THE ROLE OF MATRIX

METALLOPROTEINASES IN EQUINE FUNGAL KERATITIS.

by

SHANNON DENISE BOVELAND

(Under the Direction of K. Paige Carmichael)

ABSTRACT

Equine fungal keratitis (EFK) is among the most frequently reported equine ocular diseases. In humans and laboratory animals, neutrophil infiltration secondary to ocular fungal infection results in the release of matrix metalloproteinases (MMP-2 and -9) that degrade the cornea and exacerbate infection. To determine the effects of these enzymes on fungal-affected tissue, their immunohistochemical expression in purulonecrotic keratitis with and without intra-lesional fungi was examined. Expression of proteins responsible for MMP release and inhibition was also evaluated. We identified MMP-2 and 9, MIP-2, and TIMP-1 and -2 in the corneal epithelium, but not the stroma of the normal corneal samples. Fungal-affected and purulonecrotic corneal samples had all proteins expressed in increased amounts in the corneal epithelium, keratocytes, inflammatory cells, and vascular endothelial cells, with greater staining intensity in purulonecrotic samples without fungi. We conclude that MMP-2 and 9 play a significant role in the pathogenesis of equine fungal keratitis.

INDEX WORDS: Matrix Metalloproteinases, Macrophage Inflammatory Protein-2, Tissue Inhibitor of Matrix Metalloproteinase, Equine Fungal Keratitis, Neutrophil Infiltration

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B.S., Paine College, 1996

D.V.M., Tuskegee University, 2000

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial

Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2007

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August 2007

DEDICATION

This is dedicated to my beautiful daughter Alexis, my muse and my inspiration. You are my

everyday reminder that nothing is impossible. None of this would be possible without you.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION and LITERATURE REVIEW

Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes that function in the remodeling of extracellular matrix within tissues. MMPs are shown to be an initiator and indicator of progressive inflammation in ulcerative keratitis secondary to rheumatoid arthritis in humans.[1] Reports show MMP-2 and MMP-9 to be up-regulated in corneas of rabbits with fungal keratitis.[2] Studies also show a correlation between the presence of MMP-2 and MMP-9 in the tear film of horses with ulcerative keratitis and subsequent corneal damage.[3] There are no current studies showing this correlation in equine corneas infected with fungal keratitis.

Studies have been performed to investigate the mechanism of action of MMPs, MIP-2 (macrophage inflammatory protein-2) and TIMPs (tissue inhibitor of matrix metalloproteinases) in equine tissues. M. Kyaw-Tanner *et al* have investigated the role of MMPs in equine lamellae. MMP-2 and -9 have been identified immunohistochemically in equine epidermal hoof lamellae and are believed to be responsible for the separation and destruction of the lamellar basement membrane in equine laminitis.[4] Studies by Brooks *et al* involving MMPs documented the increased expression of these proteinases in the tear film of horses diagnosed with corneal ulcers.[3] Franchini et al documented the role of MIP-2 as a chemotatic agent for neutrophil infiltration in the lungs of horses diagnosed with chronic obstructive pulmonary disease (COPD) in the horse.[5]

MMPs are enzymes released from neutrophils during inflammation. In people, MMP expression has been shown to be an indicator of the degree of inflammation in ulcerative colitis.

1

[6] Collaboratively, MMPs and neutrophil infiltration are thought to be responsible for the severe melting stromal ulcers commonly associated with fungal keratitis in rabbits.[2, 7]

Macrophage inflammatory protein-2 (MIP-2) plays a major role in attracting neutrophils into the cornea, and when present these neutrophils release MMPs resulting in detrimental damage to the cornea. MIP-2 has been the target of several studies examining the infiltration of neutrophils and the detrimental effects that the proteolytic enzymes released from these neutrophils have on the cornea. [8, 9] Studies in rabbit models of fungal keratitis have shown that proteases secreted by both the fungal organism and the neutrophils results in stromal degradation. MIP-2 is a potent chemokine for neutrophil release in horses. In a study of chronic obstructive pulmonary disease in the horse, it was shown that MIP-2 was largely responsible for the infiltration of neutrophils into small bronchioles. [5] Therefore, the pathogenesis of fungal keratitis may be, in part, dependant on the presence of MIP-2. It is logical that inhibition of MIP-2 will ultimately inhibit the pathophysiologic cascade of corneal degradation.

Other studies involving bacterial and viral keratitis have documented that persistent levels of MIP-2 and related chemokines result in an increased duration and severity of neutrophil infiltration, contributing to increased corneal damage. [10] Another study examining the significance of MIP-2 in corneas demonstrated that mice normally resistant to corneal perforation and permanent corneal damage in the face of inoculation with *Pseudomonas aeruginosa* developed severe keratitis and corneal perforation when MIP-2 was administered. However, mice normally susceptible to corneal perforation secondary to *Pseudomonas* keratitis had less severe infection following challenge with *P. aeruginosa* when treated with anti-MIP-2 antibody. [8, 9]

2

TIMPs are the major endogenous regulators of MMP activity in tissue, and four homologous TIMPs have been identified to date. [11] The expression of TIMPs in tissue is controlled during tissue remodeling and physiological conditions in order to maintain a balance in the metabolism of the extracellular matrix. [12] TIMPs, in conjunction with MMPs, are important in maintaining a balance between extracellular matrix deposition and breakdown. [13, 14] Disruptions between this balance results in the potential breakdown and dissolution of the corneal stroma. This imbalance is believed to be responsible for the course of necrotizing herpes simplex virus keratitis in laboratory mice.[11] However, little is known about the expression and role of TIMPs in the prevention of corneal ulcer formation. Studies suggest that TIMP-1 protects against corneal destruction at the level of the basement membrane and functions in the resurfacing of the wounded corneal epithelium. [15]

Purpose of the Study

The purpose of this prospect is to study the immunohistochemical expression of MMP-2 and -9 in equine fungal affected corneas and to explore the correlation between MMPs, the presence of macrophage inflammatory protein-2 (MIP-2), the number of infiltrating neutrophils, and the resultant severity of disease in fungal affected corneas. We hope to gain a better understanding of the role that MMPs and MIP-2 play in the pathogenesis of equine fungal keratitis. It is anticipated that treatments may be developed to control MIP-2 activity, thereby decreasing the infiltration of neutrophils and their release of MMPs, resulting in less proteolytic activity within the diseased corneal stroma.

Expected Results

We suspect that neutrophils and MMPs play an important role in the pathogenesis of fungal keratitis. The neutrophils secrete MMPs responsible for some of the damage observed in this disease. Also, the chemokine MIP-2 is key to infiltration of neutrophils into the corneal stroma, contributing to the release of MMPs. Corneas from eyes clinically diagnosed with fungal keratitis are expected to have greater immunohistochemical expression of MIP-2 than normal corneas, and also have increased infiltrates of neutrophils with subsequent increases in MMPs. We also suspect that there will be a disproportionate distribution between the immunohistochemical expression of MMPs and TIMPs in corneas diagnosed with fungal keratitis and purulonecrotic keratitis when compared to normal corneas.

The specific aims of this study are: 1) to establish the presence and distribution of MMP, MIP-2 and TIMP proteins in normal equine corneas, 2) to determine the immunohistochemical expression and distribution of MMP-2 and -9, MIP-2 and TIMPs in both purulonecrotic corneas and corneas affected with fungal keratitis, and 3) to determine the correlation between MMP-2 and -9 expression and the expression of MIP-2 and TIMPs.

CHAPTER 2

IMMUNOHISTOCHEMICAL IDENTIFICATION OF MATRIX METALLOPROTEINASE

(MMP) -2 AND -9 IN EQUINE FUNGAL KERATITIS[1]

Introduction

Equine fungal keratitis (EFK) is frequently reported in the horse, while relatively rare in other domestic animals.[16] It is a serious and devastating disease with potentially sight–threatening consequences in this species. Despite increased awareness of owners and veterinarians, and intensive medical treatment, this condition still retains a guarded prognosis. EFK is usually preceded by a corneal defect, resulting in inoculation and infiltration of fungal hyphae into the corneal stroma, causing keratitis and deep stromal abscessation down to Descemet's membrane.[16] Abscessation is the result of a vast infiltration of neutrophils. The neutrophils, keratocytes and fungi release proteases and other enzymes including the matrix metalloproteinases (MMPs).[17]

MMPs are a group of zinc-dependent proteolytic enzymes that function in the remodeling of extracellular matrix within tissues. The proteins are produced by corneal epithelial cells, inflammatory cells (neutrophils, macrophages and lymphocytes), and vascular endothelial cells in both active and inactive forms. After injury, corneal repair is mediated by leukocytosis, fibroplasia, angiogenesis, re-epithelialization and extra cellular matrix deposition. Many of these responses are caused by or modified by MMPs and other proteinases.[18,19, 20] In humans, it has been shown that under pathologic conditions, MMP expression can be detected via zymography, western blot analysis, immunoprecipitation analysis, and gelatin affinity chromatography in inflammatory cells, epithelial cells and keratocytes after corneal damage.[21, 22] MMPs also play a

6

pathological role in the degradation of extracellular matrix components such as basement membrane collagen types IV, V, VII; the same types of collagen found in the corneal basement membrane and stroma.[23, 24] In people, MMPs-1, 2, 3 and 9 have been identified in the destruction and remodeling of collagenous connective tissues within the anterior uvea of the eye, as well as MMP 8 and MMP-13.[25] MMPs have been shown to be upregulated in ulcerative fungal keratitis in the rabbit, as well as the tear fluid in horses with ulcerative keratitis.[2, 26, 27]

The proteins responsible for the release and inhibition of MMPs play an important role in MMP activity in tissue.[8, 28] These proteins include macrophage inflammatory protein-2 (MIP-2), and tissue inhibitors of matrix metalloproteinases (TIMPs). Evidence suggests that macrophage inflammatory protein-2 (MIP-2) is one of the main chemokines responsible for the recruitment of neutrophils into the stroma, making MIP-2 an ideal focus for further research.[8] Studies have demonstrated that MIP-2 is the major chemokine responsible for attracting neutrophils in corneas infected with herpes simplex virus -1.[29, 10] This study proposes that the amount of MIP-2 detected will directly correlate to the amount of neutrophilic invasion and subsequent tissue damage seen in clinical cases of equine fungal keratitis. This study also explores the possible correlation between the immunohistochemical expression of inflammatory cytokines released by neutrophils within the corneal stroma, and the presence of matrix metalloproteinases (MMPs) in clinical fungal keratitis cases.

Tissue inhibitors of metalloproteinases (TIMPs) are important in the control of numerous physiological and pathological processes. These include tumor cell invasion, angiogenesis, degradation of joint cartilage, trophoblast implantation, mammary gland involution, and wound healing [28]. Regulation of the equilibrium between levels of active MMPs, and free TIMPs are thought to determine overall MMP activity by suppressing proteolysis and corneal invasion in

cases of keratitis. It has been reported that all active forms of MMPs are inhibited by TIMP-1 and TIMP-2.[28]

In the current study, immunohistochemical staining for MMP-2, MMP-9, MIP-2, and TIMP-1 and TIMP-2 was performed on normal and diseased corneal sections. The purpose of this study was to establish a correlation between these proteins in equine keratitis, with and without fungi.

Experimental Methods and Design

Tissue preparation and processing:

The tissues used in this study were obtained from archived histopathology samples from the Department of Pathology at the University of Georgia dating between 2003- 2006. Fungal corneal samples were selected based on ophthalmic examination, positive fungal culture, and histopathologic identification of fungal organisms with both routine hematoxylin and eosin and specific fungal staining. Purulonecrotic samples were selected based on ophthalmic examination, histopathologic evaluation, and negative fungal and bacterial cultures. Normal corneal tissues were used as a negative control. These samples were selected based on normal ophthalmic examinations and normal histopathologic identification. Total sample numbers included 26 fungal infected corneas, 41 purulonecrotic corneas, and 9 normal cornea samples. Immunohistochemical staining for MMP-2, MMP-9, MIP-2, TIMP-1 and TIMP-2 was performed on all corneal sections.

<u>MMP-2, MMP-9, MIP-2, TIMP-1 and TIMP-2 Immunohistochemical staining:</u>

Immunohistochemical staining for each protein was performed all corneal types based on similar methods described by other studies. [6, 42] Formalin fixed, paraffin embedded tissue blocks were sectioned at 5μm. The tissue sections were mounted on Superfrost / Plus microscope slides (Fisher Scientific, Pittsburg, PA). Sections were dried in an oven at 55º C for 30 minutes. The sections were routinely deparaffinized and rehydrated in a graded series of alcohol, ending in tap water. Epitope enhancement was performed by submerging slides in 10mM sodium citrate buffer pH 6.0. Buffer was heated in a microwave for 5 minute intervals three times. During this process the temperature was maintained between 95-100º C for a total of 30 minutes. Endogenous peroxidase activity was blocked by incubating the sections in aqueous 3% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) for 20 minutes. To prevent nonspecific binding, corneal samples were incubated with 150μl of normal rabbit serum diluted in 10mls of phosphate buffered solution {PBS- Sigma-Aldrich, St. Louis, MO}, pH 7.4) for 8 hours at room temperature. Corneal samples were incubated overnight at 4º C with the primary polyclonal goat antibodies MMP-2, MMP-9, MIP-2, TIMP-1 and TIMP-2 (R&D Systems, Minneapolis, MN) in a humidified chamber. Dilution for antibodies MMP-2, MMP-9, TIMP-1 and TIMP-2 were 1:18 in blocking solution. MIP-2 antibody (R&D Systems, Minneapolis, MN) dilution was 1:7 in blocking solution.

Sections were incubated with diluted biotinylated secondary antibody solution (VECTASTAIN® Elite ABC Kit Goat IgG, Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature in a humidified chamber. Sections were incubated with VECTASTAIN® Elite ABC reagent (Vector Laboratories, Burlingame, CA) for 30 minutes. The

tissue sections were incubated in peroxidase substrate solution (DAB/Ni substrate kit, Vector Laboratories, Burlingame, CA) for 2 minutes. Nuclei were counterstained with Meyer's Hematoxylin (Sigma-Aldrich, St. Louis, MO) for 5 minutes, and blued in running tap water for 5 minutes. The slides were routinely dehydrated, cleared and permanently mounted with Permount media (Fisher Scientific, Suwanee, GA) and covered with glass coverslips (Fisher Scientific, Suwanee, GA). Controls included omitting the 1º antibody and substituting with naïve goat serum (1:100 dilutions) or PBS.

Immunohistochemical staining was scored quantitatively in 5 high-power fields. The number of inflammatory cells was counted in each high power field. Staining characteristics were documented in the corneal epithelium, keratocytes and vascular endothelial cells.

Statistical Analysis:

MIP-2, MMP-2 and MMP-9 and TIMP-1 and TIMP-2 immunolocalization in corneal samples were statistically analyzed using SAS V 9.1 (Cary, NC). The number of immunoreactive inflammatory cells were compared between purulonecrotic and fungal corneal samples, and normal control corneas by chi-square tests ($\alpha=0.05$). The number of immunoreactive inflammatory cells were compared between purulonecrotic and fungal corneas by a student's t-test ($\alpha=0.05$). A log(X+1) transformation was used for MIP-2, MMP-9 and TIMP-2 inflammatory cell numbers to equalize unequal variances. The number of immunoreactive inflammatory cells was compared between proteins for each cornea type (purulonecrotic or fungal infected) separately using a repeated measures analysis of variance to account for multiple measurements being made on each corneal tissue sample. An unstructured covariance structure was used in the repeated measures model. Hypothesis tests were 2-sided and the significance

level was $\alpha = 0.05$. The repeated measures analysis was performed using PROC MIXED in SAS. Multiple comparisons were adjusted for using Tukey's test. Linear correlations were used to test for a correlation between immunoreactive inflammatory cell numbers for MIP-2 and both MMP-2 and MMP-9, as well as TIMP-1 and TIMP-2 for each corneal type separately. All analyses were performed using SAS version 9.1.

Results

In the normal corneal samples, immunoreactivity to all proteins was located in the corneal epithelium of all samples. Immunolocalization in the normal cornea for MMP-2 revealed diffuse immunoreactivity throughout the epithelium with a mild immunolocalization within the basal epithelium (Fig. 1). Immunoreactivity of the same corneal samples for MMP-9 showed strong intensity in the basal epithelium (Fig. 2). Immunohistochemical staining of the normal cornea for MIP-2 revealed similar staining characteristics as MMP-9 with strong immunolocalization in the mid and basal epithelial layers of cornea (Fig. 3). Immunoreactivity for TIMP-1 and -2 was present in the corneal epithelium, with moderate to strong staining of the basal epithelium (Figs. 4 and 5). There was slight to no immunoreactivity to any of the proteins in the corneal stromal, posterior epithelial (endothelium) layers, or corneal blood vascular endothelium.

Overall, the purulonecrotic corneal samples displayed immunohistochemical staining of the corneal epithelium, inflammatory cells and vascular endothelial cells with all antibodies. Immunohistochemical staining of the purulonecrotic samples for MMP-2 disclosed moderate immunoreactivity of the corneal epithelial cells, with strong staining of neutrophils, lymphocytes, keratocytes and vascular endothelial cells (Fig. 6). Immunohistochemical staining

11

of the same corneal samples for MMP-9 revealed moderate to strong immunoreactivity of the corneal epithelium, neutrophils, macrophages, vascular endothelial cells, and keratocytes (Fig. 7). MIP-2 immunoreactivity in the purulonecrotic samples was strong in the corneal epithelium with greater intensity in the basal epithelium, and included strong staining of keratocytes, lymphocytes, macrophages, neutrophils and vascular endothelial cells (Fig. 8). Purulonecrotic samples incubated with TIMP-1 antibodies revealed moderate immunoreactivity throughout the corneal epithelium, with strong immunolocalization in neutrophils, macrophages and keratocytes, and mild staining of vascular endothelial cells (Fig. 9). TIMP-2 immunoreactivity was slight in the corneal epithelium and moderate in lymphocytes, neutrophils and collagen fibers Fig. 10).

Overall, fungal corneal samples revealed strong corneal epithelial immunoreactivity when the epithelium was present. MMP-2 immunoreactivity of the same corneal samples revealed strong staining around the fungal organisms, with mild to moderate staining of neutrophils, vascular endothelial cells, and keratocytes. Slight staining of the collagen stroma was also observed, with no staining of degenerative neutrophils (Fig. 11). Fungal organisms, neutrophils, vascular endothelial cells, and keratocytes, had moderate MMP-9 immunoreactivity with slight staining of the collagen stroma, and no staining of degenerative neutrophils (Fig. 12). Fungal organisms and stromal collagen had only slight MIP-2 immunoreactivity with no staining of inflammatory cells (Fig. 13). Fungal corneal samples had moderate to strong TIMP-1 immunoreactivity in the corneal stroma, vascular endothelial cells, keratocytes and fungal organisms (Fig. 14). TIMP-2 immunoreactivity was marked in fungal organisms with mild staining of stromal collagen and strong staining of neutrophils, macrophages, keratocytes and vascular endothelial cells (Fig. 15).

<u>Statistical Analysis/Correlation of MMP-2 and -9 and TIMP-1 and -2:</u>

The numbers of positively stained inflammatory cells were statistically different for the purulonecrotic and fungal infected corneas when compared to the normal cornea for MMP-2, MMP-9, MIP-2, TIMP-1 and TIMP-2 ($p<0.0005$). The number of immunoreactive inflammatory cells was significantly higher in the purulonecrotic versus fungal infected corneas for MMP-2, MIP-2, and TIMP-1; but not MMP-9 and TIMP-2 (Table 1).

Multiple comparisons showed that the number of immunoreactive inflammatory cells was significantly different between proteins overall for the fungal infected and purulonecrotic corneal samples ($p<0.0045$). Multiple comparisons showed that the number of immunoreactive inflammatory cells for MIP-2 was significantly less than compared to TIMP-1. The number of immunoreactive inflammatory cells was significantly greater for MMP-2 than the number of immunoreactive inflammatory cells for MIP-2 and TIMP-2. Also, the number of immunoreactive inflammatory cells was significantly greater for TIMP-1 than compared to MIP-2, MMP-9 and TIMP-2 for purulonecrotic corneal samples (Table 2).

Finally, there was a linear correlation of the number of immunoreactive inflammatory cells between MMP-2 and MIP-2; MMP-9 and MIP-2; and MMP-9 and TIMP-1 in the fungal infected samples. There was a linear correlation of the number of immunoreactive inflammatory cells between MMP-9 and MIP-2; MMP-9 and TIMP-2; MIP-2 and TIMP-2; and MIP-2 and TIMP-1 for the purulonecrotic tissue samples (Table 3).

<u>Discussion</u>

This study detected immunohistologically, the presence of MIP-2, MMP-2, MMP-9, TIMP-1 and TIMP-2 in equine corneal samples. Currently, there are limited published reports documenting the immunohistochemical identification of these proteins in equine corneal samples. In this study we identified MMP-2 and MMP-9 in the normal corneal epithelium, with greater expression in the basal cell layer. No expression was seen in the stroma of normal corneas, with only slight to no detection in the basement membrane. Previous published reports have identified the proenzyme (inactive) form of MMP-2 and MMP-9 via Western blot in the normal cornea of laboratory animals.[11] In a study comparing MMP-2 and –9 in the tear fluid of corneal grafted individuals and individuals with vernal keratoconjunctivitis compared to healthy unaffected individuals, the proenzyme but not the active enzyme was detected in healthy samples.[30, 31] The immunolocalization of MMP-2 and -9 in the normal equine corneas could be a representation of the inactive form of these enzymes.

Immunohistochemical staining of normal equine corneas for MIP-2, TIMP-1 and TIMP-2 revealed similar findings of moderate to strong staining of the corneal epithelium with the strongest intensity in the basal cell layer and slight to no staining of the corneal stoma. Similar findings were documented in a study where the role of TIMP-1 was investigated in the corneas of laboratory mice infected with *Pseudomonas*.[15] Immunohistochemical staining of the normal corneas resulted in staining of the epithelium, basement membrane, stroma and endothelium, with the greatest intensity in the basal cell layer. The increase in staining intensity of the basal cell layer in the normal equine corneal epithelium supports similar findings in laboratory mice, where TIMP-1 presence was found to serve as a protective mechanism for the regeneration of

corneal epithelium in the presence of corneal ulceration.[15] The role behind the

immunolocalization of MIP-2 in the normal cornea remains to be determined. The

immunoreactivity of this antibody in the normal epithelium, including the basal cell layer,

could be an indication that this protein is constitutively present in low levels but increases

during inflammation.

In the inflamed equine corneal samples (purulonecrotic and fungal infected), MMP-2 and

-9 expression were similar; they were present throughout the corneal epithelium, instead of

predominately in the basal cell layers. In addition, inflamed corneas had MMP-2 and -9

immunoreactivity within keratocytes and vascular endothelium, as well as infiltrating

inflammatory cells (neutrophils and macrophages), with varying intensities between samples

within the corneal groups, but not between corneal groups or metalloproteinases.

Correlations between the presence of MMP-2 and MMP-9 in the tear films of horses with

ulcerative keratitis and subsequent corneal damage have been shown.[26] Strubbe *et al* showed that

MMP-2 and MMP-9 were found in significantly higher concentrations in the tear film of

ulcerated horse eyes versus normal controls.[26] The proenzyme and active forms of both MMP-2

and MMP-9 have been observed in the tear fluid of people with vernal keratoconjunctivitis, and

in the corneal epithelium of dogs with refractory superficial ulcers.[31, 32]

In human patients with pseudophakic corneal edema and ulcerated corneas,

immunohistochemical expression of MMP-9 and MMP-2 was significantly higher in the corneal

epithelial basal cell layer and epithelia-stromal boundary respectively when compared to the

normal control subjects.[33, 34] Results of other human studies involving UV-B irradiation and

MMP production have documented increased immunolocalization of MMP-2 in corneal stromal

fibroblasts as a result of UV-B exposure.[35] Reports have shown MMP-2 and MMP-9 to be upregulated in the cornea of other species such as rabbits with fungal keratitis.[7]

In our study, corneal ulceration with subsequent inflammation resulted in increased immunoreactivity of MMP-2 and MMP-9 involving corneal epithelium, corneal stroma, including keratocytes, inflammatory cells (neutrophils, plasma cells and macrophages) and vascular endothelial cells. The expression of MMP-2 and –9 in the ulcerated cornea is the result of cell migration and re-epithelialization in an attempt to heal a corneal wound. The expression of MMPs within neutrophils and other inflammatory cells support that these inflammatory cells are a contributing factor to corneal damage associated with purulonecrotic and fungal keratitis.

There was no statistical significance in the expression of inflammatory cells between the purulonecrotic and fungal infected samples. The immunohistochemical identification of MMP-2 and MMP-9 in the equine purulonecrotic and fungal keratitis samples concurs with reports documenting the expression of MMP-2 and MMP-9 by corneal epithelial cells and fibroblasts as well as neutrophils, monocytes/macrophages and eosinophils in wounded corneas and neoplasias respectively .[30, 36]

The immunolocalization of MMPs in the vascular endothelial cells is a direct result of the inflammatory process. Vascular endothelial cells from limbal venules are stimulated by vascular endothelial growth factor and other angiogenic factors to produce active MMP-2 and MMP-9, which function to digest surrounding endothelial cell basement membrane and corneal tissue extracellular matrix.[13]

In our study, there was no immunoreactivity in degenerative neutrophils of the fungal corneal samples. This could be due to decreased proteinase release or decreased activity of neutrophils through inhibition by the fungal organisms. *Aspergillus fumigatus* has been

documented to induce suppression of human alveolar macrophages and polymorphonuclear leukocytes.[37]   It is therefore possible that the decreased expression of MMP-2 and -9 in the fungal infected corneas could be the result of fungal inhibition of leukocytes. Similar results were noted in a study by Strubbe *et al* where median MMP-2 and neutrophil elastase levels were decreased in tear film samples where bacteria and/or fungal organisms were present when compared to "sterile" ulcers.[26] In addition, the decrease in immunolocalization of MMP-2 and -9 in degenerative neutrophils may be due to the release of the enzymes from the neutrophils into the corneal stroma. MMP-3 and MMP-9 were found to be released by eosinophils in lung tissue of asthma patients and skin biopsies of patients with untreated bullous pemphigoid. [38, 39]

In sections where fungal organisms were observed histologically, there was immunohistochemical staining of the organisms for both MMP-2 and MMP-9. Staining may result from the release of these proteinases from the organisms themselves, or their cell walls may have been outlined as a result of the release of MMPs from the inflammatory cells and keratocytes into the stroma.[38, 39] The former theory is supported by several findings indicating that bacterial and fungal pathogens produce MMPs and also induce corneal epithelial cells, corneal stromal fibroblasts, and neutrophils in the tear film to upregulate cytokines and induce MMP production and leukocyte infiltration, thus eliciting inflammatory, angiogenic and destructive processes.[2,13, 18, 19, 20, 40, 41, 42,]

Immunolocalization of MIP-2 in the corneal epithelium with greater emphasis in the basal cell layer of the normal equine corneas is similar to the immunohistochemical staining pattern MMPs and TIMPs. The reason behind this finding is unknown, as there are no known published reports citing the identification of MIP-2 in normal corneal samples of any species. It is possible that the immunoreactivity identified in the epithelium, especially the basal cell layer

in the normal cornea could be present in relatively low levels, then become upregulated in the face of inflammation, recruiting neutrophils to the designated area.

Studies have reported MIP-2 to be the main chemokine responsible for neutrophil recruitment and activation in keratitis associated with *Pseudomonas aeruginosa* in mice.[8, 42, 43, 44] MIP-2 has also been detected in corneal epithelial and endothelial cells, as well as keratocytes. [45] Similar results were observed in the current study. These findings support the statement that MIP-2 is an important recruiter for neutrophil infiltration in equine purulonecrotic and fungal keratitis cases.

Statistical analysis revealed a statistical significance between the presence of neutrophils as well as other inflammatory cells and the presence of immunohistochemical staining for MIP-2 in the purulonecrotic keratitis cases, supporting the claim that MIP-2 is a chemoattractant for neutrophils in equine keratitis cases.

The immunoreactivity of TIMP-1 and TIMP-2 in conjunction with MMP-2 and -9 in normal equine corneal epithelium supports the idea that endogenous proteinases normally exist in balance with inhibitory factors (TIMP), and that the proenzyme of MMP is bound and inactivated by the TIMP that is present in tissue which serves to prevent excessive degradation of normal healthy tissue.[22, 46] Also, TIMPs are important in maintaining a balance between extracellular matrix deposition and breakdown.[13, 14]

The results of this study indicate that MMPs, MIP-2 and TIMPs are proteins present in the normal intact equine corneal epithelium, with increased intensity within the basal epithelium. There was no immunoreactivity of other cells in the normal equine cornea for MMPs, MIP-2 and TIMPS. The identification of these proteins within the intact normal cornea support other studies

stating that all but MMP-9 are present in an inactive state, [13, 19, 22, 45] however, this is the first study identifying the presence of MMP-9 in the normal noninjured cornea of any species.

Statistically, the purulonecrotic and fungal keratitis cases both revealed a greater expression of MMPs and MIP-2, and a greater neutrophil infiltration compared to the normal corneal samples. However, MMP-2, MIP-2 and TIMP-1 were expressed to a greater degree in the purulonecrotic keratitis cases. The greater expression of these three proteins in the purulonecrotic samples versus the fungal affected could be based on inhibition of leukocytes from the fungal organisms. This inhibition could have resulted in a decreased infiltration, and a decreased expression of MMP-2 and -9 in the neutrophils within the fungal corneal samples, resulting in decreased MIP-2 expression.

Our hypothesis stated that because MMPs would be up-regulated in response to inflammation within the cornea, TIMP would be decreased, resulting in the damage that is observed histologically. The decreased immunoreactivity of inflammatory cells for TIMP-2 in the purulonecrotic corneal samples is similar to previous studies documenting that TIMP levels are lower in nonhealing chronic ulcers, and bullous pemphigoid skin lesions.[13, 45, 47] Statistically, the immunoreactivity was significantly greater for TIMP-1 in inflammatory cells for the fungal and purulonecrotic corneal samples but not TIMP-2. Based on this, it is speculated that during inflammation TIMP-1 became upregulated. Other studies have documented similar findings of upregulation of TIMP-1 related to increased activity and production of MMP-9 in patients with cardiopulmonary bypass.[48]

An imbalance between proteinases and proteinase inhibitor levels due to excessive levels of proteinases can cause pathologic degradation of corneal stromal collagen and proteoglycans. [49, 50, 51] Immunolocalization of purulonecrotic samples for TIMP-1 and TIMP-2 revealed staining

of the corneal epithelium, stroma/keratocytes, as well as vascular endothelial cells and inflammatory cells (neutrophils, plasma cells and macrophages) with varying intensities. Adequate amounts of TIMP in infected corneas protect degradation of newly formed basement membrane following corneal injury.[15] The presence of intact basement membrane permits the resurfacing of the epithelium.[15] Increased TIMP-1 immunoreactivity may have a protective effect by inhibiting epithelial-derived MMP-9, which inhibits the rate of wound closure, by degrading the newly synthesized basement membrane components upon which the wounded epithelium migrates.[52] This may explain why the immunoreactivity of TIMP-1 was significant in the fungal and purulonecrotic corneal samples.

Statistical analysis identified a positive correlation for inflammatory cells between MIP-2 and both MMP-2 and MMP-9 in the fungal affected corneas, as well as between MIP-2 and MMP-9 in the purulonecrotic corneas. The correlation between both MMP-2 and MMP-9 with MIP-2 in the fungal affected samples supports the hypothesis that MIP-2 is responsible for MMP immunoreactivity via the recruitment of neutrophils, as well as macrophages and lymphocytes, contributing to the extensive damage observed in equine fungal keratitis.

Statistical analysis for a linear correlation of the inflammatory cells revealed a correlation between MMP-9 and TIMP-1 in the fungal affected samples, and a correlation between MMP-9 and TIMP-2 as well as between MIP-2 and TIMP-2; and MIP-2 and TIMP-1 in the purulonecrotic samples. The correlation between MMP-9 and TIMP-1 in the fungal affected samples supports other studies documenting that TIMP-1 has a high affinity for MMP-9. However, the active forms of MMP are equally inhibited by both TIMP-1 and -2, which could explain the correlation between MMP-9 and TIMP-2 in the purulonecrotic samples.[30]

This study also identified a correlation between MIP-2 and TIMP-1 and TIMP-2 in the fungal and purulonecrotic samples. This finding could correspond with the correlation between MIP-2 and MMP-2 and MMP-9 and that TIMP expression increases directly with MMP expression during inflammation.

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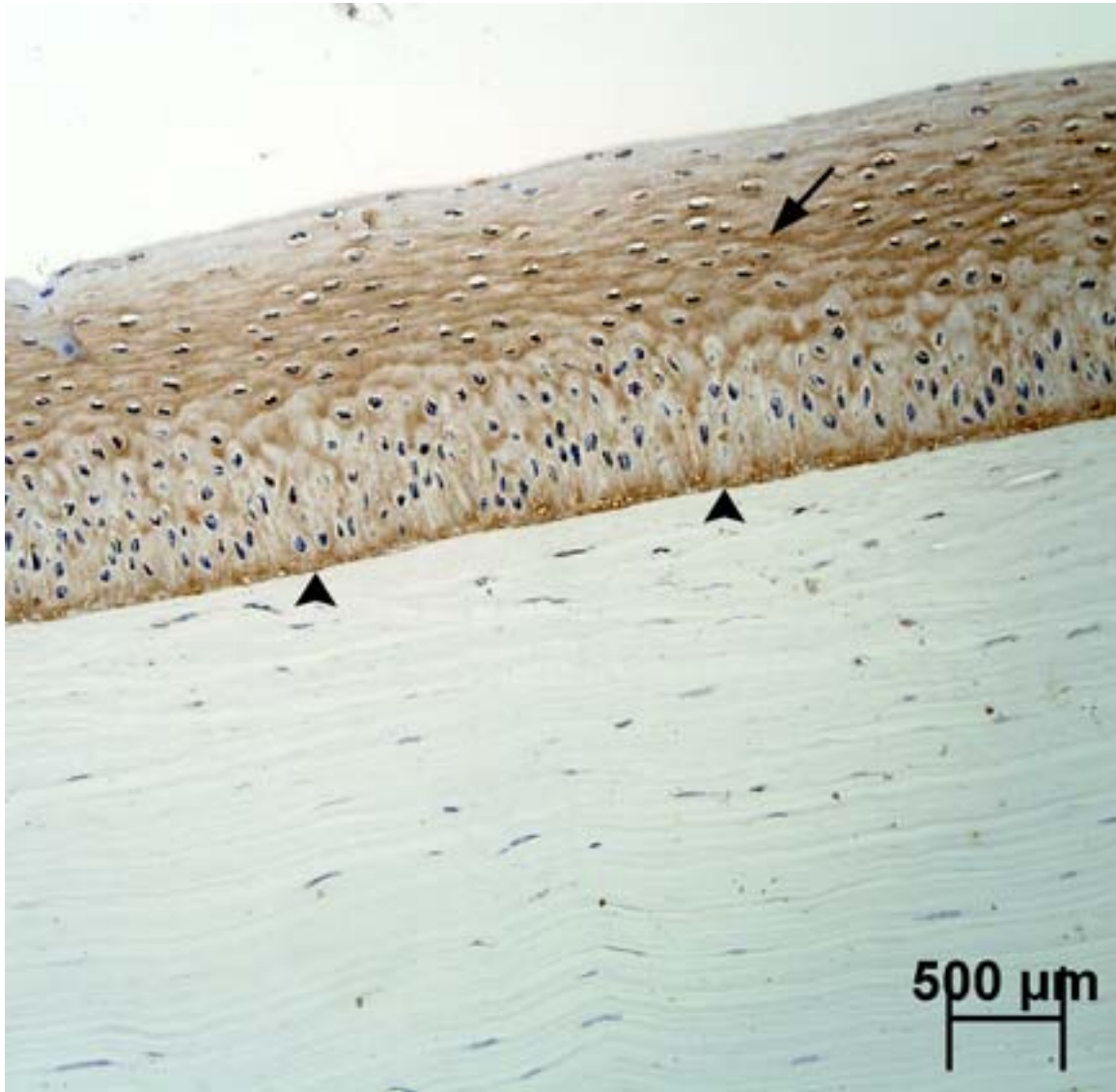
Figure 1: Photomicrograph of normal equine cornea immunohistochemically stained for MMP-2. Moderate staining throughout the corneal epithelium (arrow), with emphasis in the basal lamina (arrowheads).
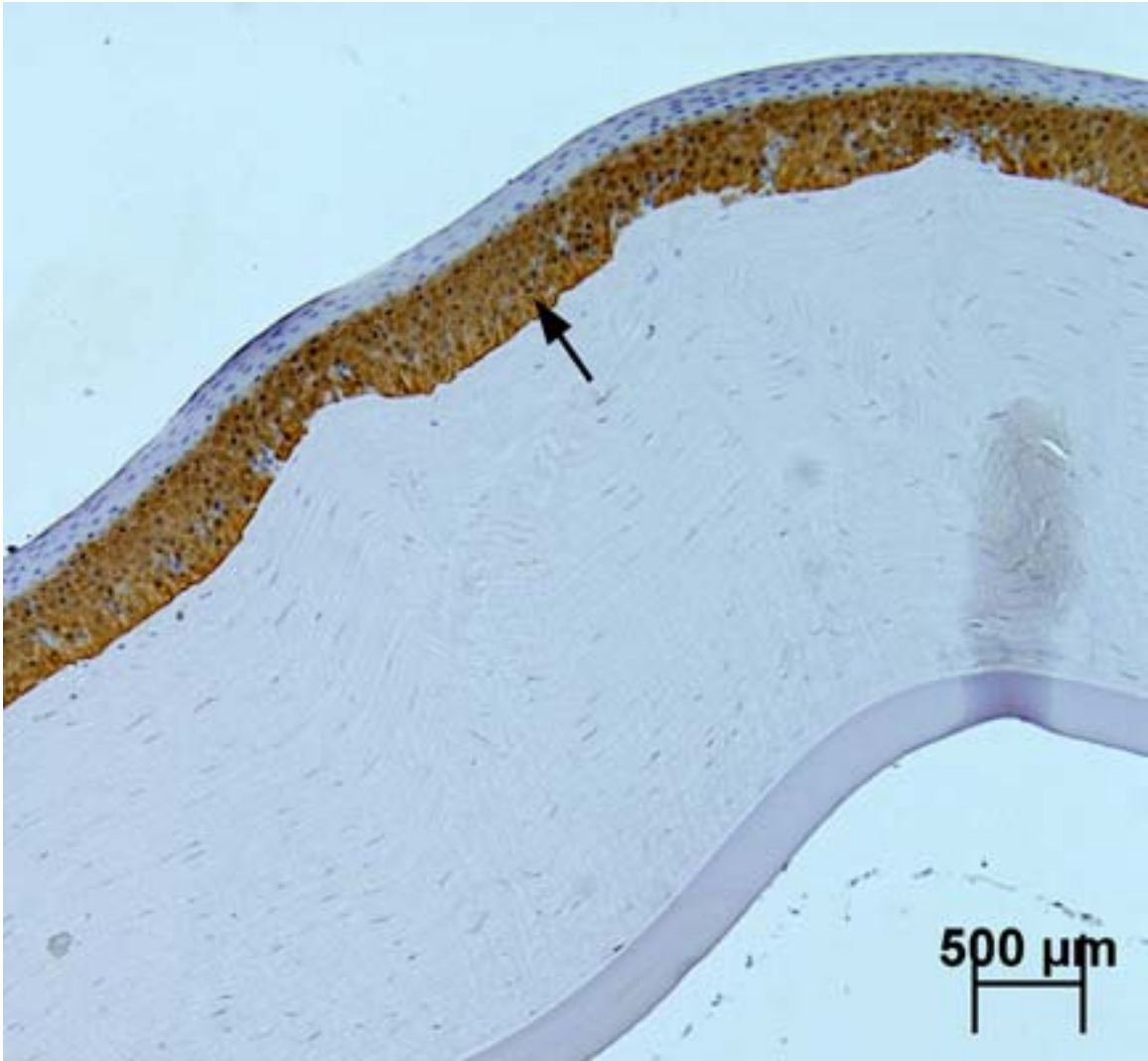
Figure 2: Photomicrograph of a normal equine cornea immunohistochemically stained for MMP-9. Strong intensity of basal corneal epithelium (arrow).
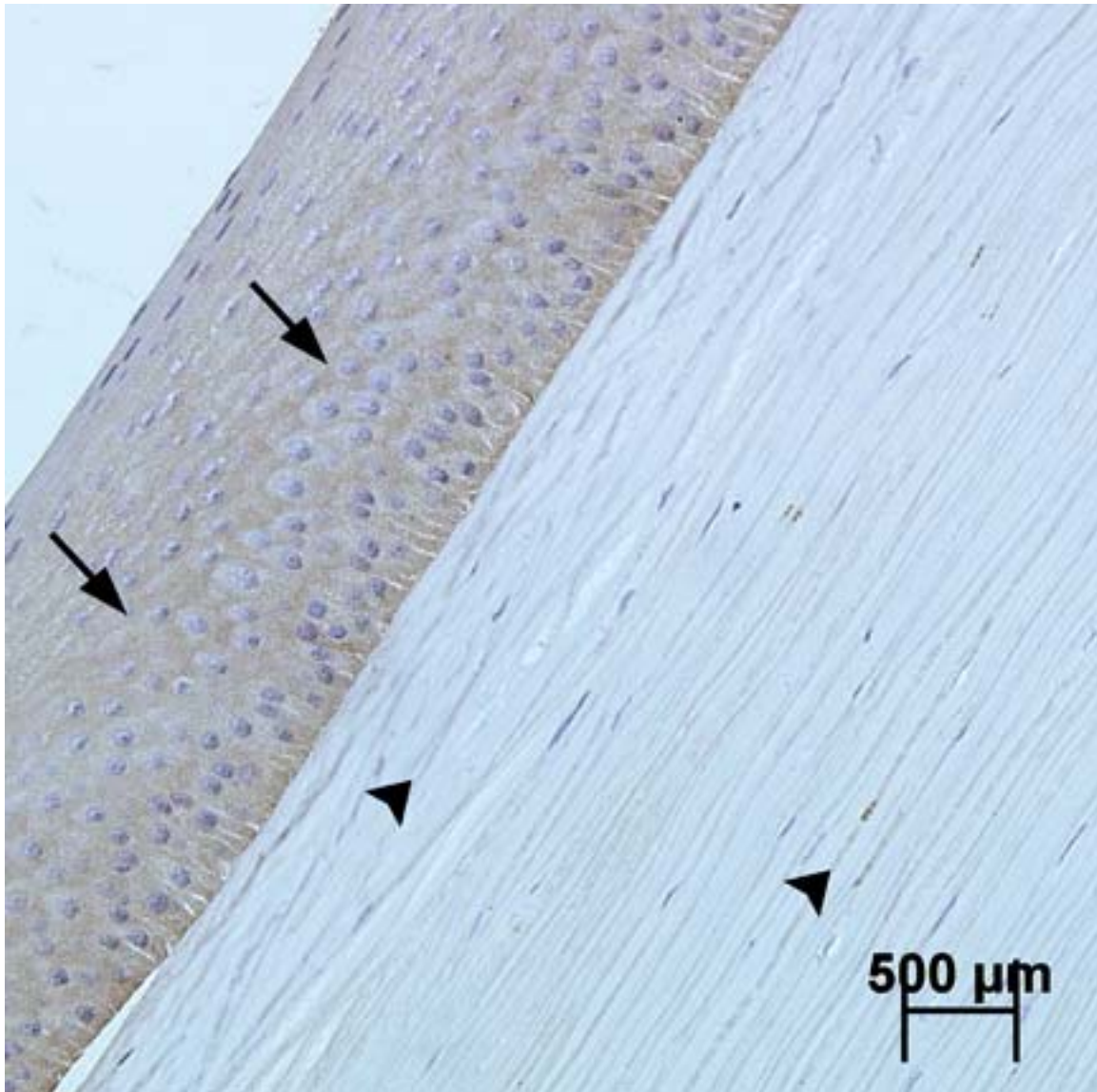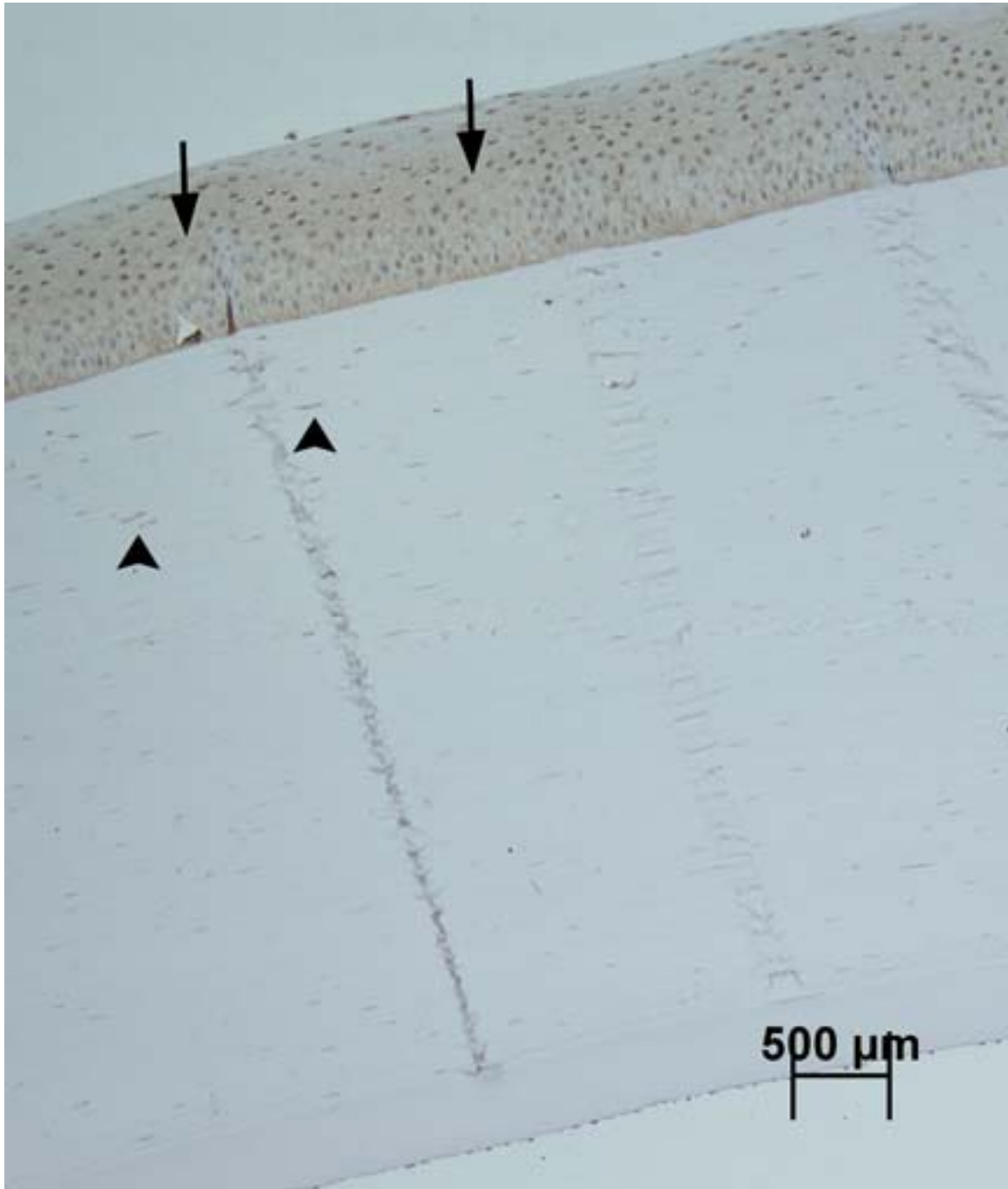
Figure 3: Photomicrograph of normal equine cornea immunohistochemically stained for MIP-2. Mild staining throughout the epithelium (arrow) and stromal fibers (arrowheads).

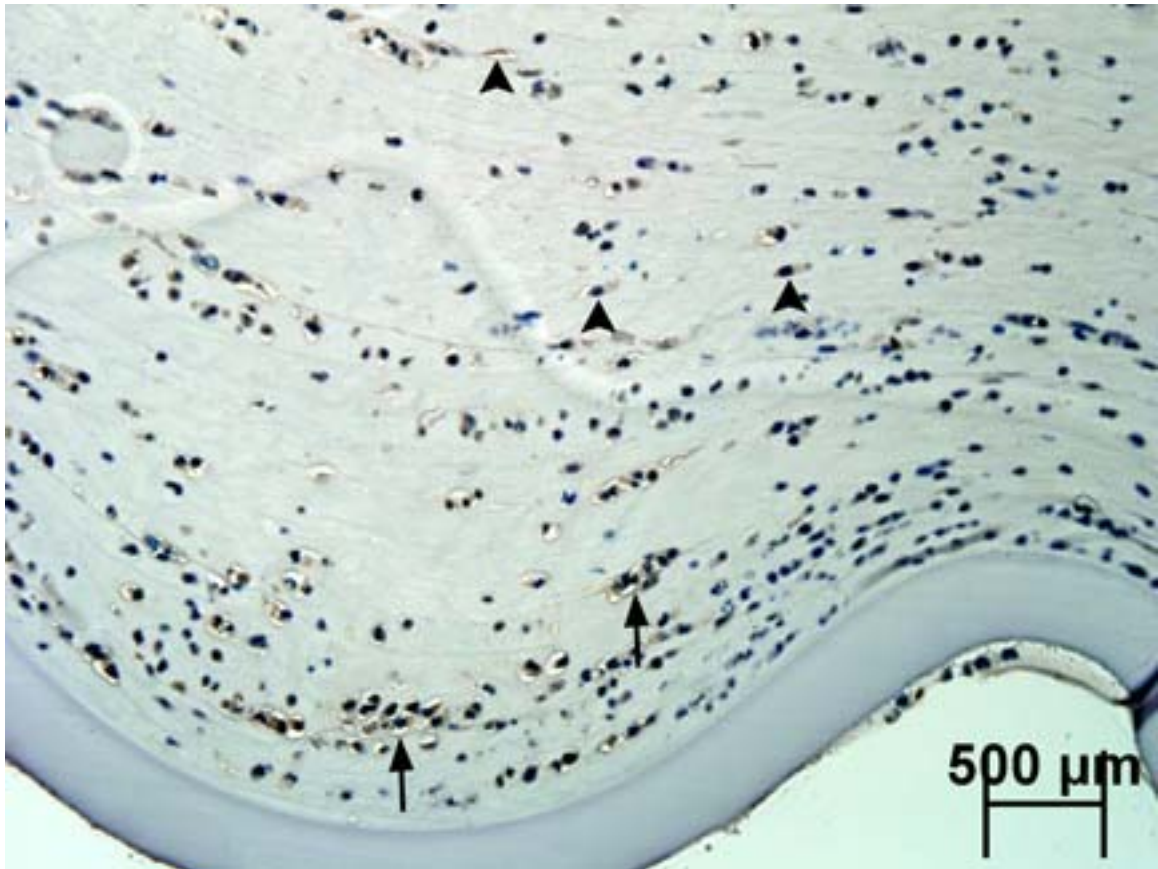Figure 4: Photomicrograph of normal equine cornea immunohistochemically stained for TIMP-1. Strong staining throughout the epithelium (arrows), mild staining of keratocytes (arrowheads).
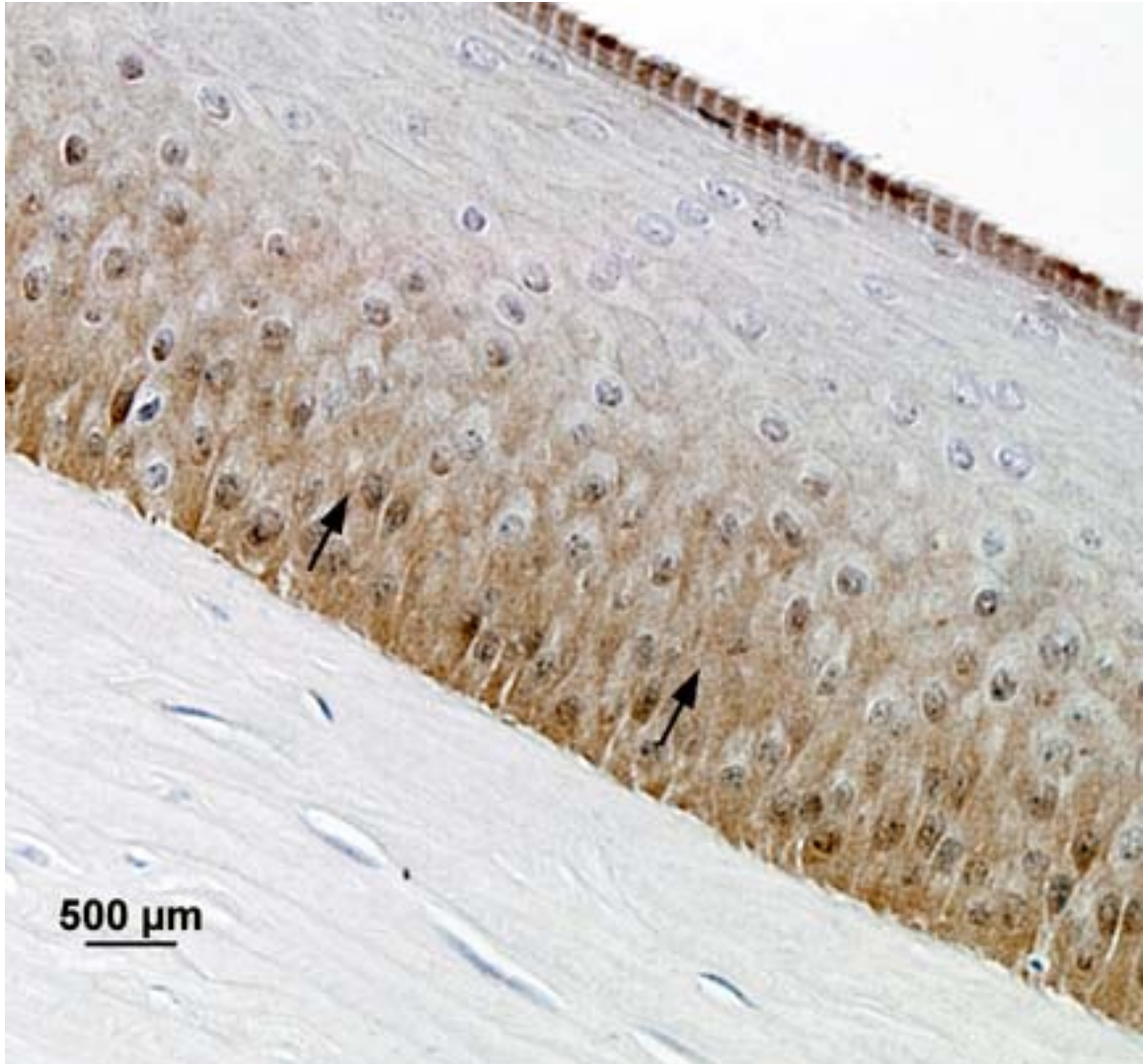
Figure 5: Photomicrograph of normal equine cornea immunohistochemically stained for TIMP-2. Strong staining of the basal epithelium (arrows).

Figure 6: Photomicrograph of purulonecrotic equine cornea immunohistochemically stained for MMP-2. Strong staining of inflammatory cells (arrows) and keratocytes (arrowheads).
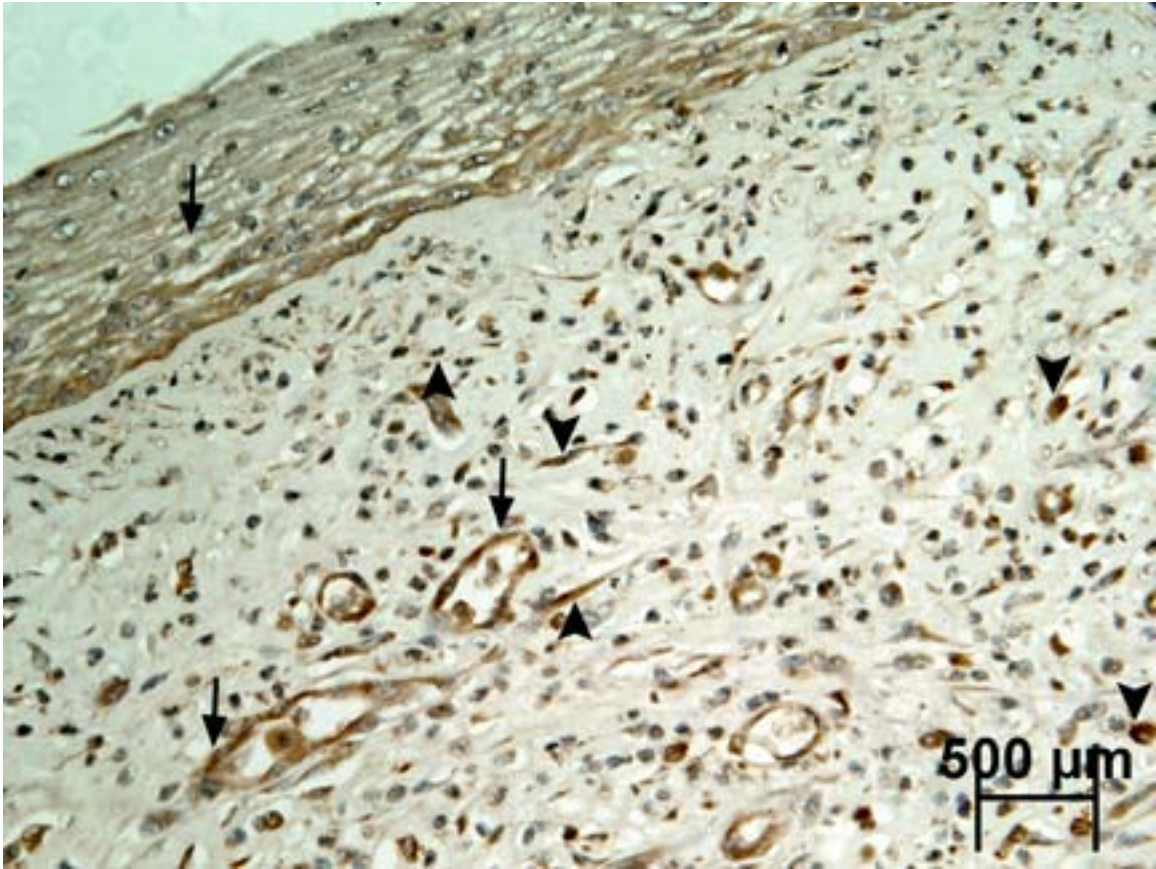
Figure 7: Photomicrograph of purulonecrotic equine cornea immunohistochemically stained for MMP-9. Strong staining of inflammatory cells and keratocytes (arrowheads). Strong staining of corneal epithelium and vascular endothelial cells (arrows).
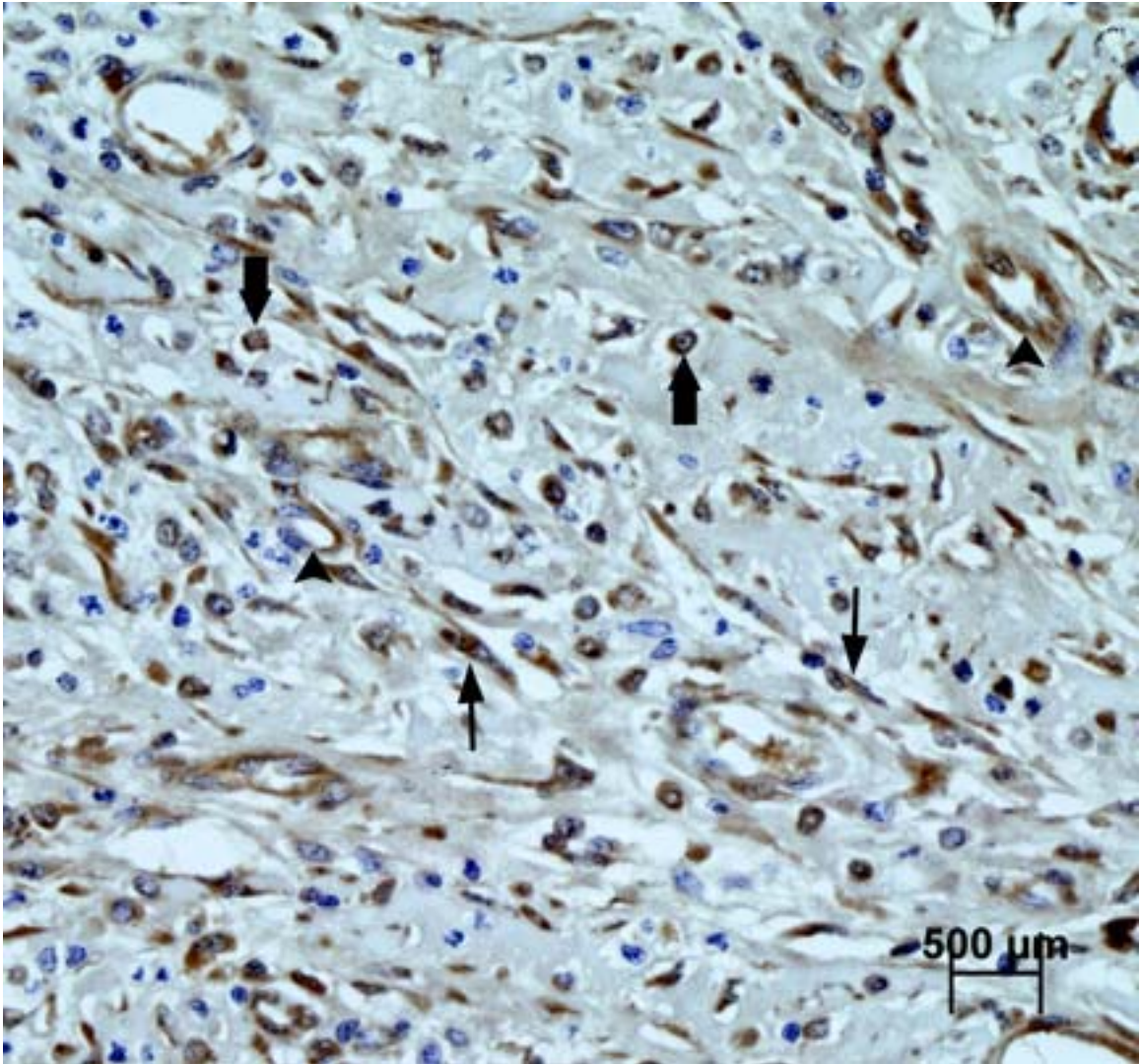
Figure 8: Photomicrograph of purulonecrotic equine cornea immunohistochemically stained for MIP-2. Strong staining of inflammatory cells (fat arrows), keratocytes (arrows) and vascular endothelial cells (arrowheads).
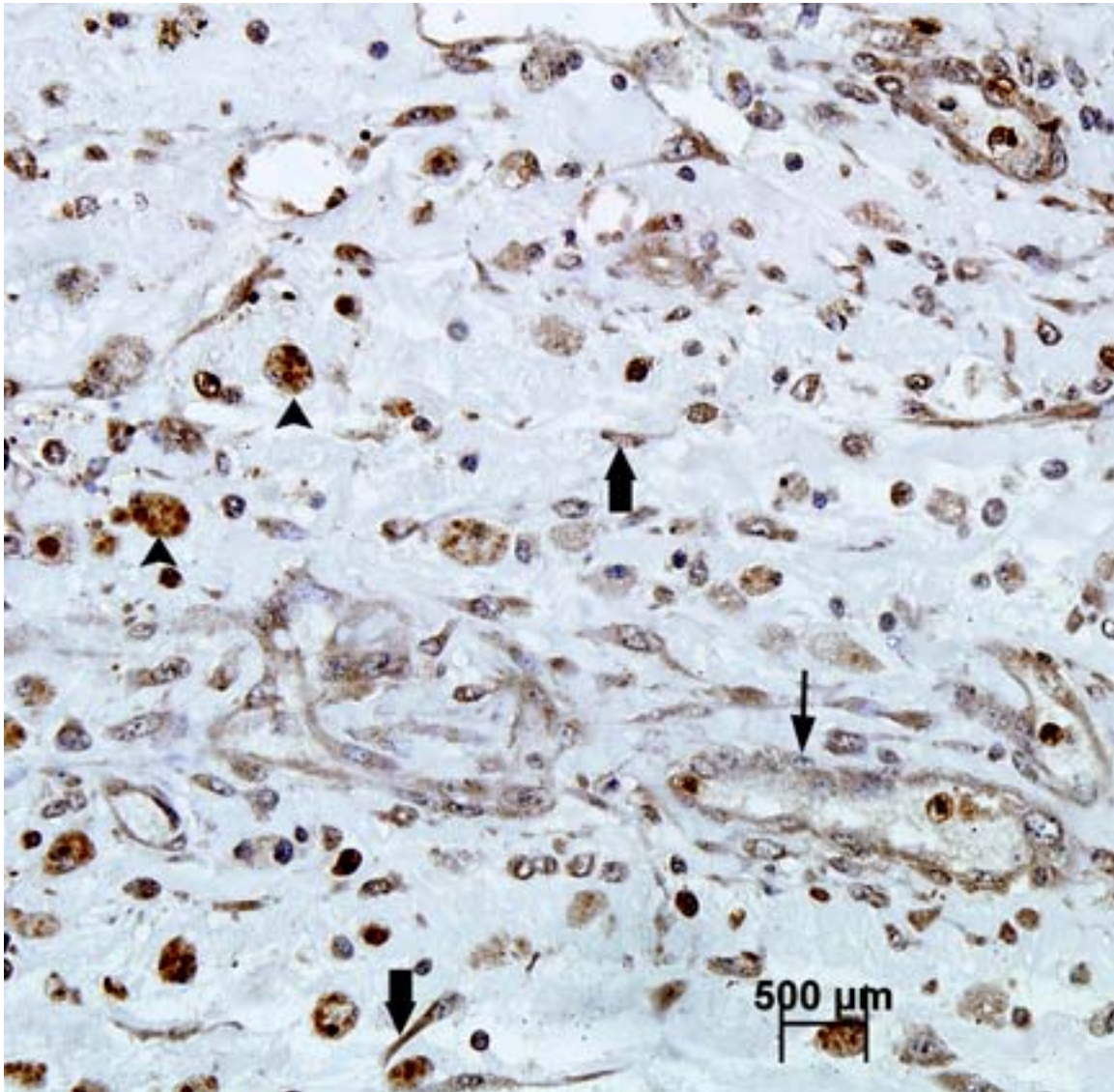
Figure 9: Photomicrograph of purulonecrotic equine cornea immunohistochemically stained for TIMP-1. Strong staining of inflammatory cells (arrowheads), keratocytes (fat arrows), with moderate staining of vascular endothelial cells (arrow).
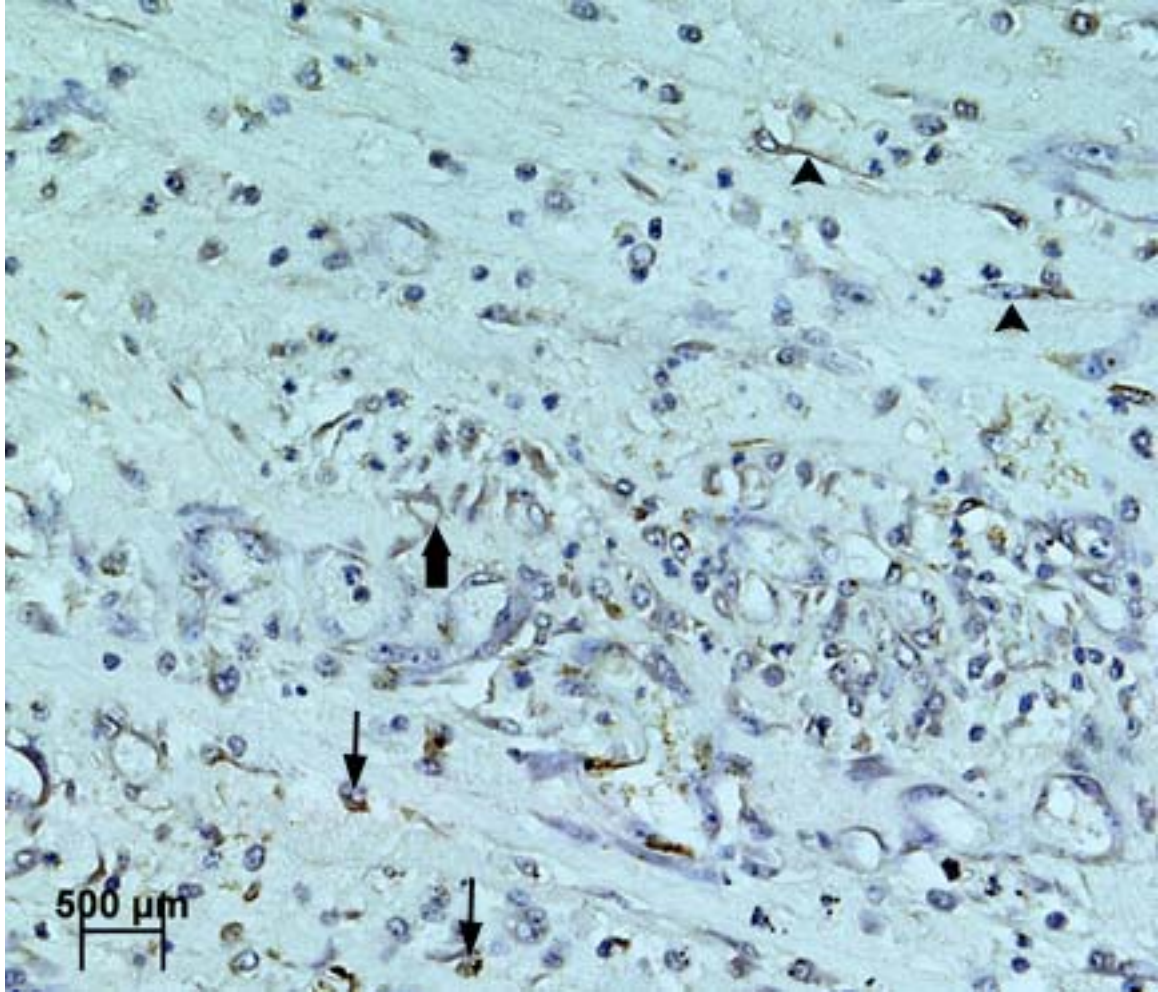
Figure 10: Photomicrograph of purulonecrotic equine cornea immunohistochemically stained for TIMP-2. Mild staining of inflammatory cells (arrows) and vascular endothelial cells (fat arrow), with moderate staining of keratocytes (arrowheads).
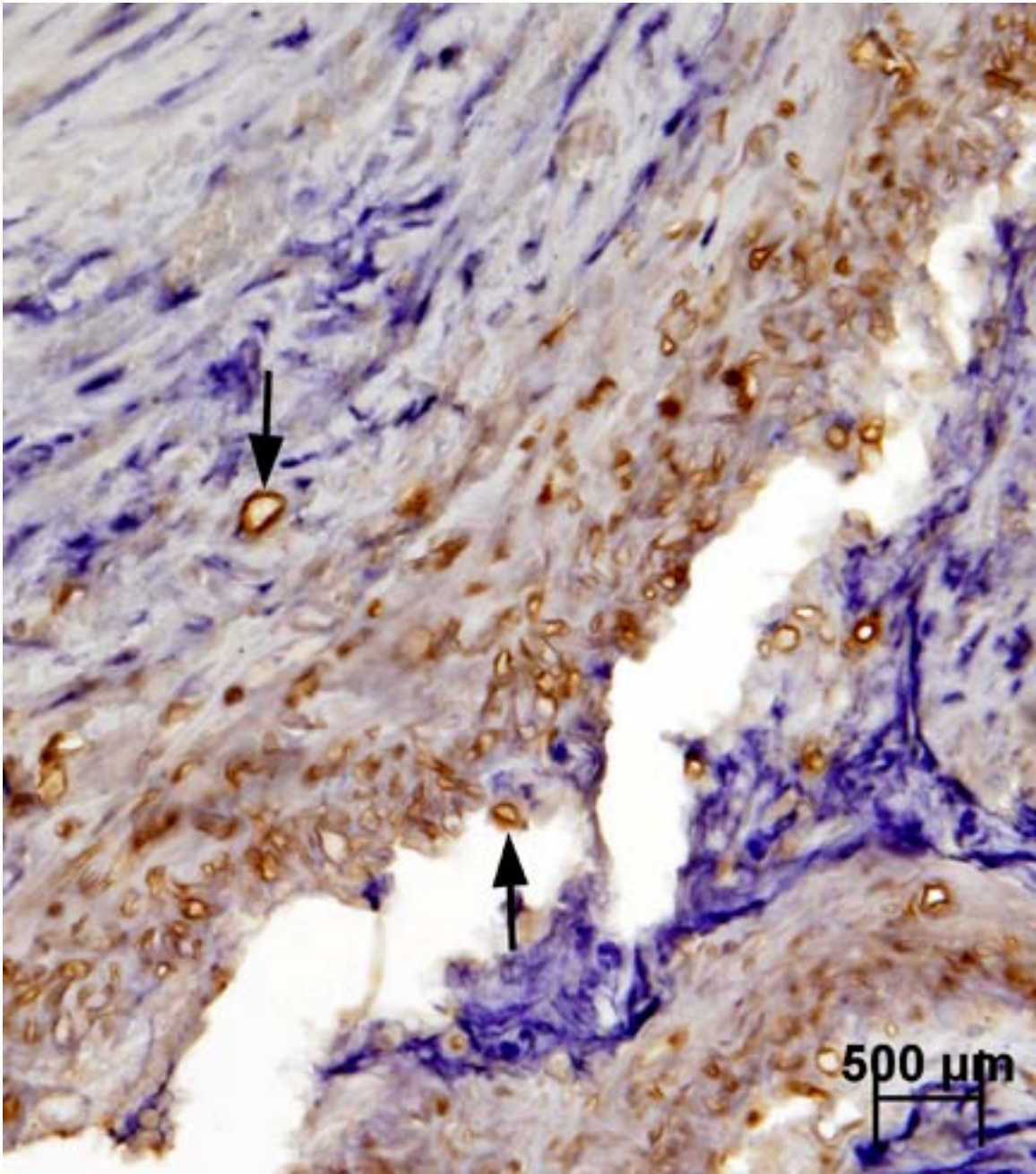
Figure 11: Photomicrograph of fungal affected equine cornea immunohistochemically stained for MMP-2. Strong staining around fungal organisms (arrows). No staining of keratocytes or degenerative neutrophils.
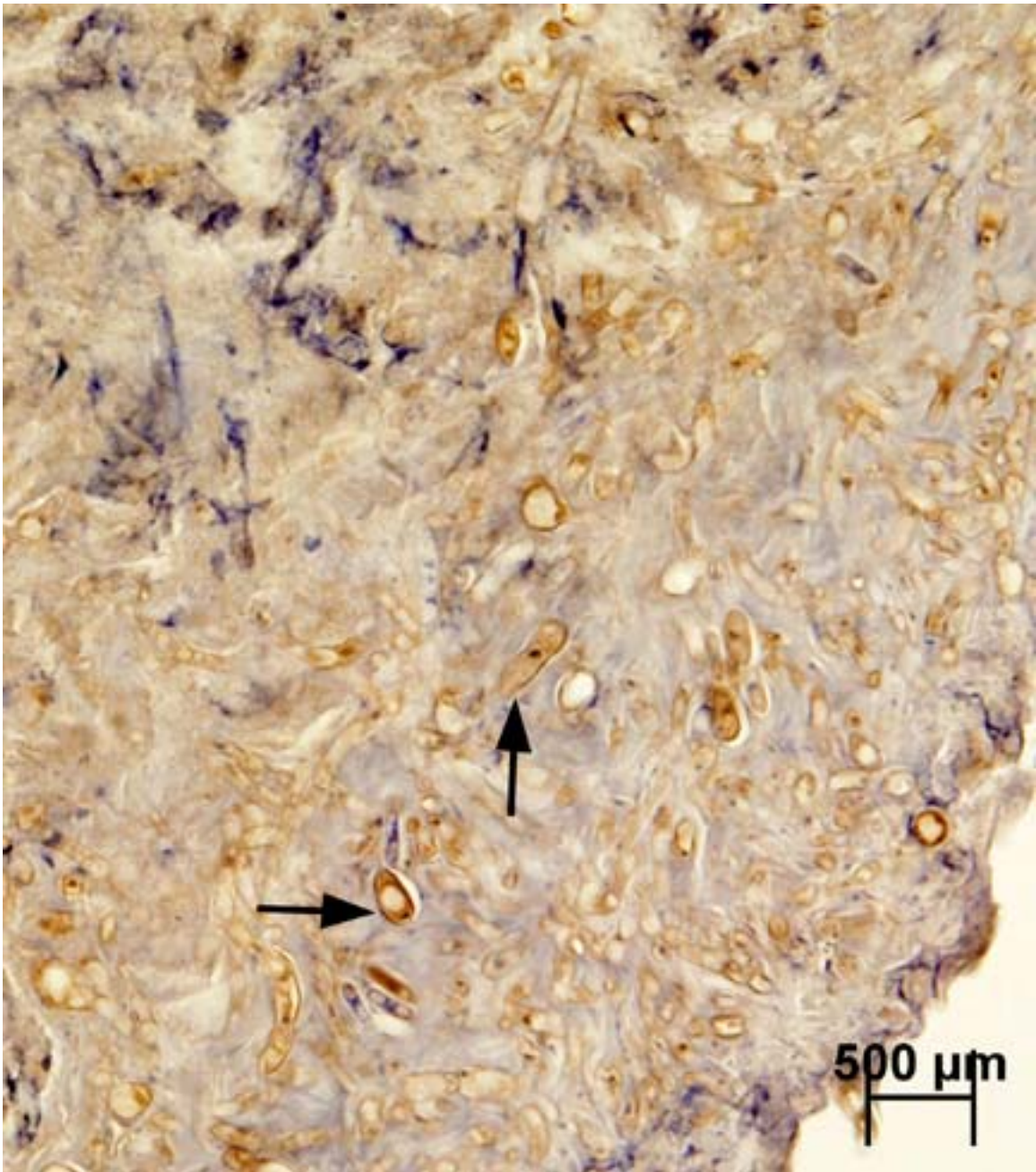
Figure 12: Photomicrograph of fungal affected equine cornea immunohistochemically stained for MMP-9. Moderate staining of fungal organisms (arrows). No staining of keratocytes or degenerative neutrophils. Mild staining of corneal stroma.
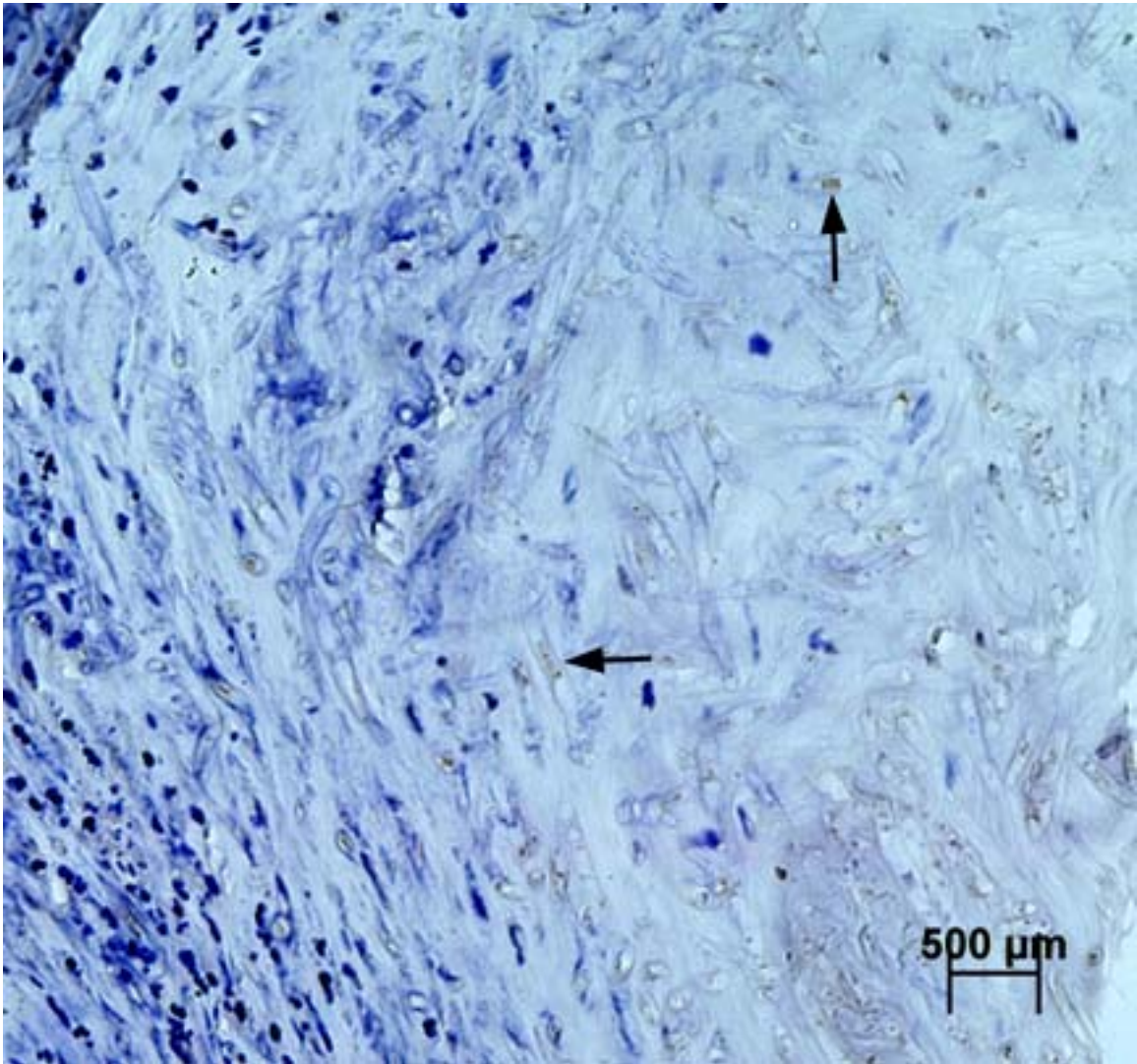
Figure 13: Photomicrograph of fungal affected equine cornea immunohistochemically stained for MIP-2. Slight staining of fungal organisms (arrows). No staining of keratocytes or neutrophils.
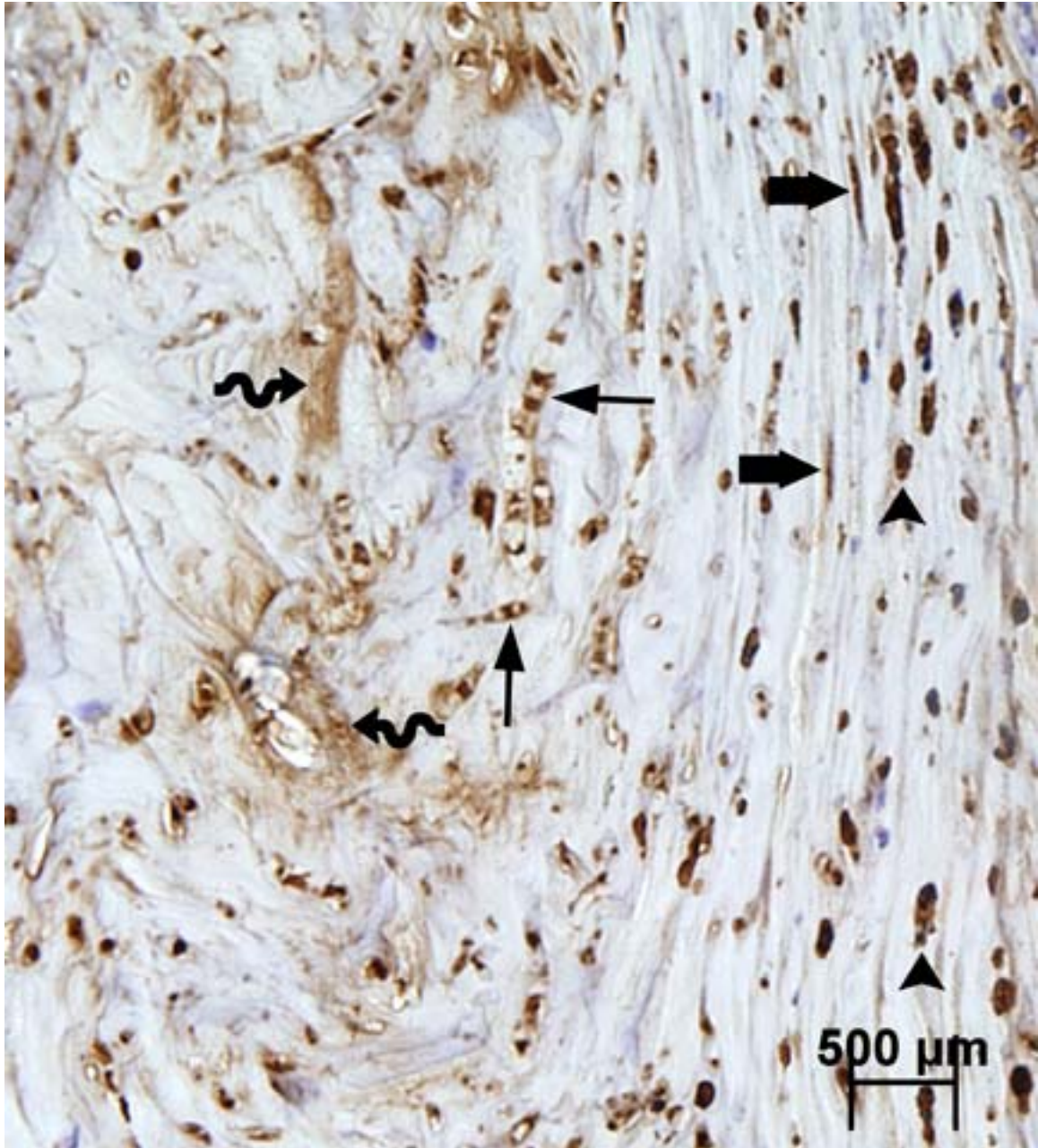
Figure 14: Photomicrograph of fungal affected equine cornea immunohistochemically stained for TIMP-1. Strong staining of fungal organisms (arrows), keratocytes (fat arrows) and inflammatory cells (arrowheads). Moderate staining of corneal stroma (curved arrow).

Figure 15: Photomicrograph of fungal affected equine cornea immunohistochemically stained for TIMP-2. Slight staining of fungal organisms (fat arrow). Strong staining of keratocytes (arrows) and inflammatory cells (arrowheads). Slight staining of corneal stroma.

Number of Inflammatory Cells
Least squares means

Antibody	Fungal Affected	Purulonecrotic
MMP-2 p=0.0449	**48.3039**	**86.2514**
MMP-9	**42.2585**	**63.8677**
MIP-2 p=0.0056	**10.1019**	**39.0311**
TIMP-1 p=0.0041	**71.2985**	**124.19**
TIMP-2	**38. 3585**	**28.9555**

Table 1: The number of inflammatory cells was higher in the purulonecrotic samples for MMP-2, MIP-2 and TIMP-1 than compared to the fungal affected samples.

Number of Inflammatory Cells
Least squares means

Antibody	A Fungal Affected p=0.0045	B Purulonecrotic p<0.0001	C Purulonecrotic p<0.0001
MMP-2	48.3039	**86.2514**	86.2514
MMP-9	42.2585	63.8677	**63.8677** p=0.0002
MIP-2	**10.1019**	**39.0311** p=0.0069	**39.0311** p<0.0001
TIMP-1	**71.2985** p=0.0013	124.19	**124.19**
TIMP-2	38. 3585	**28.9555** p=0.0005	**28.9555** p<0.0001

Table 2: The number of stained inflammatory cells was statistically different overall between the antibodies for the fungal affected (p=0.0045) and purulonecrotic samples (p<0.0001). **A.** Inflammatory cells stained for MIP-2 was significantly less than those stained for TIMP-1 for fungal affected samples. **B.** Inflammatory cells stained for MMP-2 were greater than those stained for MIP-2 and TIMP-2 in the purulonecrotic samples. **C.** Inflammatory cells stained for TIMP-1 were significantly greater than those stained for MMP-9, MIP-2 and TIMP-2 in the purulonecrotic samples.

Number of Inflammatory Cells
Linear correlation

A   B

Fungal Affected	Purulonecrotic
MMP-2 & MIP-2 r=0.59, p=0.0093	MMP-9 & MIP-2 r=0.65, p<0.0001
MMP-9 & MIP-2 r=0.72, p=0.0008	MMP-9 & TIMP-2 r = 0.73, p<0.0001
MMP-9 & TIMP-1 r =0.50, p=0.0291	MIP-2 & TIMP-2 r = 0.47, p=0.0041
	MIP-2 & TIMP-1 r = 0.40, p=0.0156

Table 3: **A.** Staining of inflammatory cells in fungal affected samples revealed a linear correlation between MMP-2 and MIP-2; MMP-9 and MIP-2 and MMP-9 and TIMP-1. **B.** Staining of inflammatory cells in purulonecrotic samples revealed a linear correlation between MMP-9 and MIP-2; MMP-9 and TIMP-2; MIP-2 and TIMP-2; MIP-2 and TIMP-1.

CHAPTER 3

CONCLUSION

Equine fungal keratitis (EFK) is a serious disease with potentially sight-threatening consequences. Despite intensive medical treatment, EFK has a guarded prognosis. It is usually preceded by a corneal defect, resulting in inoculation and infiltration of fungal hyphae into the corneal stroma, causing keratitis and/or deep stromal abscessation.

The immunohistochemical staining results of the corneal epithelium, stroma, keratinocytes, inflammatory cells (neutrophils, plasma cells and macrophages), vascular endothelial cells, as well as fungal organisms showed that MMP-2 and MMP-9 play a significant role in the normal remodeling and pathogenesis of equine purulonecrotic and fungal keratitis. The response to corneal injury has been documented to be mediated by leukocytes, fibroblasts and vascular endothelial cells, resulting in angiogenesis, re-epithelialization, granulation tissue formation and ECM deposition.[13] Many of these responses are known to be caused by or modified by MMPs and other proteinases.[18, 19, 20] The results of this study also supported that the pathogenic role of MMP-2 and MMP-9 contributed to corneal degradation, however the presence of fungi within the cornea was not taken into account as to have an increased effect on their release.

MIP-2 may be indirectly responsible for the levels of MMP-2 and MMP-9 expression in equine fungal keratitis through its role in neutrophil chemo-attraction. MIP-2 may be indirectly responsible for MMP-9 but not MMP-2 levels in purulonecrotic corneas without fungi. The reason for the lack of a correlation between MIP-2 and MMP-2 in the purulonecrotic samples is currently unknown.

The results of this study were expected to demonstrate that corneas infected with fungi have a higher concentration of MIP-2 proteins and neutrophil infiltrates with increased presence of MMP proteins. The amount of TIMP proteins expressed was expected to be inversely proportional to the amount of MMP and MIP-2 proteins, and neutrophil infiltrates in the corneas infected with fungi. The presence of MMP and MIP-2 proteins, as well as neutrophil infiltrates were expected to be higher in the corneas infected with fungal organisms than normal and purulonecrotic corneas.

Problems encountered with this study included paraffin blocks containing small pieces of tissue, which limited the number of sections that could be made. In addition, little ground work has been done to demonstrate MMPs and TIMPs in ulcerated corneas of horses by IHC staining, necessitating the concern for specificity. However, there have been studies identifying MMPs in other equine tissues with the use of in situ-zymography, Western blot analysis or immunohistochemical staining without the mention of cross reactivity. If difficulties such as cross reactivity had been documented when staining for these proteins, then western blot and/or in-situ zymography methods would have been employed with corneal tissues to achieve our stated objectives.

Our explanation of MMP, MIP-2, neutrophil infiltration and TIMP expression will further characterize the pathogenesis of equine fungal keratitis. In addition, our demonstration of MMPs, MIP-2 and TIMPs in equine fungal keratitis may lead to additional studies in the treatment of equine fungal keratitis, and the effect fungi have on the release of MMPs. To the authors' knowledge, this is the first study identifying MMP-9 in the normal cornea of any domestic species. This is also the first study to identify MMP-2 and MMP-9 in the equine cornea.