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Standardization of Human Corneal Endothelial Cell Isolation and the Use of Denuded Human Amniotic Membrane as a Scaffold for Human Corneal Endothelial cells

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Abstract

Objectives: To standardize the isolation of human corneal endothelial cells (HCECs) and to use the denuded human amniotic membrane (HAM) as a scaffold for isolated HCECs.

Methods: Human amniotic membrane was denuded using 1.2 units/ml of Dispase II at 37°C for 60 minutes followed by mechanical scraping. Corneal endothelial and Descemet's membrane sheets were peeled from human donor cadaveric eyes unfit for surgical use and enzymatically digested with 2 mg/ml of collagenase II solution at 37°C and 5% CO_2 for 2 hrs. Isolated cells were resuspended in culture medium with supplements and plated onto uncoated cultureware for four hours to eliminate fibroblasts which adhere more rapidly than endothelial cells. After preplating, the non-adherent cells were seeded onto gelatin coated dishes or onto denuded amniotic membrane in OptiMEM media supplemented with growth factors. The cells were analyzed by microscopy for adherence and polygonal morphology.

Results: Microscopy of the denuded amniotic membrane showed no epithelial cell remnants. Enzymatic digestion of cornea left behind acellular Descemet's sheets with the endothelial cells floating individually or in clusters with preplating aiding in a more fibroblast free endothelial cell isolation. Few isolated cells managed to scaffold onto the amniotic membrane and retain that adhesion during subsequent media replacements.

Conclusion: Prolonged Treatment of HAM using the mild enzyme Dispase-II results in denuding the membrane, which serves as a successful scaffold for harvested corneal endothelial cells. This approach may be further explored as a cost effective alternative for endothelial cell proliferation and as an in vitro model for corneal tissue engineering studies.

Keywords: Human amniotic membrane; Corneal endothelial cell isolation; Scaffold; Dispase-II, Corneal endothelial cell proliferation

Introduction

The clear cornea is composed of multiple cell layers that form the primary refractive surface of the eye. The human corneal endothelium lines the internal surface with a single layer of cells that plays a critical role in the regulation of corneal hydration, maintaining corneal thickness and keeping the cornea transparent [1,2]. Unlike epithelial cells that divide to repair defects, human corneal endothelium has a very limited propensity to proliferate in vivo. Endothelial cell density reduces with increasing age as the rate of cell division is unable to keep pace with the rate of cell loss. In order to replace dead or damaged corneal endothelium, the existing cells spread out so as to maintain functional integrity and sustain corneal deturgescence [3,4].

When corneal endothelial cell loss is severe, as may occur from corneal trauma, intraocular surgery, or endothelial dystrophy, the precise control of corneal hydration is lost. This results in their inability to pump fluid out of the stroma. The resultant disruption of collagen fibrils and opacification of the cornea causes loss of corneal clarity and visual acuity, and this will eventually lead to the clinical condition of bullous keratopathy [2]. Corneal transplantation with replacement of the endothelial monolayer is the only treatment option to restore a clear cornea in these circumstances [5].

Penetrating keratoplasty (PK), however, is not an ideal therapy due to many reasons such as the requirement of fresh cadaveric tissue, the inability to treat thermal and chemical burns and the adverse effect caused on eye structure, making it vulnerable to further insults and often requiring many months before vision is stabilized. Moreover PK is also not suitable for eyes with inflammation, or patients with other disease or inflammatory processes.

In addition to this there is a global shortage of transplant-grade donor corneal tissues, which greatly restricts the number of corneal transplantations performed yearly. In India alone nearly 8.4 lakh people are affected per year due to corneal disorders. Unfortunately there is a huge shortfall of availability of corneas as only about 35,000

eyes are donated each year with a requirement of 150,000 corneas on the other hand. Among these a significant number are deemed unsuitable for corneal transplantation.

In recent years, there has been tremendous interest in understanding the proliferative potential of the corneal endothelium to arrive at better solutions for the management of endothelial cell loss. The first step toward achieving this goal is to identify mechanisms responsible for the induction and maintenance of mitotic inhibition of the corneal endothelium in vivo.

The mechanisms underlying this difference in proliferative properties in vivo are poorly understood, and little is known about how the cell types affect each other's growth in vivo. However, during early embryogenesis, the three different cell types have been assumed to exert some growth regulatory influence on each other [6].

With rapid advancements in the field of endothelial keratoplasty, the selective replacement of the corneal endothelial layer is now possible, and these procedures include Descemet's stripping endothelial keratoplasty (DSEK) and Descemet's membrane endothelial keratoplasty (DMEK), the latter being a procedure whereby only the DM with attached endothelium is replaced [7-9]. Such a streamlined approach opens up new possibilities for the overall concept of a cell-tissue engineered replacement of the endothelial cell layer.

Moreover the clinical utility of procedures such as PK/DSEK is limited due to a lack of high quality donor corneas. In addition to this HCE cell lines could provide efficient models for studies of cellular specification, cellular signaling, cell replacement, in vitro reconstruction of tissue-engineered human corneal endothelium (HCE), immunology of HCE graft rejection, and molecular pathways regulating normal HCE cell homeostasis.

In order to facilitate the development of such an endeavor, a systematic procedure that enables the propagation and expansion of cultured human corneal endothelial cells (HCEC) in vitro becomes very critical. Current published isolation and cultivation methods for the establishment and propagation of HCECs vary greatly between laboratories, some with more success than others [10-13].

In theory, an ideal and effective engineering method should comprise three key steps: isolation of HCEC from the donor cornea, preservation of isolated HCEC's for a period to allow transportation, and expansion of isolated HCECs on an appropriate in vitro environment suitable for transplantation.

Regardless of donor age, corneal endothelial cells can enter and complete the cell cycle. In the presence of fetal bovine serum and fibroblast growth factor, cells from old donors can proliferate but respond more slowly and to a lesser extent than cells from young donors. However the addition of EGF to the medium stimulates cells from old donors to enter the cell cycle faster, increases the relative number of actively cycling cells, and increases the number of cells exhibiting mitotic figures. The resultant hypothesis is that it is possible to stimulate a significant proliferative response in corneal endothelial cells from old individuals. Administration of an optimal combination of stimulatory growth factors is required under conditions in which cells have been transiently released from contact inhibition [14,15].

HCEC from older donors also exhibit reduced expression of proteins that support important cellular functions such as metabolism, antioxidant protection, protein folding, and protein degradation. These differences may affect the ability to consistently obtain a sufficient number of healthy cultured HCEC for use in preparing bioengineered endothelium as an alternative method for the treatment of endothelial dysfunction.

Aim and Objectives

The aim of this study was to use denuded amniotic membrane as a scaffold for isolated human corneal endothelial cells. The objectives to be achieved were divided into amniotic membrane denudation, a standardization of isolation of corneal endothelial cells and HCEC adhesion onto the denuded amniotic membrane.

Methodology

The study was conducted during the time period of January 2012 to October 2012 after receiving approval from the Institutional ethics committee of Sri Ramachandra University. All chemicals mentioned were obtained from Sigma Aldrich or Himedia Laboratories. FBS was procured from Life technologies and human recombinant growth factors were procured from MP Biomedicals.

Amniotic membrane denudation

Human amniotic membranes (HAM) were procured from Ramayamma international eye bank, L.V Prasad eye institute, briefly stored at -80°C in Dulbecco's modified Eagle's media (DMEM) and These were then washed several times in Phosphate buffered saline (PBS) supplemented with antibiotics Penicillin/Streptomycin and Amphotericin. The Amniotic membrane was cut to appropriate sizes and placed epithelial side facing up, onto sterile 3 cm culture dishes and washed well with ample PBS containing antibiotics and allowed to dry for 3 minutes to prevent HAM from floating off in subsequent steps.

PBS containing 1.2 units/ml of dispase II was added, to cover the entire HAM which was then digested at 37°C for 60 minutes. The epithelium was scraped off using a cell scraper after which HAM was repeatedly washed with PBS again and a repeat scraping was carried out followed by ample washing. The HAM was then examined under the microscope to ensure denudation and subsequently placed onto fresh culture ware and was allowed to dry for 10 to 20 minutes at 37°C and then rehydrated with culture medium before seeding.

Standardization of isolation of corneal endothelial cells

Isolation of Descemet's membrane and endothelium: Human eyes were obtained from Sri Ramachandra eye bank, department of ophthalmology in accordance to the Helsinki declaration and the ethics committee approval. They were preserved as per the established guidelines at the Sri Ramachandra University involving proper consent and after informing the next of kin. Donor age ranged from 40 to 80 years and the interval between death and enucleation varied from 1 to 6 hrs. Corneas found unfit for surgical usage (slit lamp examination: severe corneal cloudiness, several Descemet's membrane folds, poor endothelium, donor history: death of unknown cause, active septicemia, neuralgic disease, prior intraocular or anterior segment surgery etc) were taken for the study. A total of 24 unfit human eyes were obtained for the study.

The whole globe was refrigerated at 4 degree centigrade in moist chamber storage with antibiotics following the enucleation. The corneoscleral rim excision was done under strict aseptic precautions in a laminar air flow chamber. Five percent povidone-iodine solution in a

5.0 cc sterile syringe was used to coat the entire globe followed by balanced salt solution to irrigate the eyes until all the povidone-iodine is rinsed off. The eye was removed from its sterile container using a toothed forceps and wrapped securely around the equator with the long strip of gauze. A 360° peritomy was done around the limbus and the exposed sclera carefully scraped outward with a scalpel blade (#15) to remove all remaining conjunctival tissue. An incision was made through the sclera 2 mm from the limbus using a scalpel blade (#11) and extended 360° parallel to it without perforating the choroid using corneal section scissors.

The corneoscleral rim was then placed on a Teflon block. The rim was centered in the block and a 10 mm trephine was used to just pierce through the endothelium, Descemet's membrane and posterior stroma without making a full thickness punch. A Sinsky hook was then used to ensure that the endothelium and Descemet's was detached completely (Figure 1).

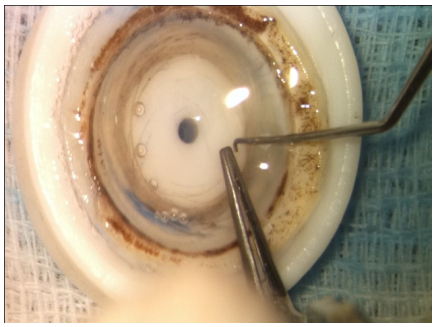

Figure 1: Peeling of corneal endothelium, Descemet's membrane complex.

Once it was confirmed that the central position of Descemet's could be stripped for 1 to 2 clock hrs a McPherson forceps was used in a fashion similar to Utrata forceps during capsulorrhexis to peel off the Descemet's membrane and endothelium. The whole process was done with BSS filling the well. The donor sample was then transferred to a test tube filled with Culture media Opti MEM media with 8% FBS, 0.08% Chondroitin Sulphate, 200 µg/ml Calcium Chloride, 50 µg/ml Bovine pituitary extract, 20 µg/ml Ascorbic acid, 0.005% human lipids, RPMI-1640 vitamin solutions, Insulin, transferrin Selinium and Antibiotics.

Protocol for HCEC isolation: The isolated Descemet's membrane (DM) and corneal endothelium (CE) were washed using ample PBS supplemented with Penicillin/Streptomycin/Amphotericin/ Voriconazole and Ciprofloxacin. The sample was then stored at 37°C with 5% CO_2 overnight in OptiMEM media with 8% FBS, 0.08% Chondroitin Sulphate, 200 µg/ml Calcium Chloride, 50 µg/ml Bovine pituitary extract, 20 µg/ml Ascorbic acid, and Antibiotics for 24 hrs. The corneas after being washed with PBS without antibiotics in a sterile petri dish were cut into smaller bits with a sterile scalpel (approximately 8 pieces). They were then digested in 3.5 cm dish or 6 well plates at 37°C and 5% CO_2 for 2 hrs with collagenase II solution at a concentration of 2 mg/ml in OptiMEM with 5% FBS. Post Digestion cell clumps were dispersed by pipetting and washed with PBS.

The sample was washed with PBS and centrifuged separately at 250 relative centrifugal forces for 10 minutes to pellet the cells. Cell pellet was resuspended in 2 ml of culture media supplemented with growth factors 5 ng/ml hEGF, 10 ng/ml huFGF and 20 ng/ml hNGF. The cells were plated onto non coated cultureware for four hours to minimize fibroblast contamination of HCEC cultures as fibroblasts adhere faster to cultureware. The non-adherent cells were collected, counted and seeded onto gelatin coated cultureware an onto denuded HAM. The culture media with growth factors was replaced every 2 days. The cells were then analyzed by microscopy to assess if they maintained their polygonal morphology.

Immunofluoresence staining of NaKATPase

72 hours after seeding the cells onto gelatin coated glass coverslips they were washed with PBS and fixed in 3.7% formaldehyde, permeabilised with 0.1% triton X 100, blocked and incubated overnight with mouse monoclonal anti alpha 1 Sodium potassium ATPase antibody (Ab7671 Abcam). Coverslips were washed well and stained with Goat anti Mouse FITC (Merck) and analyzed on a Nikon TEEclipse 2000E Inverted fluorescence microscope and images were captured using the ImagePro plus software.

Results

Amniotic membrane denudation

The standard method followed by many labs to denude the human amniotic membrane is by enzymatic removal of epithelium, using Trypsin, Dispase or a mild protease. In the present study, the procedure for standardization of amniotic membrane denudation required treatmentwith Dispase ц at 37°C for a duration of 60 min [17]. Upon microscopic examination during the denudation process, HAM showed a progressive disruption of its membrane as the enzymatic treatment duration increased. At the end of one hour mechanical scraping was carried out following which no epithelial cell remnants were seen and the underlying stromal collagen was exposed (Figures 2 and 3).

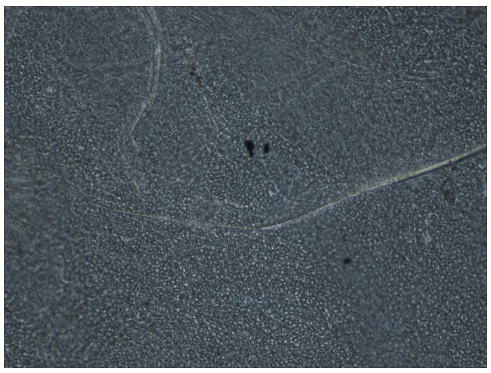

Figure 2: Human amniotic membrane (HAM) with intact epithelium (10X).

Denudation was previously attempted in the study with several other enzymatic methods based on current de-epithelisation techniques. This included treatment with 0.02% EDTA foran hour at 37°C [18]. This methodology however produced no dissociation of cells on the HAM. The usage of 1.5 ml of 0.25% trypsin solution at 37°C [19] caused a partial epithelial denudation but was found to be

too harsh as it compromised the underlying basement membrane integrity on longer exposure as well. In contrast to these enzymes Dispase showed a good denudation and most importantly, was gentle in its effect on the amniotic membrane.

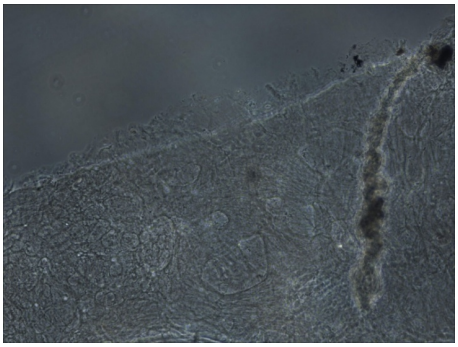

Figure 3: Denuded amniotic membrane (dAM) with underlying exposed stromal collagen after treatment with Dispase for 60 min (10X).

Isolation of HCEC

The tissue quality check of the harvested Descemet's membrane and endothelium under microscopy showed thin sheets of confluent cells with characteristic hexagonal morphology (Figure 4). The isolation of HCEC's was attempted using 2 mg/ml of collagenase-II solution (Figure 5) as per the protocol by Peh et al. [2]. At the end of 2 hours of enzymatic digestion the endothelial layer was seen to have slowly displaced of the Descemet's membrane leaving behind clear Descemet's sheets (Figure 6). These cells were seen either floating individually or in tightly packed clusters. Pre plating of these isolated cell clumps and tissue remnants on a petri dish in an attempt to separate the 2 cell masses showed that a majority of the fibroblastic cells attached readily to the surface plate (Figure 7) even in the absence of a coating media such as gelatin as opposed to the endothelial cells which did not attach (Figure 8) This method hence aided in a more fibroblast free endothelial cell isolation.

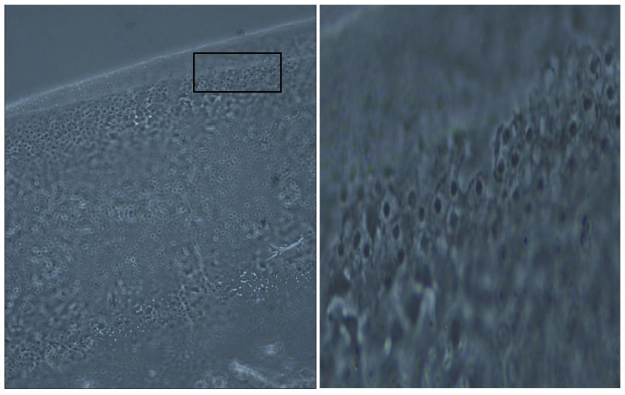

Figure 4: HCEC sheet exhibiting a typical hexagonal morphology with underlying DM (10X) as shown in the adjacent picture under higher magnification.

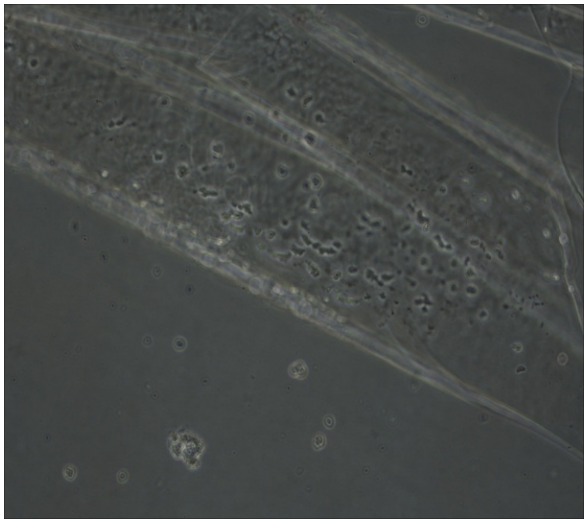

Figure 5: Partial dissolution of HCEC's from DM after collagenase II treatment and mechanical disruption by pipetting at 60 min. Few isolated endothelial cells can be seen adjacent to the sheet (10X).

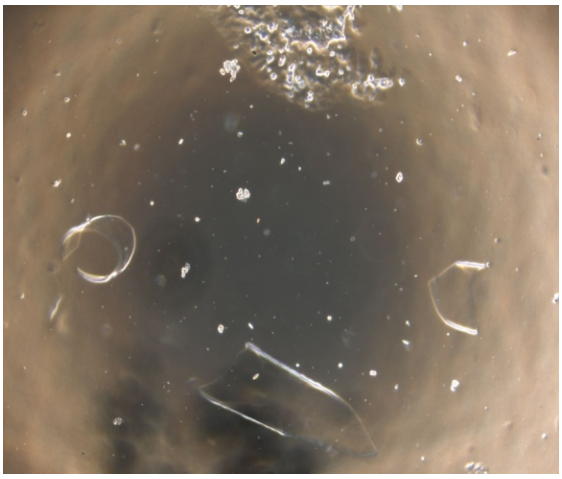

Figure 6: Clear transparent sheets of DM with floating endothelial cells seen in the periphery at 120 min (4X).

The attempt to establish a standardized protocol in order to obtain endothelial cells included a study of other enzymatic agents as well. The CE-DM strips were at first incubated in 0.02%EDTA for 60 min (based on the work of Zhu et al IOVS 2004) 11 followed by forcing the tissue and medium multiple times through the narrow opening of a pipette in order to disrupt cell junctions. This however produced only partial cell dissociation of the HCEC sheets. The usage of 0.25% trypsin 20 had a harsh effect leading to increased cell fragility and decreased viability. Partial dissolution was also obtained with dispase (based on the work of Wei Li et al. IOVS, 2007 48-2) [16] at a concentration of 10 mg/ml which had a gentler proteolytic action but required up to 3-16 hrs of digestion time.

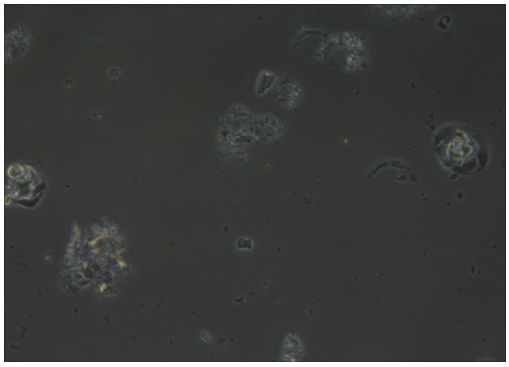

Figure 7: Pre-plating showing attached fibroblasts after 10 hrs (10X).

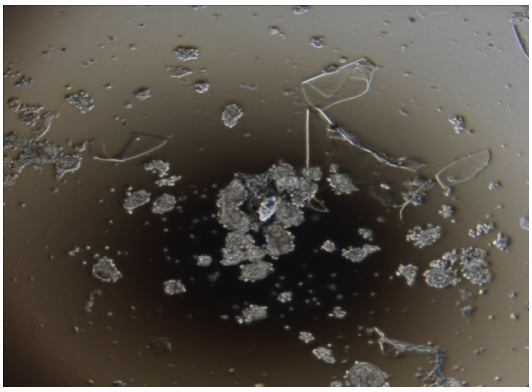

Figure 8: Non adherent endothelial cell clusters seen on pre-plating after 10 hrs (10X).

Of the various methods attempted to isolate CE and DM the peeling of endothelial sheets in a manner similar to capsulorhexsis proved to be the most effective. Another technique utilizing a microkeratome and lamellar dissector created thicker tissue samples with inclusion of posterior stroma. These required a treatment with EDTA or Dispase prior to digestion with collagenase-II in order to obtain thin sheets of CE and DM.

Immunofluorescence analysis of hcec for NaKATPase

Isolated HCEC's were seeded on gelatin coated cultureware and were examined by microscopy to evaluate their morphology. The activity of Na^+K^+/ATPase is associated to the fluid pump function critical for the proper physiological control of corneal thickness by the corneal endothelium. 72 hours after seeding, the cells were positive for Na^+K^+/ATPase which is a characteristic marker for the corneal endothelium. The majority of the cells stained positive for Na^+K^+/ ATPase (Figure 9) and also indicated that there was a minimal contamination from stromal fibroblasts.

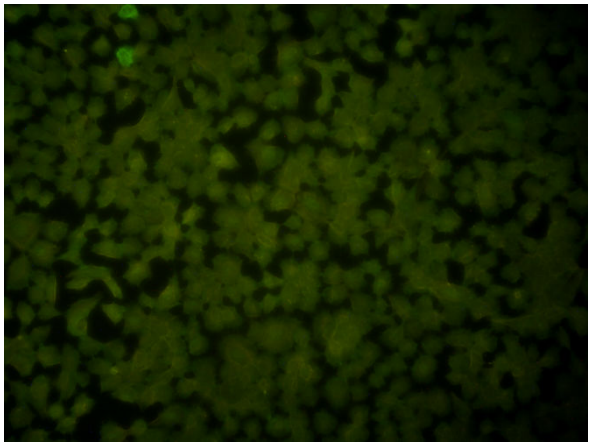

Figure 9: HCEC's on gelatin coated culture ware 72 hrs post seeding staining positive for with Na+K+ATPase - FITC (10X).

HCEC adhesion on denuded amniotic membrane

Isolated HCEC's after being seeding on denuded amniotic membrane were examined under microscopy. The cells as observed 24 hours after plating did not show any drastic morphological variation upon losing their confluence. Most of them retained a rounded morphology while few were large and irregular in shape and others friable post enzymatic dissociation. Microscopic evaluation 72 hrs and a week later showed that the isolated cells managed to scaffold onto the amniotic membrane. (Figures 10 and 11) These were seen either individually or in clusters and it was observed that they manage to retain that adhesion during subsequent media replacements carried out every 48 hrs as well.

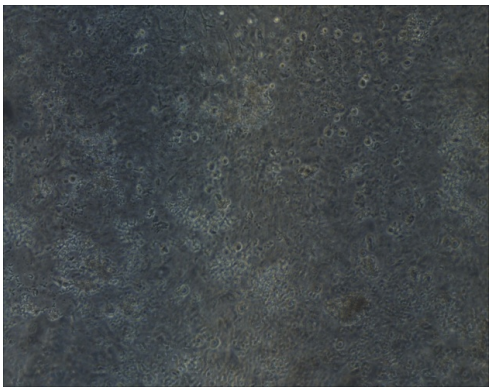

Figure 10: Individual HCEC's seen on dAM 24 hrs post isolation (10X) Individual HCEC's seen on dAM 24 hrs post isolation (10X).

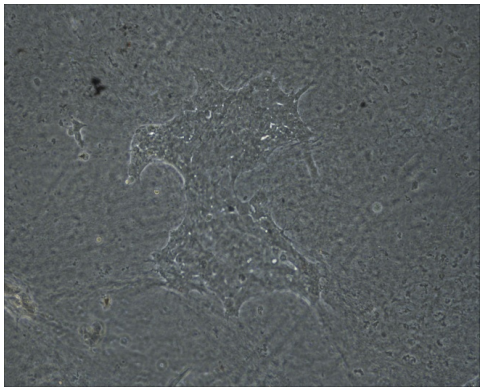

Figure 11: Adherent Individual endothelial cells and clusters on dAM after 1 week (10X).

Conclusion

Given the immense shortage of transplant grade donor corneas in the face of an ever increasing worldwide demand it has become crucial to find newer alternatives. Cell based construction strategies aimed at obtaining a viable endothelial cell populace which can replenish a dwindling cell density can be one answer.

The methodology for the research study was created with the following goals in mind. First to obtain a denuded amniotic membrane that served as a potential scaffold for corneal endothelial cells to attach.Secondly to obtain a good harvest of isolated human corneal endothelial cells and at last to seed them on the dAM.

Current studies have mentioned various methods in order to denude HAM but no clear protocol has been established. Usage of Dispase-II for enzymatic digestion yielded an exposed stromal collagen with no epithelial remnants. Past attempts utilizing several other enzymes such as EDTA, trypsin and enzyme free caused either poor dissolution or partially destroyed the underlying stromal and basement membrane integrity due to a prolonged incubation time.

Human amniotic membrane is being increasingly utilizedas substrate ocular surface reconstruction surgeries. HAM reduces ocular surface scarring and inflammation, and enhances epithelialisation. In addition, it shows limited immunogenicity and some anti-microbial properties. It has been shown that dAM in particular aids in better cell adhesion, promotes cell proliferation and as well as a more uniform cell outgrowth compared to intact AM. It was thus chosen as a scaffold for endothelial cells in this study.

Isolation of HCEC's first required stripping corneal endothelial and Descemet's membrane sheets from donor corneoscleral rims. The 24 unfit eyes utilized were obtained from the S.R.U eye bank in accordance to the Helsinki declaration and after the ethics committee approval. Donor ages varied between 40 to 80 yrs with a large majority falling into the older category (>70 yrs). Peeling of the CE-DM in a manner similar to capsulorrhexis gave sheets of ideal thickness exhibiting typical cobblestone morphology on microscopy.

The isolation was carried out with enzymatic dissolution using collagenase II at 2 mg/ml for 2 hrs followed by centrifugation. This step gave a good harvest of endothelial cells leaving behind clear, acellular Descemet's membrane. Past endeavors at using concentrations of a whole host of enzymes such as EDTA, trypsin and dispase gave a very poor isolation rate and caused cellular fragmentation. In one instance the usage of collagenase itself at a higher concentration of 5 mg/ml caused complete dissolution of not only the seeded HCEC's but the dAM as well due to residual enzymatic action. Thus proving how the effect of different enzymes at varying concentrations despite previous established studies, can have an inhibitory effect on cells.

A novel method of pre plating was utilized in order to separate endothelial cells from any fibroblastic populace present due to inclusion of posterior stroma during the stripping of CE-DM sheets.

The endothelial cells thus isolated were seeded onto dAM. Many of these cells retained a rounded morphology in contrast to some which were friable due to enzymatic proteolysis. These cells floated either individually or in clusters. Repeated microscopic examination revealed that they had adhered to the scaffold and this adherence was maintained on subsequent media replacements as well.

HCEC culture could provide an efficient model for studies of cellular specification, cellular signaling, in vitro reconstruction of tissue-engineered HCE, immunology of HCE graft rejection, and molecular pathways regulating normal HCE cell homeostasis.

References

1. Bourne WM (1998) Clinical estimation of corneal endothelial pump function. Trans Am Ophthalmol Soc 96: 229-239.
2. Peh GS, Beuerman RW, Colman A, Tan DT, Mehta JS (2011) Human corneal endothelial cell expansion for corneal endothelium transplantation: an overview. Transplantation 91: 811-819.
3. Kaufman HE, Katz JI (1977) Pathology of the corneal endothelium. Invest Ophthalmol Vis Sci 16: 265-268.
4. Edelhauser HF (2000) The resiliency of the corneal endothelium to refractive and intraocular surgery. Cornea 19: 263-273.
5. Engelmann K, Bednarz J, Valtink M (2004) Prospects for endothelial transplantation. Exp Eye Res 78: 573-578.
6. Hyldahl L (1984) Primary cell cultures from human embryonic corneas. J Cell Sci 66: 343-351.
7. Tan DT, Anshu A, Mehta JS (2009) Paradigm shifts in corneal transplantation. Ann Acad Med Singapore 38: 332-338.
8. Rose L, Kelliher C, Jun AS (2009) Endothelial keratoplasty: historical perspectives, current techniques, future directions. Can J Ophthalmol 44: 401-405.
9. Melles GR, Ong TS, Ververs B, van der Wees J (2006) Descemet membrane endothelial keratoplasty (DMEK). Cornea 25: 987-990.
10. Ishino Y, Sano Y, Nakamura T, Connon CJ, Rigby H, et al. (2004) Amniotic membrane as a carrier for cultivated human corneal endothelial cell transplantation. Invest Ophthalmol Vis Sci 45: 800-806.
11. Zhu C, Joyce NC (2004) Proliferative response of corneal endothelial cells from young and older donors. Invest Ophthalmol Vis Sci 45: 1743-1751.
12. Li W, Sabater AL, Chen YT, Hayashida Y, Chen SY, et al. (2007) A novel method of isolation, preservation, and expansion of human corneal endothelial cells. Invest Ophthalmol Vis Sci 48: 614-620.
13. Engelmann K, Friedl P (1989) Optimization of culture conditions for human corneal endothelial cells. In Vitro Cell Dev Biol 25:1065-1072.
14. Lopez JG, Chew SJ, Thompson HW, Malter JS, Insler MS, et al. (1992) EGF cell surface receptor quantitation on ocular cells by an immunocytochemical flow cytometry technique. Invest Ophthalmol Vis Sci 33: 2053-2062.
15. Senoo T, Joyce NC (2000) Cell cycle kinetics in corneal endothelium from old and young donors. Invest Ophthalmol Vis Sci 41: 660-667.

16. Li W, Sabater AL, Chen YT, Hayashida Y, Chen SY, et al. (2007) A novel method of isolation, preservation, and expansion of human corneal endothelial cells. Invest Ophthalmol Vis Sci 48: 614-620.
17. Lim LS, Riau A, Poh R, Tan DT, Beuerman RW, et al. (2009) Effect of dispase denudation on amniotic membrane. Mol Vis 15: 1962-1970.
18. Nakamura T, Endo K, Cooper LJ, et al. (2003) The successful culture and autologous transplantation of rabbit oral mucosal epithelial cells on amniotic membrane. Invest Ophthalmol Vis Sci 44: 106-116.
19. Sangwan VS, Vemuganti GK, Singh S, Balasubramanian D, et al. (2003) Successful reconstruction of damaged ocular outer surface in humans using limbal and conjunctival stem cell culture methods. Biosci Rep 23: 169-174.
20. Yue BY, Sugar J, Gilboy JE, Elvart JL (1989) Growth of human corneal endothelial cells in culture. Invest Ophthalmol Vis Sci 30: 248-253.