Intracellular pH and Glutathione Levels in Rabbit Corneal Endothelium Following Storage in Moist Chamber and MK Medium

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Rabbit corneas were stored for up to 14 days at 4°C either as the whole eye in a moist chamber or as the isolated cornea in MK medium with HEPES buffer. The intracellular pH, the glutathione content, and its oxidation state were determined in the endothelial cells of fresh and stored tissue. The endothelial pH was found to be unchanged following storage of up to 7 days by either method, but after 14 days the pH rose slightly but statistically significantly in corneas stored by both techniques. The intracellular pH was similar in endothelia of those corneas stored in MK medium and of those stored as the whole eye in a moist chamber, for all time periods studied. The intracellular total and percent oxidized glutathione of the endothelium were increased by 50 and 180%, respectively, following 7 days of moist chamber storage. Over this time period there was a 50-fold increase in total glutathione content of the aqueous humor in the stored eyes. In contrast, corneas stored in MK medium for 7 days maintained intracellular total glutathione at levels similar to those of fresh corneas. A gradual but constant decrease in percent oxidized glutathione was observed with increasing length of storage. In terms of pH and glutathione content, the MK medium provided a much more stable environment for the stored cornea than did the aqueous humor in the stored eye. Invest Ophthalmol Vis Sci 24:214-217, 1983

Previous experimental and clinical work has confirmed the efficacy of preserving corneas in an intermediate term preservative (MK medium—TC 199 plus 5% dextran) prior to penetrating keratoplasty.1-3 In this medium certain of the normal physiologic characteristics of the cornea appear to be better maintained over a period of 7 days than with whole eyes stored in a moist chamber.4 The hydration of the tissue and its sodium concentration during MK storage remained close to levels found in fresh tissue. Potassium levels of corneas stored in MK medium decreased with time, while those stored in a moist chamber showed an initial loss, followed by an increase that paralleled the increasing potassium concentration in the aqueous humor. It was also found that although active bicarbonate flux across the endothelium was reduced in MK-stored corneas, the passive bicarbonate flux in these corneas was virtually unchanged, indicating better maintenance of the endothelial barrier function under these conditions than in the moist chamber conditions.5

It was the purpose of this study to measure changes during storage in two other parameters that are known to influence corneal function, ie, pH and glutathione concentration. Gonnering et al6 have shown that the endothelium is compromised outside a range of ambient pH from 6.5 to 8.5, while Ng and Riley7 have also shown impairment of endothelial function when the glutathione redox system is severely disturbed.

Materials and Methods

Albino rabbits weighing about 2 kg were killed with an overdose of sodium pentobarbital. The eyes were enucleated and either stored, as the whole eye, in a moist chamber at 4°C, or had the cornea with a 2-mm scleral rim from the paired eye removed, this being stored at 4°C in a 20-ml vial of modified MK medium with 100 μg/ml gentamicin, 5.92 g/l HEPES buffer, and phenol red pH indicator. Following varying storage periods one of two procedures was followed.
Group I: Endothelial Intracellular pH

The DMO (5,5-dimethyl 1-2,4-oxazolidinedione) method was used according to Miller et al. with 14C labeled material. Corneas were removed from the storage medium or isolated from the stored eye and placed in a scintillation vial (8/vial) containing a bicarbonate Ringer solution of 2 μCi/ml labeled DMO (Dimethyl oxazolidine-2,4-Dione, 5,5-(2-14C), New England Nuclear, Boston, MA, 46.63 mCi/mmol specific activity), pH 7.5 and incubated for 1 hour at 37 C. The vials were sealed without any air space in order to maintain constant fluid composition. Following incubation, each cornea was removed, rinsed in nonlabeled bicarbonate Ringer of the same composition and pH as the incubation medium, and the endothelium gently blotted with filter paper. The endothelium was then scraped off, using a Gill corneal knife, and placed in a tared homogenization tube. Endothelia from four corneas were pooled for each single pH determination, and data for each storage period was computed on the basis of four individual determinations. Following weighing of the pooled endothelial sample, 100 μl of distilled water was added to the homogenization tube and the tissue homogenized. Duplicate 20 μl samples were taken, each sample was admixed with 10 ml Aquasol (New England Nuclear) and counted for 10 min using an Isocap 300 liquid scintillation counter (Tracor Analytic, Inc., Elk Grove Village, IL). The intracellular pH was then calculated using the tissue to medium count ratio. The mean and standard error of the mean were computed and comparison of the data made with the t-test.

In order to determine the pH and pCO2 of aqueous humor and MK medium following storage, samples of each respective fluid were obtained and the pH and pCO2 measured with a BMS3-MK2 blood micro system (Radiometer, Copenhagen) at 37 C.

Group II: Endothelial Intracellular Glutathione and Its Redox State

Eyes were removed from the moist chamber, and the cornea with a 2-mm scleral rim was isolated. The paired corneas stored in MK medium and those from the moist chamber were each treated similarly. Excess fluid was gently blotted from the edge of the posterior corneal surface, the cornea was placed epithelial side down, and the endothelium was removed with a knife, placed in a small vial containing 200 μl of 5 mM EDTA and promptly frozen on dry ice. Samples were stored at −80 C for not longer than 4 days and then shipped on dry ice to Dr. Riley where total glutathione and the oxidized fraction were determined by previously described methods. Previous work had documented that freshly isolated corneas prepared and shipped in this manner had total glutathione and oxidized glutathione fractions similar to those of corneas analyzed immediately after isolation in Dr. Riley's laboratory. The means of groups of paired corneas were determined, the standard error of the mean computed, and comparisons of the data made with the t-test.

For determination of glutathione content and redox states of aqueous humor or MK medium following varying storage periods, 100 μl samples were placed in 50 μl of 50 mM EDTA and were stored, shipped, and analyzed in a similar fashion.

Results

Group I: Endothelial Intracellular pH (Fig. 1)

The endothelial intracellular pH of fresh corneas was 7.31 ± 0.01, and following 3 days or 7 days of storage in either MK medium or moist chamber, there was no statistically significant change (P > 0.05). Following 14 days of storage by either method, intracellular pH was significantly higher then fresh controls (P < 0.05). Endothelial intracellular pH at 3 days, 7 days, and 14 days was statistically similar for those corneas stored in MK medium when compared to those stored as the whole eye (P > 0.05).

The pH of fresh MK medium was 7.02 ± 0.02 (Table 1) and was at a similar level of 7.04 ± 0.01 following 14 days of storage containing a single cornea. The pCO2 of fresh MK medium was 40 ± 2 and, following 14 days of storage containing a single cornea, remained at a statistically similar level.
Table 1. Total glutathione, percent oxidized glutathione (GSSG), pH and pCO₂ in aqueous humor of the fresh rabbit eye and following storage in a moist chamber, or a single cornea in 20 ml of MK medium

<table>
<thead>
<tr>
<th></th>
<th>Total glutathione (μMols/l)</th>
<th>% GSSG</th>
<th>pH</th>
<th>pCO₂ (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous humor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>15.9 ± 0.6</td>
<td>14.3 ± 4.8</td>
<td>7.52 ± 0.01</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>7d</td>
<td>785 ± 149</td>
<td>6.1 ± 3.2</td>
<td>7.36 ± 0.01</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>14d</td>
<td>1848 ± 239</td>
<td>9.1 ± 0.1</td>
<td>7.21 ± 0.03</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>MK medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>0</td>
<td>0</td>
<td>7.02 ± 0.02</td>
<td>40 ± 2</td>
</tr>
<tr>
<td>7d</td>
<td>0</td>
<td>0</td>
<td>7.04 ± 0.01</td>
<td>38 ± 2</td>
</tr>
<tr>
<td>14d</td>
<td>0</td>
<td>0</td>
<td>7.04 ± 0.01</td>
<td>37 ± 1</td>
</tr>
</tbody>
</table>

Values are the mean ± S.E.M.

The pH of fresh rabbit aqueous was 7.52 ± 0.01 and showed a decrease to 7.36 ± 0.01 and 7.21 ± 0.03 following storage of the eye in a moist chamber for 7 and 14 days respectively (Table 1). The pCO₂ of fresh aqueous was 25 ± 2 mmHg, but was reduced to 7 ± 1 mmHg after 7 and 14 days of moist chamber storage.

Group II: Endothelial Intracellular Glutathione and Its Redox State (Fig. 2).

The endothelial glutathione content of fresh corneas was 340 ± 53 ng/endothelium, 12.6% of this being in the oxidized (GSSG) form. Corneas from eyes stored in the moist chamber showed an increase in total endothelial intracellular glutathione as well as percent oxidized glutathione (GSSG) during the first week of storage. After 7 days the levels were significantly higher (P < 0.05) than fresh control corneas. However, after 14 days endothelial total and percent oxidized glutathione levels had fallen to levels similar to those found in control eyes. The concentration of glutathione in the aqueous humor of freshly enucleated rabbit eyes was 15.9 ± 0.6 μM/l (14.3 ± 4.8% GSSG) (Table 1). However, following 7 days of storage, it had increased 50-fold to 785 ± 149 μM/l, and by 14 days had increased over 100-fold to 1848 ± 239 μM/l. The oxidized fraction at 7 days was 6.1 ± 3.2% and at 14 days was 9.1 ± 0.1%.

In corneas that were stored in MK medium, the endothelial concentration of total glutathione remained statistically unchanged for the entire 14 days (Fig. 2). The percentage of glutathione in the oxidized state declined with time in storage so that after 14 days of storage only 4.0% was GSSG compared with 12.6% in fresh eyes. No detectable glutathione was found in any of the samples of fresh MK medium or in MK medium following corneal storage (Table 1).

Discussion

The technique of removing donor corneas from the eye for storage before keratoplasty was undertaken on the basis that the endothelium would be removed from the adverse environment resulting from stasis of the aqueous humor and postmortem changes in adjacent tissue. A larger ambient volume in which the cornea is bathed greatly diminishes the effects of corneal metabolism on the composition of the medium, and other metabolic changes are eliminated. The use of MK medium, containing 5% dextran, counteracts the swelling that is expected due to inhibition of the fluid pump by the low temperature. The present work shows that in MK medium with HEPES buffer pH of the endothelial cells can be maintained for up to 7 days. It is not known if MK medium with other buffer systems is as effective in maintaining intracellular pH, or if prolonged cadaver times would yield similar results. Similarly, there is no significant change in the total glutathione content.
of the endothelium in these corneas over 14 days of storage. The stability of the intracellular pH combined with the minor changes in total glutathione content and that in the oxidized state, suggests then when these corneas are returned to a normal environment at 37 C they will be able to resume metabolism without delay. It was, unfortunately, not possible to process these small amounts of tissue to yield protein or DNA content concurrently with the glutathione assays.

In the endothelium from corneas of eyes stored in moist chambers, the intracellular pH was similar to that of fresh eyes for up to 7 days of storage. At 14 days of storage intracellular pH had risen slightly, to 7.36, in spite of a fall in the pH of the aqueous from fresh levels of 7.52 to a level of 7.21 at 14 days. The glutathione content in these endothelia increased over the first week of storage, being 50% higher than control or MK corneas after 7 days. This is most probably due to the striking increase in the glutathione concentration of the aqueous humor during this period, which, presumably, results chiefly from loss of glutathione from the lens and other anterior uveal tissues. After a further week, the quantity of glutathione measured in the endothelium is diminished, a finding that most probably results from a loss of endothelial cells from the tissue since the ambient concentration increased to even higher values over this period. The marked increase in the oxidation state of glutathione at 7 days may be due to a failure of the redox system to recycle itself via glutathione reductase because of low NADPH + H + levels in the absence of glucose.

It is also apparent from these studies that the composition of the ambient MK medium during storage is more stable than that of the aqueous humor in the stored eyes. The latter exhibited wide variations in glutathione content, pH and pCO2, while these values in the MK medium were unchanged. The glutathione content of the medium, 0.16 μM as supplied, was at the lowest limit of detectability in the assay (0–0.33 μM), and the quantity presumed to leak from the cornea during storage had a negligible effect because of the large volume in the vial (0–0.6 μM). Where positive values were obtained, the glutathione was all in the oxidized state. The concentration of glutathione in the MK medium is about 1% of that in rabbit aqueous and about 10% of that in human aqueous. It seems probable that the improved preservation and clinical success reported for corneas stored in MK medium, derives in large part from its ability to provide a constant environment approaching physiological conditions. Further refinements in composition may lead to even better storage conditions for donor corneas.

Key words: cornea, corneal preservation, pH, hydrogen ion, glutathione, endothelium, MK medium, tissue preservation.

References