Synthetic media for preservation of corneal tissues deemed for endothelial keratoplasty and endothelial cell culture

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ABSTRACT.

Purpose: To compare the difference between various endothelial graft preparation methods and endothelial cell culture from tissues that are preserved in serum-based and synthetic medium.

Methods: In a randomized masked study, the tissues ($n = 64$) were preserved in Cornea Max (serum-based) and Cornea Syn (synthetic) series for 36 days at their respective preservation conditions. Following organ culture, corneal tissues ($n = 48$) were used to prepareDescemet stripping automated endothelial keratoplasty (DSAEK), preloaded ultra-thin(UT)-DSAEK, prestripped Descemet membrane endothelial keratoplasty (DMEK), free-floating DMEK, and preloaded DMEK with endothelium inward and outward grafts. These tissues were preserved for another 4days at room temperature in dextran supplemented media following which they were subjected to trypan blue, alizarin red, live/dead and Zonula Occludens-1 (ZO-1) staining. A separate set of tissues ($n = 16$) from both the series was used for human corneal endothelial cell (HCEnC) culture. At confluence, the proliferation and cell doubling rate was calculated and the cultured cells were subjected to live/dead, ZO-1, 2A12 and Ki-67 staining. Mann–Whitney test was performed with p < 0.05 deemed statistically significant.

Results: After preparation and preservation of the tissues for endothelial keratoplasty, alizarin red showed standard endothelial morphology from both the groups. Endothelial cell loss, hexagonality and uncovered areas did not show statistically significant differences (p > 0.05) between both groups. For HCEnC, cell doubling rate was 4.7 days (p > 0.05). All the antibodies were expressed in both the groups. Hexagonality, polymorphism, cell area, viable/dead cells and Ki-67 positivity were not statistically significant (p > 0.05).

Conclusions: Complete synthetic organ culture series is safe and advantageous for carrying out advanced endothelial keratoplasty graft preparation procedures and for HCEnC culture as it is free from animal or animal-derived products.

Key words: cell culture – cornea – endothelial cells – endothelial keratoplasty – endothelium – eye bank – media – preservation – synthetic

Introduction

Efficient preservation of the corneal tissue is one of the major challenges for eye banks as the quality of tissues for transplantation depends on maintenance of its cells and structure. Currently, there are two preservation systems that are followed worldwide i.e. (1) hypothermic storage, mainly used in America and Asia, maintains the cornea between 2° and 6°C for a maximum of 14 days and; (2) organ culture conservation, the most widely used method in Europe that uses medium with specific ingredients to maintain the tissue vitality and integrity up to 4 weeks (Pels et al. 2008). Foetal bovine serum (FBS) is one of the main components of organ culture medium. Although the advantages of serum are not fully known, its absence has shown harmful effects. A large amount of endothelial cell damage has been reported when porcine corneas were preserved in medium without serum (Pels & Schuchard 1983; Frueh & Bohnke 1995; Ehlers et al. 1999; Thuret et al. 2005; Parekh et al. 2015). Thus, highlighting that serum is one of the important ingredient required to maintain metabolism of the corneal cells (Ayoubi et al. 1996).

With an aim at avoiding the presence of animal or animal-derived components in organ culture system, we tested the use of Cornea Syn® [a recombinant human serum albumin (rHSA) supplemented media] for corneal storage and

compared with Cornea Max® (a FBS supplemented media) (Parekh et al. 2018). The advantages observed due to the absence of serum are manifold. First of all, animal-derived products could serve as cargo material for the introduction of animal viruses or prions into the human body. Need to reduce the use of animal-derived products started to spread with the onset of bovine spongiform encephalopathy (commonly known as mad cow disease). Indeed, products replacing serum not only remove the risk of contamination, but also take away the hazard of shortage in case of serum lack or deficiency. Another factor to consider is that serum needs batch-to-batch quality controls and assurance therefore, its use cannot be standardized. Different batches have different compositions due to the combination of environmental changes and genetic variability between animals. Three workshops were organized in Europe to discuss strategies improving development and use of serum-free defined media. The goals were to discuss possibilities to reduce or replace the use of bovine serum in cell culture media to improve the development of serum-free defined media and to state the regulatory aspects (van der Valk et al. 2004; van der Valk et al. 2010; van der Valk et al. 2018). Replacing animal or animal-derived substances has therefore been one of the important goals for the tissues deemed for human use.

A healthy corneal endothelium continuously pumps excess water out of the cornea using transmembrane ion channels; hence, the viability of the endothelial cells is crucial. It has been reported that 39% of all the corneal transplants performed are due to Fuchs' corneal endothelial dystrophy (Gain et al. 2016). Endothelial failure was previously routinely treated using full thickness corneal transplant also known as penetrating keratoplasty (PK). However, recent advances in surgical techniques allow replacement of specific layers known as endothelial keratoplasty (EK). These procedures such as Descemet stripping automated EK (DSAEK) and Descemet membrane EK (DMEK) have shown early visual rehabilitation, minimally invasive, lower rate of rejection and better visual outcomes (Tourtas et al. 2012; Greenrod et al. 2014). Hence these techniques are now taking over PK due to the advantages observed (Parekh et al. 2013).

However, due to worldwide shortage of human donor corneas, alternative approaches to obtain more corneal endothelial cells are being investigated. Many laboratories and one clinical study have shown the effect of ROCK inhibitor on human corneal endothelial cell (HCEnC) culture; however, as the tissues could be preserved in organ culture FBS, the effect of an animal-free component (rHSA) on cell culture still needs to be evaluated. The use of animal-free media for corneal preservation deemed for corneal endothelial cell culture will be advantageous while obtaining regulatory compliant certificates in the future (Peh et al. 2015b).

Thus, as the replacement of serum with rHSA could have an effect on the preparation and endothelial cell viability of the tissue for EK or HCEnC, this study investigates the effect on EK graft preparation methods and HCEnC when the tissues are preserved in serum-based and synthetic medium.

Materials and Methods

Ethical statement and donor characteristics

The corneas ($n = 64$) were collected from the Veneto Eye Bank Foundation (FBOV, Venice, Italy) with written consent from the donor's next-of-kin to be used for research in a randomized donor-matched study. The studies followed the tenets of the Declaration of Helsinki. The tissues were utilized and discarded as per the laws of Centro Nazionale di Trapianti, Rome, Italy. The corneas were suitable for research and were found to be unsuitable for transplantation due to their low endothelial cell counts (<2200 cells/mm^2). The donor age was in the range of 60–75 years with postmortem time (time from death to procurement) not more than 30 hr. No other complications or indications were recorded from the donor database.

Endothelial cell evaluation

The donor endothelium of all the tissues ($n = 64$) was stained using Trypan blue (0.25% wt/vol; Vision-Blue, D.O.R.C., Zuidland, the Netherlands) to evaluate the percentage of dead/necrotic cells. Approximately 100 μl of trypan blue was topically applied to stain the endothelium for 20 seconds followed by a washing step with sterile phosphate-buffered saline (PBS). The endothelium was exposed to a hypotonic sucrose solution (1.8%) to measure the number of endothelial cells and to examine the general morphology (pleomorphism and polymegathism) before processing. The number of empty boxes (without cells) was considered as degenerated area counted as percentage of degeneration in the given area. An in-built graticule (10 × 10) in the eyepiece of an inverted microscope (Axiovert, Zeiss, Oberkochen, Germany) was used to count the number of endothelial cells and trypan blue positive cells (TBPCs) manually and was expressed as an average of five different counts (Parekh et al. 2019a). Endothelial cell loss was measured as difference of cells found before and after graft manipulation including the number of TBPCs to ensure precise cell number after preserving the tissues in their respective series measured as cells/mm^2.

Preservation media and the experimental flow

All the preservation media was provided by Eurobio, Les Ulis, France, for the experiment. The experimental flow is illustrated in Fig. 1. In brief, the Cornea Syn series included (1) Cornea Cold that contains no serum and is used for preserving the excised cornea and to deliver it from the hospital to the eye bank at room temperature (RT), similar to Cornea Prep II; (b) Cornea Syn that contains rHSA as a replacement of FBS and the tissue is organ cultured between 31 and 37°C for up to 28 days, similar to Cornea Max and; (3) Cornea Trans, that is supplemented with dextran with no serum and it is used to regain the physiological thickness of the graft after organ culture, similar to Cornea Jet. However, Cornea Prep II, Cornea Max and Cornea Jet contain animal or animal-derived products.

Preparation of the tissues for different procedures

The tissues preserved in Cornea Max and Cornea Syn series were used for separate EK graft preparation techniques as follows.
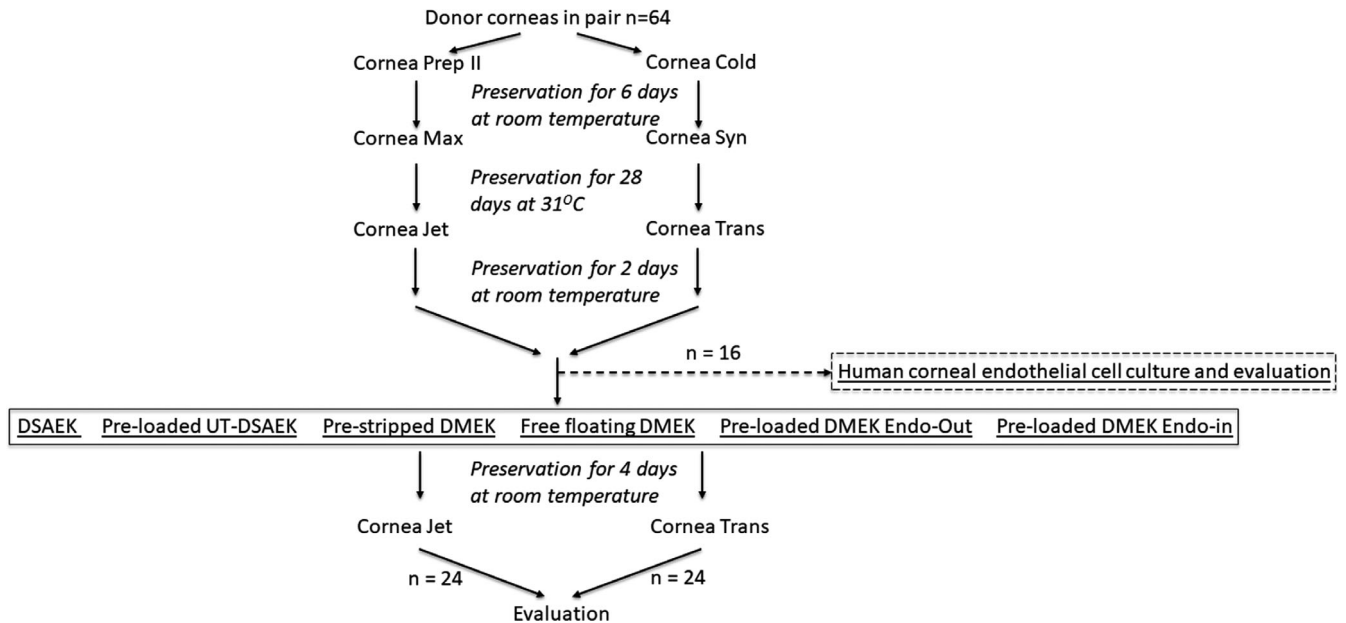
Fig. 1. Schematic representation of the experimental plan with sample size.

Descemet stripping automated endothelial keratoplasty (n = 8; four tissues from each series)

The corneal tissues were mounted on an artificial anterior chamber (Moria, Antony, France). The intra-chamber pressure was initially set at 50 mmHg (Schiøtz tonometer), but increased to 90–100 mmHg before cutting. The epithelium was carefully removed using sterile sponges. A Moria microkeratome (Evolution-3) equipped with either a 300 or 350 μm depth blade was passed over the tissue to get a posterior lamellar thickness of approximately 150 μm. The blade depth was determined from the initial corneal thickness measured using an optical coherence tomography machine (Tomey Casia SS-1000, GmbH, Erlangen, Germany). At the end, the anterior lamella of the stroma was moved back to its original place and the endothelium was examined for mortality and endothelial cell density (ECD) and preserved in the respective media for final transportation phase before the final evaluation.

Preloaded ultra-thin (UT)-DSAEK (n = 8; four tissues from each series)

Followed by similar preparation technique for DSAEK as above, an UT-DSAEK graft was prepared. The anterior stromal lamellae or silicone hydrogel contact lenses were used as a base support. The tissues were placed on a standard punching block (Moria) with the endothelial side facing the air. The tissues were trephined to obtain an 8.75 mm graft which was further transferred to the I-glide device (Eurobio) (Ruzza et al. 2015; Parekh et al. 2017c; Parekh et al. 2019e). The device was filled with the respective media and preserved. Following preservation, the endothelium was examined for final evaluation.

Prestripped DMEK (n = 8; four tissues from each series)

The tissues were centred and attached to the base of a suction punch using vacuum. An 8.5 mm trephine (Moria) was used to make a superficial incision by gentle tapping. 100 μl of trypan blue (0.25%) was topically added on the endothelium for about 20 seconds to visualize the incision. The portion of the Descemet membrane (DM)-endothelium layer outside of the incision was removed using a 120 mm straight forceps with pointed tips (Janach, Como, Italy). The endothelium was kept moist during the entire procedure using the respective media. Standardized stripping procedure was performed using a single quadrant method by peeling the DM-endothelial layer from superior end towards the inferior leaving a thin peripheral hinge at the inferior end. The stripped DM-endothelial layer was repositioned back on the stroma. ECD and mortality were checked, and the tissues were preserved in their respective media before the final evaluation.

Free-floating DMEK (n = 8; four tissues from each series)

Following DMEK preparation as described above, the tissues were stripped completely and placed in the respective media with the natural spontaneous endothelium-outwards roll (Dirisamer et al. 2012). The DMEK in this free-floating roll format was preserved in the respective media before the final evaluation.

Preloaded Endo-out DMEK (n = 8; four tissues from each series)

Following DMEK preparation as described above, the tissues were left freely floating as an endo-out roll in a sterile petri dish containing the media (Parekh et al. 2017b; Romano et al. 2018). The tissue was aspirated in a modified Jones tube and placed in a sterile vial containing the media. The tissues were preserved in their respective media before the final evaluation.

Preloaded Endo-in DMEK (n = 8; four tissues from each series)

The DMEK tissue was peeled as described in the DMEK preparation paragraph and placed back on the stroma. The prestripped membrane (9.5 mm) was re-trephined with an 8.5 mm punch (Moria) and the excess periphery was removed, maintaining

the endothelial side facing the air. The membrane was folded manually from the opposite ends using an acute forceps, touching the Descemet side to avoid as much endothelial damage as possible. The membrane was gently moved from the tissue base to a 2.2 mm intraocular lens (IOL) cartridge (Viscoject, Wolfhalden, Switzerland) preservation chamber. The membrane was pulled inside the funnel using a Grieshaber Revolution DSP 25 G end-grasping forceps (Alcon, Ft Worth, TX, USA) from the funnel opening. Funnel of the IOL cartridge was prefilled with preservation media. Descemet membrane endothelial keratoplasty membrane was pulled inside and preserved in the preservation chamber. The IOL cartridge was sealed with a rubber plug at the funnel and the rear exit. The entire unit was sealed with an in-house modified plastic clamp and preserved in a sterile vial with the media before the final evaluation (Parekh et al. 2016; Romano et al. 2018).

Alizarin red staining ($n = 12$; $n = 1$ from each condition of each series)

Endothelium of the tissues after preservation was exposed to alizarin red for approximately 3–5 min and washed with PBS to check general morphology of the preserved tissues. The cells were viewed using an inverted light microscope (Axiovert), and images were obtained using ZEN software (Zeiss, Milan, Italy) (Parekh et al. 2019d).

Hoechst, Ethidium homodimer and Calcein AM (HEC) staining to determine live/dead cells ($n = 24$; $n = 2$ from each condition of each series)

The tissues were washed with PBS after preservation prior to the assay. Five microliter of Hoechst 33342 (H) (Thermo Fisher Scientific, Rochester, NY, USA), 4 μl of Ethidium Homodimer EthD-1 (E) and 2 μl Calcein AM (C) (Live/Dead viability/cytotoxicity kit, Thermo Fisher Scientific) were mixed in 1 ml of PBS. One hundred microliter of the final solution was directly added on the tissues and incubated at RT in dark for 45 min. The tissues were flat mounted by creating 4 radial cuts and, with the solution still on the tissues, the cells were viewed using a Nikon Eclipse Ti-E (Nikon,

Burgerweeshuispad, Amsterdam, the Netherlands) using NIS Elements software (Nikon). Triple labelling showed the presence of Ethidium Homodimer stained in red representing the dead cells, Hoechst in blue representing the nuclei and Calcein AM in green marking the viable cells (Pipparelli et al. 2011).

Immunostaining for tight junctions using ZO-1 ($n = 12$; $n = 1$ from each condition of each series)

The tissues after preservation were washed with PBS and fixed in 4% paraformaldehyde (PFA) at RT for 20 min. The cells were permeabilized with 0.25% Triton X-100 in PBS for 30 min. After blocking with 10% goat serum for 1 hr at RT, the tissues were incubated overnight at 4°C with primary antibody [1:200 (FITC-

conjugated ZO1-1A12; Thermo Fisher Scientific)]. Three microliter of Hoescht was mixed in 1 ml PBS, and 100 μl of the solution was added on the tissues to stain the nucleus. After each step, the tissues were washed three times with PBS. The tissues were flat-mounted by creating four radial cuts and were covered with the mounting medium and cover slips and examined with a Nikon Eclipse Ti-E (Nikon) microscope using NIS Elements software (Nikon) for image acquisition.

Human corneal endothelial cell culture ($n = 16$; $n = 8$ from each series)

Peeling and plating the cells
Another set of corneal tissues that were preserved in Cornea Max and Cornea Syn series was used for culturing corneal endothelial cells (Fig. 1). The corneal tissues were washed with PBS,
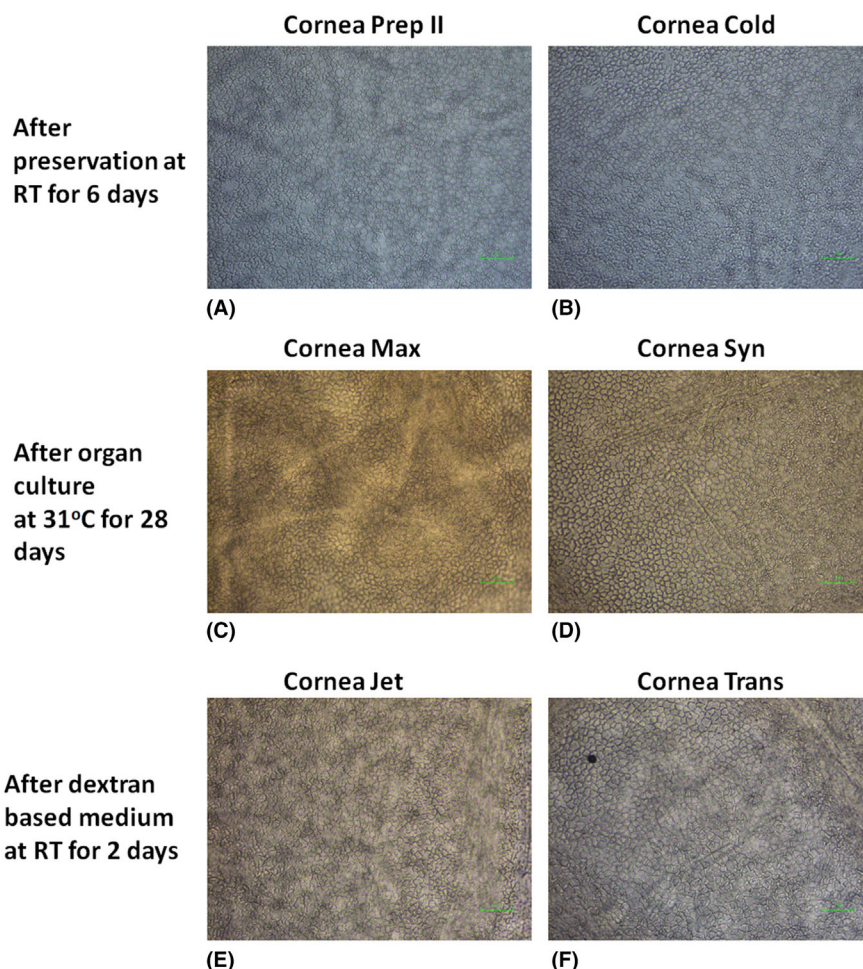

Fig. 2. Corneal endothelial cell morphology and trypan blue staining of the tissues before preserving in (A) Cornea Max and (B) Cornea Syn; after preserving at 31°C for 28 days in (C) Cornea Max and (D) Cornea Syn and finally after preserving in dextran based medium at RT for 2 days in (E) Cornea Jet and (F) Cornea Trans.

and the DM–endothelial complex were peeled in several pieces to ensure faster isolation process. The excised pieces were incubated in 2 mg/ml collagenase Type 1 (Thermo Fisher Scientific) solution for approximately 2 hr at 37°C and 5% CO_2. Collagenase and the cell clusters were then centrifuged for 5 min at 194 g using Heraeus Labofuge 400 Centrifuge (Thermo Fisher Scientific). The supernatant was removed and the cell clusters were re-suspended with TrypLE Express (1×), phenol red (Life Technologies, Monza, Italy) for 5 min at 37°C to
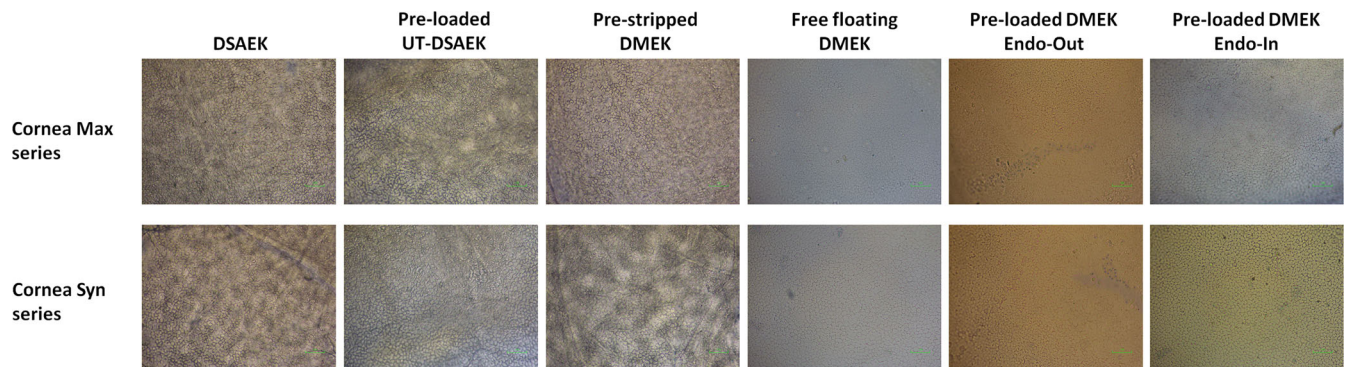

Fig. 3. Corneal endothelial cell morphology and trypan blue staining on the tissues after preparing the tissues for different endothelial keratoplasty graft preparation methods such as Descemet stripping automated endothelial keratoplasty (DSAEK), preloaded ultra-thin (UT)-DSAEK, prestripped Descemet membrane endothelial keratoplasty (DMEK), free-floating DMEK, preloaded DMEK Endo-out and preloaded DMEK Endo-in and preserving in Cornea Max and Cornea Syn, respectively.
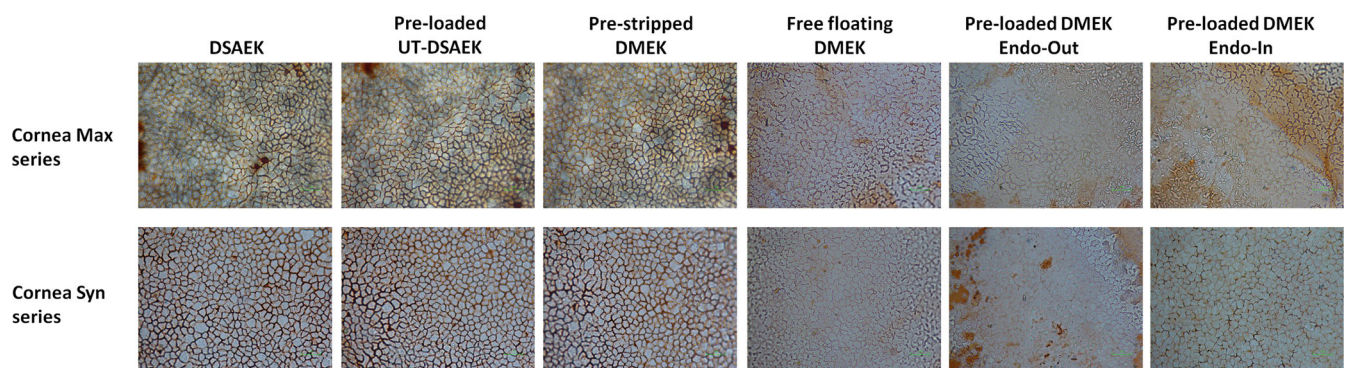

Fig. 4. Corneal endothelial cell morphology after alizarin red staining on the tissues after preparing the tissues for different endothelial keratoplasty graft preparation methods such as Descemet stripping automated endothelial keratoplasty (DSAEK), preloaded ultra-thin (UT)-DSAEK, prestripped Descemet membrane endothelial keratoplasty (DMEK), free-floating DMEK, preloaded DMEK Endo-out and preloaded DMEK Endo-in and preserving in Cornea Max and Cornea Syn, respectively.
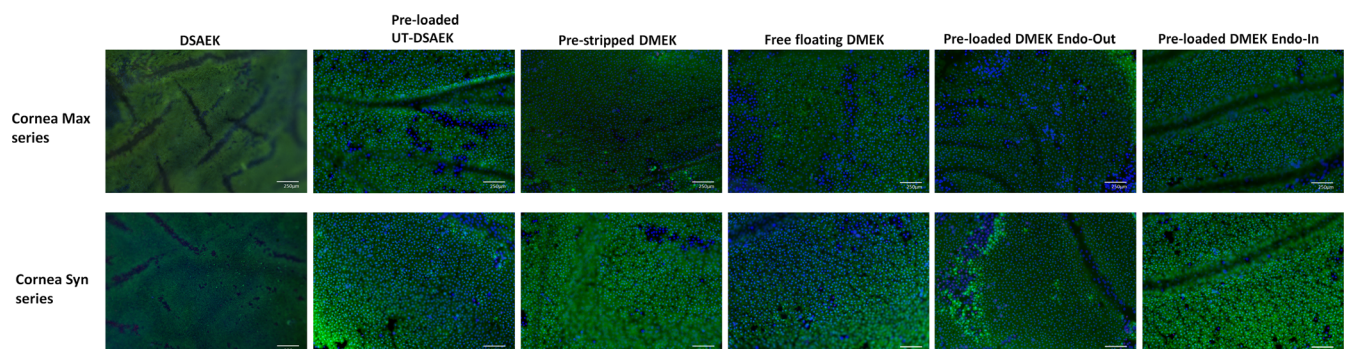

Fig. 5. Live/dead staining on the tissues using Hoechst (stains nucleus-blue), Ethidium homodimer (stains dead cells-red) and Calcein AM (stains viable cells-green) staining (HEC) after preparing the tissues for different endothelial keratoplasty graft preparation methods such as Descemet stripping automated endothelial keratoplasty (DSAEK), preloaded ultra-thin (UT)-DSAEK, prestripped Descemet membrane endothelial keratoplasty (DMEK), free-floating DMEK, preloaded DMEK Endo-out and preloaded DMEK Endo-in after preserving the tissues in Cornea Max and Cornea Syn, respectively. There was no difference observed in terms of cellular death when the tissues were preserved in either media. However, degenerated area (without cells) was observed in all the conditions due to generation of iatrogenic folds.

further dissociate into single cells. The supernatant was removed and the cells were re-suspended in 200 μl of the cell culture media which was made of 1:1 HamF12:M199, 5% FBS, 20 μg/ml ascorbic acid, 0.5% Insulin Transferrin Selenium ITS, 10 ng/ml Rec human FGF basic, 10 μM ROCK inhibitor (Y-27632) and 0.5% PenStrep (Peh et al. 2011a; Peh et al. 2011b; Peh et al. 2013a; Peh et al. 2013b; Peh et al. 2015a; Parekh et al. 2019a; Parekh et al. 2019b; Parekh et al. 2019c).

The cells were counted with TB using hemocytometer and the number of plated cells was recorded for all the cultures. Lab-Tek II chamber slides (eight chambers, 25 × 75 mm, 0.7 cm^2 culture area) from Thermo Fisher Scientific were used for plating the cells.

All the chambers were coated with 50 μl Fibronectin Collagen (FNC) coating mix [Cell attachment Reagent (FNC Coating mix) BRFF AF-10; US Biological Life Sciences, Salem, MA, USA] for at least 30–45 min at 37°C, 5% CO_2. The residual coating was removed before plating the cells. The entire 200 μl of the cell suspension from each series was added in each
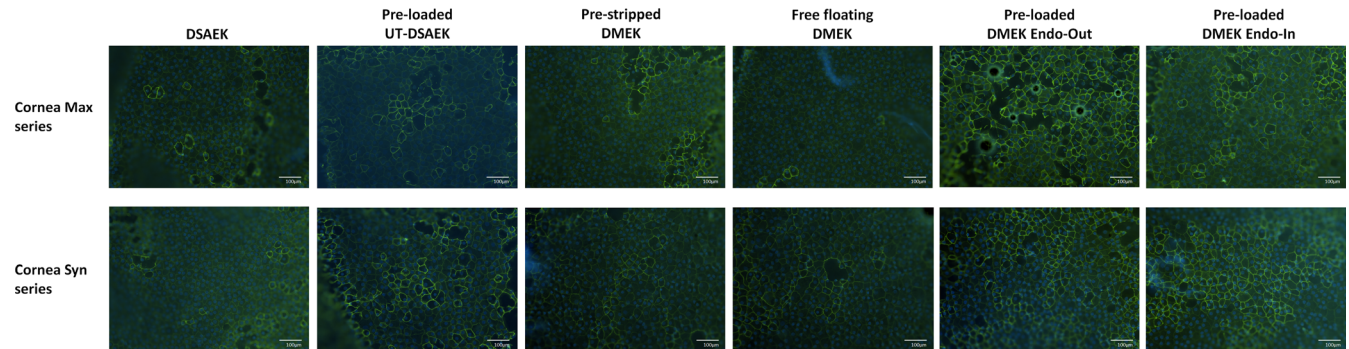

Fig. 6. Expression of Zonula Occludens-1 (ZO-1) tight junction protein on the tissues after preparing the tissues for different endothelial keratoplasty graft preparation methods such as Descemet stripping automated endothelial keratoplasty (DSAEK), preloaded ultra-thin (UT)-DSAEK, prestripped Descemet membrane endothelial keratoplasty (DMEK), free-floating DMEK, preloaded DMEK Endo-out and preloaded DMEK Endo-in after preserving the tissues in Cornea Max and Cornea Syn, respectively. The cells expressed ZO-1 and showed regular morphology with some degenerated areas from all the conditions.
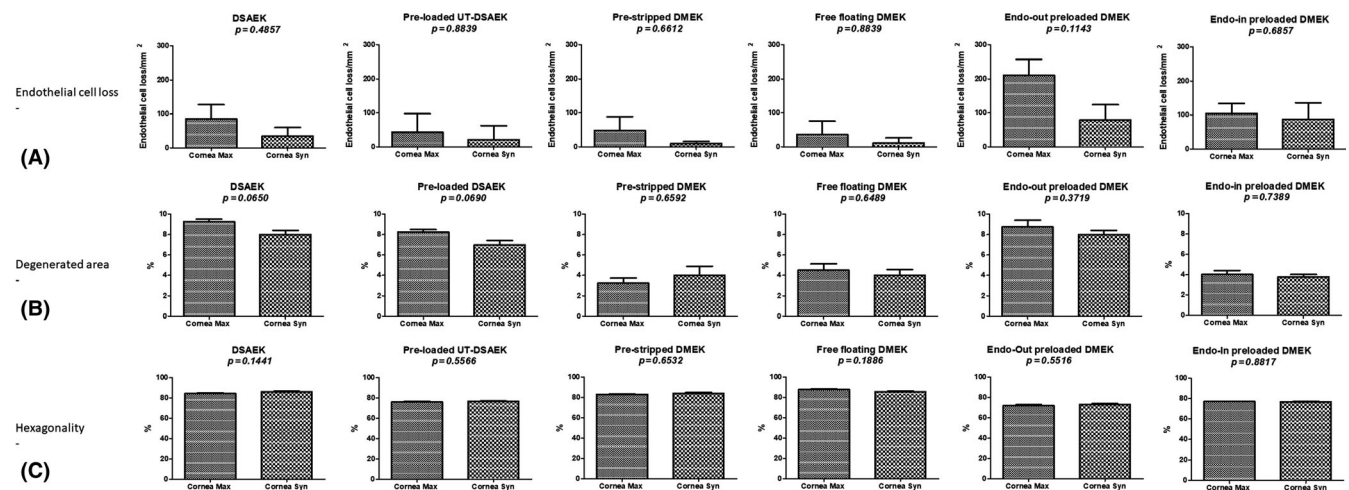

Fig. 7. Graphs representing (A) endothelial cell loss, (B) degenerated areas and (C) percentage hexagonality after preservation of different types of endothelial keratoplasty tissues in Cornea Max and Cornea Syn series, respectively.

Table 1. Percentage hexagonality, degenerated areas and endothelial cell loss observed after preservation of different types of endothelial keratoplasty tissues obtained from tissues preserved in Cornea Max and Cornea Syn series.

Average (SD)	DSAEK		Preloaded UT-DSAEK		Prestripped DMEK		Free-floating DMEK		Preloaded DMEK Endo-Out		Preloaded DMEK Endo-In	
	Cornea Max	Cornea Syn	Cornea Max	Cornea Syn	Cornea Max	Cornea Syn	Cornea Max	Cornea Syn	Cornea Max	Cornea Syn	Cornea Max	Cornea Syn
Hexagonality (%)	84 (1.2)	85 (0.9)	75 (1.7)	76 (1.2)	83 (0.8)	84 (2.1)	87 (1.7)	85 (1.2)	71 (2.3)	72 (2.1)	77 (0.8)	76 (2.0)
Degeneration (%)	10 (1.8)	7 (1.4)	8 (0.9)	5 (1.2)	3 (0.9)	4 (1.8)	4 (1.2)	4 (1.1)	8 (1.2)	8 (0.8)	4 (0.8)	3 (0.5)
ECL (cells/mm^2)	85 (84)	35 (49)	43 (108)	21 (81)	48 (81)	10 (11)	37 (76)	12 (29)	209 (94)	29 (104)	104 (60)	87 (97)

DMEK = Descemet membrane endothelial keratoplasty, DSAEK = Descemet stripping automated endothelial keratoplasty.

chamber. The cells from each donor cornea preserved in their respective preservation media were plated in each well separately. The cells were refreshed with media and were monitored every alternate day till confluence followed by end-stage characterization.

Hoechst, Ethidium homodimer and Calcein AM staining to determine live/ dead cells ($n = 4$; $n = 2$ from each series)

The confluent cells were washed with PBS. Same concentrations as listed earlier were mixed, and 100 μl of the final solution was directly added on the cells that were incubated at RT in dark for 45 min. Nikon Eclipse Ti-E (Nikon) microscope was used with NIS Elements software (Nikon) to visualize the cells while the staining solution was still on.

Immunostaining for ZO-1, 2A12 and Ki-67 ($n = 12$; $n = 2$ for each antibody from each series)

The cells were washed with PBS and fixed in 4% PFA at RT for 20 min. The cells were permeabilized with 0.25% Triton X-100 in PBS for 30 min (except for 2A12). After blocking with 10% goat serum for 1 hr at RT, the cells were incubated overnight at 4°C with primary antibody anti-Zonula Occludens-1 (ZO-1), 1:200 (FITC-conjugated; Thermo Fisher Scientific); anti-Ki-67, 1:200 (MIB-1, Milan, Italy) and anti-2A12, 1:100 (Tag-2A12, Bioprocessing Technology Institute, Singapore) (Ding et al. 2014). The cells (except for ZO-1) were incubated with goat anti-mouse FITC-conjugated secondary antibody in 10% goat serum for 2 hr at RT. Three microliter of Hoechst was mixed in 1 ml PBS, and 100 μl of the solution was added on the cells to stain the nucleus. After each step, the cells were washed three times with PBS. After detaching the walls of the Lab-Tek slide, the cells were covered with mounting medium and cover slips. The positivity of the antibodies was examined with a Nikon Eclipse Ti-E (Nikon) using NIS Elements software (Nikon).

Calculations

Endothelial cell loss from the tissues ($n = 48$)
The endothelial cell loss (ECL) was counted using the number of cells/mm^2 and TPBCs using 10×10 reticule built

in the eye piece of an inverted microscope. This was calculated before and after preservation of the tissues. The viability was also checked using Hoechst, Ethidium homodimer and calcein AM (HEC) staining.

Proliferation and cell doubling rate of the cultured HCEnCs ($n = 16$; $n = 8$ from each series)
The percentage of proliferation rate was monitored every alternate day until confluence using a 10×10 graticule (0.1 mm^2) attached in the eyepiece of an inverted microscope (Axiovert, Zeiss, Germany) at 100× magnification. The number of endothelial cells per mm^2 was counted using the same graticule to determine the cell doubling rate, and the confluency was determined by counting the number of boxes filled by the cells every alternate day represented as percentage of confluence.

Hexagonality, polymorphism, cell area, viable cells, dead cells and Ki-67 positivity ($n = 16$; $n = 8$ from each series)
All the measurements and data analysis were performed using ImageJ (FIJI) bundled with 64-bit Java 1.8.0 112. For
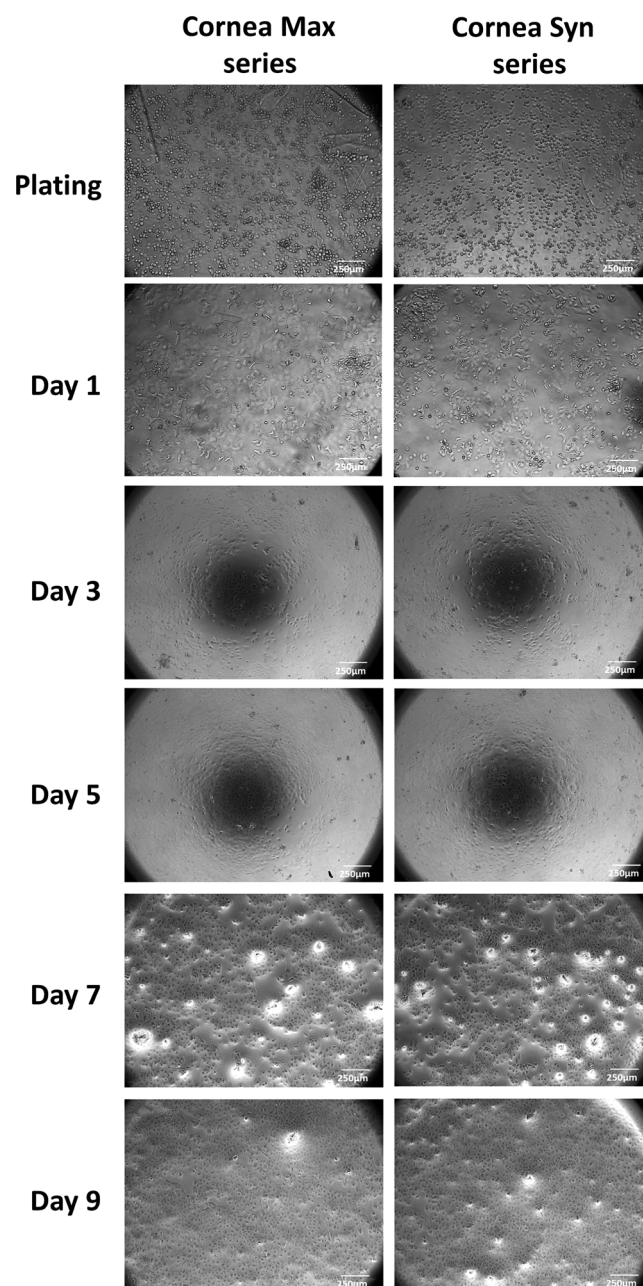

Fig. 8. Morphology and confluence of human corneal endothelial cells cultured from the tissues preserved in Cornea Max and Cornea Syn series.

ZO-1 analysis, the images were converted to overlay masks using predetermined macroinstructions to define the parameters of both hexagonality and polymorphism within a particular area (Parekh et al. 2019a). The images were auto-converted and the total number of cells in the investigated area was counted using the macros for ZO-1. The hexagonal and polymorphic cells were counted manually depending on the cellular structure comprising 6 borders per cell for hexagonal cells and less than four borders for severe polymorphic cells in the investigated area. All the measurements and data analysis were performed using ImageJ (FIJI) software. Surface areas of cells were measured based on Calcein AM uptake, where each cell was marked using a free-hand tool and measured with size limits of 150–10 000 μm^2. Viability of the cells was measured using Calcein AM, and the dead cells were measured using the positivity of Ethidium Homodimer and converted into percentage. Ki-67 positivity was assessed using random areas and analysed with an outline option. Watershed function was applied when necessary. The percentage of Ki-67-positive cells was counted based on its positive immune-signals observed in the area compared with the number of cells counted using nuclei staining (Parekh et al. 2019b; Parekh et al. 2019c).

Statistical analysis

A two-tailed nonparametric Mann–Whitney test for paired data with 95% confidence interval was used to check the statistical difference between the tissues preserved in Cornea Max series and Cornea Syn series, where $p < 0.05$ was deemed statistically significantly different using GraphPad Prism 5 (GraphPad software, San Diego, CA, USA).

Results

Tissues preserved for endothelial keratoplasty procedures

Corneal endothelial cell morphology and TBPCs of the tissues from Cornea Max and Cornea Syn series before organ culture preservation (Fig. 2A, B), after preservation at 31°C for 28 days (Fig. 2C,D) and after preservation in dextran based media at RT

for 2 days (Fig. 2E,F). After preserving the tissues prepared with different EK methods (Fig. 3) showed minimum endothelial cell mortality. Alizarin red staining showed the morphological maintenance of endothelial cells with minimum denuded areas observed from both the groups (Fig. 4). HEC staining on the tissues (Fig. 5) showed live (green) or dead (red) cells with nuclei (blue) with some denuded areas showing no cells that were mainly observed at the folds. Zonula Occludens-1 (ZO-1) staining (Fig. 6) showed positive expression in all the cells except those areas without any cells. Endothelial cell loss (ECL; Fig. 7A), degenerated areas (Fig. 7B) and percentage hexagonality (Fig. 7C) after preservation of different types of EK tissues in Cornea Max and Cornea Syn series did not show statistically significant difference (p < 0.05; Table 1) between both the groups.

Tissues preserved for endothelial cell culture

The morphology of HCEnCs did not change at different time intervals until confluence (Fig. 8) when the tissues were cultured from Cornea Max and Cornea Syn series. The cells preserved in Cornea Max and Cornea Syn proliferated at a similar rate without any

statistical significance between the groups (Fig. 9A). Post-ECD values were more than 2000 cells/mm^2 in both the groups (Fig. 9B). Cell doubling rate showed increased cell numbers from 50 000 cells to 95 000 cells by day 9 from each group without any statistical significance (Fig. 9C). Cell doubling rate was found to be 4.7 (± 0.2) days and 4.7 (± 0.18) days, and it was not found to be significantly different between the groups (Fig. 9D). Hoechst, Ethidium homodimer and Calcein AM (HEC; Fig. 10A,B), ZO-1 (Fig. 10C,D), 2A12 (Fig. 10E,F) and Ki-67 (Fig. 10G,H) staining showed high viability of cells and positive expression of the biomarkers from both the series. From Cornea Max and Cornea Syn series respectively, hexagonality was found to be 71 (± 4.6)% and 73 (± 2.6)% (Fig. 11A), polymorphism was 24 (± 2.7)% and 22 (± 3.0)% (Fig. 11B), cell area was 474 (± 22.2)μm^2 and 450 (± 28.6)μm^2 (Fig. 11C), viable cells were 98 (± 0.9)% and 98 (± 0.8)% (Fig. 11D), dead cells were 0.6 (± 0.4)% and 0.5 (± 0.4)% (Fig. 11E) and Ki-67 positivity was found to be 8 (± 0.3)% and 8 (± 0.3)% (Fig. 11F). None of the parameters showed any significant difference between Cornea Max and Cornea Syn series (p > 0.05). The tissues, when preserved in rHSA, show
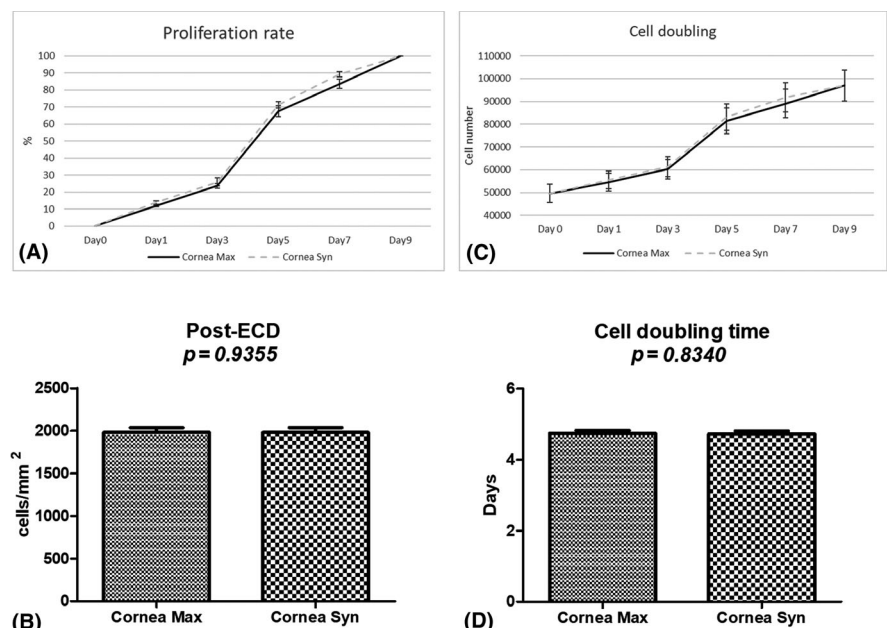

Fig. 9. (A) Proliferation rate, (B) post-ECD values, (C) cell doubling rate and (D) cell doubling time of the cells cultured from the tissues preserved in Cornea Max and Cornea Syn series, respectively.

similar outcomes when preserved in FBS-based media.

Discussion

The role of serum in cell culture media is not completely known, it could provide survival signals preventing the apoptosis of endothelium (Albon et al. 2000), it could help cells to resist stress and it could act as a scavenger, binding and neutralizing toxic compounds accumulating in the medium (Camposampiero et al. 2003). However, serum has an unpredictable biological activity and a risk associated with its use. Worldwide, the 3Rs strategy (Refinement, Reduction, Replacement) is spreading because of the necessity to reduce or replace bovine serum and to have ethical guidelines for the management of animal-derived products. It also encourages alternatives to animal models and in order to reduce the animal or animal-derived products, a synthetic medium that is completely animal-free product can be of significant relevance. So far, a lot of serum-free media have been analysed, but have not shown better endothelial cell survival as compared to serum-containing media (Pels & Schuchard 1984; Hempel et al. 2001; Stoiber et al. 2001; Møller-Pedersen et al. 2001a; Møller-Pedersen et al. 2001b; Camposampiero et al. 2003; Rieck et al. 2003). Nevertheless, the amount of commercially available serum-free media is limited or absent.

We designed an *in vitro* randomized controlled study with masked observers to test the serum-free conservation media series (Cornea Cold® – Cornea Syn® – Cornea Trans®) and compared it with the standard serum-based media series (Cornea Prep II® – Cornea Max® – Cornea Jet®). We have previously conducted a study with a similar assessment, but testing tissues for PK (Parekh et al. 2015; Parekh et al. 2018). Due to increasing interest in endokeratoplasty, we analysed tissues deemed for endothelial transplantation in a completely serum-free preservation media series and verified that tissues maintain their characteristics even after manipulation. The main outcomes we analysed were the ECL and degenerated areas. In addition, we checked the morphology, cell hexagonality of the preserved tissues and the presence of tight junctional protein by immunostaining. We found that corneas stored in either series (with and without serum) are comparable, both at the morphological and the molecular levels. The amount of endothelial cells lost after manipulation was slightly
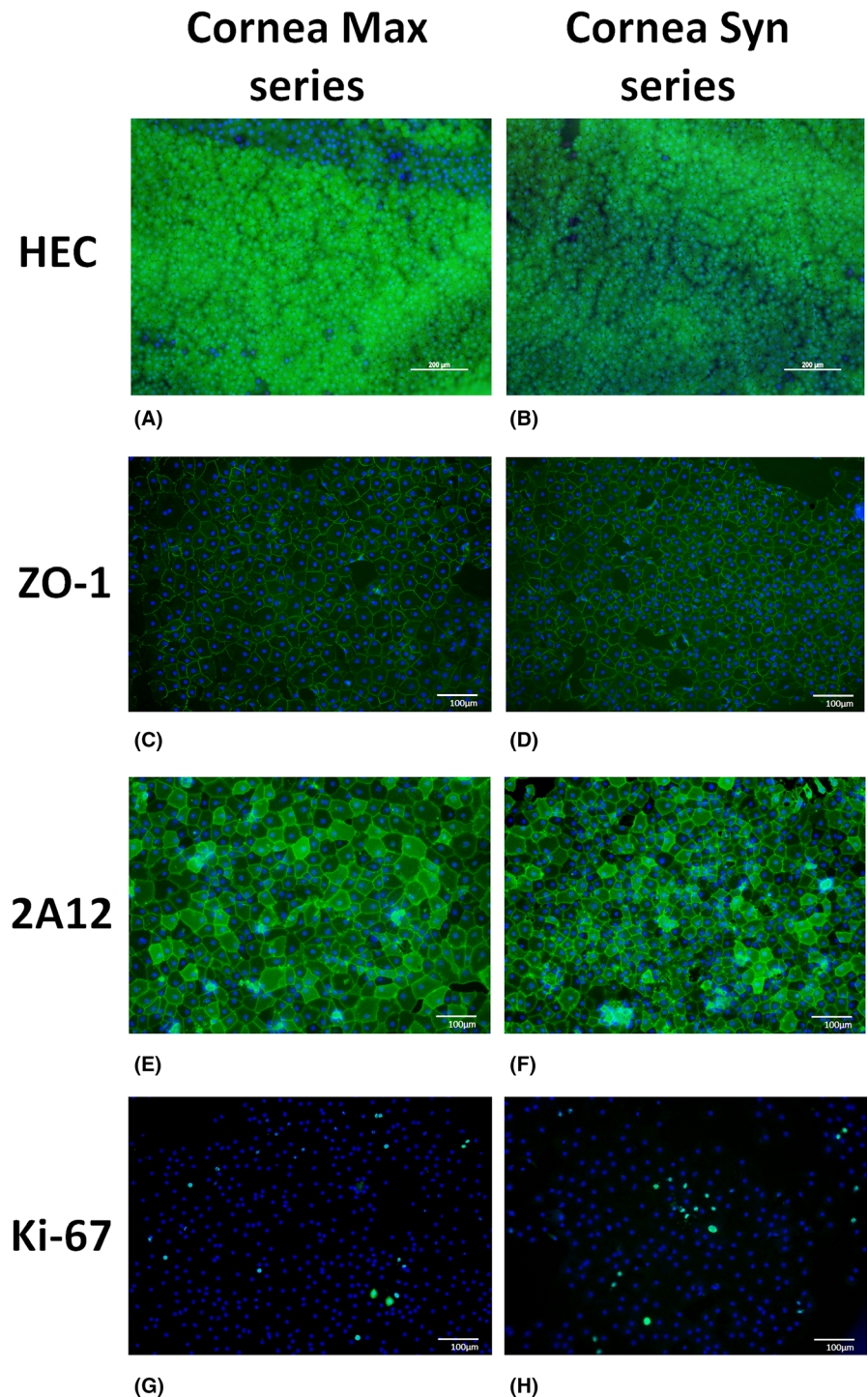

Fig. 10. Confocal microscopy images of the cultured cells stained with Hoechst (stains nucleus-blue), Ethidium homodimer (stains dead cells-red) and Calcein AM (stains viable cells-green) for (A) Cornea Max and (B) Cornea Syn. High endothelial viability with less than 1% dead cells were observed from both the groups. Zonula Occludens-1 (ZO-1) showed expression of tight junction protein in all cells both from (C) Cornea Max and (D) Cornea Syn with small amount of degenerated areas; endothelial surface marker TAG-2A12 was expressed in most of the cultured endothelial cells showing that the cells did not differentiate into another cell type after culture from both, (E) Cornea Max and (F) Cornea Syn; and proliferative marker Ki-67 showed presence of dividing cells in both, (G) Cornea Max and (H) Cornea Syn, respectively concluding that there were similar amount of proliferative cells found from the tissues preserved in either media.
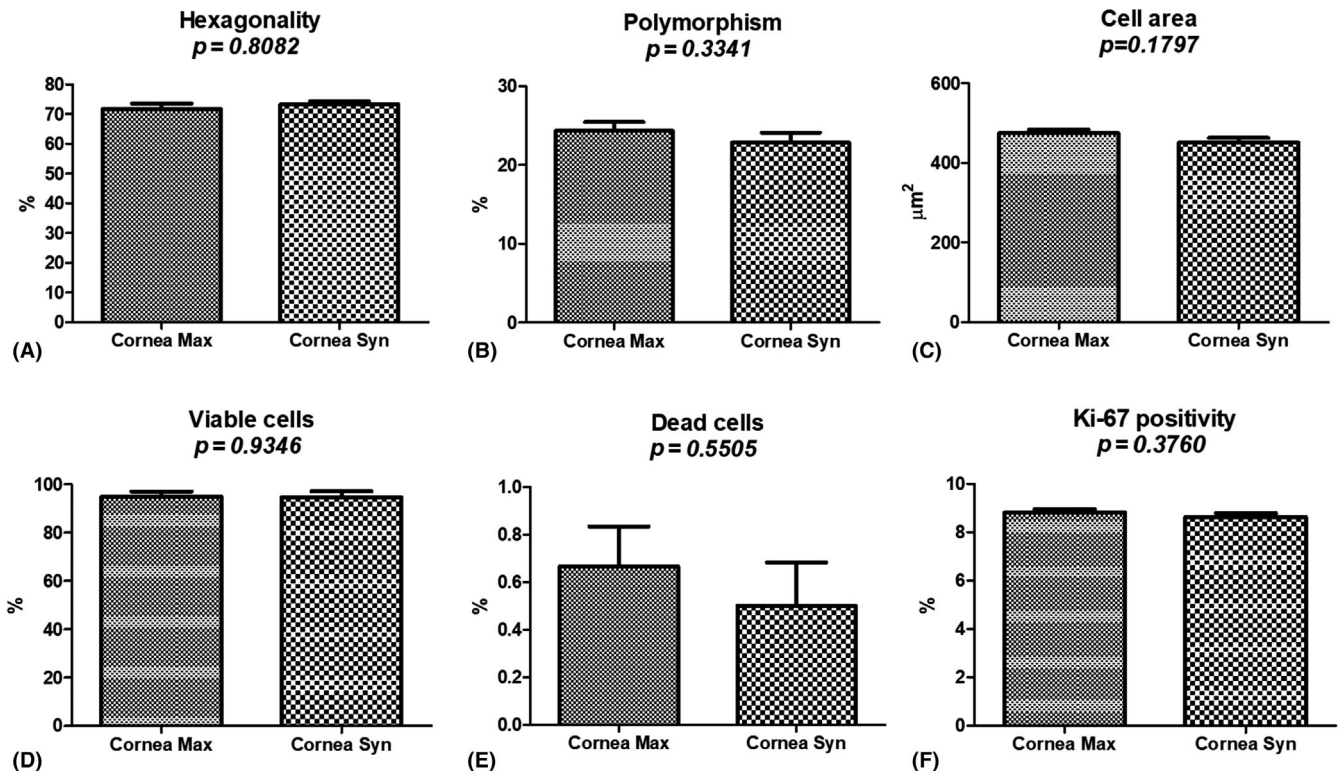
Fig. 11. Graphs from the cells cultured from the tissues preserved in Cornea Max and Cornea Syn, respectively, for (A) hexagonality, (B) polymorphism, (C) cell area, (D) viable cells, (E) dead cells and (F) Ki 67 positive cells.

lower in tissues stored in synthetic media (although statistically not significant) than in the serum-based media. Thus, we demonstrated the maintenance of the corneal quality, even under synthetic conditions simulating serum composition. This highlights that a recombinant protein substitute can maintain the cells in terms of cell morphology, viability and expression of proteins. Thus, replacement of FBS from corneal preservation solutions even if deemed for challenging techniques like EK is possible when it is replaced with rHSA. This could further advance the field of corneal preservation and EK as fully synthetic media has clear benefits over the serum-based media including those from the regulatory point of view.

Based on the satisfactory results obtained from this study, a clinical study is on-going to evaluate for the first time whether corneas stored in these serum-free media maintain their vitality and cellular activity even after transplantation. Several advantages of using synthetic media have already been noted such as: (1) reduced issues with reproducibility of batches; (2) the possibility of storing corneas will be

unconditional, even in case of serum shortage; (3) the risk of disease transmission caused by animal viruses will be eliminated; (4) the request of the health authorities will be followed without much difficulties; and (5) as the replacement does not affect any graft preservation or preparation techniques, it will be easily acceptable by the eye banks without having to undergo substantial changes in their routine protocol. Final quality of the graft is strictly dependent on a proper conservation, and therefore, media selection is of a great concern. The importance of this study is represented by the fact that results obtained, added to those acquired in our previous work (Parekh et al. 2018), validate a serum-free storage chain for all the types of keratoplasty. Various methods towards propagating primary HCEnCs have been described by several groups around the world (Bednarz et al., 2001; Joyce 2012; Kimoto et al. 2012; Okumura et al. 2013; Peh et al. 2013a; Parekh et al. 2017a). Most of the studies aim at formulating a standard procedure to improve the expansion of HCEnCs *in vitro*. These studies utilize tissues for *in vitro* cellular expansion

either from hypothermic media or organ culture preservation techniques. However, in our earlier report (Parekh et al. 2019a) we found that organ culture system is advantageous over hypothermic media for the tissues deemed for HCEnC culture *in vitro*. With recent advances in the field of endothelial cell culture and strict regulatory guidelines being governed, a completely synthetic solution must be well defined. Although the protocol for HCEnC culture significantly utilizes bovine serum albumin, collagen and fibronectin (Peh et al. 2013a) during the culture phase, it becomes difficult to exclude animal or animal-derived products and hence if the cells are not propagated under good manufacturing practices then it could lead to serious xeno-contamination. Although the use of animal or animal-derived components is a part of cell culture, we believe that using a synthetic medium for preserving the tissues would at least remove a potential source-based xeno-contamination effect. Thus, providing an animal/animal-derived free tissue for further cell culture will reduce some impact of regulatory concerns. Further to this, a synthetic media for cell

culture could also be developed to avoid any potential xeno-contamination in the future.

We thus investigated a possibility to use a completely serum-free organ culture preservation system for storing tissues for endokeratoplasty and cell culture, with the aim of freeing the corneas conservation from the necessity of serum-containing media. With several advantages of using serum-free media, we believe that Cornea Syn series will have true benefits in corneal preservation and eye banking field further increasing a compliant product with minimum risks associated with it.

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