Washing to Dilute May be the Answer to Eliminate Fungus from Donor Corneal Tissue

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ABSTRACT

Purpose: Fungal contamination can be a problem for refrigerated corneal tissue stored in Optisol-GS. We developed methods to test the elimination of fungi on donor corneal tissue.

Methods: Three yeast and two mold isolates were tested at different fungal loads with three antifungal regimens to eliminate fungi from corneoscleral tissue. In regimen 1 (antifungals), the corneoscleral rims were placed in Optisol with and without amphotericin B 2.5 µg/ml (AmpB), clotrimazole 10 µg/ml, and an antifungal synergistic mixture (ASD), respectively. In regimen 2 (antiseptics), rims were treated topically with 5% povidone iodine (PI) or 0.01% hypochlorous acid (HOCL) and placed in Optisol. Another rim set was treated with PI and placed in Optisol plus AmpB. In regimen 3 (wash-dilute), the rims underwent a wash, topical PI, wash, rest period, topical PI, wash, and placement in Optisol supplemented with ASD or AmpB. All rims were refrigerated at 6O C for 48 hours. All corneal rims were cultured and monitored for viable fungi for 7 days. The objective was 100% elimination of fungi from donor corneal tissue

Results: Regimens 1 and 2 were not fully effective for eliminating fungi. However, the wash-dilute regimen only produced yeast growth in the ASD supplemented Optisol at 1000 cfu, and mold growth in the AmpB supplemented Optisol at 100 cfu.

Discussion: Optisol supplemented with antifungals or topical antiseptics alone did not consistently eliminate fungal growth. The wash-dilute method which is a complementation of antifungals, antiseptics, and washing was best for eliminating fungi from rim tissue.

Key Words: Fungus elimination; donor corneal tissue; optisol antifungal supplementation; antifungal synergistic drugs; antiseptic fungal decontamination

Post-keratoplasty infections from donor tissue contaminated with fungi can lead to complex post-operative care and sleepless nights for both patients and ophthalmic corneal surgeons. Originally, the reported incidence of post keratoplasty fungal infections was 1.0 in 10,000 transplants[1] and has risen to 2.3 in 10,000.[2,3] Donor corneas contaminated with fungi are a possible vector for transmitting fungal infections. Vislisel reported from 3414 keratoplasty cases, fungi were isolated from 71 (2.1%) corneal rims.[4] Fungal keratitis was noted in 4 cases, but there were no cases of endophthalmitis. From our laboratory, (Shatten, submitted for publication) (2009-2019), fungi were isolated from 16 (1.2%) of 1276 cornea rim cultures. One patient developed an associated fungal keratitis. The donor corneas were used for the following procedures: DMEK, DSEK, DSAEK, scleral patch, PKP, and KPRro. The most common indication for surgery was Fuchs' Dystrophy followed by bullous keratopathy.

Although fungal infection after keratoplasty is infrequent, there is significant secondary ocular morbidity. Corneal surgeons would prefer prophylactic measures to eliminate any fungal contamination from corneal tissue. The addition

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of antifungals has been the targeted approach, but care must be taken to avoid toxicity to the corneal endothelium. Low concentrations of antifungals may be non-toxic, but ineffective. Safe antifungal concentrations have been supplemented to corneal preservation media, but these concentrations did not eliminate completely fungal contamination with yeast and molds.[5-10]

Our approach in the present study was the total elimination of both yeasts and molds at 48 hours under refrigerated conditions (4-8O C).[11] The presence of any fungi still allows for the risk of infection. Our model tests the ability of antifungal measures to eliminate fungi from corneal tissue as a fixed medium. It can be reasoned that fungi generally first contaminate the donor tissue prior to placement in preservation medium. We were not concerned with post-optisol warming cycles with our model because effective initial treatment would eliminate fungal contamination. Warming would not resurrect dead fungi.

We tested three regimens to eliminate fungi from donor corneal tissue: 1) supplementation of antifungals in Optisol medium (Bausch and Lomb, Rochester, NY), 2) the use of antiseptics to directly sterilize corneal tissue, and 3) washing to dilute (simulation of irrigation) fungal contamination from corneal tissue.

METHODS

Corneal Tissue

Excess corneoscleral donor tissues were used in this study. In general, corneoscleral rims were cultured for microbial contamination after keratoplasty by placing the rims in 10 ml of enriched thioglycollate broth (BBL™, Becton, Dickinson and Co., Sparks, MD). After 5 days of incubation at 37O C, the culture-negative rims were removed from the enriched thioglycollate broth and frozen at -80˚C for research purposes in a clinical tissue bank. The rims were excess tissue received from corneal surgeons and not from the eye bank. In addition, whole corneas deemed not suitable for surgical use, but were biologically safe, were donated by the eye bank. The CORE Eye Bank of Pittsburgh Pennsylvania has given permission for the research use of these excess corneal tissues. This study was approved by the University of Pittsburgh Institutional Review Board (IRB# Study19120005). The paucity of corneal rims limited our study to pertinent fungal isolates and antifungal testing.

Fungal Testing Isolates

Two yeast isolates of *Candida albicans* (donor tissue, endophthalmitis), one yeast isolate of *C parapsilosis* (keratitis), one mold isolate of *Fusarium species* (endophthalmitis), and one mold isolate of *Aspergillus fumigatus* (endophthalmitis) were tested. The isolates were stored in a clinical tissue bank, de-identified for patient identification, and were used to support laboratory certification.

Three Regimens of Antifungal Testing

It must be re-emphasized that our approach was to eliminate both yeasts and molds from corneal tissue, and not to decrease the load in liquid media which could leave remnants of live fungus. Although our study tested these regimens on corneoscleral rims, the real-world application of these methods would be on intact globes prior to corneal donor recovery, thus the toxicity of 5% povidone iodine or another disinfectant to the corneal endothelium was not relevant for this disinfection experiment.

For these three regimens, corneoscleral rims stored at -80˚C were thawed and placed in multi-well plates (4 per fungal isolate). As indicated previously, testing was limited to single testing due to the paucity of corneal tissue. The rims were inoculated with 4 respective loads of fungus: 0, 10^1, 10^2, and 10^3 colony forming units (CFU) in two sets. One set was used to test for antifungal effect and the other acted as the control without antifungal effect (Optisol alone). All plates were incubated at cold storage (2-8˚C) for 48 hours. Forty-eight hours storage (2-8˚C) was chosen to represent an early time that tissue would be used for keratoplasty. After 48 hours, all corneal rims were placed into 5 ml of yeast extract peptone dextrose medium (YPD); a 10 µl sample of each well was plated onto Sabouraud plates (SAB); and all plates with the remaining Optisol were incubated at 35-37˚C. Fungal growth was monitored for 7 days.

In the first regimen (antifungals), the fungal-inoculated rims were tested for growth after placement in Optisol supplemented with 2.5 µg/ml Amphotericin B (AmpB), 10 µg/ml clotrimazole (CLO), or an Antifungal Synergistic Drug (ASD) mixture (ASD = Antifungal Synergistic Drug = 0.42 µg/ml amphotericin B; 1.04 µg/ml natamycin; 4.2 µg/ml clotrimazole; 4.2 µg/ml nystatin.)[5], and Optisol as control. ASD was developed previously for eliminating fungi from donor tissue.[5]

In the second regimen (antiseptics), the fungal inoculated rims were topically treated with antiseptics, 5% povidone iodine (PI) (Alcon, Fort Worth, TX) or 0.01% hypochlorous acid (HOCL) (Avenova, NovaBay, Emeryville, CA) prior to Optisol placement. Each antiseptic was sprayed twice onto the inoculated tissue and allowed 5 minutes contact time. A single spray was considered 2 pumps of the spray bottle. In another set, the inoculated tissue was sprayed with PI for two applications 5 minutes apart (a total of 4 pumps over 10

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minutes) and placed in Optisol containing AmpB.

In the third regimen (wash-dilute), the inoculated tissue underwent a series of wash and PI steps as follows:

1) Frozen corneal rims (-80O C) were thawed and quartered. Quarter 1 received 1000 cfu of fungus, Quarter 2 received 100 cfu, Quarter 3 received 10 cfu, and Quarter 4 received no fungus. We tested 3 yeasts and 2 molds, respectively, as previously described. The inoculated quarters were placed in 6 well plates for each fungus.
2) 5 ml of balance salt solution (BSS)(non-preserved) (Alcon, Fort Worth, Texas) was added to each inoculated rim for 10 seconds. Washing involved gently swirling the corneal tissue with sterile forceps in 5 ml of BSS. Washing simulated irrigation. The washed rims were transferred to another plate.
3) The washed rims were flooded with 2 sprays of PI and allowed to remain in contact for 5 minutes. For this step, PI was placed in clean spray bottles used for Avenova.
4) The rims were transferred to another clean plate containing 5 ml of BSS for 10 seconds.
5) The washed rims were transferred to another clean plate and allowed to rest for 5 minutes. The break of 5 minutes was performed for concerns of epithelial toxicity and was recommended by the eye bank medical advisory committee.[12]
6) The washed rims were flooded with 2 sprays of PI and allowed to remain in contact for 5 minutes.
7) The rims were transferred to another clean plate containing 5 ml of BSS for 10 seconds.
8) The rims were transferred to wells containing Optisol with antifungals (ASD or 2.5µg/ml Amphotericin B)
9) The plates were refrigerated (approximately 6O C) for 48 hours.
10) At 48 hours, the treated corneal rims were tested for viable fungus as described previously.

RESULTS

Table 1 summarizes the viability of fungi on corneal tissue after antifungal and antiseptic treatment and refrigeration in Optisol.

Table 1: *The Viability of Fungi on Corneal Tissue Treated with Antifungal Drugs, Antiseptics and Stored at Refrigerator Conditions*

Fungus	Count CFU	ASD Growth	AmpB Growth	Clotrimazole Growth	5% PI Growth	HOCL Growth	PI + AmpB Growth	Optisol Growth
Candida albicans (Donor)	1000	+	+	+	-	+	+	+
	100	+	+	+	+	+	-	+
	10	+	+	+	-	+	-	+
	0	-	-	-	-	-	-	-
Candida parapsilosis (keratitis)	1000	+	+	+	+	+	-	+
	100	-	+	+	-	+	-	+
	10	-	+	+	+	+	-	+
	0	-	-	-	-	-	-	-
Candida albicans (endophthalmitis)	1000	+	+	+	+	+	+	+
	100	+	+	+	+	-	+	+
	10	-	+	-	-	-	-	+
	0	-	-	-	-	-	-	-
Fusarium species (endophthalmitis)	1000	+	+	+	-	+	-	+
	100	-	+	-	-	+	-	+
	10	-	+	-	-	+	-	+
	0	-	-	-	-	-	-	-
Aspergillus fumigatus (endophthalmitis)	1000	+	+	+	+	+	+	+
	100	+	+	+	-	+	+	+
	10	+	+	+	-	+	-	+
	0	-	-	-	-	-	-	-

(+) was indicated as turbidity in yeast peptone dextrose broth with colony growth confirmation; (-) indicated no viable growth

ASD = Antifungal Synergistic Drug = 0.42 µg/ml amphotericin B; 1.04 µg/ml natamycin; 4.2 µg/ml clotrimazole; 4.2 µg/ml nystatin. AmpB = 2.5 µg/ml amphotericin B; clotrimazole = 10 µg/ml clotrimazole; 5% povidone-iodine spray with 5 minute contact; 0.01% hyphchlorous acid (Avenova) spray with 5 minute contact; PI + AmpB 2.5 µg/ml = 2 sprays of 5% PI at 5 minute contact intervals and placement in AmpB; Optisol with no antifungals.

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For regimen 1 (antifungals), all fungi grew in Optisol supplemented with ASD, AmpB, and clotrimazole. Optisol as the control was positive for all fungal isolates at all fungal loads (1000, 100, and 10 CFU). (Table 1)

For regimen 2 (antiseptics), a single PI application was successful for eliminating Fusarium, but variable growth was present for the remaining four fungal isolates. Topical HOCL was not successful for eliminating the five fungal isolates. For the double PI treatment and placement in AmpB, *Candida parapsilosis* and *Fusarium* species were eliminated. The remaining three fungal isolates demonstrated variable growth. (Table 1)

For regimen 3 (wash-dilute), *Candida albicans* was only isolated from the ASD well containing 1000 cfu. *Aspergillus fumigatus* was only isolated from the AmpB well containing 100 cfu. (Data not included in Table 1) These results were confirmed with repeat testing.

It must be noted that the positive fungal growth was based on isolation from the YPD medium. SAB growth did not always correlate with positive fungal growth from YPD. YPD contained the entire corneal rim which may have adhered the fungi without dispersing in the Optisol. Likewise, Optisol fungal growth did not always correlate with positive fungal growth from YPD. Fungi did not grow well in Optisol at refrigeration or 30O C.

DISCUSSION

The major questions from this study and other studies is whether decreasing fungal contamination on donor corneal tissue, in a safe manner, is enough to prevent post-surgical infection. The quantity of fungi cultured from our donor rims, (Shatten, submitted for publication) ranged from a few colonies to rare, and this suggests larger loads of fungus may be required for infection. A single donor cornea with moderate growth of yeast did not result in a post-surgical infection (Shatten, submitted for publication). The current study was designed to test small loads with the hope to eliminate fungi from the tissue. Our design was unique because susceptibility was based on fungi attached to tissue and not suspended in a liquid medium. We surmised that higher concentration of fungi in liquid medium was not realistic and would be difficult to eradicate under refrigeration.

Antifungals would work best when fungi are in the growth phase which exists above refrigeration to human body temperature. In this study, Optisol supplemented with AmpB, clotrimazole, and ASD was not effective in eliminating fungi under refrigeration. Clotrimazole (10 µg/ml) alone was tested because the MICs to the 5 fungi in our study ranged between 0.06 to 2.0 µg/ml. We could not use higher concentrations of clotrimazole because of the risk of cytotoxicity.[13]

There is much interest in the use of topical PI and HOCL for eliminating fungi from donor tissue. PI has found its niche in the preliminary disinfection of microbial contamination at the time of recovery, but HOCL has not been tested, but its use has intrigued some.[12] Topical PI for a 5 minute contact was not fully effective. A double dose of PI and placement in AmpB did reduce contamination at lower loads of fungi. HOCL for a 5 minutes contact time was not effective for eliminating fungi. To reiterate, the PI contact is intended at the time of recovery so concerns about endothelial toxicity are not relevant.

Washing to dilute fungi from donor tissue along with a double PI exposure and final placement in Optisol supplemented with ASD or AmpB provided the best reduction of fungal load. It would have been a complete elimination of fungi except for growth of *Candida albicans* in a single well of ASD and *Aspergillus fumigatus* in a single well of AmpB. ASD was developed by our laboratory in the mid-1980s for the prophylaxis of donor tissue against a wide number of yeasts and fungi (Kowalski RP, et al. IOVS 1984;25(3,suppl):20).[5] As a synergistic combination of four antifungals at low concentrations, ASD was found to be non-toxic.[5] Rabbit corneal tissue preserved in McCarey-Kaufman medium supplemented with ASD was successfully transplanted to another rabbit (Dunn D, et al. IOVS 1985;26(3,suppl):68). ASD was injected safely into the anterior chamber of two patients: one with a vegetative intraocular injury to prevent infection and another after keratoplasty for a fungal corneal ulcer. ASD was also used clinically as a supplement in McCarey-Kaufman medium for six penetrating keratoplasties with no signs of post-operative toxicity (Budd RM, et al. IOVS 1986;27(3,suppl):89). Although there has not been wide concern of toxicity with 2.5 µg/ml of amphotericin B (AmpB) addition to optisol-GS, a co-author (DKD) has indicated a possible observance of clinical delayed endothelial recovery with this possible addition. It was not conclusive that AmpB was totally responsible without further analysis.

Washing to dilute may be an effective supplemental method to reduce or eliminate fungi from donor corneal tissue. This laboratory experiment included washing (simulation of irrigation) and PI twice to most closely simulate the real-world donor cornea recovery prep.[12] Salisbury et al demonstrated that doubling the exposure of PI prior to donor corneoscleral excision reduced the rate of recipient fungal infections in the setting of endothelial keratoplasty. Our study highlights the importance of irrigation itself, in addition to the double PI exposure.

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In summary, random fungal contamination of donor corneal tissue appears to remain a problem. It may require complementary approaches of antifungal supplementation of cold storage media, topical antiseptics, and mechanical washing (irrigation) to reduce fungal load and secondary post-surgical infections. Our methodology using donor corneal tissue to test for fungal elimination should be considered for future trials of decontamination.

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