EFFECTS OF CORNEAL PRESERVATION CONDITIONS ON HUMAN CORNEAL ENDOTHELIAL CELL CULTURE

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Abstract

The purpose of this study was to investigate the growth capacity of human corneal endothelial cells (HCEnCs) isolated from old donor corneas preserved in 4 different storage conditions. The following conditions were evaluated, A) cold storage (CS) (Optisol GS) for 7 days at 4^{o}C [n=6]; B) organ culture (OC) (Cornea Max) for 7 days at 31^{o}C [n=6]; C) OC for 28 days at 31^{o}C [n=6] and; D) CS for 7 days at 4^{o}C followed by OC for 28 days at 31^{o}C [n=6]. Following preservations, the Descemet membrane-endothelium complex was peeled and digested using Collagenase-Type1 and was subsequently trypsinized before being plated into 2 wells (from each cornea) of an 8-well chamber slide. Media was refreshed every alternate day. The confluence rate (%) was assessed, and overall viability was determined using Hoechst, Ethidium Homodimer and CalceinAM staining. HCEnC-associated markers ZO-1, Na^{+}/K^{+}-ATPase, CD166 (Tag1A3), PRDX-6 (Tag2A12) and proliferative marker Ki-67 were used to analyse the cultures established from each condition. Donor tissues preserved in hypothermia (condition A) resulted in 9.3%±4.0% trypan-blue positive cells (TBPCs) hence lower number of HCEnCs was plated. There were <1% TBPCs observed in conditions B, C and D. Indicatively, confluence in conditions A, B, C and D was 14.0%, 24.8%, 23.4% and 25.4% respectively (p=0.9836) at day 1. By day 9, HCEnCs established from all conditions became confluent except cells from condition A (94.2% confluence). All HCEnCs in the 4 conditions were viable and expressed HCEnC-associated markers. In conclusion, OC system has advantages over hypothermic media for the preservation of older donor corneas rejected for corneal transplant and deemed suitable for corneal endothelial cell expansion, with lower TBPCs before peeling and longer possible period of preservation over hypothermic storage system.

Keywords

Eye bank; cornea; preservation; endothelium; cell culture; storage

1. Introduction

The posterior monolayer of the human cornea comprises of endothelial cells that allows the transmission of soluble essential metabolites, and nutrients from the aqueous humour into the cornea (Srinivas, 2010), and is essential for maintaining corneal transparency (Joyce, 2003). The corneal endothelium must be maintained as the number of endothelial cells decrease throughout life (Bourne, 2003). If the endothelial cells are damaged to the stage that it impedes function, it can lead to oedema followed by opacity resulting in corneal blindness (Engelmann et al, 1999; Edelhauser, 2006). One of the main causes of endothelial failure is Fuch's endothelial dystrophy, which can be treated by a corneal transplant using a healthy cadaveric donor cornea. As per a recent report, nearly 40% of the keratoplasties in the United States are for treating corneal endothelial dysfunction. It has been estimated that approximately 12.7 million patients are on the waiting list for a corneal transplant (Gain et al, 2016; Wong et al, 2017). Hence, to address this severe shortage of donor corneas, alternative approaches need to be identified to reduce the global burden of corneal tissues.

Although the cells of the corneal endothelium are not known to regenerate within the eye (Joyce & Zhu, 2004), it has been shown that human corneal endothelial cells (HCEnCs) can be propagated using appropriate *in vitro* growth conditions, driving substantial research foci towards the development of alternative treatment options for corneal endothelial dysfunction through cell replacement therapeutics (Okumura et al, 2012). The development of a suitable graft alternative, through tissue engineering or injection of cultivated HCEnCs has already been proposed (Koizumi et al, 2012; Choi et al, 2010). The isolation and propagation techniques of HCEnCs *in vitro* have been

described (Peh et al, 2011A, 2011B) and subsequently reviewed (Parekh et al, 2013; Parekh et al, 2016).

Younger donors have advantages in terms of overall cell yield considering their high proliferative capability over older donor corneas, which tend to senesce at earlier passages (Peh et al, 2015). However, as most of the young donor corneas are usually suitable for transplantation, it is difficult to find such tissues for cellular expansion. If HCEnCs can be cultured from old donor corneas by modulating cell adhesion then it may potentially increase the donor pool for culturing HCEnCs (Parekh et al, 2017),

Apart from donor characteristics, it is also important to evaluate the media used for corneal preservation, and its effects on culturing of the HCEnCs isolated from older donor, in a systemic donor-matched manner. If the cornea is to be preserved for a longer period of time (between 14 to 21 days), then the storage period, methodology, as well as conditions must be optimized, validated and described if the corneas are intended for subsequent cell culture and expansion. Hypothermic or cold storage (CS) (2-6^{o} C) system is being used in the United States and majority of the Asian countries whereas most of the European eye banks prefer to use organ culture (OC) (31-37^{o}C) method. The maximum storage time for CS has been limited normally to 14 days whereas OC offers long-term storage for up to 4 weeks. OC has several advantages over CS in terms of microbiological checks, quality assurance of the tissues and preparation and planning time for surgery (Parekh et al, 2014; Parekh et al, 2015). Thus, this study intends to assess the culture capacity of HCEnCs when the corneas are preserved in different storage media, and in different conditions especially for those corneas that are excised from aged donors.

2. Material and Methods

2.1. Ethical Statement

The corneas [n=24] were collected from the Veneto Eye Bank Foundation (FBOV) with written consent from the donor's next-of-kin to be used for research. The methods were carried out in accordance with the declaration of Helsinki. The tissues were used under the laws of Centro Nazionale di Trapianti, Rome, Italy. The corneas were suitable for research and unsuitable for transplantation due to low endothelial cell count (<2200 cells/mm^2). No other complications or indications like Diabetes, HIV or HBV were noted from the donor database. All the tissues with endothelial cell density (ECD) between 1500-2200 cells/mm^2, with no mortality before preservation, age above 65 years without any previously known history of ocular surgery were included in the study.

2.2. Endothelial cell count and donor characteristics

ECD and mortality [trypan blue positive cells (TBPCs)] before isolation was checked using trypan blue (TB) staining (0.25%) (Thermo Fisher Scientific (Rochester, NY, USA), which is routinely used in eye banks. Approximately 100 µL of TB was topically applied to stain the endothelial cells for 20 seconds followed by washing with phosphate buffered saline (PBS). TBPCs were recorded before isolation using an in-built eyepiece reticule for inverted microscope (Axiovert, Zeiss, Oberkochen, Germany). The reticule (10X10) was also used to check the number of cells before isolation and at confluence. An average of 5 readings were taken from different sites to lower the risk of biased data. The donor characteristics of 24 corneas were obtained retrospectively from FBOV database to determine the age, gender, post-mortem time, cause of death, ECD and mortality.

2.3. Preservation media and conditions

The tissues were preserved in conditions A) CS (Optisol GS – Bausch & Lomb, New York, USA) for 7 days at 4^{o}C [n=6]; B) OC (Cornea Max – Eurobio, Les Ulis, France) for 7 days at 31^{o}C [n=6]; C) OC for 28 days at 31^{o}C [n=6] and D) CS for 7 days at 4^{o}C followed by OC for 28 days at 31^{o}C [n=6]. For Conditions A and B, the corneas were obtained from the same donor (donor-matched study) with right eye in Condition A and left eye in Condition B. For Conditions C and D, random corneas were selected with endothelial cell count of 1600-2100 cells/mm^2 with no baseline mortality.

2.4. Peel and digest method

The corneal tissues [n=24] were washed in sterile phosphate buffered saline (PBS) and the Descemet membrane-endothelial grafts were peeled in several pieces to ensure quick isolation, and a more even plating. The excised pieces were incubated in 2 mg/mL collagenase Type 1 (Thermo Fisher Scientific, Rochester, NY, USA) solution for 2-3 hours at 37^{o}C and 5% CO_2. The collagenase acts on the DM, degrading the membrane, allowing the corneal endothelium to form clusters of cells. These clusters were then centrifuged for 5 minutes at 1000 rpm, and re-suspended in TrypLE Express (1X), phenol red [Life Technologies, Monza, Italy] for 10 minutes at 37^{o}C for another 5 minutes to further dissociate the endothelial cell clusters to smaller clumps and single cells. The supernatant was removed and the cells were re-suspended in 200 µL of the cell culture media. The culture media was composed of HamF12, M199, 5% FBS, 1% ascorbic acid, 0.5% Insulin Transferrin Selenium (ITS), Rec human FGF basic (10 ng/mL), 10 µM ROCK inhibitor (Y-27632) and antibiotics (Peh et al, 2011A, 2011B; Peh et al, 2015; Parekh et al, 2017; Peh et al. 2013A, 2013B). The cells were re-suspended in a TB solution and counted using a

haemocytometer slide. The TB positive cells were excluded and the number of plated cells was recorded for all the cultures.

2.5. Plating cells

Lab-Tek II chamber slides (8 chambers, 25X75 mm, 0.7 cm^2 culture area) from Thermo Fisher Scientific (Rochester, NY, USA) was used for plating the cells. All the chambers were coated with 50 µL FNC coating mix [Cell attachment Reagent (FNC Coating mix) BRFF AF-10, US Biological Life Sciences, Salem, Massachusetts, USA] for at least 30-45 minutes at 37^oC, 5% CO_2. The residual coating was removed before plating the cells. Approximately 100 µL of the cell suspension was mixed well and added in each chamber to ensure equal distribution of cells from all the conditions. The cells from each donor cornea were isolated and plated, as mentioned above, in two wells. The cells were refreshed with media and were monitored every alternate day till confluence. The cells from all different conditions were isolated and plated as mentioned above.

2.6. Morphological analysis of the cultured HCEnCs [n=24]

The cells were visualized and the percentage of confluency was monitored every alternate day until confluence using a 10X10 reticule (0.1mm^2) attached in the eyepiece of an inverted microscope (Axiovert, Zeiss, Germany) at 100X magnification. The confluence was evaluated by counting the number of blocks filled with cells observed in the eyepiece reticule per 10X10 mm^2 field throughout the well.

2.7. Hoechst, Ethidium homodimer and Calcein AM (HEC) staining to determine live and dead cells [n=2]

Briefly, cultured cells were washed with PBS prior to the assay. 5 µL of Hoescht 33342 (H) (Thermo Fisher Scientific, Rochester, NY, USA), 4 µL of Ethidium Homodimer EthD-1 (E), 2 µL Calcein AM (C) (Live/Dead viability/cytotoxicity kit, Thermo Fisher Scientific, Rochester, NY, USA) was mixed in 1 mL of PBS. Approximately 100 µL of the final solution was directly added on the cultured cells and the cells were incubated at room temperature in the dark for 45 minutes. With the solution still on the cells, the examination was carried out at 100X magnification with a Nikon Eclipse Ti-E (Nikon, Burgerweeshuispad, Amsterdam) 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).

2.8. Immunostaining for tight junctions using ZO-1 [n=2], Na+/K+ ATPase [n=2], Ki-67 [n=2], 1A3 [n=2] and 2A12 [n=2]

The cells were washed with PBS and fixed in 4% paraformaldehyde (PFA) at room temperature (RT) for 20 minutes. The cells were permeabilized with 0.5% Triton X-100 in PBS for 30 minutes. [Note: the cells were not permeabilized with Triton X-100 for 2A12]. After blocking with 5% goat serum for 1 hour at RT, the tissues were incubated overnight at 4^{o}C with primary antibodies [anti-ZO-1, 1:200 (ZO1-1A12, Thermo Fisher Scientific, Rochester, NY, USA); anti-Na+/K+-ATPase, 1:50 (Na/K ATPase, Santacruz, Texas, USA) anti-Ki-67, 1:200 (MIB-1, Milan, Italy) and; anti-1A3, 1:100 (Tag-1A3) and anti-2A12, 1:100 (Tag-2A12) (Bioprocessing Technology Institute, Singapore) (Ding et al, 2014). The samples were incubated with goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody in 10% goat serum for 2 hours at RT. 3 µL of Hoescht 33342 was mixed in 1mL PBS and 100 µL of

the solution was added on the top of cultured cells to stain the nucleus. After each step, the cells were washed 3 times with 1X PBS. After removing the wall of the Lab-Tek slide, the cells were covered with mounting medium and cover slips. Cells were examined with a Nikon Eclipse Ti-E (Nikon, Burgerweeshuispad, Amsterdam) using NIS Elements software (Nikon).

2.9. Measurement and statistical analysis

The confluence measurements were carried out as described earlier. ImageJ (FIJI) software (ImageJ bundled with Java 1.8.0_172, NIH, USA) was used for the measurement of endothelial cell numbers, as well as to assess the hexagonality and polymorphism of the cells based on ZO-1 expression. Percentage of Ki-67 positive cells as well as cell sizes based on Calcein AM staining of HCEnCs were also evaluated. The particles were analyzed using outline option and watershed was applied if necessary for Ki-67 positive cells. For ZO-1, the area was selected and using pre-defined commands in Macros for converting the image to overlay masks, the total number of cells was automatically counted whereas the hexagonal cells and polymorphic cells were counted based on the cell structure in the particular area (with 6 borders). The same Macros were used as previously described (Parekh et al, 2017) to obtain results by simply inserting the algorithm in the ImageJ analysis. Cell surface area was determined using Calcein AM and analysed with ‘analyze particles’ with size limits of 150-1000 μm^2 to exclude background signals and large cell clusters. Data are expressed as mean ± SD.

Non-parametric Wilcoxon test for paired data and one-way ANOVA test for independent measures using SAS statistical software was utilized to check for statistical significance between different conditions where $p<0.05$ was deemed to be

statistically significant. A post-hoc correction to the significance was applied using Bonferroni.

3. Results

3.1. Donor characteristics and plating density

Paired corneas were used from the same donor for conditions A and B therefore the donor characteristics were the same for both the conditions. ECD (cells/mm^2) before the isolation of the cells is recorded in table 1. Trypan blue positive cells (TBPCs) (%) after preservation was found in condition A (Figure 1A) with limited TBPCs in conditions B (Figure 1B), C (Figure 1C) and D (Figure 1D). Approximately 9.3% ± 4.0% TBPCs was found, mostly on the folds with a few scattered cells in Condition A (Figure 1A and 1E) before peeling. Very less amount of TBPCs was recorded before peeling in the other three conditions (Figure 1F). Number of cells plated per well of a Lab-Tek II chamber slide (8 chambers, 25X75 mm, 0.7 cm^2 culture area) is half the amount of cells isolated from each cornea (table 1). All the results in table 1 were recorded for conditions A, B, C and D respectively with mean (±SD) [Range: Min-Max].

3.2. Growth dynamics of HCEnCs cultured from different preservation conditions

HCEnCs were observed at different days of culture (Figure 2) for morphology and confluency. At day 1, the growth rate (%) observed in Conditions A, B, C and D was 14.0, 24.8, 23.4 and 25.4 respectively. At day 9, apart from Condition A, that showed 94.2% confluency, all the other conditions showed 100% confluence (Figure 5A), suggesting that HCEnCs isolated following preservation in Condition A may have a slower propensity to propagate. However, the confluency rate was not statistically significantly different in either condition (p=0.9836). This may also be partly due to the presence of TBPCs, accounting to a lower overall cellular yield, and in turn, resulted in a lower initial number of plated cells observed in condition A.

3.3. Overall cellular viability of HCEnCs cultured from different preservation conditions

Cell viability was observed in all conditions (Figure 3). Cell area was determined on 20 cells per condition using Calcein AM staining and ImageJ analysis. Cell area (μm^2) was calculated and recorded in conditions A (Figure 3A), B (Figure 3B), C (Figure 3C) and D (Figure 3D) respectively without any statistical significant difference ($p>0.05$) (Figure 5B) (table 2).

3.4. Characterization of HCEnCs cultured from different preservation conditions

At the end of the culture, at day 9, ZO-1 (Figure 4) was expressed in all the conditions. Average value of hexagonality (%) and relative polymorphism (%) [polymegathism and pleomorphism] in the respective conditions are indicated in table 2 (Figure 5C). No significant difference was found in hexagonality and polymorphism. Na^+/K^+-ATPase, a function association marker for the corneal endothelial cells (Figure 4) and Tag-1A3 (Figure 4) was expressed in all the conditions suggesting the antigen target of CD166 and its presence on the cell surface of the cultured HCEnCs. Tag-2A12 (Figure 4) was also expressed in all the conditions showing the presence of cell surface epitopes PRDX-6 and confirming the cells to be HCEnCs. Ki-67, a proliferative marker showed Ki-67 positive cells (%) (Figure 4) that were counted using ImageJ and were recorded in conditions A, B, C, D as indicated in table 2 without any significant difference (Figure 5D).

4. Discussion

Alternative approaches for the treatment of corneal endothelial dysfunction have become possible with the ability to culture primary corneal endothelial cells (Peh et al, 2013A, 2013B; Shima et al, 2011; Kimoto et al, 2012; Zhu et al, 2012; Okumura et al, 2013), which enabled the development of cellular based therapy, as shown by the recent report of the corneal endothelial cell injection study (Kinoshita et al, 2018). However, the source of primary cells remains a challenge due to the limited expansion capacity of isolated primary HCEnCs, as well as the worldwide shortage of human donated corneas. It has been noted that cultivated HCEnCs derived from older donors have lower proliferative capability, where most derived HCEnCs formed cells of senescent morphology, indicating less overall cellular yield with potentially lesser functional capability than those derived from younger donors (Joyce et al, 1995).

Once the donor tissues are excised, they are sent to the eye bank and preserved in the storage media. Hypothermic preservation system offers short-term storage of corneal tissues (maximum 14 days). In Europe, many eye banks prefer OC storage method that includes the main storage medium comprising of serum and antibiotics that can preserve the corneal endothelium for up to 28 days at 31^{o}C (Pels et al, 2008). In this study, we investigated these different preservation conditions to evaluate the optimum storage method of the corneal tissues that are deemed for endothelial cell culture.

In the donor-matched comparison between corneas stored in condition A and condition B, higher amount of TBPCs were observed in the corneas from condition A. The serum in OC is substantially ‘richer in nutrients’ which helps with the viability of the corneal endothelium, as observed in condition B. Condition A showed higher TBPCs before peeling, further indicating that the starting cell population may not be

as healthy, leading to a lower cellular yield, and lower initial plating density, which may negatively impact the proliferative potential of the remaining isolated HCEnCs (Peh et al, 2013B). Preserved tissues in condition B were found to reach confluency faster than the corneas stored in condition A indicating that the endothelium may be better preserved in OC. In our observation, limited (<1%) endothelial cell death was observed in other conditions compared with condition A. Conditions C and D were a non donor-matched group, and although there may be potential donor variation, comparable results to condition B and superior outcomes to that of condition A strengthens the observation that corneas preserved in OC may be more suitable for HCEnC cultures in terms of general health of the corneal endothelium and overall cellular yield after cell isolation.

When the tissues were preserved in only OC (condition B and C) or CS followed by OC (condition D), the cells were better preserved with high viability when broadly compared to tissues preserved in Condition A, in turn, yielding higher number of cells plated and reaching confluency faster. The non-viable cells could have dislodged and were replaced by healthy neighbouring cells through physical cellular spreading or potentially through proliferation (Joyce et al, 1995) in condition D. Condition D can potentially provide an added option to further extend the shelf life of corneas stored in hypothermic condition that may have become unsuitable for transplant, and effectively increase the storage time up to 35 days with minimal mortality. Thus increasing the time frame to organize these tissues that are downgraded from transplant-grade to research-grade corneas and can be used to establish cultures of HCEnCs.

Varying factors such as health of the donor before death, the cause of death, as well as the duration from death to enucleation and the time from preservation to the establishment of culture (Peh et al, 2011A, 2011B; Beck et al, 1999; Miyata et al, 2001;

Parekh et al, 2017) have been noted to affect the cell culture. However, we did not find any significant deviation in establishing the culture of HCEnCs due to the above-mentioned factors.

Live-dead cell staining showed no dead cells with no significant change in cell areas found in either conditions (figure 3). ZO-1 showed integrity of tight junction protein. Morphological analysis showed preservation of hexagonality (>65% in the central zone) where <20% cells were noted as polymorphic. Na^+/K^+-ATPase marker showed the presence of the ionic pumps in all the conditions. The expression of cell surface CD166 and Prdx-6 have been monitored using TAG-1A3 and TAG-2A12 respectively showing a good correlation with the current standard of morphological grading of HCEnCs (Ding et al, 2014). We have optimized the number of cells plated in each well and to reduce the bias of more proliferative cells in either condition, we assessed the cells using Ki-67, which was not found to be significant between the conditions.

In conclusion, the results indicate that HCEnCs can be established from corneas preserved in all four conditions. However, to reduce further stress on the corneal endothelium and maintain its viability during isolation of the cells, it is recommended to transfer the tissues or preserve them in OC storage, as hypothermic media could have potential limitations in terms of viable or low plating density, short-term preservation leading to less amount of preparation time, and less optimal conditions and growth factors to preserve these cells. HCEnCs from old donor corneas that are readily available for research due to its less endothelial cell count and proliferative capability may ultimately provide clinicians with a new therapeutic modality in regenerative medicine and reduce the global shortage of the donor corneas for the treatment of endothelial disorders, if cultured.

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6. Figure Legends

Figure 1: Human corneal endothelial cells on the cornea before peeling, viewed using an inverted microscope under 40X magnification. A) Condition A, endothelium of the corneas preserved in hypothermia for 7 days showing trypan blue positive cells (marked with arrow), B) Condition B, endothelium of the corneas preserved in OC for 7 days at 31^{o}C showing limited TBPCs, C) Condition C, endothelium of the corneas preserved in OC for 28 days at 31^{o}C showing limited TBPCs with iatrogenic folds developed due to the preservation and swelling of the tissue. D) Condition D, endothelium of the corneas preserved for 7 days in hypothermia following by OC preservation for 28 days at 31^{o}C showing minimal mortality. E) Trypan blue positive areas found at the periphery while peeling the tissue obtained from hypothermic condition (marked in black arrow), yellow arrow indicates the already peeled area after re-staining it with trypan blue stain. F) The tissues obtained from OC preserved corneas. Yellow arrow indicates the area already peeled after re-staining with trypan blue stain.

Figure 2: Morphological analysis at different days of the culture in different culture conditions at 40X magnification. The confluence rate (%) was high in conditions B, C and D. The number of isolated cells was relatively low in Condition A.

Figure 3: HEC staining to determine live/dead cells at 100X magnification in A) condition A, B) condition B, C) condition C and D) condition D respectively. The presence of live (green) cells and nucleus (blue) were observed in different conditions without any dead cells. Confluent areas were observed at day 9 in all the conditions.

Figure 4: ZO-1 staining for intracellular tight junctions and to determine the hexagonality and polymorphic cells in different conditions at 200X magnification. The cultured HCEnCs showed expression of ZO-1 in all conditions. Na^+/K^+-ATPase was expressed in all the conditions showing hydration potential (200X magnification). 1A3 and 2A12 was observed in all the conditions (100X magnification). Expression of Ki-67 was expressed in all four conditions (100X magnification).

Figure 5: Graphical representation of the conditions and relative values. A) Confluence rate representing confluency (%) of the cells obtained from different conditions at different time points. A slow growth rate was observed when the corneas were preserved in Condition A i.e. 7 days of preservation in cold storage against the other conditions that showed similar growth trend. B) Cell area determined by counting the cell surface area of 20 cells per condition showed no significant difference between the samples. C) Hexagonality and polymorphism was monitored and calculated after ImageJ analysis however, no significant difference was found between any conditions. D) No significant difference was found in Ki-67 expression.