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***HUMAN CORNEAL ENDOTHELIAL CELL CULTURE AND  
CORNEAL TRANSPLANTATION***

**Coordinatore:** Ch.mo Prof. Stefano Piccolo

**Supervisore:** Ch.mo Prof. Giorgio Palù

**Dottorando :** Dott. Mohit Parekh

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## **ABBREVIATIONS**

Frequently used abbreviations –

PK	Penetrating Keratoplasty
EK	Endothelial Keratoplasty
DMEK	Descemet Membrane Endothelial Keratoplasty
DSAEK	Descemet Stripping Automated Endothelial Keratoplasty
ALK	Anterior Lamellar Keratoplasty
HCECs	Human Corneal Endothelial Cells
LK	Lamellar Keratoplasty
DM	Descemet's Membrane
AFM	Atomic Force Microscope
TCM	Tissue Culture Medium
TM	Transport Medium
OC	Organ Culture
FSS	Fish Scale Scaffolds
rHSA	Recombinant Human Serum Albumin
FCS	Foetal Calf Serum
GMP	Good Manufacturing Practices
FBOV	Fondazione Banca degli Occhi del Veneto Onlus
EEBA	European Eye Bank Association
EBAA	Eye Bank Association of America
PBS	Phosphate Buffered Saline
TBPC	Trypan Blue Positive Cells
TB	Trypan Blue
MEM	Minimum Essential Medium
ECL	Endothelial Cell Loss

ECD	Endothelial Cell Density
BSE	Bovine Spongiform Encephalopathy
EDTA	Ethylenediaminetetraacetic acid
CECs	Corneal Endothelial Cells
HCEPs	Human Corneal Endothelial Progenitor Cells
CEM	Corneal Endothelial Medium
ESC-CM	Embryonic Stem Cell-Conditioned Medium
ZO-1	Zonula Occludens -1
MSCs	Mesenchymal Stem Cells
HAF	Human Amniotic Fluid
ROCK	Rho-associated protein kinase
iPSCs	Induced Pluripotent Stem Cells
PFA	Paraformaldehyde
RT	Room Temperature
IOL	Intra Ocular Lens
PAS	Periodic acid–Schiff
DAPI	4,6-diamidino-2-phenylindole
DALK	Deep Anterior Lamellar Keratoplasty

## **RIASSUNTO**

La cornea è quel tessuto trasparente che riveste la superficie anteriore dell'occhio, e che consente di avere una visione ottimale e chiara. La trasparenza di questo tessuto è fondamentale e non può essere compromessa. La cornea umana è costituita da più strati, tra cui lo strato posteriore o "endotelio" è responsabile della trasparenza della cornea. L'endotelio è un monostrato di cellule che permettono agli ioni ed ai soluti di essere trasportati dall'umor acqueo alla cornea e viceversa, e che a sua volta mantiene la trasparenza della cornea conservando l'omeostasi tra la cornea anteriore e posteriore. L'endotelio non possiede capacità rigenerative. Attualmente, l'unico metodo di trattamento è la sostituzione dell'endotelio danneggiato con l'endotelio di un donatore sano. La cheratoplastica perforante, che prevede trapianti di cornea a tutto spessore, rappresentava l'unica soluzione terapeutica fino ad un decennio fa. Tuttavia, con i nuovi progressi nel campo dei trapianti di cornea, sono state identificate specifiche tecniche chirurgiche, come DMEK e DSAEK, che sostituiscono solo una parte (o uno strato) della cornea. Sono i risultati ottenuti, in termini di riabilitazione visiva, si sono rivelati vantaggiosi grazie all'utilizzo di queste procedure chirurgiche specifiche. Tuttavia, la DMEK è più impegnativa rispetto alla DSAEK in quanto non è ancora completamente standardizzata. La DMEK ha diversi vantaggi in termini di tasso di riabilitazione e risultati visivi post-operatori e quindi è necessario standardizzare questa tecnica per una maggiore diffusione di tali interventi e anche considerando che questo è l'unico trattamento possibile per la cura di pazienti affetti da disfunzioni endoteliali. Sebbene il trapianto di cornea sia in fase avanzata, a causa di una quantità limitata di cornee da donatori ai fini di trapianto, approcci alternativi come la coltura di endotelio corneale in vitro svolgono un ruolo importante. La coltura di endotelio non è l'unico problema nel trapianto di endotelio (EK) dal momento che trapiantare un innesto di 20 micron di spessore all'interno dell'occhio destinatario rappresenta una sfida ulteriore. Inoltre, la disponibilità dei donatori per la coltura di endotelio corneale è inferiore, rendendo questa strategia ulteriormente più

complicata. La tesi è quindi strutturata in modo da mettere in evidenza due questioni molto importanti nell'attuale scenario della cheratoplastica endoteliale, 1) trapianto di cornea posteriore o EK, che è l'attuale metodo di trattamento per la cheratoplastica endoteliale e 2) coltura delle cellule endoteliali della cornea umana, che rappresenta il futuro della cheratoplastica endoteliale.

Il Capitolo 1 è un'introduzione sul mondo dell' Eye Banking, sulle sue caratteristiche attuali, sullo sviluppo nel mondo moderno e sul supporto per i chirurghi, non solo in termini di nuove tecniche, ma anche di dispositivi per interventi selettivi. Si evidenzia anche la conservazione dei tessuti corneali, che è un elemento importante nel campo dell'Eye Banking. Le banche degli occhi svolgono un ruolo significativo nel settore dei trapianti di cornea, dal momento che raccolgono le cornee umane e le analizzano per il successivo trapianto. Le cornee non idonee per il trapianto possono essere utilizzate per la ricerca e quindi lo sviluppo dell'Eye Banking e la ricerca possono influenzare il campo del trapianto di cornea. Il Capitolo 2 introduce l'argomento delle colture cellulari corneali e le tecniche attuali che sono utilizzate per la coltura ed il trapianto di cellule coltivate.

Per capire il motivo e l'esigenza dell'ingegnerizzazione dei tessuti, è importante studiare la cornea umana, la sua matrice extracellulare ed il suo comportamento in diversi mezzi di coltura. Il comportamento biomeccanico di un tessuto sottile (DM) in condizioni diverse rappresenta una parte rilevante di questo studio per la futura ingegnerizzazione, che viene descritta nel Capitolo 3. È inoltre importante standardizzare il trattamento attualmente disponibile allo scopo di ridurre in futuro l'onere di pazienti con endotelio compromesso ed evitare danni o sprechi di tessuto, che attualmente avvengono nelle sale chirurgiche, fornendo tessuti standardizzati in terreni di conservazione validati, come descritto nel Capitolo 4. La DMEK è considerata il futuro della cheratoplastica endoteliale, dal momento che presenta vantaggi quali la velocità dei tempi di riabilitazione ed i risultati visivi. Il Capitolo 5 mette in evidenza l'importanza della nuova tecnica che consiste

nell'arrotolare il tessuto DMEK per consentire un facile inserimento per poi dispiegarlo nell'occhio ricevente, rispetto alla tecnica attualmente utilizzata con endotelio arrotolato in senso opposto. Attualmente, i tessuti DMEK sono o preparati in sala operatoria o allestiti in Banca degli Occhi e spediti ai chirurghi. Tuttavia, non vi è alcuna procedura standardizzata che possa contribuire ad ottenere un lembo endoteliale validato prima dell'intervento e fornire un innesto ready-to-use ai chirurghi. Il Capitolo 6 descrive una nuova tecnica di pre-caricamento di un lembo endoteliale in una cartuccia IOL disponibile in commercio che può essere utilizzato come dispositivo di conservazione, trasporto e trapianto. Questa tecnica consentirà di ridurre ulteriormente gli sprechi nei trapianti e fornirà ai chirurghi un innesto pre-convalidato, riducendo ulteriormente il tempo complessivo in sala operatoria ed i relativi costi. Quindi nella prima fase della tesi, sono stati analizzati i diversi approcci per standardizzare la tecnica DMEK.

Le HCECs sono attualmente coltivate usando cornee di donatori giovani. Ci sono due aspetti importanti, in primo luogo la disponibilità di tessuti di donatori giovani è minore rispetto a quella di donatori anziani, ed in secondo luogo non vi è, ad oggi, alcun metodo standardizzato di coltura delle HCECs. Pertanto, per ridurre la domanda di tessuti a livello mondiale, vi è una forte necessità di coltivare le HCECs derivanti da cornee di donatori anziani, che sono meno proliferative e meno resistenti in natura, ma per le quali vi è una elevata disponibilità della fonte donatrice. Il Capitolo 7 descrive lo studio sull'isolamento delle HCECs e la successiva coltura di tali cellule ottenute da cornee di donatori anziani. Una volta stabilito il protocollo, è stato eseguito uno studio completo con un alto campionamento, per dimostrare la coerenza di questa tecnica, come evidenziato nel Capitolo 8. Nel frattempo si è anche osservato che le cellule da donatori anziani possono essere coltivate utilizzando l'inibitore ROCK in combinazione con acido ialuronico (HA). HA induce una forza meccanica alle cellule per far sì che siano saldamente attaccate alla base e consentire così una maggiore proliferazione, come descritto nel Capitolo 9. La

seconda parte della tesi indaga quindi la tecnica di coltura delle HCECs da cornee di donatori anziani. Tuttavia, una volta che le cellule sono coltivate, un'altra sfida è trapiantarle nella camera anteriore dell'occhio. Ciò può essere eseguito utilizzando due strategie: la prima è quella di ad impiantare le cellule in forma di sospensione nella camera anteriore, tecnica che è già stata proposta, ma che non ha ancora fornito un'evidenza clinica; la seconda è quella di sviluppare un substrato per il trasporto delle cellule coltivate. Nel Capitolo 10, si identifica la colla di pesce (FSS) come una grande fonte di collagene e quindi come un potenziale scaffold da utilizzare per la cultura HCECs e successivo trapianto. E' inoltre importante capire le norme che regolano gli studi scientifici ed il loro uso nelle applicazioni cliniche. Pertanto, nel Capitolo 11, viene descritta l'identificazione dell' rHSA come sostituto dell' FCS per la conservazione di cornee umane. Questo contribuirà anche a creare un terreno di coltura sintetico che potrebbe essere utilizzato per la cultura HCECs in condizioni GMP in futuro.

In conclusione, si è osservato che il pre-caricamento di tessuti con endotelio rivolto verso l'interno e conservati in un terreno con destrano, potrebbe rappresentare una possibile soluzione per fornire un lembo per DMEK validato e standardizzato per il trattamento delle disfunzioni endoteliali. Le banche degli occhi svolgono un ruolo importante nello sviluppo di queste tecniche chirurgiche e relativi dispositivi, che potranno cambiare le modalità del trapianto di cornea in futuro. Una tecnica alternativa come la coltura di HCECs ha in sé il potenziale per il trattamento di disturbi endoteliali e substrati come FSS potrebbero essere utilizzati per la coltura ed il trapianto di queste cellule. Tuttavia, l'efficacia di queste cellule potrà essere validata solo dopo uno studio clinico. Considerando le questioni regolatorie, il terreno sintetico potrebbe aiutare le banche degli occhi sia per la conservazione delle cornee e dei nuovi prodotti come DMEK pre-caricati sia, in futuro, per le colture.

## **SUMMARY**

Cornea is the front transparent window of the eye which is responsible for optimal and clear vision. Transparency of this tissue is highly inevitable and cannot be compromised. Human cornea is made up of multiple layers out of which the posterior layer 'endothelium' is responsible for the transparency of the cornea. Endothelium is a monolayer of cells that allow the ions and solutes to transport from aqueous humour to the cornea and back which in turn maintains the transparency of the cornea by preserving the homeostasis between the anterior and posterior cornea. Earlier, it was observed that the endothelium had non regenerating capability however; recent studies have shown that these cells could be proliferated *in vitro*. Currently, the only method of treatment is the replacement of the diseased endothelium with the healthy donor endothelium. Penetrating keratoplasty which transplants a full thickness cornea was the only solution a decade ago. However, with the new advancements in the field of corneal transplants, specific surgical techniques like DMEK and DSAEK which replace only a part of the cornea have been identified. DSAEK replaces a part of the stroma along with the Descemet's membrane and endothelium whereas DMEK only replaces the Descemet's membrane and the endothelium and does not involve stroma. The results in terms of visual rehabilitation and outcomes have been found to be advantageous in these specific surgical procedures. However, DMEK is more challenging than DSAEK as DMEK is not yet a widespread technique, associated with steep learning curves and difficult donor tissue preparation. Despite DMEK is a challenging procedure it is becoming more popular because of the significant advantages in term of faster visual recovery, less postoperative astigmatism and reduced risk of transplant rejection, as compared to the other EK procedures. DMEK has several advantages in terms of rehabilitation rate and post-operative visual outcomes and therefore it is necessary to further refine this technique for a higher uptake of such surgeries and also considering that this is the only possible treatment for treating the patients suffering from endothelial dysfunctions. Although the corneal transplantation is

well advanced, due to a limited supply of donor corneas for the transplantation purposes, alternative approaches like culturing corneal endothelium *in vitro* play an important role. Culturing the endothelium is not the only problem in EK but transplanting a 20 micron thick graft inside the recipient eye is another challenge. Moreover, the donor availability for culturing the corneal endothelium is less, making this strategy further more complicated. The thesis is therefore structured to highlight two significantly important issues in current scenario of endothelial keratoplasty, 1) posterior corneal transplantation or EK which is the on-going method of treatment for EK and 2) Human corneal endothelial cell culture which is the future of EK.

Chapter 1 is an introduction to the world of eye banking, its current nature and development in the modern world and as a support to the surgeons not only in terms of new techniques but also devices for selective surgeries. It also highlights the preservation of the corneal tissues which is an important element in the field of eye banking. Eye banks play a significant role in the field of corneal transplants as they collect the human corneas and process them for transplantation. The corneas that are rejected for transplantation can be used for research and therefore development of eye banking and its research can change the field of corneal transplantation. Chapter 2 introduces the field of corneal cell culture and current techniques that are followed for culturing and possible transplantation of the cultured cells.

To understand the reason and requirement of tissue engineering, it is important to study the human cornea, its extracellular matrix and its behaviour in different media. The biomechanical behaviour of the thin tissue i.e. the DM in different conditions becomes a relevant part of this study for future engineering which is studied in chapter 3. It is also important to standardize the currently available treatment options to reduce the burden of endothelial compromised patients in the future and avoid damages or tissue wastage that is currently occurring in the surgical theatres by providing standardized tissues in validated

preservation medium which is studied in [chapter 4](#). DMEK promises to become a more popular technique for the replacement of unhealthy corneal endothelium as it shows advantages like early rehabilitation rate and visual outcomes. [Chapter 5](#) highlights the importance of new technique in rolling the DMEK tissue for easy insertion and unfolding in the recipient eye compared to the currently used technique with endothelium rolled in opposite direction. Presently, the DMEK tissues are either prepared in the surgical theatre or are stripped in the eye bank and shipped to the surgeons. However, there is no standardized procedure that could help validate a graft before surgery and provide a ready-to-use graft to the surgeons. [Chapter 6](#) describes about a new technique of pre-loading a graft in a commercially available IOL cartridge which can be used as a preservation, transportation and transplantation device. This technique will further reduce graft wastage and will provide the surgeons a pre-validated graft further reducing the overall time in the surgical theatre and related costs. Thus different approaches for standardizing the DMEK technique were studied in the first phase of the thesis.

HCECs are currently being cultured using young donor corneas. There are two major issues, firstly, the availability of the young donor corneas is less compared to the old donor corneas and secondly, there is no standard method of culturing the HCECs obtained so far. Therefore, to reduce the global tissue demand, there is a strong need to culture the HCECs from the old donor corneas which are less proliferative and less robust in nature but with high availability of the donor source. [Chapter 7](#) is a study on isolation of HCECs and further culture of these cells from old donor corneas. Once the protocol was obtained, a full length study was performed with high sample size to prove the consistency of this technique which is highlighted in [chapter 8](#). Meanwhile it was also noted that cells from old donors can be cultured using ROCK inhibitor in combination with Hyaluronic Acid (HA). HA induces mechanical force to the cells attaching them forcefully on the base and allows

a higher proliferation of old donor cells which was studied in chapter 9. The second part of the thesis therefore investigates the culturing technique of HCECs from old donor corneas. However, once the cells are cultured, another challenge is to transplant them in the anterior chamber of the eye. This can be performed using two strategies, first, to implant the cells as suspension in the anterior chamber which is already been proposed, but the clinical evidence is still not confirmed yet, and second, to develop a carrier to transport the cultured cells. In chapter 10, we identified fish scales as a great source of collagen and therefore have investigated it as a potential scaffold to be used for HCECs culture and transplant in the future. It is also important to understand the regulations that govern the scientific studies and its use for clinical applications. Therefore, we also identified rHSA as a source to replace FCS for preserving human corneas in chapter 11. This will also help to create a synthetic media that could be used for GMP purposes for HCECs culture in the future.

In conclusion, it was observed that pre-loading the tissues with endothelium-flapped inwards and preserved in dextran based medium could be a potential solution for providing a validated and standardized DMEK graft for the treatment of current endothelial dysfunction. Eye banks play a major role in the development of these surgical techniques and related devices which will change the face of corneal transplantation in the future. Alternatives like HCECs culture has a potential for the treatment of endothelial disorders and carriers like FSS could be used for culturing and transplanting these cells. However, the efficacy of these cells will only be validated after the clinical study. Considering the regulatory issues, synthetic medium would help both, the eye banks for preserving the corneas and its new products like pre-loaded DMEK and for cell culture in the future.

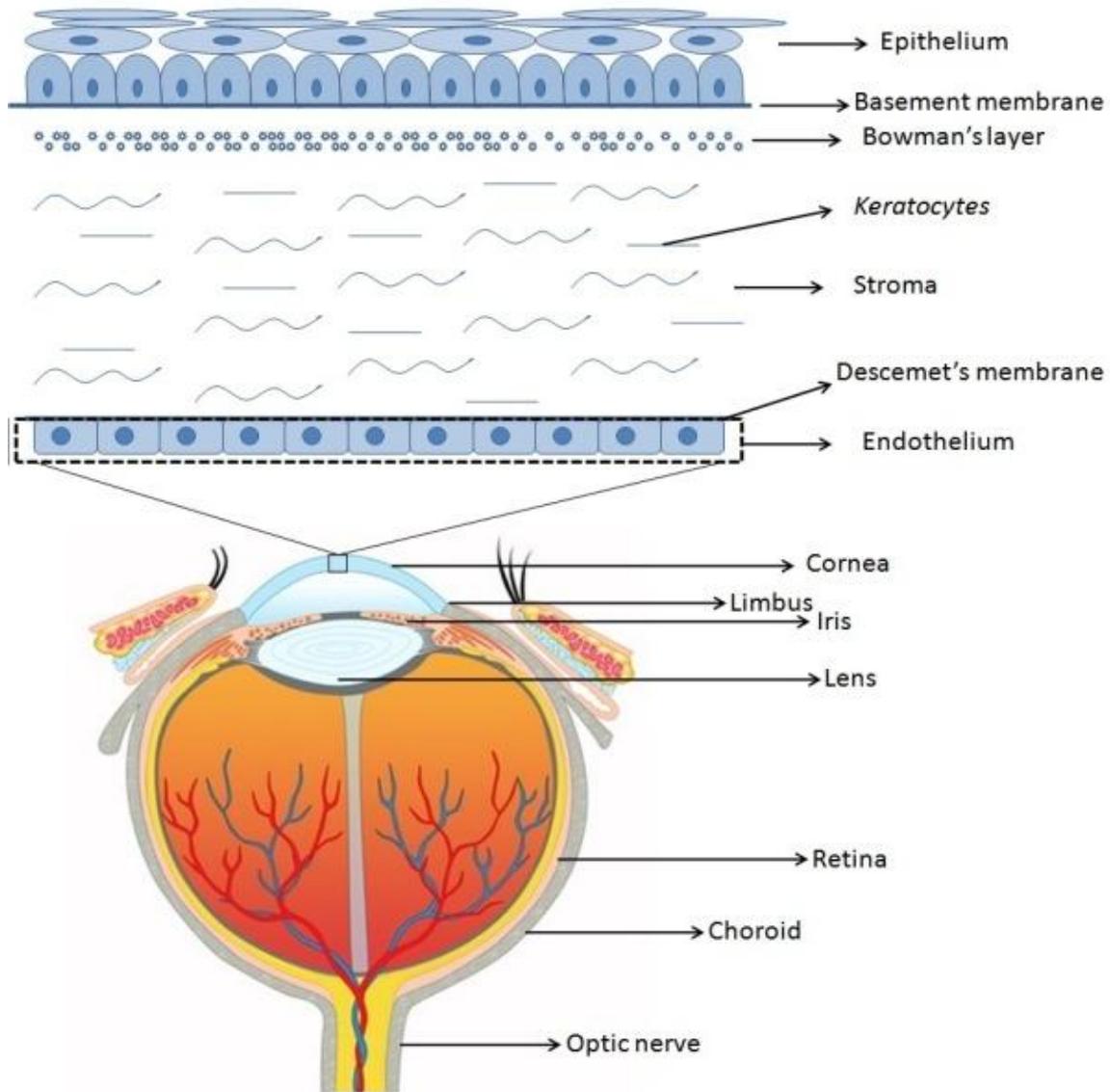
## **CORNEA**

### **BACKGROUND AND INTRODUCTION**

Human eye is a fascinating organ that reacts to light and provides vision to an individual. Its systematic organization allows the light to pass from the surrounding through cornea, lens, vitreous, retina, choroid, optic nerve and brain. This pathway is responsible for clear vision. Damage to either component may reduce vision or acquire blindness. Human cornea is the transparent front part of the eye that covers the anterior chamber. As cornea is directly exposed to the external environment it also acts as a primary protection against external living or non-living elements and does not allow any entry of foreign particles in the eye globe. It refracts the light from the surrounding to the lens and retina to hit the optic nerve. The cornea is supposed to account for approximately two-thirds of the eye's total optical power. The refractive power of the cornea is around 43 dioptres.

Transparency of the cornea is of primary importance. Human cornea is an avascular tissue (no blood vessels) which helps in reducing any immune rejection of the graft. It receives the oxygen directly through the air by dissolving in tears and diffusing it further. It receives nutrients from two parts, a) diffusion from the tear fluid through the outer surface and b) aqueous humour from the inside. Neutrophins supply nutrients via nerve fibres that innervate it. A normal human cornea has a diameter of around 11.5 mm with thickness of around 0.5-0.6 mm in the center and 0.6-0.8 mm in its periphery. The thickness may increase if the cornea is swollen. Transparency, avascularity, presence of immature resident immune cells, and immunologic privilege makes the cornea a very important and interesting tissue to study. The cornea borders with the sclera by the corneal limbus.

Human cornea has a highly organized structure to maintain the transparency of the tissue and is well determined by its functional layers as shown in figure 1.



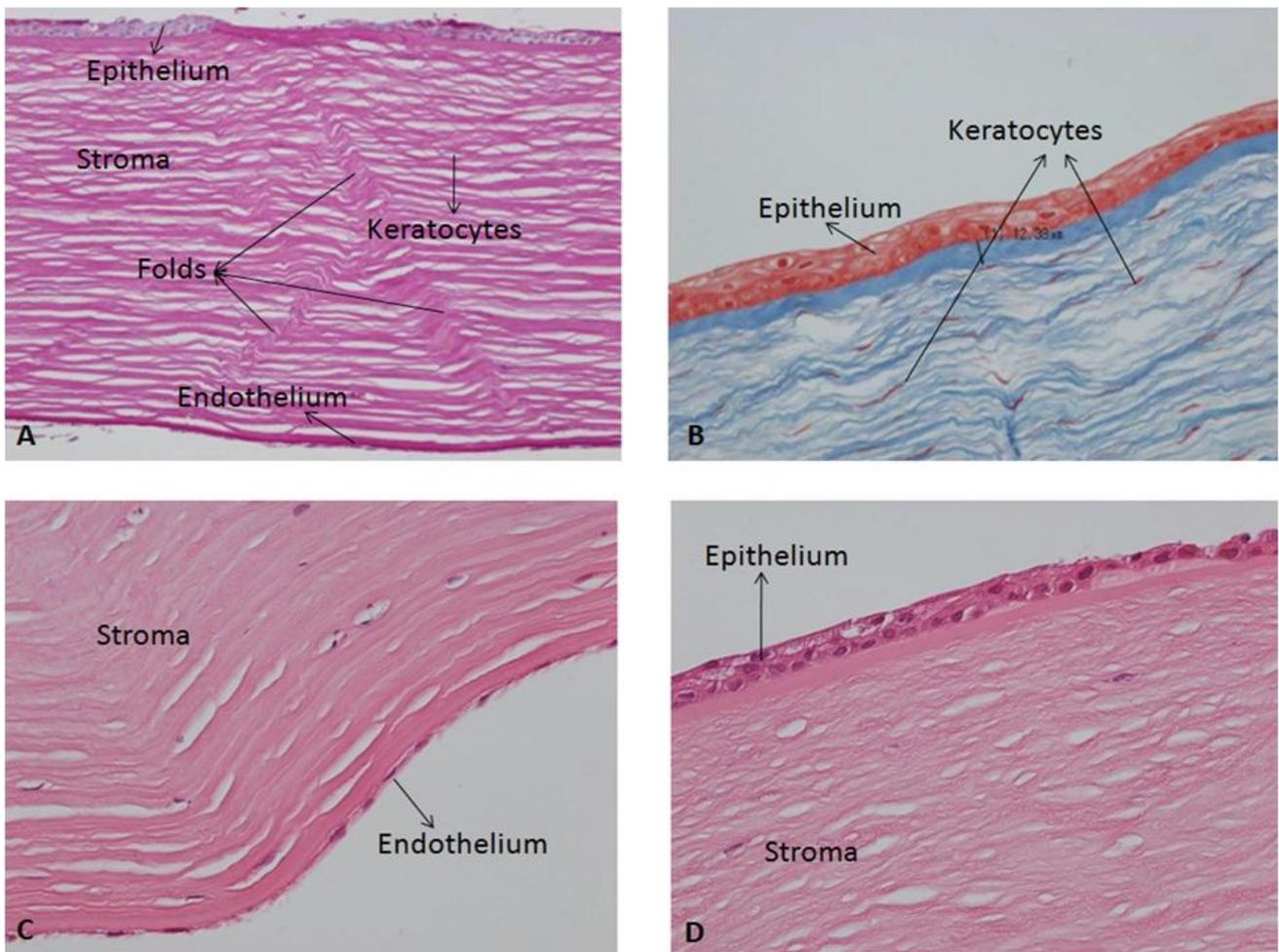
**Figure 1:** Anatomy of human eye globe showing different parts of the eye and schematic representation and structure of human cornea showing specific layers of the tissue.

The layers include -

- a) *Corneal epithelium*: it is a multicellular epithelial tissue layer (non-keratinized stratified squamous epithelium). The epithelium has a regenerative capability and it can renew itself with the help of limbal stem cells which reside in the Palisades of Vogt. Damage or edema of the epithelium reduces the smoothness of the air/tear-film interface which is the most important part of the refractive power hence minimizing the visual acuity.

- b) *Bowman's layer*: this is the basal membrane of the corneal epithelium which protects the stroma.
- c) *Stroma*: it is a thick and transparent layer which is arranged with collagen fibres along with sparsely distributed keratocytes. Keratocytes are responsible for the general repair and maintenance. This middle layer of the cornea consists of up to 90% of the total corneal thickness. The arrangements of the collagen fibrils in the stroma are supposed to be responsible for the overall transparency of the human cornea. The collagen fibrils in the stroma are also responsible for the polarization patterns of the cornea. Just anterior to Descemet's membrane, a very thin and strong layer, the Dua's layer, 15  $\mu\text{m}$  thick has been proposed recently.
- d) *Descemet's membrane*: this membrane is a thin acellular layer that serves as the basement membrane of the corneal endothelium. This layer is composed mainly of collagen type IV fibrils, less rigid than collagen type I fibrils, and is around 5-20  $\mu\text{m}$  thick, depending on the age of an individual. *Corneal endothelium*: simple squamous or cuboidal cells. These cells form a monolayer, approx 5  $\mu\text{m}$  thick that is rich in mitochondria. These cells are responsible for regulating fluid and solute transport between the aqueous and corneal stromal compartments. The corneal endothelium is flushed continuously by aqueous humor, not by blood or lymph, and has a very different origin, function and appearance from vascular endothelia. Unlike the corneal epithelium, the cells of the endothelium do not regenerate (this is still a hypothesis). Instead, they stretch out to compensate for dead cells which reduce the overall cell density of the endothelium further affecting the fluid regulation. If the endothelium does not maintain a proper fluid balance, stromal swelling due to excess fluids and subsequent loss of transparency will occur and this may cause corneal edema and interference with the transparency of the cornea and thus impairing vision.

Histological images of human cornea are shown in figure 2.



**Figure 2:** Histological analysis of human corneal tissue. A) All the layers can be seen precisely with stromal folds, b) epithelium, stroma and keratocytes, c) stroma and endothelium and d) epithelium and stroma.

#### Human corneal endothelium -

The posterior layer of the cornea is the endothelium which is a physiologically important monolayer of cells. HCECs play a major role in maintaining the corneal transparency, thickness and hydration [1,2].

The cornea takes in fluid from the aqueous humour and the small blood vessels of the limbus, but the pump function in the HCECs eject the fluid immediately once entered. The

pump may fail when the energy is deficient, or may work too slowly to compensate. This further causes swelling of the cornea which could arise at death. A dead eye can although be placed in a warm chamber in combination with sugar and glycogen that can keep the cornea transparent for at least 24 hours. The endothelium controls this pumping action, and as discussed above, damage thereof is more serious, and may increase opacity and swelling. When a cornea is needed for transplant from an eye bank, the best procedure is to remove the cornea from the eyeball, preventing the cornea from absorbing the aqueous humour.

It is noted that the total number of donated corneas in 2015 distributed by US eye banks was 79,306 [Eye Bank Association of America (EBAA) statistical report 2015]. Of these, 39,554 were destined for full thickness PK and 30,710 for EK (14,472 for Fuchs' Dystrophy and 3,208 other causes of endothelial dysfunction). This signifies that nearly 40% of corneal transplantations were carried out for endothelial dysfunction. Endothelial keratoplasty has been the most commonly performed keratoplasty procedure in the United States in the last four years and continues to increase. This means that endothelial diseases are the main indication for corneal transplantation in the United States. Although EK has a high success rate in terms of visual rehabilitation and post-op visual outcomes, shortage of donor corneas still remain a global challenge [3].

Due to the non-regenerative capability of the HCECs, its preservation and maintenance becomes a critical issue. It is noted that the HCECs have a low tendency to proliferate *in vivo*, as they are arrested in the G1 phase, and therefore these cells spread out to replace the deceased cells, thus maintaining the functional integrity and corneal deturgescence [4,5].

Accidental or surgical trauma can result into acute corneal endothelial dysfunction which results in the inability of the pumping function of the endothelium which is necessary for the drainage of excess fluids. This causes critical anomalies like stromal edema, loss of transparency and most importantly the visual acuity which usually leads to the clinical condition of bullous keratopathy. The corneal endothelium is assumed to be originated from the neural crest cells [6]. It has already been described that the neural crest cells migrate and differentiate during the developmental phases of the cornea. Periocular mesenchymal cells are originated from the neural crest cells and are responsible for the development of the corneal epithelium and the synthesis of the primary stroma. These cells further migrate to the periphery of the optic cup eventually moving between the lens and the corneal epithelium for the development of the trabecular meshwork and the corneal endothelium. Further, in the second phase of the development, the neural crest cells occupy the primary stroma and differentiate into corneal keratocytes [7].

HCECs are metabolically active and continuously functions as a fluid pump for the movement of fluid from the stroma and the anterior chamber. Thus, as described earlier, the endothelial layer plays a significant role, but lacks the capacity to regenerate and hence its viability should be maintained. Currently, surgical replacement of the diseased endothelium by a healthy donor tissue using corneal transplantation is the only solution to restore vision caused due to endothelial dysfunction.

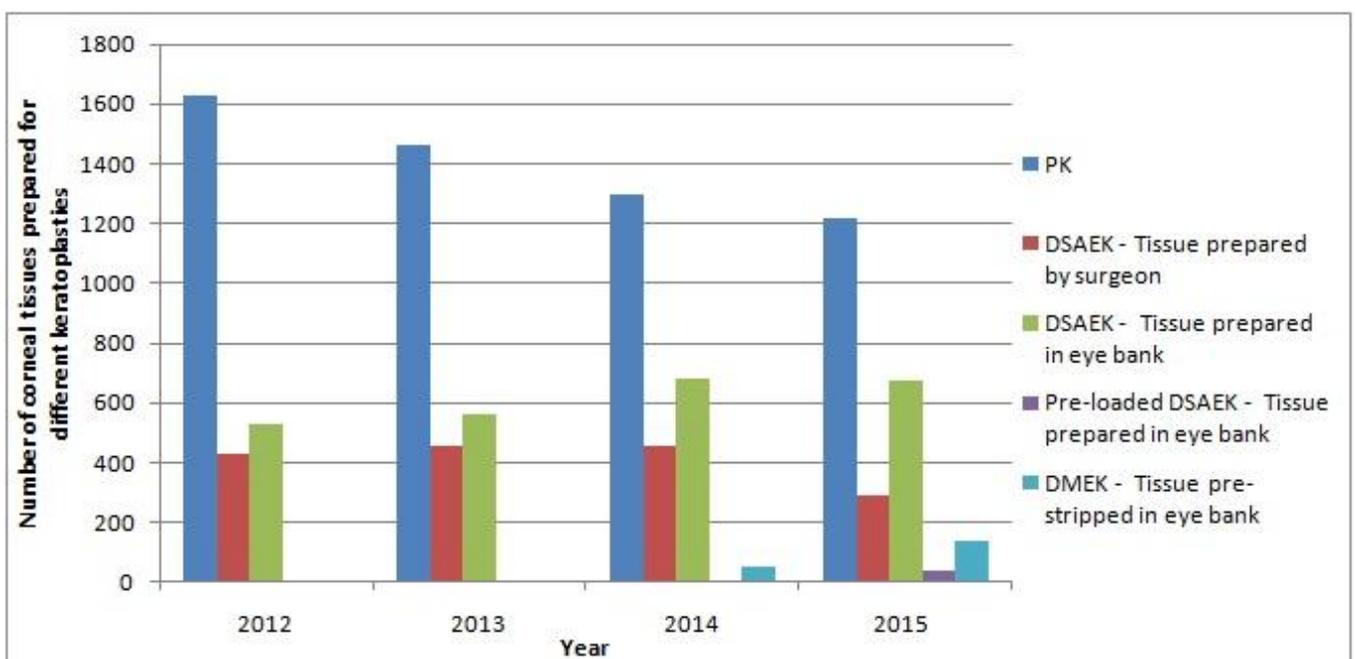
The function of the endothelium is compromised if the cell density falls below a critical threshold of 500 cells/mm<sup>2</sup>. Recently, surgical procedures such as DSAEK have focused on the transplantation of corneal endothelium, yielding better clinical results, yet the shortage in donor tissue remains a main issue. Various other methods have been

attempted to treat endothelial dysfunctions, and in recent years DMEK have also been employed [8].

However, the availability and quality of corneas are main concerns; more than 40% of the donated corneas to our institute, the Veneto Eye Bank Foundation (FBOV – Venice, Italy) are rendered unsuitable for transplantation. Additionally, more than 60% of the donors are over 60 years and due to the low endothelial cell density that most of the grafts from these age groups have, most of the grafts become unsuitable for transplantation in the preliminary phase of evaluation. Isolation, culturing of the cells and tissue engineering, thus, can play a vital role and serve as an alternative in the treatment of endothelial dysfunction. It has been observed that the HCECs at the periphery possess a higher density of precursors which have a strong proliferation capability, as compared to the central endothelium and can be differentiated into neural or mesenchymal cells. A mass production of HCECs can be achieved through young donors, which could ultimately reduce the waiting lists of the patients requiring endothelial keratoplasty. The current treatment available for the restoration of the corneal endothelium is keratoplasty, which represent more than 22% of the corneal tissues supplied by FBOV in 2012 but has been increasing ever since.

DMEK has evolved over the years since it was first introduced by Dr. Melles in 2006. Various preparation and implantation techniques have been addressed so far. However, there has not been a standard technique for DMEK. Eye banks are confronted with new requirements from corneal surgeons as DMEK is gaining popularity in terms of early rehabilitation and better visual outcome. DMEK validation and standardization both in terms of preparation and implantation is thus becoming essential. At FBOV, we have seen a gradual increase in the demand of tissues for specific purposes like pre-cut DSAEK

since 2012. FBOV has also received requests for pre-cut DMEK tissues and we have seen an upcoming interest in these tissues since 2014. In 2015, we reported pre-loaded tissues for DSAEK using a commercial device, iGlide (Eurobio, France), which has the capability to transport and transplant a pre-cut DSAEK tissue [Figure 3] [9]. In 2016 we reported a ‘proof of concept’ for pre-loading DMEK tissues and introduced a modified design for a surgical device that will help to preserve, transport and transplant a DMEK graft with less manipulation [10]. Pre-loading DMEK, as we assume, will also be useful to standardize and validate DMEKs with advantages like reduced primary graft failure rates due to de-oriented grafts, lowering high surgical skills quotient, high rehabilitation rate with better visual acuity, low surgical time, costs and logistic requirements.



**Figure 3:** Number of corneal tissues prepared for specific keratoplasties between 2012 and 2015 at FBOV, Venice, Italy. A consistent decrease in the request for PK has been noticed since 2012 where as tissues for DSAEK and DMEK prepared in the eye banks have shown increased popularity.

However, these relatively new procedures still face some obstacles, such as the worldwide shortage of transplantable donor corneas, continuing cell loss after transplantation, technical difficulty, and primary graft failure. In order to address the worldwide shortage of donor corneas, the idea of one donor cornea for each patient has been challenged, and the concept of using one donor cornea for treating multiple patients has yet to be widely accepted. Such a timely goal prompts researchers to establish optimum technologies for isolation and cultivation of HCECs. Testing several cultivation methods and scaffolds will eventually lead to better transplantation outcomes. With mark of stem cells and regenerative medicine, the next ambitious goal would be the identification and amplification of corneal endothelial stem cells to treat endothelial disorders reducing invasiveness of clinical interventions. Although, maintenance of the undifferentiated stem cell nature, promotion, amplification, molecular mechanisms and differentiation are still the key issues to learn, with the on-going studies, cultured endothelial cells for transplantation in humans seems to be a possible option in the near future.

The emerging strategies in the field of cell biology and tissue cultivation of corneal endothelial cells aim at the production of transplantable endothelial cell sheets. Cell therapy focuses on the culture of corneal endothelial cells retrieved from the donor followed by transplantation into the recipient. This strategy likely poses multiple advantages over the conventional PK and most certainly, EK, as it has evolved over the past 15 years and has become the treatment of choice for endothelial diseases. The advantages include reduction in post-operative complications like irregular astigmatism, wound leakage, corneal infection, neovascularisation and epithelial defects. Recently, the focus is on overcoming the challenge of harvesting human corneal endothelial cells and the generation of new bio-membranes to be used as cell scaffolds in surgical procedures. Current results represent important progress in the development of new strategies based

on alternative sources of tissue for the treatment of corneal endotheliopathies. Similarly, multiple techniques have been described in the literature [11]. Human corneal endothelium is a physiologically important monolayer of cells, with a simple, but crucial, role to maintain corneal clarity.

In conclusion, corneal transplantation is a popular choice of treatment for many corneal disorders. However, the shortage in availability of donor tissues limits the amount of corneal transplantation around the globe. Continuous increase in the number of patients has boosted the need for clinical grade tissues or a reasonable substitute to overcome this issue. Alternatives include cell, tissue or bio-engineering, cell culture *in vitro*, the potential use of stem cells or other related therapies. The current treatment measure to overcome the corneal disorders is transplantation of a healthy donor corneal graft with a diseased patient's cornea. There is a rapid advancement in the field of endothelial keratoplasty as it has advantages being a less-sutured surgery with quicker rehabilitation and better post operative visual outcomes. Thus, to overcome the necessity of donor tissues for selective keratoplasty, the endothelial reconstruction or regeneration is currently studied.

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# **CHAPTER 1**

## **EYE BANKING – AN OVERVIEW**

*Derived from published book chapter*

Mohit Parekh, Stefano Ferrari and Diego Ponzin. Eye Banking: An Overview. In: Mohit Parekh, Stefano Ferrari and Diego Ponzin. Eye Banking: Changing Face of Corneal Transplantation. Pages 1-18.

Corneal transplantation mainly referring to PK is performed to replace the central part of a cornea which has lost its physiologic curvature and/or transparency. LK refers to a selective surgery which is aimed at replacing the corneal stroma i.e. ALK OR the posterior corneal stroma with the Descemet's membrane-endothelium complex i.e. EK. The paradigm shifts in corneal transplantation encouraged by recent advances in LK is having a tremendous impact in the daily routine of eye banks. In particular, the field of EK has shown a dramatic impact in current eye banking and corneal transplantation procedures. The speed of adoption and implementation of this new form of selective tissue replacement is making a mark in the field of EK, and most eye banks are now pre-cutting, pre-bubbling or pre-stripping the corneal tissues for DSAEK, DMEK and DALK to improve the surgical feasibility.

Earlier, the role of eye banks was to recover, evaluate and store donor corneas and other ocular tissues for surgical use. However, in the recent years, eye banks are satisfying the demands of the clinics in terms of pre-evaluated pre-prepared grafts. If the next of kin of the donor consents, then the tissues could also be used for research purpose which is equally important to understand the mechanism and behavior of the human tissues both *in vitro* and *in vivo*. Tissue removal and tissue processing in an eye bank should be carried out with sterile instruments by operators wearing personal protective clothing and observing strict aseptic techniques. The morphologic and functional status of the endothelium is the most important indicator of donor corneal suitability for transplantation.

Various preservation media have been identified to preserve the integrity and functional mechanism of the corneal endothelium under varied temperature and conditions. Hypothermic storage at 2-6°C and organ culture at 30-37°C are the two storage practices for the cornea that are practiced around the world in wide-range. For keratolimbal

allografts, the donor limbal epithelium must be protected from trauma and desiccation, and a conjunctival rim of 3-4 mm should be left [1]. Donor sclera is prepared from ocular tissue following excision of the corneo-scleral button, or from the donor ocular globes.

Recent advances in the fields of eye banking, ophthalmology and regenerative medicine are challenging the traditional activities of eye banks. Serum-free media are being evaluated for corneal storage, autologous serum eye-drops, amniotic membrane transplantation and *ex-vivo* expanded limbal stem cells are being offered as complementary remedies for ocular surface disorders.

### **Screening of donors**

Transplantation of human cornea requires additional care and involves potential risks of transmission of host disease to recipient. In order to ensure safety, a set of contraindication has been established, after retrospective discoveries of transmission of disease, or on a cautionary basis (theoretical or significant risk of transmission). Other contraindications have also been set to improve the quality of the graft so making the criteria for donor screening a little more stringent for penetrating than for lamellar grafts.

Diseases with the potential risk of transmission by corneal transplantation comprise infections (local and/or systemic), hematologic malignancies, prion diseases and corneal disorders, the latter being more related to quality than to safety issues. Metastatic neoplasia do not exclude from donation and transplantation. The EEBA [2] and EBAA [3] have established Minimum Medical Standards and Medical Standards, respectively (details can be found at [www.europeaneyebanks.org](http://www.europeaneyebanks.org) or [www.restoresight.org](http://www.restoresight.org)). The contraindications comprise a group of systemic disorders (including the death of unknown cause), intrinsic eye diseases and prior intraocular or anterior segment surgery.

In Europe, the contraindications have been listed in the Directive 2004/23/EC that, together with Directive 2006/17/EC and Directive 2006/86/EC, set the standards for safety and quality of tissue transplantation. As set by the Directives, the serological screening for HBV, HCV, HIV and syphilis must be performed for every tissue that has been donated. Besides the search for antibodies or antigens, some nations require the execution of nucleic acid testing (NAT), a molecular technique developed to shorten the window period (the time between the infection and when a positive antibody/antigen can be revealed). Because of the window period, also the behaviors that may have put the donors at risk, such as intravenous drug use, must be evaluated. Post mortem blood can be obtained from direct heart puncture or accessible blood vessels, within 24 hours from death. Despite the low incidence, transmissible spongiform encephalopathies, such as Creutzfeldt-Jakob disease, have been transmitted via corneal transplantation. The disease is progressive and fatal. For which, any donor who died with neurological symptoms, or degenerative neurological conditions, must be excluded from donation. Theoretically lymphoproliferative disorders including leukemias, lymphomas and lymphosarcomas pose the greatest risk of transfer from donor to host, because they may invade the cornea. However, there is no report of a systemic malignancy transmission following keratoplasty. The source of information (pathologist, medical records, attending medical and nursing staff, family members or other relevant persons close to the deceased, family doctor, post-mortem report) is another important documentation to be noted. Transmission of local corneal disorders is a further risk of corneal transplantation. Early stage anterior and posterior membrane dystrophies, and keratoconus, may escape detection. A thorough tissue evaluation in the eye bank may minimize these events. Age criteria for cornea donors are not well defined and vary between eye banks and surgeons. The diameter of the infant cornea stops growing after 2 years of age, and the curvature probably reaches adult dimension during the second 6 months of life. The small diameter, thinness and

elasticity of the infant cornea may cause technical problem for the surgeon. In general, the diagnosis of the recipient and the surgeon's experience are more important than donor age in determining the long-term graft clarity. Ocular tissues should be recovered as soon as possible after death. A short post mortem interval warrants a higher yield of suitable corneas and limits endothelial loss during storage.

#### *Ocular tissue removal -*

The removal of ocular tissue for surgical use must minimize the endothelial cell loss and contamination, maximize the number and quality of cells that are ultimately grafted, and should not alter the appearance of the donor. After the physical inspection of the donor, the enucleator, with the aid of a penlight, should examine the periorbital and orbital tissues, and the anterior segment of the eye, for pathologic findings such as mucopurulent material, congenital or acquired corneal abnormalities or signs of intraocular surgery.

The donor's head must be kept elevated for the whole length of the ocular tissue removal, in order to prevent bleeding. The eyelids must be gently opened to allow a thorough irrigation of the cornea and conjunctival sac of each eye with sterile phosphate-buffered saline, then with iodine solution, followed by phosphate-buffered saline, to prevent corneal toxicity. Then, a preparation of the operative area using sterile gauze moistened in the iodine solution should be performed, starting at the medial canthus of the upper closed eyelid and moving out, around and below the lid, over the bridge of the nose, in an ever-widening circular pattern. Finally, the donor is draped to create a sterile field at the operative site. The upper eyelid of the donor's right eye is gently opened with sterile gauze and the closed lid-speculum is inserted, taking care not to touch the cornea. The conjunctiva is grasped with the forceps, near the lateral edge of the cornea at the limbus, and cut using the microsurgery scissors, continuing 360° all the way around the cornea,

removing the conjunctiva as far as possible. Closed, straight scissors are inserted under the conjunctiva, and a blunt dissection is performed by gently opening the blades. This will separate any adhesions between the conjunctiva and the anterior globe. The remaining conjunctiva is removed by carefully scraping from the limbus with a scalpel blade.

#### *Enucleation -*

Using a muscle hook, the rectus muscles are exposed and severed where they meet the sclera. The lateral rectus must be severed last, leaving a 5 mm stump on the sclera. The stump is gasped with a hemostat and the globe is lifted upwards with the aid of enucleation scissors. The optic nerve is identified and severed with the enucleation scissors, leaving a 5-10 mm stump. The globe is then lifted from the socket with the hemostat clamped to the lateral rectus muscle, while cutting away any remaining connective tissue. The globe is wrapped in sterile gauze with the cornea facing up and a small amount of PBS is poured over the cornea to moisten it. The globe is then placed in the eye jar, carefully inserting at least four rectangular, sterile ophthalmologic tampons between the gauze and the sides of the container. Once moistened, they will swell, keeping the globe in position.

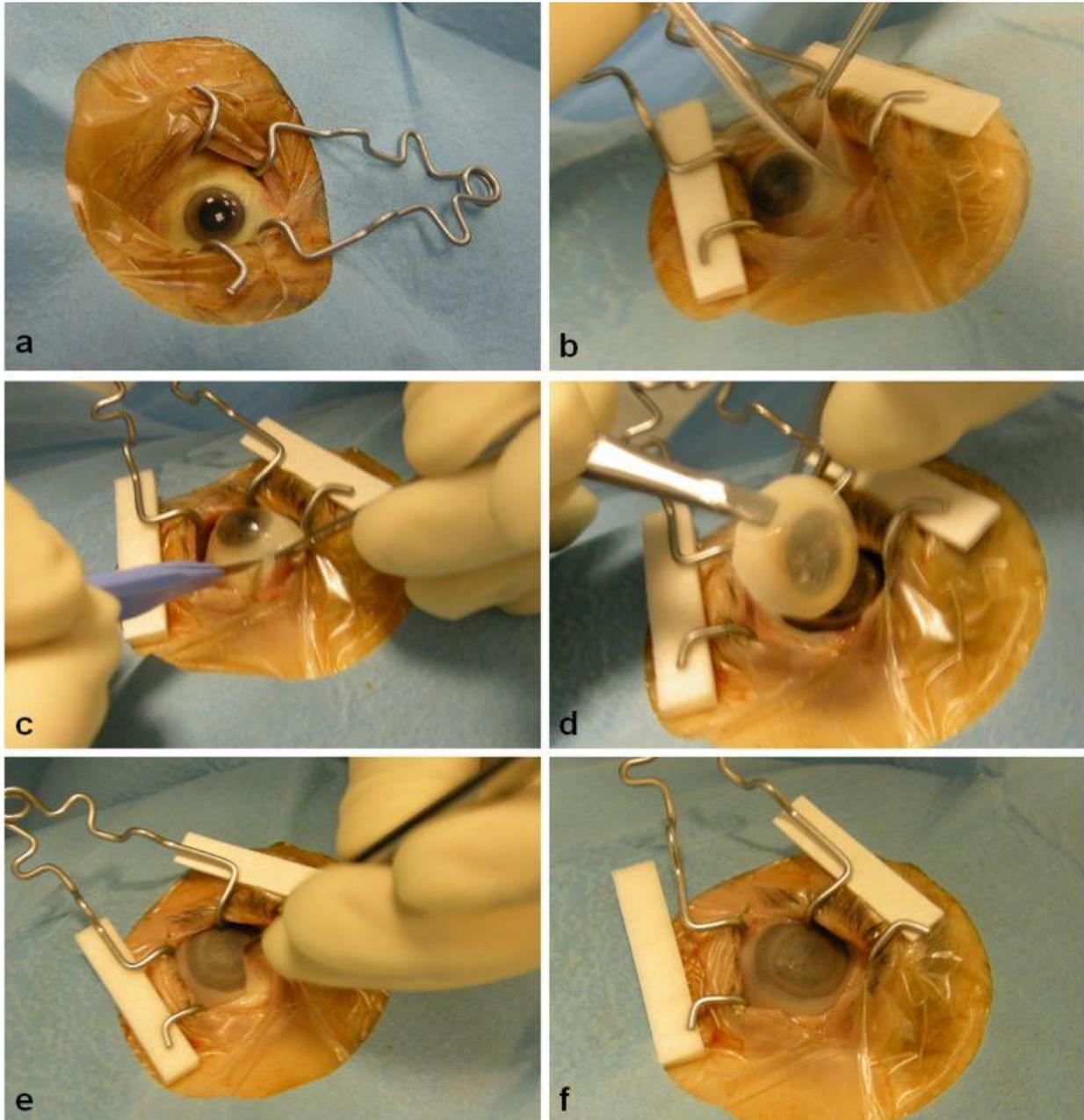
#### *In situ corneo-scleral rim excision -*

Without perforating the choroid, a scleral incision is performed using a scalpel, approximately 4 mm from and parallel to the limbus. The incision is extended 360° with microsurgery scissors, taking care to remain at least 4 mm from the limbus and avoid perforating the underlying uveal tissue. The tips of the scissor blades must not enter the anterior chamber and the corneas' normal curvature must be maintained. If the incision has been performed correctly, the corneo-scleral rim adheres to the ciliary bodies only at the scleral spur. An air bubble will appear in the anterior chamber if it is entered in the

chamber correctly. The removal is completed using one pair of small forceps to hold the scleral rim, and a second set of forceps, to push the ciliary body-choroid downward and away from the corneo-scleral button. The remaining adhesions can now be gently separated from the corneo-scleral button, avoiding distortion of the cornea shape with excessive traction. The posterior chamber of the donor eye must be examined to check the presence of the natural crystalline lens.

#### *Donor reconstruction -*

After enucleation, a moistened piece of gauze, rolled into a ball of the approximate dimensions of the globe, can be placed in the socket and covered with a plastic eye cap or a plastic prosthesis. The conjunctival remains are used for overall covering. Following in situ corneal excision, a plastic eye cap can be placed between the remains of the conjunctiva and the globe, covering part of the eye cap with the remains of the conjunctiva. The eyelids will be closed and gently manipulated to restore the donor's appearance. The donor's head should be left elevated, verifying the absence of bleeding. It is recommended to ask the mortuary staff to check the conditions of the donor later. The process is illustrated in figure 1.



**Figure 1:** In situ corneo-scleral rim excision and donor reconstruction. a) The globe is exposed using eye speculum to keep the eye lashes out of the corneal retrieval field, b and c) conjunctival remnants are cut, d) the scleral incision is performed with a scalpel blade and microsurgery scissors extended to 360°, e) the corneo-scleral rim is excised, f) a plastic cap is placed back and the conjunctival remains are used for overall covering of the eye to retain the donor eye characteristics.

## **Tissue processing**

All eye bank manipulations are carried out in sterility using a laminar flow cabinet, placed in a defined and monitored environment. Prior to any manipulation or evaluation, the ocular tissues and solutions should be allowed to reach normal room temperature, avoiding multiple repeated warming/cooling cycles.

### *Decontamination of donor eyes -*

The eyes are rinsed with sterile PBS, then immersed in sterile polyvinylpyrrolidone-iodine, sodium thiosulfate in PBS, and rinsed again in PBS, where they are left until the corneal excision is performed. This procedure has been reported to reduce the percentage of contaminated eyes. Further reduction of the load of contaminating microbes is up to the antibiotics in the corneal storage solutions.

## **Tissue evaluation**

Prior to transplantation, the corneas must be examined for the presence of biological characteristics that are required in order to ensure a good clinical outcome.

The morphological and functional status of the endothelium is a key factor for the success of corneal grafting and therefore the most important indicator of donor cornea quality. As there is no direct functional test that can be used, the cornea must be evaluated by morphological parameters [3,4]. A cornea suitable for transplantation is required to display some essential biological characteristics:

- a non interrupted epithelial layer;
- a stroma free of opacities in the optic centre and sufficient clear zone in case of an arcus lipoides;
- absence of folds of the stroma and the DM indicative for poor endothelial function;

- a viable (absence of cell degenerations) and regular (absence of dystrophies, no substantial pleomorphism or polymegathism) endothelium, with a cell density above 2000 cells/mm<sup>2</sup> (the lowest value generally accepted by most surgeons).

Corneas from eligible donors with local eye disease affecting the corneal endothelium, or previous ocular surgery that does not compromise the corneal stroma, can be used for lamellar (anterior, posterior) or patch grafts.

Eye banks currently examine donor corneas by a combination of techniques. In general, the slit lamp biomicroscopy of the anterior segment (enucleation), or the slit lamp examination of the cornea (*in situ* excision) is combined with specular microscopy (mostly in the USA), or with light microscopy [4].

#### *Slit lamp examination -*

A 10× examination with a wide slit of light for a general inspection of the corneo-scleral rim is performed first. A more in-depth examination allows an evaluation of the following:

- epithelial defects;
- corneal transparency (noting any scars, edema, or arcus lipoides);
- infiltrates or foreign bodies;
- Descemet's alterations: folds (iatrogenic, stretch striae, hypotonic folds);
- corneal guttata;
- evidence of technical problems during removal and or transport;
- the corneal endothelium; and
- adequacy of the scleral rim.

#### *Specular microscopy -*

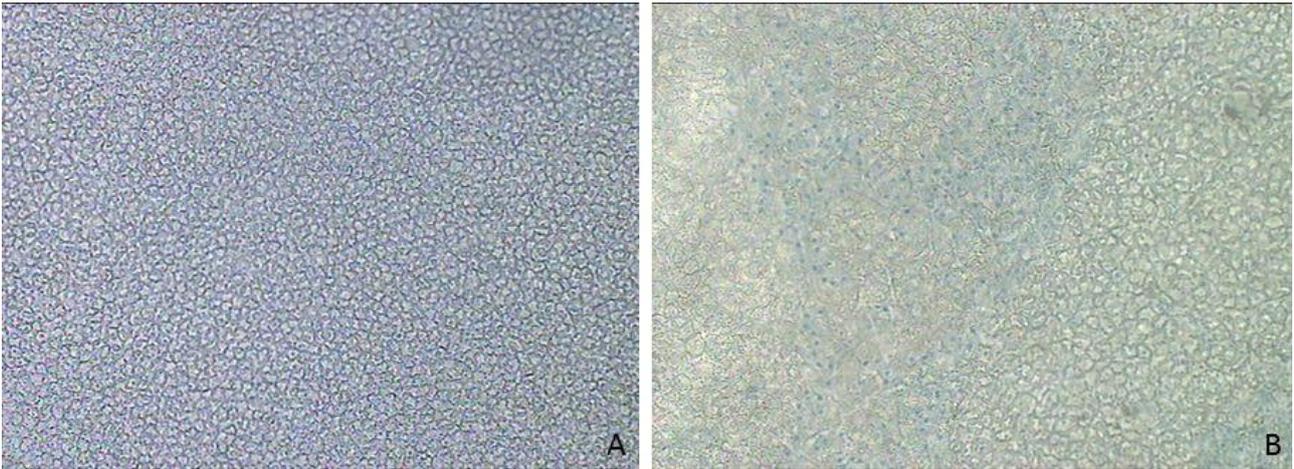
Specular microscopy can be performed on donor eyes or corneoscleral rims by non-contact, computerized endothelial microscopes. These instruments visualize the

endothelium when the cornea is optically transparent, either on the donor eye or the excised corneoscleral rim. Endothelial density can be estimated by a calibrated reticule, or calculated by built-in software.

#### *Light microscopy -*

The donor cornea is put into a sterile petri dish. The endothelial mosaic can be visualized by exposing the cells to a slightly hypotonic solution, which induces an enlargement of the intercellular spaces. The whole surface of the corneal epithelium, stroma and endothelium can be scanned with an inverted (tissue leaning on the scleral rim, immersed in swelling solution), or non-inverted phase contrast or bright-field light microscope, at a magnification of 50×, 100× and 200×. The number of endothelial cells is estimated at about 100×, with the help of a calibrated grid/reticule (10×10 mm) mounted onto one eye-piece of the microscope. The cells should exhibit a regular pattern of intercellular spaces swelling. Absent or irregular swelling, associated with a grayish appearance of the cells has been correlated to metabolic suffering of the corneal endothelium.

The presence of dead cells is studied exposing the endothelium to trypan blue. The trypan blue exclusion assay is a well established method to test the endothelial cell viability or membrane alteration(s), despite the staining is not very specific for dead and necrotic cells. The presence of TBPC in corneal endothelium is usually related to post mortem degenerative changes or injuries during tissue manipulation. The cell morphology and TBPCs are shown in figure 2.



**Figure 2:** Human corneal endothelium. a) Regular pattern of the corneal endothelium without any trypan blue positive cells and b) damaged corneal endothelium with large area of disorganized cells and presence of trypan blue positive cells.

### **Preservation of corneas**

The primary aim of corneal storage is the maintenance of endothelial viability from the time of corneal excision to transplantation. Currently there are two storage practices for the corneas, both use liquids, based on cell culture medium: the hypothermic storage at 2-6°C, adopted by many eye banks all over the world, and organ culture at 30-37°C, the current method of choice for most eye banks in Europe [5]. McCarey-Kaufmann medium, which allowed the hypothermic storage of donor corneas for 3–4 days was a huge success. As a consequence, corneal transplantation became a scheduled, rather than emergency procedure. The storage of donor corneas for an extended period allowed extensive donor screening, scheduling of operations and a more rational dispatching of donor tissue to transplant centers. Other formulations containing chondroitin sulphate in addition to dextran, that retarded corneal swelling during storage, and components promoting tissue survival were introduced afterwards.

During the same period, the organ culture technique was invented in the USA, and then subsequently modified and enhanced with methods for the evaluation of the corneal endothelium. Organ culture has been widely adopted in Europe, where it still remains the most widely used technique for the storage of donor corneas.

#### *Hypothermic storage -*

Donor corneas are stored in serum-free tissue-culture medium at a temperature of 2-6°C. At this temperature the metabolic activity of endothelial cells is minimal and pumping function is lost. Cornea swelling may be prevented by the addition of water retentive compounds to the preservation medium. One of the most commonly used is the deturgescent compound dextran either alone or in association with the glucosaminoglycan chondroitin sulphate. Storage liquids also contain antibiotics (gentamicin alone or with streptomycin) that, together with the low temperature, prevent or limit the bacterial growth. Commercially available solutions are also supplemented with a number of additives (energy sources, antioxidants, membrane stabilizing components, growth factors), but the specific contribution of such compounds for corneal storage has never been clarified.

During hypothermia, the cornea shows progressive degeneration of the epithelium and the endothelium, intercellular disruption, decreased adhesion and, eventually, cell death. Both apoptosis and necrosis occur in cells during hypothermic storage, with apoptosis appearing to predominate. The extent of endothelial loss seems to be related to the biological quality of the tissue, rather than the composition of the medium. Furthermore, the ability of a medium to prevent cornea swelling *in vitro* does not correlate with the viability and function of the endothelium. Therefore, most corneas are transplanted after 3-5 days of storage, without displaying major alterations.

The hypothermic storage method does not allow time for obtaining pre-operative microbiology controls before distribution of the tissue for transplant. Scleral rim cultures are usually performed at the time of surgery, despite the recognition that bacteriologic contamination of donor eyes does not necessarily lead to infection. As per the data collected by the Veneto Eye Bank Foundation, around 12-28% of cultures are found to be positive for bacteria and fungi, with *Staphylococcus epidermidis* as the most common contaminant. However, ocular infections after penetrating keratoplasty are rare and, in most of the cases, related to the recipient eye condition or the surgical procedure.

Overall, hypothermic storage seems to offer donor tissues of good quality comparable to that obtained by organ culture, provided that the storage time is kept short. Indeed, according to the literature, the risk of primary graft failure increases significantly after storage longer than 7 days. Furthermore, corneas stored longer than 7 days display epithelial alterations that may hinder the surgical procedure or delay the full recovery of the graft [6].

#### *Organ culture -*

The OC storage method consists of two phases – a storage period in culture medium at 30-37C°, and a de-swelling and transportation phase at 30-37C° and room temperature in the same medium supplemented with 4-8% dextran. Organ culture solutions are based on cell culture media. They generally consist of a base of Eagle's MEM or its variant Dulbecco's MEM supplemented by penicillin, streptomycin and fungicide (amphotericin B or nystatin) to counteract the growth of microbiological contaminants, and by 2-10 % FCS as a source of growth factors. A storage period of 30 days can be achieved without significant loss of endothelial cells. The evaluation of endothelium, which can show reparative phenomena during storage, is usually performed before and after storage. The

organ culture serves as a stress test and corneas not tolerating long term storage are detected by a significant endothelial cell loss and are discarded.

Irrespective of the storage method used, inspection of the endothelium after a prolonged storage is essential to prevent transplantation of corneas of poor quality. Changes in the endothelium as a result of postmortem time and other variables such as cause of donor death, donor age, circumstances of death, may affect cell viability and result in endothelial death in the long run. Cultured corneas have preservation folds caused by swelling of the stroma in the absence of osmotic agents. These folds do not affect the quality of the tissue, provided that they remain covered by viable endothelium. Before transport and surgery the swelling is reversed by the dextran present in the transport medium. The final thickness is reached after about 24 hrs and is dependent on the dextran concentration. The deswelling phase precludes that an organ cultured cornea, contrary to a hypothermically stored cornea, is ready for immediate use unless specific systems and logistics are developed for emergencies. The dextran also protects the cornea against the lower ambient temperature during transport. Organ culture offers a longer storage time, a less restricted donor supply on beforehand, corneal endothelium with a better defined quality and a pre-operative sterility control. Organ cultured corneas always display an epithelium made up of 2-3 layers of viable cells. The 30 days storage period allows an efficient use of valuable donor tissue: planning of operations is easier, allowing sufficient time for the allocation of HLA-matched corneas. The disadvantages of this method are the relative technical complexity and the need for qualified staff to perform tissue culture and selection of the corneas [7].

Samples of the storage medium of cultured corneas are routinely tested for microbiology after 3-7 days in the first phase, and after 1 day in the second phase. Cultured corneas

are also visually inspected every day. A gradual change in color of the medium is expected, but any cloudiness or significant color change of the medium is indicative of bacterial or fungal contamination. A contaminated cornea is discarded regardless of whether the microbe is pathogenic or not. Identification helps to identify trends and opportunities for process improvement [8,9]. It is still a point of debate whether the clinical outcome after grafting of corneas stored by hypothermic or organ culture techniques is the same, although the few studies comparing the effect of the storage methods on outcome demonstrate similar graft survival and post-operative decline in endothelial cell density [10-13].

### **Tissue processing for specific surgical purposes**

#### *Eye bank preparation of corneal tissue for lamellar keratoplasty -*

Lamellar keratectomy of donor corneas may be performed by eye bank technicians who have successfully demonstrated proficiency in sectioning the cornea using aseptic technique with an automated microkeratome under laminar flow. Donor selection criteria for corneas used in lamellar keratoplasty are the same as for penetrating keratoplasty with a few exceptions. Corneas with prior laser photoablation surgery or non-infectious anterior stromal scars may be suitable for posterior keratoplasty, but corneas with previous intraocular surgery scars are not recommended for use since the cornea may rupture under infusion pressure while on the artificial anterior chamber [14-16].

A 3-4 mm scleral rim is needed for corneas used in lamellar keratoplasty procedures to ensure an adequate seal on the artificial chamber of the automated microkeratome. An evaluation of the cornea by slit lamp biomicroscopy and specular microscopy should be performed prior to lamellar keratectomy. An automated microkeratome system consists of a control unit, an artificial chamber, microkeratome turbine and heads. The control unit

should be set up in close proximity to the laminar flow cabinet. A sterile field is set up under the laminar flow cabinet with the artificial anterior chamber connected to an irrigation system and turbine connected to the control unit. The cornea is placed using tissue forceps centrally onto the artificial anterior chamber which has been moistened by activating the irrigation system and the chamber is locked into place. The system must be watertight to ensure a smooth cut. The cornea is pressurized by infusing PBS through the irrigation system. A tonometer lens is placed on the corneal surface to confirm that a minimum of 65 mm Hg has been established inside the artificial chamber through the infusion of PBS. In case of anterior lenticules, the graft desired thickness is obtained by the correspondent microkeratome head. For posterior lenticules, a pachymetry reading is obtained after the removal of the epithelium, to determine which microkeratome head to use to obtain a final graft thickness in a range of 120  $\mu\text{m}$  to 250  $\mu\text{m}$ .

#### *Resection of cornea with a swinging microkeratome -*

The corneal epithelium may be gently removed before preparation, or left in place. In the former case, the subsequent swelling of the stroma during preservation could be limited. Two points are marked on the mid-periphery of the cornea using a sterile gentian violet or trypan blue marker to assist with re-aligning the cap back onto the remaining stromal bed after the cut has been made.

The blade is inserted into the microkeratome head, which is secured into the turbine and lubricated with PBS. The power source is then activated to begin the blade oscillation of the microkeratome. The microkeratome head is rotated manually across the cornea. Once the sectioning is completed, the free cap is removed from the microkeratome head and repositioned onto the corneal bed, taking care of re-aligning the marks. A wexel sponge spear is used to smooth out any bubbles between the cap and the graft bed. The pre-cut

cornea is removed from the artificial chamber using tissue forceps. Once lamellar keratectomy has been completed, the cornea should be re-evaluated by slit lamp biomicroscopy and specular/light microscopy to confirm that the tissue is suitable for the intended use [17].

#### *Storage of corneal lenticules for lamellar keratoplasty -*

Anterior corneal lenticules can be either dehydrated or freeze-dried, and stored at 2-6°C according to the eye bank's validation protocol. Alternatively, anterior/posterior lenticules can be placed in a cornea viewing chamber filled with preservation media (hypothermic storage) or in the transport medium (OC).

#### *The preparation of donor sclera -*

The donor sclera is used in allografts for a variety of procedures, most commonly to enclose orbital implants for reconstruction of anophthalmic cavities, reconstruct eyelids, cover tubes used in glaucoma surgery, repair scleral thinning, correct lid retraction and cicatricial entropion, tumour excision. Selection criteria are the same as cited for penetrating keratoplasty, except that tissue with local eye disease affecting the corneal endothelium is acceptable for use. Being a vascularised tissue, malignancies are applied as additional contraindication. Post mortem interval may be extended.

Donor sclera is prepared from remaining ocular tissue following excision of the corneoscleral button or from donor globes which have been disqualified before corneoscleral rim excision. Any abnormalities such as discoloration, thinning or atypical vessels preclude the utilization of the tissue, as well as tumors in the donor medical history. Since conjunctival tissue is an excellent carrier for microbes, remnants of muscles and conjunctiva must be removed. The intraocular material is removed by using forceps,

iris scissors, sterile gauze or cotton tipped applicators. The sclera is finally rinsed in PBS, reshaped to its original spherical form, preserved dehydrated in ethanol (70% or higher concentration) or glycerol, fixed in formalin, freeze dried, or frozen.

### **Future aspects in eye banking**

#### *Synthetic medium for corneal preservation -*

Conventional OC is typically a 3-stage protocol: the first transport medium that allows the corneal shipment from the morgue to the eye bank, the OC (storage) medium that helps to preserve the cornea for duration of 21-28 days at 31°C and the final transport medium with a deswelling agent to reduce the thickness of cornea for ease of transplantation as reported earlier in this chapter. The storage and the final transport medium contain serum of animal origin in it. Apart from serum, other nutrients of animal origin have also been investigated for prolongation of the endothelial metabolic activities, such as chicken feather, ovalbumin, and pig bone amino acids, usually used in combination with other sources of nutrient supplements. The significance of FCS is not well-known; however, it was proved that a higher rate of endothelial cell damage occurred when the porcine corneal endothelial cells were preserved in the OC medium without serum. It has previously been reported that FCS is responsible for resisting the stress levels of the cells in vitro, whereas other reports suggest that the serum-free medium causes higher ECL due to necrotic areas. Animal-derived products have the potential to introduce animal viruses or prions that if assimilated in the human body could be hazardous. Animal viruses, especially retroviruses, could integrate into the human genome and activate human oncogenes or oncosuppressor genes, while prions could lead to human forms of BSE. This is why synthetic media have been developed. However, in the literature, there is no document reporting the transmission of animal viruses due to the fact that animal serum was used and most of the safety concerns are ruled out. The potential transmission

of BSE primarily comes from donors who have donated their corneas and were at risk of having BSE (from example, UK donors at the time of mad cow disease). Theoretically, there could be a transmission of animal-derived viruses that could integrate in the genome and activate oncogenes, therefore, technically it would be safer to develop and integrate a totally synthetic/animal free media in the routine eye banking procedures [18,19].

#### *Pre-cut and pre-loaded tissues for Descemet Stripping Automated Endothelial Keratoplasty -*

Donor tissues for DSAEK can be prepared by the eye banks where the cornea is cut using a microkeratome and the entire tissue is delivered to the surgeon as a pre-cut lenticule. The anterior cap of the cornea can still be left attached to the scleral rim by its peripheral edge for ease of transportation and to lower any potential endothelial cell damage [20,21,22]. Eye bank prepared pre-cut tissues have certain advantages over those tissues that are prepared in the surgery. If the tissue is prepared by the surgeon in the operating theatre and if it fails due to irregular cut or perforation in some cases then the surgery has to be postponed or an extra cornea has to be kept ready for replacement which increases the tissue wastage in general. Moreover, technological advances like 3D (three dimensional) printing has helped ophthalmic field in various ways, one of which is designing and developing surgical tools for transplantation purposes.

#### *Device prototyping for surgical glides -*

3D printing technology can be used for initial prototyping of the surgical glides however depending on the requirement of the units, it can be custom built, sterilized and used in a surgical theatre. Initial prototyping is used in order to check the units before proceeding to mass production. This technique finds applications in multiple fields such as aerospace, art and sculpture, industrial design, engineering, medical industries, biotech (human tissue

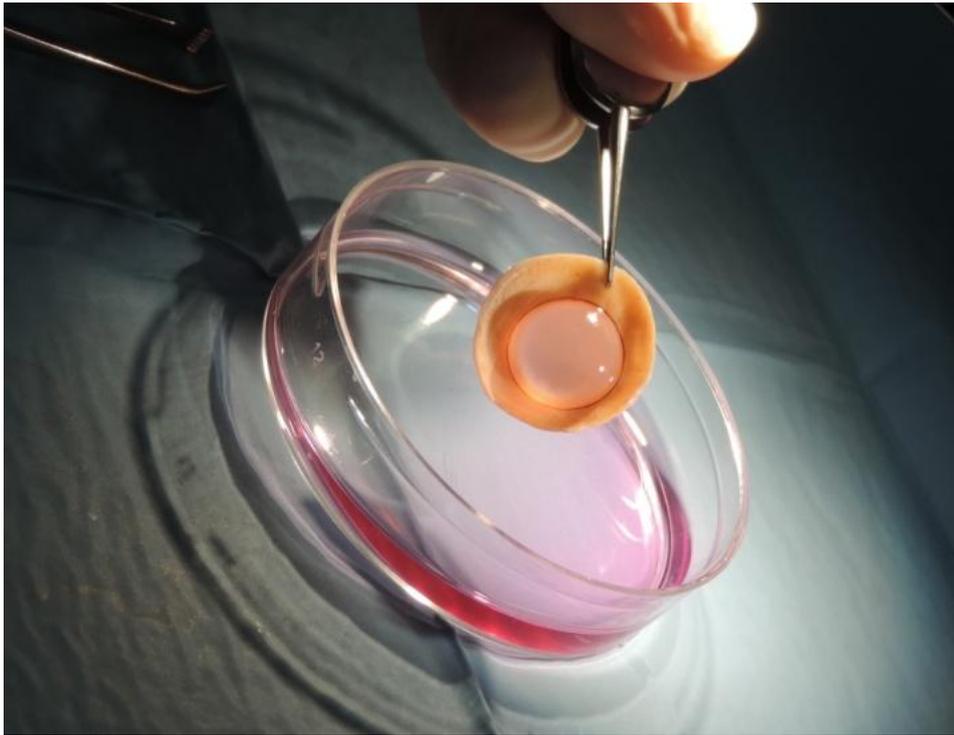
replacement) and much more. This technology could be used in order to get a functional, inexpensive, ready to use prototype. Starting from a digital file (STL - STereoLithography/Standard Tessellation Language) the device is generated by adding multiple layers of material (plastic, resin, etc.) until the entire object is formed.

#### *Preparation of the pre-loaded lenticules -*

In order to keep the procedure easy, especially when ultra thin lenticule are prepared, the posterior lenticules were preserved with a support such as the anterior lenticule of the tissue or a synthetic support such as a contact lens. The support prevents the tissue to shrink and it can be used as an anchor point to avoid any endothelial damage. The pre-cut tissue and its support are put in a standard punching block (Moria, Antony, France) with the endothelial side facing up. The tissues can be trephined with a desired diameter (8-9 mm). The posterior lenticule is picked up grasping the support and is inserted into the glide. The device is further filled with 1 mL tissue culture medium after removing the air present inside the glide in order to avoid the formation of bubbles that remain in contact with the endothelium during the storage time. The lid of the glide is closed and the glide is gently fixed in the preservation container. The container is filled with 50 mL of the preservation media with Dextran and all the grafts are ready to be delivered within 4 days from the preparation.

#### *Pre-bubbling the tissues for Descemet Membrane Endothelial Keratoplasty -*

Recently, two techniques for preserving pre-separated corneal tissues for DMEK have been published. Descemet's membrane and endothelium can be separated from the overlying stroma with a simple technique using air or liquid dissection. Air injection is usually performed with a high pressure (pneumodissection) to create the separation using a big-bubble technique. However, liquid requires medium to high pressure (figure 3).



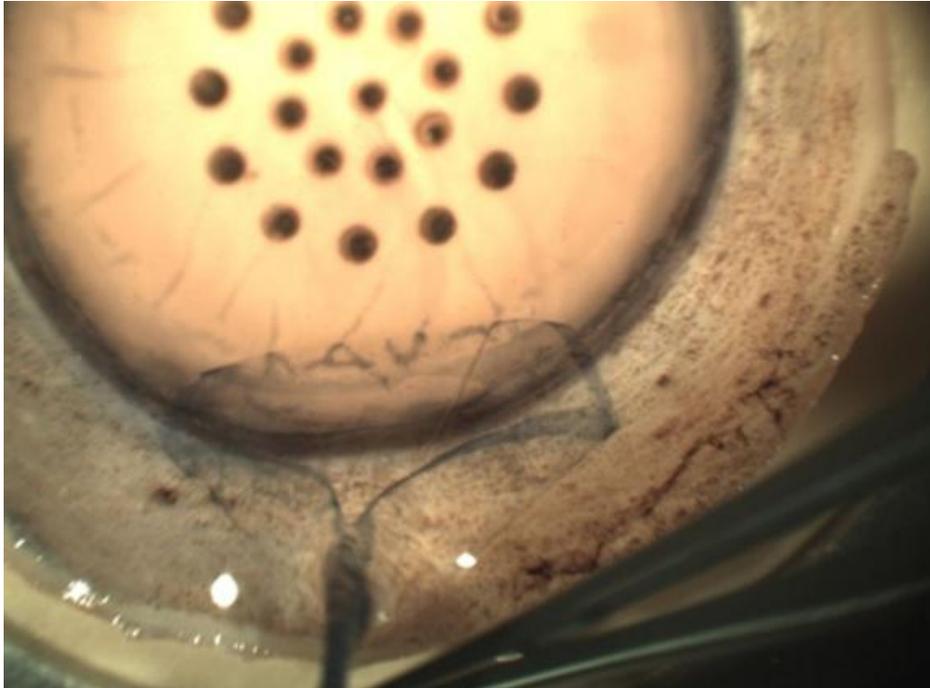
**Figure 3:** Pre-bubbled tissue prepared using submerged hydro-separation (SubHyS) method.

The tissues can then be preserved in the transport medium for 7 days. These techniques can be performed at the eye banks and the pre-bubbled tissues can be shipped to the surgical theatre allowing surgeons to reduce tissue wastage in the surgery room due to tissue preparation errors. An adequate size of graft tissue can be obtained without the need to manually handle the tissue. The technique allows storage of the tissue in tissue culture medium with low endothelial cell loss. However, either of the techniques has no significant changes seen in the endothelium apart from that the yield generated using liquid separation was slightly higher than air [23-25].

*Pre-stripping the tissues for Descemet Membrane Endothelial Keratoplasty -*

Stripping, unlike the bubble separation technique is performed by peeling the Descemet's Membrane and the endothelium away from the stroma leaving a hinge at the end of the

lenticule as showed in figure 4. This allows preservation of pre-separated endothelial grafts in the eye bank further shipped to the surgeons. This technique has showed minimum mortality rate as compared to the other currently performed techniques.



**Figure 4:** Pre-stripped tissue using stripping method.

Thus, Pre-loaded, pre-bubbled or pre-stripped tissues can be prepared in the eye bank and shipped to the surgery to ensure a validated graft by the eye bank for surgery.

#### *Human corneal endothelial cell culture -*

The field of eye banking surely requires more in depth knowledge in terms of graft manipulation however new technologies and culture methods for HCEC culture would surely prove to be a next challenge for eye banks. Considering the requirement of donor corneal tissues for clinical purposes, *in vitro* cultured cells may help to reduce the demand of tissues for endothelial keratoplasty. However, challenges concerning the requirement of clean room facilities and culture conditions along with pre-defined scaffolds or a surgical technique will need a thorough revision in the near future.

Thus, we envision that eye bank is growing not only in the field of procuring the tissues for transplantation but also in the field of research and development. Development in the preservation techniques, surgical devices, modification of tissues and designing new methodologies for ocular health care is now becoming a part of eye bank world. Standardizing the posterior lamellar graft preparation methods will reduce unnecessary manipulation of the tissue in the operating theatre and reduce the high surgical skill or risk quotient. Pre-cut tissues which would reduce the overall intervention costs and time seems to be the future of eye banking. The efforts by the eye banks on the final quality of the graft would reduce the severe efforts of manipulation by the surgeons thus providing better quality tissue for patients [26]. The tissues may be replaced by the *in vitro* cultured cells to reduce the demand of human corneal tissues in the near future and eye banks may play a dramatic role in determining the co-factors that will relate to the cellular pathophysiology and conditions.

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## **CHAPTER 2**

# **INTRODUCTION TO CORNEAL ENDOTHELIAL CELL CULTURE AND TRANSPLANTATION**

*Derived from published review article*

Parekh M, Ferrari S, Kaye S, Sheridan C, Ahmad S. Concise review: An update on the culture of human corneal endothelial cells for transplantation. Stem Cells Transl Med. 2016;5(2):258-64. [PMID: 26702128].

## **Introduction**

In recent years, corneal tissue engineering has evolved dramatically, from culturing cells *in vitro* to developing synthetic scaffolds and artificial corneas. Isolation, culturing and bioengineering have been studied to a limited extent and further research is on-going. Attempts have already been discussed in the literature for exploiting the power of native cells for the manufacturing of stroma-like extracellular matrix and for the production of cell sheets, whether it be the epithelium with amniotic membrane/fibrin or endothelial cells with intact basement membranes [1]. It has also been found that collagen-based engineered matrices support cell growth and demonstrate appropriate optical and mechanical properties. Current research is showing promising results in growing corneal constructs. The next challenge is to ensure if these constructs show appropriate thickness, transparency and strength *in vivo*. However, issues like cellular responses, wound healing and inflammatory responses need to be studied in detail clinically before any conclusions can be drawn [1].

## **Evidence for corneal endothelial stem cells**

Both *in vitro* and *in vivo* studies have been carried out which indicates the presence of corneal endothelial progenitors. Some of which include:

*BrdU* - Studies carried out using Alkaline phosphatase-Bromodeoxyuridine (AP-BrdU) have suggested the presence of corneal endothelial progenitors. In these studies the limbus was intensely stained with AP-BrdU [2,3]. This experimental method was used to detect the presence of dividing cells. BrdU retention was identified by alkaline phosphatase activity. Although this technique indicates dividing cells, it may also identify the presence of native, cellular alkaline phosphatase activity which is typically observed in both intracellular and cellular plasma membrane isoforms of this enzyme and particularly

in stem cells. These studies also showed that HCECs from the corneal periphery increased in number suggesting the presence of progenitor cells.

*Telomerase* - In the telomerase based studies, corneas were divided into three sections, a) central, b) middle and c) peripheral. The authors observed telomerase activity in the middle and the peripheral sections whereas the central section did not show the presence of telomerase activity [9-12]. It was also observed that donor age may have a potential limitation to the human wounding response or cell division in the periphery [4-7].

*Schwalbe's line cells* - A novel cell type (named Schwalbe's line cells) was identified in 1982 by Raviola and co-workers. These cells form a discontinuous cord that is usually found in the circumference beneath the Schwalbe's ring (transition region between the corneal endothelium and the anterior extension of the trabecular meshwork) [8]. An increase in cell division by the Schwalbe's ring cells after laser trabeculoplasty has been observed. This indicates that Schwalbe's line cells may hold progenitor cell-like properties. A four-fold increase in cell division in human laser-treated explants was also confirmed by Acott and co-workers [9]. It was observed that over 60% of the cell division was initially localized to the anterior non-filtering region of the trabecular meshwork and these migrated towards the burn [10-13].

*Corneal endothelial regeneration in vivo* - It has been hypothesized that a slow, regeneration of HCECs starting from the corneal periphery can occur [14]. The microanatomy of the endothelium showed anatomic organization in the periphery of the human corneal endothelium, which also suggested a continuous slow centripetal migration throughout life of HCECs from specific niches. This further led to the understanding that

the corneal endothelium can be regenerated if cells are excised from the peripheral region [14].

### **Isolation of human corneal endothelial progenitors**

*Single cell culture:* There are several methods used for HCEC isolation. Initially, collagenase or trypsin was used on whole corneas but as it inevitably caused contamination of corneal fibroblasts, a selective L-valine-free medium was used. As this medium had to be used for several passages to completely abolish the presence of fibroblast growth, L-valine free selection medium was assumed to have acted by arresting the growth of fibroblast-like cells rather than by killing them [15].

The peel-and-digest method by Peh and co-workers has resulted in successful cultures [16]. Other methods include stripping or bubble techniques to separate the DM from the stroma and then digestion of the cells using trypsin for cell culture. Stripping of HCECs can also be performed with the aid of a vacuum suction holder [13]. After peeling, the DM and endothelial layer are finally digested enzymatically using dispase or EDTA, followed by gentle pipetting. However, enzymatic digestion requires a prolonged incubation time to detach cells from the matrix, subsequently also leading to higher cellular degeneration. Thus, although several potentially successful methods do exist in the literature, it is evident that there is still the need to standardize a specific and reproducible method to isolate and culture HCECs from donor corneas [17].

*Explant culture method* - A recent study showed remarkable growth that was achieved using a serial explant culture technique. The explant was transferred to 7 new plates over a period of 6 months, generating sheets of small, primitive cells in each plate. The findings

are consistent with the theory that progenitor cells for the corneal endothelium reside within the limbus and provide new insights to HCECs culture [18].

*The HCEC sphere forming assay* - It is believed that the corneal endothelium is derived from neural crest cells during embryonic development but also has a mesenchymal origin [19,20]. Sphere colonies from corneal endothelial cells express neural and mesenchymal proteins. Mimura and Wing *et al* have also shown that these colonies have the potential to differentiate into neuronal lineages [21,22]. The sphere forming method has also been used to investigate cultivated HCECs with differences in telomere length, telomerase activity, and characteristics reflecting senescence. The sphere forming assay was performed to obtain precursors from cultured sixth passage (p6) HCECs. p6 and p7 cultured HCECs were used as the controls. It was observed that precursors obtained from the spheres had longer telomeres and higher telomerase activity than cultured p6 cells. Strong positive staining for senescence-associated  $\beta$ -galactosidase activity was detected in p6 and p7 cultured HCECs, whereas little or no staining was detected in the precursors within spheres obtained from p6-cultured HCECs or their progeny. The progeny of spheres derived from cultured HCECs were small regular cells that grew at a higher density and contained more 5-bromo-2'-deoxyuridine-incorporating cells compared with the parental cultured cells. These findings indicate that the sphere forming assay enriches precursors with longer telomeres, higher telomerase activity, and younger progeny than the original cells. Thus, the sphere forming assay may contribute to obtaining the young HCECs needed for regenerative medicine [21].

In another study, the cultured cells partially retained the properties of neural crest and periocular mesenchyme which are believed to be the source of origination of corneal endothelium via the neural crest using serum free media. The progenitors have a high

proliferative potency and possess endothelial function which was checked by Ussing Chamber and transplantation in the rabbit cornea [20].

### **The culture of human corneal endothelial cells using conditioned medium**

Many human corneal endothelial cell culture media have been described earlier with a combination of endothelial growth factors, base medium, serum, growth factors, insulin, vitamins etc [17]. There have been several studies utilising conditioned medium from other cell types to promote HCEC proliferation. These include conditioned medium from mouse embryonic stem cells, human bone marrow derived mesenchymal stem cells and human amniotic fluid.

#### *Mouse embryonic stem cell conditioned medium –*

Studies were performed to understand if mouse embryonic stem cell conditioned medium had any effect on the proliferative capacity of HCECs *in vitro*. Primary HCECs were cultured in human CEM containing 25% ESC-CM for the experimental group and CEM alone for the control group. The results showed that HCECs in the 25% ESC-CM group resulted in polygonal cells on day 2, whereas those in the CEM group showed slightly larger cells during days 3-4. HCECs in the 25% ESC-CM group could be sub-cultured until 6<sup>th</sup> passage without increasing in cell volume whereas those in the CEM group were cultured and lost their polygonal appearance by passage 2. Cells in both the groups expressed ZO-1, Na<sup>+</sup>-K<sup>+</sup> ATPase, VDAC3, SLC4A4, and CLCN3 (described further in the article). Ki67 positive cells and the percentage of cells entering the S and G2 phases were higher in the 25% ESC-CM group than in the CEM group. The 25% ESC-CM group showed a decrease in apoptotic cells and p21 protein expression. Furthermore, it was also reported that the cells cultured in 25% ESC-CM had enhanced HCEC proliferation and promoted HCECs into the cell cycle [23-27]. To summarise, in these studies primary

cultures of HCECs were cultured using 25% mouse embryonic stem cell conditioned medium. Compared to control cultures, these cultures showed cells with increasing polygonal morphology (characteristic of healthy HCECs), the cultures could be sub-cultured further (until the 6<sup>th</sup> passage), had a higher number of cells expressing Ki-67 and fewer apoptotic cells.

*Human bone marrow derived mesenchymal stem cell conditioned medium –*

Human bone-marrow-derived MSCs derived conditioned medium (MSC-CM) use has also been suggested. It has been observed that cells cultured in MSC-CM showed regular morphology, functional phenotypes of intracellular junctions and pump functions as compared to those cells that were cultured without MSC-CM. An increase in cellular proliferation was also noted with double the amount of positive cells in MSC-CM cultures. Pump proteins such as VDAC3, CLCN3, SLC4A4 and p120 were expressed in the MSC-CM cultured cells. It also regulated the G1 proteins of the cell cycle which are required for endothelial functioning [28].

*Human amniotic fluid –*

Another study was designed to evaluate the effects of HAF on the growth of HCECs and to establish an *in vitro* method for expanding HCECs. This showed that 20% HAF containing medium exhibited a greater stimulatory effect on HCEC growth and could represent a potential enriched supplement for HCEC regeneration studies [29].

The conditioned media that have been discussed so far either have an animal origin or an animal derived component, mainly serum. However, a xeno-free media would be more suitable to reduce the chances of transmission of prions or any potential disease from the animal origin. Moreover, it is always required by the regulatory authorities to show the

minimal risks of using animal derived components for human use or use a complete synthetic medium. This will further be challenging but required both in terms of healthcare and regulatory issues.

### **Cultivation of the primary human donor corneal cells using a dual media approach**

A study by Peh GS *et al* demonstrated that the outcome of culturing primary HCECs and proliferative potential can be successful if negatively impacted by lower, sub-optimal plating density. The study showed that it was possible to obtain the hexagonal morphology of the cells at the end of third passage and the count of up to  $2.5 \times 10^7$  cells with a seeding density of greater than or equal to  $1 \times 10^4$  cells per  $\text{cm}^2$  [30]. However, after defining the seeding density, the same group also described a novel dual media approach for the expansion of primary HCECs *in vitro*. Analysis of growth dynamics of the cells in proliferative or maintenance media was carried out. At the third passage, homogeneous appearance of the cells and polygonal morphology was observed using dual media approach. The cells also expressed endothelial associated markers. Using this method, 7 day exposure to maintenance media showed differential gene expression associated with cell proliferation and wound healing which eventually confirmed that this is a reproducible and consistent method for the culture of HCECs [31].

### **Human corneal endothelial cell markers**

Structural markers such as ZO-1 which is a tight junctional protein have been used widely to study the junctions of *in vitro* expanded HCECs. As the major function of the corneal endothelium is to prevent corneal swelling by the activity of membrane pumps, these are also used as functional markers for cultivated HCECs. These pumps include  $\text{Na}^+$ - $\text{K}^+$ ATPase, VDAC3, CLCN3, SLC4A4 and p120.

Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), a target of Wnt signaling has also been identified as a potential biomarker for HCECs. LGR5 has been widely used as a marker of intestine, stomach and hair follicle stem cells in mice models however human corneal tissues were used to study functional gain and loss. The peripheral region of corneal endothelial cells shows a high expression of LGR5 and the cells that express this protein have also been shown to have stem/progenitor cell characteristics. It has been observed that LGR5 is the target molecule of the Hedgehog (HH) signaling pathway in the human corneal endothelium. More importantly, this study showed a repeatable expression of LGR5 that helped to maintain endothelial cell phenotype and the potential to inhibit mesenchymal transformation (MT) through the Wnt pathway. Thus, this study highlights new findings that underline homeostatic regulation of human corneal endothelial stem/progenitor cells by LGR5 through the HH and Wnt pathways that are important for corneal endothelial cell functioning [32].

Another study reported the characterization of HCECs in developmental stages. mRNA transcriptomes in human fetal and adult corneal endothelium were studied. High expression signature genes were identified for both fetal (245) and adult (284) HCECs. Many of these genes were identified as disease target genes in hereditary corneal dystrophies, consistent with their functional significance in HCEC physiology. Immunohistochemistry showed localization of four novel markers for fetal and adult HCECs such as Wnt5a, S100A4, S100A6 and IER3. This can further be used to characterize HCECs derived from stem cells or *ex vivo* expanded cells [33].

As the currently used markers are not highly satisfactory due to their non-specific or co-expression in other cell types, a published RNA-seq data of CECs and the FANTOM5 atlas representing diverse range of cell types based on expression patterns was studied.

Five genes CLRN1, MRGPRX3, HTR1D and ZP4 were identified as novel markers for CECs. The specificities of these genes were confirmed at RNA and protein levels. These markers could further be used for purification of actual CECs and to evaluate the products derived from other cell types [34].

### **Wound healing of CECs**

Rho-associated protein kinase (ROCK) inhibitor helps in regulating the shape and movement of cells by acting on their cytoskeleton. It has been reported that ROCK inhibitor Y-27632 promotes adhesion, inhibits apoptosis, increases monkey CECs *in vitro*, and has also been suggested as enhancing corneal endothelial wound healing *in vivo* in animal models. HCECs did not show any toxicity or cell alterations when treated with ROCK inhibitor. Although it did not show any cell proliferation, ROCK inhibitor significantly enhanced cell adhesion and wound healing. Thus, as ROCK inhibitor did not show any toxicity, its benefits in corneal wound healing and adhesion may be relevant for therapeutic purposes [35].

### **Tissue engineering HCEC sheets**

*Scaffolds* - Once the cells are cultured, it should be seeded on a transportation unit. The scaffolds play an important role in keeping the cells viable and transplanting the same being a bio-mimetic or as synthetic material. Earlier reports have shown that the cultured CECs have been seeded on the denuded DM scaffolds or stromal discs [36]. Human amniotic membranes have been used as a carrier for cultured CECs [37], but due to its translucent nature, it is not highly recommended for clinical applications. Gospodarowicz *et al* [38-40] tried to combine two species by seeding bovine CECs onto rabbit corneas denuded of their endothelium. It was reported that when corneas were transplanted back into rabbits, the corneal buttons showed full transparency without any edema [38-40].

Recently, many methods for culturing CECs on synthetic or biological grafts have been established. Mimura and colleagues used a network of loosely cross-linked type I collagen fibers for CECs [41]. Corneal edema decreased rapidly after transplantation in DSAEK groups [41]. Out of the many scaffolds that are described in the literature and have proven its efficiency *in vitro* or in animal models, Koizumi and colleagues cultured monkey EC sheets on collagen type I carriers into monkeys' eyes however the corneas recovered their clarity only six months after transplantation [42]. The scaffolds for CECs transplantation can be biologic or synthetic, permanent or biodegradable. Common biological materials in scaffolds reported so far are collagen, fibronectin, and hyaluronan. However, the next step forward would be to check the feasibility of the use of these scaffolds *in vivo*.

*Nanotopography* - It has been reported that in the native environment, corneal endothelial cells interact with the nanotopography of the underlying Descemet's membrane. The study showed that nanotopography enhanced bovine corneal endothelial cell (BCEC) responses and created a monolayer which resembles the healthy corneal endothelium. Topographies of different geometries were first tested to identify those that would elicit the most significant responses. A BCEC monolayer was generated on both micro and nanoscale pillars and wells, and these topographies showed polygonal geometries with well-developed tight junction proteins. SEM revealed that cells on pillars showed a higher density of microvilli, which was similar to native corneal endothelium. BCECs on nanopillars displayed a lower coefficient of variation of area that was within the range of healthy corneal endothelium. More importantly, a BCEC monolayer cultured on nanopillars also had enhanced Na(+)/K(+)-ATPase immunofluorescence expression and mRNA upregulation and a higher Na(+)/K(+)-ATPase activity. These results suggest that

nanopillar substrate topography may provide the relevant topographical cues, which could significantly enhance the formation and function of the corneal endothelium [43].

*Hydrogels* - In a recent study, fabrication of biocompatible and biodegradable poly(ethylene glycol) (PEG)-based hydrogel films (PHFs) for the regeneration and transplantation of CECs has been described. 50- $\mu\text{m}$  thin hydrogel films have similar or greater tensile strengths to human corneal tissue. Light transmission studies revealed that the films were >98% optically transparent, while *in vitro* degradation studies demonstrate their biodegradation characteristics. Cell culture studies demonstrate the regeneration of sheep corneal endothelium on the PHFs. Although sheep CECs do not regenerate *in vivo*, these cells proliferate on the films with natural morphology and become 100% confluent within 7 days. Implantation of the PHFs into live sheep corneas demonstrates the robustness of the films for surgical purposes. Regular slit lamp examinations and histology of the cornea after 28 days following surgery revealed minimal inflammatory responses and no toxicity, indicating that the films were benign. The results of this study suggest that PHFs are excellent candidates as platforms for the regeneration and transplantation of CECs as a result of their favourable biocompatibility, degradability, mechanical, and optical properties however, *in vivo* trials will show its real potential in the future [44].

*Thermoresponsive plates* - Cellular organization of foreign grafts constructed from cultivated cells is critical to successful graft-host integration and tissue repair. This study described a novel HCEC therapeutic method, where cultivated adult HCEC sheet with uniform orientation was prepared and transplanted to the rabbit cornea. Having a correct morphology and intact barriers, the HCEC sheet was made by the temperature-modulated detachment of monolayered HCECs from thermoresponsive poly-N-isopropylacrylamide (PNIPAAm)-grafted surfaces and was delivered with proper polarity to the corneal

posterior surface by a bio-adhesive gelatin disc. Results of the in vivo studies, including the follow-up clinical observations and histological examinations, showed the laminated CEC sheet successfully integrated into the rabbit corneas denuded of their endothelial layer after the bio-degradation of gelatin carrier. More importantly, the feasibility of handling and delivery of a mono-singular layer of cell sheet in a clinical setting was a major advantage. These data indicate the feasibility of the proposed procedure in cell therapy for corneal endothelial cell loss [45].

### **HCEC transplantation**

Multiple studies have demonstrated the possibility of culturing HCECs and the current prospects are to study the transplantation potential of cultured cells in human and animal models. In one study of note, collagen sheets were used as substitute carriers for cultured HCECs. Pump functions showed 76%-95% of those of natural human donor corneas. Thickness was significantly less and no stromal edema was found post surgery in a rabbit model [46]. In another study the cells were injected in the anterior chamber with face down position in the rabbit models. Very early studies on cultured human corneal endothelial cells have been recently carried out on monkey models. Recently, 11 human patients (unpublished clinical trial) with bullous keratopathy or Fuch's dystrophy have been treated using the same technique and 20/20 vision has been attained post-operatively. Early stage experiments using Rho Kinase inhibitor drops have also showed promising results in terms of visual recovery, thickness and maintenance of intra-ocular pressure as reported during ASCRS 2015 symposium (S. Kinoshita).

### **Immunosuppression**

Although there are studies carried out on transplanting the cultured corneal endothelial cells in human recipients, it should not be left out of consideration that immunological

responses may hinder the outcomes. The corneal graft has a huge immune privilege as compared to other transplants due to absence of blood and lymph vessels in the graft and its bed, absence of MHC class II<sup>+</sup> antigen presenting cells in the graft, reduced expression of MHC-encoded alloantigens on graft cells, constitutive expression of T cell-deleting CD95 ligand on corneal graft endothelium, the existence of an immunosuppressive local microenvironment (aqueous humor), and the capacity of the graft to induce anterior chamber associated immune deviation (ACAID). Precisely, corneal endothelial cells have a distinct molecular strategy to reduce their antigenic visibility to CD4<sup>+</sup> and CD8<sup>+</sup> effector T cells, and to alter the functional program of responding T cells. This may be advantageous in terms of donor corneal transplantation. However, the immunological reactions of the *in vitro* cultured and transplanted corneal endothelial cells are still being studied [47].

### **Differentiation of other cell types into HCEC**

Studies have also shown the potential to derive CEC-like cells from human embryonic stem cells (hESCs). The differentiation potential of hESCs to HCECs through the periocular mesenchymal precursor (POMP) phase has been shown using transwell co-culture system of hESCs with differentiated human corneal stromal cells. CEC-like cells were derived from POMP cells using lens epithelial cell-condition medium. Corneal endothelial differentiation marker N-cadherin and transcription factors FOXC1 and PitX2 were expressed within 1 week of culture. The isolated cells were seeded onto posterior acellular porcine corneal matrix lamellae to construct the CEC-like cell sheets. These cell sheets were transplanted into rabbit eyes and the transparency was found to have restored gradually. The cells derived from this source displayed characteristics of native human CECs [48].

Umbilical cord blood mesenchymal stem cells have also been differentiated into HCECs and to determine whether these MSCs can 'home' to sites of corneal endothelial cell injury using an *ex vivo* corneal wound model. RNA was isolated and purified from UCB MSCs and HCECs. The ability of different culture media was determined and the morphology, immuno-localization and gene expression was determined in both tissue culture and *ex vivo* corneal endothelial wound models. MSCs attached to damaged, but not intact, corneal endothelium in *ex vivo* corneal wounds. The results have indicated the potential differentiation of cord blood derived MSCs towards HCEC-like cells [28]. However, further studies will be required to identify the specific microenvironmental conditions that would permit tissue engineering of UCB MSCs to replace damaged or diseased corneal endothelium.

ESCs and iPSCs have an extensive self-renewal capacity and the potential to differentiate into any tissue specific cell lineages. A study carried out using *trans* retinoic acid (RA) treatment during the embryoid body (EB) on mouse ESCs and iPSCs showed the promotion of neural crest cells. These cells were further differentiated into CEC-like cells using lens epithelial cell conditioned media. For the initial differentiation into neural crest cells, dose of 1  $\mu$ M RA on day 4 of EB formation showed neural crest differentiation. Plating these cells on gelatine-coated plates further led to cell migration out of EBs. Further, LEC-CM enhanced the differentiation of neural crest into CEC-like cells which was confirmed using ICC and RT-qPCR. This study showed a potential of two-step inducement procedure for treatment of corneal endothelial failure [49].

In conclusions, dysfunction in the corneal endothelium, which controls the hydration and transparency of the cornea, is one of the most common reasons of corneal transplantation. The current primary treatment to cure the endothelial failure is

replacement of the diseased corneas with a healthy donor tissue. However, globally the donor tissue supply is too low as compared to the requirement and therefore there is an enormous interest in the development of alternative treatment or therapeutic strategies to decline the current requirement of human donor corneas. A tissue-engineered corneal endothelium is of interest for corneal regeneration and for *in vitro* testing of ocular drugs. Cell therapy focuses on the culture expansion of corneal endothelial cells retrieved from the donor followed by transplantation to many recipients. Recently, research has focused on overcoming the challenge of harvesting human corneal endothelial cells and the generation of new bio-membranes to be used as cell scaffolds in surgical procedures. But a standard culture method has not been well established yet for the clinical purposes. The studies using telomerase activity, Schwalbe's line, progenitor cells indicated the presence of potential stem-like or progenitor cells in the periphery of the corneal endothelium. However, no clear evidence of corneal endothelial 'Stem Cells' have been reported so far. The progenitors are isolated from the periphery of the corneal endothelium and have been exploited for the proliferative capability with early success. Till date the endothelial cell markers were non-specific but with the identification of novel markers, the researchers are one step ahead in terms of characterizing the endothelial cells. Although a few patients have already been treated using injection method, it is also essential to facilitate the validation of cell-injection therapy, or downstream development of an alternative corneal endothelium construct through cell-tissue engineering. This, in turn, will elicit greater confidence in facilitating downstream development of alternative corneal endothelium replacement using tissue-engineered graft materials or cell injection therapy. Current results highlight important emerging advances in the development of new treatment strategies for corneal endothelial dysfunction in humans with a better hope to treat these disorders with minimal clinical manipulations [50].

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## **CHAPTER 3**

# **BIOCHEMICAL AND MECHANICAL BEHAVIOUR OF DESCEMET'S MEMBRANE**

*Derived from published research article*

Mohit Parekh, Alessandro Ruzza, Rosa Di Mundo, Stefano Ferrari, Giuseppina Recchia, Hossein Elbadawy, Giuseppe Carbone, and Diego Ponzin. Role of dextran in maintaining adhesive and stiffness properties of pre-stripped DMEK lenticules. European Journal of Ophthalmology. 2016 Nov 25:0.[ PMID: 28009413]

Before initiating the cell culture, it is important to learn the properties of the extracellular matrix of corneal endothelial cells which is the DM. The HCECs release the DM and adhere strongly on to the stroma and therefore the constituents of this material are important for cell adherence and proliferation. The elasticity of this membrane and implantation strategy at this stage would also allow exploring different possibilities to preserve the cells for the final transplantation.

### **Aim**

To investigate the adhesive and stiffness properties of pre-stripped DMEK lenticules in different preservation conditions (with and without dextran).

### **Introduction**

EK is a valid alternative to PK for the management of corneal endothelial failure [1-4]. Compared to PK, EK leaves a more secured eye with less post-operative astigmatism and faster visual rehabilitation with better optical quality. Different types of dissection techniques and instruments have been described to excise the very fragile DM and endothelium exclusively [5-9].

Currently, organ culture is the most commonly used corneal storage method in Europe. It allows long storage times of approximately 4 weeks [10]. The TCM induces stromal swelling, reduces corneal transparency, and increases stromal and DM folding [10]. To facilitate surgery, the corneal graft thickness should be similar to that of the recipient's cornea while performing PK [11]. Therefore thinning the tissue is usually achieved by using a hyperosmolar medium (i.e. 5-6% dextran T500) for a minimum of 24 hours. As DMEK involves the transplantation of just a thin monolayer of endothelial cells and the

DM, which does not swell during TCM preservation due to the absence of stroma, it is not mandatory to de-swell the pre-stripped DMEK grafts [12]. Therefore, it was important to study the effect of the preservation conditions on corneal stiffness or adhesion of the pre-stripped DMEK lenticules and the extent of manipulation during surgery.

Adherence of the graft is an important parameter considering the re-bubbling rate during DMEK surgery post-operation [13,14]. Although there are many reasons that are correlated to re-bubbling rates such as the diameter of descemetorhexis [15] and surgical trauma. It has been reported that the donor characteristics may have no direct influence on re-bubbling rates [14] however, the preservation conditions has not been evaluated so far especially considering the rigidity of the graft. Therefore, to determine the optimum preservation media and conditions for pre-stripped DMEK lenticule, it is necessary to study the expression of adhesive proteins that are retained on the tissue when preserved in specific medium. In parallel, evaluation of the elastic properties of the tissue is needed to understand the extent of manipulation during the surgical procedure. The adherent properties can be studied using antibodies specific for adhesive proteins and the elasticity can be determined either using the elastic proteins or by the elastic modulus (or Young's modulus,  $E$ ) of the DM, thus understanding whether the mechanical properties of the tissue could vary as a consequence of different preservation media. The elastic modulus ( $E$ ) of a material can be determined from nano-indentation measurements on its surface [16-19]. Due to the high compliance and thinness of the investigated biological material (i.e. DM), the nano-indentation measurements have been performed with AFM since this method allows utilizing loads as low as few nN. The space resolution is also remarkable since the curvature radius of the probe tips is of few nanometers [16].

The purpose of the reported study thus was to evaluate the effect of preservation medium and their cross-combinations to determine the adhesive and stiffness profiles of pre-stripped DMEK tissues post preservation.

## Materials and Methods

### *Ethical statement and collection of samples*

Fifteen unsuitable corneas for transplantation were collected from FBOV, Italy, with a written consent to be used for research from the donor's next of kin. The donor corneas did not show indications of any systemic disease, dystrophy or infections. The corneas were not suitable for transplant due to low endothelial cell count (<2200 cells/mm<sup>2</sup>).

### *Characteristics of the samples*

The average age of the donors was 68.46 (±5.01) with male:female ratio of 13:2 and post mortem time of 11.96 (±5.99) hours. The tissues were preserved in TCM for 20.11 (±10.49) days and were further transferred to the de-swelling/TM when required with 6% dextran T-500 for 3.5 (±1.64) days.

### *Experimental conditions*

Different conditions of the preservation medium and their combinations that were tested during this study are listed in table 1.

	<b>Condition 1 (C1)</b>	<b>Condition 2 (C2)</b>	<b>Condition 3 (C3)</b>
<b>Tissues collected from</b>	TCM	TM	TCM
	Stripping and Loading		
<b>Preserved in</b>	TCM	TM	TM
	Analysis		

**Table 1:** Different conditions of the preservation media before and after stripping the DMEK lenticule. Three samples per condition were used for immunostaining to study the adherence and elastic properties. Two DM samples per condition were used for AFM, nanoindentation method to study the rigidity of the tissues.

### *Methodology*

#### *Stripping method*

All the corneas [n=15] were centered on the base of a trephine punch using the peripheral holes of the suction area as reference. The corneal tissue was secured on the base, using vacuum, with endothelium facing the air. A 9.5 mm diameter punch (Moria, Antony, France) was used to create a superficial cut by gently tapping the endothelial surface (cut-edge) of the tissue. The endothelium was submerged with the TCM or de-swelling medium/TM as required in the experimental condition to create a thin film of fluid. The membrane was lifted using a cleavage hook throughout the circumference to limit the peripheral tearing of the very fragile DMEK tissue. Using acute forceps (e.janach, Como, Italy), the membrane was stripped with a longitudinal movement from 3 sides ensuring that no torsions were generated during this phase. The tissue was stripped leaving a hinge behind (attached at the periphery) and were re-stored to its initial place by opening the tissue using surgical sponges. The tissues were further preserved in their respective media for 4 days at room temperature.

#### *Immunofluorescence*

The tissues [n=9] were stripped leaving a hinge behind and were preserved in different conditions of preservation medium (n=3 per condition), as listed in table 1. The tissues were washed with 1X PBS and were fixed overnight using 4% PFA. Later, they were washed in increasing concentrations of sucrose solution (7.5%, 15% and 30%

respectively) and embedded in optimal cutting temperature compound (OCT) for microtome cutting. All the samples were immunostained with the following antibodies: anti-fibronectin (ab2413), anti-vitronectin [VN58-1] (ab13413) and anti-laminin (ab11575) for adhesive proteins and anti-fibrillin (ab53076), anti-collagen VI (ab118955) and anti-elastin (ab21610) for elastic proteins. All the antibodies were bought from Abcam, Cambridge, UK.

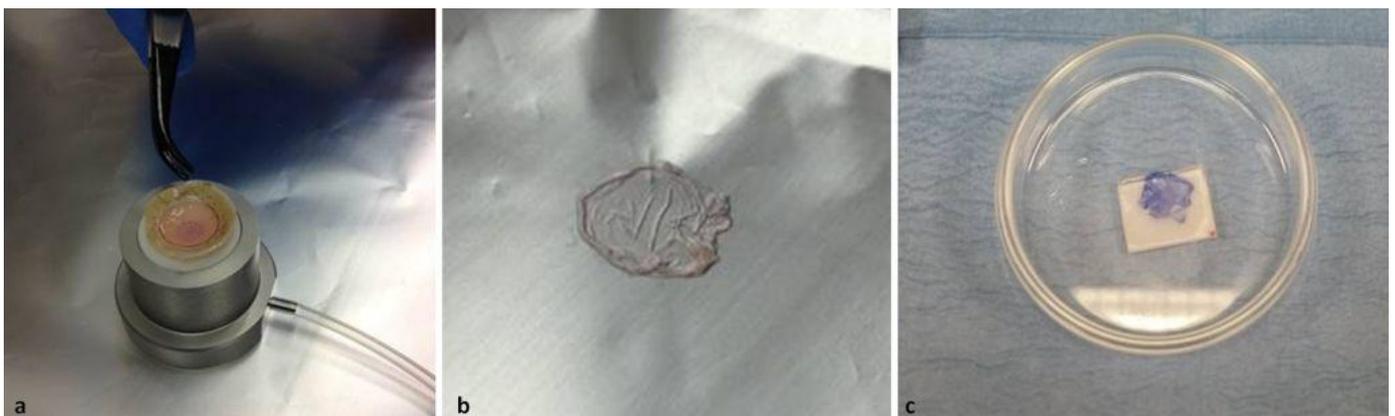
The sections were washed twice with 1X PBS for 5 minutes and permeabilized using 0.5% Triton-X for 30 minutes at RT. Using a blocking mix (PBS, bovine serum albumin, goat serum and triton-x), the sections were blocked for one hour. All the primary antibodies were added at 1:100 apart from anti-Elastin and anti-Fibrillin which were added at a concentration of 1:50 and incubated at RT for 2 hours. A FITC-conjugated secondary antibody was added at 1:100 and the sections were incubated for 1 hour at RT. The sections were mounted using Vectashield and the cover slips placed and viewed using a laser scanning confocal microscope LSM510 (Carl Zeiss, Milan, Italy) at 488 nM.

### AFM nanoindentation

#### *Sample preparation*

The tissues [n=6] were stripped leaving a hinge behind and shipped to the AFM center in different conditions of preservation medium (n=2 for each condition), as listed in table 1. A critical issue for the AFM analysis in liquid was to keep the membrane well opened and firmly adhered to a solid support (such as a cell ground) as the DM folds spontaneously in the liquid environment. Therefore a procedure to make the DM lenticules adhere to a solid support under liquid was optimized as follows. Solid support PC (Polycarbonate) slabs (1 mm thick) were chosen for this study. These slabs were roughened with sand paper (P1000 grade) in order to improve adhesion of the membrane. The tissues were picked

from the opposite end (stripped end) of the hinge and spread out onto an Aluminium (Al) foil with the endothelial side facing the air. The tissue was made visible with *Trypan Blue* stain. The Al foil was used since it keeps the tissue (DM) open and allows, by just reversing it, to lay the tissue on the Al foil. The tissue was kept open on the foil and was put in contact (inverted) with the PC slab point where a glue drop (2-4  $\mu$ l) had been positioned to expose the DM to the air. After 2 minutes (adherence time), the Al foil was detached and the tissue was then left firmly adhered with cyanoacrylic glue on the PC support with the endothelial side touching the base and the DM (non-endothelial side) facing upwards, i.e. the cantilever AFM tip. Figure 1 shows a DM sample immobilization according to the procedure mentioned above.



**Figure 1:** Set up of AFM nanoindentation. a) Pre-stripped DMEK on the vacuum block, as sent from the FBOV labs to the AFM center. Descemet Membrane (DM) was separated leaving a hinge behind. b) DM was placed on an Al foil with endothelial side facing the air. c) DM with the non-endothelial side facing the air was separated from the cornea and immobilized with cyanoacrylic glue on polycarbonate slab (stained with Trypan Blue), ready to be inserted in the liquid measurement cell once covered with deionised water. The membrane was stained with Trypan Blue to make it visible and make the operations easier.

### *AFM measurements*

Nano-indentation was performed using atomic force microscopy (AFM Ntegra from NT-MDT) in the *spectroscopy* mode. In this modality, the AFM tip was pushed into the sample (by moving the sample upwards) until a predefined deflection was obtained comparable to the set-point deflection value. Once the tip reached this point, it was retracted again. During this complete loading-unloading cycle, the position of the tip as well as the force exerted on the cantilever was accurately monitored, resulting in a force–distance (height) curve. The tissues were analyzed in liquid environment in order to avoid any modification of their properties as well as to avoid any adhesion falls due to jump-in/jump-off phenomena in acquired force-distance curves. A CSG01 (NT-MDT) probe was utilized for these experiments. This was chosen as a consequence of its very low force constant (typical  $K=0.03$  N/m), as suggested for very soft (biological) materials [16-21].

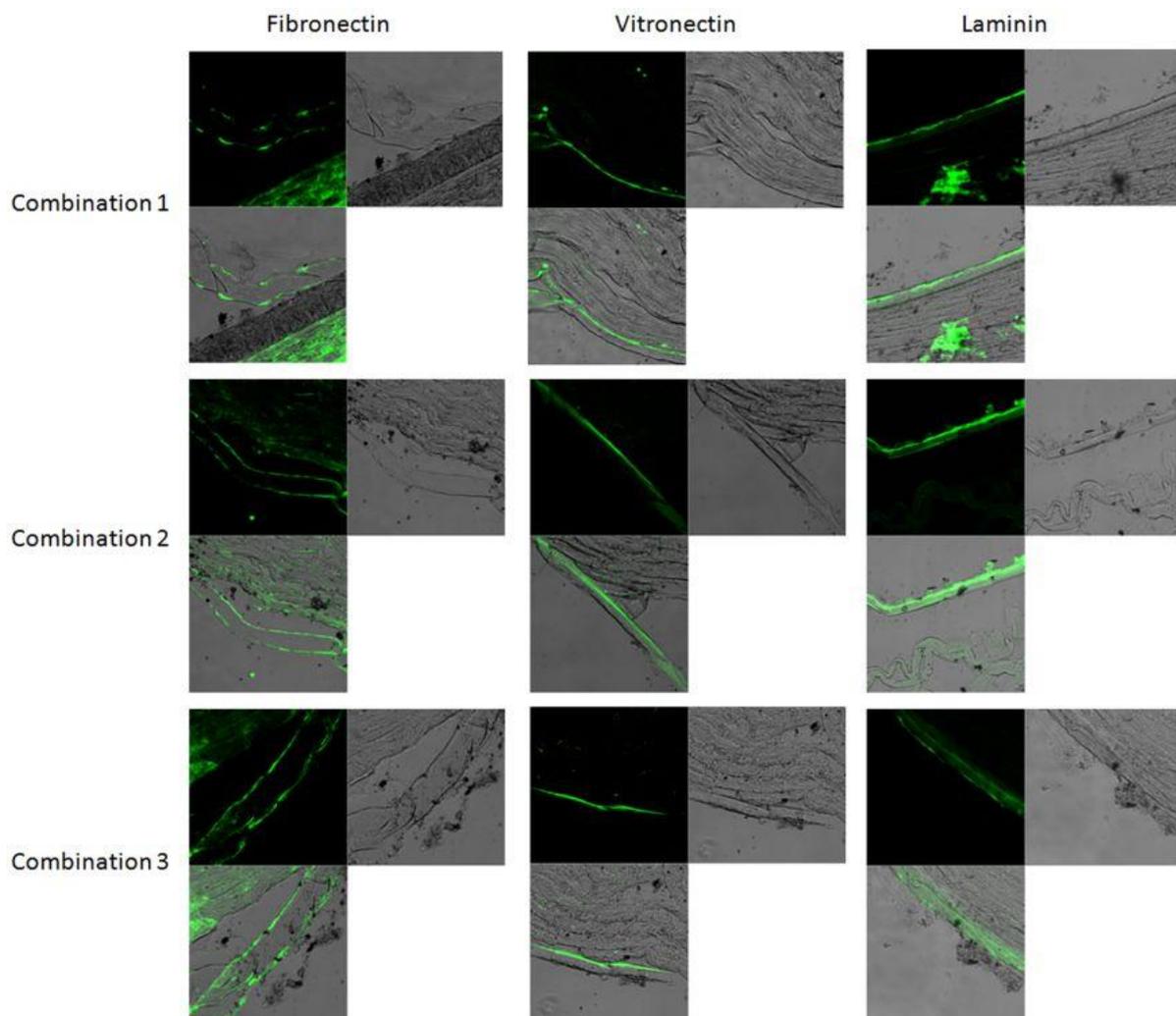
Deionized water was used as liquid after having verified that the storage liquids of the cornea samples were not suitable since they are too rich in macromolecules that adhere to the apparatus components. 30 indentations were performed on different points of each tested DM sample. Some indentations were also performed under the same experimental conditions on the surface of the PC slab in order to acquire the force-height curve on a rigid (undeformable) reference. It was previously verified that this procedure allows obtaining expected values of elastic modulus on benchmark soft materials (polyethylene and polytetrafluoroethylene).

Statistical analysis was performed using student's t-test where  $p<0.01$  was deemed statistically significant.

## Results

### *Expression of adhesive proteins in dextran based medium*

Fibronectin was localized at different points on the DM in condition 1 (C1), however it was strongly expressed throughout in condition 2 (C2) and condition 3 (C3), which contains dextran. Vitronectin showed expression in all the samples from each condition on the basement of the DM facing towards the stroma. Laminin was expressed in all the conditions at the junction between the endothelial cells and the DM but was intense in C2 (Figure 2).

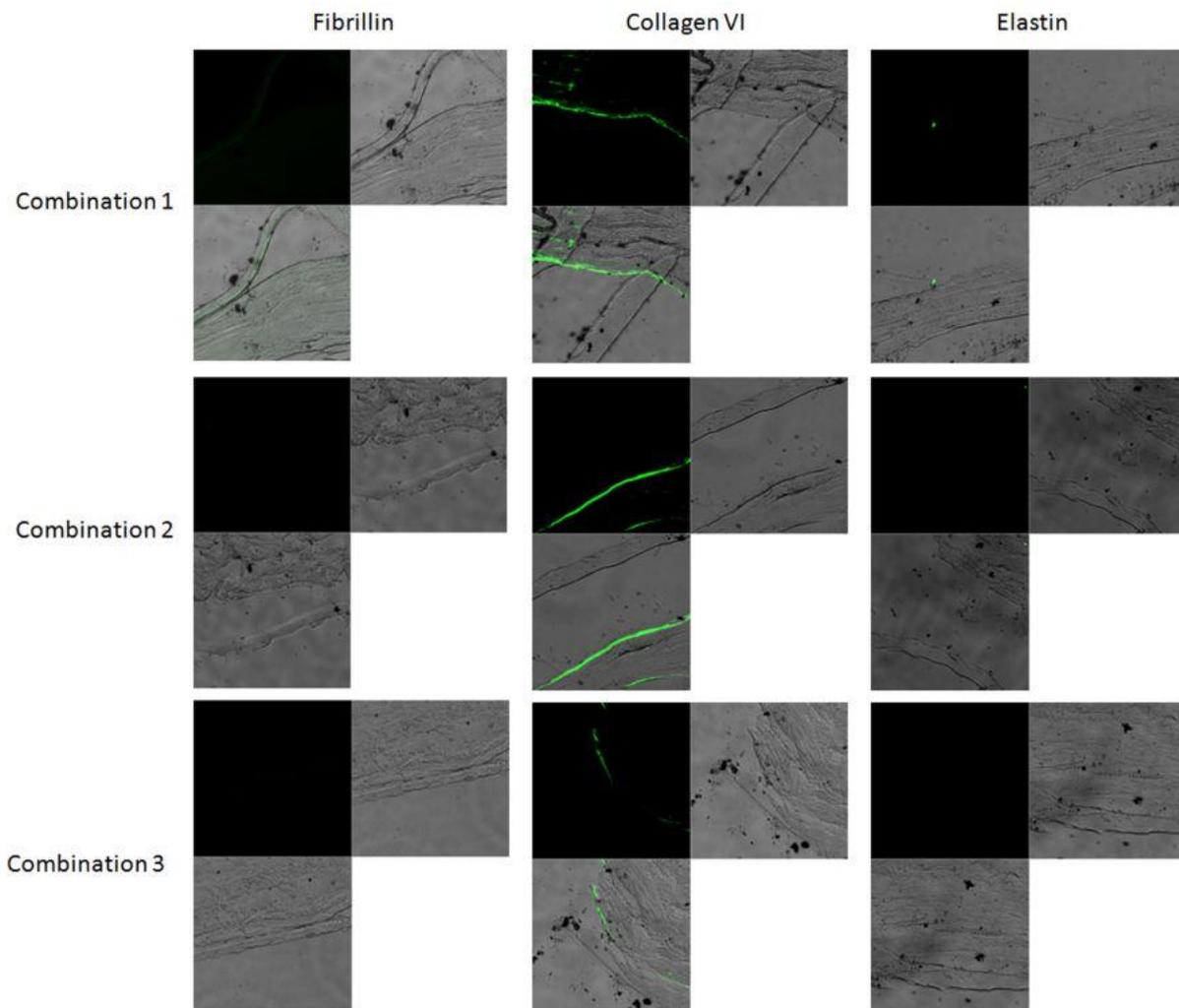


**Figure 2:** Expression of adhesive proteins on the pre-stripped DM preserved in different conditions of the preservation medium observed at 400X magnification. Fibronectin was found throughout on both, the endothelial and the stromal side of the DM apart from

condition C1 that did not show expression throughout (localized). Vitronectin was expressed on the stromal side of the DM in all the conditions. Laminin, although expressed on the endothelial side in all the conditions, was highly expressed in C2.

*Expression of elastic proteins in dextran based medium*

None of the tissues from any condition showed the expression of any elastic protein on the DM, as shown in figure 3. Collagen VI was expressed in all the conditions, but on the posterior stroma and not on the DM (figure 3). It was therefore important to study the rigidity of the tissues using nanoindentation to check the stiffness of the DM.



**Figure 3:** Elastic properties on the pre-stripped DM preserved in different conditions of the preservation medium observed at 40X magnification. None of the samples showed expression of the elastic proteins in either condition on the excised DM apart from Collagen VI that was expressed in all the conditions on the posterior stroma.

*Influence of dextran on stiffness properties of pre-stripped DMEK lenticules*

For the nanoindentation measurements, the deflection set point was fixed at 0.7 nA, which gives rise to a maximum force of about 5 nN. For the data processing and elastic modulus calculation unloading curves were utilized. It is worth pointing out that negligible hysteresis was observed during the approaching-retraction cycles, reasonably as a consequence of the absence of cells on this side (non endothelial side) of the membrane [21].

A sketch of the indentation process of the tissue is presented in figure 4a. The length of tip penetration in the sample at the highest force is the indentation depth ' $\delta$ ' which is measured as height difference with respect to the rigid (undeformable) reference.

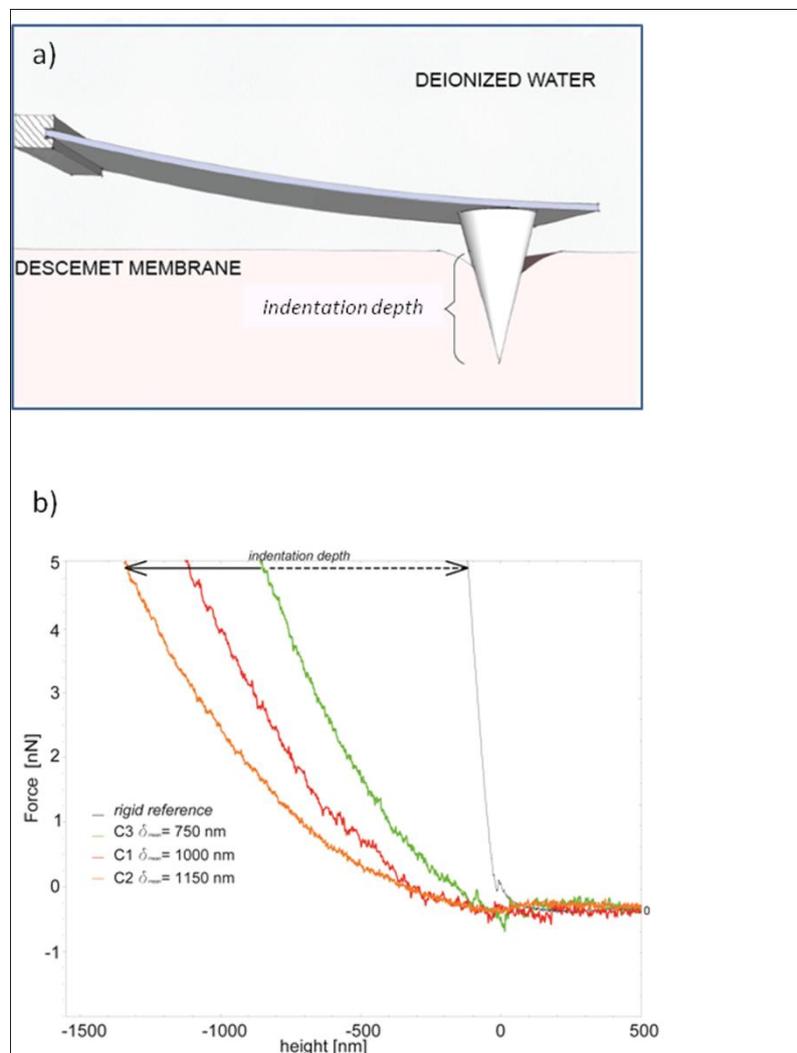
In figure 4b, the force-height curves obtained by these measurements are presented along with the curve obtained on the rigid reference. Calculation of the penetration depth ( $\delta$ ) is thus calculated as a height difference between the sample and the reference curve at the maximum force (5 nN). In particular, in this diagram the curves showing a penetration depth corresponding to the mean penetration depth value ( $\delta_{\text{mean}}$ ) for the three investigated combination is reported.

From the graph in figure 4b, the indentation spans in the range of 750-1150 nm (500-800 nm and even values higher than 1  $\mu\text{m}$  has been found in other curves). No interference of

the sample support (PC slab) is expected since the indentation depth is not higher than 1/10 of the sample depth, as it is expected to be in nanoindentation measurements [17]. By analyzing the type of relation existing between  $F$  and  $\delta$ , it has been found that the shape of the contact area tip-sample is conical (figure 4a). Thus the following equation has been used to extract the elastic modulus,  $E$  [22]:

$$F(\delta) = \frac{2E}{\pi t g \alpha (1-\nu^2)} \delta^2$$

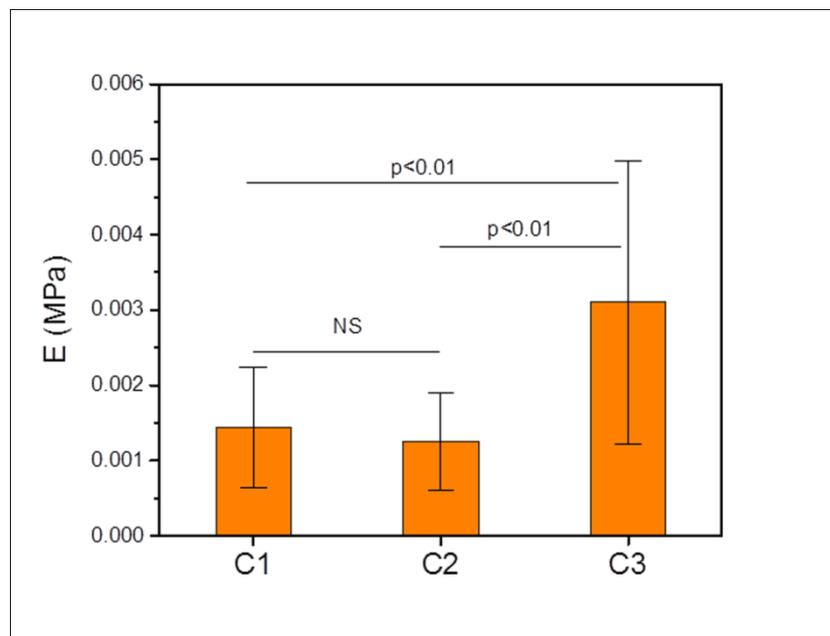
where,  $\alpha$  is the semi-vertical angle of the tip,  $\nu$  and  $E$  are the Poisson ratio and the elastic modulus of the indented tissue-respectively.



**Figure 4:** Atomic force microscopy of DM. a) Nanoindentation of the DM lenticule by the tip of an atomic force microscope and b) examples of force-height curves obtained from

these measurements from which the elastic modulus values were derived. The curves reported in this diagram represent the mean values of penetration depth ( $\delta_{\text{mean}}$ ) measured on membranes of the C1, C2, C3 combinations.

The mean elastic modulus of the tissues from conditions 1, 2 and 3 was found to be 0.0014, 0.0012 and 0.0031 MPa respectively. Only for condition 3, the mean E value was significantly different compared to the other conditions indicating a higher stiffness of the samples preserved according to condition 3 (figure 5). There was no significant difference in stiffness between C2 and C1 conditions, thus overall more flexible tissues as compared to C3. The difference between C1 and C2 was statistically insignificant ( $p=0.23$ ); however, the differences between C1-C3 and C2-C3 were statistically significant [ $p < 0.05$  ( $p=2.1 \times 10^{-9}$  and  $p=2.3 \times 10^{-8}$ ), respectively].



**Figure 5:** Mean elastic modulus values from AFM nanoindentation analysis of the tissues from conditions 1, 2 and 3 (C1, C2, C3 in the histogram). The samples from condition C3 are those showing the highest stiffness. Difference between C1 and C2, giving a level of significance  $p$  (calculated by student's T-test) of 0.23 is non-significant (NS); the

differences between C1-C3 and C2-C3 are significant both with  $p < 0.01$  ( $p = 2.1 \times 10^{-9}$  and  $p = 2.3 \times 10^{-8}$ , respectively).

This may highlight that when the tissues are excised and completely exposed (DM+Endothelium) to dextran after storage, the tissues gain stiffness as compared to those preserved in TCM or TM throughout. If the tissues are preserved in dextran based media before and after stripping, they show more compliance (C2) as compared to those that are preserved in TCM and then exposed to Dextran post stripping as seen in C3.

## **Discussion**

EK has dramatically evolved as an alternative to PK in the recent years [23]. DMEK is becoming popular in terms of faster visual recovery, less postoperative astigmatism and reduced risk of transplant rejection, as compared to the other EK procedures [24-29]. However, it is also important to understand how the preservation techniques may play a role in creating a different biophysiological environment of the cornea. Failure of graft preparation during the learning curve has been reported in approximately  $\leq 16\%$  of donor corneas [30]. The preservation conditions may play an important role in graft preparation, preservation and transplantation and on the corneal biomechanics.

TCM has advantages as longer times of preservation allow organizing the transplant better and to perform microbiological and serological tests. However, as TCM swells the cornea almost to its double, the tissues must be de-swelled to a more physiological thickness before transplantation. For this reason, tissues are preserved in a dextran based de-swelling medium. This de-swelling or TM allows the water-soluble macromolecule that produces colloid osmotic pressure to extract excess water accumulated in the stroma. The tissues are usually preserved up to 4 days to manage the de-swelling and transport the tissues to the surgical theatre avoiding any further endothelial cell damage. A functional

cell layer along with a smooth DM is essential for a successful DMEK surgery, as it allows both quick adherence of the lenticule to the underlying stroma of the recipient eye and reduces the possibility of dislocation. For DMEK, however, to become more useful and be adopted by the surgeons, it would be important to (1) reduce the wastage during graft preparation; (2) excise the tissues more reproducibly and (3) have preservation conditions that allow the tissue to reach the theatre with minimal damages. Hence we decided to check the adhesive properties of the DM when preserved in different conditions to identify whether the DM preserves its adhesiveness post preservation, which may eventually allow a more successful surgery. Higher stiffness may induce a higher tendency of graft tearing during the DM extraction and may cause high manipulations while unscrolling the graft inside the recipient eye. Stiffness of the tissue can be considered an important parameter as higher manipulation of the tissues inside the recipient eye may danger the graft survival with high endothelial cell loss.

Immunostaining experiments showed the expression of fibronectin throughout the tissues preserved in dextran based medium. Fibronectins bind cell surfaces and various compounds including collagen. They were expressed on both the anterior and posterior layer of the DM. Laminin was expressed in all the media however, it was intense in C2 (dextran based medium). Laminin binds to the cells with a high affinity receptor and is thought to mediate the attachment, migration and organization of the cells into tissues during embryogenic development by interacting with other extracellular matrix components. Therefore, we believe that dextran based media would be a more appropriate solution for preserving pre-stripped DMEK lenticules. Intense laminin expression was seen in the media containing dextran which may be useful for preservation or for binding of the endothelial cells onto the extracellular matrix, the DM. This may also prevent the endothelial cells from falling-off during the preservation and

transplantation phases. The expression profiles are shown in figure 2. No difference was found in the expression of vitronectin from either condition. It may be important to note that dextran-based medium may have a role in cell and tissue attachment during preparation, preservation, transportation and surgery. However, none of the samples showed any expression of elastic proteins and therefore nanoindentation was required to check the stiffness of these tissues. It has been reported earlier that fibronectin and vitronectin are present only on the stromal side of the DM along with some minute remnants that are present on the endothelial side of the tissue [31-35]. However, our samples showed the presence of fibronectin on both, the stromal and the endothelial sides, although the expression was not uniform throughout the entire DM.

The E values calculated from the force-height curves taken from six DM samples of different conditions were in the range of 0.001 to 0.004 MPa. Nanoindentation has previously been studied on different scaffolds and therefore it was determined to study the rigidity of the DM when preserved in different preservation media [16-19]. A higher E value was found in the samples from condition 3, indicating a slightly stiffer behavior of these samples. We assume that when the tissues are preserved in the same medium before and after stripping, they do not show any difference in the stiffness properties however, when the DM is excised and exposed from TCM to TM with dextran, the tissue adheres to more dextran and increases its rigidity, especially on the DM side towards the stroma. High rigidity may not be actually required, as it may not help to open the tissues inside the recipient eye during DMEK surgery. We believe that with higher flexibility the tissue can be manipulated easily in the recipient eye. Although there may not be any difference in the preparation method [12], the preservation of DMEK lenticules can be an important factor to manage critical surgeries.

It is essential to formulate strategies that improve graft adhesion after DMEK to reduce the re-bubbling rate. The re-bubbling rate of C1 which is similar to what is suggested by Melles et al has been found to be 5.2% [13] however, with C2 which is similar to what has been suggested by Terry et al. has been found to be around 10.6% [14]. However, Busin et al. have observed around 23.8% of re-bubbling rate from C2 [36].

It has been noted that the corneal donor characteristics does not have any potential influence on graft adhesion however, preservation of the bare DMEK lenticule has shown a higher flexibility (statistically insignificant) and lower endothelial cell fall-off from C2. Condition C3 provides a more rigid tissue, which could increase difficulties in manipulating or unrolling the tissue (endothelium flapped-out) during the DMEK surgery. Conditions C1 and C2 showed comparable rigidity. We have found that the endothelial cell fall-off is higher in cases when the DMEK is stripped from the TCM as compared to the preparation from TM which is contradictory to the literature [13]. We assume that a) due to a higher cell fall off from TCM and b) following preservation of the lenticule in TM (C3) may have allowed a higher area for the dextran to stick on the DM making it more rigid. This was not the case with C1 (TCM to TCM and therefore no interference of dextran) and C2 (preserved in Dextran with minimal cell fall-off). The tissue preservation systems are different in America (dextran based hypothermic media) and Europe (non-dextran based TCM followed by dextran based TM), thus the tissues obtained from TM may have a good preservation potential for preserving DMEK grafts.

In conclusion, although the sample size is a limitation to this study, we believe that condition C2 (i.e., tissues collected from TM, stripped and preserved in TM) showed to be, among the ones investigated, a suitable preservation solution to store pre-stripped DMEK lenticules. Although, the re-bubbling rates and the graft survival can change with different

procedures and diameter of descemetorhexis [15], the laboratory investigation states that preservation conditions like C2 may also be advantageous. In fact, tissues in C2 conditions showed maintenance of adhesive properties to the extra cellular matrix which may be useful in reducing the cell fall-off during the preservation and surgical phases. In addition, the higher compliance is likely to allow an easier manipulation of such tissues during the surgery.

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## **CHAPTER 4**

### **PRESERVATION OF PRE-LOADED DMEK LENTICULES**

### **IN DEXTRAN AND NON DEXTRAN BASED ORGAN**

### **CULTURE MEDIUM**

*Derived from published research article*

Mohit Parekh, Alessandro Ruzza, Stefano Ferrari and Diego Ponzin. Standardizing storage conditions for pre-loaded DMEK lenticules. Journal of Ophthalmology. 2016;2016:5830835. [PMID: 27994884]

## **Aim**

To evaluate the optimum preservation conditions for storing pre-loaded DMEK lenticules.

## **Introduction**

DMEK is a type of corneal surgery, which allows the transplantation of DM and endothelium [1-4]. DMEK has its own advantages as compared to PK in terms of better optical quality, early visual rehabilitation and less post-operative astigmatism with a much protected eye. As it does not involve excision of the entire cornea (optic zone) from the patient's eye like PK, it is considered a safer surgery. Various techniques have been identified for the preparation of this highly fragile tissue [5-12].

We, at the FBOV have recently started providing pre-loaded tissues for DSAEK and UT-DSAEK surgeries, a step further to pre-cut tissues [13,14]. This reduces the time and efforts in surgical theatre, increases efficiency of the DSAEK surgery and allows validated tissue to be used. Eye bank prepared DMEK tissues are usually pre-stripped, rolled or pre-bubbled and shipped to the operating room [8,12]. In our institute, these tissues are stripped and currently preserved in TM (tissue culture medium + 6% Dextran T500) which is a de-swelling medium required for transportation. As the tissue is only comprised of DM and endothelium, the requirement of dextran is not justified for preserving DMEK lenticule. However, due to the properties of dextran, which may be useful to keep the cells adherent to the extra-cellular matrix, its evaluation therefore becomes necessary.

TCM is the most commonly used corneal storage media in Europe while hypothermic-based preservation method is pursued in America and most of the world. As the tissue preservation is important to keep the endothelium viable, it becomes necessary to investigate the optimum condition to pre-load a DMEK lenticule, which is the next

advancement in the field of endothelial keratoplasty [15]. Pre-loading is likely to reduce the undesired effects that are seen while shipping the tissues as free floating or pre-stripped and allows transplanting a validated tissue. Thus, the aim of this paper is to study the optimum preservation conditions (medium with and without dextran) and to evaluate the possibility to preserve the DMEK lenticules flapped (tri-folded) in a closed chamber, i.e., to pre-load and provide a ready-to-use tissue to the surgeons for transplantation with minimal manipulations.

## **Materials and Methods**

### *Ethical statement*

Thirty human donor corneal tissues were collected from FBOV, Venice, Italy with a written consent from the donor's next of kin to be used for research.

### *Media constituents*

TCM was composed of 2% newborn calf serum with MEM-Earle as a base medium along with 25 mM HEPES buffer, 26 mM sodium bicarbonate, 1 mM pyruvate, 2 mM glutamine, 250 ng/mL amphotericin B, 100 IU/mL penicillin G, and 100 mg/mL streptomycin. TM was composed of TCM incorporated with 6% dextran T500. TCM and TM were prepared in house (FBOV, Mestre, Italy) with full regulatory compliance.

### *Pre-evaluation*

All the corneas were preserved in TCM before the study. However, to load the tissues and study the effect of the preservation medium on the tissues, ten corneas were further preserved in TM. The endothelial cells were evaluated using a hypotonic sucrose solution and the viability was checked using trypan blue staining for 30 seconds followed by washing the cells with phosphate buffered saline (PBS). Corneal thickness from the TCM

and TM groups was recorded before peeling using Optical Coherence Tomography (OCT SS-1000, Tomey, Nagoya, Japan).

### *Preservation conditions*

The tissues were collected and preserved in different conditions, as described in Table 1.

	<b>Condition 1 (C1)</b>	<b>Condition 2 (C2)</b>	<b>Condition 3 (C3)</b>
<b>Tissues collected from</b>	TCM	TM	TCM
	Stripping and Loading		
<b>Preserved in</b>	TCM	TM	TM
	Analysis		

**Table 1:** Different conditions of preservation media to store pre-loaded DMEK lenticules

C1; C2 and C3 = Condition 1; condition 2 and condition 3

### *Stripping and loading*

The procedure was carried out as described in our previously published article [15]. In brief, the [n=30] corneas were secured on a vacuum punch base and secured. The corneas were gently tapped on the endothelial side and a 9.5 mm superficial cut was created using a Moria punch (Moria, Antony, France). The endothelium was stained using Trypan Blue for 20 seconds to determine the cut area. After removing the periphery, the central DMEK lenticule was excised and placed back on the tissue with endothelium facing the air. The pre-stripped membrane was punched again to excise an 8.5 mm (Moria, Antony, France) lenticule. The peripheral remnants were removed. The lenticule was folded (tri-fold) with endothelium-in position using an acute forceps. The lenticule was then gently moved in the preservation chamber of a 2.2 IOL cartridge (Viscoject, Wolfhalden, Switzerland) and was further pulled inside the funnel of the cartridge using a 25G microincision forceps from the funnel end. The funnel was filled with the

preservation medium as listed in table 1 before the lenticule was inserted and was later filled completely. The funnel and the back entrance were closed using rubber stoppers and the entire system was preserved in the media as listed in table 1. The lenticules were preserved for 4 days at room temperature. The stopper was removed and the tissues were released out from the funnel pore using the microincision forceps and analyzed as described below.

#### *Endothelial cell evaluation*

All the lenticules [n=30] were stained after storage using trypan blue for 20 seconds and washed with PBS. The lenticules were exposed to sucrose solution in a petri plate. The ECD and mortality were counted using a 10 X 10 mm reticule (grid) inserted in the eyepiece of an inverted microscope (Primovert; Zeiss, Milan, Italy) at 100X magnification by masked observers.

#### *Glucose uptake of the preserved lenticules to determine the metabolic activity*

Glucose uptake was determined from the preservation media of all the samples [n=30] after 4 days of preservation (post-storage) in an IOL cartridge. This helped to check the metabolic activity of the endothelial cells when preserved *in vitro*. Quantitative analysis was performed using D-Glucose HK kit (Megazyme International Ireland Ltd, Bray Business Park, Bray, Co. Wicklow, Ireland) post preservation.

#### *Histological analysis to determine the presence of endothelial cells on DM, collagen fibrils or stromal residues*

The lenticules were opened up after storage before processing it for histological analysis. The presence of Descemet's Membrane, collagen fibers and endothelium was investigated [n=9; n=3 from each conditions]. The tissue was fixed in 4% PFA overnight

followed by washing it with sucrose solution at 7.5%, 15% and 30% for 15 minutes each. Final washing was carried out with PBS and the tissues were embedded in optimal cutting temperature compound (OCT) for microtome cutting. PAS staining was performed on all the samples and sections were viewed at 10X magnifications in order to check the variability and reproducibility in tissue selection and preservation performances along with stromal interference, if any.

*Antibody staining to determine the polymorphism, expression of tight junctional proteins and cell apoptosis study, for cellular integrity post preservation*

#### *TISSUE FIXATION AND PREPARATION FOR CELL APOPTOSIS AND IMMUNOSTAINING:*

The tissues (n=21; n=7 from each condition) were opened up after storage before checking cell apoptosis and immunostaining. The preserved tissues were fixed in 4% PFA at 4°C overnight.

#### *IMMUNOSTAINING WITH ZONULA OCCLUDENS-1 (ZO-1):*

Twelve tissues [n=4 from each condition], previously treated as described above, were permeabilized with 0.5% Triton X-500 in PBS for 30 minutes. After blocking with 2% goat serum, the tissues were incubated overnight at 4°C with a primary antibody (Zonula Occludens-1 [ZO-1], 1:500 dilution). The samples were incubated with goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody in 20% goat serum for 3 hours at room temperature. Mounting medium containing DAPI was used to stain the nuclei. After each step, the cells were washed 3 times with 10X PBS. Cells were examined with an LSM 510-meta laser scanning microscope (Zeiss, Milan, Italy). Examination was

performed under the ultraviolet light or by excitation at 488 nm or 547 nm, and subsequent detection of the fluorescence was obtained.

*CELL APOPTOSIS USING TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE  
DEOXYURIDINE TRIPHOSPHATE NICK-END LABELING ASSAY:*

Cell apoptosis was performed as described in the manufacturer's protocol for TACS 2 terminal deoxynucleotidyltransferase (TdT) diaminobenzidine (DAB) in situ apoptosis detection kit (Cat# 4810-30-K; Trevigen, Maryland, USA). One separate positive sample was induced with apoptosis using TACS nuclease and nine samples [n=3 from each Cs] were viewed at 100X magnifications of an inverted microscope. The images were analyzed using ZEN (Zeiss, Milan, Italy) software.

*Statistical analysis*

Student's T-Test and Wilcoxon paired data for non-parametric analysis was employed to check statistical significance between the different groups. P<0.05 was deemed statistically significant.

**Results**

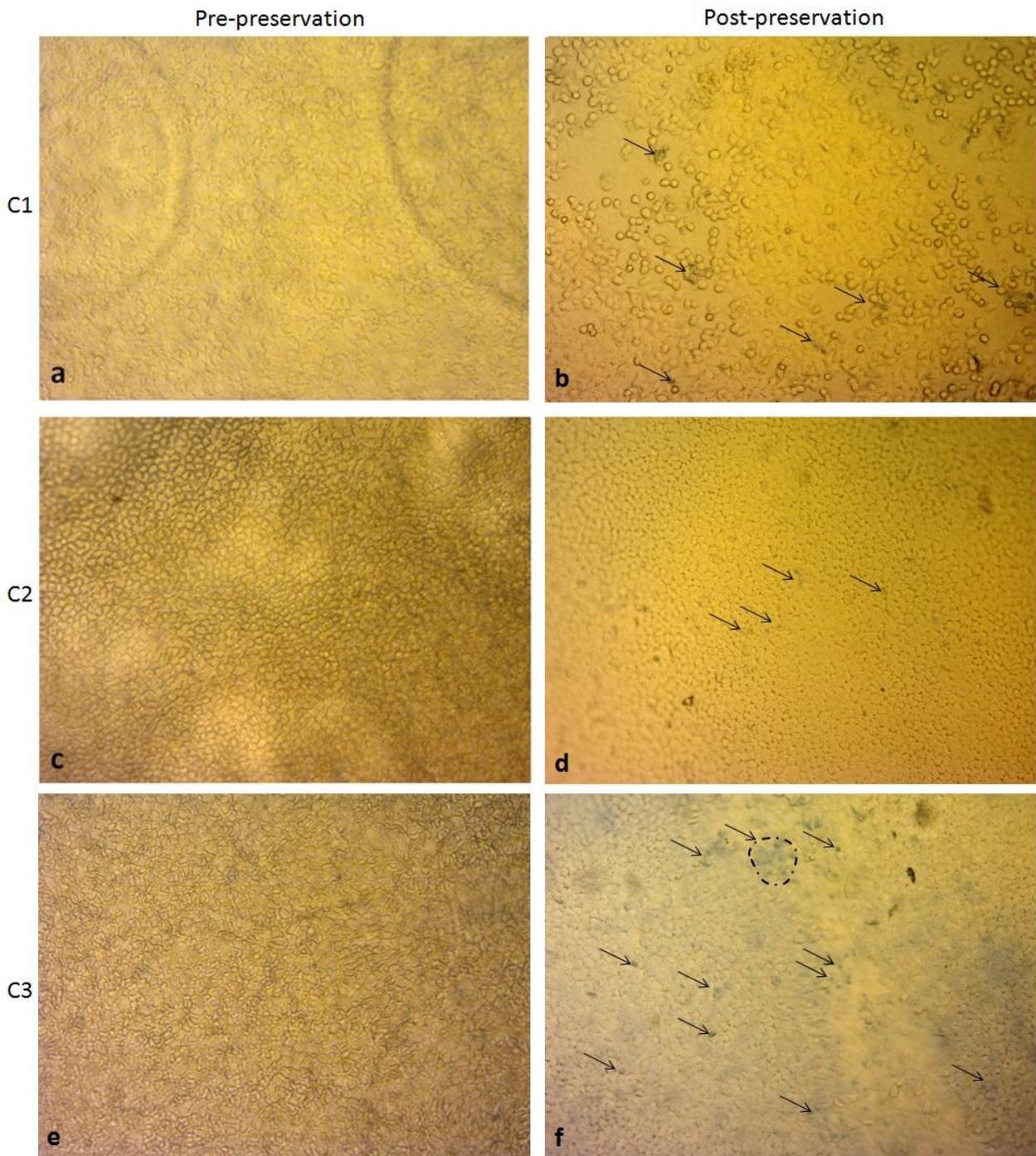
*Donor characteristics and pre-evaluation [n=30]*

The average age of the donor was 67.1 ( $\pm 6.20$ ) years with male:female donor ratio of 24:6. Average post-mortem time was 14.6 ( $\pm 6.45$ ) hours. All the corneas were previously preserved in TCM with an average preservation time of 14.27 ( $\pm 6.09$ ) days. The tissues were further preserved in TM for 3.4 ( $\pm 2.72$ ) days before stripping. The average endothelial cell density recorded was 2203.33 ( $\pm 335.52$ ) cells/mm<sup>2</sup> with initial mortality of 0.2 ( $\pm 0.54$ )%. Successful peeling was observed in 76% of cases when the tissues were collected from TCM whereas 100% tissues were peeled successfully when the tissues

were collected from TM. Average stripping time for C1 and C3 was 25 minutes and that with C2 was 19 minutes. Average loading time was 5 minutes for all the tissues. The tissues collected from TCM had a higher thickness of 963.5 ( $\pm 77.02$ )  $\mu\text{m}$  as compared to TM, which was 570.3 ( $\pm 49.47$ )  $\mu\text{m}$  thick before stripping. The tissues from TM showed thickness similar to *in vivo* corneas as they were de-swelled.

*Lower endothelial cell loss in dextran based media [n=30]*

ECD (cells/ $\text{mm}^2$ ) post preservation in C1, C2 and C3 was found to be 1130( $\pm 944.05$ ), 1950( $\pm 108.01$ ) and 1970( $\pm 512.18$ ), and the mortality (%) was 25.94( $\pm 44.23$ ), 3.8( $\pm 7.74$ ) and 21.8( $\pm 36.41$ ), respectively. It was also observed that the cells detached from the lenticule during preservation and therefore the uncovered areas (%) determined for C1, C2 and C3 were 40.7 ( $\pm 47.96$ ), 13.0( $\pm 18.55$ ) and 41.8( $\pm 37.77$ ). Loading to post preservation in C1 showed statistically significant endothelial cell loss ( $p=0.0051$ ) however, C2 ( $p=0.1092$ ) and C3 ( $p=0.0819$ ) did not show statistical significance, although the average ECL observed in C3 was high. Figure 1 shows how the lenticules obtained from corneas preserved in C1, C2 and C3, respectively, appear post-stripping (a, c, e) and post-preservation (b, d, f).



**Figure 1:** Endothelial cell density, mortality and uncovered areas of pre-loaded DMEK lenticules at 100X magnification. A, c, e) shows healthy endothelium that was found post-stripping with minimal mortality in different conditions as listed. B, d, f) shows mortality and uncovered areas in each condition. C1 and C3 showed high mortality (trypan blue positive cells marked with arrow) and uncovered areas as compared to C2, which showed minimal mortality.

*Glucose was utilized in all the media [n=30]*

Average glucose uptake by the endothelial cells in the preservation chamber for 4 days at room temperature was 0.32( $\pm$ 0.18) mg/mL from C1, 0.43( $\pm$ 0.27) mg/mL from C2 and 0.56( $\pm$ 0.18) mg/mL from C3, which is one third to half of the total amount of glucose present in the media. These results are similar to those showed in the pre-loaded DSAEK lenticules [13]. However, there was no statistical difference seen between either groups ( $p>0.05$ ).

*Presence of DM and endothelial sheet on the excised DMEK tissues from dextran based media [n=9]*

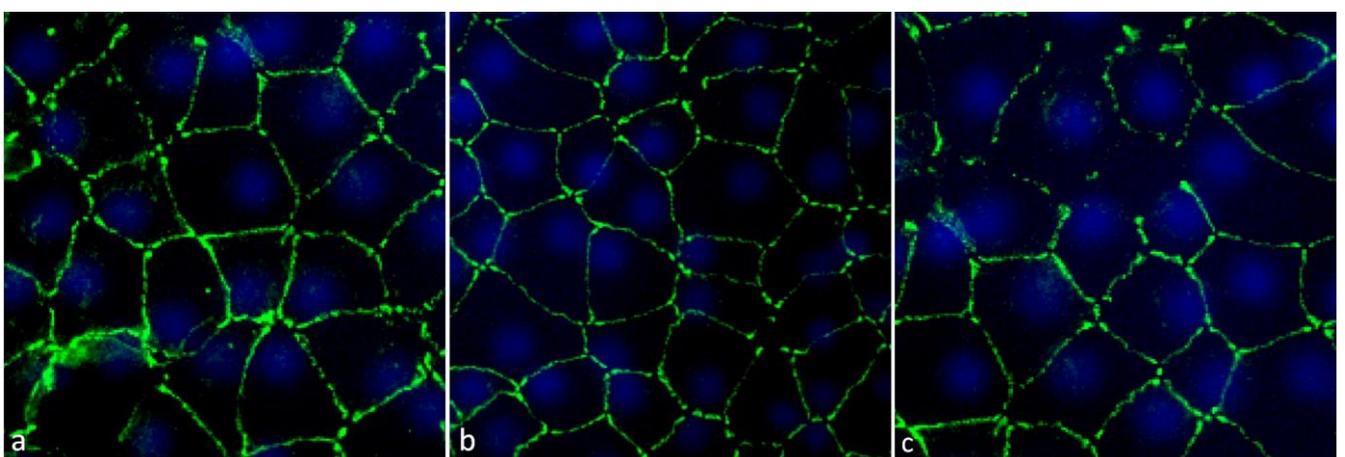
The analyzed tissues (100x magnification using PAS staining) showed no presence of endothelial cells, but only the DM (figure 2a) in C1. This may be a reason due to high 'fall-off' rate of the cells from C1. As observed in figure 1, the cells does not maintain a hexagonal shape and have turned itself into circular shape (stressed) along with high mortality as seen using trypan blue staining. Descemet's membrane and endothelium (found in one of the tissues) without any collagen fibrils or attached stromal residues in C2 is seen in figure 2b. However, lesser endothelial cells were found in lenticules from C3 (figure 2c). This phenomenon can be due to the presence of dextran in the preservation medium after loading in the cartridge. Preservation of endothelial cells with DM was found in C2.



**Figure 2:** Histological analysis on pre-loaded DMEK lenticules post preservation at 100X magnification. a) C1 showed no presence of endothelium due to major cell fall-off, b) C2 showed presence of endothelium and DM without any stromal remnants and c) C3 showed limited endothelium.

*Expression of tight junctional proteins and polymorphism on the endothelial cells after preservation [n=12]*

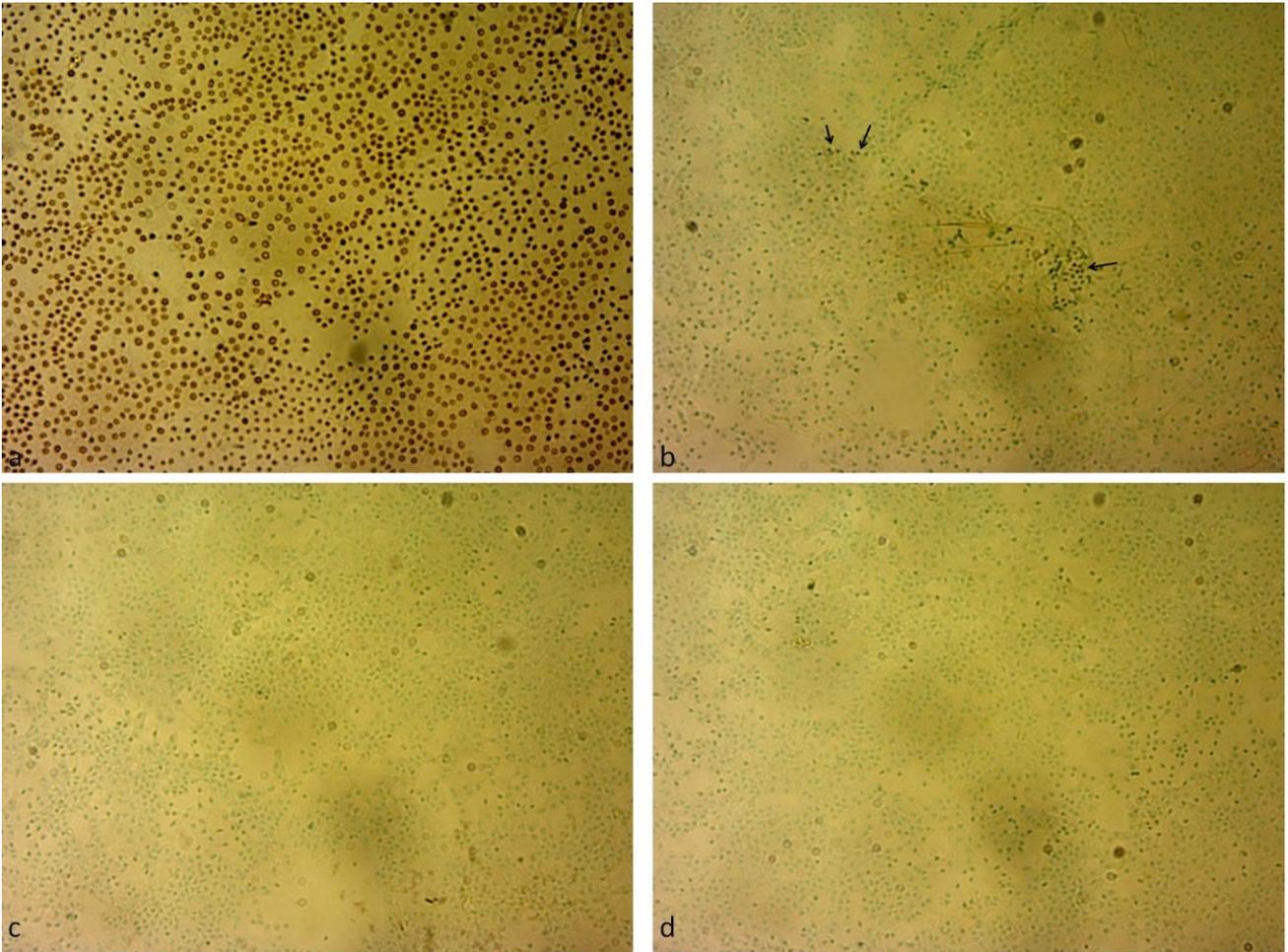
The endothelial cells showed expression of tight junction protein (ZO-1) post preservation as seen in figure 3 at 400X magnification. C1 and C3 (figure 3a and 3c respectively) showed loss of hexagonality, most of the cells were polygonal to circular. Tight junctional protein was not consistent in C3 but was intense in C1 at many areas. C2 (figure 3b) showed endothelial cell mosaic with more hexagonal shaped cells and the conservation of tight junctional proteins in the pre-loaded DMEK lenticules.



**Figure 3:** Immunostaining results using ZO-1 marker for expression of tight junctional proteins in the pre-loaded DMEK lenticules visualized at 400X magnification. Preserved cells showed expression of these proteins confirming the integrity of the endothelial cells. However, a) and c) C1 and C3 respectively showed some areas without endothelial cell borders and hence loss of tight junctions whereas b) C2 expressed tight junction protein with less polymorphism compared to C1 and C3. Hexagonality of the cells was observed in C2.

*No cell apoptosis from dextran based media [n=9]*

Control cells were induced with apoptosis using TACS nuclease (figure 4a) to compare them with the samples. C1 showed apoptosis post preservation at 100X magnification (figure 4b). However, C2 and C3 did not show any apoptosis post preservation (figure 4c and 4d). C2 and C3 were preserved in dextran-based medium and we assume that may have prevented the cells from apoptosis.



**Figure 4:** Cell apoptosis post preservation of pre-loaded DMEK lenticules. a) Induced apoptosis on the control sample at 100X magnification. b) C1 showed mild apoptosis whereas c and d) C2 and C3 did not show any apoptosis post preservation and the nucleus was visible with methyl green counter stain at 100X magnification. Hence it was determined that the preservation of the DMEK lenticule in a cartridge for 4 days does not have any drastic damage on the endothelial cells.

## Discussion

As indicated in the introduction, DMEK requires high surgical skills, but there are ways to ameliorate the quality of the tissues distributed to surgeons and help them perform an easier transplantation. One of the methods is to check whether the preservation technique may affect the quality of the tissue. Dextran has been widely used to de-swell the cornea

after the tissue has been swollen during the TCM preservation phase. The number of viable endothelial cells and a thickness that is similar to the *in vivo* conditions are important for a successful corneal transplantation.

Organ culture offers several advantages in terms of sterility checks, longer duration of preservation and hence a proper planning of the surgery [16]. Although storage in dextran-containing medium is not obligatory for preparing the tissues for DMEK surgery, the important parameters like endothelial cell density and smoothness of the DM should be considered before the surgery. It has been suggested that with higher number of functional cells, the dislocation chances of a DMEK decreases and the adherence increases [16]. To our knowledge, there has not been any study in the literature that shows the comparison of different storage media for pre-loaded DMEK preservation in a closed chamber like an IOL cartridge.

Previously, it has been shown that the preservation medium has no effect on the rate of successful peeling of DMEK tissues [16]. However, we observed that when the tissues are thick (stromal thickness), i.e., approximately over 900 $\mu$ m, the success rate changes as compared to the normal thickness of the graft, which has re-gained its original thickness (approximately 550 $\mu$ m). We observed 76% successful peeling cases with higher thickness where the tissues were preserved in TCM as compared to 100% successful cases when the tissues were preserved in TM. Peripheral tearing that resulted into the loss of tissues eventually was the major reason when the tissues were excised from TCM. The stroma was thick and the punch for creating a superficial cut does not direct a regular cut in an uneven stromal base when the tissues were taken from TCM. On the contrary, when the tissues were obtained from TM, due to the regular thickness and a more constant cornea throughout, the cut obtained was precise and the excision was accurate without any

peripheral tears. Therefore, to standardize the peeling technique, the tissues can be obtained from TM or from a dextran based media for peeling.

Further, the tissues are usually pre-bubbled, pre-stripped or pre-rolled and preserved in the respective media [8,12]. However, there has not been any study that reports about a completely excised and pre-loaded tissue. Therefore, we tried to pre-load the tissue in a closed chamber (the IOL cartridge). Tissues that were pre-loaded did not show any damage in terms of cellular morphology or molecular integrity. The glucose uptake study showed similar results as those obtained with pre-loaded DSAEK grafts, thus concluding that active metabolism and functional activities of the endothelium continues for at least 4 days at RT [13]. Histology confirmed the presence of DM and endothelium without any stromal residues in all the cases but the presence of endothelium was definite when the tissues were excised from C2. The cellular apoptosis was observed in C1, it was not due to the pre-loading but due to the preservation medium (TCM). Cell apoptosis has been reported to incur in the tissues that are preserved in TCM for a long time [17]. Therefore, Dextran seems to play an important role in maintaining the functionality of the cells while pre-loading it.

Endothelium, if flapped-in, does not have statistically more damage compared to endothelium when flapped outward (which is the natural phenomenon) [18]. However, we recommend that the tissues should be flapped-in, a) to avoid any possible friction between the endothelial cells and the cartridge during preservation, transportation and transplantation and b) to reduce the time of opening the graft inside the recipient eye [18]. The average ECL [%] (average of mortality and uncovered areas) post preservation of the pre-loaded DMEK lenticule was found to be 45.05( $\pm$ 46.61), 10.77( $\pm$ 15.99) and 34.77( $\pm$ 37.37) in C1, C2 and C3, respectively. The endothelial cell mortality cannot be

considered high because 1) the tissue is punched again at the eye bank after stripping and is folded and loaded in the cartridge where there is some endothelial cell loss observed. This step is currently carried out in the surgical theatre where the surgeons do not check the endothelial cell loss after this step, 2) the tissue is then preserved in closed chamber (IOL cartridge) in the same orientation without any free movement and some cells may be damaged due to friction, 3) the tissues obtained for this research are not suitable for transplantation because of low endothelial cell count and previous mortality therefore it may have some additional mortality due to the health of the tissue in general. The results can still be extrapolated to the transplantable grade tissues as they have high endothelial cell density (between 1800-2000 cells/mm<sup>2</sup>) for research. As per our previously reported article, there is a learning curve to pre-load a DMEK graft and after the learning curve, the average endothelial cell mortality and uncovered areas post preservation in dextran based media was 3.55% ( $\pm 5.79\%$ ) and 7.8% ( $\pm 14.13\%$ ) respectively. Overall ECL after preservation in dextran-based medium was 4.35%, which is less than the endothelial cell loss observed here that is due to the learning curve [15]. Moreover, dextran is negatively charged and therefore the media repels the endothelial cells allowing a higher cell adherence to the extra cellular matrix, the DM. This property of dextran may also help to reduce the cell fall-off and highlight that the tissue should be preserved in dextran based medium. The tissues from TCM have two major disadvantages such as, a) lower successful peeling rate and b) higher endothelial cell loss. We have also noticed that the tissues preserved in C2 have a better flexibility, lower stiffness and high cell to extra-cellular matrix adherence (data under consideration for publication) as compared to the other conditions.

In conclusion, pre-loading a DMEK tissue may facilitate DMEK to be more successful in terms of transplanting a validated graft along with a comparatively short surgical

procedure, low costs and logistic requirements. Dextran may therefore have an important role during preservation, preparation and transportation, and hence it should be considered in the medium before pre-stripping or pre-loading a DMEK lenticule.

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## **CHAPTER 5 – PART I**

### **ENDOTHELIUM-IN VS ENDOTHELIUM-OUT FOR DMEK GRAFT PREPARATION AND IMPLANTATION**

*Derived from published research article*

Parekh M, Ruzza A, Ferrari S, Ahmad S, Kaye S, Ponzin D and Romano V. Endothelium-in Vs Endothelium-out for DMEK graft preparation and implantation. Acta Ophthalmol. 2016 July 16. [PMID: 27422483]

## **Aim**

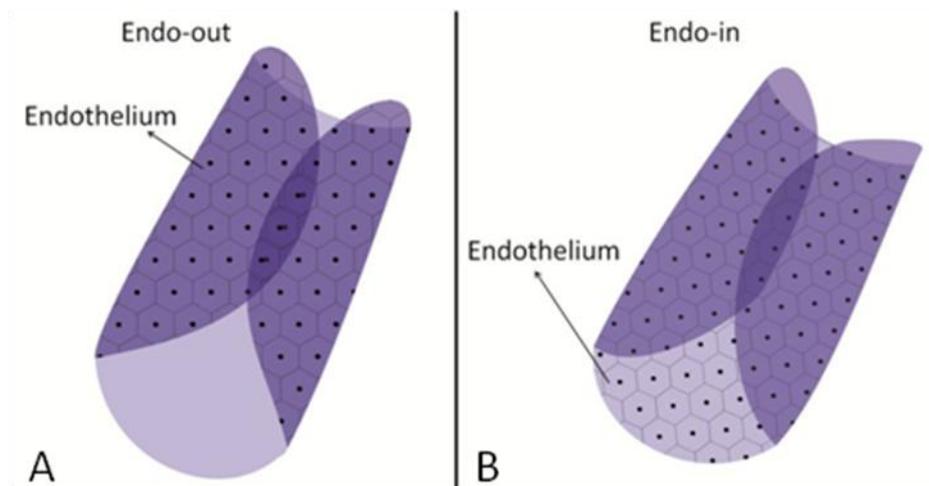
To compare the difference between endothelium flap-in and endothelium flap-out using injection method for DMEK.

## **Introduction**

Over the last few years, there has been an increase in the uptake of Descemet Membrane Endothelial Keratoplasty (DMEK) as a treatment for corneal endothelial disorders. During the years 2013-2015, our institution at the Veneto Eye Bank Foundation (Venice, Italy) has received a comparable increase in requests of pre-stripped tissues for DMEK. The Eye Bank Association of America (EBAA) reports have also shown that there is a slow increase in the uptake of DMEK since 2011 [Annual reports of EBAA, 2011-2014]. Although DMEK is a minimal invasive surgery with faster rehabilitation rate; preparation and transplantation techniques still remain a challenge. Out of the many preparation techniques that have been proposed [1-6], the stripping method has been the most widely adopted. In contrast, the challenges of unfolding and positioning the graft inside the eye have led to a wide variety in methods used to deliver the graft into the eye [7,8]. Busin et al recently reported a novel method to insert the graft into the eye using a pull through technique [9]. Injector systems for DMEK have also been evaluated [10]. The current approach in DMEK is based on inserting the graft with the endothelium exposed or rolled out. Scrolling of Descemet membrane with the endothelium out is the spontaneous form that the tissue takes within a liquid (Figure1a). This, however, makes the handling of the tissue difficult and exposes the endothelial cells to potential damage against the wall of the cartridge or the delivery tube, during loading and insertion [11]. If it were possible to roll the tissue with the endothelium on the inside, it would facilitate insertion of the tissue both within the introducer and also into the eye with less potential damage to the endothelium. One possibility is to manually roll the tissue inwards (Figure1b). The aim of

this study was to investigate and evaluate a technique to fold the graft with the endothelium on the inside (Figure 1b). We postulated that the folding of the graft with the endothelium inwards would be easier to achieve with larger grafts. There is already a good evidence that the endothelial cell density increases towards the paracentral and peripheral regions of the cornea [12,13] and it has been shown that larger (9.5mm and 9.00mm) DSAEK grafts have better survival rates [15].

Larger DMEK (9.5 mm) grafts therefore, were specifically used in this study and endothelial density measured in both the centre and periphery.



**Figure 1:** Schematic representation of the orientation of the grafts. a) endothelium flapped in a natural spontaneous folding of the graft and b) Right - endothelium flapped manually inside.

## Materials and Methods

Donor corneal tissues were obtained from The Veneto Eye Bank Foundation (Venice, Italy) following consent from the donor's next of kin. The study was conducted in the institutional setting, the laboratories of Veneto Eye Bank Foundation (Venice, Italy). The donor corneal tissue (9 tissues for each study) was randomly assigned to be used either for endothelium in (endo-in) or endothelium out (endo-out) technique using a coin toss.

### *Stripping method*

The corneas were centered on a punch base and fixed using a vacuum block. A superficial circumferential cut was created with a 9.5 mm punch [Moria, Antony, France] by gently tapping the trephine. The edge of the cut was visualized with trypan blue and the excess of peripheral membrane removed. The endothelium was kept moist during the procedure with a de-swelling media. The membrane was separated using a cleavage hook throughout the periphery and the peripheral residues were detached. The DMEK graft (9.5 mm) was stripped leaving a small peripheral attachment hinge before being re-stored. The pre-stripped grafts were then preserved in the de-swelling medium at room temperature for 4 days. This is the procedure used for storage of pre-stripped tissue issued for transplantation by our eye bank.

### *Mounting the cornea*

After removal of the DM, each cornea was mounted on the artificial anterior chamber (AAC, Moria, Antony, France) with continuous irrigation of saline. The pressure inside the AAC was controlled by adjusting the height of an infusion bottle at 20 cm (15.3 mmHg) [15]. At the 12 o'clock surgical position a 3mm limbal incision was made using the slit-knife. Three side ports were created at 10:30, 1:30 and 7:30. The orientation of the graft was checked after implantation using an inverted microscope [Primovert; Zeiss, Milan, Italy].

### *Endothelium-out (Endo-out)*

Following stripping of the DM the grafts were freed from the corneal hinge and immediately placed onto a petri-plate containing phosphate buffered saline (PBS). The graft, as expected, rolled with the endothelium outwards. Using a modified Jones tube, the

graft was aspirated inside the tube and injected into AAC. The graft was unfolded and attached using an air tamponade.

#### *Endothelium-in (Endo-in)*

The grafts were freed from the corneal hinge, but retained on the corneal base prior to folding. Using acute forceps (e.janach, Como, Italy), two opposing peripheral edges of the graft were grasped in turn and drawn towards the centre with the endothelial side facing inwards. One flap was drawn slightly beyond the centre to overly the other flap. Using the same acute forceps, the graft was pulled inside a 2.2 IOL (Intra Ocular Lens) cartridge (Viscoject, Wolfhalden, Switzerland) maintaining the graft in the same orientation (Descemet membrane side touching the bottom of the cartridge with the folds uppermost). A small drop of PBS was then added to the cartridge to keep the tissue moist prior to injection. Viscoelastic was not used. Using Grieshaber Revolution DSP 25 Ga + endgrasping forceps (Alcon, Texas, USA), the graft was pulled inside the funnel containing sterile PBS solution ready for delivery [9,16]. The cartridge was then inverted with the exposed DM side uppermost in the funnel and the folded flaps lowermost to facilitate opening of the graft inside the anterior chamber. The IOL cartridge was then inserted through one incision and the graft was injected with gentle pressure to avoid rapid pressure changing the orientation of the graft. Using an air bubble the unfolded graft was attached on the donor corneal stroma using an air tamponade.

#### *Endothelial cell count*

The corneal tissue was dismantled and the graft gently removed using PBS before staining with trypan blue for 20 seconds and washing with PBS. The graft was opened gently using PBS flow to minimize the risk of endothelial damage. With a hypotonic (sucrose) solution in a petri plate, the integrity of the graft and endothelium was examined

using an inverted microscope [Primovert; Zeiss, Milan, Italy]. The cells were manually counted in central and peripheral areas (combined and averaged) in triplicates using a 10X10 eye piece reticule and the images were captured at 100X magnification.

### *Analysis*

The following time periods were recorded. The time required to

1. strip the tissue,
2. prepare the tissue from dislocating the graft to either manually folding the tissue (endothelium inwards) and loading it into a cartridge, or aspirating it into the tube endothelium out,
3. prepare the anterior chamber for insertion of the graft: mounting the cornea on artificial chamber, control of pressure, and preparation of limbal incision and 3 side ports,
4. unfold the tissue once injected and attachment with air tamponade bubble and
5. complete the procedure.

### *Statistical Analysis*

A non parametric test (Wilcoxon test) was used to test for a difference between the two groups, endo-in and endo-out. Differences were considered statistically relevant with  $p < 0.05$ .

### **Results**

The average age of donors was 66.00( $\pm$ 6.73) years with a male:female donor ratio of 11:7. The average post-mortem time was 13.02( $\pm$ 7.33) hours and preservation time of 34.11( $\pm$ 12.48) days in tissue culture medium (TCM), followed by 6.22( $\pm$ 1.77) days in transport medium (TCM with 6% Dextran).

All the tissues were successfully peeled in one attempt (100% success rate). An average of 20.22(±5.54) minutes was required to prepare a pre-stripped DMEK graft. The different time intervals and the total time of the procedure are included in table 1. Pre, post-strip and post-loading endothelial cell density, mortality and uncovered areas are presented in table 2.

	Endo-in Mean (± SD)	Endo-out Mean (± SD)	pValues
<b>Loading time (Minutes)</b>	4.43(± 3.43)	1.68 (±0.57)	<b><u>0.04</u></b>
<b>Time to Inject (Minutes)</b>	3.56 (±1.72)	4.89(±2.99)	0.26
<b>Time to Unfold (Minutes)</b>	0.82(±1.04)	4.92(±4.21)	<b><u>0.02</u></b>
<b>Total time (Minutes)</b>	8.81 (±4.54)	11.49 (±6.77)	0.34

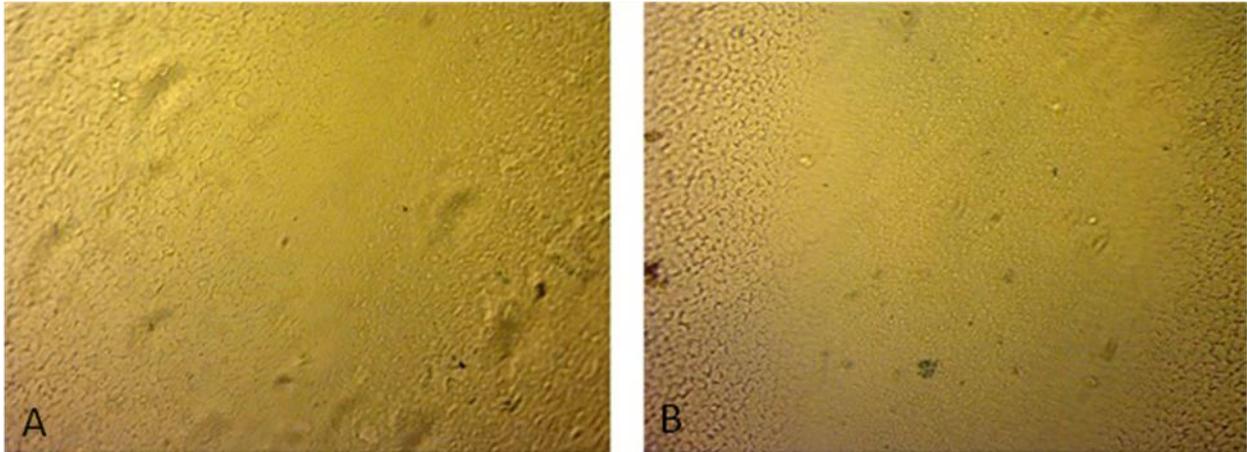
**Table 1:** Parameters which showed that time to prepare the tissues was significantly lesser with endo-out whereas time to unfold the graft was significantly lower with endo-in. Endo-in may spontaneously roll out in the eye and may help to reduce surgical manipulations. Loading time is when the pre-stripped lenticules are loaded in the Jones tube for endo-out or in a cartridge for endo-in. Time to inject is when the corneas are mounted and incisions are made for the delivery of the graft. Time to unfold the graft is when the grafts after delivery are unfolded on the stroma with DM touching the stromal side and air bubbled. Total time is the time required for the entire procedure using a pre-stripped membrane.

	Endo-in Mean (± SD) [Range]			Endo-out Mean (± SD) [Range]			P values		
	Pre stripping	After stripping	After unfolding	Pre stripping	After stripping	After unfolding	Pre-stripping	After stripping	After unfolding
Endothelial cell density (cells/mm <sup>2</sup> )	1900(±206.16) [1600-2200]	1888.90(±220.48) [1600-2200]	1700(±212.13) [1500-2000]	1911.1(±208.83) [1600-2200]	1894.4(±218.58) [1600-2200]	1766.66(±244.95) [1300-2200]	0.91	0.96	0.55
Mortality (%)	0.66(±0.96) [0.01-3]	0.09(±0.16) [0-0.5]	0.92(±1.05) [0-2.3]	0.10(±0.13) [0-0.4]	0.08(±0.14) [0-0.4]	0.79(±0.51) [0.01-1.4]	0.12	0.88	0.74
Uncovered areas (%)	0.1(±0.26) [0-0.8]	0.03(±0.07) [0-0.2]	1.22(±1.97) [0-5.6]	0.06(±0.17) [0-0.5]	0.04(±0.10) [0-0.3]	0.82(±0.90) [0-2.676]	0.75	0.79	0.59

**Table 2:** Endothelial cell density, mortality and uncovered areas did not show any statistical significance between either group before stripping, after stripping and after

unfolding the graft which signifies that endo-in or endo-out could be used without any difference in endothelial damage for DMEK surgery.

The tissue preparation time for the manual folding of endo-in ( $4.43 \pm 3.43$  minutes) was longer than for the auto-folding of endo-out ( $1.68 \pm 0.57$  minutes) [ $p=0.04$ ]. The time required to unfold the graft inside the eye, however, was significantly less for endo-in ( $0.96 \pm 1.10$  minutes compared to endo-out ( $4.92 \pm 4.21$  minutes) [ $p=0.02$ ] (table 1). There was no significant difference, however, between the methods for the time for the entire procedure, that is,  $8.94 \pm 4.43$  minutes for endo-in and  $11.49 \pm 6.7$  minutes for endo-out method ( $p>0.05$ ). There was similar amounts of endothelial cell loss post implantation (measured at center and periphery and combined) for Endo-in ( $10.53 \pm 2.82\%$ ) and Endo-out ( $7.56 \pm 14.74\%$ ), ( $p>0.05$ ). The cells were counted *in vitro* in triplicates using a larger area than would be obtained *in vivo*. We think that this would be more accurate and less prone to magnification error. In addition, we also measured ECD just after delivery, which has not been undertaken in clinical studies. There were no significant differences in endothelial cell density, mortality and uncovered areas between the two methods ( $p>0.05$ ) (table 2) and as shown in figure 2a (endo-in) and figure 2b (endo-out) either before, or following insertion of the graft. A slightly greater number and size or grade of acellular areas was observed in the endo-in method after stripping and therefore it was extrapolated to the unfolding results. This was however, variable between different donors.



**Figure 2:** Endothelial status after implantation and removal of the graft from the cornea mounted on AAC. a) Endothelial cells with minimal mortality and uncovered areas from Endo-in and b) Endo-out, showing less damage.

## Discussion

Endothelial keratoplasty using pre-cut or; pre-cut and pre-loaded grafts (DSAEK and DMEK) from the eye banks is gaining popularity [17]. DMEK offers a quicker rehabilitation rate and better visual outcomes, but requires high surgical and manipulative skills. It is evolving at a much slower rate due to the challenges that are faced by the surgeons in preparation, transplantation and managing post-operative complications. Using prepared tissue for DMEK may facilitate surgery. Although pre-stripped tissues from eye banks have been evaluated recently; unfolding the graft within the eye and dealing with post-operative complications is one of the reasons for the relatively slow uptakes of DMEK [18-20].

Currently DMEK grafts are aspirated inside a tube and injected with the endothelium on the outside of the rolled surface. This is due to the spontaneous rolling of Descemet's membrane when in fluid. The position of the endothelium increases the risk of endothelial damage during injection and during manipulation inside the eye, the latter being technically difficult. We envisioned that a DMEK roll with endothelium flap-in would unroll

spontaneously once inside the eye to form its natural endo-out structure. In addition, with endo-in, the tissue should have a minimal endothelial cell loss as the cells are not directly exposed to the plastic of the cartridge during the delivery of the graft. Using an IOL cartridge to aspirate an endo-in graft may de-orientate the graft or may lead to the graft folding back to an endo-out position [9]. In order to test this hypothesis we evaluated the method using donor corneas on an artificial anterior chamber and compared it to the conventional endo-out method.

No significant difference in cell loss was observed with either technique. There is a risk that we did not detect a difference of less than 20% with the sample size used but it was not feasible to increase the sample size of 100 to 400 to detect a 10% and 5% difference. We think that the highest cell loss from endo-in method may occur during folding of the graft in the preparation phase. In contrast, however, with the endo-out method, damage to the endothelium may be more likely during insertion and manipulation in the eye. One of the major advantages of endo-in is the reduced possibility of graft failure from the graft being placed upside down [9], which has been observed with the endothelium flapped out position [18]. In addition, folding of the graft endo-in may reduce damage against the wall of the cartridge. This may be particularly useful for pre-loading DMEK grafts by the eye bank [16].

There is good evidence that the endothelial cell density increases towards the paracentral and peripheral regions of the cornea [12,13]. In addition, it has been shown that larger (9.5mm and 9.00mm) DSAEK grafts have better survival likely due to an increase in transplanted endothelial cells. A 9.5mm grafts would also reduce the impact of zones of cell damage from the end-grasping forceps used to load the graft into the cartridge. It has been reported in DSAEK surgery that graft size is not a risk factor for the incidence of

rejection [15]. Although there are many factors that contribute to graft endothelial failure [21], the number of endothelial cells transplanted likely plays a crucial role. It is reasonable to suggest, therefore, that if graft size is increased to 9.5 mm, more of the periphery will be included and may help in maintaining an adequate number of functional endothelial cells in the long term. Although subjective, we found it easier to fold and handle 9.5 mm grafts, and that the endo-in grafts unfolded inside the artificial chamber with minimal manipulation. Although this is an *in vitro* study it provides good evidence to undertake an *in vivo* clinical study using an endo-in technique for DMEK.

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## **CHAPTER 5 – PART II**

# **PULL-THROUGH TECHNIQUE FOR DELIVERY OF A LARGER DIAMETER GRAFT USING ENDOTHELIUM-IN METHOD**

*Derived from correspondence (Submitted – under peer review)*

Vito Romano, Alessandro Ruzza, Stephen Kaye and Mohit Parekh. Pull-through technique for delivery of a larger diameter DMEK graft using endothelium-in method. Canadian Journal of Ophthalmology.

Busin et al. recently reported the results of an innovative surgical technique describing the delivery of tri-folded (endothelium-in) Descemet Membrane Endothelial Keratoplasty (DMEK) graft using a pull through technique [1]. We performed a similar [n=9], but *in vitro* study with larger DMEK grafts (9.5mm) in order to evaluate the surgical endothelial cell loss (ECL) and learning curve. We intentionally used larger DMEK grafts, because as previously seen with large ultra-thin Descemet Stripping Automated Endothelial Keratoplasty (UT-DSAEK), they increase long term graft survival [2]. Using the stripping method the tissue was tri-folded using acute forceps (E.Janach, Como, Italy) to manipulate Descemet membrane (DM) with the endothelial side facing inwards to avoid endothelial damage. Using the same forceps, the graft was gently pulled inside a 2.2 IOL (Intra Ocular Lens) cartridge (Viscoject, Wolfhalden, Switzerland) maintaining the graft in the same orientation (DM side touching the cartridge bottom and the endo-in on the top). Using a 25G end grasping forceps (Grieshaber forceps, 25G Alcon, Texas, USA), the graft was pulled inside the funnel of the IOL containing sterile phosphate buffered saline (PBS) ready for delivery [3]. After the DM-endothelium was removed, the cornea was mounted on an artificial anterior chamber (AAC, Moria, Antony, France). The pressure inside the AAC was controlled by adjusting the height of an infusion bottle at 20 cm (15.3 mmHg) [4]. At the 12 o'clock surgical position, a 3mm limbal incision was made using a slit-knife. Three side ports were created at 10:30, 1:30 and 7:30 clock positions. The cartridge was inverted so that the exposed DM side was on the top of the funnel and the flaps on the bottom to facilitate opening the graft inside the eye. The IOL introducer cartridge was then inserted through one incision and the graft was pulled from the opposite side using end grasping forceps. An air bubble was then used to attach the unfolded graft to the donor corneal stroma. The cornea was then dismounted from the AAC and the graft gently removed using PBS and stained with trypan blue for 20 seconds followed by a wash with PBS. With a hypotonic (sucrose) solution in a petri plate, the endothelium was examined

for damage, cell loss and uncovered areas using an inverted microscope [Primovert; Zeiss, Milan, Italy]. The cells were counted using a 10X10 eye piece reticule. Time required for stripping, loading, injecting, unfolding and the total surgery time were recorded. All the tissues were successfully peeled in one attempt (100% success rate). An average of  $18.78 \pm 5.65$  minutes was required to prepare a pre-stripped DMEK graft in the Eye Bank. Post-stripping, post-loading and post-delivery, mean endothelial cell density, mortality, uncovered area, time intervals, and total time of the procedure are listed in Table 1.

<b>Endothelial cell evaluation</b>	
Post Stripping ECD (cells/mm <sup>2</sup> )	2044.44±427.53
Post Stripping Mortality (%)	0.52±0.99
Post Stripping Uncovered Areas (%)	0.17±0.35
Post Loading ECD (cells/mm <sup>2</sup> )	2000.00±409.27
Post Loading Mortality (%)	0.59±0.96
Post Loading Uncovered Areas (%)	0.29±0.43
Post Delivery ECD (cells/mm <sup>2</sup> )	1588.89±321.89
Post Delivery Mortality (%)	0.79±1.00
Post Delivery Uncovered Areas (%)	17.84±24.19
<b>Timing</b>	
Time to prepare (min)	1.07±0.29
Time to inject (min)	3.51±1.00
Time to unfold (min)	5.84±3.95
Total time (min)	10.42±3.68

**Table 1:** Endothelial cell loss and surgical timing

We did not observe any de-orientation of the graft in all 9 cases. The EC loss at the end of the procedure was 22.28% which is less than that reported for DMEK surgery (endothelium out) of 35-37% at 6 months [5]. We also had similar results in a previous *in*

*vitro* study where we compared the ECL and total time of the surgery after injecting a 9.5 mm DMEK graft with endothelium flapped in or rolled out [6]. In addition, a 9.5 mm DMEK graft will transplant increased number of cells initially and may help in maintaining an adequate number of functional endothelial cells in the long term. It is worth considering that an increase from a 8.25 mm to a 9.5 mm diameter graft would result in transplanting approximately 20% more cells [2,7]. Even compared to the very low ECL of 9.9% reported by Busin et al [1], a larger (9.5mm) graft with an ECL loss of 22.28%, would however, still provide a greater number of cells compared to a 8.25mm graft with a 9.9% ECL. We also observed that, similarly to 9.5mm UT-DSAEK, larger DMEK grafts were easier to handle in terms of folding and loading compared to smaller grafts. This is also highlighted by no significant ECL during loading time. The time required for the entire procedure was  $10.42 \pm 3.68$  minutes which may give an additional advantage to the surgeons.

Despite the limitations of this *in vitro* report, these results suggest that the described technique using large (9.5mm) grafts for DMEK has an acceptable ECL and learning curve and therefore may be useful for long term survival as compared to a smaller graft.

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## **CHAPTER 6**

### **PRE-LOADED TISSUES FOR DESCemet MEMBRANE**

### **ENDOTHELIAL KERATOPLASTY**

*Derived from published research article*

Parekh M, Ruzza A, Ferrari S, Busin M and Ponzin D. Pre-loaded tissues for Descemet Membrane Endothelial Keratoplasty. Am J Ophthalmol. 2016;166:120-125. [PMID: 27066719]

DMEK tissues are important to study for multiple reasons as it allows understanding the cell loss incurred during the preparation, storage, transport, implant and post-op survival. It is therefore important to study this to standardize the current DMEK options to treat EK and further enhance the transport and implantation technique of the HCECs.

## **Aim**

To determine the feasibility of pre-loading endothelial tissues for Descemet Membrane Endothelial Keratoplasty (DMEK).

## **Introduction**

Endothelial Keratoplasty (EK) is a selective transplantation technique and an effective alternative to Penetrating Keratoplasty (PK) for the management of corneal endothelial failure. Descemet Membrane Endothelial Keratoplasty (DMEK) is a sub-type of EK which foresees the transplantation of Descemet membrane and endothelium exclusively [1-4] and offers an extremely fast recovery of vision often at the 20/20 level.

Similar to other lamellar keratoplasty procedures, DMEK is gaining popularity and eye banks are confronted with new requirements from corneal surgeons. Recently, they have started the preparation and transportation of pre-cut donor tissues with a desired diameter for Descemet Stripping Automated Endothelial Keratoplasty (DSAEK), Ultra-Thin DSAEK (UT-DSAEK) and pre-stripped tissues for DMEK [5-7].

As this reduces the efforts, time and cost in the surgical theatre, the popularity of pre-cut/pre-stripped tissues is increasing rapidly. Moreover, it allows validation of the tissue to be grafted, a quality control that cannot be done in the surgical theater, when the surgeons prepare the tissue themselves. The Veneto Eye Bank Foundation has now

started providing pre-loaded tissues, a step further to pre-cut tissues for DSAEK and UT-DSAEK surgeries [8]. This further reduces the time and efforts in the surgical theater, increases the efficiency of DSAEK surgery and allows the use of a validated tissue. DMEK tissues can be pre-stripped [7] or pre-bubbled [9] at the eye bank and shipped to the operating room with the full cornea as a base support. Instead, if the DMEK tissue is stored without corneal support, it rolls with the endothelium facing outwards in contact with the solid walls of the cartridge, thus possibly being negatively affected by the storage process. We have investigated the possibility of preserving DMEK tissue rolled with the endothelium inwards to avoid any damage caused by the contact with the cartridge wall and providing a ready-to-use tissue to the surgeons for direct transplantation. This study is a proof of concept for a pre-loaded DMEK and the clinical relevance still needs to be tested.

## **Materials and Methods**

### *Ethical statement*

Twenty human donor corneal tissues were collected from The Veneto Eye Bank Foundation, Venice, Italy to be used for research with a written consent from the donor's next of kin.

### *Donor characteristics*

The average age of the donors was 67.6( $\pm$ 7.47) years with Male:Female donor ratio of 14:6. The average post-mortem time of 15.99( $\pm$ 5.56) hours and preservation time of 14.5( $\pm$ 4.83) days in tissue culture medium [TCM] were recorded followed by 4.6( $\pm$ 6.27) days in transport medium [TM]. TCM was composed of 2% new-born calf serum with MEM-Earle as a base medium along with 25 mM Hepes buffer, 26 mM sodium bicarbonate, 1 mM pyruvate, 2 mM glutamine, 250 ng/mL amphotericin B, 100 IU/mL

penicillin G and 100 mg/mL streptomycin. TM was composed of TCM+6% Dextran T500. TCM and TM were prepared in-house (FBOV, Mestre, Italy) with full regulatory compliance. The endothelial cells were determined using a hypotonic sucrose solution and the mortality was estimated using Trypan blue staining. The endothelial cells were checked using an inverted microscope (Zeiss, Jena, Germany). The average endothelial cell density recorded was  $2070(\pm 374.31)$  cells/mm<sup>2</sup> with no initial mortality or uncovered areas.

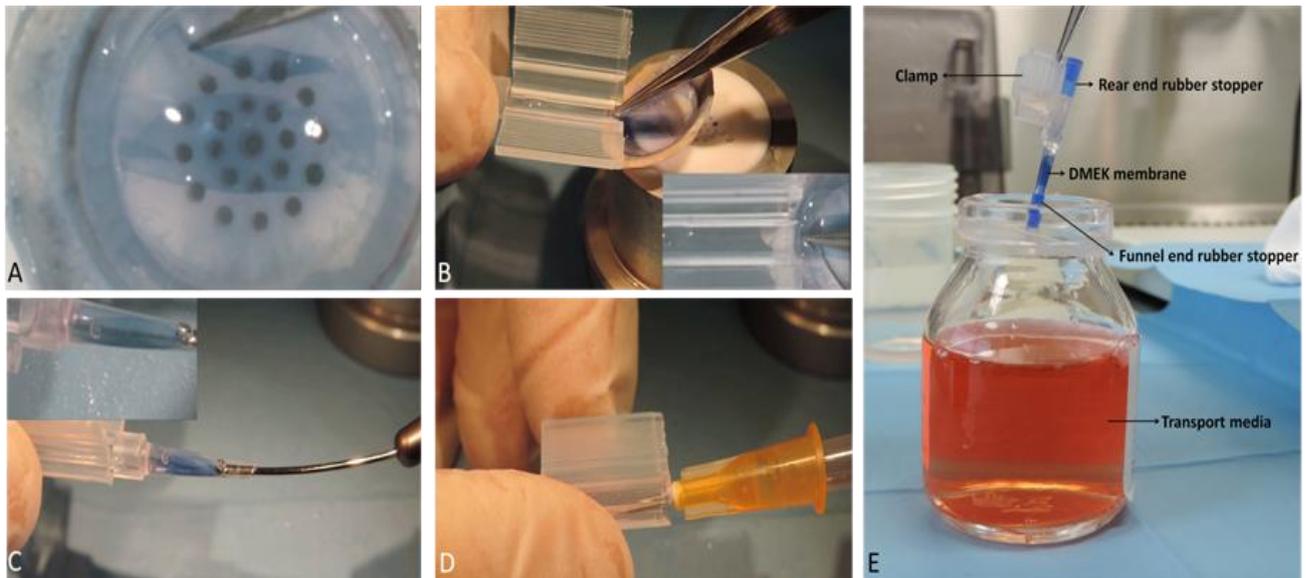
### *Stripping method*

The [n=20] corneas were centered on a punch base of the suction. Vacuum was created using a syringe and the cornea was secured on the base. A 9.5 mm [Moria, Antony, France] trephine was used to make a superficial cut using gentle tapping method. The endothelium was stained with Trypan blue for about 20 seconds to determine the area of the cut. Excess peripheral membrane was removed using 120mm acute forceps (e.janach, Como, Italy). The endothelium was kept moist during the entire procedure using TM to create a film of fluid on the top of the corneal-scleral ring. The membrane was slightly lifted using a cleavage hook and the procedure was carried on along the entire circumference of the cut to ensure limited tearing of the very fragile DMEK tissue. The peripheral membrane was stripped with a longitudinal movement using a 3 quadrant method, ensuring no torsions were generated during this phase to limit the mortality. Once the tissue was stripped completely, it was re-stored on the corneal stroma.

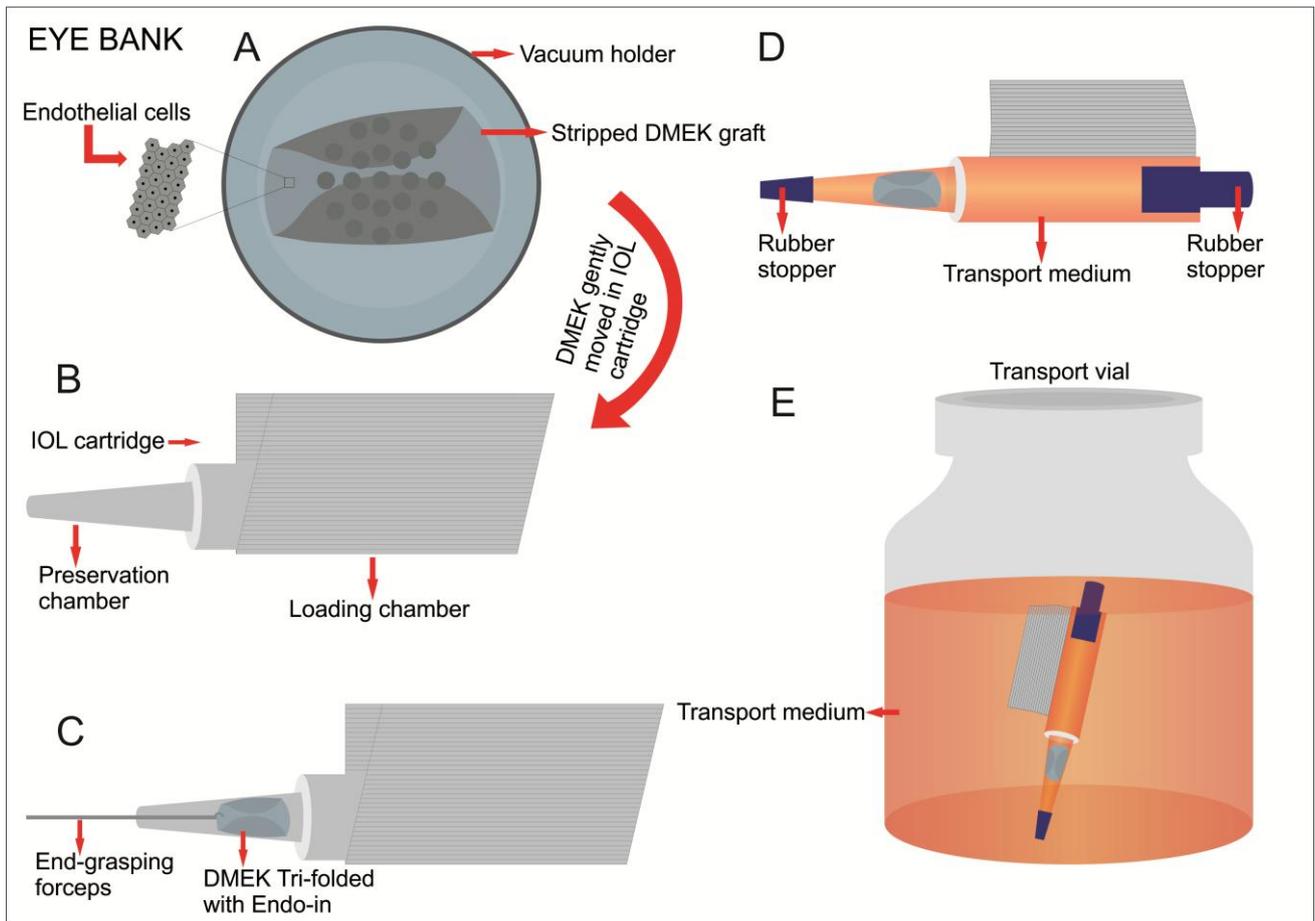
### *Loading method*

The pre-stripped membrane (9.5 mm) was trephined again with an 8.5 mm punch [Moria, Antony, France] and the excess periphery was excised maintaining the endothelial side facing the air. The membrane was folded manually from two ends using an acute forceps,

touching the Descemet side to avoid as much endothelial damage as possible (figure 1a). A small drop of liquid was added on the membrane to keep the tissue moist during the entire procedure. The purpose of the drop was only to support the moisture and not the opening of the membrane as this may change the orientation. The membrane was gently moved from the tissue base to a 2.2 IOL (Intra Ocular Lens) cartridge (Viscoject, Wolfhalden, Switzerland) preservation chamber (figure 1b). This step can be performed either using a contact lens as a scaffold as showed by Busin et al. [10] or directly from the cornea to the cartridge maintaining the architecture of the membrane. The membrane was pulled inside the funnel using a Grieshaber Revolution DSP 25 Ga + endgrasping forceps (Alcon, Texas, USA) from the funnel opening (figure 1c). A small amount of TM (<0.5 mL) was used to fill the funnel of the IOL cartridge for preservation of the DMEK membrane from its rear end (figure 1d). The IOL cartridge was sealed with a rubber plug both at the funnel and the rear exit. The entire unit was sealed with an in-house modified plastic clamp. The entire system was preserved in a sterile flask with TM for 4 days at room temperature (figure 1e). The plug was removed and the tissues were released out from the funnel pore using the microincision forceps and analyzed post preservation. Schematic representation of pre-loading the graft is shown in supplementary Figure 1 (derived from Eye World, ASCRS, October 2016, USA).



**Figure 1:** Pre-loaded Descemet Membrane Endothelial Keratoplasty preparation technique. A) using acute forceps on the Descemet side, the tissue is tri-folded with endothelium flapped in position with a small drop of tissue culture medium supplemented with dextran to keep the tissue moist. The graft should be folded half-way through from both the ends to make a tri-fold maintaining the correct orientation, b) the graft can be directly transferred from the corneal base to the intra ocular lens cartridge preservation chamber using acute forceps which should only touch the periphery of the graft, c) using an endgrasping forceps, the graft is gently pulled inside the funnel (pre-filled with transport media) [the over-flaps should be visible on the top end and the Descemet Membrane should be touching the cartridge], the graft is secured in the funnel area, d) the cartridge is filled with the transport media from the back and closed using rubber stopper to preserve the membrane and its orientation inside the funnel and e) the funnel is closed using a sterile rubber stopper and the graft is preserved using transport media in a 2.2 intra ocular lens cartridge fixed using two rubber stoppers at each end and a clamp on the top, the entire unit is preserved in 50 mL transport media to avoid any leakage and shipped.



**Supplementary figure 1:** Pre-loading DMEK grafts in the eye bank. a) Stripped DMEK tissue on vacuum block with endothelial side tri-folded inwards, b) DMEK graft is gently moved in the loading chamber of an IOL cartridge, c) and pulled further in the preservation chamber using end-grasping forceps, d) DMEK graft in the IOL cartridge filled with transport medium blocked from its rear and front end using rubber stoppers and e) the entire unit is then preserved in transport vial using transport media.

### *Endothelial cell survival*

The membranes were stained with trypan blue for 20 seconds and washed with PBS. The endothelium was checked using an inverted microscope [Primovert; Zeiss, Jena, Germany] for endothelial cell survival and tissue integrity. The cells were counted using a 10X10 eye piece reticule and the images were captured at 100X magnification.

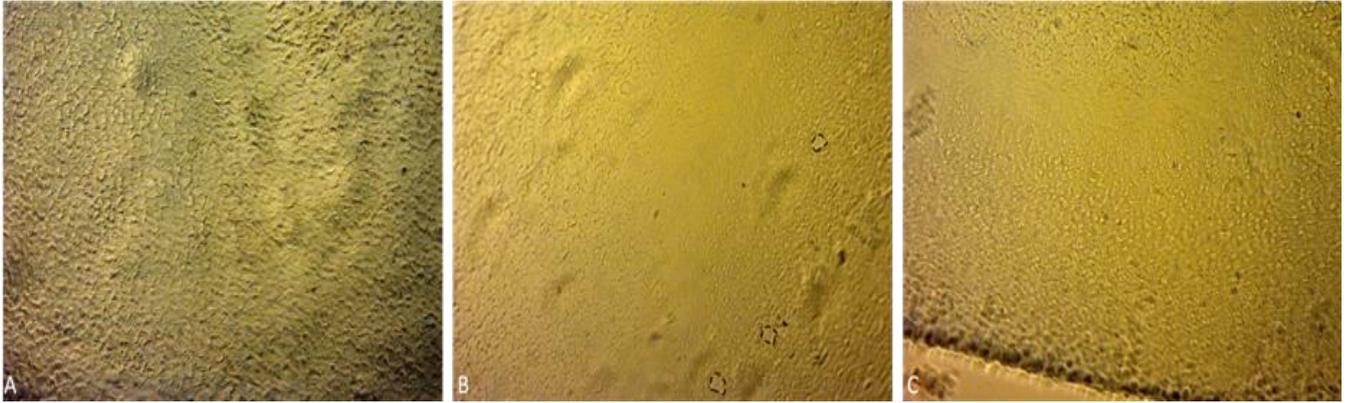
### *Glucose uptake*

Glucose uptake was determined in the preservation media in which the tissues were preserved for 4 days. The metabolic activity of the endothelial cells was checked using this technique. D-Glucose HK kit (Megazyme International Ireland Ltd, Bray Business Park, Bray, Co. Wicklow, Ireland) was used for quantitative analysis post preservation [8].

## **Results**

### *Endothelial cells survive after preserving pre-loaded DMEK grafts [n=20]*

All the final twenty experimental tissues were stripped and loaded successfully (100% cases). Average stripping and loading time was recorded at 20 and 4.5 minutes respectively. Average endothelial cell density after stripping the tissues was  $2070(\pm 374.31)$  cells/mm<sup>2</sup>, which did not differ from the initial count. However, the mortality increased to  $5.08(\pm 12.61)\%$ . There were visible uncovered areas in the tissues post stripping with an average of  $0.73(\pm 1.89)\%$  [calculated manually at a magnification of 100x using a 10 X 10 calibrated reticule mounted in the ocular of the microscope – fixed frame technique] (figure 2a). Average endothelial cell density post preservation was found to be  $1980(\pm 278.34)$  cells/mm<sup>2</sup> with mortality of  $3.55(\pm 5.79)\%$  and  $7.8(\pm 14.13)\%$  uncovered areas (area on the tissue without endothelial cells) (Figure 2 middle). Endothelial cell loss (ECL) after preservation was 4.35%. Localized uncovered areas were observed in some parts (Figure 2b). Apart from the areas where the endgrasping forceps get in contact with the endothelium for pulling the graft inside the funnel (approximately 0.25-0.5 mm<sup>2</sup>), other areas of the periphery did not show any cell loss or mortality (figure 2c). It was also observed that if the tissues are kept moist and folded just to orient the graft then minimal to no mortality can be observed.



**Figure 2:** Determination of the endothelial cell density, mortality and uncovered areas of the preserved grafts at 100x magnification. a) the endothelial status post stripping with minimal mortality and uncovered areas, b) small uncovered areas as marked in the figures and c) peripheral endothelium without any mortality or uncovered areas (this peripheral area is different than the one where the endgrasping forceps touch the endothelium)

During the learning curve, mortality was observed on the folds (figure 3a). The tissues must be handled gently as if grabbed rigorously using endgrasping forceps, the tissues may tear near the periphery (figure 3b). Less uncovered areas were recorded after learning the method using more than twenty corneas (figure 3c). The tissues should be handled gently to optimize results and the technique requires a learning curve.



**Figure 3:** Determination of the endothelial cell density, mortality and uncovered areas of the preserved grafts at 100X magnification during the learning curve. a) uncovered areas and trypan blue stained cells were observed if the tissues are rigorously handled while transferring them on the intra ocular lens (IOL) cartridge from the cornea base and preserved for 4 days at room temperature in transport media, b) a tear was observed in the periphery if the microincision forceps are touched tightly and when the tissue is not moved in the funnel gently and c) relatively lesser mortality and uncovered areas were observed with increased learning techniques.

*Pre-loaded grafts show active metabolism [n=20]*

The average glucose uptake by the endothelial cells in the preservation chamber for 4 days at room temperature was  $0.55(\pm 0.26)$ mg/mL (Minimum – 0.10 mg/mL and Maximum – 0.882 mg/mL) which is half the total amount of glucose present in the media (1 mg/mL). Glucose uptake was higher as compared to the previously shown uptake from pre-loaded DSAEK membrane [8].

## **Discussion**

DMEK requires high surgical skills both for the preparation and transplantation of the tissues. If eye banks can provide ready and validated DMEK tissues, surgeons do not have to engage with this time consuming phase of the procedure, thus saving time, eliminating possible tissue waste and facilitating surgery.

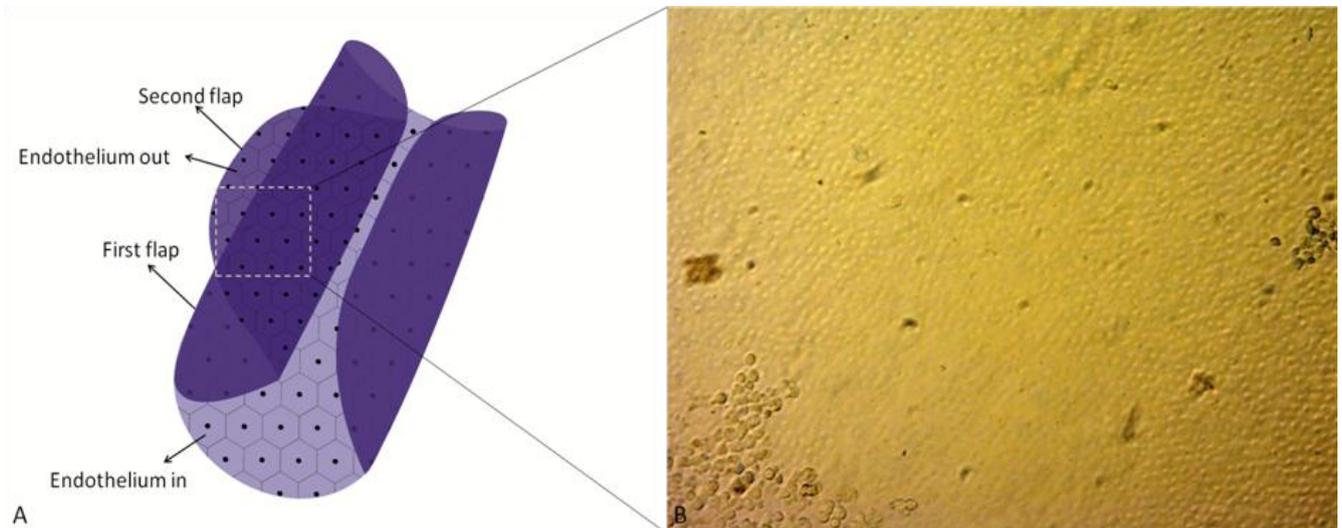
It has already been determined that the cell loss in dextran based media is higher than it has been found without dextran for PK [11-12]. However, we have observed that corneal-scleral rims obtained from the dextran based medium allow excising a regular DMEK graft with minimal endothelial cell loss. Dextran based medium helps to keep the endothelial

cells adhered to the DM and maintains the flexibility required for the tissues for unfolding it (unpublished data). Moreover, we have also obtained 100% successful peeling cases when the tissues were obtained from TM as compared to 76% successful peeling cases when the tissues were collected from TCM. Therefore we selected an inclusion criteria of the tissues prepared and preserved in dextran based medium.

Mortality was observed near the folds or when the tissues were treated rigorously by making the folds tighter and stronger to maintain the orientation, which was later realized as not so important. A small drop of liquid that keeps the tissue moist and without any tight folds can be served as the best option. Majority of the uncovered areas were found near the periphery of the tissue where the endgrasping forceps touch the endothelium to pull the graft inside the funnel. In one of the cases, a peripheral tear was generated with high mortality and uncovered area, whereas, in other case, only uncovered areas were observed on the peripheral tissue without any mortality. If the graft is gently pulled in with minimal manipulations, then the forceps generated uncovered areas and mortality decreases to the level of acceptance for grafting as described by Busin et al [10].

Most of the cases showed the correct orientation and the architecture of the graft same as before preservation however, one case during the learning curve showed an 'S' shaped fold where half of the corneal endothelium was exposed to the cartridge (figure 4a) and the cells were completely detached from that area (figure 4b) showing once again the importance of avoiding possible contact between endothelium and cartridge wall. A contact lens could be employed or the graft should be pulled gently inside the preservation chamber and the funnel area [10]. It was also observed that due to the thickness of the IOL cartridge (2.2 pore opening), the tissue does not get a chance to open up and unfold itself to endothelium out. The tissues open up to its maximum with endothelium-in and

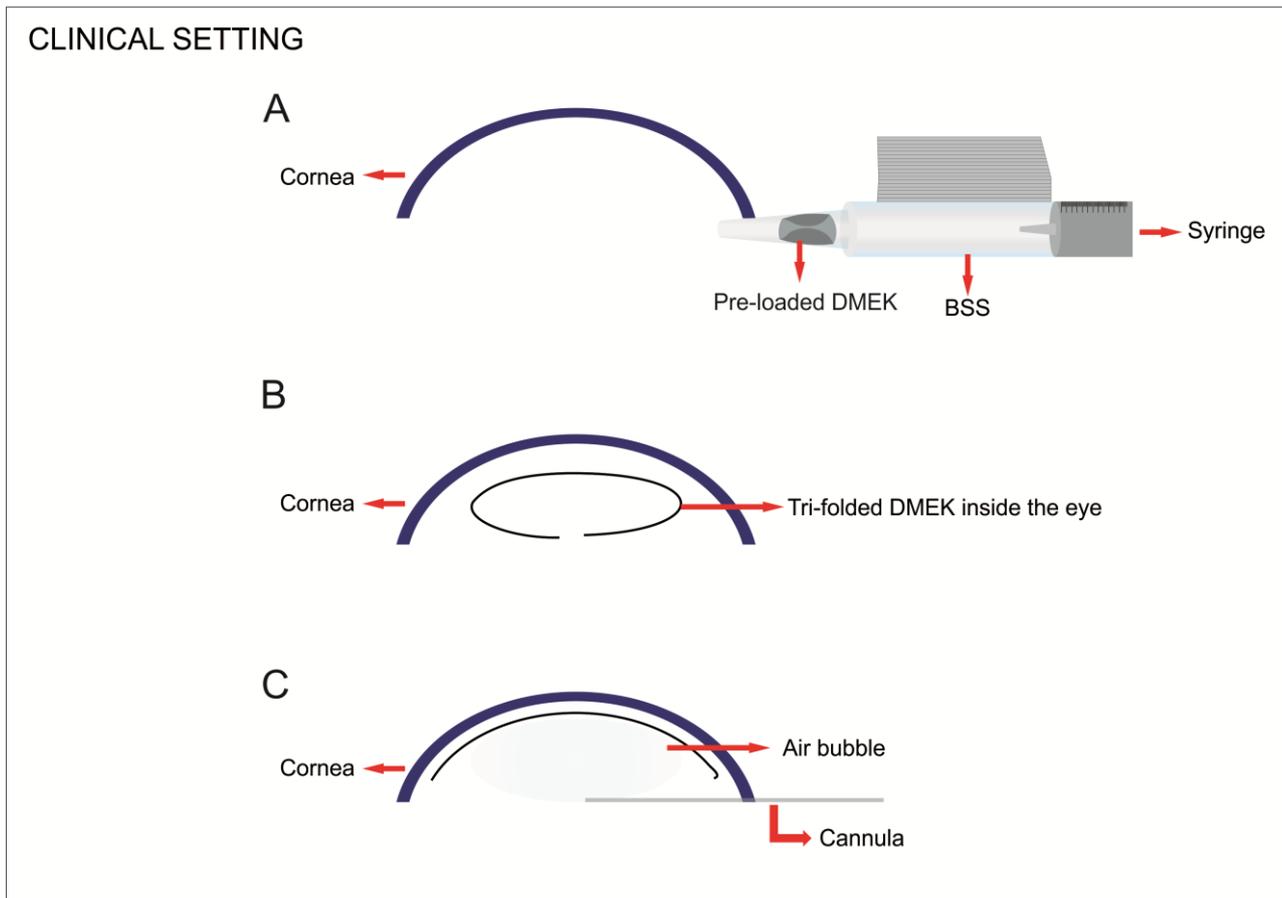
stay in the same fashion without any further opening. The 'S' shape was observed due to manual error while pulling the tissue inside the cartridge. If the tissue is properly inserted then they stay without any further damage or de-orientation.



**Figure 4:** De-oriented graft showing 'S' shape orientation. A) graphical representation of the tissue showing 'S' shape orientation with one flap exposed to the wall of the cartridge and b)- the tissue showing >90% loss of the endothelial cells (uncovered areas) on the exposed flap.

There are two transplantation strategies for DMEK, a) the injection method which is widely used around the world due to its simplicity and natural rolling of the graft and b) pull-through technique that is contemporarily used for DSAEK surgery, but has been also introduced recently for DMEK [10]. Due to the presence of serum in the preservation medium, it is not advisable to inject the tissue directly in the recipient eye as the medium will be injected as well. Therefore, a pull through technique should be preferred for a successful surgery. The preserved tissue can be released out of the cartridge using a microincision forceps and inserted into the recipient eye using bimanual pull-through technique. As the endothelium is flapped-in, it reduces a direct contact or friction of the endothelial cells with the wall of the cartridge.

The DMEK membrane in its natural tendency rolls with the endothelium-out when in contact with liquid and therefore if they are manually flapped-in (forced against the nature), once inside the eye, they open up easily and try to retain its natural formation inside the recipient's eye. Therefore, this may serve as an additional advantage over the traditional DMEK surgical technique. Moreover, the tissue preparation and loading time (approximately 25 minutes) can be saved in the surgery if the tissue is pre-loaded in an eye bank. Simultaneously, if the injection method is supposed to be used then the TM from the funnel must be removed out using a cannula and filled with balanced salt solution (BSS) and the tissue can be injected in the eye. However, for both methods, the tissue can be stained inside the funnel using trypan blue for better visibility during the surgery [Supplementary video 2]. We have also observed that the tissue opens easily with either methods but only when the endothelium is flapped inwards. The tissue can be easily washed using BSS to aspirate the TM out however this procedure should be carried out gently to avoid any de-orientation of the graft inside the funnel. Alternatively, the TM can be removed using a 1 mL syringe and the tissue can be washed with BSS. We have found that using the BSS directly to remove the TM has been the best option as it avoids the unscrolling and de-orienting the graft. Removing the TM may suck the tissue and grab it on the needle of the syringe making it difficult to keep the orientation with endo-in. Schematic representation of the process is explained in figure 5 (derived from Eye World, ASCRS, October 2016, USA).



**Figure 5:** Pre-loaded DMEK graft in a clinical setting. a) Transport medium in IOL cartridge is replaced with BSS and the DMEK graft is stained with Trypan blue. A syringe is fixed at the rear end for injecting the tissue inside the recipient eye, b) the stromal side of the tissue should face the wall of the cartridge and on the top, facing the stroma of the recipient to easily open inside the recipient's eye and c) tri-folded DMEK opens inside the eye spontaneously and it is allowed adhering to the recipient stroma using air bubble with a cannula.

It has already been proposed that the DMEK donor tissues preserved up to 2 days at 4°C does not show any significant endothelial cell loss difference [13]. However, the results described in this paper suggest that the DMEK tissues can be preserved in the IOL cartridges filled with transport media for up to 4 days at RT without a high cell loss and active metabolism. The surgical outcome further needs to be evaluated. The tissues used

in this study were obtained from relatively older donor corneas and therefore they did not unscroll to its natural tendency of endo-out. However, the tissues should be preserved around 2 mm away from the funnel pore to keep the tissue as tight as possible and avoid any de-orientation of the graft. DM-endothelium grafts for transplantation in DMEK procedures can be prepared from TM and preserved in TM for the best outcome. The tissues should be transplanted using a pull-through technique to reduce the possibility of injecting the serum inside the recipient eye, although an injection method could also be used if the tissue is washed inside the funnel. Injection method will reduce the chances of peripheral tears and uncovered areas. Pre-loading DMEK membrane will reduce surgical time, tissue wastage and costs. In addition, it will provide a validated tissue for transplantation. The study presented here is a 'proof of concept' and has not undergone any clinical evaluation therefore the complications need to be identified before undergoing any surgery.

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## **CHAPTER 7**

### **ISOLATION OF THE PIG AND HUMAN CORNEAL**

#### **ENDOTHELIUM**

## **Aim**

To understand the cell culture technique for pig and human corneal endothelial cells and determine the optimal conditions.

## **Introduction**

There are several methods that are in use for Human Corneal Endothelial Cells (HCECs) isolation. Initially, Collagenase or Trypsin was used on whole corneas but as it inevitably caused interference of corneal fibroblasts, a selective L-valine-free medium was used as described earlier. Stripping the Descemet's Membrane (DM) and the endothelial explants culture in T-2 flasks have also been used. Peel and digest method by Peh *et al.* have showed successful cultures as well. The recent protocol shows scrapping of HCECs with the DM using enzyme digestion followed by cell scrapping. Although this method may eliminate fibroblast contamination, scrapping of cells using a blade has reduced the viability of the primary cells dramatically. Other methods can include stripping or bubble techniques to separate the Descemet's membrane from the stroma and then digest the cells using trypsin for cell culture. Stripping of HCECs can also be performed with the aid of a vacuum suction holder, as described by Peh *et al* [4]. After peeling, the Descemet's membrane and endothelial layer are finally digested enzymatically, using dispase or Ethylenediaminetetraacetic acid (EDTA), followed by pipetting. However, enzymatic digestion requires a prolonged incubation time to detach cells from the matrix, subsequently leading to higher cellular degeneration. Thus, it is evident that there is still room to standardize a specific and reproducible method to isolate and culture HCECs from the donor corneas [5].

As there is no standardized protocol for isolation, two major isolation techniques were investigated which included, a) Accutase cell scrapping method and b) Peel and digest

Trypsin method. The media constituents were re-evaluated for the optimal concentrations from the literature.

## Materials and methods

### *Media for culturing HCECs*

<b>Media for HCECs</b>	<b>500 mL</b>
Ham's F12	195 mL
DMEM	195 mL
FBS	100 mL
Penstrep	5 mL
Fungizone	5 mL
Insulin	0.5 mL
Ascorbic acid	10 mg
bFGF	20 microg

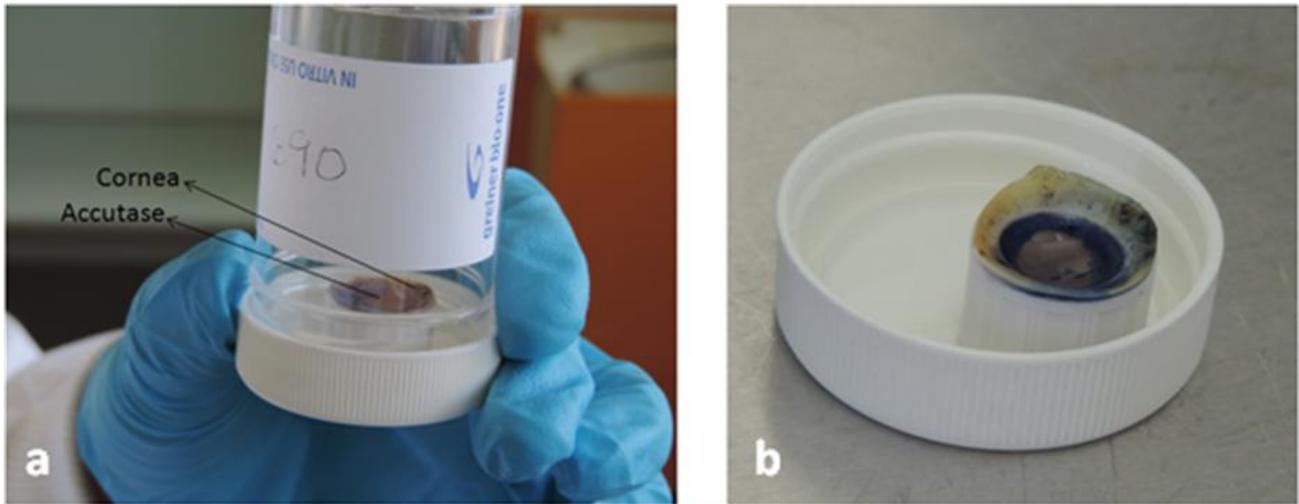
*Materials to culture pig and HCECs using **ACCUTASE CELL SCRAPING METHOD** and **PEEL AND DIGEST TRYPSIN METHOD***

Accutase
Trypsin-EDTA
Tweezers
Silicon blocks
Sterile petri dishes
Cell scrapers
T 25 flasks

6 and 24 well tissues culture coated plastic plates
Sterile PBS 1X
PFA for histology
30% Sucrose for wash
H&E staining materials
ICC antibodies for cell characterisation
Vacuum blocks
8.5 mm punch
Pierce forceps

*Method to culture HCECs using ACCUTASE CELL SCRAPPING METHOD*

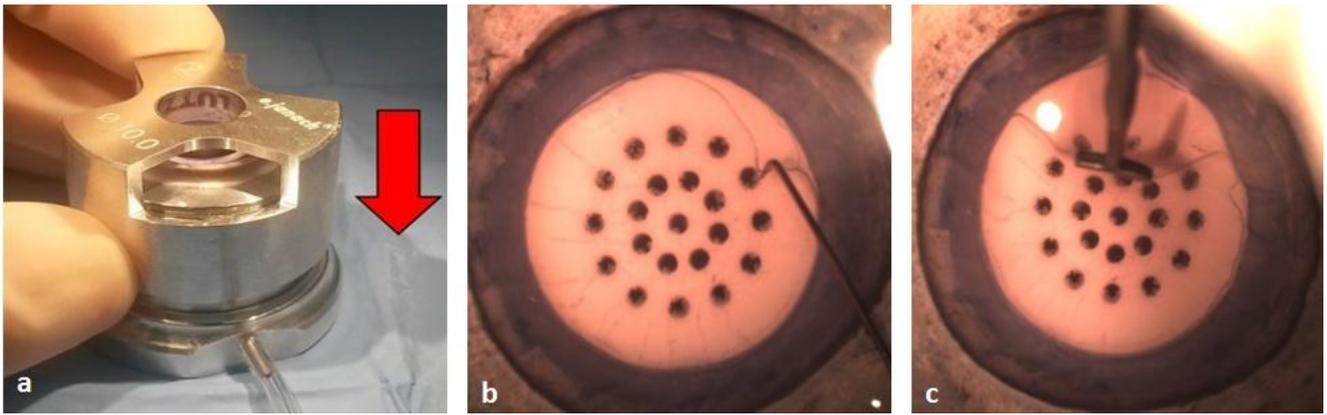
After excising the TM, the corneas were placed in a way that the corneal epithelial side faces the bottom of sterile plastic bijou tube eye holders. The endothelium was covered with Accutase enzyme (approximately 200  $\mu$ L) up to the pigmented line and the entire unit was incubated for 15 minutes at 37°C with 5% CO<sub>2</sub> and humidified atmosphere as showed in figure 1a. Using a sterile cell scraper or a plastic inoculation loop, the cells were dislodged at the peripheral endothelium. The cells mix with Accutase and hence the cells can be directly pipetted out along with Accutase directly into one well of a 24 well plate with 1.5-2.0 mL of fresh HCECs medium and incubated at 37°C. The medium was changed after 2-3 days. During the first media change, the cells were washed using PBS to remove any potential debris associated with dissection. Trypan blue staining was carried out to judge the area of the excised tissue as showed in figure 1b.



**Figure 1:** Cell culture of HCECs using Accutase cell scraping method. a) HCECs were exposed to accutase for approximately 15 minutes at 37°C in humidified atmosphere with 5% CO<sub>2</sub>. The cells were excised from peripheral and central cornea and cultured separately and b) Trypan blue staining of the HCECs after cell scraping from the peripheral region.

*Method to culture HCECs using **PEEL AND DIGEST TRYPSIN METHOD***

The Descemet's membrane and endothelium can either be separated using Submerged Hydro Separation (SubHyS) or Stripping method (central and peripheral). However, Stripping method (Figure 2) was selected for this study considering its reproducibility and access to peripheral and central endothelium which is difficult with SubHyS method as it separates an entire tissue.



**Figure 2:** Stripping method using peel and digest trypsin method. a) Initial punch using 8.5, 9 or 10 mm donor punch on vacuum block, b) identification of the central Descemet's membrane and endothelium and c) peeling the central Descemet's membrane and endothelium using Fogla forceps. The peripheral endothelium was excised using forceps and placed in Trypsin EDTA solution.

The peripheral and the central Descemet's membrane and endothelium were excised and were used for either explant culture technique or trypsin digestion method. Peel and digest Trypsin method helps to lower down the stromal interference and hence limits the fibroblast expansion but does not eliminate completely. The Descemet's membrane and endothelium was further digested in Trypsin EDTA solution after washing the lenticule in 1X PBS. The procedure is as follows:

- 1) The Descemet's membrane and endothelium (central and peripheral) [Note: The entire cornea was used as peripheral cornea did not show a higher number of extractions and therefore was difficult to identify the potential co-factors] was transferred in a tube containing 10 mL of Trypsin EDTA solution (TE). The tube containing the tissue was incubated in water bath at 37°C for 30 minutes. The tube was shaken manually every 5 minutes approximately.

- 2) At the end of the incubation time, the biopsy was transferred in a new 15 mL tube containing 5 mL of TE and a new cycle of trypsinization was carried out.
- 3) At least 5 mL of the HCECs medium was added to the tube containing the solution with the extracted cells to stop the Trypsin procedure. The tube was centrifuged for 3 minutes at 800 rpm. The supernatant was aspirated gently to avoid drying of the cell pellet. The pellet was re-suspended in approximately 0.5 mL of the medium, and the final volume was verified.
- 4) Approximately 50-100  $\mu$ L of the sample was transferred into a 2 mL eppendorf tube, followed by a transfer of 50  $\mu$ L to a second eppendorf tube containing 50  $\mu$ L of Trypan blue. One or both sides of the Haemocytometer slide was filled with the solution and the cells were counted by 2 different drafts of the cell suspension. The number of extracted cells were counted, distinguishing between viable and non-viable cells using a standard formula:

*'Number of cells/number of chambers (4 or 8) X 10,000 X Trypan blue positive cells X Volume'*

The entire extraction procedure was repeated until the cells are decreased drastically (usually 2-3 cycles). The cells were plated as soon as possible, to avoid the cells in suspension for long. Therefore, after each extraction cycle or when the sufficient number of cells was extracted, the plating of the cells in a 24 well plate was carried out. The plates were incubated at 37°C, 5% CO<sub>2</sub> and 90% of humidity for 48 hours. Media was replaced every alternate day.

The confluent cells were further re-suspended using Trypsin EDTA treatment for 5 minutes incubated at 37°C, 5% CO<sub>2</sub> and 90% of humidity. 50% of the suspension were re-

cultured and the rest were used for immunocytochemistry after cyto-spinning the cells for 5 minutes at 1000 RPM to characterize the cells.

*To check the density of the cells as a potential factor for human corneal endothelial cell culture*

10 human donor corneas (organ cultured) were pooled in together using cell scrapping method to excise total endothelium comprising periphery and central region. TM was excised and the cells were scrapped up to far periphery as described earlier. The cells were placed in 5 mL fresh media (HCECs medium). The cells were centrifuged at 200G for 4 minutes and the pellet was obtained. 3 mL of media was re-filled to collect the cells and were further cultured in serial dilutions. The dilution factors that were used for this study with cells:media were 1.5:0.5; 1:1 and 0.5:1.5 respectively.

However, with peel and digest method, the cell count obtained was higher as compared to the Accutase cell scrapping method and therefore 4 corneas were used for the peel and digest method to collect different amount of cells and plated to check if the density was an issue for primary cells to expand and check the cell-cell communication.

*Explant culture method*

The lenticules were peeled off and placed in a T-25 flask with the Descemet's Membrane side touching the flask. The media was refilled every 2 days. Only stripping technique can be used for such culture methods.

*Histological analysis*

The excised Descemet's membrane and endothelium were used for the culture and the remaining tissue was fixed in 4% paraformaldehyde (PFA) for 24 hours at 4°C. This

procedure was carried out to check the absence of Descemet's membrane and endothelium from the entire tissue and verify the separation. The tissue was washed with PBS and was treated with 7.5%, 15% and 30% sucrose consecutively for 30 minutes each. The tissues were further washed in PBS and embedded in optimal cutting temperature compound (OCT) for 30 mins at -80°C before cutting. 10-25 micron thick sections were cut for histological analysis.

#### *Histological analysis with Hematoxylin-Eosin (H&E) staining*

The sections were brought to room temperature before H&E staining. The sections were dipped in Harris Hematoxylin for 3 minutes and washed briefly in Scott's tap water thrice to remove excess Hematoxylin. The section was further dipped into differentiation solution for 2 mins and was washed in tap water briefly followed by eosin treatment and washing with tap water for 2 minutes. Evaporation procedure was carried out using 70%, 90% and 100% ethanol followed by Xylol for 1 minute each. The slides were then air dried and the sections were fixed and mounted using DPX mounting solution and were viewed under an inverted microscope at 100X magnification. The pictures were collected using Zen software and were analysed further.

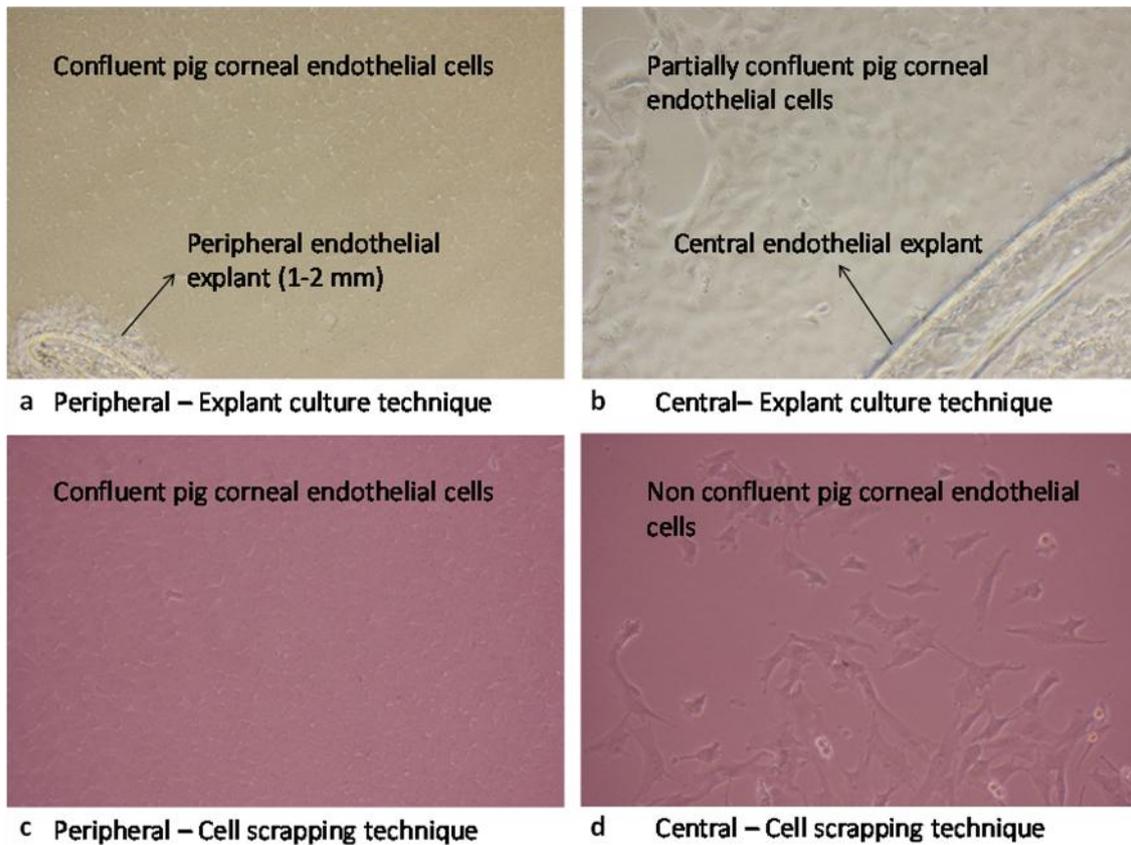
#### *Immunostaining using Zonula Occludens-1 (ZO-1)*

The cultured cells were extracted using Trypsin treatment for 5 minutes at 37°C, 5% CO<sub>2</sub> and 90% of humidity. The extracted cells were counted and were sub-cultured as first passage and cyto-spinned 1:1. The cells were placed in the cyto spin chamber and were spinned down at 1000 rpm for 5 minutes. The collected cells were fixed with 4% PFA overnight. The cells were then brought to room temperature before washing them with PBS thrice at RT for 5 minutes. Permeabilization solution (0.5% Triton-X solution) was used for 30 minutes at RT and was washed briefly with PBS. The cells were further

blocked in a humidity chamber for 2 hours at RT using a blocking mix (10mL PBS + 150  $\mu$ L goat serum + 0.1 gm bovine serum albumin + 10  $\mu$ L of Triton-X). The cells were further incubated overnight at 4°C using blocking solution and primary antibody (ZO-1, 1:100). PBS was used to briefly wash (three times 15 minutes each) the primary antibody off. Secondary antibody conjugated with FITC (1:100) was used with blocking mix for 2 hours at RT in dark. The cells were further washed with PBS for 5 minutes thrice in dark to avoid bleaching and were further mounted with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) which is a nuclear stain which can penetrate in the intact cell membrane. Cells were examined using a LSM 510-meta laser scanning microscope (Zeiss, Milan, Italy). Examination was performed under an ultraviolet light or by excitation at wavelengths of either 488nm or 594nm, and subsequent detection of the fluorescence was obtained.

## **Results**

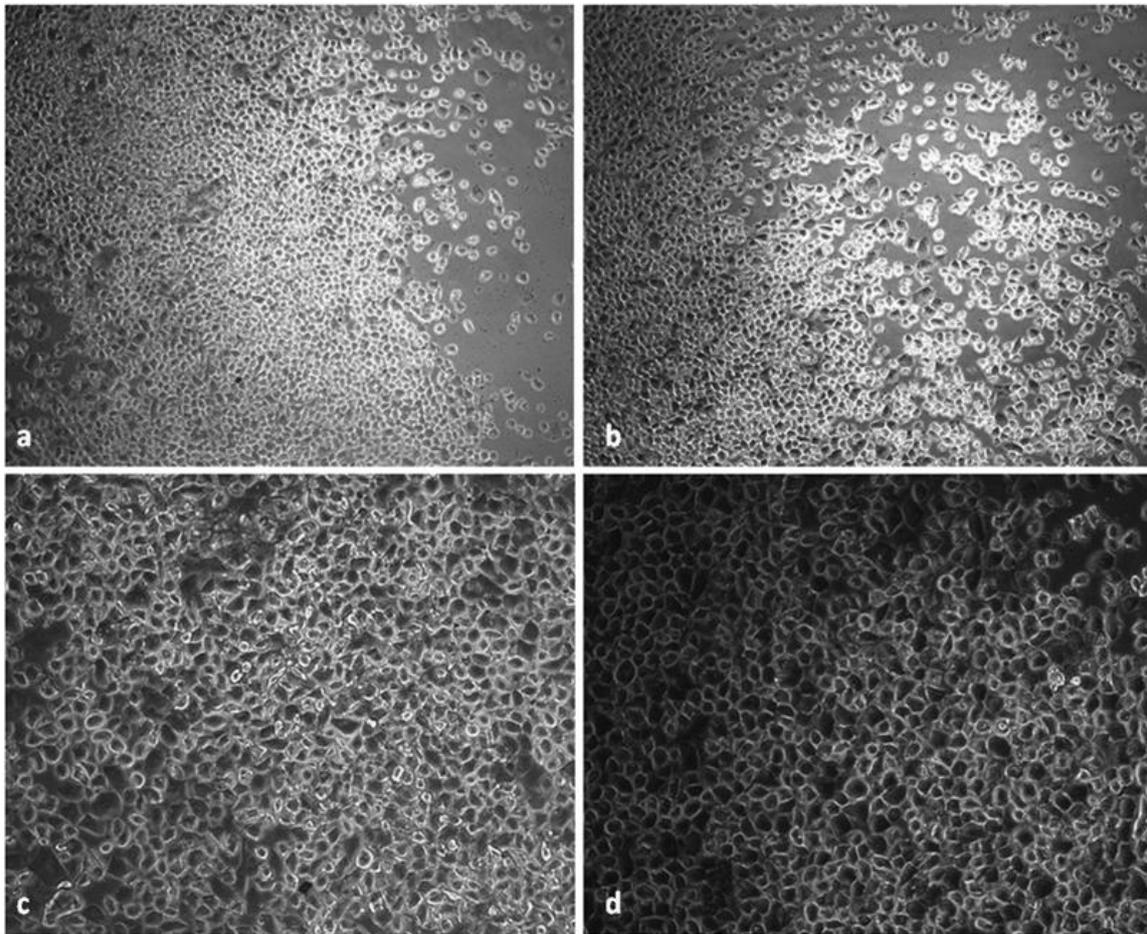
Pig corneal endothelial cell culture using explants culture and cell scrapping technique. A higher rate of cell proliferation capability was seen from peripheral region of the explants and the cell scrapping method as compared to the central region which reflects the possibility of the presence of putative stem cells in the corneal peripheral endothelium as seen in figure 3.



**Figure 3:** Pig corneal endothelial cell culture using explants and cell scrapping technique at 100X magnification of an inverted microscope. a) the peripheral explants showed confluency of the cells within two weeks of the culture where as b) the central explants showed partial confluency in the same period. c) Accutase cell scrapping method showed confluent cells after 1 week of culture, whereas d) the cells from central region did not show any confluency. The results show that the peripheral cornea may have putative stem cells.

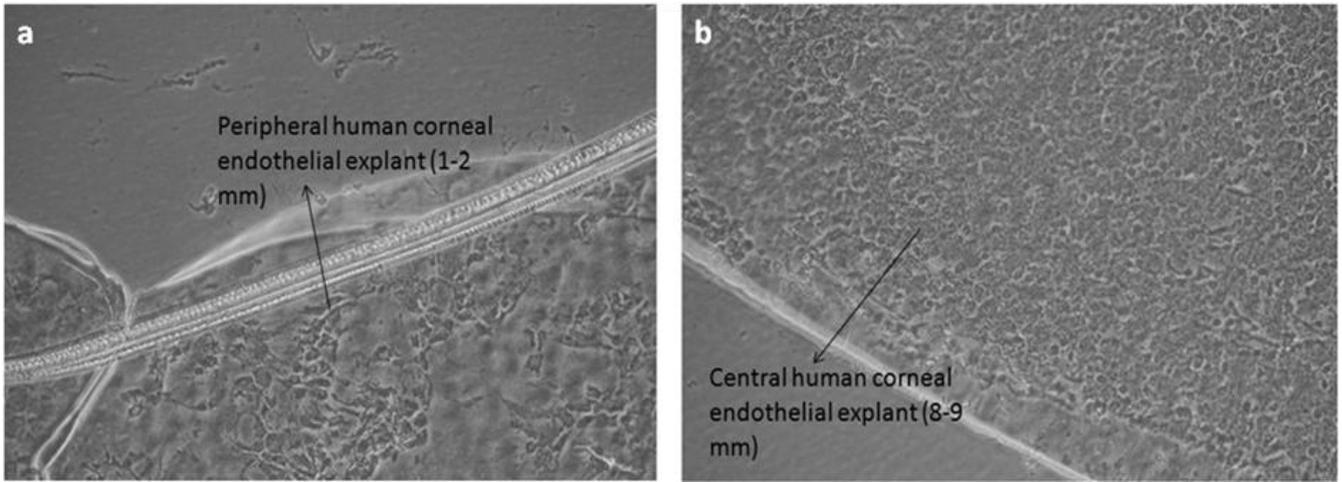
The cells from one of the cadaveric donor corneas showed proliferative capability as seen in figure 4 at different magnifications. The TM of this cornea was not removed and the cells were excised from far periphery of the corneal endothelium which is presumed to be the corneal endothelial stem cell niche. However, out of 15 corneas that were cultured, only one showed proliferative capability and therefore it is necessary to further investigate

the co-factors. This is currently being characterized and explored further. They are presumed to be HCECs only on the morphological basis.



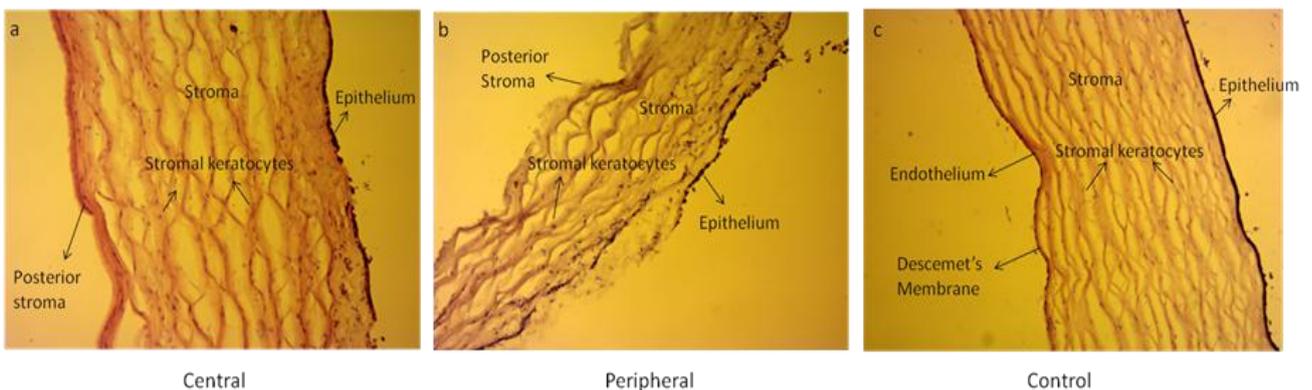
**Figure 4:** HCECs culture using Accutase cell scrapping method at different magnifications, a and b) 50X, whereas c and d) 200X, Only one of the donor corneas showed proliferative capability as seen in the figure at different magnifications. The morphology was observed to be similar to HCECs as per the literature.

Human corneal endothelial cell culture using explants culture technique. There was no cell proliferation seen using explant culture technique either from the periphery or from the central cornea as seen in figure 5. This also highlights that every donor behaves differently. None of the samples showed any proliferative capability out of five.



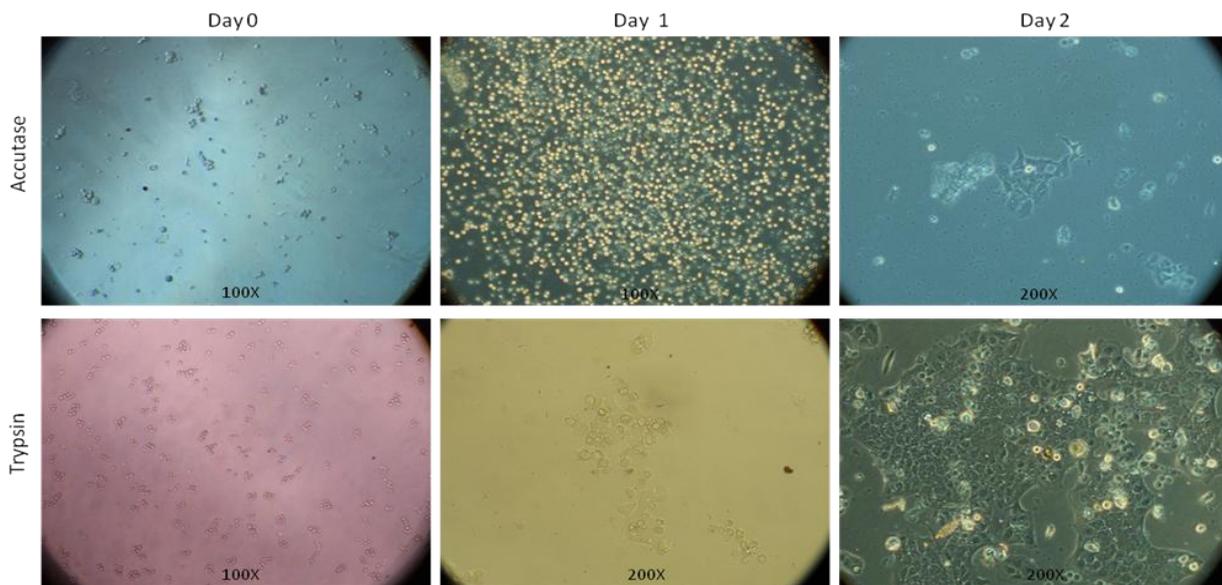
**Figure 5:** HCECs explants culture technique observed at 50X magnification. The cells did not proliferate from the a) periphery and b) central lenticule. Although the cells were observed on the tissues they did not show any growth after 2 weeks.

H&E staining showed the absence of Descemet's Membrane and Endothelium from the entire cornea further confirming the excision of HCECs from the donor tissues as seen in figure 6.

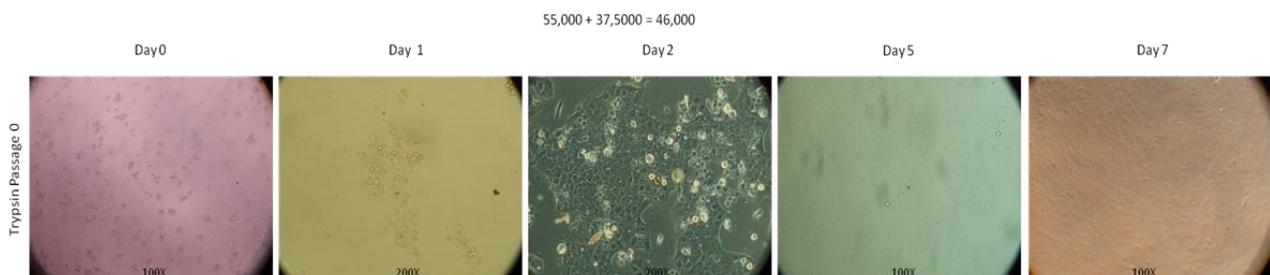


**Figure 6:** Histological analysis on the excised tissue observed using an inverted microscope at 100X magnification. a) centrally excised donor tissue without DM and endothelium, b) peripherally excised donor tissue without DM and endothelium and c) control tissue showing the presence of all the layers of a donor corneal tissue.

Two important characteristics were observed between the two methods: 1) Accutase does not allow excision of higher number of primary cells as compared to Trypsin digestion and 2) cell scrapping hampers the viability of the cells and therefore it becomes a little difficult to culture them further as observed in figure 7. The cells from the Trypsin digestion method were further cultured as they showed proliferative capability (figure 8) and were characterised later.

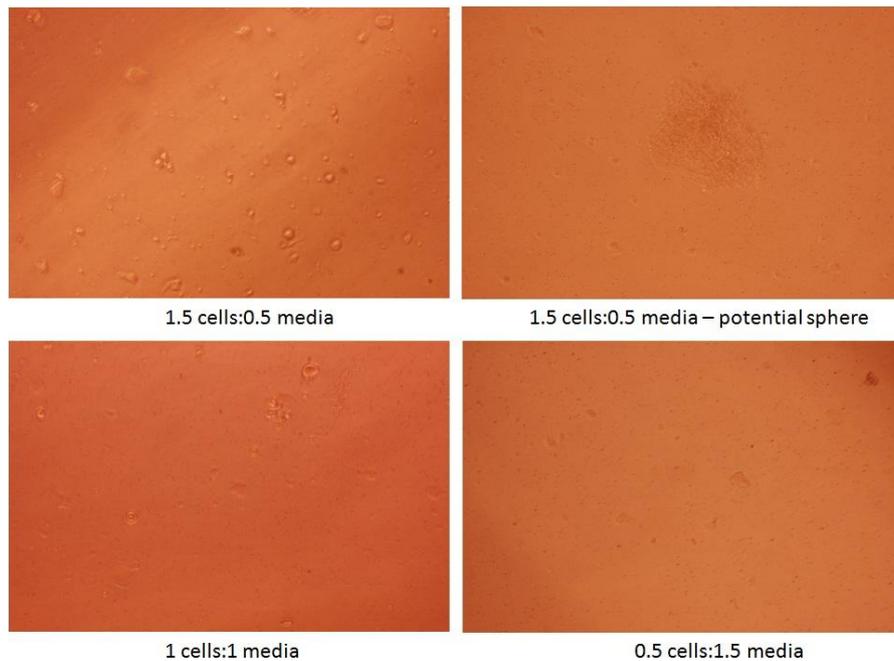


**Figure 7:** Comparison between Accutase and Trypsin digestion and isolation potential of each method for culturing human corneal endothelial cells at different magnifications and from day 0 to day 2. The figure shows that the Trypsin digestion allows a higher amount of cell isolation as compared to Accutase within first three days of the cell growth.



**Figure 8:** Cells isolated using Trypsin and cultured for 7 days before immunostaining at different magnification for better evaluation of the cell expansion *in vitro*.

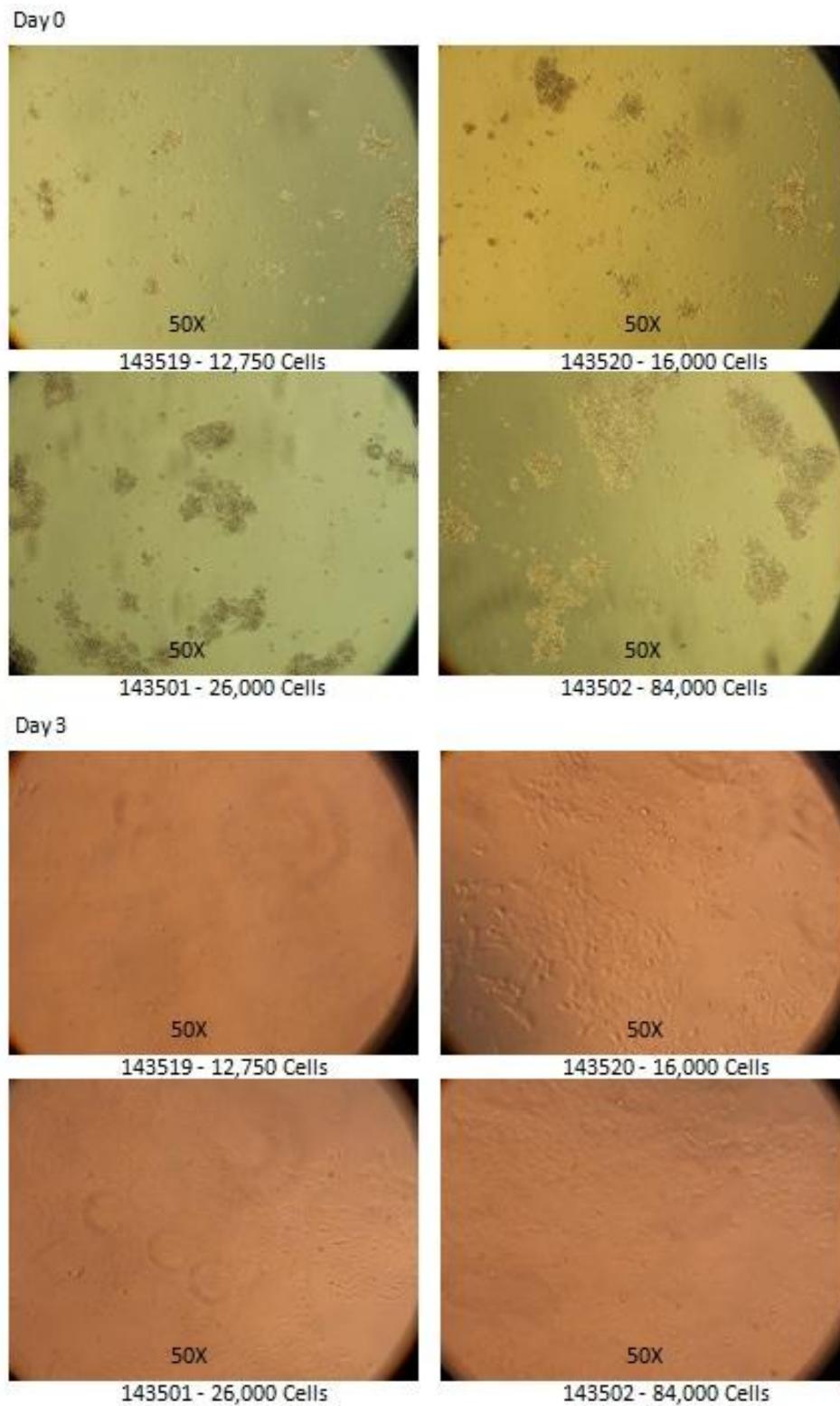
The cells obtained using Accutase cell scrapping method was too low and therefore the cells did not proliferate any further in either case as seen in figure 9.



**Figure 9:** Density gradient experiment to verify the theory of initial requirement of the cells for culture as seen using a 100X magnification of an inverted microscope using Accutase cell scrapping method. The amount of cells isolated was too low and therefore none of the cells proliferated further.

A higher amount of cells (countable under Hemocytometer slide) were obtained using Trypsin digestion method and were plated as per a higher degree of cell count obtained as seen in figure 9. It was observed that wells containing 12,750 cells did not show proliferation as compared to the one with 16,000 cells (same donor). Whereas the wells

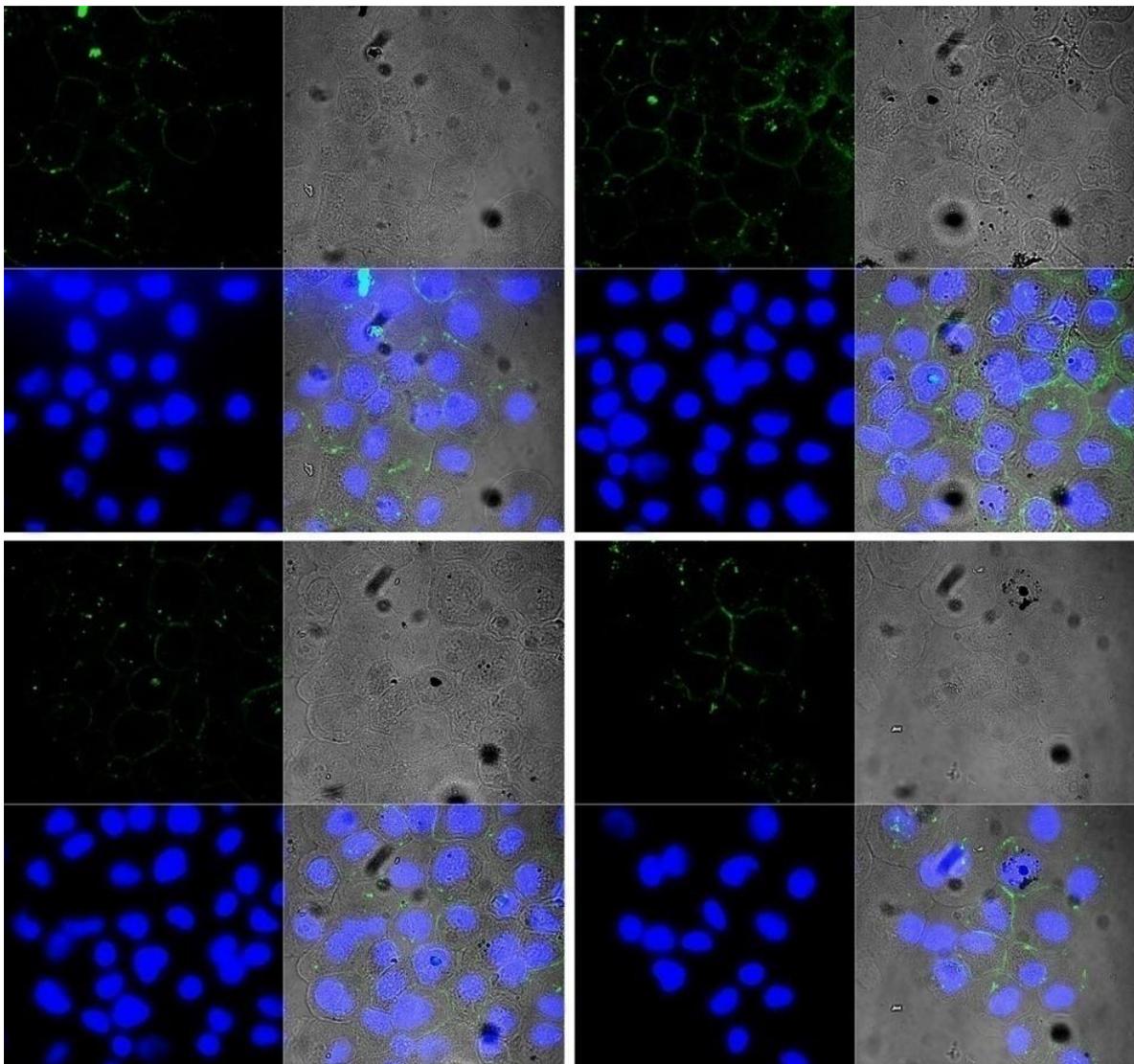
with 26,000 cells and 84,000 cells showed a high proliferative capability (same donor) as seen in figure 10.



**Figure 10:** Density gradient experiment to verify the theory of initial requirement of the cells for culture as seen using a 50X magnification of an inverted microscope using

Trypsin digestion method. Different amount of cells were isolated using this method and a higher rate of cell proliferation was observed within 3 days from the higher amount of cells that were isolated as compared to the ones with low amount of cell isolation.

The cultured cells were further characterized using ZO-1 (tight junctional protein expressed in the corneal endothelial cell borders) immunostaining. The cells expressed the ZO-1 antibody as seen in figure 11.



**Figure 11:** Immunostaining on the cyto-spinned cells using ZO-1 observed under oil immersion magnification. The marker stains the tight junctional proteins of the corneal

endothelium and therefore the presence of this marker indicates HCECs like cells from the culture and no interference of fibroblasts after day 7 of the culture.

## **Discussion**

Pig corneal endothelial cells can be cultured *in vitro* using the culturing technique as described in this article however, HCECs requires further knowledge of cell extraction, interaction and proliferation capabilities, identification of specific media and growth factors that allow these cells for a controlled growth *in vitro* and determine a standard cell culture technique using functional biomarkers for specific cell types. The early observations were -

1. Pig corneal endothelial cells can be cultured using Accutase and explants culture method using specific media formulation as described in the report. Peripheral corneal endothelium showed a high confluency as compared to the central proving the hypothesis of the presence of putative stem cells in the peripheral cornea.
2. Accutase method can be used to culture HCECs, however as only one cornea showed the proliferative capability, therefore the method needs a further validation and standardization.
3. HCECs cannot be cultured using explants culture method however other co-factors need to be evaluated before concluding this.
4. Stripping method shows a precise removal of the DM and endothelium and therefore limiting the growth of fibroblasts, however, this needs to be further evaluated to understand whether the fibroblasts appear after 2<sup>nd</sup> or 3<sup>rd</sup> passage as was seen in one of the earlier cases.
5. Trypsin digestion method showed a high amount of cell isolation, proliferation and confluency as compared to Accutase and therefore Trypsin digestion method was further used for this study.

6. Density gradient experiment did not show countable cells using Accutase method, however, the cells that were isolated using Trypsin method showed a higher number of cells and better proliferation rate up to confluency. It was also noted that cell count below 15,000 cells did not show any growth however, cell count with 16,000; 26,000; and 84,000 cells showed cell expansion. It can be hypothesized that with higher cell density, chances of acquiring putative stem cells may be high from the periphery with better cell-cell communication higher chances of expansion.
7. Immunostaining showed the presence of ZO-1 marker from the first passage however this needs to be verified with further cultures and reproducibility needs to be confirmed for the potential of this expansion technique.

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**CHAPTER 8**  
**PILOT STUDIES**

**PART 1**  
**INTRODUCTION OF RHO-ASSOCIATED PROTEIN**  
**(ROCK)**

## **Aim**

To evaluate the effect of ROCK inhibitor (Rho-Kinase) on the proliferation rate of HCECs using dual media approach.

## **Introduction to ROCK inhibitor**

It has been thought that human corneal endothelial cells, unlike those from other species, are terminal and cannot proliferate. However, there is some clinical and emerging *in vitro* evidence that this is not the case. Indeed, our preliminary data shows that human cadaveric corneal endothelial cells can be made to expand in culture. We have already seen that some donor human cadaveric tissue results in incredibly successful endothelial cell expansion whereas other samples result in aborted growth or no growth at all. In addition, the initial yield of corneal endothelial cells from tissue samples can be variable.

Many studies have shown the effect of ROCK inhibitor on corneal endothelial cell proliferation and therefore we set to replicate the results using old age donors which have been assumed to have a slow growth rate as compared to the young donors which has already been evaluated so far.

Rho-associated protein kinase (ROCK) is a kinase belonging to the AGC (PKA/PKG/PKC) family of serine-threonine kinases. It is involved mainly in regulating the shape and movement of cells by acting on the cytoskeleton. ROCKs (ROCK1 and ROCK2) occur in mammals (human, rat, mouse, cow), zebrafish, *Xenopus*, invertebrates (*C. elegans*, Mosquito, *Drosophila*) and chicken.

Alternative approaches to corneal regeneration following endothelial failure include the use of Rho-kinase (ROCK) inhibitor eye drops to enable the proliferation and healing of host endothelium, or the development of allogeneic *ex vivo* expanded corneal endothelial

cells for transplantation. A major drawback for the ROCK inhibitor strategy is that in Fuchs' endothelial dystrophy, which is a common endothelial disorder, the host endothelium is already abnormal. It therefore requires a total replacement instead of simple proliferation. This will further enable to develop corneal endothelial sheets for future clinical trials. This may also allow the mass production of HCECs from donor cornea, thereby reducing the global shortage of human corneal tissues for endothelial disease.

## **Materials and methods**

### *Ethical statement*

Human cadaveric donor corneas were collected with consent from the donor's next of kin to be used for research if not for transplantation and were preserved in tissue culture medium with age range of >60 years and endothelial cell density of <2200 cells/mm<sup>2</sup>.

### *Peel and digest method*

Wash corneas in wash buffer for 5-10 minutes. Peel the Descemet's membrane and endothelium using gentle stripping method. Tear the tissue in various pieces to ensure higher and quicker isolation of the cells. Punching method can be used but it may release keratocytes and therefore unwanted contamination of the fibroblasts. Once the tissues are washed in the wash buffer the peeling should be carried out at the earliest. Meanwhile the media should be warmed up (maintenance media). The tissues are left in the collagenase enzyme for 4-5 hours for cell digestion.

### *Plating*

The cells were washed and placed in a small eppendorf tube and spun down for 5 minutes at 1000 rpm. The supernatant was removed and TripleE was added to cut the clumps into single cells for 10 minutes at 37 degrees (as much as possible). The pellet

was mixed with the TripleE Express and the cells were dissociated. The cells were then spinned down. Meanwhile a 6 well plate was coated with FNC. The liquid coating was left in the plate for 10-30 minutes. The residual coating was removed. After removing the TripleX supernatant, the cells were mixed with maintenance medium (basal serum free media without any growth factors). 2.5 mL of maintenance media was added on the coated plate and 500  $\mu$ L was mixed with the cells. The cell suspension was added in the maintenance media to make a total volume of 3 mL. The cells were preserved at 37 degrees for 2 days. The cells were left in the maintenance media for 2 days and then transferred to the proliferation media for cell expansion. The cells were monitored up to confluence.

### *Passaging*

The cells were dissociated in TripleE Express for 5 minutes at 31 degrees. Once the cells were dislodged, they were gently washed and flushed from each side of the petri to ensure the removal of all the cells, they were then pipetted out and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed and refilled with maintenance media and mixed well. The cells were counted. The plates were coated with FNC. 150k cells were plated in a 6 well plate with proliferation media.

### *Antibody staining*

The small cover slips were UV sterilized and placed in the small 4 well plates. The cover slips were coated with FNC. The FNC was removed without touching the 7 mm cover slips (approximately 60  $\mu$ L on each cover slip). The cells were plated on the cover slips and left to attach for 4-5 hours. It usually looks like a single drop on the cover slip. Once attached the cells were flattened by adding 1 mL of maintenance media covering the entire well. No growth was required if plated at high density.

The media was removed and the cells were washed with 1X PBS (5 minutes). Fix with PFA for 10 minutes and wash twice with 1X PBS. Add blocking buffer on the cells for 30 minutes [10% Goat serum diluted in 1X PBS – Blocking buffer]. Add primary antibody [ZO-1, GPC4, Na-K ATPase, 1A3] for 1-2 hours at RT. The cells were washed twice with PBS for 5 minutes each. Add secondary antibody diluted in blocking buffer and incubate the cells for 1 hour at RT in dark. Proceed with Hoechst staining for 5 minutes at RT in dark. Wash the cells in 1X PBS and mount it with Vectashield mounting medium with DAPI.

#### *Proliferation – ClickIT BrDu assay*

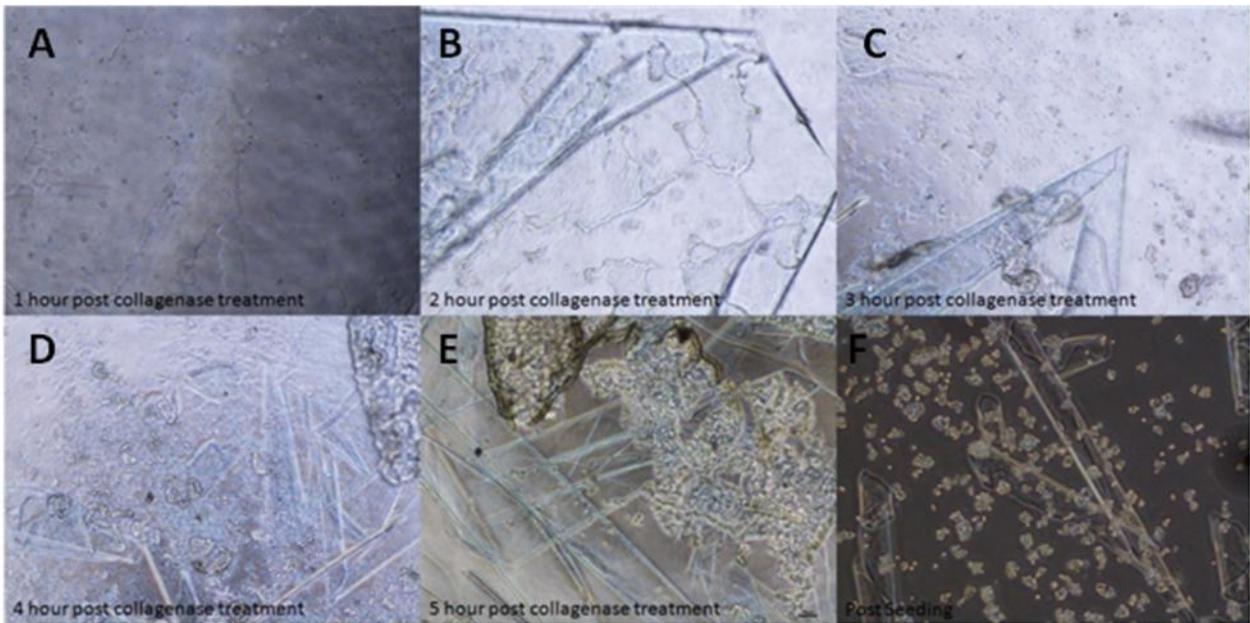
Proliferation of the corneal endothelial cells are assessed using a 5-ethynyl-29-deoxyuridine (EdU) incorporation assay, by using a Click-iT EdU Alexa Fluor 488 cell proliferation assay kit (Invitrogen, Carlsbad, CA, USA). Cultured HCECs were passaged using TE and seeded in their respective culture conditions on FNC-coated glass slides at a plating density of 2,000-5,000 cells/cm<sup>2</sup> for 24 hours. The cells were then incubated in their respective medium containing EdU (10 mM) for another 24 hours. HCECs were washed with PBS, and fixed using 4% paraformaldehyde (PFA) for 15 minutes on ice, followed by a 0.1% Triton X-100 in 3% BSA block and permeabilization step for 20 minutes, all at room temperature. Incorporated EdU was detected by fluorescent-azide coupling Click-iT reaction. Briefly, cells were incubated for 30 minutes in a reaction containing azide-conjugated Alexa Fluor 488 dye in Click-iT EdU reaction buffer supplemented with 4 mM CuSO<sub>4</sub>. Cells were washed twice with 1 mL 3% BSA in PBS, and mounted on glass slides with Vectorshield containing DAPI (Vector Laboratories, Burlingame, CA, USA). Images of cells were examined using a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss, Germany). At least 500 nuclei were analyzed per experiment and data point.

### *Live/dead/apoptosis FACS analysis*

Trypsinize the cells. Suspend and count. FITC Annexin V Apoptosis Detection Kit with PI. The cells were washed with cold BioLegend's cell staining buffer, and then resuspend the cells in Annexin V Binding buffer at a concentration of  $0.25-1 \times 10^7$  cells/mL. Transfer 100  $\mu$ L of cell suspension in a 5 mL test tube. Add 5  $\mu$ L of FITC Annexin V. Add 10  $\mu$ L of Propidium-Iodide solution. Gently Vortex the cells and incubate for 15 minutes at room temperature (25°C) in the dark. Add 400  $\mu$ L of Annexin V binding buffer to each tube. Analyze by flow cytometry with proper machine settings. PI is a fluorescent dye that binds to DNA. When excited by 488 nm laser light, it can be detected with 562-588 nm band pass filter. Flow cytometry analysis was carried out.

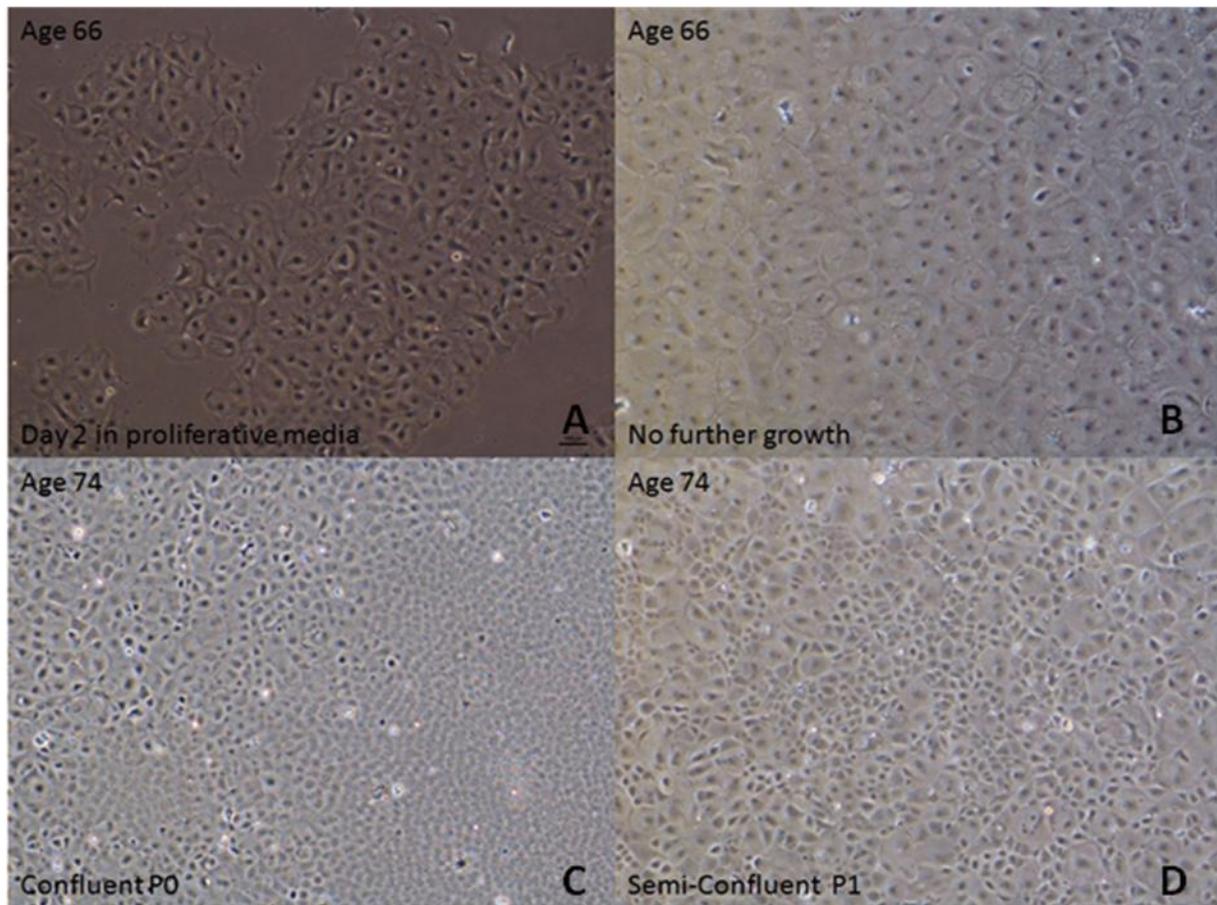
### **Results**

Collagenase digestion of the excised human corneal endothelial cells using stripping method showed total isolation of the cells within 5 hours at room temperature. The digestion phases at each hour were showed in figure 1. The cells were then trypsinized and collected as single cells from the clumps and seeded on the wells coated with FNC as shown in figure 1f. The cells were then left and monitored for adherence and proliferation with media change at every alternate day.



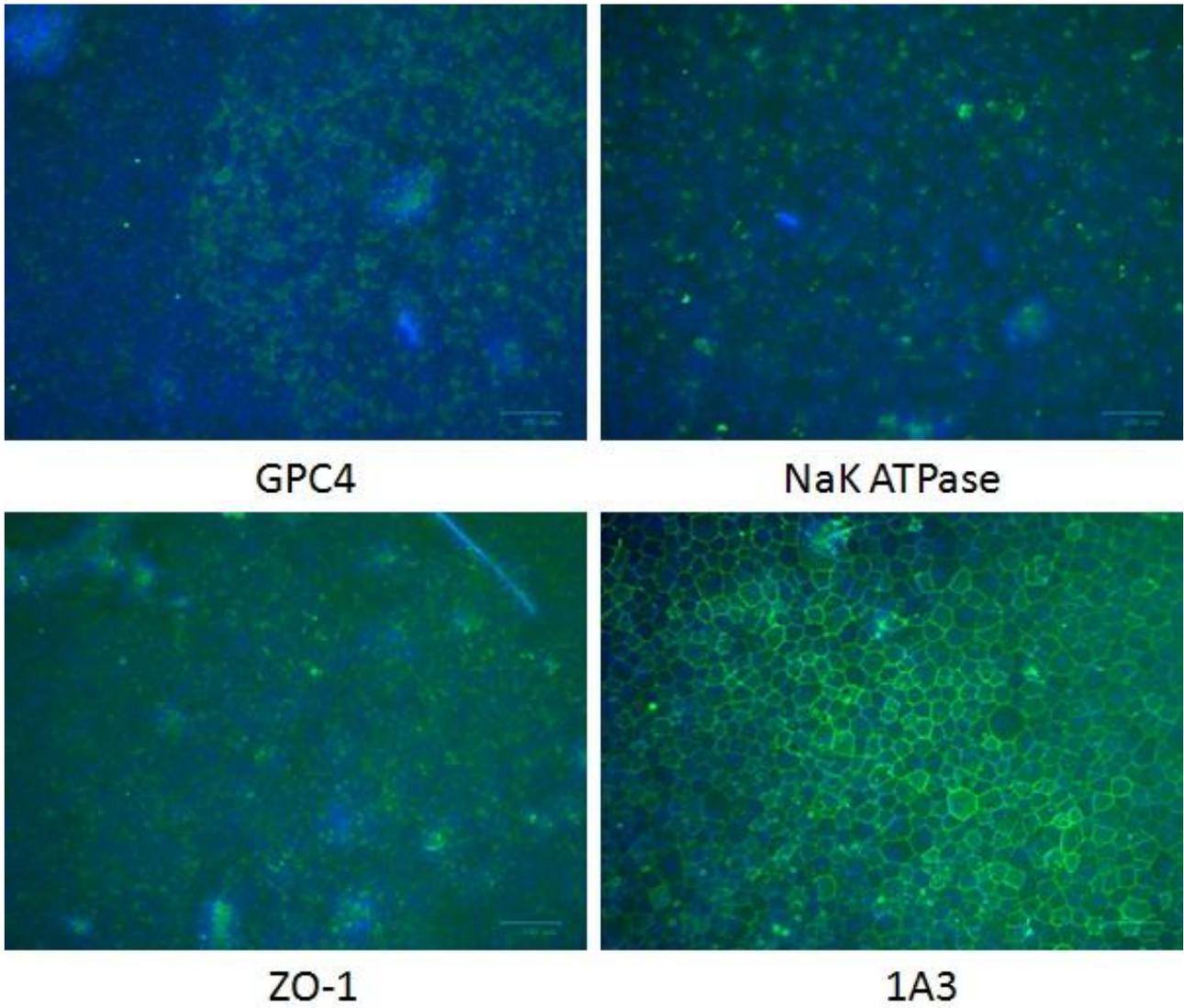
**Figure 1:** Collagenase digestion, a-e) at hourly basis showed that the cells were isolated totally within 5 hours of collagenase enzyme digestion. F) further, the cells were plated on the FNC coated wells and were left for attachment and further proliferation.

7 pairs of donor cornea showed proliferation in P0 while one pair was contaminated with fibroblasts. The cells with proliferation but no further growth in P0 are showed in Figure 2a and 2b, however the other donors showed confluency and were further passaged to P1 (figure 2c and 2d). The cells did not show any further confluency restricting the growth only up to P1.



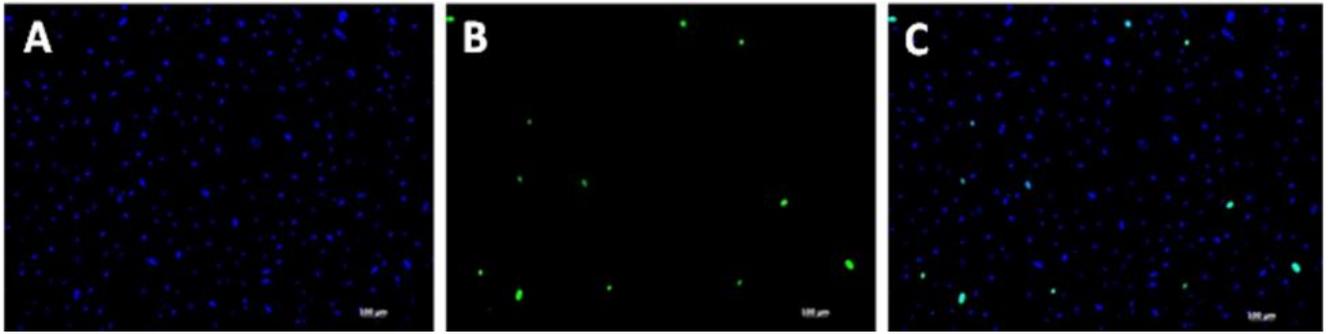
**Figure 2:** Cultured corneas in proliferative media. a and b) the cells showed proliferation but no confluency in P0. c and d) The cells showed confluency and were further passaged to P1. The cells did not show any further confluency restricting the growth only up to P1. Different donors have different characteristics and growth profile.

Immunostaining showed expression of different antibodies as seen in figure 3 which further confirmed the presence of protein profiles of cultured human corneal endothelial cells.



**Figure 3:** The antibody staining showed presence of pump proteins and tight junctional proteins. The new antibodies specifically designed for human corneal endothelium (1A3) also showed expression of the proteins.

Proliferative cells were observed in the cultures as seen in figure 4.



**Figure 4:** Proliferation study. 5% of cultured human corneal endothelial proliferative cells were observed. A) shows the presence of cell nuclei using DAPI staining, b) shows the presence of proliferative cells using FITC and c) shows the merge of two.

## Discussion

HCECs can be cultured from human cadaveric donor corneas. The donor characteristics are yet to be defined to understand the best suitable source for such kind of cultures. There are no specific corneal endothelial markers and therefore there is a need to identify a specific marker in order to conclude the culture results. Plating density should be identified and the source cells should be used accordingly.

**CHAPTER 8**

**PART 2**

**CELL ADHESION AND PROLIFERATION**

## **Aim**

To identify different methods of cell adhesion and coating mixture for corneal endothelial cell proliferation.

## **Introduction**

Cell adhesion is an important part of cell culture. HCECs are already been suggested to be non proliferative especially when they are isolated from the old donor corneas. If these cells do not attach properly to their base then there is a high chance of cells not surviving in the due course. Therefore, it was important to learn the base coating and adhesion properties. Base coating currently is carried out using FN coating mix. There are many other coating mixtures but FN has been standardized earlier for HCECs cell culture. HCECs proliferation is another important parameter after studying cell adhesion. Proliferating cells are analyzed and further used for passaging. In this study, we checked the cell adhesion and proliferation of HCECs and the effect of ROCK inhibitor.

## **Material and methods**

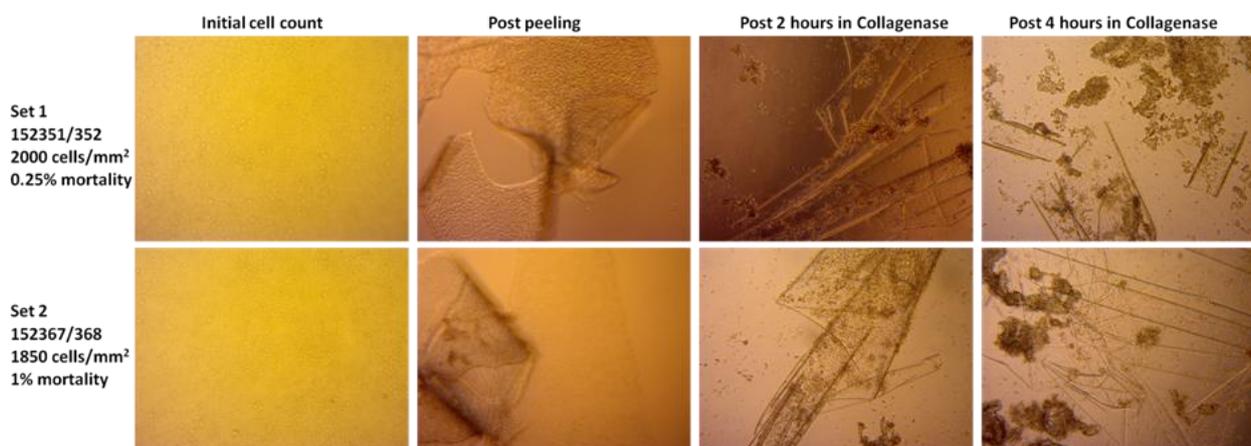
### *Ethical statement*

The corneas (n=10) were obtained from the eye bank in University of Szeged, Hungary. Five donors with age 64, 83, 66, 85 and 81 were used for this study. Corneas from each donor were excised from whole eye globes. The DMEK graft was excised using standard peeling method and the Descemet's membrane and endothelium from both the corneas were pooled together in one well. The endothelial cells were isolated from the Descemet's membrane sheet using collagenase A for approximately 2 hours. The cells were centrifuged at 1000 rpm. The supernatant was removed and the cells were trypsinized for 10 minutes at 37°C in a humidified incubator. The cells were centrifuged again for 5 minutes and the supernatant was removed. The cells were mixed with maintenance

media. The cells were mixed well and divided into two parts, a) the control well where the cells were plated with maintenance media without ROCK (rho-kinase inhibitor) with Fibronectin (FN) coating on the plastic well and b) the experiment well, where the cells were plated with maintenance media and ROCK on a FN coated plastic well. Media was replaced every 2 days and the attachment, growth and morphology was monitored

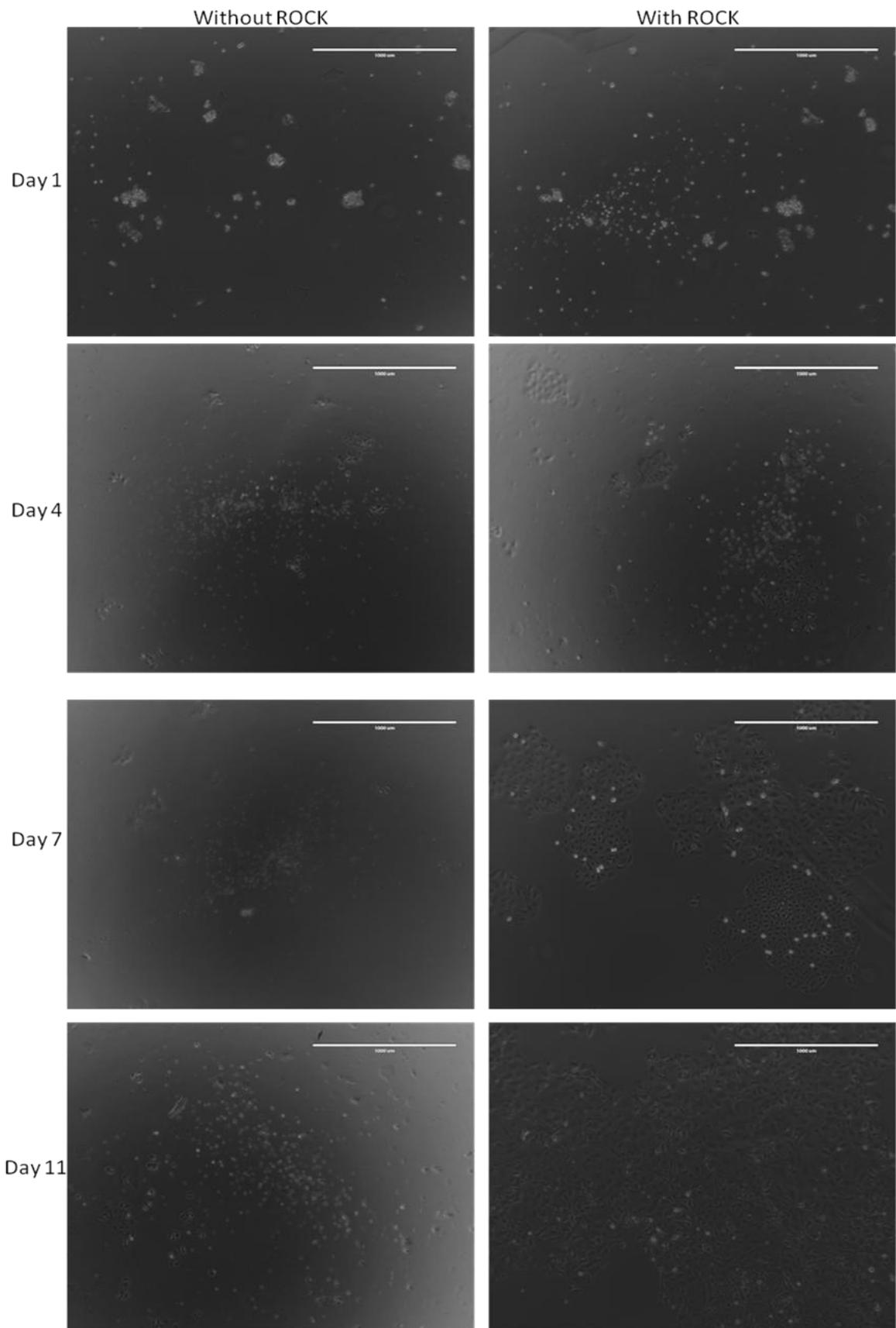
## Results

Descemet's membrane was monitored for isolation of cells. Figure 1 shows the peeled Descemet's membrane in collagenase A for digestion.



**Figure 1:** Descemet's membrane and endothelium with endothelial cells attached on it while collecting the initial cell count. Peeled DM in collagenase A with corneal endothelial cells still attached post peeling. Isolation of HCECs after 2 and 4 hours in Collagenase A.

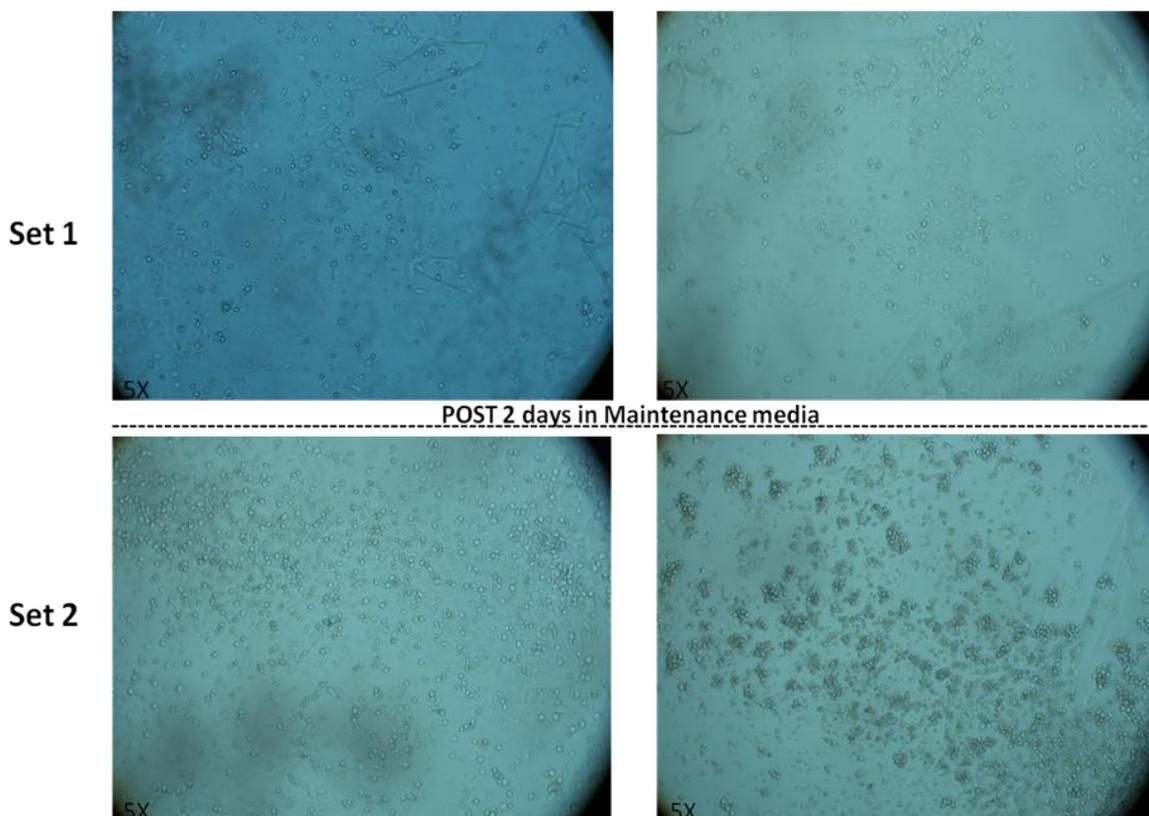
After plating the cells, the media was added and the cell growth and morphology was monitored. It was observed that with time, the cells cultured in ROCK inhibitor show higher proliferative capability as compared to the control as seen in Figure 2. The FN coating allows cell adhesion and hence proliferation of the cells. Moreover, the morphology of the cells in the early days looks polygonal out of which a few cells turned to be elongated with time.



**Figure 2:** Cell proliferation and comparative analysis of the corneal endothelial cells grown with and without ROCK inhibitor at day 1, 4, 7 and 11. It was observed that the cell proliferation was much higher when the endothelial cells are exposed to ROCK however the cells showed elongated morphology after day 11.

Another two sets of corneas were used for confirmatory analysis and similar trend was observed when the cells were cultured on the FN coated plates with ROCK inhibitor as shown in figure 3. This analysis was performed as per the new protocol. The cells were plated on FN coated wells and after maintaining them in maintenance media for two days, the media was replaced with ROCK supplemented media and monitored till confluence.

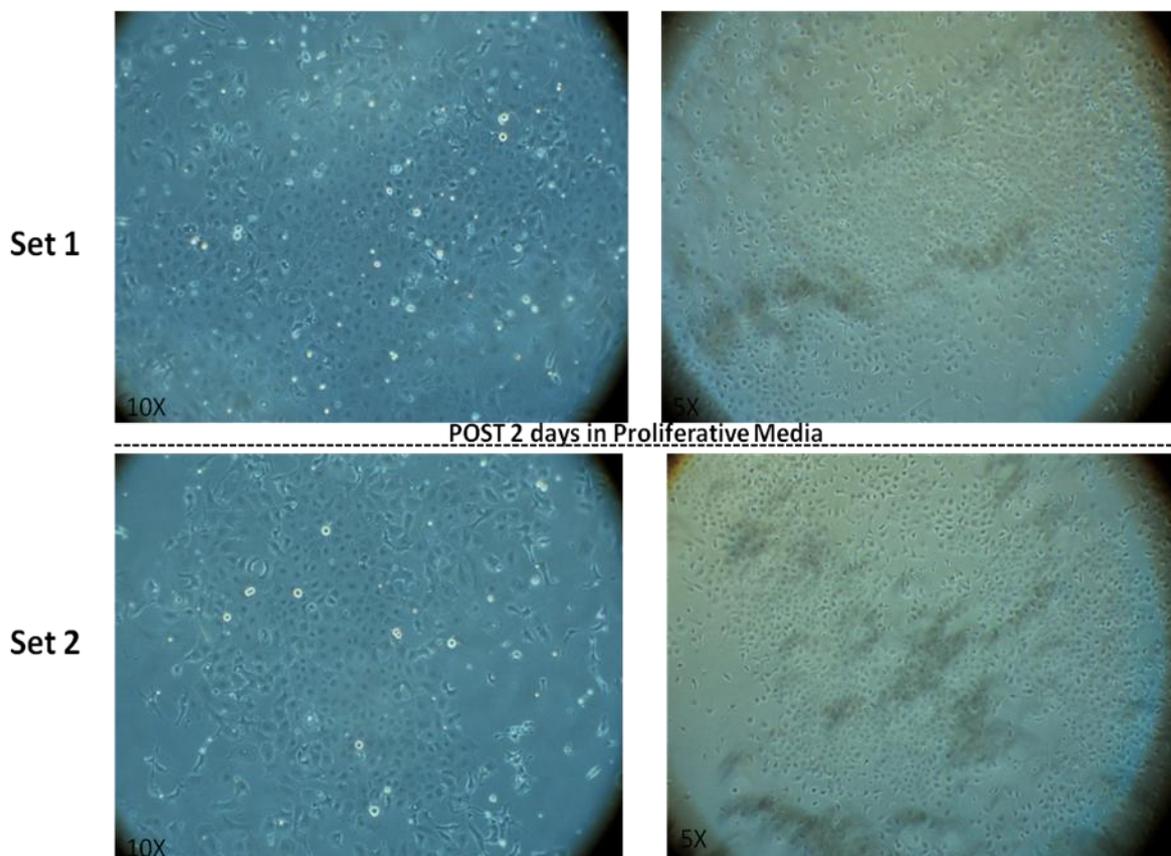
Figure 3 shows the attachment of the cells at day 2 in maintenance media from the corneas collected from set 1 and set 2 (two different donors).



**Figure 3:** Cells showing high adherence after 2 days in maintenance media

At day 4 (2 days in proliferative media – media supplemented with ROCK inhibitor), the cells showed high proliferation capability and high percentages of hexagonality which was subjectively measured as seen in figure 4. Whereas, at day 6, central well showed confluence whereas the periphery was not confluent and the cells still looked isolated.

**Figure 4:** Cells showing proliferative capability after 2 days in proliferative media. The cells also show hexagonality.



**Figure 5:** Cells showing proliferative capability after 6 days in proliferative media. The periphery was not confluent, however the central area was confluent.

## **Discussion**

From this study, it was determined that cells grown on pre-coated wells with FN show high adherence capabilities. These cells further proliferate at even a higher rate when cultured using ROCK inhibitor. Therefore, a combination of FN and ROCK was determined to be a valuable solution for the next phase.

**CHAPTER 8**

**PART 3**

**INTRODUCTION OF HYALURONIC ACID FOR FORCE**

**ATTACHMENT**

## **Aim**

To optimize a protocol for culturing HCECs using Hyaluronic acid (HA) for force attachment.

## **Introduction**

Sodium hyaluronate is a naturally occurring, high molecular mass, polysaccharide found in the extracellular matrix of the connective tissues. Sodium hyaluronate, and other viscoelastic substances including methylcellulose, chondroitin sulphate, polyacrylamide, and collagen, have been used in intraocular surgery since 1970s.

## **Materials and methods**

Corneas [n=6] were excised from whole eye globes using peeling method. The cells were isolated from the DM using collagenase A (1mg/mL) using a shaking incubator for 2 hours. The cells were centrifuged at 1000 rpm for 5 minutes in a 15 mL tube. The supernatant was removed and the cells were trypsinized for 10 minutes at humidified incubator at 37°C. The cells were centrifuged again at 1000 rpm for 5 minutes and the supernatant was removed. The cells were then mixed well with the maintenance media and plated as below.

The cells were mixed with 1000 mL of maintenance media and the final volume was 1200 mL including supernatant. The cells were mixed well and 100 µL was added in each chamber of the slide as shown below. The glass slide was pre-coated with FNC.

C	V	R	V+R
C	V	R	V+R

C = Control (FNC coating + maintenance media)

V = Viscoelastic (The cells were plated and viscoelastic was added on top of cells to cover the entire well and induce cell adherence+maintenance media)

R = ROCK inhibitor (The cells were plated with ROCK inhibitor)

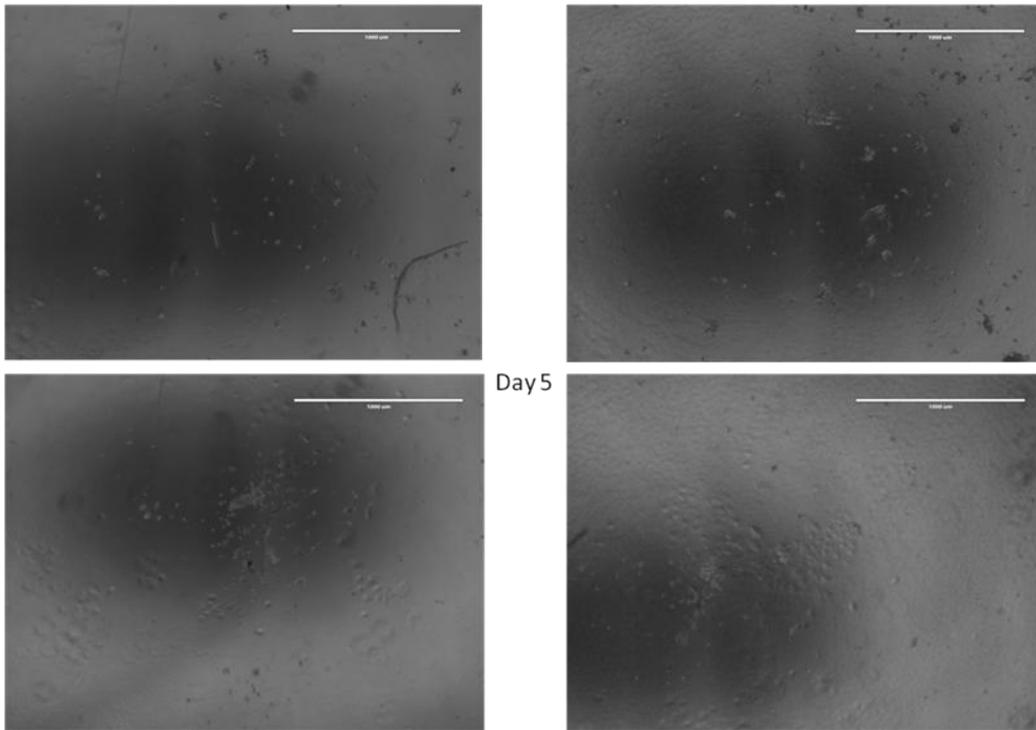
V+R = Viscoelastic + ROCK inhibitor (The cells were covered with Visco+ROCK and added without maintenance media).

The remaining 400  $\mu$ L were added on a plastic 12 well plate coated with FNC and were monitored over time.

## **Results**

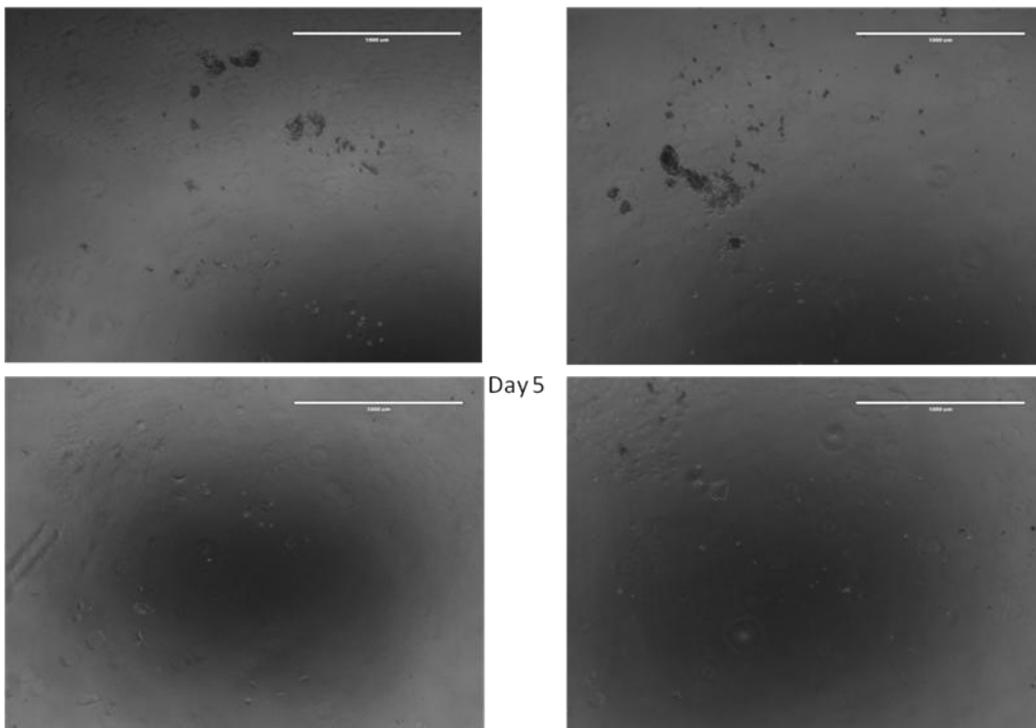
It was noted that the cells that were cultured only with maintenance media did not show a high proliferative capability at day 1 and day 5 as seen in figure 1. However, better adherence was found in the condition where HA was used, but as the cells were cultured in maintenance media, they did not proliferate at day 1 and day 5 as seen in figure 2. It was observed that when the cells were cultured with ROCK on FN coated base, the cells showed high adherence and proliferative capability as observed in figure 3. The cells adhered and proliferated the most in condition where the cells were forcefully adhered using HA, supplemented with ROCK inhibitor on FN coated base as seen in figure 4.

8 chamber glass slide – control with FNC – Day 1



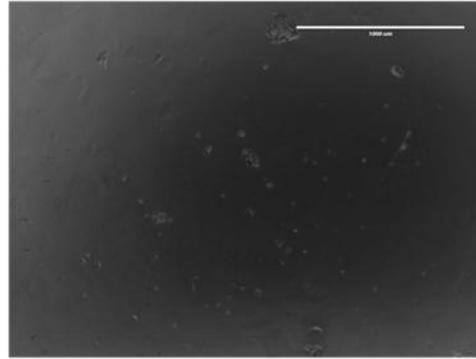
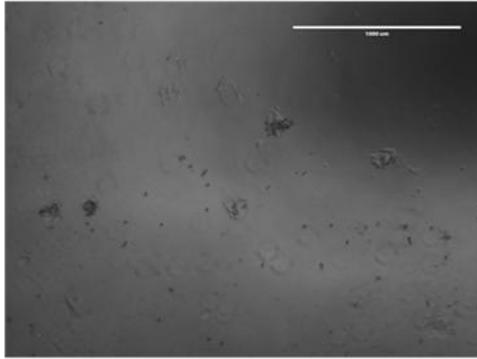
**Figure 1:** HCECs on FN coated base cultured using maintenance media.

8 chamber glass slide – viscoelastic FNC – day 1

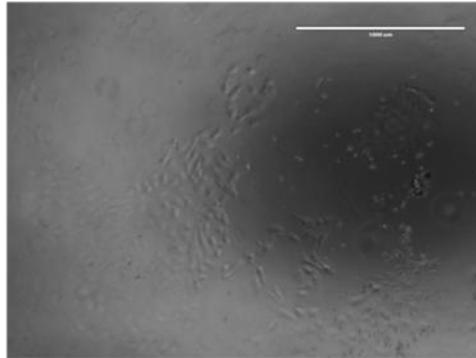
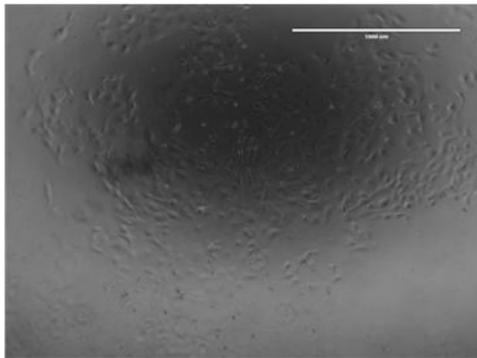


**Figure 2:** HCECs on FN coated base cultured using maintenance media but adhered firmly using HA.

8 chamber glass slide – ROCK FNC– day 1

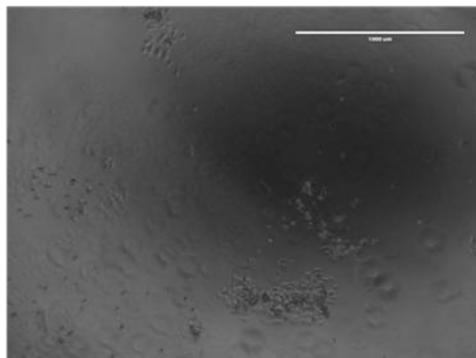
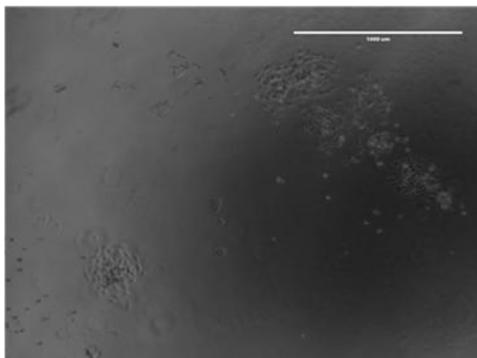


Day 5

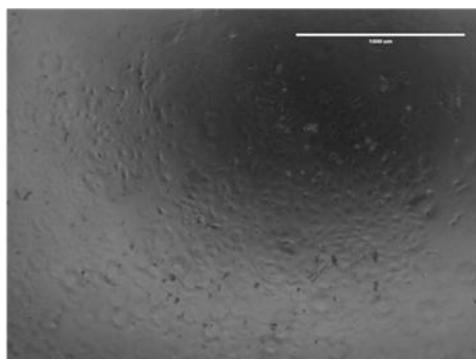
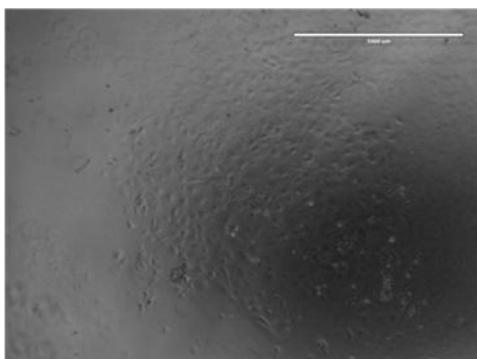


**Figure 3:** HCECs on FN coated base cultured using ROCK supplemented media.

8 chamber glass slide – ROCK+Visco FNC– day 1



Day 5



**Figure 4:** HCECs on FN coated base cultured using ROCK supplemented media and adhered firmly using HA.

## **Discussion**

From this pilot study, it was noted that it is necessary to use a base coat which allows adherence to the HCECs, along with inhibitors like ROCK which would allow further adherence to the HCECs. Viscoelastic provides a higher and faster adhesion of the cells on the plate and therefore allows a quick attachment and proliferative potential. Therefore, a full study using the results of these pilot results was carried out and detailed in the next chapter.

## **CHAPTER 9**

# **HUMAN CORNEAL ENDOTHELIAL CELL CULTIVATION** **FROM OLD DONOR CORNEAS WITH FORCED** **ATTACHMENT**

*Derived from article (Revisions submitted – under peer review)*

Mohit Parekh, Sajjad Ahmad, Alessandro Ruzza and Stefano Ferrari. Human corneal endothelial cell cultivation from old donor corneas with forced attachment. Scientific Reports.

## **Aim**

To culture HCECs from old donor corneas with forced attachment using HA.

## **Introduction**

Human cornea is made up of several layers. The endothelium that is the posterior monolayer is responsible for maintaining the required transparency of the cornea. An osmotic gradient is generated by the transmission of essential metabolites across the corneal endothelium, which transports water into the cornea. The corneal endothelium continuously pumps the water, ions and solutes out of the cornea using trans-membrane ion channels [1]. Increased water content in the cornea can lead to oedema and hence opacity which is responsible for corneal blindness [2]. Human corneal endothelial cells (HCECs) maintain the clarity and thickness of the cornea [3]. Endothelial failure is seen mostly as a cause of Fuch's endothelial dystrophy, which is one of the common reasons for corneal endothelial replacement.

Penetrating keratoplasty (PK) was the most popular choice among the surgeons to treat endothelial disorders. However, with the recent advancements, endothelial keratoplasty (EK) has shown clinically relevant results like early rehabilitation rate and better visual outcome over PK and is gradually being accepted by the surgeons due to standardized procedures [4]. The only recognized treatment for endothelial disorders so far is a corneal replacement. However, due to the donor shortage, the transplantation options also remain limited. Therefore, alternative therapeutic approaches are currently explored to provide a worldwide solution.

One of the most common approaches for therapeutic treatment and HCECs regeneration includes the use of Rho-Kinase (ROCK) inhibitor for the development of allogeneic *ex vivo*

expanded HCECs for transplantation [5]. It has been previously reported that ROCK inhibitor (Y-27632) allows adhesion of HCECs to a substrate and the inhibition of ROCK signalling may manipulate cell adhesion properties [6-8]. As the host endothelium is already abnormal in Fuch's dystrophy, a direct injection of ROCK inhibitor may not be considered as a therapeutic approach, as it needs a complete replacement. However, *ex-vivo* expansion using ROCK inhibitor may allow potential cell-based therapy. It has been reported that despite the limited regenerative potential *in vivo*, HCECs have a capacity to proliferate *in vitro* [1]. Therefore, cultured HCECs could be a prospective alternative treatment for corneal endothelial diseases. Several methods to define the media, conditions, isolation techniques etc. for cultivation of HCECs have been successfully investigated so far [6,9].

Hyaluronic Acid (HA) binds to and protects the corneal endothelial cells. HA dissolves in saline and can be aspirated easily out of the endothelium. A thin layer of HA, however, remains on the endothelial cells [10]. The hypothesis of the present study is that the viscosity of HA is higher than the media used to culture the HCECs. Therefore, using gravitational pull and viscosity of HA [11], the isolated primary HCECs can be pushed towards the base and allowed to be strongly adhered for culturing HCECs when topically applied over the cells.

Previously reported studies on culturing HCECs have been performed on younger donor corneas [12]. It has been observed that young donors have a high proliferative capability compared to older donor corneas. However, it is difficult to obtain young donors for culturing HCECs *in vitro* due to its characteristics that are suitable for transplantation. Most of the older donor corneas are easy to obtain for research due to its endothelial cell density that is less than the threshold required for transplantation. The proliferative

capability is also noticed to be less. It is a challenge to culture older donor corneas however if the HCECs from the older donors can be cultured then the availability of the source will be much higher compared to the younger donor corneas. The chapter thus highlights four different conditions to identify the role of HA and Rho kinase (ROCK inhibitor) for force adherence in culture of HCECs which may eventually lead to mass production of huge number of corneal endothelial sheets from older donor corneas, reducing the requirement of human corneal tissues globally.

## **Material and Methods**

### *Ethical Statement*

The corneas [n=48, twenty four pairs] 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 corneas were suitable for research and unsuitable for transplantation due to low endothelial cell count ( $<2200$  cells/mm<sup>2</sup>). No other complications or indications were noted in the donor corneas. All the tissues were preserved in tissue culture medium at 31°C prior to use.

### *Endothelial cell count and donor characteristics*

Endothelial cell density (ECD) and mortality before isolation was checked using trypan blue (TB) stain (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 it with phosphate buffered saline (PBS). Trypan blue positive cells (TBPCs) were recorded before isolation using an in-built eyepiece reticule for inverted microscope (Axiovert, Zeiss, Germany). The donor characteristics of 48 corneas (twenty four pairs) were obtained from the FBOV database

to determine the age, gender, post-mortem time, cause of death, preservation time, ECD and mortality.

### *Sodium Hyaluronate*

Sodium hyaluronate is a naturally occurring, high molecular mass, polysaccharide found in the extracellular matrix of the connective tissues. Sodium hyaluronate, and other viscoelastic substances including methylcellulose, chondroitin sulphate, polyacrylamide, and collagen, have been used in intraocular surgery since 1970s [13]. In this study, we used Viscoat (Alcon, Texas, USA) – 0.8 mL comprising of 3% sodium hyaluronate and 4% chondroitin sulphate with Resting State Viscosity of  $40,000 \pm 20,000$  cps that is currently being used in corneal and cataract surgery. The viscosity of HA allows the cells to settle down faster and force the cells to adhere to the coated base quicker.

### *Formulations*

The procedure of isolation and culture was slightly modified from the previously published article by Peh et al [12,14]. Two different types of media were used as described earlier [14], maintenance media and proliferative media. In brief, the maintenance medium (M5) contained human endothelium SFM, 5% FBS along with antibiotics. The proliferative medium (M4) was a mixture of HamF12, M199, 5% FBS, 1% ascorbic acid, 0.5% Insulin Transferrin Selenium (ITS), Rec human FGF basic (25 µg/mL), 10 µM ROCK inhibitor (Y-27632) and antibiotics.

### *Peel and digest method*

The method is similar to previously published article [14] with some modifications which is described in brief. The corneal tissues [n=48] were washed in sterile phosphate buffered saline (PBS) and the Descemet membrane-endothelial tissues were peeled gently (similar

to the stripping technique for Descemet membrane endothelial keratoplasty – DMEK method) in various pieces to ensure faster isolation of the cells when incubated in the enzyme. The excised pieces were incubated with 2mg/mL Collagenase Type 1 (Thermo Fisher Scientific, Rochester, NY, USA) solution for 2-3 hours 31°C, 5% CO<sub>2</sub>. While peeling, the forceps was cleaned using PBS after every single encounter with Collagenase Type 1 solution. Once the cells were isolated from the Descemet membrane, they were collected with Collagenase Type 1 and centrifuged for 5 minutes at 1000 rpm in a 15 mL falcon tube. The supernatant was removed and the cells were re-suspended with TrypLE Express (1X), phenol red [Life Technologies, Monza, Italy] to cut the clumps into single cells for 10 minutes at 37°C [if the clumps were not converted to single cells then TrypLE Express treatment was repeated for 5 minutes]. The supernatant was removed and the cells were re-suspended with 400 µL of M5 after counting them with TB in a haemocytometer slide. The plating density was recorded for all the cultures.

#### *Plating and force attaching the cells on coated slides*

Labt-Tek II chamber slides (8 chambers, 25X75 mm, 0.7 cm<sup>2</sup> 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°C, 5% CO<sub>2</sub>. The residual coating was removed before the plating. 100 µL of the cell suspension was mixed well and added in each chamber to ensure equal distribution of cells and proliferative cells in all the conditions. Four chambers were used for each donor that was pooled together. The chamber slides were used to check four conditions as shown in table 1. The cells were monitored every alternate day till confluence.

Condition 1	Condition 2	Condition 3	Condition 4
100µL of cell suspension + 200 µL of M5 up to day 2.  The media was changed after day 2 to M4 and was re-freshed every alternate day	100µL of cell suspension + 200 µL of M5 up to day 2.  Cells were force adhered using gentle addition of Viscoelastic (approximately 50-100 µL) on top of the cell suspension.  The media was changed after day 2 to M4 and was re-freshed every alternate day	100µL of cell suspension + 200 µL of M4 throughout the culture period. The media was refreshed every alternate day.	100µL of cell suspension + 200 µL of M4 throughout the culture period. The media was refreshed every alternate day.  Cells force adhered using gentle addition of Vicoelastic (approximately 50-100 µL) on top of the cell suspension.

**Table 1:** Description of plating conditions. Condition 1, 2, 3 and 4 shows different plating characteristics with and without HA and ROCK. In condition 1, no HA or ROCK was used to check the behaviour of the cells without the influence of these extra parameters. In Condition 2, only HA was added to check the adherence of the cells without ROCK. Condition 3 only utilized ROCK inhibitor from the day of plating to check the effect of ROCK inhibitor directly on the cells. Condition 4 was a mixture of HA and ROCK to understand the influence of both the parameters together.

*Morphological analysis of the cultured HCECs [n=30, fifteen pairs]*

The cells were visualized every alternate day until confluent using an inverted microscope (Axiovert, Zeiss, Germany) at 50X and 100X magnifications. The percentage confluency was monitored and recorded every alternate day manually using an eyepiece reticule.

*Glucose uptake of the cultured HCECs for functionality checks and metabolic analysis*  
*[n=48, twenty four pairs]*

Glucose uptake was determined from the preservation media of all the samples at day 3 and day 15. The amount of glucose used and lactic acid produced at day 3 (with adapting and proliferative cells) and day 15 (confluent cells) were recorded. The assay helps to check the metabolic activity of the HCECs when preserved *in vitro*. Quantitative analysis was performed using D-Glucose HK kit (Megazyme International Ireland Ltd, Bray Business Park, Bray, Co. Wicklow, Ireland).

For Hoechst, Calcein AM and Ethidium homodimer (HEC) and, Immunostaining of Ki-67 and Vinculin, the study was divided into two groups, a) n=6 (three pairs), to evaluate the early behaviour of the cells at day 3 and b) n=6 (three pairs), to evaluate the nature of confluent cells at the end of the study. ZO-1 was only evaluated at the end of the culture period (day 15).

*Hoechst, Calcein AM and Ethidium homodimer (HEC) staining to determine live, dead and dying cells [n=12, Six pairs]*

Live cells are distinguished by the presence of intracellular esterase activity, determined by the enzymatic conversion of the nonfluorescent cell-permeant Calcein AM to the intensely fluorescent Calcein. The polyanionic dye Calcein, is retained within live cells, producing an intense green fluorescence in live cells (ex/em 495 nm/515 nm). Ethidium Homodimer-1 (EthD-1) enters cells with damaged membranes and enhances fluorescence upon binding nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em 495 nm/635 nm). EthD-1 is excluded by the intact plasma membrane of live cells. Hoechst 33342 is a nuclear dye which stains the nucleus of the cell. Triple endothelial labelling with Hoechst 33342 (H), Ethidium Homodimer (E), and Calcein-AM

(C) showed expression of 'E' in red representing the dead cells, blue represents the nuclei 'H' and green marked the living cells 'C'.

The cells were washed with PBS prior to the assay to remove or dilute serum esterase activity generally present in serum-supplemented growth media such as M4 or M5. Control sample was prepared using a human donor cornea. Descemet membrane was peeled out using stripping method and the excised lenticule was washed before the assay. Some cells were damaged by purpose through gently scrapping and touching the endothelial cells using sharp forceps. 5  $\mu$ L of Hoescht 33342 (H) (Thermo Fisher Scientific, Rochester, NY, USA), 4  $\mu$ L of Ethidium Homodimer EthD-1 (E), 2  $\mu$ L Calcein AM (C) (Live/Dead viability/cytotoxicity kit, Thermo Fisher Scientific, Rochester, NY, USA) was mixed in 1 mL of PBS and mixed well. 100  $\mu$ L of the final solution was directly added on the cultured cells and incubated at room temperature in dark for 45 minutes. For the control tissue, the stripped lenticule was placed on the slide, cut at four peripheral sides for a flat mount and covered with coverslips without the mounting medium. HEC was viewed at 488, 495, 515 and 635 nm at 50X, 100X and 200X magnification.

*Immunostaining for tight junctions using Zonula Occludens-1 (ZO-1) [n=12, Six pairs], proliferation marker Ki-67 [n=12, Six pairs] and focal adhesion marker Vinculin [n=12, Six pairs]*

The cells were washed with PBS and fixed in 4% paraformaldehyde (PFA) at room temperature (RT) for 30 minutes. The cells were permeabilized with 0.5% Triton X-100 in PBS for 30 minutes. After blocking with 2% goat serum for 2 hours at RT, the tissues were incubated overnight at 4°C with primary antibodies [anti-ZO-1, 1:200 (ZO1-1A12, Thermo Fisher Scientific, Rochester, NY, USA); anti-Ki-67, 1:200 (MIB-1, Milan, Italy); anti-vinculin, 1:200 (Abcam, Cambridge, Massachusetts, USA)]. The samples were incubated with goat

anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody in 20% goat serum for 2 hours at RT. 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 an LSM 510-meta laser scanning microscope (Zeiss, Milan, Italy). Cells were examined under ultraviolet light or by excitation at wavelengths of either 488 nm or 594 nm where subsequent detection of the fluorescence was obtained.

### *Measurement and statistical analysis*

ImageJ software was used to measure the number of endothelial cells and check the hexagonality and polymorphism of the cells using ZO-1. It was also used to check the percentage of Ki-67 positive cells in the given area and to check the number of focal adhesions (Vinculin positive cells). ANNOVA and non-parametric Wilcoxon test for paired data using SAS statistical software was employed to check the statistical significance between different conditions where  $p < 0.05$  was deemed significant.

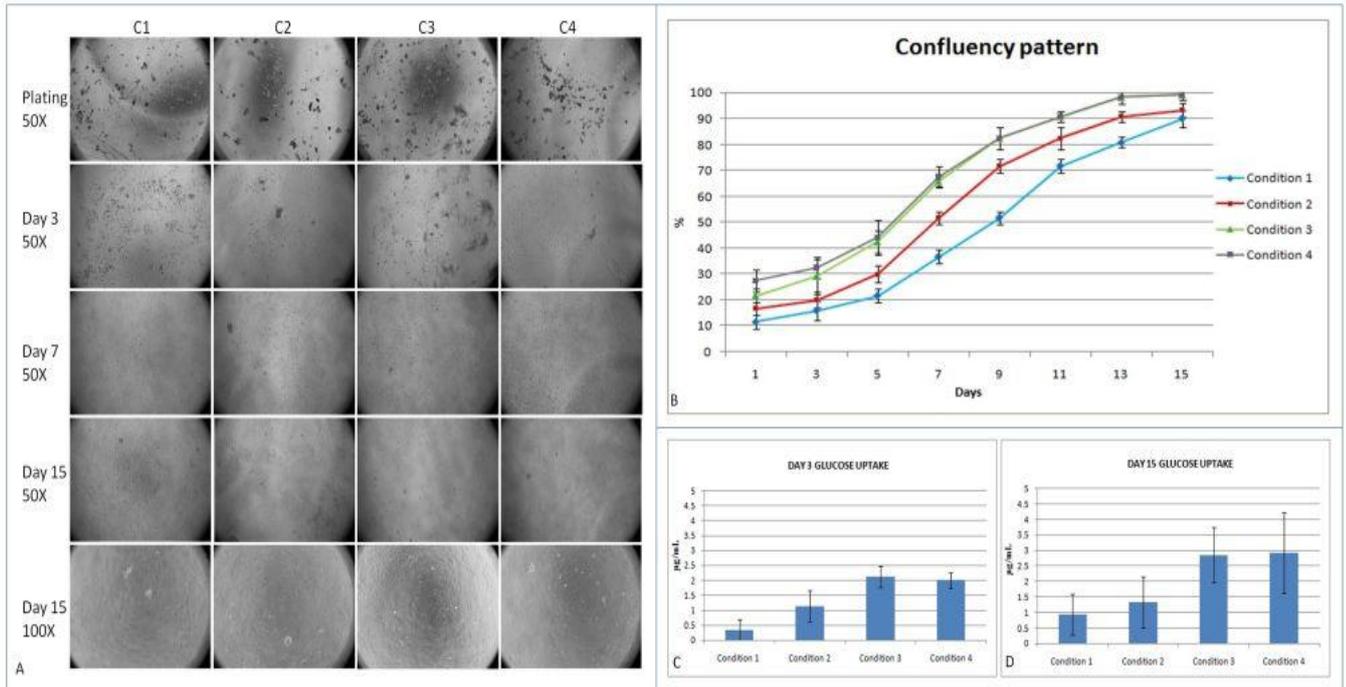
## **Results**

### *Donor characteristics and plating density [n=48, twenty four pairs]*

Average age of the donors recorded was 63.94 ( $\pm 13.79$ ; Min-48, Max-79) years comprising of 14 Males and 10 Females. The average post mortem time was 16.71 ( $\pm 6.37$ ; Min – 5.0 h, Max – 25.35) hours. The tissues were preserved in the tissue culture medium for 31.69 ( $\pm 6.67$ ; Min – 20, Max – 40) days. Average endothelial cell density before isolation was 1943.75 ( $\pm 222.02$ ; Min – 1800, Max – 2100) cells/mm<sup>2</sup> without any trypan blue positive cells (TBPCs). 92,313.58 ( $\pm 10,544.16$ ; Min – 75,988, Max – 99,734.5) cells in average per Labt-Tek II chamber slides (8 chambers, 25X75 mm, 0.7 cm<sup>2</sup> culture area) from Thermo Fisher Scientific (Rochester, NY, USA), well was plated after isolation.

*Culture of HCECs and their confluency patterns show higher proliferation from C4 [n=30, fifteen pairs]*

HCECs were observed at different days of culture (figure 1a). The cells were not passaged as only the adhesion and proliferation capability was studied and the cells were sacrificed for the immunostaining analysis at day 15. At day 3, the growth rate (%) observed in C1 (condition 1 – no HA or ROCK), C2 (condition 2 – only HA), C3 (condition 3 – only ROCK) and C4 (condition 4 – HA + ROCK) was 15.8, 20, 29.2 and 32.5, however the growth rate (%) at day 7 increased to 36.6, 51.6, 65.8 and 67.5 respectively without any statistical significance ( $p < 0.05$ ). The data reveals that when the cells are adhered forcefully, they get a boost start. The cell attachment to the matrix or its base is highly important for culture and proliferation. At day 15 the cell showed (%) 90, 93.3, 99.16 and 99.16 confluency (Figure 1b). It was observed that at day 3, C1-C4 showed statistical significance in growth rate ( $p = 0.0404$ ) and the other conditions did not show any significance (table 2). Although statistically insignificant ( $p > 0.05$ ) at day 15 (table 2), the cells showed faster proliferation in C4 in the first week of culture. Once the cells get adapted to the environment, they grow normally. It was observed that the proliferation capability of the cells after 7 days in C3 and C4 was the same. C1 and C2 took a longer time to adapt and reached almost 90% confluence at day 15.



**Figure 1:** Morphological pattern and glucose analysis at different days in different conditions. A) It was observed that the cells attach firmly in C2, C3 and C4 and start proliferation within day 3. However, by day 7, C3 and C4 show a higher number of cells and growth rate. This is due to ROCK inhibitor in C3 and HA+ROCK in C4. By day 15, confluence was observed in C3 and C4 and >90% of the culture was confluent in C1 and C2. At day 15, the cell shape and size along with confluence is better and higher in C3 and C4 as seen at 100 X magnification. B) The growth pattern is recorded in different conditions and it was determined that C3 and C4 show higher proliferation capability when firmly adhered to the base. Confluency of the HCECs cultured *in vitro* in different conditions shows up to 30% boost in C3 and C4 as compared to around 20% in C1 and C2. However, by day 15, C3 and C4 reached 99% confluency whereas C1 and C2 were at 90%. HA is not the only reason for cell attachment, a combination of HA and ROCK suits the cells to adhere both mechanically and molecularly and allow higher proliferation capability. C and D) Higher glucose uptake was observed in C3 and C4 at day 3 and also at day 15 due to high number of metabolically active cells.

*Glucose uptake showed high metabolic activity in C3 and C4 [n=48, twenty four pairs]*

It was observed that the average amount of glucose ( $\mu\text{g/mL}$ ) that was taken up by day 3 in C1, C2, C3 and C4 was  $0.34(\pm 0.33)$ ,  $1.13(\pm 0.53)$ ,  $2.13(\pm 0.36)$  and  $2.0(\pm 0.27)$  respectively. This was statistically significant ( $p < 0.05$ ) between C1-C4 and C1-C3 at day 3 (figure 1c). No other conditions showed any statistical significance at day 3. However, glucose uptake was statistically significant between all the groups ( $p < 0.05$ ) except C1-C2 and C3-C4 at day 15 (table 2) where C1, C2, C3 and C4 utilized  $0.94(\pm 0.59)$ ,  $1.33(\pm 0.58)$ ,  $2.85(\pm 0.78)$  and  $2.92(\pm 1.01)$   $\mu\text{g/mL}$  of glucose (figure 1d). This means that the cells were functional throughout as the metabolism was active due to the consumption of glucose and production of lactic acid in all the groups. It was noticed that higher amount of glucose was uptaken in C3 and C4 by day 3 as compared to C1 and C2. This is because of the presence of HA and ROCK inhibitor. Faster cell adherence and start of proliferative phase was observed in C3 and C4. Both the groups have similar media composition, hence only the similar groups did not show any significance, which also highlights that apart from force adhesion using HA, ROCK is also important for higher cell adhesion and better proliferation.

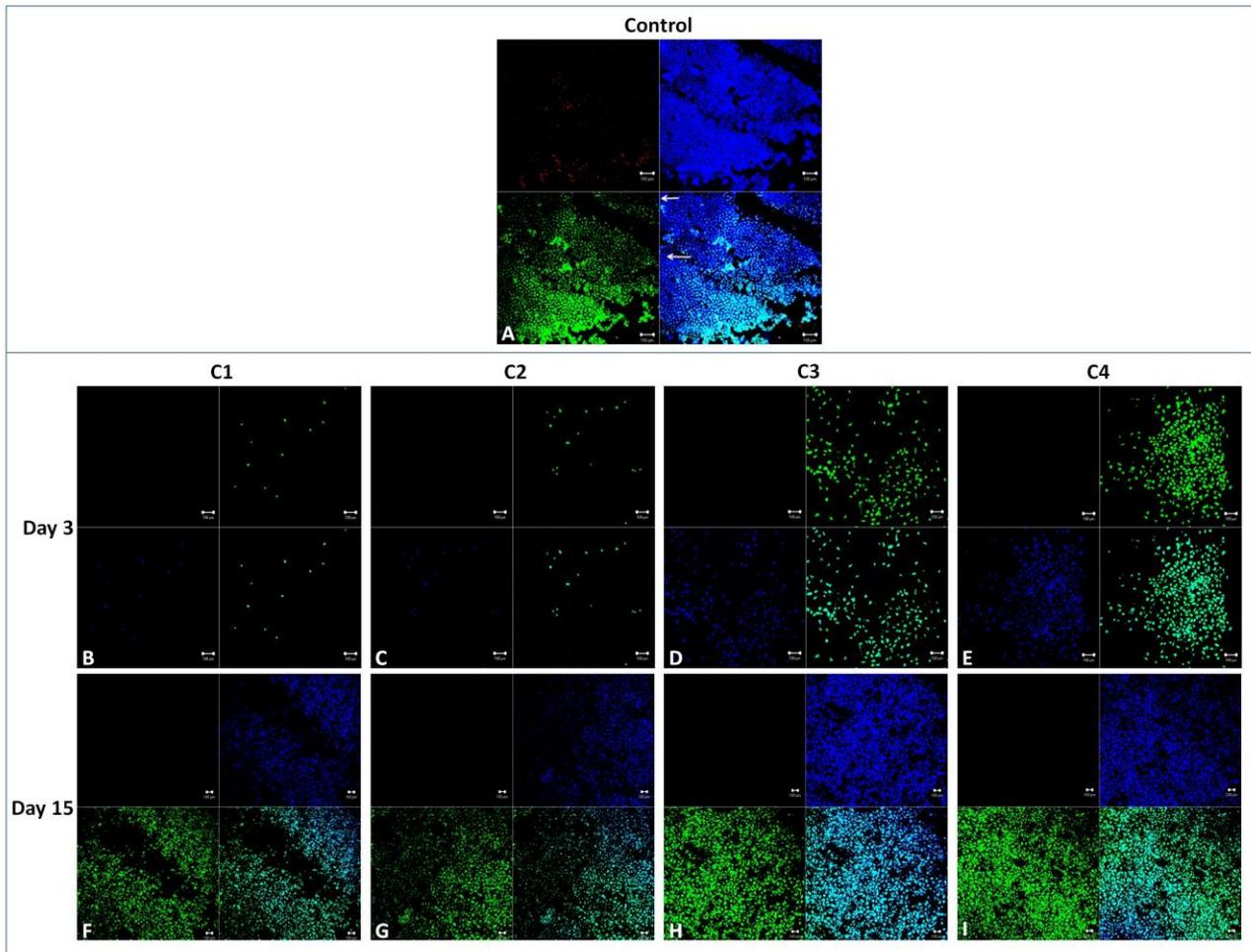
pValues	C1-C2	C2-C3	C3-C4	C1-C4	C1-C3	C2-C4
<b>Confluence day 3</b>	0.2339	0.2789	0.4294	0.0404	0.1459	0.0751
<b>Confluence day 15</b>	0.5495	0.5712	0.9198	0.1985	0.4922	0.5775
<b>Glucose uptake day 3</b>	0.2379	0.1763	0.7251	0.0406	0.0381	0.2185
<b>Glucose uptake day 15</b>	0.197	0.0007	0.888	0.0005	0.00009	0.0027
<b>% Hexagonality day 15</b>	0.5077	0.3357	0.2078	0.0488	0.2122	0.0758
<b>% Polymorphism day 15</b>	0.3209	0.2145	0.1239	0.0268	0.0419	0.0141
<b>Ki-67 day 3</b>	0.3371	0.4077	0.5657	0.5251	0.9003	0.7626
<b>Ki-67 day 15</b>	0.9709	0.4568	0.4177	0.9248	0.3957	0.9143
<b>Vinculin day 3</b>	0.1088	0.2598	0.7036	0.0395	0.1668	0.0609
<b>Vinculin day 15</b>	0.5392	0.2743	0.7225	0.1091	0.1531	0.1889

**Table 1:** Statistical data comparing different conditions and parameters. Numbers in red indicate statistical significant difference. C1, C2, C3 and C4 indicate conditions 1, 2, 3 and 4.

*Hoechst, Calcein AM and Ehidium homodimer (HEC) staining showed higher number of cells at day 3 and confluency pattern at day 15 without any dead or apoptotic cells in C3 and C4 [n=12, Six pairs]*

HEC was observed in the control group to show the presence of all the dyes for comparison (figure 2a). White arrow marks the apoptotic cells, which are nucleus without cytoplasm (attached dying cells to the extracellular matrix). At day 3, C3 and C4 showed more number of cells as compared to C1 and C2. Only viable cells were observed at day 3 (figure 2b-2e). However, at day 15, confluency was observed in C3 and C4 compared to C1 and C2 with no dead cells and few apoptotic cells marked in white, which were not

significant (figure 2f-2i). This was a qualitative analysis and therefore statistics were not performed.

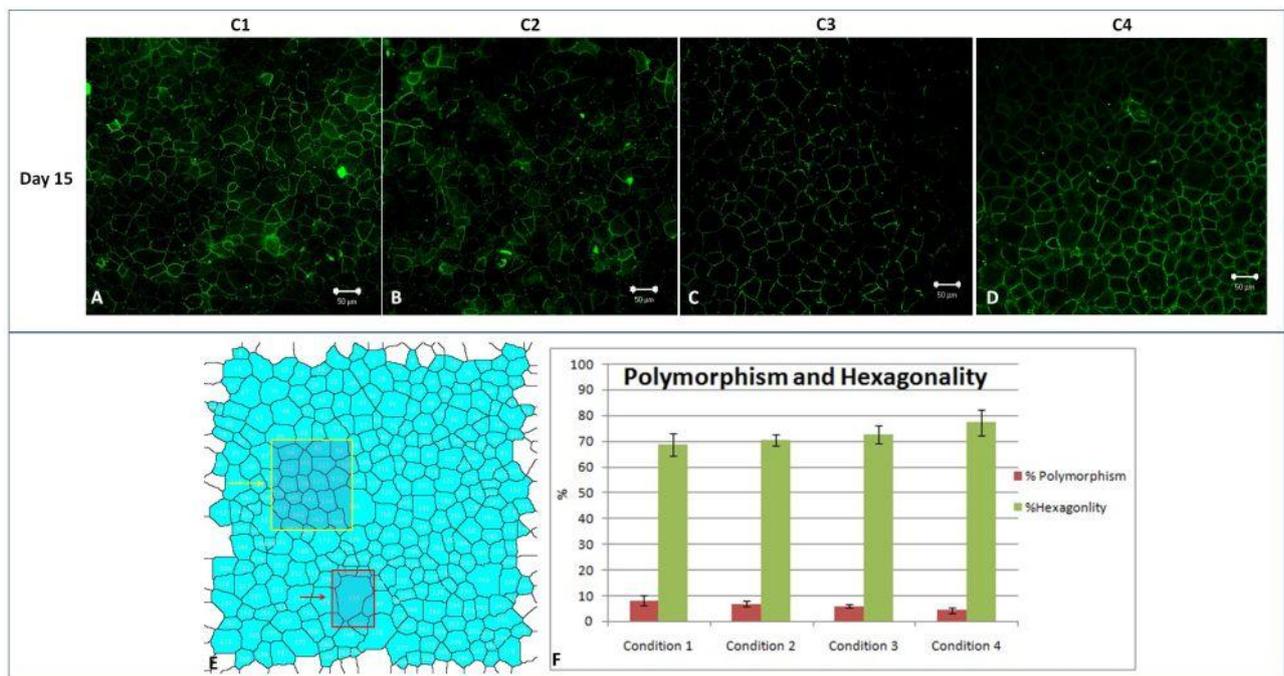


**Figure 2:** HEC staining to determine live/dead/apoptotic cells. A) Control cornea to show the presence of dead (red), live (green), apoptotic (blue without green marked with white arrow) and nucleus (blue). B-E) at day 3, higher number of cells was observed in C3 and C4 compared to C1 and C2 without any dead or apoptotic cells. F-I) at day 15 confluence of viable cells was observed in C3 and C4 compared to C1 and C2.

*Expression of Zonula Occludens-1 (ZO-1) and measurement of hexagonality and polymorphism showed significant data at day 15 [n=12, Six pairs]*

Zonula Occludens-1 (ZO-1) is a tight junction protein which is present in the intercellular borders between the cells. Apart from showing the functions of tight junction protein, it can

also be indirectly use to judge the hexagonality of the cells. ZO-1 has also been widely used in the research of HCECs. At the end of the culture at day 15, ZO-1 (figure 3a-3d) was expressed and showed significant difference between C1-C4 ( $p=0.0488$ ). Average value of hexagonality (%) (yellow box in figure 3f) in C1, C2, C3 and C4 was  $68.79(\pm 4.47)$ ,  $70.58(\pm 2.18)$ ,  $72.77(\pm 3.49)$  and  $77.27(\pm 5.18)$  whereas the polymorphism (%) (indicated as red box in figure 3f) in the respective combinations was found to be  $8.16(\pm 2.08)$ ,  $6.86(\pm 1.05)$ ,  $5.97(\pm 0.66)$  and  $4.35(\pm 1.01)$ . However, the cells from condition 1 and 2 showed slightly less hexagonal cells, which were checked using ImageJ (not significant,  $p>0.05$ , table 2, figure 3e) for other conditions. Higher polymorphism was observed between C1-C3 ( $p=0.0419$ ), C1-C4 ( $0.0268$ ) and C2-C4 ( $0.0141$ ). There was no significant difference between other conditions at the end stage (day 15) but as the cells were not fully confluent in C1 and C2, the cells showed higher polymorphism.

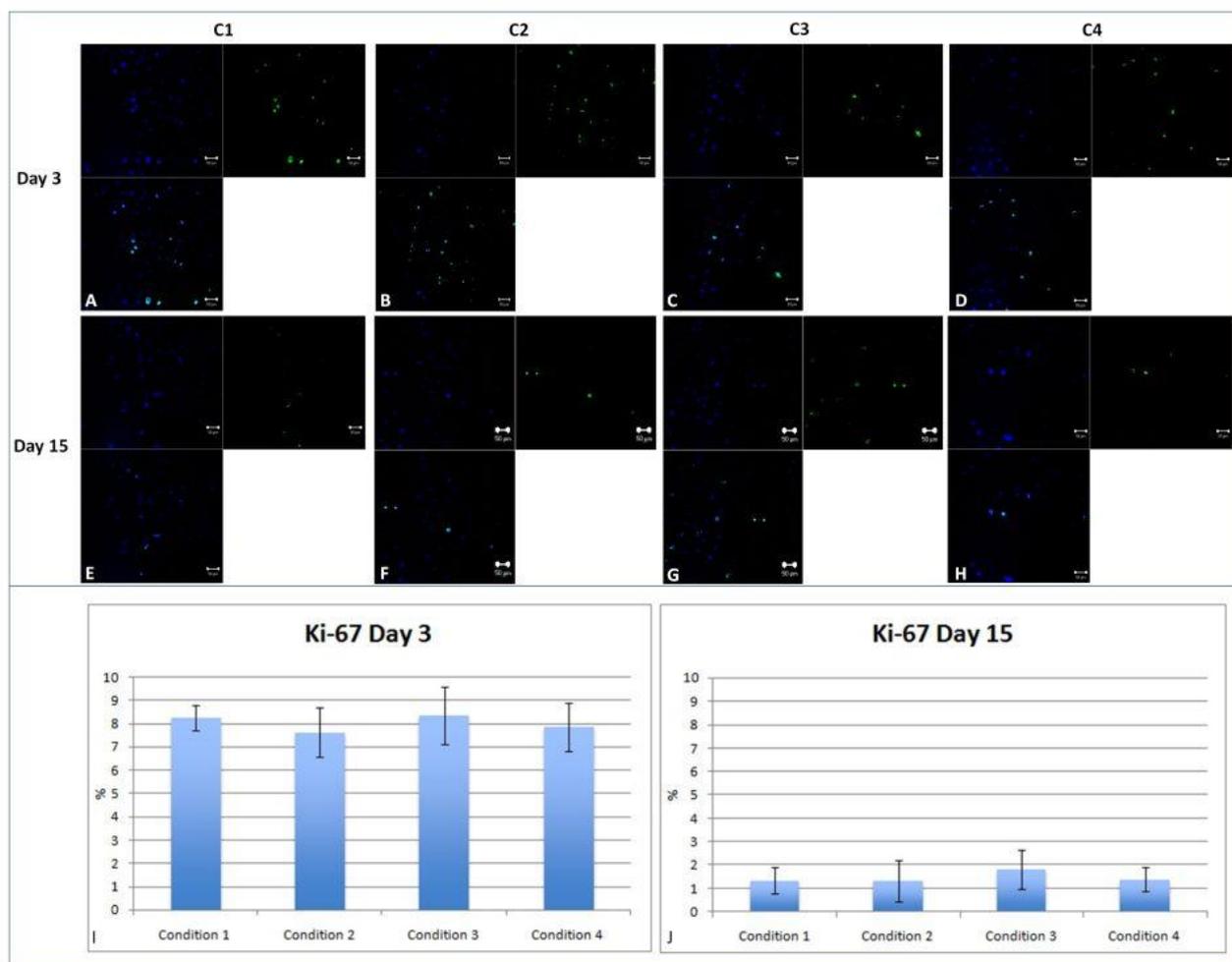


**Figure 3:** ZO-1 staining for intracellular tight junctions and to determine the polymorphism and hexagonality of the cells in different conditions. A-D) The cultured HCECs showed expression of ZO-1 in all conditions. E) The image was locked in imageJ and was

processed to overlay masks for determination of hexagonal cells and number of cells that showed high polymorphism (polymegathism and pleomorphism). Yellow blocks represent number of hexagonal cells and red block indicate highly polymorphic cell in the determined area. F) Hexagonality was higher in C4 with less polymorphic cells compared to other conditions.

*Statistically insignificant expression of Ki-67 was observed in all the conditions [n=12, Six pairs]*

Ki-67 is a proliferative marker which is used to check the amount of proliferative cells present in the culture. Ki-67 was used at the initial stage to control the presence of nearly equal amount of proliferative cells in all the conditions and to rule out the possibility of a biased study with presence of higher number of cells in either condition. We performed this study on n=3 separate tissues to optimize our plating density. It was observed that all the conditions had similar amount of proliferative cells at the beginning (day 3) (figure 4a-4d, 4i). Average Ki-67 expression (%) found on day 3 in C1, C2, C3 and C4 was 8.25( $\pm$ 0.53), 7.63( $\pm$ 1.04), 8.34( $\pm$ 1.22) and 7.86( $\pm$ 1.02) whereas on day 15, Ki-67 decreased to an average of 1.33( $\pm$ 0.58), 1.32( $\pm$ 0.90), 1.80( $\pm$ 0.82) and 1.37( $\pm$ 0.51) respectively. Number of proliferative cells or Ki-67 expression decreased in number at day 15 (figure 4e-4h, 4j), but did not show any significance ( $p > 0.05$ ) (table 2) in either condition.

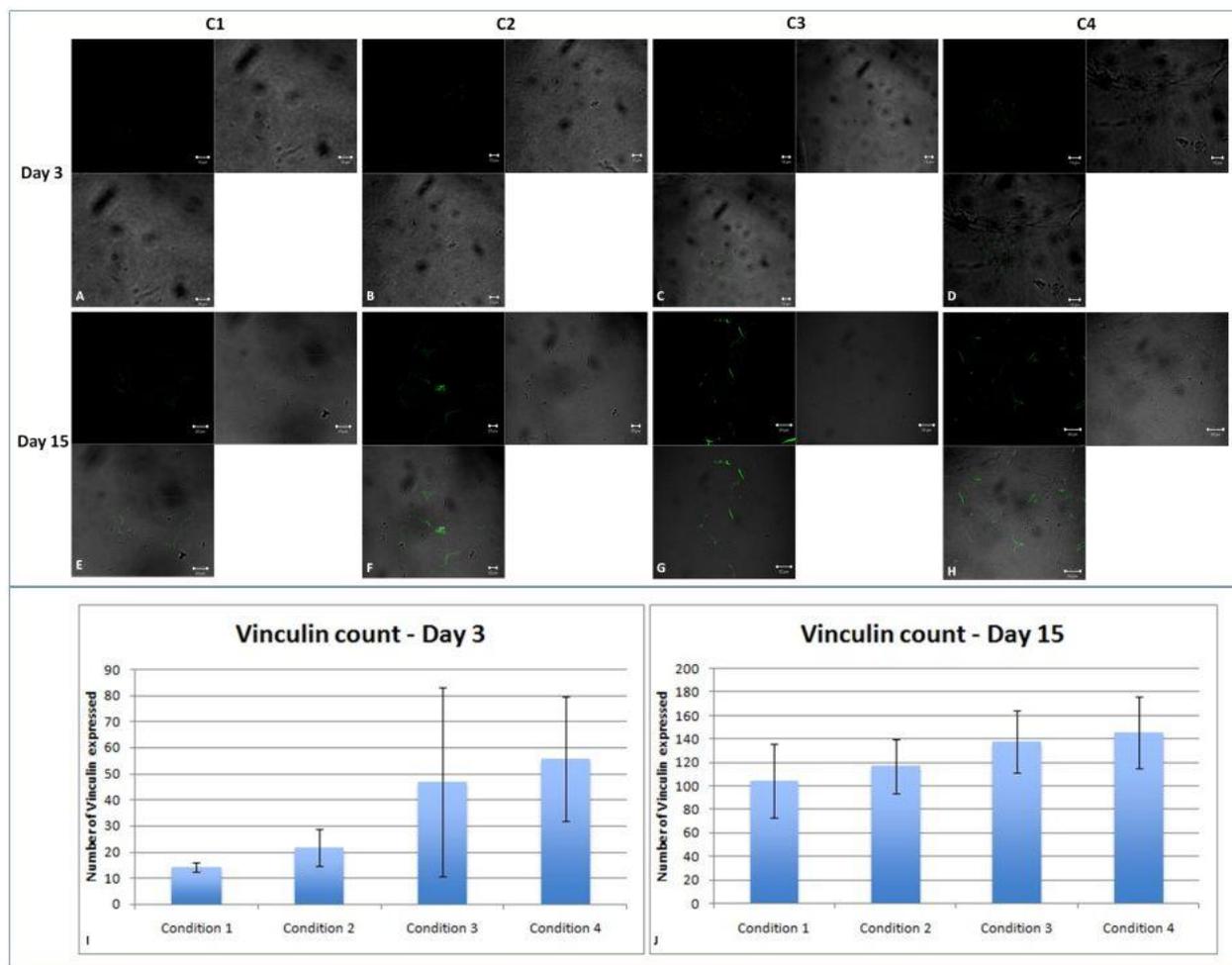


**Figure 4:** Expression of Ki-67, proliferative marker. A-D and I) Ki-67 was almost equally expressed in all four conditions, which highlights that the plating density of Ki-67 was equal in all conditions at day 3. E-H and J) at day 15, number of Ki-67 cells decreased in all four conditions without any statistical significance.

*Vinculin showed higher focal adhesion points in C4 at day 3 [n=12, Six pairs]*

Vinculin is a membrane-cytoskeletal protein in focal adhesion plaques that is involved in linkage of integrin adhesion molecules to the actin cytoskeleton. It is also associated with cell-cell and cell-matrix junctions, where it is thought to function as one of several interacting proteins involved in anchoring F-actin to the membrane. Vinculin was expressed in all the conditions at day 3 however; it was significantly expressed more in C4

compared to C1 and C2 (table 2). C1, C2, C3 and C4 showed 14.0( $\pm$ 1.83), 21.75( $\pm$ 6.95), 47.0( $\pm$ 36.34) and 55.75( $\pm$ 23.96) focal adhesion spots on day 3. C3 did not show any statistical significance compared to any groups (figure 5a-5d). Day 15 did not show any significance between any groups (figure 5e-5h) (table 2) with 104.25( $\pm$ 31.55), 117( $\pm$ 22.85), 138( $\pm$ 26.32) and 145.5( $\pm$ 30.47) focal adhesion spots on day 15. The results highlight that higher focal adhesions may be induced with forced adhesion especially when there is an involvement of mechanical pressure because of HA and molecular force due to ROCK inhibitor (figure 5i-5j).



**Figure 5:** Expression of Vinculin as a focal adhesion marker. A-D and I) Number of focal adhesions found in C4 was statistically higher compared to C1. C3 also showed high number of focal adhesion points but was not significant at day 3. This highlights that

mechanical and molecular forces may induce higher focal adhesions and integrins for better cell attachment. E-H and J) at day 15, Vinculin was expressed in all the conditions without any significant difference.

## **Discussion**

Corneal endothelial dysfunction is a major indication for corneal transplantation [15]. However, the isolation and propagation of primary HCECs have been described by various research groups using different approaches [16-20] to find alternative solutions reducing the global shortage of donor corneas. Cultivated HCECs derived from older donors have lower proliferative capability, a senescent cell phenotype, and cell morphology, which suggest less functional ability than those derived from younger donors [21]. If the HCECs can be cultured from old donor corneas, then the availability of donor corneas for isolating endothelial cells will increase and it will overcome the overall shortage of donor corneas available for transplantation [22,23].

Corneal endothelial dysfunction has been treated by full-thickness corneal transplantation (PK) since more than half a century, but more selective corneal endothelial replacement such as Descemet Stripping Automated Endothelial Keratoplasty (DSAEK) and Descemet Membrane Endothelial Keratoplasty (DMEK) were developed in the last decade [24,25]. The clinically successful outcomes of DSAEK and DMEK imply that reconstruction of the corneal endothelium is a definitive treatment that can replace full thickness keratoplasty, further corneal endothelium reconstruction by cell-based therapy is a clinically relevant approach [26]. Currently, although HCECs are isolated from donor corneas and cultured for research purposes, several limitations such as limited proliferative ability, vulnerable transformation with loss of functions, and senescence that prohibits efficient *in vitro*

expansion for clinical use have not been answered. No protocol specifically designed for clinical application has been established so far.

It is already known that inclusion of ROCK inhibitor Y-27632 elevates the adhesion property, enhances cell proliferation and suppresses apoptosis of HCECs, thus allowing the successful transplantation of HCECs to reconstruct functional corneal endothelium [27]. Another report has emphasized the effect of ROCK inhibitor in enhancing adhesion and wound healing of HCECs [7]. It has already been noted that Rho-ROCK signalling carries out a variety of cellular processes such as cell adhesion, morphogenesis, migration, and cell-cycle progression through mediating cytoskeletal dynamics. Rho regulates a variety of cytoskeletal dynamics that underlie cell morphology and adhesion through the activation of ROCK [28,29]. The low efficiency of engraftment and loss of phenotype after transplantation due to the absence of cell/cell and cell/ECM interaction *in vivo* impairs organ reconstruction in various tissues [30-32]. Researchers have therefore been exploring the use of various techniques, such as artificial scaffolds, biologically active molecules, and ECM coatings, to improve cell retention and survival [33-35].

Although the forced adhesion technique is relatively robust, the HCEC cultures can be affected substantially by the procurement process as well as donor variations. The characteristics that we have used for this study are extreme with old donor age, long preservation time and endothelial cell count that are less than the normal density required for transplantation. If these challenging cells can be cultured in the appropriate conditions then the cells from young donors with high cell density and proliferative capability will definitely show increased proliferation rate (also internally observed in one donor – 32 year old). Donor characteristics such as health of the donor before death, the cause of death, as well as the duration from death to enucleation and preservation and the time

from preservation to the establishment of culture [14,36-38] has also shown to have a potential impact on the cell culture. Beyond these potential hindrances, we report herein that the combination of HA and ROCK (C4) [39] approach in expansion of HCECs can be further enhanced, in terms of high adhesion and proliferation capability. The shorter time it took for HCECs to reach confluence was most likely due to the increased proliferation rates as observed in C4 due to high adhesion rate. It has been reported that the use of Y-27632 to increase the proliferation of HCECs may only be appropriate for cultures of HCECs established using younger donors, as the addition of Y-27632 were not found to be advantageous for cultures established using older donors [12]. Similar observation has been also reported that Y-27632 has no effect on the proliferative capacities on HCECs, which utilized corneas from donor ages  $73\pm 3$  years [7]. However, forced adhesion of the HCECs from older donor corneas may show a potential impact in cell culture of these low proliferative cells.

In this study, we investigated the differences in cellular phenotype in response to culture in different types of adherence conditions. We found that the proliferative capability of HCECs differed depending on the conditions used. Four conditions were selected to check the adhesive properties and proliferative capabilities especially for older donors. The isolation of HCECs is very much important as it determines the preliminary success of the culture. If the isolation is not performed correctly, it may lead to contamination of stromal fibroblasts or trabecular meshwork cells. We did observe such contaminations in our very first cultures (excluded from this study). Gentle peeling of Descemet membrane and endothelium piece-by-piece seems to be the best solution so far. We have previously tried using a Descemet Membrane Endothelial Keratoplasty (DMEK) donor punch (9.5 mm). This has a great advantage in terms of excising two exclusive parts of the endothelium (periphery and centre) with cell loss that only occurs at the punch site.

However although punching method can be used, there is a possibility that increase in punching pressure may pierce the stroma which may then activate keratocytes leading to unwanted contamination of the fibroblasts. Peeling a larger diameter DMEK graft (9.0mm) and incubating it with Collagenase type 1 also takes longer time for the cells to digest due to the less available area for the enzyme to cleave between the cells. It was also noted that the DMEK tissue of the younger donors are firmly adhered to the stroma as compared to the older donors which can be peeled easily if the donors have no other contraindications like diabetes. Therefore manual peeling of the cells piece by piece was determined as a suitable protocol for this study.

It has been widely reported that HCECs isolated from older donors are less proliferative than those established from younger donors, and this has been associated to significant increase of cyclin kinase inhibitors, hence resulting in an age-dependent increase in negative regulation of cell cycle [40]. However, using C4 i.e. HA combined with ROCK, it is possible to culture the older donor corneas too with a high proliferative capability. The presented data highlighted that the cells can reach confluence by day 10 when provided with conditions like C4. We used 15 pairs of older donor corneas and the confluency pattern seen was significant between C1 and C4 suggesting that C4 was superior in terms of cellular proliferation. However, C3, the condition with only ROCK did not show any significance in confluency with C4 or C1 but showed lesser proliferative capability than C4 up to a week. Moreover, all the tissues that were collected for this study had an endothelial cell density  $<2200$  cells/mm<sup>2</sup>. The plating density of younger donor corneas is around 550,000 cells/9 cm<sup>2</sup>, which is less than the plating density we have used which is around 93,000 cells/0.7 cm<sup>2</sup>. Increasing the plating density for  $<1$  cm<sup>2</sup> area (optic zone) could also be sufficient for culturing older donor corneas.

We have noticed that relating culture conditions, proliferation and confluency patterns to donor characteristics was very difficult as some donors show good proliferation rate and others don't. However, most of the donors were cultured with success using C3 and C4. Morphological analysis showed hexagonal cells when cultured in different conditions and very less amount of cells <10% showed high polymorphism. We were able to culture 4 wells (0.7cm<sup>2</sup> each), which are similar to an optic zone by pooling low-density old age donor corneas and get them to confluence with good morphology and high rate of hexagonality. As it is difficult to obtain younger donor corneas with high-density endothelial cells, the technique (C4) could be of valuable importance in terms of donor availability and culture capacity. Moreover, if the target is to culture primary endothelium into multiple grafts, then high plating density in combination with C4 could be a valuable solution especially when the tissues are isolated from older donors. With this approach, there is a practical possibility of obtaining 4 grafts from 2 corneas (1 donor).

The amount of utilized glucose can be determined during the culture by checking the metabolic activity of the cells. When the cells metabolize, they produce lactic acid from glucose and therefore the amount of glucose uptaken in the media by the cells can be evaluated. In this study we show that the amount of glucose that was utilized in the first 3 days was significantly higher in C4. This was due to a higher number of proliferating cells in the first two days. However, when the cells were confluent at day 15, all the conditions showed insignificant amount of glucose uptake, which confirmed that majority of the cells were metabolically active during the culture stage and at the confluent stage. If the cells were stressed due to the acidity levels of HA, they would have also shown high glucose uptake in C2, which was not the case. It only showed in C3 and C4, which further highlights that HA are safe to be used for cell culture.

HEC staining also showed that the cell proliferation in C3 and C4 was higher compared to C1 and C2 at day 3 and day 15 when it was confluent. A minimum amount of apoptotic cells were observed with no dead cells in the culture. The cell viability was higher in all the conditions. ZO-1 immunostaining was used to check the hexagonality and the presence of tight junctional proteins. It was observed in separate conditions (data unpublished) that ZO-1 did not express much when the cells were packed because even though the cells look confluent, the time required for the development of tight junctional protein is higher. Therefore, the cells should be allowed to culture for an extra day after confluency just to ensure that they have properly developed intra-cellular tight junctions. Cell shape is related to various cell functions, such as the communication with other cells, regulation of cell movement. Polymorphism was not high in the cells cultured in either condition at the day of confluency as all the conditions utilize ROCK inhibitor, they all proliferate up to the confluence stage. Condition 4 showed lower polymorphism as compared to the other conditions. Force adherence helps to maintain the cell shape and size. To ensure the plating density of the proliferative cells is almost the same in all the conditions, we optimized our plating conditions in different set of corneas before the experiment. It was observed that the proliferative cells found in different conditions were almost the same in all four conditions at day 3. Proliferative cells showed a decline in number at day 15 in all the conditions which is already previously known that the proliferative capabilities of the cells decreases with time.

A previous study shows that actin cytoskeleton plays a critical role in regulating the adhesive property through interaction between the actin cytoskeleton and integrin [41-43]. Another study reported that inhibition of ROCK signalling by a selective ROCK inhibitor or by the siRNA enhances adhesive property of HCECs and is consistent with the findings of those previous studies. It was also found that vinculin, which is involved in the linkage of

the integrin adhesion complex to the actin cytoskeleton [44-45] is upregulated in ROCK-inhibitor treated HCECs. However, it is noted that further investigation is required to elucidate whether the ROCK inhibitor promotes the focal adhesions through inhibiting actin polymerization and induces the upregulation of cell adhesion properties on the extracellular matrix (ECM) [5]. Vinculin expression and HEC staining revealed the same thing, that C3 and C4 show higher number of cells at day 3 and day 15. C1 and C2 showed lower number of cells when compared to C3 and C4. Vinculin expression was significantly higher in C4 at day 3. Therefore, it is assumed that force adherence in combination with ROCK may be responsible to induce more focal adhesions and hence integrins and expression of Vinculin.

Due to the availability of young donors for research, we were only successful in culturing one young donor. We observed confluency on day 7 with high hexagonality (approximately 90%) when young donors (age 32) with high plating density were cultured using forced adhesion with HA and ROCK. Old donors may not have a high proliferative capability but if they are forced to attach and proliferate, although it may take longer to confluence, they do proliferate and manage to obtain the required cell size and shape.

A surgical hypothesis for using HA is that it is currently used in the anterior chamber surgical procedures. The injection technique for introducing cultured HCECs with face down position is already been studied by Kinoshita and colleagues (data unpublished). The patients have to rest their face down for 2-3 hours which may be longer for some patients therefore, introducing HA after face down after introduction of HCECs may help the cells to adhere to the stroma faster compared to the regular adherence speed. This may have a potential clinical impact in the future.

In conclusion, the findings of this present study, which are supported by the previous data, indicate that ROCK inhibitor Y-27632 in combination with HA may enable the establishment of a cultivated-HCEC-based therapy. This novel strategy of using a force attachment of the HCECs combined with a ROCK inhibitor in older donor corneas with less proliferative and isolated cells 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.

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## **CHAPTER 10**

# **FISH SCALE DERIVED SCAFFOLDS FOR CULTURING HUMAN CORNEAL ENDOTHELIAL CELLS FROM OLD DONOR CORNEAS**

*Derived from article (Submitted – under peer review)*

Mohit Parekh, Van den Bogerd Bert, Nadia Zakaria and Stefano Ferrari. Fish scale derived scaffolds for culturing human corneal endothelial cells from old donor corneas.

Scientific Reports.

## **Aim**

To investigate a fish scale derived scaffold for culturing HCECs

## **Introduction**

Human cornea is the transparent, outermost tissue of the eye. It is the principal refractive element of the visual system and its function depends majorly on the optical clarity. HCECs are responsible for maintaining the transparency of the cornea with a mechanism known as the pump-and-leak hypothesis [1].

The monolayer of hexagonal HCECs avoids corneal edema, but also allows passive diffusion of nutrients simultaneously. Due to their amitotic nature *in vivo*, the amount of endothelial cells only decreases throughout life. However, they can be dramatically accelerated by trauma or disease. When the amount of HCECs drops below a certain threshold, corneal edema becomes unavoidable and leads to an opaque cornea. The only available treatment to overcome this disorder is corneal transplantation, whether full or partial thickness. However, it hugely depends on the availability of the donor tissue, which is currently a huge limitation. It is noted that the total number of donated corneas in 2015 distributed by US eye banks was 79,306 [Eye Bank Association of America (EBAA) statistical report 2015]. Of these, 39,554 were destined for full thickness penetrating keratoplasty (PK) and 30,710 for endothelial keratoplasty (EK) (14,472 for Fuchs' Dystrophy and 3,208 other causes of endothelial dysfunction). This means that nearly 40% of corneal transplantations were carried out for endothelial dysfunction. Although EK has a high success rate in terms of visual rehabilitation and post-op visual outcomes, shortage of donor corneas still remain a global challenge [2]. Endothelial keratoplasty has been the most commonly performed keratoplasty procedure in the United States in the

last four years and continues to increase. This means that endothelial diseases are the main indication for corneal transplantation in the United States.

Alternative medical and therapeutic approaches like *ex vivo* expansion of the cultured HCECs thus become important as well as developing a scaffold for cell culture and eventually as a carrier for transplantation. As patients suffering from corneal endothelial dysfunction take up a big proportion on the current waiting list, these tissue-engineering approaches could greatly reduce current waiting lists. Apart from various attempts for efficient endothelial cell expansion using different media [3] and inhibitors [4], different scaffolds have also been reported to act as a mechanical support and meanwhile sustain cell expansion and functionality. Among these different options, three different categories can be distinguished: (i) biological, (ii) synthetic and (iii) biosynthetic substrates [5].

In 2010, Lin et al. proposed a collagen scaffold derived from decalcified fish scales (Tilapia; *Oreochromis Mossambicus*) for use in corneal regeneration. Until now, preliminary *in vitro* studies have shown the cytocompatibility of cells on these heterogeneously patterned, biological scaffolds [5]. Its architectural features have been suggested as an important feature for cell seeding, migration and growth. Its transparency and nature (soft-tissue) makes it a superior biocompatible material for HCEC culture and transplantation. Additional *in vivo* studies performed on rats and rabbits have demonstrated its potential as a DALK alternative or to seal perforated corneas, respectively [6,7].

The design, development and preliminary evaluation of an acellular, decalcified fabricated scaffold that fits the need for the design of current corneal prostheses has been evaluated. Although, fish scale-derived collagen scaffolds have been identified as a potential scaffold for ocular surface, it has never been studied before for culturing the

HCECs. Acellularized and decalcified fish scales have appeared to favour cell proliferation and biosynthetic activity after 7 days of cultivation. This chapter intends to investigate the efficacy and efficiency of fish scales as potential scaffolds for culturing and a possibility of transplanting HCECs cultured *ex vivo*. Due to its transparent nature and wide availability, we investigated FSS to act as a HCECs scaffold more into detail.

## **Materials and Methods**

### *Ethical Statement*

The corneas [n=30, Fifteen pairs] 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 corneas were suitable for research and unsuitable for transplantation due to its low endothelial cell count (<2200 cells/mm<sup>2</sup>). No other complications or indications were noted in the donor corneas. All the tissues were preserved in tissue culture medium at 31°C prior to use.

### *Characteristics of Fish Scale Scaffolds*

Fish scale scaffolds (FSS) were shipped to the FBOV labs from Body Organ Corp. (Taipei, Taiwan). Tilapia fishes were cleaned and acellularized using previously reported articles [6-8]. To increase the pore sizes and porosity within the test samples, the acellular tissues were additionally treated with acetic acid. The resulting acellularized fish scales were rinsed extensively with and stored in sterilized phosphate-buffered saline. These scales were decalcified and shipped to the FBOV labs in sterile PBS.

### *Endothelial cell count and donor characteristics*

Endothelial cell density (ECD) and mortality (on the donor cornea) before isolation was checked using trypan blue (TB) stain (0.25%) (Thermo Fisher Scientific (Rochester, NY,

USA) which is routinely used in eye banks. Approximately 100  $\mu\text{L}$  of TB was topically applied to stain the endothelial cells for 20 seconds followed by washing it with phosphate buffered saline (PBS). Trypan blue positive cells (TBPCs) and endothelial cell density were recorded before isolation using an in-built eyepiece reticule for inverted microscope (Axiovert, Zeiss, Germany). The donor characteristics of 30 corneas (fifteen pairs) were obtained from the FBOV database to determine the age, gender, post-mortem time, cause of death, preservation time, ECD and mortality.

#### *Formulation of the medium for cell culture*

The proliferative medium (M4) was a mixture of HamF12, M199, 5% FBS, 1% ascorbic acid, 0.5% Insulin Transferrin Selenium (ITS), Rec human FGF basic (25  $\mu\text{g}/\text{mL}$ ), 10  $\mu\text{M}$  ROCK inhibitor (Y-27632) and antibiotics as described earlier [3].

#### *Peel and digest method*

The method is similar to previously published article [9] with some modifications which is described in brief. The corneal tissues [n=30] were washed in sterile phosphate buffered saline (PBS) and the Descemet membrane-endothelial tissues were peeled gently (similar to stripping technique for DMEK method) but, in various pieces to ensure faster isolation of the cells when incubated in the enzyme. The excised pieces were incubated with 2mg/mL Collagenase Type 1 (Thermo Fisher Scientific, Rochester, NY, USA) solution for 2-3 hours 31°C, 5% CO<sub>2</sub>. While peeling, the forceps was cleaned using PBS after every single encounter with Collagenase Type 1 solution. Once the cells were isolated from the DM, they were collected and centrifuged for 5 minutes at 1000 rpm in a 15 mL falcon tube. The supernatant was removed and the cells were re-suspended with TrypLE Express (1X), phenol red [Life Technologies, Monza, Italy] to dissociate the clumps into single cells for 10 minutes at 37°C [if the clumps were not converted to single cells then TrypLE

Express treatment was repeated]. The supernatant was removed and the cells were re-suspended with 400  $\mu\text{L}$  of M5 after counting them with TB in a haemocytometer slide. The plating density was recorded for all the cultures.

#### *Plating the cells on coated slides and FSS*

Lab-Tek II chamber slides (8 chambers, 25X75 mm, 0.7  $\text{cm}^2$  culture area) from Thermo Fisher Scientific (Rochester, NY, USA) and Fish Scale scaffolds (Body Organ Corp. Taipei, Taiwan) were used for plating the cells. The chambers of Lab-Tek slides and the FSS were coated with 50  $\mu\text{L}$  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°C. The residual coating was removed before the plating. Approximately 92,500 cells/0.7 $\text{cm}^2$  were plated in two wells of the Lab-Tek slides from one cornea of the same donor whereas, 185,000 cells/1.3  $\text{cm}^2$  were plated from other cornea of the same donor on one FSS. As the comparison was between the base for the HCECs, that are the Lab-Tek, and FSS, we decided to use the same donor without pooling them to normalize the plating density to the area. For FSS, the cell suspension (50 $\mu\text{L}$ ) was added and incubated at 37°C for 20 minutes allowing the cells to settle on the scaffold. Once the cells were settled and showed attachment, additional 150 $\mu\text{L}$  of the media was added and further incubated for 1 hour. After the cells show complete attachment and were not floating in the media, another 800 $\mu\text{L}$  of the media was added and incubated to monitor cell growth. For Lab-Tek slides, 50 $\mu\text{L}$  of cell suspension was added followed by addition of 150 $\mu\text{L}$  of the media and were monitored every alternate day till confluence with media refreshed every alternate day.

#### *Morphological analysis and confluence of the cultured HCECs [n=30, Fifteen pairs]*

The cells were visualized every other day until confluent using an inverted microscope (Axiovert, Zeiss, Germany) at 50X and 100X magnifications. The percentage confluency was manually counted using an in-built reticule inside the eye-piece of the microscope and monitored every alternate day.

*Glucose uptake of the cultured HCECs for functionality checks and metabolic analysis*

*[n=30, Fifteen pairs]*

Glucose uptake was determined from the preserved media of all the samples every alternate day that were stored at -20°C. The amount of glucose used and lactic acid produced by the HCECs during the culture was recorded. This assay helps to evaluate the metabolic activity of the HCECs when grown *in vitro*. Quantitative analysis was performed using D-Glucose HK kit (Megazyme International Ireland Ltd, Bray Business Park, Bray, Co. Wicklow, Ireland).

*Hoechst, Calcein AM and Ethidiumhomodimer (HEC) staining to determine live, dead and dying cells [n=6, Three pairs]*

Live cells are distinguished by the presence of intracellular esterase activity, determined by the enzymatic conversion of the nonfluorescent cell-permeant Calcein AM to the intensely fluorescent Calcein. The polyanionic dye Calcein, is retained within live cells, producing an intense green fluorescence in live cells (ex/em 495 nm/515 nm). Ethidium Homodimer-1 (EthD-1) enters cells with damaged membranes and enhances fluorescence upon binding nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em 495 nm/635 nm). EthD-1 is excluded by the intact plasma membrane of live cells. Hoechst 33342 is a nuclear dye, which stains the nucleus of the cell. The cells were washed with PBS prior to the assay to remove or dilute serum esterase activity generally present in serum-supplemented growth media such as M4. Control sample was

prepared using a human donor cornea. Descemet membrane was peeled out using stripping method and the excised lenticule was washed before the assay. Some cells were damaged purposely by gently scrapping and touching the endothelial cells using sharp forceps. 5  $\mu$ L of Hoescht 33342 (H) (Thermo Fisher Scientific, Rochester, NY, USA), 4  $\mu$ L of Ethidium Homodimer EthD-1 (E), 2  $\mu$ L Calcein AM (C) (Live/Dead viability/cytotoxicity kit, Thermo Fisher Scientific, Rochester, NY, USA) was mixed in 1 mL of PBS and mixed well. 100  $\mu$ L of the final solution was directly added on the cultured cells and incubated at room temperature in dark for 45 minutes. For the control tissue, the stripped lenticule was placed on the slide, cut at four peripheral sides for a flat mount and covered with coverslips without the mounting medium. HEC staining was viewed at 488, 495, 515 and 635 nm at 50X, 100X and 200X magnification.

*Immunostaining for tight junctions using Zonula Occludens-1 (ZO-1) [n=6, Three pairs], proliferation marker Ki-67 [n=6, Three pairs] and focal adhesion marker Vinculin [n=6, Three pairs]*

The cells were washed with PBS and fixed in 4% paraformaldehyde (PFA) at room temperature (RT) for 30 minutes. The cells were permeabilized with 0.5% Triton X-100 in PBS for 30 minutes. After blocking with 2% goat serum for 2 hours at RT, the tissues were incubated overnight at 4°C with primary antibodies [anti- ZO-1, 1:200 (ZO1-1A12, Thermo Fisher Scientific, Rochester, NY, USA); anti-Ki-67, 1:200 (MIB-1, Milan, Italy); anti-vinculin, anti-collagen VI, anti-laminin, 1:200 (Abcam, Cambridge, Massachusetts, USA)]. The samples were incubated with goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody in 20% goat serum for 2 hours at RT. After each step, the cells were washed 3 times with 1X PBS. After removing the media chambers of the Lab-Tek slide, the cells were covered with mounting medium and cover slips. Cells were examined with an LSM 510-metalaser scanning microscope (Zeiss, Milan, Italy). Cells

were examined under ultraviolet light or by excitation at wavelengths of either 488 nm or 594 nm where subsequent detection of the fluorescence was obtained. For Vinculin, focal adhesion points (dots) from ten cells were counted and the average number of focal adhesions was recorded for the analysis.

#### *Histological analysis to determine the presence of endothelial cells on FSS [n=3]*

After day 11, the FSS were washed with 1X PBS for 5 minutes and were fixed in 4% PFA overnight followed by washing it with sucrose solution at 7.5%, 15% and 30% for 15 minutes each. Final washing was carried out with PBS and the tissues were embedded in optimal cutting temperature compound (OCT) for microtome cutting. Periodic acid–Schiff (PAS) staining was performed on all the samples and sections were viewed at 200X and 400X magnifications in order to check presence of HCECs on the FSS.

#### *Measurement and statistical analysis*

ImageJ software was used to measure the number of endothelial cells and check the hexagonality and polymorphism of the cells using ZO-1. It was also used to check the percentage of Ki-67 positive cells in the given area and to check the number of focal adhesions (Vinculin positive cells) and the area of the cell. Non-parametric Wilcoxon test for paired data was employed to check the statistical significance between Lab-Tek and FSS, where  $p < 0.05$  was deemed significant.

## **Results**

#### *Donor characteristics*

Average donor age was 60.75( $\pm$ 14.55) years and Male:Female ratio was 10:5. The post mortem time of the donors recorded was 16.54( $\pm$ 5.89) hours. The tissues were preserved in tissue culture medium for 32.33( $\pm$ 6.37) days before isolation of the cells. The

endothelial cell density before isolation was found to be  $1966.67(\pm 226.97)$  cells/mm<sup>2</sup> without any trypan blue positive cells (TBPCs).

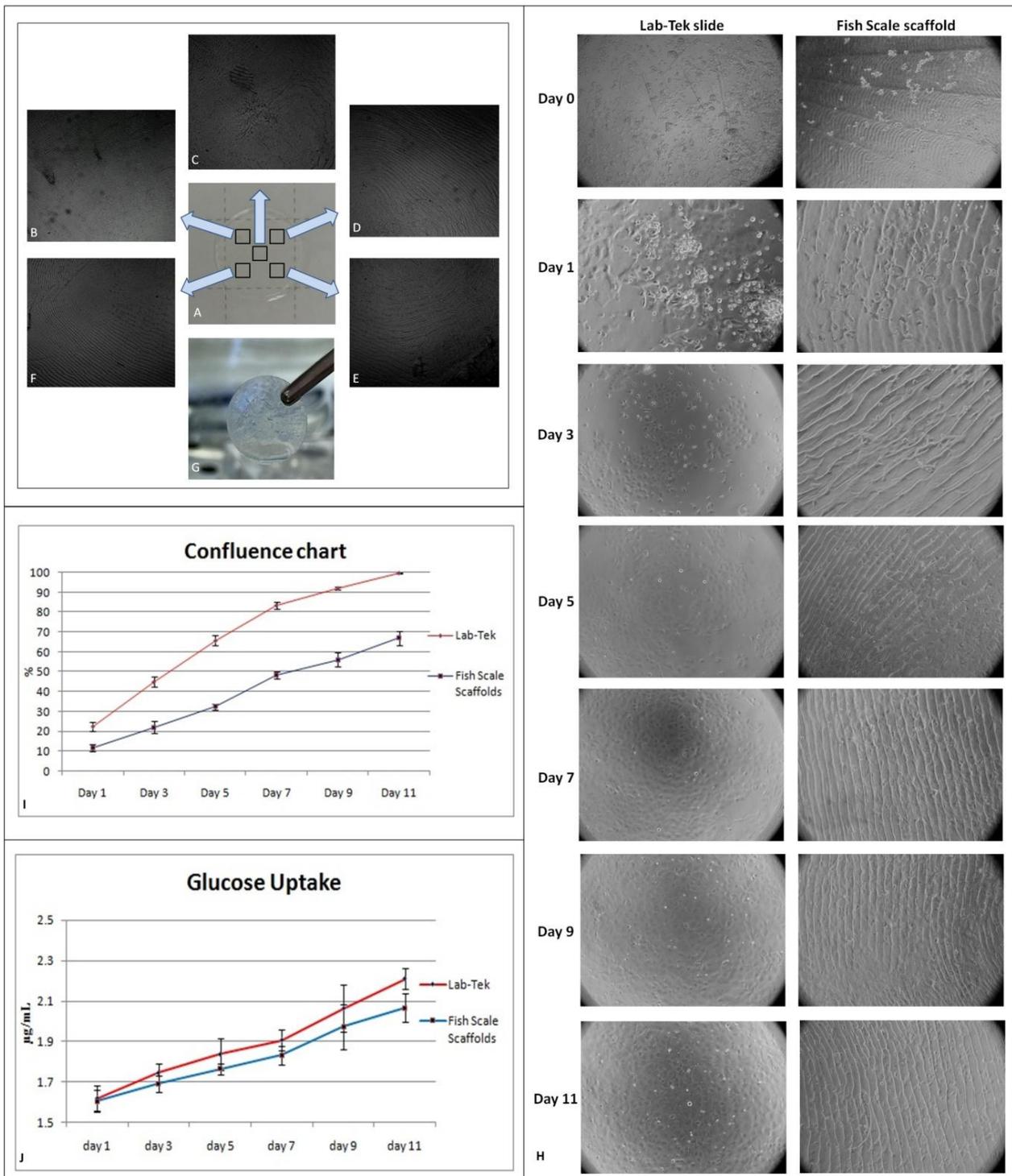
#### *Characteristics of the fish scale scaffolds (FSS)*

Each FSS was 13 mm in diameter with an average thickness of 100-120  $\mu$ m. Tensile stress was found to be  $12.68\pm 9.53$ MPa, Young's modulus was  $56.4\pm 21.91$ MPa with Elongation of  $24.72\pm 5.65$  %. There was no porosity in the scales because of its structure. Water holding capacity of the FSS was 84.12% with initial transparency of 92.67% (380-780 nm).

#### *Morphology, confluency and glucose uptake of the HCECs in Lab-Tek and FSS [n=30, fifteen pairs]*

FSS is highly transparent (figure 1a) and when observed under the confocal microscope showed narrow ridges (Figure 1b) and valleys (Figure 1b) in the upper left quadrant. Central FSS was not smooth but was relatively a flat surface compared to the other areas (figure 1c). The upper right (figure 1d) and lower left showed broad ridges and valleys (Figure 1f). However, lower right quadrant showed arc strokes (Figure 1e). FSS was highly transparent when observed before and after the culture of HCECs (figure 1g). It was noticed that when the HCECs were plated on the flat or regular surfaces, the adherence and proliferation capacity increased. The cells showed more adherence capability of the HCECs on broad ridges and in the central FSS (figure 1h). Growth rate of the cells in Lab-Tek was higher compared to that on FSS. Confluence was observed in Lab-Tek slides whereas around 65% confluency was observed in FSS at day 11 (statistically insignificant,  $p=0.0883$ ) (figure 1i). Average glucose uptake recorded from the total amount already present in the media (2.8  $\mu$ g/mL) in Lab-Tek was ( $\mu$ g/mL)  $1.61(\pm 0.06)$ ,  $1.74(\pm 0.06)$ ,  $1.83(\pm 0.07)$ ,  $1.90(\pm 0.05)$ ,  $2.06(\pm 0.11)$ ,  $2.21(\pm 0.05)$  at day 1,3,5,7,9,11 respectively

compared to  $1.60(\pm 0.05)$ ,  $1.69(\pm 0.04)$ ,  $1.76(\pm 0.02)$ ,  $1.83(\pm 0.04)$ ,  $1.97(\pm 0.11)$ ,  $2.06(\pm 0.07)$  in FSS without statistical significance ( $p=0.5181$ ) (figure 1j) at day 11.

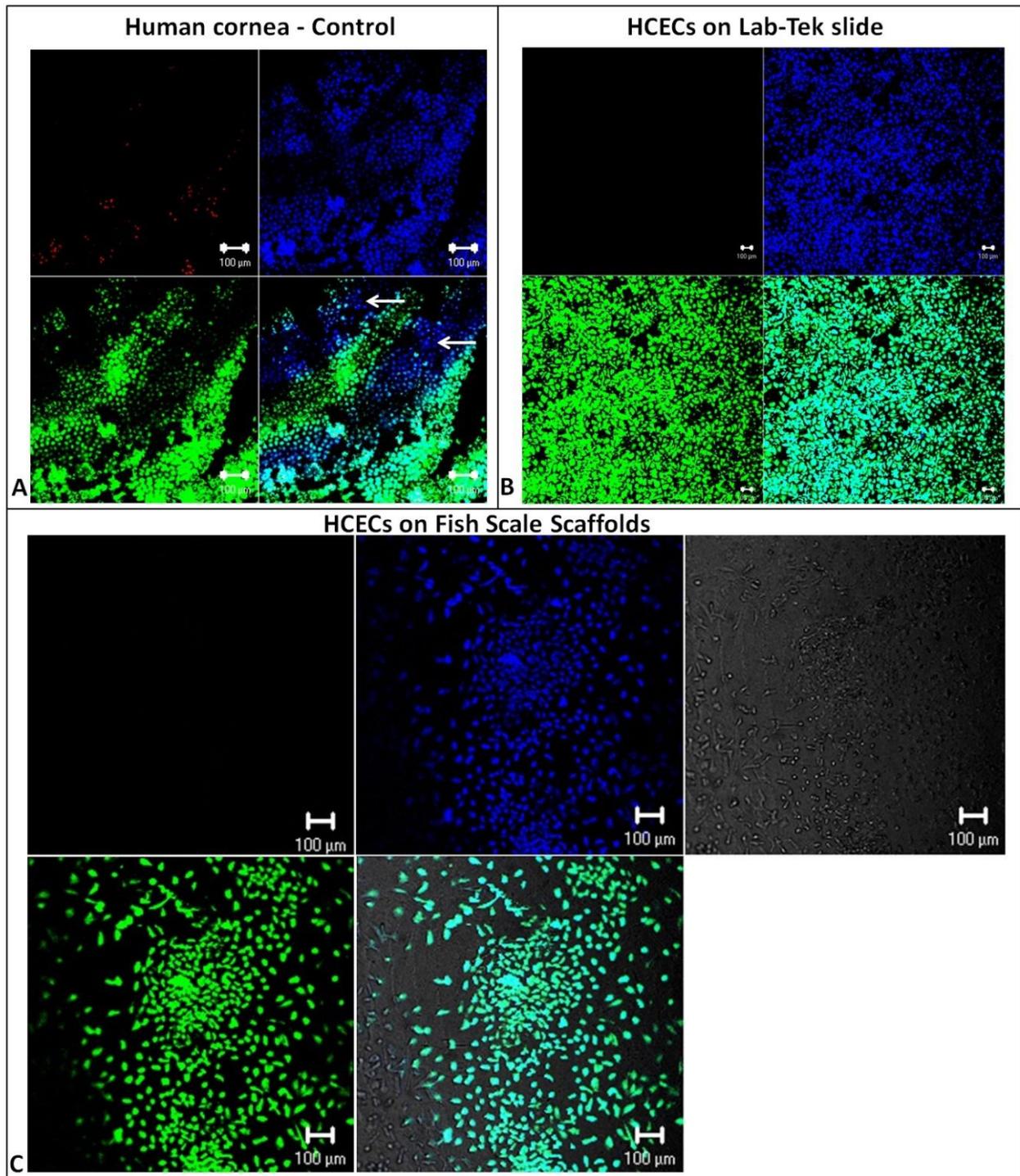


**Figure 1:** Fish scale scaffolds (FSS) showed a) high transparency and when observed in bright field of the confocal microscope, it showed b) first quadrant with narrow ridges and valleys, c) central FSS which was rough but was relatively a flat surface compared to the

other areas, d and f) second and third quadrant showing broad ridges and valleys, e) Fourth quadrant showing arc strokes, g) highly transparent FSS before and after the culture of HCECs. H) Morphological analysis between Lab-Tek and FSS grown HCECs at different magnifications on different days of culture when observed using inverted light microscope. Most of the cells were adhered and showed outgrowth when cultured on broad ridges (quadrant 2 and 3) and in the central FSS. I) Growth rate of the cells in Lab-Tek was higher compared to that on FSS. 100% Confluence was observed in Lab-Tek slides whereas around 65% confluency was observed in FSS at day 11. I) Glucose uptake was higher in Lab-Tek grown cells compared to Fish Scale Scaffolds and showed active metabolism of cells without any significant difference between the two conditions.

*HEC staining showed confluence and viability of HCECs on the Lab-Tek and cell viability on FSS*

Triple endothelial labelling with Hoechst 33342 (H), EthidiumHomodimer (E), and Calcein-AM (C) showed expression of 'E' in red representing the dead cells, blue represents the nuclei 'H' and green marked the living cells 'C'. HEC was observed in the control group to show the presence of all the dyes for comparison (figure 2a). White arrow marks the dying cells (blue without green) without metabolic activity (attached dying cells to the extracellular matrix). The human cornea used as a control to demonstrate the HEC staining showed dead (red), nucleus (blue), live (green), dying (blue without green) and merge. It also showed some apoptotic cells marked in white (figure 2a). The HCECs in Lab-Tek showed confluence with high viability as shown in figure 2b. In compliance with the confluency data, HEC staining also showed that the cells were approximately 60% confluent (figure 2c) on the FSS with almost 100% of viability in both the conditions. The cell size can also be observed in FSS compared to the one in Lab-Tek.



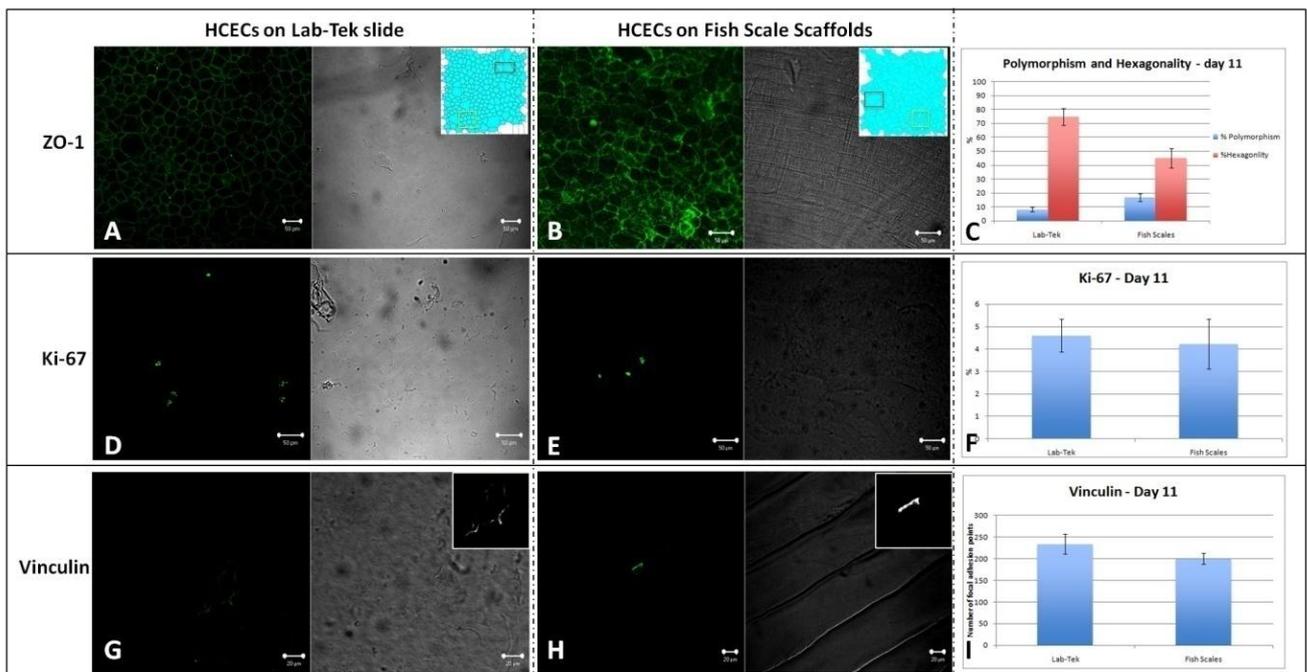
**Figure 2:** HEC staining to determine live/dead/apoptotic cells. A) Control cornea to show the presence of dead (red), live (green), dying cells (blue without green marked with white arrow) and nucleus (blue) cells. B) HCECs from old donor corneas cultured on Lab-Tek showed high viability and confluency without any dead cells. C) Relatively lesser amount

of cells was observed when HCECs were cultured on FSS. There were no dead cells observed in FSS too but again, most of the cells were cultured in quadrant 2 and 3.

*Immunostaining showed high hexagonality and low polymorphism using Zonula Occludens-1 (ZO-1) in Lab-Tek slides and showed insignificant amount of Ki-67 but significant amount of focal adhesions and Vinculin expression between both the conditions*

ZO-1 tight junction protein was expressed in HCECs from Lab-Tek (Figure 3a) and FSS (Figure 3b). Using ImageJ (inserts in Figure 3a and 3b), the hexagonality and polymorphism was counted using overlay masks. HCECs on Lab-Tek showed 8.13( $\pm$ 1.47)% polymorphism with 74.73( $\pm$ 6.04)% hexagonality whereas those cultured on FSS showed 16.68( $\pm$ 2.82)% polymorphism and 45.01( $\pm$ 6.81)% hexagonality (figure 3c). HCECs cultured on Lab-Tek slides showed significantly less polymorphism ( $p=0.0041$ ) and high percentage of hexagonal cells ( $p=0.0006$ ). This was presumed earlier, as Lab-Tek has flat surface, it allows the monolayered cells to grow and spread uniformly compared to the cells cultured on an irregular base such as FSS which has ridges and valleys. It was also noted that the cells cultured in the central area, which is more uniform and flat, showed similar morphology as it has rough surface compared to Lab-Tek slide. HCECs cultured on Lab-Tek showed 4.61( $\pm$ 0.72)% Ki-67 positive cells (figure 3d) compared to 4.22( $\pm$ 1.11)% on FSS (figure 3e) without significant difference ( $p=0.5922$ ) (figure 3f). Proliferative cells were almost equal in both the conditions, however, the confluency and morphology observed was better in Lab-Tek compared to FSS. Vinculin is a membrane-cytoskeletal protein in focal adhesion plaques that is involved in linkage of integrin adhesion molecules to the actin cytoskeleton. Using ImageJ, the binary masks of the focal adhesions (figure inserts of 3g and 3h) were captured and analysed. HCECs cultured on Lab-Tek with FNC coating mix showed 233.5( $\pm$ 22.63) number of focal adhesions (figure 3g) compared to 199.75( $\pm$ 12.18) number of focal adhesions from FSS

(figure 3h) which was statistically lower than Lab-Tek ( $p=0.0507$ ) (figure 3i) at day 11. It was observed that HCECs took longer time for cell adherence on FSS compared to Lab-Tek due to its surface characteristics, which is not smooth and regular. Vinculin may also be induced by the presence of ROCK inhibitor in the media, however, as both the conditions utilized ROCK and were coated with FNC mix, it is assumed that matrix characteristics have an effect on the adherence capability.

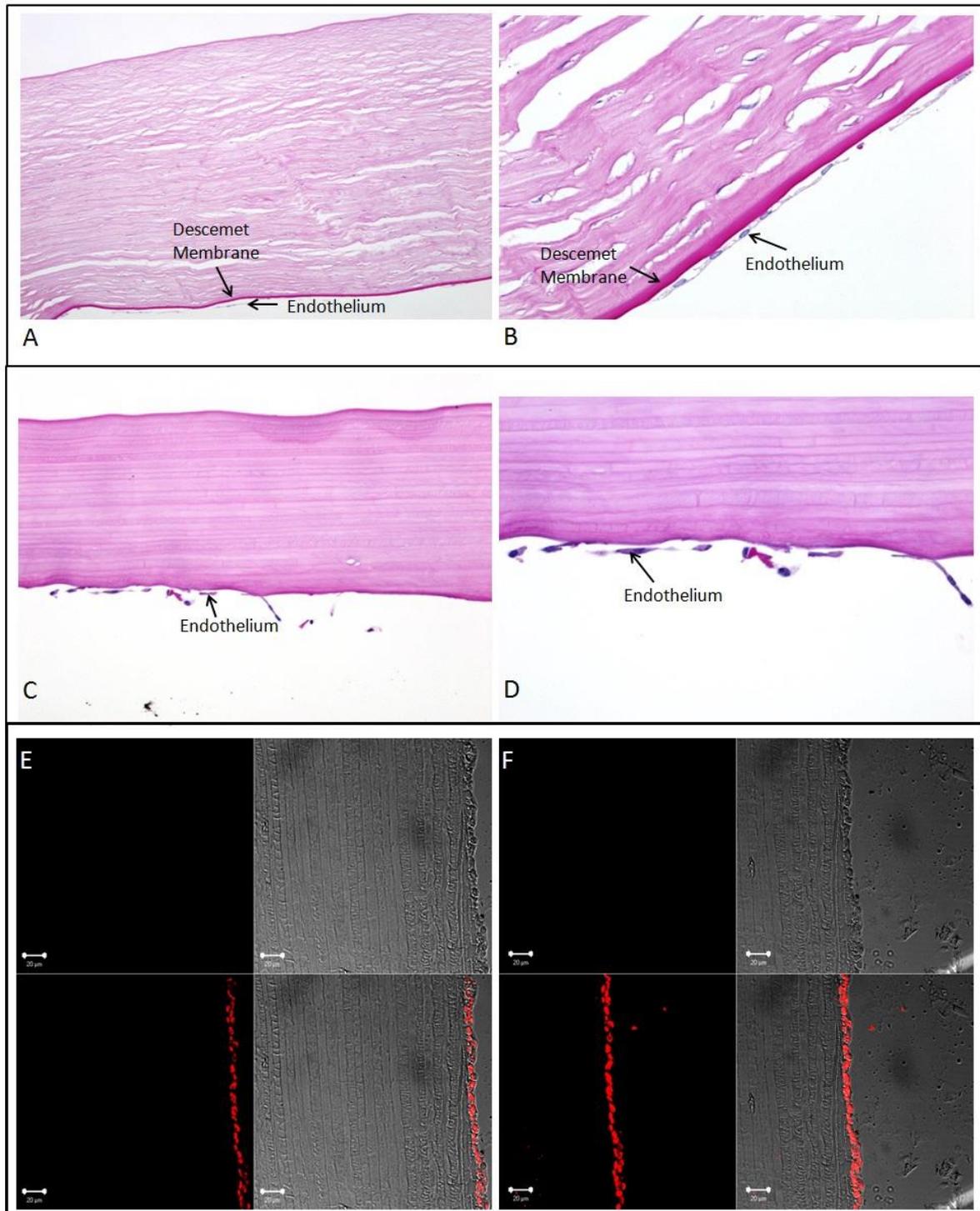


**Figure 3:** Expression of different proteins in the cultured HCECs on Lab-Tek and FSS. A) ZO-1 staining for intracellular tight junctions to determine polymorphism and hexagonality of the cells in Lab-Tek and B) in FSS. Figure inserts show the overlay masks of the expression for calculation of hexagonality and polymorphism (polymegathism and pleomorphism). C) High percentage of polymorphism and low percentage of hexagonality was observed when HCECs were cultured on FSS compared to Lab-tek slides. D) Proliferative cells did not differ when the HCECs were cultured in D) Lab-Tek and E) FSS, and F) did not show statistical significance. G) Vinculin as a focal adhesion marker showed higher number of focal adhesions when HCECs were cultured on Lab-Tek compared to that on H) FSS which was I) statistically significant. Figure inserts show the

amount of vinculin expressed (number of focal adhesions) which was counted using binary images.

*Histological analysis showed presence of HCECs as a monolayer on FSS*

As controls, Periodic acid-Schiff (PAS) staining showed all the layers of a cornea including Descemet Membrane and Endothelium for comparison at different magnifications (figure 4a and 4b). HCECs cultured on FSS grow as a monolayer and does not stratify. Moreover, it was observed that the cells are not adhered completely to the FSS due to the ridges and valleys present in the scaffold (figure 4c). At day 11, the cells did not show the secretion or presence of its own extracellular matrix (Descemet's membrane) but only uniform distribution of pure endothelium on the FSS (figure 4d). It may take extra time in the development of the Descemet's membrane. Collagen VI (figure 4e) and Laminin (figure 4f) did not express at day 11 which confirms that the cells did not produce the DM. The cells can hence be cultured on FSS but the adherence capability must be increased in order culture HCECs precisely and the FSS may have to be customized more to be flat and regular especially for HCECs.



**Figure 4:** Histological analysis using Periodic acid-Schiff (PAS) staining. A) A normal cornea showing the presence of Descemet Membrane and endothelial cells using PAS staining at 100X and B) 400X. C) FSS showing monolayer of endothelial cells at 200X magnification and D) at 400X magnification. Histology did not show any presence or development of Descmet's membrane on FSS by the HCECs but showed a monolayer of

cells attached in most of the areas. E) Collagen VI and F) Laminin as the extracellular markers did not express on the cells at day 11. Nucleus can be seen in red marked with Drac 5.

## **Discussion**

In the recent years, development in bioengineered corneal substitutes has been designed to replace the full or partial thickness cornea, which is damaged or diseased [10]. They range from fully synthetic prostheses like keratoprosthesis or tissue engineered cell based constructs [11] and hydrogels [12] and have already been introduced that permit the integration of the implant and regeneration of the host tissues. Many therapeutic strategies have been adopted to reduce the global shortage of donor availability.

Most of the previously reported studies on culturing HCECs have been performed on younger donor corneas [3]. It has been observed that younger donors have a high proliferative capability compared to older donor corneas. However, it is difficult to obtain young donors for culturing HCECs *in vitro* due to their priority use for transplantation. Stringent exclusion criteria, more often an inadequate endothelial cell density, make that most of the older donor corneas are available for research, however their proliferative capability is also noticed to be less. It is a challenge to culture older donor corneas, however if the HCECs from the old donors can be cultured then the availability of the source will be much higher compared to the younger donor corneas. This study therefore highlights the culture of HCECs from older donor corneas. The other challenge is the transplantation technique of these cells. Injection of the cells has already been proposed earlier by Kinoshita et al, however, as this technique requires a face down position, it becomes relatively challenging for the patients as well [13,14]. Another option that could have a potential clinical relevance would be development of the scaffold for culturing and

transplanting the HCECs, thereby creating composite grafts similar to current DMEK/DSAEK transplants.

Here we describe the FSS as a potential scaffold for the culture of HCECs *in vitro*. Fish is a good source of collagen as the scales are composed of connective tissue protein and collagen covered with calcium salts [6]. Although it has already been proposed that the biomechanical stiffness is low and FSS has a rapid biodegradation rate, we found that the subjective transparency was higher in the FSS after the culture phase with low biodegradation rate and biomechanical stiffness that would be more suitable for the transplantation techniques like EK. The flexibility of the FSS was enough to fold without breaking so it would be possible to insert it inside the anterior chamber similar to the currently used techniques like Descemet Stripping Automated Endothelial Keratoplasty (DSAEK) that was noted as personal observations. As a collagen multilayer structure can maintain water up to 84%, the use of FSS becomes more realistic for EK procedures. In animal models, FSS have been used so far in rat, mini pig and in rabbit models [15,16]. No immune response was revealed over the 6-month period of the rabbit study, while mild to moderate inflammation was noticed in some rats. However this model is known to be more sensitive for neovascularisation. Transparency of FSS was clear and it did not dissolve or degenerate in both of the studies [6-8].

In an earlier reported study, the morphology of the corneal cells cultured on FSS showed cells spread extensively over the surface of the scaffold [6]. However, in our study we only observed huge number of cells on the broad ridges and more on the central region of the FSS. Although the cell viability did not differ in either conditions i.e. Lab-Tek and FSS, it was determined that the FSS is cytocompatible as HCECs did not show any toxic effect and showed viability when checked using HEC staining. The cells were metabolically active as they utilized glucose present in the media. Both the conditions showed similar

amount of glucose uptake. As already noticed that FSS facilitates cell attachment and cell migration for other corneal cells, it may not be true for HCECs as these cells might require different characteristic for adhesion and migration. Therefore we coated these FSS with FNC coating mix, although they consist mainly out of collagen. Even more, during investigative studies we saw no to little cell attachment when scaffolds were uncoated. Although the old donor corneas do not have a high proliferative potential, they still showed a high growth rate when cultured on Lab-Tek compared to FSS. HCECs require flat surfaces for the development of a monolayer with high percentage of hexagonal cells otherwise the polymorphism increases as seen by ZO-1 staining. Although HCECs may grow on the FSS, its morphology may be compromised as seen against Lab-Tek. Maintenance of proliferative cells in both the conditions was similar too. Actin cytoskeleton plays a critical role in regulating the adhesive property through interaction between the actin cytoskeleton and integrin [17-19]. Previous study has reported that inhibition of ROCK signalling by a selective ROCK inhibitor or by siRNA enhances adhesive property of HCECs and is consistent with the findings of those previous studies. It is also found that vinculin, which is involved in the linkage of the integrin adhesion complex to the actin cytoskeleton [20,21] is upregulated in ROCK-inhibitor treated HCECs. However, further investigation is needed to elucidate whether the ROCK inhibitor promotes the focal adhesions through inhibiting actin polymerization and induces the upregulation of cell adhesion properties on the extracellular matrix (ECM) [4]. Vinculin is a cytoskeletal protein associated with cell-cell and cell-matrix junctions, where it is thought to function as one of several interacting proteins involved in anchoring F-actin to the membrane. Vinculin expression features the number of focal adhesions or integrin presence by the cells. It therefore highlights the adherence capability of the cells. In our study, vinculin expression or number of focal adhesions was significantly higher in Lab-Tek cultured cells compared to FSS. We assume that this was due to the flat surface of the Lab-Tek allowing a huge

area of the cells to attach after incorporating ROCK inhibitor compared to FSS. FSS is irregular and therefore shows less flat area for the cells to attach and induce focal adhesions formation even after the presence of ROCK inhibitor in both the conditions.

In conclusion, although tissue engineering is growing with the developments of cytocompatible and biodegradable materials, culture of HCECs and maintaining them on a scaffold may be challenging especially when old donor corneas are utilized. However, using more old donor corneas, rejected for transplantation due to their endothelial counts would increase the number of lab cultured corneal substitutes and decrease the global shortage of the available corneas. Although FSS has a huge potential in terms of culturing challenging cell types like primary HCECs, its characteristics and nature is suitable as a corneal substitute. Not only the overall costs and wide availability may attract researchers to culture HCECs on FSS. Also, its low immunogenic response, transparency and high flexibility may allow surgeons to transplant such grafts without adapting current ways of delivery or high dosage steroids. The study is a proof of concept for culturing HCECs from old donor corneas on FSS and requires further refinement before entering the pre-clinical phase.

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## **CHAPTER 11**

# **RECOMBINANT HUMAN SERUM ALBUMIN FOR CORNEAL PRESERVATION**

*Derived from article (Revisions submitted – under peer review)*

Mohit Parekh, Hossein Elbadawy, Gianni Salvalaio, Marie-Claude Amoureux, Denis Fortier, Diego Ponzin, Stefano Ferrari and Alessandro Ruzza. Recombinant human serum albumin for corneal preservation. ACTA Ophthalmologica.

## **Aim**

To study the performance of a complete synthetic organ culture preservation system containing recombinant human serum albumin (rHSA) for preservation of human donor corneas.

## **Introduction**

Human corneas once retrieved from the cadaveric donors should be well preserved to maintain the viability of the cells and the transparency of the tissue which are the crucial parameters for corneal transplantation. Currently, there are two main preservation systems that are pursued: cold/hypothermic storage with preservation at 2-6°C mostly used in America and Asia, and organ culture (OC) with preservation at 31-37°C which is followed in parts of Europe [1]. OC allows long-term preservation (approximately one month) as compared to hypothermic preservation which is limited to two weeks. Hypothermic media utilizes a single vial of media for the entire preservation phase whereas OC requires three phases of preservation which includes a) collection of the tissue, b) organ culture to preserve the cornea by means of media containing serum and c) transport media or de-swelling media which contains dextran to regain the original thickness of the cornea which swells during the OC phase. OC therefore has advantages in terms of microbiological checks, quality assurance and gives longer preparation time for surgery. Commercially available OC media contain fetal calf serum (FCS) for preservation of the tissues. Earlier investigations have also shown that chicken feather, ovalbumin, pig-bone amino acids etc. with other nutrients can be used for prolonging the metabolic activities of endothelial cells for corneal preservation *in vitro* [2-6]. Although the role of FCS is not yet clearly known, a higher amount of cell loss was reported when porcine corneas were preserved without serum. It has been reported that FCS helps the cells to resist the stress levels *in vitro* [7-9] although other studies suggested that serum-free

medium can cause higher endothelial cell loss (ECL) due to necrosis [10]. A few studies reported the effects of different de-swelling compounds such as poloxamer or dextran based medium [2,11]. Either of them could be used for de-swelling due to their synthetic properties.

Endothelial cell viability is the most important factor for corneal preservation and transplantation. It is also considered as a major influential factor for acceptance or rejection of a cornea for grafting [2]. Media used for OC usually contain serum and since animal derived products have a possibility to introduce animal viruses or prions, they could be hazardous if assimilated in the human body. Animal viruses, especially retroviruses, could integrate into the human genome and activate human oncogenes or onco-suppressor genes, while prions could lead to the human forms of Bovine Spongiform Encephalopathy (BSE). For this reason, as well as to decrease the variability between batches of animal derived serum, synthetic media have started to be developed.

Therefore, to reduce the uptake of animal or animal derived components, a Pharmaceutical grade recombinant human serum albumin (rHSA) was tested as a potential replacement for serum. rHSA is a recombinant protein which is biochemically synthesized and used in the development of medical products. Thus, we investigated the possibility to use rHSA to replace serum and make a completely synthetic medium in order to avoid any animal derived components.

## **Materials and Methods**

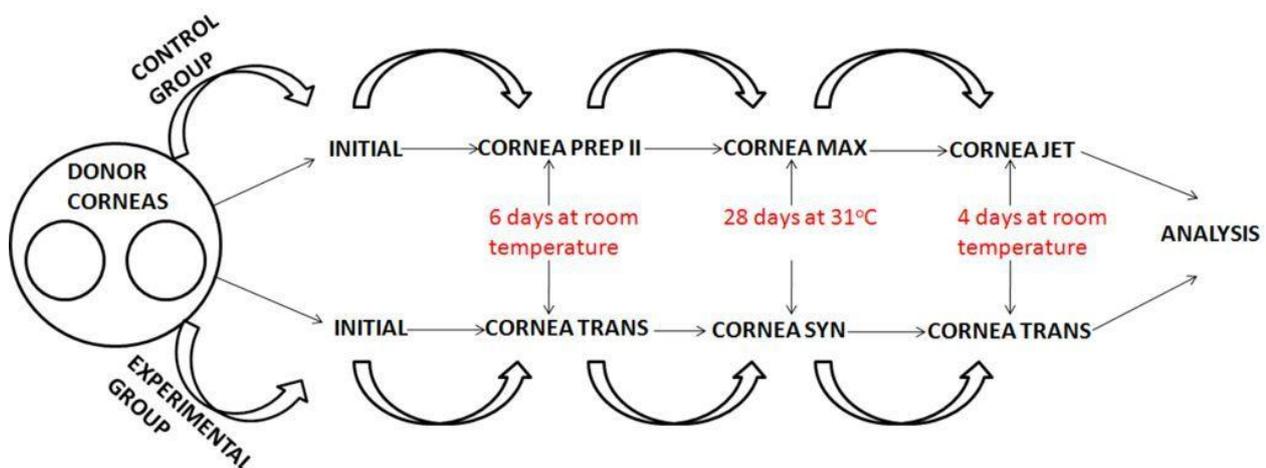
### *Ethical statement and donor characteristics*

Twenty-four pairs of human cadaveric donor corneal tissues unsuitable for transplantation and with the written consent from the donor's family were used to obtain the results

described. The average age of the corneal donors was 71.08 ( $\pm 3.39$ ) years, post-mortem time was 12.73 ( $\pm 6.82$ ) hours and male:female ratio was 14:10.

### Study design

Each cornea from the same donor was preserved in the synthetic and serum based media series respectively. The corneas were transferred from each stage to the next and the evaluation process was carried as follows: a) before preservation [Initial – Stage I], b) after preservation in Cornea Trans<sup>®</sup> and Cornea Prep II<sup>®</sup> respectively for 6 days at room temperature (stage II), c) after preservation in Cornea Syn<sup>®</sup> and Cornea Max<sup>®</sup> for 28 days at 31-37°C (stage III) and d) after preservation in Cornea Trans<sup>®</sup> and Cornea Jet<sup>®</sup> for 4 days at room temperature (RT) (stage IV). A schematic representation of the study design is shown in figure 1. Cornea Syn<sup>®</sup> is the synthetic medium with rHSA and is based on similar media constituents with a recombinant serum albumin as a replacement for serum. Cornea Trans<sup>®</sup> and Cornea Jet<sup>®</sup> contain dextran. Cornea Prep II<sup>®</sup> does not contain dextran. The other constituents of synthetic medium are not listed, as the formulation is confidential. Cornea Prep II<sup>®</sup>, Cornea Max<sup>®</sup> and Cornea Jet<sup>®</sup> are commercially available serum based media for corneal preservation marketed by Eurobio, France (control group for this study).



**Figure 1:** Schematic representation of the study design. The figure shows different stages of corneal preservation in serum based and synthetic media before preservation [Initial – Stage I], b) after preservation in Cornea Trans<sup>®</sup> [with dextran] and Cornea Prep II<sup>®</sup> [without dextran] respectively for 6 days at room temperature (stage II), c) after preservation in Cornea Syn<sup>®</sup> [with rHSA] and Cornea Max<sup>®</sup> [with serum] for 28 days at 31-37°C (stage III) and d) after preservation in Cornea Trans<sup>®</sup> [with dextran] and Cornea Jet<sup>®</sup> [with dextran] for 4 days at room temperature (RT) (stage IV).

### *Evaluation parameters*

The parameters mentioned below were checked at stage I, II, III and IV to determine the quality of the tissue periodically. All the corneas were pre-checked [Stage I] using parameters like (a) thickness, (b) transparency (c) endothelial cell density (ECD), mortality and viable endothelial cell density (VECD) and (d) morphology as below:

#### *a. Central Corneal Thickness (CCT)*

The tissues were removed from the media and mounted on artificial anterior chamber (AAC) with an infusion of sterile phosphate buffered saline (PBS) irrigation and locked using AAC stopper. The CCT was measured using Optical Coherence Tomography (OCT) (OCT SS-1000, Tomey Corporation, Nagoya, Japan] at different stages as listed above.

#### *b. Transparency*

The corneas were dismounted from the AAC. The blank reading of the petri plate was recorded. The corneas were placed in the petri plate in sterility and analyzed objectively using a transparency device [12] at different stages. The average of 3 transparency readings was considered suitable to reduce the error.

c. Endothelial cell density (ECD) and mortality using trypan blue

The corneas were removed from the petri plates and placed on a silicon cap with the endothelium side facing up. The endothelium was stained using Trypan blue (0.25%) for approximately 20 seconds, washed with PBS and placed in a sterile petri plate containing sucrose solution (1.8%) with the epithelium facing the lid. A reticule (grid) [10X10 mm] inserted in the eyepiece of the microscope was used for manual cell counting and mortality at 100X magnification [13]. Central (optic zone) and peripheral readings were recorded by two masked observers. The viable endothelial cell density (VECD) was calculated as described by Parekh et al [6].

$$VECD = VisECD - \left[ \frac{VisECD \times \left[ \frac{(\%CM \times CA) + (\%PM \times PA)}{TA} \right]}{100} \right]$$

Where, CM = Central mortality; CA = Central area [ $\pi r^2$ ] (within optic zone = approximately 4 mm radius); PM = Peripheral mortality; PA = Peripheral area [ $\pi r^2$ ] (outside optic zone = approximately 1.5 mm radius); TA = Total area [ $\pi r^2$ ] (approximately 5.5 mm radius); VisECD = Visual endothelial cell density (counted manually under 100X magnification).

d. Morphology

The morphology was also checked using the corneas in the same petri-plates and the parameters included are as described: (i) *intercellular borders (IB)* were checked by assessing disappearance, swelling or irregularity; (ii) *polymorphism* was checked using polymegathism (cell size) and pleomorphism (cell shape); (iii) the corneas were checked for *dystrophy* before preservation and (iv) *degeneration* was recorded by the disappearance of the intercellular borders and/or complete cells. After subjective evaluation, morphology was translated into objective numbers for statistical analysis. The

objective values were marked as Regular-4, Regular-Mild-3.5, Mild-3, Mild-Moderate-2.5, Moderate-2, Moderate-Severe-1.5, Severe-1, Severe-Poor-0.5, Poor-0 [6].

#### *Glucose uptake*

Glucose uptake was determined in the preservation media in which the tissues were preserved for 4 days, as previously described by Ruzza et al [14]. The metabolic activity of the endothelial cells was checked using this technique. Quantitative analysis was performed using D-Glucose HK kit (Megazyme International Ireland Ltd, Bray Business Park, Bray, Co. Wicklow, Ireland) post preservation from each stage.

#### *Statistical analysis*

The data were analyzed using SAS 9.2 software for Windows (SAS Institute Inc., Cary, NC, USA). Non-parametric Wilcoxin test for paired data was performed at each individual time point. The difference between the groups was considered statistically significant when  $p < 0.05$ .

#### *Histological analysis to determine the presence of each layer on the preserved tissues*

All the tissues were fixed in 4% paraformaldehyde (PFA) overnight and washed with 7.5%, 15% and 30% sucrose solution progressively for 15 minutes each. The tissues were further washed with PBS and embedded in optimal cutting temperature compound (OCT) compound for microtome cutting. Periodic acid–Schiff (PAS) staining was performed on all the sections which were viewed at 100X magnification.

#### *Immunostaining to determine the expression of different proteins in the preserved tissues*

The tissues were fixed and cut as described above to obtain sections. The sections were permeabilized with 0.5% Triton X-500 in PBS for 30 minutes and blocked using blocking

mix (PBS, goat serum, bovine serum and triton-x). Sections were incubated overnight at 4°C with primary antibodies, p63 [1:100], αSMA (1:50), ZO-1 (1:100) and CK12 (1:100). After washing thoroughly with PBS, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody for around two hours at room temperature in the dark. The sections were washed with PBS and air dried. Mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei. Expression was examined with an LSM 510-meta laser scanning microscope (Zeiss, Milan, Italy). Examination was performed under an ultraviolet light or by excitation at wavelengths of either 488 nm or 594 nm.

#### *Cell apoptosis using terminal deoxynucleotidyltransferase deoxyuridine triphosphate nick-end labeling assay*

The tissues were fixed and cut as described above to obtain sections. Cell apoptosis was performed as described in the manufacturer's protocol for TACS 2 terminal deoxynucleotidyltransferase (TdT) diaminobenzidine (DAB) *in situ* apoptosis detection kit (Cat# 4810-30-K; Trevigen, Maryland, USA). One positive sample was induced with apoptosis using TACS nuclease and other samples were viewed at 100X magnifications of an inverted microscope. The images were analyzed using ZEN (Zeiss, Milan, Italy) software [6].

#### *Microbiological checks*

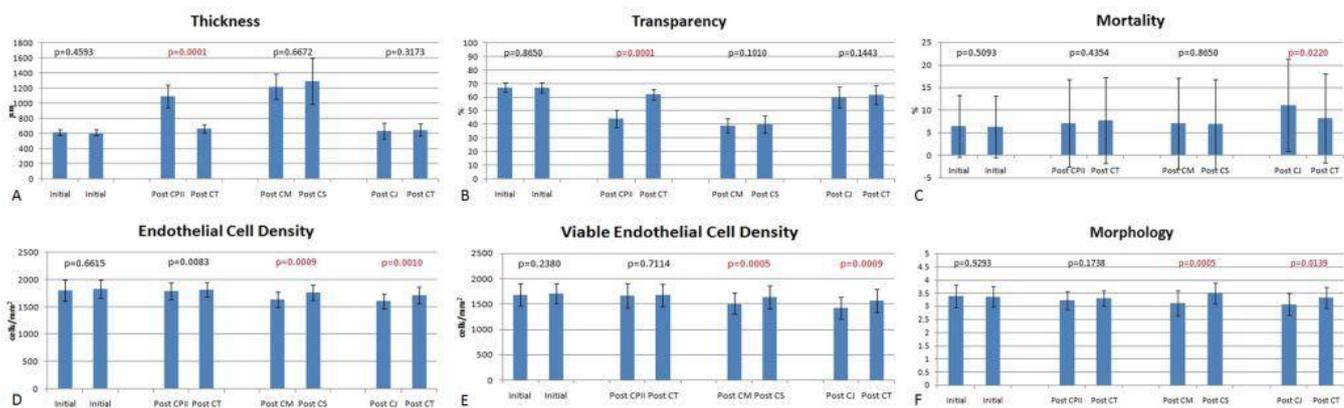
All the samples were checked for microbiology using Bactec 9240 instrument (Becton-Dickinson, USA) for aerobic and anaerobic microorganisms (bacteria and yeasts) using inoculums of the preservation medium.

## Results

None of the corneas assessed showed any microbial contamination. Power calculation for statistical analysis revealed that 24 pairs of cornea were sufficient to gain statistical significance. The parameters that were evaluated led to the results that are described below:

### *No significant difference in thickness*

A statistically significant increase of thickness was observed when the tissues were preserved in Cornea Prep II<sup>®</sup> as compared to Cornea Trans<sup>®</sup> ( $p < 0.001$ ). This is due to the presence of dextran in Cornea Trans<sup>®</sup> and not in Cornea Prep II<sup>®</sup>. However, no differences were seen in any other consecutive stage of preservation, as seen in figure 2a.



**Figure 2:** Parametrical analysis of the tissues preserved in synthetic and serum based media. A) The thickness of the tissues preserved in serum based media showed a higher thickness as compared to the ones preserved in synthetic based with dextran in phase II; b) Transparency was higher in synthetic media which is inversely proportional to the thickness measurement; c) mortality as observed after trypan blue staining, d) visual endothelial cell density which showed better endothelial preservation with minimal mortality in synthetic medium; e) viable endothelial cell density showed better preservation of endothelial cells in synthetic medium f) morphology was better in synthetic medium as compared to serum based.

Key: CP II = Cornea Prep II; CT = Cornea Trans; CM = Cornea Max; CS = Cornea Syn and CJ = Cornea Jet

*No significant difference in transparency*

A statistically significant decrease of transparency was observed when the tissues were preserved in Cornea Prep II<sup>®</sup> as compared to Cornea Trans<sup>®</sup> after stage II ( $p < 0.001$ ) which also correlates with a higher thickness at this stage. No significant difference was seen at any other stage of preservation as seen in figure 2b.

*No significant difference in mortality*

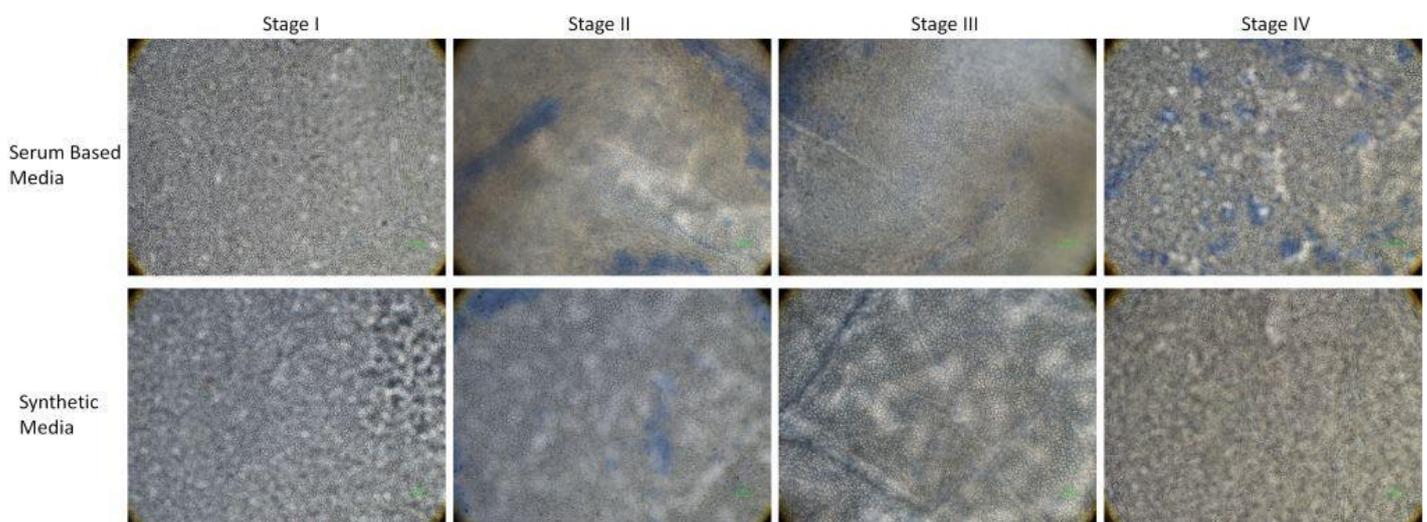
After the final preservation, tissues preserved in Cornea Jet<sup>®</sup> showed statistically higher mortality as compared to Cornea Trans<sup>®</sup> as seen in figure 2C. Increased Trypan blue positivity was monitored when the tissues were transferred from one stage to another. This was further confirmed by the analysis of the apoptosis as described later.

*Endothelial cell density (ECD) and Viable endothelial cell density (VECD) were preserved better in Cornea Syn<sup>®</sup>*

ECD values showed higher number of endothelial cells after preservation in Cornea Syn<sup>®</sup> ( $p < 0.05$ ) and Cornea Trans<sup>®</sup> ( $p < 0.05$ ) as compared to Cornea Max<sup>®</sup> and Cornea Jet<sup>®</sup> respectively. No significant cell loss was observed when the tissues were preserved in Cornea Prep II<sup>®</sup> and Cornea Trans<sup>®</sup> (Figure 1d). VECD showed statistically significant difference at stage III ( $p < 0.05$ ) and stage IV ( $p < 0.05$ ) indicating that the endothelial cells are better preserved in synthetic media as compared to the serum based media series (Figure 1e).

*Morphology was significantly better in Cornea Syn<sup>®</sup>*

Statistically, the morphology looked better after organ culture in Cornea Syn<sup>®</sup> and final transportation in Cornea Trans<sup>®</sup> as compared to Cornea Max<sup>®</sup> and Cornea Jet<sup>®</sup>. No other stage showed any significant difference as seen in figure 1F. The tissues from the synthetic series showed clear cells as compared to hazy tissues from serum based media and therefore the morphology was clearly visible. The morphology, endothelial cell density and mortality from each medium at each stage are shown in figure 3.

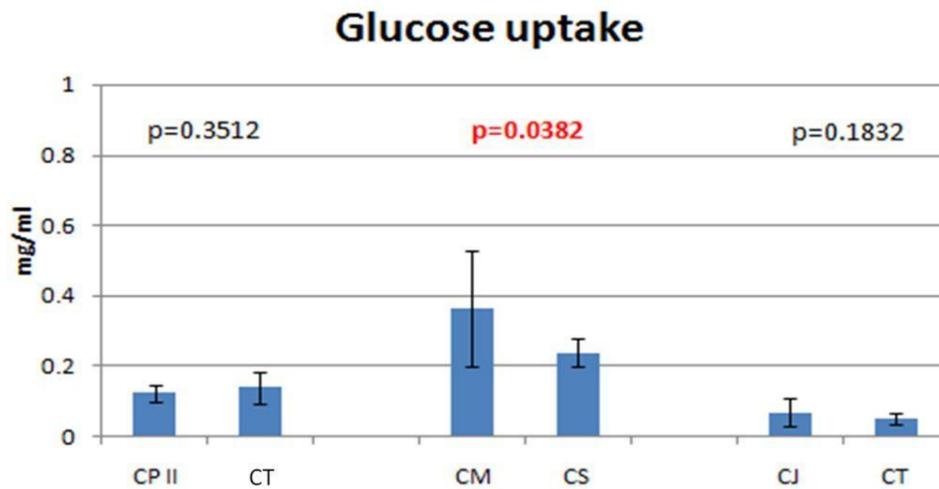


**Figure 3:** Morphological analysis. Endothelial cell density, mortality and morphological analysis performed at different stages of preservation in serum based and synthetic media (100x).

*Cornea Max<sup>®</sup> showed higher glucose uptake*

A statistically significantly higher uptake of glucose was observed in Cornea Max<sup>®</sup> as compared to Cornea Syn<sup>®</sup>. However, glucose uptake was similar in the rest of the media in their respective phases as seen in Figure 4. Glucose is converted to the pyruvic acid by producing lactic acid and ATP. In the Krebs cycle, glucose utilizes pyruvic acid and O<sub>2</sub> to produce ATP. Lower but active metabolism is usually beneficial to the cornea [15]. The

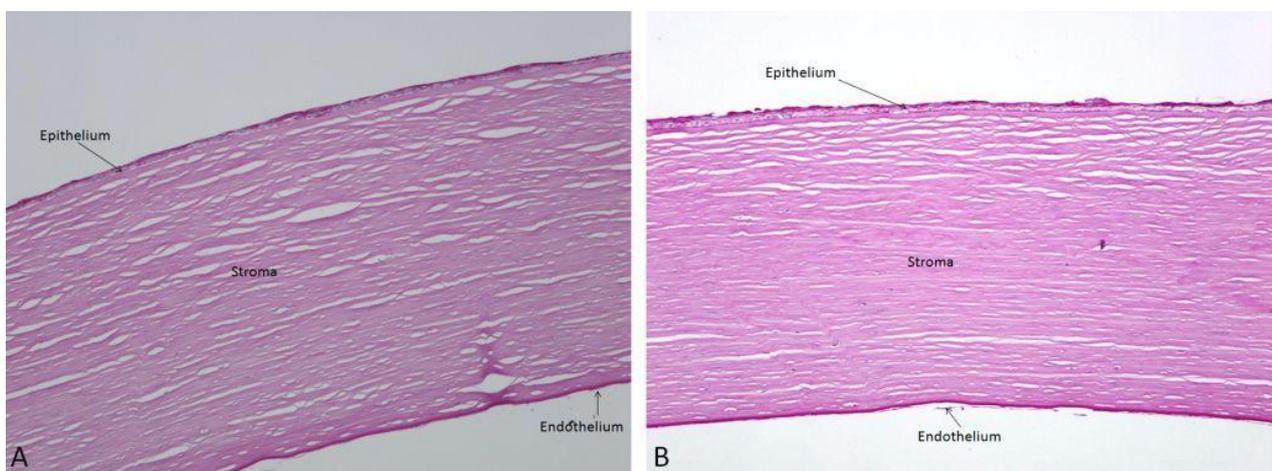
fact that Cornea Syn<sup>®</sup> consumes less glucose than Cornea Max<sup>®</sup> may potentially make it available longer for survival.



**Figure 4:** Glucose uptake analysis. Glucose uptake was lower in synthetic medium as compared to serum based when the tissues were preserved in rHSA. All the readings are in their original scale and unit.

*Histology showed presence of all the layers*

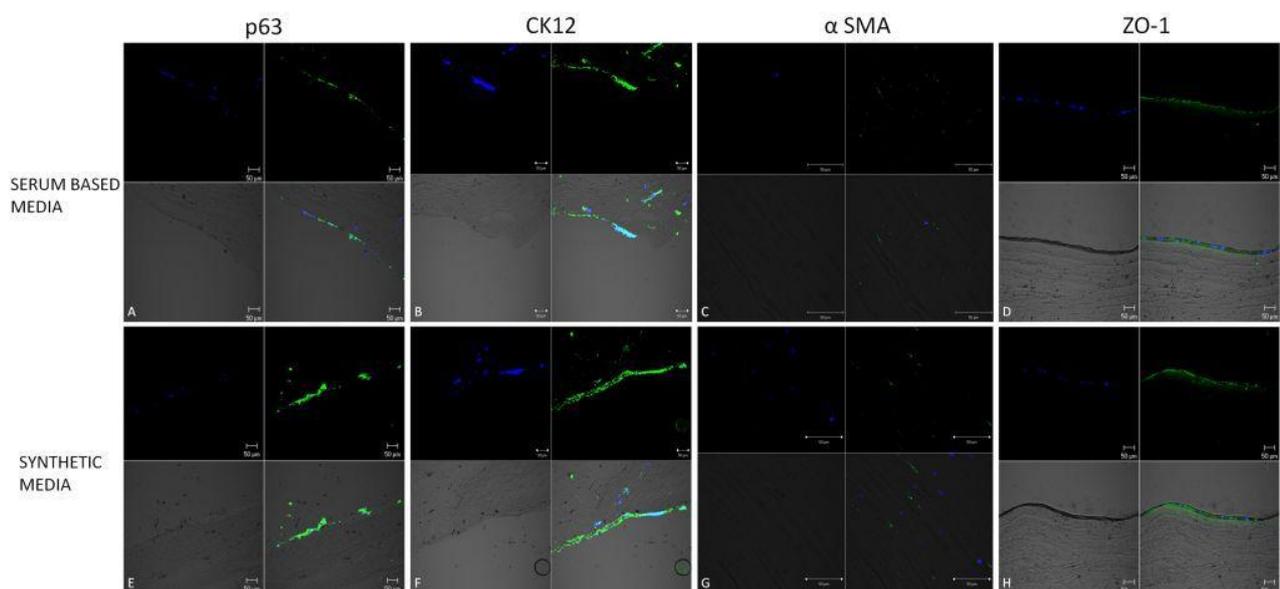
Histological analysis using PAS staining showed the presence of all the layers (epithelium, stroma and endothelium) without any significant damage on the tissues preserved in either media as seen in figure 5.



**Figure 5:** Histological analysis as observed on the sections after preservation using PAS staining at 100x magnification. a) tissue preserved in serum based media and b) tissue preserved in synthetic media, both the tissues showed all the layers of cornea without any damage to any layer.

*Immunostaining showed expression of corneal, stromal and endothelial markers*

Immunostaining using different markers showed expression of p63 (Figure 6a and 6e) near the limbus whereas CK12 (cytokeratin 12) was observed in the corneal epithelium (figure 6b and 6f) in both the media.  $\alpha$ SMA (smooth muscle actin) was scattered and observed in stroma (figure 6c and 6g). ZO-1 (zonula occludens-1) was observed in the endothelium (figure 6d and 6h) in both the media respectively. This showed that there was no difference in the protein expression when the tissues were preserved in either media.

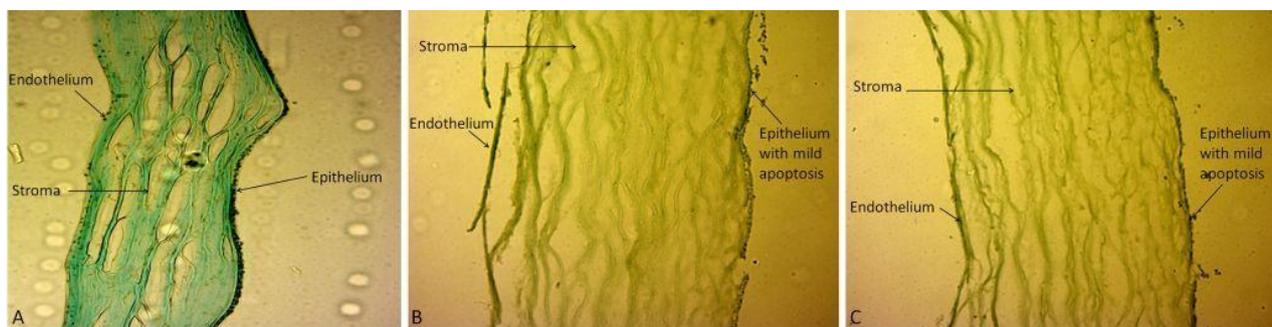


**Figure 6:** Immunostaining of the sections to determine the protein expression in synthetic and serum based series after preservation. a and e) shows presence of p63 near limbal area, b and f) shows expression of CK12 in the corneal epithelium, c and g) shows presence of  $\alpha$ SMA in the stromal region and d and h) shows the presence of ZO-1 in the

endothelium. DAPI staining is observed in all the respective cells. Immunostaining did not show any damage to the cells when preserved in synthetic or serum based media.

#### *Cell apoptosis was observed in the epithelium and not in other layers*

Mild apoptosis was observed in the epithelium in the tissues preserved in both the preservation media. No apoptosis was seen in any other layer of the tissue as observed in figure 7. Positive control is shown to compare the difference.



**Figure 7:** Cell apoptosis analysis. a) positive control which was induced with apoptosis. Cell apoptosis was observed in all cells of the section, expected mild apoptosis was observed in the epithelium of the sections when preserved in b) synthetic and c) serum based media. No other cells showed apoptosis when preserved in either medium.

#### *Microbiological checks did not show any contamination*

Microbiology performed using Bactec 9240 instrument did not show any contamination in either media showing that the antibiotics were active and it is safe for corneal preservation.

## **Discussion**

It has already been accepted that the risk of using FCS or bovine serum is minimum but as the corneas are in contact with the serum for almost a month, health authorities have huge concerns as they are intended for human grafting. There are sera that come from

pools of various animals; possibility of high variability between FCS batches makes the standardization of OC media, and its final validation, very difficult. To reduce a worldwide reduction in the use of animals for research and development, a three Rs (3Rs) strategy has been implemented. These include replacement, reduction and refinement, which also define the ethical guidelines for the use of animal models. 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 high relevance. So far, there have been a lot of serum free media that have been analyzed but have not shown better endothelial cell survival as compared to the serum containing media [16-22]. Stoiber et al [16] tested a serum-free OC medium that derives from a medium for short-term corneal storage at +4°C (Eurosol, Bausch and Lomb, Irvine, CA). Many used several serum-free media for other medical purposes [10,17-19]. Whereas others kept the MEM base but replaced the 2% of FCS with  $\beta$ -FGF [20] or ovalbumin [22]. A study by Thuret et al. used poloxamer as the deswelling agent for animal free medium [2].

We set out for a randomized controlled study with masked observers and showed that Cornea Syn<sup>®</sup> medium preserves endothelial cells after 38 days of OC better than standard serum containing media (Cornea Max<sup>®</sup>). The results in this article show statistical significance in terms of endothelial cell viability that is the major parameter of graft rejection. The highlights of our study were: a) huge sample size (n=24 pairs) allowing sincere statistical comparisons, b) paired corneas with a starting ECD greater than 1600 cells/mm<sup>2</sup>, theoretically which could be extrapolated to the corneas that are used for transplantation, c) immersion of corneas directly in the media under test, without prior placement in another medium containing animal compounds, d) reproducibility and three batches of production were checked commercially, e) parameters that are routinely practiced in eye banking procedures were checked periodically. We also incorporated

parameters like thickness and transparency, which are not currently used by all the eye banks due to the lack of sophisticated instruments and devices. Triplicate reading and immediate measurement of cell mortality reduce the error. Apart from these parameters we also included a new parameter of viable endothelial cell density (VECD), which is an inclusion of endothelial cell density and mortality previously described [6]. This demonstrates the surviving and active endothelial cells and provides the actual number of cells that are used for transplantation for accurate post-op data.

Dextran has been used widely in corneal preservation, be it hypothermic or OC. It reduces the thickness of the cornea and revert it back for ease of transplantation. Poloxamer has been used in medicine and pharmaceuticals. Zhao et al. [11] investigated the efficacy and tolerance of different poloxamers for deswelling human corneas. Dextran is also responsible for reducing the folds that are generated during the storage of the tissues, further leading to increased transparency. Therefore, we determined that a combination of preservation system that would replace the serum with rHSA and use dextran as a deswelling agent would show potential results. Although studies by various groups showed undesirable confirmation of the endothelial toxicity of using dextran [22-24], the ECD were also better preserved when a complete synthetic media series was used using rHSA and dextran based deswelling media.

In our study, we observed that rHSA does not have any negative effects when replaced with serum. The tissues showed higher transparency and lower thickness during phase II due to the presence of dextran in the synthetic series. Morphology and endothelial cell density were highly maintained in Cornea Syn® when compared to Cornea Max®. Metabolism was active and the glucose uptake was statistically significantly higher in Cornea Max® compared to Cornea Syn®. Histology showed presence of all the layers on

the cornea representing no damage to any layer anatomically. Immunostaining showed expression of p63 (limbal stem cells marker), CK12 (corneal epithelial cells marker),  $\alpha$ SMA (marker for smooth muscle actin in stroma) and ZO-1 (tight junctional protein, corneal endothelial cell marker). Cell apoptosis was only observed on the superior epithelial layer and not on any other layers of the cornea, which indicated no damage to any layer of the cornea in terms of apoptosis. Hence, at the anatomical and molecular level, the corneas did not show any significant damage. Thus, Cornea Syn<sup>®</sup> may be considered equal or slightly better in terms of corneal health when compared to conventional serum based media. With Cornea Syn<sup>®</sup>, we have not only improved the preservation system but also the duration of preservation which comes to a maximum of 38 days (including transport and organculture).

The final quality of the graft is of utmost importance and therefore the tissue selection is of a great concern. This study shows that the final quality of the tissue in either media provides a higher maintenance standard (eye bank standards) and the quality of the tissue. Therefore with all the benefits that are engaged with serum free media, with higher ECD and better morphology, rHSA containing synthetic medium Cornea Syn<sup>®</sup> along with transport in Cornea Trans<sup>®</sup> could be used for preservation of corneas in the conventional organ culture protocol.

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## **CONCLUSIONS AND FUTURE PERSPECTIVES**

The aim of this thesis was to study the posterior cornea which includes the DM and endothelium and further enhance the field of eye banking in terms of providing validated tissues for selective surgeries like endothelial keratoplasty, especially the DMEK. The second aim of the thesis was to find alternative approaches from the previous findings for the transplant of endothelial cells and reduce the global shortage of donor corneas by culturing the donor endothelium *in vitro*.

During the studies, we noticed that by defining a new way to store and transplant a DMEK graft i.e. a pre-loaded DMEK inside a preservation chamber which can be used for transportation and transplantation, challenging surgeries like DMEK will become easily accessible to more surgeons, also to those with less experience or skills. Dextran preservation will provide a standardized and validated graft preservation method with required rigidity and flexibility. This in turn will reduce further manipulations during the surgery. Currently, the surgeons request for additional tissues that are kept as stand-by substitute tissues in case of any preparation error and therefore huge graft wastage of transplantable tissues have been recorded. The pre-loaded validated grafts will be prepared by the eye bankers who have skills and prepare a lot of tissues on daily basis. This will reduce wastage of transplantable tissues and provide a ready-to-use tissue that can be directly transplanted in the recipient eye. Apart from the issue of wastage, if the tissues are damaged during preparation in the surgical theatre with the patient on bed, then it becomes a serious issue. The above mentioned details are the complications that can be overcome by using a pre-loaded DMEK tissue however such kind of devices and tools can also be useful in reporting accurate post-op clinical data. Currently, the surgeons only receive a pre-stripped graft and they have to cut the tissue, load and transplant it in the eye. The last three stages mentioned are crucial in reporting the post-op ECD count as each stage affects the ECD significantly. Therefore, a pre-loaded graft with accurate

ECD count before transplantation would significantly change the scenario of reporting the post-op data. The only ECD damage will occur during the implantation phase and this will further allow the surgeons and the eye bankers to revert and evaluate the stage which affects the ECD the most and re-formulate the procedure accordingly for a better graft in the future. Apart from new products like pre-loaded tissues, it is also necessary to change the implantation technique like endo-in which will help the surgeons to reduce the surgical time and costs dramatically. Minimal endothelial cell loss and ease of unfolding due to endo-in method will help to reduce surgical manipulations. Hence, the new techniques and products described in this thesis will provide better tissues with easiness to implant which in turn will allow high uptake of challenging surgeries like DMEK and will reduce surgical time and costs for the national health system. Eye banks will play a major role in changing the field of DMEK transplantation and will deal with everything reducing the efforts of surgeons drastically.

Although the pre-loading DMEK technique may be useful in terms of reducing graft wastage in the surgical theatre, the donor availability is a major issue. Although regions like northeast Italy or Spain that can account for high organ/tissue donation and USA that collects more than 125,000 corneas per year, the overall tissue donation rate is low. It has been observed that there is a high demand of donor corneas required for transplantation which shoots up to the requirement of 100,000 corneas/year in developing countries. These countries either face a huge patient waiting list ranging from kids to old age group. Therefore, the only possibility for these countries is to import tissues from other parts of the world. Many countries are also tabooed from donating corneas on religious backgrounds and therefore, the requirement of donated corneas keep shooting up dramatically. We foresee even a higher demand of corneal transplantation in the future. Therefore, alternative solutions like culturing the corneal endothelial cells *in vitro* could be

a possible solution for such kind of requirements. HCECs are highly challenging and the challenges keep rising when they have to be manipulated under GMP guidelines which also include the use of synthetic media. Currently, the availability of old donor corneas that do not pass the eligibility for transplant is high however these cells are very difficult to culture. The method discussed in this thesis will significantly improve the quality of the culture from old age corneas which will allow production of more products due to the available source. Although the overall costs of the final product will be high in terms of GMP regulations, our speculations of 4 DMEK grafts from 1 donor (2 corneas) [and even more with young donors] would reduce the global shortage of the donor corneas for the treatment of endothelial disorders which will have a huge impact clinically. The high flexibility and transparency of the studied scaffold may allow surgeons to transplant such grafts without adapting new techniques of delivery or high dosage steroids. Only the donor tissues will be replaced by the cultured cells *in vitro*, therefore, the products will become highly validated, with batch reproduction, total synthetic, available on requests which will help to plan the surgery with costs that can be justified to the current requirement.

Thus, in brief, the aims were successfully achieved with further validation of the methods *in vitro*. Current EK techniques can be now modified and validated and standardized products will be in the surgery soon that will help the surgeons to reduce graft wastage and will also affect the overall time and cost of the surgery positively. Alternatives like tissue engineering may be useful to reduce the patient waiting list due to the availability of the donor corneas and will provide a more reproducible GMP validated grafts. All the preservation of the grafts and bioengineered tissues will be produced in a totally synthetic medium in the near future and therefore will reduce any potential chance of xenotransplants.

This thesis highlights important details and modifications in the field of EK which is deemed to be the future of corneal transplantation. Most of the data were successfully obtained and published in peer-reviewed journals and the others are under consideration for publication.

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