Intermediate-term corneal storage

Takashi Sakimoto, John Valenti, Motokazu Itoi, and Herbert E. Kaufman

Human and rabbit corneas were evaluated for suitability for keratoplasty donor material after storage in moist chambers or soaked in various media. A new chamber was designed which allows corneas to be stored for up to two weeks at ordinary refrigerator temperature. Corneas with scleral rims were placed in the chamber at 4° C. The epithelium faced down into the medium of modified Ringer's or physiologic saline plus Dextran-40 while the endothelium faced a moist chamber. The chamber was then placed in the refrigerator and the cornea sustained one or two weeks storage at 4° C. Corneas were evaluated by means of light and electron microscopy, specular microscopy, and penetrating keratoplasty. Of 12 grafts performed using rabbit corneas stored in this new chamber, 10 grafts were successful. Human corneas were also stored in the chamber, and compared favorably under electron microscopic observation with the mate control corneas.

Key words: corneal storage, storage chamber, moist air, keratoplasty, electron microscope, osmolarity, endothelium.

In recent years, techniques, instrumentation, and material of cornea transplantation have made giant strides. Preservation of corneal tissue, however, has remained comparatively static. Much donor tissue never reaches the surgeon because, having not been sufficiently preserved, the postmortem changes are too great for the cornea to be of value in penetrating keratoplasty.

Several methods for short-term storage of donor corneas have been published, the most widely used method being storage of the enucleated eye in a moist chamber at 4° C. Other methods include storage in homologous and autologous serum and in aqueous humor-like medium with chondroitin sulfate. Since 1965, the corneal cryopreservation technique, first developed by Capella, Kaufman, and Robbins, and Kaufman and co-workers has been used with excellent clinical results. However, because of its complexity, and the difficulty in shipping this tissue, a simpler method was sought to store corneas long enough to satisfy the needs of most eye banks.

The purpose of this study, therefore, was to develop a technique easy enough to be used by any institution desiring to store corneal tissue longer than 48 hours. The technique must lend itself to easy shipment of the preserved cornea over great
Materials and methods

Sixty New Zealand albino rabbits weighing from 2.0 to 3.5 kilograms were used in this study. The donor rabbits were killed by intravenous injections of 6 ml. of Nembutal sodium and both eyes were enucleated. Corneas with 3 mm. scleral rims were removed and washed in physiologic saline, or TC-199 media for several seconds.

Specially designed “Dual Interface Chambers” (Fig. 1, A and B) were constructed in our instrument shop of plexiglass or Teflon. This is a three-piece chamber consisting of a well, a retaining rim, and a screw cap. The well holds approximately 15 ml. of the preserving medium. Chambers were first made of plexiglass, but problems were encountered with sterilization. The chamber is now made of Teflon which can easily withstand the high temperature of the autoclave.

After washing and a thorough draining, the cornea was placed epithelium down into the medium while the endothelium faced a moist chamber. The retaining rim was placed over the cornea in contact with the scleral rim. The cap was then screwed tightly in place and the chamber was placed in the refrigerator at 4° C. These corneas were examined by light and electron microscopy after 1, 3, 5, 7, 10, and 14 days of storage.

For convenience and ease of multiple preservations most work was carried out in the chamber illustrated in Fig. 1, C which is based on the same principle. Up to eight small shell vials nearly filled (6 ml.) with medium were placed over a piece of wet gauze in the beaker. The corneas were placed epithelium down onto the medium, and the sealed beaker was stored at 4° C. Four different media were used including normal saline with 5 or 10 per cent Dextran-40 (from Sigma Chemical Company, St. Louis, Mo.) and modified Krebs-Ringer’s solution* buffered with Tris. Modified Krebs-Ringer’s solution was subsequently used without glutathione, adenosine, and glucose (MKR). Osmotic pressure of the preserving solutions were standardized at 306 to 309 milliosmoles. The pH of the solutions was adjusted to 7.4.

Twenty-five human corneas which were rejected by the eye bank for reasons of extended postmortem time or age of the donor were also studied. When a pair of human eyes was received, one cornea was placed in the chamber at 4° C for one week and the mate cornea was fixed immediately for electron microscopy to serve as a control.

Several preliminary preservation studies were also done storing whole eyes in moist chambers, storing cornea-scleral tissue in moist chambers,
Fig. 2. Flat preparations of rabbit corneal endothelium stored for seven days (A) and 14 days (B) in the "Dual Interface Chamber." Stained with NADH oxidoreductase (×77, original).

and soaking corneas in the same medium used in the "Dual Interface Chambers." Corneas were stored for one week in the refrigerator.

Twelve corneal grafts, 6.5 or 8 mm., were performed in rabbit with corneas preserved in this chamber with MKR plus 5 or 10 per cent Dextran-40. The preserved cornea was submerged and washed for several seconds in sterile physiologic saline. The trephine and a cornea punch, as reported by Polack and Capella, 8 were used to cut the preserved corneal button which was immediately transplanted to the host.

Flat preparations were made with enzyme stains to give an indication of both metabolic and morphologic normality. A solution of 5 mg. nitroblue tetrazolium, 10 mg. phosphopyridine nucleotide reduced form, and 5 ml. phosphate buffer was made for NADH oxidoreductase staining. 6, 9 Corneas were incubated in this solution, fixed with glutaraldehyde, and mounted on a glass slide for light microscopy.

For electron microscopy, corneas were fixed in glutaraldehyde and post-fixed in osmium buffered with Millonig. The tissue was then dehydrated in ethanol and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate, and observed with the Zeiss EM 9S2 electron microscope.

After storage in this new chamber some corneas were mounted in the perfusion chamber of the specular microscope and perfused with modified Krebs-Ringer's solution with reduced glutathione and adenosine at 34° C. and corneal thickness measurements were recorded.

Results

Rabbit corneas stored in the "Dual Interface Chamber" remained clear throughout the entire preservation time, although slight folds were noticed on the endothelial side. The thickness of these corneas increased approximately 40 per cent after two weeks preservation.

Flat preparation of endothelial cells on the seventh day revealed nuclei and cytoplasm stained slightly more than normal, however, no severe degeneration was seen (Fig. 2, A). By the fourteenth day (Fig. 2, B) the nuclei of the endothelium had changed shape, as had the cytoplasm which stained easily for NADH oxidoreductase. No significant differences in preservation could be found by flat preparation as well as electron microscopy between corneas washed in TC-199 or saline before preservation. Also, no electron microscopic differences were noticed between rabbit corneas preserved with MKR and those preserved with saline.

The first ultrastructural changes due to preservation of these rabbit corneas were observed in the basal epithelial cells. The disappearance of mitochondrial cristae and...
enlargement of rough-surfaced endoplasmic reticulum were observed by the fifth day. From the fifth day to the fourteenth day there was little additional change in the epithelium. The basement membrane remained normal (Fig. 3).

From the first day of preservation, a characteristic cleft was seen not only in the epithelium but also in the keratocytes and endothelium. These clefts were usually found near the nucleus and endoplasmic reticulum but their formation seemed to bear no relationship to the above-mentioned cell component degeneration (Fig. 4).

The features of the endothelial cells which were facing a moist chamber during the preservation period were slightly different from those of the epithelium or stroma. Few mitochondrial changes were observed in the endothelium after one week of preservation (Fig. 5), and these did not increase even up to 14 days (Fig. 6).

A characteristic cleft which was detected in the other layers of the rabbit cornea was also observed in the endothelial cells, even after the shortest time of storage. They gradually became more evident with time. One week after preservation most endothelial cells had one or two clefts which were straight or crescent in shape (Fig. 5), and approximately two microns in length, parallel to the surface of the endothelial cells. These characteristic clefts were often observed so close to the membranes of the endoplasmic reticulum or the vacuoles that it appeared as if they were limiting membranes of the clefts (Fig. 7). The clefts, however, were also observed without the membrane structure.

In general, there were few ultrastructural changes in the endothelial nuclei at the shorter times of storage. After 10 days of preservation, nuclear chromatin was clumped. On the fourteenth day of preservation, however, many alterations could be seen such as nuclear elongation and ir-
Fig. 4. The superficial epithelial layers of a rabbit cornea preserved 14 days in the "Dual Interface Chamber." Large clefts (*) near the nuclei (Nu) can be seen. Intercellular spaces are widened and there are many interdigitating processes (arrow) (x6,100).

Fig. 5. Endothelium of a rabbit cornea preserved in the "Dual Interface Chamber" for seven days shows minor changes, i.e., a few small vacuoles and clefts (*). Mitochondria and endoplasmic reticulum remain normal (x9,400).

Electron microscopic results of corneas stored for one week by other methods. 
(1) Whole eye in moist chamber: good epithelium, swollen stroma, and very poor endothelium (very irregular cell membranes, abnormal nuclei, and vacuolized mitochondria). 
(2) Cornea-scleral tissue in moist chamber: very good epithelium, swollen stroma, and nearly normal endothelium. 
(3) Corneo-scleral tissue soaked in MKR plus 5 per cent Dextran-40: good epithelium, normal thickness, and poor regularity, enlargement of intercellular spaces, and abnormal spaces between Descemet's membrane and the endothelial cells in which a granular substance was observed (Fig. 6). This may indicate a limitation of storage time at about 14 days.
Fig. 6. Endothelium of a rabbit cornea preserved 14 days in the "Dual Interface Chamber." In spite of vacuole (V) and cleft (*) formation the mitochondria and endoplasmic reticulum remain normal. A granular substance (arrows) can be seen between the endothelium and Descemet's membrane (DM) (x6,400).

Fig. 7. Endothelium of a 14-day preserved cornea in the "Dual Interface Chamber." A large cleft (*) is parallel to smooth-surfaced endoplasmic reticulum (sER). A membrane-like structure (arrows) can be seen in the cleft. Mitochondria (Mi) and rough-surfaced endoplasmic reticulum (rER), however, remained normal (x32,000).

endothelium (broken cell membranes, irregular nuclei, and distended endoplasmic reticulum).

Of the twelve rabbit grafts (6.5 or 8 mm. in diameter) performed using corneas preserved in the chamber with MKR plus 5 or 10 per cent Dextran-40 only two failed (Table 1). Poor wound apposition was seen along with heavy scarring and heavy neovascularization. The remaining 10 corneas were clear with Descemet's folds disappearing between the second and third day after surgery. Under the electron microscope there were very few changes in the endothelium after only three postoperative days (Fig. 8). An increased number of rough-surfaced endoplasmic reticulum and free ribosomes gave the
Fig. 8. Rabbit endothelium with 10 days preservation in the "Dual Interface Chamber" and three days after penetrating keratoplasty. An increased number of rough-surfaced endoplasmic reticulum along with repopulation by other cell organelles account for the higher electron density. The cells appear nearly normal (x26,000).

Table I. Rabbit penetrating keratoplasty results. Twelve corneas were grafted after 7, 10, or 14 days preservation in the "Dual Interface Chamber." The corneas were examined light and electron microscopically at three days, seven days, one month, and six months postoperatively. The numbers in parentheses indicate failed grafts, i.e., out of 12 grafts two failed.

<table>
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<tr>
<th>Days of preservation</th>
<th>Postoperative time (Corneas removed and examined)</th>
<th>3 days</th>
<th>7 days</th>
<th>1 month</th>
<th>6 months</th>
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<td>7</td>
<td>2 (1)</td>
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<td>10</td>
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endothelium a higher electron density. Seven days after surgery mitochondria and endoplasmic reticulum of the endothelium were highly developed. Fragments of keratocytes could be seen in the anterior stroma even one month after surgery. These were the last abnormal features which could be seen. After three months all layers were normal except for the wound area, and the grafts were perfectly clear.

As illustrated in Fig. 9, a seven-day preserved rabbit cornea undergoes temperature reversal. Thickness measurements were made over a 10 hour period. After a brief thickening the corneas gradually became thinner. Ultrastructure of this rabbit endothelium after 10 hours perfusion appears nearly normal.

Good results were obtained with human corneas preserved for one week in the "Dual Interface Chamber." Fig. 10 shows the endothelium of one pair of human corneas at 14 hours postmortem. Fig. 10, A is the control cornea which was fixed immediately after receipt; Fig. 10, B is the mate cornea after one week of preservation.
Fig. 10. A, Human cornea with 14 hours postmortem time was fixed for electron microscopy immediately upon receipt to serve as a control. All mitochondria (Mi) are swollen and their cristae are almost completely absent (x19,000). B, Mate of above human cornea preserved in the "Dual Interface Chamber" for one week. The mitochondria are often small and dense (arrows). Vacuoles (V) are also seen. Cell membranes and junctions look normal. Nu = Nucleus (x19,000.)

The vacuoles of the mitochondria of the control cornea have almost disappeared in the preserved cornea. Intercellular spaces and nuclei of the preserved cornea remain in good condition. Characteristic clefts, seen previously in the preserved rabbit corneas, are also found in human corneas.

Discussion

Although various techniques for preserving corneal tissue for a short-term period have been described by several investigators,3-6 none have become widely used. Some reasons may be the unwillingness to use the highly nutritive media of most techniques and the complexity of others.

This paper described a preservation method which, because of its simplicity and easy availability of material, could very well find wide acceptance in the human cornea preservation field.

The results (good epithelium, swollen stroma, and very poor endothelium) achieved with corneas of whole rabbit eyes stored in moist chambers led to the second step, isolating corneas with scleral rims in a moist chamber. It was reasoned that if the epithelium could fare so well facing a moist chamber that the endothelium might also benefit. As it turned out, the endothelium did indeed store well facing only moist air. Its condition was far superior to corneal endothelium of whole eyes stored under eye bank conditions. Stromal swelling, however, remained a problem.

In order to minimize hydration, which
might permit the endothelium to detach, it was decided to soak the corneas in a hyperosmotic preservation solution. Modified Krebs-Ringer's solution plus 5 and 10 per cent Dextran-40 were selected. After a week at 4° C. the corneas remained clear and thin. The endothelium, however, was in poor condition with broken cell membranes, irregular nuclei, and distended endoplasmic reticulum. It appeared as if the high colloid osmotic pressure provided by the Dextran-40 was more than the delicate endothelial layer could withstand. The epithelium, though, remained in good condition.

With the knowledge that the endothelium maintained its integrity facing moist air and that the Ringer's with Dextran-40 prevented stromal swelling, the new preservation chamber came into being.

At first, nothing more than a 20 ml. beaker with 1 ml. of MKR plus Dextran-40 in the bottom, it was developed into the chamber in Fig. 1, B. The cornea was placed epithelium down while the endothelium faced the moist air. The results, as demonstrated, were excellent. Further experiments revealed that glucose, glutathione, and adenosine were not necessary for preservation in this new chamber. Thus, these ingredients were eliminated possibly reducing the risk of contamination. Additionally, in the preservation of rabbit corneas, normal saline could be substituted for MKR as the preservation medium with equal results, further reducing the complexity of the medium. In fact, during the one year of the study no gross contamination was ever seen in the media or in any of the corneas preserved. It must be pointed out that the chamber illustrated in Figs. 1, A and 1, B is specifically designed for shipment of the cornea during preservation. The preservation technique is quite simple as demonstrated with the 1,000 ml. beaker in Fig. 1, C. Corneal thickness is maintained through the epithelial surface while endothelial integrity is preserved in the moist chamber.

However, because the endothelial cells are not in direct contact with the preserving medium, factors affecting preservation such as pH, osmolarity, and high polymer and ion concentrations, are not critical to the success of preservation. Thus, more consistent results should be obtained with this preservation technique.

The results of our rabbit grafts were quite good as indicated in Table I. Better results could not be expected even with fresh tissue. Even the clefts seen in preserved tissue disappear after transplantation as seen in Fig. 8.

The temperature-reversal study also lends support to the functional condition of the endothelium. Although the curve in Fig. 9 is not the ideal for a perfectly pumping endothelium, it indicates that the cornea is still capable of temperature reversal.

Rabbit corneas with similar morphology survived well after transplant. It is, however, well known that rabbit corneas are much easier to work with, in this respect, than human corneas. The preliminary in vitro studies with human corneas reported here suggest that they can also be preserved in good condition with this technique. Thus, the final test must be human penetrating keratoplasty with corneas preserved with this new technique.

There has been an emotional attachment to the idea that “fresh” corneal tissue is somehow superior to “preserved” corneal tissue. In fact, we almost never use “fresh” tissue but rather tissue refrigerated with its endothelium bathed in the stale aqueous which is subjected to the products of necrosis and no longer kept nutritious by active circulation. This work, as well as others, suggests that the prompt removal of the cornea from this stale aqueous solution and its placement in a proper preservative solution may provide the cornea with a healthier environment for whatever time is required for the tissue, the patient, and the surgeon to make appropriate contact in the operating room as well as permitting longer tissue survival and more optimal scheduling of surgery.

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REFERENCES