Toxicity of Topical Antifungal Agents to Stratified Human Cultivated Corneal Epithelial Sheets

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Abstract

Purpose: Prolonged use of topical antifungal agents may compromise corneal epithelial integrity. Here, we used an *in vitro* model of human stratified corneal epithelium to compare the ocular toxicity profiles of 4 different antifungal eye drops.

Methods: Human corneal epithelial cell sheets were cultured in a serum-free medium containing 0.1% micafungin, 1% voriconazole, 5% pimaricin, 0.1% amphotericin B, or controls (saline or 5% glucose). Cell viability and barrier function were measured by WST-1 assay and carboxyfluorescein permeability assay, respectively. Cell migration was measured on a wound healing assay.

Results: WST-1 assay and carboxyfluorescein permeability assay revealed that amphotericin B was the most toxic drug, followed by pimaricin, micafungin, and voriconazole. Cell migration on a wound healing assay was decreased in the following order, amphotericin B, pimaricin, micafungin, and voriconazole.

Conclusions: Topical micafungin and voriconazole appeared to be the least toxic to the corneal epithelium. Drug prescription should consider not only fungal species and susceptibility but also ocular toxicity and stage of treatment.

Introduction

FUNGAL KERATITIS (KERATOMYCOSIS) is a sight-threatening infection that occurs in the cornea.¹ It has the potential to cause devastating complications, including corneal ulcer, perforation, and blindness.² Despite the increasing prevalence of fungal infections, few topical therapeutic agents for treating fungal keratitis are commercially available, and ophthalmologists often use antifungal eye drops prepared in their hospital's pharmacy department. However, fungal keratitis is intractable and requires prolonged use of antifungal eye drops. Under such circumstances, the corneal epithelial integrity might be compromised. Therefore, it is necessary to have good knowledge of the toxicity of these antifungal agents toward the ocular surface. The toxicity of several topical antifungal eye drops has been previously reported,³ but comparisons of their toxicity profiles are more rare.³

Human corneal epithelial cell sheets (HCES) have been clinically used for the reconstruction of ocular surface diseases such as limbal stem cell deficiency. Recently, we have established primary cultures of HCES in a serum-free culture system.⁴ Because the HCES form layers 4 to 7 cells thick and closely mimic the *in vivo* stratified corneal epithelium in humans, they also provide a good *in vitro* model for studying cellular toxicity. In fact, we have previously

determined the toxicity profiles of topical antiglaucoma drugs using these cell sheets.⁵

In this study, we compared the toxicity of 4 topical antifungal agents (micafungin, voriconazole, pimaricin, and amphotericin B) to HCES. Micafungin is a (1,3)- β -D-glucan synthase inhibitor.⁶ Voriconazole exerts its effect through inhibition of cytochrome P450-dependent 14 α -sterol demethylase, an enzyme responsible for the conversion of lanosterol to 14 α demethyl lanosterol in the ergosterol biosynthetic pathway.⁷ Pimaricin and amphotericin B combine with cell membrane sterols, inducing changes in the cell membrane structure. These 4 antifungal compounds are available in our hospital and cover a wide range of mechanisms of action. In this study, we describe their effects on cell viability, permeability, and migration.

Methods

Preparation of stratified cultivated HCES

HCES were cultivated as previously described.⁴ In brief, human corneas were obtained from an eye bank (SightLife, Seattle, WA). Each experiment was performed in duplicate or triplicate with a combination of cells isolated from 2 to 3 different donors. The limbal tissues were cut and incubated

overnight at 37°C in a basal culture medium [Dulbecco's modified Eagle's medium (DMEM)-F12] with 0.02% type 1A collagenase. Cells were collected from incubated tissues in tubes coated with 0.05% trypsin/EDTA for 10 min at 37°C, and then dissociated into single cells by pipetting. After washing the trypsin/EDTA, the cells were resuspended in a medium with 20 ng/mL of epidermal growth factor (EGF) and B27 supplement. Next, epithelial cells (2.4×10^4 cells/well) were seeded onto the culture inserts of 12-well culture dishes. The cells were covered with a medium in the culture insert and kept at 37°C in an atmosphere of 5% CO₂/95% air without airlifting. The medium was changed daily and the HCES were grown for 3 weeks.

Preparation of antifungal drugs

We compared the toxicity profiles of 4 antifungal drugs: micafungin (0.1%, Funguard®; Astellas, Tokyo, Japan), voriconazole (1%, Vfend®; Pfizer, Tokyo, Japan), pimaricin (5%, Pimaricin®; Senju, Osaka, Japan), and amphotericin B (0.1% Fungizone®; Bristol-Myers, Tokyo, Japan). The drug concentration was determined based on clinical use in our hospital and on previous reports.⁸ Voriconazole and micafungin were diluted in saline, and amphotericin B was diluted in 5% glucose in accordance with the manufacturer's guidelines. Saline and 5% glucose were used as controls.

WST-1 assay

Cellular viability was evaluated using a nonradioactive colorimetric assay (WST-1; Takara Bio, Inc., Shiga, Japan) according to the manufacturer's protocol. This assay, which is based on the cleavage of a tetrazolium salt, only detects living cells, and the signal generated is directly proportional to the number of live cells. After exposure to antifungal eye drops or controls for 30 min, the WST-1 reagent in a phenol red-free DMEM/F12 medium was applied for 1 h at 37°C, and the dye was measured at 450 nm by a plate reader (Victor 3V Multilabel Counter model 1420; PerkinElmer, Waltham, MA). The results were expressed as the mean $\pm$ standard deviation of the percentage of cell viability compared with that of the control-treated control.

Carboxyfluorescein permeability assay

The barrier function of HCES was evaluated using an assay that measures permeability to carboxyfluorescein, as previously described.⁵ In brief, we prepared HCES onto the upper chamber of 12-well plates (Corning® Transwell®, Cat # 3460; Corning, Inc., Lowell, MA) with 500 μ L and 1 mL of DMEM/F12 medium in the upper chamber and 1 mL in the lower chamber, respectively. After washing the upper chamber thrice with phosphate-buffered saline (PBS), antifungal eye drops or controls were added to the upper chamber for 30 min. After 3 washes with PBS, 500 μ L of 50 μ M 5(6)-carboxyfluorescein (catalog# 21877; Sigma-Aldrich Japan, Inc., Tokyo, Japan) in the DMEM/F12 medium (pH 7.4) was added to the upper chamber and incubated for 2 h. The fluorescence intensity of the outer dish was measured 2 h later at an emission wavelength of 532 nm on a plate reader (Victor 3V Multilabel Counter model 1420; PerkinElmer). The results are expressed as the mean $\pm$ standard deviation of the percentage of fluorescence intensity compared with that of the control.

Wound healing assay

An *in vitro* wound was made by scraping through a HCES using a 1.5-mm-diameter trephine and mini forceps. After a 30-min exposure to the reagents, the cells were washed twice with PBS and then incubated in the medium containing 20 ng/mL of EGF and B27 supplement for 12 h. Photographs were taken and wound closure was measured after 1, 3, 6, 9, and 12 h. The area covered by the HCES was calculated with ImageJ software (NIH, Bethesda, MD). The area covered by saline-treated HCES in 12 h was defined as 100.

Statistical analysis

Statistical comparisons between control and treatment groups for WST-1, carboxyfluorescein permeability, and wound healing data were performed using the Mann-Whitney *U* test with a Bonferroni correction (JMP software; SAS Institute, Cary, NC). Statistical comparisons between control and treatment groups for wound healing data were performed using the 2-way analysis of variance (ANOVA) test. Values of *P* < 0.05 were considered statistically significant.

Results

Antifungal agents decreased cell viability

After 30 min of exposure to the different antifungal or control agents, the cellular viability was significantly decreased with voriconazole, pimaricin, amphotericin B, and 5% glucose, but not micafungin, compared to saline-treated control cells (**P* < 0.05 vs. saline; *n* = 8 in saline, *n* = 9 in the other groups in Fig. 1). Amphotericin B-treated HCES showed the lowest cell viability ($33.59\% \pm 2.01\%$ of saline; $49.85\% \pm 2.98\%$ of 5% glucose; $\star P = 0.002$ vs. 5% glucose in Fig. 1).

FIG. 1. Effect of antifungal agents on the viability of human corneal epithelial cell sheets (HCES). Cell viability was measured using a WST-1 assay after 30 min of treatment with 0.1% micafungin (MCFG), 1% voriconazole (VRCZ), 5% pimaricin (PM), 0.1% amphotericin B (AMPH-B), and controls (saline and 5% glucose). We did a 30-min exposure as no toxicity was observed after a 10-min MCFG and VRCZ exposure in our previous tests. Cell viability was significantly decreased with voriconazole, pimaricin, amphotericin B, and 5% glucose, but not micafungin, compared with saline-treated control cells (**P* ≤ 0.05 vs. saline; *n* = 8 in saline, *n* = 9 in the other groups). Amphotericin B-treated HCES showed the lowest cell viability ($33.59\% \pm 2.01\%$ of saline; $49.85\% \pm 2.98\%$ of 5% glucose; $\star P = 0.002$ vs. 5% glucose).

FIG. 2. Effect of antifungal agents on barrier function of HCES. Barrier function was measured using a carboxy-fluorescein permeability assay after 30 min of treatment with 0.1% MCFG, 1% VRCZ, 5% PM, 0.1% AMPH-B, and controls (saline and 5% glucose). Barrier function was significantly impaired in HCES treated with pimarinic and amphotericin B compared to saline-treated control cells ($*P \leq 0.05$ vs. saline; $n=5$ in saline, $n=9$ in the other groups). The barrier function of HCES treated with 5% glucose was better than that of HCES treated with saline ($*P \leq 0.05$ vs. saline). Amphotericin B-treated HCES showed the worst membrane integrity ($241.66\% \pm 17.76\%$ of the fluorescence intensity of saline; $738.05\% \pm 54.25\%$ of the fluorescence intensity of 5% glucose; $☆P \leq 0.005$ vs. 5% glucose; $P \leq 0.005$ vs. 5% pimarinic).

Antifungal agents increased cell permeability

After 30 min of exposure to the different antifungal or control agents, the barrier function was significantly impaired in HCES treated with pimarinic and amphotericin B compared to saline-treated control cells ($*P < 0.05$ vs. saline; $n=5$ in saline, $n=9$ in the other groups in Fig. 2). The barrier function of HCES treated with 5% glucose was better compared with HCES treated with saline ($*P < 0.05$ vs. saline in Fig. 2). Amphotericin B-treated HCES showed the worst membrane integrity ($241.66\% \pm 17.76\%$ of the fluorescence intensity of saline; $738.05\% \pm 54.25\%$ of the fluorescence intensity of 5% glucose).

glucose $☆P < 0.005$ vs. 5% glucose; $P < 0.005$ vs. 5% pimarinic in Fig. 2).

Wound healing was impaired in the presence of antifungal agents

In HCES treated with saline, wounds healed nearly completely in 12 h (Fig. 3). The healed area was significantly different between drugs and time points ($P < 0.05$ by 2-way ANOVA; Fig. 4). Thirty minutes of exposure to antifungal eye drops was sufficient for reducing the wound-healing capacity of HCES by amphotericin B, pimarinic, micafungin, and voriconazole, in order of effect. Because the wound healing rate was increased in a linear manner after 3–9 h (Fig. 4), we compared the wound healing rate at this time point. Amphotericin B and pimarinic significantly disrupted the corneal epithelial healing rate ($*P < 0.05$ vs. saline; $n=9$ in saline; $n=8$ in micafungin, amphotericin B, and 5% glucose; $n=7$ in voriconazole and pimarinic in Fig. 5). The lowest healing rate was observed in amphotericin B-treated cells ($1.24\% \pm 2.45\%$ of saline; $1.35\% \pm 2.68\%$ of 5% glucose; $☆P < 0.05$ vs. 5% glucose; $P < 0.05$ vs. 5% pimarinic in Fig. 5).

Discussion

To evaluate the toxicity of topical ophthalmic formulations, cultured corneal epithelial and conjunctival cell lines have been used^{9,10} as a useful alternative to animal models, but these cell lines have a single layer and cannot mimic the *in vivo* stratified corneal and conjunctival epithelium. Barrier function, an important role of the corneal epithelium, is compromised in eye-drop-induced keratopathy.^{11–13} However, it is impossible to evaluate barrier function using monolayer cells. Although a 3-dimensional stratified corneal epithelial cell model has been established,^{14–16} this model differs from *in vivo* human corneal epithelial cells because it uses unnatural immortalized cells. Recently, we have established primary cultivated HCES in a serum-free and antibiotic-free culture system.⁴ In our department, such

FIG. 3. Pictures of HCES at wound healing. HCES at 1, 3, 6, 9, and 12 h after wound making and 30 min of exposure to 0.1% MCFG, 1% VRCZ, 5% PM, 0.1% AMPH-B, and controls (saline and 5% glucose).

FIG. 4. Effect of antifungal agents on wound healing. The wound healing was calculated as the percentage of area covered by HCES at 1, 3, 6, 9, and 12 h after a 30-min exposure to 0.1% MCFG, 1% VRCZ, 5% PM, 0.1% AMPH-B, and controls (saline and 5% glucose). The area covered after 12 h under saline treatment was defined as 100.

HCES have been used for reconstruction in patients with ocular surface diseases such as limbal deficiency. Because the HCES form 4 to 7 layers and closely mimic the *in vivo* stratified corneal epithelium in humans, they also provide an ideal system for estimating multiple endpoints, such as cellular viability, barrier function, and wound healing.

Cellular viability significantly decreased in 5% glucose-treated cells compared to saline-treated control cells under our experimental conditions (Fig. 1). The WST-1 assay is based on cleavage of a tetrazolium salt, which is catalyzed by a mitochondrial enzyme. Therefore, after application of 5% glucose, mitochondrial energy might have been used to respond to changes in osmotic pressure. Furthermore, Lamers et al. suggested that high glucose is potentially cytotoxic through increased production of high reactive oxygen species.¹⁷ The wound healing rate was slower in HCES cells treated with 5% glucose than in those treated with saline, but this difference was not statistically significant (Fig. 3). In the WST-1 assay, the number of viable HCES cells treated with 5% glucose was recovered after 2 h (data not shown). We speculated that the difference in osmotic pressure at the cell membrane might

FIG. 5. Effect of antifungal agents on wound coverage. The wound area was covered 3–9 h after the scratch was made. The area covered under saline treatment was defined as 100, and the healing rate of HCES treated with 0.1% MCFG, 1% VRCZ, 5% PM, 0.1% AMPH-B, and controls (saline and 5% glucose) was calculated on this basis. * $p < 0.05$ vs. saline; ☆ $p < 0.05$ vs. both 5% glucose and pimaricin.

have disappeared after 2 h. This might provide a possible explanation as to why the migration rate was not significantly impaired in HCES cells treated with 5% glucose.

The barrier function was better with 5% glucose treatment than with saline treatment (Fig. 2). Our previous study showed that the toxicity could be more sensitively detected by the carboxyfluorescein permeability assay than by the WST-1 assay and histological examination.⁵ However, the toxicity of voriconazole was more sensitively detected by the carboxyfluorescein permeability assay than by the WST-1 assay in this study. One possible explanation for this discrepancy is the presence of preservative in the eye drops. According to our previous study, the toxicity of antiglaucomatous drugs depends on the concentration of preservatives such as benzalkonium chloride.⁵ However, the antifungal drugs used in this study are preservative-free. Thus, we speculate that toxicity mechanisms in antifungal drugs may be different from those in antiglaucomatous drugs. Another possible explanation is that the application time of eye drops was also different from that in the previous report.⁵

The wound healing capacity was most severely impaired by amphotericin B and pimaricin. In the management of fungal keratitis, the antimicrobial activity should be the major concern. However, corneal cellular toxicity is also of major importance because fungal keratitis often progresses to corneal ulcer. When antimicrobial activity is similar, toxicity should be considered, with the less toxic antifungal drugs being preferred.

Taken together, the present data indicate that the decreasing order of ocular toxicity, with respect to cell viability, was amphotericin B, pimaricin, and voriconazole. The decreasing order of ocular toxicity to barrier and wound healing function was amphotericin B, followed by pimaricin. The low cellular toxicity of voriconazole observed here is in agreement with the findings of a previous study.¹⁸ The therapeutic concentration of amphotericin B, appeared to have higher toxicity compared with the other antifungal agents. This result is in agreement with the strong toxicity reported in a previous article.³ However, the reported damage to the barrier function is not always a negative consequence, as the fungus often invades into the deeper layers of the cornea in cases of fungal keratitis. To allow penetration of the agent into deeper layers of the cornea, breakage of the corneal barrier is necessary. Therefore, selection of the most appropriate antifungal eye drop may depend on the clinical stage of the infection. In a future study, a lower concentration of amphotericin B may be tested.

There are some limitations to this study. First, we did not assess the effects of different concentrations of the antifungal test solutions. Mahdy et al., reported that the combination therapy of subconjunctival fluconazole and topical amphotericin B (a lower concentration of 0.05%) was effective for treating keratomycosis.¹⁹ Nevertheless, the concentrations used herein correspond to those currently used in clinical practice. Therefore, we believe that our findings are relevant to the field of ophthalmology. Second, we used pimaricin in its commercially available form. This topical formulation contains inactive components and additives such as preservatives and buffers. Unfortunately, we could not identify and evaluate these components in this study. Therefore, additives in pimaricin may have contributed to the toxicity of this formulation.

In conclusion, the antifungal eye drops containing 0.1% micafungin and 1% voriconazole were less toxic than 0.1%

amphotericin B and 5% pimaricin. To the best of our knowledge, this study is the first to compare the toxicity profiles of these antifungal eye drops using the same culture system. This study may facilitate selection of appropriate antifungal agents.

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Author Disclosure Statement

The authors declare that there are no conflicts of interest.

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