Corneal epithelial changes during midterm storage*

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The increased use of donor epithelium in keratoplasty for severe corneal disease has prompted an investigation of epithelial viability during midterm storage. Glycogen, ATP, and ADP have been used as indicators of the metabolic state of epithelial cells. These substances have been measured after conventional moist-chamber storage at 4° C. for 1, 2, and 12 days and after immersion at 4° C. in McCarey-Kaufman medium for 1, 2, and 12 days. In addition, recovery of glycogen and ATP stores has been evaluated after warming of the tissue in the storage solution to 37° C. The evidence indicates that the corneal epithelium may recover these biologically important substances, despite depletion secondary to delayed cooling of donor eyes.

A prospective clinical study has been initiated at our institution to determine the short- and long-term effects of retaining donor epithelium on the healing, clarity, and rejection rate of human corneal transplants. Our preliminary observations seem to support the concept that retention of donor epithelium may be of value in avoiding prolonged epithelial defects and subsequent ulceration, particularly in severely damaged corneas. These impressions are reinforced by similar observations reported by Stuart Brown and have continued to stimulate our interest in preservation of the epithelial layer of donor corneas. It became apparent to us that the donor corneal epithelium was frequently nonviable when the tissue was used, and disintegrated either at the time of operation or was sloughed off the donor button two or three days after operation.

Previous work has indicated that donor tissue sustained a prompt loss of epithelial glycogen and adenosine triphosphate as well as rapid increase in hydration after death, unless cooling was accomplished within approximately one to two hours. In view of the sporadic nature of most corneal donations, prompt cooling of the tissue is difficult to achieve.

Results relating to the viability of donor corneal endothelium during prolonged storage indicate that the McCarey-Kaufman Corneal Bathing Medium (M-K medium) is quite effective in preserving the functional viability of endothelial cells. The
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Fig. 1. Rabbit corneal epithelial glycogen after remaining "in situ" or after moist chamber storage. Glycogen is expressed as micromoles of glucose per gram of dry weight ± the standard errors of the mean with the number of measurements in parentheses.

The purpose of the work presented here was to investigate the effect of storage in M-K medium on epithelial viability using glycogen and adenosine triphosphate levels as indicators.

Methods

Tissue preparation. Rabbits weighing between 2 and 3 kilograms were killed by an overdose of pentobarbital and the eyes were treated in one of the following four ways.

Moist chamber storage. Eyes were enucleated immediately after death and stored individually with antibiotics in a moist chamber at 4°C for 5, 18, 24, or 48 hours, and 12 days in the usual method of eye bank storage. Afterward, in some cases, the epithelium was scraped off, frozen in liquid nitrogen, weighed, dried, and then reweighed. From these figures, water content and hydration (milligrams of water per milligram of dry weight) were calculated. The glycogen content was then measured as described below. In other cases, in order to preserve the adenosine triphosphate levels in the tissue, the entire eye-ball was frozen with a second molded block which had been previously chilled in liquid nitrogen. The frozen tissue was then lyophilized and the dried epithelium scraped off as a powder, after which either glycogen or adenosine phosphate levels were measured.

In situ. The rabbits were killed, the lids were closed, and the animals were left at room temperature for 1, 2, 5, or 18 hours, after which the tissues were prepared as described above.

Midterm storage. The cornea with a 2 mm. scleral rim was excised and suspended in 20 ml. M-K medium and stored at 4°C for 1, 2, and 12 days. After storage, the cornea with scleral rim was removed from the medium, rinsed in three changes of normal saline (0.9 per cent NaCl), placed on a metal block molded to the shape of the cornea, and frozen with a second molded block which had been previously chilled in liquid nitrogen. The frozen tissue was then lyophilized and the dried epithelium scraped off as a powder, after which either glycogen or adenosine phosphate levels were measured.

Incubation in M-K medium. After the eyes had been left in situ for five hours at room temperature, the cornea with a 2 mm. scleral rim was excised and suspended in 20 ml. M-K medium, which was then put in a 37°C water bath and 5
IN MOIST CHAMBER
IN SITU

Fig. 2. Rabbit corneal epithelial adenosine triphosphate (ATP) levels after remaining "in situ" or after moist chamber storage. ATP is expressed as micromoles per gram of dry weight ± the standard errors of the mean with the number of determinations in parentheses.

per cent Co-95 per cent O2 was bubbled into it for four hours. Thereafter, the tissue was removed from the medium, rinsed, and prepared as described above and metabolite levels were measured.

Human corneas no longer considered suitable for transplantation were provided by the Massachusetts Eye Bank. These eyes had been stored in a moist chamber at 4° C. for several hours. After the eyes were received from the eye bank, the tissues were prepared and analyzed as described for the rabbit tissue.

Metabolite analysis. Glycogen was determined in the epithelium after extraction in 1.0 ml boiling 20 per cent sodium hydroxide. Precipitation twice in iced 3 ml ethanol was followed by 45-minute centrifugation at 8,000 g, drying, and then 90 minutes hydrolysis in 2 N sulfuric acid at 100° C. After neutralization, glucose was measured with the hexokinase reaction and glycogen levels are expressed in glucose units.

Adenosine triphosphate (ATP) was measured using the firefly luminescence test and adenosine diphosphate (ADP) was measured by enzymatic optical tests after extraction in 0.5 N perchloric acid and neutralization.

Results

Moist chamber storage of rabbit corneal epithelium at 4° C. results in only a slight reduction of epithelial glycogen (Fig. 1) or ATP (Fig. 2) in 48 hours. If the corneas were left in situ, ATP and epithelial glycogen were dramatically reduced to less than one-sixth normal within 5 hours (Figs. 1 and 2).

Storage at 4° C. in M-K medium maintained epithelial glycogen levels at least 50 per cent of normal for up to 12 days, while after 12 days of moist chamber storage epithelial glycogen supplies were only about 12 per cent of normal (Fig. 3). Similarly, ATP levels after 12 days storage at 4° C. in M-K medium were 67 per cent of normal compared to about 25 per cent of normal after 12 days in moist chamber storage at 4° C. (Fig. 4).

If corneas were left in situ for 5 hours, then corneoscleral rings excised and incubated 4 or 16 hours in oxygenated M-K medium at 37° C., the depleted epithelial ATP levels could be restored to normal and glycogen levels restored to 83 per cent of normal (Figs. 5 and 6).

Table I shows that ADP levels were slightly increased by short-term moist chamber, or mid-term M-K medium storage, and decreased in situ. ADP levels could also be restored to almost normal levels by incubation in oxygenated, 37° C. M-K medium after depletion secondary to 5 hours in situ warming.

Fig. 7 shows that human donor corneal epithelial glycogen levels were also increased as a result on incubation at 37° C. in M-K medium after depletion following warming of the tissue.
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**Discussion**

Preliminary data from our prospective clinical study indicates that wound healing is occasionally delayed in the absence of donor epithelium, with subsequent ulceration of the wound margins in severely damaged host corneas. However, when donor epithelium was retained on the donor button, it became apparent that the epithelium was frequently nonviable and either disintegrated at the time of operation or did not remain in the donor tissue more than two or three days after operation. This observation was consistent with those of Maumenee and Kornbleuth and Dohlman, who found that the epithelium

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Fig. 3. Comparison of rabbit corneal epithelial glycogen levels after storage in moist chamber or in M-K medium at 4°C.

Fig. 4. Comparison of rabbit corneal epithelial ATP levels after storage in moist chamber or in M-K medium at 4°C.
was sloughed from donor tissue during the early postoperative days. However, Khodadoust and Silverstein,\textsuperscript{12} using fresh tissue, found retention of donor epithelium for at least several weeks, while deOcampo and Sung\textsuperscript{13} suggested earlier that fresh tissue was necessary to prevent loss of the epithelial layer.

The problem of prompt cooling of the donor eye, which should be done within one hour after death, could not be solved effectively in view of the sporadic nature of most tissue donations. The present data indicate that even five hours at room temperature need not produce irreversible death of the corneal epithelium as long as subsequent storage is done in nutrient-rich medium, rather than in apposition to an already depleted aqueous humor. Other media such as glutathione adenosine Ringer's solution, Kinsey medium, or other frequently used in vitro corneal maintenance media might represent a better test of the tissue's ability to replenish its ATP and glycogen levels. However, use of M-K media clinically is frequently associated with warming of the tissue. Therefore, it was important to evaluate the effect of such warming on the tissue in this medium.

The viability of endothelial cells in artificial medium has been determined by a number of authors using several different measures of cellular function. Scanning and transmission electron microscopy and trypan blue staining have showed that human corneal endothelial cells maintained their integrity up to 21 days in organ culture at 37\degree C.\textsuperscript{3} More recent studies indicate, however, that cat and human corneal endothelial viability was maintained only four to six days if the tissue was organ cultured at 40\degree C.\textsuperscript{14, 15} Laboratory studies show good preservation of endothelial cell activity and function after midterm storage of donor tissue up to 14 days.\textsuperscript{16, 17}

While in the past, it has been assumed that a healthy endothelium indicated a healthy epithelium, it appears more likely that the situation is reversed since clinical observations and laboratory studies\textsuperscript{2} indicate that a healthy endothelium can be present long after the epithelium is nonviable (as in moist chamber storage). Transplantation of donor epithelium is of no value if the cells are nonviable since they are promptly sloughed away. Our current studies indicate that the epithelial cells retain sufficient quantities of ATP and glycogen for metabolic recovery after as long as 12 days in M-K medium at 4\degree C. While our experiments on midterm stor-
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Fig. 7. Human donor corneal epithelial glycogen levels after incubation at 37° C. in M-K medium.

Table I. Rabbit corneal epithelial adenosine triphosphate (ATP), adenosine diphosphate (ADP)/levels and ATP/ADP ratios after storage and incubation.

<table>
<thead>
<tr>
<th>Condition</th>
<th>ATP (μmoles/Gm. of dry weight)</th>
<th>ADP (μmoles/Gm. of dry weight)</th>
<th>ATP/ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>12.3 ± 0.5 (19)</td>
<td>3.1 ± 0.4 (20)</td>
<td>3.97</td>
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<tr>
<td>Moist chamber storage:</td>
<td></td>
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<tr>
<td>5 hours</td>
<td>12.5 ± 0.6 (6)</td>
<td>5.9 ± 0.9 (6)</td>
<td>2.12</td>
</tr>
<tr>
<td>24 hours</td>
<td>10.9 ± 0.3 (6)</td>
<td>7.9 ± 0.9 (6)</td>
<td>1.38</td>
</tr>
<tr>
<td>48 hours</td>
<td>10.3 ± 0.9 (10)</td>
<td>4.4 ± 0.8 (10)</td>
<td>2.34</td>
</tr>
<tr>
<td>12 days</td>
<td>2.9 ± 0.7 (5)</td>
<td>2.2 ± 0.6 (5)</td>
<td>1.32</td>
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<td>In situ storage:</td>
<td></td>
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<tr>
<td>1 hour</td>
<td>8.3 ± 1.2 (5)</td>
<td>1.9 ± 0.3 (9)</td>
<td>4.37</td>
</tr>
<tr>
<td>5 hours</td>
<td>1.6 ± 0.6 (7)</td>
<td>1.3 ± 0.2 (6)</td>
<td>1.23</td>
</tr>
<tr>
<td>18 hours</td>
<td>1.2 ± 0.8 (7)</td>
<td>2.2 ± 0.4 (8)</td>
<td>0.55</td>
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<td>Mid-term storage in M-K medium:</td>
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<tr>
<td>1 day</td>
<td>11.7 ± 0.7 (6)</td>
<td>3.4 ± 0.3 (6)</td>
<td>3.44</td>
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<tr>
<td>3 days</td>
<td>11.1 ± 0.8 (6)</td>
<td>8.1 ± 0.9 (6)</td>
<td>1.37</td>
</tr>
<tr>
<td>12 days</td>
<td>8.1 ± 0.9 (7)</td>
<td>12.3 ± 1.2 (7)</td>
<td>0.66</td>
</tr>
<tr>
<td>Recovery after incubation:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>12.3 ± 0.5 (19)</td>
<td>3.1 ± 0.4 (20)</td>
<td>3.97</td>
</tr>
<tr>
<td>5 hours in situ</td>
<td>1.6 ± 0.6 (7)</td>
<td>1.3 ± 0.2 (6)</td>
<td>1.23</td>
</tr>
<tr>
<td>5 hours in situ, 4 hours incubated at 37° C with oxygenated M-K medium</td>
<td>11.5 ± 0.8 (8)</td>
<td>2.5 ± 0.4 (8)</td>
<td>4.60</td>
</tr>
</tbody>
</table>

age did not impose metabolic stress on the donor tissue before storage in the medium, our recovery experiments did indicate that substantial recuperation of metabolic stores occurs after prolonged metabolic stress at room temperature.

On the basis of the above data, we would recommend that donor cornea be refrigerated within two hours after death or stored in M-K or similar medium within five hours after death for preservation of a viable donor epithelium.

The McCarey-Kaufman Corneal Bathing Medium was generously supplied by Warner Lambert Research Institute, Morris Plains, N. J.
REFERENCES