Corneal lenticule storage before reimplantation

Yu-Chi Liu,[1,2,3] Geraint P. Williams,[1,2] Ben L. George,[1] Yu Qiang Soh,[1,2] Xin Yi Seah,[1] Gary Swee Lim Peh,[1] Gary Hin Fai Yam,[1] Jodhbir S. Mehta[1,2,3]

(The first two authors contributed equally to this work.)

[1]*Tissue Engineering and Stem Cell Group, Singapore Eye Research Institute, Singapore;* [2]*Singapore National Eye Centre, Singapore;* [3]*Department of Clinical Sciences, Duke-NUS Medical School, Singapore*

Purpose: To explore the optimal lenticule storage conditions that maintain lenticule integrity and clarity.
Methods: A total of 99 lenticules obtained from myopic patients undergoing small incision lenticule extraction (SMILE) were divided into four combinations for short-term storage conditions: PBS, Dulbecco's Modified Eagle's Medium (DMEM), Optisol GS, or anhydrous glycerol. Two thirds of the lenticules were further stored for 4 weeks under eight different conditions. Clarity evaluation with transmittance measurements, cell-death assays with terminal deoxynucleo-tidyl transferase-mediated nick end labeling assay (TUNEL), collagen fibril spacing and necrotic response assessed with transmission electron microscopy (TEM), and immunohistochemistry analysis for human leukocyte antigens (HLAs) and CD45 for immunogenicity, and matrix metalloproteinase (MMP)-2 for keratocyte response, were undertaken at baseline, 48 h (short term), and 4 weeks (long term).
Results: The TUNEL and immunogenicity results were comparable among the groups. The mean percentage of TUNEL-positive cells across all groups was 24.3% ± 11.8% and 62.9% ± 20.7% at the 48 h and 4 week time points, respectively. HLA-ABC+, HLA-DR+, and CD45+ cells were extremely rare, and MMP-2 expression ranged from non-detectable to minimal, under all conditions at all time points. Transmittance at 4 weeks was significantly different among groups with the greatest maintenance of clarity seen in the lenticules stored initially in DMEM at 4 °C for 48 h followed by cryopreservation in serum-free medium or glycerol at 4 °C followed by storage at room temperature. At TEM analysis at 4 weeks, the lenticules cryopreserved in liquid nitrogen, regardless of storage solutions, had significantly narrower inter-fibrillar distance than controls, while glycerol-preserved lenticules, at either room temperature or −80 °C, maintained the inter-fibrillar distance.
Conclusions: Clarity, structural integrity, and low immunogenicity under various conditions, at 4 °C or room temperature for short-term storage, offer encouragement for lenticule storage. It can be undertaken without access to s specialized and potentially expensive laboratory setup at least within the first 48 h before transportation to larger facilities for long-term storage.

Refractive errors can be corrected by corneal stromal ablation through excimer laser and removal of the corneal epithelium (e.g., photorefractive keratectomy) or the creation of a hinged replaceable flap, either by microkeratome or femtosecond laser (laser in situ keratomileusis, LASIK) [1]. An alternative approach to the removal of the stromal tissue is the extraction of a lenticule of tissue, created by a femtosecond laser, in a procedure called refractive lenticule extraction (ReLEx) [2]. Initially, ReLEx was described as a procedure with the creation of a flap akin to LASIK, called femtosecond lenticule extraction (FLEx) [3]. However, a flapless variation of ReLEx, termed small incision lenticule extraction (SMILE), was further described by Sekundo and colleagues as an alternative keyhole procedure [4]. SMILE offers advantages over other laser refractive procedures, such as LASIK, by not only allowing the surgeon to undertake the refractive correction with one laser platform but also inducing less post-operative inflammation (especially at higher myopic corrections), faster recovery of corneal nerves, and a reduction in higher order aberrations [5-7].

Corneal lenticules extracted during SMILE offer an opportunity to utilize this surplus tissue either for autologous future reimplantation or allogeneic donor implantation. Recent animal studies have established this as a proof of concept [8,9], and subsequent clinical studies have confirmed lenticule implantation as a means of corneal patch grafting for the management of corneal microperforation or partial-thickness corneal defect [10], corneal stromal expansion for cross-linking ultrathin corneas [11], correction of low to moderate keratoconus, hyperopia, and presbyopia by transplanting the lenticule into stroma [12-16]. However, the majority of these studies used fresh lenticules for implantation, which limits the extensive use of the lenticules.

Corneal transplantation relies on the harvesting, storage, and transportation of corneal tissue. Typically, these activities

Correspondence to: Jodhbir S. Mehta, Singapore National Eye Centre, 11 Third Hospital Avenue, Singapore 168751; Phone: +65-6227-7255; FAX: +65-6227-7290; email: jodmehta@gmail.com.

are coordinated by eye banks, and this relies on the optimal recovery of tissue before transfer to an eye bank facility. Corneal graft materials preserved by cryostorage in glycerol solution are able to maintain optical transparency and biomechanical strength [17,18] and have been used for temporary or emergency therapeutic or tectonic penetrating keratoplasty when fresh corneas are not available [17-20]. Although these conditions may be suitable for long-term storage of corneal stromal tissue until reimplantation is required, the reagents, consumables, and equipment required to maintain cryological conditions may not readily be available in standard refractive surgery clinics where stromal lenticules are to be extracted following a SMILE procedure. Furthermore, although promotion of cell survival is essential for endothelial keratoplasty, in the case of stromal refractive lenticule reimplantation, the loss of stromal keratocytes is not an overriding concern, as infiltration with the patient's own keratocytes would be expected in the longer term [21]. The most important consideration for future reimplantation is the maintenance of lenticule architecture and thus, clarity.

To date, the influence of alternative storage conditions on the clarity, integrity, and antigenicity of corneal stromal lenticules for future reimplantation has not been established. It is anticipated that most refractive centers would not be within academic or research units with the necessary laboratory setup; therefore, there is a need for an intermediate inexpensive transport medium that is easily accessible as a transient carrier before longer storage. In this study, we examined various storage conditions for the preservation of corneal stromal lenticules to maintain lenticule transparency and collagen fibril structure. We also evaluated the cell death and immunogenicity expression after short- and longer-term storage.

METHODS

Human corneal lenticules: Lenticules derived from patients undergoing SMILE were obtained from the Singapore National Eye Centre Laser Vision Centre. SMILE was performed by the same surgeon (JSM) using a 500 kHz VisuMax Femtosecond (FS) laser (Carl Zeiss Meditec, Jena, Germany). All SMILE procedures were for myopic treatment as previously described [22,23]. The mean patient age was 31.2 ± 7.30 years, and the mean spherical equivalent refractive correction was -6.0 ± 1.8 diopters. The extracted lenticules were processed and stored in their respective short-term storage conditions for 48 h before they were transferred into the long-term storage conditions (see below). Approval for the study was granted by the institutional review board of SingHealth, Singapore (reference number: 2011/109/A). The

study was conducted in accordance with the Declaration of Helsinki, and informed consent was obtained. The study was conducted in accordance with the Declaration of Helsinki, adhered to the ARVO statement on human subjects, and informed consent was obtained.

Storage conditions: The flow diagram for the eight experimental groups is shown in Figure 1. The extracted lenticules were washed twice in wash buffer, containing saline, Opti-Modified Eagle's Medium (MEM; Thermo Fisher Scientific, Roskilde, Denmark), human serum-free medium (SFM), and antibiotic and antimycotic solutions. For short-term (48 h) storage, four storage conditions were used (n = 24 lenticules for each condition): (1) PBS (Thermo Fisher Scientific; 1X; 137 mM sodium chloride, 2.7 mM potassium chloride and 12 mM phosphate buffer, PH 7.2-7.6): PBS supplemented with Ca^{2+} and Mg^{2+} without any additional supplements, stored at 4 °C, (2) Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific): DMEM supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), stored at 4 °C, (3) Optisol GS (Baush and Lomb, Irvine, CA): Optisol GS without any additional supplements, stored at 4 °C, and (4) 100% glycerol (Sigma Aldrich, St. Louis, MO): anhydrous glycerol without any additional supplements, stored at room temperature. Before storage, the lenticules were first placed in 1 ml of the 48-h storage medium, respectively, for 10 min, to ensure removal of the wash buffer solution, before being transferred to a second vial containing 1 ml of the respective 48-h storage solution. For the 100% glycerol storage group, the lenticules were washed in 1 ml of pure DMEM for 10 min, as accurate micropipetting of glycerol and measurement of glycerol transmittance proved problematic in isolation.

For longer-term (4 weeks) storage, 16 lenticules from each short-term storage group, selected randomly, were washed in 1 ml of the appropriate 4-week storage solution for 10 min to ensure removal of the previous 48-h storage medium, and then were replaced in fresh 1 ml of the 4-week storage solution in a cryovial for an additional 4 weeks. The 4-week storage conditions were as follows: (1) Lenticules were stored in DMEM supplemented with 10% FBS and 10% dimethyl sulfoxide (DMSO; Sigma Aldrich) in a −80 °C freezer overnight and transferred into liquid nitrogen. (2) Lenticules were stored in SFM supplemented with 2.5% (w/v) chondroitin sulfate (Thermo Fisher Scientific), 2.5 mM HEPES (Sigma Aldrich), and 10% DMSO in a −80 °C freezer overnight and transferred into liquid nitrogen. (3) Lenticules were stored in room-temperature glycerol for the lenticules previously stored in room-temperature glycerol. (4) Lenticules were stored in −80 °C glycerol for the lenticules previously stored in room-temperature glycerol.

Optical transmittance measurement: Lenticule transmittance was measured at baseline, 48 h, and 4 weeks after storage. Lenticules were transferred individually in a 96-well plate, and 100 µl of solution (wash buffer for three control lenticules, or the respective storage solution for the stored lenticules) was added in to each well, together with an optical blank. The 4-week cryostorage lenticules were thawed first in a water bath for 10 min. Absorbance measurements were obtained using a Tecan Infinite M200 (Tecan, Männedorf, Switzerland). Absorbance, A, was measured over the wavelength range 380–780 nm at 1-nm intervals. Transmittance, T, of the lenticule and blank wells was calculated as $T = 10^{-A}$, yielding transmittance of either the blank solution (T_B) or of the solution and the lenticule (T_{B+L}). Transmittance of the lenticule itself, T_L, was then calculated as $T_L = T_{B+L} + (1 - T_B)$.

Cryosectioning: After completion of the 4-week storage interval, the lenticules were removed from storage, thawed, washed twice in their basal 4-week storage medium for 10 min (e.g., lenticule stored in DMEM + 10% FBS + 10%

DMSO was washed in DMEM without FBS or DMSO) or in the case of glycerol-stored lenticules, washed in un-supplemented DMEM. Lenticules were then embedded in tissue freezing medium (Leica, Wetzlar, Germany) and stored at −80 °C. Using a Micron HM525 cryostat (Micron, Walldorf, Germany), the tissues were cut into 7-µm-thick sections and mounted on glass slides. Sections were then employed for immunofluorescence staining and terminal deoxynucleotidyl transferase-mediated nick end labeling assay (TUNEL) assays. Sections of whole, fresh cornea were cut and mounted in a similar manner for use as staining controls.

TUNEL assay: TUNEL assays were performed on lenticules from each combination of 48-h and 4-week storage conditions according to the manufacturer's instructions (Click-iT TUNEL Alexa Fluor Imaging Assay, Thermo Fisher Scientific) to detect fragmentation of DNA associated with cell death. Briefly, slides were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and washed again. The TUNEL reaction mixture was added,

- **Group 1:** PBS 4°C for 48 hours and then DMEM LN$_2$ storage for 4 weeks

- **Group 2:** PBS 4°C for 48 hours and then SFM LN$_2$ storage for 4 weeks

- **Group 3:** DMEM 4°C for 48 hours and then DMEM LN$_2$ storage for 4 weeks

- **Group 4:** DMEM 4°C for 48 hours and then SFM LN$_2$ storage for 4 weeks

- **Group 5:** Optisol 4°C for 48 hours and then DMEM LN$_2$ storage for 4 weeks

- **Group 6:** Optisol 4°C for 48 hours and then SFM LN$_2$ storage for 4 weeks

- **Group 7:** Glycerol RT for 48 hours and then glycerol RT storage for 4 weeks

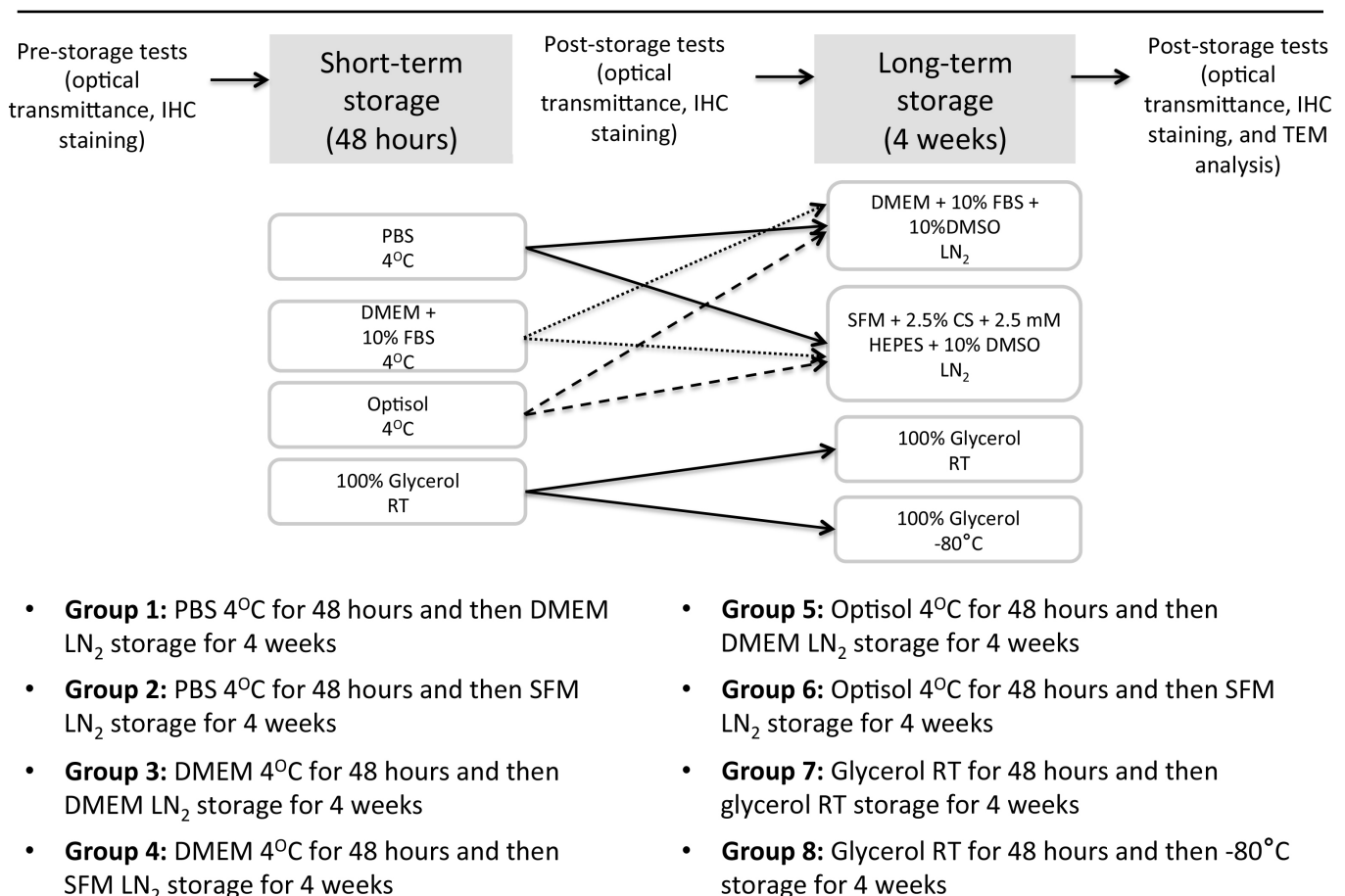- **Group 8:** Glycerol RT for 48 hours and then -80˚C storage for 4 weeks

Figure 1. Flow diagram showing the experimental design. IHC: immunohistochemistry, PBS: phosphate buffered solution; DMEM: Dulbecco's Modified Eagle's Medium; SFM: serum-free medium; FBS: fetal bovine serum; CS: chondroitin sulfate; DMSO: dimethyl sulfoxide; RT: room temperature; LN$_2$: liquid nitrogen; TEM: transmission electron microscopy.

Group	Donor age (years)	Refractive spherical equivalence (diopters)
1	33.5 ± 7.3	-5.4 ± 1.7
2	31.0 ± 5.8	-4.9 ± 1.0
3	33.3 ± 6.5	-5.4 ± 1.7
4	32.1 ± 7.5	-5.9 ± 2.2
5	32.7 ± 4.5	-5.9 ± 2.3
6	35.5 ± 4.9	-5.0 ± 1.8
7	32.1 ± 6.7	-6.4 ±1.9
8	29.6 ± 4.7	-6.3 ± 2.4
P value*	0.202	0.502

TABLE 1. LENTICULES DONOR AGE AND REFRACTIVE POWER BY GROUP.

* Comparisons among 8 groups

and the sections were sealed under plastic coverslips at 37 °C for 1 h in the dark. Then the coverslips were removed, the sections were washed again and incubated with Hoechst 33,342 (Thermo Fisher Scientific) for 5 min, and washed a final time in PBS. Sections were then covered with mounting medium (Vector Laboratories, Burlingame, CA) and coverslips and sealed. The positive TUNEL- and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI)–stained cells were manually counted on both sides of the peripheral (P1 and P2) and central areas (C) of the lenticules in three randomly selected fields of each lenticule using a magnification of 200X. The percentage of the TUNEL-positive cells was calculated as positive TUNEL cells in the P1, P2, and C areas, divided by DAPI cells in the P1, P2, and C areas.

Immunofluorescence: Slides of lenticules and whole corneal sections (positive controls) were tested for antigenicity. Sections were washed with PBS for 5 min, then fixed for 5 min at room temperature in 4% paraformaldehyde in PBS, and then washed an additional three times. Slides were blocked with 10% normal goat serum (Sigma Aldrich) in PBS for 1 h at room temperature, and primary antibodies (human leukocyte antigen (HLA) class I ABC antibody, ab70328, 1:100; HLA class II DR antibody, ab 20,181, 1:100; CD45 antibody, ab10559, 1:100; Abcam, Cambridge, UK; matrix metalloproteinases-2 (MMP-2), Abbiotec 250,752, 1:200, San Diego, CA) in 10% goat serum-supplemented PBS were applied for a further 2 h at room temperature. Slides were then washed three times and incubated with secondary antibody (AlexaFluor 488-conjugated anti-mouse, A21131, 1:750, or AlexaFluor 568-conjugated anti-rabbit, A11011, 1:750; Thermo Fisher Scientific) in 10% goat serum-supplemented PBS for 1 h at room temperature, washed three times with PBS, and incubated with Hoechst 33,348 (1:10,000; Thermo

Fisher Scientific) in PBS for 5 min, washed one final time in PBS, then mounted in VectaShield, and imaged with fluorescence microscopy with a Zeiss AxioPlan 2 (Zeiss). Positive control staining of primary antibodies was performed on the frozen sections of the corneal limbal rim region for HLA antibodies and on primary human stromal fibroblasts for the MMP-2 antibody. Another three fresh lenticules, placed in the wash buffer immediately after extraction and transferred to the laboratory for analysis within 15 min after extraction, were washed twice in wash buffer and served as negative controls for immunofluorescence analysis.

Transmission electron microscopy: Transmission electron microscopy (TEM) was performed on lenticules from each short- and long-term storage combination to assess differences in collagen fibril density and organization. Another three fresh lenticules that were washed only twice with wash buffer served as controls. Lenticules were fixed in 2% glutaraldehyde and 2% paraformaldehyde in PBS for 1 h at room temperature and then cut into small pieces (about 1 mm^2) with a scalpel blade. Pieces were then fixed for a further 1 h and washed three times for 5 min in PBS. Samples were then fixed with 1% osmium tetroxide and 1% potassium ferrocyanide for 1 h and rinsed thoroughly with distilled water. For glycerol-fixed samples, samples were fixed with 1% osmium oxide and 1% tannic acid. After extensive rinsing with distilled water, tissues were dehydrated in a graded series of ethanol and then embedded in Araldite (Electron Microscopy Sciences, Hatfield, PA). The ultrathin sections, 70–90 nm thick, were cut with a Reichert-Jung Ultracut E Ultramicrotome (C. Reichert Optische Werke AG, Graz, Austria) and were collected on copper grids, double stained with uranyl acetate and lead citrate for 8 min each, and then viewed and imaged on a JEM 1220 electron microscope (JEOL, Tokyo,

TABLE 2. MEAN SPECTRAL TRANSMITTANCE AT DIFFERENT TIME POINTS IN DIFFERENT GROUPS.

| Group | Mean spectral transmittance (%) | | | | | P value between baseline and 4 weeks |
	Baseline	48 hours	Changes in transmittance between baseline and 48 hours	4 weeks	Changes in transmittance between baseline and 4 weeks	
1	92.8 ± 1.5	93.8 ± 3.5	1.0 ± 0.4	90.3 ± 2.4	-2.5 ± 0.7	
2	92.9 ± 1.9	94.3 ± 3.1	1.4 ± 0.8	91.1 ± 2.8	-1.8 ± 0.9	0.294
3	91.7 ± 1.2	91.4 ± 3.3	-0.3 ± 0.1	91.6 ± 2.4	-0.1 ± 0.1	
4	90.7 ± 1.8	92.0 ± 0.8	1.3 ± 0.4	91.7 ± 1.9	1.0 ± 0.3	
5	93.0 ± 0.5	91.3 ± 2.8	-1.7 ± 0.7	92.0 ± 2.2	-1.0 ± 0.4	
6	92.5 ± 2.1	91.2 ± 4.6	-1.3 ± 0.3	91.9 ± 2.5	-0.6 ± 0.2	
7	90.8 ± 2.7	95.0 ± 1.5	4.2 ± 1.2	92.4 ± 1.9	1.6 ± 0.6	
8	91.9 ± 2.9	94.2 ± 1.6	2.3 ± 1.0	90.9 ± 2.5	-1.0 ± 0.3	
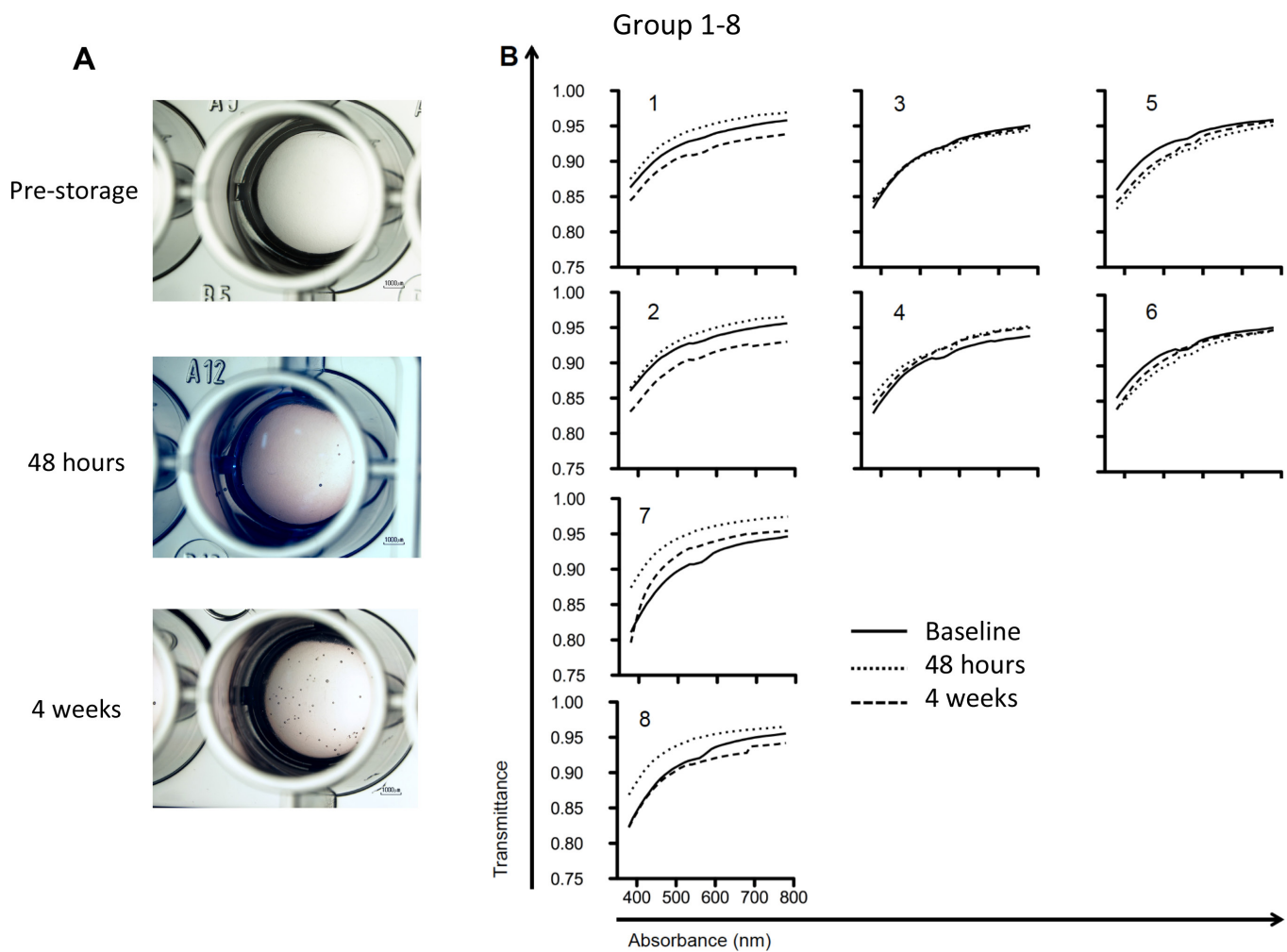
Figure 2. Spectrum-wide transmittance trends and mean transmittance over time in different groups. **A**: Representative color photographs of lenticules at different time points. **B**: Mean transmittance over the entire spectrum of visible light for the eight groups across all time points.

Japan) at 100 kV. Long-term ultrastructural changes in the lenticule collagen were further analyzed. For each storage condition, five separate TEM images at the central part of the lenticule were selected. The average fibril spacing was measured. In each imaging for a subset of transverse collagen fibrils, a reference fibril was randomly selected in five areas (center and four quadrants). The center-to-center interfibrillar distance (in terms of pixel length) was defined as the spacing between the reference fibril spot and its closest neighbors without other fibrils blocking in between [24]. The distance was measured using the ruler tool in Image J.

Statistical analysis: SPSS (ver. 18.0, SPSS Inc., Chicago, IL) was used for data analysis. One-way ANOVA (ANOVA) was employed to detect differences in transmittance, TUNEL activity, and interfibrillar distance among the different storage groups. Repeated-measures ANOVA was employed to determine the statistical significance of changes in mean transmittance over time. Sphericity was assumed if the p value was less than 0.05 for Mauchly's test of sphericity, and homogeneity of between-subjects variance-covariance was assumed if the p value was less than 0.05 for Box's M test. The multivariate approach for analysis of within-subject
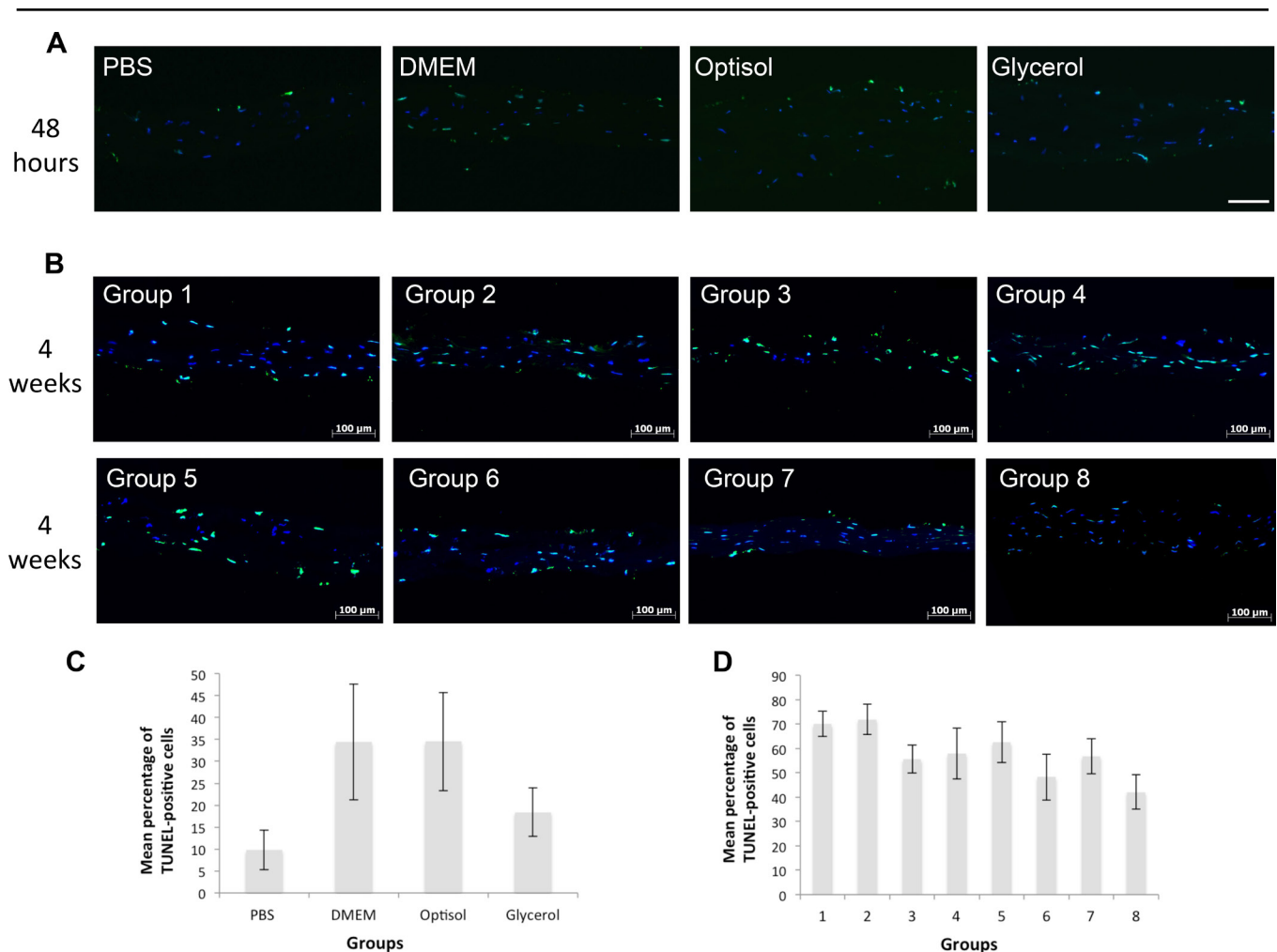

Figure 3. TUNEL cell-death detection. **A**: Representative images of lenticules stained with terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) after 48 h storage for different groups. **B**: Representative images of lenticules stained with TUNEL after 4 weeks of storage for different groups. Nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; blue). Original magnification: 200X; scale bar = 100 μm. **C**: Mean percentage of TUNEL-positive cells in all groups after 48 h. **D**: Mean percentage of TUNEL-positive in all groups after 4 weeks of storage. No statistically significant inter-group differences were found at each time point.

effects was adopted as the sphericity assumption was not valid for this data set. A p value of less than 0.05 was considered statistically significant.

RESULTS

A total of 99 human cornea lenticules were used. There was no significant difference in the spherical equivalent refractive power, representing the lenticule thickness (p = 0.052), among the groups. The lenticule donor age among groups was also comparable (p = 0.202; Table 1).

Assessments of transmittance: Spectral transmittance was compared at baseline, 48 h, and 4 weeks after storage (Figure 2A). Variations in mean spectral transmittance were observed at different time points and between the different storage groups (Figure 2B). Mean changes in the spectral

transmittance from baseline to 48 h and from baseline to 4 weeks are presented in Table 2. Most groups appeared to demonstrate small losses of clarity by 4 weeks, despite initially maintaining transmittance at 48 h. In contrast, lenticules stored short-term in DMEM followed by longer-term storage in SFM (Group 4) or glycerol at room temperature (Group 7) appeared to have improvements in transmittance after storage for 4 weeks. However, a repeated-measures ANOVA model showed that there were no statistically significant changes in transmittance after 4 weeks of storage (p = 0.294).

TUNEL assay: TUNEL-positive cells were visible at 48 h (Figure 3A) and 4 weeks (Figure 3B) in the lenticules stored under all conditions. The mean population of TUNEL-positive cells across all groups was 24.3% ± 11.8% and 62.9% ± 20.7% at 48 h and 4 weeks, respectively. For both time points,
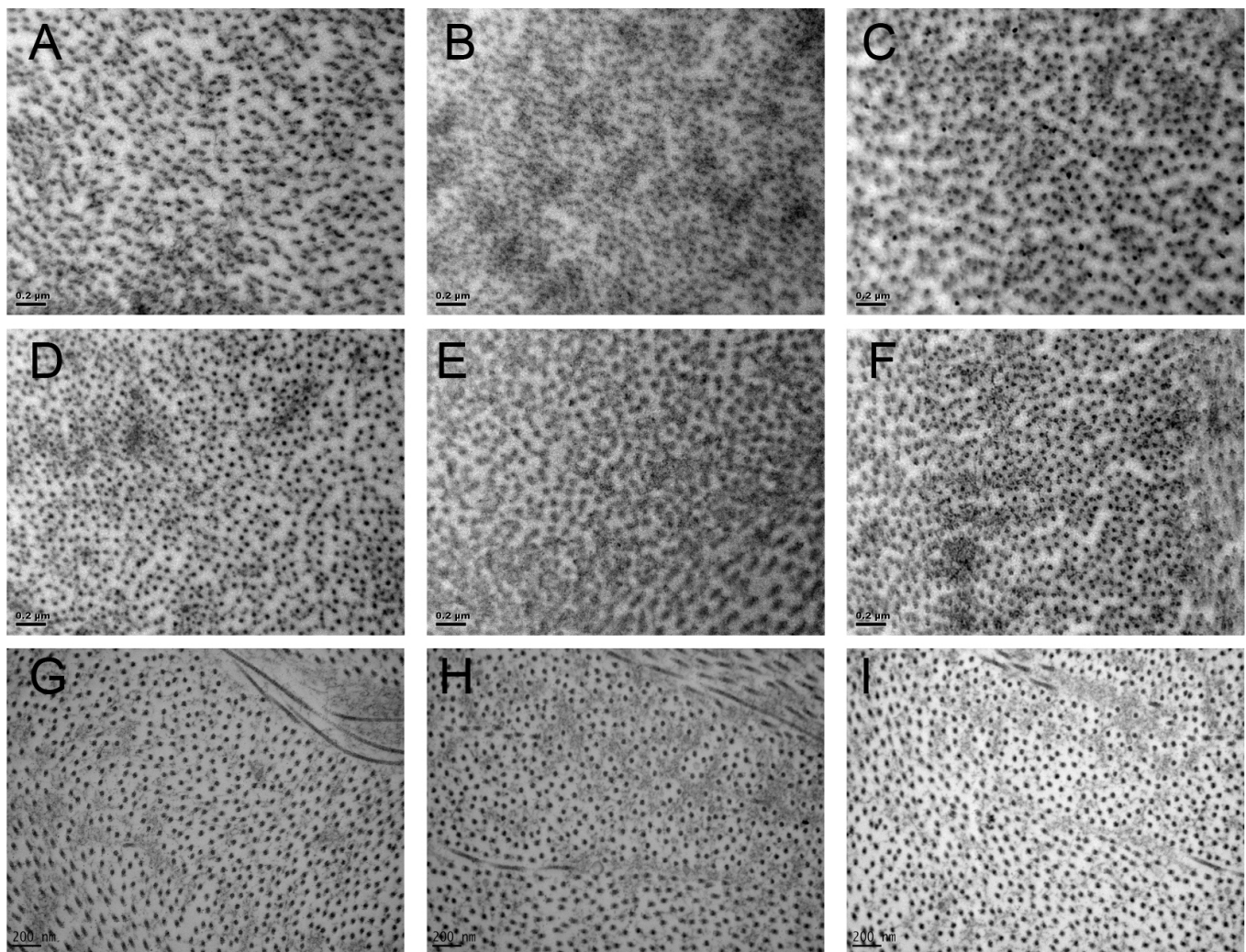

Figure 4. Transmission electron microscopy of the stromal lenticule showing transverse section of collagen fibrils following 4 weeks of storage (**A–H**: groups 1–8; **I**: control). Scale bar = 0.2 μm.
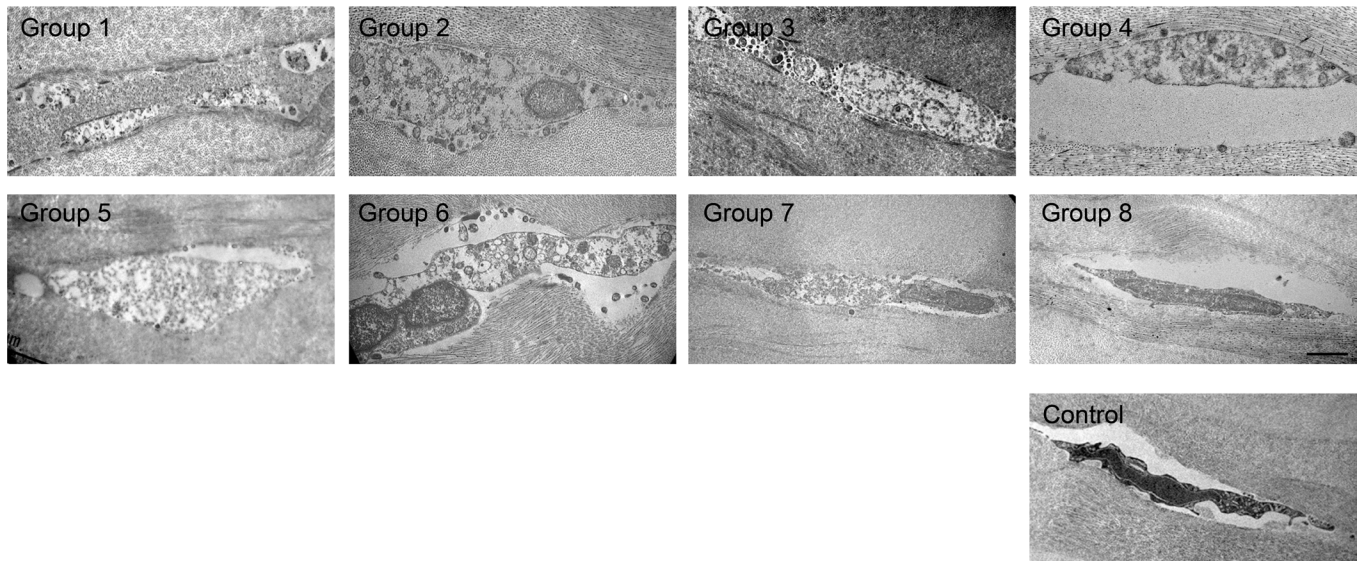
Figure 5. Transmission electron micrographs of necrotic keratocytes in the experimental and control groups following 4 weeks of storage. In the control lenticule, the chromatin in the cell nucleus was condensed, whereas in the lenticules in groups 1–8, there was irregular clumping of chromatin, cytoplasmic vacuoles, and swelling nuclei. These necrotic keratocytes were less observed in groups 7 and 8. Scale bar = 2 µm.

there was no statistically significant inter-group differences in the TUNEL-positive reaction ($p = 0.074$ and $p = 0.093$ at 48 h and 4 weeks, respectively (Figure 3C,D).

Assessments of TEM: Inter-fibrillar spacing between collagen bundles assessed using TEM are shown in Figure 4A–I. The inter-fibrillar distance in groups 1–6 ($26.7 ± 1.20$, $25.0 ± 2.70$, $27.3 ± 3.40$, $22.2 ± 1.00$, $26.9 ± 1.50$, and $25.6 ± 3.30$ pixel length, respectively) was significantly less than that in the control group ($47.0 ± 2.10$ pixel length), Group 7 ($40.3 ± 9.40$ pixel length), and Group 8 ($42.4 ± 5.20$ pixel length; all $p<0.01$). The inter-fibrillar spacing in groups 7 and 8 was comparable to that in the control group ($p = 0.672$). There was no significant difference between Group 7 and Group 8 ($p = 0.781$) and between any two groups among groups 1–6 ($p = 0.317$). We also observed necrotic keratocytes in all groups, with small amounts in groups 7 and 8. These necrotic keratocytes exhibited incomplete nuclei and cell membrane, cytoplasmic vacuoles, dispersed chromatin with irregular clumping, and swelling nuclei (Figure 5).

MMP-2, HLA-ABC, HLA-DR, and CD45 immunostaining: The expression of MMP-2, a marker for corneal stromal repair, was minimal in groups 4 and 6–8 and was not detectable in the other groups (Figure 6). The HLA-ABC+, HLA-DR+, and CD45+ cells were extremely rare in the corneal lenticules stored under all conditions at all time points (Appendix 1).

DISCUSSION

ReLEx was the first technique described that relies on the removal of a corneal lenticule, to achieve a refractive correction. Unlike LASIK, excimer laser ablation of the stromal tissue bed is circumvented by using a femtosecond laser to remove a lenticule, measuring approximately 6.5 mm in diameter and of variably central thickness depending on the type and amount of correction achieved. Furthermore, the requirement for a flap to facilitate ablation for LASIK or lenticule extraction in ReLEx has been superseded by a minimally invasive approach, SMILE [3,8].

The potential for lenticule reimplantation is apparent. We have previously shown in two feasibility studies that lenticules can be extracted, stored with FBS and DMSO (under conditions akin to those outlined for Group 3 in this study), and successfully reimplanted in a rabbit and a non-human primate model following ReLEx [8,9]. In both studies, lenticule extraction preceded 28-day storage before autologous reimplantation. Subsequently, human studies evaluating the use of allogeneic lenticules retrieved post-SMILE have shown that the lenticules could be successfully and safely implanted autogenically or allogeneically to correct hyperopia and presbyopia [12,14-16]. It has also been reported that adding a myopic lenticule to the corneal surface allows stromal tissue expansion in ultrathin corneas for crosslinking [11]. In addition, the lenticule has been used as a corneal patch for the management of microperforations and complicated corneal tears [10]. Authors have also demonstrated that

multiple lenticules could be constructed with fibrin glue for the management of deep corneal defects or full thickness perforations [25].

PBS is a generic isotonic solution while Optisol is routinely used in clinical practice for the preservation of donor cornea tissue post-harvesting due to the solution's ability to preserve corneal endothelial cells for up to 2 weeks at 4 °C, mitigating the effect of post-preservation stromal edema [26]. However, the cost of Optisol limits its generalized use in lenticule storage. DMEM with 10% FBS is a common cell culture medium and has been used in primary cell culture, passage, and cryopreservation of human fetal corneal endothelial cells [27]. SFM is another commonly used medium and has been shown that it could protect human corneal endothelial cells from apoptotic and necrotic cell death [28], as well as preserve human corneal limbal epithelial cells [29]. Compared to Optisol, glycerol preservation significantly lowers the cost of storage (Optisol GS: approximately USD

4,671.2/500 ml; glycerol: approximately USD 176.7/500 ml; SFM: approximately USD 246.5/500 ml; DMEM: approximately USD 94.5/500 ml). The present results showed that the lenticules stored in glycerol at room temperature maintained the clarity and ultrastructure features, and glycerol also offers advantages over the use of medium or Optisol that works best at 4 °C [29,30]. The ability of anhydrous glycerol to preserve donor lamellar tissue for prolonged periods of time has been extensively investigated and validated [17-19,31-33], and is attributed to its dehydrating and antimicrobial effects [17,18], which allows the maintenance of native stromal collagen ultrastructure and transmittance characteristics, and is a good candidate for donor corneal storage [31-33].

In this study, we sought to determine differences in clarity, cell death, architecture, and immunogenicity under alternative storage conditions for lenticules. We chose 48 h as the short-term time point because it is the logistic time that the lenticules are expected to be transported from refractive
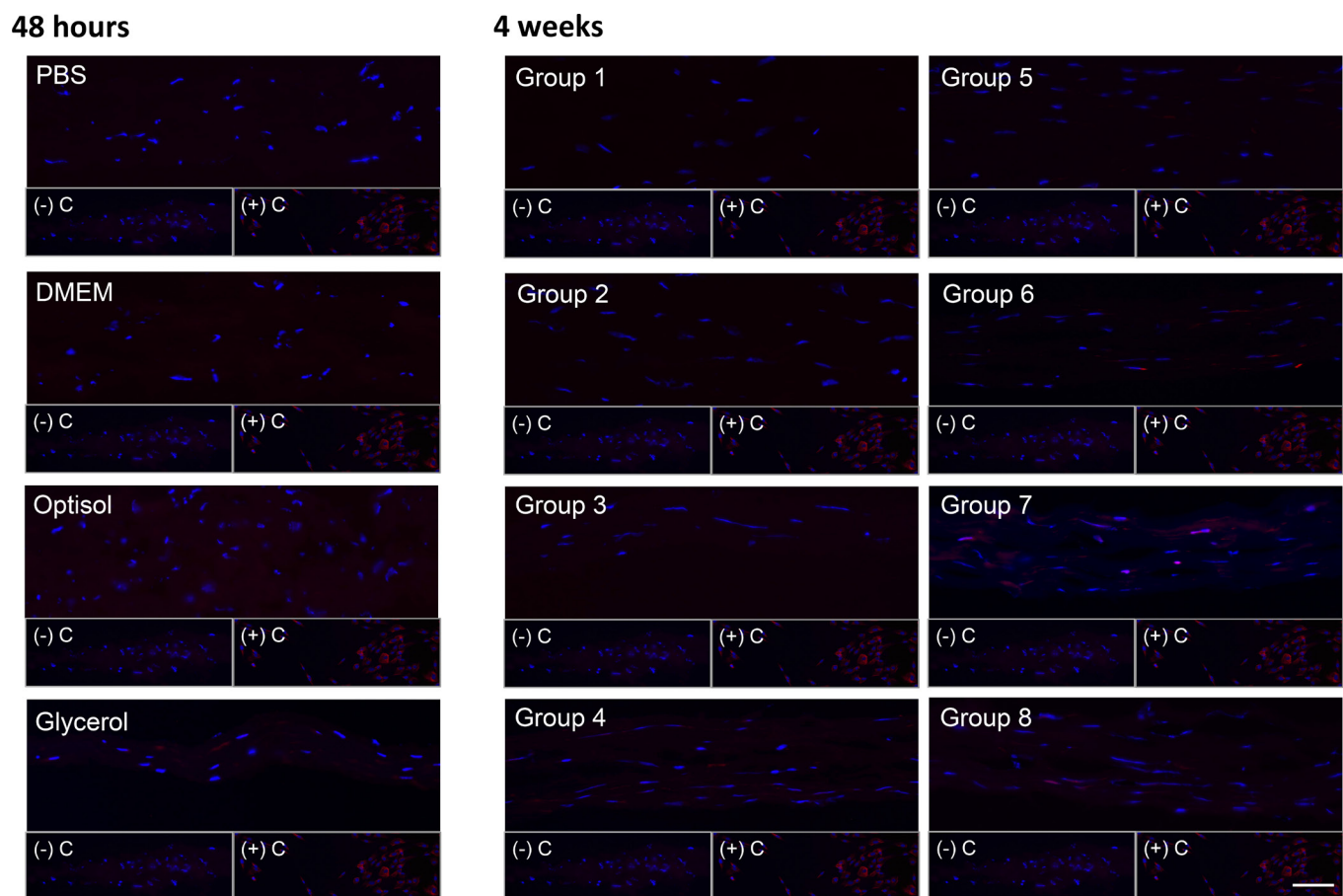
48 hours   4 weeks

Figure 6. Expression of MMP-2 at the central part of lenticules at 48 h and 4 weeks. There was minimal expression of matrix metalloproteinase (MMP)-2 in groups 4 and 6–8, and the expression was not detectable in the other groups. Negative control ((-) C): fresh lenticules; positive control ((+) C): primary human stromal fibroblasts. Nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; blue). Original magnification: 200X; scale bar = 50 μm.

clinics to academic research centers that have the setup to store lenticules for longer term or perform lenticule reimplantation. In the present study, the inter-fibrillar distance was evaluated at the central part of the lenticule, as in the future clinical application, the central part of lenticule will be within the patient's visual axis and affect the patient's visual acuity after lenticule implantation. In the TEM analysis, the lenticules preserved in liquid nitrogen (groups 1–6) had a significantly shorter inter-fibrillar distance than the glycerol-preserved lenticules. The latter allowed better maintenance of native stromal collagen ultrastructure, stored at either room temperature or −80 °C (groups 7 and 8). This might be because liquid nitrogen induced expansion of collagen fibrils due to intra-fibrillar ice formation [34], whereas glycerol, a dehydrating agent, binds strongly to water molecules and prevents them from forming ice crystals [35]. However, the transmittance was comparable across the eight groups, indicating that the difference in the collagen fibril spacing did not lead to significant changes in clarity. We found that better clarity was found under two conditions: DMEM at 4 °C followed by cryopreservation in SFM (Group 4) and glycerol at 4 °C followed by glycerol at room temperature (Group 7). These changes were not statistically significant, and the clinical significance of such small changes in transmittance (in the region of 0.001–0.04) may not be clinically relevant. Although Group 3 appeared to have reduced clarity in this study, these conditions were successfully employed for the rabbit and non-human primate models, as well as the clinical study undertaken by Ganesh and colleagues who used cryopreserved lenticules in culture media together with DMSO [13]. The data that showed the lenticules in Group 7 (glycerol storage at room temperature for 4 weeks) still maintained good clarity providing evidence to support the use of this medium at institutions that lack advanced storage facilities and transportation.

The TUNEL assay indicated comparable rates of cell death across all groups, with a mean percentage of TUNEL-positive cells of 62.9% at 4 weeks. Assessment of lenticule immunogenicity indicated the relative absence of HLA-ABC and HLA-DR, which play an important role in the pathway and mechanism of corneal graft rejection [36], as well as CD45, a marker for leukocyte common antigen that can prime T cells to initiate the corneal alloimmune response [37], following storage either at 48 h or 4 weeks. In considering the feasibility of the use of these extracted lenticules as allografts, this may confer a lower risk of allograft rejection inherent in such a procedure. In normal corneas, a low level

of MMP-2 is present [38]. MMP-2 is produced by stromal fibroblasts and participates in stromal wound healing and wound remodeling independent of corneal epithelial injury [39]. After 48 h of storage, there was only minimal expression of MMP-2 in Group 4 and groups 6–8 and no expression of MMP-2 in the other groups, indicating the minimal stromal repair response after long-term storage. The reason that the lenticules in Group 4 and groups 6–8 expressed minimal MMP-2 might be because there were more living stromal fibroblasts in these groups than other groups (Figure 3D).

A limitation of this study pertains to the maximal duration of follow-up. The functional validation of lenticules following long-term storage has been investigated for durations of up to 10–20 years, which is the typical duration between when a patient requests refractive surgery and when presbyopia management is warranted clinically. However, cryopreservation has been proven to be stable and effective method for storage of cells and tissue for more than 10 years, including successful preservation of corneal tissue [32,40].

In conclusion, the clarity, structural integrity, and low potential antigenicity under a myriad of storage conditions, at 4 °C or room temperature, offer encouragement that lenticule storage can be undertaken even without access to specialized and potentially expensive reagents. This has implications for the potential to maximize lenticular storage use and offers cost-effective solutions that may be affordable in the absence of a clear need to utilize specialized storage solution within the first 48 h before transportation to larger facilities. Further validation over a longer time-frame is required to fully explore longer-term storage.

APPENDIX 1. REPRESENTATIVE IMAGES SHOWING NEGLIGIBLE HLA-ABC, HLA-DR AND CD45 IMMUNOSTAINING AT ALL TIME POINTS.

Nuclei were counterstained with DAPI (blue). Original magnification: 200X, scale bar 100 μm. To access the data, click or select the words "Appendix 1"

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