnuts. Consequently, a broader examination of the integument in horses given laminitis-inducing substances may provide additional insight into the pathogenesis of the disease.

Normal equine integument contains few neutrophils,[5,11] and these cells may be difficult to detect under pathologic conditions because of the fact that they rapidly undergo apoptotic degeneration[10]. However, the presence of these cells in tissues may be documented by monitoring the presence of MPO, a leukocyte-specific lysosomal enzyme.[12] Stimulation of neutrophils leads to their activation and release of MPO, which catalyzes the production of reactive oxygen species that can cause local tissue damage, including disruption of the laminar basement membrane.[13] Although MPO concentrations have been used as a marker of neutrophil

presence in other equine tissues and plasma,[12,14-17] concentrations of MPO in equine laminar tissue and skin have not to our knowledge been previously reported.

The purpose of the study reported here was to compare results obtained with 2 new laboratory techniques designed to monitor changes in MPO concentration or activity. These techniques were used to characterize temporal changes in MPO in plasma, laminar tissues, and skin obtained from control horses and horses given BWHE. The hypotheses of this study were that administration of BWHE results in increased concentration and activity of MPO in plasma, laminae, and skin, compared with values from control horses; that tissue concentrations of MPO in horses given BWHE increases after the onset of leukopenia; and that concentrations of MPO in the skin correlate with values in the laminar tissues in individual horses. We elected to study the effects of BWHE to be able to directly compare our results with the results of recently published studies that have implicated activation of peripheral blood WBCs in pathophysiologic causes of acute laminitis.[3,4,18]

MATERIALS AND METHODS

Animals—Healthy horses ranging in age from 5 to 15 years were used in this study. All horses included were free of existing lameness and lacked clinical evidence of systemic inflammatory disease. No radiographic evidence of pre-existing laminitis was present on survey lateral and dorsopalmar radiographic views of the forelimb digits. A 12-gauge catheter was placed in the left jugular vein of each horse for serial blood sample collection, and the middle region of the neck was clipped prior to the start of the study. The study was approved by the University of Georgia Animal Care and Use Committee.

BWHE preparation—The BWHE was prepared as described previously.[19] Briefly, 1 kg of black walnut heartwood shavings was agitated in 7 L of water at room temperature (approx. 22°C) for 24 hours. An aqueous filtrate was then obtained by filtering the solution through cheesecloth. Six liters of the resulting BWHE was administered by nasogastric tube to horses.

Experimental protocol—Horses were randomly assigned to 1 of 4 groups as follows: control, 1.5-hour BWHE, 3-hour BWHE, and 12-hour BWHE. Horses in the control group (5 horses) received 6 L of water via nasogastric intubation and served as controls; all horses in this group were euthanatized after the 12-hour sample collection. Horses in the 1.5-hour BWHE group (5 horses) received BWHE via nasogastric intubation and were euthanatized at 1.5 hours after intubation. Horses in the 3-hour BWHE group (6 horses) received BWHE via nasogastric intubation and were euthanatized at the onset of leukopenia (approx 3 hours after administration of BWHE and defined as a ≥ 30% decrease from time 0 peripheral WBC counts). Horses in the 12-hour BWHE group (6 horses) received BWHE via nasogastric intubation and were euthanatized at the onset of Obel grade-1 laminitis (clinical signs consisting of weight shifting and bounding digital pulses without evidence of lameness at a walk) or at 12 hours after intubation, if signs of Obel grade-1 laminitis had not developed by that time.

Each horse was evaluated prior to intubation and every hour thereafter for attitude, heart rate, respiratory rate, capillary refill time, hoof temperature, digital pulses, and evidence of lameness consistent with Obel grade-1 laminitis. For horses in the control and 12-hour BWHE groups, blood samples were obtained via the jugular catheter at 0, 1, 2, 3, 4, 6, 8, 10, and 12 hours; blood samples were obtained at 0 and 1.5 hours from horses in the 1.5-hour BWHE group and at 0, 1.5, and 3 hours from horses in the 3-hour BWHE group. Blood samples were aliquoted into duplicate vacuum-evacuated tubes containing EDTA[a]. One sample was used for determination of

the peripheral WBC count, whereas the other sample was immediately centrifuged at 400 × *g*. Plasma from the latter tube was frozen at –80˚C until assayed for MPO concentration. All horses were euthanatized with a penetrating captive bolt, in compliance with guidelines outlined in the *2000 Report of the AVMA Panel on Euthanasia*.

Collection of laminar tissue and skin—Both forelimbs were disarticulated at the level of the metacarpophalangeal joint and the hooves cut into sections with a band saw. During the procedure, thermal damage was minimized by constant irrigation of the tissue with ice-cold physiologic salt solution containing the following: 118mM NaCl, 24mM $NaHCO_3$, 1mM $MgSO_4$, 0.435mM NaH_2PO_4, 5.56mM glucose, 1.8mM $CaCl_2$, and 4mM KCl. Two full thickness segments from each forelimb foot were then placed in the ice-cold physiologic salt solution. The hard keratinized portion of the hoof and distal phalanx were removed and specimens of laminar tissue removed by sharp dissection. Concurrently, a second investigator removed full thickness, 6 cm × 6 cm sections of skin from the middle region of the neck. These specimens were also placed in the ice-cold physiologic salt solution. The laminar and skin specimens were rapidly frozen in liquid nitrogen and stored at –80˚C.

Frozen tissue specimens were homogenized with a polytron tissue homogenizer[b] in western lysis buffer containing 50mM HEPES, 150mM NaCl, 1% Triton X-100, 1mM EGTA, 6mM sodium deoxycholate, 1mM Na_3VO_4, 1mM NaF, 1mM phenyl-methane-sulfonyl fluoride, aprotinin (20mg/mL), and leupeptin (20 mg/mL). After homogenization, samples were sonicated and centrifuged at 14,000 × *g* for 15 minutes at 4°C. The supernatant was aliquoted and frozen at –80°C.

Sample preparation for ELISA—Immediately prior to performing the ELISA, plasma and laminar supernatants were thawed and diluted 1:40 in a dilution buffer prepared with 20mM PBS solution (pH 7.4) containing bovine serum albumin (5 g/L) and 0.1% Tween 20. Skin supernatants were thawed and diluted 1:100 or 1:1000 in the same dilution buffer.

ELISA for measurement of MPO concentration—Myeloperoxidase was measured with a specific sandwich ELISA[8] in which the primary antibody (rabbit anti-MPO IgG) was coated onto microplate[c] wells. For standard preparation, MPO was extracted from equine neutrophils isolated from whole blood by sedimentation on density gradient followed by ion and gel filtration chromatography.[16] Equine MPO standards (ranging from 0.78 ng/mL to 50 ng/mL) and samples containing MPO were added (100 µL) to the microplate and incubated overnight at 4°C with the primary antibody. After washing with %saline (0.9% NaCl) solution containing 0.1% Tween 20, the immobilized antibody-antigen complexes were incubated for 2 hours at 37°C with the secondary antibody (guinea pig anti-MPO IgG). After washing, a third antibody produced in goats against guinea pig IgG and labelled with alkaline phosphatase[d] was added to recognize the sandwich complex (ie, primary antibody-MPO-secondary antibody). After washing, phosphatase activity was detected by incubation (30 minutes, 37°C, in the dark) with the substrate paranitrophenyl phosphate[d] (2.7mM) in DEA buffer[d] (9.7% diethanolamine, 0.02% NaN_3, 0.01% $MgCl_2$, pH 9.8). The reaction was stopped with 2.5M NaOH and the absorbance (405 nm) was read with a plate reader[e]. Control (blank) and dilutions of MPO standards and samples were made with the dilution buffer and each sample was run in duplicate. The absorbance value was directly proportional to the quantity of the sandwich complex and, therefore, to the concentration of MPO in the sample.

SIEFED assay for measurement of MPO activity—The SIEFED method, developed specifically for measurement of equine MPO, is used to measure MPO activity in biological fluids.[20] The SIEFED method consists of capture of MPO from a tissue homogenate by immobilized (microplate coated) specific antibodies followed by elimination of the homogenate by washings and an in situ detection of the enzyme activity with a sensitive fluorogenic substrate (Amplex red) and a nitrite based amplifier system. The primary antibody (rabbit anti-MPO IgG) was coated onto black microplate wells[c]. The MPO was extracted from equine neutrophils isolated from whole blood by sedimentation on density gradient followed by ion and gel filtration chromatography and used for MPO standards.[16] Equine MPO standards (ranging from 0.25 mU/mL to 6.4 mU/mL) and nondiluted samples containing MPO were added (200 µL) to the microplate and incubated for 2 hours at 37°C. After 3 washings, the peroxidase activity of MPO was detected by adding 100 µL of a 40µM Amplex red[f] (10-acetyl-3, 7-dihydroxyphenoxazine), freshly prepared in phosphate buffer (50mM) at pH 7.5 containing 10µM H_2O_2 and 10mM nitrite and fluorescence was measured with a fluorescence scanning instrument[g] at the excitation and emission wavelengths of 544 and 590 nm, respectively. Controls (blank) were prepared with the dilution buffer and each sample was run in duplicate. The fluorescence value was directly proportional to the quantity of active MPO in the sample.

Statistical analysis—Statistical analyses were performed with commercially available software.[h] concentrations and activities of MPO in laminar tissue were compared with those in skin by linear regression. Similar methods were used to compare values for each tissue obtained with the 2 methods of MPO detection, SIEFED and ELISA. Plasma and tissue MPO data were log (ln) transformed for normal distribution of data before analysis. Data for each time point for skin and

laminar specimens were compared for ELISA and SIEFED results by use of a 1-way ANOVA with Bonferroni post-test and unpaired t-test on transformed data. An unpaired t-test with the Welch correction for unequal variances was used to compare plasma concentrations for multiple time points with log (ln) transformed data. For all comparisons, a value of $P < 0.05$ was considered significant.

RESULTS

Plasma MPO concentrations determined by ELISA—Mean MPO concentrations in plasma samples obtained at time 0 did not differ significantly ($P = 0.74$) between the control and BWHE groups. In contrast, plasma concentrations of MPO in the BWHE group were significantly greater than those in the control group at 1, 2, 3, 4, 6, and 8 hours. Plasma concentrations of MPO within the control group were significantly lower than time 0 values at time points 1, 2, and 3 (**Figure 1**).

Laminar tissue MPO concentrations determined by ELISA—Concentrations of MPO in laminar specimens from the 3-hour BWHE and 12-hour BWHE groups were significantly greater than those of the control group (**Figure 2**). Concentrations of MPO in laminar tissues of the 3-hour BWHE and 12-hour BWHE groups were not significantly different.

Skin MPO concentrations determined by ELISA—Concentrations of MPO in skin specimens from the 3-hour BWHE and 12-hour BWHE groups were significantly greater than values for the control and 1.5-hour BWHE groups. No significant differences were found in MPO

concentrations between the control and 1.5-hour BWHE groups, or between the 3-hour BWHE and 12-hour BWHE groups.

Mean MPO concentrations in laminar and skin specimens obtained from horses in the control group were not significantly different (**Figure 2**). Mean MPO concentrations in skin and laminar tissues of horses 1.5 hours after administration of BWHE were not significantly different from values for control horses (**Table 1**).

Laminar tissue activity determined by SIEFED—Mean MPO activities in laminar tissue and skin specimens were determined (**Figure 3**). Activities of MPO in laminar specimens from the 1.5-hour BWHE, 3-hour BWHE and 12-hour BWHE groups were significantly greater than values for the control group (Table 1). No significant differences were found in MPO activities between the 3-hour BWHE and 12-hour BWHE groups.

Skin activity determined by SIEFED—Activities of MPO in skin specimens from the 1.5-hour BWHE, 3-hour BWHE, and 12-hour BWHE groups were significantly greater than those from the control group. No significant differences were found in MPO activities between the 3-hour BWHE and 12-hour BWHE groups.

Correlations between ELISA and SIEFED data—Positive linear correlations were identified for MPO concentrations between laminar tissue and skin obtained by use of the ELISA (R^2 = 0.51) and SIEFED (R^2 = 0.70). A comparison of individual MPO values obtained with the

ELISA and SIEFED yielded positive linear correlations for laminar tissue ($R^2 = 0.51$) and skin ($R^2 = 0.64$).

DISCUSSION

Results of our study provide convincing evidence for systemic activation of neutrophils, and their emigration into the integument in horses given BWHE. In our study, plasma concentrations of MPO at time 0 in both groups of horses were within normal limits for horses, suggesting that little intravascular release of neutrophil granular contents occurs under basal conditions. However, plasma concentrations of MPO increased by 1 hour after administration of BWHE, and remained significantly increased throughout the 8-hour period. This early increase in circulating concentrations of MPO is consistent with intravascular activation and degranulation of neutrophils, and coincides with the development of leukopenia as activated cells leave the circulation. The sustained increase in MPO concentration in plasma is indicative of continued intravascular activation of WBCs, and coincides with increased production of reactive oxygen species by WBCs after horses received BWHE. [3,5] To be fair, however, it cannot be determined whether the increased plasma concentrations of MPO were solely caused by intravascular release of the enzyme or if some MPO released in adjacent tissues re-entered the circulation. For example, recent evidence suggests that the administration of BWHE damages colonic mucosal epithelium, thus compromising the mucosal barrier. [21] This damaging effect of BWHE may be attributable to upregulation of proinflammatory mediators and cytokines that in turn could lead to neutrophil activation and release of MPO in plasma.

Concentrations and activities of MPO in skin and laminar tissue of control horses were low, consistent with recent reports that relatively few neutrophils are present in these tissues in healthy horses.[5,11] In one report, ≤ 1 $CD13^+$cells (ie, neutrophils or monocytes), were present per 40× field in laminae from 5 healthy horses and 0 to 3 $CD13^+$ cells were present in skin from 4 of the 5 horses.[5] Consequently, overlap in MPO values obtained for laminae and skin of the control horses is not unexpected. By 3 hours after administration of BWHE, however, MPO concentrations and activities were significantly increased in laminae and skin, and remained increased at the 12-hour time point. Collectively, these results provide supportive evidence for the recent observation that the number of neutrophils in laminar tissue and skin increases as an early response to BWHE administration.[5] Taken together, those findings and our results indicate that the inflammatory processes initiated by intragastric administration of BWHE are not restricted to the laminar soft tissues, but occur in the integument in general.

Degradation of the laminar basement membrane is a histologic hallmark of acute laminitis in horses. An increase in the local activity of MMP has been theorized to be the cause of this basement membrane disruption.[4,22,23] The MMPs comprise a family of enzymes that degrade and remodel various components of the extracellular matrix. Several studies have documented increases in MMP-2 and MMP-9 in laminar tissue in acute laminitis.[18,22,23] Although MMP-2 is produced by local cellular components, MMP-9 is a product of activated neutrophils.

Results of or study provide additional support for the hypothesis that activated neutrophils play a role in initiating the local inflammatory responses that occur in the laminar soft tissues during the development of acute laminitis. Neutrophils are activated by endogenous processes that ultimately induce their extravasation and degranulation, with the release of MPO. In our study the increase in tissue MPO concentrations and activities were accompanied by increases in

plasma concentrations of MPO, suggesting that intragastric administration of BWHE results in systemic activation of neutrophils. Increases in plasma MPO concentrations are found in horses with strangulating large intestinal obstructions and decreased numbers of circulating WBCs, suggesting that the increased circulating MPO concentrations originated from neutrophils that have become activated within the circulation.[15] These findings also are consistent with the recent report that horses with strangulating intestinal lesions have activated neutrophils in circulation. [24] Although the primary purpose of our study was to monitor changes in MPO concentration or activity as markers for the presence of neutrophils, a direct role of MPO in the pathogenesis of acute laminitis should not be overlooked. Myeloperoxidase generates a variety of reactive oxygen species, increases the oxidative potential of hydrogen peroxide, and is strongly associated with the development of endothelial cell dysfunction in laboratory animals and humans. [25-28] For example, MPO activity is linked with a reduction in the bioavailability of nitric oxide, inhibition of nitric oxide synthase activity, and the development of atherosclerotic cardiovascular disease in people.[25,29] Thus, it is likely that increased local activity of MPO plays a prominent role in the development of laminar microvascular dysfunction in horses developing acute laminitis.

To our knowledge, this is the first report on MPO concentrations and activities in horses with experimentally induced laminitis, and, therefore, serves as another step in the on-going effort to elucidate the pathophysiologic causes of acute laminitis. Because the increases in MPO concentration and activity occurred in the skin and laminar tissue, these findings provide support for future studies designed to determine whether MPO concentrations and activities in skin specimens can be used as biochemical markers of the inflammatory processes involved in the pathogenesis of acute laminitis. With this approach, it may be possible to monitor changes in

MPO concentration and activity in serial skin biopsies as indicators of the morphologic changes in laminar tissue. The ease with which skin specimens can be obtained, compared with the collection of laminar tissue, may facilitate identification of horses at increased risk of developing acute laminitis or for assessing the efficacy of new treatment modalities designed to minimize activation and extravasation of WBCs.

a. Becton Dickinson, Franklin Lakes, NJ

b. Glen Mills, Inc, Clifton, NJ

c Microplate wells, Thermo Labsystems, Waltham, MA

d. Sigma, St Louis, MO

e. Multiscan Ascent plate reader, Thermo Labsystems, Waltham, MA

f. Molecular Probes, Eugene, OR

g. Fluoroscan Ascent instrument, Thermo Labsystems, Waltham, MA

h. GraphPad Prism, version 3.03, GraphPad Software, San Diego, California

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Table 3.1—Mean ± SEM values for MPO tissue concentrations and activities in laminar tissue and skin.

	ELISA		SIEFED	
Group	**Lamina (ng/mL)**	**Skin (ng/mL)**	**Lamina (mU/mL)**	**Skin (mU/mL)**
Control	236.3 ± 61.73	196.4 ± 51.5	9.4 ± 1.6	7.7 ± 1.1
1.5-h BWHE	651.4 ± 335.6	516.4 ± 153.9	53.0 ± 12.7*	39.7 ± 4.8*
3-h BWHE	3342.9 ± 1726.8*	4122.5 ± 1559.0*	92.7 ± 11.1*	107.3 ± 25.2*
12-h BWHE	2455.5 ± 1504.1*	6168.5 ± 1913.2*	60.0 ± 14.4*	68.7 ± 14.3*

* Significantly different from control group.

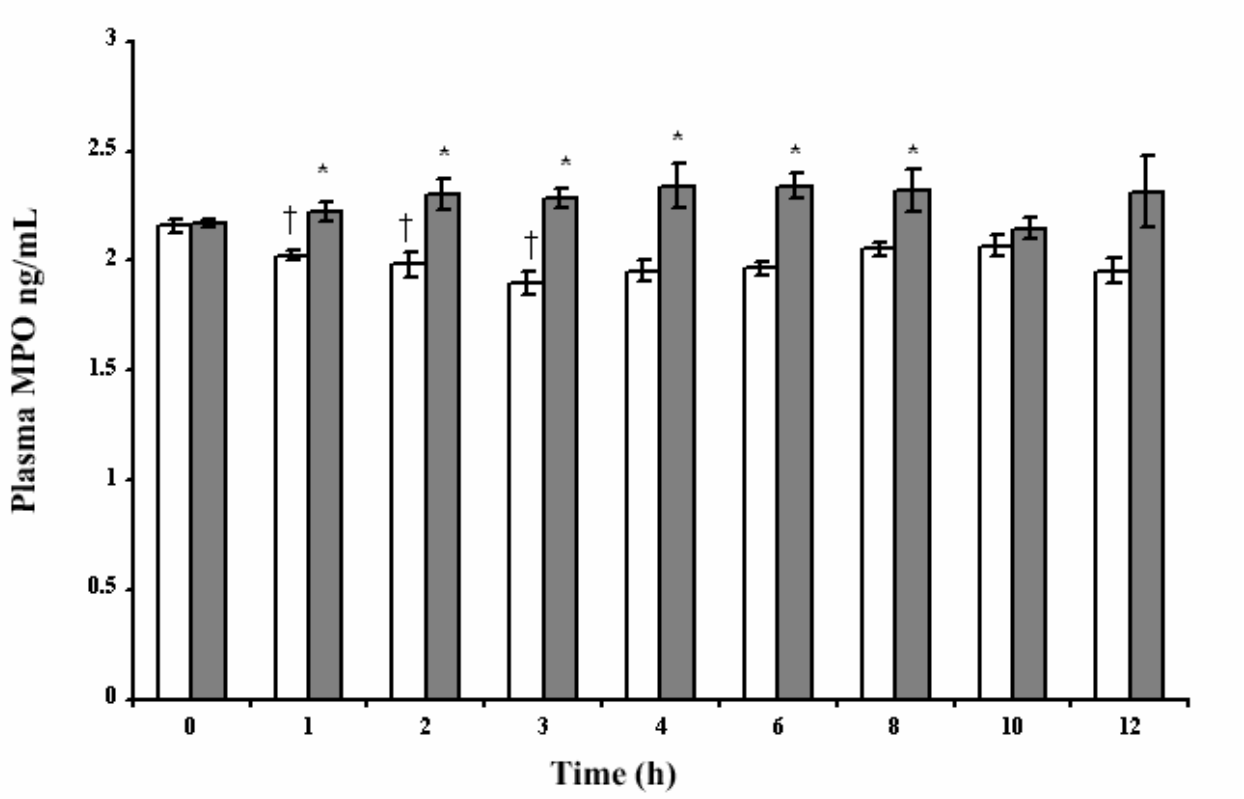

Figure 1. Mean log concentration ± SEM of MPO in plasma samples from control horses (white bars) and BWHE-treated horses (grey bars) for described time points. * represent means significantly higher than Time 0 values and † represent means significantly lower than Time 0 values. ($p<0.05$)

Figure 3.1

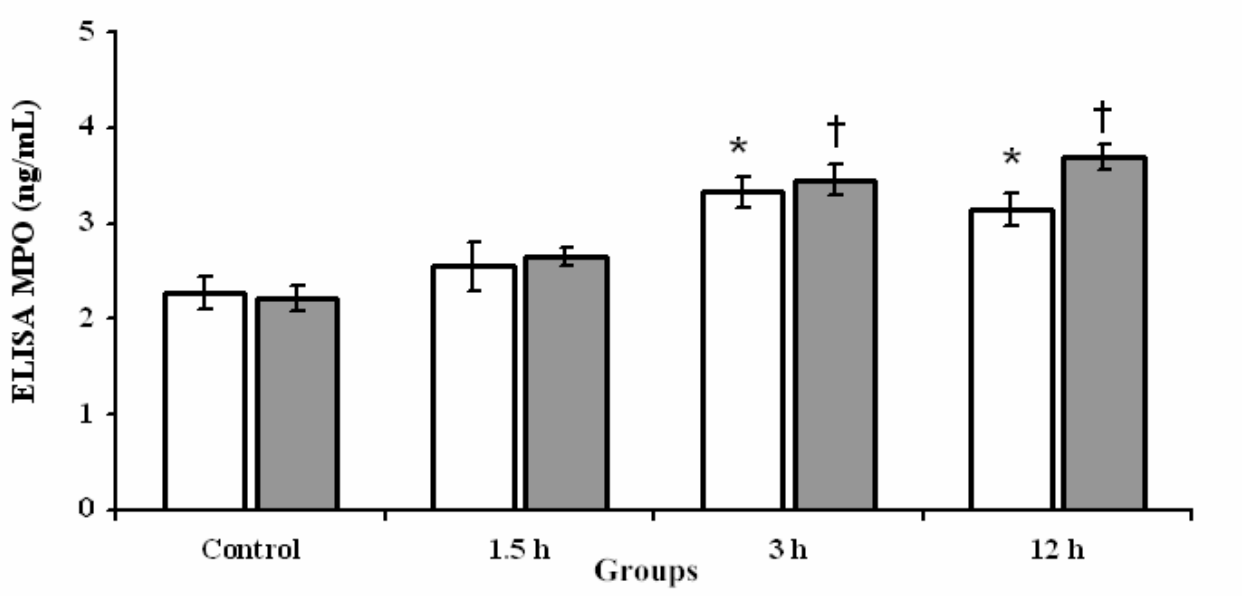

Figure 2. Mean log concentration ± SEM of MPO in laminar tissue (white bars) and skin (grey bars) from control and BWHE-treated horses for described time points determined by ELISA. * represent means significantly higher than Control values in laminar tissue and † represent means significantly higher than control values in skin. ($p<0.05$)

Figure 3.2

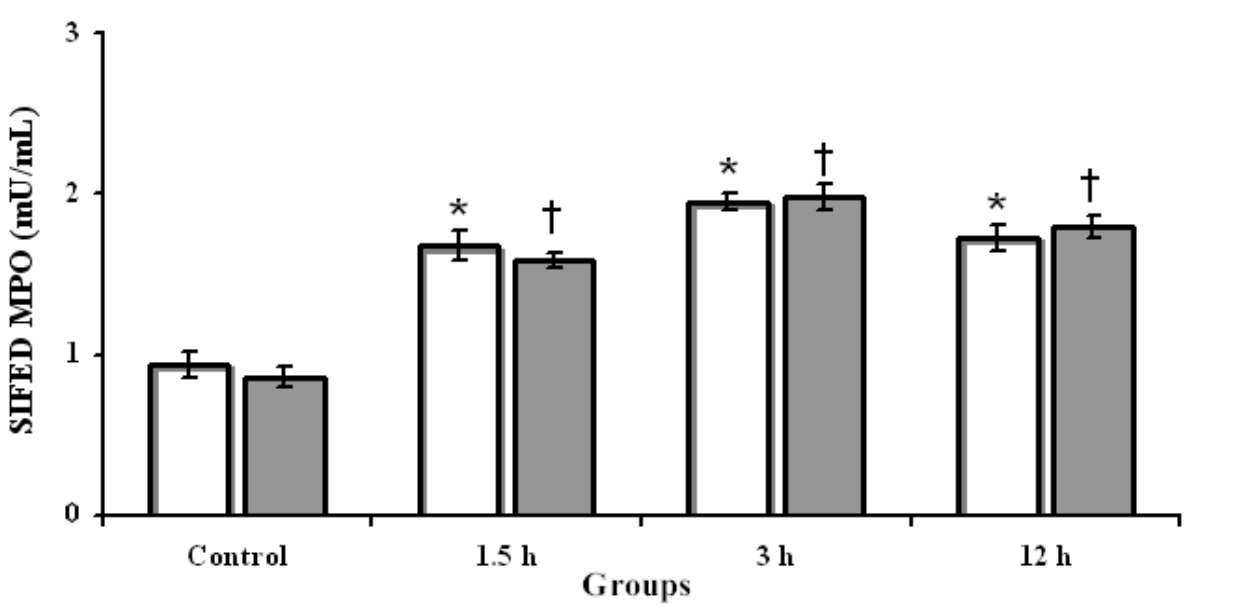

Figure 3. Mean log activity ± SEM of MPO in laminar tissue (white bars) and skin (grey bars) from control and BWHE-treated horses for described time points determined by SIEFED. * represent means significantly higher than Control values in laminar tissue and † represent means significantly higher than control values in skin. ($p<0.05$)

Figure 3.3

CHAPTER FOUR

BIOCHEMICAL MARKERS OF LEUKOCYTE PRESENCE IN HORSES GIVEN BLACK WALNUT HEARTWOOD EXTRACT AND LIPOPOLYSACCHARIDE [1]

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ABSTRACT

The pathogenesis of acute laminitis in horses is complex and poorly understood. As a component of the integumentary system, the hoof, like the skin, is composed of epidermis, dermis, and subcutaneous tissue. For this reason, it may be possible to identify alterations occurring in the skin that correlate with derangements in the laminae. Thus, examination of these two tissues may allow identification of pathologic mechanisms affecting the integument during the onset of laminitis. The equine hoof is a specialized modification of the integumentary system and includes interdigitations of the dermis and epidermis. The purpose of this study was to further define the presence of leukocytes and their enzymatic products in the skin and laminar tissues of horses administered black walnut heartwood extract and compare those findings to horses administered purified lipopolysaccharide (LPS). Data obtained in the present study indicate that CD13-positive leukocytes and MMP-9 expression and activity, increased in laminar tissue, are also increased in skin from horses with experimentally induced laminitis. Additionally, these increases were not evident in horses treated with LPS. Results of the LPS group were not significantly different from Control tissues. The results of this study show that the patterns of leukocyte emigration are similar in skin and laminar tissue collected from the black walnut extract model. Furthermore, these biochemical events are not occurring in experimental acute endotoxemia. The ease with which skin specimens can be obtained, compared with the collection of laminar tissue, may facilitate identification of horses at increased risk of developing acute laminitis or for assessing the efficacy of new treatment modalities designed to minimize activation and extravasation of WBCs.

INTRODUCTION

The pathogenesis of acute laminitis in horses is complex and poorly understood. Most research efforts aimed at elucidating the pathophysiology of laminitis have focused on local vascular derangements or on biochemical and histological changes that occur within the hoof capsule during the development of the disease.[1-5] As a component of the integumentary system, the hoof, like the skin, is composed of epidermis, dermis, and subcutaneous tissue. For this reason, it may be possible to identify alterations occurring in the skin that correlate with derangements in the laminae. Thus, examination of these two tissues may allow identification of pathologic mechanisms affecting the integument during the onset of laminitis. [6]

The main cellular components of the integument are the keratinocytes. These cells proliferate in the integument in a process known as keratinization, producing cytokeratins. More than 30 different cytokeratins have been recognized in human integument [7], each contributing to the mechanical properties of their respective tissues. The patterns of expression of cytokeratin subtypes in both healthy horses and those affected by acute laminitis were characterized for chestnut and periople.[8] Both tissues exhibited a similar cytokeratin expression pattern in the basal cells in stratum externum matrix that were significantly different between healthy horses and horses with acute laminitis. The information gleaned from that study indicates that subsets of the integumentary system mature and function in similar ways and that similar changes in expression of cytokeratins occur in these tissues in horses with laminitis.

In a recent study, intradermal skin testing revealed increased hyperreactivity responses to intradermal antigen challenge in horses with chronic laminitis when compared

to healthy control horses [9] A relationship between cutaneous and laminar lesions also exists in the skin blistering disorder of Belgian horses called junctional epidermolysis bullosa.[10] In that disease, the absence of an architectural protein in the integument, laminin 5, causes disruption of the basement membrane in both skin and laminar tissues in a manner similar to that seen in histological studies of laminar tissue from horses with laminitis.[5]

In the integument, the avascular epidermis is connected to the vascular dermis by the basement membrane and receives nutrients through the same route. The equine hoof is a specialized modification of the integumentary system and includes interdigitations of the dermis and epidermis termed laminae. These interdigitations dramatically increase surface area, dissipate the resistance to load, and increase the weight bearing strength of the hoof.[11,12] The results of previous studies of acute laminitis indicate that enzymatic degradation of the basement membrane of the hoof leads to dermal-epidermal separation and the subsequent loss of the integrity of laminar interdigitations leading to compromised weight bearing. [5,13]

Proteases such as matrix metalloproteinase-2 and -9 (MMP-2 and –9), have been implicated in the disruption of the basement membrane that occurs during laminitis.[14-17] The MMPs are secreted in a latent (pro-MMP) form, and require an activation step before they gain full enzymatic activity. MMP-2 is present in normal laminar tissue, constitutively produced by cells such as keratinocytes and fibroblasts, and is involved in normal growth and remodeling of the hoof. MMP-9 is produced by neutrophils, stored in tertiary granules and released upon cellular activation.

In recent studies of acute laminitis, the presence of MMP-2 and 9 in laminar tissue has varied depending on the origin of the disease (naturally-occurring cases, carbohydrate overload-induced or black walnut extract-induced). Using SDS-PAGE gelatin zymography, increased levels

of MMP 2 and 9 were demonstrated in laminar tissue obtained from several horses with naturally occurring acute and chronic laminitis [18] and in two horses with experimentally-induced carbohydrate overload laminitis [19]. Tissues obtained from the horses with carbohydrate overload induced laminitis also had increased expression of MMP-2, although the authors did not indicate the timepoint at which the samples were collected.[20] In contrast, laminar tissue obtained from horses after administration of black walnut extract had increased expression and activity of MMP-9 but not MMP-2, as determined by RT-PCR and gelatin zymography. [15]

The most recent laminitis research provides evidence that the activation of peripheral blood leukocytes is involved in the development of acute laminitis. The experimental induction of laminitis with black walnut heartwood extract has been associated with initiation of a peripheral leukopenia [21] followed by the presence of increased myeloperoxidase and matrix metalloproteinase-9 activity in both skin and laminar tissue.[6,15] Both enzymes are produced in neutrophils, stored in cytoplasmic granules and released upon cellular activation. These enzymes are associated with the induction of experimental laminitis, but are not the only event involving the activation of leukocytes that affect the equine patient. For example, endotoxemia, as occurs in horses with a variety of gastrointestinal diseases, is characterized by activation and emigration of leukocytes from the peripheral circulation. As well, in a recent multivariate analysis of risk factors for development of acute laminitis, endotoxemia was the only factor significantly associated with the disease.[22]

Horses are extremely sensitive to endotoxin (lypopolysaccharide, LPS) and, intuitively, researchers questioned whether gram-negative endotoxin and the subsequent overwhelming reaction of the equid to it was a directly causative factor in the development of laminitis. Regrettably but not surprisingly the pathogenesis of the disease is not that simple.

Administration of 20-30 ng/kg results in hyperthermia, tachypnea, tachycardia and neutropenia, clinical signs associated with sepsis in equids. However, the infusion of purified *Escherichia coli* endotoxin does not cause laminitis. [23]. The link is clearly present but is not directly a causative one. Regardless, any hypotheses regarding the pathogenesis of the disease must take in to account the association between endotoxemia and laminitis. It is important to discern whether biochemical events link to laminitis, such as leukocyte emigration and matrix metalloproteinase activity are also occurring in experimental endotoxemia. By comparing leukocyte emigration and matrix metalloproteinase activity in experimentally endotoxemic horses to previously published data from horses with experimentally induced Obel grade 1 laminitis, we will determine whether leukocyte emigration into the integument is similar in endotoxemia and acute laminitis.

The purpose of this study was to further define the presence of leukocytes and their enzymatic products in the skin and laminar tissues of horses administered black walnut heartwood extract and compare those findings to horses administered purified lipopolysaccharide. First, the presence of leukocyte surface antigen CD13 was evaluated in skin and laminar tissues of horses administered either black walnut heartwood extract or LPS. Second, gene expression and enzymatic activity of MMP-2 and MMP-9 were quantified in skin and laminar tissues from the same treatment groups. We hypothesized that (1) CD13 protein concentration and labeled cells, and MMP gene expression and enzymatic activity would be increased to a similar extent in skin and laminar tissues of horses administered black walnut extract and (2) horses given LPS would not show similar increases in sampled tissues.

MATERIALS AND METHODS

Animals—25 healthy horses ranging in age from 5 to 15 years were used in this study. All horses included were free of existing lameness and lacked clinical evidence of systemic inflammatory disease. No radiographic evidence of pre-existing laminitis was present on survey lateral and dorsopalmar radiographic views of the forelimb digits. Prior to the start of the study, the middle region of the neck was clipped and an iv catheter was placed in the left jugular vein. The study was approved by the University of Georgia Animal Care and Use Committee.

Black walnut extract preparation- The extract was prepared as described previously.[24] Briefly, 1 kg of black walnut heartwood shavings was agitated in 7 L of water at room temperature (approx. 22°C) for 24 hours. An aqueous filtrate was then obtained by filtering the solution through cheesecloth, and 6 liters of the resulting extract were administered by nasogastric tube.

Experimental design—Horses were randomly assigned to 3 groups as follows: Control (n=10), Black walnut heartwood extract (BWHE, n=10) and lipopolysaccharide (LPS, n=5). Horses in the Control group received 6 L of water via nasogastric intubation, and were euthanized 12-hours later. Horses in the LPS group received 20ng O55:B5 E. coli endotoxin/kg bwt as a continuous i.v. infusion in 500 ml 0.9% saline through the jugular vein catheter.

Horses in the BWHE group were euthanatized at the onset of Obel grade-1 laminitis (clinical signs consisting of weight shifting and bounding digital pulses without evidence of lameness at a walk) or at 12 hours after administration, if signs of Obel grade-1 laminitis had not developed by that time. Horses in the LPS group were euthanized at 12 hours after administration. Each horse was evaluated prior to the start of the study and every hour

thereafter for attitude, heart rate, respiratory rate, capillary refill time, hoof temperature, digital pulses, and evidence of lameness consistent with Obel grade-1 laminitis. Blood samples were taken hourly for complete blood count determination. All horses were euthanatized with a penetrating captive bolt, in compliance with guidelines outlined in the *2000 Report of the AVMA Panel on Euthanasia*.

Collection of laminar tissue and skin—Both forelimbs were disarticulated at the level of the metacarpophalangeal joint and the hooves cut into sections with a band saw. During the procedure, thermal damage was minimized by constant irrigation of the tissue with ice-cold physiologic salt solution containing the following: 118mM NaCl, 24mM $NaHCO_3$, 1mM $MgSO_4$, 0.435mM NaH_2PO_4, 5.56mM glucose, 1.8mM $CaCl_2$, and 4mM KCl. Two full thickness segments from each forelimb foot were then placed in the ice-cold physiologic salt solution. The hard keratinized portion of the hoof and distal phalanx were removed and specimens of laminar tissue removed by sharp dissection. Concurrently, a second investigator removed full thickness, 6 cm × 6 cm sections of skin from the middle region of the neck. These specimens were also placed in the ice-cold physiologic salt solution. Half of the laminar and skin tissue samples were rapidly frozen in liquid nitrogen and stored at –80˚C until assayed. The remaining tissue was fixed in 10% buffered neutral formalin for immunohistochemical analysis.

Real-Time PCR

Laminar and skin samples were prepared using a protocol for fibrous tissue. The samples were prepared by first fragmenting the frozen tissue with a razor blade followed by homogenization with a motorized mortar and pestle. (Pellet Pestle, Kontes Glass Co.,) Gene

expression was quantified in a two-step reverse transcription-polymerase chain reaction (RT-PCR). Total RNA extraction was performed using the Versagene Total RNA Purification Kit (Gentra Systems, Minneapolis, Minnesota) according to the manufacturer's protocol. Reverse transcription for production of cDNA was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems , Foster City, California). Oligonucleotide primers and Taqman probes (Applied Biosystems, Foster City, California) were designed with Primer Express software (Perkin-Elmer Biosystems, Foster City, CA) using equine MMP-2 and MMP-9 sequences obtained from GenBank.

Quantitative real-time PCR was performed in duplicate on each sample using the ABI Prism 7700 sequence detection system. Reactions were performed using the reference gene 18s rRNA (Applied Biosystems, Foster City, California) as an endogenous control .

Gelatin Zymography

Frozen tissue samples were homogenized using a polytron tissue homogenizer (Glen Mills, Inc, Clifton, NJ) in Western lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 6 mM sodium deoxycholate,1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 20mg/ml aprotinin, and 20 mg/ml leupeptin. After homogenization, the samples were sonicated and centrifuged at 14,000 g for 15 min at 4°C. A Bradford protein assay was performed on the supernatant which was then aliquoted and stored frozen at -80°C.

8% SDS-PAGE gels containing 0.1% gelatin were prepared with a 4% stacking gel. Samples were loaded at 20µg of protein per lane and electrophoresed at 100 volts for 120 minutes. Following electrophoresis, each gel was washed 2 x 30 min in 2.5% Triton X-100 on a rocking platform to remove SDS. Gels were then incubated for 16 hours in 100mM

Tris/HCl, pH=8.0, 5mM CaCl2, 0.005% Brij-35 and 0.001% NaN3 at 37C and subsequently stained in Coomassie Brilliant Blue R-250 staining solution (Bio-Rad, Richmond, CA) for 60 minutes. Gels were destained on a rocking platform in 5% methanol and 7.5% acetic acid for 2 hours. Gels were imaged and recorded using a Bio-Rad Fluor-S Max 2 MultiImager (Bio-Rad, Richmond, CA) . The specificity of the enzymatic activity was validated by incubating samples in a solution lacking calcium, necessary for gelatinase enzymatic activity. This resulted in zero enzymatic activity for the gelatin substrate (data not shown)

Western blot analysis

Samples containing 20 μg of protein from previously homogenized tissue were separated by 12% SDS-polyacrylamide gel electrophoresis, electrophoretically transferred to a membrane, and incubated with anti-equine CD13 monoclonal antibody (AbD Serotec, Raleigh, NC). Following antibody treatments, blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Pierce Biotechnology, Rockford, IL). Blots were then treated with superSignal West Dura extended duration chemiluminescence substrate solution (Pierce Biotechnology, Rockford, IL) and analyzed using a Fluor-S Max2 MultiImager system (Bio-Rad, Richmond, CA). A standard quantity of equine leukocyte-rich plasma was included on each immunoblot as a positive control.

Immunohistochemistry

Full thickness formalin-fixed tissues were paraffin-embedded and sectioned for immunohistochemical analysis with anti-equine CD13 monoclonal antibody (AbD Serotec, Raleigh, NC). Sections were deparaffinized and endogenous peroxidase activity was blocked with 1% hydrogen peroxide in water. Heat-induced epitope retrieval (HIER) with sodium citrate buffer was performed followed by blocking with 10% normal goat serum prior

to antibody incubation. Sections were incubated with primary antibody for 16 hours at 4°C, followed by biotinylated mouse anti-goat secondary antibody (AbD Serotec, Raleigh, NC) and avidin-biotin-peroxidase complex. Staining was performed using DAB as the peroxidase substrate and slides were counterstained with hematoxylin. An investigator (CLJ) was blinded and slides were evaluated for presence and number of CD-13 positive cells. The evaluation was performed by centering on a dermal vessel with the 40X objective and counting all CD-13 positive cells in that field. Five fields per tissue sample were evaluated and averaged.

Statistical Analysis

Initially the BWHE horses were subdivided into two groups, those that did not show signs of laminitis and were euthanized 12 hours after administration of the extract (BWHE-NL), and horses that were euthanized at the onset of Obel Grade 1 laminitis (BWHE-L). Statistical analysis of these two groups revealed no significant difference between them and both were significantly different from control. Therefore, the groups were recombined and are designated the BWHE group.

RT-PCR

Gene expression data for MMP-2 and MMP-9 were normalized to 18s rRNA and analyzed relative to control tissues using the $2^{-\Delta\Delta C}$T method.[25] For RT-PCR data, a two-tailed t-test was performed to analyze data from each treatment group for MMP-2 and MMP-9 for skin and lamina from each horse. For all comparisons, a value of $P \leq 0.05$ was considered significant.

Gelatin zymography

MMP-2 and MMP-9 bands were analyzed using Quantity One Quantitation Software (Bio-Rad, CA). Volume intensity was measured for each band, adjusted for background stain and normalized to a positive control (MMP-2/9 human, company) included on each gel. Results were reported as arbitrary units of optical density (ODu). MMP-2 and MMP-9 data for skin and laminar tissues from horses in the CON, BWHE and LPS groups were compared using a 1-way ANOVA with a Tukey-Kramer post-test (GraphPad Prism Software, San Diego, California). A value of $P \leq 0.05$ was considered significant.

Immunohistochemistry

CD13 data for skin and laminar tissues from horses in the CON, BWHE and LPS groups were compared using a 1-way ANOVA with a Tukey-Kramer post-test. A value of $P \leq 0.05$ was considered significant.

RESULTS

The horses in the Control group did not show signs consistent with Obel grade-1 laminitis and all were euthanized at 12 hours. Five horses in the BWHE group (BWHE-L) demonstrated signs consistent with Obel grade 1 laminitis, were euthanized between 9 and 12 hours after administration of the extract and are designated BWHE-L. The remaining five BWHE horses did not become lame (BWHE-NL) and were euthanized at 12 hours post-induction. Horses in the LPS group did not show signs consistent with Obel grade-1 laminitis and all were euthanized at 12 hours. Complete blood counts from horses in the LPS consistently revealed a leukopenia of at least 30 % similar to that seen in the BWHE induction model (data not shown).

MMP-2 and MMP-9 RT-PCR Results

A significant increase in MMP-2 gene expression (p-value <0.0001) was noted in the laminae of the BWHE group compared to the Control group. In contrast, there was not a statistically significant increase in MMP-2 gene expression in BWHE skin samples taken from the same horses. There were significant increases in MMP-9 gene expression in both laminar tissue and skin in the BWHE group (p-values of <0.0001 and 0.034, respectively). MMP-2 and -9 gene expression was significantly decreased in laminar tissue and skin from the LPS group compared to the Control group. The RT-PCR data are reported for MMP-2 and MMP-9 as fold-changes relative to control values in Table 1 and Table 2, respectively.

Zymographic Activity

There were no significant differences in mean MMP-2 zymogen activity in laminar and skin samples between the treatment groups (Figures 1 and 2). There was a very weak correlation (r^2=0.18) for MMP-2 zymogen activity levels between skin and laminar samples from individual animals.

Mean MMP-9 zymogen (92 kd) activity in laminae and skin samples from horses in the Control group were not significantly different from each other. (Figures 3 and 4) However, there was a significant increase in MMP-9 zymogen activity in the BWHE-treated laminar and skin samples when compared to the control group. For both laminar and skin samples, mean MMP-9 zymogen activity was not significantly increased in the LPS group. A weak correlation (r^2=0.42) was present between skin and laminar samples for MMP-9 zymogen activity from individual animals.

CD13 Western blot analysis

A significant increase in CD13 expression was noted in the skin and laminar samples from the BWHE group when compared to control samples (Figure 5). In contrast, no differences in CD13 concentrations were evident in laminar or skin samples from the LPS group when compared to control tissues (Figure 6).

CD13 Immunohistochemistry

CD13 –positive cells were present in laminar tissue and skin sections from each treatment group. CD13-positive cells in laminar tissue appear to be exclusively myeloid cells and are present only in dermal layers while the laminar epidermis contains no CD13 positive cells. The skin samples, like laminar tissue, contain CD13-positive myeloid cells, as well as CD13-positive cells within the sebaceous glands, dermal root sheath of hair follicles, and stratum basale of the epidermis. The distribution of CD13-positive non-myeloid cells did not vary between treatment groups while the distribution of positive myeloid cells within the tissues varied between intravascular and extravascular locations depending on each group evaluated (fig 7). The morphology of CD13-positive leukocytes included a mixed population of both neutrophils and monocytes. All skin sections evaluated contained both intravascular and extravascular CD13-positive myeloid cells but varied in number determined by treatment group. In contrast, all laminar tissue sections contained intravascular CD-13 positive cells but the presence of positively-stained cells in extravascular locations varied between groups.

Control group: In laminar tissue, CD13-positive myeloid cells were noted only within the dermal vasculature. No extravascular cells were found in the laminae but were found in low

numbers in skin samples from the Control group. These cells were located in the superficial dermis at the level of sebaceous glands and hair follicles along with intravascular myeloid cells.

LPS group: Laminar tissue contained intravascular positively-stained myeloid cells and rarely an extravascular positive cell, approximately 0-2 per tissue section. Skin from the LPS group contained low numbers of CD13-positive cells, similar in distribution and number to those cells noted in the Control group skin samples.

BWHE group: Tissues, both laminar and skin, from the BWHE group contained marked numbers of CD-13 positive myeloid cells both within and surrounding the dermal vessels. In laminar sections positive cells were located predominately along the vessels of the primary dermal laminae and in deeper sections of the dermis. In skin sections of the BWHE group, like those of the Control and LPS groups, CD13-positive myeloid cells were located in the superficial dermis at the level of sebaceous glands and hair follicles along with intravascular myeloid cells. However, in the BWHE group, the number of cells was markedly increased.

DISCUSSION

Results obtained in the present study provide overwhelming evidence that significant biochemical abnormalities associated with the development of laminitis occur in the skin as well as laminar tissue. Historically, laminitis researchers have assumed the uniqueness of laminar tissue to explain the predilection of the disease's manifestation in the laminae. While modifications based on the specialized functions of the individual tissues do exist it is

apparent that there are commonalities within integumentary tissues with regards to the development of laminitis. Several studies have shown MMP-9 expression and activity to be increased in laminar tissue.[17,19,26] The present study indicates that MMP-9 expression and activity are also increased in skin from horses with experimentally induced laminitis. Unlike the results of another study in which MMP-9 activity was not identified in laminar tissue from Control horses [15], this study demonstrated a low but evident basal level of MMP-9 zymogen in both skin and laminae of healthy horses. The difference between results of this study and others may be due to slight differences in zymography protocols allowing for increased sensitivity in the present study. Regardless, MMP transcription and activities in skin and laminar tissue of horses in the Control group were low, consistent with recent reports that relatively few neutrophils are present in these tissues in healthy horses.[27,28] Although the direct correlation in skin and laminae for ODu values measuring MMP-9 activity was moderate (r^2=0.4200), the variation in results obtained between individual animals and the small sizes of the treatment groups weakened the strength of the correlation between skin and laminae tissue samples, and there appears to be prominent distinction between control and treatment values for both tissue types. Additionally, the significant increase in MMP-9 in these tissues occurred regardless of the degree of lameness exhibited by the horse in the developmental phase of the disease.

Horses in the developmental phase of laminitis become leukopenic at the same time that an increase in neutrophils can be detected in the integument.[6,21,27] MMP-9, myeloperoxidase and other tissue-degrading enzymes are produced by and stored in neutrophils. These enzymes are both markers of neutrophil presence and may have a contributing role in the development of laminitis. While these enzymes are present at

increased levels in the skin of horses at the time of onset of laminitis, there is no evidence of clinical disease in these tissues. The difference between the two types of integument that results in changes in laminar tissues being associated alterations in function is most likely multifocal. The skin is not subjected to the biomechanical load placed on the laminae, nor is it adapted to do so. Moreover, the vasoactive properties of the laminar microvasculature appear to be unique, as evidenced by the fact that these vessels do not react in a manner similar to large peripheral digital vessels in BWHE-induced laminitis.[29,30] The predisposition of laminar veins to vasoconstriction and the increase in digital postcapillary resistance that develops after administration of BWHE are strongly suggestive of the contribution of venous disturbances to the disease process.

The significance of the generalized involvement of the integument system is amplified by the information obtained from the inclusion of the LPS group in this study. In a recent multivariate analysis of clinical risk factors for development of acute laminitis, endotoxemia was the only factor significantly associated with the disease.[22] In this study, the administration of LPS, while causing significant leukopenia, does not cause the activation and immigration of leukocytes into the integument, as occurs following BWHE administration. The large number of CD13-positive myeloid cells surrounding dermal vessels were present in the BWHE group but were not noted in either the Control or LPS group on immunohistochemical analysis. The lack of leukocytes in the LPS tissues and the decrease in MMP-9 gene expression may be due to the apparent arterial vasoconstriction that occurs in endotoxemic horses preventing the extravasation of leukocytes into the integument.[31,32] Additionally, leukocytes do not randomly extravasate from the vasculature but are, rather, induced to migrate by chemoattractants such as complement factor 5a,

leukotriene B4, platelet activating factor, and various chemokines.[33] The administration of LPS may not, at this comparative time point, affect the local upregulation of chemoattractants in the integument causing the migration of leukocytes. Therefore, the increased immigration of leukocytes, presence of the metalloproteinase CD13 and upregulation of gene expression and activity of MMP-9 seen in BWHE-induce laminitis appear to be events distinct from those which occur in acute endotoxemia.

The data from this and previous studies [6,27] support the concept that a generalized disruption of normal physiologic processes occurs in the integument in the developmental phase of laminitis. This information and the ease by which skin biopsies can be collected in horses, suggest that histopathologic evaluation of these tissue samples may allow the skin to become a useful tool for identifying generalized alterations in the integument in horses at risk for the development of laminitis and for studying the progression of laminitis in individual animals. The early identification of at-risk patients would allow more accurate prognosis and aggressive intervention.

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Table 4.1. MMP-2 mRNA concentrations in laminar tissue and skin expressed as fold changes relative to control tissues when significant ($p<0.05$).
NS – fold change not significant.

	Lamina		Skin	
	Fold ↑or ↓	p-value	Fold ↑ or ↓	p-value
LPS	↓ 6.67	<0.0001	↓ 10.0	<0.0001
BWHE	↑ 5.54	<0.0001	NS	

Table 4.2. MMP-9 mRNA concentrations in laminar tissue and skin expressed as fold changes relative to control tissues when significant ($p<0.05$).

	Lamina		Skin	
	Fold ↑ or ↓	p-value	Fold ↑ or ↓	p-value
LPS	↓ 2.94	0.0007	↓ 3.34	0.0006
BWHE	↑ 46.3	<0.0001	2.3 ↑	0.034

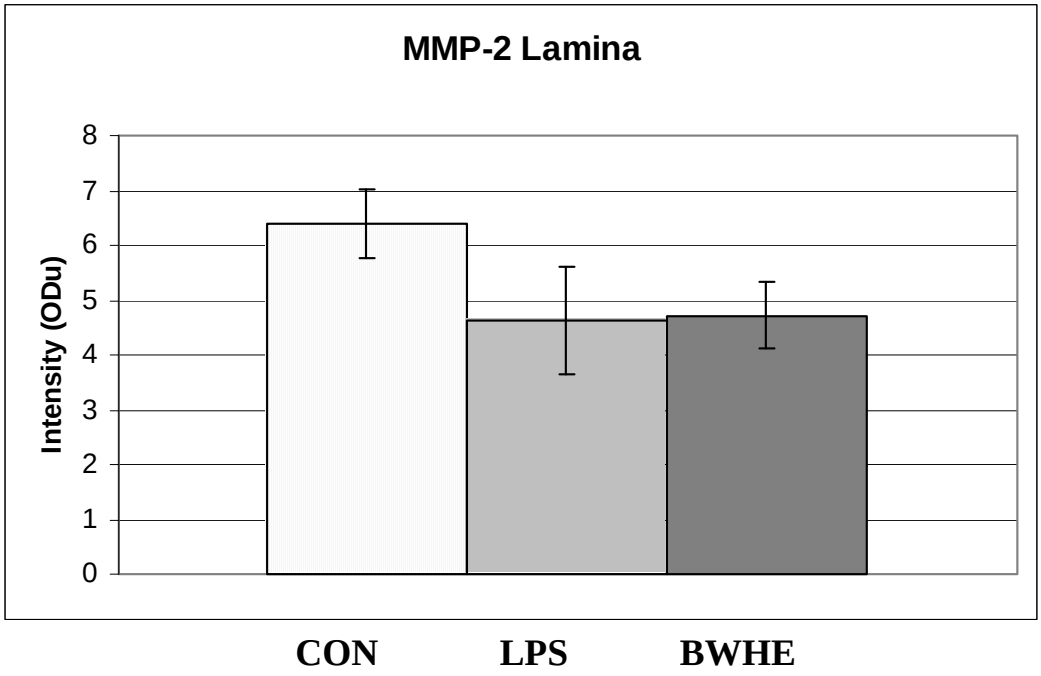

Figure 4.1. Mean intensity +/- SEM of MMP-2 zymogen activity bands in laminar tissue as determine by SDS-page gelatin zymography. * represent means significantly higher than Control values. ($p<0.05$)

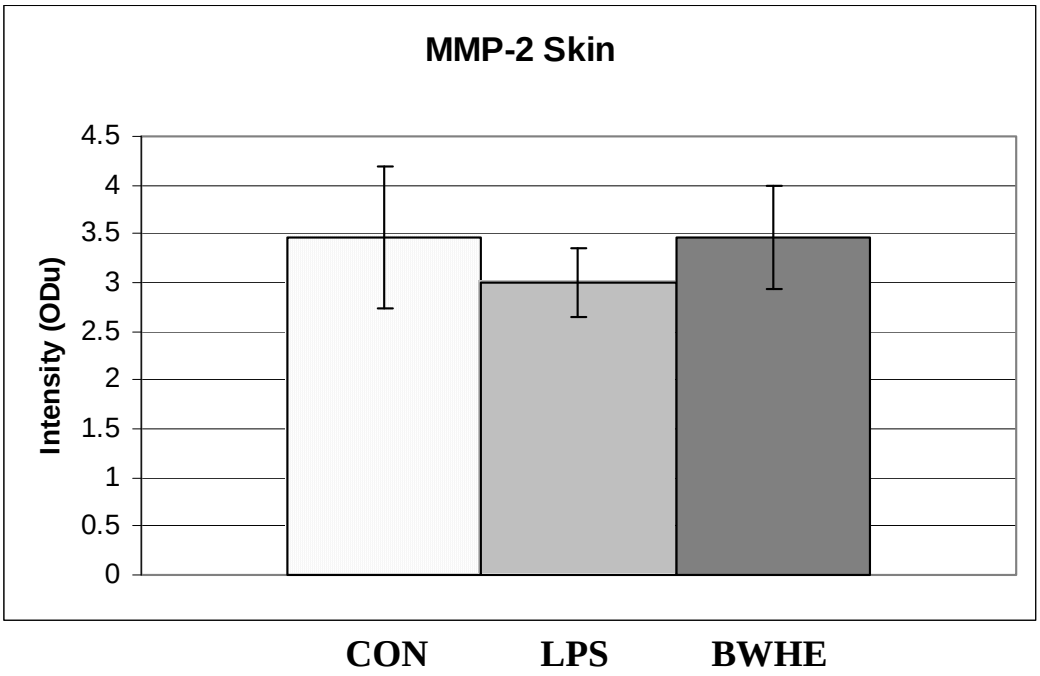

Figure 4.2. Mean intensity +/- SEM of MMP-2 zymogen activity bands in skin as determine by SDS-page gelatin zymography. There was no significant difference in mean intensity between Control, LPS and BWHE treatment groups ($p<0.05$).

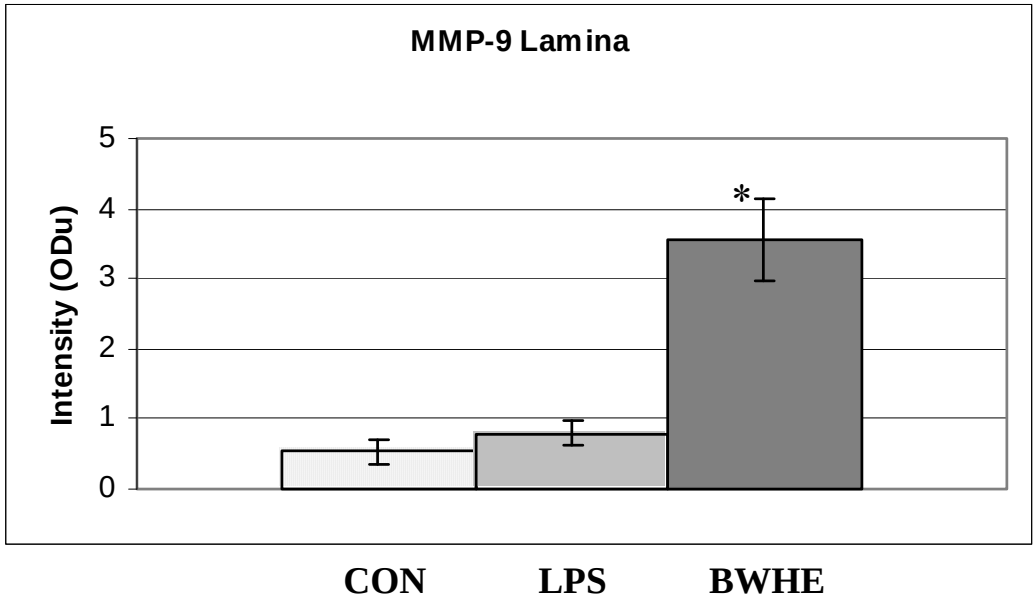

Figure 4.3. Mean intensity +/- SEM of MMP-9 zymogen activity bands in laminar tissue as determine by SDS-page gelatin zymography. * represent means significantly higher than Control values. ($p<0.05$)

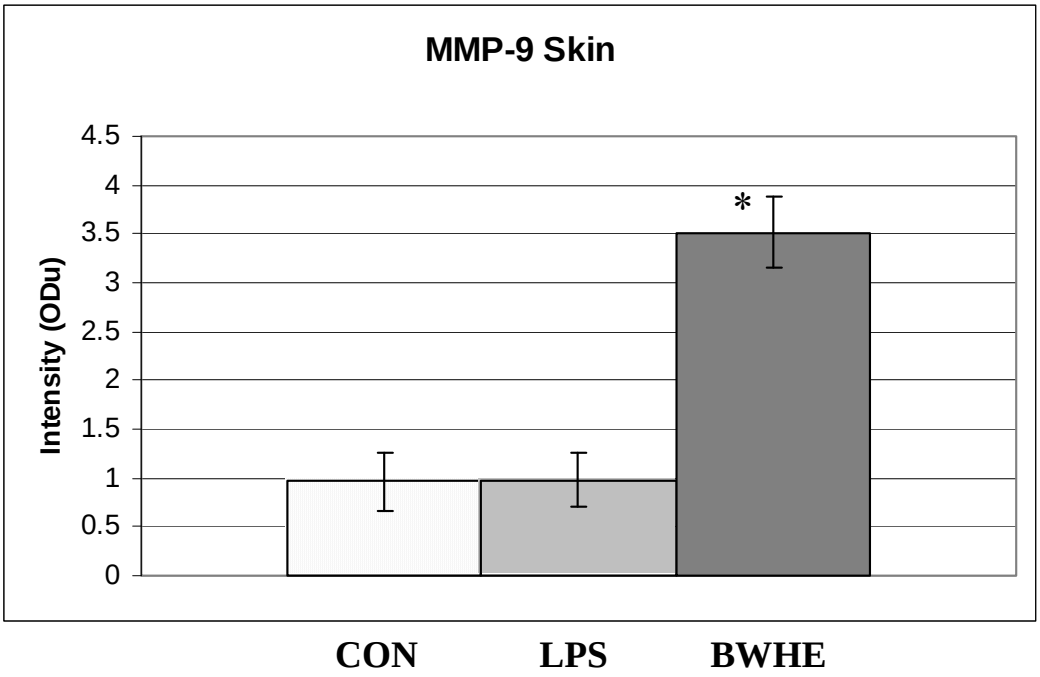

Figure 4.4. Mean intensity +/- SEM of MMP-9 zymogen activity bands in skin as determine by SDS-page gelatin zymography. * represent means significantly higher than Control values. ($p<0.05$)

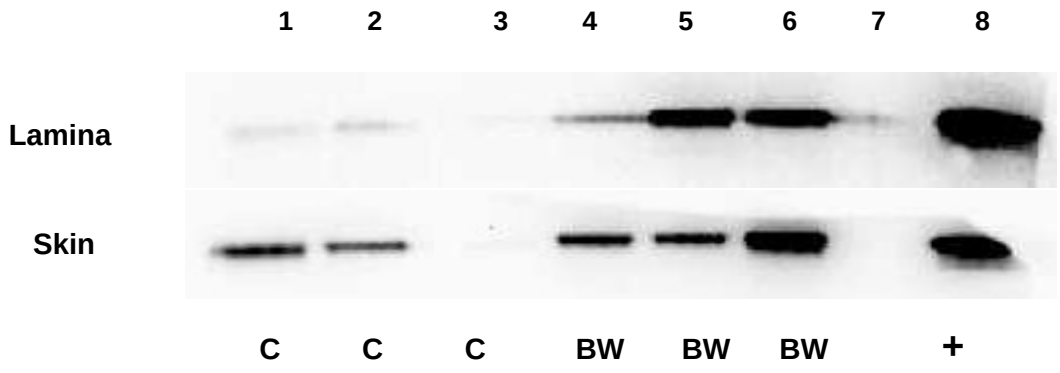

Figure 4.5. CD-13 Western hybridization. Note the difference in CD13 expression between Control (C) tissues and BWHE (BW). + (lane 8) denotes positive control.

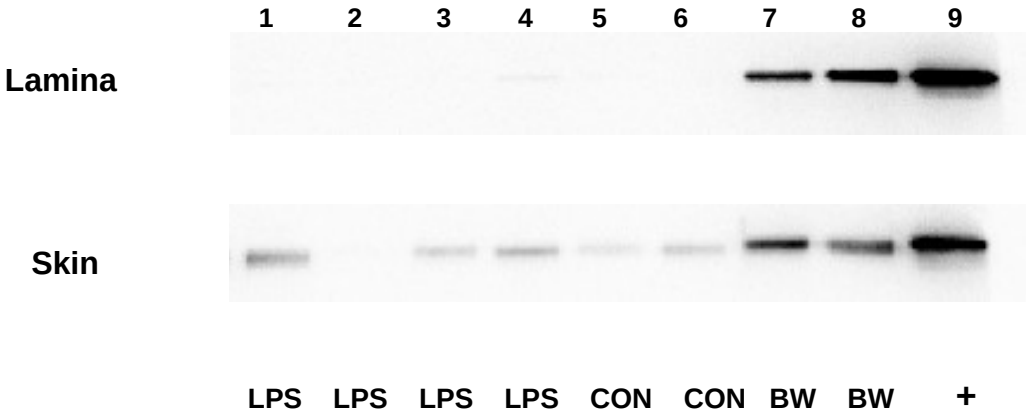

Figure 4.6. CD-13 Western hybridization. Note the similar gene expression difference in CD13 expression between Control (C) tissues and BWHE (BW). + (lane 8) denotes positive control.

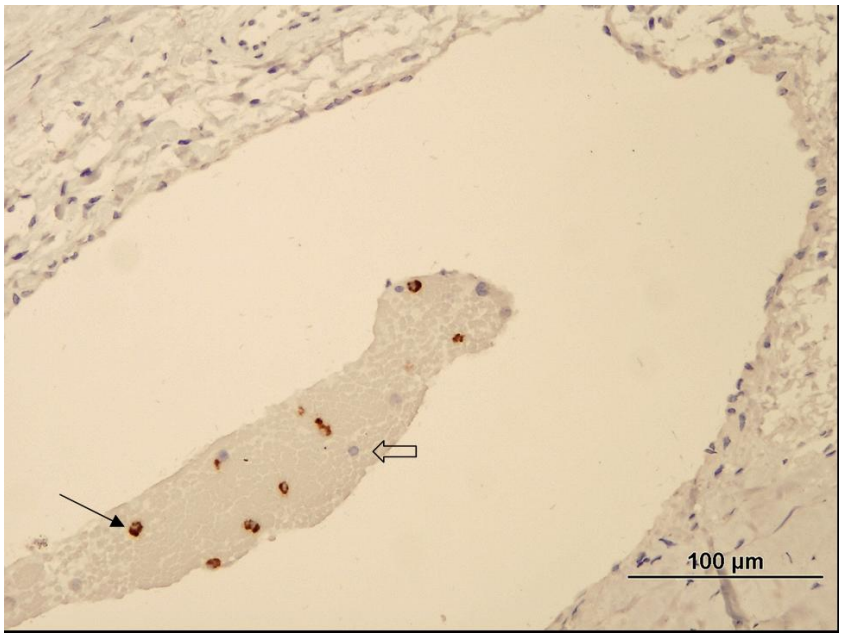

Figure 4.7. CD13-positive cells within a large venule of the dermal laminae. Note the presence of both CD-13 positive (solid arrow) and CD-13 negative (open arrow) leukocytes within the thrombus and lack of CD-13 positive cells in extravascular dermis.

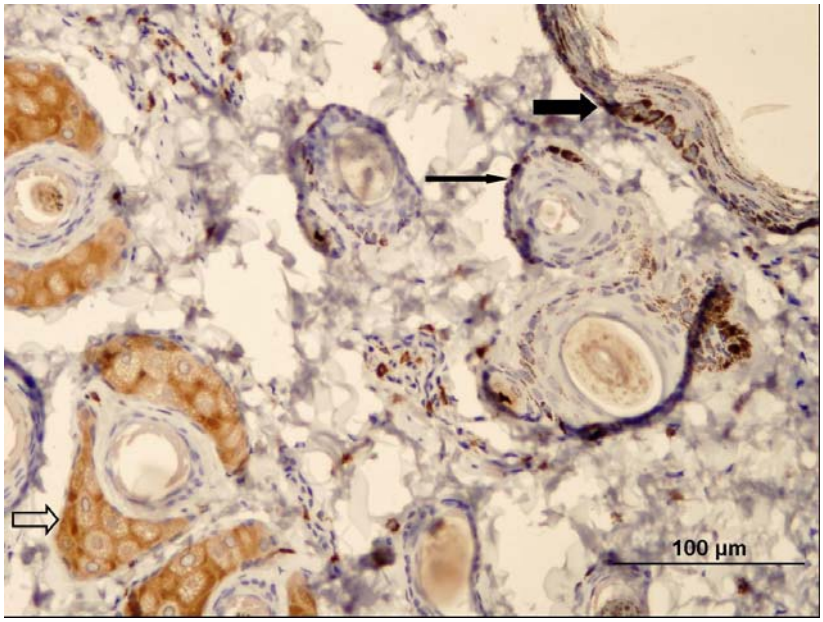

Figure 4.8. Non-myeloid CD13 positive cells in a skin sample from the Control group. Note positive staining within the sebaceous gland (open arrow), dermal root sheath of the hair follicle (narrow arrow), and stratum basale of the epidermis (wide arrow).

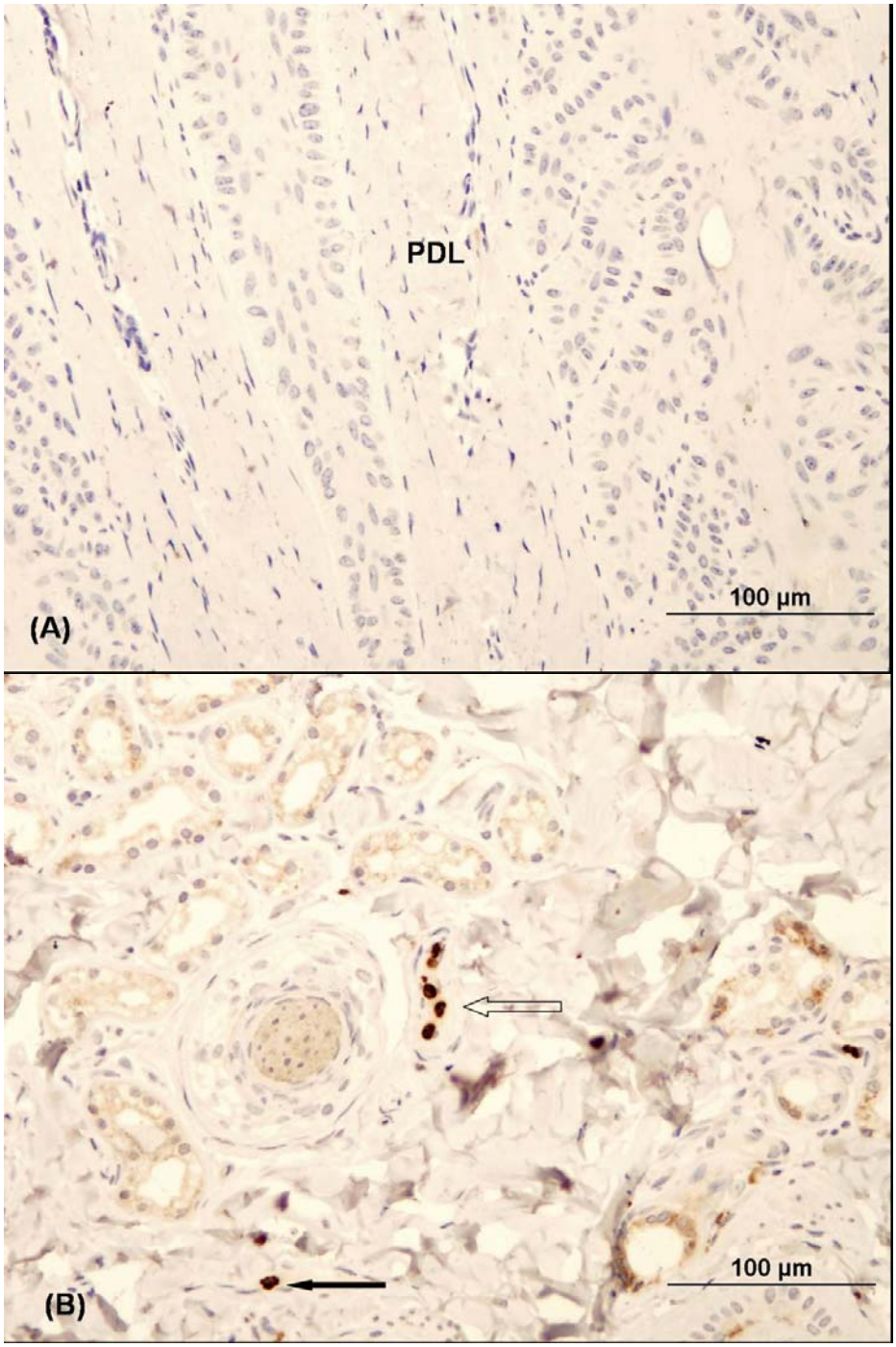

Figure 4.9. CD13 myeloid cell expression in Control laminae and skin. Laminar tissue samples (A) from the Control group contained no extravascular CD-13 positive cells. Control group skin samples contained low numbers of both intravascular (open arrow), shown in a vessel with a longitudinal orientation, and extravascular (closed arrow) CD13-positive cells.

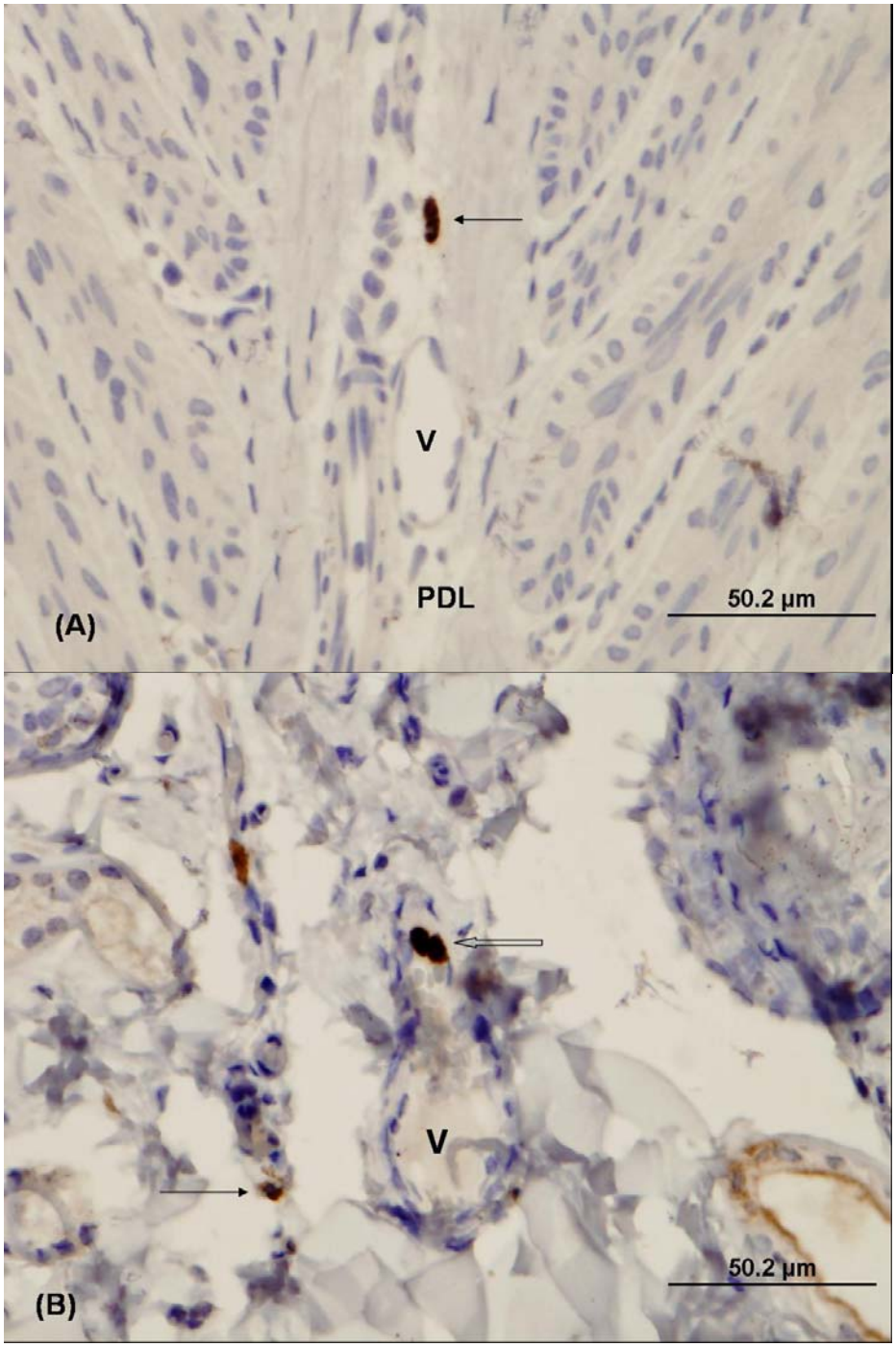

Figure 4.10. CD13 myeloid cell expression in LPS group laminae and skin. Laminar tissue samples (A) from the LPS group contained an occasional extravascular CD-13 positive cell closely associated with laminar vessels (V). LPS group skin samples contained low numbers of both intravascular (open arrow), and extravascular (closed arrow) CD13-positive cells.

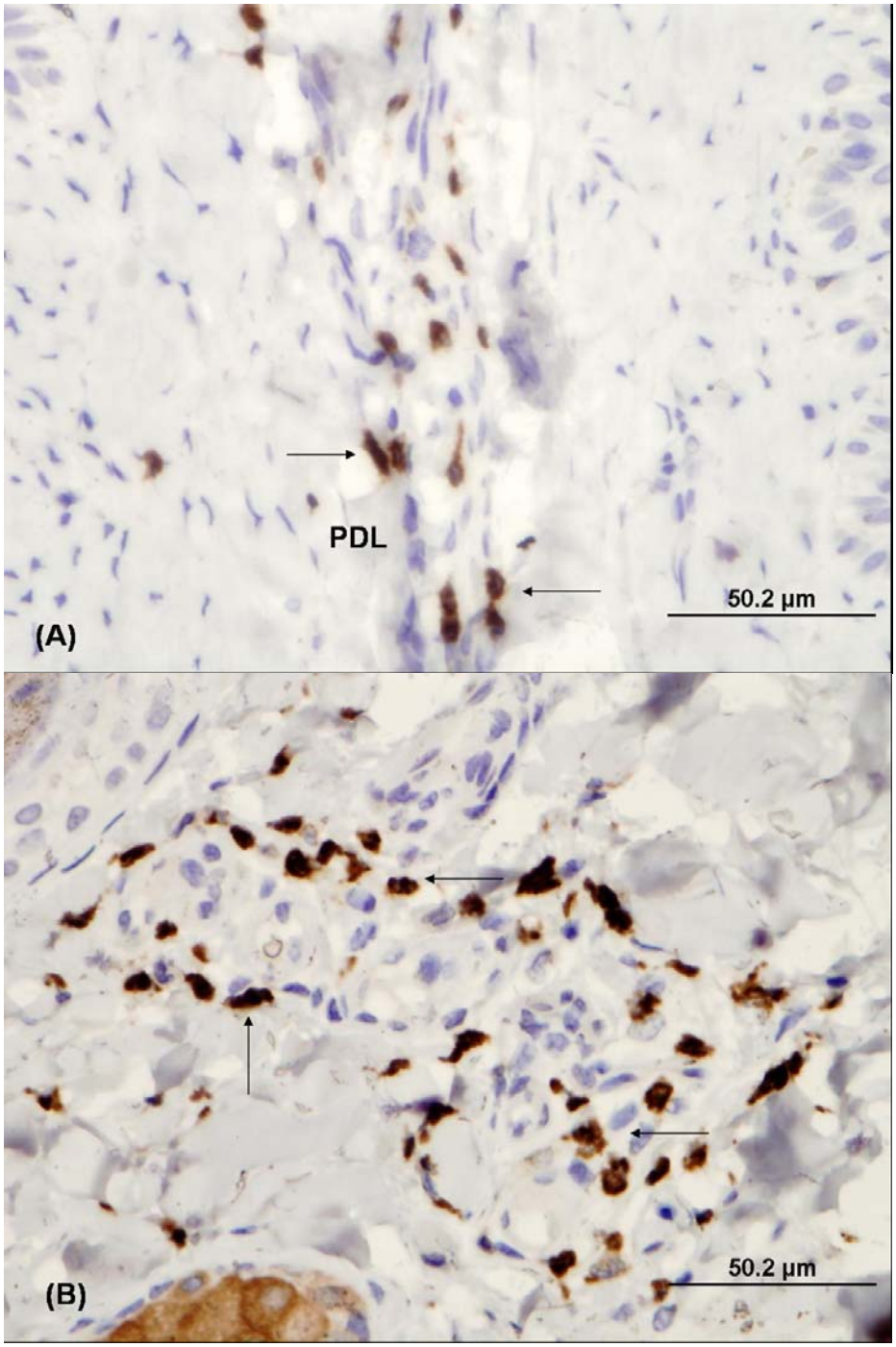

Figure 4.11. CD13 myeloid cell expression in BWHE group laminae and skin. Laminar tissue samples (A) from the BWHE group contained marked extravascular CD-13 positive cells shown in here in a primary dermal lamina (PDL). BWHE group skin samples contained marked numbers of extravascular (arrows) CD13-positive cells.

CHAPTER FIVE

BIOCHEMICAL MARKERS OF LEUKOCYTE PRESENCE IN A CARBOHYDRATE OVERLOAD MODEL OF LAMINITIS

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ABSTRACT

There has been a great deal of debate as to the best experimental model for laminitis research and it is unknown how much, if any, these models differ in the early critical steps of the disease. The purpose of this study is to further define the presence of leukocytes and their enzymatic products in the skin and laminar tissues of horses using the carbohydrate overload model of laminitis. We hypothesized that (1) CD13 protein concentration and labeled cells, and MMP gene expression and enzymatic activity would be increased in skin and laminar tissues of horses administered carbohydrate and (2) these increases would be similar to previously reported increases using the black walnut heartwood extract model. The results of this study provide strong evidence that the carbohydrate model induces an activation of leukocytes and that these leukocytes emigrate into both the skin and laminar tissues during the developmental phase of laminitis. Furthermore, CD13 protein concentration and labeled cells, and MMP gene expression and enzymatic activity increases are similar to increases using the black walnut heartwood extract model. The data from this study supports the concept that a generalized disruption of normal physiologic processes occurs in the integument in the developmental phase of laminitis.

INTRODUCTION

There has been a great deal of debate as to the best experimental model for laminitis research and it is unknown how much, if any, these models differ in the early critical steps of the disease. Three experimental models have been used to study the development of laminitis and these include the intragastric administration of an overload of unrefined carbohydrate, administration of fructan or nonstructural storage carbohydrate and administration of black walnut heartwood extract (BWHE).[1-3] The models vary in the time from administration to clinical signs, what clinical parameters change after administration and how closely the model resembles naturally-occurring laminitis conditions. Acute laminitis develops in approximately 80% of horses administered either carbohydrate or BWHE.[1-3]

Each of the experimental models of acute laminitis is based on ingestion of a substance capable of inducing the disease and, as well, the gastrointestinal tract is commonly the location of naturally-occurring primary disease. Based on previous research , hypotheses exist for the mechanisms by which these models result in laminitis; Once ingested these substances may themselves be absorbed and/or cause irritation to the colonic mucosal epithelium in the BWHE extract [1,4] or as in carbohydrate-overload, may induce alterations in the normal microflora[5-12] which then lead to the clinical signs of laminitis. However, a unified theory for the cascade of events that results in laminitis remains elusive.

A peripheral leukopenia occurs after administration of BWHE and is associated with the activation and emigration of leukocytes into the integument.[4] The result is a local increase in proteolytic enzymes such as CD13, myeloperoxidase and matrix metalloproteinase-9, present in both skin and laminar tissue.[13,14] Previous studies implicate enzymatic degradation of the basement membrane of the laminae leading to dermal-epidermal separation and the

subsequent loss of the integrity of laminar interdigitations as the functional alteration which causes gross pathologic damage and lameness.[15,16] These biochemical changes appear to be unique to the development of laminitis and do not occur in the same time frame when endotoxin is administered to horses, despite the association between clinical endotoxemia and laminitis [17].

The purpose of this study is to further define the presence of leukocytes and their enzymatic products in the skin and laminar tissues of horses using the carbohydrate overload model of laminitis. We hypothesized that (1) CD13 protein concentration and labeled cells, and MMP gene expression and enzymatic activity would be increased in skin and laminar tissues of horses administered carbohydrate and (2) these increases would be similar to previously reported increases using the black walnut heartwood extract model.

MATERIALS AND METHODS

Animals—15 healthy horses ranging in age from 5 to 15 years were used in this study. All horses included were free of existing lameness and lacked clinical evidence of systemic inflammatory disease. No radiographic evidence of pre-existing laminitis was present on survey lateral and dorsopalmar radiographic views of the forelimb digits. Prior to the start of the study, the middle region of the neck was clipped and an iv catheter was placed in the left jugular vein. The study was approved by the University of Georgia Animal Care and Use Committee.

Black walnut extract preparation- The extract was prepared as described previously.[18] Briefly, 1 kg of black walnut heartwood shavings was agitated in 7 L of water at room temperature (approx. 22°C) for 24 hours. An aqueous filtrate was then obtained by filtering

the solution through cheesecloth, and 6 liters of the resulting extract were administered by nasogastric tube.

Experimental design—Horses were randomly assigned to 2 groups as follows: Control (n=10) and Carbohydrate-overload (CHO, n=5). Horses in the Control group received 8 L of water via nasogastric intubation, and were euthanized 12-hours later. Horses in the CHO group received 17.6g/kg bwt of 85% corn starch and 15% wood flour in a volume of water resulting in a gruel consistency which was administered by nasogastric tube.

Horses in the CHO group were euthanized at the onset of Obel grade-1 laminitis or at 24 hours after intubation, if signs of Obel grade-1 laminitis had not developed by that time. Each horse was evaluated prior to the start of the study and every hour thereafter for attitude, heart rate, respiratory rate, capillary refill time, hoof temperature, digital pulses, and evidence of lameness consistent with Obel grade-1 laminitis. All horses were euthanatized with a penetrating captive bolt, in compliance with guidelines outlined in the *2000 Report of the AVMA Panel on Euthanasia*.

Collection of laminar tissue and skin—Both forelimbs were disarticulated at the level of the metacarpophalangeal joint and the hooves cut into sections with a band saw. During the procedure, thermal damage was minimized by constant irrigation of the tissue with ice-cold physiologic salt solution containing the following: 118mM NaCl, 24mM $NaHCO_3$, 1mM $MgSO_4$, 0.435mM NaH_2PO_4, 5.56mM glucose, 1.8mM $CaCl_2$, and 4mM KCl. Two full thickness segments from each forelimb foot were then placed in the ice-cold physiologic salt solution. The hard keratinized portion of the hoof and distal phalanx were removed and specimens of laminar tissue removed by sharp dissection. Concurrently, a second investigator

removed full thickness, 6 cm × 6 cm sections of skin from the middle region of the neck. These specimens were also placed in the ice-cold physiologic salt solution. Half of the laminar and skin tissue samples were rapidly frozen in liquid nitrogen and stored at −80˚C until assayed. The remaining tissue was fixed in 10% buffered neutral formalin for immunohistochemical analysis.

Real-Time PCR

Laminar and skin samples were prepared using a protocol for fibrous tissue. The samples were prepared by first fragmenting the frozen tissue with a razor blade followed by homogenization with a motorized mortar and pestle. (Pellet Pestle, Kontes Glass Co.,) Gene expression was quantified in a two-step reverse transcription-polymerase chain reaction (RT-PCR). Total RNA extraction was performed using the Versagene Total RNA Purification Kit (Gentra Systems, Minneapolis, Minnesota) according to the manufacturer's protocol. Reverse transcription for production of cDNA was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems , Foster City, California). Oligonucleotide primers and Taqman probes (Applied Biosystems, Foster City, California) were designed with Primer Express software (Perkin-Elmer Biosystems, Foster City, CA) using equine MMP-2 and MMP-9 sequences obtained from GenBank.

Quantitative real-time PCR was performed in duplicate on each sample using the ABI Prism 7700 sequence detection system. Reactions were performed using the reference gene 18s rRNA (Applied Biosystems, Foster City, California) as an endogenous control .

Gelatin Zymography

Frozen tissue samples were homogenized using a polytron tissue homogenizer (Glen Mills, Inc, Clifton, NJ) in Western lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 6 mM sodium deoxycholate,1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 20mg/ml aprotinin, and 20 mg/ml leupeptin. After homogenization, the samples were sonicated and centrifuged at 14,000 g for 15 min at 4°C. A Bradford protein assay was performed on the supernatant which was then aliquoted and stored frozen at -80°C.

8% SDS-PAGE gels containing 0.1% gelatin were prepared with a 4% stacking gel. Samples were loaded at 20µg of protein per lane and electrophoresed at 100 volts for 120 minutes. Following electrophoresis, each gel was washed 2 x 30 min in 2.5% Triton X-100 on a rocking platform to remove SDS. Gels were then incubated for 16 hours in 100mM Tris/HCl, pH=8.0, 5mM CaCl2, 0.005% Brij-35 and 0.001% NaN3 at 37C and subsequently stained in Coomassie Brilliant Blue R-250 staining solution (Bio-Rad, Richmond, CA) for 60 minutes. Gels were destained on a rocking platform in 5% methanol and 7.5% acetic acid for 2 hours. Gels were imaged and recorded using a Bio-Rad Fluor-S Max 2 MultiImager (Bio-Rad, Richmond, CA) . The specificity of the enzymatic activity was validated by incubating samples in a solution lacking calcium, necessary for gelatinase enzymatic activity. This resulted in zero enzymatic activity for the gelatin substrate (data not shown)

Western blot analysis

Samples containing 20 µg of protein from previously homogenized tissue were separated by 12% SDS-polyacrylamide gel electrophoresis, electrophoretically transferred to a membrane, and incubated with anti-equine CD13 monoclonal antibody (AbD Serotec, Raleigh, NC). Following antibody treatments, blots were incubated with horseradish peroxidase (HRP)-

conjugated secondary antibody (Pierce Biotechnology, Rockford, IL). Blots were then treated with superSignal West Dura extended duration chemiluminescence substrate solution (Pierce Biotechnology, Rockford, IL) and analyzed using a Fluor-S Max2 MultiImager system (Bio-Rad, Richmond, CA). A standard quantity of equine leukocyte-rich plasma was included on each immunoblot as a positive control.

Immunohistochemistry

Full thickness formalin-fixed tissues were paraffin-embedded and sectioned for immunohistochemical analysis with anti-equine CD13 monoclonal antibody (AbD Serotec, Raleigh, NC). Sections were deparaffinized and endogenous peroxidase activity was blocked with 1% hydrogen peroxide in water. Heat-induced epitope retrieval (HIER) with sodium citrate buffer was performed followed by blocking with 10% normal goat serum prior to antibody incubation. Sections were incubated with primary antibody for 16 hours at 4°C, followed by biotinylated mouse anti-goat secondary antibody (AbD Serotec, Raleigh, NC) and avidin-biotin-peroxidase complex. Staining was performed using DAB as the peroxidase substrate and slides were counterstained with hematoxylin. An investigator (CLJ) was blinded and slides were evaluated for presence and number of CD-13 positive cells. The evaluation was performed by centering on a dermal vessel with the 40X objective and counting all CD-13 positive cells in that field. Five fields per tissue sample were evaluated and averaged.

Statistical Analysis

Initially the BWHE horses were subdivided into two groups, those that did not show signs of laminitis and were euthanized 12 hours after administration of the extract (BWHE-

NL), and horses that were euthanized at the onset of Obel Grade 1 laminitis (BWHE-L). Statistical analysis of these two groups revealed no significant difference between them and both were significantly different from control. Therefore, the groups were recombined and are designated the BWHE group.

RT-PCR

Gene expression data for MMP-2 and MMP-9 were normalized to 18s rRNA and analyzed relative to control tissues using the $2^{-\Delta\Delta C}$T method.[19] For RT-PCR data, a two-tailed t-test was performed to analyze data from each treatment group for MMP-2 and MMP-9 for skin and lamina from each horse. For all comparisons, a value of $P \leq 0.05$ was considered significant.

Gelatin zymography

MMP-2 and MMP-9 bands were analyzed using Quantity One Quantitation Software (Bio-Rad, CA). Volume intensity was measured for each band, adjusted for background stain and normalized to a positive control (MMP-2/9 human, company) included on each gel. Results were reported as arbitrary units of optical density (ODu). MMP-2 and MMP-9 data for skin and laminar tissues from horses in the CON, BWHE and LPS groups were compared using a 1-way ANOVA with a Tukey-Kramer post-test (GraphPad Prism Software, San Diego, California). A value of $P \leq 0.05$ was considered significant.

Immunohistochemistry

CD13 data for skin and laminar tissues from horses in the CON, BWHE and LPS groups were compared using a 1-way ANOVA with a Tukey-Kramer post-test. A value of $P \leq 0.05$ was considered significant.

RESULTS

The horses in the Control group did not show signs consistent with Obel grade-1 laminitis and all were euthanized at 12 hours. All five of the CHO group became lame between 22 and 23 hours post-treatment and were euthanized at that time.

MMP-2 and MMP-9 RT-PCR Results

There was not a statistically significant increase in MMP-2 gene expression in either CHO skin or laminar samples relative to Control group values. There were significant increases in MMP-9 gene expression in both laminar tissue and skin in the CHO group (p-values of and , respectively). The RT-PCR data are reported for MMP-2 and MMP-9 as fold-changes relative to control values in Table 1.

Zymographic Activity

There were no significant differences in mean MMP-2 zymogen activity in laminar and skin samples between the treatment groups (Figures 1 and 2). Mean MMP-9 zymogen (92 kd) activity in laminae and skin samples from horses in the Control group were not significantly different from each other. (Figures 3 and 4) However, there was a significant increase in MMP-9 zymogen activity in the CHO treated laminar and skin samples when compared to the Control group.

CD13 Western blot analysis

A significant increase in CD13 expression was noted in the skin and laminar samples from the CHO group when compared to Control samples (Figure 5)

CD13 Immunohistochemistry

CD13 –positive cells were present in laminar tissue and skin sections from each treatment group. CD13-positive cells in laminar tissue appear to be exclusively myeloid cells and are present only in dermal layers while the laminar epidermis contains no CD13 positive cells. The skin samples, like laminar tissue, contain CD13-positive myeloid cells, as well as CD13-positive cells within the sebaceous glands, dermal root sheath of hair follicles, and stratum basale of the epidermis. The distribution of CD13-positive non-myeloid cells did not vary between treatment groups while the distribution of positive myeloid cells within the tissues varied between intravascular and extravascular locations depending on each group evaluated (fig 7). The morphology of CD13-positive leukocytes included a mixed population of both neutrophils and monocytes. All skin sections evaluated contained both intravascular and extravascular CD13-positive myeloid cells but varied in number determined by treatment group. In contrast, all laminar tissue sections contained intravascular CD-13 positive cells but the presence of positively-stained cells in extravascular locations varied between groups.

Control group: In laminar tissue, CD13-positive myeloid cells were noted only within the dermal vasculature. No extravascular cells were found in the laminae but were found in low numbers in skin samples from the Control group. These cells were located in the superficial dermis at the level of sebaceous glands and hair follicles along with intravascular myeloid cells.

CHO group: Tissues, both laminar and skin, from the CHO group contained marked numbers of CD-13 positive myeloid cells both within and surrounding the dermal vessels. In laminar sections positive cells were located predominately along the vessels of the primary

dermal laminae and in deeper sections of the dermis. In skin sections of the CHO group, like those of the Control group, CD13-positive myeloid cells were located in the superficial dermis at the level of sebaceous glands and hair follicles along with intravascular myeloid cells. However, in the CHO group, the number of cells was markedly increased.

DISCUSSION

The results of this study provide strong evidence that the carbohydrate model induces an activation of leukocytes and that these leukocytes emigrate into both the skin and laminar tissues during the developmental phase of laminitis. Furthermore, CD13 protein concentration and labeled cells, and MMP gene expression and enzymatic activity increases are similar to increases using the black walnut heartwood extract model reported in the previous chapter.

It has been stated that, in contrast to the response to carbohydrate overload, the clinical signs associated with BWHE-induced laminitis tend to be less severe, self-limiting and do not result in lesions that are evident histologically.[18,20] It has been suggested that this difference between the two models may be a result of the lack of increased presence of MMP-2 after administration of BWHE, when compared to changes identified in the CHO model where increased MMP-2 has been detected.[21] The results of the current study are in contrast to those reported previously, as MMP-2 gene expression was not increased in the laminar tissues in horses at Obel grade 1 laminitis after administration of carbohydrate. Furthermore, the there was no significant increase in MMP-2 at the activated protein level in the current study, leaving the necessity for MMP-2 presence for the development of clinically significant laminitis in question. Although MMP-2 may play a role later in the disease, the results of

this study suggest that this MMP is not consistently increased in the developmental phase of experimentally induced laminitis.

The models using both overload of unrefined carbohydrate and fructan are similar to the clinical condition of grain overload, all of which can produce profuse watery diarrhea, mild to moderate depression and inappetance, tachycardia and increased rectal temperature.[3] Clinical signs of laminitis are apparent approximately 24 hours after administration. The BWHE model, which takes about 12 hours to induce clinical signs, has the advantage that it does not induce secondary clinical problems, such as colic and diarrhea. It is not, however, associated with a common clinically-apparent disease. It has been established that the development of acute laminitis induced by BWHE is accompanied by a rapid decrease in total white blood cell count that usually occurs within 4 hours of administration of BWHE.[1,4]

Comparisons, as seen above, of the laminitis models have not found one to be superior to the others. When evaluated, the vascular[22-25] and biochemical changes[13,21,26] appear to be similar with a slightly different time frame. The alterations in both skin and laminar tissue occur regardless of the model used. Because the carbohydrate model closely resembles naturally occurring disease it is highly likely that these changes in the integument occur in clinical cases of naturally occurring disease. In the integumentary system, modifications based on the specialized functions of the individual tissues do exist but it is apparent that there are commonalities between these tissues with regards to the development of laminitis. The data from this and previous studies [13,14] supports the concept that a generalized disruption of normal physiologic processes occurs in the integument in the developmental phase of laminitis. As yet, the skin has not been thoroughly evaluated for histologic evidence of pathology in experimental or clinical cases of laminitis. This information and the ease by which skin biopsies can be collected in horses, suggest that histopathologic evaluation of these

tissue samples may allow the skin to become a useful tool for identifying generalized alterations in the integument in horses at risk for the development of laminitis and for studying the progression of laminitis in individual animals.

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Table 5.1. MMP-2 and MMP-9 mRNA concentrations in laminar tissue and skin expressed as fold changes in CHO tissues relative to control tissues when significant ($p<0.05$). NS – fold change not significant.

	Lamina		Skin	
	Fold ↑ or ↓	p-value	Fold ↑ or ↓	p-value
MMP-2	NS		NS	
MMP-9	↑ 12.19	<0.0001	↑ 1.59	0.047

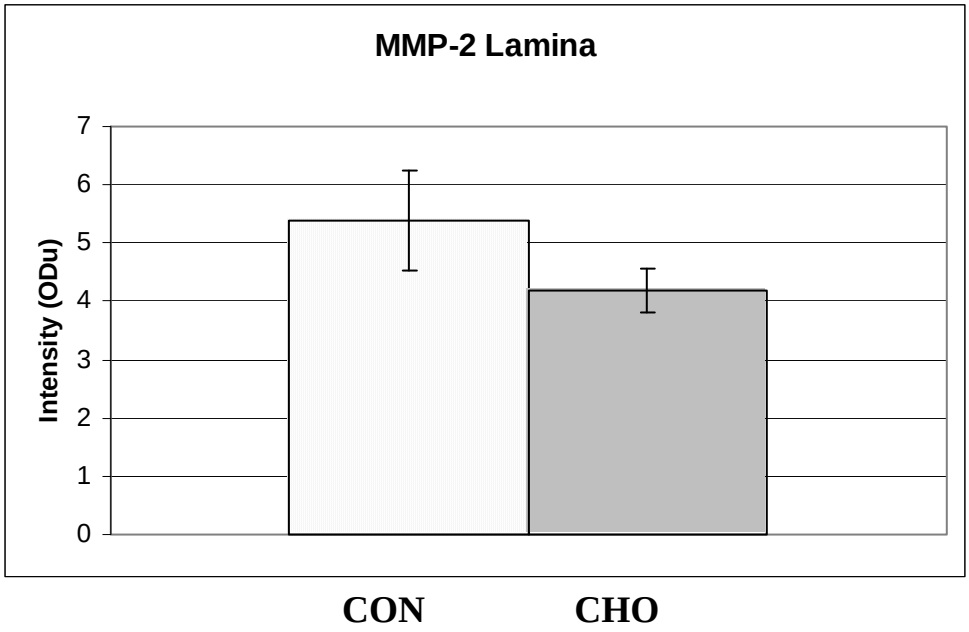

Figure 5.1. Mean intensity +/- SEM of MMP-2 zymogen activity bands in laminar tissue as determine by SDS-page gelatin zymography. * represent means significantly higher than Control values. ($p<0.05$)

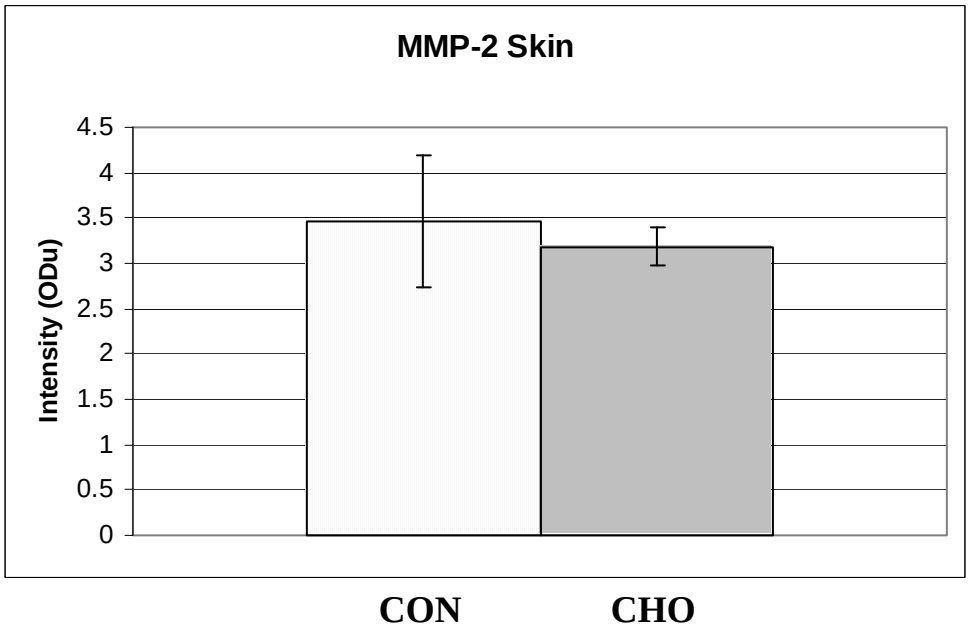

Figure 5.2. Mean intensity +/- SEM of MMP-2 zymogen activity bands in skin as determine by SDS-page gelatin zymography. There was no significant difference in mean intensity between Control, LPS and BWHE treatment groups ($p<0.05$).

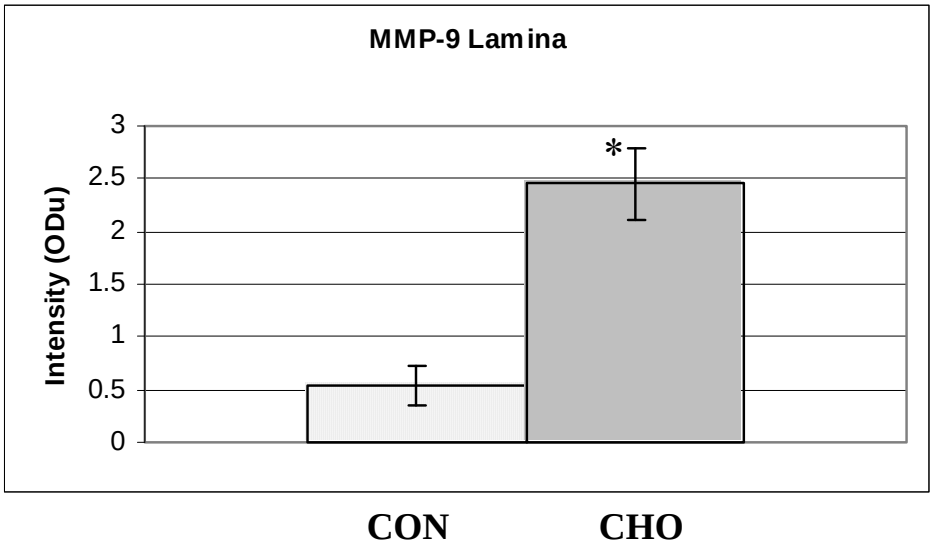

Figure 5.3. Mean intensity +/- SEM of MMP-9 zymogen activity bands in laminar tissue as determine by SDS-page gelatin zymography. * represent means significantly higher than Control values. ($p<0.05$)

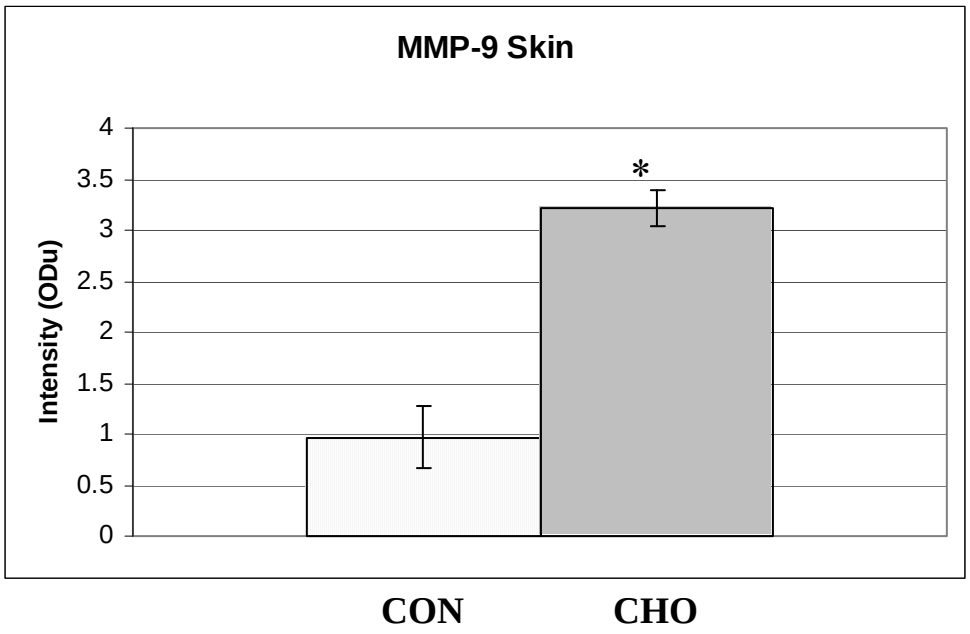

Figure 5.4. Mean intensity +/- SEM of MMP-9 zymogen activity bands in skin as determine by SDS-page gelatin zymography. * represent means significantly higher than Control values. ($p<0.05$)

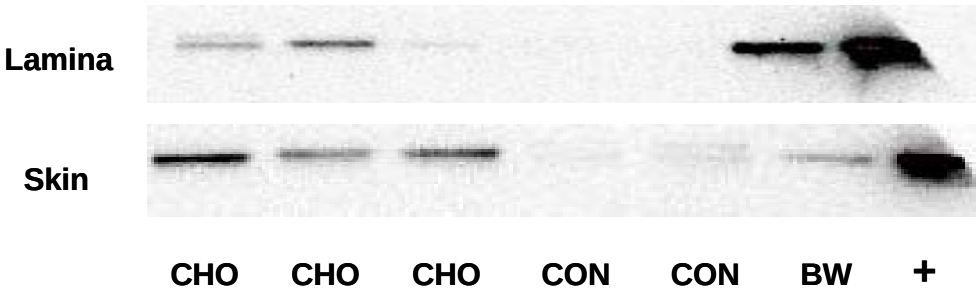

Figure 5.5. CD-13 Western hybridization. Note the difference in CD13 expression between Control (C) tissues and CHO tissues. A BWHE sample is used for comparison between CHO and BWHE samples. + (lane 8) denotes positive control.

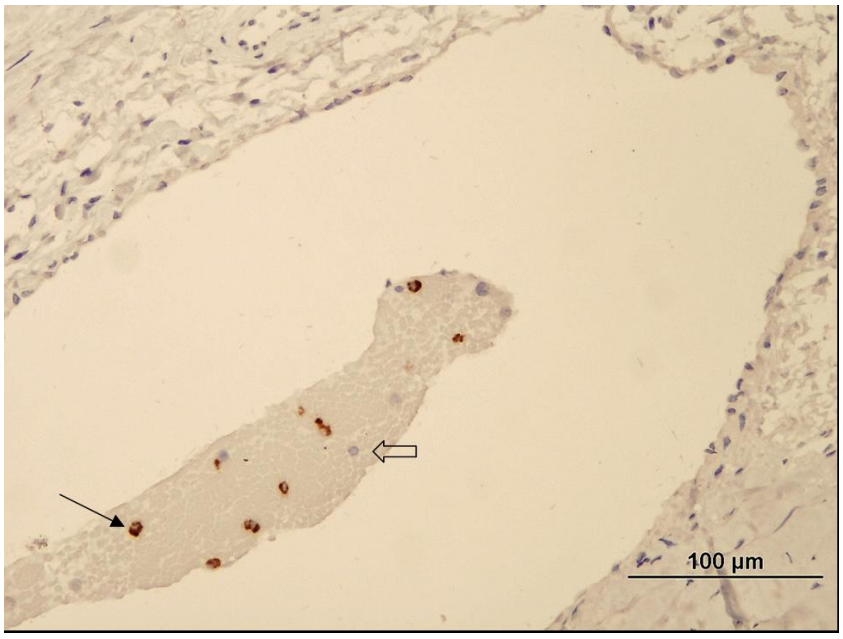

Figure 5.6. CD13-positive cells within a large venule of the dermal laminae. Note the presence of both CD-13 positive (solid arrow) and CD-13 negative (open arrow) leukocytes within the thrombus and lack of CD-13 positive cells in extravascular dermis.

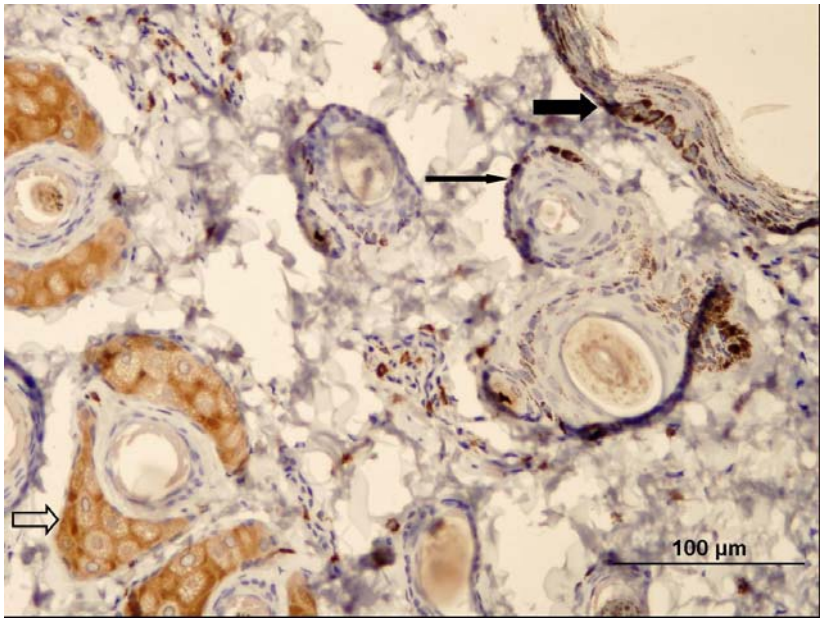

Figure 5.7. Non-myeloid CD13 positive cells in a skin sample from the Control group. Note positive staining within the sebaceous gland (open arrow), dermal root sheath of the hair follicle (narrow arrow), and stratum basale of the epidermis (wide arrow).

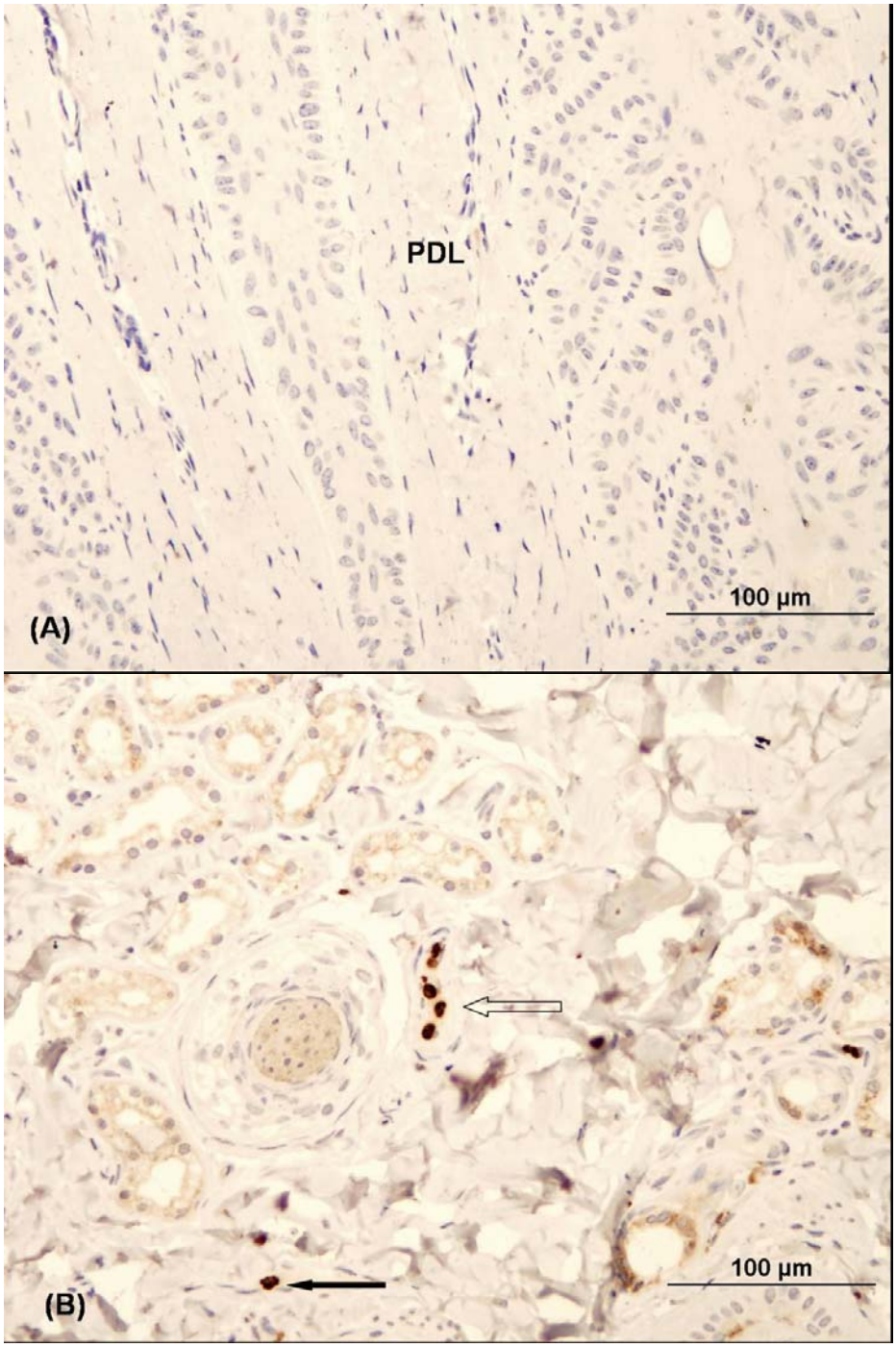

Figure 5.8. CD13 myeloid cell expression in Control laminae and skin. Laminar tissue samples (A) from the Control group contained no extravascular CD-13 positive cells. Control group skin samples contained low numbers of both intravascular (open arrow), shown in a vessel with a longitudinal orientation, and extravascular (closed arrow) CD13-positive cells.

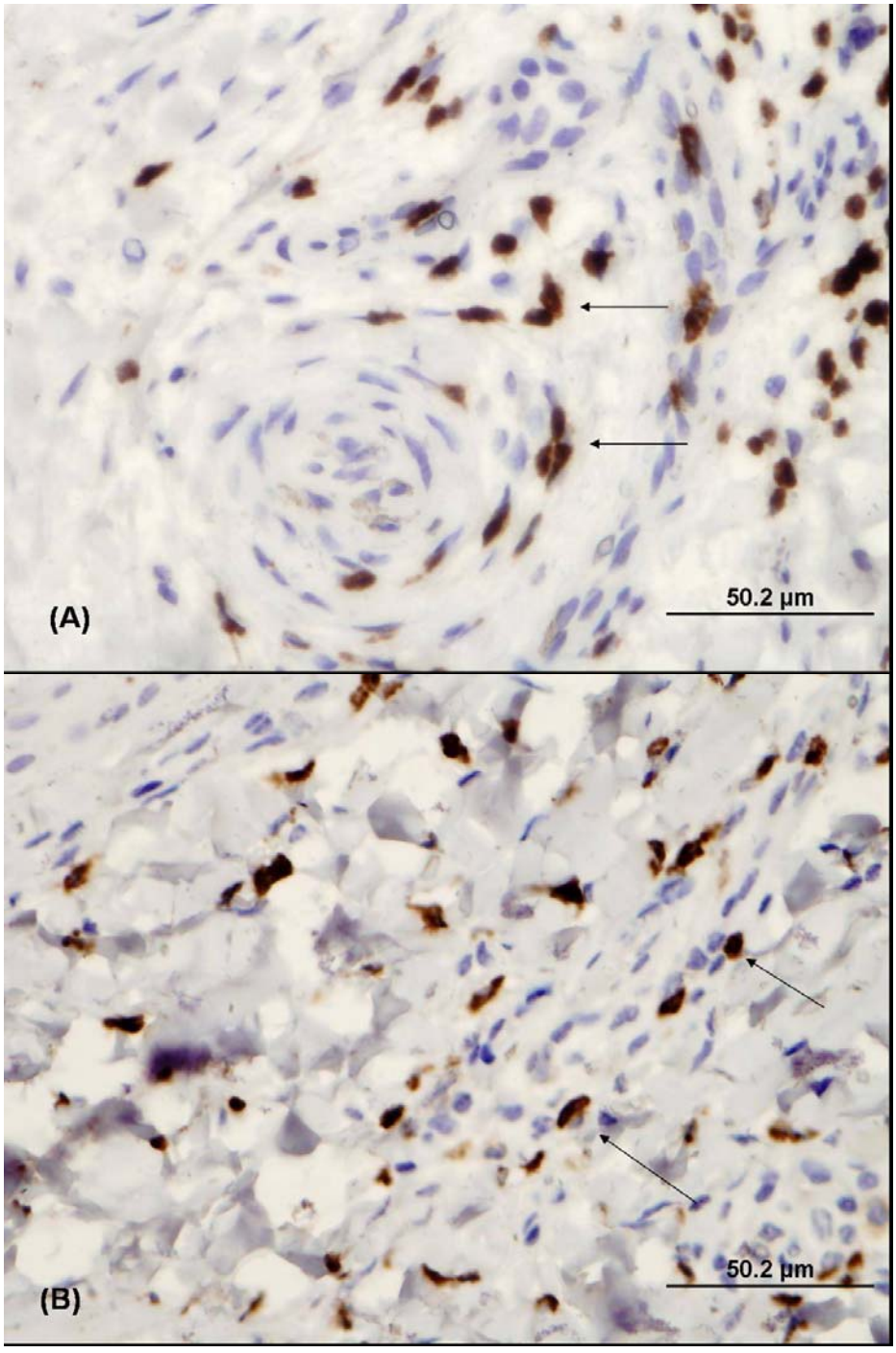

Figure 5.9. CD13 myeloid cell expression in CHO group laminae and skin. Laminar tissue samples (A) from the CHO group contained marked extravascular CD-13 positive cells. CHO group skin samples contained marked numbers of extravascular (arrows) CD13-positive cells.

CHAPTER SIX

GENERAL DISCUSSION

The main purpose of the studies reported here was to investigate the involvement of the equine integument in experimental models of laminitis. Specifically, it was important to determine if the leukocyte emigration and biochemical alterations occurring in the laminae are also occurring in the skin. The results of this study provide convincing evidence for systemic activation of neutrophils, and their emigration into the integument in horses administered either black-walnut heartwood extract or an overload of unrefined carbohydrate, the two most frequently used experimental models of laminitis. The results of these studies indicate that the inflammatory processes initiated by intragastric administration of BWHE or CHO are not restricted to the laminar soft tissues, but occur in the integument in general.

Historically, laminitis researchers have assumed the uniqueness of laminar tissue to explain the predilection of the disease's manifestation in the laminae. While modifications based on the specialized functions of the individual tissues do exist it is apparent that there are commonalities within integumentary tissues with regards to the development of laminitis.

The significance of the generalized involvement of the integument system is amplified by the information obtained from the inclusion of the LPS group in this study. In a recent multivariate analysis of clinical risk factors for development of acute laminitis, endotoxemia was the only factor significantly associated with the disease.[1] In this study, the administration of LPS, while causing significant leukopenia, does not cause the activation and immigration of leukocytes into the integument, as occurs following BWHE administration.

This information and the ease by which skin biopsies can be collected in horses, suggest that evaluation of these tissue samples may allow the skin to become a useful tool for studying the mechanisms of the disease. At present, laminar samples can only realistically be obtained after an animal has been euthanized. This reality requires that animals be euthanized for tissue collection and prevents horses from acting as their own control in a research study, i.e. no "time 0" information can be obtained. These facts coupled with the great variation which exists between individual animals, the financial burden of purchasing horses without orthopedic disease for inclusion in the studies and the ethical considerations when performing disease-induction terminal studies with horses slows the rate of research studies in this area and has undoubtedly contributed to the relative paucity of unified information regarding the mechanisms of the disease. Skin samples would allow serial samples to be collected during the induction process. In addition, the information collected in these studies could be used to dentify generalized alterations in the integument in horses at risk for the development of laminitis and for studying the progression of laminitis in individual animals. The early identification of at-risk patients would allow more accurate prognosis and aggressive intervention.

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APPENDIX A

INTRODUCTION

The presence of increased levels of matrix metalloproteinase 2 and 9 in plasma from horses with naturally occurring acute laminitis has been established.[1,2] In addition to establishing normal variation for matrix metalloproteinase (MMP) 2 and 9 in control equine laminar and skin tissue and comparing relative MMP amounts between normal and BWHE experimentally-induced laminitic animals, tissue samples were collected from horses with naturally-occurring chronic laminitis for MMP 2 and 9 analysis.

Using zymographic techniques to analyze the presence of MMPs in control animals, clinically symptomatic animals and experimentally induced laminitic animals, we hypothesized that laminar and skin tissue levels of MMPs would correlate in individual animals. We expected to see increased MMPs in laminar and skin tissue from experimental and clinical laminitis populations while control horses would have only basal levels. More variation was expected in the clinical group due to various disease stages. Both the clinical and research settings can benefit from such results finding accurate and less invasive measurements for the detection and progression of laminitis.

MATERIALS AND METHODS

Horses: The control group (n=8) consisted of horses aged 8 to 15 years found free of lameness and other inflammatory diseases. The clinical case group consisted of horses donated to the University of Georgia Veterinary Teaching Hospital with a history of chronic laminitis. The donated horses were euthanized upon admission. Skin and laminar samples were collected immediately following euthanasia. These specimens were placed in an ice-cold physiologic salt

solution. The laminar and skin tissue samples were rapidly frozen in liquid nitrogen and stored at –80˚C until assayed.

Tissue Homogenization: Frozen tissue samples were homogenized using a polytron tissue homogenizer (Glen Mills, Inc, Clifton, NJ) in Western lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 6 mM sodium deoxycholate,1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 20mg/ml aprotinin, and 20 mg/ml leupeptin. After homogenization, the samples were sonicated and centrifuged at 14,000 g for 15 min at 4°C. A Bradford protein assay was performed on the supernatant which was then aliquoted and stored frozen at -80°C.

Gelatin Zymography: 8% SDS-PAGE gels containing 0.1% gelatin were prepared with a 4% stacking gel. Samples were loaded at 20µg of protein per lane and electrophoresed at 100 volts for 120 minutes. Following electrophoresis, each gel was washed 2 x 30 min in 2.5% Triton X-100 on a rocking platform to remove SDS. Gels were then incubated for 16 hours in 100mM Tris/HCl, pH=8.0, 5mM CaCl2, 0.005% Brij-35 and 0.001% NaN3 at 37C and subsequently stained in Coomassie Brilliant Blue R-250 staining solution (Bio-Rad, Richmond, CA) for 60 minutes. Gels were destained on a rocking platform in 5% methanol and 7.5% acetic acid for 2 hours. Gels were imaged and recorded using a Bio-Rad Fluor-S Max 2 MultiImager (Bio-Rad, Richmond, CA).

RESULTS

The average MMP 2L intensity in skin of control animals was 6.41 ± 2.64 optical density units (ODu) and 1.27 ± 1.20 ODu for MMP-9L. Laminar tissue intensity averaged 6.64 ± 1.67 ODu for MMP-2L and 0.48 ± 0.44 ODu for MMP-9L**.** No significant difference was found between skin and laminar tissues in control animals in either MMP-2L or MMP-9L activity. (Figure 1 and 2)

DISCUSSION

While MMP-9L activity was significantly higher in laminar tissue of BWHE treated animals in comparison to control animals, a similar trend, although not significant was also seen in the clinical animals. These trends are not significant due to variation within the zymographic procedure. The quantity of MMPs in skin and laminar tissue were unknown and may have measured outside the range found to be valid by some studies (0 to 200 pg of MMP), limiting zymographic detection of differences in samples. Variation may be reduced by increasing incubation time and/or decreasing the amount of sample loaded into each well.

Studies in this laboratory are currently investigating the role of the active forms of MMP-2 and MMP-9 using an ELISA to specifically measure active MMPs. By increasing sensitivity and decreasing variation within one animal, the assay could help further describe the involvement of MMPs with laminitis.

The correlation of laminitis and MMP activity will give further insight into the difficult study of laminitis pathophysiology. Also, a connection between the skin and laminar MMPs detected would prove useful in the clinical setting for earlier diagnosis using skin biopsy of at-risk horses. As in most disease processes, lifestyle changes and treatments could be instituted early to slow the disease progression. The equine industry would benefit greatly, both medically and economically, from any such findings.

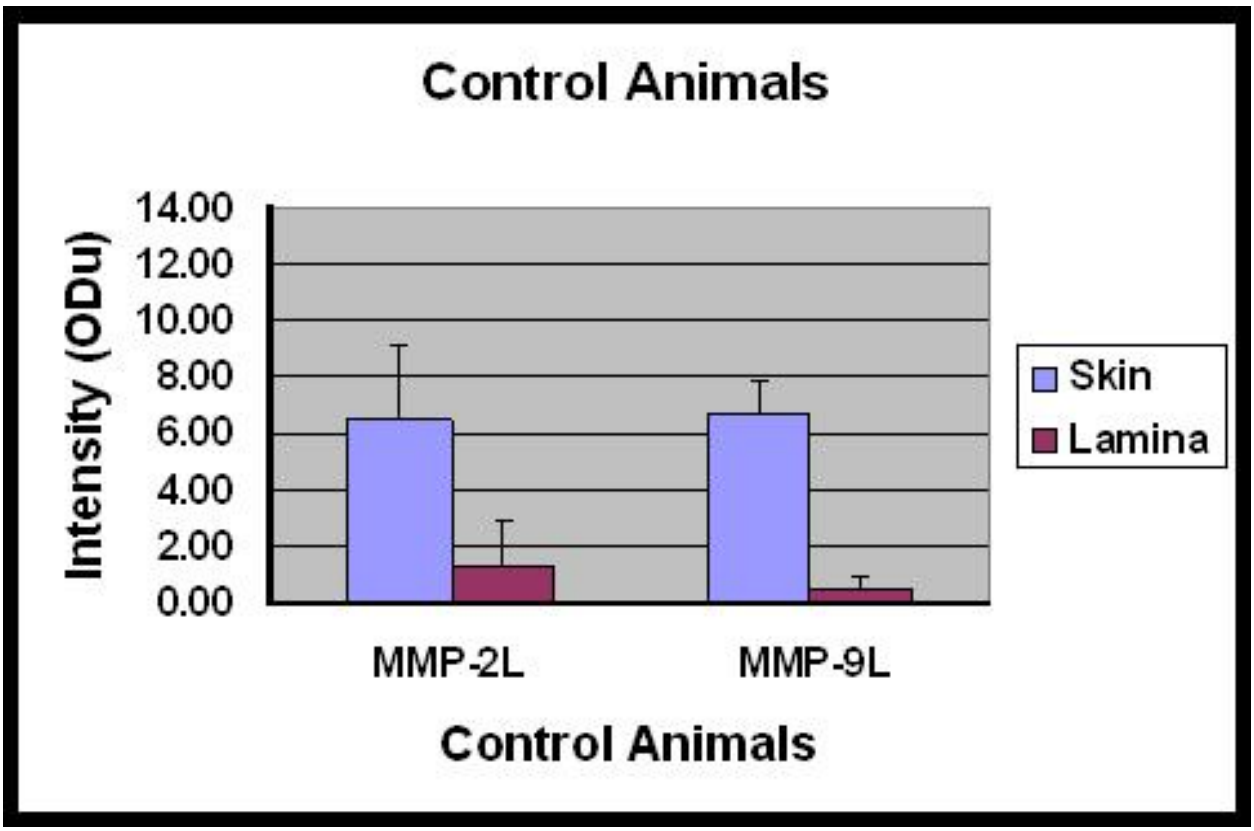
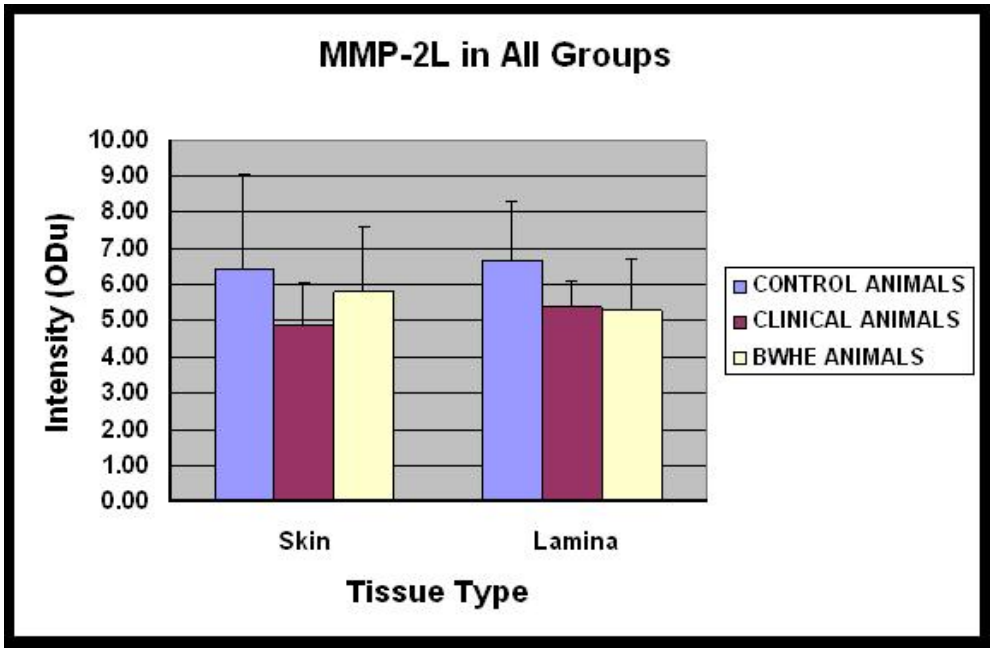

Control Animals
Intensity (ODu)
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
MMP-2L
MMP-9L
Skin
Lamina
Control Animals

Figure A.1.


MMP-2L in All Groups
Intensity (ODu)
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Skin
Lamina
Tissue Type
CONTROL ANIMALS
CLINICAL ANIMALS
BWHE ANIMALS

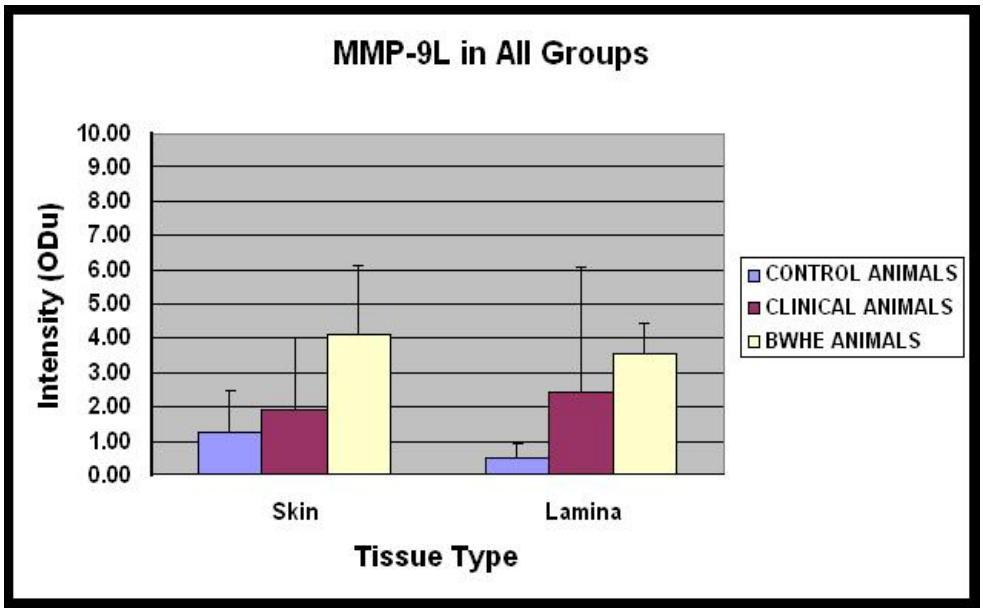
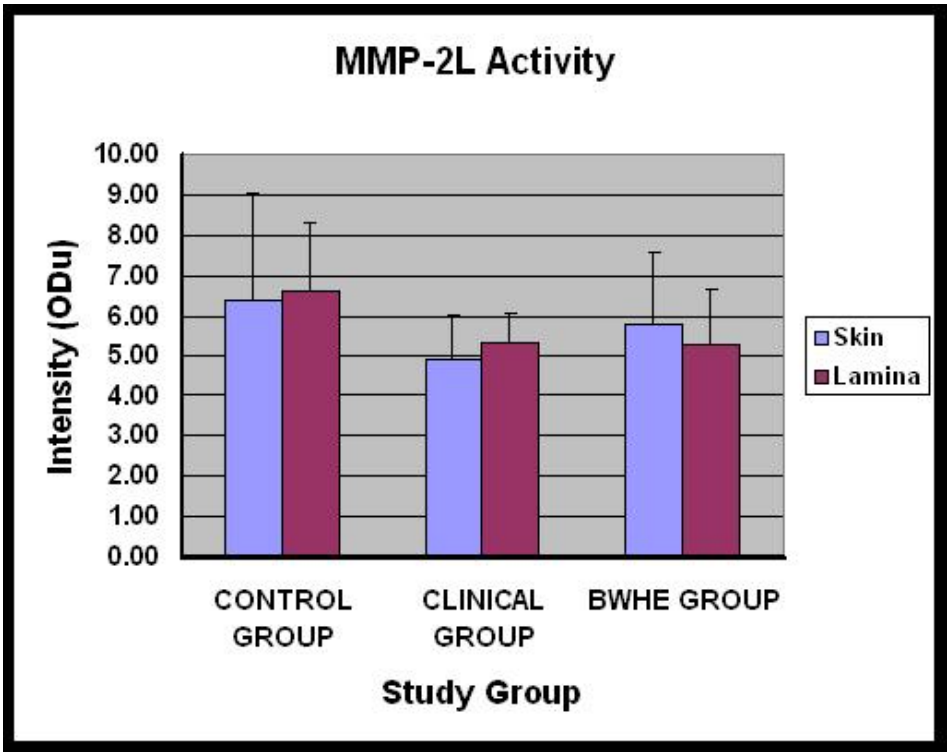

MMP-9L in All Groups
Intensity (ODu)
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Skin
Lamina
Tissue Type
CONTROL ANIMALS
CLINICAL ANIMALS
BWHE ANIMALS

Figure A.2.


MMP-2L Activity
Intensity (ODu)
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
CONTROL GROUP
CLINICAL GROUP
BWHE GROUP
Study Group
Skin
Lamina

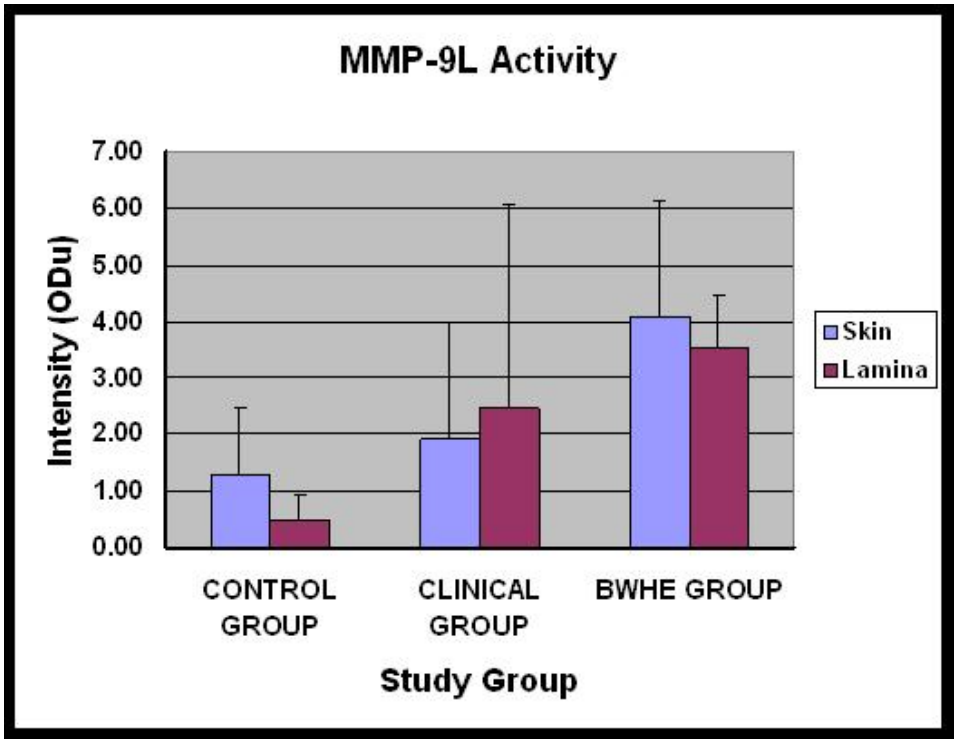

MMP-9L Activity
Intensity (ODu)
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
CONTROL GROUP
CLINICAL GROUP
BWHE GROUP
Study Group
Skin
Lamina

Figure A.3.

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APPENDIX B

INTRODUCTION

Tissue damage occurs due to interactions among cytokines, growth factors, and inflammatory cells. Products released by circulating cells play important regulatory roles in the inflammatory responses in tissue injury and repair. The equine laminar tissue is heavily vascularized and much of the damage in laminitis is related to edema and ischemia of perivascular tissues. The tissue lesions that characterize acute laminitis are consistent with dysregulation of inflammatory cell recruitment and production of reactive oxygen species, nitric oxide and enzymes (i.e., gelatinases, metalloproteinases).[1] For these reasons, the involvement of nitric oxide and reactive oxygen species in the pathophysiology of the disease was explored. Peroxynitrite was evaluated as a product of the reaction between nitric oxide and superoxide anion.

Nitric oxide (NO) is synthesized from nitric oxide synthase (NOS). Presently three isoforms of NOS have been identified: neuronal nitric oxide synthase (nNOS or NOS-I), inducible nitric oxide synthase (iNOS or NOS-II) and endothelial nitric oxide synthase (eNOS or NOS-III). Similarly, each of the three isoforms synthesizes nitric oxide from L-arginine.[2,3] Recent experiments have established iNOS as a key component of inflammatory-mediated diseases in equines. These diseases are not limited to the equine hoof but, rather, involve multiple organ systems including the integument, gastrointestinal and respiratory systems.[3]

The toxicity of NO is, in part, due to its reaction with superoxide anion generated by neutrophil degranulation, leading to the formation of the potent oxidant peroxynitrite.[4] The accepted method for detecting peroxynitrite activity *in vivo* is based upon the identification of 3-nitrotyrosine in injured tissues.[5] 3-nitrotyrosine represents the product of the interaction between peroxynitrite and tyrosine residues on proteins leading to alteration of the cellular functions regulated by these proteins. For example, peroxynitrite may cause long-term down-regulation of G protein-coupled receptors by the nitration of extracellular tyrosine residues in the extracellular domains of the receptors (see below). This collection of data suggests that the inflammatory process occurring with the onset of laminitis is likely a systemic reaction rather than a reaction localized only to the laminar tissues. Peroxynitrite is produced by macrophages, neutrophils, vascular endothelial and smooth muscle cells, and increased production of peroxynitrite occurs in endotoxemia and in inflammatory diseases such as atherosclerosis.[5,6] Peroxynitrite is a powerful oxidant of protein and non-protein sulfhydryls, and membrane phospholipids and readily nitrates free and protein-bound tyrosine residues.[7]

To further investigate this idea, preliminary immunohistochemical studies were performed on the lamellae and skin from normal and black walnut treated horses for 3-nitrotyrosine, the product of the reaction between peroxynitrite and tyrosine.

MATERIALS AND METHODS

Black walnut extract preparation- The extract was prepared as described previously. Briefly, 1 kg of black walnut heartwood shavings was agitated in 7 L of water at room temperature (approx. 22°C) for 24 hours. An aqueous filtrate was then obtained by filtering the solution through cheesecloth, and 6 liters of the resulting extract were administered by nasogastric tube.

Horses in the Control group received 6 L of water via nasogastric intubation, and were euthanized 12-hours later. Horses in the BWHE group were euthanatized at the onset of Obel grade-1 laminitis (clinical signs consisting of weight shifting and bounding digital pulses without evidence of lameness at a walk) or at 12 hours after administration, if signs of Obel grade-1 laminitis had not developed by that time. Each horse was evaluated prior to the start of the study and every hour thereafter for attitude, heart rate, respiratory rate, capillary refill time, hoof temperature, digital pulses, and evidence of lameness consistent with Obel grade-1 laminitis. Blood samples were taken hourly for complete blood count determination. All horses were euthanatized with a penetrating captive bolt, in compliance with guidelines outlined in the *2000 Report of the AVMA Panel on Euthanasia*.

Collection of laminar tissue and skin—Both forelimbs were disarticulated at the level of the metacarpophalangeal joint and the hooves cut into sections with a band saw. During the procedure, thermal damage was minimized by constant irrigation of the tissue with ice-cold physiologic salt solution containing the following: 118mM NaCl, 24mM $NaHCO_3$, 1mM $MgSO_4$, 0.435mM NaH_2PO_4, 5.56mM glucose, 1.8mM $CaCl_2$, and 4mM KCl. Two full thickness segments from each forelimb foot were then placed in the ice-cold physiologic salt solution. The hard keratinized portion of the hoof and distal phalanx were removed and specimens of laminar tissue removed by sharp dissection. Concurrently, a second investigator removed full thickness, 6 cm × 6 cm sections of skin from the middle region of the neck. The tissue was fixed in 10% buffered neutral formalin for immunohistochemical analysis.

Immunohistochemistry

Full thickness formalin-fixed tissues were paraffin-embedded and sectioned for immunohistochemical analysis with rabbit anti-human 3-nitrotyrosine monoclonal antibody

(AbD Serotec, Raleigh, NC). Sections were deparaffinized and endogenous peroxidase activity was blocked with 1% hydrogen peroxide in water. Blocking with 10% normal goat serum was performed prior to antibody incubation. Sections were incubated with primary antibody for 16 hours at 4°C, followed by biotinylated rabbit anti-goat secondary antibody (name source) and avidin-biotin-peroxidase complex. Staining was performed using DAB as the peroxidase substrate and slides were counterstained with hematoxylin.

RESULTS

The preliminary data suggested that both the skin and the lamellae stain for 3-nitrotyrosine. Figure 1 (panels A and B) demonstrates evidence of an increase in 3-nitrotyrosine staining in laminar tissue obtained from BWHE treated horses as compared to control horses.

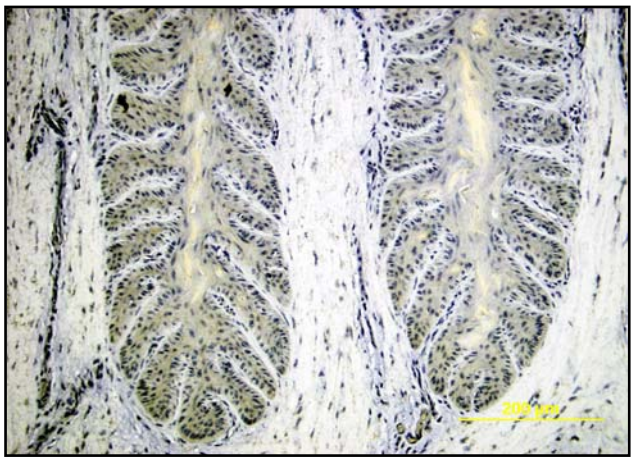

Figure B.1 (Panel A)

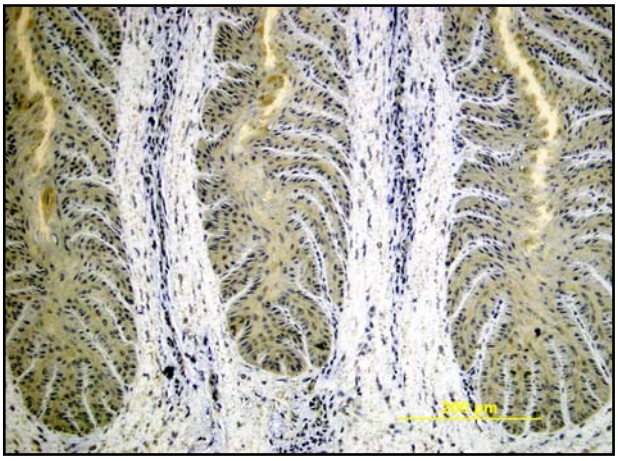

Figure 3 (Panel B)

Panel A. Immunohistological examination of laminar tissue for nitrotyrosine. Section of laminar tissue obtained from a control horse stained for nitrotyrosine (brown).

Panel B. Immunohistological examination of laminar tissue for nitrotyrosine. Section of laminar tissue obtained from a BWHE treated horse demonstrating an increase in the presence of nitrotyrosine (brown) as compared to control (above).

Figure 2 (700X below) validates the specificity of the antibody used to identify 3-nitrotyrosine in BWHE treated horses. 3- nitrotyrosine antibody was pretreated with 10mM 3-nitrotyrosine for one hour prior to exposure of tissue section. Lack of brown stain demonstrates specificity of antibody specifically to nitrotyrosine.

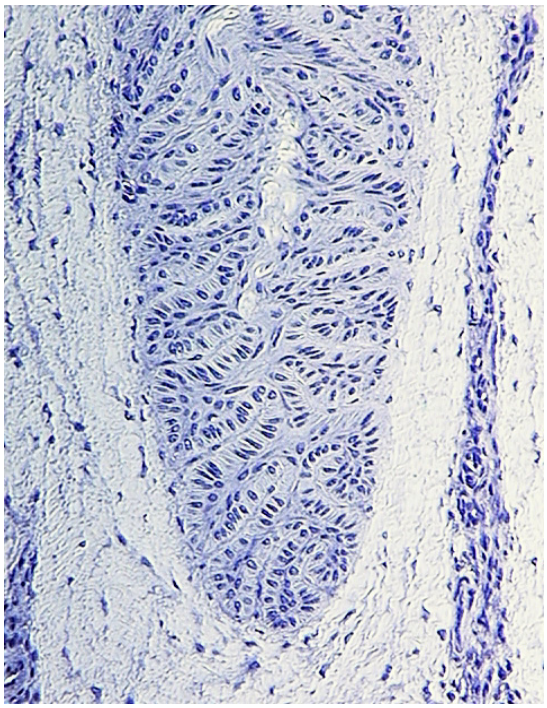

Figure B.2 Immunohistological examination of laminar tissue for nitrotyrosine.

Section of laminar tissue obtained from a BWHE treated horse demonstrating specificity of nitrotyrosine antibody.

To determine whether similar events are occurring in the skin, samples from the midcervical region of both BWHE treated and control horses were analyzed for the presence of 3-nitrotyrosine. Preliminary data derived from immunohistochemistry showing increased 3-nitrotyrosine staining in skin obtained from BWHE treated horses as compared to control are shown in figure 3 (panels A and B).

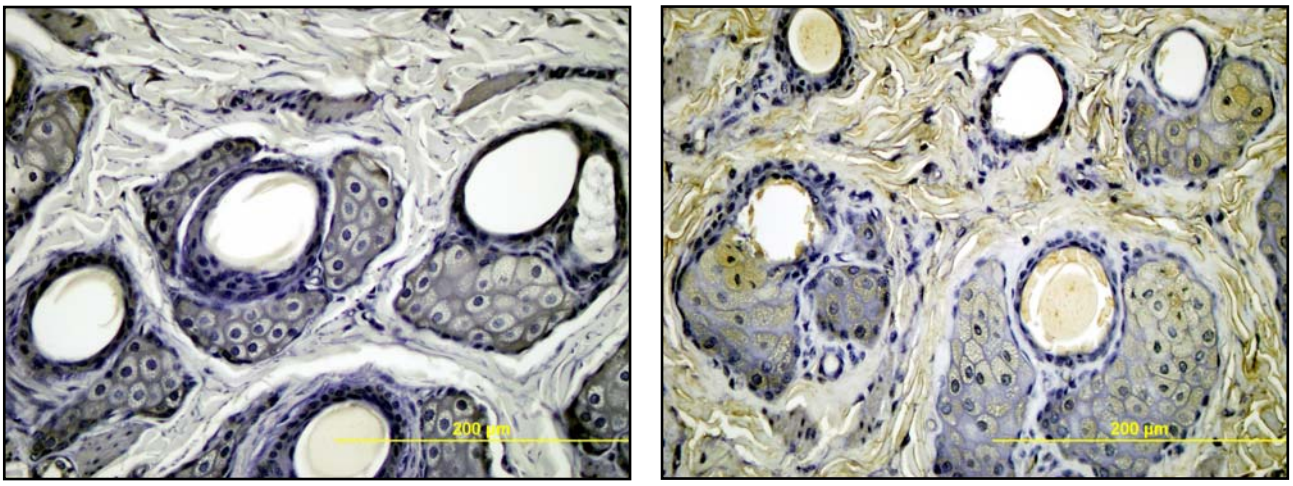

Figure B.3.

Panel A: Immunohistological examination of skin for nitrotyrosine. Section of skin obtained from a control horse stained for nitrotyrosine (brown).

Panel B: Immunohistological examination of skin for nitrotyrosine. Section of skin obtained from a black walnut heartwood extract treated horse demonstrating an increase in the presence of nitrotyrosine (brown) as compared to control skin (above).

The immunohistochemical identification of increased quantities of 3-nitrotyrosine in skin and laminar tissue obtained from horses treated with BWHE, strengthened the proposition that the skin could be sampled to investigate these early inflammatory responses. Further analysis of the immunohistochemical protocol revealed that the staining was not consistent and appeared to be non-specific in many sections. The preliminary results were not validated most likely due to the interactions with the species in which the antibodies were produced.

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