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Improvement of human corneal endothelium in culture after prolonged hypothermic storage

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PURPOSE. *To evaluate whether the organ culture method for human cornea preservation may be applied to corneas stored for several days at 4°C.*
METHODS. *The cell density, viability, and morphology of corneal endothelium were examined in 140 human corneas stored at 4°C for the minimal time required for transport to the bank and for the preliminary controls of cornea status (1.6±1.1 days) and in 46 corneas preserved at 4°C for 6.1±1.9 days in Optisol-GS. The evaluation was repeated after 19.7±9.1 days of incubation at 31°C in a culture medium containing 2% newborn calf serum.*
RESULTS. *After the hypothermic storage the corneal endothelium had a mean density of 2475±159 cells/mm^2 without significant difference between the short and the long term incubation. Several corneas of the two groups showed signs of endothelium degeneration and were positive to trypan blue test. After the incubation at 31°C, the corneas with endothelial degeneration decreased by 52.2% and those positive to trypan blue decreased by 21.7%. Polymorphism (enlarged endothelial cells) increased from 9.6% to 14.5% of the corneas. The remodeling of the endothelium led to a 6.7% decrease in cell density. These results were similar after short-term and long-term storage at 4°C.*
CONCLUSIONS. *Organ culture was effective in improving corneal endothelium when the hypothermic storage was prolonged to the upper temporal limit for this procedure (7-10 days). These results may encourage the possibility of an eye bank to allocate the available conea pool, thus decreasing the risk of discarding precious material. (Eur J Ophthalmol 2003; 13: 745-51)*

KEY WORDS. *Cornea culture, Cornea preservation, Endothelium repair*

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INTRODUCTION

Sustained demand for corneas for grafting stimulates a continuous search for a more adequate preservation procedure. According to the 2003 Directory of the European Eye Bank Association, 36,567 corneas were processed in European eye banks in 2001, with 52% delivered for transplantation (7% increase with respect to 2000). In Europe, the majority of transplanted corneas (64.1% in 2001) are preserved by storage at a physiologic temperature (organ culture method), whereas the remaining fraction is preserved at 4°C (hypothermic cornea storage).

The two methods yield distinct and well-characterized advantages (1-3). The hypothermic method consists in the incubation of corneas at 4°C in a medium containing antibiotics and a deturgescent agent to maintain cornea clarity and physiologic thickness. After a

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maximum of 7-10 days, the corneas are used or discarded. The culture method allows the preservation of corneas for up to 1 month, although utilization within 3 weeks is recommendable. Shortly after their isolation, corneas are immersed at a physiologic temperature in a culture medium supplemented with antimicrobial agents, to avoid contamination, and bovine serum, to improve the survival of endothelial cells (4). In this method, the cornea clarity is lost and swelling of the stroma occurs. To restore transparency and physiologic thickness, cultured corneas are transferred in the days before grafting to a medium containing a deturgescent agent (5).

Considering the greater complexity of the culture method, hypothermic preservation would be more convenient for an eye bank. However, the number of corneas that can be delivered for transplantation by this procedure is limited. In our institution, the hypothermic and culture methods of cornea preservation are both operative. Therefore, we made an effort to integrate the two methods, examining whether preservation of corneas in the cold for the maximal time allowed for this procedure may be followed by further preservation in culture. The data show that this sequential procedure is possible. In fact, several corneas showing endothelial defects after hypothermic preservation improved at the end of the culture period.

MATERIALS AND METHODS

Incubation media

Corneas assigned to the organ culture method of preservation after a brief storage in the cold were collected in MEM-Earle containing 6% dextrane-T500 (Eusol). Glucose, Na bicarbonate, L-glutamine, and pyruvate were also present to support the cornea metabolism. Optisol-GS has been used for long-term hypothermic storage of corneas (6). In this medium the metabolic requirements of corneal tissues are satisfied by glucose, Na bicarbonate, pyruvate, aminoacids, and purines. Ascorbic acid is also present according to the finding that this vitamin is 20 times more concentrated in the aqueous humor than in plasma. Cornea clarity is maintained by the addition of 1% dextrane-T500 and 2.5% chondroitin sulphate. The medium for cornea culture (Tissue C) was similar to Eusol except that dextran was omitted. Newborn calf serum and three antibiotics were added to insure the survival of endothelial cells and wide protection from bacteria and fungi. The detailed composition of these media is reported in Table I.

Experimental design

We stored at 4°C 249 corneas collected in our institution in July 2001. Of these, 168 were collected and stored in the medium designed for prompt utilization or early transfer to the culture medium (Eusol group). Eighty-one corneas were collected and preserved in Optisol-GS to have a group suitable for delayed utilization (within 7-10 days). During the hypothermic preservation, 28 corneas of the Eusol group and 35 corneas of the Optisol-GS group were required by the surgeons for penetrating keratoplasty. The remaining corneas (140 of the Eusol group and 46 of the Optisol-GS group) were transferred to the culture medium. Considering the limited fraction of corneas utilized during the hypothermic storage, these numbers demonstrated the advantage of having the culture procedure available at the end of the cold storage. During the culture, the medium was not changed, according to the "closed system" procedure (1). The evaluation of endothelium was performed before the addition of corneas to the culture medium and at the end of incubation at 31°C.

Evaluation of corneas

The cornea evaluation was performed as previously described (5). The epithelium, stroma, and endothelium were examined blindly by the slit lamp and the light microscope. Before the light microscope evaluation, the endothelium was exposed to 0.25% (w/v) trypan blue to test cell viability. When the cells are dead, the plasma membrane is leaky and the dye penetrates into the cytosol staining the cell nucleus. Corneas were considered trypan blue positive when stained nuclei comprised >0.1% of the total endothelial cells. Trypan blue positive cells were either dispersed in the central part of the cornea or assembled in small to large groups (5). Sometimes they formed rows along a corneal fold. After the trypan blue test, the corneas were exposed to a hypotonic sucrose solution to evaluate the morphology of the endothelium. The num-

TABLE I - COMPOSITION OF MEDIA

Composition	Eusol	Optisol-GS	Culture (Tissue-C)
Basic components	MEM-Earle	A mixture of TC-199 and MEM-Earle media	MEM-Earle
Buffer	25 mM HEPES	25 mM HEPES	25 mM HEPES
Antibiotics	0.1 mg/ml gentamicin	0.1 mg/ml gentamicin 0.2 mg/ml streptomycin	50 UI/ml nystatin 100 UI/ml penicillin G 0.05 mg/ml streptomycin
Others	26 mM Na bicarbonate 6% dextran-T500 1 mM pyruvate 2 mM glutamine 2-mercaptoethanol	26 mM Na bicarbonate 2.5% chondroitin sulfate 1% dextran-T500 1 mM pyruvate 0.1 mM nonessential aminoacids 2-mercaptoethanol ascorbic acid vitamins purines (adenine, adenosine, inosine) cholesterol	26 mM Na bicarbonate 1 mM pyruvate 2 mM glutamine 2% (v/v) newborn calf serum

Optisol-GS (Optisol supplemented with gentamicin and streptomycin) was supplied by Bausch & Lomb Surgical Inc.
Other corneal storage media (Eusol, Tissue-C) and reagents were supplied by Al.chi.mi.a. (Padova, Italy)

TABLE II - SPECIFICATIONS OF THE CORNEAS ADDED TO CULTURE AFTER THE HYPOTHERMIC STORAGE

Cornea specifications	Optisol-GS	Eusol	Total
Number of corneas	46	140	186
Donor age (yr)	57.5±15.4*	65.4±13.0	63.5±14.0
Death-to-enucleation time (hours)	7.1±3.8	8.7±5.8	8.3±5.4
Storage at 4°C (days)	6.1±1.9*	1.6±1.1	2.7±2.4
Endothelial cell density (cells/mm^2)	2446±199	2483±145	2475±159
% of corneas with signs of endothelial damage	41.3	34.2	36.0
% of corneas trypan blue positive	41.3†	19.3	24.7

Data are mean ± SD
* $p<0.001$ for the difference with Eusol (Student t-test)
†$p<0.01$ for the difference with Eusol (χ^2 test)

ber of endothelial cells was estimated with the help of a calibrated grid (fixed frame) mounted in the ocular of the microscope. The morphologic evaluation of the endothelium included the incomplete pattern of intercellular space swelling, irregular cell shape and borders, cell swelling, and the degree of polymorphism.

Statistical methods

Results are expressed as mean ± standard deviation unless otherwise specified. Comparison of mean values was made by unpaired and paired Student's t-test, as appropriate. The comparison between the Optisol-GS and the Eusol group in Table II was ana-

TABLE III - EFFECT OF CULTURE AT 31°C ON THE ENDOTHELIAL CELL DENSITY

Effects	Optisol-GS	Eusol	Total
Number of corneas	46	140	186
Culture interval (days)	22.2±9.6	18.0±8.8	19.7±9.1
Initial endothelial cell density (cells/mm^2)	2446±199	2483±145	2475±159
Final endothelial cell density (cells/mm^2)	2322±271*	2326±287*	2325±282*
Decrease in cell number (cells/mm^2)	151±178	171±251	166±236
% of cell density decrease	6.2±7.4	6.9±10.4	6.7±9.8

Values are mean±SD
*Paired, two-tailed t-test analysis of the decrease in the endothelial cell density yielded a $p<0.0001$ for the three groups

TABLE IV - EFFECT OF CULTURE AT 31°C ON ENDOTHELIUM MORPHOLOGY AND CELL VIABILITY

Effects	Optisol-GS n = 46	Eusol n = 140
Endothelial morphology		
Cornea damaged after 4°C	19 (41.3)	48 (34.2)
Cornea damaged after the culture	9 (19.6)	23 (16.4)
Cornea repaired	10	25
Cell viability (trypan blue test)		
Cornea trypan blue positive after 4°C	19 (41.3)	27 (19.3)
Cornea trypan blue positive after culture	14 (30.4)	22 (15.7)
Cornea repaired	5	5

Values are n (%). The evaluation of the endothelium included the incomplete pattern of intercellular space swelling, the irregular cell shape and borders, and the cell swelling. Corneas were considered trypan blue positive when stained nuclei comprised >0.1% of the total endothelial cells. Trypan blue positive cells were dispersed in the endothelium, assembled in groups, or assembled as rows along folds (5)

lyzed by the χ^2 test. In Table IV, the comparison before and after the incubation at 31°C was analyzed by a Mc Nemar test. p Values of <0.05 were considered to be significant.

RESULTS

This study focused on the effect of culture on corneas collected and preserved several days in Optisol-GS, a medium suitable for long-term hypothermic preservation (6). As a control we used a simple medium (Eusol) suitable for the collection of corneas and for their brief storage in the cold. Because it has been reported that culture at 31°C stimulated the repair of damaged endothelium (2, 7), we included in our experimental groups the corneas presenting mild endothelial damage after the hypothermic preservation. As shown in Table II, the corneas of the Optisol-GS group showed a minor difference in the age of donors with respect to our control corneas (the Eusol group). The death-to-enucleation time and the endothelial cell density were comparable. A fraction of corneas presented signs of endothelial degeneration (incomplete pattern of intercellular space swelling, irregular cell shape and borders, cell swelling). These alterations might be in part related to the damaging effect of a low temperature on the organization of F-actin cytoskeleton and on the intercellular adhesive interactions (8).

A fraction of corneas was also trypan blue positive. In the Optisol-GS group the total number of trypan blue positive corneas was 19 but 50% of these only had scattered positive cells comprising less than 1% of the total cells. In the Eusol group the trypan blue positive corneas were 27 with 17 corneas presenting dispersed stained cells forming less than 1% of the total cells. After 3 weeks of culture, we observed a small but significant decrease in the endothelial cell density that, however, was not detrimental to the cornea suitability for grafting (Tab. III). The decrease was similar in the Optisol-GS and Eusol groups. Considering the two groups together, the mean percent decrease was 6.7±9.8%, in agreement with the cell loss reported in other studies (5, 9, 10). The endothelium morphology changed during culture, suggesting an extensive cell remodeling. The final result was an improvement of the endothelium, irrespective of the time of the hypothermic preservation (Tab. IV). In the two groups, 52% of the corneas initially damaged were repaired, increasing the number of corneas without endothelial degeneration from 27 to 37 in the Optisol-GS group and from 92 to 117 in the Eusol group. Considering the entire cornea pool, the increase was from 119 to 154 ($p<0.001$). Consistent with a regenerative effort, we observed that at beginning of culture 9.6% of corneas had a variable degree of polymorphism (enlarged endothelial cells). After the organ culture the corneas presenting polymorphism increased to 14.5% (not reported in Tab. IV). Because corneal endothelial cells cannot replicate, cell enlargement is considered to be a measure to compensate for the cell loss (2, 7). Although not significant, the improvement of endothelium was also observed with the trypan blue test. In the Optisol-GS group the number of trypan blue positive corneas decreased by 26% after the culture. In the Eusol group the number of trypan blue positive corneas decreased by 18%. The number of corneas presenting full cell viability increased from 27 to 32 in the Optisol-GS group and from 113 to 118 in the Eusol group. In the entire cornea pool the increase was from 140 to 150. Considering the improvement of endothelial morphology and cell viability together, the pool of healthy corneas free of endothelial defects and with full cell viability increased from 119 to 149 (25%). This improvement was independent of the time of hypothermic preservation.

DISCUSSION

The preservation of corneas for transplantation became a current procedure in 1974 when McCarey and Kaufman demonstrated the possibility of cornea storage at 4°C in medium-199 supplemented with 5% dextran, bicarbonate buffer, and antibiotics (11). Dextran acts as a deturgescence agent, maintaining cornea clarity. Cold preservation of corneas is technically simple, allows the maintenance of a viable endothelium, and prevents the growth of microorganisms. The length of storage was increased without endothelial deterioration by the replacement of 80% dextran with 2.5% chondroitin sulphate (Optisol) (6). Because of this improvement, the accepted period for hypothermic cornea preservation is extended to 7-10 days. The alternative procedure for cornea preservation is incubation at 30-37°C in a medium containing several nutrients, bicarbonate buffer, antibiotics, and bovine serum. By this procedure the corneas are preserved for up to 1 month, although 3 weeks seems a recommendable time. Since its introduction in the United States following a procedure previously developed for the skin (12, 13), this method found diffusion and refinement in Europe, through the promotion of the Danish and the Netherlands eye banks (3, 5). The hypothermic method has the advantage of being simple and economical. Accurate reviews of donor history and serologic analysis minimize the possibility of transmitting diseases. The short storage time precludes an effective microbiologic control of the medium during the cornea storage; however, this control is not considered to be a major requirement, because a correlation between the bacterial content of the cornea and the occurrence of postkeratoplasty endophthalmitis has not been found (14). At variance with the hypothermic procedure, the culture method allows the possibility of a rigorous cornea selection. The prolonged incubation selects corneas with a stable endothelium, able to survive the drastic changes of its extracellular environment and presenting the capacity to repair their defects. In this procedure, continued microbiologic analyses are possible and may allow, discarding of contaminated corneas whose bacteria content is resistant to the antibiotics added to the incubation medium. Despite a lack of correlation between the bacterial content of the cornea and the occurrence of endophthalmitis, this selection seems advisable because

it has been calculated that the risk of infective complications is 12-22 times greater with a contaminated cornea (15, 16). In addition, incubation of cornea at a physiologic temperature may reduce its content of leukocytes, thus decreasing the risk of allograft reaction (3).

Our study had the purpose of exploring whether the advantages of the two procedures may be combined by using in sequence the hypothermic preservation of the corneas and the culture method. This procedure would drastically reduce the wastage of donor tissue while preserving the positive clinical outcome of penetrating keratoplasty that is equivalent in the two methods of preservation (3). To this end we examined the survival in culture of corneas stored for the longest allowed times at 4°C. As a control we used corneas cultured after the brief hypothermic storage required to collect the corneas and to perform the preliminary tests of suitability. To validate our study we used Optisol-GS for the prolonged hypothermic storage of the corneas, one of the most suitable media for this purpose. Following the standards reported in the 2003 edition of the Directory of the European Eye Bank Association, we examined corneas with an endothelial cell density greater than 2000 cells/mm^2, obtained after a short death to enucleation time. The organ culture interval was about 3 weeks. We found that in spite of a long period of cold preservation the influence of culture was similar to that observed after a brief storage. In agreement with a limited loss of endothelial cells during culture (5, 9, 10), we found a cell density decrease of 6.9±10.4% in the group with a short period of cold preservation and of 6.2±7.4 in the group stored for a longer time. The final endothelial cell count in all corneas was 2325 ± 282 cells/mm^2, with no significant difference between the two groups. These data indicate that the integrated method of cornea preservation (i.e., the long-term hypothermic procedure followed by the cornea culture) may give the advantage of conforming the properties of the donor cornea to the age and the general conditions of the recipient. For example, a cold-preserved cornea that did not go through a strict microbiologic control would better fit a young recipient with intact immune defenses against bacteria whereas a cultured cornea free of bacterial contamination would be more suitable for an immunodeficient recipient.

A remarkable result of the present study is the demonstration of endothelial repair during organ culture. This result confirms early studies demonstrating high glucose uptake in cultured corneas and high capacity to repair an artificially induced wound in the endothelium (7, 17). Elevated metabolic activity in the endothelium in the first weeks of culture also has been demonstrated, examining the synthesis of high energy compounds (18). We observed endothelial repair in corneas transferred after brief hypothermic storage (Eusol group), but also in corneas transferred after long-term cold storage (Optisol-GS group). The repair of the endothelium is attributed to several processes occurring together such as the elimination of damaged cells and the elongation of residual endothelial cells that slide over the damaged area (2). Although this is a distinct advantage of the organ culture method, it suggests the cautionary measure to limit as much as possible the time of culture as the extensive endothelial stimulation during this period may reduce a possible wound healing effort after grafting. The same tendency to repair has been observed examining the cell viability before and after the culture (trypan blue test). Table II shows that in the Optisol-GS group the corneas showing dead cells (positivity to trypan blue) were significantly higher when compared with the Eusol group. However, this difference was not due to the effect of prolonged storage in the cold. Rather, it was the result of the greater delivery to surgeons of the best corneas during the prolonged cold storage, thus concentrating the damaged corneas in the fraction destined for culture. In the Optisol-GS group, 35 of 81 corneas were delivered to surgeons during the hypothermic preservation (43.8%), whereas in the Eusol group the utilization was 28 of 168 (16.6%). We find that the trypan blue positive corneas decreased in the two groups after the culture. In the entire cornea pool the decrease was from 46 to 36. Because the trypan blue positive cells are irreversibly damaged, the decrease is likely due to detachment of the dead cells from the endothelium with compensative enlargement of contiguous cells. Close examination shows that this process involved five corneas in each group and was manifest on corneas presenting scattered trypan blue positive cells comprising less than 1% of the total endothelial cells. As a result, the decrease in the total number of endothelial cells was low and insufficient to cause a difference in the overall decrease in endothelial cell density between the two groups. In agreement, the

decrease in cell density was comparable in the two groups (Tab. III).

In summary, our study shows that the transfer in culture of corneas previously stored in the cold for the maximal time permitted by this procedure of preservation allowed prolongation of their preservation for at least 3 weeks. During this time the quality of the endothelium may even improve. This procedure may increase the possibility of an eye bank providing corneas suitable for penetrating keratoplasty.

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