Comparison of Fresh Corneal Tissue versus Glycerin-Cryopreserved Corneal Tissue in Deep Anterior Lamellar Keratoplasty

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PURPOSE. To compare the microstructural differences in fresh corneal tissue (FCT) with glycerin-cryopreserved corneal tissue (GCCT) used during deep anterior lamellar keratoplasty (DALK).

METHODS. The medical records of 48 consecutive patients who underwent DALK for stromal opacity without endothelial abnormalities were retrospectively reviewed. Patients were divided into two groups: an FCT group (n = 22) and a GCCT group (n = 26), according to the corneal tissue used. The best corrected visual acuity (BCVA), slit lamp, corneal topography, pachymetry, and laser scanning in vivo confocal microscopy examinations at 2 weeks and 1, 3, 6, 12, and 24 months after surgery were analyzed.

RESULTS. No graft rejection developed in the GCCT group, whereas stromal rejection developed in one eye in the FCT group. There were no significant differences in spherical equivalent (P = 0.37), astigmatism (P = 0.26), BCVA (P = 0.64), central corneal thickness (P = 0.73), or endothelial cell density (P = 0.49) between the two groups at 24 months. Confocal microscopy showed that GCCT was acellular, whereas dendritelike cells and keratocytes were found in the FCT group 2 weeks after surgery. The keratocyte density improved significantly in the GCCT group at 3 months after surgery, whereas it decreased significantly after surgery in the FCT group during follow-up. No significant difference in regeneration of nerve fibers was found in the subbasal layer and anterior stroma between the two groups at 24 months.

CONCLUSIONS. DALK using GCCT provides clinical results comparable to FCT. GCCT can be used safely and effectively for DALK and may minimize stromal rejection after surgery. (Invest Ophtalmol Vis Sci. 2010;51:775–781) DOI:10.1167/iovs.09-3422

Deep anterior lamellar keratoplasty (DALK) is a procedure that removes pathologic corneal stromal tissue down to Descemet’s membrane followed by transplantation of donor tissue. The advantages of DALK include elimination of endothelium rejection and prevention of long-term endothelial loss, while retaining favorable postoperative visual function.1–4 Although endothelial graft rejection after DALK has not been reported, rejection of the donor epithelium or stroma is a problem resulting in graft failure and visual loss without early intervention.5,6 Until now, most surgeons who perform DALK have preferred fresh corneal tissue (FCT), since fresh tissue is considered essential for successful corneal transplantation. However, cellular components of the FCT including the epithelium, keratocytes, and the bone marrow-derived cells are sources of major histocompatibility complex (MHC) antigens and minor H antigens7,8 that might be responsible for DALK rejection. One way to overcome this may be through transplantation of corneal substrates without their cellular components.

In previous lamellar keratorefractive procedures, such as epikeratophakia or keratophakia, the donor tissue is often glycerin-preserved or lyophilized, in which keratocytes were reported to be absent.9–12 In addition, experimental fresh epikeratophakia grafts implanted on a vascularized bed in a preensitized recipient may be rejected, whereas freeze-dried epikeratophakia lenticules are not.13 In a recent study14 in which the 6-month outcomes of DALK performed using lyophilized and preserved corneas were compared, the lyophilized corneas were used successfully during DALK to treat keratoconus with results similar to those achieved with preserved corneas. There is increasing interest in the use of acellular corneal tissue in DALK, but doubt remains over how this donor tissue can be integrated with the recipient cornea after DALK.

Laser scanning confocal microscopy is a quantitative, non-invasive confocal microscopy technique that enables assessment of corneal wound healing after keratoplasties in vivo under more physiologic conditions and highlights the behavior of the keratocytes.

The present study was performed to compare the visual outcomes, refractive results, and complications of DALK between FCT and glycerin-cryopreserved corneal tissue (GCCT) and to assess the characteristic confocal microstructural differences after surgery by using laser scanning in vivo confocal microscopy.

METHODS

Patient

We reviewed the medical records of the patients who underwent DALK at the Eye Hospital of Wenzhou Medical College between May 2005 and August 2008. Patients with infectious keratitis, trauma, bulbar keratopathy, or other ocular diseases (i.e., amblyopia, cataract, glaucoma, macular edema, or macular degeneration) were excluded. Inclusion criteria also required a minimum follow-up time of 24 months. Forty-eight eyes of 48 patients (25 men, 23 women) met the criteria and ultimately were included in the study. All patients received explanation about characteristics of both GCCT and FCT before surgery. Both types of tissues were used concomitantly. Twenty-two...
patients accepted GCCT voluntarily, and four patients received GCCT because FCT was not available. Twenty-two patients who refused GCCT were treated as a control FCT group. This research was approved by the Wenzhou Medical College Review Board, Wenzhou, Zhejiang, China, and was performed in accordance with the tenets of the Declaration of Helsinki.

Data were collected from routine examinations including the best corrected visual acuity (BCVA), slit lamp biomicroscopy, corneal topography, pachymetry using optical coherence tomography (OCT), and in vivo confocal microscopy of the cornea at 2 weeks and 1, 3, 6, 12, and 24 months after surgery.

**Donor Corneal Preparation**

Fresh donor corneas were placed in preservative (Optisol-GS, Alcon Laboratories, Inc., Fort Worth, TX) at 4°C. The preserved donor corneas were stored at −78°C in pure sterile glycerin. The average duration of cryopreservation before surgery was 7.8 months (SD, 2.1).

**Surgical Technique**

All DALKs were performed by the same surgeon (CW). The host cornea was trephinated to a depth of 60% to 80% and deepened with a sharp-tipped blade (15° slit knife) in the trephination groove to a depth of 80% to 90%. After a small stromal pocket was created with a Sinskey hook, a blunt-tipped iris spatula was gently inserted into a pocket to enlarge it using gliding rotating movements. The first layer with 80% to 90% of the cornea was removed using a curved blunt-tipped Vanna’s scissors. Subsequently, paracentesis was performed at the limbus to reduce the intraocular pressure, and sterile water for injection with no osmotic pressure was applied into the recipient bed to facilitate swelling of the stromal fibers that then can be manipulated easily with a Sinskey hook to form another stromal pocket. Host corneal tissue was removed layer by layer until Descemet’s membrane was exposed. A full-thickness graft without endothelium and Descemet’s membrane was sutured in place. The interrupted sutures were removed 3 months after surgery.

Microperforations in Descemet’s membrane developed in three eyes (two eyes in the GCCT group and one eye in the FCT group) during DALK, but only two eyes were complicated by formation of a second chamber after surgery, which resolved spontaneously within 3 days. No patient required intraoperative conversion to a penetrating keratoplasty.

Eyes in both groups were treated with topical antibiotics (levofloxacin, Tarivid; Santen Pharmaceutical Co., Osaka, Japan) and 0.1% prednisolone (Sanbetason; Santen Pharmaceutical Co.) six times daily. The eye drops were tapered over the following 3 months.

**VA Assessment**

The VA was measured by using the standard Snellen chart and BCVA withspectacle correction was recorded. The results were converted into logMAR units.

**OCT Pachymetry**

Each selected eye was imaged with the anterior segment OCT (Visante OCT Model 1000; Carl Zeiss Meditec, Dublin, CA). The Visante OCT is a noncontact, high-resolution tomographic and biomicroscopic device designed for anterior segment imaging and measurement. Analogous to an ultrasound B-scan, the Visante OCT acquires multiple A-scans and aligns them to construct two-dimensional images. Each eye was imaged three times in normal room light. The corneal thickness was imaged with the enhanced high-resolution corneal protocol (scan length, 16 mm; 256 A-scans) by one examiner. The OCT corneal measurements were used for donor, recipient, and total corneal thicknesses.

**Corneal Topography**

Astigmatism was measured by corneal topography. Three consecutive measurements (Orbscan II; Orbtec, Bausch & Lomb, Tampa, FL) were captured.

**In Vivo Confocal Microscopy**

Confocal microscopy was performed in the center of the cornea using a laser scanning confocal microscope (Heidelberg Retina Tomograph II with Rostock Cornea Module; Heidelberg Engineering, Dossenheim, Germany). The position of the fixation light was consistent for all patients. Each eye was anesthetized with one drop of 0.4% benoxinate hydrochloride (Santen Pharmaceutical Co.). A coupling agent (Viscotears, Carbomer 980, 0.2%; Novartis, North Ryde, NSW, Australia) was used between the applating lens and the cornea. The focal plane (the plane to be imaged) was moved manually. The full thickness of the central cornea was scanned using the section mode of the device.

The anterior stroma was defined as the first three clear images (without motion blur or compression lines) immediately posterior to Bowman’s layer; the posterior stroma was defined as the first three clear images immediately anterior to Descemet’s membrane; the mid stroma was defined as three images equidistant from Bowman’s layer and Descemet’s membrane in the full-thickness sections. Cell density was counted with a caliper tool (Analysis 3.1; Soft Imaging System, Munster, Germany). The amplitude of the field was 400 × 400 μm, and three images of each layer were chosen for analysis. The results were expressed in cells per square millimeter.

**Statistical Analysis**

The unpaired t-test was used to assess the statistical significance of the differences between the two groups in refractive results, cellular density, BCVA, and central corneal thicknesses at each time point. Intragroup mean changes between different time points were analyzed using the unpaired t-test. The statistical differences in the age, gender and trephination size between the two groups were analyzed using Fisher’s exact test and the Wilcoxon rank sum test. All tests were two-tailed, and P < 0.05 was considered significant.

**RESULTS**

**Patients**

The 26 patients who received GCCT were a mean age of 44.9 years (range, 22–60) with and a median follow-up time of 25.6 months (range, 24–33). Twenty-two patients who received FCT were a mean age of 45.5 years (range, 23–62). This group was followed for a median of 27.1 months (range, 24–31). No vascularization was found preoperatively in any eyes. Table 1 summarizes the characteristics and operative data of the study patients.

**Pachymetry**

The central corneal thickness in the GCCT group was significantly thinner than in the FCT group at 2 weeks (625.3 and 570.4 μm, P < 0.01), but it did not reach significance between the two groups at 24 months (523.8 and 525.4 μm, P = 0.73). The central corneal thicknesses in the two groups decreased significantly between 2 weeks and 3 months (P < 0.01 for both groups). Figure 1 shows the changes in the central corneal thickness during the follow-up. The OCT corneal images also showed that the interface between the donor and the recipient tissue was evident at 2 weeks and became indistinct 1 month after surgery (Fig. 2). The interface disappeared 3 months after surgery.

**BCVA and Refractive Results**

The average BCVA values at 24 months were 0.26 in the GCCT and 0.28 in the FCT groups (P = 0.64). In 19 (71.5%) of 26 eyes in the GCCT group, the BCVA was 0.3 or better, and in the FCT group, that level was achieved in 17 (77.3%) of 22 eyes at 24 months. The BCVA level in the two groups stabilized at approximately 6 months after surgery. Figure 3 shows the
changes in the postoperative BCVA during the follow-up period. The median spherical equivalent and astigmatism were similar in both groups at each follow-up visit. At the 24-month follow-up visit, the median spherical equivalent in the GCCT and the FCT groups were, respectively, 2.46 D (range, 1.50–8.00) and 1.81 D (range, 3.5–6.25; \( P = 0.37 \)), although the GCCT group had more myopia. The mean astigmatism was less than 2.50 D in both groups at 24 months. The BCVA, spherical equivalent, and spherical equivalent during the follow-up are shown in Table 2.

### Slit Lamp Biomicroscopy

Stromal rejection developed in one eye in the FCT group 14 months after DALK. Stromal edema and new vessels appeared in the anterior stromal layer (Fig. 4A). With early medical intervention, the new vessels could be reversed and the cornea again became clear (Fig. 4B). Rejection did not occur in any eye in the GCCT group. Four patients in the GCCT group developed small but persistent epithelial defects during the first postoperative week. These patients were extended-wear soft contact lenses and completely epithelized during the second postoperative week.

### In Vivo Confocal Microscopy

Confocal microscopy showed that GCCT was acellular, whereas dendritelike cells and keratocytes were found in the FCT group 2 weeks after surgery (Fig. 5). In the FCT group, 9 (40.9%) of 22 eyes had dendritelike cells with a density of 79 ± 42 cells/mm² in the basal epithelial cell layer at 2 weeks. The presence of dendritelike cells first was observed 1 month after surgery in the GCCT group. At 24 months, 10 of 22 eyes in the FCT group and 11 of 26 eyes in the GCCT group had dendritelike cells, and no significant difference was found in the density of the cells between the two groups (112 ± 51 cells/mm² in the FCT group and 97 ± 38 cells/mm² in the GCCT group, \( P = 0.68 \)). The keratocyte density increased significantly in the anterior stroma, mid stroma, and the posterior stromal layers in the GCCT group from 3 to 24 months after surgery (\( P < 0.01 \) for each comparison; Table 3). In contrast, the keratocyte density decreased significantly in the anterior stroma, mid stroma, and posterior stromal layers in the FCT group from 2 weeks to 24 months after surgery (\( P < 0.01 \) for each comparison). In the GCCT group, confocal scans showed small (<10-μm diameter), highly reflective, and condensed structures in the anterior keratocyte layers at 2 weeks (Fig. 5). The endothelial cell density decreased significantly from 2386.7 ± 469.0 cells/mm² at 2 weeks to 2079.7 ± 303.7 cells/mm² at 24 months after surgery in the GCCT group (\( P < 0.01 \)) and from 2492.7 ± 309.1 cells/mm² at 2 weeks to 2023.0 ± 342.3 cells/mm² at 24 months after surgery in the FCT group (\( P < 0.01 \)). No significant difference in the endothelial cell density was observed between the two groups at 24 months (Table 3; \( P = 0.23 \)). No nerve fibers were found in both groups at 2 weeks (Fig. 5). Regeneration of nerve fibers was observed in the anterior stroma from 6 months onward in both groups (Fig. 6). At 24 months, 10 of 22 eyes in the GCCT group and 8 of 22 eyes in the FCT group had regenerated stromal nerve fibers (\( P = 0.72 \)). Regeneration of nerve fibers first was observed in the subbasal layer at 12 months in the FCT group (\( n = 3 \)) and at 24 months in the GCCT group (\( n = 14 \); Fig. 6). No significant difference was found in the number of eyes with regeneration of the subbasal nerve fibers between the two groups at 24 months (14/26 eyes in the GCCT group and 10/22 eyes in the FCT group, \( P = 0.37 \)).

### DISCUSSION

In this retrospective study, we showed that DALK with GCCT provides results comparable to those obtained with FCT. GCCT can be used safely and effectively during DALK and theoretically can minimize stromal rejection after surgery.

In the present study, 77.3% of patients achieved 0.3 or better in the FCT group at 24 months, and 73.1% of patients in the GCCT group reached that BCVA level at 24 months. The BCVAs obtained with GCCT was comparable to those obtained with fresh corneas (\( P = 0.64 \)). The mean astigmatism was less than 2.5 D in both groups at the last follow-up visit. No difference was observed in astigmatism, central corneal thick.
ness, or endothelial cell density between the two groups at 24 months, indicating that GCCT provides similar clinical results to FCT. The only difference is the central corneal thickness 2 weeks after surgery in the GCCT group which was thicker than in the FCT group. This may be because the arrangement of the collagens in the stroma was meshy and distorted after the decellularization procedure, and it takes time to recover its original arrangement after surgery. GCCT stored at \(-78^\circ\text{C}\) has a long storage life, which can meet the emergent need of therapeutic DALK in countries in which there is an extreme shortage of donor corneas for transplantation.

The present study showed that GCCT is just a stromal collagen matrix for keratoplasty. Confocal scans showed that the stroma had low reflectivity and appeared to be predominantly an acellular zone between 2 weeks after surgery. In the acellular zone, small (<10 \(\mu\text{m}\) diameter), highly reflective, condensed structures were observed. These bright structures represent nuclear fragmentation, pyknosis, or both (Li HF, et al. \textit{IOVS} 1997;36:ARVO Abstract 1880). Preservation of the cornea by chemical glycerin-dehydration is a simplified technique to eliminate cells and create the acellular biological materials.

The GCCT group had a progressive increase in keratocyte density beginning 3 months after surgery. Farias et al.,\textsuperscript{14} who recently reported the results of DALK using lyophilized corneas in patients with keratoconus, found that keratocyte density increased at 6 months in the lyophilized corneas. However, they presented only the 6-month follow-up results and did not observe the microscopic confocal changes of keratocytes over time after surgery. In the present study, we found that the density of keratocytes with GCCT gradually increased during 24 months’ observation, even though it did not reach the level of FCT at the last follow-up examination. In contrast to the GCCT group, keratocyte density in the FCT group decreased significantly from 2 weeks to 24 months after surgery. Bourne\textsuperscript{18} also reported reduced keratocyte density after transplantation at every stromal level. The reason for the decreased keratocyte density in the cornea after transplantation is unclear. Increased apoptosis has been observed in transplanted corneas, particularly at the wound’s edge.\textsuperscript{19} Low endothelial cell loss was observed during the follow-up in both groups. The results of the present study tend to support the observations of Sugita and Kondo,\textsuperscript{2} who reported a 13% endothelial cell loss between 1 month and 2 years after DALK.

The results in the current series are consistent with the observation of Richter et al.\textsuperscript{20} that the first nerves in the superficial stroma of the graft center are observed 7 months after surgery. In the present study, regeneration of nerve fibers was observed in the anterior stoma from 6 months on in both groups. No significant differences were found in the number of eyes with regeneration of subbasal and stromal nerve fibers between the two groups at 24 months \((P > 0.05\) for each). This indicates that the cryopreservative procedures do not affect neural regeneration.

DALK has been advocated because the host endothelium is not replaced and the allograft rejection rate is far lower than that of penetrating keratoplasty.\textsuperscript{22,23} However, epithelial and stromal rejection still can occur and result in visual loss in DALK with fresh grafts, even in patients without risk factors for graft rejection.\textsuperscript{5,6} In the present study, one case in the FCT group also appeared to have stromal rejection after DALK. Allograft rejection may be triggered by two distinct pathways

![Figure 2](https://i0144.transform防卫.jpg)  
**Figure 2.** OCT images of the FCT group (A, C) and the GCCT group (B, D). An interface between the donor and recipient tissue is seen clearly 2 weeks after surgery and becomes indistinct 1 month after surgery. The central corneal thicknesses in both groups decrease between 2 weeks and 1 month.

![Figure 3](https://i0144.transform防卫.jpg)  
**Figure 3.** The mean postoperative changes in the BCVA in the two groups over time after DALK. The BCVA tends to improve from 2 weeks to 3 months \((P < 0.01\) in both groups) and stabilizes between 6 and 24 months \((P = 1.00\) in GCCT group and \(P = 0.61\) in the FCT group).
of allore cognition (i.e., the direct and indirect pathways). In the direct pathway, donor antigen-presenting cells (APCs) of the FCT present intact MHC class II molecules residing on their surface to T cells. In the indirect pathway, recipient APCs present processed MHC or minor antigens of FCT to T cells. In contrast, although human leukocyte antigens were detected on GCCT without live cells including resident dendritelike cells, epithelial cells, and keratocytes, the presence of antigens alone probably is insufficient to stimulate immune rejection. Therefore, cryopreservation can theoretically prevent not only direct sensitization but also indirect sensitization to donor MHC class II antigens. Zhivov et al. reported that dendritelike cells were found in the corneas of normal subjects. In the present study, using vivo confocal microscopy, we also found dendritelike cells on FCT. In contrast to FCT, GCCT was devoid of dendritelike cells (professional APCs) 2 weeks after surgery. This finding demonstrates that corneal preservation by chemical glycerin dehydration effectively depletes donor dendritelike cells to prevent direct sensitization. Dendritelike cells can be observed on GCCT from 1 month after surgery, and no significant difference was found in the density of dendritelike cells between the two groups at

### Table 2. BCVA, Spherical Equivalent, and Spherical Equivalent in the Two Groups during Follow-up

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<th>2 wk</th>
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**Figure 4.** Slit lamp biomicroscopy of the eye shows stromal rejection in the FCT group. (A) A slit lamp photograph of presumed stromal rejection 14 months after DALK for scarring shows stromal edema and new vessels. (B) With early intervention, new vessels and edema can be reversed. The final BCVA is 6/12.

**Figure 5.** In vivo confocal microscopy of the corneas in the FCT group (A–C) and the GCCT group (D–F) after DALK. (A) Dendritelike cells in the basal epithelial cell layer 2 weeks after surgery. (B) Keratocytes in the anterior stromal layers 2 weeks after surgery. (C) Keratocytes in the anterior stromal layers 24 months after surgery. (D) This GCCT cornea had no dendritelike cells 2 weeks after surgery. (E) This GCCT cornea had no keratocytes, but small (<10 μm diameter) and highly reflective and condensed structures were present in the anterior stromal layer 2 weeks after surgery. (F) Repopulation of the keratocytes in the anterior stromal layer at 24 months.
Density of endothelial cells

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<td>2492.7 ± 309.1</td>
<td>2386.7 ± 469.0</td>
<td>2448.5 ± 384.6</td>
<td>2291.2 ± 433.4</td>
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<td>GCCT</td>
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<td>49.2 ± 59.2</td>
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Data are expressed in cells per square millimeter.

24 months. APCs serve as immune sentinels to the foreign world, the repopulation of APCs on GCCT may contribute to its long-term health. Close monitoring and appropriate steroid and/or immunosuppressi
tive therapy are needed after DALK with FCT to prevent allograft rejection, particularly in high-risk patients. However, corticosteroids and immunosuppressive agents have a wide range of side effects including infection, cataract, and glaucoma. In contrast, using GCCT in DALK these side effects are avoided, because theoretically there is no need to use agents to prevent graft rejection.

In conclusion, laser scanning confocal microscopy offers exciting insight into different microstructural changes in DALK between GCCT donor cornea and FCT. In this study, GCCT was acellular at 2 weeks, which demonstrates that all cells including APCs, keratocytes, and other resident bone-marrow derived cells are not viable after glycerin-cryopreservation. This observation may have important implications for stromal rejection in DALK. Although this study was not randomized and the two groups were not matched for the type and severity of corneal pathogeneses, we still advocate GCCT for DALK, because glycerin-cryopreservation could be a simple and practical technique to minimize graft rejection and enlarge the source of donor tissue.

References