2009

Matrix metalloproteinases in the equine systemic inflammatory response: implications for equine laminitis

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MATRIX METALLOPROTEINASES IN THE EQUINE SYSTEMIC INFLAMMATORY RESPONSE: IMPLICATIONS FOR EQUINE LAMINITIS

A Dissertation

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

in

The Interdepartmental Program in
Veterinary Medical Sciences
through the Department of
Veterinary Clinical Sciences

by
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B.S., Louisiana State University, 1999
D.V.M., Louisiana State University, 2002
May 2009

To Barbaro and all the other unknown equine champions

who have courageously fought the battle

ACKNOWLEDGMENTS

I would like to acknowledge Dr. Susan C. Eades (co-major professor); Dr. Rustin M. Moore (co-major professor); Dr. Changaram S. Venugopal; Dr. Kathy L. O'Reilly; and Dr. Patrick J. DiMario, the members of my graduate committee. I sincerely thank each of them for their support, assistance, and advice, but most importantly for their patience and faith in me.

I would like to thank Dr. Britta S. Leise and Leslie A. Talley for their friendship, support, and assistance. I could not have made it through without them. I sincerely thank Catherine Koch, Michael Keowen, Pat Triche, Dr. Randy Schulz, Dr. Sam Black, and Dr. John Loftus for their technical assistance. Without them, I would have been lost. I would also like to recognize the students in the laboratory, who were always willing to help no matter what I asked of them. Most importantly, I would like to thank my family and friends who have always supported and believed in me. I hope that I have made you proud.

I extend my thanks to the Louisiana State University School of Veterinary Medicine Equine Health Studies Program for its financial support of these studies.

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LIST OF ABBREVIATIONS

AAEP	American Association of Equine Practitioners
Abs405/h2*1000	rate of change of absorbance at 405 nm
AC	affinity chromatography
ANOVA	analysis of variance
APMA	p-aminophenylmercuric acetate
A.U.	arbitrary units
AVA	arteriovenous anastomosis
BALF	bronchioalveolar lavage fluid
BM	basement membrane
BWE	black walnut extract
°C	degrees centigrade
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
CD14	cluster of differentiation 14
CHO	carbohydrate overload
cm	centimeter
CNS	central nervous system
CO_2	carbon dioxide
COX	cyclooxygenase
CRT	capillary refill time
DOXY	doxycycline or doxycycline followed by infusion of LPS
DMEM	Dulbecco's Modified Eagles Medium
DMSO	dimethylsulfoxide
ECM	extracellular matrix
E. coli	*Escherichia coli*
EDTA	disodium ethylene diamine tetraacetate
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbant assay
ET-1	endothelin-1
°F	degrees Fahrenheit
FLU	flunixin meglumine
FLUNIXIN	flunixin meglumine followed by infusion of LPS
g	acceleration due to gravity
GI	gastrointestinal
GrT	granulation tissue
HCl	hydrochloric acid
HD	hemidesmosome
HOCl	hypochlorous acid
HR	heart rate
5-HT	5- hydroxytryptamine
Hz	Hertz
IgG	immunoglobulin G
IL	interleukin
IV	intravenous

kD	kilo Dalton
kg	kilogram
L	liter
LPB	lipopolysaccharide binding protein
LPS	lipopolysaccharide
M	molar
MAP	mean arterial pressure
MD2	myeloid differentiation factor 2
mg	milligram
µg	microgram
ml	milliliter
µl	microliter
mm	millimeter
mM	millimolar
MMP	matrix metalloproteinase
MMPI	matrix metalloproteinase inhibitor
mRNA	messenger ribonucleic acid
MT-MMP	membrane-type matrix metalloproteinase
N	Newtons
NaCl	sodium chloride
NaN_3	sodium azide
NADPH	nicotinamide adenine dinucleotide phosphate
NFκB	nuclear factor κB
ng	nanogram
NGT	nasogastric intubation
nm	nanometer
NO	nitric oxide
NSAID	non-steroidal anti-inflammatory drug
OF	oligofructose
OXY	oxytetracycline
OXYTET	oxytetracycline followed by infusion of LPS
P3	third phalanx
p38/MAPK	p38 mitogen activated protein kinase
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDL	primary dermal laminae
PGE_2	prostaglandin E_2
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethyl sulfonyl fluoride
RBC	red blood cell
RECK	reversion-inducing-cysteine-rich protein with kazal motifs
RAO	recurrent airway obstruction
ROS	reactive oxygen species
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute (developer of medium)
RR	respiratory rate
SD	standard deviation

SDL	secondary dermal laminae
SDS	sodium dodecyl sulfate
SEL	secondary epidermal laminae
SEM	standard error mean
SIRS	systemic inflammatory response syndrome
T	time
TELF	tracheal epithelial lining fluid
Temp	body temperature
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of matrix metalloproteinase
TLR 4	Toll-like receptor 4
TMB	tetramethylbenzidine
TNF-α	tumor necrosis factor-α
TXA_2	thromboxane A_2
TXB_2	thromboxane B_2
U	units
V	volts
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide
v/v	volume/volume
Zn^{2+}	zinc

ABSTRACT

Laminitis is a crippling and often life-threatening disease of the equine foot. Soft tissue damage characteristic of this disease has been associated with increased MMP activity. Therefore, it seems likely that MMPIs could be potential therapeutic agents for laminitis. Further characterization of equine MMPs and evaluation of the effectiveness of MMPIs in the horse are needed.

Equine MMP-9 was harvested from neutrophils, purified by affinity chromatography, and evaluated using western blotting and gelatin zymography. The Biotrak MMP-9 Activity Assay was evaluated for use with equine samples using equine neutrophil MMP-9 as a standard, and was determined to have insufficient sensitivity for equine MMP-9. Therefore, zymography was used for evaluating MMP activity in all studies.

The abilities of doxycycline, oxytetracycline, and flunixin meglumine to inhibit LPS-induced equine MMP-2 and MMP-9 activities *in vitro* were investigated using a digital laminar explant model. The structural integrity of the explants was tested using an Instron biomechanical testing device, and MMP activity in the explants medium was evaluated using zymography. Doxycycline, oxytetracycline, and flunixin meglumine all successfully inhibited equine MMP-9 to varying degrees. However, only doxycycline and oxytetracycline increased the structural integrity of the explants. Explant structural integrity was inversely correlated with MMP-2 concentrations in the medium.

Based on the *in vitro* results, a non-terminal *in vivo* model for investigating MMPIs in the horse was validated. The administration of IV endotoxin to normal adult horses resulted in significant increases in MMP-2 and MMP-9 activities, as assessed by zymography. This *in vivo* model of MMP induction was used to determine the effects of doxycycline, oxytetracycline, flunixin meglumine, and pentoxifylline on equine MMP inhibition. Pentoxifylline and

oxytetracycline appeared to be potent MMP-9 and modest MMP-2 inhibitors in the horse. Flunixin meglumine and doxycycline were potent inhibitors of equine MMP-2, but only weak inhibitors of equine MMP-9. These findings warrant the evaluation of pentoxifylline and oxytetracycline as MMPIs in the prevention/treatment of equine laminitis.

CHAPTER 1. INTRODUCTION/REVIEW OF LITERATURE

1.1 Introduction

The extracellular matrix (ECM) plays a critical role in normal tissue structure and function. It is constantly undergoing remodeling via the actions of proteases, in particular, matrix metalloproteinases (MMPs). During normal physiological events, MMPs are highly regulated and necessary for homeostasis. However, in pathologic states, their enzymatic actions may go unchecked and lead to substantial tissue destruction. Extensive evidence exists for the role of MMPs in the pathophysiology of both human and animal diseases. Thus, MMPs have become targets for therapeutic intervention by the development of MMP inhibitors (MMPIs).

Liberation of endotoxin from the cell wall of Gram-negative bacteria often leads to endotoxemia and the subsequent induction of a systemic inflammatory response. Multiple inflammatory mediator cascades are initiated that ultimately result in tissue destruction and death if left untreated. Some of the many mediators responsible for the pathological changes associated with endotoxemia include MMPs. The experimental administration of endotoxin has been used in many species to study alterations in various mediator activities, including MMPs. Although endotoxemia has been experimentally induced in horses, MMP activity has not been investigated.

Laminitis is a crippling and often life-threatening disease of the equine foot. The pathophysiology of laminitis remains unclear; however, recent studies suggest that MMPs are responsible for the soft tissue damage associated with this disease. Therefore, it seems likely that MMPIs could be potential therapeutic agents for laminitis. The induction of laminitis for research is costly, emotionally difficult for researchers, and causes pain and discomfort for the horses. Endotoxemia may be an effective model of MMP induction in the horse which can be used for evaluating MMPIs for use in the prevention/treatment of equine laminitis.

1.2 Matrix Metalloproteinases

The function of MMPs cannot be fully understood without first understanding the structure and function of the ECM. The main structural unit of the ECM is collagen. Collagens provide the scaffolding necessary for structural integrity of tissues. There are over 20 distinct collagens. Collagens types I, II, III, V, and XI assemble into fibrils that provide tissues such as bone, cartilage, tendon, and skin with tensile strength. The basement membrane (BM) of the ECM is composed mostly of type IV collagen and laminin.[1,2] The BM also contains other proteins such as type V collagen, growth factors, proteases, proteoglycans, and glycoproteins.[2] Type IV and V collagens are organized into non-fibrillar multilayer networks (as are types VIII and X) that are resistant to nonspecific proteolytic degradation.[1,2] Proteoglycans adhere to the collagen framework and function to support cell adhesion and bind growth factors.

The ECM functions to maintain tissue structure and integrity, regulate cellular migration, and serve as a reservoir for various cytokines and growth factors.[3] Multiple stimuli induce remodeling of the ECM during homeostasis, normal development, and pathologic conditions. Remodeling involves both ECM degradation and synthesis. Proteolytic breakdown is accomplished via numerous proteases, especially MMPs.[1]

Proteases can be divided into exopeptidases and endopeptidases, or proteinases. Endopeptidases are hydrolytic enzymes that are grouped according to the catalytic group at their active site. Metalloproteases contain over 200 mostly zinc-dependent enzymes, of which only 25 or more are MMPs. The metzincin superfamily of metalloproteases contains the matrixin family, also known as MMPs.[4]

Matrix metalloproteinases are a family of over 25 zinc-dependent endopeptidases that degrade ECM components. They were first discovered when enzymes from tadpole tails undergoing metamorphosis hydrolyzed a native collagen matrix.[5] All MMPs share general basic

characteristics. They are secreted (except for the membrane-type) or anchored to the cell surface in the pro enzyme form as zymogens that require cleavage for activation.[4] They require Ca^{2+} for stability and function at neutral pH.[6,7]

Proteases must contain at least two conserved motifs, a pro and catalytic domain, to be classified as MMPs.[8] Their structure usually contains a signal sequence followed by the N-terminal pro-domain.[7] The pro domain is approximately 80 amino acids long and contains a cysteine residue. The active, or catalytic, domain is approximately 170 amino acids long and contains three histidines which ligate a Zn^{2+} active site.[9] The inactive pro-enzymes are held in the latent form by a "cysteine switch" mechanism which consists of the interaction between the thiol moiety of the pro-domain cysteine residue and the Zn^{2+} of the active site. This interaction blocks access of the active site to substrate and must be disrupted for activity.[7] At the C-terminal, there is a hemopexin-like domain which aids in substrate recognition.[10] Some MMPs also have specialized domains that further determine substrate specificity and allow recognition and interaction with other proteins.[10]

Matrix metalloproteinases not only perform matrix catalysis, but also regulate cell-cell and cell-matrix interactions and activate other proteins. They act on membrane proteins or proteins within the extracellular space.[8] Their expression, location, and substrate availability determine the proteolytic activity of MMPs in physiologic and pathologic inflammatory processes.[11] They are present in tissues for homeostasis, but can be induced for repair or remodeling and during disease states by activated cells.[8] Matrix metalloproteinases help to regulate physical barriers, modulate inflammatory mediators, and establish chemokine gradients for movement of leukocytes in inflamed tissue. Specifically, they degrade endothelial cell junctional proteins to allow leukocyte migration into areas of infection or inflammation. They help promote re-epithelialization and restoration of epithelial barriers against bacteria and other

invading pathogens. They also promote or suppress inflammation by proteolytic activation and de-activation of cytokines and chemokines.[11]

Matrix metalloproteinases are grouped according to their substrate specificity and primary structure (Table 1.1).[7] Collagenases degrade fibrillar forms of interstitial collagen and include interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), and collagenase-3 (MMP-13). Gelatinase A and B (MMP-2 and MMP-9) degrade denatured collagen. Stromelysins 1, 2, and 3 (MMP-3, 10, and 11) act on non-collagen components of the ECM.[10] The matrilysins (MMP-7 and MMP-26) are the smallest of MMPs. There are 7 membrane-type MMPs (MMP-14, 15, 16, 17, 23, 24, 25) which degrade ECM components as well as activate other MMPs.[7,12]

The gelatinases are the most widely studied group of MMPs. Matrix metalloproteinase-2 is a 72-kD protein in its pro-enzyme form known as Gelatinase A. It degrades gelatin (denatured collagen), along with type-IV, V, VII, and X collagen, elastin, laminin, and fibronectin.[13] Gelatinase A is constitutively expressed by many structural cell types including endothelial cells, osteoblasts, and fibroblasts.[12] Its activation is unique in that it requires binding to tissue inhibitor of MMP-2 (TIMP-2). Once bound, this complex is then activated by MMP-14, a membrane type MMP (MT-MMP).[14] Gelatinase B, or MMP-9, is a 92-kD protein in its pro-enzyme form. It also degrades gelatin, types IV and V collagen, elastin, fibronectin, and plasminogen.[13] Unlike MMP-2, MMP-9 expression is generally induced. It is synthesized by leukocytes, predominately neutrophils, fibroblasts, and keratinocytes. Neutrophils store preformed MMP-9 in tertiary granules for immediate release that does not require *de novo* synthesis.[7]

Regulation of MMP activity is tightly controlled on several levels to prevent rampant tissue destruction, including mRNA/protein expression, pro-enzyme activation, and inhibition/degradation. Transcriptional regulation occurs through the cyclic adenosine

Table 1.1 – MMP nomenclature organized by group.

MMP NOMENCLATURE

Group	Name	MMP Number
Collagenase	Interstitial Collagenase	MMP-1
	Neutrophil Collagenase	MMP-8
	Collagenase-3	MMP-13
	Collagenase-4	MMP-18
Gelatinase	Gelatinase A	MMP-2
	Gelatinase B	MMP-9
Stromelysin	Stromelysin-1	MMP-3
	Stromelysin-2	MMP-10
	Stromelysin-3	MMP-11
Matrilysin	Matrilysin	MMP-7
	Matrilysin-2	MMP-26
Membrane-Type	MT1-MMP	MMP-14
	MT2-MMP	MMP-15
	MT3-MMP	MMP-16
	MT4-MMP	MMP-17
	MT5-MMP	MMP-24
	MT6-MMP	MMP-25
Others	Metalloelastase	MMP-12
	RASI-I	MMP-19
	Enamelysin	MMP-20
	CA-MMP	MMP-23
	Epilysin	MMP-28

monophosphate/p38/mitogen activated protein kinase (cAMP/p38/MAPK) pathway, and can be induced or inhibited by various cytokines and growth factors, such as interleukin-1 (IL-1), IL-2, tumor necrosis factor-α (TNF-α), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF).[3,6,12,15-22] Other inflammatory mediators such as nitric oxide (NO), endothelin-1 (ET-1), eicosanoids such as prostaglandin E_2 (PGE_2), and neuropeptides such as vasoactive intestinal peptide (VIP) have also been shown to induce or inhibit synthesis of MMP-2 and MMP-9.[15,21,23-26] Matrix metalloproteinase gene expression can also be influenced by cell-cell interactions.[27]

Pro-enzyme activation of MMPs can occur through direct proteolytic cleavage of the pro-domain via proteases such as plasmin, trypsin, elastase, tissue kallikrein, cathepsin-G, chymase, and other MMPs.[10,28,29] Activation may also occur by exposure to organomercurials, oxidated glutathione, and reactive oxygen species (ROS).[6,30] Heavy metal ions, such as mercurial compounds, can activate MMP zymogens by reduction of the pro-domain thiol.[4] Oxidants can activate MMPs through oxidation of the pro-domain thiol and subsequent release of the Zn^{2+} active site allowing autolytic cleavage.[31] Hypochlorous acid (HOCl), a ROS by-product of leukocyte myeloperoxidase, and peroxynitrite, a by-product of NO release, have been shown to activate MMP-2 and MMP-9.[4,32-36] Several MMPs have also been found to undergo allosteric activation without pro-domain removal when in contact with the appropriate substrate.[31]

Regulation of MMP activity occurs post-activation by interaction with endogenous MMP inhibitors and degradation of the enzyme by ROS. Endogenous inhibitors include the non-specific protease inhibitor α_2-macroglobulin, TIMPs, and reversion-inducing-cysteine-rich protein with kazal motifs (RECK).[31,37] Circulating α_2-macroglobulins bind MMPs to prevent substrate attachment. The complexes are then cleared from the circulation by macrophage endocytosis.[31] In normal tissues, MMP activity is highly regulated by the MMP/TIMP

balance.[3,38] To date, four TIMPs have been discovered and characterized (TIMP-1 through TIMP-4). TIMP-1, 2, and 4 are in a soluble form, whereas TIMP-3 is associated with the ECM. They all bind non-covalently in a 1:1 ratio with high affinity to both pro and active MMP catalytic sites resulting in either prevention or loss of activity.[7,39] TIMP-1 binds specifically with MMP-9 and TIMP-2 with MMP-2, as previously stated. In addition to inactivating MMPs, TIMPs also affect cell growth by inducing apoptosis.[10] RECK is a membrane anchored glycoprotein that inhibits MMP-2, MMP-9, and MMP-14 (MT-MMP-1). It has been suggested that RECK regulates MMP activity through direct inhibition of protease activity, regulation of cellular release, and cell surface sequestration.[39] Degradation can occur via the same ROS that activate MMPs through modification of amino acids that are essential for catalytic activity.[31]

Several techniques are available for the detection of MMP-2 and MMP-9; however, gelatin zymography is most often used. Zymography utilizes electrophoresis to separate proteins in a polyacrylamide gel impregnated with gelatin.[40] Any MMP-2 or MMP-9 present in a sample will degrade the gel at the appropriate molecular weights. Staining of the gels with Coomassie blue allows MMP activity to be visualized as a clear band in the blue gel (Figure 1.1). Zymography is highly specific for MMP-2 and MMP-9 activities, because they are the only MMPs known to degrade gelatin; however, it is only semi-quantitative. Relative values can be established for MMP concentrations by digitally scanning zymography gels and measuring band intensity and size using a computerized densitometry program. Both pro and active forms of the enzymes can be detected using zymography. The samples are diluted and subjected to electrophoresis in buffers containing sodium dodecyl sulfate (SDS), which removes any inhibitors present and activates the enzymes. The SDS is rinsed away following electrophoresis, allowing the enzymes to renature and degrade the gel. Other procedures available for evaluation

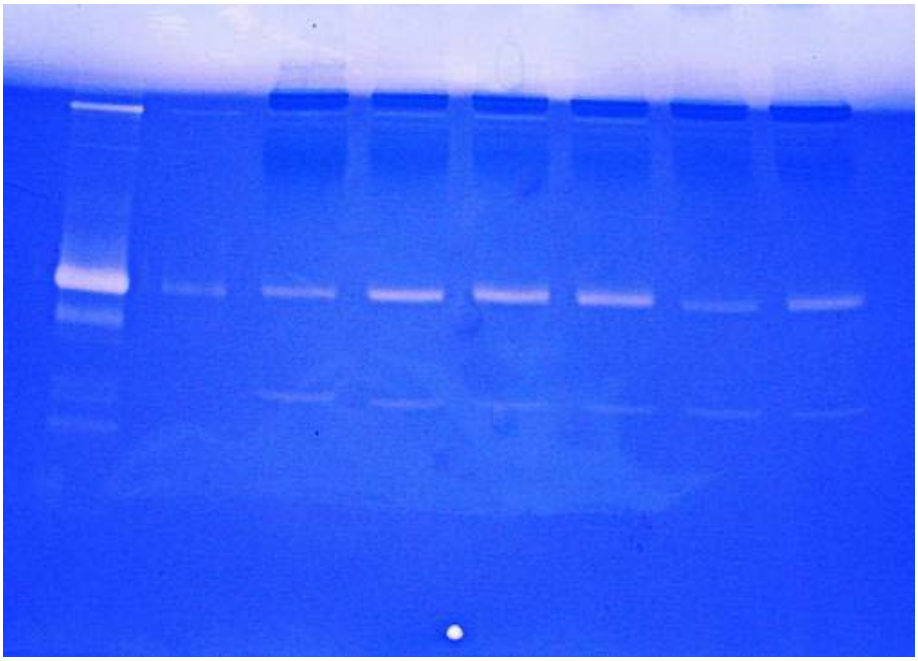


Figure 1.1 – An example of a zymogram. The clear areas indicate the presence of MMPs.

of MMPs include enzyme-linked immunosorbant assays (ELISAs), fluorogenic assays, activity assays, immunohistochemistry, and mRNA expression analysis.[41,42]

Numerous studies have shown that MMPs are crucial for normal tissue development, remodeling, and homeostasis. They are active in many processes such as ovulation, blastocyst implantation, embryonic development, postpartum uterine involution, bone remodeling, angiogenesis, and apoptosis.[43-49] Several MMPs modulate the activities of growth factors, cytokines, and other mediators. Both MMP-2 and MMP-9 activate TNF-α and IL-1β.[11,50] In turn, IL-β can also be degraded by the gelatinases.[51] Big ET-1 is cleaved into the potent vasoconstrictor ET-1by MMP-2, a process which can be blocked by TIMP-2.[52] Vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), and EGF are all released and activated through the enzymatic actions of MMPs.[53-55]

While MMPs are crucial for normal biological processes, they are mainly disease-associated enzymes. Almost all forms of cancer are associated with increases in MMPs, because they are necessary for tumor metastasis and angiogenesis.[56,57] Various neurological conditions result from unregulated MMP activity at the blood brain barrier, which allows pathogens and inflammatory cells access to the central nervous system (CNS).[58,59] Increased MMP activity is also associated with osteoarthritis, asthma, cardiovascular disease, and numerous other diseases.[6,10,60-64]

In horses, MMPs have been identified in association with several pathologic conditions. Matrix metalloproteinase-9 is involved in equine respiratory disease, in particular in recurrent airway obstruction (RAO).[65] Several studies have shown increased MMP-9 concentrations in bronchioalveolar lavage fluid (BALF) and tracheal epithelial lining fluid (TELF) of horses with RAO, and that elevations in MMP-9 are correlated with neutrophil numbers.[66-68] Increased MMP-8 and MMP-13 activities are also present in association with RAO, and can be inhibited

by synthetic MMPIs.[69,70] Increases in equine synovial fluid MMP-1, 2, 3, 9, and 13 concentrations are associated with osteoarthritis.[71-76] Matrix metalloproteinase-13 mRNA expression is up-regulated in equine superficial digital flexor tendonitis lesions, and MMP-9 is suggested to be important in this disease as well.[77,78] Increases in gelatinase activity have been associated with colic, corneal ulceration, and endometriosis in horses.[79-81] Studies have shown increased MMP-2 and MMP-9 concentrations in laminar tissues of both experimentally-induced and naturally-acquired equine laminitis.[82-84] Increased mRNA expression of MMP-2 and MMP-14 has also been found in laminar tissues of laminitic horses.[85,86]

As the role of MMPs in various pathological conditions has become more apparent, attention has been focused on MMPIs as therapeutic agents. Synthetic MMPIs, such as batimastat, marimastat, and others have been developed as potential treatments for diseases such as cancer and arthritis. However, in clinical trials, these compounds have been proven to be either ineffective or cause adverse side effects believed to be due to their non-specific MMP inhibition.[87] The predominant side effect is a musculoskeletal syndrome (MSS) characterized by pain and immobility in the shoulder joints, arthralgias, contractures in the hands, and an overall decreased quality of life.[88] Consequently, the only drug approved in human medicine for its effects on MMP inhibition is the tetracycline antibiotic doxycycline, which is used in the treatment of periodontal disease.[88]

Several classes of drugs are capable of inhibiting MMPs including corticosteroids, retinoic acid, chelating agents, bisphosphonates, statins, certain antibiotics, non-steroidal anti-inflammatory drugs (NSAIDs), and phosphodiesterase inhibitors.[23,88] Glucocorticoids and retinoic acid have both been shown to inhibit MMP transcription.[12,13,38,89,90] Dexamethasone, triamcinolone, and prednisolone inhibit MMP-1, 2, 3, and 13 in equine chondrocyte cultures.[71,91] Chelating agents, N-acetylcysteine, and disodium ethylene diamine tetraacetate (EDTA), are

known to inhibit MMPs by binding Zn^{2+} and Ca^{2+}. Both agents are used in the treatment of ulcerative keratitis.[92,93] Bisphosphonates, while developed for use in diseases involving bone and calcium metabolism, also have MMP inhibitory effects in various diseases including equine RAO.[70,94,95] The mechanism of action of bisphosphonate MMP inhibition is unclear, but is suggested to involve chelation of cations and down-regulation of mRNA and protein expression.[70] Statins are often used in cardiovascular disease and have been shown to inhibit MMPs through decreased expression and secretion.[96,97] Clarithromycin and erythromycin, both macrolide antibiotics, have MMP inhibitory effects in cardiac allograft recipients and cultured smooth muscle cells through decreased MMP expression.[98,99]

In addition to their antibiotic effects, tetracyclines inhibit MMPs through decreased transcription, chelation of Zn^{2+} and Ca^{2+}, and increased degradation of the enzymes.[100,101] Doxycycline, a semi-synthetic tetracycline, has been shown to be an effective MMPI in various tissues and diseases including endothelial cells, pleural fluid, corneal epithelium, cerebral ischemia, and endotoxemia-induced cardiac dysfunction.[102-106] As previously mentioned, it is the only MMPI approved for human use. In horses, doxycycline is used in the treatment of various diseases including Potomac horse fever caused by *Neorickettsia risticii*, a disease associated with depression, fever, diarrhea, and laminitis.[107] It has been reported to cause cardiovascular collapse and death when administered intravenously (IV) to horses; however, several studies have demonstrated the safety of oral administration of doxycycline.[108-110] Oxytetracycline, a tetracycline analogue, is used in the horse for its antibiotic properties, but also for the treatment of flexural deformities in foals. It has been shown to decrease MMP-1 expression in equine myofibrils.[111] Chemically modified tetracyclines have been developed by removing the antimicrobial portion of the compound; thus, leaving the MMP inhibitory portion.[112] Research suggests that they may be of potential use in horses with RAO.[70]

NSAIDs are cyclooxygenase (COX) inhibitors, thus preventing the formation of eicosanoid inflammatory mediators in the arachidonic acid cascade. They decrease MMP-2 and MMP-9 activities by decreasing mRNA expression.[113,114] Cyclooxygenase-2 increases MMP-2 and MMP-9 expression that can then be decreased in response to both non-selective and selective NSAIDs.[114-117] Other research suggests that NSAIDs up-regulate mRNA expression of RECK.[118] Due to their MMP inhibiting abilities, various NSAIDs are being used to prevent tumor migration and metastasis in cancer. Although many studies have shown the MMP inhibitory effects of NSAIDs in other species, there is very little data assessing their ability to inhibit equine MMPs. Flunixin meglumine is an NSAID that is commonly used in horses for gastrointestinal pain and inflammation. The only research published do date suggests that *in vitro*, flunixin meglumine does not inhibit MMP-2 or MMP-9 obtained from equine cell culture.[91]

Phosphodiesterase inhibitors regulate MMP expression by increasing intracellular cAMP concentrations that disrupt phosphorylation pathways and prevent gene transcription.[113,119] Pentoxifylline is a methyl xanthine derivative and phosphodiesterase inhibitor commonly used for its rheological effects on peripheral vascular disease in people.[120] It produces potent anti-inflammatory effects through reduction of TNF-α and IL-6 concentrations and enhancement of the anti-inflammatory cytokine IL-10.[121] Pentoxifylline has also been shown to decrease neutrophil infiltration and activation.[119,122] It is used in the horse for its anti-inflammatory effects in the treatment of endotoxemia and other systemic inflammatory conditions.

1.3 The Systemic Inflammatory Response/Endotoxemia

Systemic inflammatory response syndrome (SIRS) has been defined by the American Society of Chest Physicians and the Society of Critical Care Medicine as an inflammatory response that includes more than one of the following clinical manifestations: 1) hyper- or

hypothermia, 2) tachycardia, 3) tachypnea, or 4) leukocytosis or leucopenia.[123] This systemic response is most often associated with endotoxemia originating from Gram-negative bacteria. Endotoxin (lipopolysaccharide, LPS) is one of the most potent bacterial toxins known, and is responsible for inciting the severe inflammatory response seen during endotoxemia in the horse, as well as other species.[124] The microvascular inflammatory response to endotoxin results in activation of mononuclear phagocytes which release numerous cytokines and enzymes. This leads to endothelial and leukocyte activation with increased leukocyte and platelet adherence and microthrombi formation.[125] Neutrophils are capable of being activated intravascularly, and are easily activated *in vitro* by LPS causing expression of integrin adhesion molecules, decreased deformability, size variation, and degranulation.[126,127] Endothelial cells swell, increasing capillary and venular permeability resulting in tissue edema. Diapedesis and degranulation of neutrophils results in further tissue damage and activation of inflammatory mediators. This systemic inflammatory response begins a vicious cycle in the microcirculation, and if left unchecked, usually results in cardiac dysfunction, progressive hypotension, coagulopathies, and organ dysfunction/failure.[125]

Endotoxin is a component of the outer cell membrane of all Gram-negative bacteria that can be released during rapid proliferation or cell death. It consists of three components. The inner lipid A portion is highly conserved among Gram-negative bacteria and imparts the toxic effects. Each LPS molecule also contains a core oligosaccharide and an outer O-specific polysaccharide that imparts serotype specificity for different bacterial strains.[128]

The endotoxin-induced inflammatory process is initiated by the binding of endotoxin to lipopolysaccharide binding protein (LBP) at the lipid A moiety. Lipopolysaccharide binding protein acts as a shuttle to facilitate the transport of endotoxin to various cell types including mononuclear phagocytes, neutrophils, and endothelial cells; however, monocytes and

macrophages are the principal cells involved in the initial response to endotoxin.[129] The inflammatory response is initiated when LBP transfers endotoxin to the cell surface receptor CD14. This receptor is present in both a soluble form in the blood and a membranous form on the surface of monocytes, macrophages, and neutrophils. The soluble form allows other cells such as endothelial and epithelial cells to react to endotoxin. The endotoxin/LBP/CD14 complex increases cell sensitivity to endotoxin, but cannot cross the cell membrane to stimulate second messenger systems or signal transduction pathways.[128] The LPS signal is carried intracellularly by the pattern recognition receptor Toll-like receptor 4 (TLR 4). This occurs with the help of myeloid differentiation factor 2 (MD2) on the cell surface. Cell stimulation by LPS leads to activation of the transcription factor nuclear factor κB (NFκB) and the MAPK pathway. Activation of these pathways ultimately results in the synthesis of various pro- and anti-inflammatory mediators responsible for the clinical manifestations of endotoxemia.[129]

The inflammatory cascade induced by endotoxin includes release of pro-inflammatory cytokines, chemokines, adhesion molecules, proteolytic enzymes, and acute phase proteins, as well as production of ecosanoids.[125] Tumor necrosis factor-α is the initial mediator of endotoxin's effects, and is produced by macrophages in response to LPS.[124] This cytokine typically reaches peak levels in the horse 1-2 hours after LPS exposure.[130,131] It is responsible for a myriad of effects including stimulating the release of other cytokines and neutrophil activation. Interleukin-6 is produced by monocytic phagocytes, fibroblasts, and endothelial cells, and is a potent inducer of acute phase proteins.[124,129] Interleukin-6 activity is increased in endotoxemic horses from 30 minutes to 8 hours post-infusion of LPS.[130,132] Interleukin-1β is also released from machrophages in response to LPS. It is a potent attractant for neutrophils, as well as performing many functions similar to TNF-α.[124] Endotoxin has also been shown to up-regulate IL-1β and TNF-α mRNA in equine mononuclear cell culture.[133]

Endotoxemia also results in the activation and release of proteolytic enzymes, in particular MMPs. Gelatinolytic activity (MMP-2 and MMP-9) is increased in patients with clinical Gram-negative sepsis.[134] Plasma MMP-9 concentrations are increased between 1 hour and 8 hours post-infusion of LPS to human volunteers and between 30 minutes and 6 hours after LPS administration to baboons.[134-136] Septic shock non-survivors have higher plasma concentrations of MMP-9 and greater expression of MMP-9 mRNA compared with survivors, with a positive correlation between plasma LPS and MMP-9 concentrations.[137] Matrix metalloproteinase-9 activity is increased in the liver, spleen, kidney, brain, heart, and aorta of mice administered LPS.[105,138,139] Endotoxin also stimulates release of MMP-2 in endothelial cells and rat aorta, as well as MMP-9 in peritoneal macrophages.[138,140-142] Interleukin-1β stimulates the release of MMP-2 from rat aorta.[138] Lipopolysaccharide, TNF-α, IL-8, and granulocyte colony-stimulating factor all stimulate rapid release of MMP-9 from neutrophil granules.[134] Reactive oxygen species released from neutrophil granules help regulate the activity of vascular MMPs and are generated in response to LPS.[143,144] In particular, peroxynitrite, a by-product of NO release, can activate MMPs and is increased along with NO in LPS-treated rats.[140,145,146] Increases in MMP-9 activity correlate inversely with mean arterial pressure (MAP) in endotoxemic rats.[147] Furthermore, LPS and IL-1β both induce hyporeactivity in rat aorta that can be ameliorated by doxycycline, a potent MMPI, suggesting that MMPs play an important role in the cardiovascular effects of endotoxemia.[138]

In healthy horses, the gastrointestinal tract contains large amounts of endotoxin owing to the large quantities of Gram-negative bacteria needed for fermentation of food material.[129] There are several mechanisms present to prevent LPS from reaching the systemic circulation during normal states. Mucosal epithelial cells, the mucous layer covering them, and other resident bacteria restrict the movement of endotoxin within the intestinal lumen.[128] Occasionally in

healthy animals, small amounts of the toxin escape into the portal circulation. These molecules are quickly removed by either hepatic macrophages known as Kupfer cells or circulating anti-endotoxin antibodies to prevent a systemic inflammatory response. However, under disease conditions, endotoxin can overwhelm these mechanisms and play a major role in the systemic inflammatory response in the horse.[124]

Endotoxemia in the horse most often occurs secondary to compromise of the intestinal tract barrier. The quantity of endotoxin in the circulation can become so great that it cannot be sufficiently removed and thus overwhelms the normal protective mechanisms. Endotoxin can also cross hypoperfused or inflamed intestine, enter the peritoneal cavity, and gain access to the systemic circulation via the thoracic duct.[128]

There are many disease processes of the horse that may lead to the development of endotoxemia. Acute abdominal disease, colitis, post-operative ileus, enteritis, peritonitis, pleuropneumonia, metritis, and grain overload are all capable of inducing secondary endotoxemia in the horse. Most commonly, equine endotoxemia results from an acute gastrointestinal tract disturbance. Endotoxin has been detected in the plasma of approximately 25% of horses admitted to teaching hospitals for acute gastrointestinal tract disease.[148] Experimentally, LPS decreases cecal blood flow and increases intestinal permeability, thus allowing endotoxin access to the systemic circulation.[149] In an experimental model of small intestinal ischemia/reperfusion injury, circulating endotoxin was detected at 60 and 120 minutes after reperfusion.[150] Clinical studies have shown that 50% of horses are endotoxemic during colic surgery, and that circulating LPS can be detected in a large proportion of horses up to 5 days after colic surgery.[151,152]

Intravenous infusion of LPS has been used extensively in the past as a method for inducing experimental endotoxemia. Typically following administration of low-dose LPS (10 –

35 ng/kg), horses become mildly colicky and develop fever, tachypnea, tachycardia, an initial neutropenia with a rebound neutrophilia, thrombocytopenia, increased capillary refill time (CRT), hemoconcentration, and lactic acidosis.[130,131,153] Leukopenia is one of the cardinal signs of endotoxemia and can occur between 30 minutes and 4 hours post-infusion.[154,155] Higher doses of LPS (over 10 µg/kg) have resulted in mucous membrane cyanosis, hyper- and hypoglycemia, hyper- and hypothermia, and extreme coldness of the lower limbs.[156,157]

Although experimentally-induced endotoxemia does not exactly replicate or mimic clinical disease, it allows for investigation of various aspects that cannot be studied or controlled in the clinical arena. Endotoxemia has been shown to cause decreased gastrointestinal tract motility in horses, specifically decreased intestinal muscular activity due to edematous degeneration and coagulative necrosis of smooth muscle cells of the intestinal muscularis layer and possibly degeneration of the central, autonomic, and myenteric nervous systems.[158,159] Lipopolysaccharide stimulates COX-2 activity in equine digital arterial smooth muscle cells.[133] Prostaglandin E_2 decreases gastric contraction amplitude and rate in endotoxemic ponies.[160] This decrease in gastric activity can be prevented by pre-treatment with phenylbutazone, a known COX-1 inhibitor.[161] Insulin resistance is caused by endotoxemia in humans and rats. In horses, administration of endotoxin resulted in decreased insulin sensitivity for 24 hours along with a compensatory pancreatic response.[153]

One major sequelae to diseases accompanied by endotoxemia in horses is the development of laminitis. Endotoxin has been detected in both cecal contents and plasma of horses with experimentally-induced carbohydrate overload (CHO) laminitis, and has been associated with Obel Grade 3 lameness in this model.[162,163] Lactic acid-induced decreases in the cecal population of Gram-negative bacteria correlates with the over 5 times increase in cecal fluid endotoxin levels.[162,164] Severe compromise of the cecal mucosa found in experimental

CHO allows numerous toxins, including endotoxin, to enter the circulation and stimulate the release of pro-inflammatory cytokines, as demonstrated by the increased plasma endotoxin levels seen in CHO-induced laminitis.[163,165] Evidence of endotoxemia including elevated temperature, tachycardia, and limb edema has also been observed in horses developing laminitis after accidental CHO.[166] Experimentally-induced endotoxemia leads to decreased digital arterial and venous blood flow, decreased digital laminar blood flow, and decreased coronary band and hoof wall temperature.[131,167] Increases in plasma 5-hydroxytryptamine (5-HT) and thromboxane B_2 (TXB_2) occur with *in vivo* administration of LPS and with *in vitro* stimulation of equine platelets.[131,168] These substances have been suggested as possible vascular mediators in the development of laminitis.[168] Endotoxemia has also been implicated as an important risk factor for the development of acute laminitis in horses hospitalized for medical or surgical conditions.[169] Despite the association between clinical endotoxemia and laminitis, experimentally-induced endotoxemia does not reproducibly induce laminitis. Nevertheless, the clinical occurrence of laminitis subsequent to diseases associated with endotoxemia suggests that LPS does play a role, either directly or indirectly, in the development of laminitis.

1.4 Equine Laminitis

Acute laminitis is an excruciatingly painful and severely debilitating disease of the soft tissues of the equine digit. In many instances, it can lead to the separation of the epidermal and dermal laminae, resulting in rotation and/or sinking of the third phalanx within the hoof capsule.[170] This disease is common to all breeds world-wide and often results in chronic pain, lameness, and sometimes death. Laminitis is frustrating to both veterinarians and owners as current therapy consists mainly of NSAIDs and therapeutic shoeing, which are often ineffective. One of the major reasons for the limited scope of treatment is that the pathogenesis of laminitis is not fully understood despite years of intense research.

A recent survey by the American Association of Equine Practitioners (AAEP) found that equine practitioners believe laminitis is the most important disease afflicting horses and the number one disease requiring further research efforts and funding.[171] This is most likely due to the prevalence, morbidity and mortality, and economic and emotional costs of the disease, as well as the incomplete understanding of its pathogenesis. Laminitis is considered one of the most important diseases in the equine industry with an estimated 15% of horses developing laminitis during their lifetime, of which 75% of those admitted to referral hospitals eventually require euthanasia.[172] Annual monetary losses related to laminitis have been conservatively estimated at greater than $13 million associated with its diagnosis, treatment and loss of horses subsequent to complications.[173]

In the horse, the distal phalanx (P3, coffin bone) is contained within the hoof capsule, and is attached to the hoof wall via soft tissue attachments. Theses attachments, or laminae, extend from the inner surface of the hoof wall to form the primary epidermal (insensitive) laminae (PEL) and from the periosteum of P3 as primary dermal (sensitive) laminae (PDL). It is estimated that there are approximately 600 PEL per hoof.[174] These epidermal and dermal laminae interdigitate and, in effect, suspend the distal phalanx within the hoof capsule (Figure 1.2). Both the PEL and PDL have secondary laminae that also interdigitate and increase the surface area of attachment between the hoof wall and P3.[175] A BM separates the avascular secondary epidermal laminae (SEL) from the vascular secondary dermal laminae (SDL). Laminin, a glycoprotein, is distributed throughout the BM and is important for the differentiation and attachment of epidermal basal cells.[175] Hemidesmosomes (HD) attach the epidermal basal cells to the *lamina densa* of the BM through anchoring filaments.[175] The proximal epidermal laminae located near the coronary band and periople are proliferative, but the majority of the epidermal laminae are nonproliferative and function to suspend P3.[176]

Figure 1.2 – A hoof from a normal horse illustrating the hoof wall (A), epidermal laminae (B), dermal laminae (C), and third phalanx (D). Note that the third phalanx is parallel to the hoof wall.

The medial and lateral palmar digital arteries supply oxygenated blood to the foot. Blood flows through various arteries on its way to the laminar microvasculature. Each PDL is supplied by a papillary artery flowing into a capillary bed which feeds the lamina and then passes out by way of a papillary vein.[174] Deoxygenated blood eventually leaves the foot via the medial and lateral palmar digital veins. Each papillary artery and vein have numerous (approximately 500/cm2) arteriovenous anastomoses (AVAs), or shunts, between them that may regulate hoof temperature in cold environments.[174]

During acute laminitis, the laminae are inflamed and may become necrotic and separate. If this occurs, P3 is able to rotate or sink distally within the hoof capsule leading to intense pain and hoof structural alteration (Figure 1.3). Consistent histological changes within the laminae early in laminitis are degradation of the BM and detachment from the epidermal basal cells in the SEL.[83,175] Also, numerous neutrophils accumulate at the SDL tips, many of which have penetrated the BM and have moved into the SEL.[83,177,178]

Laminitis may be a primary disease, (e.g. due to excessive concussive force), but most often occurs secondary to other illnesses. Grain overload, colitis, enteritis, pleuropneumonia, metritis, colic, and contralateral limb lameness often precede the onset of laminitis.[179] With the exception of contralateral limb lameness, the aforementioned diseases may all be associated with endotoxemia. Studies have shown that gastrointestinal (GI) disease is the most common problem noted prior to the onset of laminitis, and that endotoxemia is a significant risk factor for the development of laminitis.[169,180] Although there appears to be an association clinically between endotoxemia and laminitis, experimentally-induced endotoxemia does not reproducibly result in laminitis. However, laminitis can be experimentally induced using either a CHO model, a black walnut extract (BWE) model, or an oligofructose (OF) model.[181-183]

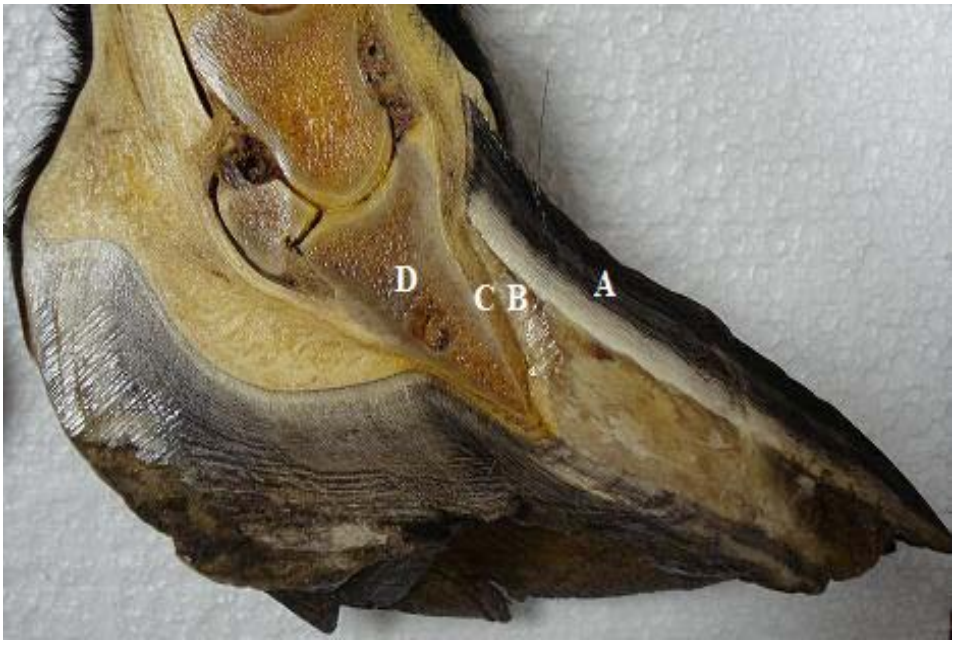


Figure 1.3 – A hoof from a horse with chronic laminitis illustrating the hoof wall (A), epidermal laminae (B), dermal laminae (C), and third phalanx (D). Note that the third phalanx has rotated distally from the hoof wall, and the epidermal and dermal laminae are thickened.

Due to the variable etiologies for the development of laminitis, the pathophysiology remains elusive. There are several different hypotheses regarding its pathogenesis.[184] A vascular theory hypothesizes that dysfunction of the digital vasculature results in interstitial edema and ischemia followed by laminar necrosis and separation.[185] There is conflicting evidence as to whether vasodilation or vasoconstriction occurs at the onset of laminitis. Early work using arteriovenograms to evaluate blood flow before and after CHO indicated that blood flow in the terminal arch of the foot was significantly decreased or absent after CHO.[186] Other research has suggested that venoconstriction mediated via either ET-1, TXA_2, or 5-HT is responsible for the laminar ischemia.[168] Evaluation of Starling forces in horses with CHO and BWE indicate that increases in capillary pressure owing to increased vascular resistance leads to interstitial edema within the digit.[187,188] Decreased hoof wall temperature, suggesting a decrease in digital vascular perfusion, and decreased lamellar microvascular blood flow occur during the developmental phase of carbohydrate overload induced laminitis.[189,190] Other studies report conflicting data. Early data refuted the theory of decreased perfusion suggesting that increased digital blood flow occurred in the CHO model due to decreased vascular resistance.[191] Scintigraphy used to evaluate blood flow following CHO indicated there was no reduction of blood flow and that flow was significantly increased in the laminae.[192] Others have reported that increases in hoof wall temperature occur at the onset of clinical signs with no prior indication of decreased blood flow, and that cryotherapy prevented histological evidence of laminitis and lameness.[83,193] It has been suggested that vasodilation occurs initially leading to opening of AVAs within the digital microvasculature to protect the laminar capillaries from the high pressures of increased flow. However, this leads to ischemia of the laminar tips if vasodilation is prolonged as in laminitis.[194] Regardless of the initial event, it is generally accepted that vascular changes are intricately involved in the pathogenesis of laminitis.

Other research has characterized laminitis as a local manifestation of a systemic inflammatory response.[195] Pro-inflammatory mediators, namely IL-1β, IL-6, IL-8, and COX-2 are up-regulated in the laminae during the early stages of BWE laminitis.[196-199] Normal laminae contain little or no neutrophils or macrophages in the perivascular region of the SDL.[178] However, in BWE laminitis, there is an influx of leukocytes, mostly neutrophils, into the dermal laminae at the onset of leukopenia that increases to significance by the onset of lameness.[178] Neutrophil activation is further demonstrated by increases in plasma, laminar, and skin myeloperoxidase prior to the onset of lameness in BWE laminitis.[200]

Coinciding with the inflammatory component of laminitis is the theory that intestinal mediators are released into the circulation that initiate SIRS and activate inflammatory cells to release cascades of mediators.[201] Among these mediators are enzymes that regulate formation and degradation of the extracellular matrix, especially MMPs. In the horse, MMP-2 and -9 have been isolated within the laminae of the digit.[202] As stated previously, they are members of the gelatinase family and have the ability to degrade type IV collagen. Hemidesmosomes separate from the *lamina densa* due to degradation of anchoring filaments and allow laminar separation to occur *in vitro* when explants are exposed to the MMP activator p-aminophenylmercuric acetate (APMA), a mercurial compound.[203] APMA-induced laminar separation can also be prevented by the MMPI batimastat.[204] Laminar explants of horses with acute laminitis have increased concentrations of MMP-2 and MMP-9.[83] Thus, MMP-2 and MMP-9 appear to be associated with the BM destruction and laminar separation characteristic of acute laminitis. Mungall et al described an "in vitro laminitis model" in which bacterial broths increased MMP-2 and MMP-9 concentrations and increased laminar separation in equine digital laminar explants.[205] Bacterial proteinases such as thermolysin increase laminar explant separation as well as activate proMMP-2 and proMMP-9 *in vitro*.[202] This, along with increases in Gram-positive bacteria in

the cecum of horses during CHO laminitis, led some researchers to believe that *Streptococcus bovis* toxins are responsible for the initiation of MMP-associated laminar destruction.[205]

The roles of MMP-2 and MMP-9 have also been evaluated *in vivo*. Both enzymes are activated in laminar tissues of horses with naturally-acquired laminitis when compared to laminae of normal horses.[82] Also, plasma MMP-9 concentrations and transcription of MMP-2 in laminar tissues are increased during experimentally-induced laminitis using a CHO model.[85,206] Recently, increased transcription of MMP-14 in the laminae of horses with OF laminitis has been reported, which is required for activation of MMP-2.[86] As previously mentioned, MMP-9 concentrations are increased in laminar tissue of BWE horses; however, pro and active forms of MMP-2 are present in both control and BWE horses.[84] Serum collagen IV concentrations are increased in horses with naturally-acquired laminitis suggesting the breakdown of ECM by MMPs.[207]

Several inflammatory cells release MMP-9, in particular neutrophils. Activation of MMPs during laminitis could be associated with leukocyte recruitment and induction of inflammation. Decreases in circulating leukocytes as well as increases in production of ROS by leukocytes have been found in horses with BWE laminitis.[195] Small amounts of MMP-9 are produced by keratinocytes and epithelial cells and have been found in normal SEL.[208] Neutrophil accumulation within the laminae is followed by increases in MMP-9 concentrations and mRNA expression in laminar tissues coinciding with the onset of Obel grade 1 lameness, suggesting that neutrophil infiltration, MMP-9 accumulation, and lameness are linked.[84,196] Release of MMP-2 from platelets leads to platelet aggregation, and there is a correlation between MMP-2 inhibition and decreased platelet aggregation.[209] This suggests that MMP-2 may be involved in increased platelet aggregation and platelet-neutrophil aggregates found in CHO

laminitis.[210] Therefore, the neutrophil infiltration and release of inflammatory mediators within the digital laminae during laminitis may lead to increased release of both MMP-2 and MMP-9.

Metabolic disturbances are also suggested to precede the development of laminitis. Starch-rich diets and high fat supplements fed to inactive horses leads to increases in abdominal adipose tissue and predisposition to the development of a "peripheral Cushing's syndrome" characterized by insulin resistance, glucose intolerance, and laminitis.[211] Laminitis-prone ponies are hyperinsulinemic and insulin resistant compared with control ponies.[212] Clinical and histological laminitis can be induced in normal ponies by prolonged hyperinsulinemia with euglycemia.[213] Chronic administration of the glucocorticoid dexamethasone leads to increased insulin resistance and predisposition for the development of laminitis.[214] *In vitro,* glucose deprivation induces laminar separation by reducing HD numbers, suggesting a mechanism by which insulin resistant horses may develop laminitis.[203]

The exact pathophysiology of acute laminitis is actively being pursued in the equine scientific community. Although there are still many gaps in the multiple etiologic pathways that may ultimately result in laminitis, each of the components suggested above most likely plays a role in the development of this disease. Laminitis of alimentary origin can be used to illustrate how these components may be interconnected (Figure 1.4) In this model, it is suggested that altered GI permeability leads to the elaboration of gut-derived substances which are absorbed into the portal circulation causing profound systemic effects. This leads to local changes within the digit including vascular, inflammatory, enzymatic, and metabolic effects, each of which can perpetuate the other, leading to a vicious circle of events. All of the local digital effects result in laminar structural alterations that can lead to laminar biomechanical weakening. Weakening of the laminae ultimately results in rotation/sinking of the distal phalanx within the hoof capsule and the clinical manifestations of acute laminitis.

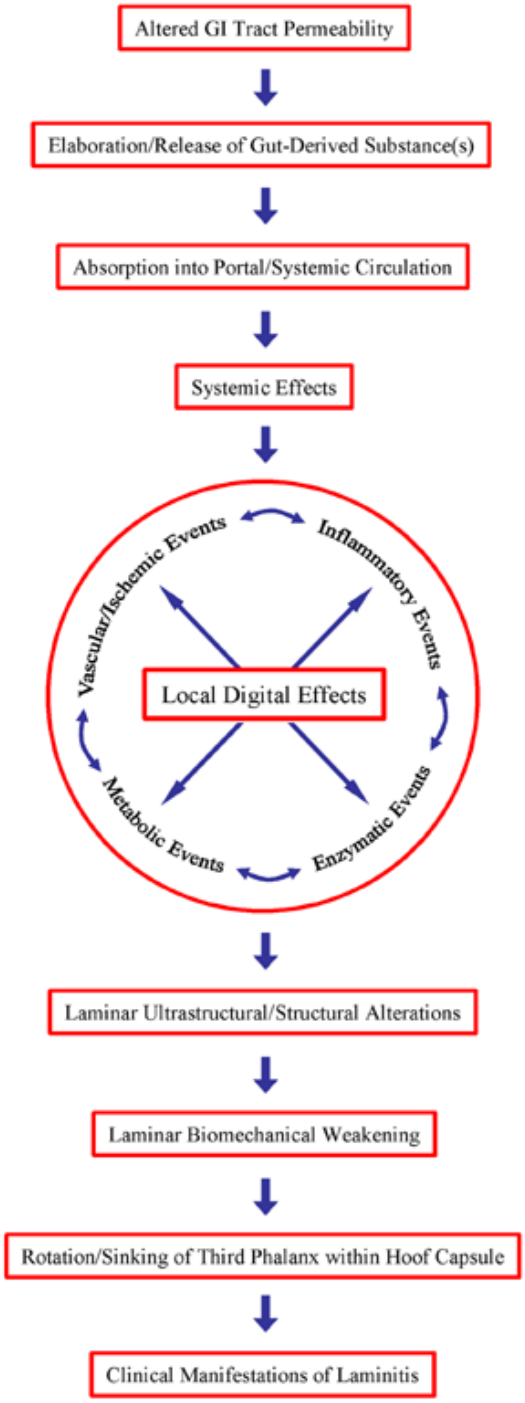


Figure 1.4 – A schematic of the events associated with the development of acute laminitis of alimentary origin.

While etiologies are varied and the definite pathophysiology of acute laminitis remains elusive, current methods of treatment remain unchanged. NSAID administration along with application of frog supports are the cornerstone of therapy, with phenylbutazone being the most commonly used medication.[180] Other medications used include the NSAID flunixin meglumine, the vasodilators acepromazine, isoxsuprine, and nitroglycerin, and the anti-inflammatory agents dimethylsulfoxide (DMSO) and pentoxifylline.[180,215] Acepromazine administered intravenously or intramuscularly leads to modest increases in digital arterial blood flow in normal horses.[215,216] Isoxsuprine has been reported to improve lameness associated with CHO laminitis when administered IV, but has no effect on digital blood flow when administered orally.[215,217] Nitroglycerin has been shown to both improve and have no effect on digital blood flow.[190,218-221] Pentoxifylline has also been shown to have no effect on digital blood flow when administered orally to normal horses.[215] Therapeutic shoeing, deep digital flexor tenotomy, and radical hoof wall resections are treatments used for cases of chronic laminitis.

The lack of effective treatments for acute laminitis necessitates further investigation of possible therapeutic agents. The recent elucidation of the suspected role of MMPs in the pathophysiology of this disease suggests that MMPIs may be effective in its treatment and warrants their evaluation.

1.5 Summary of Literature and Hypotheses for Present Studies

Matrix metalloproteinases are enzymes responsible for the normal remodeling and pathologic destruction of the ECM. During inflammation and disease, the increased release and unregulated activation of MMPs lead to rampant tissue destruction and organ failure. Regulation of MMPs using exogenous MMPI is a new avenue of exploration for the treatment of various pathological conditions.

Endotoxemia incites a systemic inflammatory response characterized by activation of inflammatory mediator cascades. The increased release and production of MMPs play a significant role in the pathologic changes associated with this condition. The administration of endotoxin, the toxic portion of Gram-negative bacteria responsible for initiating SIRS, has been shown in many species to directly and indirectly induce MMP synthesis. Experimental induction of endotoxemia in the horse results in increases in various inflammatory mediators; however, MMP activity has not been investigated.

Acute laminitis in the horse is characterized by breakdown of the BM between the SEL and SDL and subsequent separation of the laminae within the digit. This is believed to occur following the proteolytic actions of MMP-2 and MMP-9 on the laminar BM. Therefore, reductions in MMP activity via MMPIs may be beneficial. The experimental induction of equine laminitis can be achieved through several methods; however, while necessary, this is costly both monetarily and emotionally. The development of a non-terminal model of MMP induction in the horse could be used to assess the effectiveness of MMPIs before evaluating them in experimentally-induced laminitis. I propose that experimentally-induced endotoxemia can be used for such a model. The hypotheses of the studies presented in this dissertation include:

Study 1 – Equine neutrophils can be stimulated to release MMP-9 by PMA. The commercially available human Biotrak MMP-9 Activity Assay can be validated for use with equine samples.

Study 2 – Incubation of digital laminar explants with endotoxin will significantly increase medium MMP-2 and MMP-9 concentrations and significantly decrease laminar structural integrity. The addition of doxycycline, oxytetracycline, flunixin meglumine, and combinations thereof will significantly decrease medium MMP-2 and MMP-9 concentrations

and significantly increase laminar structural integrity. Laminar structural integrity will be inversely related to medium MMP-2 and MMP-9 concentrations.

Study 3 – Intravenous infusion of endotoxin will significantly increase digital venous plasma MMP-2 and MMP-9 concentrations in healthy adult horses when compared with digital venous plasma concentrations in horses administered an IV infusion of saline solution. Pre-treatment with oral doxycycline, IV oxytetracycline, IV flunixin meglumine, or IV pentoxifylline will significantly decrease digital plasma MMP-2 and MMP-9 concentrations in healthy adult horses administered an IV infusion of endotoxin.

CHAPTER 2. EVALUATION OF MATRIX METALLOPROTEINASE -9 ACTIVITY IN EQUINE NEUTROPHIL SUPERNATANT USING ZYMOGRAPHY AND AN ACTIVITY ASSAY

2.1 Introduction

Matrix metalloproteinases are proteolytic enzymes that not only degrade ECM components, but also activate various inflammatory mediators and regulate cell-to-cell interactions.[8,10,11] All MMPs are secreted in a latent, pro-enzyme form that usually requires cleavage for activation.[4] Matrix metalloproteinases are required for normal tissue development and remodeling and play important roles in various human pathological conditions from neurologic disease to cancer.[4,56,59] In recent years, MMPs have been implicated in several equine diseases, including RAO, OA, tendonitis, corneal ulceration, and laminitis.[68,71,77,79,84]

The most commonly studied MMPs to date are the gelatinases, MMP-2 and MMP-9. While proMMP-2 (72 kD) is constitutively expressed by various structural cell types, proMMP-9 (92 kD) is mostly induced during inflammatory states and released from neutrophils.[7] Most other cells do not store MMP-9, and secretion typically follows induction by de novo synthesis; however, neutrophils accumulate the zymogen within tertiary granules and release large quantities upon degranulation.[7] Neutrophil accumulation has been correlated with MMP-9 activity in several equine diseases, including equine laminitis and arthritis.[74,84] Increases in MMP-2 and MMP-9 concentrations have been found in laminar tissues of both experimentally-induced and clinically laminitic horses.[82,85] Plasma concentrations of MMP-9 are also increased in horses with CHO laminitis.[206]

There are several methods available for assessing MMP activity. Gelatin zymography is the traditional method used for measuring gelatinase activity; however, it is only semi-quantitative. ELISAs are quantitative, but can only measure either the pro or active form of MMPs. An MMP activity assay has been developed which is highly specific and sensitive, quantitative, and can determine both pro and active forms of the enzymes.

The purpose of this study was to collect and purify MMP-9 from equine neutrophils for use as a standard in the validation of the Biotrak MMP-9 Activity Assay[a] for use with equine samples. The presence of MMP-9 in the neutrophil supernatant was confirmed by western blotting using equine granulation tissue as a positive control. Gelatin zymography and the activity assay were used to evaluate MMP-9 activity in the neutrophil supernatant.

2.2 Materials and Methods

2.2.1 Isolation/Stimulation of Equine Neutrophils – This study was approved by the Institutional Animal Care and Use Committee of Louisiana State University. Jugular venous blood was collected (60 ml each) from 9 adult horses into vacutainers containing preservative-free heparin. The blood was mixed with room temperature 0.9% NaCl (saline) at a 1:2 dilution. The cell suspension was carefully poured onto 10 ml Ficoll-Hypaque[b] solution in a 50 ml conical tube. The tubes were centrifuged at 400 X g for 40 minutes at 20°C. The upper saline and Ficoll-Hypaque layers were then aspirated, leaving the neutrophil/red blood cell (RBC) pellet. The pellet was resuspended in 20 ml phosphate buffered saline (PBS). An equal volume of dextran/saline solution (3% Dextran T-500[c] in 0.9% saline) was added. The mixture was incubated in an upright position for approximately 20 minutes at room temperature. The neutrophil-rich upper layer was aspirated, the cells pelleted by centrifugation at 250 X g for 10 minutes at 5°C, and the pellet resuspended in10 ml of 0.9% saline. Residual RBCs were removed by subjecting the cells to hypotonic lysis by resuspending the pellet in 20 ml of cold 0.2% NaCl for 30 seconds. Isotonicity was then restored by adding 20 ml of ice-cold 1.6% NaCl. The cells were centrifuged at 250 X g for 6 minutes at 5°C and the supernatant discarded. This was repeated until the neutrophil pellet was free of RBCs. The cells were counted and resuspended in serum-free RPMI medium at a concentration of 1×10^7 cells/ml. The neutrophils were stimulated to release MMP-9 by incubation with phorbol 12-myristate 13-acetate[d] (PMA) at

a dose of 50 ng/ml for 30 minutes at 37°C. Following stimulation, the cells were centrifuged at 250 X g for 10 minutes at 5°C, and the supernatant was collected and frozen at -70°C.

2.2.2 Protein Extraction of Equine Granulation Tissue – Protein was extracted from a sample of equine granulation tissue for use as an equine positive control in western blotting. A small piece of granulation tissue was frozen in liquid nitrogen and pulverized. The pieces of tissue were placed into a 15 ml conical tube. Three milliliters of extraction buffer (0.05M Tris, 0.001M NaEDTA, 0.15M NaCl, 20mM phenylmethyl sulfonyl fluoride (PMSF), 5 µg/ml aprotinin, and 0.5% Triton X-114 at pH 8.0) were added to the conical tube. The mixture was vortexed and left on ice for 2 hours. The mixture was then centrifuged at 12,000 X g for 30 minutes at 4°C.

2.2.3 Western Blot Analysis of Supernatant MMP-9 – The equine neutrophil supernatant was concentrated 5-fold (5X) via centrifugation at 8000 rpm for 20 minutes using Centriprep YM30[e] tubes. The waste was removed and the tubes centrifuged once more. The 1X and 5X neutrophil supernatant, equine granulation tissue extraction, and human proMMP-9 standard were diluted 1:2 with 2X sample buffer (0.0625M Tris-HCl, 25% glycerol, 4% SDS, 0.01% bromophenol blue at pH 6.8). Fifteen microliters of each sample and the standard were added to a commercially available 12% Tris-HCl polyacrylamide gel[f] and subjected to electrophoresis for 45 minutes at 200 V. The gel was removed and placed in transblot buffer (25mM Tris, 192mM glycine, and 20% methanol (v/v) at pH 8.3) for 15 minutes. The gel was then placed into a blot apparatus and subjected to electrophoresis for 90 minutes at 90 V to transfer the separated proteins onto blot paper. The blot was allowed to incubate overnight in 10% milk in NET buffer (0.05M Tris, 0.001M NaEDTA, and 0.15M NaCl at pH 7.4). After incubation, the blot was rinsed in NET buffer for 30 minutes. A polyclonal rabbit anti-human MMP-9 antibody[g] was diluted to 18 µl/ml using 10% milk in NET buffer. The blot was

incubated with MMP-9 antibody for 1 hour and then rinsed with NET buffer for 15 minutes 4 times. Anti-rabbit IgG + horseradish peroxidase conjugate[h] diluted in 10% milk in NET buffer (1:1000) was incubated with the blot for 1 hour. The blot was rinsed again with NET buffer for 15 minutes 4 times. The chromogen, tetramethylbenzidine (TMB), was added to the gel to allow visualization of the protein bands.

2.2.4 Affinity Chromatographic Purification of Equine MMP-9 – Neutrophils were collected from 9 additional horses, stimulated with PMA to release MMP-9, and the supernatant collected as previously described. The supernatant was split into Centriprep YM30 tubes and centrifuged at 4500 rpm for 1 hour at 4°C. The waste was removed and the tubes refilled with chromatography starting buffer (0.05M Tris-HCl, 0.5M NaCl, 0.005M $CaCl_2$, 0.05% Brij-35, and 0.02% NaN_3 at pH 7.6) for 3 washes. After washing, the supernatant was further concentrated to 25X the original concentration.

A 5 ml bed volume of gelatin sepharose beads[i] was added to a 10 ml chromatography column. The column was rinsed with 30 ml of chromatography starting buffer. The 25X supernatant sample was applied to the column, re-collected, and applied to the column a second time. Fifteen milliliters of elution buffer (0.05M Tris-HCl, 1M NaCl, 0.005M $CaCl_2$, 0.05% Brij-35, 0.02 NaN_3 and 5% DMSO (v/v) at pH 7.6) were applied to the column, and 1 ml elution fractions were collected.

2.2.5 Zymographic Analysis of MMP-9 Activity – Zymograms were performed using commercially available 10% gelatin polyacrylamide gels.[j] Equine neutrophil supernatant elution samples, 25X neutrophil supernatant, and pro and active MMP-9[k] standards were diluted 1:2 with 2X sample buffer (62.5 mM Tris-HCl, 25% glycerol, 4% SDS, and 0.01% bromophenol blue at pH 6.8). Ten microliters of each sample and 7 µl of the standard were loaded onto the gels. Following 1 hour of electrophoresis at 166 V, the gels were washed with renaturing buffer

(2.5% Triton X-100) for 30 minutes and incubated for 16-20 hours at 37˚C in development buffer (50mM Tris-HCl, 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35 at pH 7.5). After incubation, the gels were stained with 0.25% Coomassie brilliant blue in a mixture of aqueous 50% methanol: 10 % acetic acid (v/v) and destained in aqueous 20% methanol: 10% acetic acid (v/v). Gelatinolytic activity was detected as transparent bands against a dark blue background. Relative values in arbitrary units (A.U.) were established for MMP-9 concentrations by digitally photographing the gels and measuring band intensity and size using Image J[l] densitometry software.

2.2.6 Activity Assay Analysis of MMP-9 Activity – Linear dilutions of equine neutrophil supernatant elution samples and plasma from normal adult horses were evaluated using the Biotrak MMP-9 Activity Assay. The assay detects both endogenous levels of active MMP-9 and total MMP-9 (pro + active) by activating any proMMP-9 in the sample with APMA. Briefly, samples and human proMMP-9 standards (0, 0.125, 0.25, 0.5, 1, 2, 4, 8, and 16 ng/ml) were incubated in duplicate in microtiter wells coated with human anti-MMP-9 antibody. Any MMP-9 present became bound to the wells, and any other components in the samples were removed by washing. APMA was added to all standards and each sample well designated for total MMP-9 quantification. A pro detection enzyme was added to each well that was then cleaved and activated by any MMP-9 present. The activated detection enzyme was then able to cleave a chromogenic peptide substrate resulting in a color change. The color was read at 405 nm using a spectrophotometer. The concentration of MMP-9 in each sample was interpolated from a standard curve.

2.3 Results

2.3.1 Western Blot Analysis of Supernatant MMP-9 – The presence of proMMP-9 was detected in both the 1X and 5X equine neutrophil supernatants and in the equine granulation tissue extract (Figure 2.1).

2.3.2 Zymographic Analysis of MMP-9 Activity – All supernatant elutions contained proMMP-9 (92 kD) and proMMP-9 dimer (220 kD) (Figure 2.2).

2.3.3 Activity Assay Analysis of MMP-9 Activity – A linear MMP-9 standard curve was produced ranging from 0 – 16 ng/ml (Figure 2.3). MMP-9 was detected in the linearly diluted equine neutrophil supernatant elutions; however, the assay became saturated at approximately 4 ng/ml equine MMP-9 (Figure 2.4). No MMP-9 was detected in any equine plasma sample.

2.4 Discussion

In this study, MMP-9 was successfully harvested from PMA-stimulated equine neutrophils and verified using western blotting. Neutrophil supernatants were further purified using affinity chromatography, and gelatin zymography was used to assess MMP-9 activity present in the eluted supernatant samples. Both the monomeric proMMP-9 (92 kD) and the dimeric proMMP-9 (220 kD) forms were present. The Biotrak MMP-9 Activity Assay was able to detect equine MMP-9 in the neutrophil supernatant. However, the activity assay was only approximately 25% as sensitive for equine MMP-9 compared with human MMP-9, for which the assay was developed.

There is currently no commercially available equine MMP-9 standard; therefore, MMP-9 was harvested from equine neutrophils. A rabbit anti-human MMP-9 antibody was used to detect MMP-9 in the equine neutrophil supernatant and equine granulation tissue. Wounds with granulation tissue are in a state of remodeling and, therefore, should contain large amounts of

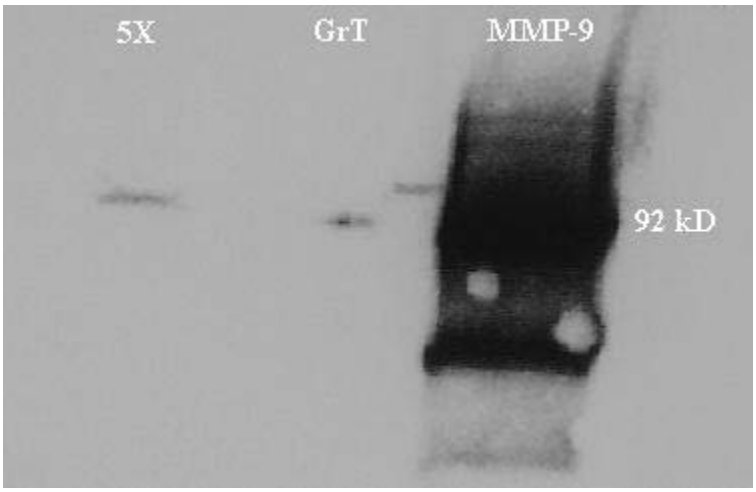


Figure 2.1 – Western blot analysis of concentrated equine neutrophil supernatant (5X), protein extraction from equine granulation tissue (GrT), and human proMMP-9 (MMP-9) using a rabbit anti-human MMP-9 antibody. A dark band represents the presence of MMP-9.

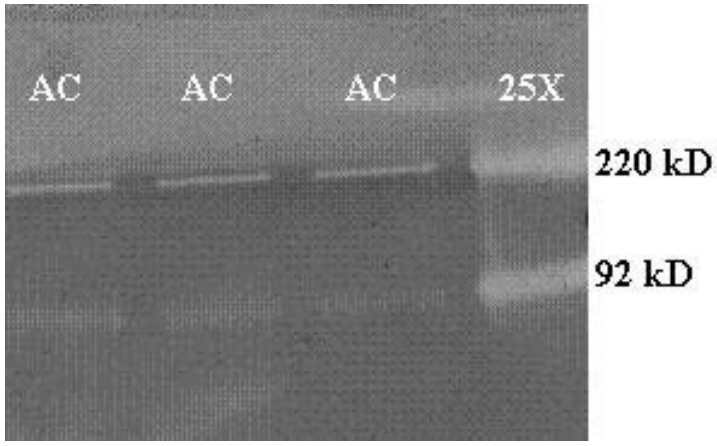


Figure 2.2 – Zymogram of equine neutrophil supernatant purified by affinity chromatography (AC) and 25X concentrated equine neutrophil supernatant (25X). Clear bands represent the presence of proMMP-9.

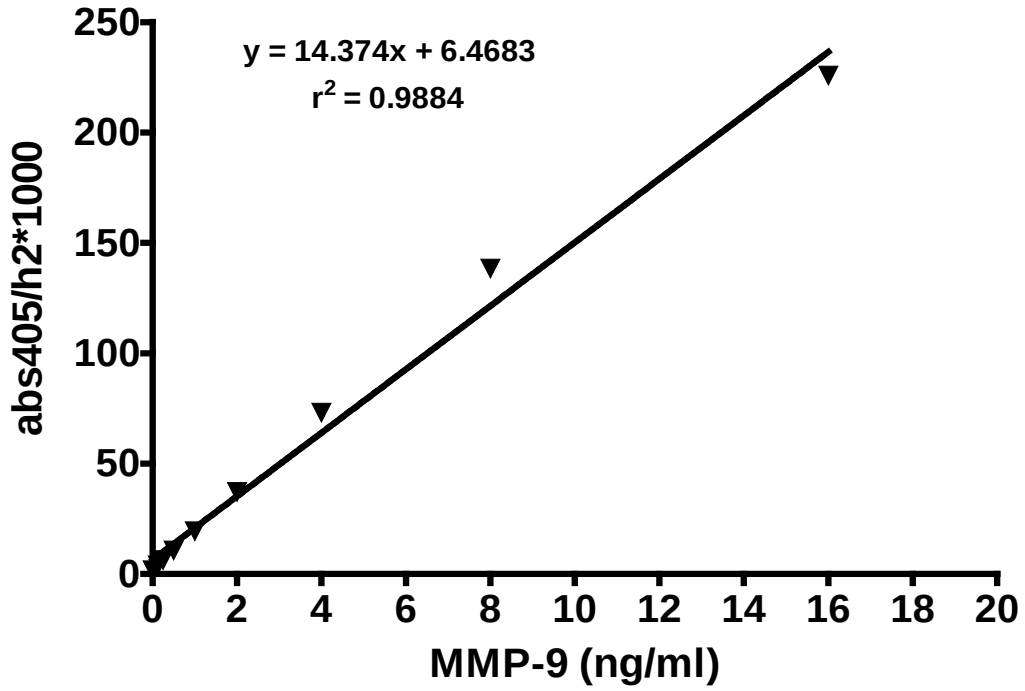


Figure 2.3 – Human MMP-9 (ng/ml) standard curve produced by the Biotrak MMP-9 Activity Assay. Color change in the assay is directly proportional to MMP-9 activity and is represented by the rate of change of absorbance at 405 nm (abs405/h2*1000).

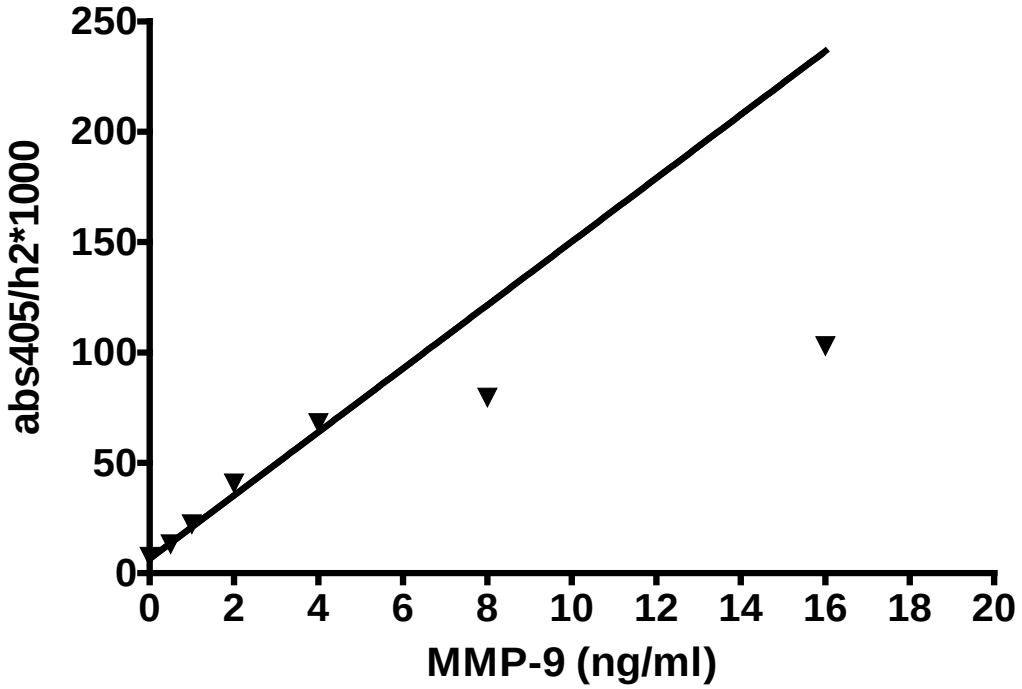


Figure 2.4 – Concentration of MMP-9 (ng/ml) in linear dilutions of equine neutrophil supernatant elutions determined by the Biotrak MMP-9 Activity Assay. Color change in the assay is directly proportional to MMP-9 activity and is represented by the rate of change of absorbance at 405 nm (abs405/h2*1000).

MMPs. For this reason, equine granulation tissue was selected as a positive control for the MMP-9 antibody used in the western blotting. Although the western blot indicated that the 5X concentrated neutrophil supernatant contained MMP-9, the supernatant was further concentrated to 25X to ensure that enough MMP was present in the chromatography elution for detection by the activity assay. The monomeric and disulfide-bonded homodimeric forms of proMMP-9 seen on the zymogram in the neutrophil supernatant are both physiologic forms of the same enzyme and have been identified in many different tissues, cell culture supernatants, and biological fluids.[222]

PMA-induced neutrophil degranulation occurs by activation of phosphokinase-C and NADPH-oxidase. Stimulation of equine neutrophils with PMA resulted in significant release of MMP-9 into the supernatant, confirming previous reports that equine neutrophils store this enzyme.[223] This has significant implications for equine diseases in which the elevated presence of MMP-9 correlates with neutrophil abundance, such as laminitis, RAO, and arthritis.

Gelatin zymography is the traditional method for assessment of both MMP-2 and MMP-9. This method is highly sensitive and specific for the gelatinases and quite economical. However, it is only qualitative or semi-quantitative, as only relative values of MMP activity can be calculated from densitometry readings. The Biotrak MMP-9 Activity Assay is highly specific for MMP-9 and equally as sensitive as an ELISA. Like an ELISA, the activity assay is truly quantitative; however, it is superior due to its ability to determine both pro and active forms of the enzyme.

Unfortunately, the activity assay had a low sensitivity for equine MMP-9. This may have been due to the human monoclonal MMP-9 antibody used in the assay, although it reportedly cross-reacts with mouse and rabbit samples.[224] Equine MMP-9 may have similar but not identical epitopes as human MMP-9. Thus, all of the equine MMP-9 may not have been bound

by the antibody, decreasing the amount of enzyme that could be detected. Components of the chromatography elution buffer may also have been interfering with the assay.

Although the activity assay was able to measure MMP-9 in the purified neutrophil supernatant, it could not detect any MMP-9 in the normal equine plasma samples. Normal human plasma contains 4.4 – 27.2 ng/ml MMP-9, as measured by the activity assay.[224] It would seem likely that normal equine plasma would also contain measurable MMP-9 concentrations. The incubation time was extended to increase the sensitivity of the assay, but MMP-9 was never detected in any equine plasma samples. This seems most likely due to the lowered sensitivity of the activity assay for equine MMP-9. Also, other components of the plasma, such as endogenous inhibitors, may have been preventing the antibody from binding MMP-9 in the samples.

Due to the high cost of the activity assay and the time already invested in its assessment, any further evaluation for its use with equine samples was forgone. Supernatant from an equine tumor cell line had also been collected for evaluation of the Biotrak MMP-2 Activity Assay; however, it was also aborted. Gelatin zymography was chosen instead for MMP-2 and MMP-9 evaluation of all equine samples in the following studies.

2.5 Product Information

[a]Biotrak MMP-9 Activity Assay System RPN2634, Amersham Biosciences, Pascataway, NJ

[b]Ficoll-Paque Plus 17-1440-02 , GE Healthcare Biosciences Corp, Pascataway, NJ

[c]Dextran T-500, Amersham Biosciences, Pascataway, NJ

[d]Phorbal 12-myristate 13-acetate P1585, Sigma-Aldrich, St. Louis, MO

[e]Centriprep YM-30 4322, Millipore Corporation, Billerica, MA

[f]12% Tris-HCl Ready Gel 161-1156, Bio-Rad Laboratories, Hercules, CA

[g]MMP-9 Ab-10 RB-1590-P1, Lab Vision, Thermo Scientific, Freemont, CA

[h]Goat anti-rabbit IgG (H+L) TR-001-HR, Lab Vision, Thermo Scientific, Freemont, CA

[i]Gelatin Sepharose 4B 17-0956-01, GE Healthcare Biosciences Corp, Pascataway, NJ

[j]10% Gelatin Ready Gel 161-116, Bio-Rad Laboratories, Hercules, CA

[k]MMP-9 Proenzyme PF038 and MMP-9 Human Recombinant PF024, Calbiochem, La Jolla, CA

[l]Image J, U.S. National Institutes of Health, Bethesda, MD

CHAPTER 3. MATRIX METALLOPROTEINASE ACTIVITY AND STRUCTURAL INTEGRITY OF EQUINE DIGITAL LAMINAR EXPLANTS EXPOSED TO ENDOTOXIN AND MMP INHIBITORS

3.1 Introduction

Endotoxin is a cellular membrane component of all Gram-negative bacteria that is responsible for inciting a systemic inflammatory response. It is released following bacterial cell death or rapid proliferation, and is usually removed from the circulation by hepatic Kupfer cells or anti-endotoxin antibodies.[124] However, during certain diseases, large amounts of endotoxin can overwhelm normal defense mechanisms and gain access to the systemic circulation resulting in the activation of multiple inflammatory cascades.[128]

Matrix metalloproteinases are endopeptidases that degrade ECM proteins and regulate various cell-cell and cell-matrix interactions.[4] They are involved in normal tissue homeostasis, but are most active during inflammation and disease.[8] The enzymes are secreted in a pro-enzyme, or zymogen, form that must be cleaved for activation.[225] The gelatinases, MMP-2 and MMP-9, degrade denatured collagen, or gelatin, as well as other components of the ECM. While MMP-2 is constitutively expressed by various structural cell types such as fibroblasts and endothelial cells, MMP-9 is mostly expressed by inflammatory cells, in particular neutrophils.[7] Endotoxin can stimulate the release of MMP-2 and MMP-9 from various cells and tissues *in vitro*.[138,140,141]

Laminitis is an excruciatingly painful and often life-threatening disease of the equine digit that occurs when the epidermal and dermal laminar attachments within the foot become inflamed and, in severe cases, fail. Histopathological evaluation of acutely laminitic horses have shown that epidermal laminar necrosis and disintegration of the laminar basement membrane are primary lesions.[226,227] Recent studies have suggested that activation of MMPs within the digit is responsible for the laminar separation.[206] Both MMP-2 and MMP-9 have been localized to the equine digit. Several studies have found increased expression of both MMP-2 and MMP-9 in laminar tissues during experimentally-induced and naturally-acquired laminitis.[82,85]

Matrix metalloproteinase inhibitors are currently being studied in human medicine as potential therapeutic agents for many MMP-associated diseases such as rheumatoid arthritis, cardiovascular disease, and cancer.[56,58,60] Tetracyclines down-regulate expression of MMP-2 and MMP-9, and may also prevent activation of the zymogen forms.[23,88] Doxycycline, a semi-synthetic tetracycline, has been shown to be an effective MMPI in various tissues and diseases including endothelial cells, pleural fluid, corneal epithelium, cerebral ischemia, and endotoxemia-induced cardiac dysfunction.[102-106] Oxytetracycline, a tetracycline analogue, has been shown to decrease MMP-1 expression in equine myofibrils.[111] NSAIDs also inhibit MMP-2 and MMP-9 through decreased transcription.[114] Due to the apparent involvement of MMPs in equine laminitis, MMPIs may be beneficial in preventing or lessening the laminar destruction associated with this disease.

The purpose of this study was to evaluate the ability of doxycycline, oxytetracycline, and flunixin meglumine to inhibit equine MMPs *in vitro*. Endotoxin was used to induce MMP-2 and MMP-9 in equine laminar explants. The structural integrity of the explants was assessed using a mechanical testing device. The culture medium was analyzed for MMP activity using gelatin zymography.

3.2 Materials and Methods

3.2.1 Horses – This investigation was approved by the Institutional Animal Care and Use Committee of Louisiana State University. Laminar tissues were harvested from horses undergoing euthanasia for unrelated research projects. Twelve clinically healthy, adult horses of varying sex (6 mares, 6 geldings) and breed (4 Thoroughbreds, 4 Paso Finos, 3 Peruvian Pasos, and 1 Quarter Horse), ranging in age from 4 to 21 years old (median, 12 years) were used for this study. The horses were deemed to be free of medical problems related to inflammatory diseases, endotoxemia, or diseases of the digit as determined through complete physical examination,

complete blood count, lameness examination including hoof testing, and radiographic evaluation of the digit.

3.2.2 Experimental Design – Laminar explants were harvested from both forefeet of each horse and were used for 3 separate studies. For each study, explants were incubated and stimulated in triplicate with various treatments for 24 hours at 37°C. After incubation, the medium was collected from each explant and frozen at -70°C until analyses for MMP activity were performed. The remaining explants were then subjected to biomechanical testing for assessment of structural integrity. Treatments for each of the studies consisted of the following.

Study 1: (1) medium (control); (2) LPS[a] (*E. coli* O55:B5)100 ng/ml; (3) LPS + doxycycline[b] (DOXY) 10 ng/ml; (4) LPS + DOXY 100 ng/ml; (5) LPS + DOXY 1 µg/ml; and (6) LPS + DOXY 10 µg/ml. N = 8 horses were used for this study. The LPS 100 ng/ml dose for explants stimulation was determined through preliminary data.

Study 2: (1) medium (control); (2) LPS 100 ng/ml; (3) LPS + oxytetracycline[c] (OXY) 100 ng/ml; (4) LPS + OXY 1 µg/ml; (5) LPS + OXY 10 µg/ml; and (6) LPS + OXY 100 µg/ml. N = 8 horses were used for this study.

Study 3: (1) medium (control); (2) LPS 100 ng/ml; (3) LPS + flunixin meglumine[d] (FLU) 8 ng/ml; (4) LPS + FLU + OXY 100 ng/ml; and (5) LPS + FLU + DOXY 100 ng/ml. N = 6 horses were used for this study.

3.2.3 Harvesting of Explants – The laminar explants were harvested using a modification of the methods described by Pollitt.[205] Immediately following euthanasia with sodium pentobarbital[e] (100 mg/kg, IV), both forefeet were removed just distal to the metacarpophalangeal joint. Each hoof was then trimmed proximally and caudally with a band saw to facilitate further cutting. The hooves were scrubbed with an antibacterial disinfectant and the solar surface pared with a hoof knife prior to transecting. Using a table saw sterilized with

disinfectant, the outer ¼ of either the medial or lateral aspect of the hoof was removed in a sagittal plane. Five more sagittal cuts were made to create five 6-mm slices of the digit. The slices were rinsed and placed in a sterile saline solution. A small diamond band saw was used to cut 6 mm X 6 mm blocks of laminar tissue from each slice consisting of the hoof wall, epidermal laminae, dermal laminae, and distal phalanx (Figure 3.1). The laminar explants were then incubated in Dulbecco's Modified Eagles Medium[f] (DMEM) supplemented with piperacillin[g] (64 µg/ml), tazobactam[h] (8 µg/ml), amikacin[i] (5 µg/ml), and nystatin[j] (100 U/ml) and stimulated with the treatments described above at 37˚ C in humidified air supplemented with 5% CO_2.

3.2.4 Instron Biomechanical Testing – An Instron[k] biomechanical testing device was used to determine the structural integrity of each laminar explant. Pneumatic clamps were securely affixed to the hoof wall and to the bone of each segment and mounted in the testing frame (Figures 3.2 and 3.3). Segments were loaded at a constant elongation rate of 25 mm/second until failure. Data were collected at 20 Hz by analog/digital conversion and stored. The site of failure was recorded and confirmed with light microscopy of specimens. For each segment examined, stress-strain curves were generated and used to derive the maximum load to failure.

3.2.5 Zymographic Analyses of MMP Activities – All zymograms were performed using commercially available 10% gelatin polyacrylamide gels.[l] Medium samples and pro and active MMP-2[m] and MMP-9[n] standards were diluted 1:2 with 2X sample buffer (62.5 mM Tris-HCl, 25% glycerol, 4% SDS, and 0.01% bromophenol blue at pH 6.8). Ten microliters of each sample and 7 µls of each standard were loaded onto each gel. Following 1 hour of electrophoresis at 166 V, the gels were washed with renaturing buffer (2.5% Triton X-100) for 30 minutes and then incubated for 16-20 hours at 37˚C in development buffer (50mM Tris-HCl,

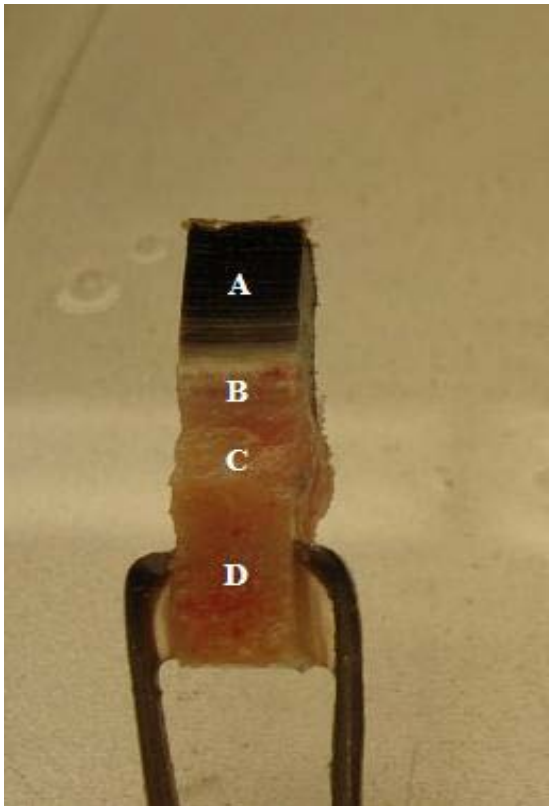


Figure 3.1 – Example of a laminar explant consisting of hoof wall (A), epidermal laminae (B), dermal laminae (C), and distal phalanx (D).

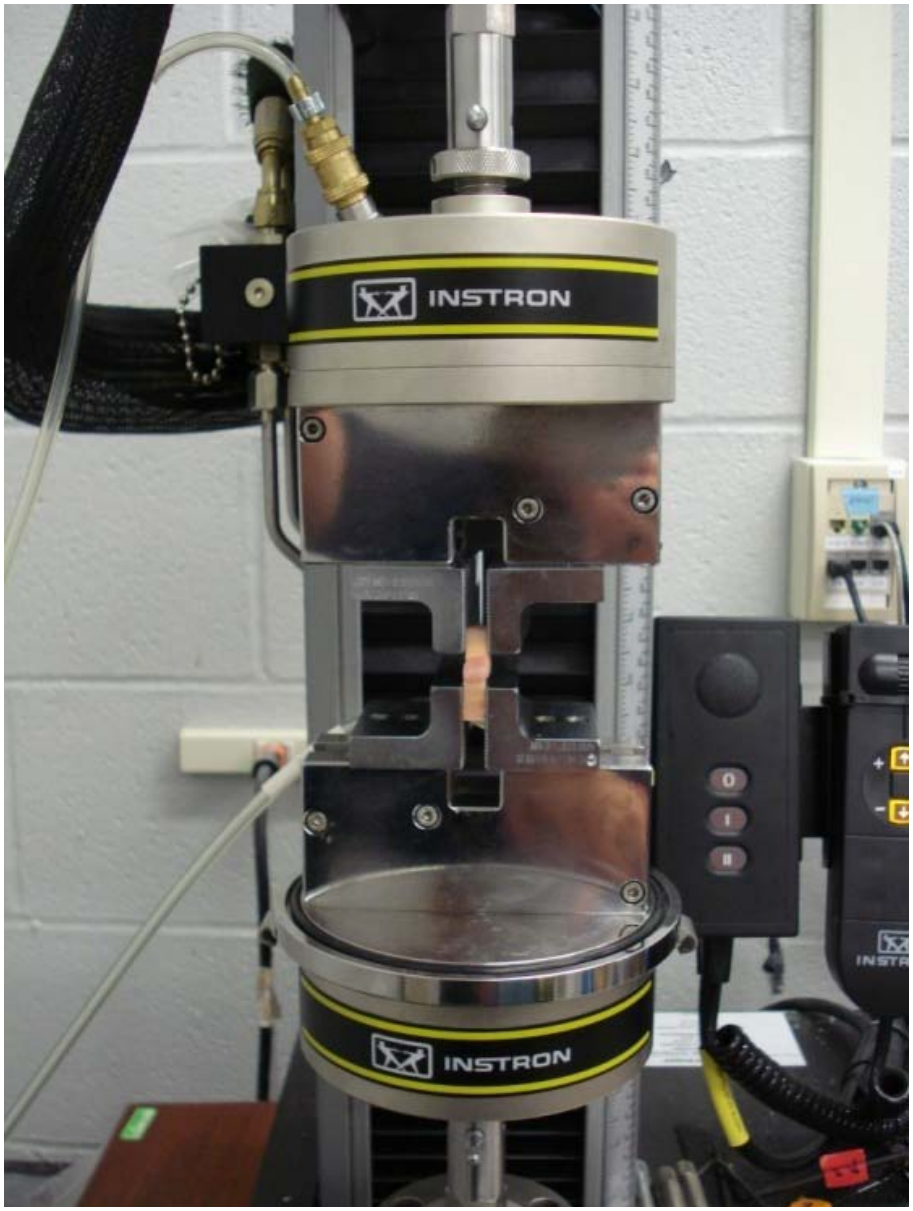


Figure 3.2 – A laminar explant mounted in the Instron biomechanical testing device.

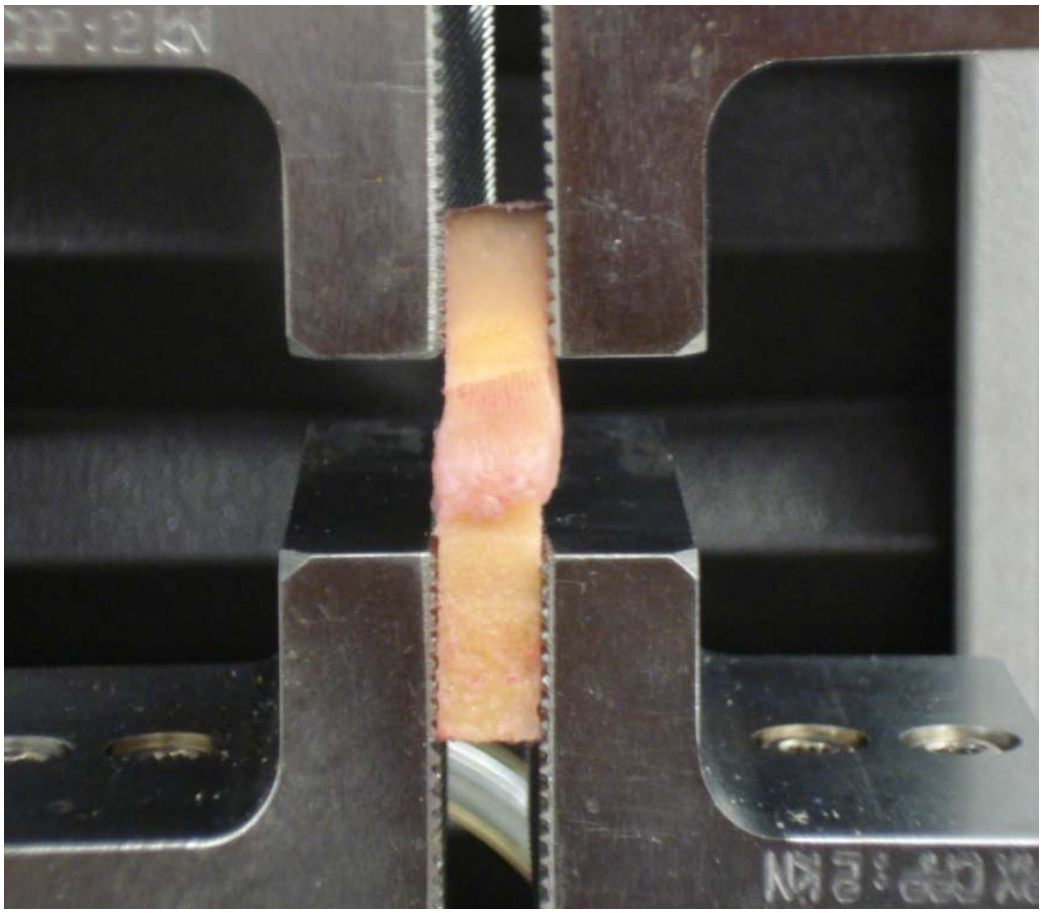


Figure 3.3 – A closer view of a laminar explant mounted in the pneumatic clamps of the Instron biomechanical testing device.

200 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35 at pH 7.5). After incubation, the gels were stained with 0.25% Coomassie brilliant blue in a mixture of aqueous 50% methanol: 10 % acetic acid (v/v) and destained in aqueous 20% methanol: 10% acetic acid (v/v). Gelatinolytic activity was detected as transparent bands against a dark blue background. Relative values in arbitrary units (A.U.) were established for MMP-9 concentrations by digitally photographing the gels and measuring band intensity and size using Image J^o densitometry software.

3.2.6 Statistical Analyses – All data were tested using the Shapiro-Wilk statistic and found to have normal distributions. Normally distributed variables were analyzed using an ANOVA and post-hoc comparisons were made using least squares means. Data were summarized and graphed as mean ± SEM. Correlations between Instron data and MMP zymography data were assessed using the Pearson correlation coefficient. A $p \leq 0.05$ was considered significant for all data. SAS^p statistical software was used for all statistical analyses.

3.3 Results

3.3.1 Instron Biomechanical Testing – In Study 1, LPS + DOXY 1 µg/ml (68.33 ± 4.42) explants had significantly greater load to failure than LPS (59.28 ± 3.09) and LPS + DOXY 10 ng/ml (58.33 ± 4.06) explants. Explants treated with LPS + DOXY 100 ng/ml (67.54 ± 3.43) were also significantly stronger than those treated with LPS + DOXY 10 ng/ml (Figure 3.4). There were no significant differences between control explants and any treatment group. In Study 2, LPS + OXY 100 ng/ml (74.49 ± 6.19) explants had significantly greater load to failure than LPS (60.32 ± 3.05) explants (Figure 3.4). There were no significant differences between control explants and any treatment group. In Study 3, there were no significant differences between any explants in any group (Figure 3.4).

3.3.2 Zymographic Analyses of MMP Activities – Lucent bands of enzyme activity corresponding to proMMP-2 (72 kD) and proMMP-9 (92 kD) were detected in all laminar

explants medium samples. The active forms of MMP-2 (66 kD) and MMP-9 (83 kD) were not detected. In study 1, proMMP-2 concentrations were significantly lower in LPS + DOXY 100 ng/ml (0.183 ± 0.010), LPS + DOXY 1µg/ml (0.177 ± 0.011), and LPS + DOXY 10 µg/ml (0.143 ± 0.010) explants medium compared with control (0.214 ± 0.019), LPS (0.231 ± 0.023), and LPS + DOXY 10 ng/ml (0.222 ± 0.020) explants medium (Figure 3.5). ProMMP-9 concentrations were significantly lower in LPS (0.157 ± 0.013), LPS + DOXY 10 ng/ml (0.165 ± 0.010), LPS + DOXY 100 ng/ml (0.156 ± 0.011), LPS + DOXY 1 µg/ml (0.142 ± 0.013), and LPS + DOXY 10 µg/ml (0.125 ± 0.011) explants medium compared with control (0.190 ± 0.015) explants medium. Furthermore, LPS + DOXY 10 µg/ml medium had significantly lower proMMP-9 concentrations than LPS and LPS + DOXY 10 ng/ml medium (Figure 3.5).

In Study 2, proMMP-2 concentrations were significantly lower in LPS + OXY 100 ng/ml (0.164 ± 0.012) and LPS + OXY 1 µg/ml (0.191 ± 0.015) explants medium compared with control (0.222 ±0.020) and LPS (0.246 ± 0.024) explants medium (Figure 3.6). ProMMP-9 concentrations were significantly lower in LPS (0.158 ± 0.011), LPS + OXY 100 ng/ml (0.124 ± 0.010), LPS + OXY 1 µg/ml (0.152 ±0.013), LPS + OXY10 µg/ml (0.137 ± 0.008), and LPS + OXY 100 µg/ml (0.114 ±0.007) explants medium compared with control (0.191 ± 0.015) explants medium. Furthermore, LPS + OXY 100 ng/ml and LPS + OXY100 µg/ml medium had significantly lower proMMP-9 concentrations than LPS and LPS + OXY 1µg/ml medium (Figure 3.6).

In Study 3, proMMP-2 concentrations were significantly lower in LPS + FLU + OXY (0.199 ± 0.014) explants medium compared with control (0.285 ± 0.030) and LPS (0.320 ± 0.031) explants medium. Medium from explants treated with LPS + FLU (0.244 ± 0.018) and LPS + FLU + DOXY (0.244 ± 0.022) also had significantly lower proMMP-2 concentrations than medium from explants treated with LPS (Figure 3.7). ProMMP-9 concentrations were

significantly lower in LPS + FLU (0.127 ± 0.012) and LPS + FLU + DOXY (0.130 ± 0.010) explants medium compared with control (0.175 ± 0.016) and LPS (0.162 ± 0.012) explants medium (Figure 3.7).

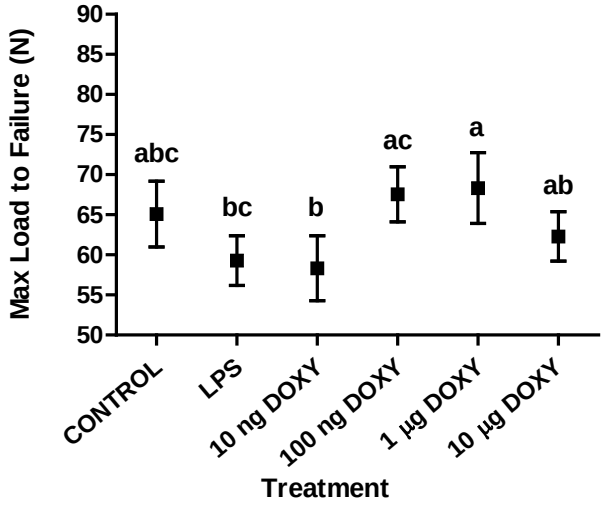
3.3.3 Correlations Between Laminar Integrity and MMPs – Laminar explant maximum load to failure had a – 20.6% correlation with proMMP-2 concentrations in the medium. This was significant at $p < 0.001$. There was no correlation between medium proMMP-9 concentrations and explant maximum load to failure.

3.4 Discussion

The MMPIs doxycycline and oxytetracycline increased the structural integrity of equine digital laminar explants; however, flunixin meglumine had no effect. Doxycycline, oxytetracycline, and flunixin meglumine all decreased MMP-2 and MMP-9 release from equine digital laminar explants to varying degrees. Structural integrity and MMP-2 medium concentrations were negatively correlated, but MMP-9 had no correlation with explant strength.

Hemidesmosomes attach epidermal basal cells to the *lamina densa* of the BM separating the epidermal and dermal laminae within the digit. Pollitt et al found that degradation of the BM, loss of HDs, and detachment of epidermal basal cells are early pathologic events in acute laminitis.[177,227] Mungall et al previously described an "in vitro laminitis model" in which bacterial broths increased MMP-2 and MMP-9 concentrations and increased laminar separation in equine digital laminar explants.[205] Bacterial proteinases such as thermolysin increase laminar explant separation as well as activate proMMP-2 and proMMP-9 *in vitro*.[202] Glucose deprivation induces laminar separation *in vitro* by reducing HD numbers.[203] Hemidesmosomes separate from the *lamina densa* owing to degradation of anchoring filaments and allow laminar separation when explants are exposed to the MMP activator APMA, a mercurial compound.[203] Thus, MMP-2 and MMP-9 appear to be associated with BM destruction and laminar separation.

Figure 3.4 – Mean (± SEM) maximum loads to failure (Newtons, N) for laminar explants in Study 1, Study 2, and Study 3 incubated with medium (CONTROL), LPS (LPS), LPS + doxycycline (DOXY), LPS + oxytetracycline (LPS + OXY), LPS + flunixin meglumine (LPS + FLU), LPS + flunixin meglumine + oxytetracycline (FLU + OXY), or LPS + flunixin meglumine + doxycycline (LPS + FLU + DOXY). Treatment groups with like letters are not significantly ($p \leq 0.05$) different.


Max Load to Failure (N)
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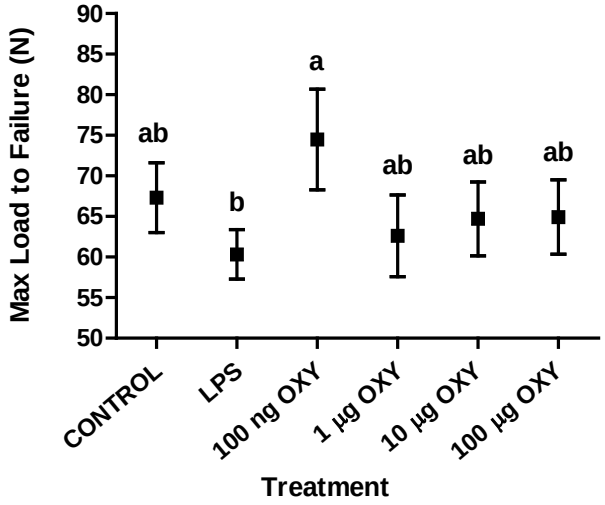
85
80
75
70
65
60
55
50
abc
bc
b
ac
a
ab
CONTROL
LPS
10 ng DOXY
100 ng DOXY
1 µg DOXY
10 µg DOXY
Treatment


Max Load to Failure (N)
90
85
80
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65
60
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50
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ab
ab
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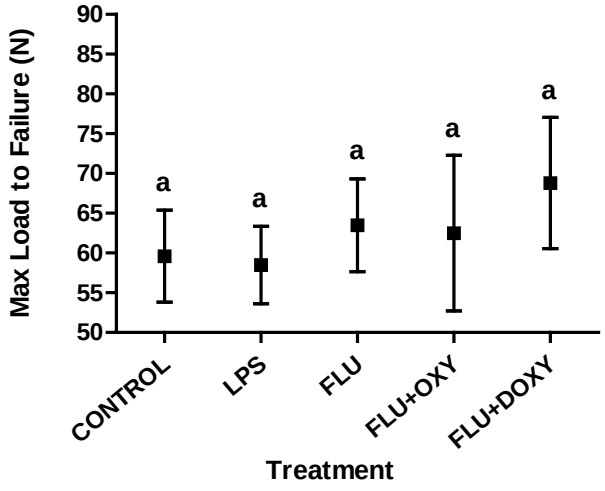
CONTROL
LPS
100 ng OXY
1 µg OXY
10 µg OXY
100 µg OXY
Treatment


Max Load to Failure (N)
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50
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a
a
CONTROL
LPS
FLU
FLU+OXY
FLU+DOXY
Treatment

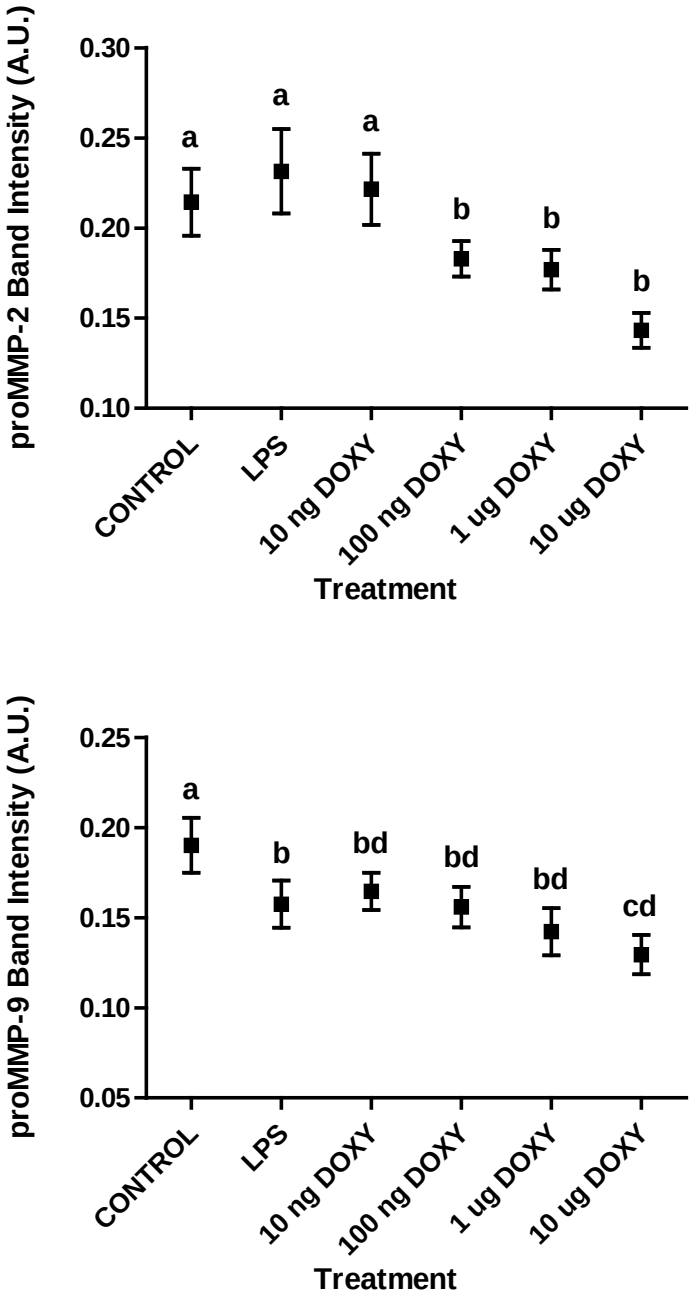

Figure 3.5 – Mean (± SEM) proMMP-2 and proMMP-9 band intensities (arbitrary units, AU) in the medium of laminar explants in Study 1 incubated with medium (CONTROL), LPS (LPS), or LPS + doxycycline (DOXY). Treatment groups with like letters are not significantly ($p \leq 0.05$) different.

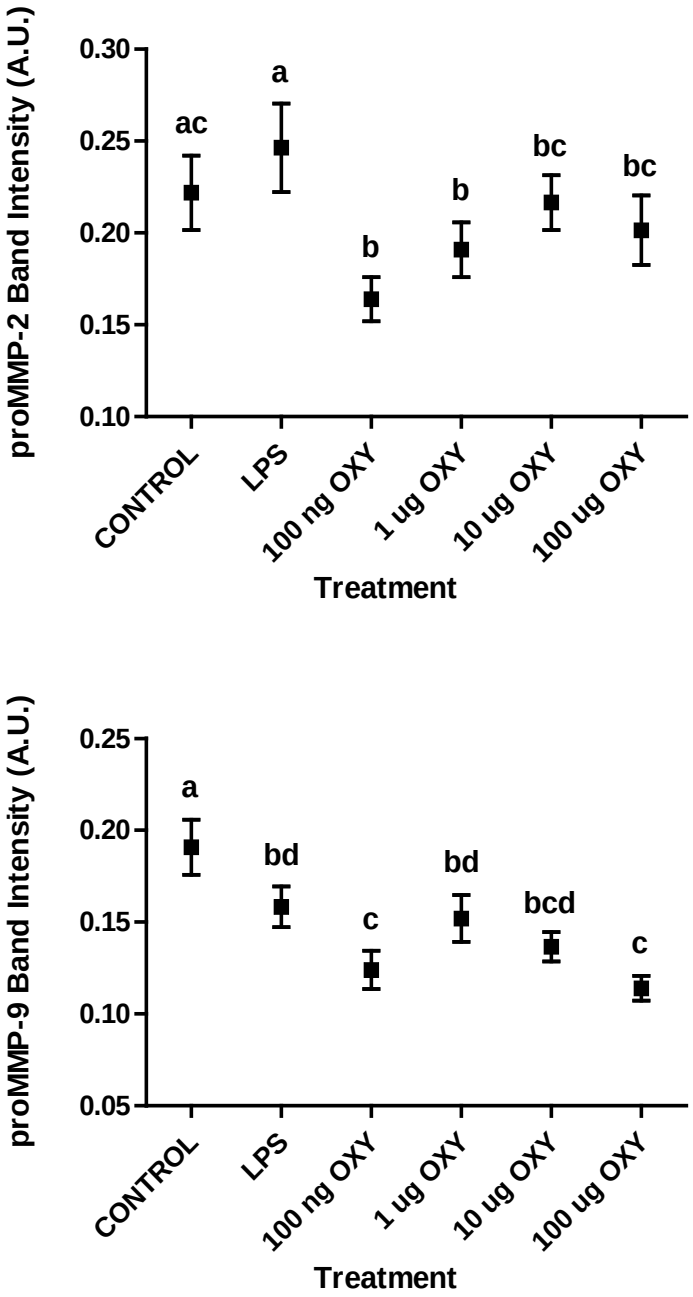

Figure 3.6 – Mean (± SEM) proMMP-2 and proMMP-9 band intensities (arbitrary units, AU) in the medium of laminar explants in Study 2 incubated with medium (CONTROL), LPS (LPS), or LPS + oxytetracycline (LPS + OXY). Treatment groups with like letters are not significantly ($p \leq 0.05$) different.

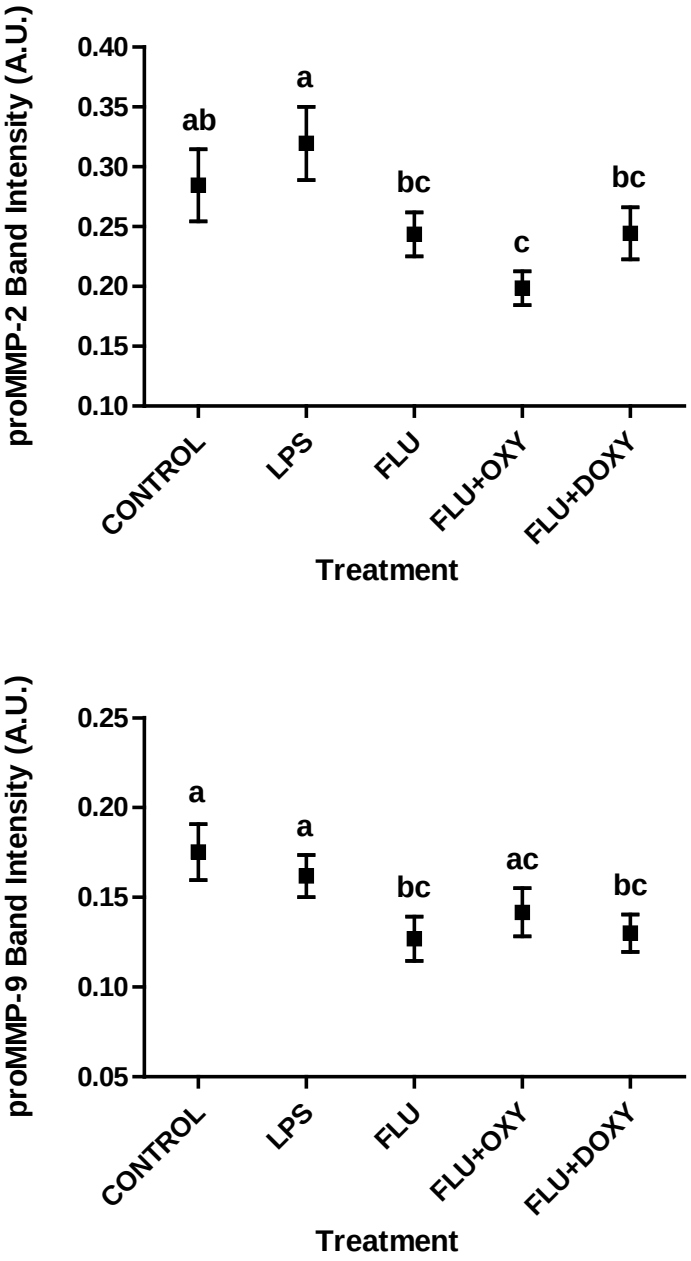

Figure 3.7 – Mean (± SEM) proMMP-2 and proMMP-9 band intensities (arbitrary units, AU) in the medium of laminar explants in Study 3 incubated with medium (CONTROL), LPS (LPS), LPS + flunixin meglumine (FLU), LPS + flunixin meglumine + oxytetracycline (FLU + OXY), or LPS + flunixin meglumine + doxycycline (FLU + DOXY). Treatment groups with like letters are not significantly ($p \leq 0.05$) different.

Incubation of laminar explants with *E. coli* bacterial broth has been reported to induce laminar separation.[205] In the study described here, stimulation of explants with *E. coli* endotoxin caused a decrease in the strength of explants compared with controls, however, this was not a significant decrease. Explants in previous studies were incubated for 48 hours, but explants in this study were only incubated for 24 hours. The incubation time was shortened due to the possible tissue deterioration that may begin to occur by 48 hours. Perhaps the decreased incubation time did not allow for maximum stimulation by endotoxin.

The addition of the MMPIs doxycycline or oxytetracycline significantly increased laminar explant integrity compared with endotoxin alone, suggesting that MMP inhibition may play a role in preventing laminar separation. This is further supported by significantly decreased MMP-2 and MMP-9 concentrations in the medium of explants exposed to doxycycline and oxytetracycline compared with endotoxin alone. Decreases in MMP-2 medium concentrations were also correlated with increases in explant strength.

Incubation of explants with flunixin meglumine had no effect on explant strength although significant decreases in both MMP-2 and MMP-9 medium concentrations were observed. The combination of flunixin meglumine and oxytetracycline further decreased MMP-2 concentrations. This is in agreement with other reports of MMP-2 and MMP-9 inhibition mediated by NSAIDs.[115,117]

The response of the laminar explants to endotoxin stimulation was different from that previously reported. Several cell types and tissues produce increased concentrations of MMP-2 and -9 when stimulated with endotoxin, such as endothelial cells, leukocytes, astrocytes, synovial cells, and aorta.[134,138,140-142,228,229] In the present study, MMP-2 concentrations in endotoxin-treated explants were only slightly greater than controls and endotoxin induced a significant decrease in MMP-9 concentrations. This *in vitro* laminar explant model may not be suitable for

extrapolating the effects of endotoxin on MMP-9 activity. Unlike MMP-2 which is expressed constitutively in tissues, MMP-9 is predominantly released by activated neutrophils.[7] Several studies have shown that MMP-9 and neutrophils are either present at very low levels, or completely absent from normal equine digital laminar tissue.[84,178] The isolated environment of the laminar explant would seemingly allow more significant changes to be seen in MMP-2 activity compared to MMP-9, as the majority of MMP-9 activity would come from the small number of neutrophils present in the hoof at the time of tissue collection. There are no reports of LPS-induced decreases in MMP-9 activity. This is a novel observation, and cannot be explained.

The results of this *in vitro* study suggest that doxycycline, oxytetracycline, and flunixin meglumine all have MMP inhibitory effects of varying degrees within the equine digit. Of the three, oxytetracycline appears to have the greatest effect and the most potential for use as an MMPI in the horse for the treatment/prevention of laminitis.

3.5 Product Information

[a]Lipopolysaccharide from *E. coli* O55:B5 L2880, Sigma-Aldrich, St. Louis, MO

[b]Doxycycline hyclate, Ranbaxy Pharmaceuticals, Princeton, NJ

[c]Liquamicin LA-200, Pfizer Animal Health, St. Louis, MO

[d]Banamine, Schering-Plough Animal Health, Union, NJ

[e]Beuthanasia-D Special, Schering-Plough Animal Health, Union, NJ

[f]DMEM – high glucose D6429, Sigma-Aldrich, St. Louis, MO

[g]Piperacillin sodium salt P8396, Sigma-Aldrich, St. Louis,MO

[h]Tazobactam sodium salt T2820, Sigma-Aldrich, St. Louis, MO

[i]Amiglyde-V, Fort Dodge Animal Health/Wyeth, Madison, NJ

[j]Nystatin 194534, MP Biomedicals, Solon, OH

[k]Instron, Norwood, MA

[l]10% Gelatin Ready Gel 161-1167, Bio-Rad Laboratories, Hercules, CA

[m]MMP-2 Proenzyme PF037 and Active PF023, Calbiochem, La Jolla, CA

[n]MMP-9 Proenzyme PF038 and Human Recombinant PF024, Calbiochem, La Jolla, CA

[o]Image J, U.S. National Institutes of Health, Bethesda, MD

[p]SAS v 9.0, SAS Institute, Cary, NC

CHAPTER 4. MATRIX METALLOPROTEINASE ACTIVITY IN THE DIGITAL CIRCULATION OF HORSES FOLLOWING AN INTRAVENOUS INFUSION OF ENDOTOXIN AND PRE-TREATMENT WITH MATRIX METALLOPROTEINASE INHIBITORS

4.1 Introduction

Systemic inflammatory response syndrome occurs when inflammation is accompanied by more than one of the following: 1) hyper- or hypothermia, 2) tachypnea, 3) tachycardia, and 4) leukocytosis or leukopenia.[123] In equine patients with gastrointestinal tract disease or Gram-negative sepsis, endotoxemia initiates a systemic inflammatory response by activating various inflammatory cascades involving cytokine induction and leukocyte activation, thereby inducing fever, tachycardia, and neutropenia.[129]

Matrix metalloproteinases are ECM proteases that are most active during inflammation and disease.[8] Various structural cells constitutively express MMP-2; however, MMP-9 is mostly expressed by inflammatory cells, in particular neutrophils.[7] Both enzymes have been implicated in various disease processes including endotoxemia.[56,59,135] In humans, MMP-9 is increased in patients with septic shock as well as experimental endotoxemia.[134,136,137]

Equine laminitis is an excruciatingly painful disease involving the dermal and epidermal laminae of the equine foot. Recent theories suggest that intestinal mediators initiate SIRS; thus, activating inflammatory cells to release cascades of mediators, including MMPs.[201] Increased MMP activity within the laminae results in loss of laminar integrity and may lead to the development of laminitis.[206] Several studies have found increased expression of both MMP-2 and MMP-9 in laminar tissues during experimentally-induced and naturally-acquired laminitis, as well as increased plasma MMP-9 concentrations.[82,85,206]

Several classes of drugs have MMP inhibitory effects including tetracyclines, retinoic acids, corticosteroids, NSAID, and phosphodiesterase inhibitors.[23,88] Tetracyclines have been shown to down-regulate expression of MMP-2 and MMP-9, and may also prevent activation of the zymogen forms.[112] Doxycycline significantly reduces MMP-9 activity *in vitro* and *in vivo* in a mouse endotoxemia model.[102-106] In horses, doxycycline is used in the treatment of Potomac

horse fever caused by *Neorickettsia risticii*, a disease associated with depression, fever, diarrhea, and laminitis.[107] Anecdotally, clinicians report that horses suffering from Potomac horse fever that are treated with doxycycline develop laminitis less frequently than those treated with other drugs. Oxytetracycline, a tetracycline analogue, is used in the horse for its antibiotic properties, but also for treatment of flexural deformities in foals via MMP-1 inhibition.[111] There are no other reports of MMP inhibition in horses by oxytetracycline.

Both NSAIDs and phosphodiesterase inhibitors decrease MMP-2 and MMP-9 activities by decreasing mRNA expression.[113,114] Flunixin meglumine is an NSAID that is commonly used in horses for gastrointestinal pain and inflammation. Pentoxifylline, a phosphodiesterase inhibitor, is used in the horse for its anti-inflammatory effects in the treatment of endotoxemia and other systemic inflammatory conditions. The effects of flunixin meglumine and pentoxifylline on *in vivo* MMP inhibition in the horse have not been investigated.

Due to the apparent involvement of MMPs in equine laminitis, MMPIs may be beneficial in preventing or lessening the laminar destruction associated with this disease. In Chapter 3, LPS-induced MMP activity was inhibited in equine laminar explants *in vitro* by doxycycline, oxytetracycline, and flunixin meglumine. The purpose of this study was to determine if the administration of endotoxin would increase plasma MMP-2 and/or MMP-9 concentrations in the horse. Experimentally-induced endotoxemia was then used as an *in vivo* model of MMP induction for evaluating potential MMPIs in the horse, including doxycycline, oxytetracycline, flunixin meglumine, and pentoxifylline.

4.2 Materials and Methods

4.2.1 Horses – This study was approved by the Institutional Animal Care and Use Committee of Louisiana State University. Twenty-nine clinically healthy, adult horses of varying sex (11 mares, 18 geldings) and breed (17 Thoroughbreds, 8 Quarter Horses, 3 American

Paint Horses, and 1 Arabian), ranging in age from 3 to 19 years old (median, 7 years), and weighing from 379 to 560 kg (median, 491 kg) were used for this study. The horses were deemed to be free of medical problems related to inflammatory diseases, endotoxemia, or diseases of the digit as determined by complete physical examination, complete blood count, lameness examination including hoof testing, and radiographic evaluation of the digit.

4.2.2 Instrumentation – On the morning of the study, horses were placed into stocks and intravenous catheters were placed percutaneously following routine aseptic preparation of the skin and subcutaneous infiltration of a local anesthetic. A 14-gauge, 13.3-cm Teflon catheter was inserted into the left jugular vein for administration of saline solution, LPS, oxytetracycline, flunixin meglumine, or pentoxifylline. A 20-gauge, 5.1-cm Teflon catheter was inserted into the left lateral palmar digital vein for blood collection.

4.2.3 Experimental Design – This investigation consisted of 2 studies. Study 1 determined if the administration of LPS could induce MMPs in the digital circulation of the horse. Study 2 used the MMP induction model developed in Study 1 to evaluate the effectiveness of several MMPI in the horse.

Study 1 – Horses were randomly allocated into either a control group (n=5) which received saline solution or a treatment group (n=5) which received LPS. Control horses were administered 1 L 0.9% saline solution IV over 30 minutes. *E. coli* 055:B5 LPS[a] was administered IV at a dosage of 35 ng/kg in 1 L 0.9% saline solution over 30 minutes.

Study 2 – Horses were randomly allocated into one of the following treatment groups which received either doxycycline[b] (n=5) at 10 mg/kg via nasogastric tube, oxytetracycline[c] (n=5) at 20 mg/kg IV, flunixin meglumine[d] (n=5) at 1.1 mg/kg IV, or pentoxifylline[e] (n=4) at 8.5 mg/kg IV in 1L 0.9% saline solution over 30 minutes in addition to LPS. The MMPI treatments were

administered every 12 hours beginning 12 hours prior to LPS administration. Horses in these groups were compared with horses that received LPS in Study 1.

For both studies, baseline (T = 0) clinical variables were recorded and digital venous blood samples were collected into heparinized tubes immediately prior to infusion of LPS or saline solution. After administration of LPS or saline solution, clinical parameters were recorded and digital venous blood samples were collected at T = 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, 16.0, and 24.0 hours. The blood samples were centrifuged for 10 minutes at 2,000 rpm, and the plasma collected and stored in 1 ml aliquots at -70˚C until analyses for MMP activities were performed. The IV catheters were removed 24 hours after LPS or saline solution administration. The horses were observed for an additional 24 hours and returned to pasture.

4.2.4 Clinical Signs of Disease – Heart rate (beats/min), respiratory rate (breaths/min), rectal temperature (°F), mucous membrane color, CRT (seconds), and behavior were monitored and recorded at the above mentioned times.

4.2.5 Zymographic Analyses of MMP Activities – All zymograms were performed using commercially available 10% gelatin polyacrylamide gels.[f] Digital venous plasma samples were diluted 1:10 with buffer (25mM Tris, 192 mM glycine, and 0.1% SDS at pH 8.3). The diluted plasma samples, as well as pro and active MMP-2[g] and MMP-9[h] standards, were diluted 1:2 with 2X sample buffer (62.5 mM Tris-HCl, 25% glycerol, 4% SDS, and 0.01% bromophenol blue at pH 6.8). Ten microliters of each sample and 7 µl of each standard were loaded onto each gel. Following 1 hour of electrophoresis at 166 V, the gels were washed with renaturing buffer (2.5% Triton X-100) for 30 minutes and then incubated for 16-20 hours at 37˚C in development buffer (50mM Tris-HCl, 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35 at pH 7.5). After incubation, the gels were stained with 0.25% Coomassie brilliant blue in a mixture of aqueous 50% methanol: 10 % acetic acid (v/v) and destained in aqueous 20% methanol: 10% acetic acid

(v/v). Gelatinolytic activity was detected as transparent bands against a dark blue background. Relative values in arbitrary units (A.U.) were established for MMP concentrations by digitally photographing the gels and measuring band intensity and size using Image J[i] densitometry software.

4.2.6 Statistical Analyses – All data were tested using the Shapiro-Wilk statistic and found to have normal distributions. Normally distributed variables were analyzed using a mixed-effects ANOVA with horse as a random variable. Post-hoc comparisons were made using least squares means. $P \leq 0.05$ was considered significant. Data were summarized and graphed as mean ± SD. SAS[j] statistical software was used for all statistical analyses.

4.3 Results

4.3.1 Clinical Signs of Disease – The mean values for heart rate, rectal temperature, and respiratory rate for each group are listed in Table 4.1. Heart rate and rectal temperature did not significantly change from baseline (T=0) in horses receiving saline solution; however, respiratory rate increased significantly and remained elevated after 0.5 hours. Tachycardia, increased rectal temperature, and mild colic occurred in all horses receiving LPS alone or in combination with an MMPI, consistent with a systemic inflammatory response. Horses receiving LPS alone or in combination with an MMPI had modest, intermittent increases in respiratory rate. The administration of LPS significantly increased heart rate from 0.5 through 6.0 hours and rectal temperature from 1.5 through 6.0 hours compared with the administration of saline. Horses receiving either oxytetracycline or flunixin meglumine had decreased heart rates and rectal temperatures compared with horses receiving only LPS. Alterations in clinical variables returned to normal by 24.0 hours in all horses.

4.3.2 Zymographic Analyses of MMP Activities – Lucent bands of enzyme activity corresponding to proMMP-2 (72 kD) and proMMP-9 (92 kD) were detected in all digital venous

Table 4.1 – Mean heart rates (HR, beats/min), rectal temperatures (Temp, °F), and respiratory rates (RR, breaths/min) over time (hours) for horses administered either an intravenous infusion of saline solution (SALINE), LPS (LPS), doxycycline followed by LPS (DOXY), oxytetracycline followed by LPS (OXYTET), flunixin meglumine followed by LPS (FLUNIXIN), or pentoxifylline followed by LPS (PTX). †Significant ($p \leq 0.05$) difference from baseline (T=0) value within treatment group. *Significant ($p \leq 0.05$) difference from LPS group.

Time	SALINE			LPS			DOXY		
	HR	Temp	RR	HR	Temp	RR	HR	Temp	RR
T=0	41 ± 7	100.2 ± 0.9	15 ± 3	43 ± 3	99.7 ± 0.7	13 ± 4	39 ± 7	100.2 ± 0.4	15 ± 4
T=0.5	43 ± 9*	100.3 ± 1.0	25 ± 10†*	54 ± 7†	100.1 ± 0.6	15 ± 3	49 ± 5†	100.6 ± 0.4	18 ± 6
T=1.0	44 ± 2*	100.2 ± 0.8	21 ± 9†	54 ± 8†	100.7 ± 1.1	17 ± 3	58 ± 8†	101.0 ± 0.3	20 ± 3†
T=1.5	43 ± 3*	100.3 ± 0.5*	22 ± 4†	60 ± 6†	101.5 ± 1.3†	19 ± 5†	60 ± 9†	101.5 ± 0.8†	17 ± 3
T=2.0	40 ± 6*	100.1 ± 0.4*	26 ± 8†*	59 ± 4†	102.7 ± 1.2†	15 ± 3	51 ± 4†	101.9 ± 1.2†	17 ± 4
T=3.0	37 ± 4*	100.1 ± 0.5*	27 ± 12†*	47 ± 8	103.1 ± 1.4†	18 ± 7	46 ± 4†	102.5 ± 1.8†	16 ± 5
T=4.0	42 ± 7*	100.4 ± 0.3*	24 ± 5†	50 ± 3†	102.7 ± 1.3†	19 ± 8†	46 ± 2†	102.1 ± 1.2†	15 ± 2
T=6.0	42 ± 4*	100.2 ± 0.4*	24 ± 6†*	51 ± 10†	101.2 ± 0.6†	15 ± 3	50 ± 5†	101.0 ± 0.8	16 ± 7
T=8.0	41 ± 3	100.3 ± 0.4	22 ± 4†	48 ± 6	100.6 ± 0.4†	15 ± 5	46 ± 6†	100.2 ± 0.5	22 ± 10†*
T=12.0	41 ± 5	99.9 ± 0.4	21 ± 3†	47 ± 8	100.5 ± 0.6†	14 ± 2	43 ± 3	100.1 ± 0.6	18 ± 8
T=16.0	39 ± 7	100.1 ± 0.4	18 ± 7	44 ± 8	99.9 ± 0.7	14 ± 2	39 ± 3	99.5 ± 0.7	16 ± 5
T=24.0	38 ± 2	99.8 ± 0.3	21 ± 7†*	41 ± 3	99.3 ± 0.6	13 ± 2	45 ± 8	99.7 ± 0.6	15 ± 4

Time	OXYTET			FLUNIXIN			PTX		
	HR	Temp	RR	HR	Temp	RR	HR	Temp	RR
T=0	40 ± 3	99.2 ± 0.9	12 ± 2	34 ± 8*	99.8 ± 0.3	14 ± 6	40 ± 0	99.6 ± 1.1	20 ± 13
T=0.5	51 ± 12†	100.1 ± 0.8	20 ± 7†	42 ± 8†*	100.5 ± 0.4	14 ± 3	48 ± 7	100.0 ± 1.2	22 ± 12
T=1.0	67 ± 7†*	100.0 ± 0.7	19 ± 4†	49 ± 8†	100.5 ± 0.3	15 ± 4	56 ± 4†	100.5 ± 1.5	24 ± 12
T=1.5	57 ± 7†	101.0 ± 0.5†	16 ± 5	50 ± 7†	100.7 ± 0.4†	19 ± 4†	52 ± 11†	101.1 ± 1.7†	24 ± 10
T=2.0	49 ± 8†*	101.1 ± 0.8†*	13 ± 4	48 ± 7†*	100.8 ± 0.4†*	14 ± 2	58 ± 8†	101.4 ± 1.6†	18 ± 4
T=3.0	42 ± 2	101.6 ± 0.7†*	14 ± 2	40 ± 9	101.3 ± 1.0†*	17 ± 7	51 ± 8†	101.8 ± 1.9†	23 ± 7
T=4.0	40 ± 4*	100.9 ± 1.5†*	15 ± 4	38 ± 11*	100.8 ± 0.8†*	16 ± 4	47 ± 7	102.1 ± 1.0†	23 ± 8
T=6.0	45 ± 3	99.9 ± 0.9*	14 ± 5	50 ± 9†	100.6 ± 0.7†	22 ± 8†	46 ± 10	101.0 ± 0.5†	19 ± 6
T=8.0	42 ± 5	100.4 ± 0.9†	12 ± 3	47 ± 3†	100.4 ± 0.4	18 ± 6†	47 ± 7	100.1 ± 0.6	25 ± 11*
T=12.0	42 ± 2	100.8 ± 0.3†	14 ± 4	49 ± 5†	100.6 ± 0.4	15 ± 5	52 ± 9†	100.4 ± 1.1	23 ± 9
T=16.0	46 ± 10	100.0 ± 0.6	11 ± 2	43 ± 6†	99.8 ± 0.5	13 ± 5	47 ± 7	99.7 ± 1.4	17 ± 8
T=24.0	42 ± 4	98.7 ± 0.6	15 ± 3	49 ± 4	99.6 ± 0.4	14 ± 4	43 ± 6	99.4 ± 0.6	19 ± 2

plasma samples of all horses. The active forms of MMP-2 (66 kD) and MMP-9 (83 kD) were not detected.

Study 1 – Administration of LPS significantly decreased proMMP-2 and increased proMMP-9 concentrations in the digital venous plasma over time compared with baseline values (Figures 4.1 and 4.2). Both proMMP-2 and proMMP-9 concentrations remained unchanged from baseline in digital venous plasma samples from horses receiving saline solution (Figures 4.1 and 4.2). ProMMP-2 concentrations were significantly increased for the LPS group at 16.0 and 24.0 hours compared with horses administered saline solution (Figure 4.1). ProMMP-9 concentrations were significantly increased for horses administered LPS at 0.5, 1.0, 1.5, 2.0, 4.0, 12.0, 16.0, and 24.0 hours compared with horses administered saline solution (Figure 4.2).

Study 2 – ProMMP-2 concentrations remained unchanged compared with baseline values for the DOXY, OXYTET, FLUNIXIN, and PTX treatment groups; however, proMMP-9 concentrations increased over time compared with baseline values despite treatment with MMPI in these groups (Figures 4.3 – 4.10). Doxycycline significantly decreased proMMP-2 concentrations in the digital venous plasma at 1.0, 1.5, 2.0, 3.0, 4.0, 16.0, and 24.0 hours and also decreased proMMP-9 concentrations at 0.5, 1.5, 6.0, and 8.0 hours compared with horses receiving only LPS (Figures 4.3 and 4.4). ProMMP-2 concentrations were significantly decreased for the OXYTET group at 0.5, 1.0, 16.0, and 24.0 hours compared with the LPS group (Figure 4.5). The OXYTET group also had significantly decreased proMMP-9 concentrations compared with the LPS group at 0, 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 hours (Figure 4.6). Flunixin meglumine significantly decreased proMMP-2 concentrations at 0.5, 1.0, 1.5, 2.0, 4.0, 16.0 and 24.0 hours and significantly decreased proMMP-9 concentrations at 0.5 and 1.5 hours compared with horses administered LPS alone (Figures 4.7 and 4.8). Administration of pentoxifylline significantly decreased proMMP-2 digital venous plasma concentrations at 0, 0.5, 1.0, 16.0 and

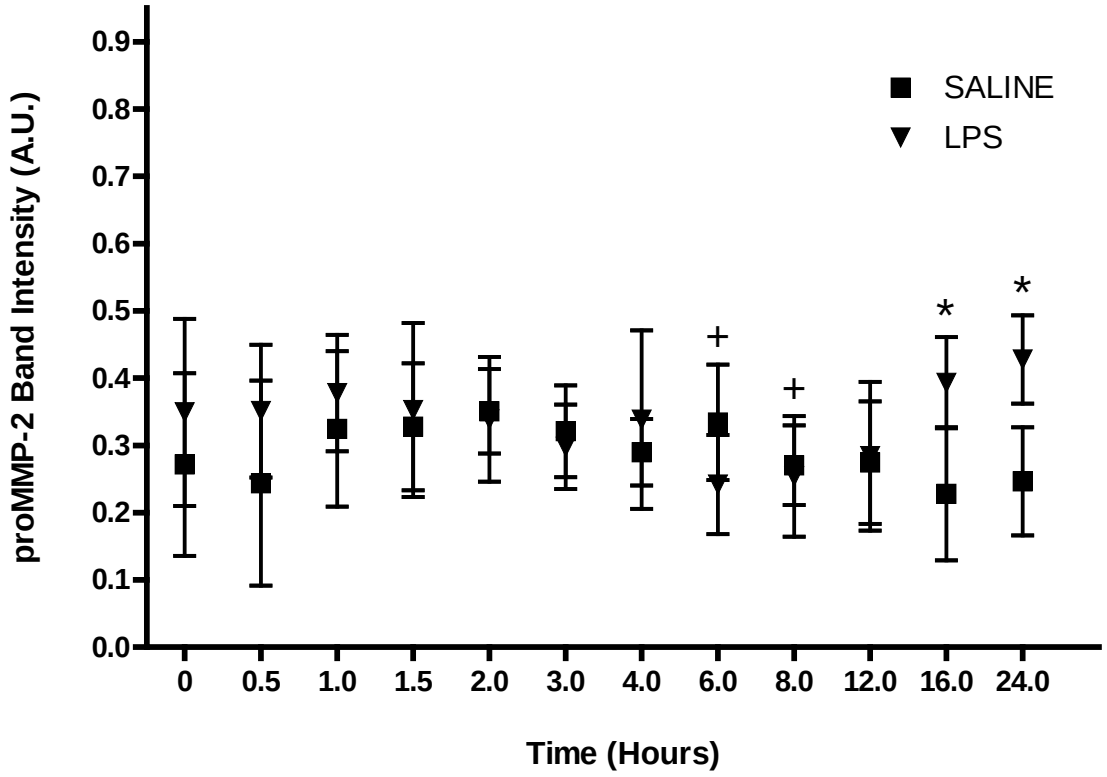

Figure 4.1 – Mean (± SD) proMMP-2 band intensities (arbitrary units, A.U.) in the digital venous plasma of horses receiving an IV infusion of LPS (LPS) or saline solution (SALINE). +Significant ($p \leq 0.05$) difference from baseline ($T = 0$) values for LPS group. *Significant ($p \leq 0.05$) difference between treatment groups.

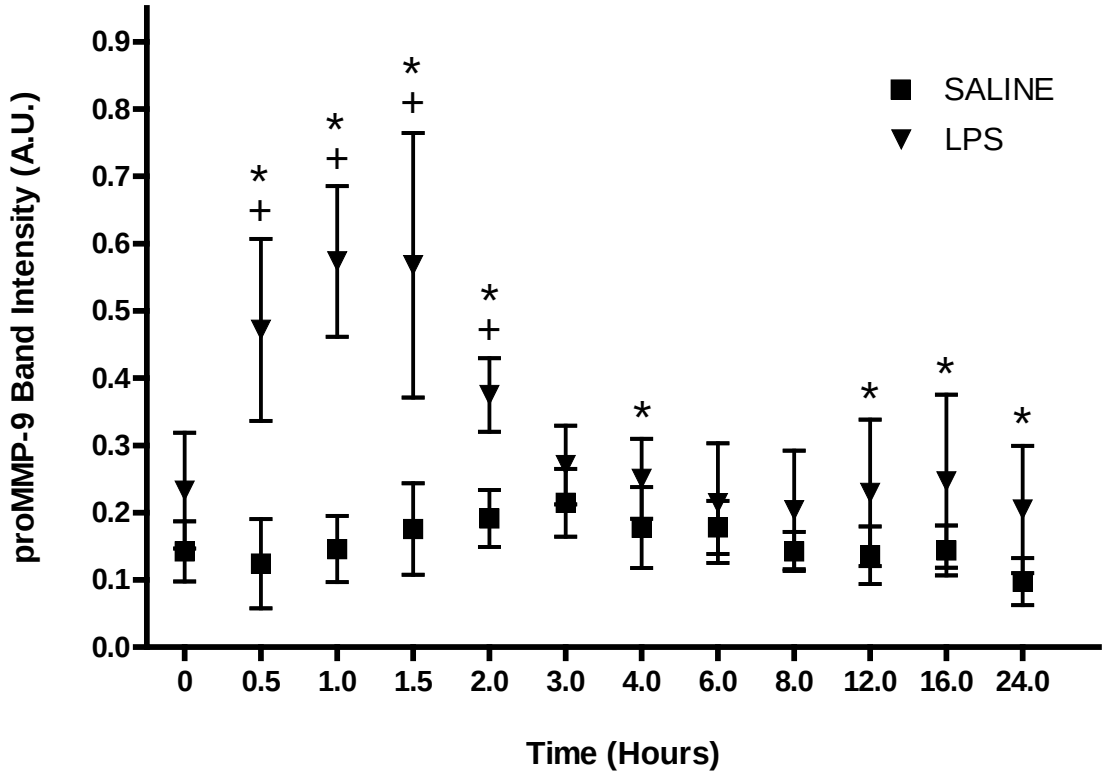

Figure 4.2 – Mean (± SD) proMMP-9 band intensities (arbitrary units, A.U.) in the digital venous plasma of horses receiving an IV infusion of LPS (LPS) or saline solution (SALINE). +Significant ($p \leq 0.05$) difference from baseline (T = 0) value for LPS group. *Significant ($p \leq 0.05$) difference between treatment groups.

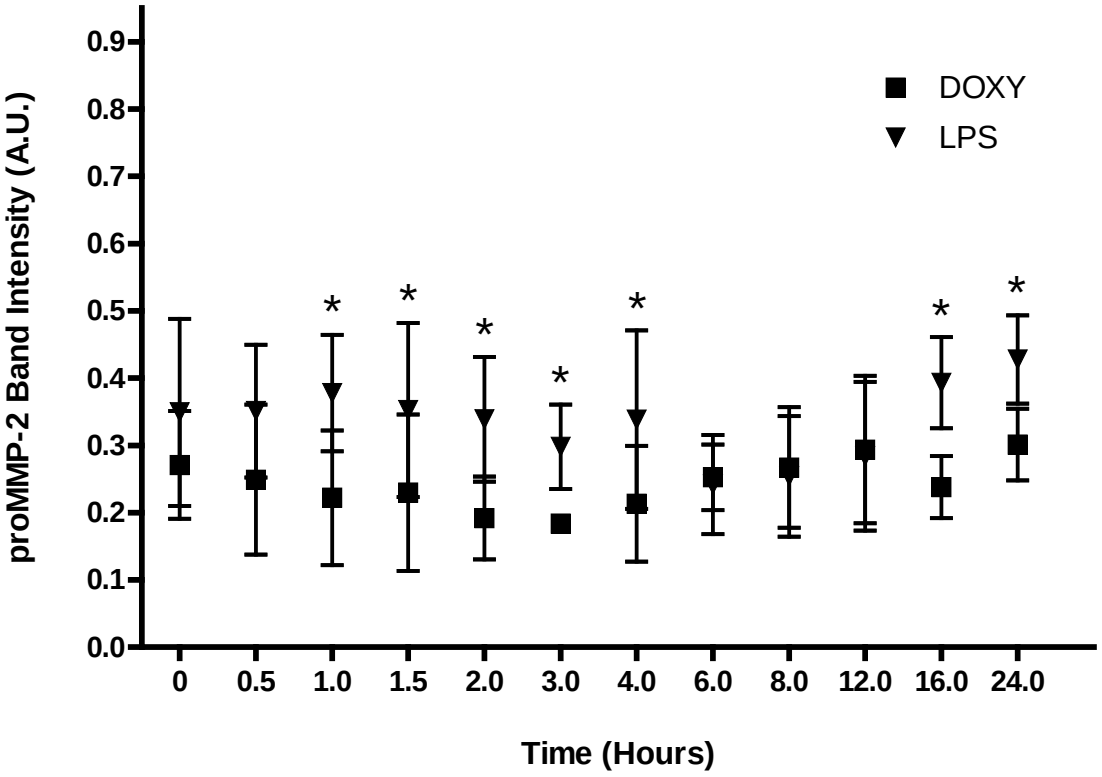

Figure 4.3 – Mean (± SD) proMMP-2 band intensities (arbitrary units, A.U.) in the digital venous plasma of horses receiving an IV infusion of LPS (LPS) or doxycycline followed by an IV infusion of LPS (DOXY). *Significant ($p \leq 0.05$) difference between treatment groups.

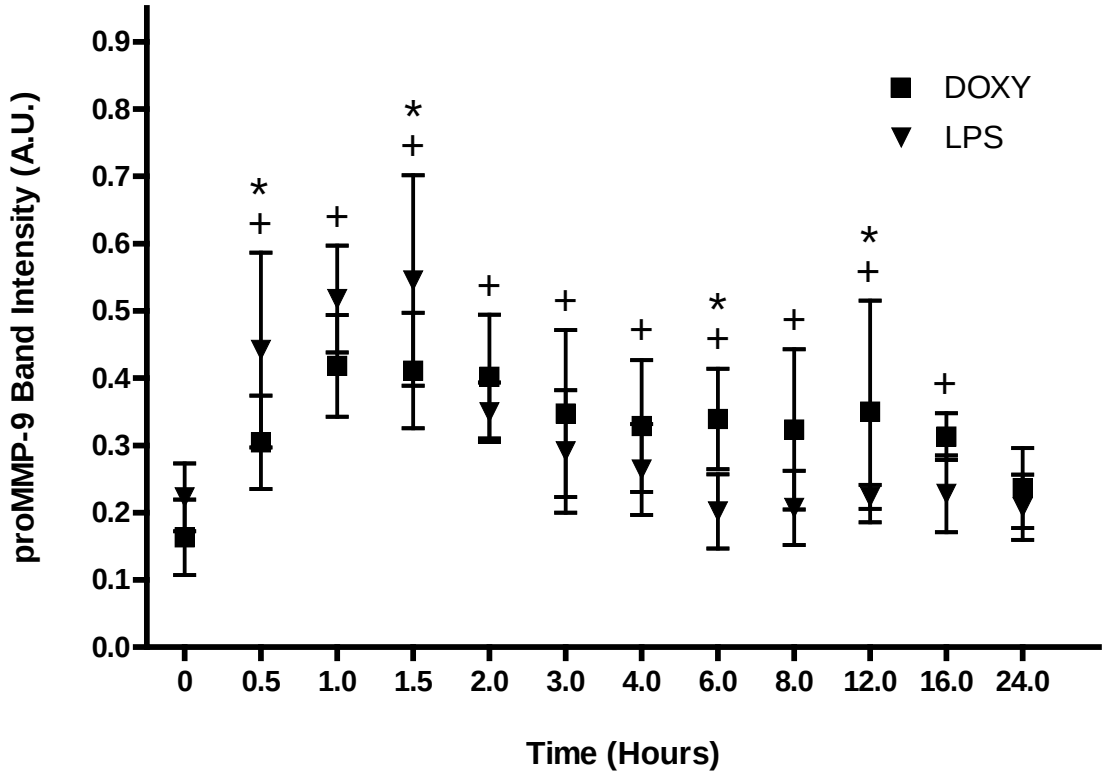

Figure 4.4 – Mean (± SD) proMMP-9 band intensities (arbitrary units, A.U.) in the digital venous plasma of horses receiving an IV infusion of LPS (LPS) or doxycycline followed by an IV infusion of LPS (DOXY). +Significant ($p \leq 0.05$) difference from baseline (T = 0) value for DOXY group. *Significant ($p \leq 0.05$) difference between treatment groups.

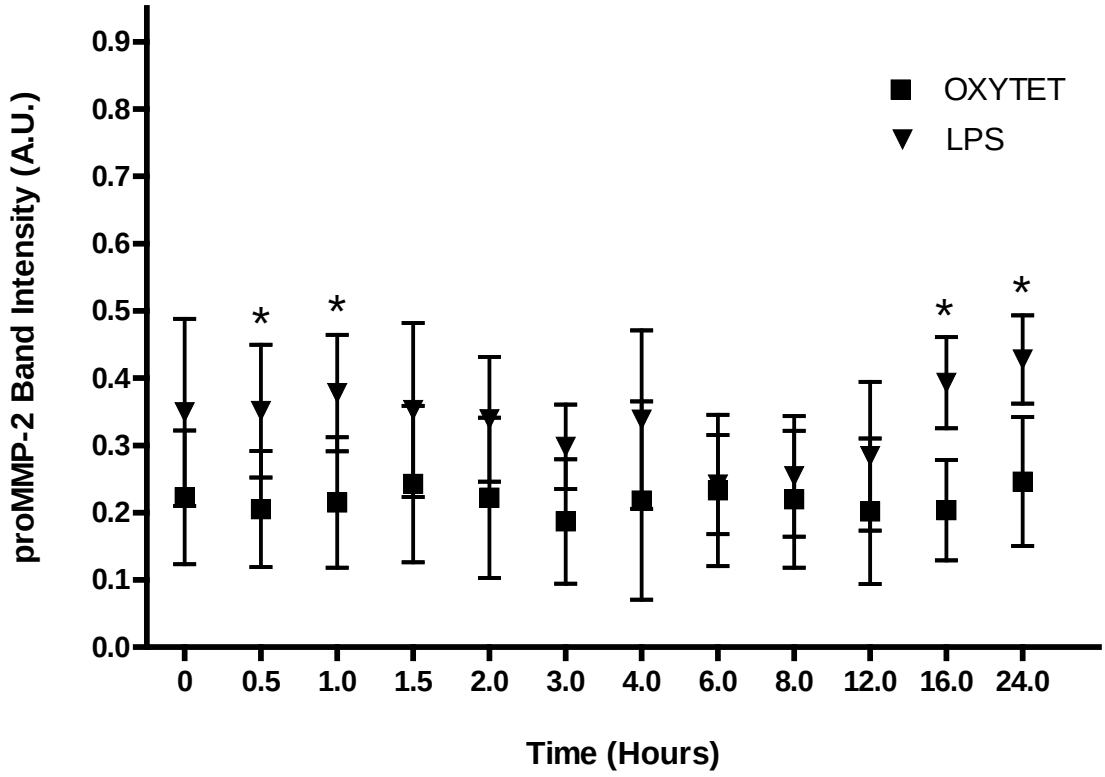

Figure 4.5 – Mean (± SD) proMMP-2 band intensities (arbitrary units, A.U.) in the digital venous plasma of horses receiving an IV infusion of LPS (LPS) or oxytetracycline followed by an IV infusion of LPS (OXYTET). *Significant ($p \leq 0.05$) difference between treatment groups.

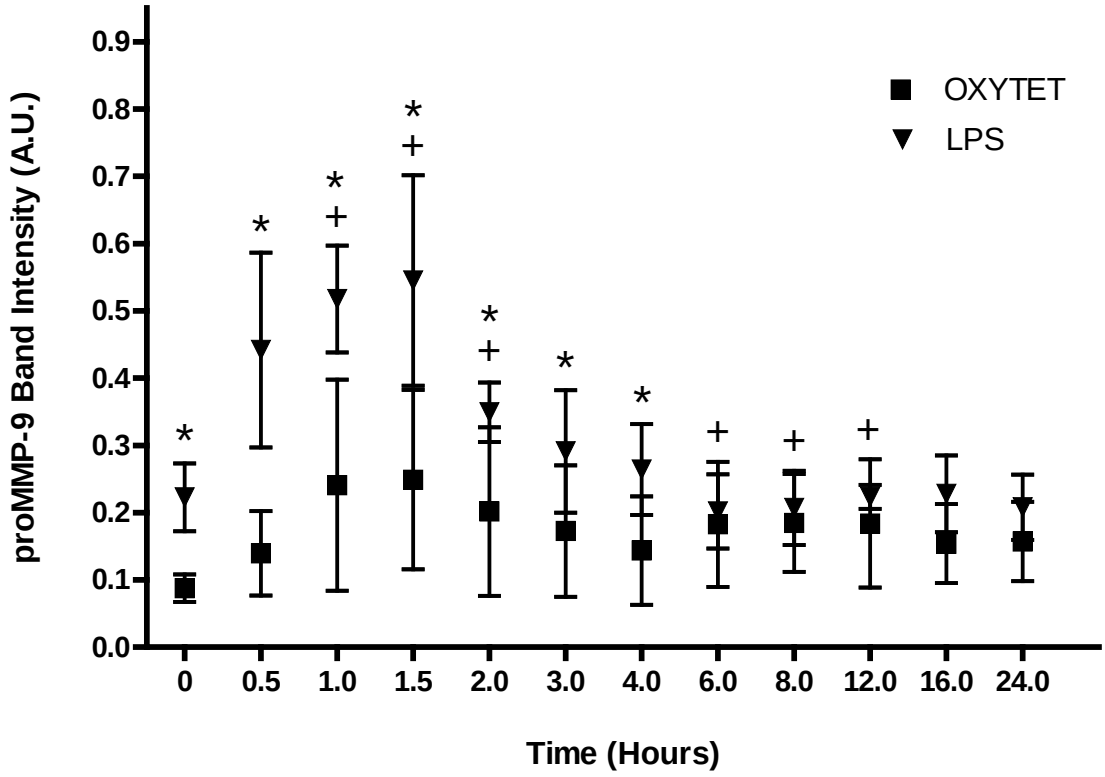

Figure 4.6 – Mean (± SD) proMMP-9 band intensities (arbitrary units, A.U.) in the digital venous plasma of horses receiving an IV infusion of LPS (LPS) or oxytetracycline followed by an IV infusion of LPS (OXYTET). +Significant ($p \leq 0.05$) difference from baseline ($T = 0$) value for OXYTET group. *Significant ($p \leq 0.05$) difference between treatment groups.

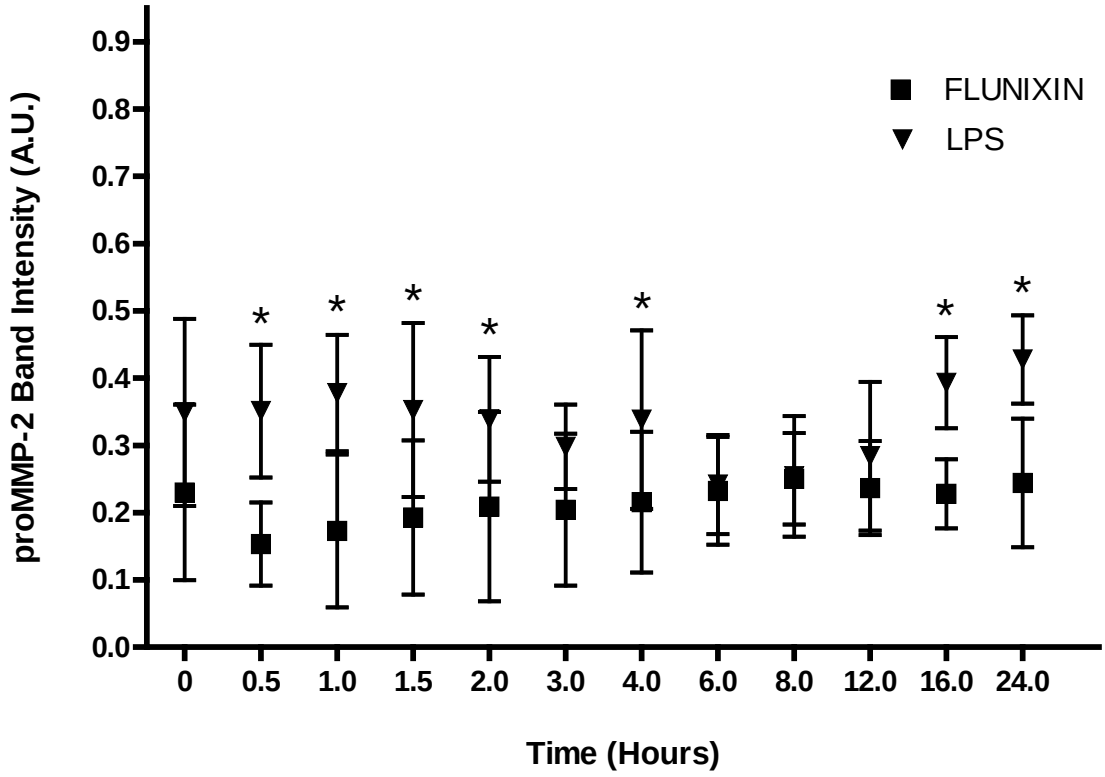

Figure 4.7 – Mean (± SD) proMMP-2 band intensities (arbitrary units, A.U.) in the digital venous plasma of horses receiving an IV infusion of LPS (LPS) or flunixin meglumine followed by an IV infusion of LPS (FLUNIXIN). *Significant ($p \leq 0.05$) difference between treatment groups.

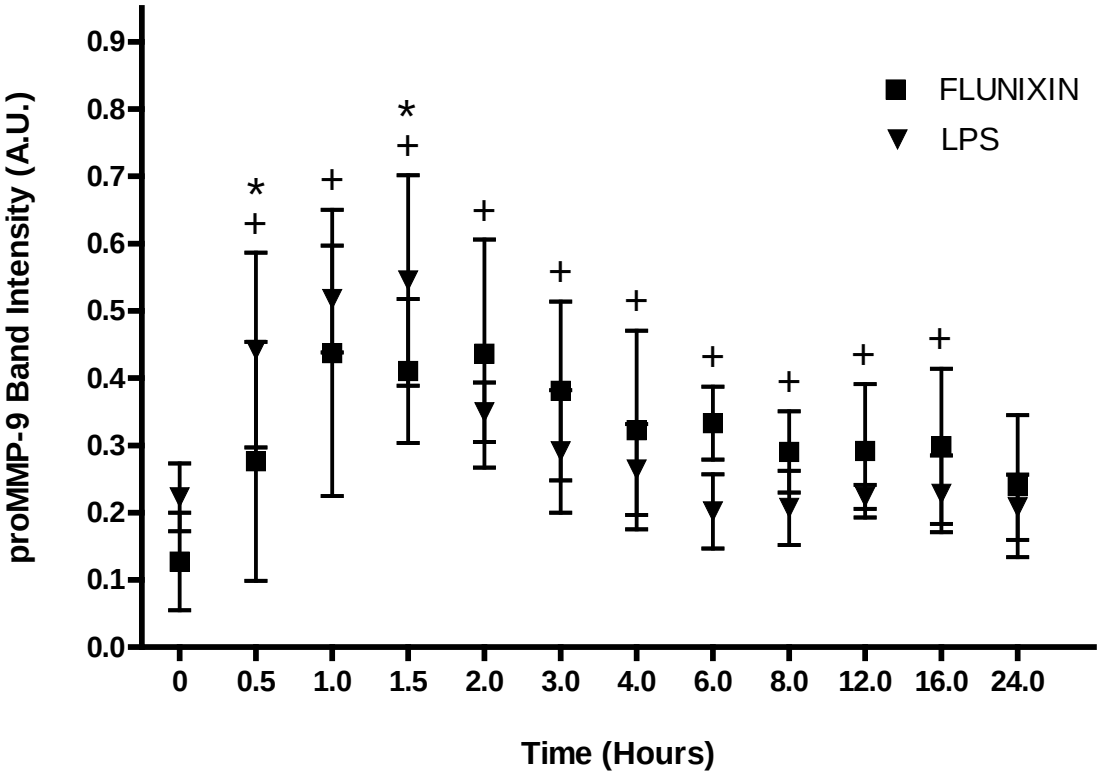

Figure 4.8 – Mean (± SD) proMMP-9 band intensities (arbitrary units, A.U.) in the digital venous plasma of horses receiving an IV infusion of LPS (LPS) or flunixin meglumine followed by an IV infusion of LPS (FLUNIXIN). +Significant ($p \leq 0.05$) difference from baseline (T = 0) value for FLUNIXIN group. *Significant ($p \leq 0.05$) difference between treatment groups.

24.0 hours and significantly decreased proMMP-9 concentrations at 0, 0.5, 1.0, 1.5, 2.0, and 3.0 hours compared with horses receiving only LPS (Figures 4.9 and 4.10).

4.4 Discussion

Intravenous infusion of endotoxin significantly increased MMP-2 and MMP-9 concentrations in the digital circulation of healthy adult horses. Horses receiving LPS developed clinical signs associated with endotoxemia similar to previous reports.[130,131] The administration of doxycycline, oxytetracycline, flunixin meglumine, or pentoxifylline prior to LPS administration resulted in significant decreases in MMP-2 and MMP-9 digital venous plasma concentrations of varying degrees.

Early in the pathogenesis of experimentally-induced laminitis, a systemic inflammatory response occurs that is localized to the digit including increased pro-inflammatory cytokine induction, neutrophil infiltration, and MMP synthesis and release.[178,195-197,200] Both MMP-2 and MMP-9 activities are increased in digital laminar tissue of horses with clinical and experimentally induced laminitis.[82,84,85] Also, increases in MMP-9 concentrations have been found in the systemic circulation of experimental CHO laminitis.[206] Thus, it would seem likely that synthetic inhibitors of MMPs may be useful in the treatment of this disease. The validation of endotoxemia as a model of MMP induction in the digital circulation of the horse enabled further study of MMPIs as potential treatments for laminitis without requiring the euthanasia of numerous horses.

The non-proteolytic latent form of both MMP-2 and MMP-9 were identified in plasma samples, but not the active forms. This is similar to results from experimental infusion of endotoxin in humans and in other animals.[134,136] Reportedly, in healthy tissue most of the potential MMP activity is present in the latent form.[82] ProMMPs can undergo allosteric

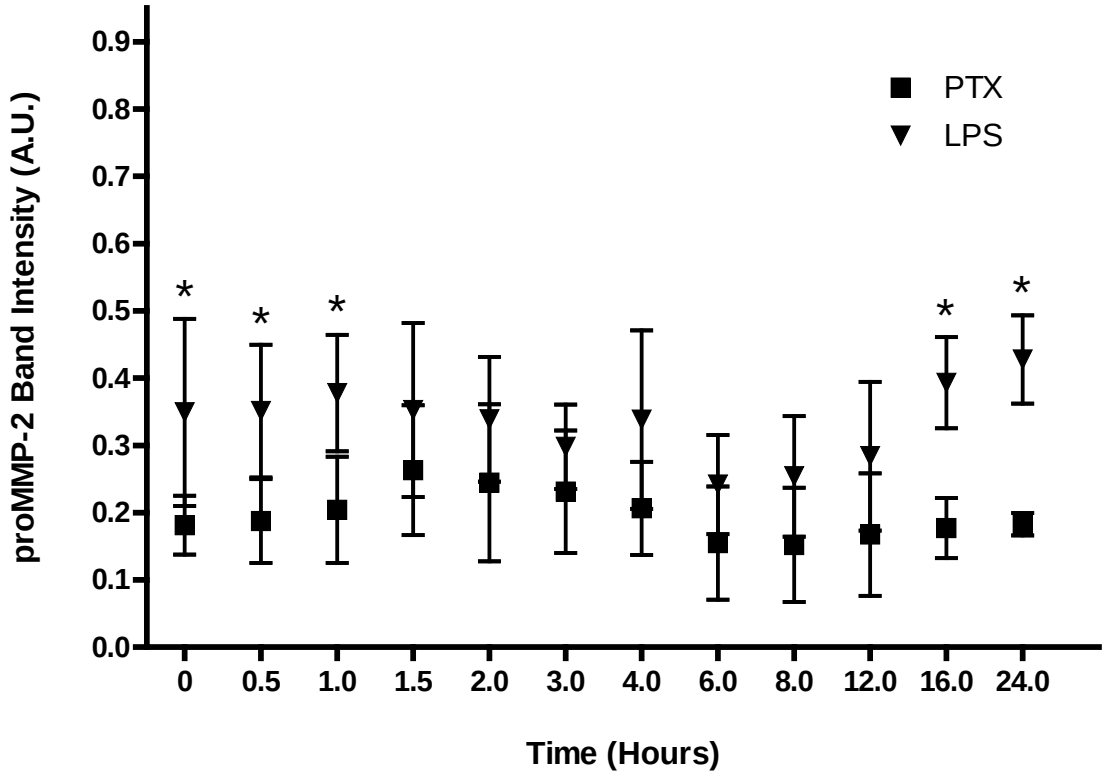

Figure 4.9 – Mean (± SD) proMMP-2 band intensities (arbitrary units, A.U.) in the digital venous plasma of horses receiving an IV infusion of LPS (LPS) or pentoxifylline followed by an IV infusion of LPS (PTX). *Significant ($p \leq 0.05$) difference between treatment groups.

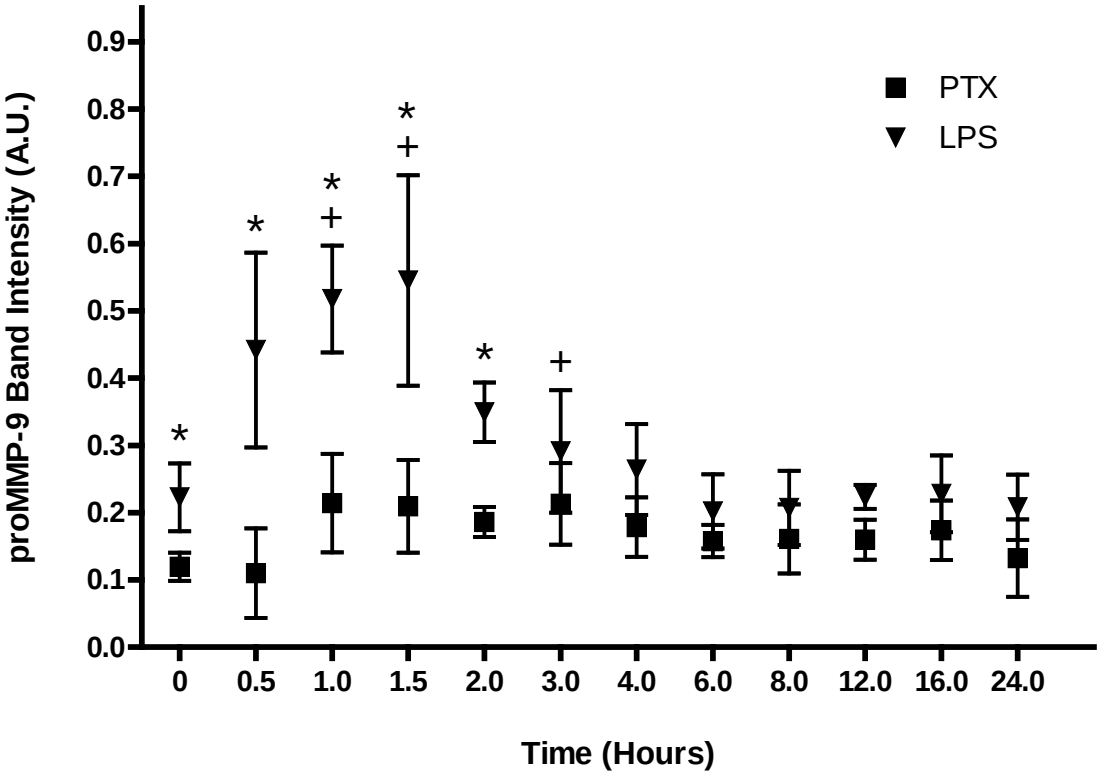

Figure 4.10 – Mean (± SD) proMMP-9 band intensities (arbitrary units, A.U.) in the digital venous plasma of horses receiving an IV infusion of LPS (LPS) or pentoxifylline followed by an IV infusion of LPS (PTX). +Significant ($p \leq 0.05$) difference from baseline (T = 0) value for PTX group. *Significant ($p \leq 0.05$) difference between treatment groups.

activation without proteolysis of their active domain if they are in contact with the appropriate substrate.[31] Therefore, the zymogen forms may be up-regulated and released into the plasma, but may be either awaiting activation by other proteases or seeking the appropriate substrate. In addition, the active MMPs may have already become bound to substrate in tissues and be unavailable for measurement in the plasma.

It is not surprising that endotoxin administration had only minor effects on MMP-2 activity compared with its effects on MMP-9 activity. Endotoxemia incites a severe inflammatory response that initiates numerous mediator cascades, many of which are MMP activators and substrates.[15,16,22,125] Although MMP-9 can be constitutively expressed to some extent, it is mostly induced in response to inflammatory mediators and released by neutrophils.[7] Therefore, MMP-9 seems the mostly likely of the two gelatinases to be upregulated by endotoxin exposure. Other studies have also shown that endotoxin induces predominantly MMP-9.[230] The constitutive nature of MMP-2 and the lack of response seen here upon exposure to LPS, lend further evidence to the suggestion that MMP-2 is mostly involved in homeostasis and may possibly even play a protective role. While the increases in proMMP-2 at 16.0 and 24.0 hours in the LPS group were significant compared with all other groups, they were not significantly increased compared to the LPS baseline value and seem to be merely fluctuations.

All of the MMPIs evaluated in this study were found to inhibit MMPs in the horse. Flunixin meglumine appeared to have the greatest inhibitory effect on MMP-2 plasma concentrations followed by doxycycline, pentoxifylline, and oxytetracycline. In contrast, pentoxifylline was the most potent MMP-9 inhibitor, with oxytetracycline having only slightly lesser inhibitory effects. Both doxycycline and flunixin meglumine had very little inhibitory effects on MMP-9 in the horse.

The NSAID flunixin meglumine, a non-selective COX inhibitor, had inhibitory effects on both MMP-2 and MMP-9 in the horse *in vivo*. These findings differ slightly from other *in vitro* results which indicated that neither flunixin meglumine nor phenylbutazone, another NSAID, inhibited equine MMP-2 or MMP-9 obtained from equine cell culture.[76] The effects of NSAIDs on MMP inhibition have been extensively studied. Cyclooxygenase-2 increases activation of MMPs, and NSAIDs, or COX inhibitors, decrease MMP-2 and MMP-9 expression.[114-117] Other research suggests that NSAIDs up-regulate mRNA expression of RECK, a membrane anchored endogenous MMPI.[118] Increased expression of RECK leads to decreases in MMP-2 activity and suppression of MMP-9 release.[231] Although RECK is inversely related to MMP-2 activation, it is not related to MMP-9 activation. However, tumors with high RECK expression have a trend toward decreased MMP-9 expression.[39] Therefore, flunixin meglumine's potent inhibitory effects on MMP-2 as opposed to its weak effects on MMP-9 may be attributed to increased expression of RECK and its apparent affinity for MMP-2 inhibition.

The results of this study suggest that pentoxifylline is a potent MMP-9 and modest MMP-2 inhibitor in the horse. Phosphodiesterase inhibitors regulate MMP expression by increasing intracellular cAMP concentrations that disrupt phosphorylation pathways and prevent gene transcription.[113,119] Pentoxifylline is a methyl xanthine derivative and phosphodiesterase inhibitor commonly used for its rheological effects on peripheral vascular disease in people.[120] Pentoxifylline has also been shown to decrease neutrophil infiltration and activation in the lung and liver of rats administered endotoxin.[119,232] Neutrophils are the predominant source of MMP-9; therefore, the neutrophil inhibiting effects of pentoxifylline may account for a portion of the decrease in MMP-9 activity demonstrated here.

Horses administered pentoxifylline had significantly lower baseline MMP-2 and MMP-9 concentrations compared with horses administered endotoxin alone. Likewise, oxytetracycline

had significantly lower baseline MMP-9 concentrations. Various cells constitutively produce MMP-2 and, to a small extent MMP-9.[7] The MMPI treatments were administered 12 hours prior to endotoxin infusion; therefore, it is logical that baseline MMP concentrations could be decreased because of inhibition already present at the time of LPS infusion. Inhibition of basal MMP concentrations suggests that pentoxifylline and oxytetracycline are more potent MMPI in the horse than doxycycline and flunixin meglumine. Furthermore, MMP-9 is usually induced by inflammatory mediators and released from neutrophils, as previously stated. The above mentioned neutrophil inhibitory effects of pentoxifylline may also account for the decreases in baseline plasma MMP-9 concentrations.

Although doxycycline is a potent MMP-9 inhibitor in other species and the only drug approved for MMP inhibition in humans, it surprisingly had only minimal effects on endotoxin-induced MMP-9 activity in this study. However, it was a potent MMP-2 inhibitor, along with flunixin meglumine. This is unusual because other reports indicate that doxycycline predominantly inhibits MMP-9 and in some cases has no inhibitory effect on MMP-2.[101,230] Matrix metalloproteinase inhibition by tetracyclines is believed to occur by chelation of Zn^{2+} ions at the binding site in the catalytic domain of MMPs.[112] Other studies indicate that they regulate MMP gene expression by affecting mRNA stability.[233] Doxycycline may also decrease MMP-9 secretion through up-regulation of its endogenous inhibitor, TIMP-1.[101]

Doxycycline has been reported to cause cardiovascular collapse and death when administered IV to horses; therefore, in this study, the drug was administered via nasogastric intubation (NGT). Endotoxemia leads to decreased gastric and intestinal motility through the activation of COX and subsequent production of PGE_2.[149,160] It is possible that NGT administration of doxycycline in this study may have led to decreased absorption and decreased MMP inhibition. To determine if decreased absorption occurred, plasma doxycycline

concentrations were determined. Plasma doxycycline concentrations peaked 0.5 hour after NGT administration with a mean of 0.97 ± 0.34 µg/ml. This is consistent with reported maximum steady-state doxycycline serum concentrations of 0.94 µg/ml achieved after oral administration in horses at 10 mg/kg every 12 hours.[234] Therefore, the low degree of MMP inhibition obtained in this study does not appear to be due to decreased intestinal absorption. However, the maximum steady-state serum concentrations reported above correspond with the MIC for certain bacteria. Perhaps serum concentrations of doxycycline required for adequate MMP inhibition are much greater than the MIC; therefore, the dose used in this *in vivo* study would not have been sufficient for maximal MMP inhibition. Furthermore, this would also explain why doxycycline had greater MMP inhibitory effects in the confined explant culture environment. The equivalent *in vivo* dose required would be approximately 5X the maximum safe dosage; therefore, it would not be an advisable dose to investigate *in vivo*.

Oxytetracycline had a much greater effect on equine MMP inhibition than doxycycline. It was both a potent MMP-9 inhibitor and a modest MMP-2 inhibitor. The effects of oxytetracycline on MMP-2 and MMP-9 inhibition have not been studied previously. It appears that oxytetracycline has greater inhibitory effects on MMP-9 than MMP-2, as is seen with other tetracyclines.[101,230]

Tetracyclines are excreted through the biliary duct into the intestine; therefore, there is a risk for the development of diarrhea following administration at higher doses due to intestinal microbial alterations.[234] Several studies have demonstrated the safety of oral administration of doxycycline at both 10 mg/kg every 12 hours and 20 mg/kg every 24 hours.[108-110] Oxytetracycline has been used safely in horses at dosages ranging from 5 – 40 mg/kg IV.[235] In this study, none of the horses administered doxycycline at a dose of 10 mg/kg or oxytetracycline at a dose of 20 mg/kg IV every 12 hours developed diarrhea.

Establishing non-terminal models for the study of disease is important in any field of research, but especially in equine research. This study has demonstrated that experimentally-induced endotoxemia can be used as a non-terminal, *in vivo* model for the induction and investigation of MMPs in the horse. Furthermore, pentoxifylline, oxytetracycline, flunixin meglumine, and doxycycline were all found capable of inhibiting equine MMPs. Flunixin meglumine and doxycycline appeared to be weak MMP-9 and potent MMP-2 inhibitors. However, pentoxifylline and oxytetracycline were both potent MMP-9 and moderate MMP-2 inhibitors in the horse. Past studies suggest that MMP-9 may play a more important role in laminitis; therefore, pentoxifylline and oxytetracycline warrant further study for use as treatments/preventatives for equine laminitis.

4.5 Product Information

[a]Lipopolysaccharide from *E. coli* O55:B5 L2880, Sigma-Aldrich, St. Louis, MO

[b]Doxycycline hyclate, Ranbaxy Pharmaceuticals, Princeton, NJ

[c]Liquamicin LA-200, Pfizer Animal Health, St. Louis, MO

[d]Banamine, Schering-Plough Animal Health, Union, NJ

[e]Pentoxifylline, HDM Pharmacy, Lexington, KY

[f]10% Gelatin Ready Gel 161-1167, Bio-Rad Laboratories, Hercules, CA

[g]MMP-2 Proenzyme PF037 and Active PF023, Calbiochem, La Jolla, CA

[h]MMP-9 Proenzyme PF038 and Human Recombinant PF024, Calbiochem, La Jolla, CA

[i]Image J, U.S. National Institutes of Health, Bethesda, MD

[j]SAS v 9.0, SAS Institute, Cary, NC

CHAPTER 5. SUMMARY

Acute laminitis is a debilitating disease affecting the equine foot, of which the pathogenesis is incompletely understood despite years of research. Recent studies have suggested that MMP-2 and MMP-9 may be responsible for the laminar destruction seen with this disease. Laminitis often occurs secondary to many diseases such as endotoxemia, which has also been associated with increases in MMP activity. In human medicine, MMPIs have been investigated as potential treatments for various MMP-associated diseases; therefore, MMPIs may be helpful in the treatment/prevention of equine laminitis. This doctoral research had three main goals: 1) to collect MMP-9 from equine neutrophils and to validate a human MMP activity assay for use with equine samples as a more complete evaluation of MMP activity compared with zymography; 2) to validate experimental endotoxemia as a non-terminal model for MMP induction in the horse; and 3) to use the MMP induction model to evaluate the effectiveness of various MMPIs in the horse.

Equine MMP-9 was successfully harvested from neutrophils and characterized. The Biotrak MMP-9 Activity Assay had low sensitivity to equine MMP-9; therefore, it could not be used for quantification of MMPs in these studies. *In vitro*, doxycycline and oxytetracycline significantly increased digital laminar explant structural integrity. Furthermore, doxycycline, oxytetracycline, and flunixin meglumine all significantly decreased MMP-2 and MMP-9 concentrations in the medium of digital laminar explants incubated with endotoxin. Laminar structural integrity and medium MMP-2 concentration were inversely correlated. Intravenous infusion of endotoxin to healthy adult horses significantly increased digital venous plasma MMP-2 and MMP-9 activities compared with IV infusion of a saline solution. Pre-treatment with oral doxycycline or IV flunixin meglumine significantly decreased digital plasma MMP-2 concentrations, but merely blunted MMP-9 concentrations in healthy adult horses. However, pre-treatment with IV pentoxifylline and IV oxytetracycline profoundly inhibited the effects of

endotoxin on plasma MMP-9 concentrations and modestly inhibited plasma MMP-2 concentrations in healthy adult horses.

An established model of experimentally-induced endotoxemia was successfully validated for use as a non-terminal *in vivo* method of MMP induction in healthy adult horses. This model was used to determine that doxycycline and flunixin meglumine are potent MMP-2 inhibitors in the horse, whereas pentoxifylline and oxytetracycline are far more potent MMP-9 inhibitors. The *in vitro* results agree that oxytetracycline was a superior MMP-9 inhibitor; however, it was also significantly inhibited MMP-2 in the laminar explants. Flunixin meglumine also appeared to be a significant MMP-9 inhibitor *in vitro*, but not *in vivo*. These results demonstrate the variations that can be seen when tissues are isolated in the culture environment. Unfortunately, pentoxifylline was not evaluated *in vitro*. It would be interesting to determine if pentoxifylline's MMP inhibitory actions would be the same in explant culture as in the digital circulation of the horse.

MMPIs may have significant roles in the future of veterinary medicine, especially in regard to equine laminitis. The validation of a non-terminal model for the study of MMPIs in the horse has great significance. It will not only allow pre-screening of potential drugs for use in experimental CHO laminitis studies, but can also be used in the study of other equine diseases associated with increases in MMPs. The data presented here suggest that the MMP inhibitory effects of pentoxifylline and oxytetracycline should be evaluated in an experimental equine laminitis model as potential treatments/preventatives.

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VITA

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