Comparison of Different Methods of Glycerol Preservation for Deep Anterior Lamellar Keratoplasty Eligible Corneas

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PURPOSE. To compare different methods of glycerol-preserved corneas intended for deep anterior lamellar keratoplasty (DALK).

METHODS. We analyzed transparency, transmittance, thickness, biomechanics, morphology, and antigenicity of donor corneas preserved by four different glycerol-based methods (n = 6 per group) for 3 months, as follows: tissues in anhydrous glycerol without aluminosilicate molecular sieves at room temperature (GRT); tissues in anhydrous glycerol with aluminosilicate molecular sieves at room temperature (SRT); tissues in anhydrous glycerol without aluminosilicate molecular sieves at –78°C (G78); and tissues in anhydrous glycerol without aluminosilicate molecular sieves at –20°C (G20).

RESULTS. Slit lamp images and transmittance curves obtained by spectrophotometer show that the G78 cornea was the most transparent tissue. Stress-strain behavior indicated that corneas in the G78 group were the most pliable, and SRT corneas were the stiffest. Electron microscopy analysis indicated that corneal cytoarchitecture and keratocyte integrity was destroyed in all glycerol-preserved corneas. Disorganized stromal collagen fibers were evident in groups stored at RT. Especially in SRT corneas, parallelism was lost, fibrils were extremely tortuous and discontinuous, and widespread fibril degeneration could be found. Antigenicity of tissue, assessed via immunohistochemistry for CD45-positive cells, HLA-ABC and HLA-DR, was not found. Antigenicity of tissue, assessed via immunohistochemistry for CD45-positive cells, HLA-ABC and HLA-DR, was not found.

CONCLUSIONS. Anhydrous glycerol preservation without molecular sieves in a –78°C freezer was the best method to obtain DALK-eligible tissues that were both transparent and pliable. (Invest Ophthalmol Vis Sci. 2012;53:5675–5685) DOI: 10.1167/iovs.12-9936
As follows: tissues in anhydrous glycerol without aluminosilicate molecular sieves at room temperature (GRT); tissues in anhydrous glycerol with aluminosilicate molecular sieves at room temperature (SRT); tissues in anhydrous glycerol without aluminosilicate molecular sieves at ~78°C (G78); tissues in anhydrous glycerol with aluminosilicate molecular sieves at ~20°C (G20). Donor corneas used for GRT in this study came from two different sources: Global Sight Network (GSN), Birmingham, Al, and Wenzhou Eyebank, Wenzhou, Zhejiang, China. Donor corneas for SRT came from GSN only. All GPCs in the four groups were kept in storage for 3 months. Fresh donor corneas served as a comparison group for these GPCs.

**Experimental Methods**

Frozen corneas were thawed before rehydration and further analysis. All glycerol-preserved tissues were rehydrated at room temperature for 30 minutes in balanced salt solution (BSS) before the following laboratory experiments were conducted. Assays were chosen to assess five properties important to the usability of glycerol-preserved tissue as an allograft material for DALK. All experiments were conducted under the pre-established flow in a time-locked manner. Such, each GPC in different groups was tested individually from slittlamp observation, light transmittance to thickness, then biomechanical behavior or immunohistochemical staining and electron microscope observation (one cornea cut in half for two testings). The experimental flow chart and the number of samples in each assays is shown in Figure 1.

**Transparency.** Using a spectrophotometer (UV-Visible-NIR Spectrophotometer U-4100; Hitachi High-Tech, Tokyo, Japan), total light transmittance (the sum of parallel and dispersed light transmittance) of the corneal tissues was measured from 280 to 800 nm. The percent transmission was determined with a resolution of 1 nm at a rate of 600 λ/min. In each donor sample, the area of central cornea was measured from 280 to 800 nm. The percent transmittance (the sum of parallel and dispersed light transmittance) of one cornea cut in half for two testings. The experimental flow chart and the number of samples in each assays is shown in Figure 1. All GPCs in the four groups were tested individually from slit-lamp observation, light transmittance to thickness, then biomechanical behavior or immunohistochemical staining and electron microscope observation (one cornea cut in half for two testings). The experimental flow chart and the number of samples in each assays is shown in Figure 1.

**Morphology of Collagen Fibrils.** Corneal tissues were first thoroughly rinsed with 0.1 mol/L sodium cacodylate buffer (pH 7.4) and then preserved by immersion in 2% glutaraldehyde in 80 mM sodium cacodylate (pH 7.4, 320–340 mOsm/kg) overnight at 4°C. All subsequent steps were performed at room temperature unless noted. After thorough fixation, 1 × 3-mm pieces were cut from the central region, rinsed in cacodylate buffer, and postfixed by immersion in freshly prepared 1% osmium tetroxide in 100 mM cacodylate buffer for 1 hour under subdued lighting. Samples were again washed in cacodylate buffer and dehydrated through a graded alcohol series (30% ~100% in six steps). Samples were then infiltrated with propylene oxide (3 changes at 10-minute intervals) and a 3:1 vol/vol mixture of propylene oxide and Spurr's resin (product number 4300, Electron Microscopy Sciences, Fort Washington, PA) for 3 hours. This was followed by overnight immersion in a 1:1 vol/vol mixture of propylene oxide and Spurr's resin, followed by transfer to 100% Spurr's resin overnight. Tissue samples were oriented in embedding molds and left for overnight polymerization at 60°C. Thick transverse sections (0.5–1 μm) were cut and stained with toluidine blue for light microscopic observations and to determine tissue orientation. Ultra-thin sections were obtained and mounted on parallel-bar copper grids (115 μm bar spacing) (product number 2415C-XX, SPI Supplies, West Chester, PA). Sections were double-stained first in 3.5% aqueous uranyl acetate for 20 minutes at 60°C, followed by Reynold's lead citrate for 10 minutes. Grids were examined in a transmission electron microscope (H-7650; Hitachi, Tokyo, Japan). Micrographs were chosen at random for analysis.
Antigenicity. Sections of formalin-fixed, paraffin-embedded tissue blocks were used for immunohistochemical analysis. Sections were de-paraffinized, followed by antigen retrieval with epitope retrieval solution (10 mmol citrate buffer, pH 6.0; Dakocytomation Inc., Carpinteria, CA) in an autoclave (121°C, 20 minutes). Endogenous peroxidase was blocked by a peroxidase blocking solution (ChemMate; Dakocytomation). Primary antibodies (HLA Class I ABC antibody [ab70328]; 1:100; HLA Class II DR antibody [ab20181]; 1:100; CD45 antibody [ab10559], 1:1000; Abcam, Cambridge, UK) were applied to sections at 4°C overnight. Thereafter, sections were incubated with streptavidin-biotin complex (Simple Stain MAX-PO kit; Nichirei, Tokyo, Japan) for 30 minutes. Sections were treated with 3,3'-diaminobenzidine (Dakocytomation) for 5 minutes and counterstained with hematoxylin. Slides were examined by light microscopy, a positive reaction being indicated by a brown color. Sections for which primary antibodies were omitted were used as negative controls (not shown). Sections of fresh thymus tissue served as positive controls (not shown).

RESULTS

Transparency

As shown in Figure 2, both GRT and SRT corneas transmitted less light than the two frozen GPCs (G78 and G20) at all wavelengths. Transmission of the G78 corneas was closest to that of fresh cornea of all the groups, and it decreased gradually with shorter wavelengths. This result is consistent with slit-lamp observation (Fig. 3), which revealed that G78 corneas were the most transparent, and SRT corneas were extremely cloudy and edematous. As summarized in the Table, average percent transmission between visible light wavelengths differed significantly among the among the four groups (P < 0.05). The transmission value for one fresh cornea, which had

![Figure 2. The percent transmission curves for glycerol-based corneas in four groups and one fresh cornea. Each point on each curve represents an average of the five corneal tissues in each group. The entire light spectrum is shown on the horizontal axis. Red curve, G78 group; blue curve, G20 group; green curve, GRT group; magenta curve, SRT group; black curve, one fresh cornea for control.](image)

![Figure 3. Slit-lamp images of glycerol-based corneas in four preservation groups show the G78 cornea is transparent just as fresh tissue, but the SRT cornea looks cloudy and extremely edematous.](image)
been preserved in intermediate-term media for 12 days, was 66.6 % as control.

**Thickness**

Mean center thicknesses of glycerol-preserved corneas are shown in Figure 4. The mean corneal thickness in the SRT group was dramatically increased to $1651 \pm 112.5 \, \mu m$ after 30 minutes of rehydration. There were statistically significant differences among groups SRT, GRT, and G78, but not between G20 and G78 groups ($P = 0.469$). Relative to the other three groups, the differences of GRT and SRT corneas were all significant ($P < 0.05$). There was no statistically significant difference between G20 and G78 groups ($P = 0.469$).

**Biomechanical Behavior**

Stress-strain behavior of corneal specimens was derived using shell theory. In all cases, stress-strain behavior followed an exponential function that was determined to be best fit by the average value within each group. Mean stress at each strain level was obtained for each of the four groups. As shown in Figure 5A, results demonstrated clear stiffening associated with higher preservation temperature. At the same strain levels, including low and high, there were consistent increases in stress associated with the high-preservation temperature. This test indicated that G78 corneas were the most pliable, and SRT corneas were the stiffest. The stress-strain behavior of a fresh corneal specimen is shown in Figure 5B as a reference.

**Ultrastructure**

Electron microscopy analysis indicated that keratocytes in a normal cornea usually lie between collagen lamellae. The cell body contains a large nucleus. After glycerol preservation, keratocytes were destroyed and collapsed to a certain extent in all four groups. As demonstrated, in GPC samples, organelles of keratocytes were absent, and the nucleus and cytoplasm were compact (Fig. 6).

Electron microscopy of the stroma reveals collagen fibrils cut perpendicularly and tangentially. Fibrils in a fresh cornea have uniform diameters and form compact parallel arrays (Figs. 7A, 8A). In contrast, the regular arrangement of collagen fibrils exhibited vast differences among 4 kinds of glycerol preservation methods with the same rehydration time. In G78 corneas, regularly arranged collagen fibrils re-approximated their original spacing when the tissue was rehydrated. Collagen fibrils also maintained parallelism and organization (Figs. 7B, 8B). In G20 corneas, fibrils maintained parallelism, but the spaces between the lamellae varied. There were blank regions, often called lakes, where fibrils are missing (Fig. 7C). In GRT corneas, parallelism was partially maintained, spaces between the lamellae became larger, fibrils were tortuous, and numerous lakes were found. Moreover, threadlike high-density shadows around the fibrils, represent-
ing degenerating fibrils, were apparent (Fig. 8C). In SRT corneas, parallelism was lost, fibrils were extremely tortuous and discontinuous, and widespread fibril degeneration could be found (Figs. 7D, 8D).

**Antigenicity**

After glycerol preservation, HLA-ABC was readily detected on corneal epithelium and limbus and rarely detected on endothelium and keratocytes in the anterior stroma beneath epithelium (Figs. 9A, 9B). HLA-DR was seen in corneal epithelium and was more abundant near the limbus. HLA-DR was not found in stromal keratocytes or in the endothelium (Figs. 9C, 9D). CD45-immunoreactivity was present in fewer cells than the other markers and was located dispersed on the limbus only (Fig. 10). Altogether, positive reactions for classes I and II, and CD45 were reduced in all glycerol-preserved corneas and were mainly located on corneal epithelium and limbus rather than stroma. For the two RT groups, immunoreactivity was further reduced.
A goal of the study was to determine how to best exploit properties of glycerol for preparing DALK-ready tissues. We showed organelles of keratocytes were absent, nucleus and cytoplasm were compact, and cellular antigens were lost through all four methods of glycerol preservation. Preservation in anhydrous glycerol without molecular sieves, stored at −78°C, was the best method to obtain DALK-eligible tissues.

Glycerol is a chemical compound commonly called glycerin. This colorless, odorless, and viscous liquid has three hydrophilic hydroxyl groups that impart both its solubility in water and its hygroscopic nature. Glycerol acts as a cryoprotectant for frozen tissue storage, because it binds strongly to water, prevents water molecules from joining a growing ice crystal, and forestalls the formation of damaging ice crystals. An early goal of cryo-biology was survival of healthy corneal endothelial cells after glycerol cryo-protection and freezing. Glycerol has excellent antibacterial, antifungal, and antiviral properties, well documented by literature on skin and bone banking. As a dehydrating agent, glycerol permits the use of GPC as scaffolding for procedures not requiring viable cell layers; however, tissues preserved using different formulations of glycerol have noticeably different properties. For example, skin preserved in 85% glycerol/15% water, a virucidal concentration, is also more supple than skin preserved in

**Figure 7.** Transmission electron micrograph of collagen fibrils in the midcentral stroma illustrating the arrangement of individual collagen fibrils within various lamellae (A, fresh cornea; B, G78; C, G20; D, SRT) (bar, 500 nm).
anhydrous glycerol. Bone grafts kept in 98% glycerol at room temperature exhibited similar bone matrix preservation to cryopreserved bone; no bacteria or fungi were found in the samples and there was no difference between the distributions of histomorphologic variables and cell populations. Thus, preservation protocols must be optimized for target tissue and intended surgical purpose.

Our results show, after the same rehydration time, the extent of corneal edema was remarkably greater in RT groups than cryo-preserved groups. This led to a significant decrease of transparency and increase of thickness for RT corneas. Especially for SRT cornea, the mean corneal thickness was dramatically increased to 1651.0 ± 112.5 μm after 30 minutes of rehydration, almost triple that of normal fresh cornea. Excessive stromal edema also influenced the corneal biomechanical behavior, which demonstrated stiffening associated with higher preservation temperature. GPC stored at −78°C was the most pliable cornea as compared with other preservation temperatures.

To investigate the effect of different preservation temperatures on stromal edema, we compared the ultrastructure of collagen lamellae using electron microscopy. The results show collagen fibers maintained parallelism and organization in G78 corneas. In SRT corneas, however, parallelism was lost and

![Figure 8](https://iov.s.arvojournals.org/lookup/suppl/doi:10.1167/iovs.12-10518/-/DCSupplemental/Figure8a.png)

**Figure 8.** Transmission electron micrograph of collagen fibrils in the midcentral stroma illustrating the parallelism of the collagen fibers (A. fresh cornea; B. G78; C. GRT; D. SRT) (bar, 500 nm).
Figure 9. The glycerol-preserved cornea (G78) show positive reaction (brown) for HLA-ABC (A, limbus; B, epithelium and anterior stroma) and HLA-DR (C, limbus; D, epithelium) (light microscopy; bar, 20 μm).
The fresh cornea and glycerol-preserved cornea show positive reaction (brown) for CD45 (A, fresh, limbus; B, fresh, epithelium and anterior stroma; C, G78, limbus; D, G78, epithelium) (light microscopy; bar, 20 μm).
fibris were extremely tortuous and discontinuous; moreover, widespread degeneration could be found around the fibris. We attribute the extreme edema of SRT cornea to destruction of corneal collagen structure and degeneration of collagen fibers. However, one limitation of this study is that the original corneal thickness was unmeasured and we cannot comment on the relative hydration of each cornea. Ideally, DALK-ready tissues, which are not dependent on living cells, would exhibit the following characteristics: an adequate thickness of stromal lamellae conferring appropriate strength and rigidity similar to fresh cornea, and suitable for intra-operative suture to host bed; quick restoration of transparency postoperatively, to provide an acceptably good visual acuity; and resistance of corneal stromal structure to postoperative melt. On the basis of our current data, we conclude that G78 cornea was the most eligible GPC for DALK.

Lamellar keratoplasty using GPC was established by the pioneering experimental and clinical studies of J.H. King,7,8,22,23 who first preserved corneas in 95% glycerol under vacuum to ensure an anhydrous state. Later, equivalent dehydration was achieved by including in the glycerol molecular sieves (sodium and calcium aluminosilicates) that, as physical adsorptive agents, removed water to an extremely low vapor pressure. With new surgical techniques and instrumentation innovation, corneal transplantation is now at a level of sophistication with a major paradigm shift from traditional penetrating keratoplasty (PK) to newer and innovative forms of lamellar keratoplasty aimed at targeted replacement of only diseased tissue layers.24 A recent editorial25 concluded that DALK is rapidly gaining acceptance as the procedure of choice for such patients whose endothelium is not compromised. DALK does not require donor endothelial cells. Therefore, it is the leading application for the use of acellular long-term preserved tissue. Of importance to this application, DALK performed with acellular corneas using lyophilization11 or glycerin-cryopreservation9,10 yields results equal to or superior to fresh corneas. GPC could decrease the possibility of immune rejection in the setting of DALK, which is mainly ascribed to its de-cellularized nature, as demonstrated in our previous clinical studies.3,10 Cells in the fresh corneal tissue, such as epithelium, keratocytes and bone marrow-derived cells, are important in corneal allograft response. De-cellularization has been successfully demonstrated to reduce tissue immunogenicity, and low antigenicity of the corneal tissue could reduce graft rejection and offer long-term graft survival. As demonstrated by Zhang et al.,26 anti-CD45 antibody plus complement-mediated targeting of ex vivo donor tissue was a highly efficient way to deplete corneal passenger leukocytes. Corneas de-cellularized by other methods such as lyophilization and treatment with detergents and proteases11,27,28 are also being explored for their potential as lamellar grafts; however, none of these methods have been used clinically up to now. Recently, Utine et al.29 reported that sterile gamma-irradiated corneas may be considered in lieu of fresh donor corneas for lamellar corneal patch grafts for lack of immunogenicity. However, these tissues required a new preoperative management for fresh corneas and relatively expensive equipment. In the present study, the results of immunohistochemistry showed that positive reaction for HLA-ABC, HLA-DR, and CD45 was reduced in all GPCs and was mainly located on corneal epithelium and limbus. The antigenicity of stroma was low or almost lost, which was consistent with our electron microscopy findings showing the keratocytes were collapsed and destroyed after glycerol preservation. This is more advantageous for DALK-ready tissues, whose epithelium and endothelium will be removed before surgical operation. Thus, among different long-term corneal-preservation techniques, the application of glycerol was a simple yet effective technique to achieve decellularization.30

The use of GPC in lamellar keratoplasty could increase the pool of corneas acceptable for transplantation. This potential advantage must not be overlooked in the emerging economies, where donor corneas and eye banks are limited. In 2010, US members of the Eye Bank Association of America, while distributing 59,271 corneas for transplantation, also recovered 9471 medically eligible tissues that were determined not usable for transplantation, because of low endothelial cell density, stromal scar, or small clear zone.31 GPCs from this latter source, preserved by GSN using the SRT method tested in the current study, are also being used as patch grafts for glaucoma drainage device surgery.32,33 For that purpose, GPC tissue clarity is adequate for laser lysis of sutures, and resistance to erosion is good up to 1 year postoperatively (Wigton E, Swanner J, Joiner DW, et al., manuscript submitted, 2012); however, in this study, the tissues with best potential for DALK were frozen quickly after immersion in glycerol and stored frozen. This preparation method would be most readily implemented in settings with a local eye bank, given the current regulations governing international tissue distribution. In conclusion, anhydrous glycerol preservation induced de-cellularization and reduced antigenicity. Anhydrous glycerol preservation without molecular sieves in a –78°C freezer was the best method to obtain DALK-eligible tissues that were both transparent and pliable. All methods of glycerol preservation exhibited loss of cellular antigens that may decrease rejection risk.

References


