In vitro efficacy of an ophthalmic drug combination against corneal pathogens of horses

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Objective—To evaluate the in vitro efficacy of an ophthalmic drug combination against common corneal pathogens of horses.

Sample Population—Representative isolates of 3 bacterial and 2 fungal corneal pathogens of horses.

Procedures—Pathogens were subjected to minimum inhibitory concentration (MIC) testing of a drug combination that consisted of equal volumes of natamycin 3.33%, tobramycin 0.3%, cefazolin 5.5%, and equine serum. Proteinase inhibitory activity of the drug combination was assessed by use of a fluorescence microplate assay with gelatin and collagen I as substrates. The MICs of the drug combination were compared with those for each of the component medications and antiproteinase activity of the drug combination was compared with that of serum by use of paired t tests and a 2-way ANOVA, respectively.

Results—The drug combination was at least as effective as each medication separately for inhibiting microbial growth of all pathogens tested and was significantly more effective against β-hemolytic Streptococcus spp, Aspergillus spp, and Fusarium spp than the relevant medications separately. Serum and the drug combination both had significant antigelatinase activity, and serum had significant anticollagenase activity. Antiproteinase activity of serum was a concentration-dependent event, which enabled serum to achieve significantly greater activity than the drug combination after 3.5 and 4 hours of incubation for the gelatin and collagen I assays, respectively.

Conclusions and Clinical Relevance—Drug combinations have the attractive potential of minimizing the time, stress, and fatigue associated with topical treatment regimens consisting of multiple drugs used separately for horses with keratitis. (Am J Vet Res 2008;69:101–107)

Ulcerative keratitis is a prevalent vision-threatening disease in horses. Thirty-five percent of all horses examined at the Ophthalmology Service of the University of Florida Veterinary Medical Center between January 1987 and October 2002 had ulcerative keratitis. Microbial culture and cytologic examination of corneal specimens commonly identify causative bacteria or fungal agents. Horses with moderate to severe ulcerative keratitis require antimicrobial treatment every 2 to 6 hours, with a gradual reduction in frequency of treatment as healing progresses.

Corneal melting, which is characterized by a gelatinous or soft appearance of the cornea, is often associated with infected corneal ulcers. The gelatinous appearance results from enzymatic destruction of the corneal ECM proteins. Uncontrolled corneal melting can progress to corneal perforation in as little as 24 hours and is therefore considered an emergency. Collagenases and gelatinases are 2 classes of proteinases whose excess is implicated as the cause of this ECM destruction in horses. They are derived exogenously from infective microbes and endogenously from resident corneal cells (keratocytes) and leukocytes that travel in the tear film. Collagenases degrade intact collagen fibrils, which are the principal support structure of the cornea. Gelatinases degrade denatured collagen; therefore, they may exacerbate melting after ECM injury is initiated. The mainstay of antimelting treatment at our facility is allogeneic serum because of its broad-spectrum antigelatinase and antibacterial activity. The bioavailability of serum, similar to that of other nonenhanced ophthalmic medications, is presumed to be low because 90% of the product is lost as a result of dilution in the tear film and turnover within minutes after application. Accordingly, rapid corneal melting is treated aggressively with topical application of serum as frequently as every hour.

Melting or infected corneal ulcers induce a substantial secondary uveitis. The combination of anti-

**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>CHP</td>
<td>Clostridium histolyticum proteinase</td>
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<td>MIC</td>
<td>Minimum inhibitory concentration</td>
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<td>MFI</td>
<td>Mean fluorescence intensity</td>
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microbials, antiproteinases, and anti-inflammatory medications often necessitates 24-hour frequent nursing care that can be taxing for clients and veterinary staff. Increasingly, veterinarians are combining medications into cocktails or ulcer mixtures to decrease treatment delivery time, decrease the stress associated with frequent patient handling, and increase treatment compliance. Such mixtures have been delivered via automated infusion pumps for further efficiency. Anecdotally, results have been favorable, but the efficacy of the drugs when mixed into a combination product has not been thoroughly investigated.

The study reported here was conducted to investigate the hypothesis that a drug combination would be as effective as its separate component medications against relevant corneal pathogens in vitro. The drug combination consisted of serum and 3 drugs commonly used at our facility for treatment of horses with severe, melting ulcerative keratitis. The antimicrobial efficacy of the drug combination against bacterial and fungal pathogens was compared with that for each of the antimicrobials separately. Antiproteinase effect of the drug combination against CHP was compared with that of serum alone.

**Materials and Methods**

**Sample population**—Bacterial corneal pathogens of horses were identified by searching the University of Florida database for the period from January 2003 through December 2006. Fungal corneal pathogens of horses were identified similarly, except the time frame for the database search was January 2005 through December 2005. Representative isolates of the 3 most commonly identified bacterial and 2 most commonly identified fungal species were shipped to an MIC testing laboratory.

**Drug combination and stability**—The drug combination was created by aseptically mixing equal volumes of natamycin 3.33%, cefazolin 5.5%, tobramycin 0.3%, and neat equine serum into a multiple-dose vial. The natamycin 3.33% and cefazolin 5.5% were prepared by diluting commercially available preparations of each drug in sterile water and artificial tears, respectively. The vial was stored in the dark at 4°C throughout the study. The pH of the drug combination was evaluated daily for 7 days by use of a pH meter. Optical clarity of the drug combination was subjectively scored on a scale of 1 to 4 (1 was clear and 4 was opaque) by the same investigator (NCS) daily for 7 days. Tobramycin 0.3% was used as a control sample; evaluation of pH, scoring of optical clarity, and storage of tobramycin were performed as for the drug combination.

**Antimicrobial efficacy**—Antimicrobial efficacy of each antimicrobial and the drug combination were quantified in vitro via triplicate MIC testing conducted in accordance with standard protocols and by use of custom-designed plates. The drug combination had been prepared ≤ 24 hours before MIC testing and stored at 4°C until used.

Once MICs of the drug combination were determined for each microbe, the MIC of each component medication was calculated by multiplying the MIC of the drug combination by 0.25 because each component comprised 25% of the drug combination. These back-calculated MICs were then compared with MIC values determined for each of the component medications when tested separately.

**Antiproteinase activity**—Proteinase inhibitory activity of the drug combination and serum were quantified by use of a commercially available gelatinase-collagenase assay conducted in accordance with manufacturer protocols. Briefly, solutions of fluorescein-quenched gelatin from pig skin or collagen I from bovine skin were placed in microplate wells. Clostridium histolyticum proteinase, a product with both collagenase and gelatinase activity, was then added to these wells at 0.2 U/mL for the gelatin assays (to induce gelatinase activity) and 1.0 U/mL for the collagen assays (to induce collagenase activity) on the basis of data obtained empirically by the authors. The antiproteinase of interest (ie, serum or the drug combination) was then added as a third component to the microplate wells in concentrations ranging from 12.5% to 100%. The various concentrations were prepared via dilution with the reaction buffer provided in the assay kit. Reaction buffer alone was used as an antiproteinase negative control sample, and 1,10-phenanthroline was used as an antiproteinase positive control sample. Destruction of proteins, with consequent release of fluorescein molecules, was quantified by measuring the MFI every 15 minutes for 5 hours with an automated microplate reader. Mean ± SD fluorescein excitation and emission were set at 485 ± 20 and 528 ± 20 nm, respectively. Background emissions were determined by calculating the mean MFI of 3 empty wells on each plate; background emissions were subtracted from all sample measurements before statistical analyses. All incubations were performed in duplicate. The incubation start time was designated as 0 hours. Results of other experiments have been used to validate the assay for use with equine samples, verify high intra-assay precision, and verify that results do not differ significantly when the drug combination is stored for up to 2 weeks.

**Statistical analyses**—Daily pH values, optical clarity scores, and MIC data were compared by use of paired t tests. Antiproteinase data were analyzed by use of a 2-way ANOVA. Values of P < 0.05 were considered significant.

**Results**

The database search yielded 266 corneal bacterial isolates representing 38 bacterial species. The 3 most commonly identified species (reported here as the number of isolates and percentage of total bacterial isolates) were β-hemolytic Streptococcus spp (47 [18%]), Pseudomonas aeruginosa (39 [15%]), and Staphylococcus aureus (25 [9%]). The database search yielded 28 corneal fungal isolates representing at least 5 fungal species. The 2 most commonly identified species were Aspergillus spp (10 [36%]) and Fusarium spp (2 [7%]).

**Antimicrobial efficacy**—The drug combination was at least as effective as each component medication.
for inhibiting microbial growth for all species of pathogens tested. The drug combination was significantly (P < 0.001) more effective than tobramycin alone for inhibiting β-hemolytic Streptococcus spp and natamycin alone for inhibiting Aspergillus spp and Fusarium spp (Figures 1 and 2). Mean ± SD values of the individual medications versus the calculated MICs of the medications as components of the drug combination, respectively, were calculated. For S aureus, cefazolin MICs were 1.33 ± 0.58 and 1.70 ± 1.51 µg/mL and tobramycin MICs were 10.83 ± 18.33 and 0.09 ± 0.08 µg/mL. For β-hemolytic Streptococcus spp, the MICs for cefazolin were 1.33 ± 0.58 and 0.83 ± 0.00 µg/mL and for tobramycin were 0.25 ± 0.00 and 0.05 ± 0.00 µg/mL. For P aeruginosa, cefazolin MICs were >512.00 ± 0.00 and 144.40 ± 232.23 µg/mL and tobramycin MICs were 43.00 ± 73.61 and 7.88 ± 12.67 µg/mL. For Aspergillus spp, natamycin MICs were 128.00 ± 0.00 and 16.65 ± 0.00 µg/mL, whereas for Fusarium spp, natamycin MICs were 32.00 ± 0.00 and 8.33 ± 0.00 µg/mL.

Antiproteinase activity—Several methods were used to evaluate antiproteinase activity of the medications. Results were determined for each method.

PROTEINASE ACTIVITY MEASURED VIA THE COMMERCIAL ASSAY
A direct relationship was detected between the CHP concentration and MFI activity (data not shown). All assays revealed a direct relationship between MFI and time, with notable differences in the MFI range, depending on the substrate. From 0 through 5 hours with gelatin as the substrate and a CHP concentration of 0.2 U/mL, mean ± SD MFI ranged from 95.00 ± 1.73 to 554.00 ± 4.00 (a range of 459 units). During the same time frame with collagen I as the substrate and a CHP concentration of 1.0 U/mL, MFI ranged from 77.50 ± 6.36 to 130.00 ± 11.31 (a range of 52.5 units).

SERUM ANTIPROTEINASE ACTIVITY
Relative to results of incubations with no inhibitory enzyme (those containing only protein substrate and CHP), incubations that contained serum had decreased MFIs. This was true for all serum concentrations tested and for both gelatin and collagen assays. Values differed significantly (P = 0.01) from hours 1 through 5 for all serum concentrations in the gelatin assay. For the collagen assay, only the 100% serum concentration decreased a significant decrease in MFI. Overall, an inverse
relationship was evident between serum concentration and MFI for the gelatin assay and, less distinctly, for the collagen assay, which suggested a concentration-dependent effect for the antiproteinase activity of serum (Figure 3). Relative to results of incubations with no inhibitory enzyme, incubations that contained the drug combination had decreased MFIs. This was true for all concentrations tested and for both gelatin and collagen assays, although the decreases were only significant for the gelatin assay. Similar to the serum dilutions, concentration and MFI were inversely related when gelatin served as the substrate, which suggested a concentration-dependent effect for the antigelatinase activity of the drug combination (Figure 4). Contrary to results for all other assays, there was a direct relationship between the drug combination concentration and MFI for the collagen assay.

#### Drug Combination Antiproteinase Activity

Relative to results of incubations with no inhibitory enzyme, incubations that contained the drug combination had decreased MFIs. This was true for all concentrations tested and for both gelatin and collagen assays, although the decreases were only significant for the gelatin assay. Similar to the serum dilutions, concentration and MFI were inversely related when gelatin served as the substrate, which suggested a concentration-dependent effect for the antigelatinase activity of the drug combination (Figure 4). Contrary to results for all other assays, there was a direct relationship between the drug combination concentration and MFI for the collagen assay.

#### Serum versus the Drug Combination in the Gelatin Assay

Mean MFIs of all time points for incubations that contained serum at 100% concentration and the drug combination at 100% concentration were significantly (P = 0.025) by 33.7%, and not significantly (P = 0.161) by 25.1%, respectively, relative to results for incubations with no inhibitory enzyme. For the first 3.5 hours of these incubations, there was no significant difference in MFIs between those that contained serum and those that contained the drug combination. After 3.5 hours, MFIs of
incubations that contained serum were significantly lower than MFIs of incubations that contained the drug combination (Figure 5). Similar to results for the gelatin assay, comparison between incubations that contained the drug combination at 100% concentration and serum at 25% concentration revealed no significant differences in mean MFI reduction at any time point (Figure 3).

**Stability of the drug combination**—No significant difference in mean ± SD pH was detected between the drug combination and tobramycin 0.3% (7.62 ± 0.17 and 7.71 ± 0.10, respectively). The drug combination rapidly settled out of suspension. When the drug combination was left undisturbed, particles would settle out within minutes. Manual shaking resulted in a fairly uniform pale-yellow opacity that did not vary throughout the duration of the study, and each daily optical clarity score was 4.

**Discussion**

The study reported here provided evidence that a drug combination consisting of equal volume of natamycin 3.33%, cefazolin 5.3%, tobramycin 0.3%, and equine serum was equally effective in vitro as each of the component medications against the common corneal microbes of horses tested. The MICs of the drug combination did not differ significantly from those of the component medications for any bacterial or fungal species tested, except that in some instances they were significantly lower. The increased efficacy of the drug combination may have been attributable to an increased spectrum of activity or drug synergy, with the latter being a reported phenomenon between cephalosporins and aminoglycosides. The reason the drug combination was not universally more effective against bacteria was likely related to the high SD values in some groups, which was a consequence of including a few highly resistant isolates. The cause of the dramatic reduction in antifungal MICs when these medications were components of the drug combination is unknown. Interestingly, the addition of EDTA to antifungal medications can significantly decrease their MICs for filamentous fungal organisms. Perhaps proteinase inhibition that results from EDTA and serum is involved in inhibiting fungal growth. Pairwise testing of the component medications contained in the drug combination (eg, natamycin plus cefazolin, natamycin plus tobramycin, natamycin plus serum, and the various other dual combinations) against each isolate is indicated to truly determine the source of the increased antifungal and antibacterial efficacy of this drug combination. Overall, analysis of the antimicrobial results revealed that the drug combination was at least as effective as each of the component medications for all bacterial and fungal species tested.

The fluorometric assay used in this study allowed quantification of both the antigelatinase and anticollagenase types of antiproteinase activity. More specifically, the assay quantified enzymatic destruction of dye-quenched proteins. As protein was degraded, fluorescein dye was released and its emission measured. Therefore, protein destruction was quantified by measurement of the MFI. When antiproteinase agents (ie, serum or the drug combination) were added to the assay, their activity was quantified in terms of reductions in MFI. Serum and the drug combination each had
significant antigelatinase activity for hours 1 through 5. Similarly, both serum and the drug combination had anticollagenase activity throughout the 5-hour incubation period, although this effect was only significant for the 100% concentration of serum.

We chose to focus comparisons between the antiproteinase activity of serum and the drug combination for only those assays that contained these agents at 100% concentrations because this mimics the forms in which these agents are currently being used (experimentally) in vivo. Both serum and the drug combination had significant antiproteinase effects for up to 3.5 hours in the gelatin assay and 4 hours in the collagen assay. After these time points, however, serum reduced proteinase activity significantly more than did the drug combination. We hypothesized that the dampened inhibition of the drug combination after 3.5 to 4 hours was likely attributable to the decreased concentration of serum in the drug combination (23% because it was 1 of 4 components), relative to that of serum when tested alone (100% concentration). This is supported by the fact that serum has a concentration-dependent effect on collagenase inhibition, as measured by hydroxyproline release in vitro, as well as by the concentration-dependent effect of serum revealed in both the gelatin and collagen assays in the study reported here (Figure 3).

To test this hypothesis, further evaluations were conducted between serum and the drug combination at dilutions for which the serum concentrations were equal (100% for the drug combination and 25% for serum). Analysis of the results revealed no significant differences in mean reduction of MFI between the serum-equilibrated concentrations of the components, which supported our hypothesis that the effect was dependent on the serum concentration.

We suspected that the lack of significant anticollagenase activity by the drug combination was related to the low limit of detection afforded by the fluorometric assay for the collagen I substrate. The MFI range was 52.5 units for the collagen assay, compared with a range of 459 units for the gelatin assay (Figures 3 and 4). Such a dramatic difference in the degree of enzymatic destruction between collagen type I and gelatin is not surprising given the superior molecular fortitude of collagen I. However, such a low and narrow MFI range may have allowed autofluorescence of the drug combination to overwhelm detection of the drug combination’s anticollagenase activity. The direct relationship between the drug combination concentration and MFI in this assay supports autofluorescence artifact. In contrast, all assays that contained serum, a more translucent inhibitory agent, consistently had a logical concentration-dependent and indirect relationship between drug combination and MFI. A higher concentration of CHP in the collagen I assay may have abrogated any autofluorescence artifact and resulted in detection of a significant anticollagenase effect of the drug combination. However, the concentration of CHP used in the study (1.0 U/mL) emphasized the fact that combining serum with other medications can significantly decrease the anticollagenase activity of serum.

The shortcomings of in vitro studies have been documented. However, for the study reported here, it is worth mentioning that there may be important differences between laboratory and clinical proteinase activity. First, the concentrations at which proteinases truly cause corneal melting in vivo have not been determined and therefore may not be adequately represented by the concentrations of CHP used in this study. Second, CHP was used in this study for its ability to act as a broad-spectrum proteinase that targets native collagen and other ECM proteins (including gelatin). However, the degree to which the proteinase activity of CHP correlates with the proteinase activity in animals with corneal melting is also unknown. Additional studies on the source, type, and concentration of proteinases that result in corneal melting in vivo would be necessary to make such comparisons. The species differences represented by the assays (ie, porcine gelatin and bovine collagen I) are not likely of major consequence because the structure of collagen is preserved among species.

We chose to focus comparisons between the antiproteinase activities of the drug combination and proteinase activity of serum have focused largely on its preservation of gela
tin or nonspecific collagen substrates. Collagen type I was chosen as a substrate in the study reported here because it is the predominant protein in corneas. To our knowledge, this is the first study to provide evidence on the ability of serum to specifically preserve collagen type I. The degree to which serum inhibited destruction of collagen type I (collagenase-specific destruction) in this study was 49% via fluorescence microplate assay. This is similar to the inhibitory capacity for serum against the proteinase activity of C histolyticum on whole equine corneas, as determined via a hydroxyproline release assay. This correlation suggested, albeit indirectly, that proteinase destruction of equine corneas was largely attributable to collagenase activity on collagen type I. This is of potential clinical relevance because improved knowledge of the specific substrates and enzyme classes involved in corneal melting will better enable clinicians to adopt an evidence-based approach to antiproteinase therapy.

Consistency of pH supports stability of the drug combination. However, the fact that the combination rapidly settled out of suspension is noteworthy. This may adversely affect treatment efficacy when delivered via automated subpalpebral lavage infusion pumps because active drug particles may never reach the subpalpebral lavage tubing. Manual shaking would be recommended before each treatment with this drug combination. Negative interactions between the medications have not been completely ruled out but were considered unlikely given sufficient antimicrobial and antiproteinase activities of the drug combination.

To our knowledge, the study reported here is the second in which in vitro antimicrobial efficacy of topical ophthalmic drug combinations has been described. In the other study, investigators evaluated the effects of various combinations of tobramycin 0.3%, gentamicin 0.3%, miconazole 1%, and atropine 1% against the growth of Pseudomonas spp and Aspergillus spp. The authors in that study found no significant difference between the antimicrobial efficacies of drug combinations, compared with the efficacy for each drug separately.

The aforementioned studies, as well as the study reported here, provide preliminary data to support
the use of drug combinations for infected corneal ulcers as well as for concurrent conditions such as corneal melting and uveitis. However, several important issues should be addressed before these combinations are considered justified for use in vivo. Of paramount importance is the judicious use of topical antimicrobials. Drug combinations consisting of multiple antimicrobials are indicated only in patients with corneal and susceptibility results that support such treatments or in patients at risk of corneal perforation (ie, patients with deep stromal or melting ulcers) that require treatment before such results can be obtained. Drug combinations also pose a risk of increased corneal toxicity relative to separate medications. The degree of toxic effects varies among drug combinations on the basis of their components and preservatives and should be weighed against potential benefits before administration. Finally, effects of the delivery route for drug combinations should be considered. Continuous infusions via automated pumps connected to subpalpebral lavage tubing are already in sporadic clinical use. Although the results of 1 preliminary study are promising, the impact of drug combinations on tear film dynamics, and therefore on corneal health, is unknown.

Drug combinations have the attractive potential of minimizing the time, stress, and fatigue associated with topical treatment regimens for horses with severe, melting ulcerative keratitis. The in vitro efficacy revealed in the study reported here supported anecdotal reports of clinical efficacy with drug combinations, although the antiprotease effect of serum is a concentration-dependent effect. To our knowledge, this study provided evidence for the first time of the inhibitory effect of equine serum on collagen type I destruction in vitro and introduced the possibility of improved antifungal efficacy of natamycin when delivered in combination with other keratitis medications.

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