Original Article

Efficacy of voriconazole and amphotericin B in corneal preservative media

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Purpose: To evaluate the efficacy of voriconazole and amphotericin B in McCarey–Kaufman (MK) media. **Methods:** MK media vials were supplemented with either voriconazole at 1, 2, 20, 50, 100 µg/mL or amphotericin B at 0.5, 1, 2, 10, 20 µg/mL. The standard inoculum of the American Type Culture Collection (ATCC) strain of *Candida albicans*, *Aspergillus flavus*, and *Fusarium keratinoplasticum* was added to the set of vials. The efficacy outcomes were calculated as 'viable fungal colony counts' determined from the samples taken on Days 0 and 4. MK media containing fungal inoculum but without antifungal supplements were used as control. **Results:** In the voriconazole arm, on Day 4, a reduction in the colony count was observed for *Candida albicans* (1 µg/mL, 36%; 100 µg/mL, 100%), *Aspergillus flavus* (1 µg/mL, 53.8%; 100 µg/mL, 80.4%), and *Fusarium keratinoplasticum* (1 µg/mL, 39.0%; 100 µg/mL, 72.2%). Similarly, in the amphotericin B arm, on Day 4, a reduction in the colony count was observed for *Candida albicans* (0.5 µg/mL; 99.9%; 20 µg/mL, 100%), *Aspergillus flavus* (0.5 µg/mL, 65.2%; 20 µg/mL, 84.8%), and *Fusarium keratinoplasticum* (0.5 µg/mL, 90.1%; 20 µg/mL, 100%). **Conclusion:** Compared to voriconazole, the addition of amphotericin B significantly reduces fungal contamination in MK media.

Key words: Antifungal, corneal preservative medium, MK medium

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Acute endophthalmitis is a devastating complication after intraocular surgery. Keratitis and endophthalmitis have also been reported after corneal transplantation procedures. In many of these cases, the probable source of infection is the donor cornea. A concordance between the organisms cultured from the donor rim and postoperative endophthalmitis has been reported.[1-5]

The popular corneal storage media (CSM) in the United States is Optisol-GS (Bausch and Lomb, Rochester, NY)[6]; whereas in Europe, the organ culture media supplemented with amphotericin B (Biochrom AG, Berlin, Germany) is more commonly used.[7-10] It has been found that the fungal recovery rate in antifungal supplemented organ culture media used in Europe is lower than the non-antifungal-supplemented CSM in the United States (0% vs. 0.59%, $P = 0.025$), with comparable graft failure rates.[11,12]

With a recent increase in the number of cases of fungal infection, especially after lamellar surgeries, corneal surgeons feel the need for antifungal drugs in the CSM. This issue has become particularly important because most of the currently available CSM are supplemented with only antibacterial agents and without antifungal agents.

The efficacy and safety of voriconazole have been studied in Optisol-GS CSM.[13] It significantly reduced the rate of the positive rim fungal culture with no signs of endothelial toxicity. The addition of amphotericin B to Optisol-GS CSM has shown an improved activity against the contamination by the *Candida* species.[14] However, this efficacy was associated with significant endothelial toxicity. Similarly, the preservation in Optisol-GS CSM mixed with moxifloxacin and voriconazole has shown to induce significant toxicity on the endothelial cells of the porcine corneas compared to the control group.[15] Studies have also concluded that the previously frozen amphotericin B (2.5 µg/mL, stored at −20°C for 4 weeks) in Optisol-GS has been found to be highly effective against *C. albicans* resulting in >90% Colony Forming Unit (CFU) reduction by 6 h and >99% reduction by 72 h. Thus, this study not only proves amphotericin B to be efficacious but also addresses the logistic burden of the eye bank by utilizing frozen-then-thawed amphotericin B.[16] On the other hand, studies have found amphotericin B (0.255 µg/mL) to be ineffective in eliminating yeast from the corneal tissue stored in the Optisol-GS media (2°-8°C).[17]

McCarey–Kaufman (MK) is the common CSM used in India and in many developing countries. Although the MK media contains antibiotics (gentamicin), it does not contain any antifungal agent to arrest the growth of fungi. Various strategies have been developed to reduce infection. The earlier studies have focused their experiments on the *Candida spp* only. The aim of this study is to evaluate the efficacy of the antifungals, viz.

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voriconazole and amphotericin B, in MK media against *Candida albicans*, *Aspergillus flavus,* and *Fusarium keratinoplasticum*.

Methods

Fungal strain and preparation

We used the ATCC strains of the following three fungi for these experiments: *Aspergillus flavus*-9643, *Fusarium keratinoplasticum*-36031, and *Candida albicans*-90028. These three fungi were sub-cultured on Sabouraud dextrose agar (SDA) at 26°C for *Fusarium keratinoplasticum* and *Aspergillus flavus* molds, and at 37°C for *Candida albicans*. *Candida albicans* was incubated for 24–48 h, and the other molds were incubated for ~ 7 days till mature powdery spores were seen on the surface of the growth. The standard suspension of *Candida* from the colonies on SDA, and spores of *Fusarium* and *Aspergillus* were prepared in distilled water to have 0.5 MacFarland turbidity (equal to 5×10^6 CFU/mL). This standard inoculum was added to each of the vials supplemented with antifungals, including the positive controls but excluding the negative controls, in 20 mL of MK media in such volume so that the final concentration of the fungus would be 2.5×10^3 CFU/mL in the MK media vials. The inoculated vials were refrigerated at 2–8°C and viable colony counts from these vials were done on Day 0 (immediately after the inoculation and before refrigerating) and on Day 4.

Drug concentration and preparation

Vials of MK media, containing 20 mL each of the corneal preservative medium and stored at 4°C, were brought to room temperature before aseptically supplementing with different concentrations of voriconazole (powder from Sigma-Aldrich, Mumbai, India) and amphotericin B (pharmacological grade powder for IV injection, Mycotin, United Biotech Pvt Ltd) for testing the antifungal activity. The minimum inhibitory concentration (MIC) for clinical isolates of *Aspergillus flavus, Fusarium solani,* and *Candida albicans* for voriconazole and amphotericin B, as previously published, was taken as the standard.[18] According to Sabatelli *et al*.,[18] MIC90 for voriconazole and amphotericin B for *Aspergillus flavus* and *Fusarium solani* was 2 µg/mL each for both the drugs, and for *Candida albicans*, it was 0.5 and 1 µg/mL, respectively. Accordingly, in our experiments, the MK media vials were supplemented with various concentrations of these two antifungals sequentially, as shown in Table 1. Although MIC for *Candida spp* was lower than the other two molds for both the antifungals, the higher MIC for the molds was taken as the standard to maintain uniformity in the testing, as a single concentration of either or both the antifungals in the MK media was finally used. For each of these three sets of supplemented vials for each antifungal, a positive control (only fungal inoculum in MK media without antifungals) and negative control (only MK media without antifungals or fungal inoculum) were included.

Table 1: Drug concentration of voriconazole and amphotericin B supplements in MK medium

Multiple of MIC90 of Voriconazole	Voriconazole (µg/mL)	Multiple of MIC90 of Amphotericin B	Amphotericin B (µg/mL)
0.5x	1	0.25x	0.5
1x	2	0.5x	1
10x	20	1x	2
25x	50	5x	10
50x	100	10x	20

Experimental design

The above-inoculated vials were taken out from the refrigerator on the above-mentioned days (Days 0 and 4) and 1 ml of the inoculated MK media was immediately removed aseptically from each of the vials while refrigerating the remaining media at 2–8°C. Out of 1 mL, a 10 µL volume was immediately sub-cultured onto the SDA plates. All the above plates were then incubated at 26°C for the molds and 37°C for the yeasts for 48 h, before reporting viable colony counts on SDA. The experiments were repeated in triplicate and the mean values were calculated.

Results

The percentage reduction of colony counts (of *Candida albicans, Aspergillus flavus,* and *Fusarium keratinoplasticum* compared to the mean unsupplemented positive control at Day 0) on Day 0 and Day 4 for various concentrations of voriconazole and amphotericin B are shown in Table 2. The colony counts on Day 0 and Day 4 of *Candida albicans*, *Aspergillus flavus,* and *Fusarium keratinoplasticum* supplemented with various concentrations of voriconazole are shown in Table 3.

Candida albicans

With respect to voriconazole, there was no growth of *Candida albicans* in the concentration of 100 µg/mL on Day 0 and Day 4. On Day 4, the reduction in the colony count in the vials inoculated with 2 and 1 µg/mL was 62 and 36%, respectively. Significant percentage reduction of colony counts was observed between the concentrations: 2 µg/mL versus 1 µg/mL and 20 µg/mL versus 2 µg/mL on Days 0 and 4 [Table 2].

When tested with amphotericin B more than 99% reduction in the colony counts was observed in all the concentrations on Day 4. Significant difference was observed in the percentage reduction of the colony counts between the concentrations: 20 µg/mL versus 10 µg/mL, 10 µg/mL versus 2 µg/mL and 2 µg/mL versus 1 µg/mL on Day 0 only.

Aspergillus flavus

The reduction in the colony counts increased with time in all the concentrations of voriconazole. The maximum reduction in the colony count on Day 4 (80.4%) was observed with 100 µg/mL. A significant percentage reduction of colony counts was observed between the concentrations: 20 µg/mL versus 2 µg/mL and 100 µg/mL versus 50 µg/mL on Day 0.

There was a maximum reduction (84.8%) in the colony count on Day 4 with the concentration of 20 µg/mL of amphotericin B. The reduction was less than 70% when the concentration was ≤2 µg/mL on Day 4. A significant difference was observed in the percentage reduction of the colony counts between the concentrations: 10 µg/mL versus 2 µg/mL on Day 0 and 2 µg/mL versus 1 µg/mL on Day 4.

Fusarium keratinoplasticum

The maximum reduction in the colony count of 72% was observed with the voriconazole concentration of 100 µg/mL

Table 2: Chi-square test for comparison of two proportions (i.e., percentage reduction of colony counts of *Candida albicans, Aspergillus flavus*, and *Fusarium keratinoplasticum* compared to unsupplemented positive control at Day 0) for different drug concentrations

		Candida albicans		Aspergillus flavus		Fusarium keratinoplasticum	
		Day 0	Day 4	Day 0	Day 4	Day 0	Day 4
Voriconazole		(*n*=210)		(*n*=75)		(*n*=80)	
[A_V]	100 µg/mL	100.0%	100.0%	68.0%	80.4%	68.5%	72.2%
[B_V]	50 µg/mL	100.0%	99.4%	47.1%	74.7%	49.3%	64.3%
[C_V]	20 µg/mL	96.7%	95.9%	34.3%	61.3%	37.8%	54.8%
[D_V]	2 µg/mL	24.7%	62.4%	15.6%	55.1%	18.7%	53.1%
[E_V]	1 µg/mL	3.2%	36.0%	12.9%	53.7%	12.9%	39.0%
[A_V] vs [B_V]	*P*	----	0.2615	0.0099	0.4044	0.0139	0.2846
	(95% CI)	(-1.8%-1.8%)	(-1.2%-2.8%)	(5.0%-35.3%)	(-7.6%-18.8%)	(3.9%-33.2%)	(-6.5%-21.8%)
[B_V] vs [C_V]	*P*	0.0080	0.0180	0.1118	0.0796	0.1437	0.2223
	(95% CI)	(0.8%-6.6%)	(0.5%-7.1%)	(-2.8%-27.6%)	(−1.5%-27.5%)	(−3.8%-26.1%)	(−5.6%-24.0%)
[C_V] vs [D_V]	P	<0.0001	<0.0001	0.0084	0.4430	0.0075	0.8297
	(95% CI)	(64.8%-77.6%)	(26.2%-40.5%)	(4.8%-31.7%)	(−9.4%-21.3%)	(5.1%-32.1%)	(−13.5%-16.7%)
[D_V] vs [E_V]	*P*	<0.0001	<0.0001	0.6373	0.8638	0.3161	0.0745
	(95% CI)	(15.2%-27.9%)	(16.9%-35.2%)	(−8.7%-14.1%)	(−14.2%-16.9%)	(−5.7%-17.2%)	(−1.3%-28.6%)
Amphotericin B		(*n*=263)		(*n*=83)		(*n*=98)	
[A_A]	20 µg/mL	96.7%	100.0%	55.6%	84.8%	47.1%	100.0%
[B_A]	10 µg/mL	89.7%	100.0%	48.4%	82.4%	36.5%	99.0%
[C_A]	2 µg/mL	36.2%	100.0%	28.8%	74.0%	32.7%	95.9%
[D_A]	1 µg/mL	13.8%	99.9%	17.6%	56.8%	17.8%	92.5%
[E_A]	0.5 µg/mL	14.6%	99.9%	10.0%	65.2%	10.9%	90.1%
[A_A] vs [B_A]	*P*	0.0014	-	0.3547	0.6772	0.1335	0.7548
	95% CI	(2.7%-11.5%)	(−1.4%-1.4%)	(−7.8%-21.8%)	(−9.0%-13.7%)	(−3.1%-23.8%)	(−3.6%-3.9%)
[B_A] vs [C_A]	*P*	<0.0001	-	0.0097	0.1913	0.577	0.0514
	95% CI	(46.1%-59.8%)	(−1.4%-1.4%)	(4.7%-33.2%)	(−4.2%-29.7%)	(−9.4%-16.8%)	(−0.6%-9.9%)
[C_A] vs [D_A]	*P*	<0.0001	0.6083	0.0884	0.0202	0.0166	0.3098
	95% CI	(15.1%-29.4%)	(−1.3%-1.6%)	(−16.0%-23.6%)	(2.7%-30.7%)	(2.7%-26.5%)	(−3.6%-10.8%)
[D_A] vs [E_A]	*P*	0.7929	1.0000	0.1570	0.2687	0.1694	0.5521
	95% CI	(−5.2%-6.8%)	(−1.5%-1.5%)	(−3.0%-18.3%)	(−6.3%-22.6%)	(−3.0%-16.8%)	(−5.8%-10.8%)

Table 3: Colony count on Day 0 and Day 4, of Candida albicans, Aspergillus flavus, and Fusarium keratinoplasticum supplemented with various concentrations of voriconazole and amphotericin B

	Candida albicans		Aspergillus flavus		Fusarium keratinoplasticum	
	Day 0	Day 4	Day 0	Day 4	Day 0	Day 4
Voriconazole						
100 µg/mL	0	0	24.0	14.7	25.3	22.3
50 µg/mL	0	1.3	39.7	19.0	40.7	28.7
20 µg/mL	7.0	8.7	49.3	29.0	50.0	36.3
2 µg/mL	158.3	79.0	63.3	33.7	65.3	37.7
1 µg/mL	203.7	134.7	65.3	34.7	70.0	49.0
Amphotericin B						
20 µg/mL	8.7	0	37.0	12.7	51.7	0
10 µg/mL	27.0	0	43.0	14.7	62.0	1.0
2 µg/mL	167.7	0	59.3	21.7	65.7	4.0
1 µg/mL	226.3	0.3	68.7	36.0	80.3	7.3
0.5 µg/mL	224.3	0.3	75.0	29.0	87.0	9.7

on Day 4. A significant percentage reduction of the colony counts was observed between the concentrations: 20 µg/mL versus 2 µg/mL on Day 0.

More than a 90% reduction in the colony count was observed on Day 4 for all the concentrations of amphotericin B. A significant difference was observed in the percentage reduction of the colony counts between the concentrations: 2 µg/mL versus 1 µg/mL on Day 4.

Discussion

Despite strict protocols followed by the eye banks for sterility, there are reports of postoperative infections.[1-5] Although bacterial infection is more commonly reported compared to fungal infection, the infection due to the latter poses more challenges.[1,4,19,20] Also, the outcome due to fungal infection is known to be worse than the bacterial infection. The *Candida* species are responsible for a majority of the reported cases of post-keratoplasty keratitis and endophthalmitis.[2,4,21,22] The donor-related *Candida* infection has also been reported after lamellar keratoplasty.[23,24] *Candida* constitutes 8.6% of the culture-positive cases from the cultures of donor corneoscleral rim.[22] Therefore, there is a need for an antifungal as a supplement in the corneal preservative medium.

The microbial profile obtained from the culture of donor corneoscleral rim constitutes ocular flora of conjunctiva. The prevalence of the fungi may increase with the cornea recovered from the patients on the ventilator and ocular surface disease. Except for Europe and Australia, most countries use hypothermic storage media at 4°C as a mode of preservation. The organ culture medium supplemented with amphotericin B, stored at 30–37°C is the preferred medium in the European Countries. The bacterial and fungal contamination is low in the cornea stored in the organ culture medium.[25] However, the commonly used medium like Optisol-GS and MK lack antifungals. Voriconazole and amphotericin B have been studied as an additive to Optisol-GS in experimental studies.[13-15] The earlier studies have demonstrated the efficacy of voriconazole on *Candida*. However, the efficacy of the antifungal has not been studied in the MK media.

Voriconazole, a second-generation triazole, shows a broader *in vitro* susceptibility profile against *Candida*, *Aspergillus*, *Fusarium*, *Scedosporium*, and *Paecilomyces*.[26,27] It inhibits the formation of ergosterol and damages the cell wall. It has been successfully used for fungal keratitis and endophthalmitis. Amphotericin B is a polyene group of antifungals and works by disrupting the cell wall of the fungus. It is effective against *Candida*, *Aspergillus*, and *Fusarium*. Among the polyene class of antifungals, amphotericin B shows the most potent activity with low MIC values for most of the clinically relevant fungi.[27]

Since filamentous fungi are more prevalent than *Candida* in tropical countries, we planned to conduct our study for the two filamentous fungi in addition to *Candida albicans*. Layer *et al*.[14] used four concentrations of voriconazole starting with 1–50 µg/mL and observed for 14 days. They did not find any difference in the colony count with the voriconazole-supplemented Optisol-GS. However, we have used concentrations up to 100 µg/mL. There was no growth of *Candida albicans* when the concentration was 100 µg/mL. This might be due to a higher concentration. In our experiment, >95% reduction was observed when the concentration was 20 µg/mL and more. This might be due to the different compositions of the preservative media. Voriconazole, at 100 µg/mL concentration, has been shown to reduce significant fungal growth in another study where half of the corneoscleral rim was placed in voriconazole supplemented with Optisol-GS compared to unsupplemented Optisol-GS.[13] The reduction of colonies was less for *Aspergillus flavus* and *Fusarium keratinoplasticum* in comparison to *Candida albicans*. It correlates with the MIC levels of the drugs for different organisms.[27]

When compared to voriconazole, amphotericin B has a superior activity. More than 99% reduction was noticed in all the concentrations on Day 4 for *Candida albicans*. It is comparable with an earlier study in terms of the superiority of amphotericin B over voriconazole.[14] Though the reduction in the colony count was less in *Aspergillus flavus* and *Fusarium keratinoplasticum* compared to *Candida albicans*, it was better than voriconazole. Amphotericin B has a superior activity for *Fusarium keratinoplasticum* than *Aspergillus flavus*. Amphotericin B being fungicidal might be the reason for better efficacy compared to voriconazole.

The decision of adding antifungal is dependent on the toxicity and efficacy of any antifungal, in addition to the cost. Gibbons *et al*.[28] have studied the cost-effective analysis of supplementing hypothermic cold storage media with antifungal drugs. Amphotericin B was found to be more cost-effective than voriconazole, caspofungin, and a combination antifungals, especially for endothelial keratoplasty grafts. Another study was conducted to compare the cost-effectiveness of amphotericin B supplementation versus current costs to treat post-endothelial keratoplasty (EK) fungal infection. It was concluded that amphotericin B supplementation is cost-effective and can prevent more than 69.62% of the post-EK fungal infections.[29] Based on this current experiment, the lowest concentration with a higher efficacy was observed with amphotericin B (0.5 µg/mL). Future research on endothelial toxicity (currently experiments are under process) with the above concentrations can guide us about the correct dosage and choice of the drug.

Conclusion

The addition of amphotericin B to the MK media may significantly reduce fungal contamination considering the continuous reduction of colony counts at all the concentrations till the end of the study period.

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Conflicts of interest

There are no conflicts of interest.

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