Effects of Systemic Flunixin Meglumine, Topical Oxytetracycline, and Topical Prednisolone Acetate on Tear Film Proteinases in Normal Horses

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Thesis submitted to the Faculty of the Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science
in
Veterinary Medical Sciences

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April 22, 2004
Blacksburg, Virginia

Keywords: Matrix Metalloproteinase, Equine, Tear Film

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Abstract

The purpose of this study was to determine the effects of three medical treatments, topical oxytetracycline, topical prednisolone acetate, and systemic flunixin meglumine, on the activity of two proteinases, matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9), in equine tear film. The study design consisted of twelve ophthalmically normal horses separated into three groups of four in a cross-over study design. Each group was treated for 5 days with flunixin meglumine (500mg IM bid), topical 1% prednisolone acetate (0.2ml tid), or topical oxytetracycline (0.2ml tid), followed by a 9-day washout period. All topical medications were applied to the left eye and the right eye was treated with a placebo. Tears were collected before the first treatment on day one and the morning following the last treatment on day 5. Tear film proteinase activity was measured using gelatin zymography and measurements of optical density. Statistical analysis of the difference between the treated and untreated eyes and the eyes before and after treatment was performed using mixed effects model for ANOVA. When eyes were compared after treatment, there was no significant difference between treated and placebo eyes for MMP-2 or MMP-9 for any of the treatments. When post-treated eyes were compared to pre-treated eyes, there was a significant decrease in MMP-2 activity in the left eye of horses treated with flunixin meglumine (P=0.0259). There were no differences in MMP-2 and MMP-9 activity for the other treatments. In conclusion, topical 1% prednisolone acetate and topical oxytetracycline did not

significantly change MMP-2 or MMP-9 activity in normal equine tear film. Systemic flunixin meglumine had an inhibitory, but questionable, effect on MMP-2 activity in normal equine tear film. This project was funded by Patricia Bonsall Stuart Award for Equine Research.

Table of Contents

Introduction

Ulcerative keratitis is a common condition in horses, accounting for 18% to 39% of equine ophthalmic patients presenting to referral institutions.[1-3] The high incidence of corneal ulceration in horses is a consequence of several factors including large, prominent, laterally positioned eyes, naturally aggressive physical activity, and ubiquitous exposure to bacterial and fungal pathogens.[1] While many corneal ulcers are superficial and heal without incident, keratomalacia or corneal 'melting' can develop under certain circumstances causing corneal rupture and iris prolapse in as little as 24 hours.[4,5] In a retrospective study of 32 horses with corneal perforation and iris prolapse, 10 (31%) had evidence of keratomalacia noted on initial presentation.[5] Of these cases, only 1 (10%) resulted in a visual eye following treatment. In comparison, five of seven eyes with corneal perforation secondary to ulcerative keratitis and no evidence of keratomalacia (71%) retained vision.[5] Overall, 10 of the 22 eyes (45%) that developed iris prolapse, either secondary to trauma or ulcerative keratitis, and had no evidence of keratomalacia, had a positive visual outcome.[5] Based on these findings, the authors deemed keratomalacia a negative prognosticator for retaining vision in horses with iris prolapse.

Keratomalacia is the result of rapid proteolytic degradation of the corneal stroma. The primary mediators of corneal stromal degeneration are proteinases derived from

exogenous sources (bacteria and fungi) and endogenous sources (leukocytes and corneal cells).[6-11] Bacterial pathogens associated with production of proteinases and subsequent keratomalacia include *Pseudomonas*, *Streptococcus*, and *Staphylococcus*.[5,7,12,13] Fungal species commonly isolated from malacic ulcers include *Aspergillus* and *Fusarium*.[5,6,8,12] Endogenous sources of these proteinases include damaged epithelial cells, keratocytes, and degranulating neutrophils and macrophages.[9-11] These proteinases act on collagen, proteoglycans, and other components of the stromal extracellular matrix to cause rapid destruction of the corneal stroma.

Major categories of proteinases that have been identified include matrix metalloproteinases (MMP) and serine proteinases.[14,15] MMPs are zinc-dependent enzymes that include gelatinases, collagenases, and stromelysins.[16] Two gelatinases of interest are MMP-9 (gelatinase B), with a molecular weight of 92 kD, and MMP-2 which has a reported molecular weights of 72kD and 66kD.[14,16,17] These enzymes have been shown to have activity against denatured collagen types I, II, and III and native types IV, V, VII collagen.[16,18] MMP-9 appears to have an affinity for and profound degradative effect on the epithelial basement membrane.[18] In the cornea, stromal keratocytes are the main source for MMP-2. Corneal epithelial cells and polymorphonuclear (PMN) leukocytes are the primary secretors of MMP-9.[14,19,20]

The matrix metalloproteinases comprise a large family of structurally and functionally similar zinc-dependent endopeptidases that are found in a variety of species

from plants to vertebrates.[21,22] In humans, 25 different MMP's have been identified (MMP-1 through MMP-28, omitting 4-6).[21] Vertebrate MMP's are synthesized as latent proteinases which are transported to the cell surface where they are either membrane bound or are secreted.[21] The latent proteinase consists of a 80 amino acid pro-domain and an adjacent catalytic domain consisting of 175 amino acids. Matrix metalloproteinases 2 and 9 differ from this standard with an additional 175 residue fibronectin type II domain inserts. The linker between the pro-domain and the catalytic domain is susceptible to proteolytic cleavage, thus converting from a latent to active state.[21] The catalytic domain exhibits a flattened ellipsoid shape with an active-site cleft in the flattened side that contains the catalytic zinc. Descriptions of MMP function vary from the degradative effect of MMP-1, MMP-2, and MMP-9 on denatured and native collagen[16,18,23], the affinity of MMP-9 to epithelium basement membrane[18], to role of MMP-3 in the conversion of the pro-MMP-9 to the active form.[24]

Matrix metalloproteinases have been evaluated in normal and diseased eyes of humans, rats, mice, guinea pigs, dogs, and horses.[2,13,14,24-29] The endogenous proteinases found in normal eyes perform important functions in corneal stromal physiology.[30,31] MMP-2 is expressed in undamaged corneal stroma where it may have a custodial function to degrade collagen molecules that become damaged.[18] Evaluation of normal human corneal grafts maintained in culture medium were found to produce measurable levels of active MMP-2. The authors contended that the quantity of active MMP-2 produced led to sufficient damage of the type IV collagen component of the epithelium

basement membrane to preclude successful transplantation if stored for prolonged periods of time.[32] Levels of MMP-9, while present in normal corneas, are at much lower concentration than MMP-2.[14] Both MMP-2 and MMP-9 are considered important in corneal healing. After corneal wounding, expression of MMP-2 is increased with much of the enzyme in the active form.[18] The increased levels of MMP-2 persist for up to seven months after the cornea has healed, suggesting that MMP-2 is involved in the remodeling of the stromal collagen.[18] By contrast, MMP-9 increased early in wound healing and is considered to participate in controlling resynthesis of the basement membrane for the corneal epithelium.[18,33] Based on the timing of expression and location of MMP-9, it may also be involved in the degradation of the epithelial basement membrane that precedes corneal ulceration.[14,18] These proteinases, in conjunction with resident surface flora, anatomic barriers, local T-lymphocyte cellular response, local humoral factors and local antibodies, act as a defense mechanism to protect the cornea from microbial pathogens.[31,34,35] The degradative effects of the proteinases are balanced by local anti-proteinase factors such as α-1 anti-trypsin, α-2 macroglobulin, and tissue inhibitor of metalloproteinase (TIMP).[36-38]

In corneal ulcerative disease processes, the balance between proteolytic and anti-proteolytic activity is altered by excessive levels of proteinases, either from endogenous or exogenous sources, which may result in the rapid degradation of the corneal stromal collagen and extracellular matrix.[11,30,39] A demonstration of damaging effects of the alteration of this balance can be shown in a study of human herpetic keratitis using a

murine model.[40] Samples harvested before infection had measurable levels of TIMP-1 and TIMP-2. Two days after inoculation, coinciding with epithelial lesions, both MMP-2 and MMP-9 activity were increased and there was no measurable TIMP-1 and TIMP-2 activity. However, by day 7, the epithelial lesions had healed, coupled with an increase in TIMP-1 and TIMP-2 expression with a corresponding decrease in both MMP-2 and MMP-9 activity. By day 14 post-infection, there was significant corneal pathology consisting of necrotizing stromal keratitis and epithelial ulceration. At that time, MMP-2, MMP-9, and TIMP-2 activity was present, but the ratio of MMP to TIMP activity was elevated compared to the healthy cornea. It was proposed by the authors of this study that the ratio of MMPs to TIMPs may be important for the course of necrotizing herpes simplex virus keratitis.[40] In another mouse model of experimental keratitis, corneal injury resulted in increases in both MMP-2 and MMP-9 and a significant down-regulation of TIMP-1.[41]

Alterations of MMP activity has also been measured in humans with corneal disease. Accumulation of active MMP-9 in tear film was measured in human patients with peripheral ulcerative keratitis, keratoconus, herpetic keratitis, and systemic and non-systemic dry eye conditions.[32] A Japanese study comparing human patients with ocular surface disorders versus those with normal corneas, found measurable amounts of active MMP-2 and MMP-9 in diseased eyes compared with only the proforms of these enzymes detected in healthy eyes.[42]

The importance of TIMP, which inhibits MMP-9 activation by binding to the proenzyme,[43] does not appear to be consistent. A study of 15 patients with ocular rosacea, a dry eye condition in humans, evaluated levels of MMP-9 and TIMP-1 and compared them to values collected from normal corneas. Affected eyes had significantly increased levels of both TIMP-1 and the proform and activated form of MMP-9.[24] However, the ratio of TIMP-1 to pro-MMP-9 was not significantly different and was actually increased. This is contradictory to a previous findings that theorized that a ratio of TIMP-1 to pro-MMP-9 of less than 1 was needed to promote activation of MMP-9.[44] An explanation for this discrepancy, proposed by the authors, was the production of another enzyme, neutrophil elastase, by the diseased corneas. While not evaluated in the study, neutrophil elastase has been reported to degrade TIMP-1 and has been measured in eyes with corneal disease.[2,45,46] The authors continue to theorize that once the TIMP-1 is inactivated, the pro-MMP-9 is amenable to activation.[24]

Matrix metalloproteinase has also been evaluated in the tear film of normal corneas and corneas with ulcerative keratitis in horses.[2,3] Strubbe et al [2], measured the activity of two matrix metalloproteinases, MMP-2 and MMP-9, and neutrophil elastase in tear film of 23 horses with ulcerative keratitis, described as keratomalacia, and compared these findings with the levels from the contralateral, non-diseased eye, and from 33 normal eyes. The enzyme activity, based on densitometry values, of MMP-2 in ulcerated corneas was 83% greater than normal control eyes and 30% greater than the contralateral, non-affected, eye. MMP-9 in the ulcerated eyes was increased 232% over the normal

control eyes and 159% greater than the contralateral eye. Neutrophil elastase in ulcerated eyes was 272% the level of normal control eyes and 138% the level of the contralateral eye. The authors found that MMP-2 and MMP-9 levels were significantly higher in ulcerated eyes than in normal, control eyes. When the authors evaluated the contralateral eyes from affected horses they found when compared to the control eyes, MMP-2 levels were significantly elevated in the contralateral eyes. When these contralateral eyes were compared to the ulcerated eyes, only MMP-9 was significantly increased in the ulcerated eyes versus the contralateral eyes. The authors propose that the unexpected finding of a relative increase in MMP-2 in the normal fellow eye of horses with ulcerative keratitis could be secondary to a systemically mediated response eliciting an inflammatory response in the contralateral eye. Another possible explanation was that these diseased horses had naturally increased MMP-2 levels and this predisposed them to progressive ulcerative keratitis. Evaluation of proteinase inhibitors, such as TIMP, was not performed in this study, but the increased levels of neutrophil elastase may have rendered these enzymes functionally inert and thus could have been a contributing factor to the progressive nature of the ulcerative disease in these horses.[24] A second novel finding in this study was that younger control horses had significantly higher tear film MMP-9 activity when compared to older (> 10 years) control horses.[2] The authors suggested that this may be a result of a decrease in corneal epithelial activity with increasing age of the horse.

The initiation of the increased expression of the MMP's and the activation seems to be multifactorial.[23,24,33,41,47,48] Cytokines, such as interleukin-1 beta (IL-1 beta), transforming growth factor-beta (TGF-beta), tumor necrosis factor-alpha (TNF-alpha) and platelet-activating factor (PAF), eicosanoid metabolites, such as the prostaglandins PGD2, PGE2, and PGF2α, and other proteinases, such as MMP-3, have been implicated as initiators of the degradative proteinase cascade. Cultured human corneal epithelial cells that were exposed to many different cytokines demonstrated a marked elevation of MMP-9 by IL-1 beta and TNF-alpha, in a dose dependent manner.[47] MMP-2 activity was not significantly modulated by any of the cytokines. In a murine model of corneal trauma, eyes pre-treated with an antibody to IL-1 beta had significant reduction of MMP-2 and MMP-9 compared with eyes not pre-treated with the antibody.[41] In this same model, mice treated with the IL-1 beta antibody also had markedly reduced corneal damage and significantly reduced MMP-9 activity once bacteria was introduced than eyes that were not pre-treated.[41] Interleukin-1 was also shown to decrease TIMP-1 expression in bovine articular chondrocytes.[49] Rabbit corneal epithelial cells that were subjected to TGF-beta exhibited increased MMP-9 activity.[33]

The role of prostaglandins on MMP activity has been demonstrated in a variety of tissues. Stimulation of cyclooxygenase-2 (COX-2) of rat fetal hepatocytes resulted in the release of activated MMP-2 and MMP-9.[48] A similar response was noted when these cells were stimulated with PGE2.[48] PGE2 expression was also linked to MMP-2 and MMP-9 activity in human prostate tumor cells.[50] The effects of eicosanoid metabolites

on MMP expression in corneal tissue was studied by Ottino, et al. They reported that when corneal tissue was incubated with PAF, PGD2, PGE2, and PGF2α, MMP-9 activity increased significantly.[23]

The role of other matrix metalloproteinases, namely MMP-3, on activation of the proform of MMP-9 was evaluated by Sorbin, et al.[24] In this study, MMP-3 levels were measured in human patients with diseased corneas. MMP-3 was especially elevated in patients with ulcerative conditions, but was absent from normal corneas. This is consistent with findings in rabbit corneal epithelial cultures.[14] When MMP-3 was added to epithelial cell cultures and incubated for 4 hours, the proform of MMP-9 was completely converted to the 83 kDa active form.[24] Therefore, in conditions of corneal pathology, not only is MMP-9 expression increased due to a multitude of factors, but the stimulation of MMP-3 increases MMP-9's activity.

In an attempt to address the imbalance between proteinases and anti-proteolytic factors, and arrest the progression of the keratomalacia, many different proteinase inhibitors have been advocated for the treatment of ulcerative keratitis.[3,51,52] Traditional examples of pharmacologic anti-proteinases include metal chelators such as sodium and calcium ethylenediamineteraacetic acid (EDTA)[53], acetylcysteine[54,55], citrate[56], cysteine[55], penicillamine[57], tetracycline[24,25,54,58], and synthetic[54,59] and natural proteinase inhibitors, such as α-1 anti-trypsin[60] and α-2 macroglobulin[61] found in serum. Chelating agents bind zinc and calcium ions, forming a complex with the MMPs and reducing the

enzymatic activity.[62] The proteinase inhibitors function by binding to the proform of the enzyme, preventing activation of the enzyme.[43]

Ollivier et al, reported 0.1% doxycycline reduced combined MMP-2 and MMP-9 activity in tears collected from horses with ulcerative keratitis by 96.3%.[3] An evaluation of the effects of doxycycline on human corneal epithelial cells in culture showed a 70% reduction of MMP-9 activity when treated with 10 μg/ml concentration.[25] While the tetracyclines are traditionally described as chelating agents, there is abundant evidence that they have further effects on MMP expression such as restriction of gene expression of neutrophil collagenase and epithelial gelatinase, suppression of α-1 anti-trypsin degredation, and oxygen radical scavenging.[63] Evaluation of the effect on bovine chondrocytes treated with tetracycline found evidence that the inhibition of MMP was mainly due to down-regulation of the respective gene expression.[64] Doxycycline was found to decrease the synthesis and bioactivity of IL-1 beta in cultured human epithelial cells.[65] In a study that evaluated the effects of different cytokines on MMP activity, it was reported that doxycycline suppressed the stimulatory effect of IL-1 beta and TNF-alpha on MMP-9 expression. While the level and activity of MMP-9 was reduced, the MMP-9 mRNA was not affected, suggesting that the doxycycline modulation of MMP-9 was related to epithelial cell production of the enzyme.[47] Based on the demonstration that doxycycline suppresses MMP activity through interference with inflammatory mediators such as IL-1 beta, one may deduce that tetracycline is acting more as an anti-inflammatory agent. Solomon et al, observed that doxycycline's suppression of IL-1 beta

was equally as potent as corticosteroids.[65] The contention that tetracycline's modulating effect on MMP may be due to its anti-inflammatory properties is further bolstered by the demonstration that MMP-9 expression in doxycycline treated human corneal epithelial cells was not significantly different from those treated with methylprednisolone.[25]

The effects of steroids on certain proteinases have been evaluated in a variety of species.[25,49,66,67] In a bovine articular chondrocyte model, dexamethasone was reported to inhibit collagenase activity.[49] However, in this same study, dexamethasone also significantly decreased TIMP-1 levels. In a study of equine articular cartilage, high concentrations of dexamethasone and methylprednisolone acetate demonstrated inhibitory effects on MMP-2, but not MMP-9.[67] Prednisolone was shown to suppress MMP activity in rabbit corneas in-vitro.[66] In a human study of recalcitrant recurrent corneal erosions, MMP-9 expression in supernate collected from cultured corneal epithelial cells treated with methylprednisolone was reduced by 56% and patients treated with a combination of systemic doxycycline and topical prednisolone healed within 2 to 10 days.[25] The suppressive effects of corticosteroids may be due to direct inhibitory effects on MMP, either via decreasing expression or blocking activation, or secondary to their anti-inflammatory effects.[25,67,68]

While the effect of non-steroidal anti-inflammatory drugs (NSAIDs) on tear film proteinases has not been extensively investigated, there have been contradictory reports on the effectiveness of this class of medication on different MMPs.[23,48,50,67,69-72] Human

prostate tumor cells that were exposed to non-toxic doses of ibuprofen, a general COX inhibitor, and NS398, a highly selective COX-2 inhibitor, had significant reductions in pro-MMP-2, MMP-9, and pro-MMP-9 levels.[50] However, unlike with steroids, there was no effect on TIMP. In human lung cancer cells, NS398 inhibited MMP-2 mRNA and MMP-2 levels, but had no effect on MMP-9.[72] In a study of equine chondrocytes, even at levels that could not be attained in-vivo, flunixin meglumine and phenylbutazone had no inhibitory effect on MMP-2 or MMP-9.[67] COX-2 inhibitors have been evaluated in rabbit corneal cultures.[23] Stressed corneas treated with NS398 and nimesulide, a COX-2 inhibitor, demonstrated significant reduction of MMP-9 activity in a dose dependent manner, approaching control levels at higher levels of NS398. However, there are indications that NSAIDs could be potentially contraindicated if used topically.[73,74] Rats that underwent corneal epithelium debridement and were then treated with 0.1% dichlofenac or 0.5% ketorolac developed significant increases in MMP-1, MMP-2, and MMP-8 compared to rats receiving a placebo.[73] Evaluation of corneal samples from human patients that developed ulcerative keratolysis associated with perioperative diclofenac demonstrated elevated levels of MMP-1, MMP-2, MMP-8, and MMP-9.[74] Due to the anterior uveitis and pain commonly associated with ulcerative keratitis[1], many horses with corneal ulcers are treated with a systemic NSAID's. However, their effect on tear film proteinases in horses is unknown.

More novel agents have been proposed as anti-proteolytic treatments.[66,67,75] A profile of rabbit corneas' response to a keratoprosthesis and the efficacy of potential

treatments found that medroxyprogesterone significantly decreased MMP activity compared to controls both in-vitro and in-vivo.[66] A 50% reduction in the loss of sample equine corneal mass was attained when the corneas were incubated with tetanus anti-toxin and collagenase versus corneas incubated with collagenase alone.[75] Polysulfated glycosaminoglycan (PSGAG) significantly inhibited both MMP-2 and MMP-9 in equine chondrocytes.[67] The effectiveness of PSGAGs as corneal anti-proteolytic treatment may also be demonstrated in a study of canine indolent ulcers.[28] In this clinical study, dogs with indolent ulcers treated topically with 5% PSGAG experienced a reduction in proteolytic activity versus the pre-treated value. The authors theorized that the therapeutic effect of the PSGAG was the inhibition of plasmin that degraded the fibrin/fibronectin complex needed for epithelial cell migration over the defect. However, humans with recurrent corneal erosions have significant increases in MMP-9 activity[24,25], and MMP-9 has been implicated as a causative factor in delayed corneal wound healing and re-epithelialization.[76] Thus the beneficial effect noted by Willeford et al could, at least, be partially associated with reduced MMP-9 activity.

Tools used to detect proteinase activity include Western blot assay[23,40,66,72,73], immunofluorescence[74], immunohistochemistry[40,74], hydroxyproline concentration[75], viscometry[77], and gel electrophoresis, also called zymography.[18,23,40,66,73] The first use of zymography to detect matrix metalloproteinase was demonstrated with RAS oncogene-transformed fibroblasts in 1988.[78] Zymography identifies the presence of a specific enzyme by its capacity to digest a substrate which is copolymerized in a polyacrylamide

gel.[18] Gelatin, a commonly used zymography gel, uses denatured type I collagen as a substrate. An SDS-buffer is added to an equal volume of the sample in order to denature the enzyme. Electrophoresis is used to separate enzymes based on molecular weight. The enzyme is then renatured and gelatinse activity is allowed to take place on the denatured collagen. Once the gel is stained, the position of the enzyme can be determined as cleared areas in the stained gelatin background and compared to the positions of molecular weight standards.[18]

While the efficacy of corneal anti-proteolytic treatments has been well demonstrated in humans and rabbits, documentation of beneficial effects in horses has been lacking until recently. One study evaluating the effect of several different anti-proteinases on MMP-2 and MMP-9 in pooled equine tear documented a significant inhibition of these enzymes with a multitude of anti-proteinases including 0.2% EDTA, 0.1% doxycycline, 10% N-acetylcysteine, 0.1% ilomostat, equine serum, and 0.1% and 0.5% α-1 proteinase inhibitor.[3] Another in-vitro study reported an inhibition of bacterial collagenase when incubated with normal equine corneas when anti-proteinases, such as equine serum, tetanus antitoxin, and acetylcysteine, were added.[75] However, to the authors' knowledge, no corneal anti-proteolytic treatment has been assessed in horses *in-vivo*. The purpose of this study was to evaluate three medications, systemic flunixin meglumine, topical oxytetracycline, and topical prednisolone acetate on the activity of MMP-2 and MMP-9 in the tear film of normal horses.

Methods and Materials

Animals

Tear samples were collected from the eyes of twelve breeding mares with an average age of 10.8 years. The horses were housed on a breeding farm in Florida. The horses were kept on pasture during the experiment. The horses were evaluated and deemed ophthalmically normal using slit lamp biomicroscopy and direct ophthalmoscopy. The study was conducted under the guidelines of the animal care and use committee of the Virginia-Maryland Regional College of Veterinary Medicine.

Collection of Tear Samples

Tears were collected on the morning of the first day immediately before any treatment was given and on the morning following the last day of treatment, approximately 12 hours following the last treatment. Prior to collection, horses were sedated with xylazine[a] (0.5mg/kg, IV). Approximately 60 µl of tears were collected from lower conjunctival fornix of each eye using a capillary tube with an atraumatic tip and minimizing contact with the conjunctival surfaces. The tears were centrifuged and the supernate was transferred into polypropylene microcentrifuge tubes.[b] The samples were stored at –80^{o}C until analysis.

Study Design

The horses were placed into three treatment subgroups of four horses each and were treated in a randomized cross-over design. There were three treatment phases of 5 days each with a 9 day washout period between the phases. During each treatment phase one group of horses was treated with either topical 1% prednisolone acetate (PA)[c], topical oxytetracycline ointment(OX)[d], or systemic flunixin meglumine(FM)[e] and a topical placebo, either artificial tears[f] or petroleum ointment[g].

All topical treatments were applied to the left eye and all placebos were applied to the right eye. The treatments and placebos were applied as follows: PA – 0.2 ml OS tid and artificial tears 0.2 ml OD tid, OX – 0.2 ml OS tid and a petroleum ointment 0.2 ml OD tid, FM – 500 mg IM bid and a petroleum ointment 0.2 ml OD tid.

Zymograms

Aliquots (10 µl) of tear sample were mixed with an equal volume of SDS-sample buffer.[h] An aliquot of the mixed sample (15 µl) was loaded into wells of 10% zymogram gelatin gels.[i] Prestained molecular weight standards[j], active and latent human MMP-2 standard[k,l], and active and latent human MMP-9 standard[m,n] were also added to each gel. One gel was used for each horse.

The gels were electrophoresed at a constant voltage of 125V until the dye line had reached the bottom of the gel, approximately 2 hours. The gels were then rinsed with

distilled water and the MMPs were reactivated by gently shaking in a renaturing solution of 2.7% Triton X-100[o] for 1 hour at 37°C. The gels were then incubated for 24 hours at 37°C on a rotary shaker in developing buffer.[p]

Afterwards, the gels were rinsed and stained by incubation with Coomassie blue[q] for 1 hour. The gels were then destained in a solution of 5% acetic acid and 7.5% methanol for 5 to 10 minutes.

Proteolytic activity appeared as colorless bands against the surrounding dark-blue background. Identification of the bands was determined by the distance the band had migrated compared to the molecular weight and human standards.

Image Analysis

Digital photographs of the stained gelatin zymograms were attained by use of an imaging densitometer[r] and analyzed by use of quantification software.[s] An area under the curve (AUC), corresponding to the optical density multiplied by the width of the band, was calculated for each band. The higher the AUC the more proteolytic activity was present. The AUC of active and latent MMP-2 and active and latent MMP-9 was determined for each eye at the start and the end of each treatment phase. (Table 1)

Statistical Analysis

Ratios of AUC were determined for MMP-2 and MMP-9 by adding the active and latent AUC of the MMP divided by the sum of the AUC human standard active and latent MMP on that gel. A mixed effects model for analysis of variance was performed on the data using a statistical program.[t]

Results

Animals – There were 12 horses included in this study. The mean age was 10.8 years (range, 3 years to 21 years). All subjects were Thoroughbred mares.

Baseline – The difference between OS & OD before treatment was evaluated. There was no significant difference in the activity of MMP-9 or MMP-2 for any of the treatment groups. (Table 2)

Treatment effect – The difference between the treated eye (OS) and the placebo eye (OD) was evaluated at the end of the treatment phase. There was no significant difference in the activity of MMP-9 or MMP-2 for any of the treatments. (Table 3)

Within treatment effect – The difference in the treated eye (OS) after treatment compared to before treatment was evaluated. There was a significant decrease in MMP-2 activity with FM ($P=0.0259$). There was no significant difference of MMP-2 activity for the other treatments. No difference in MMP-9 activity was detected for any treatment group. (Table 4)

Within placebo effect – The difference between the placebo eye (OD) after treatment compared to before treatment was evaluated. A significant increase in MMP-9 activity was found in the prednisolone acetate therapy group in eyes treated with

artificial tears (P=0.0482). The effect of the other placebos on MMP-9 activity and all placebos on MMP-2 activity was not significant. (Table 5)

Discussion

The presence of measurable proteinase activity in the normal equine eyes was consistent with previous findings.[2] As these were non-diseased eyes, the finding that there was no significant difference between the right and left eyes before treatment was expected. This result also acts as a confirmation that the 9 day wash-out period between treatments was adequate.

Interpretation of the lack of significant difference in MMP levels between treated and placebo eyes depends on what treatment is being evaluated. The fact that the topical medications, whose effects are more pronounced locally, did not cause significant changes in MMP levels compared to placebo would indicate that the treatment was ineffective at inhibiting MMP-2 and MMP-9 activity over the period evaluated in this study. However, with regards to FM, which was administered systemically, any inhibitory effect should have been bilateral, thus affecting both eyes equally. The expected result would be no significant difference in proteinase activity between the two eyes, but possibly a change in both eyes compared to baseline MMP levels.

When assessing proteinase level in the left eye before and after treatment, the lack of significant difference for the topical medications indicates either a lack of efficacy of these medications to inhibit MMP-2 or MMP-9 *in-vivo*, or an inability to maintain the

inhibition for 12 hours following cessation of treatment. These findings are contrary to what has been previously reported *in-vitro*.

A number of antiproteolytic agents have been identified. Tetracyclines are theorized to inhibit MMPs by chelating zinc and calcium ions, forming a complex with the MMPs and reducing the enzymatic activity.[62] Other proposed mechanisms of inhibition include restriction of gene expression of neutrophil collagenase and epithelial gelatinase, suppression of α-1 anti-trypsin degradation, and oxygen radical scavenging.[63] In humans, doxycycline added to cultured epithelial cells led to a 70% decrease in MMP-9 activity.[24,25] Pooled tear samples from horses with ulcerative keratitis demonstrated a decrease in combined MMP-2 & MMP-9 activity when 0.1% doxycycline was added.[3] Rabbits receiving oral doxycycline were found to have significantly decreased collagenase activity in an alkali-burn cornea model.[79] Topical methylprednisolone given to humans patients with refractory corneal ulcers resulted in a significantly shorter healing time, possibly related to decreased MMP activity.[25] In that study, supernate from cultured epithelial cells were found to have marked reduction in MMP-9 activity when cultured with 0.01% methylprednisolone. It is unclear if this action of glucocorticosteroids is due to a direct inhibitory effect or is secondary to their anti-inflammatory properties.[25] Glucocorticosteroids have potent immunomodulatory effects, including downregulation of production of inflammatory cytokines such as interleukin-1 and tumor necrosis factor α, that stimulate MMP production.[68]

There are several possible explanations for the contrasting findings of this study compared to others. Many of the reports that have documented inhibitory effects of medications on MMP activity were performed in disease or simulated-disease conditions and are often performed *in-vitro*.[3,25,66,75,79] In normal eyes, there may be a basal level of MMP production that cannot be reduced with treatment. Another possibility is that there may have been a dilution effect of tears on the topically applied medications, such that the regimen performed failed to attain an adequate concentration of medication to produce a significant effect. An example of this was noted in pooled equine tear samples in which 0.1% solution of an α-1 proteinase inhibitor produced considerably less effect than a 0.5% solution.[3] A third possible explanation for the lack of effect of topical medications on MMP activity may be the prolonged period between treatment and sampling. There may have been a temporary effect of the treatments, but due to the 12 hour lag between the last treatment and collection of samples, that effect may have waned. A final theory may be a transitory effect. The medications may have had an immediate local effect that tapered quickly due to continued local production of MMPs. Most studies that have documented decreased MMP levels after addition of anti-proteinase medications were done *in-vitro*, with limited to no ability for proteinase production.[3,24,25,66,75,79] In a study in rabbits, despite the inhibitory effect of certain treatments *in-vitro*, including tetracycline and prednisolone, when assessed in a live model, they were deemed ineffective.[66] A similar result as this study was noted in beagles, where topical 1% doxycycline, among others proteinase inhibitors, failed to reduce MMP-2 or MMP-9 activity compared to artificial tears.[80] If antiproteolytic

effects are indeed transitory, one would expect a systemic medication to perhaps have a more long-lasting effect than a topically applied substance. In studies that have documented effective proteinase inhibition by doxycycline in humans and rabbits, the medication was administered orally.[25,79]

Systemic administration of flunixin meglumine produced a significant decrease in MMP-2 activity when the post-treatment left eye was compared to pre-treatment. Multiple NSAIDs have been shown to modulate collagenase activity.[69] The finding of preferential action on MMP-2, in this study, corresponds with the results of a study of cyclooxygenase type 2 (COX-2) inhibition in human lung cancer cells.[72] Those authors reported that when exposed to both NS398, a selective COX-2 inhibitor, and indomethacin, a non-selective COX-2 inhibitor, the cells produced significantly less MMP-2 while there was no effect on MMP-9 expression. However, in an experiment using murine cancer cells, aspirin and sodium salicylate suppressed MMP-9 while having no effect on MMP-2.[71] As a further contrast, in human hepatoma cells, aspirin and NS398 significantly reduced MMP-9 activity[70], and ibuprofen and NS398 significantly inhibited both MMP-2 and MMP-9 activity in human prostate tumor cells.[50] While COX-2 promotion of MMP has been extensively documented in cancer cells, there is evidence that MMP activity in the cornea is also mediated by this pathway. Stimulation of MMP-9 activity in corneal cultures was shown to be induced by platelet-activating factor, while inhibition of COX-2 decreased MMP-9 levels.[23] The proposition that FM reduced MMP-2 activity is contradictory to the findings of an *in-vitro* study using equine

chondrocytes.[67] The authors found no effect of FM on MMP-2 or MMP-9 even at high concentrations that could not be achieved *in-vivo*.

The expected finding with parenteral administration of FM would be a similar effect on both eyes. However, when the pre- and post-treated right eye was evaluated, no significant effect was found on either MMP activity. One potential explanation for this discrepancy was that petroleum ointment administered to the right eye three times a day during the treatment may have been irritating, causing mild inflammation and an increase in MMP activity that negated the effects of the FM. Support for this explanation is the finding of statistically significant increased MMP-9 activity in the placebo eyes from the prednisolone acetate group treated with artificial tears.

In conclusion, this study found that topical oxytetracycline and prednisolone acetate administered three times daily did not significantly alter either MMP-2 or MMP-9 activity in normal horses over the time period evaluated in this study. Twice daily flunixin meglumine given IM had an inhibitory effect on tear film MMP-2 in non-placebo treated eyes of normal horses. Future exploratory avenues include effects of higher frequency application, tear film sampling at a shorter interval following treatment, in vivo studies of antiproteolytic regimes in horses with ulcerative keratitis, and determination of whether the route of medication, topical or systemic, has a significant impact on proteinase activity.

Footnotes

a. Sedazine® 100mg/ml, Fort Dodge Animal Health, Fort Dodge, IA.

b. Eppendorf tubes, Brinkmann Instruments Inc, Westbury, NY.

c. 1% Prednisolone Acetate Ophthalmic Suspension USP, Alcon Laboratories, Fort Worth, TX.

d. Terramycin®, Pfizer Inc, New York, NY.

e. Flunixamine™, Fort Dodge Animal Health, Fort Dodge, IA.

f. Akwa Tears®, Akorn Inc, Buffalo Grove, IL.

g. Paralube® ointment, E. Fougera & Co, Melville, NY.

h. Novex tris-glycine SDS native sample buffer (2X), Invitrogen, Carlsbad, CA.

i. 10% Novex zymogram gelatin gel, Invitrogen, Carlsbad, CA.

j. See Blue prestained standards, Invitrogen, Carlsbad, CA.

k. Active MMP-2 enzyme, Oncogen, Boston, MA.

l. Proenzyme MMP-2, Oncogen, MA.

m. Active MMP-9 enzyme, Oncogen, Boston, MA.

n. Proenzyme MMP-9, Oncogen, Boston, MA.

o. Novex zymogram renaturing buffer (10X), Invitrogen, Carlsbad, CA.

p. Novex zymogram developing buffer (10X), Invitrogen, Carlsbad, CA.

q. Coomassie rapid stain, Diversified Biotech, Boston, MA.

r. GS-710 imaging densitometer, Bio-Rad Laboratories, Hercules, CA.

s. Quantity One quantification software, 4.2.1 β version, Bio Rad Laboratories, Hercules, CA.

t. SAS version 8.2, SAS Institute Inc, Cary, NC.

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List of Abbreviations

MMP – Matrix Metalloproteinase

PMN – polymorphonuclear

TIMP – tissue inhibitor of metalloproteinase

IL-1 – interleukin-1

TGF – transforming growth factor

TNF – tumor necrosis factor

PAF – platelet-activating factor

PG – prostaglandin

COX – cyclooxygenase

EDTA – ethylenediamineteraacetic acid

NSAID – non-steroidal anti-inflammatory drug

PSGAG – polysulfated glycosaminoglycan

PA – 1% prednisolone acetate

OX – oxytetracycline

FM – flunixin meglumine

Bid – twice daily

Tid – three times daily

IM – intramuscular

IV – intravenous

OS – left eye

OD – right eye

AUC – area under the curve

Table 1

Average AUC – Average of the area under the curve of each MMP for all treatments.

Treatment	MMP	COS	COD	TOS	TOD
Prednisolone Acetate	MMP2	0.028269	0.025446	0.031928	0.035294
	MMP9	0.46257	0.549406	0.638291	0.922945
Oxytetracycline	MMP2	0.024612	0.027784	0.025962	0.029652
	MMP9	0.701983	0.526674	0.392769	0.48835
Flunixin Meglumine	MMP2	0.035756	0.030797	0.026798	0.034582
	MMP9	0.62928	0.430821	0.648324	0.62928

COS – OS before treatment
COD – OD before treatment
TOS – OS after 5 days of treatment
TOD – OD after 5 days of treatment

Table 2

Baseline – The difference between OS & OD before treatment (COS-COD).

Treatment	MMP	t-Value	P value
Flunixin Meglumine	MMP-2	1.11	P=0.2819
	MMP-9	0.72	P=0.4827
Prednisolone Acetate	MMP-2	0.19	P=0.8552
	MMP-9	-0.60	P=0.5577
Oxytetracycline	MMP-2	-0.70	P=0.4926
	MMP-9	1.20	P=0.2428

Table 3

Treatment Effect – The difference between treated eye (OS) and the placebo eye (OD) at the end of 5 days of treatment (TOS-TOD).

Treatment	MMP	t-Value	P value
Flunixin Meglumine	MMP-2	-0.76	P=0.4570
	MMP-9	0.11	P=0.9167
Prednisolone Acetate	MMP-2	-0.50	P=0.6230
	MMP-9	-1.58	P=0.1292
Oxytetracycline	MMP-2	-0.55	P=0.5902
	MMP-9	-0.53	P=0.6010

Table 4

Within Treatment Effect – The difference in the treated eye (OS) after treatment compared to before treatment (TOS-COS).

Treatment	MMP	t-Value	P value
Flunixin Meglumine	MMP-2	-2.43	**P=0.0259**
	MMP-9	0.45	P=0.6573
Prednisolone Acetate	MMP-2	0.74	P=0.4697
	MMP-9	0.70	P=0.4929
Oxytetracycline	MMP-2	0.74	P=0.4674
	MMP-9	-1.23	P=0.2333

Table 5

Within Placebo Effect – The difference between the placebo eye (OD) after treatment compared to before treatment (TOD-COD).

Treatment	MMP	t-Value	P value
Flunixin Meglumine	MMP-2	0.55	P=0.5871
	MMP-9	1.12	P=0.2769
Prednisolone Acetate	MMP-2	1.38	P=0.1852
	MMP-9	2.10	**P=0.0482**
Oxytetracycline	MMP-2	0.27	P=0.7899
	MMP-9	-0.22	P=0.8313

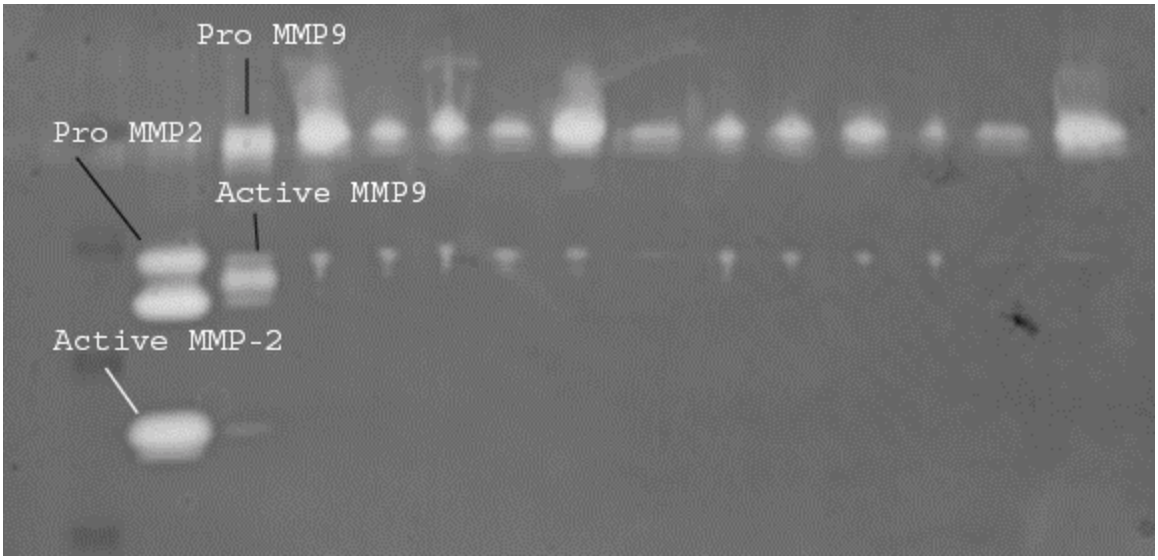

Figure 1: Sample Gel

Vita

Marc Edward Rainbow was born in New Orleans, LA, on January 30, 1969. Marc graduated from high school in Fort Walton Beach, FL in 1987. After graduating from high school, Marc attended the Georgia Institute of Technology in Atlanta, GA and graduated in 1991 with Bachelor of Science Degree in Laser and Optical Physics. Upon graduation, Marc received his commission in the U.S. Air Force and was stationed in Witchita Falls, TX and Albuquerque, NM during the time period 1991-1996.

Marc left active military service in 1996 to attend the College of Veterinary Medicine at the University of Georgia in Athens, GA. Upon completion of the degree in 2000, Marc completed a rotating small animal internship from 2000-2001 at the Texas A&M College of Veterinary Medicine, College Station, TX. Marc came to the VMRCVM to pursue a residency in ophthalmology and concurrently begin work towards a Master of Science. Marc will complete requirements for both programs in July 2004.