

Comparison of the Effects of Temperature and Dehydration Mode on Glycerin-Based Approaches to SMILE-Derived Lenticule Preservation

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Purpose: The aim of this study was to explore the optimal method of small-incision lenticule extraction (SMILE)-derived lenticules, subjected to long-term preservation using glycerol, under a range of temperatures, and using an array of dehydration agents.

Methods: In total, 108 myopic lenticules were collected from patients undergoing the SMILE procedure. Fresh lenticules served as a control group for this study, whereas all other lenticules were separated into 8 groups, which were preserved at 4 different temperatures (room temperature [RT], 4, -20, and -80°C) with or without silica gel in anhydrous glycerol. Evaluated parameters included thickness, transmittance, hematoxylin and eosin staining, transmission electron microscopy, and immunohistochemistry analyses.

Results: After a 3-month preservation period, lenticular thickness in these different groups was significantly increased, particularly for samples stored at RT. The mean percentage transmittance of lenticules stored at -80°C with or without silica gel was closest to that of fresh lenticules. Hematoxylin and eosin staining revealed sparsely arranged collagen fibers that were more scattered in preserved lenticules relative to fresh lenticules, particularly in RT samples. Transmission electron microscopy revealed that the fibril bundles densities in lenticules stored at RT were significantly less than those stored at other temperatures. Immunohistochemistry analyses revealed reductions in or loss of CD45 and human leukocyte antigens in all preserved lenticules relative to control samples.

Conclusions: Of the tested approaches, the preservation of SMILE-derived lenticules over a 3-month period was optimal at -80°C with or without silica gel in anhydrous glycerol.

Key Words: small-incision lenticule extraction, corneal stromal lenticules, glycerin-based, preservation

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Small-incision lenticule extraction (SMILE) is an increasingly common form of corneal refractive surgery wherein an intrastromal lenticule and a side cut are created with a femtosecond laser, yielding an intact refractive lenticule that can serve as a biomaterial with good optical transmittance, desirable mechanical properties, and excellent biocompatibility.¹ The implantation of such stromal lenticules has been successfully implemented in clinical settings to correct for hyperopia,^{2,3} presbyopia,^{4,5} and keratoconus,^{6,7} and for corneal patch grafting as a means of managing corneal perforations and other ocular diseases.^{8–10} However, fresh lenticules are generally used in these studies, greatly constraining their utility. It is, thus, vital that reliable, novel approaches to lenticule preservation be developed.

The reimplantation of cryopreserved lenticules has been shown to be both safe and effective as a means of treating a range of ocular diseases,^{11,12} and these preserved lenticules did not exhibit any apparent differences in subsequent wound healing when compared with fresh lenticules.¹³ Glycerol is commonly used for prolonged preservation of human tissues and organs, including red blood cells, skin, and bone

tissue.^{14–16} Different glycerol-based cryopreservation solutions, however, exhibit distinctive properties. Li et al¹⁷ and Tripathi et al¹⁸ previously compared different methods of glycerol-based corneas intended for deep anterior lamellar keratoplasty (DALK). To the best of our knowledge, this is the first study to assess the long-term effects of different temperatures and dehydration agents on the glycerol-mediated preservation of SMILE-derived lenticules.

MATERIALS AND METHODS

Subjects and Study Design

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethical Review Board of Fudan University Eye and ENT Hospital Shanghai, China. Informed consent was obtained from all patients.

In total, 108 corneal stromal lenticules were obtained from 64 myopic patients (44 bilateral cases and 20 unilateral cases) undergoing SMILE procedures at the Eye and ENT Hospital of Fudan University Shanghai, China. Of these lenticules, 53 (49%) corresponded to the right eye and 55 (51%) to the left eye.

An overview of the experimental flowchart used in this study is shown in Figure 1. In brief, extracted corneal lenticules were separated into 9 groups. The control group (group 9) was composed of fresh lenticules, whereas lenticules in the other 8 groups were preserved for 3 months using different glycerol-based approaches with or without the addition of silica gel (Sinopharm Chem Reagent Co, Ltd, Shanghai, China). Specifically, lenticules were preserved in anhydrous glycerol (Sigma Aldrich, St. Louis, MO) with (group 1) and without (group 2) silica gel at room temperature (RT); in anhydrous glycerol with (group 3) and without (group 4) silica gel at 4°C; in anhydrous glycerol with (group 5) and without (group 6) silica gel at -20°C; and in anhydrous glycerol with (group 7) and without (group 8) silica gel at -80°C. Thickness and transmittance were measured for all samples, as were hematoxylin and eosin (H&E) staining, transmission electron microscopy (TEM), and immunohistochemistry (IHC) analyses.

Corneal Lenticules Preparation

The fresh lenticules were preserved at 4°C in Optisol-GS (Bausch & Lomb, St. Louis, MO). The storage time in Optisol-GS before examination was less than 6 hours. Before detection, all lenticules were washed 3 or more times in the balanced salt solution. Besides, these glycerol-preserved lenticules required rehydration in the balanced salt solution for 20 to 30 minutes.

Surgical Technique

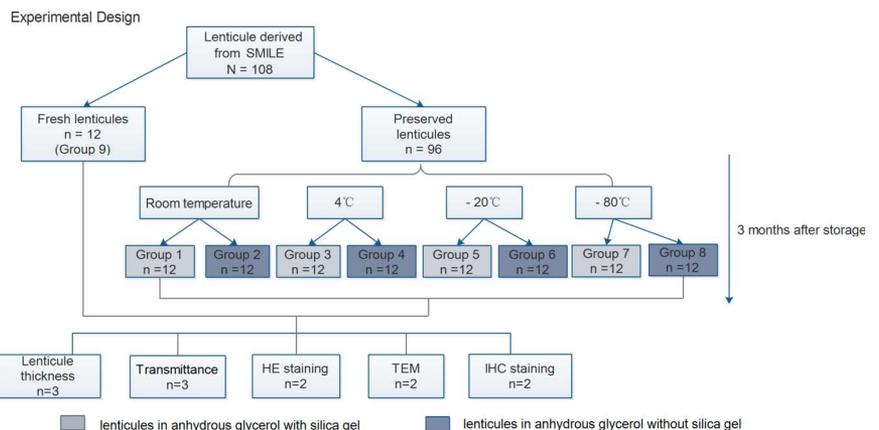
A single surgeon (X.T.Z.) conducted all SMILE procedures in this study as reported previously.¹⁹ Corneal stromal lenticules were generated with a 500-kHz VisuMax femtosecond laser (Carl Zeiss Meditec, Jena, Germany) at a pulse energy of 130 nJ. Lenticule diameters ranged from 6.5 to 6.8 mm, with the cap thickness of 110 to 120 μm and the cap diameter of 7.5 to 7.8 mm (1 mm larger than the lenticule). A small 2-mm wide side cut was made in the 12-o'clock position.

Measurements of Thickness

The lenticules were dried repeatedly on the soft absorbent paper. The center thickness of the corneal lenticule was monitored by taking the average of 3 measurements using an electronic thickness gauge (Model ET-3; CREATECH/REHDER DEV, Castro Valley, CA), the measurement precision of which was between positive and negative 2 μm. The lenticule tissue was tiled on a steel ball carrier and a probe automatically lowered to the anterior surface of the lenticule by a motorized drive. Once this probe touched the tissue, the real-time data would be displayed in the liquid crystal display monitor and would be recorded if it did not change within 3 seconds.

Measurements of Spectral Transmittance

Lenticule optical absorbance (*A*) was assessed with a Tecan Sharp instrument (Tecan, Männedorf, Switzerland) in the visible range from 380 to 780 nm. Lenticules were tiled on a 96-well plate, and the surrounding fluid was removed using a sterile sponge. Data were collected at the 10-nm



intervals, with spectral transmittance (T) being calculated as 10^{-A} .

Hematoxylin and Eosin Staining

Extracted corneal lenticules were fixed with 4% paraformaldehyde and embedded in paraffin, followed by deparaffinization using xylene, dehydration with an ethanol gradient, and washing twice with phosphate-buffered saline. Mounted samples were sliced into the 5- μ m sections and were stained for 3 to 5 minutes with hematoxylin after which they were rinsed with 1% acid alcohol. Sections were then dehydrated with an ascending ethanol gradient and were stained for 5 minutes with eosin. Sections were then again dehydrated as mentioned earlier, treated with dimethylbenzene, and sealed with neutral gum.

Transmission Electron Microscopy

Samples were minced into small ($1 \times 1 \times 3$ mm) pieces, after which they were fixed for 2 hours in a PBS solution containing 2.5% glutaraldehyde. Samples were then washed thrice with PBS (15 min/wash) and thrice in triple-distilled water (5 min/wash). Sections were then fumigated and calcined by carbonized osmium tetroxide for 0.5 to 2 hours. After 2 subsequent 100% acetone wash steps, samples were permeated with a combination of 100% acetone and epoxy resin and were fully embedded in a pure epoxy resin. After 3% uranyl acetate and lead citrate staining, ultrathin sections (70 nm) were imaged with a Tecnai G2 Spirit BioTWIN electron microscope (FEI, Portland, OR) at 120 kV.

Ultrastructural changes in analyzed lenticules were then evaluated in 10 randomly selected TEM images per group, exhibiting transverse section of collagen fibrils at the same magnification level, with the average fibril density and the number of fibrils in these sections being determined using ImageJ software.

Immunohistochemistry

Standard IHC protocols were used for this study. In brief, formalin-fixed paraffin-embedded tissue sections were deparaffinized, rehydrated, and antigen retrieval was conducted in an autoclave in citrate buffer with a pH of 6.0. A 3% hydrogen peroxide solution was used to block endogenous peroxidase activity, after which sections were blocked with 3% BSA for 30 minutes followed by incubation with primary antibodies (anti-HLA class I ABC [ab70328]; 1:100; anti-HLA class II DR [ab20181]; 1:100; anti-CD45 [ab10559]; 1:1000; Abcam, Cambridge, United Kingdom) at 4°C overnight. Secondary antibodies were then used to probe sections for 50 minutes, after which 3,3'-diaminobenzidine substrate was applied, and hematoxylin was used for counterstaining. Primary antibodies were omitted for negative control samples.

Statistical Analysis

SPSS v23.0 (SPSS Inc, Chicago, IL) was used for all statistical testing, and figures were prepared with GraphPad

Prism (v7.0; GraphPad Inc, San Diego, CA). Data are means \pm SD. The Kolmogorov–Smirnov test was used to assess data normality, with normally distributed data being assessed using Student t test, 1-way analysis of variance, and Kruskal–Wallis test as appropriate. Multiple comparisons were controlled for through Dunnett post hoc tests. A P value < 0.05 was the significance threshold.

RESULTS

In total, we analyzed 108 corneal stromal lenticules. There were no significant differences among lenticule groups regarding donor age, spherical equivalent, optical zone, or maximum thickness of lenticule (all $P > 0.05$, Table 1).

Thickness Analysis

Changes in thickness before and after preservation are compiled in Table 2. After a 3-month storage period, lenticular thickness among groups increased significantly, particularly in groups 1 and 2 (all $P < 0.05$).

Assessment of Transmittance

The mean spectral transmittance in different groups is summarized in Table 3. Relative to fresh lenticules, lenticules in groups 1 to 6 exhibited significant decreases in average transmittance ($P < 0.001$, $P < 0.001$, $P < 0.001$, $P = 0.005$, $P < 0.001$, and $P = 0.007$, respectively), whereas differences in groups 7 and 8 were not significant ($P = 1$ and $P = 1$, respectively).

Histological Analyses

H&E staining indicated that the morphological characteristics of preserved lenticules in these different groups were roughly similar. However, relative to fresh lenticules, the corneal lenticule tissue in groups 1 and 2 was notably looser with marginal haziness. Collagen fibers largely appeared to be well aligned, but some exhibited clear edema. Multiple cavitation bubbles of varying sizes were also evident, with 2 or more nuclei being apparent in one large bubble (Fig. 2).

Transmission Electron Microscopy

Figure 3 highlights the transverse collagen fibril section observed by TEM in every group. The number of fibrils at the same area in groups 1 to 9 was 1342.60 ± 148.02 , 1198.90 ± 126.24 , 1549.44 ± 248.57 , 1436.25 ± 180.57 , 1495.00 ± 124.27 , 1302.11 ± 65.32 , 1546.60 ± 95.86 , 1458.80 ± 139.39 , and 1508.30 ± 244.49 , respectively. The number of collagen fibrils in group 2 was significantly smaller than that in groups 3, 5, 7, and 9 ($P = 0.008$, $P = 0.011$, $P < 0.001$, and $P = 0.034$, respectively).

Longitudinal collagen fibril sections in preserved and fresh lenticules are shown in Figure 4, exhibiting general preservation of parallel and organized fibrils. Ruptured or absent fibrils were evident in groups 1 and 2. In addition, we detected necrotic keratocytes in each group (Fig. 5). Necrotic

TABLE 1. Baseline Characteristics of Donor Stromal Lenticules by Group

Group	Conditions	Donor Age (yrs)	SE (Diopters)	Optical Zone (mm)	Max Thickness (µm)
1	RT W SG	25.33 ± 5.71	-5.33 ± 1.66	6.70 ± 0.28	118.75 ± 13.61
2	RT WT SG	26.00 ± 5.21	-6.27 ± 1.45	6.68 ± 0.24	117.88 ± 11.00
3	4D W SG	26.22 ± 4.0	-6.08 ± 0.96	6.73 ± 0.11	119 ± 12.52
4	4D WT SG	24.62 ± 5.40	-6.27 ± 0.87	6.78 ± 0.05	121.11 ± 12.45
5	M20 W SG	24.44 ± 4.56	-6.26 ± 1.18	6.70 ± 0.18	120.56 ± 15.31
6	M20 WT SG	25.71 ± 4.89	-6.00 ± 0.80	6.79 ± 0.06	122.14 ± 11.98
7	M80 W SG	25.00 ± 4.35	-6.74 ± 1.42	6.67 ± 0.18	124.89 ± 14.05
8	M80 WT SG	23.83 ± 3.06	-6.39 ± 1.03	6.68 ± 0.19	122.81 ± 10.06
9	Fresh lenticules	26.5 ± 4.12	-6.17 ± 0.65	6.71 ± 0.10	121.75 ± 10.14
<i>P</i>		0.924	0.722	0.372	0.45

D, degrees; SE, spherical equivalent; M, minus; SG, silica gel; W, with; WT, without.

keratocytes were characterized by irregular nuclear chromatin clumping, organelle dissolution, cytoplasmic vacuolization, and membrane rupture.

Antigenicity

HLA-ABC, HLA-DR, and CD45 positivity were reduced or absent in preserved lenticules when compared with fresh lenticules (see Supplemental Figures 1–3, Supplemental Digital Contents 1–3, <http://links.lww.com/ICO/B275>, <http://links.lww.com/ICO/B276>, and <http://links.lww.com/ICO/B277>, respectively).

DISCUSSION

The SMILE procedure has become increasingly popular in recent years, and there has been a corresponding rise in the number of studies^{2–10} of the autologous and allogeneic transplantation of SMILE-extracted corneal lenticules. These preceding researches primarily use “fresh” and “unpreserved” lenticules, which limits the clinical applications of lenticules.

For the potential tissue donors, screening of communicable diseases is essential if the lenticule will be used as an allograft, which can take several days. Moreover, if it is possible to preserve the stroma lenticules with different designs, diameters, and thicknesses for a long period and build a catalog, it could better to meet the individual clinical requirements for the treatment of refractive error correction and other ocular diseases. As such, in-depth analyses of optimal approaches to the preservation of such SMILE-derived stromal lenticules will ensure the more reliable application of this approach in clinical practice.

Both glycerol and silica gel have been used previously for dehydration in the context of corneal preservation. Glycerol is a viscous, colorless, odorless liquid containing 3 hydrophilic hydroxyl groups that can readily permeate cells wherein they can interfere with the lattice-based formation of ice crystals. The hydrophobic hydrocarbon chains present within glycerol can also inhibit the permeation of water into cells, thereby protecting them.

We, in this study, evaluated the relative thickness, transmittance, ultrastructural properties, and antigenicity of SMILE-derived lenticules preserved under different temperatures and the effects of silica gel addition on these properties. We determined that the central thickness of these lenticules increased significantly after storage, particularly at RT. The

TABLE 2. Thickness Measurements Before and After Preservation Among Groups

Group	Conditions	Thickness at Baseline (µm)	Thickness after Preservation for 3 mo (µm)	<i>P</i>
1	RT W SG	122.17 ± 4.58	147.67 ± 3.88	<0.001
2	RT WT SG	117.17 ± 3.00	144.17 ± 3.49	<0.001
3	4D W SG	112.50 ± 2.26	119.84 ± 1.83	0.001
4	4D WT SG	117.50 ± 2.88	128.17 ± 7.78	0.012
5	M20 W SG	120.21 ± 3.71	124.66 ± 3.33	<0.001
6	M20 WT SG	120.83 ± 3.19	126.81 ± 3.87	0.002
7	M80 W SG	118.50 ± 5.17	123.33 ± 5.96	0.094
8	M80 WT SG	116.78 ± 4.89	122.00 ± 8.08	0.147
9	Fresh lenticules	113.42 ± 4.17		

D, degrees; M, minus; SG, silica gel; W, with; WT, without.

TABLE 3. Mean Spectral Transmittance in Each Group

Group	Conditions	Mean Spectral Transmittance (%)
1	RT W SG	67.98 ± 10.58*
2	RT WT SG	65.53 ± 10.98*
3	4D W SG	76.35 ± 6.89*
4	4D WT SG	77.40 ± 6.60†
5	M20 W SG	77.33 ± 4.76*
6	M20 WT SG	78.17 ± 4.75†
7	M80 W SG	81.26 ± 4.39
8	M80 WT SG	81.55 ± 4.59
9	Fresh lenticules	82.49 ± 3.90

**P* < 0.001, versus group 9 fresh lenticules.

†*P* < 0.01, versus group 9 fresh lenticules.

D, degrees; M, minus; SG, silica gel; W, with; WT, without.

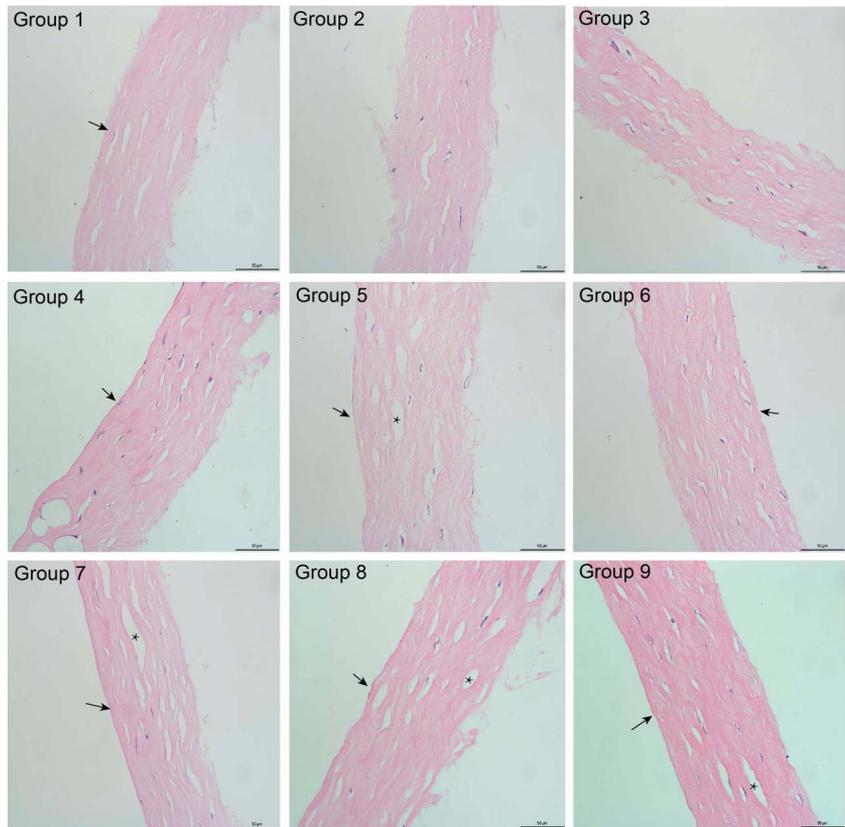


FIGURE 2. Representative images of H&E staining showed the histological changes of lenticules among different groups. The anterior surface of the lenticule (arrows) is relatively regular, and there are cavitation bubbles (asterisks) between layers (bar, 50 μ m). (The full color version of this figure is available at www.corneajrnl.com.)

reason might be that some of the water molecules in the lenticules were replaced by glycerin molecules, which could absorb a lot of water during rehydration, making the lenticules highly swollen. To maintain corneal lenticule transparency, it is important to preserve the regular collagen architecture therein.²⁰ Relative to fresh lenticules, we found that the average transmittance of lenticules preserved at RT, 4°C, and -20°C were significantly decreased, whereas no significant differences were observed for samples stored at -80°C relative to control group. Changes in collagen structure were additionally confirmed using TEM and indicated that lenticules preserved at -80°C with or without silica gel exhibited good transparency. This is in line with previous studies.^{17,18} H&E staining also exhibited roughly similar preserved lenticule morphology across groups, although small changes were evident in TEM images.

TEM images revealed that the collagen fiber density of lenticules stored at RT was significantly lower than that of lenticules stored at or below 4°C. Collagen fiber breakage was also evident. We have previously compared 3 different approaches to the preservation of refractive lenticules for 1 month at 4°C (glycerol, silica gel, and silicone oil)²¹ and found that the mean number of collagen fibrils in glycerol-preserved samples was reduced relative to fresh lenticules, likely because of incomplete dehydration and a relatively short storage time. Li et al¹⁷ and Tripathi et al¹⁸ explored the effect of temperature and dehydration on glycerol-preserved corneas for DALK and determined that RT storage was associated with highly discon-

tinuous and abnormally distorted collagen bundles in corneas imaged using TEM, in line with our findings. Liu et al²² suggested that the interfibrillar spacing of lenticules stored in anhydrous glycerol at RT for 1 month was similar to that observed for fresh lenticules, but this may be associated with the relatively brief preservation time in their study. We detected necrotic keratocytes in all groups. Earlier studies²²⁻²⁴ have similarly confirmed that such necrotic keratocytes can be identified within preserved corneal lenticules.

Because intrastromal lenticules exhibit reduced immunogenicity when compared with the full corneal layer, intrastromal lenticule transplantation is associated with a reduced risk of rejection when compared with other transplantation techniques, including lamellar and penetrating keratoplasty procedures. Even so, an immune-mediated rejection remains a procedure-related risk. HLA-ABC and HLA-DR are key HLA class I and II antigens associated with the processing and presentation of antigens to immune cells, whereas CD45 is a transmembrane molecule associated with immune cell development and functionality. We determined that preserved lenticules exhibited reduced or absent immunogenicity based on the expression of these markers. Chen et al^{25,26} previously used acellular glycerol-cryopreserved corneal tissue for grafting during DALK in high-risk corneas and determined that this approach yielded outcomes comparable with the use of fresh tissue, preventing allograft rejection. Both in vitro and in vivo studies have shown that decellularized and x-ray-irradiated lenticules can decrease the

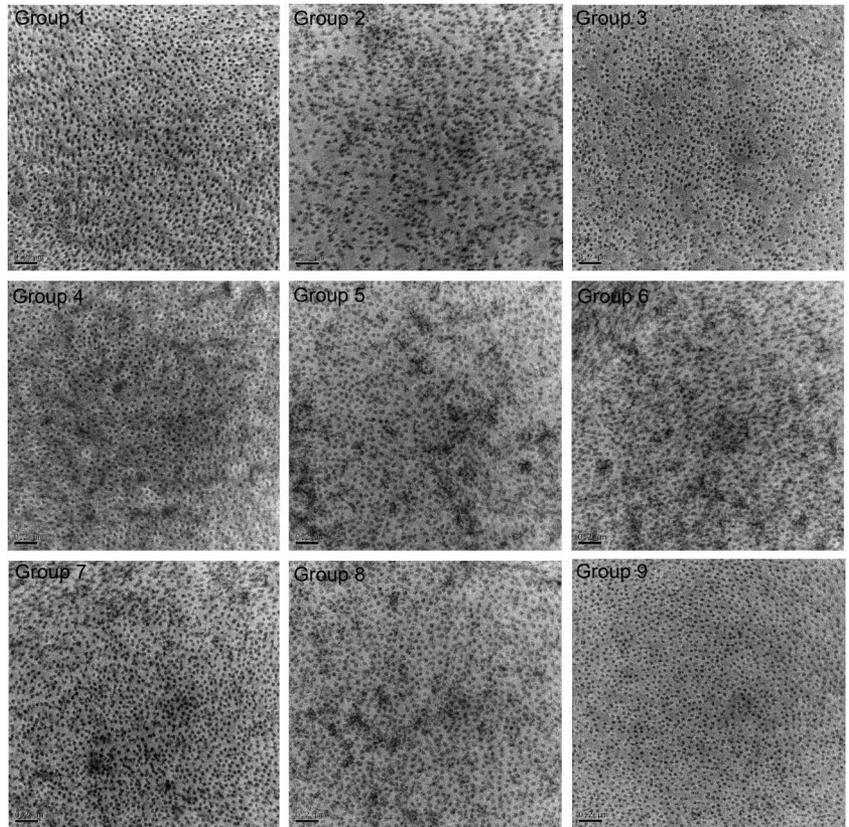


FIGURE 3. Transmission electron micrograph of transverse section of collagen fibrils in each group (bar, 0.2 μm).

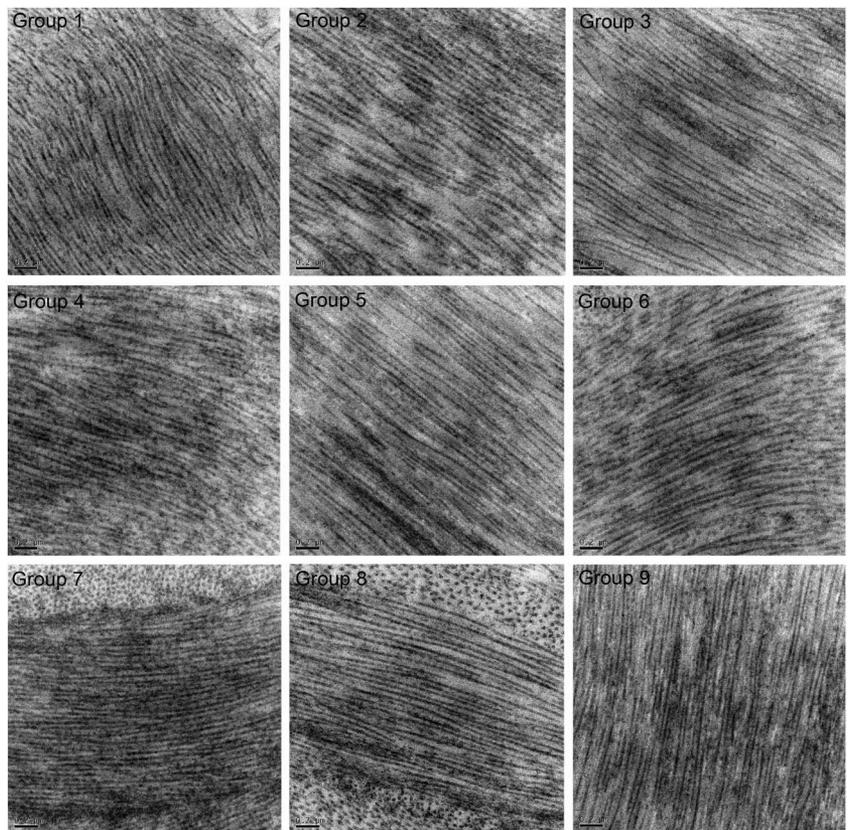


FIGURE 4. Transmission electron micrograph of longitudinal section of collagen fibrils in each group (bar, 0.2 μm).

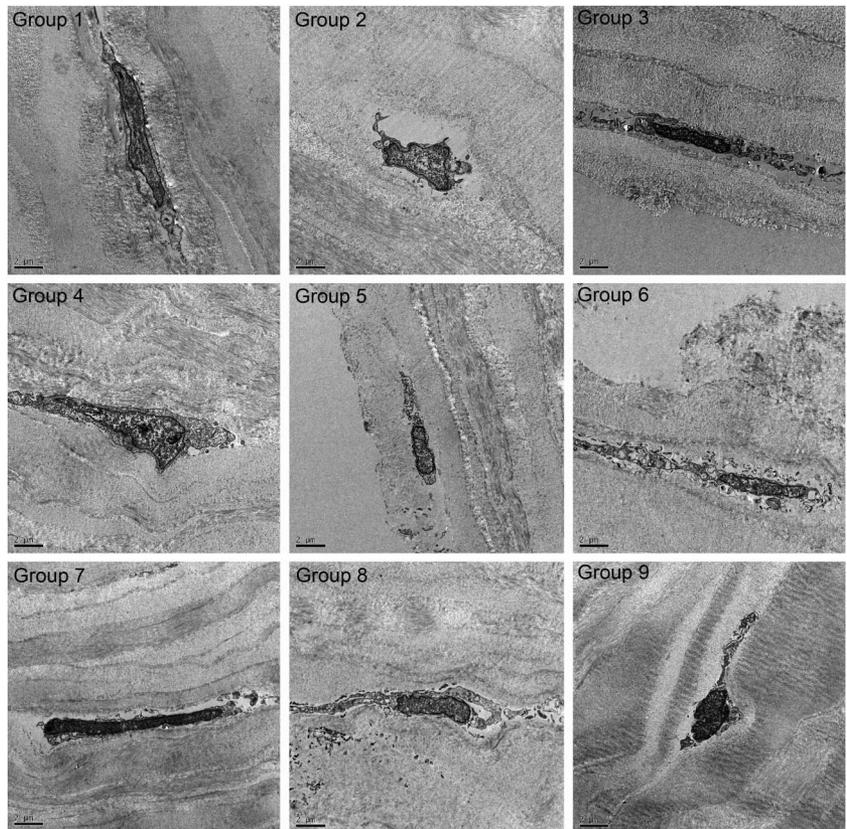


FIGURE 5. Transmission electron micrograph of keratocytes in each group (bar, 2 μm).

immunogenicity of corneal tissue.^{27,28} However, the dense collagen structures within lenticules can inhibit recellularization after decellularization in vitro,²⁹ and X-ray irradiation necessitates access to expensive equipment that can limit its clinical application.

There are multiple limitations to this analysis. First, human corneal lenticules were only analyzed in vitro, and additional animal studies are, therefore, warranted to validate these findings. Second, the biomechanical properties of lenticules were not evaluated in this study; further researches are needed to investigate the tensile strength of the lenticules.

In summary, the preservation of stromal lenticules was optimally achieved at -80°C , followed by -20 and 4°C with or without silica gel in anhydrous glycerol, because these conditions were able to better preserve optical transparency and collagen architecture such that they more closely resembled those of fresh lenticules, while all exhibiting minimal immunogenicity.

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