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Cytotoxicity of Voriconazole on Cultured Human Corneal Endothelial Cells▽

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The purpose of the present study was to evaluate the toxicity of voriconazole on cultured human corneal endothelial cells (HCECs). HCECs were cultured and exposed to various concentrations of voriconazole (5.0 to 1,000 μg/ml). Cell viability was measured using a Cell Counting Kit-8 (CCK-8) and live/dead viability/cytotoxicity assays. Cell damage was assessed using phase-contrast microscopy after 24 h of exposure to voriconazole. To analyze the effect of voriconazole on the intercellular barrier, immunolocalization of zonula occludens 1 (ZO1) was performed. A flow cytometric assay was performed to evaluate the apoptotic and necrotic effects of voriconazole on HCECs. Cytotoxicity tests demonstrated the dose-dependent toxic effect of voriconazole on HCECs. Voriconazole concentrations of ≥100 μg/ml led to a significant reduction in cell viability. The morphological characteristics of HCECs also changed in a dose-dependent manner. Increasing concentrations of voriconazole resulted in fading staining for ZO1. Higher concentrations of voriconazole resulted in an increased number of propidium iodide (PI)-positive cells, indicating activation of the proapoptotic pathway. In conclusion, voriconazole may have a dose-dependent toxic effect on cultured HCECs. The results of this study suggest that although voriconazole concentrations of up to 50 μg/ml do not decrease cell viability, intracameral voriconazole concentrations of ≥100 μg/ml may increase the risk of corneal endothelial damage.

Voriconazole is a triazole that has a broad spectrum of antifungal activities (18, 21, 30). The antifungal activity arises from a complex multimechanistic process initiated by the inhibition of a cytochrome P450 involved in the biosynthesis of ergosterol, namely, CYP51, which catalyzes the oxidative removal of the 14α-methyl group of lanosterol or eburicol (9). This eventually causes depletion of ergosterol and disrupts the integrity and function of fungal cell membranes, eventually leading to cell lysis (10, 30). This antifungal agent is a synthetic derivative of fluconazole modified by adding a methyl group to the propyl backbone and by substituting a triazole moiety with a fluoropyrimidine group; this results in a higher affinity for fungal 14-α-demethylase, leading to more potent activities and excellent efficacy in patients with a wide range of ocular fungal infections (8, 9, 13, 28, 34). The drug is also shown to be effective in invasive or serious fungal infections as well as serious infections refractory to other antifungal agents and thus has been approved by the U.S. Food and Drug Administration and European Medicines Agency for the treatment of invasive aspergillosis and serious infections caused by *Fusarium* species and *Scedosporium apiospermum* that are refractory to other antifungal agents (28). The use of voriconazole in treating ocular infections, such as fungal keratitis or endophthalmitis, is increasing, and several researchers have reported good efficacy even in recalcitrant ocular fungal infections (1, 4, 18, 21, 24, 27, 28). The drug is currently in widespread use and is administered systemically and/or topically (1, 24, 31, 32).

Intracameral voriconazole injection is conceivably the most direct and effective method for achieving higher aqueous concentrations (29), and anecdotal case reports have shown its efficacy (21, 27, 28). However, intracameral use of voriconazole is still an "off-label" use, and a safe therapeutic dosage has not been established. Therefore, considering the increasing use of voriconazole by intracameral injection, we conducted this study to evaluate the potential cytotoxic effects of various concentrations of voriconazole on cultured human corneal endothelial cells (HCECs).

MATERIALS AND METHODS

This study conformed to the tenets of the Declaration of Helsinki. Voriconazole (Vfend; Pfizer, Inc., New York, NY) was obtained in pure powder form, reconstituted in sterile water to obtain a concentration of 10 mg/ml (1%), and serially diluted in phosphate-buffered saline (PBS).

HCEC culture. Human corneal endothelial cells were cultured as described previously (7, 15). In brief, HCECs from remnant donor tissue after corneal transplantation attached to Descemet's membrane were harvested on the 5th day after death. The tissue donor age was 33 years. The endothelial cells and Descemet's membrane complex were incubated for 1 h in 0.02% EDTA solution, stirred vigorously with a flame-polished pipette to disrupt cell junctions, centrifuged for 5 min at 3,000 × *g*, and seeded onto culture plates coated with FNC coating mix (Athena Enzyme Systems, Baltimore, MD) containing bovine fibronectin (10 μg/ml) and bovine type I collagen (35 μg/ml). The cells were cultured in Opti-Mem-I medium (Gibco/BRL Life Technologies, Grand Island, NY) supplemented with 8% fetal bovine serum (FBS; Cambrex Bio Science, Walkersville, MD), 200 mg/liter calcium chloride (Sigma Chemical Co., St. Louis, MO), 0.08% chondroitin sulfate (Sigma Chemical Co., St. Louis, MO), 20 μg/ml ascorbic acid (Sigma Chemical Co., St. Louis, MO), 100 μg/ml pituitary

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extract (Invitrogen, Grand Island, NY), 5 ng/ml epidermal growth factor (Sigma Chemical Co., St. Louis, MO), 20 ng/ml nerve growth factor (Sigma Chemical Co., St. Louis, MO), 10 μg/ml gentamicin (Invitrogen, Grand Island, NY), 100 IU/ml penicillin (Cambrex Bio Science, Walkersville, MD), 100 IU/ml streptomycin (Cambrex Bio Science, Walkersville, MD), and 2.5 μg/ml amphotericin (Cambrex Bio Science, Walkersville, MD) under 5% CO_2. The medium was changed every 2 days. At confluence, the cells were split 1 to 3, and passage 3 cells were used for experiments.

Cytotoxicity tests. A Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan) and live/dead viability/cytotoxicity kits (Invitrogen, Carlsbad, CA) were used to measure the cytotoxicity of voriconazole on HCECs.

The CCK-8 assay was used to measure cytotoxicity under starved conditions, which are based on the conversion of a water-soluble tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8), to a water-soluble formazan dye upon reduction by dehydrogenases in the presence of an electron carrier (12). HCECs (5×10^4 cells/ml) were grown in 96-well plates for 48 h and treated with voriconazole over a range of concentrations (0 [control] or 5, 25, 50, 100, 250, 500, or 1,000 μg/ml). After 24 h, the HCECs were washed, and the extent of cell growth was assessed using a CCK-8 assay (Dojindo, Kumamoto, Japan). CCK-8 solution (10 μl) was added to each well, followed by incubation for 2 h at 37°C. The absorbance at 450 nm was determined by a multiplate reader (Lambda Bio-20; Beckman). Cell viability was expressed as a percentage of that of the control (untreated) cells. For each concentration of voriconazole, mean values of the mean absorbance rates from eight wells were calculated.

To assess the cytotoxicity of voriconazole on HCECs under nonstarved conditions, cell viability was investigated using a live/dead viability/cytotoxicity kit (Invitrogen, Carlsbad, CA); the contents of the kit include calcein acetoxymethyl ester (AM) and ethidium homodimer-1. Nonfluorescent cell-permeant calcein AM is converted to intensely fluorescent calcein in the presence of intracellular esterase activity; the calcein is well retained within live cells and produces green fluorescence. Ethidium homodimer-1 penetrates cells with membrane damage and binds to nucleic acid to produce red fluorescence in dead cells. Staining was performed according to the manufacturer's instructions. Adequate negative (cells without voriconazole) and positive (cells treated with 0.3% Triton X-100 detergent; Serva, Heidelberg, Germany) controls for cell death were run with each set of experiments. Briefly, HCECs (5×10^4 cells/ml) were exposed to various concentrations of voriconazole for 24 h and subsequently washed with PBS. After the live/dead viability/cytotoxicity kit was applied to each sample, the HCECs were incubated for 45 min. Cell viability was subsequently analyzed using fluorescence microscopy (Axioskop-2; Carl Zeiss, Oberkochen, Germany). The numbers of green and red cells were counted per eight fields at ×200 magnification. The percentage of cells with green fluorescence (interpreted as viable cells) was then calculated.

Morphological changes in HCECs after exposure to voriconazole. HCECs (5×10^4 cells/ml) were exposed to different concentrations of voriconazole for 24 h, and then the morphology of HCECs was assessed using phase-contrast microscopy. Special attention was paid to find signs of cellular damage, such as pleomorphism, disruption of the intercellular junctional complexes, swollen or prominent nuclei, cytoplasmic vacuolization, a shrunken cytosol, rupture of nuclear and plasma membranes, or nuclear fragmentation. The extent of cellular damage in the various concentrations of voriconazole was compared with that in the control group.

Immunohistochemistry. Immunohistochemical staining of zonula occludens 1 (ZO1), a tight junction-associated protein, was used to determine whether the HCECs maintain tight junctions under voriconazole exposure. HCECs (5×10^4 cells/ml) were exposed to various concentrations of voriconazole for 24 h, and the HCECs were fixed in 4% paraformaldehyde and 3% sucrose in PBS (pH 7.4) for 15 min at room temperature. The HCECs were then incubated with a primary antibody, rabbit polyclonal anti-ZO1 (1:100 dilution; Zymed Laboratories, San Francisco, CA), overnight at 4°C and washed with PBS three times. The cells were then incubated for 1 h at room temperature with the secondary antibody alkaline phosphatase/RED, rabbit/mouse (Dako, Glostrup, Denmark). Finally, cell monolayers were examined under a fluorescence microscope (Axioscop-2; Carl Zeiss, Oberkochen, Germany).

Flow cytometric assay for the apoptotic and necrotic effects of voriconazole on HCECs. The flow cytometry assay was performed to investigate the sensitivity of HCECs to voriconazole-induced apoptosis by discriminating "early" apoptotic cells from necrotic "late" apoptotic and vital cells. For simultaneous detection of apoptosis and necrosis, a costaining technique with Annexin V conjugated with fluorescein isothiocyanate (FITC) (Merck Biosciences, Bad Soden, Germany), in tandem with the DNA-binding dye propidium iodide (PI), was performed as described previously (33). Briefly, HCECs were grown to confluence and incubated for 24 h in 24-well plates using the same conditions as those in the previous experiments. After centrifugation and washing in cold PBS, HCECs for costaining were resuspended in binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2.5 mM $CaCl_2$; pH 7.4) at a concentration of 10^6 cells/ml. A volume of 0.5 ml, containing 5×10^5 cells, was transferred to a culture tube, and 1.25 μl FITC-conjugated Annexin V was added. After centrifugation at 1,000 rpm for 5 min and removal of the supernatant, the cells were gently resuspended in 500 μl cold binding buffer, followed by the addition of 10 μl PI. Positive controls were provided for both apoptotic and necrotic (10% ethanol) cell death. For the simultaneous scoring of the differential cellular response, aliquots of 10^4 cells each were immediately processed for fluorescence-activated cell sorting (FACS) with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Excitation parameters were set at a λ_{Ex} of 488 nm, and fluorescence emission was detected at a λ_{Em} of 518 and 620 nm for Annexin V-FITC and PI, respectively. Data were analyzed with CellQuest software (BD Biosciences, Mountain View, CA). Flow cytometry data are plotted as the mean number of events for PI- and Annexin V-positive and -negative cells in each quadrant.

Statistical analysis. All data are expressed as the means ± standard deviations (SD). Student's *t* test was used to compare mean values between two groups. A *P* value of <0.05 was considered statistically significant. We used SPSS software for Windows (V15.0; SPSS Inc., Chicago, IL) for all statistical analyses.

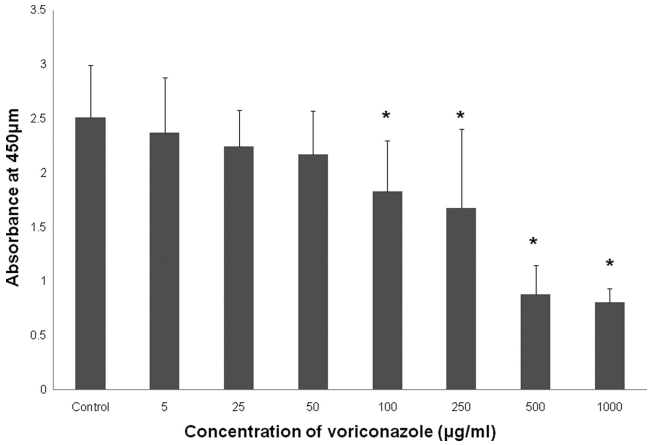


FIG. 1. Results of the Cell Counting Kit-8 (CCK-8) assay showing a dose-dependent toxic effect of voriconazole on HCECs. After 24 h, voriconazole at concentrations of ≥100 μg/ml led to a significant reduction of cell viability (*, $P < 0.05$).

RESULTS

Cytotoxicity test. The CCK-8 assay demonstrated a dose-dependent toxic effect with increasing concentrations of voriconazole on HCECs under starved conditions. No significant toxicity was observed at concentrations of up to 50 μg/ml. However, at concentrations of ≥100 μg/ml ($P = 0.027$), significant toxicity on HCECs was observed. The mean absorbance rate was 2.17 ± 0.40 at 50 μg/ml and 1.83 ± 0.62 at 100 μg/ml; this indicates a reduction of the number of viable cells by 13.5% and 27.1%, respectively, in comparison with the control with 2.51 ± 0.45 (Fig. 1).

In the live/dead viability/cytotoxicity assay, the percentages of viable cells (green fluorescence) were as follows: control group, 95.37% ± 0.96%; 5 μg/ml, 94.52% ± 0.28%; 25 μg/ml, 93.15% ± 2.90%; 50 μg/ml, 92.54% ± 0.40%; 100 μg/ml, 88.03% ± 7.10%; 200 μg/ml, 84.84% ± 7.91%; 500 μg/ml, 81.52% ± 5.80%; and 1,000 μg/ml, 75.30% ± 8.58%. The difference was not significant up to 50 μg/ml ($P = 0.070$); however, significant differences were observed at ≥100 μg/ml ($P = 0.022$). This dose-dependent reduction of

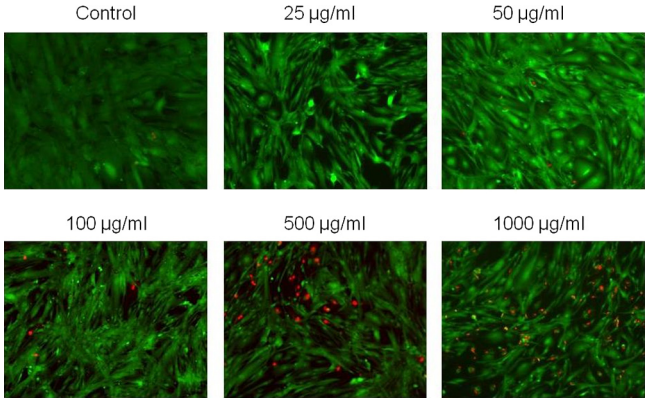


FIG. 2. Results of the live/dead cell assay, showing a dose-dependent toxic effect on HCECs. In the presence of increasing concentrations of voriconazole for 24 h, increased numbers of dead cells can be observed.

viable cells is in agreement with the results of the CCK-8 cytotoxicity test (Fig. 2).

Morphological changes in HCECs. After exposure to various concentrations of voriconazole, morphological changes of HCECs were detected in a dose-dependent manner. Increasing concentrations of voriconazole resulted in typical signs of cellular damage, including pleomorphism, rupture of the nuclear or plasma membrane, nuclear fragmentation, a shrunken cytosol, or disruption of the intercellular junctional complexes (Fig. 3).

Immunohistochemistry. Exposure to greater doses of voriconazole resulted in diminished ZO1 staining, indicating damage to tight junctions. At voriconazole concentrations of ≥100 μg/ml, a marked reduction in immunopositive areas was observed (Fig. 4). These findings are in accordance with those of the phase-contrast microscopy.

Flow cytometric assay. With increasing concentrations of voriconazole, the number of Annexin V- and PI-positive cells

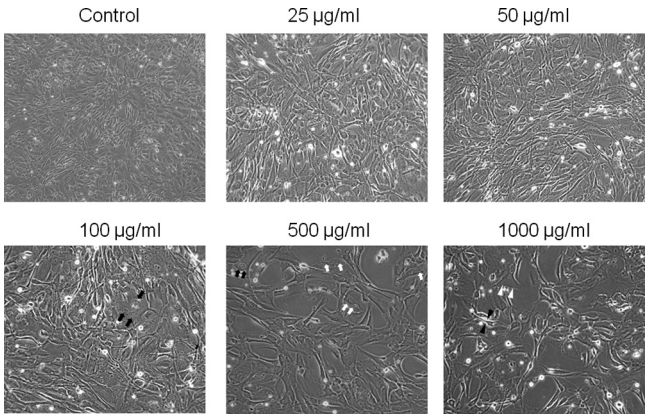


FIG. 3. Representative photographs of HCECs taken using phase-contrast microscopy. Higher concentrations of voriconazole were associated with increased signs of cellular damage, including pleomorphism, rupture of the nuclear (white arrow) or plasma (white arrowhead) membrane, nuclear fragmentation (black arrow), a shrunken cytosol (black arrowhead), and disruption of the intercellular junctional complexes.

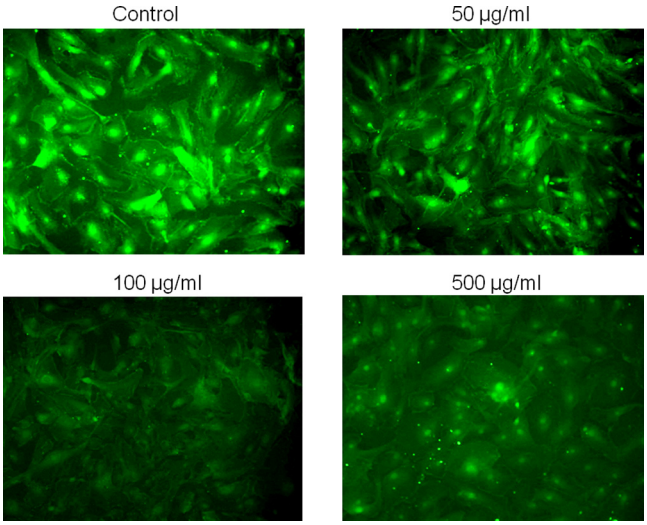


FIG. 4. Representative photographs of immunostaining with ZO1 for different concentration groups after culture for 24 h in the presence of various concentrations of voriconazole. Increasing concentrations of voriconazole led to diminished ZO1staining, suggesting damage to tight junctions.

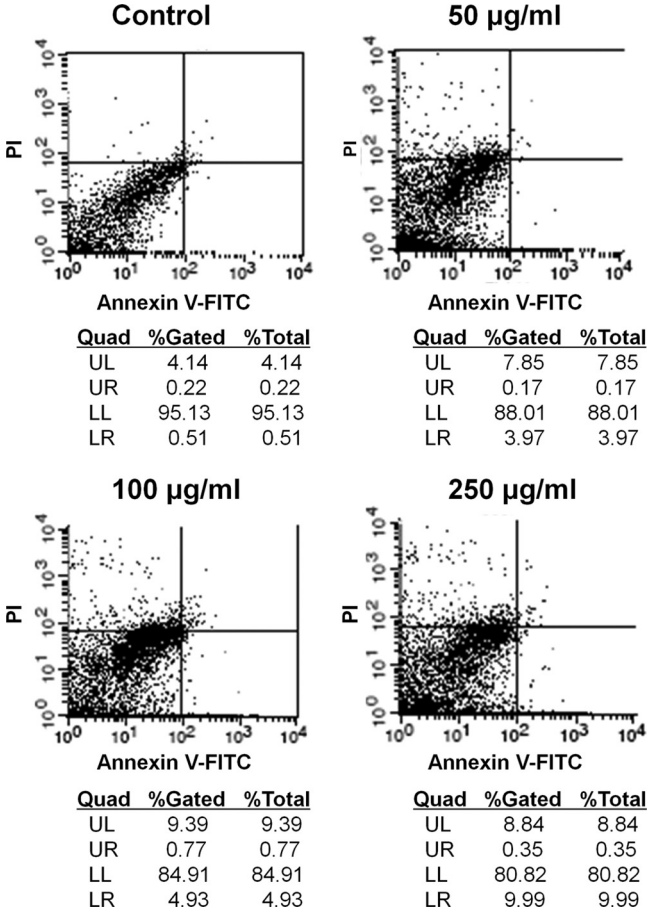
increased in a dose-dependent manner; this suggests the activation of the proapoptotic pathway, leading to subsequent apoptosis. The results of the flow cytometric assay are shown in Fig. 5.

DISCUSSION

Voriconazole has excellent antifungal activity, and most fungi, including *Fusarium* species and *Scedosporium apiospermum*, are more sensitive to voriconazole than to the other azole derivatives (5, 22). The MICs range from 0.06 to 2.08, 0.5 to 2, 2 to 8, and 0.5 −2.0 μg/ml for *Candida*, *Aspergillus*, and *Fusarium* species and *Scedosporium apiospermum*, respectively (5, 23, 25, 26, 30, 32). Topical and/or systemic administration may be a useful option in most cases of fungal keratitis, as studies have demonstrated that the therapeutic level of voriconazole concentration of 2.93 to 6.49 μg/ml can be achieved with topical and/or systemic administration (11, 31, 32).

However, in refractory cases or in cases in which fungi have high MICs, systemic and topical administration of voriconazole might be insufficient to attain drug concentrations above the therapeutic level. Even with *Candida* species, 109 of 1,763 isolates (6.2%) were reported to have voriconazole MICs of ≥4 μg/ml in refractive cases or immunocompromised patients, suggesting an increasing demand for the direct injection of voriconazole (13). Besides, in cases of deep mycosis, such as fungal hypopyon, intracameral injection may be required, because topically administered drugs poorly penetrate the intact corneal epithelium (35).

Moreover, systemic administration can cause adverse effects. The drug is, although infrequently, reported to be associated with clinically significant hepatotoxicity, mainly due to the fact that it is extensively metabolized by the hepatic cytochrome P450 isoenzymes CYP2C19, CYP2C9, and CYP3A4 (6, 14, 32). However, studies have shown that voriconazole toxicity has no relationship with CYP 2C19 status, although the



Quad	%Gated	%Total
UL	4.14	4.14
UR	0.22	0.22
LL	95.13	95.13
LR	0.51	0.51

Quad	%Gated	%Total
UL	7.85	7.85
UR	0.17	0.17
LL	88.01	88.01
LR	3.97	3.97

Quad	%Gated	%Total
UL	9.39	9.39
UR	0.77	0.77
LL	84.91	84.91
LR	4.93	4.93

Quad	%Gated	%Total
UL	8.84	8.84
UR	0.35	0.35
LL	80.82	80.82
LR	9.99	9.99

FIG. 5. Representative Annexin V and PI dot plots for different concentration groups. After 24 h of culture, higher concentrations of voriconazole lead to increased numbers of dead or dying cells (PI-positive cells, upper left [UL] and upper right [UR] quadrants in each plot). The number of Annexin V-positive cells (lower right [LR] quadrant in each plot) also increased in a dose-dependent manner, suggesting the activation of the proapoptotic pathway. LL, lower left.

CYP2C19 genotype can influence voriconazole plasma concentrations (3, 20). Rash and photosensitivity are also frequently reported (9, 19). In addition, systemic voriconazole should be the last option in pregnant women, as triazoles are classified as category D drugs for use during pregnancy (32).

The present study evaluates the toxicity of voriconazole on HCECs *in vitro*. Our results demonstrate that voriconazole exhibits a dose-dependent toxic effect on HCECs with significant cell death at concentrations of ≥100 μg/ml. Cytotoxicity tests, morphological evaluation, immunolocalization of ZO1, and flow cytometry all showed this dose-dependent toxicity. The results of the flow cytometric assay, namely, that the number of Annexin- and PI-positive cells increased with increasing concentrations of voriconazole, suggest that the activation of the proapoptotic pathway may play an important role in the toxic effect of voriconazole on HCECs. Previous studies also demonstrate that apoptosis plays a crucial role in the process of corneal endothelial cell loss and drugs that are toxic to corneal endothelial cells may conceivably induce apoptosis in these cells, thus leading to profound cell loss (4, 36). Therefore, further investigation into the mechanism underlying the induction of apoptosis is necessary.

Lin et al. (21) reported the successful use of intracameral voriconazole injection (12.5 μg/0.05 ml) in three cases of fungal endophthalmitis. Reis et al. (27) also reported one case of ocular invasive fusariosis treated with intracameral voriconazole administration (10 μg/0.1 ml). The initial intracameral voriconazole concentrations in those reports correspond to approximately 40 and 15 to 20 μg/ml, respectively, with the assumption that the human aqueous volume would be 0.3 ml; both are below the level that is shown to increase the risk of HCEC damage in the present study. Gao et al. (8) demonstrated that that intravitreal voriconazole concentrations of up to 25 μg/ml cause no electroretinographic changes or histologic abnormalities in the rat retina, which is corroborated by the results of our study. Kernt et al. (17) also reported that no significant toxicity was found for voriconazole on primary retinal pigment epithelial (RPE) cells and optic nerve head astrocytes when administered in concentrations up to 250 μg/ml, suggesting that voriconazole has low toxicity in therapeutic dose.

The results of the present study are in contrast to those in the report by Kernt et al. (16), namely, that voriconazole of concentrations up to 10 mg/ml had no significant toxicity on HCECs, trabecular meshwork cells, or RPE cells when administered for 24 h. However, the immortalized simian virus 40 (SV40)-transfected HCECs and RPE cells used in their study have an extended life span and enhanced proliferation capacity; these characteristics might, at least in part, account for the stronger resistance to damage from voriconazole than that seen with normal, nonmodified cells (2). The report by Gao et al. (8) that voriconazole concentrations of ≥50 μg/ml caused focal necrosis in rat retina also supports our assumption.

Nonetheless, the concentration of 50 μg/ml, the highest concentration that was demonstrated to be safe is 5 times the MIC of *Fusarium* and far higher than the MICs of all fungus species that are known to cause ocular infection, which suggests that intracameral injection of voriconazole can be a safe and effective treatment method (23, 25, 26, 30, 32).

The present study has the limitation that the results of *in vitro* experiments can never be directly translated into *in vivo* situations, for the following reasons. (i) Through several passages, the phenotypic properties of the HCECs and their sensitivity to toxic agents can change. (ii) In the anterior chamber, multiple mechanisms exist that protect the corneal endothelium (e.g., epithelial, fibroblast, and platelet-derived growth factors) (36). (iii) Due to metabolism in the anterior chamber, the intracameral voriconazole concentration conceivably decreases gradually; thus, exposure to sustained high concentrations of the drug for 24 h may not be common in *in vivo* conditions. Shen et al. (29) demonstrated the rapid decline in the aqueous concentration of voriconazole after intracameral injection in rabbit eyes and reported that the half-life of voriconazole in the anterior chamber of rabbit is only 22 min. (iv) In cases of intracameral fungal infection, the HCECs are damaged by fungi and are thus conceivably more vulnerable to toxicity caused by voriconazole than normal HCECs. Therefore, lower doses than those expected to be safe for normal HCECs would be needed to avoid further damage to the corneal endothelium. In addition, because 1% voriconazole solu-

tion also includes 160 mg/ml of sulfobutyl ether–beta-cyclodextrin sodium as a vehicle, intracameral injection of voriconazole solution also involves intracameral infusion of the corresponding amount of the vehicle. Therefore, we should rule out the possibility of the impact of the vehicle on HCECs, and further studies are needed to investigate the effect of sulfobutyl ether–beta-cyclodextrin on HCECs. Nonetheless, the results of this study as well as those of the previous studies suggest that the commercially available form of voriconazole may be safe in therapeutic concentrations of up to 50 μg/ml (8, 16, 17, 21, 27).

In conclusion, the present study reveals the dose-dependent toxicity of voriconazole on cultured HCECs. High concentrations of voriconazole result in increased loss of cell viability. The results of this study suggest that intracameral voriconazole concentrations of ≥100 μg/ml may increase the risk of corneal endothelial damage and thus should be used with caution, especially in cases of deep penetration of keratomycosis into the intracameral space, which can lead to a compromised corneal endothelium. Further studies are required to determine the *in vivo* toxicity of voriconazole on corneal endothelial cells and to elucidate the detailed mechanisms leading to the loss of HCECs and intercellular tight junction breakdown after exposure to voriconazole.

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REFERENCES

1. **Al-Badriyeh, D., L. Leung, G. E. Davies, K. Stewart, and D. Kong.** 2009. Successful salvage treatment of Scedosporium apiospermum keratitis with topical voriconazole after failure of natamycin. Ann. Pharmacother. **43:** 1139–1142.
2. **Bednarz, J., M. Teifel, P. Friedl, and K. Engelmann.** 2000. Immortalization of human corneal endothelial cells using electroporation protocol optimized for human corneal endothelial and human retinal pigment epithelial cells. Acta Ophthalmol. Scand. **78:**0–136.
3. **Berge, M., et al.** 2011. Effect of cytochrome P450 2C19 genotype on voriconazole exposure in cystic fibrosis lung transplant patients. Eur. J. Clin. Pharmacol. **67:**253–260.
4. **Cho, K. S., E. H. Lee, J. S. Choi, and C. K. Joo.** 1999. Reactive oxygen species-induced apoptosis and necrosis in bovine corneal endothelial cells. Invest. Ophthalmol. Vis. Sci. **40:**911–919.
5. **Cuenca-Estrella, M., B. Ruiz-Diez, J. V. Martinez-Suarez, A. Monzon, and J. L. Rodriguez-Tudela.** 1999. Comparative in-vitro activity of voriconazole (UK-109,496) and six other antifungal agents against clinical isolates of Scedosporium prolificans and Scedosporium apiospermum. J. Antimicrob. Chemother. **43:**9–151.
6. **den Hollander, J. G., et al.** 2006. Incidence of voriconazole hepatotoxicity during intravenous and oral treatment for invasive fungal infections. J. Antimicrob. Chemother. **57:**1248–1250.
7. **Engelmann, K., M. Bohnke, and P. Friedl.** 1988. Isolation and long-term cultivation of human corneal endothelial cells. Invest. Ophthalmol. Vis. Sci. **29:**56–1662.
8. **Gao, H., et al.** 2004. Intravitreal voriconazole: an electroretinographic and histopathologic study. Arch. Ophthalmol. **122:**87–1692.
9. **Ghannoum, M. A., and D. M. Kuhn.** 2002. Voriconazole—better chances for patients with invasive mycoses. Eur. J. Med. Res. **7:**242–256.
10. **Ghannoum, M. A., and L. B. Rice.** 1999. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. Clin. Microbiol. Rev. **12:**1–517.
11. **Hariprasad, S. M., et al.** 2004. Determination of vitreous, aqueous, and plasma concentration of orally administered voriconazole in humans. Arch. Ophthalmol. **122:**47.
12. **Ishiyama, M., et al.** 1996. A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. Biol. Pharm. Bull. **19:**1518–1520.
13. **Johnson, E., A. Espinel-Ingroff, A. Szekely, H. Hockey, and P. Troke.** 2008. Activity of voriconazole, itraconazole, fluconazole and amphotericin B in vitro against 1763 yeasts from 472 patients in the voriconazole phase III clinical studies. Int. J. Antimicrob. Agents **32:**1–514.
14. **Johnson, L. B., and C. A. Kauffman.** 2003. Voriconazole: a new triazole antifungal agent. Clin. Infect. Dis. **36:**630–637.
15. **Joyce, N. C., and C. C. Zhu.** 2004. Human corneal endothelial cell proliferation: potential for use in regenerative medicine. Cornea **23:**S8–S19.
16. **Kernt, M., and A. Kampik.** 2009. Intracameral voriconazole: in vitro safety for human ocular cells. Toxicology **258:**84–93.
17. **Kernt, M., A. S. Neubauer, H. M. De Kaspar, and A. Kampik.** 2009. Intravitreal voriconazole: in vitro safety-profile for fungal endophthalmitis. Retina **29:**2–370.
18. **Kramer, M., et al.** 2006. Intravitreal voriconazole for the treatment of endogenous Aspergillus endophthalmitis. Ophthalmology **113:**1184–1186.
19. **Lazarus, H. M., J. L. Blumer, S. Yanovich, H. Schlamm, and A. Romero.** 2002. Safety and pharmacokinetics of oral voriconazole in patients at risk of fungal infection: a dose escalation study. J. Clin. Pharmacol. **42:**395–402.
20. **Levin, M. D., et al.** 2007. Hepatotoxicity of oral and intravenous voriconazole in relation to cytochrome P450 polymorphisms. J. Antimicrob. Chemother. **60:**04–1107.
21. **Lin, R. C., N. Sanduja, and S. M. Hariprasad.** 2008. Successful treatment of postoperative fungal endophthalmitis using intravitreal and intracameral voriconazole. J. Ocul. Pharmacol. Ther. **24:**5–248.
22. **Linares, M. J., et al.** 2005. Susceptibility of filamentous fungi to voriconazole tested by two microdilution methods. J. Clin. Microbiol. **43:**0–253.
23. **Maxwell, M. J., et al.** 2003. Evaluation of Etest method for determining fluconazole and voriconazole MICs for 279 clinical isolates of Candida species infrequently isolated from blood. J. Clin. Microbiol. **41:**1087–1090.
24. **Perfect, J. R., et al.** 2003. Voriconazole treatment for less-common, emerging, or refractory fungal infections. Clin. Infect. Dis. **36:**22–1131.
25. **Pfaller, J. B., S. A. Messer, R. J. Hollis, D. J. Diekema, and M. A. Pfaller.** 2003. In vitro susceptibility testing of Aspergillus spp.: comparison of Etest and reference microdilution methods for determining voriconazole and itraconazole MICs. J. Clin. Microbiol. **41:**26–1129.
26. **Pfaller, M. A., et al.** 2003. Evaluation of the Etest and disk diffusion methods for determining susceptibilities of 235 bloodstream isolates of Candida glabrata to fluconazole and voriconazole. J. Clin. Microbiol. **41:**1875–1880.
27. **Reis, A., et al.** 2000. Successful treatment of ocular invasive mould infection (fusariosis) with the new antifungal agent voriconazole. Br. J. Ophthalmol. **84:**2–933.
28. **Sen, P., L. Gopal, and P. R. Sen.** 2006. Intravitreal voriconazole for drug-resistant fungal endophthalmitis: case series. Retina **26:**935–939.
29. **Shen, Y. C., et al.** 2009. Pharmacokinetics of intracameral voriconazole injection. Antimicrob. Agents Chemother. **53:**2156–2157.
30. **Shen, Y. C., et al.** 2007. Clearance of intravitreal voriconazole. Invest. Ophthalmol. Vis. Sci. **48:**2238–2241.
31. **Thiel, M. A., A. S. Zinkernagel, J. Burhenne, C. Kaufmann, and W. E. Haefeli.** 2007. Voriconazole concentration in human aqueous humor and plasma during topical or combined topical and systemic administration for fungal keratitis. Antimicrob. Agents Chemother. **51:**9–244.
32. **Vemulakonda, G. A., et al.** 2008. Aqueous and vitreous concentrations following topical administration of 1% voriconazole in humans. Arch. Ophthalmol. **126:**18–22.
33. **Vermes, I., C. Haanen, H. Steffens-Nakken, and C. Reutelingsperger.** 1995. A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J. Immunol. Methods **184:**39–51.
34. **Warrilow, A. G., et al.** 2010. Azole binding properties of Candida albicans sterol 14-alpha demethylase (CaCYP51). Antimicrob. Agents Chemother. **54:**4235–4245.
35. **Yilmaz, S., M. Ture, and A. Maden.** 2007. Efficacy of intracameral amphotericin B injection in the management of refractory keratomycosis and endophthalmitis. Cornea **26:**398–402.
36. **Yoeruek, E., et al.** 2008. Toxic effects of recombinant tissue plasminogen activator on cultured human corneal endothelial cells. Invest. Ophthalmol. Vis. Sci. **49:**1392–1397.