Efficacy and Safety of Antifungal Additives in Optisol-GS Corneal Storage Medium

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Importance Optisol-GS, the most common corneal storage medium in the United States, contains antibacterial but no antifungal supplementation. Most postkeratoplasty endophthalmitis and keratitis cases are now of a fungal origin.

Objective To assess the efficacy and safety of voriconazole and amphotericin B in reducing Candida species contamination of Optisol-GS under normal storage conditions.

Design, Setting, and Participants In vitro laboratory study using 15 pairs of research-grade donor corneas and 20-mL vials of Optisol-GS.

Interventions Twenty vials of Optisol-GS were supplemented with either voriconazole at 1×, 10×, 25×, or 50× minimum inhibitory concentration (MIC) or amphotericin B at 0.25×, 0.5×, 1×, or 10× MIC. Known concentrations of Candida albicans and Candida glabrata were each added to a set of vials. Safety studies were performed by separating 15 pairs of donor corneas into unsupplemented Optisol-GS or Optisol-GS plus an antifungal.

Main Outcomes and Measures Efficacy outcomes were viable fungal colony counts determined from samples taken on days 2, 7, and 14 immediately after removal from refrigeration and after warming to room temperature for 2 hours. Safety outcomes included percentage of intact epithelium and endothelial cell density on days 0, 7, and 14, as well as percentage of nonviable endothelial cells by vital dye staining on day 14.

Results Growth of C albicans and C glabrata was observed in all voriconazole-supplemented vials. In contrast, there was no growth of either organism in amphotericin B–supplemented vials, except at 0.25× and 0.5× MIC on day 2, when viable counts of C glabrata were reduced by 99% and 96%, respectively. Compared with paired controls, with the exception of Optisol-GS plus amphotericin B at 10× MIC, donor corneas in supplemented Optisol-GS appeared to have no difference in endothelial cell density reduction, percentage of intact epithelium, or percentage of nonviable endothelial cells.

Conclusions and Relevance The addition of amphotericin B to Optisol-GS may significantly improve activity against contamination with Candida species, the primary cause of fungal infection after corneal transplantation. This study found significant endothelial toxic effects at the maximal concentration of amphotericin B.
Postoperative endophthalmitis is a rare but serious complication of penetrating keratoplasty (PK), with rates ranging from 0.05% to 0.77%. The Eye Bank Association of America (EBAA) recently reported an increasing trend in the incidence of fungal infection after corneal transplantation and found twice as many fungal infections after endothelial keratoplasty than after PK, although neither observation reached statistical significance. The percentage of postkeratoplasty endophthalmitis cases attributable to fungal vs bacterial infection has increased markedly, from 10% in 1991 to 63% in 2010. Candida species, in particular Candida albicans and, to a lesser extent, Candida glabrata, have accounted for nearly all reported cases of fungal infection after corneal transplantation in the United States.

Optisol-GS (Bausch & Lomb Inc) is the most commonly used corneal storage medium in the United States. It contains the antibiotics gentamicin sulfate and streptomycin sulfate, and its widespread adoption in the 1990s likely contributed to the relative decrease in post-PK bacterial endophthalmitis. The storage medium, however, currently does not include an antifungal additive. In addition, its colorimetric indicator, phenol red, does not reliably detect C albicans contamination.

The organ culture medium commonly used in Europe contains the antifungal amphotericin B. A previous US study examined voriconazole as an additive to Optisol-GS and found that there was a significant reduction in the rate of positive fungal donor rim cultures with voriconazole-supplemented Optisol-GS compared with Optisol-GS alone. The aim of the present study was to assess the efficacy and safety of voriconazole and amphotericin B in reducing C albicans and C glabrata contamination of Optisol-GS under normal storage conditions.

**Methods**

**Efficacy Study**

Twenty 20-mL vials of Optisol-GS were divided into 2 groups of 10 vials each. Each group of 10 vials was created as follows: (1) 1× minimum inhibitory concentration (MIC) voriconazole (Optisol-GS plus voriconazole at a concentration of 1 μg/mL), (2) 10× MIC voriconazole (10 μg/mL), (3) 25× MIC voriconazole (25 μg/mL), (4) 50× MIC voriconazole (50 μg/mL), (5) 0.25× MIC amphotericin B (0.0625 μg/mL), (6) 0.5× MIC amphotericin B (0.125 μg/mL), (7) 1× MIC amphotericin B (4 μg/mL), (8) 10× MIC amphotericin B (40 μg/mL), and (9 and 10) 2 vials of unsupplemented Optisol-GS to serve as positive (plus inoculum) and negative (no inoculum) controls. The MICs of voriconazole and amphotericin B were determined based on published MICs of each antifungal for Candida species, including C albicans and C glabrata. Given the low adverse effect profile of voriconazole, the upper limit of its range of MICs (1 μg/mL) was used as the basis to determine the MIC multiples. Given the well-known toxicity of amphotericin B, the lower limit of its range of MICs (0.25 μg/mL) was used to determine the lower MIC multiples (0.25× and 0.5× MIC), and the upper limit (4 μg/mL) was used to determine the higher MIC multiples (1× and 10× MIC). Both antifungal additives were obtained as ophthalmic solutions (voriconazole, 1%, and amphotericin B, 0.15%) from Leiter’s Compounding Pharmacy, and they were serially diluted in Optisol-GS to obtain the appropriate concentrations. The MICs of the experimental voriconazole and amphotericin B were confirmed to fall within expected published ranges by the Clinical and Laboratory Standards Institute broth microdilution methods. Because amphotericin B is photosensitive, all vials were kept protected from light except during direct sampling.

Inocula of C albicans (ATCC strain 90028) and C glabrata (no ATCC strain with known MICs for voriconazole and amphotericin B; obtained from the Fungus Testing Laboratory, The University of Texas Health Science Center at San Antonio) were prepared according to the Clinical and Laboratory Standards Institute protocol. Isolates were grown on Sabouraud agar for 24 hours at 35°C. Colonies were harvested from the plates and suspended in sterile water to obtain turbidity comparable to that of a 0.5 McFarland standard or 1 × 10^6 to 5 × 10^6 CFU/mL. The suspensions were added at an appropriate volume to create a working suspension of 2.5 × 10^3 CFU/mL in Optisol-GS vials 1 through 9, leaving vial 10 as a negative control (unsupplemented Optisol-GS, no inoculum). Inocula of C albicans were added to the first group of vials and inocula of C glabrata were added to the second group.

Initial fungal concentrations were confirmed by serial dilution, subculture, and viable colony counts. The verification studies to confirm the MICs of the experimental isolates were performed by the Fungus Testing Laboratory at The University of Texas Health Science Center. All vials of Optisol-GS were refrigerated at 2°C to 8°C per manufacturer recommendations. On days 2, 7, and 14, vials were taken from the refrigerator, and 1 mL of solution was removed. Two samples, one 10-μL sample and one 100-μL sample diluted 1:10 with sterile water to minimize any antifungal carryover, were immediately taken from the 1 mL of solution and cultured onto Sabouraud agar. The sampling process was repeated after the 1 mL of solution had been allowed to warm to room temperature for 2 hours. All plates were then incubated at 35°C for 36 hours, at which time viable colony counts of the culture plates were measured and mean counts were calculated.

**Safety Study**

Fifteen pairs of research-grade donor corneas were obtained from SightLife. Three pairs of corneas were used to test each of the 5 different antifungal concentrations (voriconazole at 50× MIC or amphotericin B at 0.25×, 0.5×, 1×, or 10× MIC). A randomly selected cornea was placed in antifungal-supplemented Optisol-GS, and the other cornea from the pair was placed in unsupplemented Optisol-GS as a control. Randomization was performed by coin toss. All corneas were stored at 2°C to 8°C per manufacturer recommendations. Donor corneal percentage of intact epithelium via slitlamp examination and endothelial cell density (ECD) via specular microscopy were determined on days 0, 7, and 14 according to standard EBAA corneal transplant evaluation protocol by SightLife technicians (C.H. and others) masked to the treatment assignment. Paired t-tests were used to compare the mean change in the percentage of intact epithelium and the mean change in ECD between controls and supplemented Optisol-GS from day 0 to day 7 and from day 0 to day 14. For statistical analyses, a conserva-
Vital dye staining of the donor corneal endothelium was performed on all corneas at day 14. The stains used were 0.4% trypan blue (Sigma-Aldrich Corp), a vital dye that stains severely damaged and dead endothelial cells, and 1% alizarin red (GFS Chemicals, Inc), which stains cell borders and denuded Descemet membrane. Staining was conducted based on a protocol recently published by Park and associates.18 Each donor cornea was placed in a culture dish with the endothelium facing up. The endothelium was covered with several drops of 0.4% trypan blue for 60 seconds. The trypan blue was gently poured off, and the cornea was rinsed with phosphate-buffered saline. The cornea was then immersed in 0.5% alizarin red (1% solution diluted 1:1 with phosphate-buffered saline) for 90 seconds, followed by rinsing with phosphate-buffered saline. Using Westcott scissors and forceps, 4 radial incisions were made through the donor rims up to the limbus to flatten the corneas, which were then transferred to microscope slides with the endothelium facing up. A slide cover was placed and the endothelium was examined under a Nikon Eclipse E400 light microscope at 100× magnification, attached to a Nikon digital camera (DXM 1200F). Five photographs of different fields of view within the central and paracentral cornea were taken for each cornea. In each photograph, viable and nonviable cells (stained with trypan blue) were counted in a given area, and the mean percentage of nonviable cells was calculated for each cornea. Paired t tests were used to compare the percentage of nonviable cells (number of trypan-staining cells divided by the total number of cells) between controls and supplemented Optisol-GS from day 0 to day 14.

**Results**

**Voriconazole Supplementation**

Growth of *C albicans* (Figure 1) and *C glabrata* (Figure 2) was observed at all time points in all the voriconazole-supplemented
vials. In the vials inoculated with *C. albicans*, the voriconazole-supplemented vials revealed relatively similar colony counts to Optisol-GS alone. In samples warmed to room temperature, the voriconazole-supplemented vials intermittently revealed even greater colony counts than Optisol-GS alone.

For *C. glabrata*, each concentration of voriconazole supplementation resulted in lower colony counts compared with Optisol-GS alone. On average, colony counts were reduced by 70% in the voriconazole-supplemented vials. There was no clear dose-related response in colony reduction at higher concentrations of voriconazole with either organism.

**Amphotericin B Supplementation**
In the vials inoculated with *C. albicans*, there were no viable colonies in any of the amphotericin B-supplemented vials in any samples taken (Figure 1). For *C. glabrata*, there was minimal colony growth on day 2 in the 2 vials with the lowest concentration of amphotericin B supplementation, 0.25× MIC and 0.5× MIC (Figure 2). However, viable counts were reduced by 99% and 96%, respectively. Otherwise, there were no viable colonies in any other samples taken from the amphotericin B-supplemented vials. On average, *C. glabrata* colony counts were reduced by 99% in the amphotericin B-supplemented vials.

**Corneal Safety Results**
Compared with paired controls, there appeared to be no difference in the mean change in the percentage of intact epithelium in any of the groups of antifungal-supplemented Optisol-GS (Table 1). There was a significant reduction in ECD from day 0 to day 7 with amphotericin B at 10× MIC compared with paired controls, with a mean loss of 500 vs 100 cells/mm² (*P* = .04) (Table 2). There was a similar trend, although not statistically significant, with amphotericin B at 1× MIC, with a mean loss of 138 vs 84 cells/mm² in paired controls (*P* = .07). There appeared to be no difference in ECD reduction between paired controls and antifungal-supplemented Optisol-GS.

The percentage of nonviable endothelial cells as determined by vital dye staining was significantly higher with amphotericin B at 10× MIC compared with paired controls (78% vs 10% nonviable cells; *P* = .02) (Table 3). There appeared to be no difference in the percentage of nonviable endothelial cells between paired controls and antifungal-supplemented Optisol-GS.

### Table 1. Mean (SD) Change in Amount of Intact Epithelium

<table>
<thead>
<tr>
<th>Agent</th>
<th>Day 0 to Day 7, %</th>
<th>Drug</th>
<th>Control</th>
<th>P Valuea</th>
<th>Day 0 to Day 14, %</th>
<th>Drug</th>
<th>Control</th>
<th>P Valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voriconazole 50× MIC</td>
<td>−1.0 (0.0)</td>
<td>−1.3 (0.6)</td>
<td>.42</td>
<td>−2.3 (2.3)</td>
<td>−1.3 (0.6)</td>
<td>.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphotericin B 0.25× MIC</td>
<td>0</td>
<td>0</td>
<td>&lt;.01</td>
<td>−1.3 (1.2)</td>
<td>−1.3 (1.5)</td>
<td>&gt; .99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphotericin B 0.5× MIC</td>
<td>−3.7 (5.5)</td>
<td>−3.7 (5.5)</td>
<td>.32</td>
<td>−3.7 (5.5)</td>
<td>−3.7 (5.5)</td>
<td>.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphotericin B 1× MIC</td>
<td>−0.7 (0.6)</td>
<td>−0.7 (0.6)</td>
<td>.42</td>
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<td>−0.7 (0.6)</td>
<td>.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphotericin B 10× MIC</td>
<td>0</td>
<td>−0.7 (1.2)</td>
<td>.42</td>
<td>−0.7 (1.2)</td>
<td>−0.7 (1.2)</td>
<td>.42</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: MIC, minimum inhibitory concentration.

### Table 2. Mean (SD) Change in Endothelial Cell Count

<table>
<thead>
<tr>
<th>Agent</th>
<th>Day 0 to Day 7, Cells/mm²</th>
<th>Drug</th>
<th>Control</th>
<th>P Valuea</th>
<th>Day 0 to Day 14, Cells/mm²</th>
<th>Drug</th>
<th>Control</th>
<th>P Valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voriconazole 50× MIC</td>
<td>−4.3 (60.3)</td>
<td>−72.8 (233.7)</td>
<td>.60</td>
<td>−259.2 (10.3)</td>
<td>−259.2 (10.3)</td>
<td>.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphotericin B 0.25× MIC</td>
<td>−567.3 (705.4)</td>
<td>−509.9 (497.5)</td>
<td>.74</td>
<td>−291.8 (204.4)</td>
<td>−291.8 (204.4)</td>
<td>.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphotericin B 0.5× MIC</td>
<td>180.9 (243.6)</td>
<td>107.6 (94.4)</td>
<td>.27</td>
<td>−362.7 (102.8)</td>
<td>−362.7 (102.8)</td>
<td>.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphotericin B 1× MIC</td>
<td>−138.4 (140.2)</td>
<td>84.3 (59.8)</td>
<td>.07</td>
<td>−473.1 (288.4)</td>
<td>−473.1 (288.4)</td>
<td>.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphotericin B 10× MIC</td>
<td>Indeterminate</td>
<td>−99.9 (141.8)</td>
<td>.04</td>
<td>−206.3 (233.9)</td>
<td>−206.3 (233.9)</td>
<td>.04</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: MIC, minimum inhibitory concentration.

### Table 3. Vital Dye Staining Results

<table>
<thead>
<tr>
<th>Agent</th>
<th>Nonviable Cells on Day 14, Mean, %</th>
<th>Drug</th>
<th>Control</th>
<th>P Valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voriconazole 50× MIC</td>
<td>4.8</td>
<td>5.3</td>
<td>.83</td>
<td></td>
</tr>
<tr>
<td>Amphotericin B 0.25× MIC</td>
<td>9.5</td>
<td>4.1</td>
<td>.37</td>
<td></td>
</tr>
<tr>
<td>Amphotericin B 0.5× MIC</td>
<td>35.9</td>
<td>28.8</td>
<td>.80</td>
<td></td>
</tr>
<tr>
<td>Amphotericin B 1× MIC</td>
<td>41.6</td>
<td>14.5</td>
<td>.36</td>
<td></td>
</tr>
<tr>
<td>Amphotericin B 10× MIC</td>
<td>77.9</td>
<td>9.5</td>
<td>.02</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: MIC, minimum inhibitory concentration.

* Paired *t* test.

b On the basis of 2 corneas; the third had an indeterminate cell count.
Discussion

Given the morbidity associated with postkeratoplasty endophthalmitis and the increasing frequency of a fungal origin, the question of antifungal supplementation in corneal storage media remains an important issue. The present study indicates that the addition of amphotericin B to Optisol-GS may significantly improve activity against contamination with Candida species, the primary cause of fungal endophthalmitis after corneal transplantation.

The efficacy of amphotericin B in Optisol-GS was anticipated, considering that most European eye banks use a storage medium for organ culture at 30°C to 37°C that includes amphotericin B at a concentration of 0.25 μg/mL. Several studies have found lower rates of contamination and postoperative infection with organ culture compared with the North American practice of hypothermic storage at 2°C to 8°C. This difference may be due to amphotericin B supplementation and the routine microbiological screening performed before transplantation with organ culture storage. According to the 2003 annual report of the European Eye Bank Association, the mean incidence of endophthalmitis after PK using hypothermic storage was 0.56% compared with only 0.012% using organ culture storage. In addition, far fewer cases of fungal endophthalmitis have been reported in the literature after organ culture storage compared with after hypothermic storage.

Our results are in contradiction to a previous study by Ritterband and colleagues, which found a significant reduction in the rate of positive fungal donor rim cultures with voriconazole-supplemented Optisol-GS compared with Optisol-GS alone (0 vs 7 of 533 positive rim cultures; \(P = .02\)). However, there were important differences in methods between the previous study and this study. The previous study assessed corneal donor rims that had been stored for 24 hours and performed cultures only if turbidity was noted. In contrast, we stored samples for a longer period (14 days) and performed cultures on all media vials. Furthermore, Ritterband and colleagues used a concentration of voriconazole (100 μg/mL) 2 times higher than our highest concentration (50 μg/mL). Although it could be argued that the higher concentration would be more effective, our highest concentration was already at least 50 times higher than the published MICs required to inhibit the growth of 90% of organisms of voriconazole for C albicans and C glabrata.

One reason amphotericin B may have outperformed voriconazole in reducing Candida species contamination in our study is the mechanism of action of each drug. Amphotericin B is a polyene considered to be fungicidal, whereas voriconazole is a triazole that is fungistatic against most Candida species. Our time-kill plots resemble other time-kill plots for amphotericin B and azole antifungals, demonstrating the fungicidal activity (defined as >99% reduction in colony counts) of amphotericin B and the fungistatic activity (defined as <99% reduction in colony counts) of voriconazole.

Interestingly, our study found a slight decrease in colony counts with Optisol-GS alone. Perhaps this was an effect of hypothermic storage on Candida species proliferation because hypothermic storage reduces microbial activity by reduction of metabolic rate in the cold. It is unlikely to be attributable to any components in Optisol-GS itself. One early study evaluating antibiotic supplementation to Optisol-GS found no reduction of inoculated C albicans levels in Optisol-GS supplemented with streptomycin and gentamicin and stored at room temperature.

Although our study found amphotericin B to be effective, we also found significant endothelial toxic effects at the maximal concentrations of amphotericin B supplementation. We did not find significant toxic effects at lower concentrations of amphotericin B or with voriconazole, although our small sample size prevents generalization of this finding. Amphotericin B is already known to have significant adverse effects with both systemic and ocular use. Conversely, voriconazole has previously been found to have no significant toxic effects when used at effective concentrations topically and intravitreally. Voriconazole-supplemented (100 μg/mL) Optisol-GS has been reported to be safe in previous studies. Ritterband and colleagues reported no signs of endothelial toxic effects to donor corneas by electron microscopy or vital dye staining, and Kim and colleagues reported no endothelial toxic effects by electron microscopy or endothelial cell count via specular microscopy.

One major limitation of our study is the sample size of our toxicity evaluations. Because only 3 pairs of corneas per supplementation concentration were studied, there were insufficient data to show a significant difference. Furthermore, the identification of dead or damaged cells with vital dye staining has an element of subjectivity because some cells faintly stain with trypan blue. There is no definitive conclusion regarding the classification of such cells in the published literature. Finally, we did not evaluate the stability of the antifungal additives. As such, no conclusion can be drawn regarding the timing of antifungal addition to the media, which ideally would be at the time of Optisol-GS manufacturing rather than at tissue procurement. Amphotericin B is known to be unstable in light, which would necessitate light-protected media vials. Alternately, perhaps exposing amphotericin B-supplemented vials to light after a certain period would degrade the antifungal, theoretically decreasing its toxicity after its efficacy has been exerted.

Conclusions

A recent statement by the EBAA Medical Advisory Board did not recommend antifungal supplementation of corneal storage media, partly because of insufficient evidence regarding efficacy and safety. We hope this study helps to provide such evidence. Although our study suggests that a low concentration of amphotericin B might be a safe and efficacious addition to storage media, a larger study is warranted to confirm these findings.
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Acquisition, analysis, or interpretation of data: Layer, Maxwell, Hoover, Keenan, Jeng.
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