Evaluation of various compounds to inhibit activity of matrix metalloproteinases in the tear film of horses with ulcerative keratitis

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Objective—To examine in vitro effects of various antiproteolytic compounds on activity of matrix metalloproteinase (MMP)-2 and -9 in the tear film of horses with active corneal ulcers.

Sample Population—Samples of tear film obtained from the eyes of 34 horses with active ulcerative keratitis.

Procedure—Horses were sedated, and tear samples were collected from the lower fornix of 34 ulcerated eyes by use of capillary tubes. The protease inhibitors 0.2% EDTA, 0.1% doxycycline, 10% *N*-acetylcysteine (NAC), 0.1% solution of a modified dipeptide that contains hydroxamic acid (ie, ilomostat), 0.1% α1-proteinase inhibitor (PI), 0.5% α1-PI, and 100% fresh equine serum (ES) were used to treat pooled samples. Amount of latent and active MMP-2 and -9 was measured by optical density scanning of gelatin zymograms of treated and untreated tear samples.

Results—Pooled tear samples obtained from ulcerated eyes contained the latent and active forms of MMP-2 and -9. Compared with MMP activity in untreated samples, total MMP activity (sum of all bands detected) observed on the gelatin zymogram gels was reduced by 99.4% by EDTA, 96.3% by doxycycline, 98.8% by NAC, 98.9% by ilomostat, 52.4% by 0.1% α1-PI, 93.6% by 0.5% α1-PI, and 90.0% by ES.

Conclusions and Clinical Relevance—We documented that EDTA, doxycycline, NAC, ilomostat, α1-PI, and ES inhibited MMP activity in vitro. Because these compounds use different mechanisms to inhibit various families of proteases in the tear film of horses, a combination of these protease inhibitors may be beneficial for treatment of corneal ulcers in horses. (*Am J Vet Res* 2003;64:1081–1087)

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Ulcerative keratitis is a common and often vision-threatening condition in horses. The Ophthalmology Service of the University of Florida Veterinary Medical Teaching Hospital diagnosed ulcerative keratitis in 527 horses between January 1987 and October 2002. This constituted 35% of all horses evaluated for ophthalmic problems during this period. Superficial, noninfected ulcers in horses generally heal quickly and without complication, whereas stromal degradation in deep or infected ulcers can rapidly and dramatically progress to corneal perforation in horses within 24 hours.[1] A pronounced fibrovascular response is also prominent during corneal healing in horses. An understanding of the pathophysiologic processes as well as early diagnosis and aggressive treatment are important for quick resolution of ulcerative corneal diseases in horses and to speed healing, reduce scarring, and prevent corneal rupture.

Proteolytic enzymes perform important physiologic functions in normal tissues, such as turnover and remodeling of the corneal stroma. Activities of proteolytic enzymes are normally balanced by natural protease inhibitors, thus preventing excessive degradation of normal healthy tissue. Excessive amounts of proteases can create an imbalance between proteases and protease inhibitors, and increased amounts of proteases are believed to cause pathologic degradation of collagen and proteoglycans in the cornea.[2,3]

The equine cornea manifests the most severe degree of ulcer-associated stromal collagenolysis seen in animals.[1] This rapid degradation of the corneal stroma in horses with corneal ulcers appears to be caused by various proteolytic enzymes acting on collagen, proteoglycans, and other components of the stromal extracellular matrix. Microorganisms, inflammatory cells, corneal epithelial cells, and fibroblasts all produce and release proteolytic enzymes.[4-7]

Two important families of enzymes that affect the cornea are the **matrix metalloproteinases (MMPs)** and serine proteases (including neutrophil elastase).[6,8,9] Two MMPs (MMP-2 [72-kd gelatinase A] and MMP-9 [92-kd gelatinase B])[8,10] are of major importance in terms of remodeling and degradation of the corneal stromal collagen. The origin and purpose of MMP-2 and -9 appear to differ at the corneal level. Matrix metalloproteinase-2 is synthesized by corneal keratocytes and performs a surveillance function in the normal cornea, becoming locally activated to degrade collagen molecules that occasionally become damaged as a result of normal wear and tear.[2,7,11] Alternatively, MMP-9 is produced by

epithelial cells and **polymorphonuclear neutrophils (PMNs)** following corneal wounding.[7,8]

Proteases have been evaluated in the tear film of normal and diseased eyes of humans and other animals.[6,8,12-17] In severely damaged corneas, protease activities in the tear film were significantly increased.[6,13,14,16,18] In another study[13] in horses, higher amounts of MMP-2, MMP-9, and neutrophil elastase were found in the tear film of ulcerated eyes, compared with values for the tear film of eyes in age-matched normal control horses.

Protease activity in the tear film is believed to speed up degradation of stromal collagen, leading to rapid progression of ulcers. Normalizing proteolytic activity in the tear film is an objective of the treatment of corneal ulcers in horses. Thus, protease inhibitors have been recommended for treatment of ulcerative keratitis to reduce the progression of stromal ulcers, speed epithelial healing, and minimize corneal scarring.[1,19-24]

However, studies on activity of specific enzymes are limited. Accordingly, recommendations for use of topically administered ***N*-acetylcysteine (NAC)**, potassium EDTA, and serum in horses with corneal ulcers have mostly been based on anecdotal clinical reports or extrapolations from data obtained by use of these compounds in other species.[1,12,19,25-27] The availability of newer compounds, such as doxycycline a modified dipeptide that contains hydroxamic acid (ie, ilomostat), and **α1-proteinase inhibitor (PI)**, which initially have shown considerable promise as MMP or serine-protease inhibitors in other species,[28-33] outlines the need for specific studies on possible regulation of enzyme activity in tears of horses with corneal disease. These new compounds have potential as potent protease inhibitors in horses with corneal ulcers. Therefore, in the study reported here, we evaluated the effectiveness of various protease inhibitors for reducing the activity of MMP-2 and -9 in vitro in samples of tear film obtained from horses with ulcerative keratitis.

Materials and Methods

Animals—Tear samples were collected from the eyes of 34 horses with ulcerative keratitis. Each horse was evaluated by members of the Ophthalmology Service of the University of Florida Veterinary Medical Teaching Hospital during 2002. The horses had been diagnosed with various conditions, including bacterial or fungal keratitis, sterile ulcerative keratitis, and ulcerative keratitis of unknown etiologic origin.

Collection of tear samples—Horses were sedated, and akinesia of the upper eyelid was achieved prior to collection of tear samples. Samples were obtained before any diagnostic procedure or application of treatment; however, immediately after collection of tear samples, thorough ophthalmic examinations, including slit-lamp biomicroscopy, tonometry, and ophthalmoscopy, were performed. Samples were collected only from eyes with ulcerative keratitis, as identified by positive results for retention of fluorescein dye. All tear samples were collected from the lower fornix via by capillary force by use of capillary tubes with an atraumatic tip, as described elsewhere.[34] Time of collection of each sample was not recorded. All samples were immediately centrifuged, transferred into polypropylene microcentrifuge tubes,[a] and stored at –80°C until analysis. Tear samples collected from the eyes of the 34 horses were pooled to yield a volume of 1,500 µL.

Determination of MMP activity—Gelatin zymography and measurement of **optical density (OD)** were used to evaluate MMP activity in untreated pooled tear samples and conduct inhibition tests of MMP activity in treated tear samples. Aliquots (10 µL) of the pooled tears were mixed with an equal volume of SDS-sample buffer.[b] Then, 15 µL of the mixture was loaded into wells of precast 10% zymogram gelatin gels.[c] Untreated samples were constituted on each gel in 3 lanes that remained untreated. Treated samples were constituted in 3 lanes that were treated with a protease-inhibitor compound (triplicate pattern). Prestained molecular-weight standards[d] and gelatinase-zymography standards for active[e,f] and latent[g,h] forms of human MMP-2 and -9 were also assayed on each gel.

Inhibitory compounds tested in the study included EDTA, doxycycline, NAC, ilomostat, α1-PI, and **fresh equine serum (ES)**. A solution containing 0.2% EDTA was obtained by filling an evacuated, EDTA-containing, 10-mL blood-collection tube[i] with 10 mL of developing buffer. Developing buffer was added, as necessary, to 1% doxycycline,[j] 20% NAC,[k] and 1% α1-PI to obtain concentrations of 0.1% doxycycline, 10% NAC, 0.1% α1-PI, and 0.5% α1-PI, respectively. Ilomostat (2 mg) was dissolved in 50 µL of dimethyl sulfoxide, then 20 mL of developing buffer was added to achieve a final concentration of 0.1% ilomostat. Ilomostat and 1% α1-PI were provided by 1 of the investigators (GSS).

Blood samples were collected from the jugular vein of a horse into dry, sterile, 7-mL blood-collection tubes that did not contain an anticoagulant but that did contain gel and clot activator.[l] Serum was separated by centrifugation of the tubes at 1,000 × *g* for 8 minutes. Serum was harvested and maintained at room temperature (20°C); it was used undiluted for in vitro inhibition testing on the day on which it was obtained.

Gels were electrophoresed at a constant voltage of 125 V for approximately 2 hours. After electrophoresis, gels were rinsed in distilled water and then gently shaken in a renaturing solution of 2.7% Triton X-100[m] for 1 hour at 37°C to reactivate MMP activities. Following the renaturation phase, gels were cut to form 2 sets of triplicate lanes. One set of triplicate lanes was not treated with an inhibitor (untreated samples), whereas the other set was treated with 1 of the inhibitors (treated samples). Gels were then incubated on a rotary shaker in developing buffer[n] for 24 hours at 37°C to allow the MMPs to digest the gelatin substrate. Inhibitors, except for ES, were added to the developing buffer during this 24-hour digestion phase to provide inhibitory effects. To test for inhibitory effects of ES, gels were incubated in undiluted pure ES for 2 hours at 37°C prior to being incubated in the developing buffer for 24 hours at 37°C.

After the digestion phase, gels were rinsed and stained by incubation with Coomassie blue[o] for 1 hour. Gels were then destained with a solution of 5% acetic acid 7.5% methanol to maximize contrast between lytic areas and nondigested areas.

Bands of proteolytic activity appeared uncolored against a dark-blue background. Inhibition of protease activity was manifested as a lack of band formation. Identity of putative proteases was determined by analysis of the distance that bands migrated on the gels, compared with the distance for migration of molecular weight and protease standards.

Investigators were careful to obtain a homogenous pool of tears for use in evaluating the untreated samples and samples treated with the various inhibitors. An equal volume of the pooled tear sample was loaded into each well; therefore, the same amount of protein and MMPs were contained in the untreated and treated samples. Thus, a difference in MMP activity observed in a gel between an untreated and a treated sample did not result from a difference in the concentration of loaded MMPs; instead, it was considered to be the difference

in proteolytic activity attributable to the inhibitor. We considered the comparison between the untreated and treated samples to be valid, and we believed that the gels represented the actual in vitro inhibitory effects of the tested compounds.

Image analysis—Digital photographs of stained gelatin zymograms were created by use of an imaging densitometer[p] and analyzed by use of quantification software.[q] Sensitivity of the analyzer was adjusted for each gel, and bands in each lane of the gels were then automatically detected by the analyzer. Background data was then subtracted for each triplicate set of lanes. Image analysis produced an intensity pattern of each lane by use of the OD function for the distance of migration from the top of the gel. The higher the amount of proteolytic activity, the more gelatin substrate was digested, which was reflected by a concomitant decrease in the amount of stain. **Area under the curve (AUC)** corresponded to the OD multiplied by the width of the band was calculated for each band. Variables used for comparisons in the study were the AUC, which allowed an accurate estimation of proteolytic activity in each sample (untreated and treated), as well as the amount of inhibition obtained with the various inhibitors. Image analysis provided a report that listed all the bands detected on a gel and the AUC for each band.

Statistical analysis—Ratios of AUC with inhibitor (ie, treated sample) to AUC without inhibitor (ie, untreated sample) for global proteolytic activity (ie, sum of all bands detected in a lane), as well as for activity of each protease (or band) detected, were calculated and compared by use of multiple-regression analysis. A statistical program[r] was used for estimations. For all analyses, results were considered significant at values of $P < 0.05$.

Results

Detection and identification of proteases in pooled tears by use of gelatin zymography—All triplicate lanes of untreated pooled tears had 7 bands (**Fig 1 and 2**). On the basis of the migration of the bands and standards on the gels, we determined that bands 4, 5, 6, and 7 corresponded to activity of the latent form of MMP-9, active form of MMP-9, latent form of MMP-2, and active form of MMP-2, respectively. Thus, pooled tears obtained from ulcerated eyes of horses contained the latent and active forms of MMP-2 and -9.

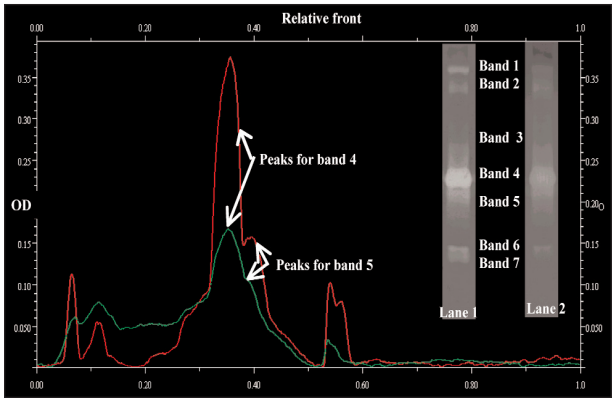

Figure 1—Image analysis of representative zymogram gels used to determine protease activity in pooled tears obtained from horses with an active corneal ulcer. Each gel on the right side of the figure (lane 1, untreated control sample; lane 2, treated with 0.1% α1-proteinase inhibitor [PI]) was scanned by use of an imaging densitometer. Notice that 7 bands were detected on each gel. Intensity patterns generated by the image analysis software for the 2 lanes (lane 1, red tracing; lane 2, green tracing) are indicated on the left side of the figure. The higher the intensity of the band, the higher the optical density (OD) and, therefore, the higher the proteolytic activity. For each band, AUC (which is calculated as the OD multiplied by the width of the band) was determined. Use of the AUC allowed an accurate estimation of proteolytic activity in the pair of samples (untreated and treated) and comparison of the AUCs allowed estimation of the inhibition rate for 0.1% α1-PI in relation to the untreated sample. Relative front = Proportional distance of migration from the top of the gel.

Inhibition of global proteolytic activity—When compared with MMP activity in the untreated tear samples, global proteolytic activity observed on the gels was reduced by 99.4% by EDTA, 96.3% by doxycycline, 98.8% by NAC, 98.9% by ilomostat, 52.4% by 0.1% α1-PI, 93.6% by 0.5% α1-PI, and 90.0% by ES (**Fig 3 and 4; Table 1**). We did not detect significant differences in inhibition rates among the various inhibitors tested, except for 0.1% α1-PI, which had a significantly ($P <$ 0.001) lower amount of inhibitory activity than that of any of the other compounds.

Inhibition of proteolytic activity for each protease—We also assessed the inhibitory effects of these various compounds on each protease in the pooled tears (Fig 3; Table 1). It is interesting that inhibition

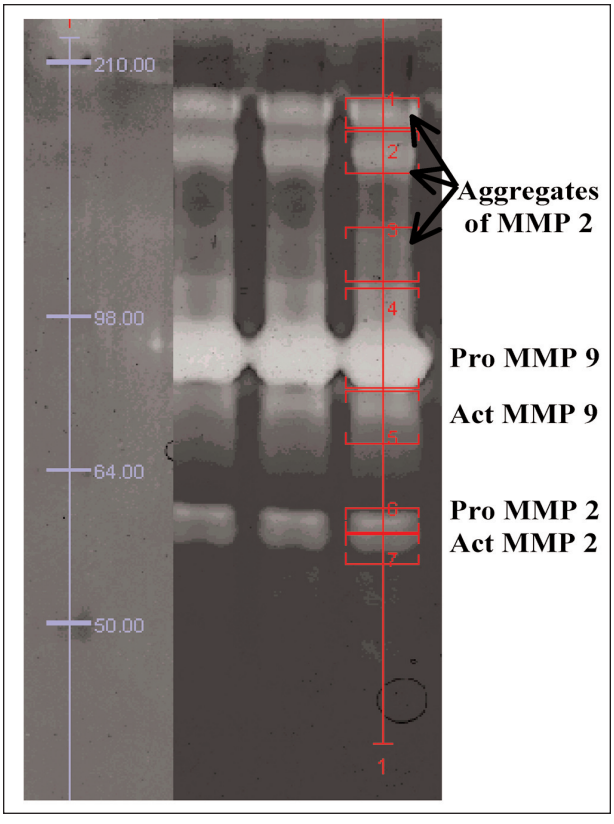

Figure 2—Gelatin zymogram of untreated pooled tears obtained from ulcerated eyes of horses. Pooled tears were loaded on each gel in triplicate and incubated without inhibitor (untreated samples) or with various protease inhibitors (treated samples) to assess effects on proteolytic activity of the proteases in the pooled tears. Molecular weight markers were included on the left side of the gel. Each of the 7 bands detected are indicated (red brackets) in the untreated lane on the right side. On the basis of their location on the gel and molecular weight, specific proteases were identified. MMP = Matrix metalloproteinase. Pro MMP-9 = Latent form of MMP-9. Act MMP-9 = Active form of MMP-9. Pro MMP-2 = Latent form of MMP-2. Act MMP-2 = Active form of MMP-2.

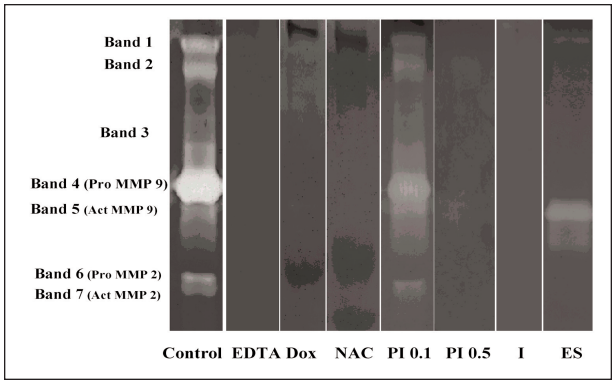

Figure 3—In vitro inhibition (mean value for triplicate samples) of global proteolytic activity, as well as proteolytic activity for each protease, was determined for various protease inhibitors by use of pooled tears obtained from ulcerated eyes of horses. Samples in each lane were treated as follows: untreated sample (control), 0.2% EDTA (EDTA), 0.1% doxycycline (Dox), 10% *N*-acetylcysteine (NAC), 0.1% α1-PI (PI 0.1), 0.5% α1-PI (PI 0.5), 0.1% solution of a modified dipeptide that contains hydroxamic acid (ie, ilomostat [I]), and fresh equine serum (ES).

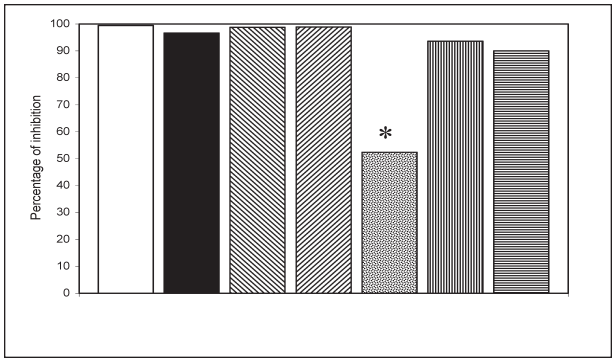

Figure 4—Percentage of in vitro inhibition (mean value for triplicate samples) of global proteolytic activity determined by use of gelatin zymography for various protease inhibitors in pooled tears obtained from ulcerated eyes of horses. Inhibitors used were as follows: 0.2% EDTA (white bar), 0.1% doxycycline (black bar), 10% NAC (diagonal lines from upper left to lower right of bar), 0.1% α1-PI (diagonal lines from upper right to lower left of bar), 0.5% α1-PI (dotted bar), I (vertical lines), and ES (horizontal lines). *Inhibition rate for this compound differed significantly ($P < 0.001$) from the inhibition rates for the other compounds.

was greatest for the latent form of MMP-9 and least for the active form of MMP-2 for EDTA, NAC, and 0.5% α1-PI, whereas inhibition was greatest for the active form of MMP-2 for doxycycline, 0.5% α1-PI, ilomostat, and ES. However, there was not a significant difference in inhibition rates for each protease among any of the tested compounds.

Discussion

Analysis of the results we obtained for the untreated (control) samples confirmed those reported elsewhere,[12] in which MMP-2 and -9 are found in substantially high amounts in the tear film of horses with ulcerative keratitis. The majority of host-derived and microbial enzymes in the tear film are MMPs.[9,10,30,35] Enzymes of bacterial or fungal origin (ie, exogenous proteases) can contribute directly or indirectly to ulcerative keratitis through the activation of corneal proteases (ie, endogenous proteases or proteinases).[1,3,35-37] In the study reported here, we assessed only the inhibitory effects of the various compounds on the global proteolytic activity that can be detected in ulcerated eyes of horses without distinguishing between proteolytic activity attributable to endogenous proteases and activity attributable to exogenous proteases. Thus, we did not take into account the specific cause of the corneal ulcers.

Protease inhibitors have been recommended and used in human and veterinary ophthalmology for many years. Their use remains controversial, because the efficacy and local toxic effects of these compounds are frequently questioned. In the study reported here, we documented a high amount of in vitro inhibition of equine MMP activity by the use of EDTA, doxycycline, NAC, ES, ilomostat, and α1-PI. For each of these protease inhibitors, the proposed mechanisms of inhibition, the current recommendations for use in human and veterinary ophthalmology, and the adverse effects that have been reported with each can be used to justify treatment of horses with ulcerative keratitis.

We observed a large reduction in the in vitro activity of equine MMPs with 0.2% EDTA. Doxycycline,

Table 1—Percentage of in vitro inhibition of global proteolytic activity, as well as proteolytic activity for each protease, determined for various protease inhibitors by use of pooled tears obtained from ulcerated eyes of horses

Band	EDTA	Dox	NAC	0.1 PI	0.5 PI	I	ES
1	96.3	86.1	94.3	46.8	94.5	89.1	90.7
2	99.9	96.3	99.2	22.5	87.0	95.1	87.4
3	96.6	96.5	97.8	44.6	96.3	92.1	63.6
4	99.8	98.4	99.8	55.3	94.9	99.7	91.4
5	98.6	95.9	98.7	46.6	92.3	99.1	82.4
6	100	95.0	97.0	78.8	90.5	98.4	95.9
7	98.5	98.8	96.3	77.8	70.7	100	96.6
Global	99.4	96.3	98.8	52.4*	93.6	98.9	90.0

Values reported represent mean value for triplicate samples. Band 4 represented the latent form of matrix metalloproteinase (MMP)-9. Band 5 represented the active form of MMP-9. Band 6 represented the latent form of MMP-2. Band 7 represented the active form of MMP-2.

*Inhibition rate for this compound differed significantly ($P < 0.001$) from the inhibition rates for the other compounds.

EDTA = 0.2% EDTA. Dox = 0.1% Doxycycline. NAC = 10% *N*-acetylcysteine. 0.1 PI = 0.1% α1-proteinase inhibitor. 0.5 PI = 0.5% α1-proteinase inhibitor. I = 0.1% Solution of a modified dipeptide that contains hydroxamic acid (ie, ilomostat). ES = Fresh equine serum.

EDTA, and NAC are metal-chelating agents, and they inhibit MMPs by chelating zinc or calcium, ions that MMPs require as a cofactor and stabilizing ion, respectively.[2,19] By chelating calcium ions, EDTA interferes with the stability of the MMPs and also decreases the stimulation for the migration of PMNs to the site of a corneal ulcer. The PMNs release powerful proteases that are also responsible for breakdown of stromal collagen. The EDTA interferes with attachment of opsonized zymosan to the cell membrane of PMNs, leaving the PMNs in a resting, inactivated, granulated state.[38] In addition, EDTA has been recommended for the treatment of ulcers associated with corneal collagenolysis[19,21,39]; however, corneal tolerance and efficacy of EDTA are subjects of controversy.[40,41] In rabbits, treatment of ulcerated corneas with 0.1% EDTA after keratectomy did not significantly affect the rate of reepithelialization.[42] Furthermore, EDTA seems to be tolerated well when used at concentrations ranging from 0.05 to 0.2% for treatment of corneal ulcers in horses.[1] However, the long-term use of EDTA may impair formation of tight junction complexes between epithelial cells as a result of the requirement of calcium ions for stable tight junctions.

In the study reported here, we documented in vitro inhibition of equine MMPs by 0.1% doxycycline. Tetracyclines inhibit MMP activity independent of their antimicrobial properties.[33] The proposed mechanism of action of these antimicrobial agents is that tetracyclines bind to zinc and calcium ions that are essential for the MMPs. Tetracyclines form a binding complex with the MMPs, resulting in reduced enzyme activity.[29,33,43] Tetracyclines, especially doxycycline, inhibit the synthesis of MMPs in human vascular endothelial cells.[29] Tetracyclines, such as minocycline and doxycycline, inhibit the breakdown of various connective tissues (ie, skin, bone, and cornea) mediated by excessive collagenolytic activity,[33] and they have been used specifically for ophthalmic treatments.[29,33] Doxycycline promotes healing of persistent ulcers and epithelial defects in humans[28,31] and inhibits alkali-induced corneal ulcers in rabbits.[44] Tetracyclines are recommended for use in ophthalmic conditions in animals,[19,25] but we are not aware of any information on the use of doxycycline in horses.

In our study, 10% NAC proved to be effective for inhibiting in vitro MMP activity in the equine tear film. This supports the fact that NAC is commonly used as an MMP inhibitor to treat humans and other animals with corneal ulcers.[19,45] Application of NAC at concentrations of 2 to 10% every 1 to 4 hours has been recommended for treatment of dogs[19,25,46] and horses.[19,25,47,48] More specifically, a combination of ES and 10% NAC has been recommended for the treatment of horses with severe corneal ulcers.[1] A thiol, NAC contains a sulfhydryl group that binds irreversibly or removes the intrinsic metal cofactor (ie, zinc) of the MMPs. It may also reduce 1 or more disulfide bonds of an enzyme.[45,49] Also, NAC inhibits MMP production at the transcriptional level.[50] In 1 study,[50] NAC suppressed MMP-9 synthesis in macrophages. In another study,[51] the effects of acetylcysteine on rabbit conjunctival and corneal surfaces were examined.[51] Investigators did not detect signs of ocular toxicosis for various concentrations tested, but 20% NAC caused superficial necrosis and dose-related disruption in the mucus layer of the tear film. Adverse effects were not found for the rate of reepithelialization of the rabbit corneas in which 10 and 20% NAC were applied as treatment for a superficial epithelial ulcer.[52]

In the study reported here, we detected high amounts of in vitro inhibition of MMP activity attributable to ES. The α_2-macroglobulin is a nonspecific protease inhibitor that reduces the activity of proteases of all 4 major protease classes (ie, serine [including neutrophil elastase], aspartic, thiol, and metalloproteinase in human and rabbit corneas).[2,4,23] The α_2-macroglobulin is a tetrameric molecule composed of 2 pairs of identical disulfide-linked subunits. Each subunit contains a region that binds the enzymes and allows the proteases to cleave peptide bonds. This cleavage leads to a change in conformation of the α_2-macroglobulin, resulting in entrapment of the protease within the inhibitor.[2,4,49] This particular mechanism tenaciously binds 2 protease molecules/α_2-macroglobulin molecule; thus, α_2-macroglobulin is 1 of the strongest known inhibitors of MMPs. Although α_2-macroglobulin mRNA and protein are found in the cornea,[2,14,53] this multifunctional inhibitor is found at much higher quantities in the blood.[14] For this reason, topical application of autologous serum[s,t] (1 or 2 drops every 1 to 2 hours) has been recommended for the treatment of corneal ulcers in humans[54] and other animals.[1,19,25] Blood collected into dry, sterile containers that do not contain anticoagulants will clot rapidly and yield serum that can be separated by centrifugation.[19] The serum can be used immediately or stored in a refrigerator until needed; in our experience, its inhibitory effect remains high even after several days of storage. However, it is recommended that serum be discarded if not used within 5 days, because it may provide an excellent medium for bacterial growth should it become contaminated.[1,19,26]

We observed a dose-dependent decrease in proteolytic activity of the MMPs in the pooled tears of horses for α1-PI at concentrations of 0.1 and 0.5%. It is possible that the dose-related reduction of activity of the MMPs attributable to this serine protease inhibitor is not a result of direct inhibition of MMPs by α1-PI; instead, it may result from α1-PI acting as a substrate for the MMPs and competing with the gelatin contained in the gels. Also called α1-antitryspin, α1-PI is found in and synthesized by the cornea,[24,55] but it is found at a higher concentration in the blood.[19,24] Furthermore, α1-PI is part of the serpin family of inhibitors that inhibit serine proteases, such as neutrophil elastase.[2,4] The α1-PI exerts its action on proteases through a mechanism similar to that for α_2-macroglobulin. It has the ability to form tight complexes with proteases and, therefore, reduces protease activity.[23] It has been documented that α1-PI is a critical substrate for MMP-9 in vivo, and MMP-9 acts upstream to regulate neutrophil elastase activity by inactivating α1-PI.[56]

During the past decade, efforts have been made to design synthetic inhibitors of proteases.[30,32,57,58] Among

those that have been reported, ilomostat appears to be promising for the treatment of rapid degradation of the corneal stroma.[32] It is more effective in vitro against MMPs in rabbits, compared with the classic chelating agents.[59,60] Ilomostat also decreases *Pseudomonas* alkaline protease activity in vitro[30] and is effective in reducing corneal destruction following alkali burns in rabbits[32] or intrastromal injection of pseudomonal culture broth.[30] In the study reported here, we reported that use of 0.1% ilomostat caused a high amount of inhibition of in vitro activity of MMPs in the equine tear film. Because the structure of MMPs is highly conserved among animal species, it is reasonable to expect that the inhibitory effects for this synthetic MMP inhibitor in rabbits would be seen in other domestic animals.

Multiple studies have found increased amounts of proteases in tears from humans and other animals with active corneal ulcers. Several agents have been proposed as treatments to reduce the activity of tear proteases, but we are not aware of any reports comparing the relative efficacy of the agents. This places practitioners in a difficult position of not knowing which treatment may be superior. In the study reported here, the relative efficacy for each of 5 protease inhibitors and ES were determined with regard to inhibition of MMP activity in pooled tears obtained from horses with active corneal ulcers. Analysis of our results indicates that EDTA and ilomostat, followed by NAC and doxycycline, are the most effective inhibitors in vitro and are likely to cause the fewest adverse effects. Because these compounds use different mechanisms to inhibit various families of proteases in equine tears, a combination of these inhibitors may be indicated for the treatment of severe corneal ulcers in horses. Objectives for the appropriate use of these compounds early in the course of corneal disease in horses would be to decrease the amount of time required for recovery and rehabilitation, reduce scarring, and potentially alleviate the need for surgical treatment of the cornea. Our results indicate that some readily available substances are effective inhibitors of proteases in tears of eyes with active corneal ulcers, which may provide practitioners with a possible valuable adjunctive treatment for vision-threatening diseases of the eyes.

[a]Eppendorf tubes, Brinkmann Instruments Inc, Westbury, NY.
[b]Novex tris-glycine SDS native sample buffer (2X), Invitrogen, Carlsbad, Calif.
[c]10% Novex zymogram gelatin gel, Invitrogen, Carlsbad, Calif.
[d]See Blue prestained standards, Invitrogen, Carlsbad, Calif.
[e]Active MMP-2 enzyme, Oncogen, Boston, Mass.
[f]Active MMP-9 enzyme, Oncogen, Boston, Mass.
[g]Proenzyme MMP-2, Oncogen, Boston, Mass.
[h]Proenzyme MMP-9, Oncogen, Boston, Mass.
[i]Vacutainer EDTA, Becton-Dickinson, Franklin Lakes, NJ.
[j]Doxy 100, American Pharmaceutical Partners Inc, Los Angeles, Calif.
[k]Acetylcysteine 20%, Abbott Laboratories, North Chicago, Ill.
[l]Vacutainer SST Plus, Becton-Dickinson, Franklin Lakes, NJ.
[m]Novex zymogram renaturing buffer (10X), Invitrogen, Carlsbad, Calif.
[n]Novex zymogram developing buffer (10X), Invitrogen, Carlsbad, Calif.
[o]Coomassie rapid stain, Diversified Biotech, Boston, Mass.
[p]GS-710 Calibrated imaging densitometer, Bio-Rad Laboratories, Hercules, Calif.
[q]Quantity One quantification software, 4.2.1 β version, Bio-Rad Laboratories, Hercules, Calif.
[r]Proc GLM, version 8.01, SAS Institute Inc, Cary, NC.
[s]Stangogiannis C, Stangogiannis E, Orellana M, et al. Epithelial promoting agents vs. autologous serum in reepithelialization after experimental mechanical debridement in vivo (abstr), in *Proceedings*. Annu Meet Assoc Res Vis Ophthalmol 2002;1680.
[t]Bron AM, Lafontaine PO, Garcher C. Treatment of persistent corneal epithelial defects by autologous serum (abstr), in *Proceedings*. Annu Meet Assoc Res Vis Ophthalmol 2002;1693.

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