Cornea

Cutting and Decellularization of Multiple Corneal Stromal Lamellae for the Bioengineering of Endothelial Grafts

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Purpose. Engineered corneal endothelial grafts able to provide numerous functional endothelial cells for the restoration of corneal transparency would be a worthwhile way of replacing donor tissue, which is extremely scarce. The grafts are simply constructed: a biocompatible thin and transparent carrier colonized by a monolayer of cultured endothelial cells (ECs). Here we describe a process able to obtain appropriate carriers by recycling human corneas unsuitable for graft in their original state, but liable to provide multiple thin lamellae when cut with a femtosecond laser as used in refractive surgery.

Methods. We selected a robust method of stromal decellularization. To demonstrate that neither this process nor long-term storage hindered cell adherence, lamellae were endothelialized with an EC line.

Results. The constructs achieved up to very high EC density (the main quality criterion for regular donor corneas) while remaining transparent and thin. We verified that they could be inserted in the anterior chamber of a human eye, like a conventional endothelial graft. Human decellularized cornea will likely be directly compatible with the recipient cornea and comply with the requirements of health regulatory authorities.

Conclusions. This study demonstrates that thin human corneal lamellae could have high potential as carriers in next-generation therapy for endothelial dysfunctions.

Keywords: cornea, endothelial keratoplasty, bioengineering, femtosecond laser, decellularization

As one corneal graft is performed of 70 needed worldwide, donor cornea appears extremely scarce.[1] Many strategies have been adopted or envisaged to reduce the supply/demand gap, from corneal donation campaigns to the most complex gene therapies intended to increase local immunosuppression and prolong graft survival. One such therapy, endothelial keratoplasty (EK), in which bioengineered graft replaces donor cornea, would be very efficient. For more than 10 years, surgeons have clearly demonstrated that endothelial diseases could benefit more from EK than from penetrating keratoplasty. Endothelial keratoplasty is now used for almost one-third of all grafts, and its indications are developing worldwide with the dissemination of surgical techniques and of graft-preparation guidelines. Indeed, these guidelines are increasingly centralized in eye banks. Nevertheless, one EK requires one donor cornea. Endothelial-graft bioengineering appears much easier than the extremely complex reconstruction of whole corneas using bioengineering techniques, which has not greatly evolved since its surprising proof of concept published 17 years ago,[2] and is deemed a viable technique in the short term for supplying tissue for EK.[3]

A bioengineered endothelial graft consists of a carrier endothelialized by functioning endothelial cells (ECs) mimicking the posterior graft cut or dissected from a donor cornea. There is no need to reconstruct a mechanically strong full-thickness stroma with perfect corneal shape and refractive properties. The ideal EC carrier is already well defined:

1. It must be thin enough to avoid forming an interface with the recipient stroma, but strong enough to allow easy handling during the in vitro steps and surgery. More than 10 years of worldwide experience with EK shows clearly that Descemet stripping automated EK (DSAEK) and Descemet membrane EK (DMEK) can be used interchangeably to treat endothelial dystrophies, even if each has its own specificities; DSAEK requires less surgical skill and is therefore more widespread. Endothelial-graft bioengineering with stromal lamellae as a carrier, mimicking the graft used for DSAEK, therefore has reasonable potential for spreading the new technique to a large population.
2. The carrier must be transparent and its refractive index close to 1.38 (mean index of the stroma[4]).
3. It must allow free passage of nutrients, gas, and metabolic waste.

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4. It must be corneo-compatible; that is, either integrating with the posterior stroma without triggering inflammation or fibrosis formation, or degrading without a trace.
5. It must promote EC growth so as to reach confluence at high endothelial cell density (ECD), with adherence strength compatible with surgical insertion into the recipient eye through a small incision.
6. In addition, Good Manufacturing Practice synthetic materials or inert biological ones must be used to obtain authorization by health authorities as advanced-therapy medicinal products.

In our laboratory, we chose to recycle human corneas deemed unsuitable for keratoplasty by eye banks. Except for those discarded for uncontrolled infectious risks, most are excellent candidates because human corneal tissue meets all of the requirements listed above. Finally, human cornea was chosen instead of porcine cornea,[5,6] although the latter resource is unlimited, to facilitate acceptance by health authorities. Xenogenic cardiac valves made from porcine and bovine tissue are the most frequently implanted animal tissues, proving that it is not an absolute barrier. The two situations are not, however, fully comparable, for at least two reasons: relevant cardiac diseases are lethal, and chemical fixation of cardiac valves with glutaraldehyde eliminates most infectious risk, except for prions. We recently reported the possibility of obtaining four to seven thin lamellae from one stored human cornea, using a commercial femtosecond laser (FSL) usually used for refractive surgery or corneal graft.[7] In the present study, we selected a method of decellularization and showed that the thin decellularized stromal lamellae (DSL) could be endothelialized with a human corneal EC line and inserted into the anterior chamber as a DSAEK.

Methods

Human Corneas

Thirty-two human corneas unsuitable for transplantation were obtained from the eye bank of Saint-Etienne, as authorized by French bioethics laws. All procedures conformed to the tenets of the Declaration of Helsinki for biomedical research involving human subjects. Mean donor age was 78 ± 9 (62, 95; min, max) years, which is typical in Europe. The corneas were procured by in situ excision with 16 to 18 mm diameter trephination, as per the procedure recommended in France for corneas intended for transplantation. They were then immediately placed in 100 mL organ culture (OC) medium (CorneaMax; Eurobio, Les Ulis, France) and stored at 31°C in a dry incubator, as per the OC preservation procedure of most European eye banks. Mean storage time was 30 ± 13 (7, 51) days. Fresh eyeballs were procured from bodies donated to science (Laboratory of Anatomy, Faculty of Medicine of St-Etienne) as permitted by the French bioethics law. Donors volunteer their body and give written consent to the Laboratory of Anatomy; no further specific approval by the ethics committee is required.

Femtosecond Laser Cutting

Twenty corneas were used for FSL cutting. Mean donor age was 75 ± 8 (62, 91) years, and mean storage time was 39 ± 9 (17, 51) days before cutting. The process of FSL cutting was described previously.[7] Briefly, the corneas were not deswelled before cutting and their thickness was measured using an anterior-segment optical coherence tomograph (AS-OCT) (Casia SS 1000; Tomey, Tokyo, Japan) just before cutting. The cornea was mounted on an artificial anterior chamber. Six horizontal cuts (for seven lamellae) were then made using a Ziemer Z6 FSL (Ziemer, Port, Switzerland), which used a planar epithelial applanation lens. The cuts were made in order of decreasing depth, to prevent the disruption of light scattering by gas bubbles formed during the previous cut. This FSL delivered 250 fs pulses at 1040 nm with a frequency in the MHz range and energy of some nano joules, and with typical spot size smaller than 1 μm. Laser displacement was performed by a rotary scanner creating a 0.8-mm large comb, displaced on the cornea by translation stages, resulting in a 9-mm-diameter lamellar cut. Each lamellar cut lasted approximately 45 seconds. After laser cutting, a mechanical trephination was performed using a Hessburg-Barron punch trephine of 8-mm diameter (Katena Products, Denville, NJ, USA). Each lamella was then detached under an operating microscope by a senior surgeon. The process was done in sterile conditions. A first series of lamellae was immediately decellularized with various methods (see below). In a second stage, lamellae were stored in 70% ethanol in water to assess whether decellularization could be delayed without damaging collagen structures, especially for future industrialization. The ethanol was intended to block enzymatic activities and prevent protein degradation during cell death and collagenase release, while limiting the risk of microbiological contamination.

Table 1. Methods of Decellularization of Stromal Lamellae

No.	Method
1	Sonification at 37 kHz for 45 min in PBS[9,10]
2	3 freezing/thawing rapid cycles in liquid nitrogen and 37°C[11]
3	Freezing in liquid nitrogen followed by hypoxia in nitrogen[12]
4	1.5 M NaCl for 24 hours followed by 0.02% EDTA + 0.05% trypsin for 24 h[13]
5	0.1% SDS for 24 h[14]
6	1% SDS for 10 min (3 cycles) on an orbital shaker (140 rpm) at RT[5]
7	Method 6, abundant rinse, then DNase I for 30 min

Trypsin (T3924; Sigma); SDS (L4509; Sigma); DNase I (1010395; Qiagen, Hilden, Germany).

Decellularization Methods and Long-Term Storage

Corneal lamellae were decellularized using seven methods described in Table 1. The first six had been reported in the literature for human or animal corneas. The seventh, combining a detergent (SDS) to destroy cell membranes and DNase to cleave DNA, was original in this indication but already described for bovine pericardium.[8] Each method 9 through 14 was tested three times on lamellae from different donor corneas.

After selection of the best decellularization protocol (see below), 40 new DSLs were generated, of which 19 were preprocessed with 70% ethanol. They were rinsed five times in sterile water and placed in sterile Petri dishes with watertight lids (50 × 9 mm, 351006; Falcon, Corning, NY, USA) and excess water was removed by sterile microsponges. The dishes containing partially dehydrated lamellae were covered by parafilm (PM-996, PARAFILM; Parafilm, Neenah, WI, USA) and stored at 4°C. Twenty-one DSLs were stored for 24 months.

Assessment of Decellularization Efficiency

Presence of cell debris was assessed using double-staining with DiOC6 and Hoechst. $DiOC_6(3)$ (3,3′-dihexyloxacarbocyanine iodide, D-273; Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA) stained the cytoplasm by labeling the different organelles[15] and Hoechst 33342 (Sigma, Saint-

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Quentin Fallavier, France) stained nuclear DNA. Lamellae were incubated for 15 minutes at room temperature (RT) in a solution containing 2.5 μg/mL of $DiOC_6$ and 5 μg/mL of Hoechst 33342 in PBS. Cytoplasmic and nuclear debris were then visualized with three fluorescence microscopes. Entire lamellae were observed using a macroscope (macro-zoom microscope) equipped with fluorescence (MVX10; Olympus, Tokyo, Japan) to assess whether decellularization was homogeneous on whole lamella. High-magnification images (×10, ×40, ×60) were also acquired using a fluorescence microscope (IX81; Olympus) to detect cell debris. Images of the three-dimensional projection were obtained with a confocal microscope (IX83, FLUOVIEW FV1200; Olympus) to assess whether decellularization was efficient over the thickness of the lamellae. The assessment was semiquantitative, with a goal of eliminating all visible cytoplasmic and nuclear debris. Each method was tested independently three times. Only lamellae closest to ideal requirements were selected for further experimentation. The best method was tested twice more to verify its reproducibility.

The effect of preprocessing with 70% ethanol was assessed on the best decellularization method (see the Results section). We first verified how ethanol alone altered keratocytes using triple labeling with Hoechst, Ethidium, and Calcein-AM that respectively stained all nuclei, nuclei of dead cells, and the cytoplasm of living cells with intact esterase activity. Briefly, lamellae were rehydrated in PBS, then incubated with a solution containing 5 μg/mL Hoechst 33342, 4-μM Ethidium homodimer (FP-FI9821; Interchim, Montluçon, France), and 2 μM Calcein-AM (FP-CE9790; Interchim) for 30 minutes at RT. Second, the efficiency of decellularization of lamellae preprocessed in ethanol was assessed using the method described above.

Assessment of Transparency and Thickness of Decellularization Lamellae

Transparency was estimated using a custom-made noninvasive device based on a recently reported principle and that is routinely used in our eye bank.[16] The principle is to measure the capacity to transmit contrasts of a back-lit chart composed of parallel black lines over a white background. It is similar to the measurement of the modulation transfer function used in optics to assess performance of optical systems, but for only one fixed spatial frequency. A score of 100 indicated an optical system with perfect contrast transmission, and 0 meant total opacity. Lamellae were assessed just after FSL cutting, before decellularization. Decellularized lamellae, stored at 4°C for 24 hours, were rehydrated in water for 5 minutes and transparency was remeasured. After 24 months' storage, the same lamellae, rehydrated in the same conditions, were measured again.

Lamella thickness was measured before and after decellularization, and after 24 months' storage, using the same AS-OCT as for the whole corneas before cutting.

Electron Microscopy

Cutting-surface roughness was assessed by scanning electron microscopy (SEM). The specimens were fixed in 0.1 M cacodylate (12300; Electron Microscopy Sciences, Hatfield, UK), buffered 2% glutaraldehyde (16220; Electron Microscopy Sciences), pH 7.4, at 4°C for 24 hours. They were washed with 0.2 M cacodylate and then water. The fixed specimens were dehydrated through ascending concentrations of acetone up to pure acetone, then dried using a critical point dryer (E3000; Quarum Technologies, East Sussex, UK). After being coated with gold-palladium in a mini sputter coater (Polaron SC 7620; Quarum Technologies) and mounted on metal stubs, the specimens were observed using an SEM (S-3000N; Hitachi, Tokyo, Japan) at an electron-accelerating voltage of 5 kV.

Cell debris and collagen organization of stromal lamellae were examined by transmission electron microscopy (TEM). Lamellae were fixed in 1% glutaraldehyde/0.5% paraformaldehyde (P/0840/53; Fisher Scientific, Loughborough, UK) in 0.02 M Na_2HPO_4 (Prolabo, Paris, France)/0.08M KH_2PO_4 (P3786; Sigma) buffer (pH 7.4), postfixed in 0.1 M cacodylate-buffered 1% osmium tetroxide (19180; Electron Microscopy Sciences) for 1 hour, dehydrated in a graded series of ethanol, and embedded in Epon resin (Oxford Instruments, Oxford, UK). Ultrathin sections of 90 nm were cut and stained with 7% uranyl acetate (73943; Sigma) in methanol and lead citrate (C6522; Sigma). Pictures were taken with a TEM (H-800; Hitachi) equipped with a charge-coupled device camera (XR40; AMT, Danvers, MA, USA). Three lamellae from different corneas were prepared and analyzed.

Endothelialization

The DSLs were rehydrated in balanced salt solution (BSS) containing 1/100 antibiotic-antimycotic cocktail (A5955-100; Sigma). The lamellae were carefully detached using toothless forceps from the Petri dishes and rinsed in BSS without antibiotic-antimycotic. The lamellae were then placed on the bottom of a 24-well cell culture plate (3526; Costar, Corning, NY). To maintain lamellae adherent onto the plastic surface and to increase cell adherence on the lamellae, 100 μL FNC coating mix (0407; AthenaES, Baltimore, MD, USA) was added and incubated for 5 minutes at RT. The FNC coating mix was removed and the coated lamellae, in the wells, were air-dried for 10 minutes at RT. One milliliter cell culture medium (human endothelial serum-free medium [Gibco, 11111-044; Life Technologies, Waltham, MA, USA] containing 10 ng/mL human FGF-2) was added carefully without moving the lamellae and incubated for 30 minutes at 37°C in a 5% CO_2 incubator. The human corneal EC line HCEC-B4G12 was used.[17] To reach a theoretical ECD of 6000 cells/mm^2, 1.14 million cells in 0.5 mL of cell culture medium were added carefully. The culture medium was renewed after 1 and 3 days, and the neo-endothelium was assessed after 5 days. Six lamellae were endothelialized 24 hours after decellularization, and four after 20 months of storage.

To assess the capacity of decellularized lamellae to stimulate EC adherence, two other biological surfaces were endothelialized in parallel: ex vivo human whole corneas and compressed collagen discs. Whole corneas mimic the situation of a suspension of cultivated ECs being injected directly into the recipient's anterior chamber. This cell therapy method was reported in monkeys by Koizumi et al.[18] and is currently under clinical investigation by Koizumi. Discs of compressed type 1 collagen were already proposed as a carrier during endothelial bioengineering.[19]

Twelve organ-cultured human corneas, stored in CorneaMax (Eurobio), were used. The ECs of six corneas were removed using sterile ophthalmic microsponges without damaging the Descemet membrane (DM). Efficacy of denudation was verified using Trypan blue, which stained the DM homogeneously. Mean donor age was 83 ± 9 (72, 95) years, and mean storage time was 17 ± 10 (7, 30) days. The DM of the other six corneas was carefully peeled off with forceps. Mean donor age was 85 ± 7 (76, 95) years, and mean storage time was 15 ± 7 (7, 28) days. Tissue was handled aseptically under an operating microscope. Corneas without ECs or DM were rinsed for 5 minutes with BSS containing antibiotic-antimycotic cocktail, then placed on the bottom of a 24-well cell culture plate. A total of 100 μL FNC coating mix was added

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and incubated for 5 minutes at RT, and the excess mix was removed with a microsponge without leaving the surface to dry. The cell-seeding procedure was the same as for the stromal lamellae.

Six thin discs of compressed collagen were generated as per the manufacturer's instructions. The reagent kit (016-OR94; Lonza, Cologne, Germany) contained collagen solution (2 mg/mL rat tail collagen type I), minimum essential medium (MEM) (10X), and neutralizing solution. The mix proportion was 10:80:6:4 for collagen solution: MEM (10X): neutralizing solution: cell culture medium. The ingredients were mixed gently to avoid bubble formation and on ice to block polymerization. A volume of 1.5 mL of the mixture was added to each well of a 24-well culture plate. After 15 minutes at 37 °C, excess liquid was removed by specific absorbers during 15 minutes at RT in a laminar flow hood. Compressed collagen discs were gently extracted from the well bottoms. The endothelialization process was the same as for the decellularized lamellae.

The quality of the endothelialization of the four carriers was assessed using three complementary methods: Alizarin red staining, SEM, and TEM (as described above). Alizarin red is a nonvital dye currently used to assess the morphology of corneal ECs because it perfectly outlines cell boundaries.[20] It binds to calcium concentrated in tight junctions of apical ECs. Endothelialized carriers were rinsed with BSS, excess liquid was removed, and 250 μL 0.5% Alizarin red solution in 0.9% NaCl, pH 5.2, was added to cover the tissue for 2 minutes. Alizarin red was removed and 2 mL 4% paraformaldehyde (PFA) in PBS, pH 7.4, was added to the well for 2 minutes. Excess dye deposits were rinsed twice with PBS. A volume of 1 mL ethanol was added to each well and removed after 30 seconds. The cells were rehydrated in PBS, and the nuclei were then counterstained with 5 μg/mL Hoechst 33342 in PBS. Some lamellae were observed directly in culture-plate wells. Other lamellae, compressed collagen discs, and whole corneas were mounted on a glass slide and observed with a transmitted light microscope. Endothelial cell density was determined using Cell^P (Cell Imaging, Hamburg, Germany). Briefly, after proper calibration using a certified ruler, EC nuclei were counted in five images per cornea (objective ×40), using a conventional variable-frame technique and with thresholding of the Hoechst fluorescence. Detected nuclei were verified and manual corrections performed whenever necessary. The mean ECD of the five images was considered.

In addition, endothelialized lamellae were detached from the culture plate and placed in a plastic cartridge resembling those currently used for insertion of endothelial graft into the anterior chamber of the recipient eye. The lamella was passed through the narrow opening of the cartridge and unrolled on a glass slide for further microscopic observation. In two cases, we also reproduced a surgical procedure by injecting an endothelialized lamella into the anterior chamber of a freshly enucleated human eyeball (body donated to science). After unfolding of the bioengineered endothelial graft, the anterior chamber was filled with air, the wound was sutured, and images were acquired with an AS-OCT.

Immunostaining

The differentiation status of ECs on DSL was assessed by immunostaining of ZO-1 and Na^+/K^+ ATPase using an optimized method previously described.[21,22] Briefly, endothelialized lamellae or ECs in cell culture plates were fixed in pure methanol for 30 minutes at RT. After rehydration in PBS, the nonspecific binding sites were then blocked by incubation in PBS containing 2% heat-inactivated goat serum and 2% BSA for 30 minutes at 37°C. The primary antibodies, ZO-1 (40-2200; Zymed, Carlsbad, CA, USA) and Na^+/K^+ ATPase (05-369; Millipore, Billerica, MA, USA) were diluted at 1/200 in the blocking buffer and incubated for 1 hour at 37°C. The secondary antibodies were diluted at 1/500 in the blocking buffer and incubated for 45 minutes at 37°C. These consisted of Alexa Fluor 488 goat anti-mouse (A-11001; Invitrogen, Eugene, OR, USA) or Alexa Fluor 555 goat anti-rabbit (A-21429; Invitrogen). Nuclei were finally counterstained with 5 μg/mL Hoechst 33342 (Sigma) in PBS at RT for 5 minutes. Three rinses in PBS were performed between all steps, except between saturation of nonspecific protein binding sites and incubation with primary antibody. The endothelialized lamellae were finally placed on glass slides, covered with Vectashield Mounting Medium (H-1000; Vector Laboratories, Burlingame, CA, USA), and gently covered with a large glass coverslip retained immobilized by adhesive tape. The ECs on cell culture plates were observed directly with an inverted fluorescence microscope. Immunolabeling was performed on three independent experiments.

Statistics

Data were presented as median (10th–90th percentile) and compared with nonparametric rank tests, with $P < 0.05$ deemed significant. Analyses were performed with IBM SPSS Statistics V23 (IBM Corporation, Chicago, IL, USA).

Results

Decellularization of FSL Cut Stromal Lamellae

A mean of six lamellae per cornea were obtained. The efficiency of the seven decellularization methods varied considerably. The physical methods were inefficient. The hyperosmolar NaCl solution, followed by EDTA and trypsin, left most of the cell debris intact. Only SDS was able to eliminate cytoplasmic debris but was not efficient enough to eliminate nuclear debris (Figs. 1A, 2). The latter were then efficiently removed by DNase I. Ultrastructural analysis by TEM confirmed the absence of cell debris after decellularization with SDS and DNase I (Fig. 3). Preprocessing with 70% ethanol stopped enzymatic activity after 20 minutes, left dead cells in place, and did not interfere with subsequent decellularization by SDS and DNAse I (Fig. 1B).

Thickness, Transparency, and Ultrastructure After Decellularization and Storage

After decellularization with 1% SDS and DNase I, thickness of hydrated lamellae significantly decreased by 39.0% (0.1–63.7) (median (10°–90° percentiles, $n = 40$), and their transparency significantly increased by 4.8% (–0.8 to 12.0; $n = 39$) ($P <$ 0.001 for both) (Fig. 3). There was no difference between lamellae stored in 70% ethanol before decellularization and those treated directly (data not shown). The process respected the ultrastructure of collagen fibers while efficiently removing keratocytes. The cut surfaces appeared even, compatible both with cell cultures and with adherence to the stromal bed in the recipient cornea. By comparison, compressed collagen discs ($n = 10$) had a lower transparency than decellularized lamellae ($n = 40$): respectively 44.8% (42.7–57.2) versus 86.5% (78.9–92.2) ($P < 0.001$), and comparable thickness: 91.5 μm (69–114) versus 105 μm (69–150) ($P = 0.079$). After 20 months' storage, despite maximum rehydration before measurement, thickness significantly decreased by 46.7% (18.1–57.8; $n = 21$) again, while transparency kept increasing by 7.6% (–1.3 to 14.5; $n = 22$) ($P < 0.001$ for both). The ultrastructure (TEM) of collagen

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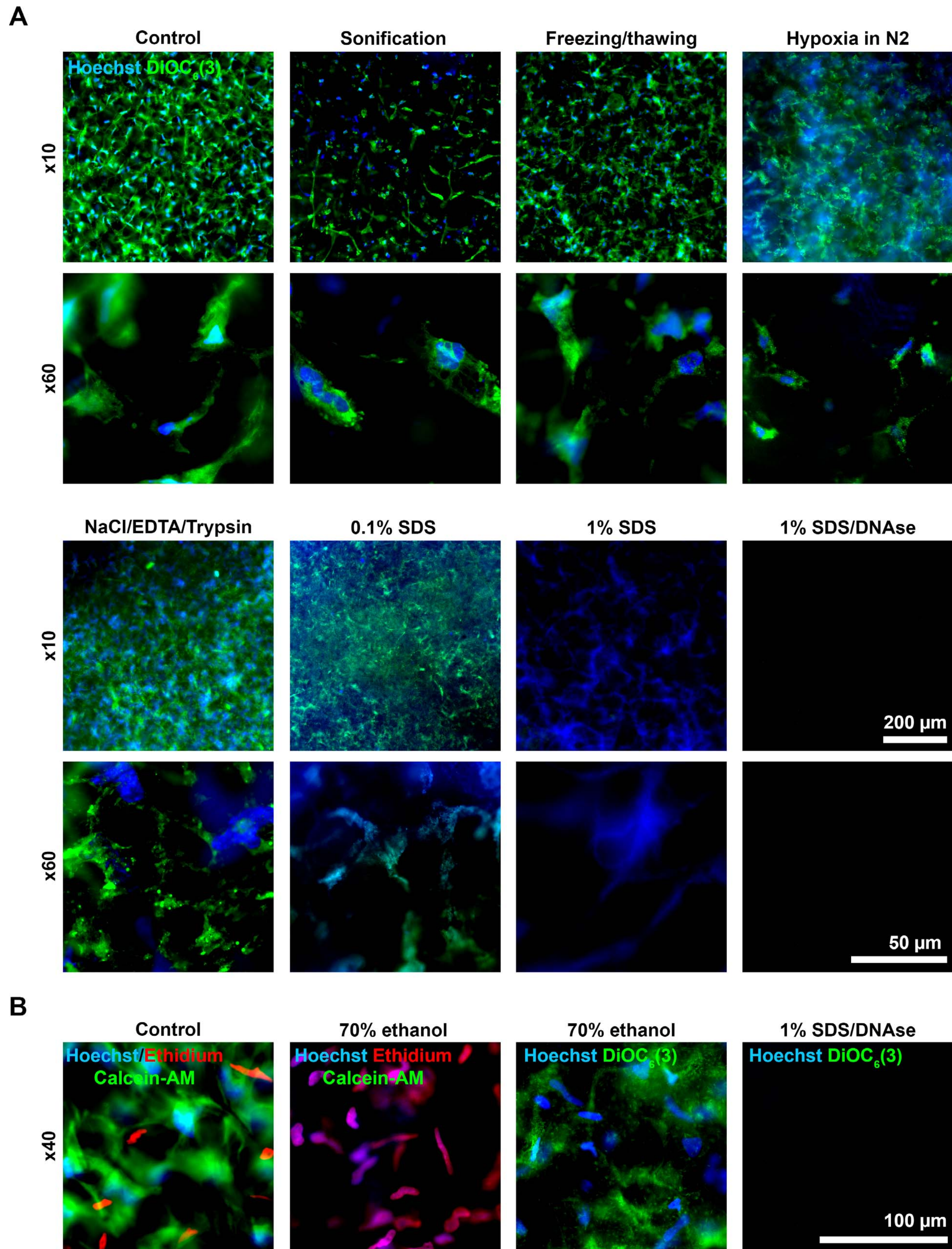

FIGURE 1. Comparison of decellularization processes. (**A**) Efficiency was assessed by double staining with Hoechst 33342 (DNA) and $DiOC_6(3)$ (organelles). A method was deemed efficient only if no residual staining could be observed. Low magnification (×10) showed homogeneity of the processes, whereas high magnification (×60) showed details of remaining structures. Only the sequence comprising 1% SDS followed by 0.1U DNAse I was fully efficient. (**B**) Assessment of preprocessing of lamellae with 70% ethanol. Ethanol killed all keratocytes without destroying them. Subsequent decellularization by SDS and DNAse I remained efficient.

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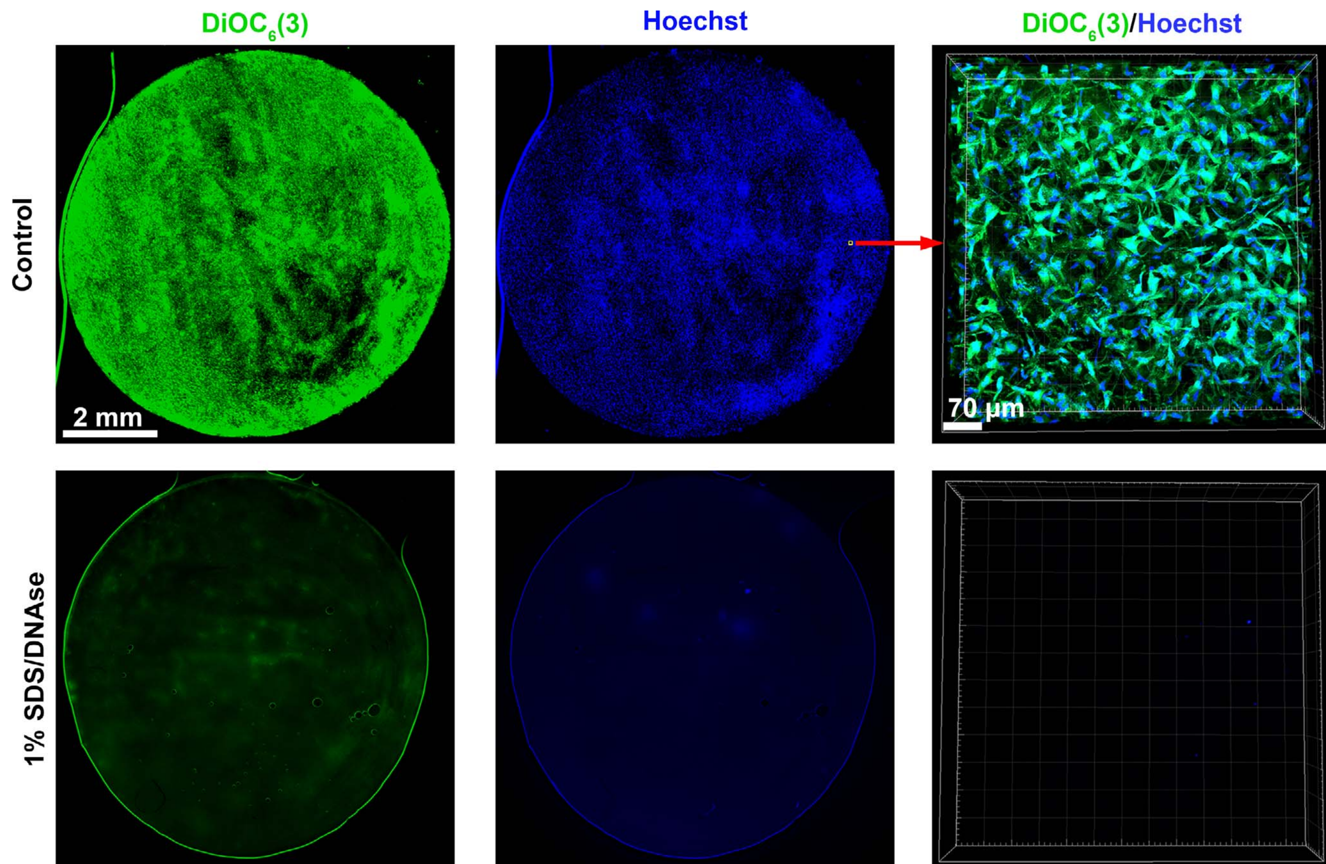

FIGURE 2. Verification of the efficiency of the selected decellularization process (1% SDS followed by 0.1 U DNAse I). The examination with a fluorescence macroscope allowed assessing the lamella as a whole (*left columns*), and confocal stacks and three-dimensional projection allowed assessing the whole lamella thickness (*right column*).

fibers seemed unchanged, with normal fiber diameter and organization in parallel bundles, without rupture. Some cross-sections showed a decrease in spacing between collagen fibers (data not shown).

Endothelialization and Surgical Handling

Five days after cell seeding onto the DSL, a monolayer of tightly packed ECs attached directly to the collagen fibers was observed in the 10 lamellae (6 fresh, 4 stored for 20 months). Alizarin red stained the boundaries only of confluent ECs adhering to the lamellae, but not of confluent ECs attached to the bottom of culture-plate wells (Fig. 4). The surgical handling of endothelialized lamellae showed no difference with an ultrathin DSAEK, in particular without tearing. Median ECDs were 6273 (4622–7364) cells/mm^2 (n = 6 fresh) and 6734 (6051–7570) cells/mm^2 (n = 4 stored) (P = 0.476). For the three other carriers, ECDs were 2144 (1690–2370) cells/mm^2 (n = 6) for whole corneas with DM, 2438 (1709–2019) cells/mm^2 (n = 6) for whole corneas without DM, and 5370 (4111–7065) cells/mm^2 (n = 6) for compressed collagen discs (Fig. 5).

DISCUSSION

As EK is now widely used to treat endothelial dysfunctions, replacing donor cornea by bioengineered corneal endothelial graft could theoretically allow a 30% increase of tissue available for the other types of graft: penetrating and anterior lamellar keratoplasties. This will become possible only when reliable methods of mass EC culture, mass production of cell carriers, and endothelialization are available using only a small number of donor corneas. The present study describes a simple process to prepare several thin decellularized carriers from one single human cornea cut with an FSL. The lamellae can be stored for later use and, after endothelialization, allow reformation of a continuous monolayer of tightly packed cells with up to very high ECD and morphologic characteristics close to those of native ECs.

To date, two types of carrier have been reported: biological tissue and reconstructed matrix. Thin carriers assessed in the past 35 years for endothelial bioengineering are summarized in Table 2. Only one process, published by Choi et al.,[23] used human decellularized corneal lamellae. Up to four lamellae per cornea were cut using a microtome with a 110-µm head. Using an FSL, we were able to obtain significantly more lamellae. Femtosecond lasers are widely used in ophthalmology and we used standard cutting parameters. The performance of commercial FSLs will also likely improve over time, and facilitate cutting of multiple lamellae. In addition, we selected an original combination of SDS and DNase that efficiently removed proteins, lipids, and DNA. Sodium dodecyl sulfate was previously shown to be efficient for whole cornea decellularization without altering mechanical properties at 0.5% concentration for 24 hours.[24] Here we used a higher concentration but for only three 10-minute cycles. The use of DNase was previously reported for the decellularization of whole porcine corneas but only in combination with 0.1 M NaOH, which resulted in severe tissue degradation.[25] In our work, SDS + DNase I was the only combination to efficiently remove all keratocytes without altering collagen-fiber organization.

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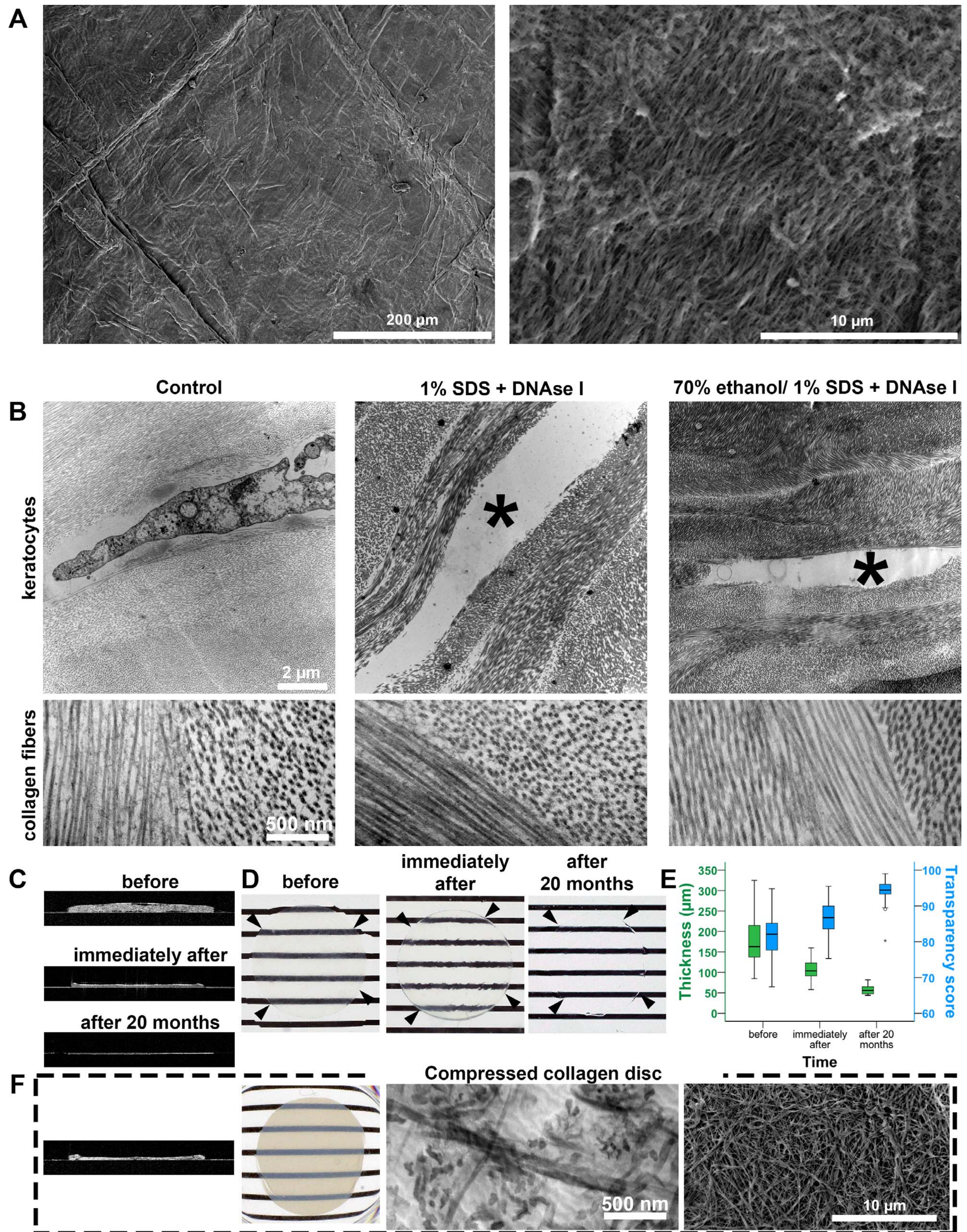

FIGURE 3. Decellularization with 1% SDS and 0.1 U DNase I with or without preprocessing in 70% ethanol. (**A**) Scanning electron microscopy at ×200 and ×5000 showed that decellularized lamellae had a smooth surface compatible with cell culture. (**B**) The ultrastructure of collagen fibers was not damaged by the decellularization process, and no cell debris was left. *Asterisks* indicate spaces left empty by keratocytes. (**C**) Thickness measurement by OCT. (**D**) Transparency. Lamellae were placed on a back-lit chart. *Arrowheads* mark the contours of the lamella, which in some cases was hardly visible. (**E**) Change in thickness and transparency, induced by decellularization, of 40 lamellae. (**F**) For comparison, the inset (*dash line*) represented the OCT images, the transparency assessment, the ultrastructure, and the surface, by SEM, of a compressed collagen disc.

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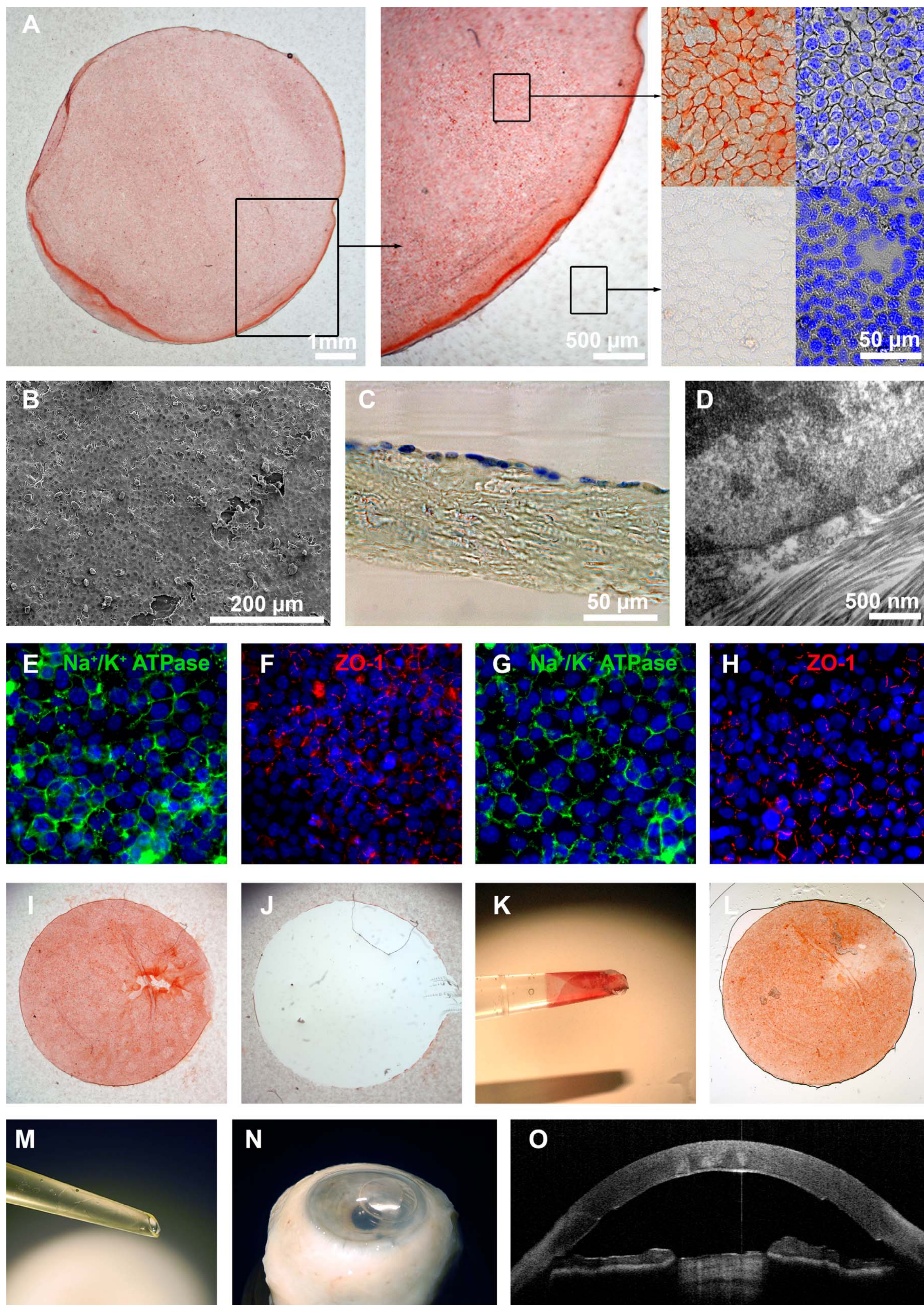

FIGURE 4. Endothelialization of the DSL. (**A**) Alizarin red staining of a lamella observed directly in the cell culture plate. Only cells grown on the collagen substrate presented the characteristic staining pattern of ECs. (**B**) Scanning electron microscopy showed an even cell layer. (**C**) Semithin sections confirmed the reconstruction of a cell monolayer (Toluidine blue staining). (**D**) Transmission electron microscopy (×50,000) showed the direct attachment of a cell on the sectioned collagen fibers. (**E**, **F**) Immunolabeling for Na^+/K^+ ATPase and ZO-1 of the endothelial cell line cultivated on a plastic culture plate. (**G**, **H**) Same markers after endothelialization of the DSL. (**I**) Another endothelialized DSL after staining with Alizarin red

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(the paracentral white/red area was an artifact caused by heterogeneous Alizarin red deposits) (**J**) The lamella could be easily detached from the culture plate. (**K**) The lamella could be rolled into a disposable cartridge. (**L**) After passing through the narrow opening of the cartridge, it was unrolled on a glass slide and showed that the neo-endothelium remained attached to the collagen. (**M**) After endothelialization, the reconstructed endothelial graft was inserted in a disposable injector, and a graft was simulated into a cadaver human eyeball (**N**). (**O**) The AS-OCT showed that the very thin lamellae perfectly followed the posterior curvature of the recipient cornea.

Immediately after decellularization, FSL-cut lamellae were approximately 100-µm thick. After 2 years' storage, despite maximal rehydration, their thickness had reduced significantly to nearly 50 µm. We suppose that this decrease was due to subtle changes in the proteoglycan content, as suggested by the reduction of interfiber spaces highlighted by TEM. Nevertheless, this thickness seems a good compromise, allowing easy handling but thin enough to give good visual results. Comparison with DSAEK or ultrathin DSAEK grafts prepared in living corneas is difficult because the behavior of decellularized stroma after implantation remains hypothetic. Integration and colonization by the host keratocytes, resorption of degraded collagen molecules, or both, could alter the thickness of the lamella remaining in the host cornea.

The use of human corneas presents advantages over xenogenic tissue or synthetic matrix:

1. They are, by definition, fully corneo-compatible (provided decellularization does not leave toxic residues). Our decellularization process uses only SDS and DNase I. The possibility to cultivate ECs on the scaffold demonstrates that thorough rinsing eliminates chemical residues.
2. It is nonxenogenic (no risk of zoonose or of systematic rejection mediated by xenogenic antigens).
3. The recycling of human corneas usually discarded by eye banks raises no ethical concerns. In a first approach, lamellae could be prepared from donor corneas that present no microbiological risk but are discarded because of endothelial insufficiency. Although percentages vary between countries and eye banks, this could apply to approximately 20% of procured corneas. We recently estimated that at least 100,000 corneas are discarded each year, of which 20% could be recycled.[1] Consequently, with a mean of five lamellae per cornea (the most pessimistic assumption), 100,000 bioengineered EKs could theoretically be reconstructed; that is, far more than the estimated number of EKs performed worldwide (30% of the 184,576 corneal grafts identified). Given that process yield will unlikely reach 100% of suitable bioengineered grafts, and that only a limited number of centers will be involved, alternative sources of human corneas could be necessary: additional viral-inactivation techniques, such as gamma irradiation,[47] could allow the use of corneas discarded for donor seropositivity, and stromal lenticules removed during FSL refractive surgery using small-incision lenticule extraction (SMILE) also could be collected.[48] These points should facilitate authorization by health authorities due to a benefit/risk ratio more favorable than with xenogenic tissue or synthetic matrix. In addition, we demonstrated that they can be reendothelialized directly on the sectioned collagen fibers, with a very high ECD. Noticeably, ECs seems to have higher ECD on denuded decellularized stroma than on whole living corneas, suggesting that interactions between keratocytes and ECs stabilize the endothelial mosaic and prevent from obtaining very high ECD. In addition, contrary to whole corneas that retain their concave shape and present storage-induced Descemetic folds, the surface of DSLs is flat and smooth, two characteristics liable to facilitate EC adherence and survival. Furthermore, the interaction between human stromal fibers and ECs seems to promote the formation of mature tight junctions presenting a normal staining pattern with Alizarin red, unlike ECs grown on plastic.
4. The natural transparency of human corneas is unaltered by our decellularization process. Transparency is crucial, particularly for patients who have sustained only moderate visual impairment. Of the carriers previously proposed, amniotic membrane and compressed collagen disc are not fully transparent and thus cannot be used for all patients.
5. They have a natural curved shape that prevents fold formation, as demonstrated by Kimoto et al.[36]
6. The mechanical characteristics of decellularized lamellae will likely be similar to those of lamellae cut by surgeons or eye banks in ex vivo corneas during DSAEK. Consequently, handling by technicians in the cell therapy unit and by surgeons will remain easy.

The use of human corneas also presents drawbacks: quality could vary between donors; quality could also vary with position within the stroma (anterior, intermediate, posterior) because of gradual changes in collagen-lamellae organization from epithelium to endothelium[49]; and preparation of multiple lamellae requires an expensive FSL. Nevertheless, we demonstrated that their preparation could be rationalized, with the possibility to delay the decellularization process after FSL cutting, simply by immersing the lamellae in 70% ethanol to stop enzymatic activity of keratocytes and to prevent degradation by proteases released during cell necrosis. A cutting program can therefore be organized with an ophthalmology center equipped with an FSL, and tissue stored until decellularization. In addition, we demonstrate the possibility of long-term storage (more than 1 year) at 4°C until reendothelialization.

Further studies will assess whether lamellae cut in the anterior, intermediate, and posterior stroma, and whether lamellae cut in donor cornea belonging to different age groups, behave differently in terms of endothelialization, surgical handling, and adherence to the recipient cornea. Although the present work suggests that the decellularization process decreases the differences between lamellae, it will be necessary to record all these parameters in future preclinical validations steps.

Endothelialization with nonimmortalized ECs will be essential to envisage translation to clinical applications. Reaching very high ECD with untransformed primary cultures of EC, to create endothelial graft with fetal ECD, also is a challenge. It will certainly increase graft survival by allowing a better resistance to surgical trauma, to immune rejection episodes, and to the subsequent slow chronic cell loss. The strength of adherence between the neo-endothelium and the stroma is another critical point, as the bioengineered graft must withstand the usual insertion techniques. Furthermore, animal experiments using a validated model of endothelial dysfunction will be required to validate the functionality of the bioengineered grafts.

Thanks to their high availability, decellularized human corneal lamellae cut by FSL could play a major role in the development of advanced-therapy medicinal products de-

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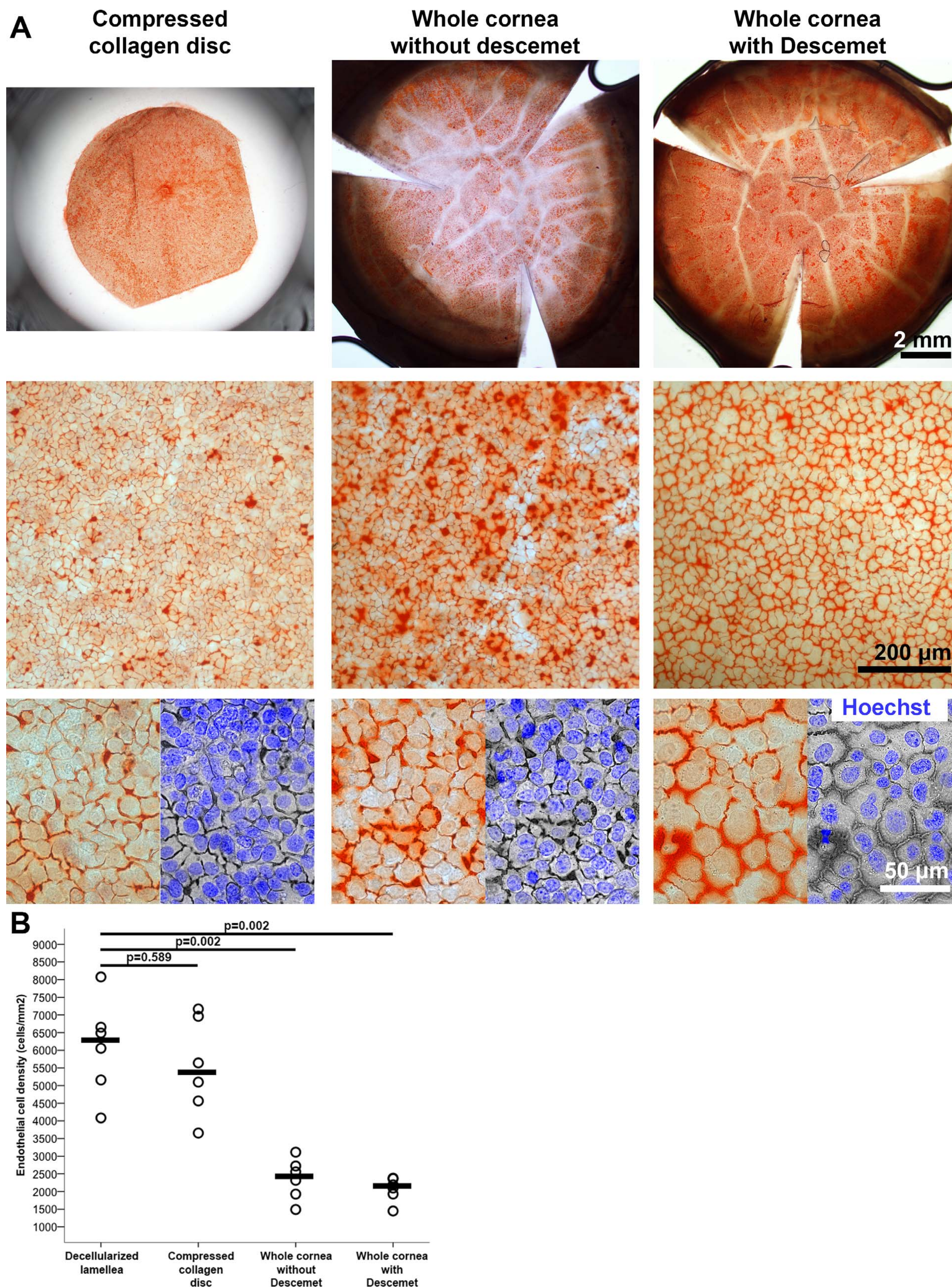

FIGURE 5. Endothelialization of the other carriers and comparison with the DSL. (**A**) Flat mounts, showed at increasing magnification (×0.79, ×10, and ×60), of the three carriers after Alizarin red and Hoechst staining to visualize cell contours and cell nuclei. For whole corneas, three radial cuts were needed for flat mounting. (**B**) Comparison of the endothelial cell density obtained for compressed collagen discs, ex vivo human whole cornea with intact DM, and ex vivo human whole cornea of which the DM was peeled off, compared with DSL ($n = 6$ for each or all four of the carriers). All individual data are represented as *circles*, along with their median (*black bar*).

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TABLE 2. Types of Carrier Already Assessed for Endothelial Bioengineering

Year, First Author Reference Country/Town	Nature of the Carrier	Endothelialization	Assessment
Synthetic			
1980, Jumblatt MM[26] USA/Stanford	1–6-μm-thick gelatin membrane, cross-linked with glutaraldehyde	Rabbit	Animal exp/rabbit
1994, Mohay J[27] USA/Louisville	100-μm-thick hydrogel lens	Rabbit and cat	Animal exp/rabbit and cat
2004, Mimura T[28] Japan/Tokyo	40–50-μm-thick cross-linked collagen I sheet	Human	Animal exp/rabbit
2006 & 2007, Hsiue GH[29]; Lai JY[30] Taiwan/Taipei	700–800-μm-thick gelatin (denatured bovine bone collagen) disc	Human	Animal exp/rabbit
2007, Koizumi N[31] Japan/Kyoto	Collagen I sheets (Vitrigel; Asahi Techno Glass, Tokyo, Japan)	Monkey	Animal exp/monkey
2011, Madden P[32] Australia/Brisbane	5-μm-thick Silkworm (*Bombyx mori*) fibroin membrane	Human (cell line)	Lab exp/IHC
2011, Watanabe R[33] Japan/Sendai	50-μm-thick gelatin hydrogel sheet derived from porcine skin	Human	Lab exp/IHC
2012, Levis HJ[19] UK/London	75-μm-thick compressed rat tail–derived collagen I disc	Human (primary and cell line)	Lab exp/IHC+SEM
2012, Wang TJ[34] Taiwan/Taipei	Chitosan:polycaprolactone (75:25) blend membrane	Bovine	Lab exp/IHC
2013, Lai JY[35] Taiwan/Taoyuan	700-μm-thick cross-linked porous gelatin hydrogels (derived from porcine skin)	Rabbit	Lab exp/viability*
2014, Kimoto M[36] Japan/Kobe+Tokyo	20-μm-thick spherically curved gelatin hydrogel sheet (derived from porcine skin)	Monkey	Animal exp/monkey
2014, Yoshida J[37] Japan/Tokyo	20-μm-thick spherically curved porcine-derived atelocollagen vitrigel	Human	Lab exp/alizarine red*
2014, Ozcelik B[38] Australia/Victoria	50-μm-thick biodegradable Polyethylene glycol–based hydrogel film	Sheep	Animal exp/sheep
Biological			
1993, Lange TM[39] USA/Louisville	Bovine Descemet membrane	Rabbit	Animal exp/rabbit
2004, Ishino Y[40] Japan/Kyoto	Denuded amniotic membrane	Human	Animal exp/rabbit
2007, Wencan W[41] China/Wenzhou	Basement membrane of human amniotic membrane	Cat	Animal exp/cat
2009, Yoeruek E[42] Germany/Tübingen	Human lens capsule retrieved during cataract surgery	Human	Lab exp/IHC
2009, Honda N[43] Japan/Tokyo	**Manually dissected human corneal stromal disc (2–3 per cornea), not decellularized**	**Human**	**Animal exp/rabbit**
2010, Choi JS[23] USA/Winston-Salem	**Microtome cut, 120–200 μm thick human stromal lamellae (3–4 per cornea), decellularized (2%triton X-100+ 0.1%NH4OH)**	**Human**	**Lab exp/IHC+SEM +Alizarin red**
2012, Kopsachilis N[44] Germany/Erlangen	Human lens capsule from donor eyes	Human	Lab exp/IHC+SEM
2012, Bayyoud T[45] Germany/Tübingen	Posterior lamella of bovine cornea, decellularized (0.1% EDTA+10KIU aprotinin+0.3% SDS)	Human	Lab exp/IHC
2015, Diao YM[46] China/Beijing	Porcine Descemet membrane	Not done	Lab exp/IHC/ immunogenicity/ safety

We excluded carriers not intended for lamellar grafts, such as full-thickness decellularized corneas. For endothelialization, unless specified otherwise, primary cultures of corneal ECs of the cited species were used. In bold, the two articles reporting use of human corneal lamellae. exp, experiment; IHC, immunohistochemistry; lab, laboratory.

* Implanted in animal for biocompatibility assay but without ECs.

signed to replace donor-derived tissue in treating corneal endothelial dysfunctions.

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