Factors Influencing the Suitability of Organ-Cultured Corneas for Transplantation

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Purpose. To assess the influence of donor and storage factors on the suitability of organ-cultured corneas for penetrating keratoplasty (PKP) using multifactorial regression analysis.

Methods. Corneas (mean donor age, 57 years; standard deviation, 21 years) were stored by organ culture at 34°C for up to 5 weeks (mean, 22 days; standard deviation, 6 days). The endothelium was assessed by light microscopy, and corneas with <2200 cells/mm² were considered unsuitable for PKP.

Results. Of the 9250 corneas stored between 1992 and 1994, 59% were issued for PKP, 5% were discarded because of bacterial or fungal contamination, and 30% were unsuitable for PKP owing to endothelial deficiencies. Donor age had the strongest influence on suitability for PKP: >80% of corneas from donors younger than 40 years of age were issued for PKP compared with only 45% of corneas from donors 80 years of age and older. There was an overall decline in the percentage of corneas suitable for PKP with increasing storage time, but the rate of this decline was inversely related to donor age. Cause of death and post mortem times to enucleation and to storage had only a small influence on suitability for PKP.

Conclusions. Criteria based on endothelial assessment rather than on donor age allow corneas from donors of all ages, stored by organ culture for extended periods, to be used for PKP. Organ culture also allows corneas with bacterial or fungal contamination to be identified and discarded before they are grafted. Invest Ophthalmol Vis Sci. 1997; 38:16-24.

Organ culture was first used successfully for corneal storage in the early 1970s, and it is now the method of choice for many European eye banks. Organ culture has been used in the Corneal Transplant Service Eye Bank in Bristol since 1986. By the end of 1994, 12,000 corneas had been supplied by this eye bank to more than 200 hospitals in the United Kingdom. The efficacy of storing corneas by organ culture for several weeks has been studied. Based on graft survival and postoperative loss of endothelial cells, organ-cultured corneas are comparable to corneas stored for much shorter periods at 4°C.

One reason for the predominance of organ culture in Europe is that it offers an extended storage period of several weeks, which has helped to improve the availability of tissue for routine and emergency grafts. In addition, organ culture, unlike hypothermic storage, offers the opportunity to detect bacterial or fungal contamination of the tissue during storage, thereby greatly reducing the risk of transplanting infected corneas. The corneal endothelium also is examined routinely at the end of the storage period to ensure suitability of the tissue for transplantation.

Clearly, not every cornea donated for transplantation, whether stored by organ culture or at 4°C, is suitable for penetrating keratoplasty (PKP), and more than one third of corneas stored in Bristol are found to be unsuitable, primarily because of deficiencies of the endothelium. We wanted, therefore, to determine the influence of factors such as donor age, cause of death, post mortem times, and storage time in organ culture on the suitability of corneas for PKP. Multifactorial regression methods were used to allow simultaneous analysis of the influence of these various factors.

METHODS

Procurement of Eyes

From 1992 to 1994, 9250 corneas were stored in the Corneal Transplant Service Eye Bank in Bristol. No
donor age limits were set, and enucleation times up to 24 hours or more were accepted. Eyes were sent to the eye bank from approximately 200 hospitals throughout the United Kingdom. Most corneas were received as whole eyes in moist chambers, but 5.7% were received as corneoscleral discs in a 4°C storage solution (M-K medium or Optisol [Chiron Ophthalmics, Irvine, CA]). The eye bank was notified of all donors through the UK Transplant Support Service Authority (UKTSSA), the UK's national organ matching and distribution organization, which organized the transport of eyes to the eye bank from donor hospitals. Eyes were retrieved from cadaveric donors in accordance with the Human Tissues Act 1961 and the Corneal Tissue Act 1986, and lack of objection by relatives to the use of the corneas for transplantation was always established.

**Corneal Storage**

Corneas were stored by organ culture at 34°C. Before removal of the corneoscleral discs, eyes were cleaned by several washes in sterile saline (0.9% wt/vol NaCl), followed by immersion in 1% (wt/vol) polyvinylpyrrolidone-iodine (PVP-I) for 2 minutes, 0.1% (wt/vol) sodium thiosulfate (to neutralize the iodine) for 1 minute, and another wash in sterile saline. Corneas were excised with a 4-mm rim of sclera, a suture was placed through the scleral rim, and the cornea was suspended in 80-ml tissue culture medium in a 100-ml glass DIN infusion bottle. The bottle was closed with a silicone rubber stopper. These procedures were carried out in a class II biologic safety cabinet.

The medium was composed of Eagle's minimum essential medium with Earle's salts buffered with HEPES and containing 26 mmol/L NaHCO₃, 2% fetal calf serum, 2 mmol/L glutamine, penicillin (100 U/mL), streptomycin (0.1 mg/ml), and amphotericin B (0.25 µg/ml). The medium was not changed during the course of the storage period.

After 7 days of organ culture, a sample of the medium was taken to test for bacterial and fungal contamination using tryptic–soy–agar plates and brain–heart–infusion broth. More than 85% of infected corneas were revealed at this stage, and the remainder were detected by direct growth of the contaminant in the organ culture bottle. All infected corneas were removed from the eye bank and referred to a medical microbiologist for identification of the bacteria and fungi.

**Endothelial Assessment**

Two days before the scheduled date of a graft, corneas were removed from the organ culture medium, and their suitability for PKP was assessed by endothelial examination by light microscopy after staining by trypan blue and hypotonic sucrose. The assessment was based primarily on endothelial cell density (estimated from cell counts made with the aid of an eyepiece graticule), and the minimum acceptable for PKP was set at 2200 cells/mm². The presence (number and distribution) of blue-stained cells, loss of cells, variations in cell size and shape, and degree of folding of Descemet's membrane also were taken into account. On the basis of these quantitative and qualitative criteria, corneas were judged to be either suitable or unsuitable for PKP. Corneas suitable for PKP were classified further as good (≥2200 cells/mm²), very good (≥2500 cells/mm²), or excellent (≥3000 cells/mm²). Corneas unsuitable for PKP because of endothelial deficiencies (that is, excluding contaminations and medical contraindications) were made available for lamellar grafts or epikeratoplasty.

Corneas suitable for PKP were placed in organ culture medium containing 5% dextran (average molecular weight was 500 kDa) to reverse the stromal edema that occurred during organ culture and then were returned to the 34°C incubator. The next day, a sample of the dextran medium was removed from each culture for microbiologic testing, and the corneas were sent to the recipient hospitals. Corneas usually were grafted 2 days after the endothelial assessment, although use of the cornea within 4 days is considered acceptable.

**Distribution of Corneas**

All corneas stored in the eye bank were distributed through UKTSSA, which received all requests for tissue and organized transport to the 200 recipient hospitals that used the service. Corneas for routine grafts were allocated on demand, but only for named patients with a scheduled date of graft. With the proviso that corneas reached or exceeded the minimum criteria for suitability for PKP, allocation was made principally on the basis of age matching, and donors were never more than 20 years older than recipients. Corneas from tissue-typed donors were offered for the best-matched patients on the UKTSSA waiting list, and, if accepted, a suitable graft date was arranged.

**Statistical Analysis**

All relevant donor information and storage data were stored on the UKTSSA computer. These included donor age, sex, cause of death, whether the donor had been tissue typed, time intervals between death and enucleation and between enucleation and organ culture, whether the corneas had been placed in 4°C storage medium before organ culture, and time in storage.

Multifactorial regression methods were applied to the data to allow the influence of the various donor and storage factors to be analyzed simultaneously. Logistic regression was used when the response variable
had only two categories (for example, contaminated or not contaminated, suitable for PKP or unsuitable for PKP). Odds ratios are quoted for significant ($P < 0.05$) factors. They show for a given factor the likelihood (or risk), compared with a baseline, that corneas would fall into one of the two response categories: For donor age, the baseline is age <20 years; for time in organ culture, the baseline is storage time <15 days; for a given cause of death, the odds ratio is the risk compared with all other causes of death; and for times to enucleation or to organ culture, the odds ratio is the risk associated with each hour of elapsed time. Odds ratios greater than one indicate increased risk compared with the baseline: The higher the odds ratio, the greater the risk, although the relationship is not linear (that is, an odds ratio of two does not mean twice the risk). Conversely, odds ratios less than one indicate reduced risk compared with the baseline. Three separate logistic regression analyses were performed to determine the influence of the various factors on the likelihood of contamination, the likelihood of corneas being considered unsuitable for PKP, and the likelihood of corneas being graded very good or excellent. Data also were analyzed by multifactorial regression using assessment grade as the response variable. This was considered justified because the assessment grade was based primarily on endothelial cell density. Assessment grade was coded as follows: 4 = excellent; 3 = very good; 2 = good; and 1 = unsuitable.

Only corneas with complete data that met all inclusion criteria were included in the various analyses. No adjustment was made for statistical dependence between two corneas from the same donor; accordingly, the distributions of age, sex, cause of death, tissue typing, and post mortem times were based on corneas rather than on donors. The level of significance was set at 5%. Odds ratios are shown with 95% confidence intervals (95% CI), and means are given with their standard deviations (SD).

RESULTS

From 1992 to 1994, 9250 corneas were received by the Corneal Transplant Service Eye Bank in Bristol. Mean donor age was 57 years (SD, 21 years), the male:female ratio was 59:41, and 21% of corneas came from tissue-typed donors. Donor age distribution (Fig. 1) shows that 45% of corneas came from donors younger than 60 years. The most common causes of death (Fig. 2) were cardiovascular disease (28%), cerebrovascular accident (22%), and cancer (21%). Enucleation was performed within 10 hours (SD, 8 hours; $n = 9109$), and corneas, excluding those that had been in a 4°C storage medium, were placed in organ culture within 29 hours (SD, 10 hours; $n = 8400$) of death. Corneas that were received in 4°C storage medium had a longer mean death to organ culture time of 74 hours (SD, 49 hours; $n = 498$). The mean storage time in organ culture was 22 days (SD, 6 days; $n = 8580$).

Of the 9250 corneas received, 59% subsequently were issued for PKP, 38% failed to meet the criteria for PKP and were discarded, and 3%, also unsuitable for PKP, were issued for lamellar grafts or epikeratophakia (that is, 41% were unsuitable for PKP). By far, the major reason corneas were unsuitable for PKP was endothelial deficiency, which applied to 71% of the corneas not issued for PKP (Fig. 3).

![FIGURE 1. Frequency distribution of corneas classified by donor age (n values in parentheses).](image1)

![FIGURE 2. Frequency distribution of corneas classified by donor cause of death (n values in parentheses).](image2)

*Figures 1 and 2 are provided as Supplementary Material.*
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Risk of Contamination

The overall loss of corneas through microbiologic contamination was 5% of all corneas stored, and the ratio of bacterial:fungal infections was 2:1. Analysis showed that the donor's cause of death had a marked influence on the risk of contamination during organ culture (Fig. 4). When the cause of death was cardiovascular disease or cerebrovascular accident, the risk of corneal contamination was reduced compared with other causes of death (see odds ratios, Fig. 4), whereas corneas from donors in the "infections" category (for example, septicemia) were more likely to be contaminated. Corneas from tissue-typed donors, the great majority of whom would have been solid organ donors and, hence, artificially respired, were no more at risk of loss through infection than corneas from untyped donors.

Longer periods of time between death and enucleation were associated with a slightly increased risk of infection (odds ratio, 1.03; 95% CI, 1.01 to 1.04; \( P = 0.0001 \)). On the other hand, time from enucleation to organ culture had little influence on the likelihood of contamination (odds ratio, 1.004; 95% CI, 0.999 to 1.009; \( P = 0.09 \)).

Endothelial Assessment

A breakdown of corneas by assessment grade is given in Figure 5, which shows that 67% of corneas subjected to endothelial examination (that is, excluding those lost through contamination or medical contraindications) met or exceeded the criteria for PKP.

Cause of Death and Postmortem Times. When the donor's cause of death was cerebrovascular accident or cardiovascular disease, corneas were more likely to be suitable for PKP than for other causes of death (Table 1). On the other hand, when the cause of death was cancer or "other," the corneas were less likely to fall into the very good or excellent grades. Donor corneas that were tissue typed (that is, mainly solid organ donors) also were more likely to be suitable or to be very good or excellent (Table 1), which suggested that there were confounding factors, such as young age and short death to enucleation time, that predisposed these corneas to suitability.

Longer death to enucleation time and longer enucleation to organ culture time were associated with increased risk of corneas either not suitable or not attaining very good or excellent grades. The effects, however, were slight (Table 1); time to enucleation had a greater influence than time to organ culture.

Donor Age. Donor age had a strong influence on the endothelial assessment grade (Fig. 6). Although
TABLE 1. Influence of Cause of Death and Postmortem Times on Endothelial Assessment

<table>
<thead>
<tr>
<th>Cause of Death</th>
<th>Suitable for PKP</th>
<th>Very Good/Excellent</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVA</td>
<td>1.18 (1.02–1.36)</td>
<td>NS†</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>1.29 (1.15–1.45)</td>
<td>NS</td>
</tr>
<tr>
<td>Cancer</td>
<td>NS</td>
<td>0.78 (0.68–0.91)</td>
</tr>
<tr>
<td>Other</td>
<td>NS</td>
<td>0.79 (0.63–0.99)</td>
</tr>
<tr>
<td>Tissue-typed</td>
<td>1.52 (1.29–1.79)</td>
<td>1.22 (1.06–1.39)</td>
</tr>
<tr>
<td>Death to enucleation</td>
<td>0.988 (0.982–0.995)</td>
<td>0.989 (0.981–0.996)</td>
</tr>
<tr>
<td>Enucleation to organ culture</td>
<td>0.994 (0.992–0.997)</td>
<td>0.996 (0.992–0.999)</td>
</tr>
</tbody>
</table>

PKP = penetrating keratoplasty; OR = odds ratio; CI = confidence interval; CVA = cerebrovascular accident; NS = not significant.

* Odds ratios indicate the likelihood (risk) that corneas will fall into the indicated category: ORs >1 and <1 indicate increased and decreased likelihood, respectively, of corneas being suitable or very good/excellent, although the relationship is not linear. The OR associated with a given cause of death is the risk compared with all other causes of death, but for times to enucleation and to storage ORs are the risk associated with each additional unit of time (hours).

† Not significant at the 5% level.

more than 80% of corneas from donors younger than 40 years were suitable for PKP, only 45% of corneas from donors older than 80 years met or exceeded the minimum criteria. There was an even more profound effect of donor age on the likelihood that corneas would be judged very good or excellent. Seventy-five percent of corneas from donors younger than 20 years of age were in this category compared with only 11% from donors older than 80 years.

In addition, an effect of donor age on the decline in quality during storage was apparent. The risk of unsuitable corneas from donors 80 years of age or older was no greater after >28 days of storage than after <15 days in organ culture. Corneas from younger age groups, however, all showed an increased risk of unsuitability with storage time (Table 2). This finding was supported by multifactorial regression analysis using assessment grade as the response variable, which showed a significant interaction between donor age and storage time (P = 0.0001). Figure 7 shows the change in assessment grade with storage time stratified by age: As donor age increases, the rate of decline in grade decreases. Half-times (t_{1/2}) for the decline in assessment grade were calculated from exponential decay curves fitted to the data. The t_{1/2} almost doubles from 40 to 70 days as donor age increases from <20 to 80+ years. The vertical displacement of the regression lines confirms the strong influence of donor age on assessment grade at any given time point.

Storage Time in Organ Culture. Time in organ culture influenced both the likelihood that corneas would be suitable for PKP and whether they were judged to be very good or excellent (Fig. 8). More than 80% of corneas stored for ≤2 weeks were suitable for PKP, but this declined to 57% with >4 weeks of storage. Only 23% of corneas were judged very good or excellent after 4 weeks of storage compared with 53% of corneas stored for ≤2 weeks.

It was noted, however, that corneas from older donors tended to be stored for longer periods of time than those from young donors. It can be seen from Figure 9 that of the corneas stored for ≤2 weeks, only 35% came from donors older than 60 years of age, whereas 65% of corneas stored for >4 weeks came from these older donors. Given the strong influence of donor age on endothelial grade, the extent of the changes displayed when the data are presented in unifactorial fashion, as in Figure 8, are, consequently, misleading. Odds ratios from the multifactorial logistic regression analysis do take this effect of donor age into account and confirm that longer storage times are indeed associated with increased risk of unsuitability or of not attaining very good or excellent grades; the size of the effect, however, is overestimated in Figure 8. Correcting for the change in donor age distribution allowed the true effect of storage time to be estimated. These calculations suggested that the percentage of corneas suitable for PKP declined from 83% to 66% (note 57% in Fig. 8A) after >28 days of storage and that the percentage of very good or excellent corneas declined from 53% to 34% (note 23% in Fig. 8B) during the same period.

DISCUSSION

Risk of Contamination

One of the criticisms leveled at organ culture as a method of corneal storage is the risk of microbial
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TABLE 2. Influence of Donor Age on Likelihood of Corneas Being Unsuitable for PKP After >28 Days of Storage (Baseline <15 Days of Storage)*

<table>
<thead>
<tr>
<th>Donor Age (years)</th>
<th>Odds Ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;20</td>
<td>4.63 (1.28–16.67)</td>
<td>0.019</td>
</tr>
<tr>
<td>20–39</td>
<td>6.14 (2.21–17.10)</td>
<td>0.0005</td>
</tr>
<tr>
<td>40–59</td>
<td>2.12 (1.15–3.92)</td>
<td>0.016</td>
</tr>
<tr>
<td>60–79</td>
<td>2.44 (1.53–3.89)</td>
<td>0.0002</td>
</tr>
<tr>
<td>80+</td>
<td>1.68 (0.73–3.85)</td>
<td>0.224</td>
</tr>
</tbody>
</table>

PKP = penetrating keratoplasty; CI = confidence interval.
* Odds ratios > 1 indicate increased likelihood.

contamination. Most eyes received by any eye bank carry bacteria, fungi, or both, on the ocular surface. Cleaning the eyes before excising corneoscleral discs, and the antibiotics and antifungals in the organ culture medium, help to keep losses from infection at a low level (5% in the current study). Indeed, one advantage of organ culture is that the antibiotics work much more efficiently at 34°C than in hypothermic storage solutions at 4°C. Moreover, should any organisms fail to be removed or killed either by the cleaning process or by the antibiotics, it is likely that they would be detected during the storage period by their growth in the organ culture medium. Hence, the corneas could be discarded before transplantation. This is not the case with hypothermic storage: Indeed, scleral rims of 12% to 28% of corneas transplanted after storage at 4°C are culture positive for bacteria or fungi. Thus, the risk of transplanting a contaminated cornea is much higher with hypothermic storage than with organ culture. Although the overall incidence of postoperative endophthalmitis is low, two studies have shown that it is more likely to occur when the scleral rim is culture positive, suggesting that the overall risk of endophthalmitis is lower with organ-cultured corneas. Indeed, Kloess et al, in their report on endophthalmitis after PKP, advocated the use of broad-spectrum antibiotics, and they recommended that corneas be stored "... under conditions at which the antibiotics are active, but corneas are well preserved." Currently, organ culture comes closer to this ideal than does hypothermic storage.

It has been reported that scleral rims from donors with septicemia at the time of death are more likely to be culture positive after 4°C storage as a
result, it was recommended (for hypothermic storage methods) that eyes should not be retrieved from these donors. Our finding that corneas from donors who died of septicemia were more likely to be discarded because of contamination during organ culture, presumably owing to a much higher initial microbial load, is therefore not surprising. On the other hand, when the cause of death was cerebrovascular accident or cardiovascular disease, we found that the risk of loss through contamination and artificial respiration of tissue-typed donors. The only other factor that influenced the likelihood of contamination was the death to enucleation time, with longer times associated with increased risk.

**Endothelial Assessment**

Cause of death and post mortem times, both to enucleation and to organ culture, had relatively little influence on either the suitability of corneas for PKP or on whether corneas were graded very good or excellent. Storage time had a greater influence, but our data support the contention that organ culture for up to 4 weeks is well tolerated by the corneal endothelium.

Donor age was by far the major factor affecting endothelial assessment grade, which is entirely consistent with the known decline in endothelial cell density with age. What was unexpected, however, was the apparently slower decline in endothelial quality during storage with increasing donor age. Because the initial cell density at the start of organ culture was inversely dependent on donor age, this finding could simply suggest that the number of endothelial cells declined continuously in an exponential manner—that is, the rate of cell loss at any given time was proportional to cell density. Consequently, the absolute loss of cells during storage should be inversely related to donor age, and the half-time for the exponential decline should be independent of donor age. However, the half-times for the decline in assessment grade, calculated separately for each donor age range, did not support this interpretation: Instead of remaining constant for the different age ranges, the half-times for donors older than 60 years were substantially higher than those for young donors. This implies that endothelial cells of older donors are more stable during organ culture than are those of young donors. This suggests further investigation, perhaps by monitoring actual rates of endothelial cell loss during storage.

![Figure 8](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933195/)

**FIGURE 8.** Influence of storage time in organ culture on (A) suitability of corneas for penetrating keratoplasty and on (B) whether corneas were graded very good or excellent (n values in parentheses are total numbers of corneas for each storage time). Odds ratios (95% CI) are shown for significant factors (baseline = storage time <15 days).

![Figure 9](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933195/)

**FIGURE 9.** Frequency distributions of corneas classified by donor age and stratified by storage time (n values in parentheses).
organ culture and by examining in more detail the qualitative reasons for discarding corneas.

Given the increased proportion of corneas from older donors that failed to meet the criteria for PKP, setting a maximum donor age might be considered justified purely on logistical grounds. Figure 5 shows that 33% of corneas assessed by endothelial microscopy had deficiencies that precluded PKP. If we had accepted corneas only from donors younger than 80 years of age, the percentage of discarded corneas would have been reduced by 3%, but we would have lost the 412 corneas from these older donors that actually were issued for PKP. We do not advocate setting a donor age limit provided that the corneal endothelium is examined routinely in sufficient detail to determine cell density and to identify deficiencies or abnormalities.

Deciding the suitability of corneas for PKP based on endothelial cell density relies on a property of corneas that has a direct bearing on the success of a graft both in the short term and, given the continuing postoperative decline in endothelial cell density and the accelerated cell loss during rejection episodes, in the long term. However, the precise relation between risk of graft failure (while controlling for all other relevant factors) and donor cell density has yet to be determined, and setting a minimum cell density acceptable for PKP must be arbitrary. It is clear that successful grafts may be achieved with corneas with cell densities lower than the 2200 cells/mm² minimum used in this eye bank, just as many corneas from donors older than the 60- to 65-year age limit set by some other eye banks can be grafted successfully. The difference, however, in the availability of corneas for PKP using criteria based on cell density versus donor age is considerable. If we had set an age limit of 60 years, 2488 of the 5483 corneas that we judged suitable for PKP during the study period would not have been made available for transplantation. On the other hand, some of the 725 corneas from donors younger than 60 years that we discarded because of endothelial deficiencies may well have been issued for PKP if we had used an age limit rather than endothelial criteria—but this hardly compensates for the considerable loss of suitable corneas from the older donors.

There remains, however, controversy about the use of corneas from older donors for PKP. Some studies show increased rates of postoperative endothelial cell loss with increasing donor age. Bourne et al found higher cell loss with increasing donor age 1 year after transplantation, but this effect of donor age was not apparent at 3 years. Musch et al reported that the percentage of cell loss at 1 year was lowest (that is, 16.5%) in corneas from donors younger than 25 years; the difference between corneas from donors 25 to 49 years of age and those from donors older than 50 years (cell losses of 24.7% and 27.1%, respectively) was not statistically significant. Neither of these studies provides strong justification for setting a maximum age limit for eye donors. On the other hand, the Corneal Transplant Follow-up Study in the United Kingdom, which followed the progress of 2311 grafts (92% organ cultured), did not find a significant effect of donor age on graft survival at 1 year using proportional hazards regression, which simultaneously controlled for recipient and other factors. Indeed, graft survival was independent of all donor and storage factors. We have demonstrated, albeit in a limited study, that postoperative cell loss from organ-cultured corneas using an exponential decay model, which gave a half-time for cell loss of 3.4 years (estimated cell loss at 1 year, 20%), is similar to that in corneal grafts stored for much shorter periods at 4°C. These two studies suggest that the evaluation method we use is appropriate and allows corneas from donors of all ages to be stored for extended periods by organ culture.

Key Words

corneal assessment, corneal endothelial cells, corneal organ culture, corneal storage, corneal suitability for penetrating keratoplasty

Acknowledgments

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