Improved corneal storage

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The corneas of New Zealand albino rabbits were isolated with a sclero-corneal rim for storage in a modified tissue culture medium (M-K medium) at 4° C. The corneas were either mounted in the specular microscope for daily observation of the endothelial layer and corneal thickness while being bathed in the storage medium at 4° C., or sealed in vials with the storage media. The vials were stored at 4° C. for 5, 9, and 14 days at the end of which the corneas were mounted in the specular microscope and the temperature was reversed for an endothelial viability test. Along with specular micrographs of the endothelial layer and corneal-thickness measurements, electron microscopy was performed to evaluate the viability of the endothelial cells. The results have indicated that a viable endothelium is present in rabbit corneas stored for up to 14 days in the M-K medium. The corneal storage technique requires no special equipment, utilizes readily available storage media components, and is easily executed with little chance of technician error. It not only permits prolonged storage, but it is superior to the stagnant aqueous of the enucleated eye.

Key words: eye bank storage, cornea, endothelium, viability, rabbit, tissue culture media, specular microscope, temperature reversal, ultrastructure.

Eye banks store donor corneas as enucleated eyes in a 4° C. moist chamber with its stagnant aqueous humor for not longer than 48 hours. The endothelium is exposed to aqueous which has products from metabolic wastes and necrosis of tissue. In 1965, Capella, Kaufman, and Robbins made a major step forward with a technique for long-term preservation of viable cryopreserved corneas. In this way, corneas were successfully transplanted even after 422 days of storage in liquid nitrogen. Yet, cryopreservation requires special equipment, a trained technician, and donor tissue, which is less than six hours postmortem. In 1963, Stocker, Levenson, and Georgiade stored rabbit corneas in rabbit serum at 4° C. for two weeks with excellent endothelial cell-culture growth and for three weeks with successful corneal grafts. A clinical evaluation by Stocker in 1968, of the serum-stored human corneas has resulted in successful penetrating keratoplasties even after 101 hours of storage. The procedure has the great inconvenience of requiring the recipient's serum instead of homologous serum in order to avoid immune reactions, hepatitis, etc., and the sterile handling of this makes the technique inconvenient.
Fig. 1. A typical series of photographs taken with the specular microscope of a rabbit corneal endothelium while being bathed in the M-K medium. Cell integrity was maintained throughout the eleven-day storage period.

Both better and safer storage of eye bank corneas for one week would greatly increase the amount and quality of donor tissue available to the surgeon and the convenience of scheduling corneal transplants. It would be most useful if it required no special equipment, was easily executed with little chance of technician error, and kept corneal tissue viable for one week. It is the purpose of this paper to report on the laboratory results of a technique which satisfies these requirements.

Methods

New Zealand white rabbits (2 to 3 kilograms) were killed with sodium pentobarbital and the eyes enucleated. Neosporin was flooded over the eyes for five minutes. Afterwards, the cornea with a 2 to 3 mm. scleral rim was isolated from the rest of the eye and handled according to the experimental design to be described below.

Modified tissue culture medium (M-K medium). Many storage media were evaluated in preliminary studies, but only the most successful medium will be discussed in this paper. A mixture of Medium-199 (Microbiological Associates Inc.), 5 per cent dextran (Sigma Chemical Co., dextran 40), and 100 units per milliliter of a streptomycin and penicillin mixture (Microbiological Associates Inc.) was the final choice. The medium had an osmolarity of 290 mOsm. and pH 7.4.

The mixture can be made by adding 5 gm. of dextran to 100 ml. of Medium-199, or else prepare a 10 per cent dextran solution and add a unit volume of Medium-199 which has a two times normal concentration. The Medium-199 is available with or without the neutral red indicator.

Daily evaluation of stored corneas: thickness and endothelial appearance. The storage medium was evaluated according to its ability to maintain a relatively normal corneal thickness and an intact endothelial layer during storage for up to two weeks. For observation experiments, isolated rabbit corneas were mounted in the specular microscope perfusion chamber which was located in a cold room (4° C.). An intravenous perfusion bottle system was used to deliver, with a positive pressure of 10 to 15 mm. Hg, the M-K medium to the endothelial surface of the isolated cornea. The epithelial surface of the cornea was bathed with the medium and sealed with a glass slide to prevent evaporation. A few milliliters of fresh M-K medium were flushed across both endothelial and epithelial surfaces each day of the observation-storage period in the specular microscope. Photographs of the endothelial layer along with corneal thickness measurements were recorded daily.

At the termination of the storage period, the corneas were removed from the specular microscope and fixed with 2 per cent glutaraldehyde in a phosphate buffer. Then they were postfixed with 1 per cent osmium tetroxide in a phosphate buffer and embedded in Epon 812 for electron microscopy.

Temperature reversal viability test after storage. Paired rabbit corneas with their scleral rims were isolated aseptically and stored for 5, 9, and 14 days at 4° C. in sterile 20 c.c. screw cap glass bottles containing the M-K medium. For each of the selected storage periods, three pairs of corneas were evaluated. After the storage period, one cornea from a pair was incubated to study temperature reversal and the other cornea was processed for histology as the stored control cornea. A perfusion chamber for the specular microscope was used to hold the temperature reversing corneas so that a constant rate perfusion pump could deliver a glutathione-bicarbonate Ringer solution at the rate of 60 ml per minute to the endothelial surface. A water-bath system kept these corneas at 34° C. The corneal thickness measurements and endothelial cell-layer photographs were recorded. At the termination of each perfusion study, the temperature-reversed corneas and their paired control corneas were processed for electron microscopy. The paired control corneas enabled evaluation of the corneas immediately after removal from the M-K medium (4° C.).

Results

Daily evaluation of stored corneas: thickness and endothelial appearance. Rab-
Fig. 2. The daily change in corneal thickness of the cornea studied in Fig. 1 reveals a gradual, but not excessive, corneal swelling.

Fig. 3. The same rabbit cornea which was held in the specular microscope (Fig. 1) was fixed with glutaraldehyde after eleven days of storage in the M-K medium. The endothelial cells had slightly wavy posterior surfaces and a normal-looking ultrastructure.

Bit corneas mounted in the specular microscope chamber could be stored in the M-K medium for about two weeks without loss of endothelial cell integrity. A series of daily specular micrographs from the same cornea (Fig. 1) revealed an intact cell pattern for eleven days. The corresponding corneal thickness (Fig. 2) of the cornea illustrated in Fig. 1, shows a gradual increase in thickness throughout the eleven-day period. The endothelial cell pattern (Fig. 1) remained constant for the first five days while the corneal thickness (Fig. 2) increased from 0.375 mm to 0.420 mm. During the sixth, seventh, and eighth days the cell pattern was less distinct because of the slight posterior stromal swelling, but cellular integrity appeared excellent. The thickness during this period was constant at 0.430 mm. The
Fig. 4. Typical temperature reversal of a rabbit cornea stored for five days in a vial of the M-K medium. Superimposed on the graph are the corresponding specular micrographs of the endothelium.

Fig. 5. Typical temperature reversal of the rabbit cornea stored for nine days in a vial of the M-K medium. Superimposed on the graph are the corresponding specular micrographs of the endothelium.

next three days revealed what might be interpreted from the specular micrographs as a swelling of the endothelial cells or an unevenness of the cell surfaces. The endothelial cell ultrastructure (Fig. 3) from the same cornea illustrated in Fig. 1, reveals that the posterior surface was unusually wavy. There were only slight changes in the mitochondria and a few vacuoles within the cytoplasm. Otherwise, the cell structure was normal.

Temperature reversal viability test after
storage. Rabbit corneas which were stored in the M-K medium had successful temperature reversals after 5, 9, and 14 days of storage. Fig. 4 shows the temperature reversal effect on the thickness of a cornea stored for five days. Superimposed on the graph are the corresponding specular photomicrographs of the endothelium. Fig. 5 contains the results from a nine-day stored cornea and Fig. 6 from a 14-day stored cornea. A comparison of the first two hours on the graphs (Figs. 4, 5, and 6) will illustrate that with each storage period there is a progressively longer initial delay before reversing in thickness. After the initial delay, the thickness thinning rates are 0.010 mm. per hour for the five-day corneas, 0.010 mm. per hour for the nine-day corneas, and 0.015 mm. per hour for the 14-day corneas.

The endothelial cell ultrastructure of the same corneas from Figs. 4, 5, and 6 are illustrated, respectively, in Figs. 7 through 9. The structural change between the temperature-reversed corneas and their paired stored control corneas were consistent for each of the storage periods. In the control corneas, the posterior surface of the endothelial cells was wavy but intact. The cytoplasm, nucleus, and endoplasmic reticulum appeared normal, but there were some vacuoles and edematous mitochondria. After temperature reversal, these ultrastructural changes were reversed, resulting in completely normal endothelial ultrastructure. The endothelial cells of corneas stored 5, 9, or 14 days are almost indistinguishable when the ultrastructure is evaluated.

Discussion
Corneas mounted with a clamp and stored in the specular microscope for many days are in a less advantageous environment than those corneas stored in bottles. Thus, the duration of storage was shorter. In order to keep the spherical shape of the cornea in the microscope chamber a positive pressure by the perfusion bottle was constantly forcing water against the endothelium and presumably into the stroma, causing an unwanted stress and probably the gradual increase in corneal thickness. Also, the nature of the specular microscope technique results in a non-sterile environment for the cornea, but the specular microscope provided an easy method of evaluating the condition of the endothelial layer by observing it on a daily basis while the corneas were being stored.

Temperature-reversal studies are considered to be a functional test for the
viability of the corneal endothelium. Corneal hydration is in a dynamic balance between the constant imbibition pressure, i.e., swelling pressure, of the stromal mucopolysaccharides and the metabolically active transport system, which is located in the endothelium. When the metabolism is reduced at 4°C, the cornea will swell. If a viable endothelium is present, the corneal thickness will reverse as the corneal temperature and metabolism are returned to normal. This viability test requires the tissue to function in vitro for six to ten hours without the aid of its natural environment, i.e., aqueous humor. It is a more rigorous viability test than the corneal transplant in that it is not dependent on the possible migration of endothelial cells from the recipient to the donor button.

The lag period illustrated in these experiments, i.e., the slight swelling and plateau before temperature reversal, might reflect diffusion of the dextran into endothelial cells and stroma. This is possible but unlikely since the dextran molecule is large, 40,000 molecular weight.

The M-K media-stored corneas studied had temperature-reversal thinning rates of 0.010 to 0.015 mm. per hour which were
Improved corneal storage

Fig. 8. Pairs of rabbit corneas were stored for nine days in vials of the M-K medium. After the storage period, one cornea (A) was fixed for electron microscopy and the other cornea (B) was temperature reversed (see Fig. 5) before being fixed.

less than those of the controls reported by McCarey, Edelhauser, and Van Horn\(^8\) (0.021 mm. per hour) and Sherrard\(^11\) (0.025 mm. per hour). The reason for the difference in thinning rates can be related to the amount of corneal swelling prior to the temperature reversals. The corneas stored in moist chambers are free to imbibe water whereas the corneas in the M-K media were inhibited from swelling because of the colloidal osmotic pressure of dextran. Before temperature reversing, the M-K media-stored corneas had a control baseline thickness of 0.420 mm. and a swelling of 0.040 mm. after five days of storage, 0.080 mm. after nine days, and 0.200 mm. after 14 days. Whereas, the moist chamber-stored corneas which Sherrard\(^11\) reported had a baseline thickness of 0.384 mm. and a swelling of 0.168 mm. after 18 hours of storage. Thus, the endothelial pump stimulation and pumping rate was less for the M-K media corneas than for the moist-chamber corneas. It can be noted that the corneas after 14 days of storage had a low thinning rate relative to the amount of swelling, but this can be expected after such a long storage duration. Temperature reversal has proved that a viable endothelium does exist in
rabbit corneas stored for up to 14 days in an M-K medium. This is further reinforced by the intact specular micrographs of the endothelium and the normal appearing ultrastructure.

The storage technique is not solely aimed at extending the donor eye storage time from 48 hours to seven days, but rather to introduce an improved method of storing all donor eyes even if the cornea is to be transplanted within 48 hours. The cornea of an intact enucleated eye which is stored in a moist chamber at 4° C. is being gradually poisoned. The concept behind this statement may be called "the aqueous sewer." This term refers to the gradual change in ionic composition from the build-up of waste products in the aqueous humor. The anterior chamber is a 0.28 ml. stagnant pool of fluid which is bordered by the iris, lens, and corneal endothelium. Any metabolic waste products produced, between the time of donor death and the time of the donor eye equilibration with 4° C., will remain in the aqueous humor. This lag time can be anything between one hour and ten hours or more, during which time the donor eye tissue

Fig. 9. Pairs of rabbit corneas were stored for 14 days in vials of the M-K medium. After the storage period, one cornea (A) was fixed for electron microscopy and the other cornea (B) was temperature reversed (see Fig. 6) before being fixed.
is continuing some metabolic processes. Thus, the advantage of the storage technique described in this paper is to isolate the cornea from the eye and bathe it in a large volume of fresh media.

The storage technique described is both practical and requires no special equipment. The M-K medium components are readily accessible and contain no toxic agents. The dextran is present to osmotically restrict excess water from swelling the cornea during storage. The required temperature is 4° C. which can be easily attained by a refrigerator or a Styrofoam chest with ice.

Although rabbit cornea can be stored intact for 14 days. Preliminary work with human corneas, however, is very encouraging. The final objective of this work is to store viable human corneas for one week.

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REFERENCES