

# Amphotericin B Supplementation of Cold Storage Media to Treat Fungal Contamination of Donor Cornea Transplant Tissue

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## ABSTRACT

**Purpose:** An increase in the incidence of post-endothelial keratoplasty fungal infections, most commonly *Candida* species, have occurred in the USA. The purpose of this study is to determine whether the addition of amphotericin b to cold cornea storage media would reduce or eliminate *Candida albicans* from the storage media.

**Methods:** 80 human donor corneas were placed in individual vials of Optisol GS (Bausch and Lomb, US). *Candida albicans* was added to the treated groups (N=20). The corneas remained in the storage media for 3 days, which was followed by a 2-hour warming period (to simulate the tissue processing for DSAEK or DMEK). This was followed by an additional 1 day of storage. A fungal assay was then performed. The groups: Group 1) The control group and had no amphotericin b 0.225 ug/ml added to the media. Group 2) Amphotericin b 0.225 ug/ml was added to the storage media when the donor cornea was initially placed in the media. Group 3) Amphotericin b 0.225 ug/ml was added to the storage media after the simulated warming period (day 3). Group 4) Amphotericin b 0.225 ug/ml was added at the initial time point and after the simulated warming period.

**Results:** Each treatment group reduced the number of fungal colony counts by a statistically significant amount, compared to the control group. Adding amphotericin b at both time points (group 4) resulted in the greatest reduction in colony counts.

**Conclusion:** Amphotericin b 0.225 ug/ml was effective at reducing the number of fungal colony counts in cold cornea storage media but did not eliminate viable *Candida* organisms.

The landscape of keratoplasty has changed within the past decade. In 2012, endothelial keratoplasty (EK) surpassed penetrating keratoplasty (PK) as the most frequently performed keratoplasty in the United States (US). According to the 2015 Eye Bank Association of America (EBAA) statistical report,<sup>1</sup> 27,208 EK procedures and 19,160 PK procedures were performed in the US. With this evolution in technique, an increase in case reports<sup>2-13</sup> of lamellar infectious interface keratitis and endophthalmitis due to fungal infections, most commonly *Candida* species, have been noted; large database studies have confirmed the increased incidence of endothelial keratoplasty related infectious keratitis.<sup>14-16</sup> The incidence of fungal infections has continued to grow in more recent years from 0.014% in a 2013 report<sup>14</sup> to 0.023% in a 2016 report;<sup>15</sup> the latter report corresponds with the steady climb in EK volume. Although the increase in fungal infections was first noted with PK in the early 2000s,<sup>17,18</sup> studies<sup>14,16,19</sup> have found the risk of fungal infection to be more likely in patients undergoing EK when compared to PK. In the United States of America, the most commonly used corneal storage media is Optisol-GS (Bausch + Lomb, Bridgewater, NJ), which contains gentamicin and streptomycin, but no antifungal additive. One aspect of the corneal preservation time study sought to indirectly determine whether corneal transplant related infections were associated with increased storage times. The study found that an increased

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corneal storage time was not associated with an increased incidence of corneal donor rim cultures.<sup>20</sup> The donor corneal tissue used in EK surgery involves more complex tissue processing by the eye bank, since only the posterior lamellae of the cornea is transplanted during the EK surgery, in contrast to the full thickness donor cornea, which is used in PK. The additional warming period required for EK tissue processing has been purported to allow fungus to proliferate since no antifungal agent is present in corneal storage media that is commercially available in the United States. Fortunately, the antibacterial activity of Optisol-GS is most effective at warmer temperatures.<sup>21</sup> Furthermore, the lamellar interface may be more amenable to fungal growth due to its relative hypoxia, as well as its sequestered nature, foiling normal ocular immunologic mechanisms.<sup>12,22</sup>

Amphotericin B has shown efficacy in eradicating *Candida albicans* in cold storage media.<sup>23,24</sup> Duncan et al<sup>24</sup> determined a concentration of 0.255 ug/ml to effectively eliminate *Candida albicans* within 48 hours without corneal toxicity. Given the high treatment burden and devastating risk of infection-related graft loss and even loss of the eye, studies targeting antifungal supplementation of cold storage media are essential. Furthermore, there exists a knowledge gap regarding the optimal timing and dosage of amphotericin B to cold storage media during tissue processing. The aim of this study is to determine the effectiveness and optimal timing of supplementing Optisol GS, the most commonly used cornea storage media used in the United States, with Amphotericin B 0.225 ug/ml to reduce or eliminate *Candida albicans* from cold cornea storage media after placement of the donor cornea.

**METHODS**

Eighty 20 ml vials of Optisol GS storage media were divided into four groups [Group 1: control group without amphotericin; Group 2: amphotericin b 0.225 ug/ml added at time point zero (TPZ) when the donor cornea is initially placed in the cold storage media; Group 3: amphotericin b 0.225 ug/ml added after the warming period (which simulates the tissue processing phase for DSAEK or DMEK corneal graft preparation); Group 4: amphotericin b 0.225 ug/ml added at time zero AND after the warming period (which simulates the tissue processing phase for DSAEK or DMEK)].

*Candida albicans* was added to each sample vial at a concentration of 2.5 X 10<sup>3</sup> CFU/ml at time point zero, with a research quality human donor cornea. Table 1 outlines the schedule for amphotericin b addition to the Optisol GS corneal storage media.

In group 1: the control group for Optisol GS), *Candida albicans* was added to the media but no amphotericin b was added to the Optisol GS. The cornea remained in the storage media for 1 day (to simulate initial recovery of tissue and storage overnight) and then left at room temperature for 2 hours (to simulate the initial tissue evaluation). This was followed by an additional storage

**Table 1:** Timetable of the Addition of Amphotericin b 0.225 ug/ml to the Corneal Storage Media

	Initial Placement of Cornea in Storage Media (TPZ)	Simulated Processing (3 days post-TPZ)
Group 1	Control group. No amphotericin b was added to the corneal storage media.	Control group. No amphotericin b was added to the corneal storage media
Group 2	*Amphotericin b added to the corneal storage media when the cornea was initially placed in the media (time point zero: TPZ)	No additional amphotericin b added to the corneal storage media
Group 3	No addition of Amphotericin b at TPZ	*Amphotericin b added to the corneal storage media
Group 4	*Amphotericin b added to the corneal storage media at TPZ	*Amphotericin b added to the corneal storage media

(\* denotes the addition of Amphotericin b 0.225 ug/ml to the corneal storage media)

for 2 days, which was followed by a 2-hour warming period (to simulate the tissue processing phase for DSAEK or DMEK) and an additional 1 day of storage. At this point the fungal assay was performed by plating the media on Sabouraud agar. Next, viable colony counts of the culture plates were performed and mean counts (up to 110 CFU) were calculated.

Group 2: A human donor cornea was added to the Optisol GS storage media at time point zero, which represents a typical eye bank protocol when the cornea would be initially placed in the storage media. *Candida albicans* and amphotericin b was also added to the storage media at time point zero. The cornea then remained in the storage media for 1 day (to simulate initial recovery of tissue and storage overnight) and then left at room temperature for 2 hours (to simulate the initial tissue evaluation). This was followed by an additional storage for 2 days, which was followed by a 2-hour warming period (to simulate the cornea tissue processing for DSAEK or DMEK) and an additional 1 day of storage. At this point the fungal assay was performed.

Group 3: Consists of the Optisol GS media with a human donor cornea and *Candida albicans* added at time point zero. The cornea then remained in the storage media for 1 day (to simulate initial recovery of tissue and storage overnight) and then left at room temperature for 2 hours (to simulate the initial tissue evaluation). This was followed by an additional storage for 2 days, which was followed by a 2-hour warming period (to simulate the tissue processing for DSAEK or DMEK). At this time point, amphotericin b was added to the storage vials, followed by an additional 1 day of storage. After this stage, the fungal assay was performed.

Group 4: Represents the placement of amphotericin b in the Optisol GS storage media, which contained a human donor cornea and *Candida albicans*, at time point zero. The cornea then remained in the storage media for 1 day (to simulate initial recovery of tissue and storage overnight) and then left at room temperature for 2 hours (to simulate the initial tissue evaluation). This was followed by an additional storage for 2 days, which was followed by a 2-hour warming period (to simulate the tissue processing

for DSAEK or DMEK). At this point, amphotericin b was added to the storage media again, followed by an additional 1 day of storage. At this time point, the fungal assay was performed.

NCSS data analysis software (<https://www.ncss.com>) was used to examine the statistically significant difference between the control and the treatment groups.

**RESULTS**

Eighty samples were divided into 4 groups, (3 treatment groups and 1 control group) each group consisting of 20 samples. A table of the treatment groups are included in table 1. As expected, the addition of amphotericin b to the Optisol GS corneal storage media decreased the number of colony counts of *Candida albicans* in all treatment groups, compared to the control group, which did not include any amphotericin b. The two groups, which had amphotericin b added to the Optisol GS at time zero, demonstrated a greater decrease in *Candida albicans* colony counts, compared to the treatment group in which amphotericin b was added to the corneal storage media after the simulated

groups, which provides multiple comparison tests for all pairwise differences between the means, demonstrated statistically significant differences between all 3 treatment groups and differences between the treatment groups and the control group (Table 3). This finding was confirmed by the ANOVA Kruskal-Wallis Multiple-Comparison Z-Value Test (Dunn’s Test), which provides comparison tests for all pairwise differences between the medians and is shown in table 4.

The results demonstrate that the addition of amphotericin b, to the corneal storage media has its greatest effect of reducing the number of *Candida albicans* colony forming units, when it is added at both time point zero and after the simulated tissue processing time point (3 days post-TPZ). The second most ef-

**Table 2: Descriptive Statistics**

Group	Count (n)	Mean	Median	Standard Deviation
1	20	110	110	0
2	20	72.5	70	7.163504
3	20	94.5	90	10.50063
4	20	39.75	35	23.14002

**Table 3: Tukey-Kramer Multiple-Comparison Test**

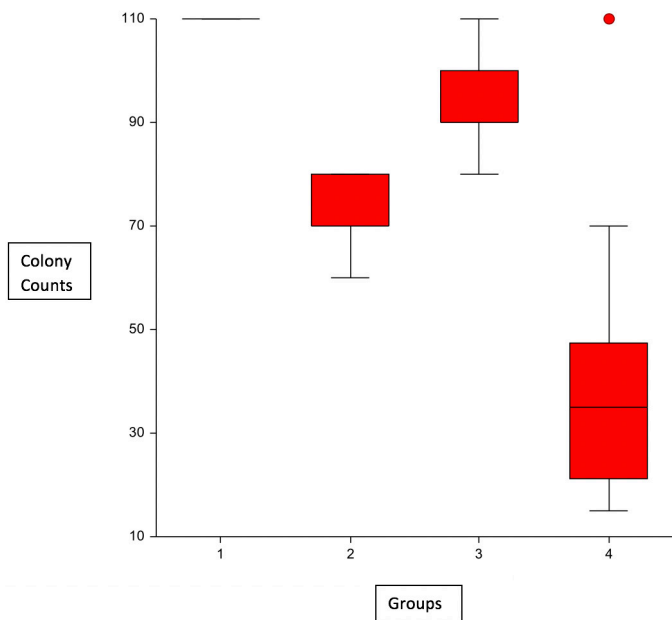
Group	Count	Mean	Different From Groups
1	20	110	2, 3, 4
2	20	72.5	1, 3, 4
3	20	94.5	1, 2, 4
4	20	39.75	1, 2, 3

ANOVA  
Response: recoded colony counts  
Term A: group x  
Alpha=0.050 Error Term=S(A) DF=76 MSE=174.2599 Critical Value=3.7216

**Table 4: TKruskal-Wallis Multiple-Comparison Z-Value Test (Dunn’s Test)**

Group:	1	2	3	4
1	0.0000	5.3446	2.3862	7.5297
2	5.3446	0.0000	2.9585	2.1850
3	2.3862	2.9585	0.0000	5.1435
4	7.5297	2.1850	5.1435	0.0000

ANOVA  
Regular Test: Medians significantly different if z-value > 1.9600  
Bonferroni Test: Medians significantly different if z-value > 2.6383



**Figure 1: Box Plot of the *Candida albicans* Colony Counts by Amphotericin b Treatment Group**

tissue processing period (3 days after time point zero). This is shown in a box plot in Figure 1.

The mean, median and standard deviation are summarized in Table 2. Multiple ANOVA studies demonstrate that the means and medians of each group are significantly different. The ANOVA Tukey – Kramer multiple comparison test of the treatment

fective treatment involved the addition of amphotericin b at time point zero. The least effective treatment involved the addition of amphotericin b after the simulated processing time point (3 days post-TPZ). None of the treatment groups resulted in zero colony counts of *Candida albicans*, at the culmination of the study.

## DISCUSSION

As the annual number of endothelial keratoplasty procedures begins to equal or exceed the number of penetrating keratoplasties in the United States, the incidence of infectious interface keratitis has increased, with the predominant infectious agent being identified as a *Candida* species of fungus.<sup>14-16</sup> Because of the increase in this complication, the addition of an antifungal agent to the storage media is being investigated.

In Europe and many other parts of the world, organ culture techniques (warm storage) are commonly used for eye banking corneal tissue storage, while most eye banks in North America use cold (hypothermic) storage for their corneal transplant tissue. There are advantages and disadvantages of each technique. One advantage of cold storage is that hypothermic conditions suppress bacterial and fungal growth. Unfortunately, the additional warming period, which is necessary for the tissue processing of the endothelial keratoplasty grafts, provides bacterial and fungal organisms an opportunity to multiply in the storage media.<sup>21</sup> Optisol GS is the most commonly used cold corneal storage media in the United States, which contains gentamicin and streptomycin antibiotics but no antifungal agents.<sup>25</sup> Organ culture (warm) media must contain antifungal agents. The most common antifungal agent used is amphotericin b.<sup>26,27</sup> Duncan and colleagues investigated the *in vitro* biocompatibility of adding amphotericin b to Optisol-GS corneal storage media. They demonstrated that 0.255 ug/ml did not alter the corneal endothelial cell viability; however, other studies have shown significant corneal endothelial cell damage and reduced corneal endothelial cell viability at higher concentrations (10 x MIC).<sup>28</sup> Our study used the concentration of amphotericin b that was studied by Duncan *et al* and a common concentration used in many European organ culture formulations, such as Tissue-C (Alchimia, Ponte S. Nicolo, Italy).

This study was limited by only counting a maximum of 110 colony forming units of *Candida albicans*, due to the confluence of colonies reached on the culture plates, which prohibited accurate *Candida* colony counts beyond 110 colony forming units. Therefore, any percentile comparison of the reduction of viable *Candida albicans* would likely under-state the difference between treatment groups. However, there was a statistically significant difference between all groups as we measured them. Of the treatment groups, the addition of amphotericin b at the later tissue processing stage was least effective at reducing the number of colony forming units of *Candida*. The most effective treatment group was the dual amphotericin treatment group, in which amphotericin was added when the cornea was initially placed in the Optisol GS media and after the simulated processing time point (3 days post-TPZ). Our study did not assess the possible toxicity of the higher concentration of amphotericin b at 0.45 ug/ml present in group 4. The second most effective treatment regime involved placing amphotericin b in the Optisol when the cornea was initially placed in the media. The effectiveness of the amphotericin dual time point treatment can be explained by including twice the concentration of amphotericin b compared to the other 2 treatment groups; and because the *Candida* organisms

were exposed to the antifungal agent for a longer period of time, compared to treatment group 3. This is likely why the group which had amphotericin placed in the storage media at time point 0, which exposed the fungal organisms to the antifungal agent for a longer period of time, compared to the group which only had the *Candida* exposed to the amphotericin b after the processing period, demonstrated a greater reduction in *Candida* colony forming units after the total storage period. It is important to note that even the group with the greatest reduction in colony forming units, the group which had amphotericin placed in the media at time point 0 and after the simulated processing period, still had viable fungal organisms present. It is unclear if the significant reduction in fungal organisms, after adding amphotericin b to the Optisol, is enough to prevent all cases of post keratoplasty keratitis. In fact, when amphotericin b is added to organ culture media, it also fails to completely eliminate *Candida* fungal organisms. This was demonstrated in a study by Hagenah, Böhnke, Engelmann and Winter, who used 10 times the concentration of amphotericin b as used in our study and after their tissue was washed in an iodine-based solution for 3 to 5 minutes, found 3 out of 1,134 eye bank donor corneas still contaminated with viable fungi.<sup>29</sup>

In conclusion, this study demonstrated that adding amphotericin b 0.225 ug/ml was effective at reducing the amount of *Candida albicans* in the Optisol GS storage media. Adding amphotericin b was most effective at reducing the number of *Candida* colony forming units when it was added at both the preliminary time point, when initially placing the cornea in the storage media, and after processing the tissue for endothelial keratoplasty. Although this study employed the most frequently used concentration of amphotericin b, none of the treatment regimens completely eliminated the *Candida albicans* organism from the cold cornea storage media. Further studies are necessary to confirm the safety and efficacy of adding this amount of amphotericin b to the cold storage media and ultimately a clinical study in human eyes.

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