Animal Compound–Free Medium and Poloxamer for Human Corneal Organ Culture and Deswelling

Gilles Thuret,1,2 Chloe Manissolle,2 Lydia Campos-Guyotat,2 Denis Guyotat,2 and Philippe Gain1,2

PURPOSE. Eliminating fetal calf serum (FCS) from corneal organ culture (OC) media has long been a challenge. This study was an assessment of a new animal compound–free (ACF) medium for corneal storage and of its combination with poloxamer for end-of-storage corneal deswelling.

METHODS. A randomized controlled study with masked assessment compared the ACF medium to standard commercialized media containing 2% FCS and their combination with poloxamer for deswelling. Paired human corneas were randomly allocated at procurement, one to the ACF medium and the other to the FCS media, and then assessed at day (D)2 and D30 of OC storage and after 48 hours of deswelling. Comparison criteria were endothelial cell density (ECD) and morphometry by a corneal analyser, quality of endothelial visualization (using saline), EC mortality (trypan blue), corneal thickness, corneal transparency, and folding. Fifty-six corneas (28 pairs) with ECD of 2000 cells/mm² or more were enrolled. Data were compared using paired tests with P < 0.01 deemed significant.

RESULTS. Parameters were similar at baseline (D2) between groups. Daily EC loss during the 30 days of storage was reduced with the ACF compared with standard (−0.31% ± 0.50% vs. −0.88% ± 0.38%, P < 0.001). With poloxamer 188 (Lutrol F68; BASF, Ludwigshafen, Germany), EC loss was substantially reduced (−1.43% ± 3.60% vs. −15.41% ± 10.13%, P < 0.001) and morphometry better preserved, despite thickness reduction, transparency improvement and folding reduction comparable to dextran. After 30 days of storage in ACF medium and deswelling in poloxamer 188, ECD was 30% higher (2466 ± 447 cells/mm² vs. 1729 ± 281 cells/mm², P < 0.001). ACF medium alone and combined with poloxamer 188 considerably facilitated EC visualization at D30 and after deswelling.

CONCLUSIONS. The ACF medium combined with poloxamer 188 for deswelling showed superiority over standard FCS medium in its ability to preserve EC viability and facilitate endothelial visualization. This innovative use of poloxamer for deswelling appears far less toxic than does dextran. (Invest Ophthalmol Vis Sci. 2005;46:816–822) DOI:10.1167/iovs.04-1078

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Organ culture (OC) at +31°C to 37°C is the corneal storage technique of choice in Europe, used exclusively by the 21 French eye banks and most other European banks. According to the 2004 directory of the European Eye Bank Association,1 61% of 34,140 corneas were OC-stored in 2002. Prolonged storage facilitates hospitalization of the recipient and cornea exchange, allows microbiologic safety checks to be performed to minimize the risk of infectious disease transmission, and allows the quality of the corneal endothelium to be checked before delivery of the cornea.2 If endothelial cell density (ECD) declines below 2000 cells/mm² at end of storage, the threshold applied by most eye banks,3 the cornea is not used for penetrating keratoplasty. Likewise, excessive loss of endothelial cells (ECs), marked anomalies in endothelial morphology (polymegathism or pleomorphism), and excessive cell mortality (traditionally assessed by vital staining with trypan blue) are further causes of cornea discarding. Thus, an endothelial problem causes 10% to 20% of corneas to be discarded at the end of OC.3

OC storage media, whether commercial or produced by eye banks, derive directly from traditional cell culture media. They are very similar in composition, with a base of Eagle’s minimal essential medium (MEM) or its variant Dulbecco’s MEM, supplemented by 2% of fetal calf serum (FCS), which provides many growth factors. Some media also contain other animal compounds such as chicken feather and pig bone amino acids, and compounds such as proteins, lipids, hormones and inorganic trace elements. These media prolong the metabolic activities of human ECs, which thus survive for several weeks.2,4,5 The importance of FCS in the survival of human ECs in OC is poorly covered in the literature, essentially by indirect arguments. FCS is thought to protect cultured porcine corneal ECs against endotoxins.6 Porcine corneal OC in a serum-free medium causes massive EC loss after 1 week of storage, with near-total EC mortality at 4 weeks.7 More generally, FCS is thought to better resist cell stresses.8 The only direct evidence of the beneficial role of FCS is provided by Bednarz et al.9 who showed that human corneal OC in serum-free MEM causes very high EC loss after 2 weeks due to the appearance of necrotic areas.

Whatever the actual role of FCS, the presence of a bovine substance of not fully defined composition, in a medium that spends several weeks in contact with tissue intended for human grafting, is a major problem for health authorities. Although it is accepted that the risk is minute, given the use of controlled herds in New Zealand and Australia, these sera come from pools of many animals. Total health safety cannot be guaranteed regarding prions (in particular variant Creutzfeldt-Jakob disease), animal viruses, and bacteriophages.10,11 In France, these media have the uncertain status of "subsidiary therapeutic products,"—that is, they belong in neither the medicinal product category nor the implantable medical device category. For example, in 1999, corneal graft activity was suspended in France for several days while the Agence Française de Sécurité Sanitaire des Produits de Santé (French Health Products Safety Agency) checked the origin and compliance of FCS batches used by OC media manufacturers.
Moreover, the possibility of high variability between FCS batches makes the standardization of OC media, and so their final validation by the health authorities, very difficult. Indeed, a difference in serum quality with a direct adverse effect on the quality of graft tissue in recipients has already been reported in the literature.12

There is an important and urgent need, in the view of clinicians and legislators, to develop and clinically validate an OC medium that is FCS free and, more generally, animal compound free (ACF). To date, several serum-free media have been assessed ex vivo9,13–17 but did not achieve better EC survival than the usual media containing serum. Moreover, no serum-free medium has had clinical results published nor has any been used by the ophthalmological community.

The purpose of the present controlled study was to present a new ACF OC medium that achieves better long-term endothelial preservation than conventional media containing 2% FCS and to describe its innovative combination with a deswelling macromolecule other than dextran.

**Materials and Methods**

**Storage: ACF Medium versus Fetal Calf Serum Medium**

For reasons of industrial secrecy, our laboratory was not fully party to the composition of the ACF medium (Stem Alpha, Saint-Clément Les Places, France). However, it was based not on conventional cell culture media such as RPMI, Dulbecco’s MEM, Eagle’s MEM, M199, or Ham’s but on synthetic molecules. It contained carbon hydrates, a HEPES buffer, metabolic precursors, trace elements, minerals, vitamins, an antioxidant system, phenol red as a pH indicator, 0.1 mg/mL streptomycin, 100 IU/mL penicillin, and 0.25 μg/mL amphotericin B. Notably, it contained no substance of animal or human origin and no growth factor.

The control storage media are both marketed in France and both contain 2% FCS: Inosol (Chauvin Bausch and Lomb, Labège, France) and CorneaMax (Eurobio, Les Ulis, France). Their very similar compositions allowed comparable EC survival, as shown by a previous controlled study on paired corneas.18

**Deswelling: Poloxamer versus Dextran**

The deswelling medium was the storage medium supplemented by poloxamer 188 (Lutrol F68; BASF, Ludwigshafen, Germany), the macromolecule replacing the dextran T500 used in conventional media. Of high molecular weight (8400 kDa), it is widely used in the pharmaceutical industry, including ophthalmic pharmaceuticals, for the composition of eye drops19 and contact lens cleaning solutions.20 Our laboratory had previously studied its efficacy in deswelling the corneal stroma and its endothelial nontoxicity in conventional media (data not shown). This novel use is protected by a patent application by our university (Université Jean Monnet, St-Etienne, France).

The control deswelling media were the two manufacturers’ hyperosmolar versions, both containing 5% dextran T500: Exosol (Chauvin Bausch and Lomb) and Corneaujet (Eurobio).

**Randomization of Organ Culture**

For this study, 35 pairs of scientific human corneas were procured at the anatomy laboratory of the Saint-Etienne Faculty of Medicine from non–heart beating donors who had donated their body to science. Body donation to science is a well-established, respectable practice in France, quite different from organ or tissue donation for therapeutic use. It provides medical faculties with bodies for students and surgeons, and with precious tissue for research. Corneas were retrieved by in situ corneoscleral excision, according to the protocol recommended in France.21 Each of the two corneas was immediately immersed in 100 mL medium: one in the control medium and the mate in the ACF medium. Fifteen pairs were stored in the Inosol/ACF medium pair and 20 in the CorneaMax medium/ACF medium pair. At no point during monitoring did any analyzed parameter differ between the corneas stored in Inosol and those stored in CorneaMax (data not shown). The control group was thus deemed consistent. The media, which were all the same color, were packaged under a laminar flow hood in identical hermetic sterile Lexan-poly carbonate bottles (Nalge Nunc International, Rochester, NY) and masked by an independent person. Allocation of each cornea to the ACF medium or control medium was randomized. The numbering system rendered unpredictable the allocation of media according to the numbers marked on the bottles. The full procedure, from procurement to statistical analysis of the results, was thus conducted by investigators who were blind to the medium used.

The OC scheme exactly reproduced the storage procedure routinely used by many eye banks. The corneas were stored at +31°C in sealed bottles in a dry incubator for 30 days, the maximum time currently authorized for commercial media. The medium was renewed first at day (D)2 and then at D16. At D30, the corneas were placed in the relevant deswelling medium for 48 hours.

**Comparative Assessment of Corneas**

**Endothelial Mortality, Visibility, Cell Density and Morphometry.** Three endothelial examinations were conducted: at D2 (start of storage), D30 (end of storage), and D32 (after deswelling). Only cornea pairs with >2000 cells/mm² at D2 continued in storage and were considered for the remainder of the study (10 pairs in Inosol/ACF, 18 pairs in CorneaMax/ACF). The endothelium was observed using the usual techniques: assessment of instantaneous cell death by incubation for 1 minute with 0.4% trypan blue (Sigma-Aldrich, St-Quentin Fallavier, France), then for 4 minutes with 0.9% sodium chloride (Sigma-Aldrich), to render the intercellular spaces visible for counting and endothelial morphometry. EC visualization quality with this protocol was graded with a three-level scoring system described elsewhere,18 and took account of cell borders, background noise, and the surface area of ECs visible in a microscopic image. The score was deemed ‘good’ if visualization of the cell borders was very good, there was low or no background noise, and cells were visible on two thirds or more of the image area; “average” if visualization of the cell borders was good, background noise was moderate, and cells were visible on one third to two thirds of the image area; and “poor” if the cell borders were hard or impossible to visualize, background noise was high, and cells were visible on one third to two thirds of the image area and “poor” if the cell borders were hard or impossible to visualize, background noise was high, and cells were visible on one third to two thirds of the image area.

**Stromal Thickness, Transparency, and Folding.** Measurements were obtained at D30 and after deswelling, but not at D2, to prevent any risk of microbiological contamination at the start of storage due to contact with the ultrasonic sensor.

Stromal transparency and folding were evaluated at the same time according to the following protocol: the two paired corneas were photographed side by side on a back lit chart formed by eight black lines of increasing thickness. Two masked observers then scored the two parameters according to a two-level system. Discordant appraisals were reviewed. Transparency was “good” if all the lines were clear or
Coefficient of variation of cell area (%) D2 28.6
Cell mortality (trypan blue) (%) D2 0.02
Cell loss during 48 hours of deswelling (%) —
Daily cell loss during storage (%) —

Hours (range, 5–72).

432 cell/mm² (99% confidence interval [CI], 273–591) in favor of the ACF group, or 18% more ECs than in the control group. Eighty-six percent (24/28) of ACF group corneas retained an ECD higher than the delivery threshold of 2000 cells/mm², against 46% (13/28) of those in the control group (P = 0.002; Fig. 1). After deswelling in poloxamer, the difference increased to 737 cells/mm² (99% CI, 585–889), or 30% more ECs than with deswelling in dextran.

At the end of storage and deswelling, total cell loss was 8.8% (99% CI, 5.0–12.6) in the ACF+poloxamer group against 36.2% (99% CI, 33.5–38.8) in the control+dextran group (P < 0.001). The percentage of corneas having maintained an ECD higher than 2000 cells/mm² decreased only slightly in the ACF+poloxamer group (82%, 23/28) but declined to 18% (5/28) in the control group (P < 0.001; Fig. 1).

EC Mortality. Cell death was comparable between the two groups during storage (D2 and D30) and after deswelling (D32).

Morphometry. During storage (D2 and D30), the coefficient of variation of cell area and the percentage of hexagonal cells were comparable between the two groups. After deswelling in poloxamer, both parameters were significantly better than after deswelling in dextran.

Quality of Endothelial Visualization. At the start of storage (D2) endothelial visualization was comparable between the two groups, whereas at D30 it was markedly better in the ACF group (Table 2). After deswelling in poloxamer, the difference was still marked. The endothelium was rarely visible in the dextran group, as shown in Fig. 2.

Stroma

Thickness. At the end of storage (D30), pachymetry was comparable between the two groups (Table 3). After deswelling in poloxamer, corneas tended to be slightly thinner, by a mean of 26 μm (99% CI −4 to +56), than those in the dextran group (P = 0.022, not significant).

Transparency and Folding. At the end of storage (D30), transparency and folding were comparable between the two groups (Table 3). After deswelling, both parameters improved in both groups but remained comparable, as shown in Figure 3.

Table 1. Endothelial Assessment of the 28 Pairs of Corneas, at the Start (D2) and End (D30) of Storage and after 2 Days of Deswelling

<table>
<thead>
<tr>
<th>Measured Parameter</th>
<th>Timing</th>
<th>ACF Medium+Poloxamer*</th>
<th>FCS Medium+Dextran*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelial cell density (cells/mm²)</td>
<td>D2</td>
<td>2702 ± 445 (2007 to 3829) 2737</td>
<td>2716 ± 444 (2001 to 3682) 2665</td>
<td>0.724</td>
</tr>
<tr>
<td></td>
<td>D30</td>
<td>2495 ± 407 (1577 to 3194) 2521</td>
<td>2063 ± 368 (1302 to 2728) 1972</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>After deswelling</td>
<td>2466 ± 447 (1441 to 3217) 2478</td>
<td>1729 ± 281 (1206 to 2345) 1703</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cell mortality (trypan blue) (%)</td>
<td>D2</td>
<td>0.02 ± 0.08 (0 to 0.4) 0</td>
<td>0.03 ± 0.08 (0 to 0.3) 0</td>
<td>0.726</td>
</tr>
<tr>
<td></td>
<td>D30</td>
<td>0.02 ± 0.004 (0 to 0.3) 0</td>
<td>0.03 ± 0.007 (0 to 0.4) 0</td>
<td>0.345</td>
</tr>
<tr>
<td></td>
<td>After deswelling</td>
<td>0.01 ± 0.03 (0 to 0.14) 0</td>
<td>0.04 ± 0.15 (0 to 0.8) 0</td>
<td>0.068</td>
</tr>
<tr>
<td>Coefficient of variation of cell area (%)</td>
<td>D2</td>
<td>28.6 ± 3.7 (21.0 to 39.4) 27.8</td>
<td>29.8 ± 5.1 (25.1 to 46.6) 28.5</td>
<td>0.172</td>
</tr>
<tr>
<td></td>
<td>D30</td>
<td>25.3 ± 3.0 (21.3 to 31.7) 24.8</td>
<td>26.2 ± 4.2 (18.6 to 37.5) 25.8</td>
<td>0.267</td>
</tr>
<tr>
<td></td>
<td>After deswelling</td>
<td>25.8 ± 4.2 (20.3 to 35.1) 25.0</td>
<td>30.9 ± 4.6 (19.8 to 40.7) 31.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hexagonal cells (%)</td>
<td>D2</td>
<td>56 ± 7 (41 to 73) 56</td>
<td>52 ± 7 (36 to 65) 52</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>D30</td>
<td>52 ± 6 (43 to 62) 51</td>
<td>47 ± 7 (32 to 67) 48</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td>After deswelling</td>
<td>50 ± 5 (41 to 58) 51</td>
<td>44 ± 5 (36 to 54) 44</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The results as a whole are expressed as the mean ± SD (range) median.

* Added solely for deswelling.

only the finest line was blurred or “mediocre” if two or more lines were blurred. Folding was “minimal” if the lines showed little or no deformation, and “high” if they showed high deformation or appeared broken.

Statistical Analysis

The normality of the data distribution was tested using both the Kolmogorov-Smirnov test and the Shapiro-Wilk normality test, with the cutoff for non-normality set at P < 0.05. The quantitative variables were compared with a paired t-test in the case of normal distribution and otherwise by a nonparametric test (Wilcoxon). The image quality scores and transparency scores were compared by the χ² test in 2 × 2 grids. Given the high number of statistical analysis performed, P < 0.01 was deemed significant.

Results

Donor Characteristics

Donors were 16 women and 12 men with a mean age of 78 ± 12 years (range, 38–94). Death to retrieval time was 27 ± 19 hours (range, 5–72).

Endothelium

EC Density. At D2, ECDs in the two groups were comparable (Table 1). At D30, there was a significant difference of 432 cell/mm² (99% confidence interval [CI], 273–591) in favor of the ACF group, or 18% more ECs than in the control group. Eighty-six percent (24/28) of ACF group corneas retained an ECD higher than the delivery threshold of 2000 cells/mm², against 46% (13/28) of those in the control group (P = 0.002; Fig. 1). After deswelling in poloxamer, the difference increased to 737 cells/mm² (99% CI, 585–889), or 30% more ECs than with deswelling in dextran.
DISCUSSION

This randomized controlled study, with blind assessment of the results, clearly shows that the ACF medium preserved ECs after 30 days of OC better than did standard media containing 2% FCS. In addition, the innovative use of the poloxamer 188 macromolecule for stromal deswelling, instead of dextran T500, achieved a comparable reduction in corneal thickness but with far lower cell loss. The defined ACF medium and the poloxamer, entirely derived from biochemical synthesis, eliminated any risk of anthropozoonosis transmission and variability problems linked to the use of FCS or any other molecule of animal origin.

Development of a defined ACF OC medium has been a long-standing goal. Three approaches have been considered. Stoiber et al. tested for OC a serum-free medium derived from a medium for short-term corneal storage at +4°C (Eurosol; Bausch and Lomb, Irvine, CA). Other teams selected, from several serum-free media, an ACF medium developed for the multiplication of ECs of umbilical veins (Endothelial Serum-Free Medium; Life Technologies/Invitrogen, Carlsbad, CA). Last, other researchers kept the MEM base but replaced the 2% of FCS with β-FGF or ovalbumin. We took a different approach, combining our laboratory’s expertise in corneal banking and that of an ACF medium manufacturer with no experience in ophthalmology. Having tested nearly 25 prototypes (data not shown), we developed a defined medium specifically suited to corneal OC. The results obtained by the aforementioned teams with FCS elimination were difficult to compare among themselves and with our results because the methodology and assessment criteria differed. Only three studies directly compared human corneas that had undergone randomized storage either in a conventional medium (MEM + 2% FCS) or an FCS-free medium. In terms of ECD preservation, the Eurosol medium produced results similar to those of the conventional media during 5 weeks of OC—the Endothelial Serum-Free Medium and the MEM + ovalbumin during the first 3 weeks only. Our series is the first to show, through a controlled study, an unequivocal benefit from preserving ECs with an ACF medium. Cell preservation at D30 of storage, which was 18% greater than with FCS/dextran, represents an unprecedented improvement in OC technique. It is important to note that our test had several interesting methodological features: a high number of corneas (n = 28 pairs) allowing statistical comparisons; inclusion only of pairs of corneas with a starting ECD greater than 2000 cells/mm² and so theoretically fit for clinical use; immersion of corneas directly in the media under test, without prior placement in another medium containing animal compounds; strict reproduction of routine OC conditions (with medium renewal under laminar flow hood at D2 and D14, as routinely practiced in

Figure 1. Box plot of ECDs and percentage of the 28 pairs of corneas that could in theory have been delivered for penetrating keratoplasty. Dotted line: the threshold of 2000 cells/mm² typically accepted for cornea transplantation. The boxes represent the interquartile distances with the median (horizontal solid bar). Circle: density > 1.5 times the interquartile space. Left: storage in ACF medium (D2 and D30) with deswelling in poloxamer. Right: storage in FCS medium (D2 and D30) with deswelling in dextran.

Table 2. Quality of Endothelial Visualization of the 28 Pairs of Corneas at the Start (D2) and End (D30) of Storage and after 2 Days of Deswelling

<table>
<thead>
<tr>
<th>Timing (Score)</th>
<th>ACF Medium+Poloxamer*</th>
<th>FCS Medium+Dextran*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2 (G/A/P), %</td>
<td>64/36/0</td>
<td>68/32/0</td>
<td>0.778</td>
</tr>
<tr>
<td>D30 (G/A/P), %</td>
<td>89/11/0</td>
<td>7/54/39</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>After deswelling (G/A/P), %</td>
<td>86/14/0</td>
<td>0/4/96</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Quality was graded as good (G), average (A) or poor/impossible (P) and is expressed as percentages.

* Added solely for deswelling.
most French eye banks); triple measurement series, at the start and end of storage and after deswelling; measurement of ECD, an essential assessment criterion, by an image analysis system and on a large number of cells, thus ensuring objective and accurate results; immediate measurement of cell mortality; analysis of morphometry, which some eye banks are starting to consider important; and assessment of transparency and stromal folding, which ophthalmic surgeons deem paramount.

Before this study, our experiments entailed selection of a family of macromolecules (poloxamers) for stromal deswelling to replace the dextran typically used or the hydroxyethyl starch suggested by certain investigators but not used by the community.26–28 One of these, poloxamer 188, promoted stromal deswelling that was as effective as dextran in reducing thickness (by 39% in both cases), resulting in similar transparency and folding. Most important, this effective deswelling was accompanied by 10 times less EC loss and better preserved morphometric parameters. The present assessment, both quantitative (ECD) and qualitative (morphometry), provides undeniable confirmation of the endothelial toxicity of dextran, a long-term issue.29–32 As Lin et al.,33 have pointed out this toxicity is probably accentuated on endothelia rendered fragile, notably by prolonged storage, as in our study (30 days). It is also noteworthy that had ECD been measured in conditions of special osmotic dilation (see below) during deswelling, as is the policy in certain eye bank, only 18% of the dextran group corneas would have been delivered against 82% of the poloxamer group corneas. This is a key point, because in most European countries, the number of corneas delivered by eye banks consistently falls short of demand.

In a previous controlled study, we stressed the role of the storage medium in endothelial visualization.18 In the present study, observation of the endothelium was made markedly easier by storage in ACF medium. This should constitute a substantial advance in graft tissue quality control and in ECD measurement, which we have shown to have limitations in France.34,35 Moreover, the use of poloxamer allows visualization of the endothelium at the end of deswelling, which is very rare with dextran, at least in typical conditions of osmotic dilation with 0.9% sodium chloride. However, an endothelial examination at that point would be, as we mentioned earlier, a far better assessment of the number of ECs actually provided.
to the recipient. Such an examination, which is routinely performed in some European eye banks (personal sources), requires dilation of the intercellular spaces by a hypotonic buffered saline solution with a toxicity that is difficult to assess. The use of poloxamer instead of dextran may make it possible to eliminate this solution and to observe the endothelium in typical conditions.

An animal experiment is under way that entails the first application of the ACF medium to a living organism. In addition, the Agence Française de Sécurité Sanitaire des Produits de Santé has just authorized the use of this medium and poloxamer in humans as part of a multicenter controlled comparative clinical trial, due to start soon (Projet Hospitalier de Recherche Clinique référence AOL 2004-289). This trial will compare the preservation of ECs in recipients of corneas stored in ACF medium and then deswelled by poloxamer and of those stored in FCS media and deswelled by dextran.

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References