

Effects of antifungal drugs and delivery vehicles on morphology and proliferation of equine corneal keratocytes in vitro

Rachel L. Mathes, DVM, MS; Adrian J. Reber, PhD; David J. Hurley, PhD; Ursula M. Dietrich, Dr Med Vet

Objective—To evaluate the effects of topical antifungal drugs and delivery vehicles on the morphology and proliferation rate of cultured equine keratocytes.

Study Population—16 corneas obtained from 8 apparently ophthalmologically normal horses < 0.5 hours after euthanasia for reasons unrelated to the study.

Procedures—Primary cultures of equine keratocytes were obtained from corneal stroma and were exposed to several concentrations of 3 commonly used, topically applied antifungals: natamycin, itraconazole, and miconazole. In addition, effects of drug delivery vehicles DMSO, benzalkonium chloride, and carboxymethylcellulose and a combination vehicle composed of polyethylene glycol, methylparaben, and propylparaben were also evaluated. Morphological changes and cellular proliferation were assessed 24, 48, and 72 hours after application.

Results—At the highest concentrations tested, all antifungals caused marked cellular morphological changes and inhibited proliferation. At low concentrations, natamycin and miconazole induced rounding, shrinking, and detaching of the cells with inhibition of cellular proliferation. Natamycin caused the most severe cellular changes. Itraconazole, at the low concentrations, caused minimal morphological changes and had a minimal effect on proliferation. All vehicles tested had significantly less effects on cellular morphology and proliferation when compared with the antifungals, except for the combination vehicle, which caused severe morphological changes and inhibited proliferation, even at low concentrations. The DMSO had minimal effects on cellular morphology and proliferation, even at high concentrations.

Conclusions and Clinical Relevance—Itraconazole had significantly less cytotoxic effects on equine keratocytes in culture than did natamycin or miconazole. Natamycin had severe cytotoxic effects in vitro. (*Am J Vet Res* 2010;71:953–959)

Fungal keratitis is a serious, vision-threatening disease that is common in horses, relative to other domestic species.^{1–3} In addition to bacterial inhabitants, horses have fungal organisms on the surface of their corneas and conjunctiva, in the healthy state. Ninety-five percent of horses reportedly have fungi typically present on their conjunctiva, in contrast to only 40% of cats and 22% of dogs.⁴ In healthy physiologic conditions, the cornea is protected by mechanical and physical barriers to help prevent pathogen-mediated entry. When a break occurs in this usual defense, however, invasion of bacteria and fungi into the cornea can easily occur.^{1,5,6} Horses likely develop fungal keratitis readily because of their ocular fungal flora and their proximity to fungi in their environment. Many features of fungal keratitis are unknown, in part because this disease appears unique to horses and data cannot be extrapolated

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From the Departments of Small Animal Medicine and Surgery (Mathes, Dietrich) and Population Health (Reber, Hurley), College of Veterinary Medicine, University of Georgia, Athens, GA 30602.

Supported by the University of Georgia Veterinary Ophthalmology Research Fund.

Address correspondence to Dr. Mathes (rmathes@uga.edu).

ABBREVIATIONS

BC	Benzalkonium chloride
BrdU	Bromodeoxyuridine
CM	Carboxymethylcellulose
DMSO	Dimethyl sulfoxide
M	Methylparaben
P	Propylparaben
PG	Polyethylene glycol 40 castor oil

from other domestic species. In addition, most research on fungal keratitis in horses has been based on in vivo studies^{2,3,6–10} with inherent limitations such as uncontrolled patient variables or limited patient numbers.

Equine fungal keratitis is difficult to treat, in part because of the strong pathogenic potential of fungal organisms.^{11,12} Fungi may cause proteinase-mediated corneal destruction or keratomalacia, allowing the organisms to invade into the corneal stroma.¹² Proteinases may be produced by the host's immune system or the fungal organisms.^{13–16} In addition to causing stromal destruction, fungi are believed to have a specific affinity for the corneal endothelial basement Descemet membrane, causing the organisms to travel deep into the posterior corneal stroma.¹⁷ Fungal keratitis, as a result,

often manifests as a deep stromal defect with extensive corneal involvement.^{18,19}

Topically applied ophthalmic antifungal drugs are the primary medications for treatment of fungal keratitis.^{20,21} Drugs from the azole and polyene families are the only antifungals used for topical ophthalmic administration.^{5,22–25} Both classes of drugs interfere with routine synthesis or function of the fungal cell wall.^{5,20,23} Natamycin, a polyene, is the only commercially available ophthalmic antifungal medication available for topical ocular application in the United States. It has the broadest spectrum of activity against fungi^{20,26} and has greater efficacy against all isolates of *Fusarium* spp when compared with the activities of other antifungals.^{6,26}

Other commonly used antifungal medications for topical application include miconazole, itraconazole, fluconazole, voriconazole, and ketoconazole.^{26–28} These products must be compounded for use in eyes.²⁹ Miconazole was effective against all isolates of *Aspergillus* spp and had good activity against other fungal isolates in a susceptibility study.²⁶ Miconazole also has good corneal penetration when applied topically.^{30,31} Itraconazole has a broader spectrum of activity against fungal organisms than ketoconazole and fluconazole and is highly effective against *Aspergillus* spp.^{21,25,26} Itraconazole is also reportedly effective for treatment of clinical equine keratomycosis.²⁵ Voriconazole, a new azole antifungal, has been recently investigated for use in horses, but its use topically is somewhat limited because of cost and unknown safety.^{23,32}

Little is known about the potential local corneal toxic effects of antifungals when administered topically in horses. Itraconazole (1%) ointment suspended in DMSO caused no corneal histologic or gross abnormalities in 6 ophthalmologically normal horses when applied topically.²⁴ A pharmaceutical study³² involving voriconazole was conducted to assess ocular toxic effects on the basis of clinical descriptions of epiphora, blepharospasm, and conjunctival hyperemia; however, no direct corneal toxic effects were evaluated. The purpose of the study reported here was to evaluate the direct effects of commonly used antifungals (natamycin, itraconazole, and miconazole) and their typical drug delivery vehicles on equine corneal keratocyte morphology and cellular proliferation in vitro.

Materials and Methods

Primary equine keratocyte culture—Equine keratocytes were obtained in primary cell culture according to described methods.³³ Briefly, 16 corneas were aseptically collected from 8 apparently ophthalmologically normal horses < 0.5 hours after euthanasia for reasons unrelated to the study. The corneal layers were enzymatically separated first by incubation for 8 minutes in 0.5% trypsin^a at 37°C in 5% CO₂ to separate the endothelium from the stroma and epithelium. The stroma and epithelium were subsequently incubated in neutral protease^b (2.4 U/mL) for 1 hour at 37°C in 5% CO₂. After mechanical removal of the epithelium with a sterile cell scraper, the stromal layer was sectioned into several 3- to 4-mm explants and 2 to 3 explants were placed into each well in 6-well collagen-coated plates.^c Afterward, a solution of Dulbecco modified Eagle medium

and Ham F-12 nutrient mixture^c supplemented with 5% fetal bovine serum,^d 200 U of penicillin/mL, 200 µg of streptomycin/mL,^e 0.5 µg of amphotericin B/mL,^a 20 ng of epidermal growth factor/mL,^f 5.6 µg of insulin/mL,^f and 4mM L-glutamine^d was added to each well, and the plates were incubated at 37°C in 5% CO₂. The explants were removed after 3 days of incubation, and medium in the wells was changed every 1 to 3 days. After the cells became confluent in approximately 7 days, 0.5% trypsin was added to each well and plates were incubated at 37°C and 5% CO₂ for 8 minutes until the cells were detached and separated. Medium was added to the cells to stop the enzymatic reaction, and the cell suspension was centrifuged for 7 minutes at 800 X g. The supernatant was removed, and the cell pellet was resuspended in medium and plated in a 25- or 75-cm² tissue culture flask. Keratocytes were passaged in the described manner when confluency was reached in the flasks. Keratocytes were identified morphologically and immunocytochemically with anti-vimentin antibody staining as described elsewhere.³³ Passages 2 through 8 were used for the study.

Equine keratocyte morphological evaluation—For morphological evaluation, keratocytes were plated into 24-well tissue culture plates^c at a density of 2 X 10⁴ cells/well. After plating, the cells were allowed to adhere and grow for 48 hours. At this time, the selected antifungals in their respective vehicles and the vehicles alone were added in media at the following concentrations: BC^g (20, 2, and 0.2 µg/mL); CM^h (600, 60, and 6 µg/mL); PG^h (1.15%, 0.115%, and 0.0115%), M^h (72.5, 7.25, and 0.725 µg/mL, respectively), and P^h (7.5, 0.75, and 0.075 µg/mL, respectively); DMSO^d (6,000, 600, and 60 µg/mL); natamycin^h (5,000, 500, and 50 µg/mL); itraconazole^h (1,000, 100, and 10 µg/mL); and miconazole^h (1,000, 100, and 10 µg/mL). All substances had a pH of 7.0 to 7.5.

Morphology of the cells was evaluated at 24, 48, and 72 hours after addition of treatments. Each application and grading was performed 4 times on separate cell passages (passages 2 through 8). All trials were performed with a control well consisting of media and an equal volume of PBS solution^d added at the highest treatment volume. The highest concentration of all antifungals and delivery vehicles represented the physiologic concentration after topical administration, accounting for tear film dilution.^{34–39} Concentrations of all drugs and vehicles were diluted 10-fold for each lower concentration.

Morphological changes were scored on the basis of the following grading scale: 0 = no evidence of any morphological changes, no cell detachment from the plate, and normal cell adherence and appearance in culture; 1 = normal cell adherence to the plate and < 30% of cells with evidence of rounding or loss of typical spindle appearance; 2 = 10% to 20% cell detachment from the plate and 30% to 60% of cells with evidence of rounding or loss of normal spindle appearance; 3 = 20% to 50% detachment from the plate and > 60% of cells with evidence of rounding, shrinking, or loss of normal spindle appearance; 4 = 50% to 90% detachment of cells from plate, severe rounding of all cells, and complete loss of spindle appearance in all cells; and 5 =

100% detachment from plate, no recognizable keratocytes in culture, loss of cells, and severe shrinking and rounding. It was not possible to blind the investigators to the treatments because the addition of antifungals and carriers made easily distinguishable differences in media appearance between treatment groups. This was because of viscosity differences in the drug vehicles and poor solubility of the antifungals.

Equine keratocyte cellular proliferation—For evaluation of cellular proliferation, keratocytes were plated into 96-well tissue culture plates^c at a concentration of 5×10^3 cells/well. The cells were allowed to adhere and grow for 48 hours before application of the specific treatments. After this, 100 μ L of the treatments was added to each well at the following concentrations: BC (20, 2, and 0.2 μ g/mL); CM (600, 60, and 6 μ g/mL); PG (1.15%, 0.115%, 0.0115%, 0.00115%, and 0.000115%), M (72.5, 7.25, 0.725, 0.0725, and 0.00725 μ g/mL, respectively), and P (7.5, 0.75, 0.075, 0.0075, and 0.00075 μ g/mL, respectively); DMSO (6,000, 600, and 60 μ g/mL); natamycin (5,000, 500, 50, 5, and 0.5 μ g/mL); itraconazole (1,000, 100, and 10 μ g/mL); and miconazole (1,000, 100, 10, 1, and 0.1 μ g/mL). The highest concentration of all antifungals and delivery vehicles represented the physiologic concentration after topical administration, accounting for tear film dilution. Concentrations of all drugs and vehicles were diluted 10-fold for each lower concentration. Antifungals and vehicles that inhibited cellular proliferation at 1,000-fold were diluted further in 2 more 10-fold dilutions to reach a point at which cellular proliferation was not inhibited. All trials were performed with a control well consisting of medium and an equal volume of PBS solution^d added at the highest treatment volume. Each proliferation evaluation was performed at least 3 times on different passages (passages 2 through 5) and evaluated at 24, 48, and 72 hours after treatment application. Early passages were used for proliferation to help eliminate variability between inherent proliferation properties of early versus late passage cells.

To evaluate cellular proliferation, 20 μ L of 1:500 5'-BrdUⁱ was added to each well and allowed to incubate for 24 hours at each time point. Cells were processed in accordance with the manufacturer'sⁱ protocol. Briefly, cells were fixed for 30 minutes. After multiple washings, the cells were then incubated with mouse anti-BrdU monoclonal antibody (1:200 dilution) for 1 hour. This was followed by another series of washes and incubation with peroxidase goat anti-mouse IgG antibody (1:2,000 dilution) for 30 minutes. After another series of washes, prediluted 3,3',5,5'-tetramethylbenzidine peroxidase was added for 30 minutes. After cells were incubated in the dark, stop solution was added and the plates were read at 450 nm with a spectrophotometric microtiter plate reader. For each trial, wells that contained cells but did not have BrdU added were included in assay processing. This baseline reactivity was subtracted from all values. Results are expressed as a percentage of control sample proliferation, after subtraction of the baseline reactivity value.

Statistical analysis—Morphological characteristics were compared among groups by use of 1-way ANOVA

with a Bonferroni correction for multiple intergroup comparisons. Degree of cellular proliferation was evaluated by use of 1-way ANOVA with a Bonferroni correction for multiple comparisons among groups at the 10-, 100-, and 1,000-fold dilutions. Because natamycin and miconazole dilutions were carried beyond those of the other treatments for the cellular proliferation experiments, data for these 2 groups of treatments were compared with an unpaired *t* test at the 10,000- and 100,000-fold dilutions. Results were considered significant at a value of $P \leq 0.05$.

Results

Growth and morphology of equine keratocytes—Keratocytes were small, spindle cells in culture, with a high growth rate. These cells were morphologically and immunocytochemically confirmed to be a monoculture.³³

Effects of applied substances—Cellular morphology was graded at all time points for all concentrations of antifungals and delivery vehicles. Each experiment was repeated 4 times, and the scores were averaged for comparison among groups. Morphological scores for control wells were consistent with grade 0 at all time points for all experiments. Morphological changes in keratocytes were compared between each antifungal in its vehicle and the vehicle alone to ensure that any changes were attributable to the active drug and not to vehicle effects (Figure 1). The scores for natamycin and itraconazole treatments were significantly higher than scores for their respective vehicles when compared at each concentration tested. The scores for miconazole and its vehicle, PG-M-P, were not significantly different, making it impossible to determine whether the cellular effects were due to the active drug or to the vehicle. Miconazole was therefore suspended in a new vehicle, CM and DMSO, to determine whether the morphological effects were due to the active drug or the carrier (Figure 2). Keratocyte treatment with the new vehicle resulted in significantly lower scores when compared with the scores for miconazole, indicating that the morphological changes were due to miconazole and not to its carrier.

Antifungal morphological effects were then compared with each other at all concentrations. Only the morphological scores for miconazole in the new vehicle (CM-DMSO) were used for comparison among antifungals. This was done to ensure that morphological scoring was not confounded by drug vehicle effects from the combination carrier PG-M-P. Itraconazole had the least effect on cellular morphology at all time points when compared with natamycin and miconazole. Natamycin caused the most severe morphological changes to the cells. All antifungals yielded marked morphological changes with cell rounding, shrinking, and detachment at the highest concentration tested (Figure 3). At all time points (24, 48, and 72 hours after addition of treatments), the 100-fold dilution of itraconazole had significantly less effects on cellular morphology than did natamycin and miconazole at the same concentration. At the 1,000-fold dilution, natamycin yielded significantly greater morphological effects than did itraconazole and miconazole at all time points.

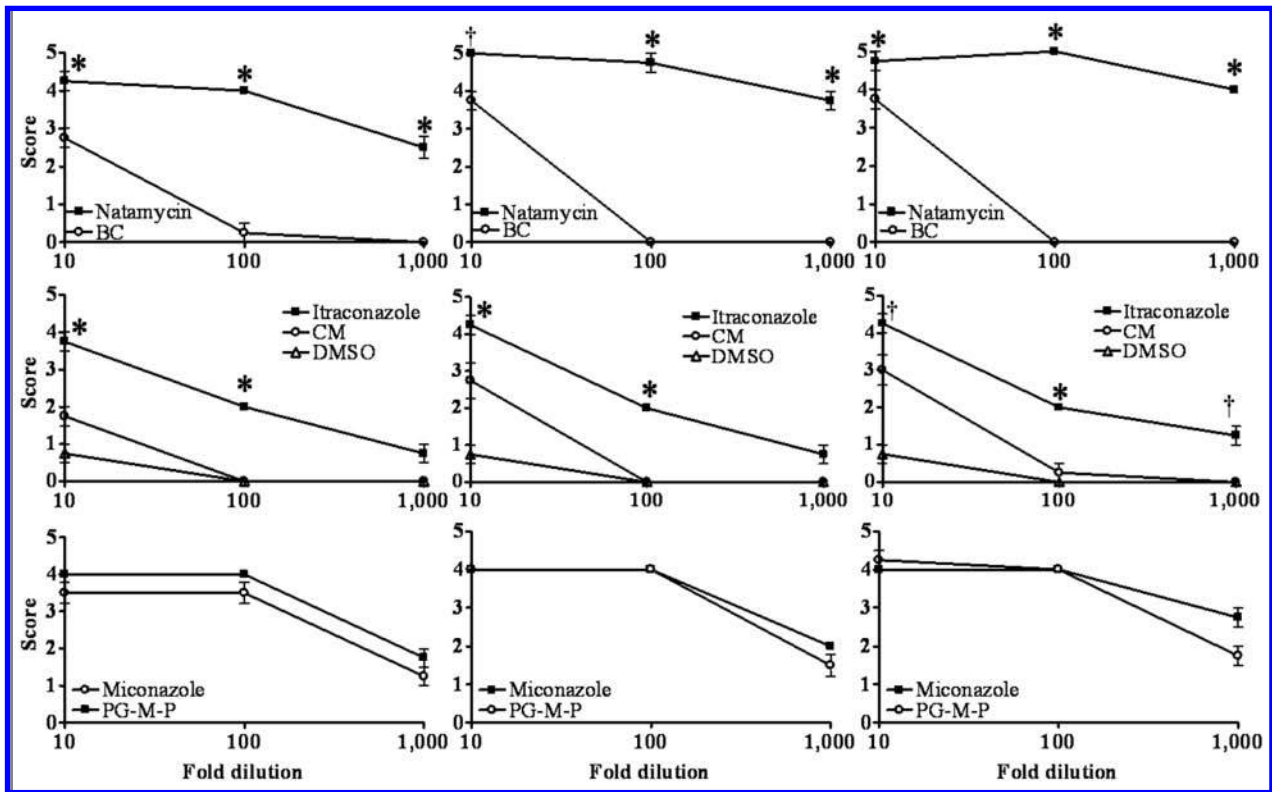


Figure 1—Mean \pm SEM morphological scores for equine keratocytes exposed to natamycin, itraconazole, and miconazole (original concentrations: 50,000, 10,000, and 10,000 $\mu\text{g}/\text{mL}$, respectively) in their respective vehicles (BC, CM and DMSO, and PG-M-P, respectively) or to their vehicles alone at clinically relevant concentrations, adjusting for tear film dilution. Morphological changes were scored on a 5-point scale (0 = no evidence of any morphological changes, no cell detachment from the plate, and normal cell adherence and appearance in culture; 5 = 100% detachment from plate, no recognizable keratocytes in culture, loss of cells, and severe shrinking and rounding) for each dilution at 24 (left column), 48 (middle column), and 72 (right column) hours after treatment application. *†Values differ significantly (* $P < 0.001$, † $P < 0.01$) between the antifungal and its vehicle or vehicles at the indicated dilution.

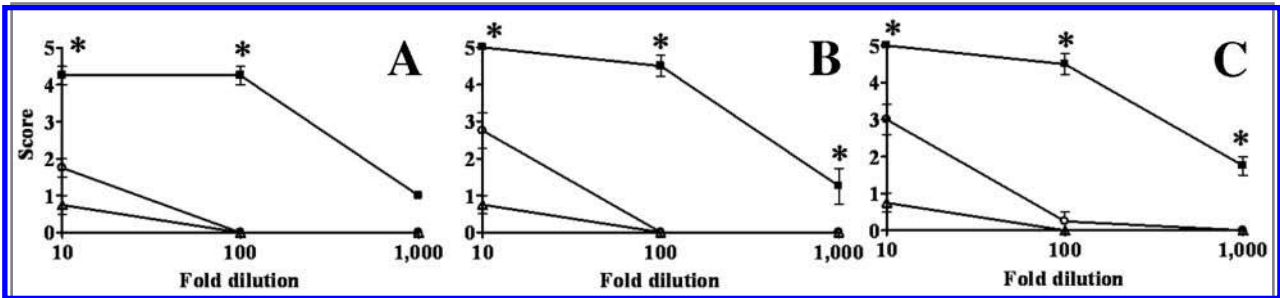


Figure 2—Mean \pm SEM morphological scores for equine keratocytes exposed to miconazole (original concentration: 10,000 $\mu\text{g}/\text{mL}$) suspended in CM and DMSO, compared with scores for exposure to CM and DMSO alone, at various dilutions. Morphological changes were scored for each dilution at 24 (A), 48 (B), and 72 (C) hours after treatment application. The highest concentration tested (10-fold dilution) is consistent with the antifungal concentration in a topical ocular application, accounting for tear film dilution. See Figure 1 for key.

Effects of antifungals and delivery vehicles on cellular proliferation—Cellular proliferation was also evaluated at specified time points for each substance at different concentrations. Effects of antifungals in the respective vehicles were compared with vehicles alone to ensure that effects on proliferation were due to the active drug and not to the carriers. Results for comparisons of vehicle and antifungal effects were similar to the results for comparisons of morphological changes to the cells after substance application (Figure 1). Natamycin and itraconazole vehicles alone had significantly less of an effect on cellular proliferation than did the

drugs in their respective vehicles (data not shown). The miconazole vehicle, PG-M-P, did not have significantly different inhibition of proliferation when compared with miconazole in PG-M-P. Miconazole was therefore suspended in the alternative carrier, CM-DMSO, as was done for morphological assessments, and proliferation data were collected for this substance. Miconazole effects on proliferation were significantly higher when compared with the alternative carrier (data not shown). Cellular proliferation data were compared among all antifungal groups by use of the data for cell exposure to miconazole in CM-DMSO, as was done for morpho-

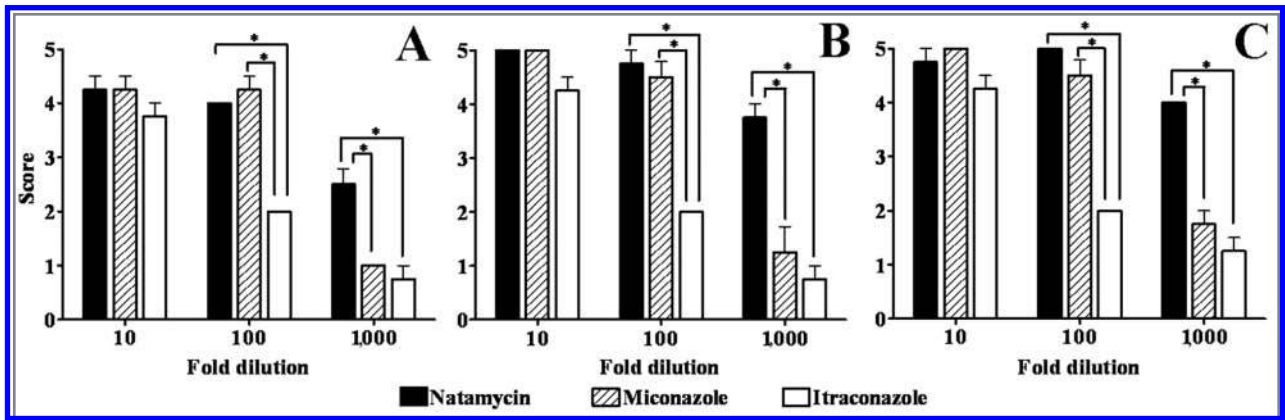


Figure 3—Mean ± SEM morphological scores for equine keratocytes exposed to natamycin, miconazole, and itraconazole at 24 (A), 48 (B), and 72 (C) hours after treatment application. *Indicated values differ significantly ($P < 0.001$).

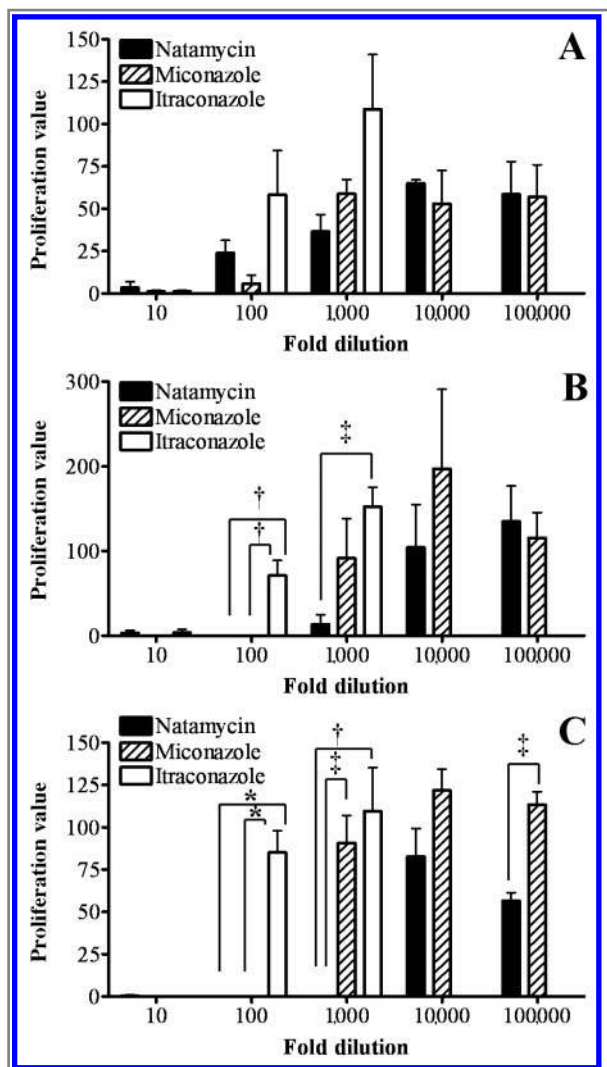


Figure 4—Mean ± SEM cellular proliferation values (percentage of control sample proliferation) for equine keratocytes exposed to natamycin, itraconazole, and miconazole (original concentrations: 50,000, 10,000, and 10,000 $\mu\text{g}/\text{mL}$, respectively) at clinically relevant concentrations, adjusting for tear film dilution. Degree of proliferation was assessed 24 (A), 48 (B), and 72 (C) hours after treatment application. *†‡Indicated values differ significantly (* $P < 0.001$, † $P < 0.01$, and ‡ $P < 0.05$).

logical comparisons to eliminate confounding vehicle effects.

The 10-fold concentration of all antifungals virtually eliminated all cellular proliferation when applied to the keratocytes at all time points (Figure 4). At 24 hours after application, itraconazole inhibited proliferation less than did natamycin and miconazole at the 100- and 1,000-fold dilutions; however, these results were not significant. Natamycin and miconazole dilutions were increased to reach a point at which cellular proliferation was not inhibited to allow comparison between these 2 antifungals. At 24 hours after application, natamycin and miconazole inhibited proliferation to a degree less than that of the control treatment, even at 10,000- and 100,000-fold dilutions. At 48 hours, itraconazole had minimal effects on cellular proliferation, whereas natamycin and miconazole markedly inhibited proliferation at 100-fold dilutions. At 1,000-fold dilutions, itraconazole did not affect proliferation, whereas natamycin caused marked inhibition. At further dilutions, natamycin and miconazole did not affect cellular proliferation at 48 hours. Similar results were evident at 72 hours after application, with itraconazole having the least effect on cellular proliferation when compared with natamycin and miconazole. At the lowest concentration (100,000-fold dilution), miconazole exposure resulted in significantly less inhibition of cellular proliferation than did natamycin exposure.

Discussion

Equine keratocytes have several characteristics in vitro that make them ideal for drug application studies. The cells have predictable small, spindle cell morphology in culture, whether subconfluent or confluent, and readily form a monolayer.^{33,40} These 2 characteristics allow for definitive grading criteria when evaluating changes after drug application. Keratocytes can also readily undergo multiple passages without changes to their morphology or immunocytochemical staining properties.³³ In addition, keratocytes have a high growth rate, allowing for cellular proliferation evaluation after drug application as a measurement of cell health. Although it would have been ideal to additionally evaluate equine corneal epithelial cells in the study

reported here, our purified equine corneal epithelial cells did not lend themselves to this type of evaluation because of their slow growth, clustering, and short life span in culture.³³ Other studies^{40,41} have demonstrated similar problems with purified corneal epithelial cells.

Clinically, cases of equine keratomycosis are almost always associated with stromal disease and are usually ulcerative.^{2,12,20,26} Because of this, topically applied antifungals are usually in direct contact with keratocytes during treatment of fungal keratitis. Antifungal penetration increases dramatically when the corneal epithelium is removed,⁴² and epithelial debridement is often performed in horses with fungal keratitis to optimize drug delivery.² Keratocyte health would presumably be of utmost importance, therefore, in the treatment of horses with keratomycosis.

Drug concentrations in the present study were selected to be clinically relevant concentrations of topically applied drugs. Ophthalmic medications that are topically applied are immediately diluted because of characteristic properties of the tear film.³⁵⁻³⁹ This dilution is considerable, and it is estimated that anywhere from 1% to 10% of a topically applied drug is actually available on the corneal surface immediately after application.^{35,36,39} Whereas surfactants and suspending agents may increase the viscosity of the drug suspension, allowing for potentially longer contact time, there is still very little drug remaining in contact with the corneal cells after 4 to 23 minutes.^{36,38} Because of irritation, tear production increases in animals with corneal disease and after application of topical medication.^{35,36,43} Because of this, the highest applied concentration of antifungals and vehicles in our study reflected the concentration of drug that would be found in contact with corneal cells immediately after application, taking into account tear dilution properties. This concentration is 10% of the ophthalmic drug preparation. The first concentration was then subsequently diluted 10-fold for the lower concentrations. Because the drugs and vehicles maintained constant contact with the cells, the lower concentrations used were likely more indicative of the clinical exposure of the keratocytes to an antifungal, even with frequent topical administration.

Antifungals and delivery vehicles used in clinical settings were selected for this study to evaluate *in vitro* cellular effects. When antifungal effects were compared with drug vehicle effects, we found that 1 vehicle, PG-M-P, caused marked morphological changes, even at the low concentrations tested (Figure 1). Polyethylene glycol is a commonly used surfactant and is likely desirable as an antifungal vehicle because its viscosity helps increase topical drug contact time with the corneal surface. Methylparaben and P are commonly used as preservatives. The ingredients in this vehicle are similar to those used in many compounding pharmacies.²⁹ The PG-M-P vehicle was believed to be representative of what would be used in a clinical setting because similar techniques are used to optimize delivery of antifungals because of the low drug solubility. Benzalkonium chloride, which is another common preservative, and CM, a suspending agent, both had minimal to no cellular effect at the lower concentrations tested. Various surfactants and suspending agents are used as delivery

vehicles to optimize contact time when antifungals are topically applied. Our results suggested that certain delivery vehicles may have less cytopathologic effects than others, although more research is required to fully determine *in vivo* effects. However, our findings underscore the importance of discriminant selection of delivery vehicles on the basis not only of their surfactant and suspending properties but also of their potential deleterious cellular effects.

Because many antifungals have low solubility in aqueous solutions, we tested a commercially available solvent, DMSO, for its effects on the keratocytes. Dimethyl sulfoxide increases the penetration of itraconazole to facilitate drug delivery in the cornea^{24,25} and likely has this effect for other antifungals. In our study, DMSO had very little effect on keratocytes, even at the high concentrations tested. Because DMSO increases drug delivery and has minimal cytopathologic effects on equine keratocytes *in vitro*, it may be an ideal antifungal delivery vehicle for topical ocular administration.

In our study, itraconazole had markedly lower cytopathologic effects in comparison with miconazole and natamycin. At the low concentrations, miconazole was less cytotoxic than natamycin. For cellular proliferation experiments, miconazole and natamycin were diluted further to reach a point at which the drug concentration used did not have a significant effect. This was done to more effectively compare the 2 drugs at lower concentrations. Although more research is needed to fully evaluate the *in vivo* cellular effects of antifungals, this study demonstrated measurable cytopathic effects of antifungals on keratocytes at clinically relevant concentrations. Although selection of an antifungal agent can be based on many factors, cytopathologic effects should be considered, particularly with repeated administration of these agents for extended periods.

The purpose of the study reported here was to evaluate the effects of antifungals and their vehicles on cultured equine keratocytes. Compared with *in vivo* testing, *in vitro* drug testing has many benefits including high reproducibility, lower cost, elimination of patient-related concerns, decreased personnel requirements, enhanced control of variables, and the possibility of larger sample sizes. Equine corneal keratocytes may be grown in culture and used to evaluate direct drug effects by use of criteria directly related to cell health in culture, such as cell morphology and proliferation. Our study showed that itraconazole had significantly less cellular effects than the other 2 antifungals tested. It also revealed that different drug vehicles have markedly different cellular effects. Effects of antifungals and vehicles on corneal cell health should be accounted for when selecting a drug for *in vivo* application because cytotoxic drug effects may decrease corneal healing.

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- a. Invitrogen, Carlsbad, Calif.
 - b. Dispase II, Gibco, Burlington, ON, Canada.
 - c. DMEM-F12, Fisher Scientific, Norcross, Ga.
 - d. Fisher Scientific, Norcross, Ga.
 - e. Mediatech Inc, Manassas, Va.
 - f. Sigma Chemical Co, St Louis, Mo.
 - g. Winthrop-Breton Laboratories, New York, NY.

- h. Wedgewood Pharmacy, Swedesboro, NJ.
- i. Exalpha Biologicals Inc, Watertown, Mass.

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