Banking of Donor Tissues for Descemet Stripping Automated Endothelial Keratoplasty

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Purpose: The demands for precut lamellar grafts for Descemet stripping automated endothelial keratoplasty rose in our eye bank from 74 in 2007 to 408 in 2010. To meet this expanding requirement, we explored the possibility to preserve these preparations in organ culture.

Methods: Organ cultured corneas, stored in a medium containing 6% dextran, were mounted on a Moria artificial anterior chamber, deprived of the epithelium and then cut with a microkeratome. The posterior lamella was protected by positioning the anterior stromal cap, trephined at a diameter of 8.5 mm and stored at 31°C in the medium containing dextran. The endothelium was examined with trypan blue and alizarin staining and tested for its glycolytic activity (conversion of glucose into lactate).

Results: Incubation for a period of 1 week caused a small increase in the cell loss observed after trephination (from 6.2% to 10.6%). After 2 weeks, the decrease in endothelial cell density was 19.9% but the endothelial organization remained intact. The rate of glycolysis remained unchanged during the 2 weeks of preservation, with the majority of glucose uptake accounted for by lactate production. The thickness of the lenticules remained constant, ranging from 170 to 180 μm during the preservation.

Conclusions: The lamellar grafts for Descemet stripping automated endothelial keratoplasty may be stored in organ culture for 2 weeks without damaging the endothelium or increasing the overall thickness.

Key Words: cultured corneas, DSAEK, graft tissues, glycolysis in graft tissues, thickness in graft tissues

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Endothelial keratoplasty has largely replaced full-thickness penetrating keratoplasty as the surgical treatment for corneal endothelial dysfunctions.[1,2] The progress in this procedure has resulted in a variety of donor preparations differing in thickness and residual stromal content. The most appropriate would be a stroma-free lenticule formed only by Descemet membrane and the endothelium.[3] This preparation would eliminate the donor stroma to recipient stroma interface, thus favoring rapid clarification and prompt visual rehabilitation. However, the difficulty in the preparation of the donor tissue and the delicacy of the surgical procedure required for the insertion has delayed its diffusion. Today the most used preparation is a donor tissue formed by the endothelium, Descemet membrane and a thin layer of posterior stroma [Descemet stripping automated endothelial keratoplasty (DSAEK)]. This preparation offers a satisfactory compromise between the possibility to handle the graft tissue and the necessary thickness required for a rapid visual recovery. In specialized ophthalmic centers, the donor grafts for DSAEK are prepared by the surgeon immediately before the keratoplasty. Recently, eye banks have begun to prepare precut corneas that may yield a lamellar graft for DSAEK after trephination at the desired diameter. These preparations are becoming increasingly popular because they extend the possibility to perform endothelial keratoplasty to those surgeons who do not have the necessary equipment to prepare the tissues themselves. Furthermore, they avoid last minute unsuccessful graft preparation in the operating theatre, with the resultant postponement of the surgery.[4–6] After adequate training, eye bank technicians are aptly skilled to prepare graft materials with constant and controlled properties, namely a sufficient endothelial cell density to ensure long-lasting pump function, and a thickness appropriate to avoid a large increase in corneal thickness after the insertion. Previous studies indicate that the appropriate thickness range for a graft tissue for DSAEK is 120–180 μm, but the current trend is to reduce this value.[7,8] In our bank, the preparation of graft tissues for DSAEK is actively performed using an automated microkeratome. In 4 years of activity, we delivered 983 precut lamellar grafts that were transplanted by 128 surgeons with only a single reported failure attributable to the donor tissue. To further improve the eye bank contribution, we began to explore the possibility of preserving the lamellar donor tissue for DSAEK in an organ culture medium. In this article, we present our first data demonstrating that preparations for DSAEK can be stored in culture at 31°C for 2 weeks without detriment to the endothelium.

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MATERIALS AND METHODS

Cultured Donor Corneas

In this study, we used human corneal–scleral discs unsuited for keratoplasty because of insufficient cell density or

serological reasons. All donors had a negative ophthalmic history and were aged between 25 and 75 years, with 1 exception (4-year-old donor). The corneal–scleral discs had a diameter of 16 mm, to be properly adapted to the artificial anterior chamber. After preparation, the discs were stored at 31°C in our conventional organ culture medium composed of minimum essential medium-Earle supplemented with 25 mM HEPES, 26 mM sodium bicarbonate, 5.5 mM glucose, 2 mM L-glutamine, 1 mM pyruvate, 2% (vol/vol) of newborn calf serum, 10 IU/mL of penicillin, 0.1 mg/mL of streptomycin, and 0.25 μg/mL of amphotericin. To prepare the posterior lenticules, the corneas were deswelled for 24 hours in the same medium supplemented with 6% (wt/vol) dextran 500 and mounted in the artificial anterior chamber (Moria, Antony, France). The anterior chamber pressure was set at 50 mm Hg as measured with a Schiøtz tonometer. Immediately before sectioning, the pressure was increased to 90–100 mm Hg and the system was closed.

Lenticule Preparation

The corneal epithelium was carefully removed by gentle scraping with a cellulose strip and the central thickness of the cornea was measured using an ultrasonic pachymeter (Nidek, Gamagori, Japan). A Moria microkeratome (Evolution-3), equipped with either a 300- or 350-μm-deep blade depending on the thickness of the cornea, was passed over the tissue with a targeted posterior lamella thickness of 150 μm. The cutoff for using the 2 blades was a corneal thickness of 550 μm. Because a value of 150 μm was below the sensitivity of the ultrasonic pachymeter, the final thickness of the posterior lenticule was calculated by subtracting the value of the anterior flap from that of the whole cornea. The thickness of the anterior flap was directly measured using a sensitive mechanical pachymeter obtained modifying a Mitutoyo thickness gauge, based on a spring-loaded spindle.[9] In the last part of this study, an anterior segment Fourier-domain optical coherence tomographer (OCT) became available (Casia SS-1000; Tomey GmbH, Tomey Corporation, Erlangen, Germany) allowing a further validation of our measurements.

At the end of the procedure, the anterior lamellar flap was again positioned on the stromal side of the posterior lenticule, and the endothelium was examined for mortality and cell density. The preparation was then transferred to a standard cutting block, to be punched with a trephine, endothelial side up, at the planned diameter that in our case was generally 8.5 mm. The final preparation, made by the posterior lenticule with the associated anterior cap, was immersed, endothelial side up, in a 25-mL closed bottle filled with 10 mL of the medium containing dextran and stored at 31°C. At the end of incubation, the lenticule was removed from the bottle and the endothelium was examined for the general morphology and density of viable cells. The anterior cap was separated from the posterior lenticule and discarded. The thickness of the lenticule was directly measured with the mechanical pachymeter before placing the preparation in an oven for the determination of the dry weight, to eliminate the variability due to the water content. In 20 lenticules, the analysis of the endothelium was omitted to allow the determination of the thickness in the absence of the hypotonic sucrose treatment required for the endothelial evaluation. After the removal of the lenticules, the preservation medium was carefully stirred and tested for the final volume, glucose, and lactate content.

Endothelial Cell Evaluation

The endothelial layer of corneas and lenticules was exposed to a hypotonic sucrose solution to evaluate the number of endothelial cells and their general organization. Trypan blue (0.25% wt/vol) was used to detect the nuclei of nonviable cells. The cell number was estimated at a magnification of ×100 with the help of a 10 × 10 calibrated graticule mounted on the ocular lens of the microscope (fixed-frame technique). The endothelial cell density was expressed as the mean of 5 different counts, each performed in a different endothelial region. The endothelium was then exposed for 2–3 minutes to 0.2% (wt/vol) alizarin red sodium sulfonate solution to inspect the cell borders.[10,11]

Glycolysis Evaluation

Glucose was tested in the incubation medium after phosphorylation to glucose-6-phosphate and oxidation by glucose-6-phosphate dehydrogenase and NADP. The resulting NADPH was measured at 340 nm (catalog no. 10716251035; Boehringer/R-Biopharm, Mannheim, Germany). Determination of NADH was used to measure the formation of lactate in the medium after its oxidation to pyruvate by NAD and lactate dehydrogenase (catalog no. 10139084035).

Statistical Analysis

Data are expressed as means ± SD. The change in cell density and thickness before and after the preservation for each experimental group was analyzed with the Wilcoxon signed-rank test, and experimental groups were compared with the Wilcoxon rank sum test. Statistical significance was set at $P < 0.05$. The statistical analyses were performed with SAS 9.2 (SAS Institute, Inc, Cary, NC).

RESULTS

As shown in Table 1, the corneas used in this study had a death-to-recovery interval of less than 24 hours and a death-to-preservation interval of less than 72 hours. The preparations were used within the accepted time for the maintenance of the endothelium (5 weeks).[12] To reduce the swelling caused by the culture, the corneas were incubated 24 hours in the preservation medium supplemented with 6% (wt/vol)

TABLE 1. Donor and Cornea Characteristics

	Mean ± SD
Donor age, yr (n = 59)	60.5 ± 14.7
Death-to-recovery interval, h (n = 59)	10.7 ± 5.8
Death-to-preservation interval, h (n = 59)	30.5 ± 9.8
Time of culture at 30°C, d (n = 59)	21.3 ± 7.7
Corneal thickness by pachymeter, μm (n = 46)	564.7 ± 76.2
Corneal thickness by OCT, μm (n = 46)	519.8 ± 39.0

dextran before the lenticule preparation. The measurement of corneal thickness by both the ultrasonic pachymeter or OCT demonstrated that the deswelling procedure restored the physiological value.[13]

As a preliminary control, we verified that the microkeratome-induced cornea cut in the absence of trephination, would not decrease the endothelial cell density (Table 2). Trephination without cutting was more damaging because it caused a statistically significant decrease of 6.2% ($P = 0.031$). A similar value was reported in a study of trephination-induced cell damage.[14] When the complete procedure of preparation was adopted (cutting, trephination, addition of the protective anterior cap, and incubation at 31°C for 7 days), the decrease in cell density was 10.6% ($P < 0.001$). This decrease was not statistically significant with respect to the loss detected after trephination alone ($P = 0.118$). When the incubation was prolonged for 2 weeks, the decrease in cell density was 19.9% ($P < 0.001$), an increase of 13.7% with respect to the trephination. Cell mortality was not detected. One or 2 weeks of storage at 31°C did not modify the endothelial organization (Fig. 1). Since we mainly used corneas unsuited for transplant because of insufficient endothelial cell density, areas of polymegathism were detectable either before or after the preservation of lenticules. However, the polymegathism was of a limited extent and did not increase during the storage. The effect of preservation on the thickness of lenticules is shown in Table 3. To prevent any damage to the endothelium, the initial thickness of lenticules was determined by calculating the difference between the thickness of the intact cornea and that of the excised anterior stroma. At the end of incubation, the thickness of the posterior lenticule was measured directly with a mechanical pachymeter (see Materials and Methods). Moreover, the final endothelial evaluation was omitted to avoid the hypotonic sucrose treatment normally performed in this procedure. The data indicated that the lenticules incubated in a dextran-containing medium had a minimal tendency to swell. When the hypotonic sucrose treatment was applied, a statistically significant swelling (54.8% ± 48.1%, $P = 0.003$) was detected, suggesting a rapid lenticule hydration during this brief treatment (5–10 minutes). When the OCT became available, we ran a series of tests to validate the measurement of the thickness of posterior lenticules. OCT allowed us to measure the thickness of these lenticules after the cornea was cut with the microkeratome, but before the trephination because our instrument required the tissues to remain connected to the artificial anterior chamber through its scleral ring. Twelve lenticules were prepared according to the general procedure, and their thickness was defined either by calculating the difference between the thickness of whole cornea and the excised anterior layer of stroma or with OCT. In the first case, the mean value was 144.2 ± 25.0 μm (median 137 μm, range 97–196 μm). In close agreement, the OCT tests yielded a mean value of 144.5 ± 17.0 μm (median 145 μm, range 115–174 μm).

To further explore the influence of incubation on the integrity of the endothelium, we examined the rate of glycolysis, its major energy source.[15] To this end, we made determinations of glucose uptake and lactate release in the incubation medium (Fig. 2). Because the epithelium was removed, and the stroma is known to have low metabolic activity, the observed glycolysis could be attributed almost entirely to the endothelial cells.[16] In addition, the storage of corneas in culture before the preparation of posterior lenticules was expected to make uniform their metabolic status.[17] The glucose uptake was about 1 μmol/mg dry weight per day and remained constant during the 2 weeks of incubation. The production of lactate followed the glucose uptake, also showing a linear rate. The ratio between lactate production and glucose uptake was 1.84 ± 0.63 in the first week and 1.47 ± 0.08 in the second weeek, confirming that aerobic glycolysis was the dominant metabolic pathway in the glucose utilization. These results indicated that in the conditions of temperature and glucose supply adopted during the storage, the metabolism of glucose inside the endothelial cells was regular during all the period of preservation. To further validate this result, in a separate series of experiments, we tested the glycolysis in trephined, epithelium-free corneal buttons of 8.5 mm diameter not subjected to microkeratome cutting. The glucose uptake was 27.56 ± 5.39 μmol in 7 days of incubation (n = 6). When the major part of the stroma was removed by the microkeratome cutting, the glucose uptake remained at 24.36 ± 8.31 μmol (n = 9). The difference between the 2 values was within the limits of the expected variability. In agreement, the corresponding values for lactate production were 41.02 ± 11.29 and 40.25 ± 9.15 μmol, respectively. These data confirmed the low glycolytic activity of corneal stroma and showed that the action of the microkeratome did not influence the metabolic status of the endothelium and its preservation during the 7 days of incubation.

DISCUSSION

The precut lamellar grafts for DSAEK delivered by our bank rose from 74 in 2007 to 218 in 2008, 283 in 2009, and

TABLE 2. Endothelial Cell Density in the Preparation and Storage of Lenticules for DSAEK

Treatment of Corneas	Endothelial Cell Density (Cells/mm²) (Mean ± SD)				
	Before	After	Decrease	%	*P*
Cutting (n = 29)	2255.2 ± 385.1	2255.2 ± 385.1	0 ± 0	0 ± 0	—
Trephination (n = 10)	2320.0 ± 332.7	2180.0 ± 373.6	140.0 ± 143.0	6.2 ± 6.6	0.0313
Cutting, trephination, 7-d incubation (n = 17)	2358.8 ± 412.4	2105.9 ± 417.5	252.9 ± 177.2	10.6 ± 6.8	0.0001
Cutting, trephination, 14-d incubation (n = 12)	2108.3 ± 299.9	1691.7 ± 296.8	416.7 ± 133.7	19.9 ± 7.0	0.0005

Cell mortality was not detected in all preparations.

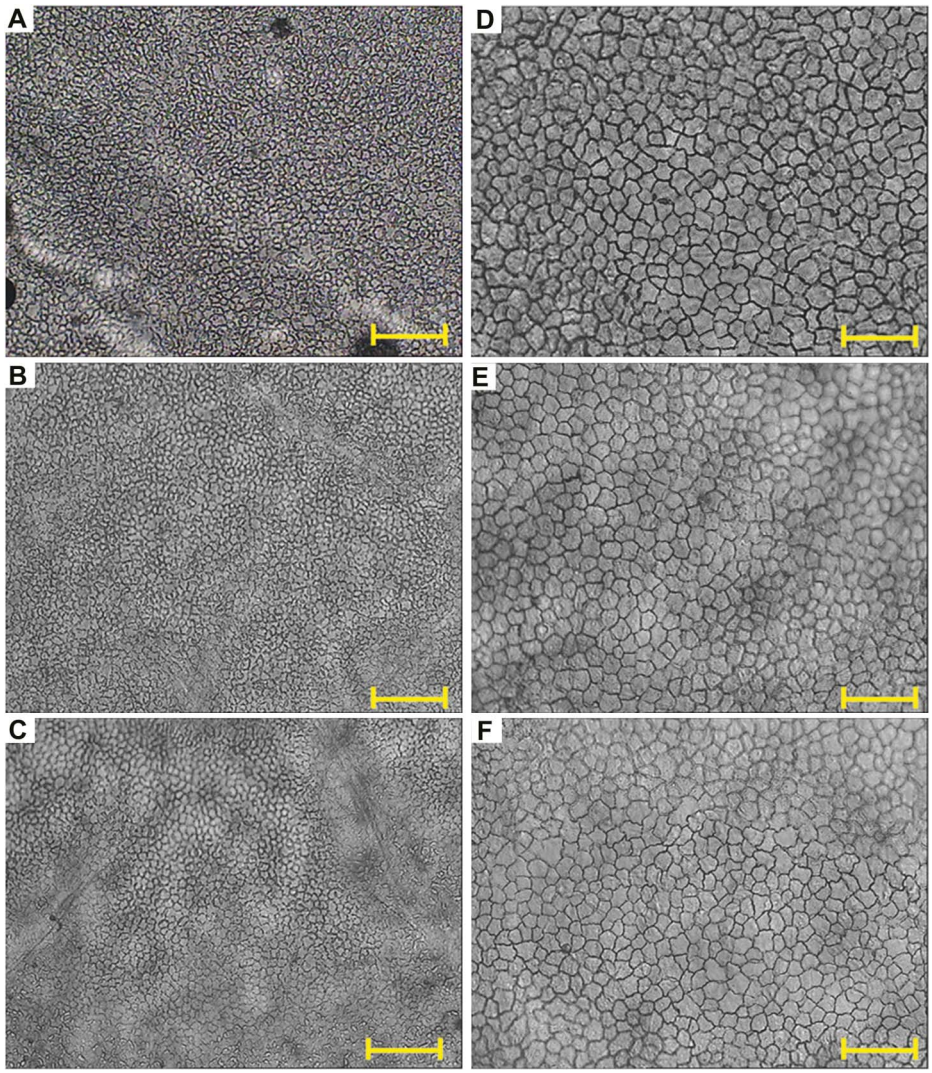

FIGURE 1. Endothelial organization in preserved lenticules. The endothelium was examined by hypotonic sucrose treatment (left side, ×100; magnification bars, 100 μm) and by 0.2% alizarin staining (right side, ×200; magnification bars, 50 μm). A and D. Lenticules before the preservation. B and E. Lenticules after 7 days of preservation. C and F. Lenticules after 14 days of preservation.

408 in 2010. At the end of 2011, the requests had been more than 500. These data illustrate the increasing popularity of this surgical procedure in the treatment of endothelial dysfunction. To meet this expanding demand, we tested the possibility of maintaining a stock of lenticules in the laboratory in culture ready to be used at any surgeon request. In addition, so as to facilitate the supply of graft tissues, we also tested whether the donor tissues could be stored as ready-to-use trephined lenticules, instead of precut corneal–scleral rims that need to be trephined in the operating theatre. With our procedure, the surgeon would only need to separate the posterior lamellar graft from the anterior protecting flap and immediately start the procedure of insertion. We choose to store our preparations at 31°C because the experience of cultured corneas indicates that this temperature is optimal for the survival of endothelium. In a previous study, donor grafts for DSAEK were preserved in hypothermic storage for 2 days.[4] Furthermore, in attempts to separate the Descemet membrane and the endothelium from the stroma by air dissection, we observed that endothelial tissue separated from the stroma by an air injection could be stored in the culture medium for 7 days.[18] Accordingly, the present data show that the trephined lamellar grafts can be stored at 31°C for 2 weeks without changes in the general endothelium organization and with only a moderate loss of endothelial cells. Confirming the proper conditions of corneal endothelium during the preservation, we observe a steady rate of glycolysis during the 2 weeks of storage. A crucial point during the preservation of lenticules for DSAEK is the need to avoid the swelling of the preparation because this event would result in excessive endothelium compression during the insertion step of the surgery, with high risk of damage. To avoid swelling, we add 6% dextran in the storage medium. As a result we have a thin lenticule (final thickness, 170–180 μm) that did not change during 7 or 14 days of preservation. We also noticed that, when dextran was removed to perform the evaluation of cell density at the end of preservation, the use of the hypotonic sucrose solution required for this test caused a prompt absorption of water that increased the lenticule thickness by almost 60% in a few minutes. Recent data show that 2 minutes of

TABLE 3. Swelling of Lenticules During Storage

Treatment of Lenticules	Thickness (μm) (Mean ± SD)				*P*
	Before	After	Increase	%	
Incubation of 7 d (n = 10)	162.8 ± 26.8	173.0 ± 30.6	10.2 ± 22.9	7.1 ± 14.7	0.1719
Incubation of 14 d (n = 10)	176.8 ± 80.3	179.0 ± 81.1	2.2 ± 32.4	2.9 ± 23.6	0.4473
Incubation of 7 d followed by hypotonic treatment (n = 13)	167.2 ± 33.3	257.3 ± 88.8	90.1 ± 80.1	54.8 ± 48.1	0.0034

To avoid endothelial damage, the thickness of the lenticules before the incubation was calculated from the difference between the thickness of the whole cornea and that of the excised anterior lamellar cap. After the storage, the thickness was directly measured with a spring-loaded thickness gauge.[9] The hypotonic treatment (for 5–10 minutes) was that of the sucrose solution used for the evaluation of endothelium.

incubation in isotonic saline medium is sufficient to cause high hydration of fragments obtained from corneal stroma.[19] Also, the swelling of corneal stroma is faster when induced through the posterior surface, likely because of the high concentration of proteoglycans bearing keratan sulfate side chains.[20–22] These results indicate that the tissue for DSAEK should never be stored in the absence of an appropriate osmotic agent in the time preceding the surgical operation.

The addition of dextran as a means to reduce the stromal edema is a common procedure in the final step of corneal culture, before the delivery to the operating theatre. In addition, 1% dextran is present in the composition of Optisol GS, a well-known cold storage medium for corneas. Although dextran is sometimes regarded as potentially dangerous for the endothelium, we did not observe any signs of dextran-induced toxic effects.[23–26] Dextran is actively taken up by the endothelial cells where part of it accumulates in the lysosomes and part is secreted into the stroma.[27] Because the amount of stroma is small in the preparation for DSAEK, it is likely that the accumulation of dextran is lower in comparison with the whole cornea.

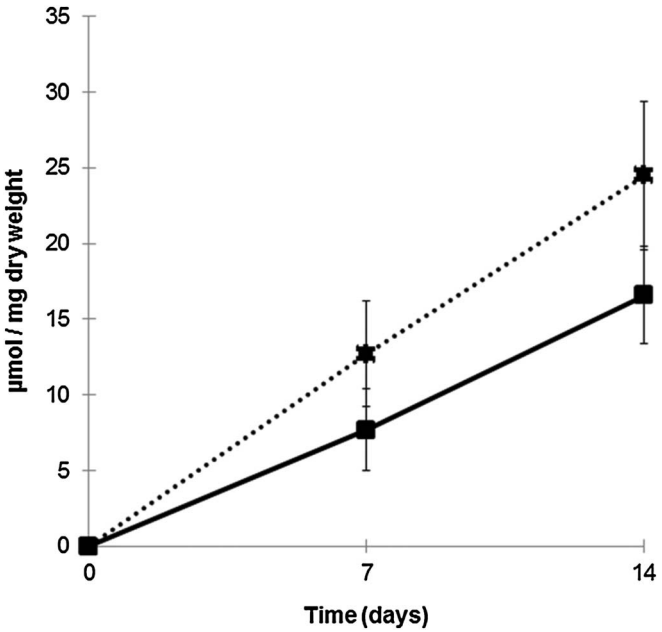

FIGURE 2. Glycolysis in preserved lenticules. Lenticules were incubated in 10 mL of a dextran-containing incubation medium for the indicated time. The initial glucose concentration was 5.5 mM. The final concentration was corrected for the slight volume reduction (10%–20%) because of evaporation. Dotted line indicates lactate production. Continuous line indicates glucose uptake. Mean ± SD of 9 (7 days) or 6 (14 days) experiments.

An interesting finding of this study, and open to further study, is the observation of a constant rate of glycolysis in the donor tissues for DSAEK. Considering this result, we would like to propose that the measurement of glucose uptake and its conversion to lactate become a method of choice to evaluate the preservation in culture of these tissues. In comparison with the traditional morphological evaluation, the rate of glycolysis is a more sensitive and complete indicator of endothelium integrity because it is not dependent on the personal evaluation of an operator and the analysis is not restricted to a small part of the monolayer surface. Clearly, the rate of glucose uptake depends on the totality of the active endothelial cells, thus giving an indication of their status. The analysis of glucose metabolism would also provide information on the availability of essential nutrients in the incubation medium, signaling the need for the appropriate additions. Conversely, the incubation of a lamellar tissue such as that prepared for DSAEK in media of a defined composition, may become a reliable procedure to study the metabolism of endothelial cells. In contrast to intact corneas, the lenticules for DSAEK are sufficiently thin to allow rapid fluxes of metabolites across the cells and are sufficiently protected from rolling over themselves by the thin layer of stroma remaining on the side of the Descemet. At variance with other preparations, such as the isolated endothelial cells, the endothelium in DSAEK maintains its physiological contacts with the basal membrane and continues to have fully operative intercellular communications.

In conclusion, our data indicate that the activity of eye banks in ocular tissue storage may extend to the lamellar preparations used in DSAEK. Our report suggests that these preparations may be stored in organ culture at 31°C for up 2 weeks without apparent damage to the endothelial integrity. Further investigations in patients receiving these preparations seem sufficiently justified to examine the fate of these preserved grafts once they have been inserted in the recipient eyes.

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