Thermal Cycling Effects on the Stored Rabbit Cornea

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Specular microscopy has proven itself a useful tool for evaluation of donor corneas. In many eye banks, corneas are stored at 4°C and warmed to room temperature for specular microscopic evaluation. On occasion it would be desirable to put the cornea back into storage provided there were no detrimental effects of the recooling and subsequent rewarming. The effects of repeated cooling and rewarming (thermal cycling) on the corneal endothelium were determined using rabbit corneas stored in M-K medium at 4°C. Some corneas were left in the refrigerator for a 7-day storage interval while others were removed daily, warmed to room temperature, evaluated morphologically and biochemically with the specular microscope and the redox fluorometer, respectively, and then placed back in the refrigerator. At the end of the 7-day storage period there were no statistically significant differences in either the biochemical signals or the specular appearance between thermally cycled corneas and corneas that were continuously stored at 4°C for the same period of time. Repeated warming, specular microscopic observation, and cooling of the cornea appear not to be detrimental to the corneal endothelium. Invest Ophthalmol Vis Sci 30:1584-1587, 1989

Materials and Methods

Preparation of the Corneas

New Zealand albino rabbits, weighing 3–4 kg, were anesthetized with an intramuscular injection of 0.5–1.0 ml of 1:1 ketamine HCl (30 mg/kg of body weight) and xylazine (6 mg/kg of body weight). Retrobulbar anesthesia using 1 ml of 1% xylcaine was then given and the eyes were enucleated. The animals were then sacrificed with an overdose of sodium pentobarbital. This procedure eliminated possible metabolic effects of pentobarbital, a known metabolic inhibitor in the cornea. Corneoscleral buttons were excised using standard eye bank procedures and were preserved in MK medium in the specular microscope corneal chambers (Cooper Vision, Irvine, CA; Corneal Storage Viewing Chamber, CV 2003). The animals were handled in accordance with the ARVO Resolution on the Use of Animals in Research. Each pair of corneas was divided into two groups and studied as follows. Group 1, the control or “continuous” group, consisted of ten corneas that were cooled from room temperature to 4°C on the initial day of the experiment, kept continuously at 4°C for 7 days and then rewarmed to room temperature. Group 2, the “daily” group (thermally cycled), consisted of ten fellow corneas that were cooled from room temperature to 4°C on the initial day, re-
warmed daily to room temperature for 2 hr and then recooled.

Specular Microscopy

Using an Eye bank Specular Microscope (Bio-Optics, Arlington, MA; LSM-2100C), the endothelium was observed and photographed in both groups at 2 hr after enucleation and again after each cornea had been rewarmed to room temperature on the seventh day.

Redox Fluorometry

The autofluorescence of reduced pyridine nucleotides (PN) and oxidized flavoproteins (Fp) within the rabbit cornea were noninvasively measured as a function of depth using an ocular redox fluorometer that has been previously described. In brief, this instrument combines a corneal specular microscope, a photon-counting fluorometer, and a computer and illuminates a small tissue volume with light of either 366 nm or 440 nm. The fluorescence excited by these wavelengths at 460 nm and 540 nm, respectively, is measured and appropriately corrected to give the PN and Fp signals. In these experiments the measurements were made continuously at room temperature by alternately measuring the PN and Fp signals for 0.1 sec per reading during a total measurement period of 2 hr. Measurements were made immediately after enucleation and 2 hr after removing the tissue from the refrigerator on the seventh day. The measurement area on the endothelium that was used was about 1 cell × 10 cells in size.

Morphometric Analysis

Morphometric analysis was also performed on the specular microscopic photographs obtained from groups 1 and 2. More than 200 cells were digitized and analyzed for each cornea under each condition using an Endothelial Image Analysis System (Bio-Optics), by digitizing cell corners using the “corners” and “histo” programs. The endothelium was analyzed for a variety of parameters, including cell area, cell perimeter, cell shape, number of sides and cell side lengths. The mean, standard deviation, coefficient of variation and skewness of each parameter were calculated automatically.

Statistical Analysis

A student paired test as well as an analysis of variation for independent groups with repeated measurements was performed using the Boston University computer to determine whether or not the changes measured were significant.

Results

Figure 1 shows specular micrographs taken at the initial day and the seventh day of storage. The cells appear normal and there are no visible differences in
Table 1. Morphometric analysis on both the "continuous" and "daily" groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Continuous</th>
<th>Daily</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean cell area (µm²)</td>
<td>Day 0</td>
<td>Day 7</td>
</tr>
<tr>
<td></td>
<td>225 ± 23</td>
<td>230 ± 31</td>
</tr>
<tr>
<td></td>
<td>253 ± 20.8</td>
<td>255 ± 18.2</td>
</tr>
<tr>
<td>Coefficient of variation of mean</td>
<td>0.162 ± 0.029</td>
<td>0.174 ± 0.014</td>
</tr>
<tr>
<td>Mean cell perimeter (µm)</td>
<td>Day 0</td>
<td>Day 7</td>
</tr>
<tr>
<td></td>
<td>56.8 ± 1.8</td>
<td>57.3 ± 2.1</td>
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<tr>
<td></td>
<td>60.2 ± 2.3</td>
<td>60.8 ± 2.5</td>
</tr>
<tr>
<td>Mean cell side length (µm)</td>
<td>Day 0</td>
<td>Day 7</td>
</tr>
<tr>
<td></td>
<td>9.47 ± 0.56</td>
<td>9.59 ± 0.62</td>
</tr>
<tr>
<td></td>
<td>10.02 ± 0.33</td>
<td>10.16 ± 0.28</td>
</tr>
<tr>
<td>Mean cell shape (circle = 1)</td>
<td>Day 0</td>
<td>Day 7</td>
</tr>
<tr>
<td></td>
<td>0.869 ± 0.004</td>
<td>0.868 ± 0.002</td>
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<tr>
<td></td>
<td>73.0 ± 5.9</td>
<td>70.2 ± 4.0</td>
</tr>
<tr>
<td>Hexagonality (%)</td>
<td>Day 0</td>
<td>Day 7</td>
</tr>
<tr>
<td></td>
<td>0.868 ± 0.007</td>
<td>0.864 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>74.0 ± 4.5</td>
<td>67.0 ± 9.1</td>
</tr>
</tbody>
</table>

Fig. 2. PN/Fp ratio in both continuous and daily groups at the initial and seventh day.

Fig. 3. Changes in PN/Fp ratio from room temperature to 4°C.

appearance between the control or "continuous" group and the "daily" group.

The PN/Fp ratio changed from 0.77 ± 0.14 to 0.70 ± 0.06 in the "continuous" group and changed from 0.76 ± 0.12 to 0.69 ± 0.09 in the "daily" group between the initial and the seventh day. Redox measurements, shown in Figure 2, showed no significant difference at the $P = 0.05$ level between the two groups on day 0 and day 7 as well as the difference between day 0 and day 7 for each group.

Under the separate experiments, corneas were cooled from room temperature to 4°C for several hours. After cooling started, the PN values increased, the Fp values decreased, and the PN/Fp ratio increased progressively. After rewarming, the PN value decreased, the Fp value increased, and the PN/Fp ratio decreased to approximately its original level. The value of the PN/Fp ratio change is shown in Figure 3.

Table 1 shows the results of morphometric analysis of each parameter done on both the "continuous" and the "daily" groups. The mean cell area between the initial day and the seventh day increased from 225 ± 22.6 to 230 ± 30.9 for the "continuous" group, and from 253 ± 20.8 to 255 ± 18.2 for the "daily" group. The coefficient of variation of cell area increased from 0.162 ± 0.029 to 0.185 ± 0.032 for the "continuous" group and 0.174 ± 0.014 to 0.198 ± 0.026 for the "daily" group. The student paired $t$-test showed that there were no significant differences either in these factors or in the cell shape, number of sides, cell perimeter and cell side length between day 0 and day 7 between the "continuous" and "daily" groups. The analysis of variation also confirmed that there was no difference at the $P = 0.05$ level in either the mean cell area, the standard deviation of mean cell area, the mean side lengths or the standard deviation of the mean side lengths for the "continuous" group and the "daily" group.

Discussion

It is generally accepted that the specular microscopic appearance of the endothelium is a good indicator of endothelial viability. Furthermore, morphometric analysis of specular microscopic pictures can provide considerable quantitative information.
concerning endothelial changes resulting from ocular surgery or diseases and can also reveal subtle changes not apparent by other methods. Morphometric abnormalities are also seen in corneas that have reduced pump function.9,10

Corneal redox fluorometry in combination with specular microscopy provides a noninvasive method to evaluate the biochemical state of the corneal tissues.3

The experimental results show that cooling corneal tissue to 4°C changed not only the specular microscopic image,11,12 but also the biochemical state of the cornea as measured by corneal redox fluorometry.12 As corneas were cooled to 4°C, the specular image degraded considerably and many abnormal structures, including unclear cell borders, black cells and dark areas are observed. Under the same conditions, the PN and Fp signals and the PN/Fp ratio also changed, indicating a slowing down of metabolism. However, these changes were reversible and immediately after rewarming started the cell appearance and the metabolic state rapidly returned to normal.12 The light intensity and the illumination area used are both so small that even continuous measurement for the 2 hr period did not warm the tissue above room temperature.

Our study shows that there were no significant differences between the initial day and the seventh day in the continuous and daily rewarming groups, in either the morphometric or biochemical states. Repeated thermal and metabolic alteration due to cooling and rewarming up to 2 hr daily did not adversely effect the endothelial appearance, the morphological characteristics or the metabolic state of the corneal endothelium for a storage period of 1 week in MK medium.

Our findings are in disagreement with those of Rootman, Hasany and Basu,2 who concluded from morphometric studies of thermally cycled human corneas that repeated rewarming to 37°C had a deleterious effect on the corneal endothelium. The quality of the images they obtained was, they state, poor. Furthermore, the methodology they used for obtaining morphometric information from the specular images contains systematic and random errors and is unsuitable for obtaining valid qualitative information.

The absence of detrimental effects associated with the warming, evaluation and recooling of eye bank stored corneas has considerable clinical and health care significance. Corneal evaluation and selection can be performed prior to surgery and the corneas then recooled until use; this will enable not only the best tissue at hand to be used but also will allow improved patient scheduling.

Key words: corneal endothelium, temperature cycling, redox fluorometry, specular microscopy, eyebank storage

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References