Original Article

Anti-fungal efficacy of combination of amphotericin B with colistin and gentamicin in McCarey-Kaufman corneal preservation medium

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Purpose: To curtail the potential of donor corneal tissue disseminating fungi to the recipient's eye, we evaluated the addition of amphotericin B to McCarey-Kaufman (M-K)—corneal storage medium supplemented with colistin. **Methods:** Amphotericin B was examined for its ability to inhibit the growth of *Candida albicans* and *Aspergillus flavus* using a microbroth dilution test and checkerboard assay in combination with only gentamicin and a combination of colistin, gentamicin, and amphotericin B. The safety on epithelium and endothelium was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. **Results:** The minimal inhibitory concentration of gentamicin was found to be >256 µg/ml against both *C. albicans* and *A. flavus*, whereas that of amphotericin B was found to be in a range of 0.25–0.5 and 1–2 µg/ml for *C. albicans* and *A. flavus*, respectively. According to the checkerboard assay, 80% (4/5) of *C. albicans* isolates and 100% (5/5) of *A. flavus* isolates responded synergistically to the combination of amphotericin B and gentamicin, but only 20% (1/5) of *C. albicans* isolates showed an additive effect. None of the tested isolates displayed antagonism. The combined effect of the three drugs also did not display any antagonistic effect. Additionally, the MTT assay reveals no toxic effect of the antimicrobials used on corneal epithelial and endothelial cells. **Conclusion:** *In vitro* experiments demonstrate that amphotericin B is not toxic to either epithelium or endothelium and is a promising additive to the M-K medium supplemented with colistin.

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Postoperative endophthalmitis is an infrequent but serious complication following keratoplasty, whose reported incidence ranges from 0.05 to 0.77%.[1-4] Although the most common cause of endophthalmitis is of bacterial nature, a recent significant surge in fungal infections in the United States was reported by Eye Bank Association of America (EBAA).[5,6] The reports showed increased rates of infection following endothelial keratoplasty (EK) compared to penetrating keratoplasty (PK). The data from our previous study also indicates a significantly higher risk after EK than with PK. The estimated incidence of endophthalmitis was 0.34 and 0.15% after EK and PK, respectively ($P = 0.049$).[7] This difference can be attributed to the warming cycle involved in EK that is absent in PK, which gives the microbes a chance to proliferate. Therefore, preoperative measures have gained attention to lower the risk of postoperative infections.[7,8] Compared to postkeratoplasty bacterial infections, fungal infections tend to have a relatively delayed clinical presentation. There is a poor correlation between postoperative bacterial infection and donor rim cultures. However, studies have also shown increased rates of positive fungal donor rim culture in postoperative fungal infections.[5,6,9]

The most predominant fungal contaminant in the corneal preservation media was observed to be *Candida* spp. and to a lesser extent, filamentous fungi such as *Aspergillus* spp.[10-13] Majority of the commonly used hypothermic corneal preservation media have antibacterial agents such as gentamicin/streptomycin but lack an antifungal agent.[14]

Despite being a rare occurrence, fungal endophthalmitis can have serious complications that can cause vision loss.[15] Additionally, literature shows that *C. albicans* contamination cannot be detected in the corneal preservation media, unlike other bacteria that can be detected by the change in media color.[14] In view of the increased risk of fungal infections after keratoplasty in the United States, research has focused on adding an antifungal agent such as amphotericin B/voriconazole into the preservation media.[8] These antibiotics have been tested in Optisol-GS medium, which is a popular preservation medium. Amphotericin B significantly improved the activity against the *Candida* spp.; however, a significant degree of endothelial toxicity was noted at the maximum concentration.[16] The corneal preservation media used in European countries is organ culture

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media that contains amphotericin B as an antifungal agent. The incidence of fungal infections was observed to be lower in the case of organ culture media with amphotericin B.[17]

In our previous study,[18] we have tested the efficacy of colistin (Polymixin E) in inhibiting multidrug-resistant bacteria and yeast like *Candida* spp. We found that colistin was safe and effective in arresting the growth of multidrug-resistant organisms like *Pseudomonas aeruginosa* and *Staphylococcus aureus* when used in combination with gentamicin, which is the sole antibiotic present in the McCarey- Kaufman (M-K) medium. Colistin was shown to have antifungal activity in previously published reports[19]; however, we did not find colistin to be effective against *Candida* spp. and *Aspergillus* spp. Hence, we wanted to study the safety and efficacy of the addition of amphotericin B in the M-K medium supplemented with colistin.

Methods

This is an experimental *in vitro* investigative study. The corneal scrapings of patients treated in clinics were used to collect the clinical isolates of *C. albicans* and *A. flavus* [Tables 1 and 2]. Both yeast and fungal cultures were grown and maintained in potato dextrose agar. To ascertain the minimum inhibitory concentration of amphotericin B against clinical isolates of *C. albicans* and *A. flavus*, the microbroth dilution test and checkerboard assay were carried out. Additionally, the amphotericin B and colistin interaction with gentamicin that is already present in the corneal storage solution M-K medium was assessed.

Table 1: Patient details of samples collected for *C. albicans*

Isolates	Age (years) and Sex	Sample	Diagnosis
1	43-Female	Corneal scraping	Fungal keratitis
2	72-Male	Corneal scraping	Fungal keratitis
3	41-Female	Corneal scraping	Fungal keratitis
4	53-Male	Corneal scraping	Fungal keratitis
5	84-Male	Corneal scraping	Fungal keratitis

Culturing of yeast and fungal isolates

Clinical isolates of *Candida albicans* (n = 5) and *Aspergillus flavus* (n = 5) were obtained [Tables 1 and 2]. The isolated yeast and fungi were subcultured on potato dextrose agar (HIMEDIA, India) and incubated at 37°C for 24–48 h for yeasts and one week for molds till mature spores were observed. The suspensions of yeast and fungi were made by suspending three to five well-isolated colonies of *C. albicans* and spores of *A. flavus* in RPMI-1640 medium (HIMEDIA, India) and adjusting the turbidity to a McFarland Standard of 1–5 × 10^6 CFU/ml. The McFarland standard was further diluted to a final concentration of 0.5–2.5 × 10^3 and 0.4–5 × 10^4 CFU/ml for yeast and fungi, respectively.

Drug preparation for susceptibility testing

The antimicrobial agents used in the study were amphotericin B (Samarth, India), gentamicin (Abbott, India), and colistin (Cipla, India). The standard forms of the drugs were procured, and stock solutions of each drug were made at a concentration that was twice that of the final working concentration. Following that, the antibiotic solutions were stored at 5°C until use.

Microbroth dilution test to determine the minimal inhibitory concentration (MIC) of amphotericin B and gentamicin alone

The MICs were determined following the CLSI (CLSI M38-A2) guidelines. In brief, the microbroth dilution assay was performed in a round-bottomed 96-well plate. The drugs tested were amphotericin B and gentamicin, the final working concentrations of which were prepared by serially diluting them in RPMI-1640 medium. Amphotericin B was tested at a concentration ranging between 0.0625–2 and 0.0625–4 µg/ml for *C. albicans* and *A. flavus*, respectively, and gentamicin was tested at a concentration ranging between 2 and 256 µg/ml for both *C. albicans* and *A. flavus*. The 96-well plate was prepared in such a way that each row of the plate contains 100 µl of antimicrobial agent at varying concentrations along with 100 µl of the prepared fungal and yeast inoculum having a density of 0.4–5 × 10^4 and 0.5–2.5 × 10^3 CFU/ml, respectively. A sterility control (only RPMI media) and a growth control (RPMI media

Table 2: Patient details of samples collected for *A. flavus*

Isolates	Age (years) and Sex	Sample	Diagnosis	Antibiotic susceptibility tests
1	36-Male	Corneal scraping	Microbial keratitis	Triazoles-S* Echinocandins-S Amphotericin B-S Flucytosine-S
2	65-Female	Corneal scraping	Chronic endophthalmitis	Triazoles-S Echinocandins-S Amphotericin B-S Flucytosine-S
3	49-Female	Corneal scraping	Blunt trauma	Triazoles-S Echinocandins-S Amphotericin B-S Flucytosine-S
4	4-Male	Corneal scraping	Fungal keratitis	Triazoles-S Echinocandins-S Amphotericin B-S Flucytosine-S
5	73-Female	Corneal scraping	Microbial keratitis	Triazoles-S Echinocandins-S Amphotericin B-S Flucytosine-S

*S, susceptible

with yeast/fungal inoculum) were also included. The inoculated plates were then incubated at 37°C and the readings were noted after 48 h. The lowest concentration of the drug that showed complete inhibition of the organism was considered the minimum inhibitory concentration.

Checkerboard assay to determine drug interaction

A checkerboard assay was performed to compare the potency of antimicrobial drugs when used in combination in comparison to their individual effect. Serial four-fold dilutions of the individual drugs were prepared. The amphotericin B and gentamicin were tested at a concentration ranging between 0.0625–2 and 0.0625–8 µg/ml, respectively, for *C. albicans*. Similarly, a concentration of 0.0625–4 and 0.03215–4 µg/ml of amphotericin B and gentamicin, respectively, were used for *A. flavus*. The concentration ranges of the individual drugs employed in the checkerboard assay were based on the MIC values obtained in the microbroth dilution test. To obtain the final concentration, one of the drugs was dispensed along the abscissa and the other along the ordinate such that each well contained a combination of both the drugs. Each of these wells was added with the yeast and the fungal inoculum that was diluted to a final concentration of 0.5–2.5 × 10^3 and 0.4–5 × 10^4, respectively. Sterility and growth control were also included. The plates were incubated at 37°C for 24 h for *Candida* spp. and 37°C for 48 h for *Aspergillus* spp. The fractional inhibitory concentration (FIC) of the drug combination was calculated against each organism using the equation $\sum FIC = FIC_A + FIC_B = A/MIC_A + B/MIC_B$. Here A and B are the MIC of each drug in combination, whereas MIC_A and MIC_B are the MIC of each drug alone. A combined FIC score of <0.5 implies a synergistic effect, where the combined effect of the drugs is greater than the sum of their individual effect. An FIC score between 0.5 and 4 indicates additive effect/indifference with the combined effect of the drugs being equal to the sum of their individual effects, and an FIC score >4 indicates antagonism, indicating the combined effect of the drugs is lesser than the effect of either drug individually.

Determination of interaction between three drugs—gentamicin, colistin, and amphotericin B

A checkerboard assay was performed to check if any inhibitory effect would be observed when colistin and amphotericin B were used together with gentamicin in M-K media. The concentration of gentamicin was kept constant at 100 µg/ml, which is the actual concentration employed in the M-K medium while varying concentrations of amphotericin B and colistin were added along the ordinate and abscissa. The concentration of colistin and amphotericin B used was in a range of 8–0.03 and 0.25–4 µg/ml, respectively, for *C. albicans*. Similarly, the concentration of colistin and amphotericin B used was in a range of 4–0.125 and 0.25–4 µg/ml, respectively, for *A. flavus*.

Cytotoxic bioassay to assess the epithelial and endothelial toxicity

3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay, a standard colorimetric assay was performed to screen for toxicity of the drugs. A 96-well plate, seeded with 10^4 epithelial/endothelial cells was treated with different concentrations of a combination of colistin and amphotericin B diluted in M-K medium and was incubated for 24 h. Subsequently, MTT solution was added to the cells and incubated for 1 h. The MTT crystals are reduced to purple color formazan by adding dimethyl sulfoxide. This reduction was quantified using a spectrophotometer at 575 nm. Cell viability was calculated using the following formula:

Cell viability percentage = Mean absorbance of test well/mean absorbance of control well ×100

Statistical analysis was performed following the experiment using one-way analysis of variance following post-hoc Tukey's test using Prism 5 software. Any *P* value ≤0.05 was considered statistically significant.

Results

The MICs of amphotericin B were found to be in a range of 0.25–0.5 and 1–2 µg/ml against *C. albicans* and *A. flavus*, respectively, while the MICs of gentamicin were found to be >256 µg/ml against both *C. albicans* and *A. flavus*. Tables 3 and 4 illustrate the MIC values of antimicrobial agents individually and in combination. The checkerboard assay suggests that the combination of amphotericin B and gentamicin has a synergistic effect in 80% (4/5) of the *C. albicans* and 100% (5/5) of *A. flavus* isolates, whereas an additive effect was observed only in 20% (1/5) of the *C. albicans* as shown in Table 5 and Figs. 1 and 2. Antagonism was not observed in the case of any isolates tested. Also, no antagonistic effect was observed when three drugs were combined together in a checkerboard assay against any of the *C. albicans* and *A. flavus* isolates used. According to the MTT assay, colistin and amphotericin B do not exhibit any cytotoxic effects on both epithelial (*P* = 0.2) and endothelial cells (*P* = 0.057) at any of the assay's utilized concentrations [Fig. 3], indicating that there is no significant difference between the control and the test.

Table 3: The MIC and FIC of amphotericin B and gentamicin individually and in combination against clinical isolates of *C. albicans*

Strain no	MIC† of amphotericin B by BMD (µg/ml)	MIC of gentamicin by BMD‡ (µg/ml)	∑FIC§ amphotericin B + gentamicin
L-36	0.5	>256	0.50
L-160	0.5	>256	0.50
L-202	0.25	>256	1.00
L-3682	0.5	>256	0.25
L-4059	0.5	>256	0.50

†MIC, minimal inhibitory concentration; ‡BMD, broth microdilution; §FIC, fractional inhibitory concentration

Table 4: The MIC and FIC of amphotericin B and gentamicin individually and in combination against clinical isolates of *A. flavus*

Strain no.	MIC† of amphotericin B by BMD (µg/ml)	MIC of gentamicin by BMD‡ (µg/ml)	∑FIC§ amphotericin B + gentamicin
L-2188	1	>256	0.50
L-2315	2	>256	0.25
L-2144	2	>256	0.37
L-2138	2	>256	0.50
L-2334	1	>256	0.50

†MIC, minimal inhibitory concentration; ‡BMD, broth microdilution; §FIC, fractional inhibitory concentration

Discussion

Postkeratoplasty infections are a concern as they can have sight-threatening consequences. Despite practicing stringent protocols during cornea recovery, there always exists a risk of infection after transplantation related to donor corneas. Literature shows a varied spectrum of fungi responsible for postkeratoplasty endophthalmitis/keratitis, but a majority of them were due to *Candida* spp. and a lesser extent due to filamentous fungi like *Aspergillus* spp.[10-13] To circumvent the issue of fungal infections, supplementation of a potent antifungal to the corneal preservation medium becomes necessary. Due to high risk of bacterial contamination and infection, currently gentamicin is extensively added to the preservation media. However, the fungal contamination of donor corneas especially in tropical countries like India cannot be ignored.[20] This study highlights the practical feasibility and importance of the addition of amphotericin B along with gentamicin in the corneal preservation media to avoid the potential risk of postkeratoplasty fungal infection, which could pose a serious threat to vision recovery. To provide a comprehensive overview of the current research, we have included a table [Table 6] summarizing the key details from similar studies examining different antifungals.[16,21,22]

Amphotericin B is the commonly used antifungal agent in the organ culture medium in European countries. Previous studies reported that amphotericin B/voriconazole can be a potential antifungal agent to eliminate fungal contamination.[7,8] Although studies have shown that voriconazole is an effective antifungal agent to arrest the growth of *Candida* spp. and *Aspergillus* spp., our initial experiments with voriconazole showed an antagonistic effect when combined with colistin.[18] Because our aim was to add an antibacterial agent colistin that is effective against multidrug-resistant organisms along with an antifungal agent to the M-K corneal storage medium, we could not continue to use voriconazole. Therefore, amphotericin B was employed, which did not exhibit any such antagonism when combined with colistin.

Table 5: The nature of interaction of the drug combinations (gentamicin and amphotericin B) for both yeast and fungal isolates

Organism	% Synergism	% Additive effect	% Antagonism
C. albicans	80	20	0
A. flavus	100	0	0

We conducted this study to evaluate the efficacy of adding amphotericin B to the M-K medium that was already supplemented with colistin. Additionally, the safety of their combination on both corneal epithelial and endothelial cells by cytotoxicity assay was studied. The cytotoxicity assay was performed in triplicates for each combination of increasing concentration of antimicrobials. The desired concentration to be tested was 2 µg/ml of colistin and 1 µg/ml of amphotericin B, based on the MIC of each of these antimicrobials in the microbroth dilution test. However, we intentionally included higher concentrations of antimicrobial combinations to explore their potential effects on corneal tissue. This approach would allow us to obtain a comprehensive understanding of dose-dependent relationship between the antimicrobial drugs and corneal damage.

Amphotericin B belongs to the polyene class of antifungals and is considered a gold standard to treat infections caused by *Candida* spp. Amphotericin B functions by targeting the ergosterol found in the cell membrane of fungi and yeast. It enters the lipid bilayer by binding to the ergosterol with its hydrophobic regions. As a result, the membrane develops multimeric pores that make it permeable to small cations, accelerating the loss of intracellular ions and ultimately causing cell death. Amphotericin B also works by producing reactive oxygen species that induce oxidative damage.[23]

A recent study conducted by Schwarz *et al*.[24] revealed that MIC values of amphotericin B against both *C. albicans* and *A. flavus* were equivalent to those of our current findings,

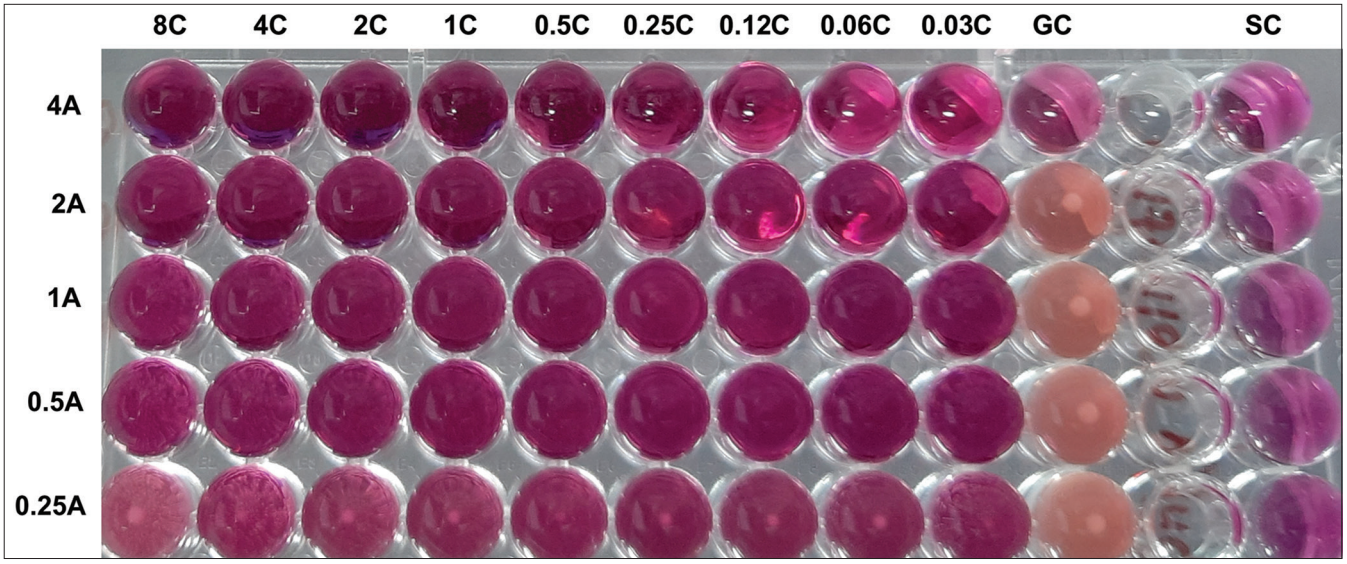

Figure 1: A representative image of checkerboard assay—A minimum concentration of 0.5 µg/ml amphotericin B (similar to its MIC) with any combination of colistin does not show growth of *C.albicans*, suggesting that colistin does not cause any inhibitory effect on *Candida* spp. when used in combination with gentamicin and colistin. A—amphotericin B; C—colistin; GC—growth control; SC—sterility control

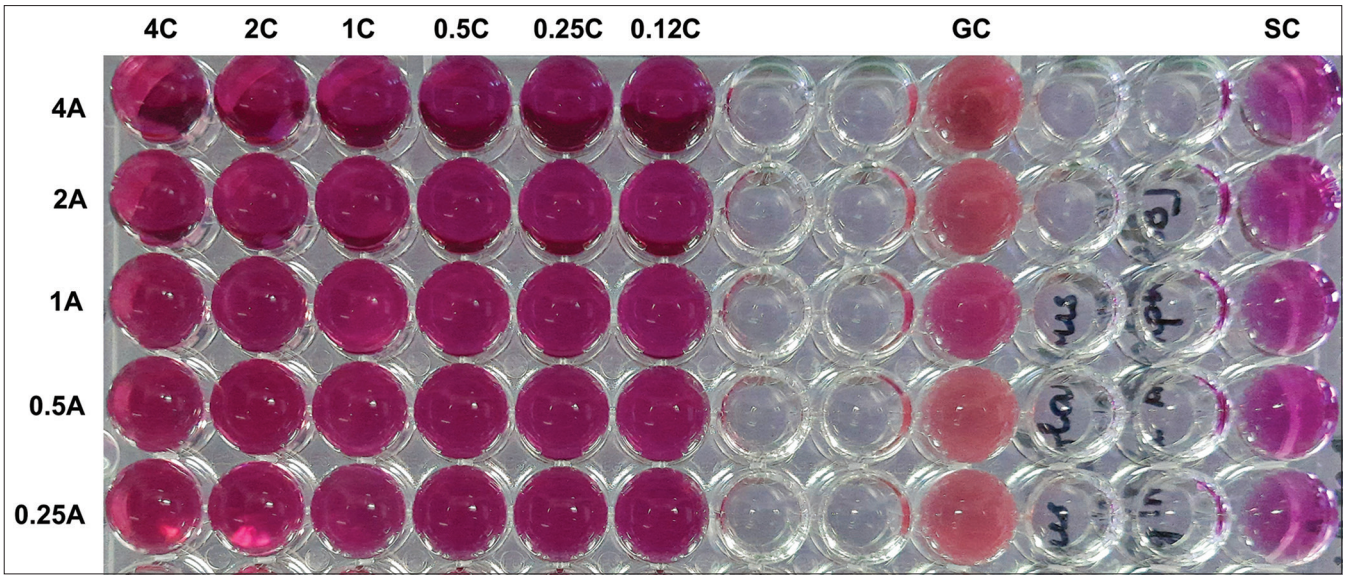

Figure 2: A representative image of checkerboard assay—No growth of *A. flavus* was observed at any of the concentrations used, suggesting that amphotericin B might have a better activity when combined with gentamicin. The image also shows that no inhibitory effect was caused by colistin. A—amphotericin B; C—colistin; GC—growth control; SC—sterility control

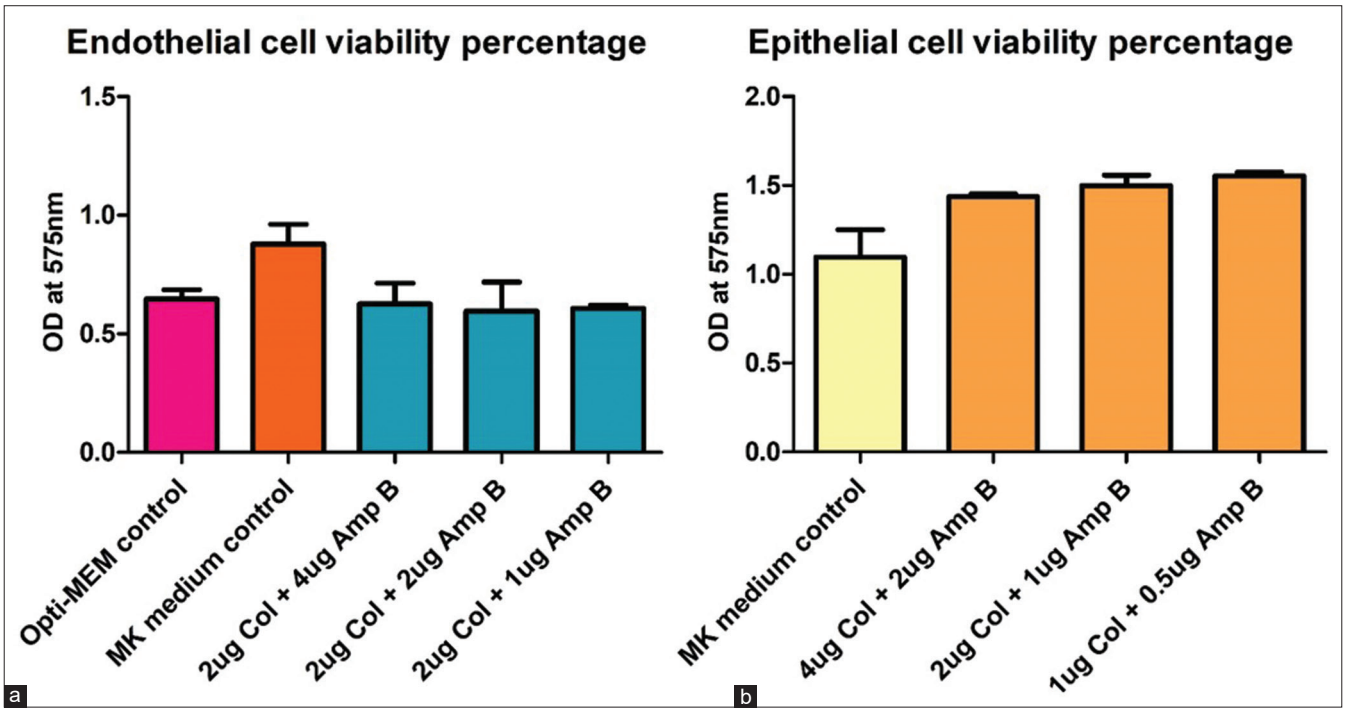

Figure 3: Graphs depicting the cytotoxicity of a combination of colistin and amphotericin B on human corneal epithelial (b) and endothelial cells (a) ($P \neq 0.05$). Col—colistin; Amp B—amphotericin B; M-K medium—McCarey-Kaufman medium; Opti-MEM—optimized-minimal essential medium

demonstrating its effectiveness against both species. However, the nature of the interaction between colistin and amphotericin B showed a slight deviation, where a 100% additive effect was observed in the case of *A. flavus* in their study, whereas our data showed a 100% synergistic effect against *A. flavus*. Because no antibiotic can be used without assessing its safety, an *in vitro* cytotoxicity test was performed to evaluate the same.

The practical feasibility of fortifying the M-K medium as per our findings, along with its cost-effectiveness in relation to rarity of post-PK fungal endophthalmitis, is a crucial consideration. With the increasing popularity of lamellar keratoplasty techniques, such as anterior and posterior lamellar procedures, the occurrence of fungal interface infections, though less than bacterial infections, cannot be disregarded. Hence investigating preventive measures becomes important.

We investigated the cost-effectiveness of additional antibiotic supplementation in the M-K medium. A single vial containing 50 mg of amphotericin B was acquired at a cost of Rs. 326. This vial is used to prepare a 10 ml of 5 mg/ml stock solution. Subsequently, for each 20 ml vial of M-K media, 4 µl (0.004 ml) of the above-prepared stock solution of amphotericin B was added.

Table 6: Salient features of prior similar studies examining the role of different antifungals

Study	Antifungal drug	Organisms	Preservation media	Key findings
Layer *et al.*	Voriconazole and amphotericin B	*C. albicans*, *C. glabrata*	Optisol-GS	The vials containing amphotericin B as a supplement exhibited no growth of either organism, except at 0.25 × and 0.5 × MIC on Day 2, reducing the viable count of *C. glabrata* by 96 and 99%, respectively.
Das *et al.*	Voriconazole and amphotericin B	*C. albicans*, *A. flavus*, and *F. keratinoplasticum*	MK media	In comparison to voriconazole, amphotericin B demonstrates a significant reduction in fungal contamination within MK media. At a concentration of 0.5 µg/ml, amphotericin B exhibits a decrease in colony count, resulting in a reduction of 99.9% for *Candida*, 65.2% for *Aspergillus*, and 90.1% for *Fusarium*
Dal Pizzol *et al.*	Cyclohexamide	*C. albicans*, *C. glabrata*, and *C. parapsilosis*	Optisol-GS	Cycloheximide in Optisol-GS showed a decline in yeast concentration from day 2 of preservation. It exerted a fungicidal activity against *C. glabrata* and a fungistatic activity against *C. albicans* and *C. parapsilosis*

Based on the above calculations, the cost of supplementing 0.004 ml of amphotericin B into each vial would cost less than a Rupee (13 paise), which is acceptable to the current cost of the M-K medium (Rs. 472).

Conclusion

In vitro experiments demonstrate that amphotericin B is not toxic to either epithelium or endothelium and is a promising additive to the M-K medium supplemented with colistin.

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Conflicts of interest

There are no conflicts of interest.

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