Use of Poloxamers for Deswelling of Organ-Cultured Corneas

Min Zhao,1,2 Gilles Thuret,1,2 Simone Piselli,1 Aurelien Pipparelli,1 Sophie Acquart,3 Michel Peoc’h,1 Jean-Marc Dumollard,1 and Philippe Gain1,2

PURPOSE. Dextran T500, routinely used as a deswelling supplement in organ culture (OC), has been suspected of being toxic to corneal endothelial cells (ECs). This study was conducted to evaluate the innovative use of poloxamers compared with dextran for deswelling OC corneas.

METHODS. Five poloxamers (P124, P188, P237, P338, and P407) were dissolved respectively in a standard OC medium to reach 350 mOsmol/kg. In vitro cytotoxicity of these media was tested by MTT assay on human corneal epithelial and endothelial cell lines and on primary human corneal fibroblasts. Paired human corneas stored in OC for at least 21 days were assigned for 48 hours to a poloxamer medium or to a standard deswelling medium containing 5% dextran T500. Corneal EC density, morphometry, visualization, mortality, stromal thickness, transparency, and folding were evaluated before and after deswelling. Corneas were finally cut into three parts for histologic and ultrastructural observation.

RESULTS. Besides similar corneal transparency improvement and thickness deswelling, poloxamers (except P124) reduced EC loss and facilitated endothelial visualization, but improved stromal folding less than dextran. The similar ultrastructures observed in the two groups were epithelial shedding, normal collagen fiber diameter and organization, uptake of deswelling agents by ECs, vacuolization but normal organelles in ECs and keratocytes, and endothelial surface modifications.


Organ culture (OC) is the most common corneal storage method in Europe, used for more than two-thirds of donor corneas.1,2 The extended storage time of 4 to 5 weeks facilitates graft quality and safety controls in eye banks and operation scheduling. However, all OC media have the drawback of triggering rapid stromal swelling, resulting in doubled corneal thickness, reduced corneal transparency, and increased stromal and Descemet’s membrane folding.3,4 To facilitate suturing and accelerate postoperative visual recovery, the cornea must be thinned before surgery by immersion in the same OC medium supplemented with a water-soluble macromolecule that produces colloid osmotic pressure to extract excess water accumulated in the stroma.

Dextran are polyglucose biopolymers of 1000 to 2000 kDa that have a high affinity for water because of their abundant hydrophilic hydroxyl groups. Technical (T) dextrans are high-purity dextrin fractions with selected average molecular weights. Dextran T250 and T500 (500 kDa) were first introduced by Sperling in 1979, and T500 is to date the only macromolecule routinely used for corneal deswelling. Initially thought usable throughout corneal storage,6 dextran was in practice rapidly restricted to the final 48 to 72 hours of storage. However, the initially described6–8 innocuous effect of dextran-containing media on corneal endothelial cells (ECs) has been questioned. An average of 8.4% EC loss was reported after 1 to 4 days of deswelling in an OC medium supplemented with 5% dextran T500.9 We have reported a 15% cell loss after 2 days of deswelling in commercial media containing 5% dextran T500,10 a result consistent with that of Rieger et al.9 in similar conditions using a home-made medium containing 5% dextran T500.10 Daily EC loss in dextran-containing media consequently appears much higher than average daily EC loss of 0.9% in dextran-free media used in the first phase of OC.4

Two other macromolecules have more recently been evaluated for corneal deswelling to replace dextran in OC. Hydroxyethyl starch (HES) 130 and 450, vegetable-origin polysaccharides initially used for plasma expansion, have been assessed ex vivo in pig and human corneas.11–12 HES130 has been assessed as a supplement throughout OC. It restricts water accumulation during long-term storage, making pregraft deswelling unnecessary. Its effect on ECs, however, has not been directly compared with dextran. No clinical application has yet been reported. The second macromolecule assessed belongs to the poloxamer family. Poloxamers are synthetic nonionic triblock copolymers comprising a central hydrophobic polyoxypropylene molecule flanked on each side by a hydrophilic chain of polyoxyethylene. They were introduced in the 1950s by BASF (Ludwigshafen, Germany). Their molecular weights range from 2,000 to 18,000. The physicochemical characteristics of different poloxamers depend on the proportion and the size of both subunits, as well as on their concentration in the solution. In the pharmaceutical field, poloxamers, per se, have therapeutic properties used to treat vasoocclusive crisis of sickle cell disease,13 but they are mostly used simply as drug excipients. In ophthalmology, poloxamers have been proposed for inclusion in the formulation of certain eye drops to improve their pharmacokinetic properties.14–16 They are also used as an antiadhesive agent against bacteria in contact lens-cleaning solutions.17 Poloxamer hydrogel has been assessed as a well-tolerated injectable intraocular lens in the rabbit.18 Findings in our study of an animal compound-free (ACF) medium combined with poloxamer 188 for corneal deswelling suggest that is superior to the standard fetal calf serum (FCS) medium containing dextran T500 in preserving occlusive crisis of sickle cell disease,13 but they are mostly used simply as drug excipients. In ophthalmology, poloxamers have been proposed for inclusion in the formulation of certain eye drops to improve their pharmacokinetic properties.14–16 They are also used as an antiadhesive agent against bacteria in contact lens-cleaning solutions.17 Poloxamer hydrogel has been assessed as a well-tolerated injectable intraocular lens in the rabbit.18 Findings in our study of an animal compound-free (ACF) medium combined with poloxamer 188 for corneal deswelling suggest that is superior to the standard fetal calf serum (FCS) medium containing dextran T500 in preserving...
EC viability during deswelling. A clinical trial comparing these media is ongoing. Nevertheless, the aforementioned study was not designed solely to assess the effect of poloxamer independently of ACF.

Consequently, the purpose of the present study was to assess the innovative use of five poloxamers for human corneal deswelling in OC by comparison with dextran T500, with a standard OC medium containing 2% FCS. Compared with dextran, four of the five poloxamers assessed were found superior in reducing EC loss and had similar efficacy in corneal deswelling and a comparable effect on corneal ultrastructure.

MATERIALS AND METHODS

Poloxamers and OC MEDIA

Five pharmaceutical-grade poloxamers were obtained from BASF: Pluronic F87, molecular weight [MW] 2200, Pluronic F68, MW 8400, P237 (Pluronic F87, MW 7959), Pluronic F108, MW 14600, and P407 (Lutrol F127, MW 12600). CorneaMax (Eurobio, Les Ulys, France) is a commercial OC medium based on HEPES-buffered Iscove's modified Dulbecco's medium containing sodium bicarbonate, 2% FCS, amino acids, penicillin-streptomycin, and phenol red. Its mean pH is 7.25 (range, 7.0–7.5), and its osmolality is 320 mOsmol/kg (range, 300–340). CorneaJet (Eurobio) is a commercial deswelling medium with the same composition as CorneaMax but supplemented with 5% dextran T500. These media are widely and routinely used in France and Europe.

In Vitro Experiments

Establishment of Concentration/Osmolality Curves. For each poloxamer, two series of 10%, 5%, 2.5%, and 1.25% (wt/vol) dilutions in CorneaMax were performed: one nonfiltered and the other after sterilizing filtration through a 0.22-μm filter (Millipore, Bedford, MA) to assess a possible phenomenon of adsorption in the filter membrane. The osmolalities of these solutions were measured in duplicate (Advanced Model 2020 Osmometer; Radiometer, Copenhagen, Denmark). To investigate the stability of solutions in different storage conditions, each solution underwent four series of measurements: immediately after preparation at room temperature, after 1 week of storage at +31°C, and after 1 month of storage at +4°C and at −20°C. A target osmolality of 350 mOsmol/kg was decided—that is, 10% below the highest osmolality that ECs are known to resist.19 The curves allowed us to determine the experimental concentration used for each poloxamer in this study.

In Vitro Cytotoxicity Assays on Three Corneal Cell Types. The human corneal endothelial cell line obtained after transfection with simian virus 40 was a kind gift of Katrin Engelmann and Jurgen Bednarz of the University of Hamburg (Germany).20 The cells were grown at 31°C in a humidified atmosphere of air and 5% CO2 in a medium F9921 consisting of M199/F12 (1:1) (Sigma-Aldrich, St-Louis, MO), 10% FCS, amino acids, penicillin-streptomycin, and phenol red. Its mean pH is 7.25 (range, 7.0–7.5), and its osmolality is 320 mOsmol/kg (range, 300–340). CorneaJet (Eurobio) is a commercial deswelling medium with the same composition as CorneaMax but supplemented with 5% dextran T500. These media are widely and routinely used in France and Europe.

Deswelling of Organ-Cultured Corneas

Organ Culture. The use of human corneas adhered to the tenets of the Declaration of Helsinki. Twenty pairs of human scientific corneas were obtained either from the Saint-Etiene Eye Bank (corneas unsuitable for transplantation for reasons other than endothelial deficiency) or procured from the anatomy laboratory of the Saint-Etiene Faculty of Medicine (cadavers donated to science). Donors were 11 women and 9 men aged 74 ± 13 years (range, 48–91), postmortem time of 22 ± 16 hours (range, 4–66) and storage time of 23 ± 3 days (range, 21–32). A Kruskal-Wallis test showed that the characteristics of the five groups did not differ (data not shown).

All corneas were stored in CorneaMax for at least 21 days (average storage time in France) at +31°C in a dry incubator, as per the storage procedure routinely used by eye banks. At the end of OC, endothelial quality was assessed (see below). Only pairs of corneas with an end-storage endothelial cell density (ECD) of more than 2000 cells/mm2 were included in the present study. These were then randomly transferred either to CorneaJet or to the poloxamer medium (CorneaMax + poloxamer) for 48 hours of deswelling. This deswelling time was chosen because it is the one most frequently reported by European eye banks.1,2 P124 was highly toxic to all three cell types in vitro (see results) and so was not assessed on human corneas. Endothelial quality, corneal central thickness (CCT), stromal transparency, and Descemet’s membrane folding in the two groups were compared before and after deswelling. Histology and ultrastructure were observed after deswelling.

Five additional pairs of corneas were stored in similar conditions and the endothelium assessed in the same way. They were then returned to a new bottle of CorneaMax (i.e., without dextran or poloxamer) for 48 hours, and the endothelium was again assessed. This group served as a control without deswelling. It simultaneously assessed the impact on ECD of handling and of using two cell culture types.
CCD camera (SC 75 CE mono CCD; Sony, Tokyo, Japan). Eight non-overlapping fields (1245 × 950 μm) were captured randomly within the 8-mm central cornea. Irrespective of apparent ECD, the best three images in terms of overall image quality were chosen. EC visualization, a critical parameter in endothelial quality assessment, was graded using a three-level score (good, average, or poor) described elsewhere and took account of cell borders, background noise, and the surface area of visible ECs. As ECs are rarely visible in saline after deswelling in dextran, we stained the cell membranes of corneas in both groups with 4% alizarin red (Sigma-Aldrich) to analyze the post-deswelling EC parameters. ECD and morphometry (coefficient of variation [CV] of cell area and hexagonal cell percentage) were determined with an endothelial analyzer (Sambacornea; Sambatechnologies, Meylan, France) on at least 300 cells.

Histology and Electron Microscopy. After the final endothelial and stromal assessment (lasting <15 minutes), the corneas were cut into three parts for examination by histology, transmission electron microscopy (TEM), and scanning electron microscopy (SEM). The OC corneas served as the control before deswelling.

Histology. Specimens were fixed in 10% formalin in PBS, dehydrated in increasing concentrations of ethanol, cleared with a methylcyclohexane solution, infiltrated with paraffin, and embedded in a paraffin block for sectioning. Semithin cross-sections were then cut and stained with hematein-eosin-safran according to routine protocol. Photographs were taken with an inverted fluorescence microscope (IX81; Olympus, Tokyo, Japan). For each specimen, epithelial thickness measurement (30 measurements) and keratocyte enumeration per 500-μm length of full-thickness cross-section were conducted on three images (×40 objective, Cell* imaging software; Olympus).

Transmission Electron Microscopy. Specimens were fixed in 1% glutaraldehyde/0.5% paraformaldehyde in 0.1 M mono Na/diK buffer (pH 7.4), postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour, dehydrated in a graded series of ethanol and embedded in Epon resin. Ultrathin (90 nm) sections were cut and stained with uranyl acetate and lead citrate. Photographs of endothelium, keratocyte, and posterior collagen fibers were taken with a transmission electron microscope.
Scanning Electron Microscopy. Specimens were fixed in 2% glutaraldehyde in a 0.1 M cacodylate buffer, dehydrated in acetate, and critical-point dried with carbon dioxide used as the transitional fluid. The preparations were then adhered to carbon stubs with the endothelial side uppermost and coated with a thin layer of palladium in a sputter coater (model SC 7620; Polaron, Watford, UK) before being examined and photographed with a scanning electron microscope (model S3000N; Hitachi) operating at 5 kV. Digital images were analyzed by three masked observers who graded the number of microvilli, cilia, intercellular interdigitations, and damaged cells according to a two-level score (low-high).

Statistical Analysis
Unless otherwise indicated, data are expressed as the mean ± SD. The quantitative variables were compared by a paired Student’s t-test in the case of normal distribution and otherwise by a Wilcoxon nonparametric test. The quality scores were compared by the χ² or the Fisher exact test in 2 × 2 grids. Considering the small number of pairs (five) for each poloxamer and that all poloxamers except for P124 tended to behave similarly, only statistics for the global poloxamer family were generated. To clarify the results, details are also given for each poloxamer independently, without statistics. Commercial software (SPSS ver. 11.3; for Windows; SPSS Inc, Chicago, IL) was used, and P < 0.05 was deemed significant.

RESULTS
Concentration–Osmolality Curves
Osmolality increased with the concentration of poloxamer in the solution. The first part of each curve was almost linear (Fig. 1). Filtration did not influence osmolality, and there was no significant difference between the freshly prepared mixture and that in different storage conditions. The concentration of each poloxamer was accordingly determined as 350 mOsmol/kg: P124 at 3%; P188, P237, and P407 at 3.5%; and P338 at 4%.

In Vitro Cell Toxicity Assays
P124 was highly toxic to the three cell types, with viability decreasing to 6% after only 24 hours of exposure. No toxicity was observed with CorneaMax. No toxicity was observed with the four other poloxamers, whatever the cell type and incubation time. Keratocyte viability decreased to 70% after 48 hours of exposure in CorneaJet, whereas no toxicity was observed in endothelial or epithelial cell lines (Fig. 2).

Ex Vivo Comparative Experiments on Human Corneas: Dextran T500 versus Poloxamers
Conneal Endothelium. Endothelial Cell Density. A significant difference of 109 cells/mm² (95% confidence interval [CI] 95% 49–170) in favor of the poloxamer group was observed after deswelling, though there was accidentally a slightly higher count of 62 cells (CI 95% 22–102) in the dextran group before deswelling. Poloxamers induced significantly less EC loss than dextran (9.7 ± 3.4 vs. 16.1 ± 3.2; P < 0.001; Table 1). Ninety percent (18/20) of corneas in the poloxamer group retained an ECD higher than the delivery threshold of 2000 cells/mm², against 70% (14/20) of those in the dextran group (P = 0.025). All four poloxamers tended to behave identically.

Morphometry. Before deswelling, cell-area CV and hexagonal cell percentage were comparable between the two groups. After deswelling, the CV was significantly lower in the poloxamer group, whereas the hexagonal cell percentage was slightly higher in the dextran group (Table 1).
### Table 1. Corneal Endothelial and Stromal Assessment before and after 48 Hours of Deswelling in Dextran versus Poloxamers

<table>
<thead>
<tr>
<th>Time Point/Parameter</th>
<th>Dextran (n = 20)</th>
<th>Poloxamers (n = 20)</th>
<th>P</th>
<th>Dextran (n = 5)</th>
<th>P188 (n = 5)</th>
<th>Dextran (n = 5)</th>
<th>P237 (n = 5)</th>
<th>Dextran (n = 5)</th>
<th>P338 (n = 5)</th>
<th>Dextran (n = 5)</th>
<th>P407 (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Before deswelling</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECD (cells/mm²)</td>
<td>2588 ± 247</td>
<td>2526 ± 224</td>
<td>0.010</td>
<td>2660 ± 285</td>
<td>2584 ± 203</td>
<td>2528 ± 200</td>
<td>2463 ± 156</td>
<td>2622 ± 201</td>
<td>2590 ± 248</td>
<td>2541 ± 335</td>
<td>2468 ± 305</td>
</tr>
<tr>
<td>CV (%)</td>
<td>23.0 ± 5.5</td>
<td>23.8 ± 3.2</td>
<td>0.350</td>
<td>22.8 ± 4.4</td>
<td>24.4 ± 0.9</td>
<td>22.2 ± 2.2</td>
<td>22.5 ± 3.8</td>
<td>24.5 ± 3.0</td>
<td>23.9 ± 1.8</td>
<td>22.3 ± 3.7</td>
<td>24.3 ± 1.8</td>
</tr>
<tr>
<td>Hexagonal cells (%)</td>
<td>51.8 ± 5.0</td>
<td>49.9 ± 6.2</td>
<td>0.156</td>
<td>50.2 ± 5.0</td>
<td>49.4 ± 7.0</td>
<td>49.3 ± 2.9</td>
<td>48.1 ± 6.2</td>
<td>52.3 ± 6.4</td>
<td>50.6 ± 6.0</td>
<td>55.4 ± 3.9</td>
<td>51.3 ± 7.3</td>
</tr>
<tr>
<td>Mortality (%)</td>
<td>0.12 ± 0.15</td>
<td>0.15 ± 0.17</td>
<td>0.507</td>
<td>0.04 ± 0.07</td>
<td>0.08 ± 0.16</td>
<td>0.08 ± 0.08</td>
<td>0.09 ± 0.09</td>
<td>0.27 ± 0.21</td>
<td>0.25 ± 0.19</td>
<td>0.10 ± 0.10</td>
<td>0.18 ± 0.21</td>
</tr>
<tr>
<td>EC visibility (G/A/P) (n)</td>
<td>6/12/2</td>
<td>6/12/2</td>
<td>1</td>
<td>3/2/0</td>
<td>3/2/0</td>
<td>1/3/1</td>
<td>1/3/1</td>
<td>1/3/1</td>
<td>1/3/1</td>
<td>1/3/1</td>
<td>1/3/1</td>
</tr>
<tr>
<td>CCT (µm)</td>
<td>1178 ± 147</td>
<td>1165 ± 147</td>
<td>0.341</td>
<td>1185 ± 162</td>
<td>1183 ± 171</td>
<td>1171 ± 144</td>
<td>1120 ± 144</td>
<td>1174 ± 148</td>
<td>1169 ± 118</td>
<td>1181 ± 184</td>
<td>1187 ± 187</td>
</tr>
<tr>
<td>Transparency (G/M) (n)</td>
<td>2/18</td>
<td>2/18</td>
<td>1</td>
<td>0/5</td>
<td>0/5</td>
<td>1/5</td>
<td>1/5</td>
<td>0/5</td>
<td>0/5</td>
<td>1/4</td>
<td>1/4</td>
</tr>
<tr>
<td>Folding (M/H) (n)</td>
<td>0/20</td>
<td>0/20</td>
<td>1</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td><strong>After deswelling</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECD (cells/mm²)</td>
<td>2172 ± 231</td>
<td>2281 ± 216</td>
<td>0.002</td>
<td>2214 ± 287</td>
<td>2280 ± 243</td>
<td>2158 ± 182</td>
<td>2245 ± 233</td>
<td>2157 ± 108</td>
<td>2276 ± 181</td>
<td>2159 ± 350</td>
<td>2225 ± 243</td>
</tr>
<tr>
<td>CV (%)</td>
<td>51.2 ± 3.0</td>
<td>28.0 ± 4.4</td>
<td>0.002</td>
<td>50.9 ± 2.2</td>
<td>28.5 ± 6.7</td>
<td>30.7 ± 4.0</td>
<td>27.2 ± 2.9</td>
<td>31.1 ± 2.4</td>
<td>28.2 ± 3.9</td>
<td>32.4 ± 3.7</td>
<td>28.2 ± 4.7</td>
</tr>
<tr>
<td>Hexagonal cells (%)</td>
<td>46.8 ± 3.5</td>
<td>44.9 ± 3.3</td>
<td>0.048</td>
<td>46.3 ± 3.8</td>
<td>46.5 ± 4.3</td>
<td>47.4 ± 4.0</td>
<td>42.8 ± 1.3</td>
<td>46.9 ± 3.7</td>
<td>46.0 ± 2.8</td>
<td>46.6 ± 3.5</td>
<td>44.2 ± 3.6</td>
</tr>
<tr>
<td>Mortality (%)</td>
<td>0.03 ± 0.06</td>
<td>0.05 ± 0.08</td>
<td>0.091</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.05 ± 0.06</td>
<td>0.03 ± 0.07</td>
<td>0.00 ± 0.00</td>
<td>0.07 ± 0.09</td>
<td>0.08 ± 0.10</td>
<td>0.09 ± 0.10</td>
</tr>
<tr>
<td>EC visibility (G/A/P) (n)</td>
<td>1/1/18</td>
<td>8/5/7</td>
<td>&lt;0.01</td>
<td>0/0/5</td>
<td>3/2/0</td>
<td>0/1/4</td>
<td>1/1/5</td>
<td>0/0/5</td>
<td>2/2/1</td>
<td>1/0/4</td>
<td>2/0/3</td>
</tr>
<tr>
<td>CCT (µm)</td>
<td>613 ± 58</td>
<td>672 ± 96</td>
<td>0.004</td>
<td>610 ± 28</td>
<td>627 ± 65</td>
<td>611 ± 61</td>
<td>642 ± 72</td>
<td>608 ± 54</td>
<td>629 ± 67</td>
<td>615 ± 93</td>
<td>787 ± 88</td>
</tr>
<tr>
<td>Transparency (G/M) (n)</td>
<td>18/2</td>
<td>18/2</td>
<td>1</td>
<td>5/0</td>
<td>4/1</td>
<td>5/0</td>
<td>4/1</td>
<td>4/1</td>
<td>4/1</td>
<td>5/0</td>
<td>5/0</td>
</tr>
<tr>
<td>Folding (M/H) (n)</td>
<td>18/2</td>
<td>4/16</td>
<td>&lt;0.01</td>
<td>5/0</td>
<td>1/4</td>
<td>4/1</td>
<td>1/4</td>
<td>5/0</td>
<td>1/4</td>
<td>4/1</td>
<td>0/5</td>
</tr>
<tr>
<td>EC loss (%)</td>
<td>16.1 ± 3.2</td>
<td>9.7 ± 3.4</td>
<td>&lt;0.001</td>
<td>16.9 ± 2.1</td>
<td>11.9 ± 2.8</td>
<td>14.6 ± 1.2</td>
<td>9.0 ± 3.8</td>
<td>17.6 ± 4.0</td>
<td>8.1 ± 4.3</td>
<td>15.3 ± 4.5</td>
<td>9.7 ± 2.2</td>
</tr>
<tr>
<td>Thickness reduction (%)</td>
<td>47.7 ± 5.6</td>
<td>41.9 ± 8.1</td>
<td>0.002</td>
<td>48.0 ± 5.4</td>
<td>46.6 ± 3.6</td>
<td>47.4 ± 6.8</td>
<td>42.2 ± 7.4</td>
<td>47.5 ± 8.5</td>
<td>45.6 ± 9.2</td>
<td>47.9 ± 2.5</td>
<td>35.2 ± 4.6</td>
</tr>
</tbody>
</table>

A, all four poloxamers analyzed together; B, C, D, E, details for each poloxamer without statistics, given that data had already been analyzed. Quantitative data are expressed as the mean ± SD. Qualitative data are expressed as the number. ECD, endothelial cell density; CV, coefficient of variation of cell area; Mortality, instantaneous EC mortality using 0.4% trypan blue; G/A/P, good/average/poor; CCT, central corneal thickness; G/M, good/mediocre; M/H, minimal/high.
**EC Mortality.** Instantaneous cell death was comparable between the two groups before and after deswelling (Table 1).

**Endothelial Visibility.** Before deswelling, cell visibility was comparable between the two groups. After deswelling, transparency improved and was comparable between the two corneas. However, folding improved more markedly in dextran than in the P338 medium (bottom). P188, P237, and P407 behaved in the same way.

**Corneal Stroma. Central Corneal Thickness.** Before deswelling, CCT was comparable between the two groups. After deswelling, corneas in poloxamer were significantly thicker than in dextran, but the difference was only 61 μm (Cl95 26–96). All poloxamers tended to behave similarly except for P407, which appeared less efficient (Table 1).

**Transparency and Folding.** Before deswelling, transparency and folding were comparable between the two groups. After deswelling, transparency improved and remained comparable in the two groups (Table 1). Folding improved more markedly in the dextran group (Fig. 3).

**Histology and Electron Microscopy**

Of the 20 pairs of corneas, 14 were processed for histology and electron microscopy (four each for P188 and P237, Table 2. Histologic and Ultrastructural Examination of Corneas Deswollen in Dextran versus Poloxamers

<table>
<thead>
<tr>
<th>Technique/Parameter</th>
<th>Dextran (n = 14)</th>
<th>Poloxamers (n = 14)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial thickness (μm)*</td>
<td>20 ± 8</td>
<td>23 ± 9</td>
<td>0.328</td>
</tr>
<tr>
<td>Number of keratocytes (n/500 μm)</td>
<td>126 ± 16</td>
<td>129 ± 15</td>
<td>0.220</td>
</tr>
<tr>
<td><strong>TEM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC thickness (μm)</td>
<td>3.20 ± 1.15</td>
<td>3.61 ± 1.44</td>
<td>0.314</td>
</tr>
<tr>
<td>Density of collagen fibers (n/μm²)</td>
<td>425 ± 86</td>
<td>368 ± 119</td>
<td>0.099</td>
</tr>
<tr>
<td>Diameter of collagen fibers (nm)</td>
<td>28.7 ± 2.6</td>
<td>29.7 ± 4.0</td>
<td>0.345</td>
</tr>
<tr>
<td>Interfibrillar distance (nm)</td>
<td>54.4 ± 4.4</td>
<td>59.0 ± 8.2</td>
<td>0.075</td>
</tr>
<tr>
<td><strong>SEM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microvilli (L/H) (n)</td>
<td>11/3</td>
<td>12/2</td>
<td>1</td>
</tr>
<tr>
<td>Cilia (L/H) (n)</td>
<td>7/7</td>
<td>9/5</td>
<td>0.445</td>
</tr>
<tr>
<td>Intercellular interdigitations (L/H) (n)</td>
<td>7/7</td>
<td>10/4</td>
<td>0.246</td>
</tr>
<tr>
<td>Damaged cells (L/H) (n)</td>
<td>12/2</td>
<td>14/0</td>
<td>0.481</td>
</tr>
</tbody>
</table>

Quantitative data are expressed as the mean ± SD. Qualitative data are expressed as the number. TEM, transmission electron microscopy; SEM, scanning electron microscopy; EC, endothelial cell; L/H, low/high.

* n = 12 in dextran and poloxamer groups for epithelial thickness analysis.

**Figure 3.** Stromal transparency and Descemet’s membrane folding of the same paired corneas before and after deswelling: example of dextran T500 versus P338. Transparency and folding were comparable before deswelling (top). After deswelling, transparency improved and was comparable between the two corneas. However, folding improved more markedly in dextran than in the P338 medium (bottom). P188, P237, and P407 behaved in the same way.
three each for P338 and P407). Controls consisted of 3 OC corneas without deswelling. Considering the small number for each poloxamer, details are given only for the 14 pairs together (Table 2).

**Histology.** *Epithelium.* One pair in P338 and one in P407 lost their epithelia during cross-sectioning. Consequently 12 pairs of corneas were available. Epithelial shedding was observed in both groups. The central epithelium contained one to four layers (Fig. 4). There was no difference in epithelial thickness between the two groups (Table 2). The mean thickness of the epithelia of the control corneas was 20 ± 3 μm.

**Stroma.** Collagen fiber organization was of similar appearance in both groups. Keratocytes were long, slender, tapering profiles (Fig. 4). There was no difference in the number of keratocytes per 500-μm length of cross section between the two groups (Table 2). The number of keratocytes in control corneas was 131 ± 16.

**Transmission Electron Microscopy.** *Endothelium.* The EC before deswelling measured 5.28 ± 1.53 μm. After deswelling, EC thickness decreased and was similar in both groups (Table 2). Many cytoplasmic vacuoles were present in the ECs, some of which contained dense materials that were not observed before deswelling. The nuclei, organelles and intercellular junctions appeared intact. Very few of the ECs showed severe injuries, such as lysed organelles and damaged cytoplasm membrane (Fig. 5).

**Posterior Collagen.** The posterior collagen fibers in both groups appeared to have uniform diameter and normal organization after deswelling (Fig. 4). The diameter and density of collagen fibers were comparable in the two groups (Table 2). Collagen fiber density correlated with interfibrillar distance (r = 0.956, P < 0.001).

**Keratocyte.** Keratocyte profiles in the posterior stroma were broad in shape and showed irregular and indented nuclei surrounded by many organelles. Before and after deswelling, a large number of vacuoles were present in the keratocytes. No dense material was observed after deswelling (Fig. 4).

**Scanning Electron Microscopy.** We observed a polygonal pattern of EC mosaic after deswelling in the two experimental groups and the controls without deswelling. The cell surface was flat after deswelling and flanked with elongated microvilli and cilia. Sometimes, two or three cilia were present on one EC surface, irrespective of the macromolecule used. The intercellular interdigitations were elaborate in all groups before deswelling. Lysed cells seemed more frequent in the dextran group than in the poloxamer group (not significant) (Table 2, Fig. 5).

**Control Group for Endothelial Assessment without Deswelling**

A mean difference in ECD of 6.4% ± 0.9% was observed between the two cell counts (from 2216 ± 343 to 1983 ± 338, P = 0.005). CV increased from 24.0% to 27.7% (P = 0.017), while hexagonal cell percentage decreased from 51.5% to 44.3% (P = 0.028).

**DISCUSSION**

Poloxamers with a safety profile are widely used for pharmaceutical and medical applications, and their biocompatibility has been proven.18,28,29 Despite various ophthalmic applications, they have never been used for eye banking except for P188 in two ex vivo studies: first in OC4 and then in hypothermic storage.30 In the present study, we investigated the efficacy and tolerance of five poloxamers for deswelling human corneas during 48 hours after prolonged storage in an OC medium containing 2% FCS that is widely used throughout Europe. The inclusion of paired human corneas of quality comparable to those actually grafted allowed a strict compar-

---

**TABLE 2.** Deswelling parameters of the experimental groups.

<table>
<thead>
<tr>
<th>Poloxamer</th>
<th>Stroma Thickness (μm)</th>
<th>Keratocyte Density (keratocytes/500 μm)</th>
<th>ECD (%)</th>
<th>CV (%)</th>
<th>Hex. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P188</td>
<td>111 ± 5</td>
<td>134 ± 12</td>
<td>23.4 ± 2</td>
<td>25.0 ± 2</td>
<td>47.6 ± 4</td>
</tr>
<tr>
<td>P338</td>
<td>112 ± 4</td>
<td>132 ± 10</td>
<td>23.5 ± 2</td>
<td>27.7 ± 4</td>
<td>44.3 ± 6</td>
</tr>
<tr>
<td>P407</td>
<td>111 ± 6</td>
<td>131 ± 12</td>
<td>23.6 ± 2</td>
<td>26.8 ± 4</td>
<td>45.2 ± 5</td>
</tr>
<tr>
<td>P605</td>
<td>110 ± 3</td>
<td>130 ± 10</td>
<td>23.7 ± 2</td>
<td>28.5 ± 4</td>
<td>44.0 ± 5</td>
</tr>
<tr>
<td>P920</td>
<td>112 ± 5</td>
<td>133 ± 14</td>
<td>23.4 ± 2</td>
<td>26.0 ± 4</td>
<td>46.2 ± 6</td>
</tr>
</tbody>
</table>

Note: ECD = endothelial cell density; CV = coefficient of variation; Hex. = hexagonal cell percentage.
ison of these macromolecules with the reference molecule, dextran T500.

Apart from P124, which proved toxic, we identified an effect that was common to the remaining four poloxamers. They exert sufficient osmotic colloid pressure to deswell corneas significantly and increase corneal transparency while better preserving the ECs, nearly halving cell loss compared with dextran. Further, our study again confirms the toxicity of 48 hours of deswelling in 5% dextran T500 on corneal ECs after prolonged OC storage. There seemed to be minor differences among the poloxamers in their ability to reverse stromal edema. P188 and P338 appeared to promote the deswelling more efficiently. The water-retention capability of poloxamers depends on the length of hydrophilic polyoxyethylene chains as well as on the polyoxyethylene-polyoxypropylene proportion, described as the hydrophilic-lipophilic balance. P188 and 338 have a higher hydrophilic–lipophilic balance, which may explain their apparently greater efficacy in corneal deswelling.

Poloxamers have another advantage over dextran: they allow better EC visualization with 0.9% NaCl incubation. Endothelial visualization is essential for corneal EC assessment during OC, and it is well known that endothelial visualization after incubation with NaCl is not possible for cornea in dextran-containing medium, probably because of its high viscosity. Eye banks that now assess corneas during or after deswelling in dextran have to use hypotonic solution to obtain satisfactory endothelial delineation. Endothelial visibility after deswelling in poloxamer is therefore an advance in graft quality control, allowing a final endothelial evaluation at that point to measure more accurately the actual ECD provided to the recipient.

Reduction of folding, a parameter rarely assessed to date, probably because of the lack of a measurement method, seemed disappointing with the poloxamers. One explanation is that poloxamers are surfactants that cause the medium to spread in a thin layer over the endothelial surface and thus highlight the folds, whereas the viscous dextran medium covers the folds, rendering the surface smooth and the folding artificially less visible. Nevertheless, this does not seem to affect graft quality, given the improved transparency and reduced corneal thickness after deswelling.

The morphologic study showed similar structural behavior in the two groups. Uptake of macromolecules by ECs, vacuolization in ECs and keratocytes, and shedding of epithelial cells observed in the poloxamer group have previously been described in corneas deswollen in dextran and do not appear

---

**FIGURE 5.** Scanning (A, B, E, F, I, J) and transmission (C, D, G, H, K, L) electron micrographs of corneal endothelium. (A, B) The endothelial surface before deswelling was undulating and showed a polygonal pattern of ECs. Prominent microvilli, cilia, and interdigitated apical flaps of cell borders were seen. (C, D) The ECs contained numerous vacuoles and swollen mitochondria. (E, F, I, J) After deswelling in dextran and poloxamer, the endothelial surface became flat, and the ECs maintained their polygonal pattern. The appearance of microvilli, cilia, and intercellular interdigitations was similar to that before deswelling. (G, H, K, L) Some vacuoles in ECs contained dense materials that were not observed before deswelling. Original magnifications: (A, E, I) ×800; (B, F, J) ×3,500; (C, G, K) ×10,000; (D, H, L) ×30,000. Arrows: primary cilia.
prohibitive for graft quality. Because the accumulation of deswelling agents in the cornea is time dependent and dynamic from surface to stroma, the 48-hour deswelling time used in our study prevents their further accumulation in the cornea, thus substantially limiting their toxicity. The collagen fiber density obtained in our study was lower than that reported by Muller et al. In this study, we examined only the posterior stroma of corneas deswollen for 48 hours after prolonged OC, whereas Muller et al. evaluated the whole stroma of corneas preserved for at least 7 days in a dextran-containing medium without previous storage in dextran-free medium. Because corneal swelling and deswelling occur mainly in the posterior stroma, the different swelling state of the stroma in the two studies may explain our differing results. Elongated primary cilia and numerous microvilli were observed on human corneal endothelium after OC storage, compared with the few microvilli and very occasional cilia reported in fresh corneas. Further, we found prominent surface structures after deswelling. Primary cilium is probably a ubiquitous component from surface to stroma, the 48-hour deswelling time flow or with chemical or osmotic regulation of ECs. The primary cilia and numerous microvilli were observed on human corneal endothelium after OC storage; compared with the few microvilli and very occasional cilia reported in fresh corneas. Further, we found prominent surface structures after deswelling. Primary cilium is probably a ubiquitous component from surface to stroma, the 48-hour deswelling time flow or with chemical or osmotic regulation of ECs. The presence of numerous microvilli has been attributed to stromal cell border modification. Possible hypotheses are active metabolic pump function or environmental stress.

For the first time, we reveal that there may be a large difference in ECD between a count using NaCl combined with trypan blue and one using alizarin red 48 hours later. The possible link between EC loss and endothelial counting in OC has been investigated only by Sperling, who suggested that trypan blue and 0.45% and 0.9% NaCl were atrumatic. However, in his study a smaller number of ECs were counted, with a manual method. Van Doooren et al. showed that exposure of corneal endothelium to 0.1% trypan blue for less than 30 minutes was not harmful to the ECs. Although our experiment used a higher concentration of dye, the endothelium was exposed for only 1 minute, which seems unlikely in itself to induce 6.4% EC loss. The cell loss observed may be related to different endothelial visualization induced by NaCl or alizarin red during EC counting and/or to actual toxicity of corneal handling, exposure to NaCl and trypan blue, oxidative stress, and several minutes of microscope lighting. Further studies are required. Whatever the explanation, this finding implies that actual cell loss induced exclusively by poloxamers or dextran should be lower than that observed.

In conclusion, during OC at 31°C in medium containing 2% FCS, P188, P237, P338, and P407 at 350 mOsM/kg appeared superior to 5% dextran T500 for human corneal deswelling in the last 48 hours before grafting, in their ability to preserve the ECs and improve EC visibility after deswelling. These poloxamers were equally effective in deswelling the cornea and increasing stromal transparency. The poloxamers induced moderate morphologic injuries similar to those observed in the dextran group. Of our four candidates, P188 and P338 are likely to be more efficient for corneal deswelling. Our study, combining in vitro and ex vivo experiments on human corneas, provides a reasonable level of confidence to allow advancement to a clinical trial.

Acknowledgments

The authors thank Isabelle Anselme, Electron Microscopy Center (Saint-Etienne University) and Samar Salloum, Biochemistry Department (Saint-Etienne University Hospital) for technical assistance.

References


