

Antifungal Efficacy and Safety of Cycloheximide as a Supplement in Optisol-GS

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Purpose: The incidence of fungal infection after corneal transplant has increased significantly in recent years, especially *Candida* spp. This study aimed to evaluate the efficacy and safety of the addition of cycloheximide in Optisol-GS media in decreasing the growth of *Candida* spp. strains.

Methods: This in vitro laboratory efficacy study measured fungal colony growth in 24 vials of Optisol-GS that were divided into 6 groups of 4 vials each, as follows: (1) MIC/2 cycloheximide, (2) MIC cycloheximide, (3) MICx5 cycloheximide, (4) MICx10 cycloheximide, from MIC values obtained for each strain, (5) unsupplemented optisol-GS as a positive control (added inoculum), and (6) unsupplemented optisol-GS as a negative control (no inoculum). In each group was added *Candida albicans*, *C. glabrata* and *C. parapsilosis*, except in the negative control. The evaluated variables were fungal colony growth from the Optisol-GS vials, corneal endothelial cell density and endothelial cell viability at different concentrations of cycloheximide.

Results: In the efficacy study, all strains showed a reduction in fungal cell growth from the second day at all evaluated concentrations of optisol-GS supplemented with cycloheximide, even at subinhibitory concentrations (MIC/2). For *C. glabrata*, the colony count was reduced to 99%. No evidence of corneal endothelial toxicity was found at any concentration, in the safety study, compared with the paired control.

Conclusion: The addition of cycloheximide to optisol-GS decreased the fungal growth, demonstrating fungicide action against *C. glabrata* and fungistatic action against *C. albicans* and *C. parapsilosis*. This drug did not demonstrate toxicity to the corneal endothelium at different concentrations.

Keywords: cornea, eye banking, infection, pharmacology

Introduction

Although fungal infection after corneal transplantation is a rare complication, the high visual impact and morbidity of these infections have led to the study of the possibility of supplementation of corneal preservation media with antifungal drugs.^{1,2} At present, none of the commercially hypothermic preservation media contain any antifungal action agent and the colorimetric indicators of microbial contamination do not consistently detect fungal contamination.³ Organ culture at 34°C is the most frequent method used for corneal storage in Europe, while in North America, hypothermic storage at 4°C is the preference of most eye banks. A survey involving 1.6 European eye banks, using a database of 16,862 corneas distributed for endothelial keratoplasty over 5 years from 2012, compared the incidences of fungal infection. As a result, the study demonstrated an infection rate of 0.5% in the cases in which the corneas were preserved in hypothermic media, compared to an incidence of 0.02% when the corneas were preserved in organ

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culture media ($p < 0.0001$).⁴ This difference is likely because the organ culture media has been added into the formula 0.25 µg/mL amphotericin B, which is different from the hypothermic media, which has no antifungal additive.⁴

Approximately all cases of fungal infection after corneal transplants were caused by *Candida* species, in particular *Candida albicans*, and *C. glabrata*. In addition, there was an increase in this microorganism after endothelial keratoplasty.^{1–7} In 2010, the Eye Bank Association of America (EBAA) created a subcommittee to investigate the benefit of fungal supplementation in Optisol-GS storage media in the United States of America (USA).³ This subcommittee concluded an increasing trend in the incidence of post keratoplasty fungal infection. However, supplementation with antifungals is not recommended because of the lack of evidence proving the efficacy, safety, and cost-benefit of supplementation. Some studies evaluated the efficacy, safety, and light stability of amphotericin B and voriconazole addition in hypothermic storage at many different concentrations and found endothelial toxicity at a high concentration of amphotericin B and lack of effect at a low concentration of voriconazole.^{5,6}

Cycloheximide is an antibiotic with significant antifungal properties. It is produced by some streptomycin-producing strains of *Streptomyces griseus* and acts by inhibiting protein synthesis.^{7,8} Fungal resistance selection is an important trend and makes therapy difficult. There is already evidence of resistance to voriconazole and other azole derivatives in the treatment of fungal ulcers.^{9,10} Cycloheximide is efficient for mutagenic strains resistant to multiple drugs,¹¹ and also has the advantage of having a low cost. In addition to presenting a broad spectrum of antimicrobial action, cycloheximide is widely used as a preservative agent in culture media to inhibit fungi and bacteria. Thus, this drug can be a good antifungal agent in the Optisol medium for corneal preservation.^{12–14} There are still no studies evaluating its efficacy and safety in the treatment of fungal ulcers or its supplementation in hypothermic preservation media. This study aimed to evaluate the efficacy and safety of the addition of cycloheximide in Optisol-GS media in decreasing the growth of the main *Candida* spp. strains.

Materials and Methods

Fungal Strains

In this study, three *Candida* spp. strains of clinical relevance were tested: *C. albicans* (CA MS2), *C. glabrata* (CG 05) and *C. parapsilosis* (ATCC 22019). The strains

were analyzed phenotypically by Vitek Yeast Biochemical Card (BioMerieux Vitek – Hazelwood, Missouri, USA). All strains were obtained from the mycology collection of the Laboratory of Applied Mycology of the Federal University of Rio Grande do Sul (Porto Alegre, Brazil). The standard strain (ATCC 22019) was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and used as a control.

Antifungal Susceptibility Testing

The minimum inhibitory concentration (MIC) of the fungal strains selected for this study was determined by the broth microdilution method, according to the M27-A3 protocol.^{15,16} The solution containing cycloheximide was prepared in RPMI 1640 media (Roswell Park Memorial Institute 1640; Sigma-Aldrich, St. Louis, Missouri, USA) pH 7.0 buffered with MOPS (morpholinopropanesulfonic acid; Dynamic, Diadema, São Paulo, Brazil), at twice the concentration to be tested, ranging from 250 to 0.97 µg/mL. The fungal inoculum was prepared in a 0.85% sterile saline solution (Dynamic; Diadema, São Paulo, Brazil), containing cell culture with 24 h of growth on Sabouraud Dextrose Agar (SDA – HiMedia, India) at 35°C. The fungal suspension was adjusted to 0.5 McFarland scale, approximately $1.0\text{--}5.0 \times 10^6$ CFU/mL, in a spectrophotometer (GT220, Global Trade Technology) at 530 nm. Then, two subsequent dilutions, at 1:50 and 1:20 were performed to obtain a cell concentration of approximately $1.0\text{--}5.0 \times 10^3$ CFU/mL.

The assay was performed on 96-well sterile polystyrene microplates. After the microdilution of the cycloheximide, 100 µL of each *Candida* inoculum suspension were added to the microplates, obtaining the same inoculum concentration ($0.5\text{--}2.5 \times 10^3$ CFU/mL) in all wells of the microplate. This ensured the same microorganism dose in all cycloheximide concentrations evaluated. Thus, the microplates were incubated at 35°C for 48 h. The MIC values were determined as the lowest concentrations capable of inhibiting 100% of fungal visual growth. The same procedures were performed to test the corneal preservation media, Optisol-GS (Bausch and Lomb, USA), this was used to replace the RPMI 1640 media.

Efficacy Study

For each *Candida* spp. strain tested, twenty-four sterile vials of Optisol-GS were used. The vials were divided into 6 groups, as follows: (1) MIC/2 cycloheximide (2) MIC cycloheximide, (3) MICx5 cycloheximide, (4) MICx10 cycloheximide, from MIC values obtained for each strain, (5) unsupplemented Optisol-GS as a positive control (added inoculum) and (6)

unsupplemented Optisol-GS as a negative control (no inoculum). All the groups were evaluated in quadruplicate (using 4 vials per group). In each group was added a *Candida* spp. strain, except in the negative control. In this study, *C. albicans*, *C. glabrata*, and *C. parapsilosis* were tested. The strains were grown on SDA for 24 h at 35°C. Colonies were harvested from the plates and suspended in sterile water to obtain turbidity equivalent to 0.5 McFarland standards ($1-5 \times 10^6$ CFU/mL). Then, the fungal suspensions were diluted to approximately 2.5×10^3 CFU/mL for groups 1 to 5. All vials of Optisol-GS were refrigerated at 2 to 8°C according to manufacturer recommendations. After 2, 7 and 14 days of incubation, 1 mL of solution was removed, and 10, and 100 μ L aliquots were diluted 1:10 with sterile water to minimize any cycloheximide carryover. Then, the samples were immediately taken from 1 mL of solution and cultured in SDA. Then, all plates were incubated at 35°C for 36 h for subsequent counting of viable fungal colonies on the plates. The methodology followed Layer et al¹⁷ with modifications.

Two-way ANOVA followed by Dunnett's test was performed to assess the effectiveness of cell reduction of *Candida* cells when different concentrations of cycloheximide was added to Optisol and compared to the control. $P < 0.05$ was considered statistically significant.

Safety Study

Five pairs of donor corneas not suitable for transplantation due to positive serology were obtained from Hospital de Clínicas de Porto Alegre Corneal Eye Bank. All donor corneas were donated voluntarily, with written informed consent, and the study methodology followed the guidelines of the Helsinki and Istanbul declaration. Ethical committee approval and institutional research board clearance were obtained from Hospital de Clínicas de Porto Alegre. Two pairs of corneas were used to test cycloheximide at MIC and 3 pairs to test cycloheximide concentration at MICx10. Randomly, by sortation, the Optisol-GS flask with the rafted cornea was supplemented with cycloheximide, and the other cornea from the pair was maintained without cycloheximide as a control. The endothelial cell density (ECD) of all corneas was evaluated via specular microscopy (Konan KSS-EB10) on day 0 and after 3 and 14 days of cycloheximide supplementation. Paired *t*-test was used to compare the mean change in ECD between controls and supplemented Optisol-GS from 0, 3, and 14 days. In cases when ECD could not be performed due to corneal oedema or inability to identify the cells borders, a value of 500 cells/mm² was used to perform the statistical analyses, assuming to be secondary to severe endothelial damage.

All donor corneal endothelium was evaluated with two different vital dyes staining at day 14. The first was 0.4% trypan blue (Sigma-Aldrich Corp), a vital dye that stains severely damaged and dead endothelial cells, and the second was 0.5% alizarin red (GFS Chemical, Inc), which stains cell borders and denudes Descemet membrane. The staining protocol used was based on the protocol published by Park et al,¹⁸ that consisted of placing each donor cornea in a culture dish with the endothelial facing up. The endothelium was covered with 0.4% trypan blue for 60 seconds. Trypan blue was then gently poured off, and the cornea was rinsed with phosphate-buffered saline (PBS). The cornea was then immersed in 0.5% alizarin red for 90 seconds, followed by rinsing with PBS. An 8 mm trephine was used to cut the central cornea and then transferred to microscope slides with the endothelium facing up. The endothelium was examined under an Olympus BX51 light microscope (Olympus, Germany) at 200x magnification, attached to an Olympus digital camera. Five different photographs within the central and paracentral cornea were taken for each cornea. Two blinded examiners counted, in a given area in each photograph, viable and nonviable cells (stained with trypan blue). The mean percentage of nonviable cells (the number of trypan blue cells divided by the total number of cells) was calculated for each cornea and each examiner (Figure 1). The intraclass correlation coefficient was used to assess whether there was a correlation of means between the two examiners. Paired *t*-test was used to compare the average of the 2 examiners of percentage of nonviable cells between controls and supplemented Optisol-GS.

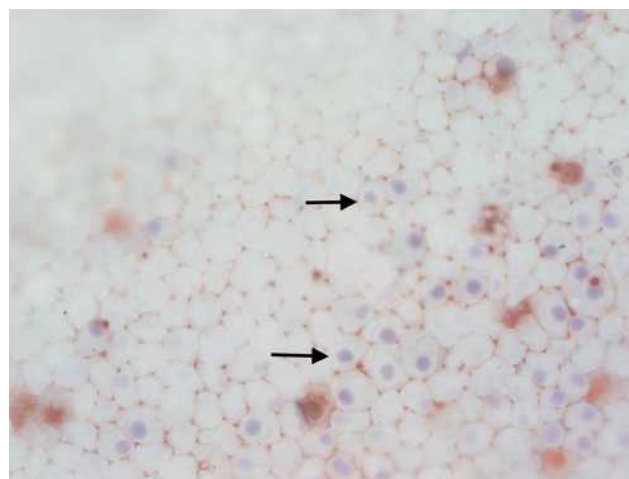


Figure 1 Corneal endothelium vital dyes stained with 0.4% trypan blue and 0.5% alizarin red (arrows indicate positive trypan blue).

Table 1 Determination of MIC Values ($\mu\text{g/mL}$) for Cycloheximide in RPMI Media and in Optisol-GS at Time Zero ($t=0$) and After 14 Days of Incubation ($t=14$)

Strain	RPMI Media	Optisol-GS ^{t=0}	Optisol-GS ^{t=14}
CA MS2 ^a	62.5	62.5	125.0
CG 05 ^b	1.95	1.95	3.90
ATCC 22019 ^c	3.90	1.95	3.90

Note: ^a*Candida albicans*, ^b*C. glabrata*, ^c*C. parapsilosis*.

Abbreviations: MIC, minimum inhibitory concentration; Concentration of cycloheximide tested in RPMI media and Optisol-GS: 250.0 $\mu\text{g/mL}$; RPMI media, Roswell Park Memorial Institute 1640 media.

Results

All evaluated *Candida* species were sensitive to cycloheximide in the RPMI media and to Optisol-GS supplemented with this antimicrobial at zero time ($t=0$), demonstrating MIC values between 1.95 and 62.5 $\mu\text{g/mL}$ for both media. After 14 days ($t=14$) of incubation in Optisol-GS supplemented with cycloheximide there was an increase in MIC values of this drug against all strains tested (MIC = 3.90–125 $\mu\text{g/mL}$). In addition, after 14 days of incubation, supplementation with cycloheximide in both RPMI and Optisol-GS media was more effective against *C. glabrata* and *C. parapsilosis* strains (MIC = 3.90 $\mu\text{g/mL}$) (Table 1).

Efficacy Study

The three *Candida* spp. strains showed a reduction in fungal cell growth from the second day of incubation in Optisol-GS supplemented with cycloheximide, at all evaluated concentrations, even at subinhibitory concentrations (MIC/2) (Figure 2). For the *C. glabrata* (Figure 2B), there

was an expressive reduction in the colony counts from the 2nd day. This reduction became more pronounced until the 14th day, mainly at MICx5 and MICx10 concentrations, in which the colony count was reduced to 99%. For *C. albicans* (Figure 2A), there was a reduction in fungal growth after the 2nd day, which remained constant until the 14th day at all concentrations evaluated. *C. parapsilosis* (Figure 2C) showed an expressive reduction in growth from the 7th day, remaining constant until the 14th day, with a reduction viable cells ranging between 77 and 92%.

For all strains there was a significant reduction in the number of viable cells at all cycloheximide concentrations evaluated when compared to the untreated control ($P < 0.05$), demonstrating the anti-*Candida* efficacy of this drug.

Safety Study

From day 0 to day 3 of supplementation and day 14 at both concentrations (MIC and MICx10), there was a reduction in ECD. However, there was no difference in ECD reduction between supplemented Optisol-GS with cycloheximide and paired controls, as shown in Table 2. The evaluation with vital dye staining demonstrated similar percentage of nonviable endothelial cells between their paired controls and antifungal supplemented Optisol-GS at both concentrations (Table 2).

Discussion

Post-keratoplasty fungal infections have increased significantly in recent years.^{3,19–21} These increase in cases are probably associated with endothelial keratoplasty (EK) and *Candida* spp have been the most common isolates,

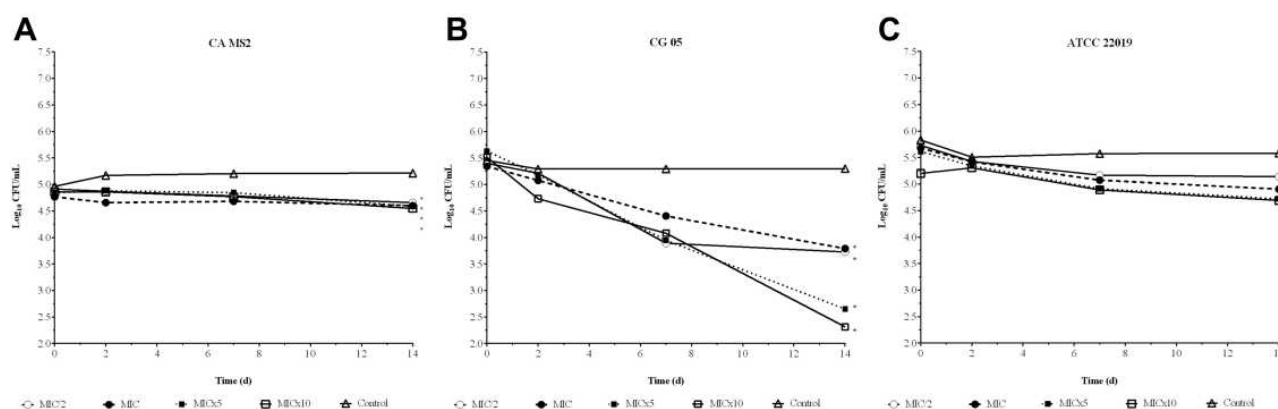


Figure 2 Time-kill plot of colony forming unit concentration (CFU/mL) over time (0, 2, 7, and 14 days) of: (A) *C. albicans* (CA MS2), (B) *C. glabrata* (CG 05) and (C) *C. parapsilosis* (ATCC 22019) in Optisol-GS supplemented with cycloheximide at MIC/2, MIC, MICx5 and MICx10. The asterisks *Indicate statistical difference when compared to the untreated control (* $P < 0.05$).

Table 2 Changes in Safety Study Variables

Study Variables	Cycloheximide Minimum Inhibitory Concentration (MIC)	
	MIC (µg/mL)	MICx10 (µg/mL)
Mean in ECD (SD) day 0, No of cells		
Study cornea	2483 (96)	2150 (1247)
Control	2537 (164)	2816 (212)
P value ^a control x supplemented (95% CI)	0.818 (-2283-2391)	0.410 (-2109-3443)
Mean in ECD (SD) day 3, No of cells		
Study cornea	896 (560)	1632 (1666)
Control	500 (0)	1553 (1125)
P value ^a (95% CI)	0.500 (-5427-4635)	0.862 (-1796-1638)
Mean in ECD (SD) day 14, No of cells		
Study cornea	500 (0)	1176 (739)
Control	500 (0)	1937 (1515)
P value ^a (95% CI)		0.232 (-1172-2695)
Mean nonviable endothelial cells at 14d, %		
Study cornea	82 (8)	57 (22)
Control	64 (42)	34 (16)
P value ^a (95% CI)	0.705 (-475-439)	0.352 (-106-60)
Mean viable endothelial cells at 14d, %		
Study cornea	18 (8)	42 (22)
Control	36 (42)	66 (16)
P value ^a (95% CI)	0.705 (-439-475)	0.352 (-60-106)

Note: ^aCalculated using the paired t-test.

Abbreviations: ECD, endothelial cell density; SD, standard deviation; 95% CI, 95% confidence interval of the difference.

especially *C. albicans* and *C. glabrata*.^{2,20} The other reason for this increase can be the corneal tissue processing, especially for EK, which requires removal of the tissue from the storage medium and increasing the tissue temperature. In addition, some surveys associated a greater fungal infection risk after EK when the tissue was prepared in the eye bank compared with tissue prepared in the operating room²⁻⁴ or with surgeons prepared tissue.¹

Hypothermic media are the most commonly used media in the USA and Latin America. In these media, the prevalence of cases of fungal infection was higher than bacterial infection, the mean incidence fungal rim culture is approximately 1.5%, with *Candida* spp being the most common contaminant. Fungal infection was more likely when grafts were prepared from corneas that had been stored in hypothermic media (0.5%) compared with organ culture (0.02%) ($p < 0.0001$) as shown by Lau et al.⁴ Other papers report lower rates of donor rim contamination and postoperative infection when organ culture media were used.^{17,22,23} These differences are possibly attributed to the absence of antifungals in the hypothermic media.

Ritterband et al published the first study about the addition of voriconazole (100 µg/mL) to Optisol-GS media. The rate of positive cultures in the Optisol-GS media without the supplementation of voriconazole was 12.3% (66/533), of which 7 (10%) were fungi *C. glabrata* and *C. albicans* were the most prevalent strains. In the rims with voriconazole addition, the positivity culture rate was 11.1% (59/533), but there was no fungal growth, this difference was statistically significant ($p = 0.015$). Endothelial toxicity was evaluated with vital dyes of trypan blue and red alizarin. The percentage of non-viable cells was 0.17% in the group with the addition of voriconazole and 0.22% without the addition, which was not significantly different ($p < 0.05$). In the stability test, the effectiveness of voriconazole supplementation in eliminating fungal growth was between 6 and 7 days.⁶ Interestingly, our study found a time-kill plot earlier than voriconazole, starting within the second day, especially, in *C. glabrata* and *C. parapsilosis*. For *C. albicans*, these findings occurred slightly later, around the seventh day.

Other authors have evaluated the supplementation of amphotericin B and voriconazole at different concentrations in Optisol-GS.¹⁷ The efficacy of both supplementations was evaluated in 20 vials of Optisol-GS and divided into groups, as follows: (1) MIC voriconazole (1 µg/mL), (2) MIC x 10 voriconazole (10 µg/mL), (3) MIC x 25 voriconazole (25 µg/mL), (4) MIC x 50 voriconazole (50 µg/mL), (5) MIC x 0.25 amphotericin B (0.0625 µg/mL), (6) MIC x 0.5 amphotericin B (0.125 µg/mL), (7) MIC amphotericin B (4 µg/mL), (8) MIC x 10 amphotericin B (40 µg/mL), (9) Optisol-GS media inoculated (positive control) and (10) only Optisol-GS media (negative control). The study tested two *Candida* spp. strains (*C. albicans* and *C. glabrata*). Due to the known toxicity of amphotericin B, concentrations below the MIC were chosen. Voriconazole, on the other hand, was chosen over the MIC due to studies that reported the absence of toxicity, including the paper of Ritterband et al⁶ which added MICx100 to Optisol-GS. In the group that added *C. albicans* to voriconazole there was a similar growth of colony-forming units in all concentrations of voriconazole and in the positive control group and in the group that added *C. glabrata* there was a reduction of only 70% of the colony-forming units, with no relation to the concentration. In the amphotericin B group, there was no fungal growth of *C. albicans* at any concentration tested. For *C. glabrata*, fungal growth occurred only at the lowest concentrations (MICx0.25 and MICx0.5), but colony-forming units were reduced by 99% and 96%, respectively. Safety was achieved through the analysis of the endothelium by specular microscopy and through the vital dyes of trypan blue and red alizarin. Compared to the control, there was only a significant decrease in endothelial cell count against amphotericin B MICx10 ($p = 0.04$) and a trend at MIC ($p = 0.07$). On the other hand, in the other concentrations and in the voriconazole group there was no significant difference. The percentage of nonviable cells was also significant only at MICx10 in amphotericin B (78% amphotericin B x 10% control $p = 0.02$). This study corroborates with a study that evaluated the effectiveness of amphotericin B at 0.25 µg/mL, which is widely used in European eye banks to supplement the organ culture medium.^{24,25} On the other hand, this study is contradictory to Ritterband et al,⁶ which demonstrated a decrease in the incidence of positive cultures of corneal rims with supplementation with voriconazole. However, these authors used concentrations two times higher (100 µg/mL) that could justify this difference.

In our study, supplementation of Optisol-GS media was performed with cycloheximide, an antimicrobial with a known antifungal action, which is inexpensive and works quickly. In

the efficacy study, a reduction in fungal cell growth from the 2nd day of incubation in Optisol-GS media, in all evaluated concentrations, even in sub-inhibitory concentrations (MIC/2), was observed. For *C. glabrata*, there was an expressive reduction of 99% in the colony count, on the 14th day at MICx5 and MICx10 concentrations. This indicates that the cycloheximide has a fungicidal action (defined as a reduction >99% in CFU/mL) dependent on the drug concentration and time. For *C. albicans* and *C. parapsilosis*, the cycloheximide presents only a fungistatic action over the time elapsed, since a reduction of less than 99% in CFU/mL was observed. In low concentrations, such as MIC, the cycloheximide has a fungistatic action against all *Candida* species that can explain the increase in the MIC values of this drug after 14 days of exposure to yeasts (Table 1).

Our results demonstrate the excellent antifungal activity of cycloheximide against the main strains involved in fungal infections after corneal transplantation, even at subinhibitory concentrations (MIC/2). Moreover, our findings suggest that the Optisol-GS supplemented with cycloheximide may have a better performance than Optisol-GS supplemented with voriconazole or amphotericin B, particularly for *C. glabrata*. Furthermore, its antifungal effect seems to be present even at lower concentrations, overcoming the voriconazole results,^{6,17} and the absence of toxicity even at higher concentrations, surpassing the amphotericin B findings.^{5,17,25}

The small sample size of our toxicity evaluation was one major limitation of our study and new studies must be carried out to strengthen our findings. However, our results suggest a possible benefit in adding cycloheximide to Optisol-GS media, considering the high morbidity of a fungal infection after corneal transplantation. Another limitation of the study is the non-testing of the different dosages of cycloheximide using yeast inoculated into the corneal scleral tissue rather than yeast alone in storage media to better simulate the realistic scenario. The *Candida* species seems to create a biofilm in mammalian cells, becoming more resistant to azoles drugs and more virulent, explaining higher positive yeast cultures in the Optisol-GS with the corneal scleral tissue than the Optisol-GS alone.²⁵

In future perspectives, we suggest studies that assess the synergism of two drugs with antifungal action to decrease the concentration and toxicity of one antifungal alone as already suggested by Kowalski et al.²⁶

Conclusion

Cycloheximide in Optisol-GS decreased significantly the yeast fungal concentration from the second day of preservation. This antifungal agent also demonstrated fungicide

action against *C. glabrata* and fungistatic action against *C. albicans* and *C. parapsilosis*. The addition of cycloheximide at different concentrations in Optisol-GS did not demonstrate toxicity to the corneal endothelium even at high concentrations. Due to its fast action and low cost, cycloheximide can be considered a possible additive to the hypothermic corneal preservation medium.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

All authors declared they have no conflicts of interest for this work.

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