Tolerance of Corneas to Multimolar Dimethyl Sulfoxide at 0°C
Implications for Cryopreservation
Michael J. Taylor and Charles J. Hunt

Attempts to improve current methods of cryopreservation of corneas, whether by conventional freezing and thawing or by vitrification in the absence of ice, will require the use of high concentrations of cryoprotectants. In this study we extend our previous investigation of the tolerance of rabbit corneas to multimolar concentrations of the cryoprotectant dimethyl sulfoxide (Me₂SO) added and removed at 0°C in CPTES, a hyperkalaemic preservation solution containing the impermeant anionic buffer N-Tris(hydroxymethyl)methyl-2-aminoethane sulphonate (TES). Isolated corneas were exposed to 1, 2 or 3 mol/1 Me₂SO at 0°C to minimize any effect due to temperature-dependent chemical toxicity and attention was given to the procedure for diluting Me₂SO from the tissue in order to minimize osmotic stress to the endothelium. Endothelial integrity following these procedures was assessed both by the ability to control stromal hydration during perfusion on the specular microscope and by the structural integrity when examined by light and electron microscopy. The presence of an active endothelial pump and good morphology were demonstrated in corneas exposed to 1 and 2 mol/1 Me₂SO; serial dilution of the cryoprotectant was more beneficial than a single-step direct dilution. Corneas immersed directly into 3 mol/1 Me₂SO were irreparably damaged irrespective of the method of dilution. Sequential addition of 1 M, then 2 M and finally 3 M cryoprotectant followed by serial dilution was, however, tolerated by the endothelium and minor alterations to the structural integrity of the endothelial layer were rapidly repaired. The osmotic nature of these observations are analyzed and discussed. Invest Ophthalmol Vis Sci 30:400-412, 1989

We have previously advocated the use of a hyperkalaemic balanced salt solution (CPTES) containing the impermeant anionic buffer N-Tris(hydroxymethyl)methyl-2-aminoethane sulphonate (TES) as a bathing medium for the low-temperature preservation of corneas. As with other so-called "intracellular-type" solutions, CPTES was designed to restrict the ionic imbalances that occur when temperature is reduced and ionic pumps are switched off. The impermeant anion is included to reduce cellular swelling during low-temperature preservation. CPTES therefore provides an effective "vehicle" solution for introducing and removing cryoprotectants from corneas before and after sub-zero storage.

Improved methods of corneal cryopreservation will require the use of high concentrations of cryoprotectant either in procedures involving freezing or in attempts to avoid damaging ice crystallization during deep sub-zero storage by employing techniques involving vitrification. The previously published study showed that the incorporation and removal of 1 mol/l dimethyl sulfoxide (Me₂SO) at 0°C is well tolerated by corneas, which retained both the structural and functional integrity of their endothelium. The investigation of endothelial tolerance to multimolar concentrations of Me₂SO is examined here during the addition and removal of 1, 2 or 3 M Me₂SO in CPTES medium. As before, corneal viability was assessed both in terms of endothelial function using specular microscopy to monitor control of stromal hydration and structural integrity using light and electron microscopy.

Materials and Methods
Preparation of Corneas
Eyes were enucleated from New Zealand white rabbits (2–3 kg) which had been killed by an i.v. overdose of sodium pentobarbitone, in accordance with the ARVO Resolution on the Use of Animals in Research. Before dissection the initial thickness of each cornea was measured in situ by mounting the
enucleated eye under the specular microscope. The mean thickness of the corneas used in these experiments was 315 μm (SD = ±22). Excision of the cornea was then carried out using the method of Dikstein and Maurice and throughout subsequent handling each cornea was held on a plastic support ring to prevent wrinkling and distortion.

Experimental Procedures

Immediately after excision the isolated corneas were immersed endothelial-side uppermost in 5 ml of medium prepared as previously described and precooled to 0°C. CPTES solutions containing Me₂SO were prepared on a weight for volume basis and calculated estimates of their osmolalities are given in Table 1. Corneas were immersed directly in either 1 M, 2 M or 3 M Me₂SO-CPTES and held for 30 min at 0°C. Since immersion of corneas directly into 3 M Me₂SO-CPTES (osmolality = 4740 mosm/kg; see Table 1) would be expected to induce a substantial osmotic shock to the endothelial cells (see Table 2), another group of corneas was transferred sequentially at 10 min intervals from 1 M Me₂SO-CPTES to 2 M Me₂SO-CPTES and finally to 3 M Me₂SO-CPTES at 0°C.

Removal of the cryoprotective additive (CPA) was done either gradually (serial dilution) or abruptly (direct dilution) as previously described. Serial dilution involved a stepwise reduction of the concentration of Me₂SO in the bathing medium by the gentle addition of quantities of precooled CPTES at 5 min intervals. For corneas bathed in either 2 M or 1 M Me₂SO-CPTES the concentration of CPA was halved at each step by adding equal volumes of CPTES until the concentration of Me₂SO was reduced to 0.25 mol/1. Serial dilution of 3 M Me₂SO-CPTES involved the stepwise addition of appropriate volumes of CPTES to reduce the concentration of Me₂SO to 2.14, 1.07, 0.5 and finally 0.25 Mol/l. After 5 min in 0.25 M Me₂SO-CPTES, corneas were transferred directly to 5 ml of glutathione-bicarbonate Ringers' solution (GBR) at 0°C for 5 min, and then to a fresh sample of GBR and allowed to warm to room temperature for a further 5 min. Direct dilution was achieved by transferring corneas from the bathing solution with CPA to 5 ml of GBR containing no CPA at 0°C and from then they were treated in the same way as corneas in the other groups. Finally, corneas were mounted for perfusion under the specular microscope or processed for light and electron microscopy.

Evaluation of Corneal Viability

Specular microscopy: Corneal transparency, hydration and thickness are all inextricably linked and

<table>
<thead>
<tr>
<th>Solution (molarity of Me₂SO indicated)</th>
<th>Osmolality* (mOsm/kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 M Me₂SO</td>
<td>620</td>
</tr>
<tr>
<td>0.5 M Me₂SO</td>
<td>900</td>
</tr>
<tr>
<td>1.0 M Me₂SO</td>
<td>1430</td>
</tr>
<tr>
<td>2.0 M Me₂SO</td>
<td>2610</td>
</tr>
<tr>
<td>3.0 M Me₂SO</td>
<td>4740</td>
</tr>
</tbody>
</table>

* Calculated using equations described by Pegg for obtaining the melting points for the ternary system Me₂SO/NaCl/H₂O.
† Measured osmolality of CPTES salts = 301 mOsm/kg.

under the direct control of the endothelium. Specular microscopy is a useful biophysical method for assessing the viability of the endothelium as it allows the thickness of the cornea to be monitored during in vitro perfusion in addition to providing information on the morphological integrity of the endothelial cells. Corneas were mounted under the specular microscope and perfused at 2 ml/hr using a hydrostatic pressure of 15 cm H₂O, as previously described. Stromal thickness was monitored during 4 hr perfusion at 34°C with GBR solution, the composition of which has been described previously. In some cases the presence of a functioning endothelial pump was tested by interrupting the normal GBR perfusion and changing the perfusate to bicarbonate and carbon dioxide-free Ringers' solution, which has been shown to switch off the endothelial pump responsible for maintaining corneal hydration.

Table 2. Calculated proportional volume changes of corneal endothelial cells during changes of bathing media

<table>
<thead>
<tr>
<th>Solution Change (molarity of Me₂SO indicated)</th>
<th>Minimum or maximum proportional cell volume change during change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>Me₂SO addition</td>
<td>1.0 M</td>
</tr>
<tr>
<td>0.0 M</td>
<td>1.0 M</td>
</tr>
<tr>
<td>0.0 M</td>
<td>2.0 M</td>
</tr>
<tr>
<td>0.0 M</td>
<td>3.0 M</td>
</tr>
<tr>
<td>1.0 M</td>
<td>2.0 M</td>
</tr>
<tr>
<td>2.0 M</td>
<td>3.0 M</td>
</tr>
<tr>
<td>Me₂SO dilution</td>
<td>2.0 M</td>
</tr>
<tr>
<td>3.0 M</td>
<td>2.0 M</td>
</tr>
<tr>
<td>2.0 M</td>
<td>1.0 M</td>
</tr>
<tr>
<td>1.0 M</td>
<td>0.5 M</td>
</tr>
<tr>
<td>0.5 M</td>
<td>0.25 M</td>
</tr>
<tr>
<td>0.0 M</td>
<td>0.0 M</td>
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<tr>
<td>2.0 M</td>
<td>0.0 M</td>
</tr>
<tr>
<td>1.0 M</td>
<td>0.0 M</td>
</tr>
</tbody>
</table>

* Calculated using the analysis of Pegg et al for the osmotic properties of rabbit corneal endothelial cells.
† Measured osmolality of CPTES salts = 301 mOsm/kg.
Light microscopy: In addition to the evaluation of endothelial morphology by specular microscopy, some samples were examined by light microscopy after supravital staining with trypan blue and alizarin red S by the technique we have described previously.11

Electron microscopy: Corneas were processed for transmission electron microscopy as described elsewhere.1 Isolated corneas were fixed in 3% glutaraldehyde in 0.16 M cacodylate buffer (pH 7.2), 325 mOsM/kg"^-1) while attached to the plastic support rings. Post-fixation was subsequently carried out on diced tissue using 1% osmium tetroxide in cacodylate buffer followed by a further fixation step in 0.5% aqueous uranyl acetate. The tissue was then dehydrated in ethanol, embedded in Araldite, sectioned and examined in a Zeiss EM9S2B electron microscope after staining with ethanolic uranyl acetate and lead citrate.

Analysis

As described in the previous study,1 measurements of stromal thickness for individual corneas were made at the same time intervals during perfusion and the change in thickness was normalized with respect to its initial thickness immediately after enucleation. Adoption of this procedure allowed mean percentage changes (± 1 SEM) in stromal thickness for a group of corneas treated in a similar way to be presented as a mean normalized response curve. The rate of stromal swelling during perfusion was calculated from a regression analysis of the rate of stromal swelling beyond the initial 60 min settling period which was severely impaired after dilution by either procedure. It was difficult to continue measurements of stromal swelling after supravital staining with trypan blue and alizarin red S by the technique we have described previously.11

Results

The ability of the corneal endothelium to control stromal hydration after the various treatments is indicated by the swelling rate indices derived from the stromal thickness curves. These indices, which were obtained by regression analysis, are listed in Table 3 and compared using the Tukey-Kramer method.12

Endothelial Function after Exposure to 2 M Me2SO-CPTES at 0°C

Stromal thickness curves for corneas following immersion in 2 M Me2SO-CPTES at 0°C are shown in Figure 1. As observed in the previous study, all corneas swelled during the first 60 min of perfusion; thereafter corneal thickness was maintained at a near constant level during in vitro perfusion with GBR. Maintenance of stromal thickness is an indication of normal endothelial function which is verified by the reversible swelling induced by means of the interrupted perfusion with CO2-free medium. The presence of an active dehydrating pump was clearly demonstrated for individual corneas from which the 2 mol/l MeSO was diluted by the serial stepwise procedure (Fig. 1C). However, a less efficient reversible response was recorded for the corneas bathed in 2 M Me2SO-CPTES and transferred directly by a single transfer to GBR (Fig. 1A).

Electrode Function after Exposure to 3 M Me2SO-CPTES at 0°C

Direct immersion in 3 M MeSO: Corneas immersed directly in 3 M Me2SO-CPTES for 30 min at 0°C followed by either serial or direct dilution of the CPA swelled rapidly and continuously during perfusion with GBR. Figure 2 shows the mean percentage increases in stromal thickness for both groups of corneas; it is clear that regulation of stromal hydration was severely impaired after dilution by either procedure. It was difficult to continue measurements of stromal hydration beyond 3 hr of perfusion in these experiments because the tissue became progressively more opaque as it thickened, thus preventing accurate estimates of thickness by specular microscopy. This problem is reflected in the larger standard error bars at the greater thicknesses.

Stepwise addition of CPA up to 3 M MeSO: Figure 3 shows the responses of corneas exposed sequentially to 1 M, then 2 M and finally 3 M Me2SO-CPTES and then returned to isotonic GBR by either the serial or direct dilution procedures. In contrast to the corneas immersed directly into 3 M MeSO, these corneas did not swell continuously but settled, after the initial 60
Table 3. Regression coefficients (slopes) of stromal thickness curves and comparisons of the differences between slopes*

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>CPTES</th>
<th>1M-D</th>
<th>1M-S</th>
<th>2M-D</th>
<th>2M-S</th>
<th>3M-D</th>
<th>3M-S</th>
<th>Regression coefficient (±SE)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPTES (Controls)</td>
<td>—</td>
<td>2.35†</td>
<td>0.13</td>
<td>2.01†</td>
<td>1.1</td>
<td>28.42†</td>
<td>42.63†</td>
<td>0.46 ± 0.27 n = 3</td>
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<tr>
<td>1 M/Me₂SO-CPTES</td>
<td></td>
<td>1.88</td>
<td></td>
<td>2.48†</td>
<td>0.34</td>
<td>3.45†</td>
<td>26.07†</td>
<td>40.28†</td>
</tr>
<tr>
<td>Direct dilution (1M-D)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>2.81 ± 0.34 n = 3</td>
</tr>
<tr>
<td>1 M/Me₂SO-CPTES</td>
<td>1.55</td>
<td>1.80</td>
<td></td>
<td>2.14†</td>
<td>0.97</td>
<td>28.55†</td>
<td>42.76†</td>
<td>0.33 ± 0.24 n = 3</td>
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<tr>
<td>Serial dilution (1M-S)</td>
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<tr>
<td>2 M/Me₂SO-CPTES</td>
<td>1.72</td>
<td>1.94</td>
<td>1.62</td>
<td></td>
<td>3.11†</td>
<td>26.41</td>
<td>40.62†</td>
<td>2.47 ± 0.29 n = 5</td>
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<td>Direct dilution (2M-D)</td>
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<td>2 M/Me₂SO-CPTES</td>
<td>1.28</td>
<td>1.57</td>
<td>1.16</td>
<td>1.37</td>
<td></td>
<td>29.52†</td>
<td>42.73†</td>
<td>-0.64 ± 0.13 n = 6</td>
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<tr>
<td>Serial dilution (2M-S)</td>
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<tr>
<td>Direct dilution (3M-D)</td>
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<td></td>
</tr>
<tr>
<td>3 M/Me₂SO-CPTES</td>
<td>4.98</td>
<td>5.06</td>
<td>4.95</td>
<td>5.00</td>
<td>4.87</td>
<td>10.26</td>
<td></td>
<td>43.09 ± 1.13 n = 4</td>
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<tr>
<td>Serial dilution (3M-S)</td>
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* The table is presented as a matrix which allows comparison of the regression data between any two of the experimental groups. The calculated differences between slopes are tabulated in the upper section and these are conveniently compared with the minimum significant differences calculated by the Tukey-Kramer method12 and given in the lower section. Where appropriate differences that are statistically significant are indicated (†P < 0.01).

1 Stromal swelling rate (% change in thickness/hr)

In each case the stromal swelling rate was extremely low and not significantly different from the group of control corneas. The extent of swelling during perfusion was, however, different for each group depending upon the concentration of Me₂SO in the final bathing medium as shown in Figure 4 for groups in which Me₂SO was diluted using the serial procedure. Since the rate of swelling in these corneas is almost zero the intercepts of the regression lines provide an estimate of the degree of post-dilution swelling sustained before control of corneal hydration and thickness is eventually reestablished. Corneas bathed in CPTES alone swelled by 15.6% of their initial in situ thickness before settling to a near constant level for the remainder of the perfusion, as previously discussed; corneas immersed in up to 1 M, 2 M or 3 M Me₂SO increased in thickness by 26.0, 43.6 and 56.3%, respectively.

Assessment of Endothelial Structural Integrity

Specular morphology of unstained and unfixed endothelium: Specular micrographs taken at various
times during the perfusion of corneas that had been treated in a variety of ways are shown in Figure 5.
A normal endothelial pattern maintained for several hours during in vitro perfusion is illustrated in Figure 5A for a control cornea which was bathed in CPTES alone without the addition of dimethyl sulfoxide. Some of the changes in endothelial morphology detected after exposure to 1 M Me₂SO-CPTES followed by a direct dilution of the cryoprotectant are shown in Figure 5B. Darkened cells, which may represent swollen cells, are presumed to be dead or dying cells (Fig. 5Bi) since they are gradually replaced after about an hour of perfusion by the flattening and spreading of surrounding viable cells; this produces centers of cellular reformation as shown in Figure 5Bii. An increased prevalence of enlarged or giant cells was also apparent after several hours perfusion, as shown in Figure 5Biv and Figure 6 (see below); this is probably a further manifestation of endothelial repair.

Another characteristic feature observed during the early stages of perfusion of corneas after exposure to solutions containing dimethyl sulfoxide is the prevalence of numerous dark spots or "vesicles" which appear predominantly at the cell margins (see Figs. 5Ci, Di). This feature, which we have reported previously, disappears within 30 min of perfusion (Fig.
5Ci) and is thought to represent the specular image of reversible osmotically induced changes to normal endothelial cell conformation. Within 1 hr of perfusion many of the morphological changes seen under the specular microscope disappeared and, as illustrated in Figure 5B–E, a normal endothelial pattern was reestablished in corneas exposed to 1 and 2 mol/l Me₂SO.

The endothelium in corneas exposed to 3 mol/l Me₂SO by the stepwise addition procedure showed many of the morphological changes described above. The specular micrographs in Figure 5F show that features which are thought to be characteristics of osmotically induced changes (Fig. 5Fi, ii) disappear within 1 hr of perfusion, as shown by the normal endothelial cell pattern in Figure 5Fiii.

Light microscopy after staining for membrane integrity: Additional details of the morphological changes in corneal endothelium exposed to 2 M Me₂SO-CPTES followed by serial dilution of the cryoprotectant are shown in Figure 6. Staining with trypan blue and alizarin red S immediately after dilution (Fig. 6A, B) revealed a number of changes to some cells within the endothelial layer; a few individual cells had been lost from the layer exposing the underlying Descemets' membrane (Fig. 6A, arrow). Other individual cells showed apparent changes of shape; in these cases the cells appeared rounded with prominent dark areas at the periphery (Fig. 6B). These features are probably the result of osmotically induced changes in cell volume, but the reason why only some individual cells within the monolayer exhibit these changes is not obvious. Nevertheless, after 1 hr of perfusion with GBR these marked alterations to the endothelial layer were clearly repaired by the "filling-in" by surrounding viable cells, giving rise to focal centers of reformation as illustrated in Figure 6C (arrow). It was also noted that these changes were zonal in that some areas of the endothelium had very few reformation "rosettes" (see Fig. 6D).

After 4 hr perfusion all defects in the endothelial layer had been repaired and the endothelium appeared normal but now included a number of large, irregularly shaped cells which were assumed to occupy the site of replaced defective cells (Fig. 6E, F).

Vital staining of corneas after exposure to 3 mol/l Me₂SO showed that several cells had been lost from the endothelial layer when examined immediately after staining.
after the serial dilution schedule (Fig. 7A, B). However, after 4 hr post-dilution perfusion, corneas that had been treated in this way exhibited an intact and fully confluent endothelial layer which possessed characteristic signs of active endothelial cell replacement (Fig. 7C, D).

Ultrastructure: After equilibration and removal of 1 M Me₂SO, only minimal osmotic damage was observed (Fig. 8A, B). This was confined mainly to the mitochondria and was more apparent after the single-step removal of the cryoprotectant than after serial dilution. The details of the structural changes have been reported elsewhere.¹

Corneas exposed to 2 M Me₂SO-CPTES at 0°C followed by dilution generally showed more severe ultrastructural changes than those exposed to the lowest concentration of cryoprotectant. The effect of the dilution schedule on endothelial structure was even more apparent here than after dilution with 1 M Me₂SO. Corneas exposed to a single-step dilution exhibited some cellular edema as well as swollen RER and golgi lacunae. Nuclei presented an ovoid appear-
Fig. 6. Morphological changes in corneal endothelium after exposure to 2 M Me₂SO·CPTES followed by serial dilution of the cryoprotectant. Endothelial cells were stained with trypan blue and alizarin red S either immediately after dilution (A, B), or after perfusion with GBR for 1 hr (C, D) or 4 hr (E, F) at 34°C following dilution. Morphological features are described in the text. Magnification bars = 100 μm except in B, where bar = 50 nm.

ance with an apparent loss of chromatin, though nuclear membranes were intact. Many condensed, shrunken mitochondria were present together with mitochondrial profiles showing fragmented membranes (Fig. 8C). In contrast, corneas exposed to the serial dilution schedule showed far less osmotic damage. Nuclear changes were largely absent, and RER and golgi arrays were no longer as swollen or vesiculated. Mitochondrial changes, though still prevalent, were confined mainly to slight swelling of the inner compartment and the intracristal space. This resulted in a loss of matrix density and a disordering of the longitudinally arranged cristae (Fig. 8D) similar to that seen at the lowest concentration. Such changes are readily reversible. 14,15

Corneas exposed to the highest concentration of cryoprotectant used in this study followed by dilution showed significant structural damage regardless of the dilution schedule used (Fig. 8E, F). The endothelium was edematous and there was pronounced swelling of RER and golgi, producing large intracellular vesicles. Nuclear changes were again evident and mitochondria showed extensive, often irreparable damage with swollen matrices and radially arranged cristae; in many there were only fragmented membranes remaining. Such changes are regarded as irreversible. 16 At the posterior surface, bleb-like structures were often seen comprised of cell cytoplasm contiguous with the main body of the cell. Even after dilution from 3 M Me₂SO, apical junctions remained closed and the intercellular space was of normal width, though few maculae occludentes were visible. The basal attachment of the cells to Descemet’s membrane immediately following dilution was unaffected by either dilution schedule or concentration of cryoprotectant, though after exposure to 2 and 3 M Me₂SO, an occasional disrupted cell was observed.

Discussion

Although it is generally held that extracellular ice formation is innocuous to slowly frozen cells in dilute suspensions, evidence is accumulating that the for-
Fig. 7. Endothelial morphology in corneas following the stepwise sequential exposure to 1, 2 and then 3 mol/l dimethyl sulfoxide. Corneas were returned to isotonic GBR medium using the serial dilution procedure. The endothelium in these corneas was stained with trypan blue and alizarin red S either immediately after completion of the dilution schedule (A, B) or after an additional 4 hr perfusion with GBR at 34°C (C, D). Notable features include the prevalence of "voids" within the endothelial monolayer, which appear as dark areas of equivalent size to single cells (arrows in A and B); these are the sites where individual cells have been lost from the endothelial layer and the underlying Descemet’s membrane has stained with alizarin red S. Most of the voids have been replaced during 4 hr perfusion at 34°C by the infiltration of surrounding cells, giving rise to characteristic areas of cellular reforma-
tion, as described in the text. A particularly active area of endothelial cell conformational change is depicted in panel D. Magnification bar = 100 μm.

A preference for dimethyl sulfoxide over other cryoprotectants such as glycerol and polyethylene glycol for protecting corneal endothelial cells during freezing and thawing was shown several years ago by the work of Smith et al. However, their investigation, which used the survival of cells in tissue culture as a means of assessing the viability of the endothelium, was limited to a study of the effect of up to 15% (w/v) Me2SO (approximately 2 mol/l). In recent years serial measurements of corneal thickness by specular microscopy have been used extensively as a more direct means of assessing the efficiency of the endothelium in controlling stromal hydration (see ref. 4). Effective control of corneal thickness is known to depend upon two properties of the endothelium: one is the physical barrier function which is a passive property dependent upon close association between intact endothelial cells to ensure restricted movement of water into the stroma, and the other is a metabolic pump which actively translocates fluid from the stroma to the aqueous humour. We have therefore
used specular microscopy as the primary test of endothelial integrity following exposure to 1, 2 and 3 mol/l Me₂SO.

In these experiments corneas were manipulated while supported on plastic support rings which minimized the risk of mechanical trauma. Furthermore, these procedures for handling the corneas during the addition and removal of cryoprotectants were chosen to emulate the methods that might be adopted during a cryopreservation protocol. An alternative procedure for studying the endothelial layer during exposure to Me₂SO would be to perfuse the corneas with cryoprotectant solutions under the specular microscope. However, the complications of uniform tem-
perature control at 0°C and satisfactory precision of fluid exchange, avoiding unstirred layers during the addition and removal of Me$_2$SO, convinced us to adopt the procedures described above.

**Endothelial Tolerance to High Concentrations of Me$_2$SO**

Tolerance of cells to a permeating cryoprotectant such as Me$_2$SO requires not only a low chemical toxicity of the compound but also the ability of the cells to withstand osmotically induced excursions in cell volume brought about during the addition and removal of the CPA. Therefore, true chemical toxicity and osmotic shock are invariably coupled when reference is made to the "toxicity" of a cryoprotectant compound. The former is temperature-dependent and is therefore minimized by using the lowest temperature possible for equilibrating the tissue with CPA, while the latter may be minimized or avoided by careful attention to the design of protocols used to administer and remove the additive. The osmotic responses of cells to anisosmotic conditions are governed by several concomitant factors, including the permeability of the cells to water and CPA, the reflection coefficient, the temperature coefficients of these parameters, the temperature and the intracellular and extracellular concentration of the additive (see refs. 27, 28). Since data for the permeation of Me$_2$SO in corneal tissue is not yet available, conditions for exposing corneas to multimolar concentrations of Me$_2$SO were chosen on a semiempirical basis, as discussed previously. The merits of stepwise dilution, rather than immersing the corneas directly into isotonic GBR to remove the Me$_2$SO, were clearly confirmed in this study and shown to be beneficial for the ultimate integrity of the tissue. Corneas immersed directly into 2 M Me$_2$SO-CPTES followed by serial dilution of the Me$_2$SO were able to regulate their thickness during perfusion in a manner which was equivalent to control corneas that had been bathed in CPTES alone at 0°C and had not been exposed to Me$_2$SO. Furthermore, these swelling rates, which were <5 μm/hr and even negative in the case of the corneas exposed to 2 molal Me$_2$SO followed by serial dilution, are equivalent to those shown by freshly isolated corneas perfused in vitro for several hours with GBR. Apart from the loss of a very small number of individual cells, the endothelial layer was shown by light microscopy to be completely intact. Ultrastructural examination showed only minor alterations to cellular organelles and such changes are known to be reversible, as discussed above. Direct immersion of corneas into 3 M Me$_2$SO-CPTES, however, followed by either sequential or direct dilution, clearly exceeded the tolerable limit beyond which the endothelia were unable to regain their "barrier-function." Structural studies revealed the extensive breakdown of the endothelial monolayer and severe ultrastructural damage induced by these procedures.

Recently Pegg et al. have published an analysis of the osmotic properties of rabbit corneal endothelium and their study demonstrated a tolerated range of osmolality of NaCl of 0.64-4.4 X isotonic, corresponding to a calculated acceptable excursion in cell volume of approximately 50-140% of the isotonic cell volume. They then calculated the maximum possible transient change in volume that would accompany given step changes in Me$_2$SO concentration, assuming instantaneous water movement, zero CPA movement and a reflection coefficient of 1. We have applied this calculation to the experimental conditions of this study and Table 2 shows the calculated instantaneous proportional volume changes of corneal endothelial cells for the various steps in the procedures for adding and removing Me$_2$SO. The analysis predicts that direct dilution from solutions containing 2 or 3 mol/l Me$_2$SO would subject the endothelial cells to severe swelling exceeding 5X their normal isotonic volume. Osmotic lysis of the cells is likely to account, therefore, for the injury manifest using these procedures. In this and a previous study serial dilution was clearly shown to be advantageous, and the analysis shows that only in the final step when tissue is transferred from 0.25 mol/l Me$_2$SO plus isotonic salts to isotonic GBR medium does the cell volume exceed 150%, and then only slightly. Pegg et al conclude from their calculations that this final dilution step may cause unacceptable excursions in cell volume, but our experiments show that this degree of volume expansion is tolerated with retention of normal endothelial structure and function. Their experiments are likely to give conservative limits, since exposure was for 5 min at 20°C, whereas anisotropic volumes are transient where permeating CPAs are used.

Direct immersion of corneas into 3 M Me$_2$SO-CPTES (4.74 osmolar) followed by the serial dilution regimen did not yield viable corneas; Table 2 shows that the minimum cell volume under such circumstances is likely to be about 0.37 of isotonic volume, which clearly exceeds the tolerable limit. It was shown, however, in additional experiments that corneas could tolerate immersion in 3 mol/l Me$_2$SO if the tissue was exposed to increasing concentrations of Me$_2$SO in a stepwise manner: the proportional volume change data in Table 2 show that during such a procedure the endothelial cell volume does not shrink below 0.47 and this may therefore be regarded
as being within the tolerable limit of cell shrinkage for these cells. The apparent toxicity of 3 mol/l MeSO shown in the direct immersion experiments is likely, therefore, to be largely osmotic in nature.

One other interesting comparison can be made from these data: the stromal swelling rate for the group of corneas exposed sequentially to 1 M, then 2 M and finally 3 M MeSO at 10 min intervals followed by a direct dilution is not significantly different, at the 95% level of confidence, from the swelling rate obtained for corneas immersed directly into 2 M MeSO-CPTES for 30 min at 0°C. Two possibilities exist to explain this observation: one is that the cells may be able better to tolerate excessive swelling during dilution if the degree of shrinkage is limited during sequential addition of the MeSO. Alternatively, the response may be related to the time of exposure to 3 M MeSO. Although the total time for addition of the cryoprotectant in the two experiments was equal (30 min), the time of immersion in the final concentration of 3 mol/l MeSO was only 10 min in the experiments where the CPA concentration was increased in sequential steps. Since data for the kinetics of permeation of MeSO into corneal endothelial cells are not yet available it must be conceded that it is possible that the cells did not attain full equilibration with 3 mol/l MeSO during this schedule. For example, if the intracellular concentration of MeSO had reached only about 2 mol/l during the sequential addition procedure, then a response similar to the single-step dilution might be expected. Comparison of the respective regression coefficients shows that the stromal swelling rates for these two groups were of a similar magnitude and were not significantly different (1.92 ± 0.16 (n = 4) and 2.47 ± 0.29 (n = 5), respectively) from each other.

Deviations of endothelial cell volume from normal isotonic values will dramatically effect the barrier property of the endothelium, which relies upon close cell-to-cell apposition. Figure 4 shows that exposure to increasingly hypertonic solutions of dimethyl sulfoxide produces increasingly higher degrees of stromal swelling before control of hydration is eventually reestablished. A temporary breakdown of the barrier function may also be due to the necrosis and loss of individual cells from the endothelial layer, as we have discussed previously.4 Such deficiencies in cellular confluence can be repaired very quickly by the coalescence of adjacent viable cells during perfusion.30-33 as illustrated in Figure 5Bi, 5Eii, and 6C.

Regulation of solute and water concentration gradients across the plasma membrane of endothelial cells during the removal of a permeating cryoprotectant such as MeSO is the basis for minimizing osmotic stresses, and this study has clearly demonstrated the benefit of gradual rather than abrupt dilution. It should also be noted that such procedures inevitably influence the thickness of the whole tissue since a concentration gradient across the endothelial layer is dissipated by the movement of both solute and water at different rates between the bathing medium and the stroma. It is likely that the empirically derived steps for the serial dilution of MeSO do not allow sufficient time for the stroma to return to its equilibrium thickness following the transient swelling induced by each transfer step to hypotonic medium. The overall slower elution of MeSO from the stroma during the stepwise dilution procedure, compared with the single-step direct protocol, can therefore give rise to thicker corneas at the end of the diluting period. This is confirmed by the data from this and our previous study,1 and Figure 3 illustrates the effect clearly for corneas exposed to 3 mol/l MeSO. Further studies are planned to measure the kinetics of MeSO permeation in corneal tissue and these data will be used to model the time-course of stromal thickness changes during procedures calculated to maintain endothelial cell volume within prescribed limits during the dilution of the permeating solute.7

The use of very high concentrations of cryoprotectant is mandatory for future attempts to improve methods of corneal cryopreservation by methods which aim to limit or prevent the damaging effects of ice. Careful control of osmotic conditions to restrict the degree of shrinkage and swelling during the addition and removal of permeating cryoprotectants is likely to dramatically improve the upper limit of tolerance of the endothelial layer to CPA and, therefore, improve the chances of achieving better cryopreservation.

Key words: cornea, corneal preservation, cryoprotectant toxicity, dimethyl sulfoxide, osmotic tolerance

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References


