

Assessment of performance and safety of Corneal Chamber hypothermic storage medium and PSS-L corneal rinsing solution in human and porcine corneas

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ABSTRACT

Purpose To prove the safety and performance of the hypothermic corneal storage medium "Corneal Chamber" and the rinsing solution "PSS-L" in support of the new *Conformité Européenne* (CE) certification process in accordance with the Medical Device Regulation.

Methods Fifteen (n=15) human donor corneas and 11 (n=11) porcine corneas were evaluated for the following parameters: endothelial cell density (ECD) and mortality, percentage of hexagonal cells (HEX%), coefficient of cellular area variation (CV%) and corneal transparency at Day 0 and after 14±1 days of storage in Corneal Chamber medium at 2–8°C. Then, the same parameters were assessed after rinsing of corneas in PSS-L for 1 min at room temperature. Evaluation of gentamicin sulfate carryover after corneal storage and PSS-L rinsing was performed by ultra-high performance liquid chromatography analysis on human corneas homogenates.

Results Human and porcine corneas stored in Corneal Chamber medium showed a good overall quality of the tissue according to the quality parameters evaluated. In particular, mean ECD, HEX% and CV% did not show statistically significant changes at the end of storage and endothelial mortality increased to 3.1±3.3 and 7.8±3.5% in human and porcine corneas, respectively. Tissue rinsing with PSS-L did not affect the quality parameters evaluated before and gentamicin sulfate residues were absent in human corneas.

Conclusions Corneal preservation in Corneal Chamber medium at 2–8°C for 14 days and the corneal rinse with PSS-L are safe and effective procedures allowing the preservation of the corneal quality parameters as well as the complete elimination of gentamicin sulfate from the tissues before transplantation. Cite Now

INTRODUCTION

Corneal transplantation is one of the most commonly performed allogenic transplant worldwide, with a consequently substantial shortage of donor corneal tissue.¹

Different methods were developed to achieve the best possible preservation of cornea physiological properties in terms of corneal transparency, endothelial, epithelial

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ "Corneal Chamber" and "PSS-L" are *Conformité Européenne* (CE)-marked medical devices developed, respectively, for the hypothermic storage and rinsing of human corneas. The new European Medical Device Regulation (MDR EU 2017/745) requires manufacturers to ensure the safety and performance of their medical devices with suitable verification and validation tests.

WHAT THIS STUDY ADDS

⇒ The present study showed that human corneas stored in Corneal Chamber medium at 2–8°C for 14 days, followed by PSS-L rinsing, maintain corneal quality parameters that are crucial for successful transplantation. The results were also confirmed by a previously validated porcine corneal model.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This work is relevant as it confirms the safety and performance of the two medical devices Corneal Chamber and PSS-L, in support of MDR compliance. In order to better clarify that Corneal Chamber and PSS-L are two medical devices studied in this work.

and limbal stem cells viability, as well as the absence of microbiological contamination.^{2,3} Most of the tissue for corneal transplantation is processed by eye banks that can use either hypothermic storage (2–8°C up to 14 days) or storage under corneal culture or so-called 'organ culture' conditions (31–37°C up to 28 days). Both techniques have their own advantages and disadvantages.⁴ Organ culture allows a longer storage period. However, due to the absence of a deswelling agent in the storage media, it leads to corneal swelling that needs to be reversed before transplantation and requires more expertise and additional instruments, making it a relatively complicated technique. Hypothermic storage allows a shorter storage time of up to 14 days,



corneal quality evaluations are performed only before the storage, but this method is easier to implement in practice. Most importantly, the limited evidence suggests similar graft survival and functional state independent of the storage method used, even if only a few studies performed a direct comparison.⁴ Nowadays, hypothermic corneal storage is used by most in the USA, while organ culture is more prevalent in Europe.⁵

Current guidelines,^{6,7} independently of the storage method, prioritise the maintenance of corneal and in particular endothelial, quality and safety. Maintaining the physiological features of preserved human corneas before transplantation is critical not only for eye bank technicians, clinicians and scientists, but also for manufacturers of corneal storage media. Even though the surgeon plays a pivotal role in assessing the suitability of the tissue for transplantation, all personnel involved in cornea processing share responsibility for achieving a successful outcome. In particular, as pointed out by the new EU 2017/745 Medical Device Regulation (MDR),⁸ the manufacturer is requested to ensure the safety and performance of its medical devices, by eliminating or reducing risks for safety as far as possible, through safe design and manufacture, with suitable verification and validation tests.^{9,10}

The hypothermic storage medium 'Corneal Chamber' (AL.CHI.M.I.A. S.R.L., Italy) has been developed for hypothermic cornea storage medical device for cornea preservation at 2–8°C for up to 14 days. Corneal Chamber medium (CCM) contains several components, including dextran, nutrients and energetic sources, that aimed to preserve donor tissue until transfer to the corneal culture medium, as well as gentamicin sulfate. The high bacterial contamination rates of donor cornea during all preservation phases despite the povidone-iodine decontamination procedure¹¹ suggest a potential benefit of gentamicin sulfate presence in the storage medium because it maintains antimicrobial activity even at low temperatures,¹² and further increases its effectiveness during warming to room temperature (RT) before use for transplantation.¹³ All these components, including gentamicin sulfate, shall be removed before transplantation and the phosphate-buffered saline solution for corneal rinsing (PSS-L, AL.CHI.M.I.A. S.R.L., Italy) was developed for this purpose. PSS-L is a *Conformité Européenne* (CE)-marked medical device that has undergone rigorous testing and evaluation to ensure the product safety and efficacy for its specific applications (eg, donor globe and corneal rinsing). In particular, lot-to-lot uniformity is guaranteed by means of stringent control of the industrial processes, accompanied by quality control testing of both raw materials and industrial batches.

The present study aimed to evaluate whether after hypothermic storage in CCM followed by PSS-L rinsing, the human cornea is suitable for transplantation and no residues of the storage medium remain in the tissue. Due to the scarce availability of human corneas for research purposes, porcine corneas were also included in the

study, according to a recently developed model that accurately predicts the response to storage conditions and treatments of human corneas.¹⁴ Quality parameters of human and porcine corneas at the beginning (Day 0) and after 14±1 days (Day 14) of hypothermic storage in CCM were evaluated, including endothelial cell density (ECD), percentage of hexagonal cells (HEX%), coefficient of variation in cell area (CV%), endothelial mortality (%) and corneal transparency. The abovementioned corneal quality parameters were also evaluated following rinsing with PSS-L (Day 14^{PR}; PR: post rinsing) at the end of storage. To verify that no antimicrobial remained in the corneal tissue after PSS-L rinse, the content of gentamicin sulfate in human corneal homogenates was measured by ultra-high performance liquid chromatography (UHPLC). The results of the performed study give information about the safety and performance of the medical devices already used in clinical settings. Moreover, the study provides new information about the previously developed and optimised animal model¹⁴ for evaluating the efficacy of corneal storage methods.

MATERIALS AND METHODS

Ethical considerations and tissue procurement

Fifteen (n=15) human donor corneas 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. Procurement and processing of human donor corneas followed Italian laws and complied the Declaration of Helsinki and the European Eye Bank Association guidelines.^{6,15} Patients or the public were not involved in the design, or conducting, or reporting, or dissemination plans of this research study.

The tissues were processed at the Fondazione Banca degli Occhi del Veneto (FBOV, Venice, Italy) following internal standard operating procedures. Donor corneas, undergoing the first evaluation within 72 hours after the procurement, were placed in CCM (AL.CHI.M.I.A. S.R.L., Ponte San Nicolò, Italy) intended for hypothermic storage, containing gentamicin sulfate. CCM has an equal formulation to corneal storage medium Eusol-C (AL.CHI.M.I.A. S.R.L., Ponte San Nicolò, Italy): these two media differ only in terms of reference market (Corneal Chamber reference market: Europe; Eusol-C reference market: USA). The human tissue inclusion criteria for this study corresponded to ECD≥1700 cells/mm², absence of severe polymorphism, endothelial mortality <5% and Central Corneal Thickness (CCT) <700 μm.

Eleven (n=11) porcine eye bulbs from young (6–8 months) domestic pigs (*Sus scrofa domestica*) were obtained from a local slaughterhouse and transported in ice to AL.CHI.M.I.A. S.R.L. (Italy) laboratories within 2 hours after death. Porcine eye bulbs were decontaminated and corneas were extracted as described by Rodella *et al.*¹⁴

The experimental protocol described in the following paragraphs is summarised in online supplemental figure 1.

Tissues quality evaluation following hypothermic storage in CCM

ECD, HEX%, CV%, endothelial mortality and corneal transparency of both human and porcine corneas were assessed at the beginning (Day 0) and after 14±1 days (Day 14) of hypothermic storage in CCM at 2–8°C.

ECD, HEX% and CV% were semi-automatically quantified with a specular microscope Konan CD-15 integrated with the software CellChekD+ (Konan Medical USA, Irvine, California, USA).¹⁶ To analyse human and porcine endothelia, CCM glass vials containing the tissues were incubated for 30–60 min in a 35°C incubator, followed by 30–60 min at RT.^{17,18} Once the endothelial layer was properly visible, a picture was taken in the central endothelium and at least 75 adjacent cells (in immediate proximity to one another, without gaps or interruptions) were manually selected and analysed by the Konan software.

Light microscopy analysis of ECD, as well as endothelial mortality evaluations were performed according to the method described by Stocker *et al.*,¹⁹ with an inverted-phase light microscope (Nikon Eclipse Ti, 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) after staining the corneas with TB-S (Trypan Blue 0.25%, AL.CHI.M.I.A. S.R.L.) and using a hypotonic solution (SR-S Sucrose 1.4%; AL.CHI.M.I.A. S.R.L.).

Light microscopy ECD was calculated using 100× magnification at the central optical area of the cornea, considering at least six squares from a digital grid made from 0.01 mm² squares (NIS-Elements Software; Nikon). The average of cells counted in the six squares was used to express ECD as cells/mm². Corneal transparency was measured using a Lux-Meter (PCE-174, PCE Instruments, Capannori, Italy) set to 0–400 lux, following the method previously described.²⁰

Immunofluorescence analysis on human tissues

After hypothermic storage in CCM at 2–8°C followed by washing with PSS-L (Day 14^{PR}), human lamellar Descemet Membrane (DM) Endothelial Keratoplasty (DMEK) grafts were stripped from n=3 human corneas, positioned on a coverslip with endothelium facing up and fixed for 30 min in 4% (wt/v) paraformaldehyde in phosphate-buffered saline (PBS). Lamellae were then permeabilised in Triton X-100 (Merck-Sigma #T8787) 0.3% (v/v) in PBS for 20 min and blocked in Goat Serum (Gibco Life Technologies #16210–064) 10% (v/v) in PBS for 60 min. Incubation with the primary antibodies anti-ZO-1 (rabbit, polyclonal 1:200, Invitrogen #61–7300) and anti-alpha 1 Sodium Potassium ATPase (Mouse, monoclonal, 1:200, Abcam #ab7671) was performed on blocking buffer at 35°C for 60 min. After PBS washes, conjugated secondary antibodies Goat anti-Rabbit IgG—Alexa Fluor 488 (1:200,

Thermo Fisher # A11034) and Goat anti-Mouse IgG—Alexa Fluor 594 (1:200, Thermo Fisher # A11032) were incubated at RT for 60 min. Following PBS washes, coverslips were mounted with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) Fluoromount-G (Electron Microscopy Sciences #17984–24) for nuclei staining and let at RT overnight before fluorescence microscopy (Nikon Eclipse Ti, Nikon, Tokyo, Japan) analysis.

Human cornea evaluation after PSS-L rinse

At the end of hypothermic storage (Day 14), human corneas were rinsed in PSS-L (PBS solution for corneal rinsing, AL.CHI.M.I.A. S.R.L.) by immersion for 1 min at RT. Immediately after rinsing (PR: post rinsing) additional evaluations of ECD (both specular and light microscopy), HEX%, CV%, endothelial mortality and transparency were performed (Day 14^{PR}). Eight out of 15 PSS-L rinsed corneas were then evaluated for gentamicin sulfate carryover. The remaining tissues were employed for immunofluorescence analysis (as described above) or were stained with the non-vital dye alizarin sodium sulfonate 0.2% (Merck-Sigma, ref: A5533) for additional morphological evaluation in inverted-phase light microscopy using 100× magnification and DMEK grafts were prepared following the gold standard FBOV procedure.²¹ DMEK grafts were analysed using the Eclipse Ti inverted-phase microscope (Nikon, Japan) equipped with Nikon objectives (Plan Flour 40×/0.30 oil ∞/0.17 WD 0.24 and ocular lenses CFI 10×/22).

Gentamicin sulfate carryover analysis on human homogenates

After PSS-L rinsing (Day 14^{PR}), central corneal buttons (8.25 mm) were prepared from human (n=8) corneas using a corneal punch (Moria, Antony, France). Corneal buttons were weighted and homogenised in 3 mL acetonitrile per button. The homogenate was centrifuged for 15 min at 4000 rpm and the supernatant was collected. Gentamicin sulfate concentration (expressed in µg/mL) was determined in triplicate for each button by UHPLC Dionex 3000 (UPLC pump model DPG-3600RS, autosampler model WPS-3000TRS, SRD-3600 Solvent Track, 6 degasser channel, oven for UPLC columns model TC C-3000RS, DAD-3000RS Photodiode Detector, Chromeleon Data Integration System model 6.80 SR9 build 2673). The analytical procedure was validated according to Q2(R1) Guideline of International Council for Harmonisation (ICH) of Technical Requirements for Pharmaceuticals for Human Use.²²

Cytotoxicity assay

To further evaluate the safety of the device according to MDR (UE) 217/745, CCM and PSS-L were tested for cytotoxicity by direct contact according to ISO 10993-5:2009²³ using the BALB 3T3 (ATCC CCL-163, Manassas Virginia, USA) cell line, cultured as previously described.^{24,25} BALB 3T3 cells' viability was quantified using the Neutral Red Uptake assay (NRU, Sigma-Aldrich, Italy) assay.²⁶

For cytotoxicity analysis of CCM, the latter was supplemented with 5% Heat-Inactivated Newborn Calf Serum (HI-NBCS, Merck-Sigma, ref: N4762) and 0.25% Dulbecco's Modified Eagle Medium (DMEM) 10x (Merck-Sigma, ref: D2429); negative control consisted in DMEM supplemented with 5% HI-NBCS and 0.25% DMEM 10x; positive control consisted in DMEM supplemented with 5% HI-NBCS and sodium dodecyl sulfate (SDS) 0.2 mg/mL.

For cytotoxicity analysis of PSS-L, the latter was tested on the BALB 3T3 cell line as it is. Negative control consisted of Dulbecco's phosphate-buffered saline (DPBS, Merck-Sigma, ref: D8662) with $MgCl_2$ and $CaCl_2$. Positive control consisted of DPBS with SDS 0.2 mg/mL.

All samples and control were tested in a final volume of 0.3 mL of culture medium, directly applied on the cell layer for 1-hour contact time. According to ISO 10993-5, a sample was considered cytotoxic when causing a reduction of cell viability greater than 30%.

Moreover, cells were evaluated by light microscopy (Leica DM IL LED, inverted-phase microscope equipped with Leica 10x objective HI PLAN I 10x/0,22PH1) before sample removal and after cell fixing in NRU assay. Changes in general morphology, vacuolisation, detachment, cell lysis and membrane integrity were assessed²⁴ and graded according to the online supplemental table 1 with a numerical grade greater than 2 considered as a cytotoxic effect.

Statistics and data analysis

The minimal sample size (both for human and porcine corneas) for the evaluation of qualitative corneal parameters (ECD, mortality, HEX%, CV% and transparency) was based on preliminary data considering $ECD \pm SD$ of corneas stored in CCM at Day 0. An estimated of $n > 3$ corneas per group were required to confirm the non-inferiority between Day 0 and Day 14 and between Day 0 and Day 14^{PR}, considering a continuous outcome non-inferiority limit of 20%, a power of 90%, an alpha error probability of 0.05, using the online calculator Sealed Envelope.²⁷

Statistical analysis was performed using Excel Microsoft Office 2019 (Microsoft, Redmond, Washington, USA) software provided with the plugin Real Statistics Resource Pack (<https://real-statistics.com/>). The normality of data distribution was evaluated using the Shapiro-Wilk test. Wilcoxon signed-rank test for paired samples was employed to compare not normally distributed data. Normally distributed data were compared using Student's two-tailed t-test for paired samples. Differences yielding $p < 0.05$ were considered statistically significant. For multiple comparisons analysis of variance test was used for continuous data normally distributed, followed by t-test with Bonferroni correction for multiple comparisons, while for data that resulted not-normally distributed, the non-parametric test of Friedman was used, followed by the post hoc Nemenyi test.

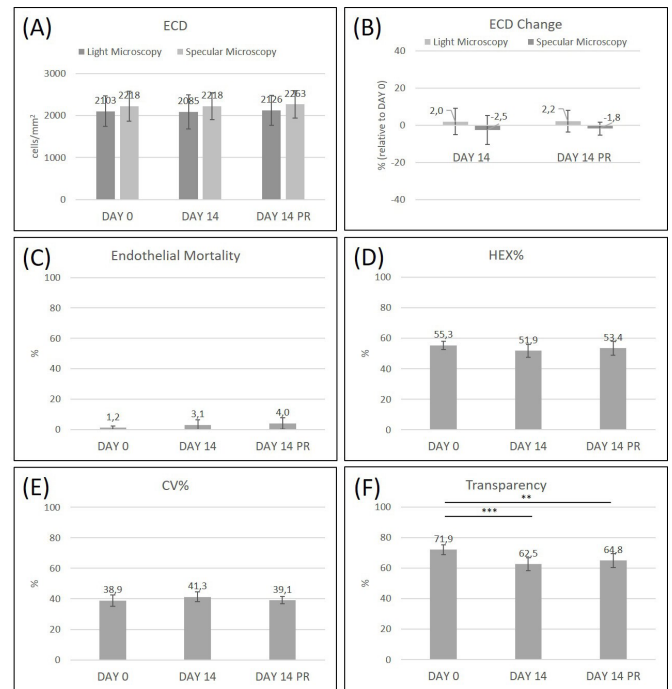


Figure 1 Endothelial cell density (ECD, A) and percentual ECD change (B, both light microscopy— $n=11$ —and specular microscopy— $n=7$ —), endothelial mortality (C, $n=15$), HEX% (D, $n=7$), CV% (E, $n=7$) and transparency (F, $n=15$) of human corneas before (Day 0) and after (Day 14) hypothermic storage in Corneal Chamber Medium (CCM) for 14 days at 2–8°C, and after rinsing of corneas in PSS-L for 1' at room temperature (Day 14^{PR}). **: $p < 0.01$, ***: $p < 0.001$. CV, coefficient of cellular area variation; HEX, hexagonal cells.

RESULTS

CCM and PSS-L efficacy and safety in human corneas

Figure 1A shows mean ECD (both with light microscopy and specular microscopy) at Day 0, Day 14 (after storage in CCM at 2–8°C for 14±1 days) and Day 14^{PR} (after storage in CCM for 14 days and PSS-L rinse). Figure 1B shows the percentage of ECD changes (light and specular microscopy) at Day 14 and Day 14^{PR}. No statistically significant differences in ECD values were observed between all the time points, both in the light and specular microscopy measurements (ECD and ECD change, online supplemental table 2).

The differences between ECD obtained with specular microscopy and ECD calculated with light microscopy were not statistically significant at all time points (two-tailed Student's t-test for independent samples: Day 0: $p=0.5135$; Day 14: $p=0.3445$; Day 14^{PR}: $p=0.4565$).

Figure 1C shows the percentage of endothelial mortality in human corneas at Days 0, 14 and 14^{PR} obtained after trypan blue (TB) staining. Endothelial mortality slightly increased between Day 0 (1.2±1.2%) and the end of hypothermic storage in CCM (Day 14, mortality: 3.1±3.3%) and the rinsing of corneas with PSS-L (Day 14^{PR}, mortality: 4.0±3.6%). However, these differences were not statistically significant (Friedman test for multiple comparisons, online supplemental table 2).

Figure 1D,E show morphological parameters HEX% and CV% of human corneas measured by specular microscopy, which remained unvaried during the experiment and no statistically significant differences were observed between all time points (Friedman test for multiple comparisons, online supplemental table 2).

Figure 1F depicts corneal transparency of human corneas at Day 0 (71.7±3.2%), Day 14 (62.9±4.1%) and Day 14^{PR} (64.5±4.2%) after storage in CCM at 2–8°C. Even if the cornea appeared transparent to a naked eye evaluation, the difference in the percentage of light transmission as detected by the lux-meter between all the time points was statistically significant (post hoc Nemenyi test following Friedman test, Day 14 vs Day 0: p<0.0001; Day 14^{PR} vs Day 0: p=0.0055; Day 14^{PR} vs Day 14: p=0.3102, online supplemental table 2).

Figure 2A shows representative images of light (left) and specular (right) microscopy of human corneas at Day 0, and after storage in CCM at 2–8°C for 14±1 days, before (Day 14) and after (Day 14^{PR}) PSS-L rinse. Occasional TB staining on Day 14 and Day 14^{PR} corresponded to endothelial folds.

Figure 2B shows the non-vital staining of human corneas with Alizarin Red (AR, left) and human DMEK grafts (right) performed on Day 14^{PR}. Both techniques allowed an additional assessment of endothelial morphology and cellular margins, which appear with regular shape and size.

Immunofluorescence analyses on DMEK grafts (figure 3) demonstrate that endothelial cells express ZO-1 and alpha 1 sodium potassium ATPase proteins after 14 days of corneal hypothermic storage followed by PSS-L rinsing.

CCM efficacy and safety in porcine corneas

Table 1 shows the averages of corneal parameters (ECD, ECD change, endothelial mortality, HEX%, CV%, transparency) of porcine corneas before (Day 0) and after (Day 14) storage in CCM 2–8°C. No statistically significant differences were observed for ECD, ECD change, HEX% and CV% (two-tailed Student's t-test for paired samples) between Day 0 and Day 14. Statistically significant increase of mortality (Day 0: 2.6±2.5%; Day 14: 7.8±3.5%; two-tailed Student's t-test for paired samples: p=0.0024) and decrease of transparency (Day 0: 78.3±4.6%; Day 14: 67.6±4.0%; Student's two-tailed t-test for paired samples: p<0.0001) was observed after storage of porcine corneas in CCM at 2–8°C (table 1).

Figure 2C shows representative images of light (left) and specular (right) microscopy of porcine corneas before (Day 0) and after (Day 14) storage in CCM at 2–8°C for 14 days. During storage, endothelial folds positive for TB staining occurred in porcine endothelia.

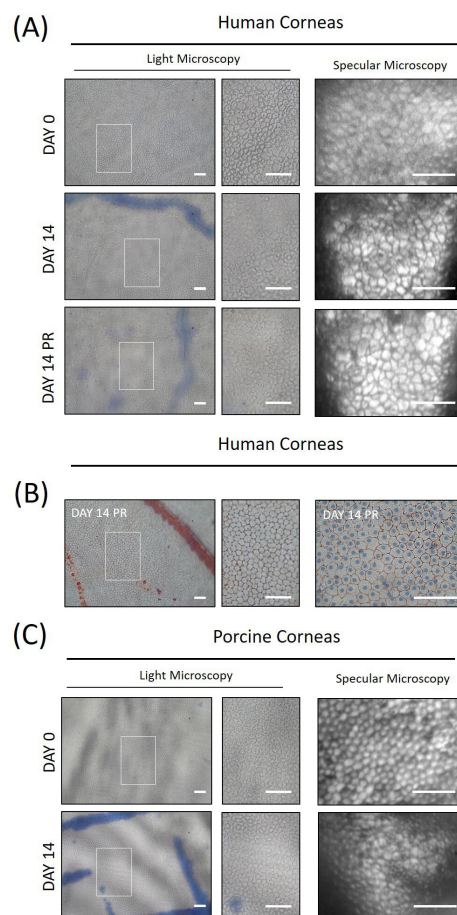


Figure 2 Representative pictures of human (A, B) and porcine (C) corneal endothelium before (Day 0) and after (Day 14) hypothermic storage in CCM for 14 days at 2–8°C, as well as post rinsing of human corneas into PSS-L for 1' at room temperature (Day 14^{PR}). Left panels (A, C): light microscopy evaluation after trypan blue staining at the different time points of human (A) and porcine (C) endothelia (scale bar: 100 µm). Central panels (A, C): magnifications of light microscopy left panels (scale bar: 100 µm). Right panels (A, C): specular microscopy evaluation at the different time points of human (A) and porcine (C) endothelia (scale bar: 100 µm). (B) Representative images of human corneal endothelium stained with Alizarin Red dye (left, scale bar: 100 µm) and of human Descemet membrane endothelial keratoplasty grafts (right, scale bar: 100 µm) after 14 days of hypothermic storage in CCM followed by PSS-L rinsing (Day 14^{PR}). CCM, Corneal Chamber medium.

Gentamicin sulfate carryover after hypothermic storage in CCM and PSS-L rinsing of human corneas

UHPLC analysis showed that gentamicin sulfate was below the limit of detection (2.4 µg/mL) in human central 8.25 mm corneal buttons after storage in CCM for 14±1 days at 2–8°C followed by rinsing of corneas in PSS-L for 1 min at RT (Day 14^{PR}), confirming its absence.

Cytotoxicity assay

Online supplemental table 3 shows the results of the in vitro cytotoxicity test by direct contact in BALB

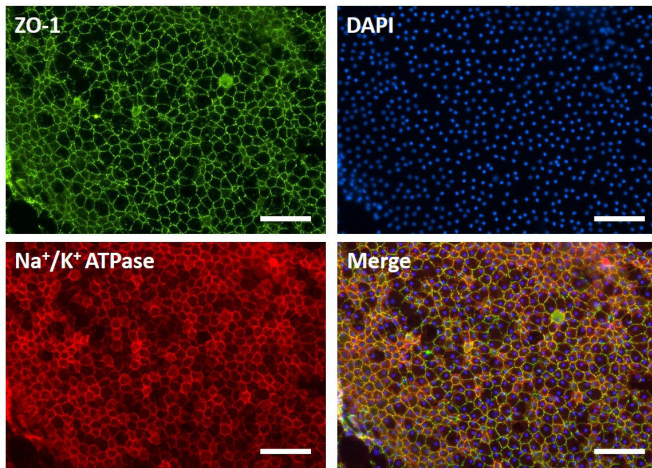


Figure 3 Immunofluorescence analysis on Descemet membrane endothelial keratoplasty grafts showing expression of the structural tight junction associated protein ZO-1 and functional alpha-1 Na⁺/K⁺ ATPase pump on human corneal endothelium after 14 days of hypothermic storage in Corneal Chamber medium followed by PSS-L rinsing (scale bar: 100 µm).

3T3 cells according to ISO 10993–5, and highlights the safety of CCM and PSS-L, as both resulted in non-cytotoxic.

Table 1 Endothelial cell density (ECD, both light microscopy and specular microscopy), endothelial mortality, HEX%, CV% and corneal transparency of porcine corneas stored 14 days in Corneal Chamber medium 2–8°C

| Parameter | Time point | Porcine corneas (n) |
|---|------------|---------------------|
| ECD light microscopy (cell/mm ²) | Day 0 | 3249±209 (9) |
| | Day 14 | 3272±181 (9) |
| <i>p</i> within group (Day 14 vs Day 0)* | | 0.7224 |
| ECD specular microscopy (cell/mm ²) | Day 0 | 3140±173 (11) |
| | Day 14 | 3138±219 (11) |
| <i>p</i> within group (Day 14 vs Day 0) * | | 0.9738 |
| Endothelial mortality (%) | Day 0 | 2.6±2.5 (9) |
| | Day 14 | 7.8±3.5 (9) |
| <i>p</i> within group (Day 14 vs Day 0)* | | 0.0024 |
| HEX (%) | Day 0 | 53.0±3.7 (11) |
| | Day 14 | 52.5±5.4 (11) |
| <i>p</i> within group (Day 14 vs Day 0)* | | 0.1184 |
| CV (%) | Day 0 | 32.5±4.4 (11) |
| | Day 14 | 36.1±4.8 (11) |
| <i>p</i> within group (Day 14 vs Day 0)* | | 0.7803 |
| Corneal transparency (%) | Day 0 | 78.3±4.6 (11) |
| | Day 14 | 67.6±4.0 (11) |
| <i>p</i> within group (Day 14 vs Day 0)* | | < 0.0001 |

The number of samples is indicated in parentheses. Values are expressed as mean data±SD.
*Two-tailed Student's t-test for paired samples.
CV, coefficient of cellular area variation; HEX, hexagonal cells .

DISCUSSION

This study aimed to provide important information on the efficacy and safety of hypothermic corneal storage in CCM and subsequent PSS-L rinsing. A comprehensive quantitative evaluation with a variety of parameters was performed at three time points: before (Day 0) and after (Day 14) 14 days of hypothermic storage, as well as after PSS-L rinsing (Day 14^{PR}), performed immediately after the end of hypothermic storage. Such comparison allowed to estimate the influence of storage medium and PSS-L rinsing on corneal quality parameters which are crucial in the suitability for transplantation. In addition, a set of corneas after PSS-L rinsing were homogenised to evaluate the presence of gentamicin sulfate residues on corneal tissues, in order to verify the completeness of the removal of the storage medium components.

Both light and specular microscopy revealed no statistically significant differences in ECD, HEX% and CV% at all time points for both human and porcine corneas.

It is crucial to emphasise that ECD remained stable for all the duration of the hypothermic storage despite the corneas obtained for the analysis were not compatible with the transplantation criteria.⁶ Noteworthy, the obtained HEX% and CV% data were comparable to those observed by Kanavi and colleagues in corneas suitable for transplantation.²⁸ In our analysis endothelial mortality slightly increased at the end of storage in both human and porcine corneas, by an average of 1.9% and 5.2%, respectively. This percentage was comparable²⁹ or lower³⁰ than those observed in studies with other hypothermic storage media at 14 days. In the presented analysis, corneal transparency decreased in human and porcine corneas after 14 days, by an average of 9.4% and 10.7%, respectively, in line with similar changes previously reported.^{14 20 21}

The described changes in corneal quality parameters are expected: previous reports show that changes in corneal endothelial functionality start early during hypothermic storage and progress proportionally to storage time.^{31–33}

The present study evaluated a storage duration of 14 days, which is the maximum hypothermic storage time defined by the manufacturers, that does not compromise long-term post-transplant outcomes too.^{34 35} However, in real practice the duration of hypothermic storage is shorter (averages 4.5 and 5.4 days in the USA and Europe, respectively).³⁶ Noteworthy, the results of the recently published Cornea Preservation Time Study (CPTS)³⁷ suggest that shorter cornea preservation time is associated with better graft outcomes. Importantly, the present study used human corneas unsuitable for transplantation, which have lower quality, as confirmed by lower prestorage average ECD (2218 cells/mm²) in the current analysis, compared with those observed in corneas deemed suitable for transplantation in a large-scale multiethnic study³⁸ and in preoperative assessment in the CPTS.³² Thus, in real clinical settings, changes in endothelial mortality and corneal transparency in

hypothermic storage in CCM could result in better outcomes in terms of corneal quality.

Furthermore, we evaluated by immunofluorescence analysis the endothelial cells expression of the tight junction-associated protein ZO-1 and alpha 1 sodium potassium ATPase pump after human corneal storage in CCM for up to 14 days followed by PSS-L rinsing, showing preserved pump and barrier functional activity.^{39 40} We could not confirm these data in porcine DMEK grafts, primarily due to the technical challenges associated with porcine tissue preparation, arising from the increased adhesiveness of porcine DM to the stroma in young (aged <1 year) animals, when compared with their human counterparts.⁴¹

Staining with Alizarin Red also confirmed preserved endothelial morphology with regular shape and size of cellular margins after PSS-L rinsing. Our data confirmed the safety of PSS-L use for corneal rinsing before transplantation, as all corneal quality parameters were maintained after PSS-L rinse for 1 min at RT. Moreover, it was verified that after PSS-L rinsing no residues of gentamicin sulfate were present in the CCM-stored human corneas.

Noteworthy, the safety of CCM and PSS-L medical devices was further demonstrated by the results obtained during the in vitro cytotoxicity test according to ISO 10993-5.²³

Considering the shortage of human donor corneas, sources of tissues alternative to human corneas to be employed as an experimental model are of outmost importance for the study of storage media and conditions. Particularly, porcine corneas are similar to human corneas and easily available from the food industry. A methodological basis for the evaluation and comparison between human and porcine corneas has been developed¹⁴ that facilitates further research and has been applied in the present analysis. Together with the data obtained here, it demonstrated that, similarly to humans, porcine corneas had comparable changes in ECD, HEX%, CV% and corneal transparency, and slightly higher endothelial mortality.

The performed analyses have some limitations. First, the sample size was relatively small even if calculated to provide the necessary statistical power. Second, human corneas unsuitable for transplantation were employed in this study, including tissues with lower endothelial quality prior to the beginning of storage in CCM. Nevertheless, even with these suboptimal tissues, there was no deterioration in a set of parameters characterising endothelial integrity and other parameters had only modest negative dynamics that were comparable to the literature data. Third, the present study does not include the evaluation of long-term post-transplant outcomes, which represent the ultimate goal of patient-centred analysis. These limitations could be overcome in a future large prospective clinical study.

In summary, the current study is relevant as it allowed to set and to validate the preservation procedure with

CCM and PSS-L solutions, confirming their safety and effectiveness, in support of specific CCM and PSS-L MDR certification and therefore related MDR compliance.

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