Killing efficacy of a new hypothermic corneal storage medium against the micro-organisms frequently found in human donor cornea intended for transplantation

Laura Giurgola,1 Claudia Gatto,1 Claudia Honisch,2 Orietta Rossi,1 Eugenio Ragazzi,3 Jana D’Amato Tothova1

ABSTRACT

Objective To study the in vitro killing efficacy of Kerasave (AL.CHI.M.I.A Srl), a medium provided with amphotericin B tablet for hypothermic storage of human donor corneas, against relevant contaminants associated with postkeratoplasty infections.

Methods and Analysis The antimicrobial activity of Kerasave was determined after 0, 3 and 14 days of incubation at 2°C–8°C, inoculating Kerasave and the control medium with 105–106 colony forming units (CFU) of Candida albicans (CA), Fusarium solani (FS), Aspergillus brasiliensis (AB), Staphylococcus aureus (SA), Enterococcus faecalis (EF), Bacillus subtilis spizizenii (BS), Pseudomonas aeruginosa (PA), Enterobacter cloacae (EC) and Klebsiella pneumoniae (KP). Log10 reductions at different time intervals were determined by assessing the number of CFU using the serial dilution plating technique.

Results After 3 days, Kerasave induced the highest log10 decreases in the concentrations of KP, PA, CA and EC (5.37, 4.15, 2.97 and 2.67, respectively; all p<0.001). The log10 decreases of SA and EF were 2.27 and 2.11, respectively (all p<0.001). The lowest log10 decrease was observed in BS, AB and FS concentrations (0.25, 0.30 and 0.67, respectively; p<0.001 for BS and AB and p=0.004 for FS). After 14 days, the microbial count of CA, FS and SA, EF, PA and EC further decreased (p=0.006 for FS; p<0.001 for the others).

Conclusion Kerasave effectively reduced or kept unchanged the microbial concentration of almost all tested strains after 3 days. Thus, this novel medium represents a valuable tool to control the microbial contamination of human donor corneas during hypothermic storage for up to 14 days before transplantation.

INTRODUCTION

The human donor cornea is the most commonly transplanted tissue worldwide. The use of lamellar grafts (eg, Descemet’s stripping automated endothelial keratoplasty and Descemet’s membrane endothelial keratoplasty) for replacing only the diseased portion of the cornea while sparing the healthy tissue has led to a significant improvement in graft survival and patient outcome.1–3 However, corneal transplantation is still associated with a certain risk of postkeratoplasty infections.4–6 Particularly, a trend towards an increase in the incidence of postkeratoplasty fungal infections was observed in the USA.6,7 These cases are mainly related to the procedures of lamellar keratoplasties.8–10

Most European eye banks store corneal tissues in organ culture media at 31°C, containing both antibiotics and antifungal agents to help reduce the risk of microbial proliferation.11–13 Contrarily, eye banks in North America preserve donor corneas at 2°C–8°C, and the currently available hypothermic
storage media in the US market are supplied with antibacterial agents but do not contain antifungal agents. For this reason, the possibility of implementing hypothermic corneal storage media with antifungals has a growing interest.

Kerasave (AL.CHI.MI.A Srl, Italy) is a newly developed medical device and Conformité Européenne (CE)-marked hypothermic storage medium containing amphotericin B, which is intended for storage of human donor corneas at 2°–8°C for up to 14 days. Kerasave’s Food and Drug Administration approval is pending at the time of this submission.

Previous studies provided evidence of the efficacy and safety of antifungal agents, including amphotericin B, in reducing Candida species contamination of Optisol-GS (Bausch & Lomb, New Jersey, USA) under hypothermic storage conditions. More recently, Tran et al. investigated the efficacy and safety of various concentrations of amphotericin B, including 2.5 µg/mL, under hypothermic storage conditions. Our previous studies evaluated the antymycotic efficacy of Kerasave at 2°–8°C against Candida albicans, C. tropicalis and C. glabrata.

The present study aimed to assess the in vitro killing efficacy of Kerasave at 2°–8°C against some clinically relevant contaminants of donor corneas, such as Gram-positive Staphylococcus aureus, Enterococcus faecalis and Bacillus subtilis spizizenii; Gram-negative Pseudomonas aeruginosa, Enterobacter cloacae and Klebsiella pneumoniae; and the fungi C. albicans, C. glabrata and Aspergillus brasiliensis.

MATERIALS AND METHODS

Experimental design

Figure 1 presents the experimental design of the time-kill assay of the present study.

Media and devices

Kerasave is a Dulbecco’s Modified Eagle’s Medium-based culture medium containing 6% dextran, 100 µg/mL gentamicin sulfate and 200 µg/mL streptomycin sulfate. It is supplied with an amphotericin B tablet to be added to the 20 mL medium before use (final concentration of 2.5 µg/mL). All vials of Kerasave and blisters containing amphotericin B tablets were stored at 2°–8°C and protected from light. Each Kerasave sample was prepared under sterile conditions immediately before use by dissolving one tablet of amphotericin B in the 20 mL vial containing the Kerasave solution.

RESEP is a syringe-like, patented, CE-marked device containing a resin mixture to remove antimicrobials from the samples. It has been validated for total elimination of antimicrobials from corneal storage media, including Kerasave (online supplemental tables 1 and 2). The validation of the method for removing antimicrobial residues from Kerasave was performed according to the experimental design reported in online supplemental figure 1. In this study, 6 mL of each sample were withdrawn using the RESEP syringe and stirred at room temperature (RT) for 45 min. Afterwards, the samples were transferred into sterile vials.

Preparation of inocula and growth controls

S. aureus (American Type Culture Collection, ATCC 6538), E. faecalis (ATCC 29212), B. subtilis spizizenii (ATCC 6633), E. cloacae (ATCC 35030), K. pneumoniae (ATCC 10031), A. brasiliensis (ATCC 16404), P. aeruginosa (ATCC 9027) and C. albicans (ATCC 10231) were obtained from Microbiologics (Minnesota, USA). F. solani (Deutsche Sammlung von Mikroorganismen, DSM 1164) were supplied by Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Germany.

Inocula of S. aureus, E. faecalis, B. subtilis, E. cloacae, A. brasiliensis, P. aeruginosa, C. albicans, K. pneumoniae and F. solani were prepared from lyophilised pellets according to the manufacturer’s protocols or from fresh culture according to McFarland standards to obtain 1.0×10⁷ colony forming units (CFU/mL in physiological solution.

Preparation of 10⁷ CFU/ml inocula of SA (ATCC 6538), EF (ATCC 29212), BS (ATCC 6633), EC (ATCC 35030), KP (ATCC 10031), AB (ATCC 16404), PA (ATCC 9027), CA (ATCC 10231) and FS (DSM1164)

Figure 1 Time-kill experimental design. In the time-kill study, 20 mL of Kerasave and growth controls were inoculated with 10⁷ CFU of tested microorganisms and incubated at 2°–8°C for up to 14 days. The effective concentration of each microorganism was determined after 0, 3 and 14 days of incubation. AB, Aspergillus brasiliensis; BS, Bacillus subtilis spizizenii; CA, Candida albicans; CFU, colony forming unit; EC, Enterobacter cloacae; EF, Enterococcus faecalis; FS, Fusarium solani; KP, Klebsiella pneumoniae; PA, Pseudomonas aeruginosa; SA, Staphylococcus aureus; ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen.

Kerasave time-kill study

Direct inoculation of 100 µl inoculum (10⁷ CFU) in Kerasave

Direct inoculation of 100 µl inoculum (10⁷ CFU) in growth control samples

Incubation at 4°C for up to 14 days

Determination of killing efficacy at Day 0, Day 3, and Day 14

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Media and devices

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(0.9% (w/v) NaCl); Merck-Sigma-Aldrich, Darmstadt, Germany.

Tryptone soya broth (20 mL; Bioculturalab, Italy) was used as growth control for C. albicans, A. brasiliensis and F. solani, and thioglycolate medium (20 mL; Bioculturalab) was used as growth control for S. aureus, B. subtilis, P. aeruginosa and K. pneumoniae. Nutrient broth (Nutrient Broth, 20 mL; BioLife, Italy) medium was used as growth control for E. faecalis and E. cloacae.

**Time-kill method**

Triplicates of Kerasave samples and growth controls were inoculated with 100 µL of inoculum to obtain a final strain concentration of $10^5$–$10^6$ CFU/mL for each time point. Effective initial strain concentration was determined by counting the number of viable CFU in the 100 µL inoculum using the serial dilution plating technique within 3 hours from spiking (day 0).

On day 3 and day 14, all Kerasave samples, except those containing F. solani, were treated with RESEP before plating to eliminate the antimicrobial residues. Then, the samples were serially diluted in physiological solution and plated on agar plates for viable colony counting. C. albicans and A. brasiliensis were plated on Sabouraud Chloramphenicol Agar Medium (BioLife); S. aureus, B. subtilis, P. aeruginosa and K. pneumoniae were plated on Trypticase Soy Agar Medium (BioLife); and E. faecalis and E. cloacae were plated on nutrient agar dehydrated medium (BioLife).

On day 3 and day 14, 1 mL of Kerasave containing F. solani was serially diluted in physiological solution and plated on potato glucose agar dehydrated medium (Merck-Sigma-Aldrich). All growth controls were antimicrobial-free, so treatment with RESEP was skipped. At the selected time points, 1 mL of each growth control was diluted in physiological solution and plated on Petri dishes containing appropriate agar medium as described above. For each condition (i.e., after 3 days and 14 days of incubation at 2°C–8°C of Kerasave samples and growth controls, respectively), the concentration of each strain was determined by counting the number of viable CFU in 1 mL of each sample using the serial dilution plating technique.

Agar plates containing S. aureus, B. subtilis, P. aeruginosa, K. pneumoniae, E. faecalis and E. cloacae were incubated at 30°C–35°C for 24 hours. Plates containing C. albicans and A. brasiliensis were incubated at 20°C–25°C for up to 72 hours and plates containing F. solani at 25°C for 5–7 days.

The number of CFU/mL in the tested sample corresponding to each plate at each time point was determined using the following formula: CFU/mL=$\text{CFU plate/dilution factor}$.

**Statistical analysis**

In previous studies, the effects observed between the two experimental conditions, Kerasave versus control, were considered ‘high’ according to the definition by Cohen. A preliminary power analysis determined three replicates for each experimental condition as appropriate, considering the low dispersion of the obtained values. Moreover, the use of triplicate samples is an accepted ‘rule of the thumb’ when sustained by a careful evaluation of characteristics and costs of the experimental design. The mean log$_{10}$ reduction was calculated for the tested samples on day 3 and day 14 relative to the initial concentration on day 0 according to Zelver et al using Microsoft Excel V.2010. Mann-Whitney U test was employed to evaluate the log$_{10}$ reduction by Kerasave against each test organism compared with control on day 3 and day 14. A p value less than or equal to 0.05 was considered statistically significant.

**RESULTS**

Table 1 presents the mean concentration (CFU/mL) and the log$_{10}$ decrease of all tested strains obtained in growth controls and Kerasave after inoculation with approximately $10^5$–$10^6$ CFU; the time-kill curves are presented in figure 2.

Kerasave reduced the concentration of all strains after 3 days of incubation at 2°C–8°C (table 1 and figure 2). After 3 days, the highest log$_{10}$ decrease values were observed for P. aeruginosa and K. pneumoniae, whereas the lowest values were observed for A. brasiliensis, B. subtilis and F. solani (table 1). After 3 and 14 days of incubation at 2°C–8°C, differences in the log$_{10}$ decrease between Kerasave and controls were statistically significant according to Mann-Whitney U test (p<0.01; table 1) for all tested strains, except for A. brasiliensis (p=0.6396, Mann-Whitney U test), for which a negative log$_{10}$ decrease was obtained (table 1), corresponding to microbial growth.

After 14 days in Kerasave, the microbial concentration further decreased for all strains, except for B. subtilis, whose extent remained unchanged, K. pneumoniae, which was eradicated on day 3, and A. brasiliensis, which showed microbial growth (table 1 and figure 2).

Overall, the microbial concentration remained almost unvaried for all strains in the growth controls, except for A. brasiliensis, E. faecalis and E. cloacae, for which evident growth was observed after 14 days (table 1 and figure 2).

The validation study conducted on RESEP showed effective elimination of the antibiotics from Kerasave and microbial recovery after 45 min of treatment with RESEP at RT (online supplemental tables 1 and 2), thus complying with the acceptability criteria adopted.

**DISCUSSION**

Kerasave is a newly developed medium intended for storage of human donor corneas at 2°C–8°C for up to 14 days. This medium has the peculiarity of being supplied with an antifungal amphotericin B tablet.

Unlike our previous study, in which only Candida spp were tested at shorter time intervals, the present study investigated nine micro-organisms incubated in Kerasave up to 14 days to simulate the eye bank common practice and cover the intended use of the device. Indeed,
The release of the cornea for transplantation usually does not exceed 7 days of storage at 4°C, and the maximum time of use of the device indicated by the manufacturer corresponds to 14 days. Therefore, in the present study, data were not acquired beyond 14 days. All the tested microorganisms showed significantly lower microbial concentrations in Kerasave than in the growth controls for all the tested time points, except for *A. brasiliensis* on day 14, which was comparable with the control. Our findings indicated that the medium effectively eliminated

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<th>Strain and incubation time (days)</th>
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<td>3.92×10⁶±5.24×10⁴</td>
<td>2.35×10⁷±3.23×10⁵</td>
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<td>Data are expressed as mean±SEM. *P value corresponds to Mann-Whitney U test on the log₁₀ decrease values between Kerasave and growth controls. ATCC, American Type Culture Collection; CFU, colony forming unit; DSM, Deutsche Sammlung von Mikroorganismen.</td>
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seven out of nine (78%) tested microorganisms. This was accomplished despite the low storage temperature of 4°C, which is considered to be a condition resulting in poor antimicrobial efficacy.11 33

The bactericidal activity is achieved when a 3 log_{10} decrease is obtained.34–36 In the time-kill assay, a very high initial microbial concentration inoculum (10^5–10^6 CFU/mL) is used to allow quantification of the killing efficacy. The high inoculum concentration used in our study does not reflect a clinical situation as it is significantly higher than the bacterial concentration observed in the blood of patients with sepsis (10^2–10^3 CFU/mL),37 therefore, complete elimination of the tested micro-organism is not expected in these assays. In our study, the 3 log_{10} decrease indicates bactericidal activity of Kerasave for some micro-organisms. Complete microbial elimination (5–6 log_{10} reduction) would correspond to the performance of disinfectants and sterilisation processes, which is not expected to be achieved by Kerasave.

A log_{10} reduction ≥3 was achieved after 3 days of incubation in Kerasave for C. albicans, P. aeruginosa and K. pneumoniae strains, and was achieved on day 14 for S. aureus, E. faecalis and E. cloacae. The elimination of C. albicans was in line with the results of our previous study, in which C. albicans killing was assessed after 10 days of incubation.18 In this study, Kerasave was shown to be effective during the 14 days of storage. For three strains (A. brasiliensis, F. solani and B. subtilis), a low or negative log_{10} decrease was observed, which indicated low microbial elimination or slight microbial growth over time. For both A. brasiliensis and F. solani, which are filamentous fungi, a low killing efficacy of 2.5 µg/mL amphotericin B contained in Kerasave was expected as the minimal inhibition concentration values for amphotericin B reported in the literature were 0.03–4 µg/mL and 4–8 µg/mL for A. brasiliensis and F. solani, respectively.38–41 In a previous study, Duncan et al42 found that the supplementation of the hypothermic corneal storage medium with amphotericin B at 0.25 µg/mL effectively reduced the growth of F. solani. Along with the lower concentration of amphotericin B, there are some other differences in the experimental conditions between this and our study, that is, a lower concentration of the inoculum (2.5×10^3 CFU/mL vs 10^5–10^6 CFU/mL) and a lower extent of growth reduction. Nevertheless, the authors also concluded that amphotericin B appeared to be a reasonable candidate drug for an antifungal additive to a corneal storage medium. In a recent study, Kaymar et al43 found that amphotericin B at 2.5 µg/mL was ineffective in eliminating small loads of Candida and Fusarium species (up to 10^6 CFU/mL). However, the goal of that study was to achieve complete elimination of fungi limited

Figure 2 Time-kill curves. The graphs show the mean values of microbial concentration, expressed as CFU/mL, observed in the Kerasave samples and growth controls on day 0, day 3 and day 14 for each tested micro-organism. CFU, colony forming unit. ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen.
to single testing due to the scarcity of corneal tissue. Unlike our study, the growth reduction was not quantified. Thus, the differences in the experimental design might explain why the authors concluded that the eradication of fungal contamination of donor corneal tissue might require complementary approaches of anti-fungal supplementation of hypothermic storage media. However, based on the results of Kapur et al’s study, which examined the effect of the hypothermic corneal storage medium with and without tissue growth of similar species, it appears that the presence of corneal tissue does not affect viable counts.

The concentration of amphotericin B in Kerasave represents the optimal balance between the efficacy and safety for preserving the donor cornea and was selected under the intended use of Kerasave, which corresponds to corneal storage rather than corneal disinfection. Although corneal disinfection as intended for disinfectants (povidone-iodine solutions) is out of Kerasave’s intended use, Kerasave temporarily prevented the growth of *F. solani*, for which a moderate 1.25 log₁₀ decrease was observed after 14 days. In any case, we can consider Kerasave effective in decreasing or eliminating contamination from *Candida* spp, which is the most commonly encountered pathogen. Other fungi were not commonly encountered in donor corneal transplantation.

Surprisingly, a very low log₁₀ decrease of 0.18 was observed for *B. subtilis* after 14 days in Kerasave, indicating a bacteriostatic rather than bactericidal effect for this strain under the tested conditions. Data in the literature reported *B. subtilis* to be sensitive to gentamicin (4.0 mg/L; 0.125 μg/mL) and resistant to streptomycin in the literature. The low killing efficacy was confirmed when we repeated the time-kill assay at 4°C and 31°C after 3 days of incubation to evaluate whether the incubation temperature could influence the killing efficacy of Kerasave (data not shown). At 31°C, a 0.6 log₁₀ decrease was observed in Kerasave after 3 days, whereas marked growth was observed in controls (~2.8 log₁₀ decrease; data not shown). We thus confirmed the bacteriostatic effect of Kerasave for *B. subtilis* under tested conditions. Moreover, we hypothesised that *B. subtilis spizizenii* can turn into sporulation, enabling survival under adverse conditions. However, microcopy studies to check for possible *B. subtilis spizizenii* sporulation were not conducted as they were beyond the scope of the present study.

With regard to growth controls, most strains maintained the initial concentration or showed growth along with incubation (*A. brasiliensis*, *E. faecalis* and *E. cloacae*). Previous studies compared Kerasave with other corneal storage media. Contrarily, in this study, we chose to use growth media as control samples to confirm microbial growth of the strains under tested conditions and not in comparison with other corneal storage media since the validity of the time-kill assay and evaluation of the recovery of viable micro-organisms require optimal microbial growth and absence of any factors that would interfere with it. As discussed by Tran et al, approaches that do not use a step to neutralise the antimicrobials present in the culture samples used for the quantification of the micro-organisms can lead to the presence of some residual amounts of drugs, which can prevent growth on culture plates. Kerasave contains different antimicrobials, and their interference with microbial growth is predictable. Therefore, to avoid false-negative results, RESEP was used before plating the Kerasave samples on agar plates. The treatment of Kerasave samples with RESEP was validated prior to its use in the time-kill assay using the ultra-high-performance liquid chromatography which revealed total removal of antimicrobials from Kerasave and complete recovery of viable micro-organisms was obtained in RESEP-treated or untreated samples (online supplemental tables 1 and 2).

The RESEP treatment was not performed on *F. solani* containing samples since growth inhibition of *F. solani* by Kerasave was not observed. Thus, the dilution plate technique was sufficient to obtain a reliable result in the time-kill study.

Safety study of Kerasave was out of the scope of the present study as it has been previously investigated in cells via a cytotoxicity test, biocompatibility animal studies according to International Organization for Standardization standards and in donor corneas.

This study completes the data obtained in our previous work on the efficacy of the Kerasave medium, confirming the killing of the most common corneal contaminants by Kerasave under hypothermic corneal storage conditions even after a short incubation time (*C. albicans*, *P. aeruginosa* and *K. pneumoniae*) and preventing the microbial growth of all the other tested strains. In this study, the killing efficacy of Kerasave was directly related to the time of incubation, as observed for *S. aureus*, *E. faecalis* and *E. cloacae*. The killing efficacy of Kerasave could be limited in case of contaminations by micro-organisms resistant to the antimicrobials present in Kerasave (amphotericin B, streptomycin sulfate and gentamicin sulfate), and eye banks should consider that a short storage interval and storage temperature of 4°C could prevent the eradication of high contamination from donor tissues. Finally, the hypothermic corneal storage medium Kerasave is a promising tool for enhancing the safety of the recipient of the transplanted cornea.

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Contributors LG and JD’AT planned and designed the study, analysed and interpreted the data, drafted the manuscript, and critically revised the manuscript. CG, LG, OR and CH performed the experiments. CH and ER analysed and interpreted the data and critically revised the manuscript. JD’AT is responsible for the overall content as guarantor.
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Ethics approval This study does not involve human participants.

Provenance and peer review Not commissioned; externally peer reviewed.

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