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Porcine Cornea Storage Ex Vivo Model as an Alternative to Human Donor Tissues for Investigations of Endothelial Layer Preservation

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Purpose: Due to the growing shortage of human corneas for research, we developed a porcine cornea storage model with qualitative features comparable to human tissues.

Methods: We established a decontamination procedure for porcine eye bulbs to ensure corneal storage at 31°C to 35°C for up to 28 days without contamination. We compared human and porcine corneas under hypothermic (2–8°C) or culture (31–35°C) conditions for central corneal thickness (CCT), corneal transparency, endothelial morphology, endothelial cell density (ECD), and a novel method to quantify whole endothelial mortality. We also examined portions of lamellar tissues consisting of Descemet's membrane and endothelial cells under the microscope after Alizarin red staining.

Results: Our decontamination procedure reduced corneal contamination from 94% (control corneas without decontamination) to 18% after 28 days of storage at 31°C to 35°C. ECD, CCT, transparency, and morphology were significantly higher in porcine corneas than in human corneas at day 0. Nevertheless, the qualitative parameters of porcine and human corneas showed comparable trends under both investigated storage conditions for up to 14 days.

Conclusions: The presented corneal storage model provides a reliable alternative to human tissues for preliminary corneal investigations.

Translational Relevance: The porcine cornea storage model can be used to investigate the efficacy and safety of new media, substances, or storage conditions. Furthermore, the method developed to assess the percentage of endothelial mortality is tissue conservative and can be used in eye banks to monitor endothelial mortality during storage of tissues intended for transplantation.

Introduction

Maintaining the physiologic features of human corneas preserved in organ culture before transplantation is critical for eye bank technicians, clinicians, and scientists.[1]

Human corneas from deceased donors are routinely processed and evaluated in eye banks to be used for transplantation. The use of human donor tissues for research purposes is strictly regulated and strongly limited in some countries. Generally, only the tissues unsuitable for transplantation may be used for research purposes in Europe.[2,3] Moreover, the shortage of donor corneas for transplantation[4,5] has been exacerbated by the SARS-CoV-2 pandemic, further limiting the availability of human tissues for research.[6,7] Consequently, researchers have had to seek alternative and reliable models.

In vivo animal models have advantages but also come with costs and raise ethical concerns regarding animal welfare.[8] Alternatively, some ex vivo models may be used as an appropriate tool that simulates the human eye while avoiding the use of live animals.

Porcine eye bulbs, for instance, are readily available from the food industry, and their use poses no ethical issues since these tissues are usually discarded as waste.[8,9] Furthermore, porcine corneas are biologically and morphologically similar to human corneas.[10–14] Similar to humans, the porcine corneal endothelium consists of a monolayer of hexagonal cells ensuring physiologic corneal thickness, which is essential for corneal clarity and vision.[11,15,16] As a result, the ex vivo use of porcine corneas has raised great interest for both clinical and nonclinical purposes.[9,10,14,17–19]

This study aims at setting up an ex vivo model based on storage of porcine corneas to be used as an alternative to human tissues for research purposes. The model includes evaluating corneal quality parameters relevant to determine tissue suitability for transplantation, such as endothelial cell (EC) density (ECD), EC mortality and morphology, and corneal transparency. Additionally, it could represent an important tool to preliminarily investigate corneal storage conditions potentially affecting the corneal endothelium quality and safety.

To this aim, an effective porcine eye bulb decontamination procedure was established. Second, a method to quantify whole endothelium mortality was developed. Third, qualitative corneal parameters, including ECD, EC morphology, central corneal thickness (CCT), and corneal transparency, were compared in human and porcine corneas. Finally, porcine lamellar fragments consisting of Descemet's membrane (DM) and EC were prepared and compared with human tissue for Descemet membrane endothelial keratoplasty (DMEK).

Materials and Methods

Ethical Considerations and Tissue Procurement

This study complied with the Declaration of Helsinki. Human donor corneal tissues ($n = 29$, aged 65–79 years) unsuitable for transplantation were used for the present study, following a written consent from the donor's next of kin and in agreement with the Italian National Transplant Centre (Centro Nazionale Trapianti, Rome) guidelines. The procurement and processing of human donor corneas were performed under Italian laws and following the European Eye Bank Association guidelines.[20,21]

The tissues were processed at Fondazione Banca degli Occhi del Veneto Onlus (FBOV, Venice, Italy) following internal standard operating procedures, and the first evaluations were performed within 72 hours after the procurement. The tissue inclusion criteria corresponded to ECD $\geq$ 1,800 cells/mm^2, absence of severe polymorphism, and CCT <800 µm. Donor corneas were stored in corneal culture medium (Tissue-C; AL.CHI.MI.A. S.R.L., Ponte San Nicolò, Italy) at 31°C to 35°C or, alternatively, in hypothermic storage medium (Eusol-C; AL.CHI.MI.A. S.R.L.) at 2°C to 8°C. Eusol-C (reference market: United States) has an equal formulation of Corneal Chamber (AL.CHI.MI.A. S.R.L.; reference market: Europe).

Sixty-two ($n = 62$) porcine eye bulbs from domestic pigs (*Sus scrofa domestica*), between 6 and 8 months old, were recovered at a local slaughterhouse and transported on ice to AL.CHI.MI.A. S.R.L. laboratories within 2 hours after death. All experiments and procedures involving animals adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The scheme of the experimental design described in the following chapters is shown in Supplementary Figure S1.

Decontamination of Porcine Eye Bulbs and Preparation of Corneal Tissues

Thirty-four ($n = 34$) of 62 porcine tissues were used for evaluation of efficacy of the decontamination protocol.

Porcine eye bulbs were divided into two groups: group A ($n = 17$) and control group B ($n = 17$). Group A eye bulbs were cleaned and decontaminated by immersion in 10 mL 5% povidone-iodine (PVP-I; IodioPharm, Milan, Italy) under orbital shaking (250–300 rpm) at room temperature (RT) for 10 minutes. The bulbs were then transferred in sterile sodium thiosulphate (Merck Sigma, Darmstadt, Italy; 217263-250G) 0.1% *w/v* in phosphate-buffered saline (PBS) for PVP-I inactivation for 2 minutes and then rinsed in Dulbecco's PBS (DPBS; Merck Sigma D8662-500ML). In the control group B, the decontamination with PVP-I and rinsing with sodium thiosulphate were not performed. Afterward, the corneas from both groups were dissected under aseptic conditions, leaving approximately 2 mm of the scleral rim, rinsed with sterile DPBS, and incubated in DPBS containing penicillin/streptomycin (Merck Sigma P4333-100ML) at RT for 1 hour before the initial tissue evaluation at day 0.

Porcine corneas from both groups were stored for 28 days in Tissue-C at 31°C to 35°C. The Tissue-C medium contains penicillin G, streptomycin, and amphotericin B. The tissues were considered microbiologically contaminated when the media's turbidity was visually detected and confirmed by two different operators after 4, 7, 14, 21, and 28 days of storage at 31°C to 35°C.

The safety of the decontamination procedure on whole corneal endothelium was evaluated in six porcine corneas immediately after corneal dissection and after 3 days of storage in Tissue-C at 31°C to 35°C, with the developed method to assess quantitively EC mortality (see below).

Quantification of Endothelial Mortality in Porcine and Human Corneas

Nine ($n = 9$) porcine corneas and three ($n = 3$) human corneas were stored in hypothermic storage medium (Eusol-C; AL.CHI.MI.A. S.R.L.) at 2°C to 8°C for 14 days; three ($n = 3$) porcine corneas and three ($n = 3$) human corneas were stored in hypothermic storage medium added with 0.2 mg/mL sodium dodecyl sulfate (Merck Sigma 05030-500ML-F) at 2°C to 8°C as cytotoxic controls.

The whole corneal endothelium mortality was quantified for each cornea at days 0, 3, 7, and 14 after endothelium staining with 100 to 120 µL 0.2% trypan blue (TB) (TB-S; AL.CHI.MI.A. S.R.L.) for 1 minutes at RT, followed by corneal rinsing by immersion in DPBS. Corneas were then placed in a Petri dish with the endothelium facing upward, and a picture of TB-stained endothelium was acquired using a high-resolution Panasonic DMC-TZ100 camera (Panasonic Corporation, Kodoma, Osaka, Japan). All abovementioned procedures were performed under aseptic conditions using a laminar flow hood. Digital pictures of TB-stained corneal endothelium were thus analyzed using a predefined command (macro; Supplementary Text 1) of ImageJ (Fiji) software,[22] which was designed to manually select a polygonal region of interest (ROI), including the whole corneal endothelial area (ROI > 100 mm^2 and >80 mm^2 of porcine and human corneas, respectively). The software quantified the whole endothelial mortality as the percentage of TB-stained area relative to the total ROI area. In addition, a circular ROI with a diameter of 8 mm was used to calculate central area mortality, expressed as a percentage of the whole endothelial area. Peripheral mortality was calculated as the difference between total and central mortality. The total TB-stained area and the diameter of central button were calculated using a calibration ruler present in the picture.

Comparison of ECD, Endothelial Morphology, Corneal Transparency, and CCT in Porcine and Human Corneas

ECD, EC morphology, CCT, and corneal transparency were compared between porcine ($n = 8$) and human ($n = 12$) corneas under hypothermic storage (2–8°C) at days 0, 7, and 14 of storage, whereas under corneal culture conditions (31–35°C), ECD and EC morphology only were assessed and compared between porcine ($n = 8$) and human ($n = 11$) corneas at days 0, 7, and 14 of storage.

ECD and EC morphology in both human and porcine corneas were evaluated by the method described by Stocker et al.[23] using an inverted-phase microscope (Nikon Eclipse T*i*; Nikon, Tokyo, Japan) equipped with Nikon objectives (Plan Flour 40×/0.30 oil ∞/0.17 WD 0.24; Plan Flour 10×/0.30 ∞/1.2 WD 15.2 and ocular lenses CFI 10×/22) with endothelia immersed in hypotonic 1.4% sucrose solution (SR-S; AL.CHI.MI.A. S.R.L) after staining the corneas with TB for 1 minute at RT.

ECD was measured on pictures acquired using 100× magnification at the central optical area of the cornea, considering at least five squares of 0.01 mm^2 (NIS-Elements Software; Nikon).

During this step, corneas were also evaluated for EC morphology according to the method described by Parekh and colleagues.[24] The corneal transparency evaluation was performed using a Light Meter (PCE-174; PCE Instruments, Cappanori, Italy) set to 0 to 400 lux, following the method previously described by Parekh et al.[25] The CCT was measured using Tomey optical coherence tomography (3D CAS-OCT SS-1000; Casia, Tokyo, Japan) according to the manufacturer's instructions. The CCT value was calculated as the average of three measurements in the central corneal area. Specular microscopy examination was performed using Konan CD-15 CellChekD+ (Konan Medical USA, Inc., Irvine, CA, USA).

Preparation of Human DMEK Lamellar Tissues and Porcine Endothelial Lamellar Tissue Portions

Corneas were stained with the nonvital dye 0.2% alizarin sodium sulfonate (Merck-Sigma, A5533) as previously described by Taylor and Hunt.[26]

Human DMEK lamellar tissues were obtained following the gold-standard procedure for DMEK tissue preparation in the FBOV Eye Bank.[27]

In porcine tissues, a trephine was employed to make a superficial cut in the endothelium using a gentle tapping method. The cut was identified by staining with TB for about 20 seconds, then washed with DPBS. Corneal concavity was filled with DPBS, and a cleavage hook was used to detach small fragments of lamellar tissues consisting of DM and EC. The floating porcine lamellar fragments were then collected using a micropipette, distended on a glass slide, and closed with a coverslip before microscopical examination.

Human DMEK lamellar tissues and porcine lamellar fragments were analyzed using the Eclipse Ti inverted-phase microscope (Nikon) equipped with Nikon objectives (Plan Flour 40×/0.30 oil ∞/0.17 WD 0.24 and ocular lenses CFI 10×/22).

Mean areas of human and porcine endothelial nuclei (from at least three different corneas each, $n = 15$ nuclei per tissue) were measured with Fiji software on human DMEK lamellar tissues and porcine lamellar fragments.

Statistics and Data Analysis

Statistical analysis was performed using Excel 2010 Microsoft software (Microsoft Corp., Redmond, WA, USA). Statistical evaluation of contamination rates was determined with Fisher's exact test. The mean and the standard deviation of endothelial mortality were calculated for each experimental group. Normality of data distribution was evaluated using the Kolmogorov–Smirnov normality test. Student's t-test or one- and two-way analyses of variance followed by a post hoc Bonferroni test were used to compare data on mortality, ECD, transparency, and CCT data obtained from human and porcine corneas. The Mann–Whitney U test was used to compare the morphology features between porcine and human corneas using the online calculator (https://astatsa.com/WilcoxonTest/). Differences yielding $P < 0.05$ were considered statistically significant.

Results

Tissue Decontamination Efficacy

The Table reports the contamination rate of porcine corneas in groups A and B at each time point. The contamination rate in group A was significantly lower than in control group B at all the time points. After 28 days of storage at 31°C to 35°C in group A, contamination was found in 3 of 17 corneas (18%). However, in group B, 9 of 17 corneas (53%) were contaminated on day 4, and contamination increased to 16 of 17 corneas (94%) after 28 days of storage at 31°C to 35°C.

No difference in EC mortality was observed before, immediately after decontamination, and after 3 days of corneal culture at 31°C to 35°C ($n = 6$, Supplementary Fig. S2).

Table. Cumulative Contamination Rates for Group A (Decontaminated Eye Bulbs, $n = 17$) and Group B (No Bulb Decontamination, $n = 17$) and Statistical Analysis for Each Time Point

Evaluation Time	Group A ($n = 17$) Cumulative Contamination Rate	Group B ($n = 17$) Cumulative Contamination Rate	P Value (Fisher's Exact Test)
Day 4	(1/17) 6%	(9/17) 53%	0.0066
Day 7	(1/17) 6%	(12/17) 71%	0.0002
Day 14	(2/17) 12%	(14/17) 82%	0.0001
Day 28	(3/17) 18%	(16/17) 94%	<0.0001

For both groups, the number of total cumulative contaminations over time is indicated in parentheses at each time point.

Comparison of EC Mortality Between Porcine and Human Corneas Stored at 2°C to 8°C

Figure 1 shows EC mortality time course in porcine and human corneas stored in hypothermic storage medium and cytotoxic control groups at 2°C to 8°C for 14 days. On day 0, no statistically significant difference was observed between human and porcine corneas in both the hypothermic storage medium and cytotoxic control groups ($P = 0.5648$ and $P = 0.0823$, respectively, Student's t-test). Additionally, the porcine and human corneas showed a similar EC mortality trend in both the hypothermic storage medium group and cytotoxic control groups on days 3, 7, and 14 (Supplementary Figs. S3A, S3B). EC mortality did not statistically differ between porcine and human corneas in both the hypothermic storage medium (days 0 and 3: $P = 1$; day 7: $P = 0.348$; day 14: $P = 0.228$; post hoc Bonferroni test, following two-way analysis of variance [ANOVA]) and cytotoxic groups (day 0: $P = 0.764$; days 3, 7, and 14: $P = 1$; post hoc Bonferroni test, following two-way ANOVA).

The EC mortality in human corneas showed an increasing trend over the storage time at 2°C to 8°C (Figs. 1B, 1D), and a statistically significant difference in EC mortality was observed between day 0 and day 7 only (day 7: $P = 0.0193$; day 3: $P = 0.1144$; day 14:

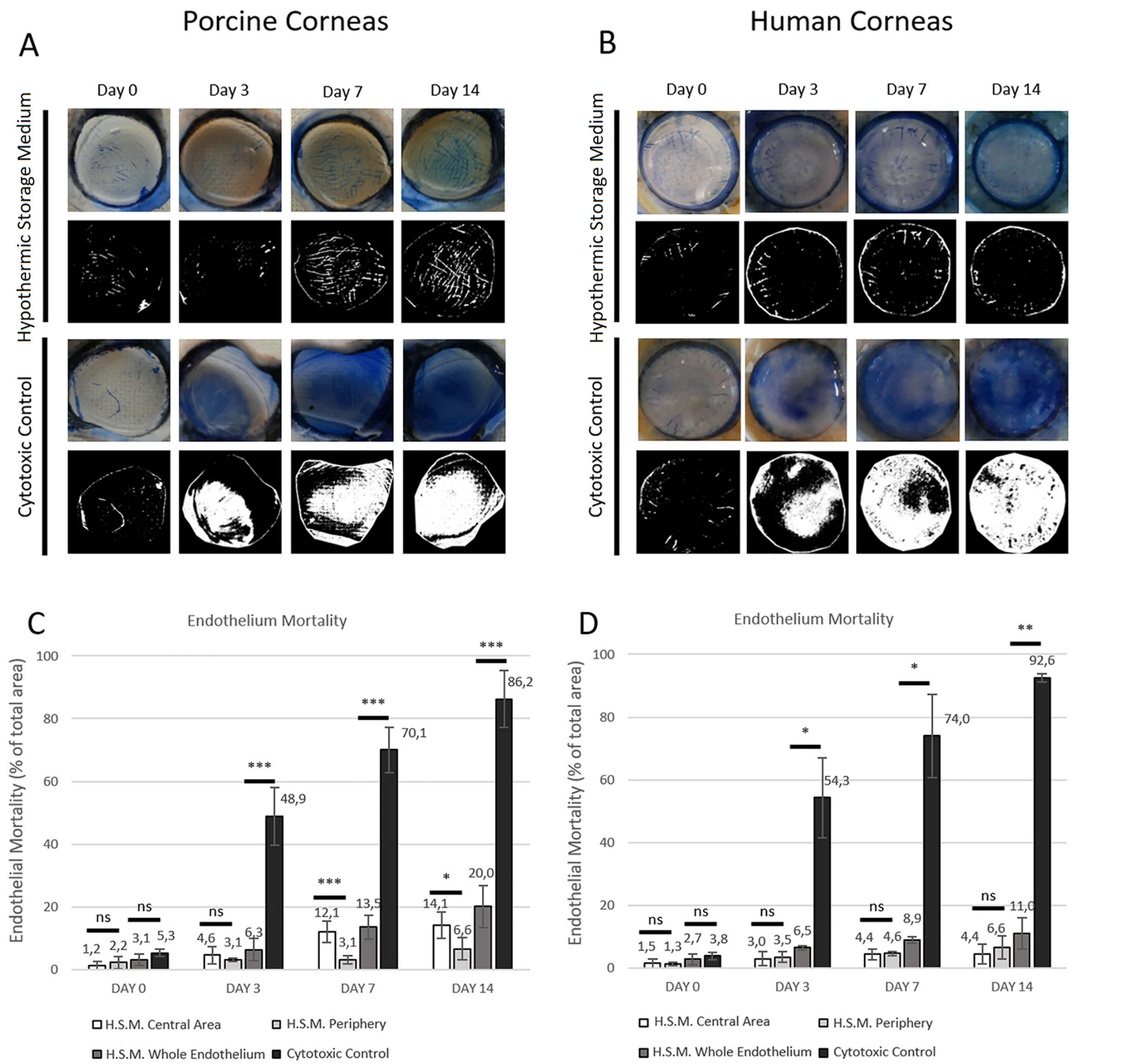

Figure 1. Representative pictures of porcine (A) and human (B) corneal endothelium after TB staining after different days of hypothermic storage (*upper panels*) and the Fiji processed pictures for TB-stained area quantification (*lower panels*, in *black* and *white*) of corneas stored 14 days in hypothermic storage medium (porcine: $n = 9$; human: $n = 3$) and cytotoxic control group (porcine: $n = 3$; human $n = 3$) at 2°C to 8°C. Photographs were taken at days 0, 3, 7, and 14 of hypothermic storage. (C, D) Mean and standard deviation of endothelial mortality in porcine (C) and human (D) corneas stored under hypothermic storage conditions and in a cytotoxic control group at different time points. H.S.M., hypothermic storage medium; ns, not significant. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

$P = 0.3056$; post hoc Bonferroni test, following one-way ANOVA).

The EC mortality in porcine corneas increased with storage time at 2°C to 8°C (Fig. 1C). Endothelial folds progressively appeared and became visible after 7 days of storage at 2°C to 8°C. Endothelial folds overlapped with TB-stained area and no evident TB-stained areas were observed outside the folds (Fig. 1A). A statistically significant difference in EC mortality was observed between day 0 and days 7 and 14 ($P = 0.0744$ for day 3; $P < 0.0001$ for days 7 and 14; post hoc Bonferroni test, following one-way ANOVA).

In the cytotoxic control groups of both human and porcine corneas, the area of EC mortality increased progressively with storage time (Figs. 1C, 1D). The EC mortality was significantly higher, compared to day 0, at all tested time points in both human ($P = 0.0172$ for day 3, $P = 0.0115$ for day 7, and $P < 0.0001$ for

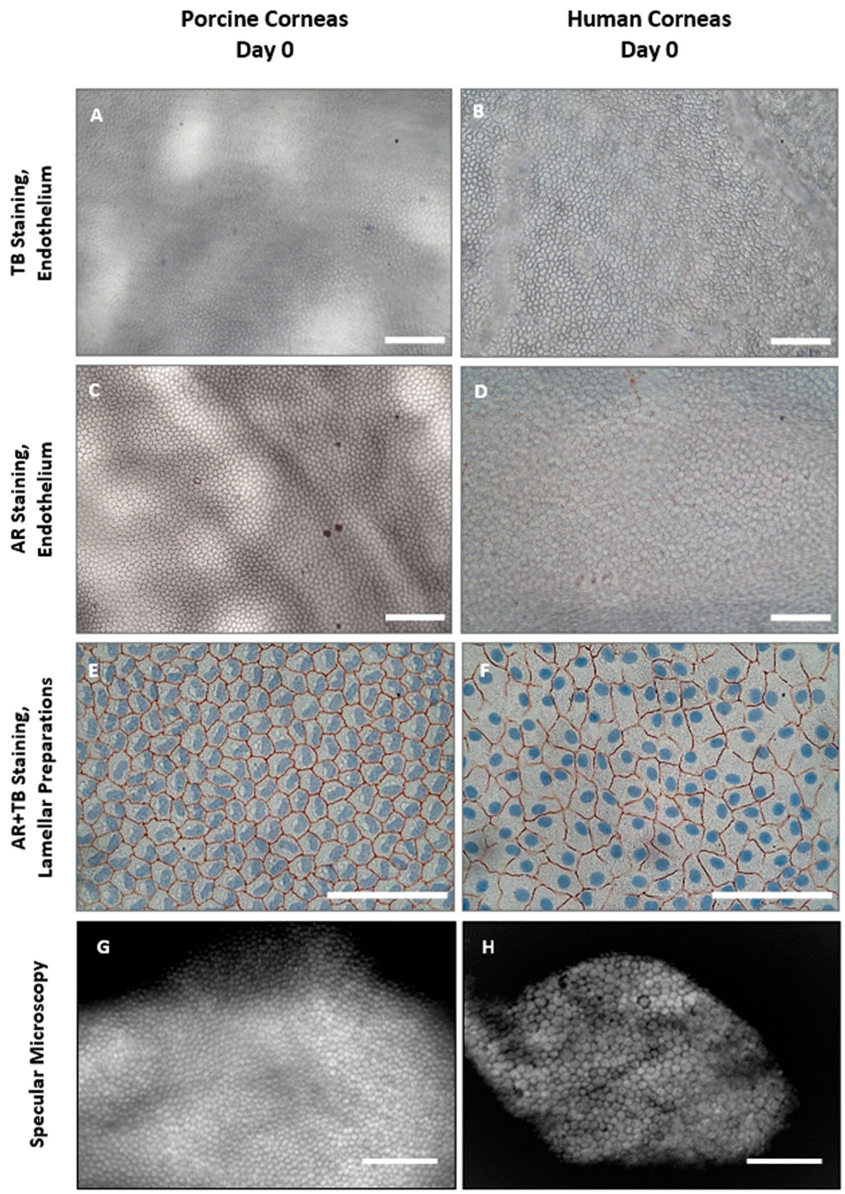

Figure 2. TB and AR staining images of fresh porcine and human corneas. (A–D) Representative 100× images of porcine (A, C) and human (B, D) corneas stained with TB and AR dyes before the beginning of the storage (day 0). Tissue staining allowed to properly assess the ECD and EC morphology of the endothelial hexagonality mosaic in both species, since cellular margins appeared sharp and clearly visible (scale bar: 200 µm). (E, F) Representative 400× images of porcine (E) and human (F) DM lamellar tissues. TB and AR dyes were used to stain nuclei and cellular membranes, respectively. This tissue preparation allowed high-magnification examination of both porcine and human endothelia (scale bar: 100 µm). (G, H) Representative images of porcine (G) and human (H) endothelia (scale bar: 200 µm) obtained with specular microscopy at day 0.

day 14; post hoc Bonferroni test, following one-way ANOVA) and porcine corneas ($P = 0.0013$ for day 3 and $P = 0.0001$ for days 7 and 14; post hoc Bonferroni test, following one-way ANOVA). The EC mortality was significantly higher in the cytotoxic control group than in the hypothermic storage medium group at days 3, 7, and 14 in both human (day 3: $P = 0.0238$; day 7: $P = 0.0145$; day 14: $P = 0.0018$; Student's *t*-test) and porcine tissues (days 3, 7, and 14: $P < 0.0001$; Student's *t*-test).

The mean measured area of porcine and human corneal endothelia corresponded to 122 ± 17 mm^2 ($n = 12$) and 89 ± 10 mm^2 ($n = 6$), respectively.

No statistically significant differences were observed between central and peripheral EC mortality in human corneas at all time points (Fig. 1D; $P = 0.8298$ for

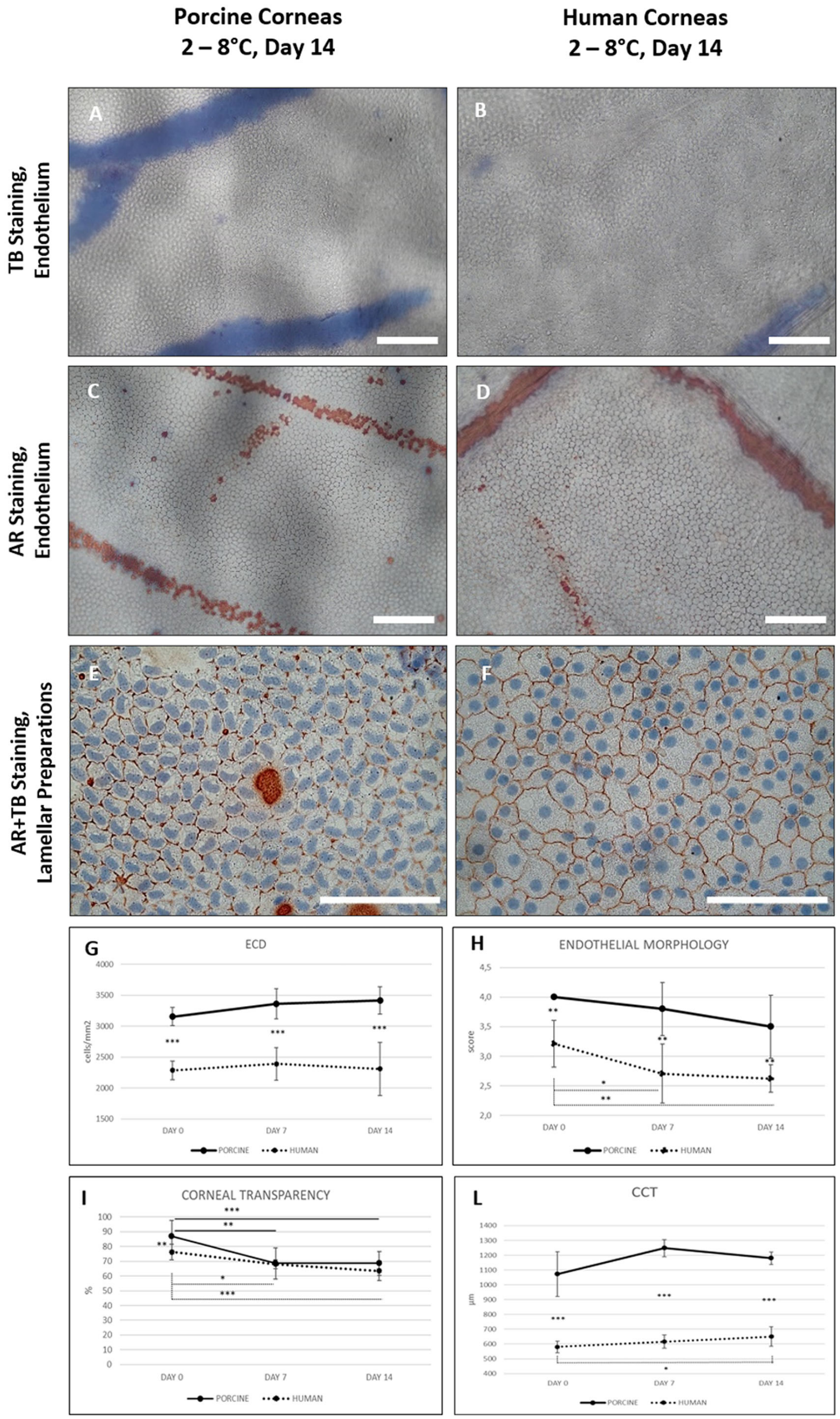

Figure 3. TB and AR staining images of porcine and human corneas after 14 days of storage at 2°C to 8°C. (A–D) Representative 100× images of porcine (A, C) and human (B, D) corneas stained with TB and AR dyes after 14 days in hypothermic storage medium at 2°C to 8°C (scale bar: 200 µm). (E, F) Representative 400× images of porcine and human DM lamellar tissues (scale bar: 100 µm). (G–L) Graphs showing the ECD (G), EC morphology (H), corneal transparency (I), and CCT (L) in porcine (*black line*) and human (*dotted lines*) corneas at selected time points during hypothermic storage medium at 2°C to 8°C. Bars are standard deviations. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

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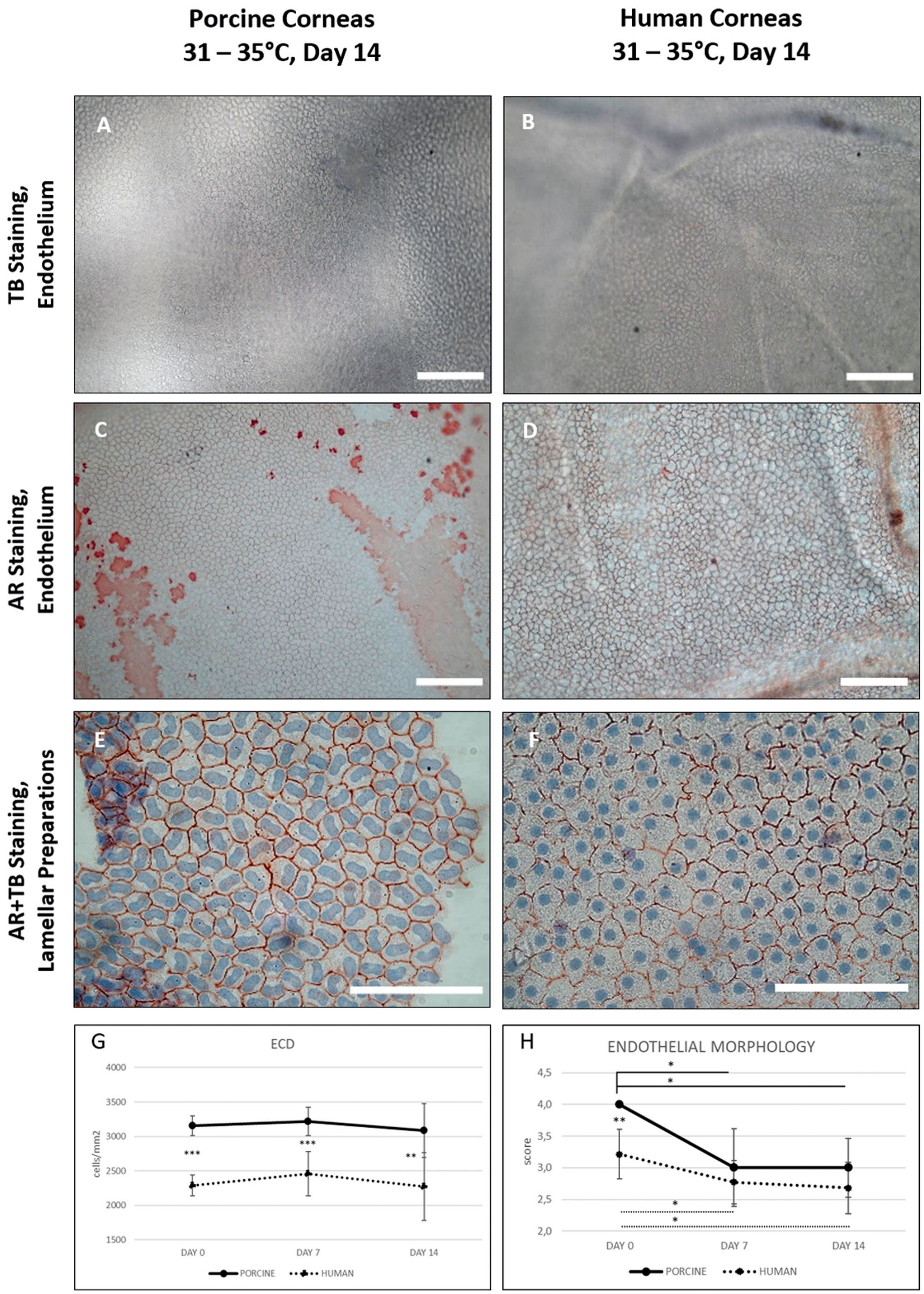

Figure 4. TB and AR staining images of porcine and human corneas after 14 days of storage at 31°C to 35°C. (A–D) Representative 100× images of porcine (A, C) and human (B, D) corneas stained with TB and AR dyes after 14 days in corneal culture medium at 31°C to 35°C (scale bar: 200 µm). (E, F) Representative 400× images of porcine and human DM lamellas (scale bar: 100 µm). (G, H) Graphic showing the ECD (G) and EC morphology (H) in porcine (*black line*) and human (*dotted lines*) corneas at selected time points during storage in corneal culture medium at 31°C to 35°C. Bars are standard deviations. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

day 0, $P = 0.7701$ for day 3, $P = 0.8424$ for day 7, $P = 0.4859$ for day 14; Student's t-test).

On the other hand, in porcine corneas, the EC mortality was significantly higher in the central area than in the peripheral area at days 7 and 14 (Fig. 1C; $P = 0.3717$ for day 0, $P = 0.2811$ for day 3, $P < 0.001$ for day 7, $P = 0.0155$ for day 14; Student's t-test).

Comparison Between Porcine and Human Corneas at 2°C to 8°C and 31°C to 35°C: ECD, EC Morphology, Corneal Transparency, and CCT

Figure 2 shows representative microscopical examination images of human and porcine corneas stained with TB (Figs. 2A, 2B) and Alizarin red (AR; Figs. 2C, 2D) at day 0. The TB and AR staining allowed assessment of the ECD and EC morphology. Cellular margins appeared sharp and visible in both species (Figs. 2E, 2F). TB- and AR-stained porcine lamellar fragments (Fig. 2E) and human DMEK lamellar tissues (Fig. 2F) allowed a high magnification of nuclei and cellular membranes. Porcine EC appeared smaller than human EC and showed reduced cytoplasmic area and large oval nuclei (mean nuclear area, 111 ± 10 μm^2), while human endothelial cells were larger with large cytoplasm and round-shaped nuclei whose areas (mean nuclear area, 92 ± 12 μm^2) were significantly smaller than porcine ones ($P < 0.0001$; Student's t-test).

Figure 3 illustrates representative images of porcine (A, C, and E) and human (B, D, and F) corneas after 14 days of storage at 2°C to 8°C. EC mosaical/hexagonal morphology appeared visible in both species. All the parameters, including ECD, EC morphology, and EC mortality, were successfully assessed after both TB and AR staining (Supplementary Table S1).

Under hypothermic storage conditions (2–8°C), the ECD was significantly higher in porcine corneas than in human tissues at all tested time points (Fig. 3G and Supplementary Table S1). No significant differences in ECD were observed over storage time in both human and porcine corneas (Supplementary Table S1).

The EC morphology score was statistically higher in porcine corneas than in human tissues at all time points. In human corneas only, the EC morphology score significantly decreased at day 14 compared to day 0 after the storage at 2°C to 8°C (Fig. 3H and Supplementary Table S1).

Corneal transparency was significantly higher in porcine corneas than in human tissues at day 0 (Supplementary Table S1, Fig. 3I), and both porcine and human tissues showed a similar slightly decreasing trend in transparency along storage time.

The CCT of porcine corneas was significantly higher than in human corneas at all tested time points, and the CCT slightly increased in both groups during hypothermic storage (Fig. 3L and Supplementary Table S1). However, the only statistical significance compared with day 0 was reached in human corneas at day 14 (Supplementary Table S1).

Figure 4 illustrates light microscopy pictures of porcine (A, C, and E) and human (B, D, and F) corneas stored at 31°C to 35°C for 14 days. The EC monolayer of these tissues was assessable in both species after both TB and AR stainings. Supplementary Table S2 shows ECD and morphology data obtained in both corneal models. The ECD values of porcine corneas stored at 31°C to 35°C were significantly higher than those of human tissues at all tested time points (Fig. 4G and Supplementary Table S2). Similar to the corneas stored under hypothermic conditions, no significant difference in ECD was observed over 14 days of storage time in both human and porcine corneas stored at 31°C to 35°C for 14 days (Supplementary Table S2).

A progressive decrease in EC morphology score was observed in both porcine and human corneas (Fig. 4H and Supplementary Table S2). The EC morphology score of porcine corneas was higher than that of human corneas only at day 0 (Supplementary Table S2). The EC morphology scores progressively and significantly decreased over time of storage in both groups (Supplementary Table S2).

Discussion

This study developed an ex vivo porcine cornea storage model with characteristics comparable to human corneas. First, a new decontamination procedure of porcine eye bulbs was set up, and the safety of the protocol was assessed quantifying the whole endothelium mortality with a new developed method. Second, quantitative and qualitative parameters (i.e., ECD, EC morphology, corneal transparency, and CCT) of porcine and human corneas were compared under hypothermic and corneal culture conditions for up to 14 days to simulate standard procedures in eye banks.[28,29] Finally, a procedure was developed to prepare porcine lamellar fragments consisting of DM and EC that could in turn be analyzed by light microscopy at high magnification (400×) for a fine-tuned examination of EC morphology, allowing microscopical comparison between porcine and human DMEK lamellar tissues.

The decontamination procedure of porcine eye bulbs was optimized to set up an ex vivo porcine model for at least 14 days of corneal storage. While human donor corneas are procured in environments controlled for microbial contamination, porcine corneas used in the present study were recovered in slaughterhouse without environmental controls. Moreover, the porcine colonizing microorganisms may differ from human pathogens,[12,30,31] and these factors strongly impact the initial tissue bioburden. The adopted orbital shaking as well as the time of contact (10 minutes) between PVP-I and the porcine eye bulb, which is longer than what is usually performed in human eye bulbs,[32] aimed at maximizing the exposure of microbial agents to the decontaminating effect of PVP-I without impacting the tissue quality. This allowed a significant decrease in the contamination rate of porcine corneas stored at 31°C to 35°C for up to 28 days. The decontamination protocol was more challenged under corneal storage conditions (31–35°C) that correspond to the optimal temperature for microbial growth and therefore to higher contamination risk compared to hypothermic storage. The decontamination procedure significantly decreased the contamination rate of porcine corneas in line with those generally observed in human corneas in eye banks[33–36] during the first 2 weeks of storage. The employed decontamination procedure was safe as no EC mortality was induced after decontamination.

Noteworthy, a quantitative method was developed to determine whole endothelium mortality (with a Fiji macro). Porcine and human corneas showed similar trends in whole endothelium mortality without significant variations over time of storage, indicating that the porcine model may well predict EC viability in human tissues under hypothermic storage conditions.

The analysis of the 8-mm central endothelial area, corresponding to the standard DMEK graft diameter,[37] showed a difference in EC mortality localization between the two species. Specifically, while no difference was observed in human corneas, porcine corneas showed significantly higher EC mortality in the central area compared to the peripheral area after 7 and 14 days of hypothermic storage. The higher number of endothelial folds observed in porcine corneas compared to human tissues may explain why EC mortality was higher in the central area of porcine corneas after 7 and 14 days of storage. This is consistent with what was observed by Kunzmann et al.,[10] whose results suggests that swelling of porcine corneas during corneal storage could induce EC mortality due to shear stress exerted by the scleral rim during the prominent CCT increase.

Previous studies have reported EC mortality ranging from 0.5% to 11%[4,24,38] after 14 days of hypothermic storage, and the present study's results, although using a different method that investigates a macroscopic rather than a microscopic image, are comparable to these findings. In addition, the present method allows analysis of EC mortality in the totality of the endothelial area, including the periphery, while microscopic evaluation usually examines fields in the central area.

The significantly higher ECD values found in porcine corneas than in human corneas under both hypothermic and corneal culture conditions, as shown by this study, are consistent with previous findings.[18,39,40] However, human and porcine corneas showed similar ECD trends under both preservation temperatures, thus indicating that the porcine cornea may reliably predict the trends observed in human tissues.

The initial EC morphology scores were higher in porcine corneas than in human ones under both storage conditions. The corneal endothelium of pigs and primates is morphologically similar,[39,41] suggesting that the difference may be due to different donor ages (i.e., porcine corneas were recovered from young [aged 6–8 months] animals, whereas the human tissues were obtained from deceased adult [aged 65–79 years] donors). Aging is usually associated with degeneration of the corneal endothelial cell mosaic in humans.[42,43] The storage of ex vivo corneas is known to induce a deterioration of the endothelial morphology in humans,[24,43,44] which could explain the observed decrease in morphology scores for both human and porcine corneas under both storage conditions.

Corneal transparency and CCT were evaluated only for the tissues stored under hypothermic conditions due to the presence of a deswelling agent in hypothermic storage medium. The lack of deswelling agents in the corneal culture medium is associated with an expected increase in CCT, corneal edema, and opacity.[21] Similar to previous studies in human corneas during hypothermic storage,[4,24,45,46] both human and porcine CCT slightly increased at 2°C to 8°C over a storage time of 14 days, with the increase being significant only in human corneas at day 14.

The transparency of both porcine and human corneas decreased during storage, consistent with previous reports in human corneas.[24] Porcine corneas initially had higher transparency than human corneas, likely due to the young age of donor animals.[43] However, a higher decrease in transparency was observed during storage for porcine corneas, possibly due to the increase in CCT.

Preparing porcine whole DMEK lamellar tissues is challenging due to the strong adhesivity between stroma and DM, as well as to the elastic properties of the donor grafts (obtained from young animals, aged 6–8 months), which increased the difficulty of the surgical manipulation.[47] Liu and colleagues[48] successfully achieved DMEK lamellar preparations from porcine corneas employing older (10–12 months) animal tissues but were unsuccessful with younger ones. In order to overcome this age-related technical challenge, we developed a method of preparing what we called porcine lamellar fragments consisting of DM and EC, which allowed for examination and comparison with human DMEK lamellar tissues. The TB- and AR-stained porcine lamellar fragments were investigated with light microscopy analysis at high tissue magnification (400×) for a fine-tuned examination of endothelial cells with detailed visualization of nuclei and cellular membranes. Human and porcine endothelia could therefore be compared at the macroscopic and cellular levels, highlighting some morphologic peculiarities in porcine corneas that slightly differed from the human ones (e.g., larger area of the endothelium, smaller size of the endothelial cells, with reduced cytoplasmic area and larger oval nuclei). Therefore, porcine lamellar tissue preparation may be a helpful tool for microscopy assessment of endothelial cells as a response to different storage conditions.

Noteworthy, porcine and human endothelia could be evaluated and compared also with specular microscopy at day 0, showing comparative results to those observed by light microscopical examination after TB and AR staining. This supports the possibility that the comparison between endothelial quality parameters of human and porcine corneas can be performed with both light microscopy and specular microscopy.

One limitation of this study is the damage induced to the corneal epithelium by PVP-I incubation during the eye bulb decontamination procedure, which resulted in detachment of the epithelium after a few days of storage (data not shown). Thus, the presented decontamination protocol is not suitable for epithelial studies.

In summary, the present study established a porcine corneal storage model as an alternative to human corneas for future investigations. This model can be used to screen the efficacy and safety of storage media and substances under different corneal storage conditions. It allows for the screening of ECD and EC morphology at magnifications typically used in eye banks, as well as at higher magnifications to investigate the endothelial cells at the cellular level using the preparation of portions of lamellar tissues.

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