

Storage of Porcine Cornea in an Innovative Bioreactor

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PURPOSE. To quantify cell survival and tissue structure preservation of porcine cornea stored in a bioreactor (BR) that recreates a transcorneal pressure gradient equivalent to intraocular pressure (IOP) and renews the medium.

METHODS. A BR comprising endothelial and epithelial chambers was machined in a biocompatible material. The porcine cornea, securely held, separated the chambers. Medium flow and pressure inside the endothelial chamber were maintained by a peristaltic pump. In the epithelial chamber, the corneal surface was alternatively exposed to air and a specific medium. Two transparent windows allowed thickness measurement by optical coherence tomography without opening the BR. Porcine corneas stored in the BR-on (pressure 20 mm Hg, flow 5 μ L/min, temperature 31°C) were compared with (1) BR-off (no pressure or flow); (2) organ culture; and (3) Petri dish with agar on the endothelial side. Epithelial and limbal structure and differentiation, corneal transparency and thickness, and endothelial viability were compared after 7 days of storage and with fresh corneas.

RESULTS. Corneas stored in the BR-on were thinner and more transparent than those stored with the other methods. The BR-on preserved a stratified and differentiated (K3/K12+) corneal epithelium and undifferentiated basal limbal cells with stemness markers (K3/K12-, ABCB5, K14, p75+), as well as endothelial integrity.

CONCLUSIONS. By recreating equivalent IOP and medium renewal, the BR obtained unprecedented storage quality of porcine corneas and preserved their main epithelial, limbal, and endothelial characteristics.

Keywords: cornea, porcine, bioreactor, epithelium, storage

Ex vivo storage of the cornea in its physiological state is the holy grail of both eye bankers and researchers.

In eye banks, both storage methods currently used worldwide (cold storage in the United States, organ culture in Europe)¹ were introduced 40 years ago, mainly for their ability to ensure endothelial survival. They have rendered huge service, but corneas sustain irreversible alteration during storage, notably endothelial cell (EC) loss hundreds of times faster than during donor life.²⁻⁴ The decrease in the endothelial reserve very likely influences graft survival. The survival of the epithelium is also altered,⁵ but its minor role during corneal transplantation makes it a small concern for eye bankers. Stromal swelling during corneal storage is only partially reversed by osmotic molecules (e.g., dextran).⁶ The overall increase in stromal thickness is likely to delay visual recovery (compared to fresh grafts), as complete deswelling takes at least 1 month.^{7,8}

Fundamental applied and preclinical research in all fields involving corneal tissue (from ophthalmology, through cosmetics, to general drug testing) would also greatly benefit from a

corneal storage method that maintains the integrity of the three corneal layers and their close interrelationship. Such a system could use corneas from food-industry animals that are normally discarded. These tissues could be collected and used immediately post mortem for preclinical tests, limiting the use of laboratory animals and providing an alternative assay in line with animal experimentation rules.⁹ Animal cornea availability allows many experiments at reduced cost. Animal corneas are thus an unlimited research resource in theory, but in practice there is no satisfactory animal model of medium- to long-term corneal storage for easily available species: Ex vivo animal corneas, much more than human ones, quickly and dramatically swell and lose transparency after immersion in storage media. In the past, various irritancy assays were developed: excised corneas stored in a Petri dish using agar to fill the endothelial side of the cornea,¹⁰⁻¹² artificial anterior chambers such as those used for the Ex Vivo Eye Irritation Test (EVEIT)¹³ or the bovine or porcine corneal opacity and permeability test.^{14,15} None of these models extends corneal storage with concomitant epithelial and endothelial preservation, which is



critical given the reported interactions between these two layers; epithelial cell differentiation¹⁶ and epithelial basement membrane formation¹⁷ are influenced by EC function.

Corneal physiology is complex and partly unexplained. Control of cornea shape, transparency, and thickness requires an endothelial pump; epithelial integrity, especially with mature watertight continuous tight junctions between the most superficial cells; and normal intraocular pressure (IOP). The biochemical composition of the stromal extracellular matrix explains its natural swelling when immersed in water. This swelling pressure (SP) (60 mm Hg in humans and pigs) is counterbalanced by the IOP (15 ± 5 mm Hg), which constantly pushes the posterior corneal surface forward, and by the constant water efflux exerted by the endothelial pump. This pump controls the imbibition pressure (IP), and the equilibrium can be summarized by the equation $IP = IOP - SP$.¹⁸ After donor death, and even more so after corneal excision, IOP loss and the concomitant gradual loss of endothelial pumping function explain the rapid swelling of the cornea. Moreover, preservation of corneal epithelial cells and limbal epithelial stem cells requires a specific culture medium, intermittent air exposure,¹⁹ and possibly shear stress.²⁰ Models with intact and functioning epithelial cells are needed to study normal and pathologic epithelial wound healing, and to develop a reliable eye irritancy assay.

To provide a more efficient storage method, we designed a corneal bioreactor (BR) that recreates the IOP in the endothelial compartment and the renewal of nutrient medium. A first version aimed to improve the condition of pregraft human corneas. A second version added intermittent epithelium exposure to air. In the present work, we assessed the integrity of the three corneal layers after 7 days' storage in this second version of our BR, which stores the cornea in a more physiological environment than other methods.

MATERIALS AND METHODS

Corneal Bioreactor

The BR applies principles of previously described perfusion chambers and anterior artificial chambers,^{12,21-26} but also has several innovations (Fig. 1). It was designed to maintain a sterile closed environment enabling long-term storage. The cornea is tightly secured to the BR base, using the scleral rim as a watertight seal to separate the epithelial and endothelial chambers; each chamber is connected to a distinct culture medium control system. A peristaltic pump controlled by a pressure sensor and a microcontroller continuously renews the culture medium (CM) at a rate of 5 μ L/min, while creating a pressure 20 mm Hg higher than atmospheric pressure in the endothelial chamber. The CM was a minimum essential medium-based corneal organ CM containing 2% fetal calf serum (CorneaMax; Eurobio, Les Ulis, France). The epithelial chamber is connected to a peristaltic pump controlled by a microcontroller, alternating exposure of the epithelium to air and immersion in a CM (hereafter called air-lifting). A specific epithelial CM (hereafter called supplemental hormonal epithelial medium [SHEM]) consisted of Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA)/Ham's F12 (Sigma, Saint-Quentin-Fallavier, France) (2:1) supplemented with 10% fetal bovine serum (Eurobio), 10 ng/mL epidermal growth factor, 1% antibiotic antimycotic solution (Sigma), 0.1 nM cholera toxin (Sigma), 5 mg/mL insulin (Sigma), 5 μ g/mL transferrin (Sigma), 0.18 nM Adenin (Sigma), 0.4 μ g/mL hydrocortisone (Sigma), 1 μ g/mL bovine pituitary extract (Thermo Fisher Scientific), and 2 nM 3,3',5 Triiodo-L-thyronine (EMD Millipore, Burlington, MA, USA). The cornea

was exposed to air and to SHEM for 30 and 1 seconds, respectively. The epithelial CM (25 mL) was changed every 2 days. In some of our experiments, the SHEM was replaced by CorneaMax. The BR with aforementioned operating endothelial and epithelial CM control systems is referred to as BR-on. Two transparent windows, on each side of the cornea, enabled optical control during experiments. The BR was designed to be compatible with current ophthalmology imaging systems, such as slit lamp, optical coherence tomography (OCT) systems, and specular microscopy. For the following experiment, prototype BRs were machined in a biocompatible material and included optical quality windows (0.9-mm-thick sapphire windows; France Fourniture Horlogerie, Vence, France). The complete system, except its control panels, was placed in a 31°C dry 5% CO₂ incubator.

Animals were used according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eyeballs of 6-month-old pigs were obtained from a local slaughterhouse within 4 hours after death and transported to the laboratory on ice. Extraocular muscles and conjunctival tissue were removed. Eyeballs were decontaminated by two successive immersions for 5 minutes in 10% and 5% povidone-iodine solutions (Betadine; Meda, Mergnac, France) and rinsed in balanced salt solution (Alcon, Rueil Malmaison, France) supplemented with 1/200 antibiotic antimycotic solution (Sigma). In five cases, central corneal thickness (CCT) was measured with OCT (see below) on the intact eyeball just before corneoscleral excision. The corneas were then excised under a laminar flow hood, in aseptic conditions, using forceps and a surgical scalpel blade, leaving a 3- to 5-mm scleral rim.

To check whether water could enter the ex vivo cornea via the lesions caused by iris root stripping and thus contribute to stromal swelling, the iris root was preserved in a subgroup of corneas. However, partial resection of the iris around the pupil was performed with scissors, as a preliminary experiment had shown harmful contact between the endothelium and the iris left intact.

Storage in the BR-on was compared with three other methods: (1) BR-off—corneas were placed in a BR base without CM control system (i.e., without pressure equivalent or continuous endothelial CM renewal, and without exposure to air of the epithelial chamber); endothelial (2 mL) and epithelial (10 mL) culture media were renewed every 2 days; (2) organ culture (OC), with corneas totally immersed in CM, as in eye banking; (3) agar system—corneas were mounted in a Petri dish on agar on the endothelial side and bathed with epithelial CM. The latter is not a corneal storage method, but has been validated for maintaining epithelial integrity.⁷ Each method is detailed below and summarized in Table 1. All corneas were stored for 7 days, longer than ever previously reported for whole porcine corneas. A group of 10 corneas was also studied fresh to provide baseline data.

Organ Culture. Corneas were immersed in plastic vials (Gosselin, Hazebrouck, France) containing 25 mL CorneaMax and incubated at 31°C, in an atmosphere of 5% CO₂ and 90% relative humidity, as the plastics could be permeable to CO₂.

Agar System. After excision and complete iris removal, corneas were placed on a concave silicon support, epithelial side down. The endothelial side was filled with 2% agar (Sigma) in phosphate-buffered saline (PBS) at 35°C in sterile conditions. Once the agar solidified, the cornea was placed in a six-well plate (Corning Costar, Sigma), epithelial side up, immersed in epithelial CM, and incubated at 31°C, 5% CO₂ and 90% relative humidity.

End-of-Storage Analysis. After 7 days' storage, all corneas were assessed in parallel for integrity of the central corneal and limbal epithelium, stromal characteristics, and endothelial viability.

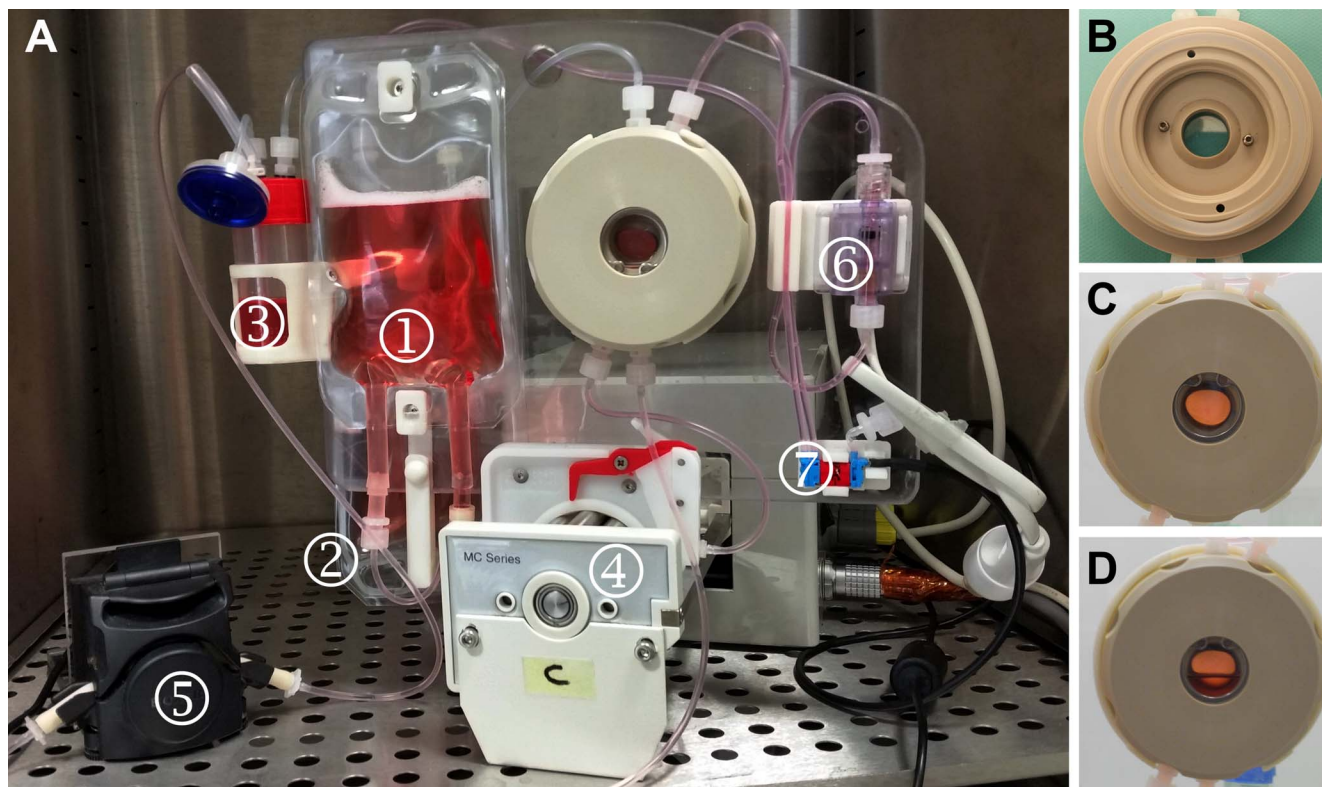


FIGURE 1. Main characteristics of the corneal bioreactor (BR) in a CO₂ incubator. (A) Overview of the BR in its cassette including the bag for fresh endothelial medium (1) and its waste (2), flask for epithelial medium (3), peristaltic pumps (4, 5), pressure sensor (6), and micro solenoid valve (7). (B) Empty BR base with endothelial chamber and posterior window. (C) Porcine cornea in a BR after lid locking. (D) BR operating in corneal epithelium immersion phase.

Epithelial Integrity. The corneas were cut in two. One half was fixed in 4% paraformaldehyde for 24 hours at room temperature (RT), dehydrated through ascending concentrations of ethanol, and embedded in paraffin. Cross sections 7 μm thick were cut, rehydrated, and stained with hematoxylin, eosin, and saffron. Bright-field tagged image format file (TIFF) images of the cross sections were acquired using a microscope (IX81; Olympus, Tokyo, Japan). The other half of the cornea was embedded in optimal cutting compound (CellPath, Newtown, UK) and frozen using 2-methylbutane (Sigma) and liquid nitrogen. Samples were stored at -20°C. Tissue sections (14 μm thick) were cut using Cryostat Microm HM550 (Thermo Fisher Scientific) and spread out on Surgipath X-tra Adhesive (Leica Biosystems, Nussloch, Germany). After rehydration of tissue sections in distilled water for 15 minutes, nonspecific binding sites were blocked by incubation for 30 minutes at

37°C with blocking buffer, based on PBS supplemented with 2% heat-inactivated goat serum (Eurobio) and 2% bovine serum albumin (Thermo Fisher Scientific). Slides were incubated at 37°C for 1 hour with the primary antibody. Primary antibodies were supplied as listed in Table 2. Nonspecific rabbit and mouse immunoglobulin G (IgG; Zymed, Carlsbad, CA, USA) were used as primary antibodies for negative controls. These two controls were performed for each cornea. Secondary antibodies were Alexa Fluor 488 goat anti-mouse or anti-rabbit IgG or/and Alexa Fluor 555 goat anti-mouse or anti-rabbit IgG (Invitrogen, Eugene, OR, USA). Secondary antibodies diluted by 1:500 in blocking buffer were incubated for 1 hour at 37°C. Lastly, nuclei were counterstained with TO-PRO-3 Iodide (1/500) (Thermo Fisher Scientific) in blocking solution for 5 minutes at RT. Three rinses in PBS were performed between each step except between blocking of nonspecific protein

TABLE 1. Study Design

Denomination	Iris	Epithelial Medium	Air-Lifting	Endothelial Medium	Endothelial CM Flow and IOP, mm Hg	n
BR-on with iris SHEM	With	SHEM	Yes	CMax	Yes/20	7
BR-on without iris SHEM	Without	SHEM	Yes	CMax	Yes/20	5
BR-off SHEM	With	SHEM	No	CMax	None	4
BR-off CorneaMax	With	CMax	No	CMax	None	5
BR-off CorneaMax for desiccation experiment	Without	CMax	No	CMax	None	3
Organ culture with iris	With	CMax	No	CMax	None	6
Organ culture without iris	Without	CMax	No	CMax	None	6
Agar system	Without	SHEM	No	None	None	5

CMax, CorneaMax; n, number of replicates.

TABLE 2. Primary Antibodies Used for Immunostaining

Target Protein	Role (Expected Cell Compartment)	Animal Source	Dilution	Reference and Manufacturer
ABC5	Stemness (plasmic membranes)	Rabbit	1/200	NBP1-50547, Novus, Littleton, CO, USA
p75	Stemness (plasmic membranes)	Rabbit	1/200	AB8874, Abcam, Cambridge, UK
K14	Stemness (cytoplasmic)	Mouse	1/200	AB9220, Abcam
Laminin-5	Basement membrane	Rabbit	1/200	AB14509, Abcam
K3/K12	Differentiated corneal epithelial cells (cytoplasmic)	Mouse	1/200	AB68260, Abcam
ZO-1	Tight junctions (apical plasmic membranes)	Rabbit	1/200	40-2200, Invitrogen, Waltham, MA, USA
e-Cadherin	Epithelial cells (all plasmic membranes)	Mouse	1/200	33-4000, Invitrogen

binding sites and incubation with the primary antibody. Finally, the slides were mounted using Vectashield medium (Vector Laboratories, Burlingame, CA, USA). Images were captured with a confocal microscope (IX83 Fluoview FV-1000, Olympus), equipped with the Olympus Fluoview software.

Stroma. To analyze transparency, each cornea, in a Petri dish, was placed between a backlit chart and a camera.²⁷ Overall transparency underwent computerized analysis. Results are expressed as a percentage ranging from 0% to 100%, respectively, designating complete opacity and perfect transparency. CCT was assessed with an anterior segment optical coherence tomograph (OCT; CASIA SS-1000; Tomey, Tokyo, Japan). The cornea in the BR was placed in front of the OCT in a specially designed holder. Thickness was measured on the central cross section. The anterior and posterior limits of the cornea were automatically selected and manually corrected if necessary. The mean of five measurements performed on the central 3 mm was calculated.

Pan-Endothelial Viability. Viable endothelial cell density (vECD) was determined as previously reported.^{28,29} Briefly, corneas were placed endothelial side up on a specific concave support to prevent corneal folds; iris root was stripped when present; then the concavity was filled with 150 μ L Hoechst 33342 (8 μ M; Sigma), calcein-AM (2 μ M; Interchim, Montluçon, France), and ethidium homodimer (4 μ M; Interchim) in PBS and incubated for 45 minutes at RT. Corneas were then gently rinsed in PBS. TIFF images were acquired using a fluorescent macro zoom microscope (MVX-10, Olympus), run by imaging software (CellSens Dimension, Olympus). A $\times 0.8$ objective allowed observation of the whole porcine cornea in a single field. A z-stack of eight images spaced 600 μ m apart, from the trabeculum to the endothelium center, was acquired with a filter for fluorescein isothiocyanate (FITC) fluorescence (ex: 450–490/em: 500–550 nm) for calcein and with a fluorescence filter for cyanine3 (CY3) (ex: 528/em: 617 nm) for ethidium. The in-focus images of the whole endothelium stained with calcein-AM and ethidium were reconstructed using the 3-D Extended Depth of Field (<http://bigwww.epfl.ch/demo/edf>, in the public domain) in ImageJ (National Institutes of Health, Bethesda, MD, USA). This image was used to calculate the area covered by viable, calcein-positive cells and with ethidium-positive cells. Images of Hoechst 33342-stained nuclei were acquired using a fluorescence filter for 4',6-diamidino-2-phenylindole (DAPI) (ex: 325–375/em: 450–490) with a $\times 10$ objective on five nonoverlapping fields (a central image and one in each quarter). These images were used to determine the number of ECs per surface unit (mean of the five regions of interest). Image analysis was performed using the Cornea J plugin.²⁹ vECD was obtained by multiplying the average ECD calculated with Hoechst 33342-stained nuclei by the percentage of surface covered by viable ECs (calcein-positive).

Dessication

To assess whether corneas remained hyperhydrated or had become hypertrophic or hyperplastic at the end of storage, three

were dehydrated with air until they reached minimal thickness. Initially, they were stored in a BR-off for 7 days, fully immersed in CorneaMax. In these conditions, they reached maximum thickness (see Results) but the scleral rim, attached to the BR, could not retract. This mount also allowed easy OCT monitoring of corneal thickness and shape, contrary to OC, which precluded repeated measurements. At the end of storage, the pressure in the endothelial chamber was restored to 20 mm Hg. After the 30 minutes needed to reach a steady state, the lid was removed and the epithelium was left exposed to ambient air. The cornea was then dried with a continuous low flow of pressurized air until it reached a stable thickness on three consecutive measurements. CCT was assessed by OCT at the end of corneal storage in the BR-off, and then every 5 minutes.

Statistical Analyses

Continuous nonnormally distributed variables were described by their median (10th–90th percentiles). The nonparametric Kruskal-Wallis test was used to compare quantitative variables between more than two independent groups, and the Mann-Whitney test for two independent groups. Post hoc analysis was performed using Dunn's multiple comparison tests to compare active BR and three other storage methods. The null hypothesis was rejected by a type I error < 0.05 ($\alpha < 0.05$). Statistical analyses were performed using SPSS 23.0 (SPSS, Inc., Chicago, IL, USA), and figures were produced using Excel (Microsoft, Redmond, WA, USA).

RESULTS

Baseline Characteristics of Porcine Corneas

On postmortem excised corneas, epithelium status, transparency, CCT, and endothelial viability were considered the baseline criteria (Figs. 2–4). Central corneal epithelium was stratified with six to nine layers of differentiated cells expressing cytokeratin 3 and 12 (K3/K12) and E-cadherin+, lying on a basement membrane positive for laminin-5. The most superficial cells expressed zonula occludens-1 (ZO-1) (Fig. 3). Limbal epithelium was stratified with six to nine layers of differentiated cells (K3/K12+) except basal limbal epithelial cells (K3/K12–), which expressed stem cell markers ABCB5+, K14+, and p75+ (Fig. 4).

Bioreactor

Corneas stored in the BR-on without their iris were significantly thicker than corneas with the iris root left intact, with CCT of 2737 (1716; 3441) vs. 1253 (1035; 1559) μ m, $P < 0.001$. The former had diffuse edema associated with massive peripheral edema in three of five cases, as shown in Figure 2. Maintaining corneal shape by the system of scleral mechanical locking prevented contact between iris remnants and the peripheral endothelium. Consequently, all corneas subsequently stored in

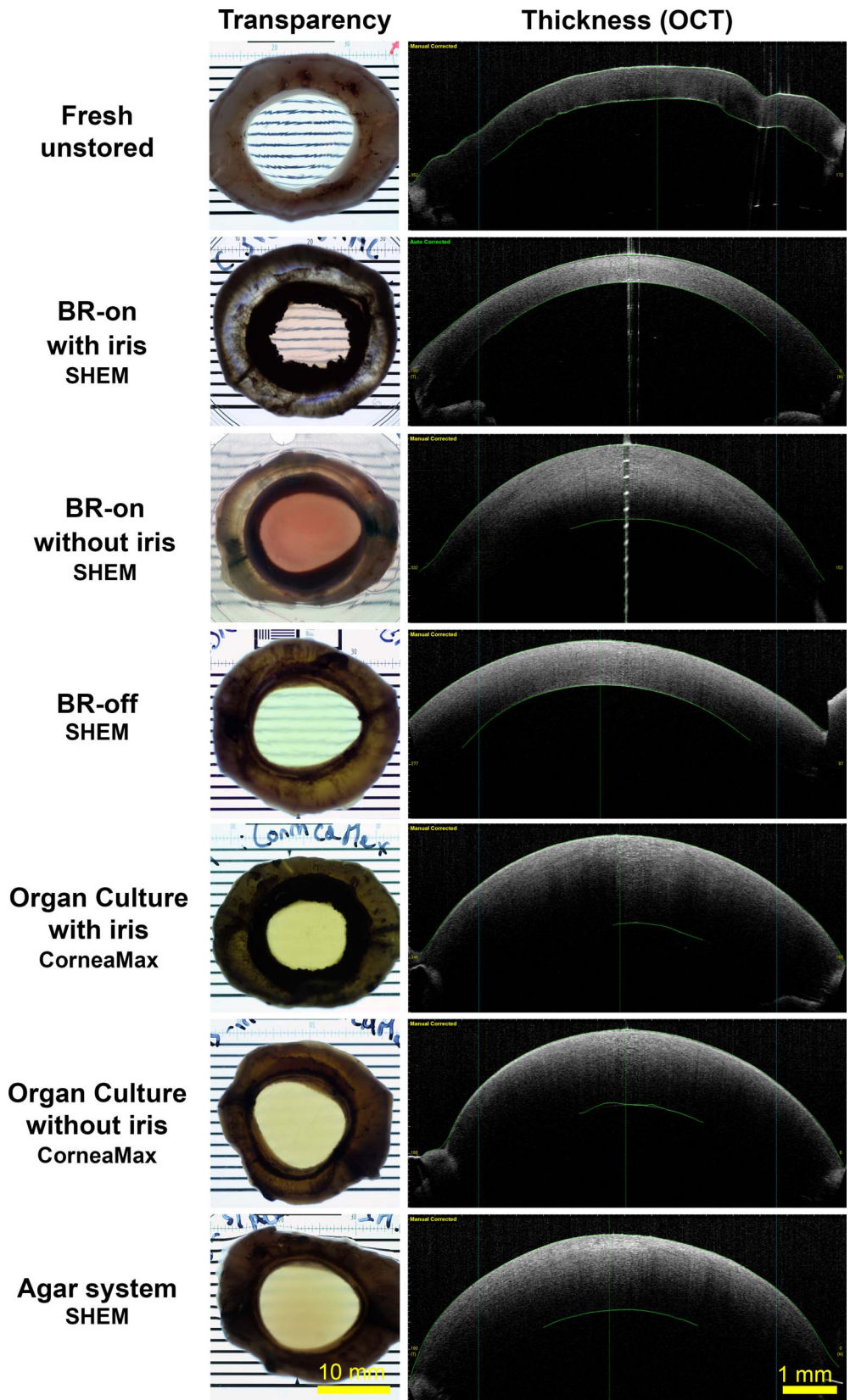


FIGURE 2. Transparency and central OCT scan of porcine corneas stored for 7 days in six different conditions. Fresh corneas were the control. Due to their natural lack of rigidity, they lost their dome shape. Except for corneas stored in the agar system, the endothelium was always in contact with a commercial organ culture medium (CorneaMax). For corneas in the BR and in the agar system, the epithelial side was immersed in supplemental hormonal epithelial medium (SHEM). For organ culture, corneas were simply immersed in CorneaMax. The BR-on was set with 20 mm Hg and 5 μ L/min of medium renewal in the endothelial chamber, and the epithelium was alternatively exposed to air and to SHEM for 30 and 1 seconds, respectively.

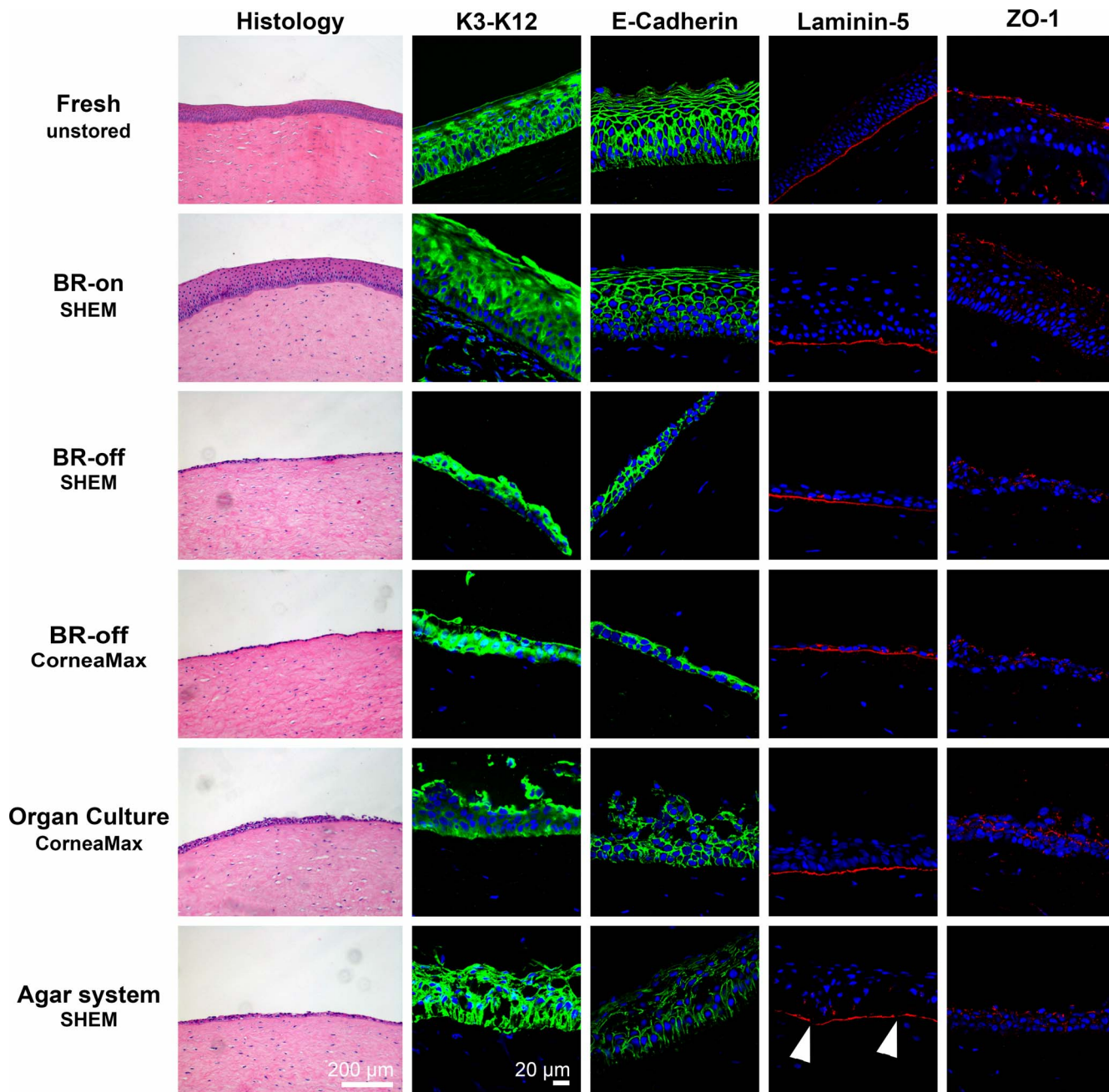


FIGURE 3. Histologic cross sections (hematoxylin, eosin, and saffron) and immunostaining of central epithelium of fresh porcine corneas (control) and after 7 days' storage in the bioreactor (BR)-on with 20 mm Hg and SHEM in the epithelial chamber, BR-off with either SHEM or CorneaMax in the epithelial chamber, organ culture in CorneaMax, and on agar with SHEM. Corneas in the BR had their iris root left intact. K3-K12 (green), E-cadherin (green), laminin-5 (red), ZO-1 (red). Nuclei were stained with TO-PRO-3 Iodide (blue). Arrowheads highlight irregularities in the basement membranes consistently observed only with the agar system. No staining was observed in negative controls (data not shown).

the BR and presented below had their iris root left intact. In these conditions, corneas stored in the BR-on had higher transparency and lower CCT compared to corneas stored using other methods (Fig. 5). Corneal and limbal epithelium organization was preserved; a stratified corneal (3 to 10 layers) and limbal (2 to 9 layers) epithelium was observed; limbal basal cells also had a small diameter and a high nuclear-cytoplasmic ratio. Immunohistochemical characteristics of the corneas stored in the BR-on were similar to those of postmortem corneas (i.e., a differentiated corneal epithelium lying on a regular basement membrane with tight junctions in the most superficial cells) (Fig. 3); limbal basal epithelial cells were

undifferentiated and expressed stem cell markers (Figs. 3, 4). Mean vECD was 7% lower in the BR-on than in fresh corneas ($P = 0.088$), but 19% lower in the BR-off ($P = 0.001$). Corneas stored in the BR-off (with CorneaMax or SHEM in the epithelial chamber) had lower transparency and higher CCT (Figs. 2, 5) than corneas stored in the BR-on. Corneal epithelium stratification varied, ranging from no epithelium to an epithelium five cell layers thick when stored in CorneaMax and nine layers thick in SHEM (Fig. 3); limbal epithelium stratification ranged from one to six cell layers thick when stored in CorneaMax or SHEM (Fig. 4); stratification was slightly improved in corneas stored in SHEM in the epithelial chamber compared with CorneaMax.

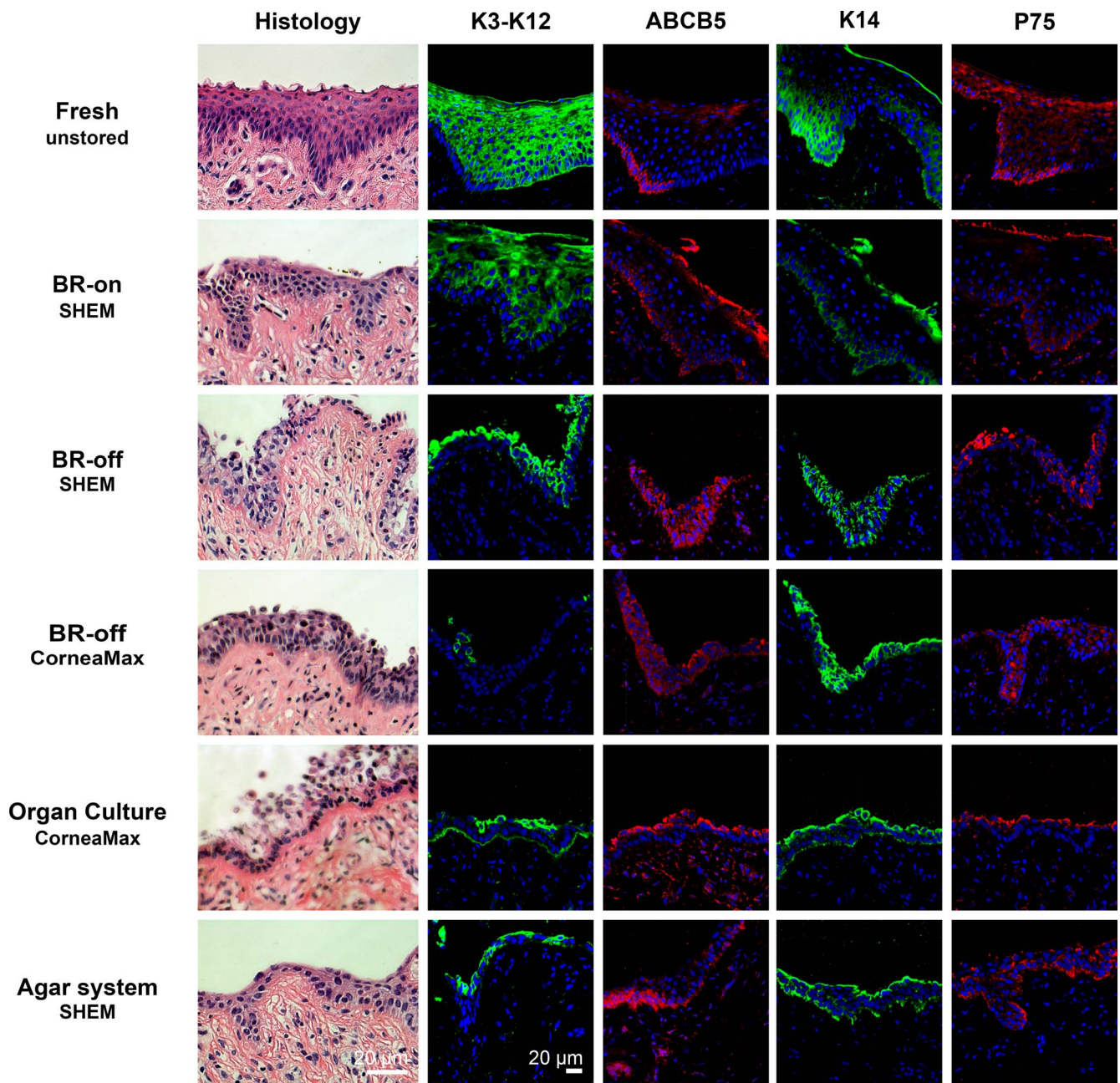


FIGURE 4. Histologic cross sections (hematoxylin, eosin, and saffron) and immunostaining of the limbus of fresh porcine corneas (control) and after 7 days' storage in the bioreactor (BR)-on with 20 mm Hg and SHEM in the epithelial chamber, BR-off with either SHEM or CorneaMax in the epithelial chamber, organ culture in CorneaMax, and on agar with SHEM. Corneas in the BR had their iris root left intact. K3-K12 (green), E-cadherin (green), laminin-5 (red). Nuclei were stained with TO-PRO-3 Iodide (blue). No staining was observed in negative controls (data not shown).

Corneal epithelium, when present, was differentiated (K3/K12+), with a preserved basement membrane (laminin-5); tight junction (ZO-1) expression was irregular (Fig. 3); clusters of basal limbal cells also preserved stem cell markers (Fig. 4).

Organ Culture

Unlike what was observed with the BR-on, corneas stored with the iris root left intact were significantly thicker than iris-less corneas, with 3305 μm (2901-3726) vs. 2790 μm (2498-3137) $P < 0.001$. They also constantly presented dramatic EC mortality in their periphery, due to contact with iris remnants:

A peripheral ring of approximately 2-mm width contained no ECs, and residual cells touching this zone were mostly stained with ethidium. As a result, all corneas subsequently stored in OC and presented below were iris-less. These corneas nevertheless lost all transparency and were dramatically swollen, some even losing their natural shape. Instead, they sometimes appeared as biconvex lenses with deep endothelial folds. Endothelial viability was reduced (Figs. 2, 5). The corneal epithelium was heterogeneous and mostly absent, except for a few areas of one to five layers of differentiated epithelium (K3/K12+; Fig. 3). A limbal epithelium one to five cell layers thick persisted, basal epithelial limbal cells were undifferentiated,

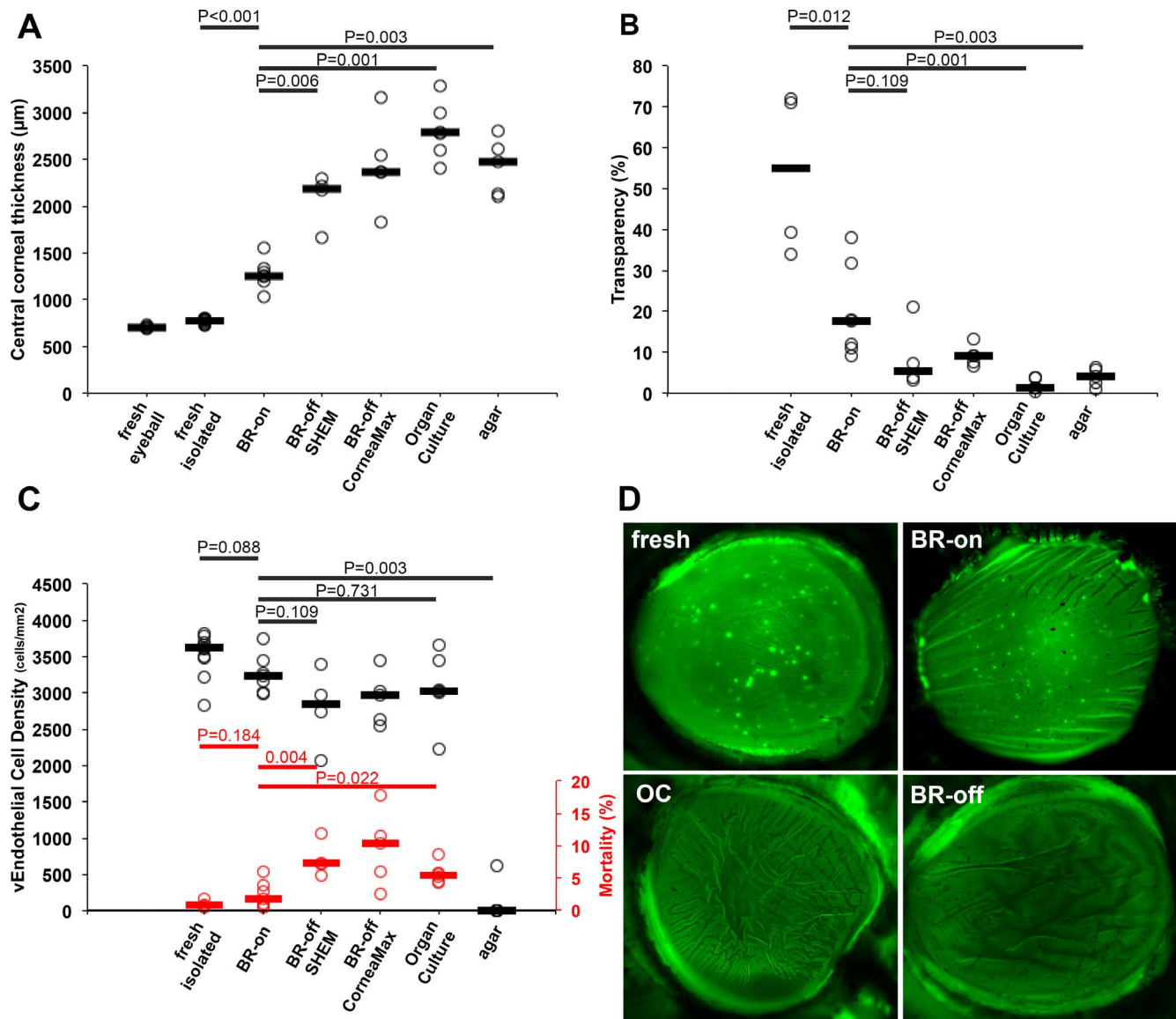


FIGURE 5. (A) Corneal thickness, (B) transparency, (C) viable endothelial cell density and % mortality of fresh porcine corneas and after 7 days' storage in five experimental conditions. CMax, commercial organ culture medium CorneaMax. Mortality was not quantified for the agar group because almost all cells had desquamated. (D) Endothelial viability determined by calcein-AM staining. In these four examples, the percentage of viable endothelium is 97% for the fresh cornea, 94% for the BR-on, 84% for OC, and 88% for the BR-off. In OC and in the BR-off, deep endothelial folds with stress lines are distributed on the whole cornea. In the BR-on, folds are absent in the center, and only mechanical folds appearing during cornea unlocking are visible in the periphery, with almost no cell mortality. Bright dots are artifacts, harmless for image analysis.

and cell clusters expressed stem cell markers (ABC5, K14, and p75) (Fig. 4).

Agar System

Corneas were also stored iris-less. These had reduced transparency and increased thickness (Figs. 2, 5). As expected, no ECs survived (except in one case, with extremely low vECD). The corneal epithelium was heterogeneous, and stratification ranged from one to four cell layers thick expressing K3/K12 (Fig. 3). A limbal epithelium two to five cell layers thick persisted; basal limbal epithelial cells were undifferentiated (K3/K12-), with clusters of cells labeled with stem cell markers ABC5, K14, and p75+ (Fig. 4). Labeling of the basement membrane (laminin-5) was irregular and

interrupted in a few areas; this feature was not observed with other storage methods (Fig. 3).

Desiccation

Corneas were placed in the BR-off for 7 days, with a resulting CCT of 2281 (2274; 2284) µm (n = 3). Thirty minutes after restoration of the IOP equivalent, CCT was 2206 µm (2093; 2266). After approximately 120 minutes of exposure to dry pressurized air, CCT was 707 µm (617; 885) (P = 0.02 between end of storage and desiccation experiment). Corneal desiccation significantly and quickly reduced the CCT of corneas stored in the BR-off, indicating that increased CCT is linked to edema and not to a deposition of material in the stroma. Also, CCT of corneas stored in the BR-off tended to be lower than

that of OC stored corneas, suggesting that scleral retraction may be involved in the observed stromal thickening of OC stored corneas.

DISCUSSION

Porcine corneas stored for 7 days in our BR-on with 20 mm Hg in the endothelial chamber, continuous renewal of the culture media, and epithelial air-lifting retain characteristics close to those of postmortem corneas for each of their three layers: epithelial, stromal, and endothelial.

Organ culture is clearly inappropriate for storing porcine corneas. The loss of IOP and of the epithelial barrier partly explains the dramatic stromal edema and subsequent EC loss in the present study. Corneas of pigs available from slaughterhouses are on average 6 months old, and are far less rigid than human corneas³⁰ after corneoscleral excision. Porcine cornea loses its shape, unlike human cornea. In OC, corneas of both species become edematous, but human cornea doubles in thickness and retains its shape, whereas porcine cornea becomes more than four times thicker and its shape is no longer recognizable. As SP is similar in both species,³¹ the dramatic swelling capacity of porcine cornea may be due to a low level of natural collagen cross-linking compared with adult human corneas, for which age-related nonenzymatic cross-linking affecting the stromal collagen fibrils occurs.³² In addition, corneal swelling starts immediately after scleral cutting (+70 μ m in the present study), suggesting that the shrinking of naturally taut elastic collagen fibers plays a role in the increase in thickness.

The massive stromal edema observed in the BR-on only with iris-less corneas suggests that iris root stripping creates an abnormal entry route for water when IOP is restored; notably, this phenomenon was not observed in OC, as a pressure gradient was lacking. On the contrary, when the anatomy of the iridocorneal angle is preserved, the pressure restored in the BR partly offsets the SP and thus significantly limits stromal swelling; corneal swelling is also limited by the integrity of the epithelial barrier linked to tight junctions in the most superficial epithelial cells. Stored corneas thus remain thin without the addition of macromolecules, typically used by eye banks in conventional OC, to increase medium osmolarity. This result may have eye banking applications, as hyperosmolar deswelling media required during OC can be toxic to the endothelium.^{33,34} In addition, reduction of corneal edema-related endothelial folds is likely to minimize EC loss, given the relationship between endothelial folds and EC apoptosis.^{3,35} Further, continuous renewal of the CM in the BR could also improve EC survival. In the present study, vECD in the BR was not significantly higher than for OC, but the sample was small and storage time only 7 days. Using the same methods as in a clinical trial, the present data will be used to calculate prospectively the number of corneas to be included in a forthcoming experiment focused solely on endothelial survival, in order to compare endothelial survival in the BR and OC. Lastly, reduced stromal swelling in the BR was also logically associated with increased transparency. The BR has made possible ex vivo storage of a transparent porcine cornea for 7 days. This could be useful for ex vivo irritancy testing for longer than with the very short-term bovine or porcine corneal opacity and permeability tests.

Corneas stored in the BR-off tended to be slightly thinner than OC stored corneas. The only difference being that the scleral rim is tightly secured in the BR, we suggest that this limits the possibility of considerable tissue shrinkage observed in OC. Restoring the tautness of those scleral fibers that are

interwoven with the corneal lamellae may help maintain corneal shape and thickness.

Corneas in the BR did not fully regain their physiological thickness. The possibility to obtain a further reduction of thickness by removing excess water through artificial drying of the cornea from the epithelial side reasonably consolidates the hypothesis of residual stromal edema and eliminates that of aberrant stromal hyperplasia. Four mutually nonexclusive hypotheses can be proposed to explain the residual edema: (1) abnormal passage of water via residual damage to the endothelium (not 100% viable); (2) hypofunctional endothelial pumps and/or an increase in intercellular permeability caused by imperfect composition of the OC medium (the culture media used in eye banks were developed more than 30 years ago, and should be revised to benefit from progress in cellular biology and EC physiology); (3) corneoscleral shrinkage begins immediately after trephination, before the rim is locked into the BR; (4) the 5% CO₂ atmosphere may favor anaerobic corneal metabolism directly involved in stromal edema.³⁶

We implemented the second version of the BR, which included a system to alternately expose the epithelium to CM and air, thus recreating part of its physiological environment. The role of air exposure on the stratification of corneal epithelium, already demonstrated,¹⁹ was also found in the BR. But no previous ex vivo model allowed both endothelium and epithelium to survive for 7 days. The specific BR environment, combining epithelium CM and intermittent air exposure, enabled preservation of a stratified and differentiated corneal epithelium and of limbal architecture with undifferentiated basal cells (K3/K12-) expressing stem cell markers (ABCB5+, K14+, p75+). Other authors developed rocking systems to alternate air and CM exposure on corneas in Petri dishes with agar, and obtained epithelial preservation for up to 4 weeks,^{11,37,38} but the epithelium seemed less stratified and the integrity of the epithelial basement membrane and of the limbus was not studied. In addition, the air-lifting system of the epithelial chamber could induce a shear stress on the limbus that could be involved in epithelial stem cell regulation.²⁰ Preservation of the endothelium and epithelium is a prerequisite for developing a corneal wound-healing model that takes account of indirect interactions between these two layers described in the literature. Differentiation of epithelial cells is indeed influenced by the presence of endothelial cells.³⁹ Cases of limbal stem cell deficiency secondary to severe endothelial dysfunction were reported, which also supports the hypothesis of endothelium-epithelium interactions.⁴⁰ Moreover, basement membrane formation, one of the key regulators of epithelial wound healing and of epithelial-stromal interactions,⁴¹ was associated in vitro with the presence of endothelial cells,¹⁷ as demonstrated in a coculture model of epithelial cells in the presence or absence of endothelial cells. A drawback of the agar system is the absence of endothelium.^{10,11,38} In our series, with this system, the basement membrane was irregular and also absent in some areas, whereas it is continuous and uninterrupted in other models with viable endothelial cells. Some irritancy assays use corneas mounted on an artificial anterior chamber^{24,37,42} that preserves the endothelium, but these systems do not allow extended storage of corneas and are thus unsuitable for studying pathologic wound-healing processes. Tappeiner et al.⁴³ induced an epithelial wound in porcine corneas while preserving the whole eyeball, but only for 26 hours. The modified interactions between endothelium, stroma, and epithelium may explain the reduced stratification of epithelium preserved in the agar system and rocking systems described by Deshpande et al.¹¹ and Piehl et al.¹² compared to in vivo; moreover, no immunolabeling was done in these models to identify epithelial stem cell preservation. The present new

model thus seems suited to studying normal and pathologic corneal epithelial wound healing and to develop an irritancy assay for screening new therapeutic molecules. In addition, the present study shows that the BR is an innovative experimental platform. It enables corneal storage in sterile conditions but also direct tissue visualization through two transparent windows without interrupting the experiment. Optical measurements such as OCT and transparency assessment can thus be performed in situ. The pressure inside the endothelial chamber can be adjusted if necessary, and the media bathing the endothelium and the epithelium can be different and have separately adjustable flows, as in the present study. In addition, media samples can be collected easily at various points in the circuit for specific analysis.

By restoring a near physiological environment to the cornea (IOP, flow, epithelial air-lifting, specific CM for each side), this BR version preserves porcine corneas for 1 week in conditions never previously achieved, with excellent EC survival, reduced thickness, shape maintenance, and integrity of the epithelium and limbus. Though still improvable, this model could be used for preclinical experiments. Research is under way to extend storage duration and to create a pathologic wound-healing assay.

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