HUMAN WHARTON'S JELLY-DERIVED MESENCHYMAL STEM CELLS PROMOTE CORNEAL EPITHELIAL GROWTH ON POLYHYDROXYALKANOATE FOR CORNEAL REGENERATION

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by

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LIST OF SYMBOLS

ºC	Degree Celsius
%	Percentage
mg	Milligram
g	Gram
µL	Microlitre
µm	Micrometre
mm	Millimetre
cm	Centimetre
γ	Gamma
L	Liter
β	Beta
min	Minute
ng	Nanogram
nm	Nanometer
pH	Potential hydrogen
x g	Relative centrifugal force
v/v	Volumeper volume
w/v	Weight per volume

LIST OF ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
3HB	3-hydroxybutyric acid
3HHx	3-hydroxyhexanoates
3HV	3-hydroxy-valerate
4HB	4-hydroxybutyric acid
ABCB5	ATP Binding Cassette Subfamily B member 5
ABCG2	ATP-binding cassette subfamily G member 2
ALK	Anterior lamellar keratoplasty
bFGF	Basic fibroblast growth factor
c DNA	Complementary DNA
CCR6	Chemokine receptor 6
CdM	Conditioned medium
CdM	Conditioned medium
CE	Corneal epithelial
CK14	Cytokeratin 14
CLET	Cultured Limbal Epithelial Cells Transplantation
COMET	Cultivated oral mucosal epithelial transplantation
CSSC	Corneal stromal stem cells
CX43	Connexin 43
DKSM	Defined keratinocyte serum-free medium
DMEK	Descemet's membrane endothelial keratoplasty
DMEM-LG	Dulbecco's modified eagle medium-low glucose
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline

DSEK	Descemet's stripping endothelial keratoplasty
ECM	Extracellular matrix
ECM	Extra cellular matrix
ELISA	Enzyme-linked immunosorbent assay
F Actin	Actin filaments
FBS	Fetal bovine serum
g DNA	genomic DNA
GVHD	Graft Versus Host Disease
hWJ-MSCs	Human Wharton- Jellyderived mesenchymal stem cells
HA	Hydroxyalkanoates
HCEC	Human corneal epithelial cells
HLA Class I	Human Leukocyte Antigen class I
HLA Class II	Human Leukocyte Antigen class II
HLE	Human limbal epithelial cells
HTCEC	Human telomerase-immortalized cornealepithelial cell line
IDO	Indoleamine 2,3 dioxygenase
IFN-γ	Interferon gamma
IL1β	Interleukin 1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
iPSC	Human induced pluripotential stem cells
iPSCs	Human-induced pluripotent stemcells
ISCT	International Society for Cellular Therapy
ITGA2	Integrin-alpha 2
ITGA6	Integrin-alpha 6
ITGA6	Integrin alpha 6
ITGB4	Integrin-beta 4

K12	Keratin 12
K12	Keratin 12
K3	Keratin 3
Ki67	Nuclear protein Ki67
KPros	Keratoprotheses
LSCD	Limbal stemcells deficiency
LSCs	Limbal stemcells
MATN2	Matrilin-2
MATN4	Matrilin-4
MHC	Major histo compatibility complex
MMP	Matrix metalloproteinases
MSCs	Mesenchymal stemcells
NHS	N-hydroxysuccinimide
NK cells	Natural Killer Cells
NO	Nitric oxide
P(3HB-co-4HB-co-5HV-co-3HHx)	Poly(3-hydroxybutyrate-co-4-hydroxybutyrate-co-5-hydroxyvalerate-co-3-hydroxyheaxoate)
P63	Tumor protein 63
PAX6	Paired box 6
PDGF	Platelet-derived growth factor
PDMS	Polydimethylsiloxane
pERK	Phospho-extracellular responsive kinase
PET	Persistent epithelial defect
PGA	Poly-glycolic acid
PGE2	Prostaglandin E2
PHA	Polyhydroxyalkanoate
PHBHHx	Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)

PHBV	Poly (3-hydroxybutyrate-co-3-hydroxyvalerate)
PK	Penetrating Keratoplasty
PLA	poly-lactic acid
PLGA	Poly (D,L-lactide-co-glycolide)
PLGA	poly(lactic acid-coglycolic acid)
RHCIII	Recombinant human collagen
RPE	Retinal pigment epithelium
Rx1	Retinal homeobox protein
SDF-1	Stromal derived factor
SEM	Scanning electron microscopy
SIX3	Six homeobox 3
sVEGFR-1	Soluble VEGF receptor 1
TE	Tissue engineering
TGF-β1	Transforming growth factor-beta 1
THBS1	Thrombospondin-1
TIMP3	Tissue inhibitor of metalloproteinase 3 MMP-Inhibitor
TNF-α	Tumour necrosis factor-alpha
TrkA	Tropomyosin receptor kinase A
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
WJ-MSCs	Wharton's Jelly derived Mesenchymal stemcells

LIST OF APPENDICES

SEL TUNJANG MESENKIMA DARIPADA JELI WHARTON MANUSIA MENGGALAKKAN PERTUMBUHAN SEL EPITELIA ATAS POLIHIDROKSIALKANOAT BAGI PENJANAAN SEMULA KORNEA

ABSTRAK

Kornea adalah lapisan luar mata yang memainkan peranan penting dalam pemfokusan cahaya dan ia juga mempunyai peranan sebagai pelindung fizikal daripada faktor yang boleh mencederakan mata. Rawatan penyakit kornea pada masa kini menggunakan ubatan, pembedahan keratoplasti separa (partialkeratoplasty) atau pemindahan kornea. Pemindahan kornea mempunyai pelbagai limitasi seperti penolakan graf, pemindahan penyakit dan kekurangan bekalan tisu. Kejuruteraan tisu dan teknologi perubatan regeneratif untuk strategi rawatan penyakitkornea pada masa hadapan adalah melibatkan penggunaan polimer yang selamat dan mudah diperolehi, biokompatibel dan biodegradasi, bersama dengan penggunaanmolekul dan sel stem yang aktif secara biologi. Polyhydroxyalkanoate (PHA) telah dibiosintesis dan dicirikan dengan menguji komposisi monomer dan sudut sentuhan air (water contact angle). Degradasi *in vitro* telah dikaji dengan menggunakan enzim lipase. Degradasi *in vivo* telah diuji dengan mengimplan filem PHA dalam mata tikusSprague Dawley (SD) dan kemudian diselidik di bawah SEM. Kesan imunomodulatori sel stem mesenkima (WJ-MSC) yang diperolehi dari Wharton-jellydinilai pada sel epitelium kornea 'telomerase-immortalised' manusia (HTCEC) yang telah dirangsang dengan IFN-g secara *in vitro*. Kesan WJ-MSC pada protein '*focal adhesion'* telah diperiksa menggunakan kaedah immunositokimia. Ekspresi gen kornea pada PHA dinilai menggunakan qPCR. Degradasi PHA secara *in vitro* menggunakan lipase menunjukkan bahawa PHA telah terdegradasi sepanjang ujian dijalankan. Biodegra-

dasi PHA *in vivo* pula menunjukkan rekahan lakuna pada permukaannya berbanding dengan sampel kawalan. Media terkondisi (CdM)daripada WJ-MSC telah meningkatkan viabiliti sel HTCEC yang dikultur bersama CdM (121%) berbanding kawalan (100%), $p<0.005$. WJ-MSC telah meningkatkan ekspresi protein "focal adhesion" dalam ko-kultur bersama HTCEC/WJ-MSC berbanding kultur tunggal HTCEC ($p<0.05$). Viabiliti sel, morfologi dan ekspresigen HTCEC adalah konsisten dengan sampel kawalan, dan fungsi sel epitelia terkawalatur oleh kehadiran CdM. Dengan kehadiran WJ-MSC, didapati penurunan ekspresi kedua-dua molekul kelas HLA I dan II. WJ-MSC yang dikultur bersama dengan HTCEC meningkatkan rembesan IL-1β dan TGF-β1 dalam HTCEC/MSC, lebih daripada kultur tunggal HTCEC. Gen-genkornea (ITGB1, ABCG2, ABCB5, CK3, CK12, CX43, dan ΔNP63) meningkat dengan ketara dengan kehadiran CdM, berbanding HTCEC yang dikultur pada PHA, $p <0.05$). Pendekatan kejuruteraan tisu menggunakan HTCEC, PHA, dan WJ-MSC untuk pertumbuhan semula dan pembaikan kornea telah berjaya dilaksanakan. Kajian dan penemuan baharu ini menjadi titik permulaan yang baik untuk kaedah penjanaan semula sel epiteliakornea dan penemuan ubatan baharudalam rawatan penyakit mata. Keupayaan suppresi-imun WJ-MSC boleh dimanipulasiuntuk memperbaiki kaedah rawatan penyakit kornea, terutamanya yang melibatkan pemindahan tisu alogenik.

HUMAN WHARTON'S JELLY-DERIVED MESENCHYMAL STEM CELLS PROMOTE CORNEAL EPITHELIAL GROWTH ON POLYHYDROXYALKANOATE FOR CORNEAL REGENERATION

ABSTRACT

The cornea is the outer layer of the eye that plays an essential role in light focusing and it also has a protective role as a physical barrier from factors that can insult the eye. The current treatments of corneal diseases include either medical therapies, partial keratoplasty surgery or corneal transplantation. Corneal transplantation has limitations such as graft rejection, disease transmission, and scarcity of tissue supply. Tissue engineering and regenerative medicine technology for future corneal diseases treatment strategies involve the use of safe, commonly available, biocompatible, and biodegradable polymer, together with the biologically active molecules and stem cells. Polyhydroxyalkanoate (PHA) was biosynthesized and characterized by testing the monomer composition and water contact angle. *In vitro* degradation was explored using lipase enzyme. *In vivo* degradation was investigated by implanting a PHA film in the Sprague Dawley (SD) rat's eye and then viewed under SEM. The immunomodulatory effect of human Wharton-jelly derived mesenchymal stem cells (WJ- MSC) on IFN-g stimulated human telomerase- immortalized corneal epithelial cells (HTCEC) was tested *in vitro*. The effect of WJ- MSC on focal adhesion proteins were examined using immunocytochemistry. Corneal gene expression on PHA was evaluated using qPCR. *In vitro* PHA degradation by lipase degradation showed that PHA was degraded *in vitro*. *In vivo* PHA biodegradation showed lacunar cracks on its surface in comparison to the control sample. Conditioned media (CdM) from WJ-MSC improved the cell viability in the presence of CdM (121%) compared to the control (100%), $p < 0.005$. WJ-MSCs improve the focal adhesion protein exp-

ression on HTCEC/WJ-MSCs co- culture compared to HTCEC single culture ($p<0.05$). HTCEC viability, morphology, and gene expression were consistent with the controls and upregulated in the presenceof CdM. In the presence of WJ-MSCs, there was a downregulation in the expression of both HLA class I and class II. WJ-MSCs co-cultured with HTCEC increased secretion of IL-1β and TGF-β1 in HTCEC/MSC more than single HTCEC culture. Corneal genes (*ITGB1*, *ABCG2*, *ABCB5*, *CK3*, *CK12*, *CX43*, and *ΔNP63*) were upregulated significantly in the presence of CdM, compared to HTCEC grown on PHA, $p < 0.05$). The novelty of the project and the findings of this study present a good starting point for a regenerative and drug delivery development in managing eye problems. In this project we use tissue engineering approach including HTCEC, PHA, and WJ-MSCs for corneal regeneration and repair. Immune suppressive abilities of WJ-MSCs could be manipulated to improve the outcome of corneal diseases, particularly involving allogeneic tissues.

CHAPTER 1

INTRODUCTION

1.1 Overview

According to the World Health Organization (WHO) fact sheets on visual impairment and blindness, there are 285 million visually impaired people worldwide (Abner et al., 2002). Out of this, 39 million are blind, while 246 million have low vision. Diseases affecting the cornea are the second most important cause of blindness after cataract. The epidemiology of "corneal blindness" covers many patho-physiologies that mainly arise from infectious and inflammatory conditions. Corneal blindness carries morbidity which affects the quality of life and is often associated with an increased economic burden (Tran et al., 2020).

Corneal transplantation is an effective treatment strategy for corneal diseases. There is limited availability for tissue or organ repair due to a limited supply of organ donors and autografts; and potential disease transmission and grafts rejection (Cascalho and Platt, 2006). Therefore, the search for the perfect biodegradable and compatible constructs as a platform for cellular, gene, immune or drug deliveries warrant intensive investigations. Biologically compatible materials which serve as *in vivo* devices with unique properties are needed as substrates or scaffolds for many types of cellular therapy (Chan and Leong, 2008).

Mesenchymal stem cells (MSCs) are rich in paracrine secretions of many bioactive molecules. They have shown remarkable immunosuppressive activity that makes themsuitable for allogeneic transplantation purposes (de Girolamo et al., 2013).

A study reported that MSC-conditioned medium (CdM) promotes corneal epithelial (CE) cellular viability and improved protein expressions of limbal stem cells. Furthermore, human wharton's-Jelly derived mesenchymal stem cells (hWJ- MSCs) and corneal epithelial co-cultures have improved characterization and growth kinetics of the corneal epithelial cells (Azmi et al., 2020). Combined cellular therapy have shown beneficial effects in other system such as cardiomyocytes differentiation and cardiac stem cells expression (Hatzistergos et al., 2010). Recent findings showed evidence that WJ- MSCs enhanced rejuvenation of aged cardiac stem cells in mice (Ng et al., 2019).

In a preliminary *in vitro* biocompatibility study, polyhydroxyalkanoate (PHA) polymers showed promising results with good cell adhesion, proliferation, and viability of WJ-MSCs (Ang et al., 2020). Thus, combining WJ-MSCs and CE cells with biological scaffolds such as PHA polymers are innovative strategies to create a novel biomimetic tissue- engineered construct for corneal epithelial regeneration and wound healing.

1.2 Rationale of the study

Corneal abnormalities will lead to severe damage to the cornea, and it may lead to loss of vision. Corneal transplantation treats corneal blindness especially corneal allografts (Oie and Nishida, 2013). Although the cornea is one of the most successful organs in terms of successful transplantation because it is considered as animmune-privileged site (Singh et al., 2019), there are limitations like the risk of graft rejection, and the transmission of diseases, and scarcity of donors (Dubord et al., 2013; Samson et al., 2002).

Looking for an affordable alternative for the current treatment strategies will be one of the solutions to overcome these limitations. Studies need to be done to explore the role of safe natural biomaterials like PHA in corneal regeneration. Also, understanding the behavior of corneal epithelial cells in the presence of PHA is very important.

Regenerative medicine is a field of science that advocates tissue engineering using biologics for tissue and organ replacements. Tissue engineering involves using biomaterials and combining them with cells to provide alternatives to fix and treat damaged cells and tissues (Berthiaume et al., 2011). PHA is one of the polymers used for many purposes including in the medical field (Lueft et al., 2017). MSCs are one of the strongest players in regenerative medicine and tissue engineering field (Shyam et al., 2017). This project adopts a multidisciplinary approach towards a safe, biocompatible, and affordable treatment strategy for corneal epithelial regeneration and wound healing from 'bench to bedside'.

1.3 Research questions

i Would human WJ-MSCs Cd M promote HTCEC growth on PHA?

i. What is the immunomodulatory effect of WJ-MSCs on cytokine-stimulated cornealepithelial cells *in vitro*?

ii. Is PHA biological scaffold compatible with HTCEC?

iv. Would HTCEC maintain its viability and gene expression on PHA?

1.4 Research hypothesis

1.4.1 Null hypothesis (H_0)

WJ-MSCs or its CdM does not improve the HTCEC growth on PHA.

1.4.2 Alternative hypothesis (H_1)

WJ-MSCs or its CdM improves HTCEC growthon PHA.

1.5 Objectives of the study

To examine the role of WJ-MSCs and its CdM in promoting HTCEC growth on PHA for corneal regeneration.

1.5.1 Specific Objectives

1) To investigate the immunomodulatory effect of WJ-MSCs on telomerase- immortalized human corneal epithelial cell line (HTCEC) stimulated with pro-inflammatory cytokine interferon-gamma (IFN-γ) in an *in vitro* co-culture model.
2) To study the effect of WJ-MSCs on corneal focal adhesion molecules using immunocytochemistry.
3) To biosynthesize and characterize PHA- P(3HB-*co*-4HB-*co*-5HV-*co*-3HHx) and test the *in vitro* and *in vivo* degradation of the polymer.
4) To test the *in vitro* and *in vivo* biodegradation of PHA.
5) To studythe effectiveness of WJ-MSCs-CdM on cell viability and gene expression of HTCEC growing on PHA using presto blue assay and qPCR.

1.6 Project framework

PHA was biosynthesized as powder. It was characterized for its physical and chemical characteristics like monomer composition, molecular weight, wettability, and biodegradation (*in vitro* and *in vivo*). This study used two types of cells: HTCEC and WJ-MSCs. HTCEC is a robust model for *in vitro* study of corneal epithelial (Shaharuddin et al., 2017). The WJ-MSCs are a good source of cell population for cell therapy and the conditioned medium for this research. The immunomodulatory effect of the WJ-MSCs on HTCEC was investigated in the lab by checking the HLA expression, actin filaments (F-Actin), and vinculin co-localization and transforming growth factor-beta 1 (TGF-β1) and interleukin 1 beta (IL1β) expression in cytokine-stimulated HTCEC model. On the final stage the biocompatibility of HTCEC on PHA was investigated with the aid of the WJ-MSC- CdM. HTCEC cell viability, adhesion, and corneal gene expression as a reflection of corneal epithelial growth are also evaluated. Figure 1.1 represents the general framework of the study.

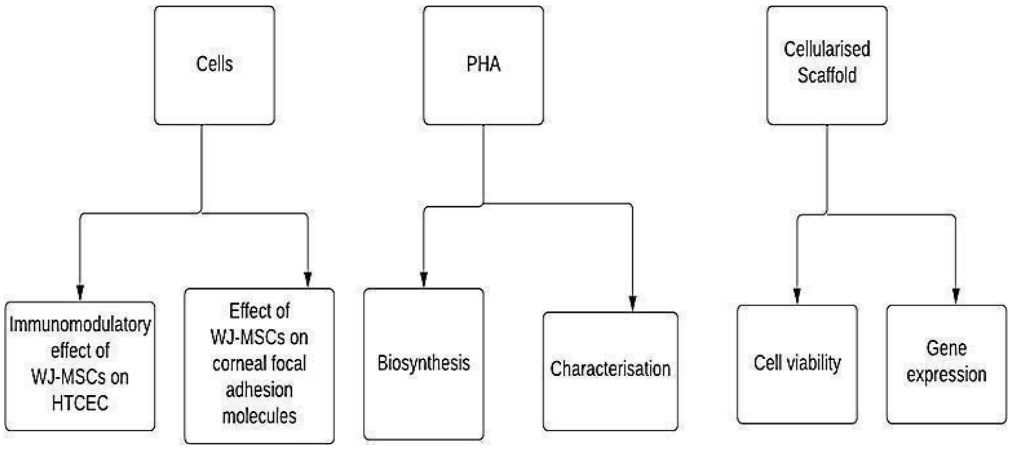

Figure 1.1 Project framework

CHAPTER 2

LITERATURE REVIEW

2.1 General structure of the cornea

Cornea is the anterior most structure of the eye which acts as a physical barrier and protection to the eye. Its major role is allowing light to pass through the eye and focus on the macula (Espana and Birk, 2020) as diagrammatically presented in Figure 2.1.

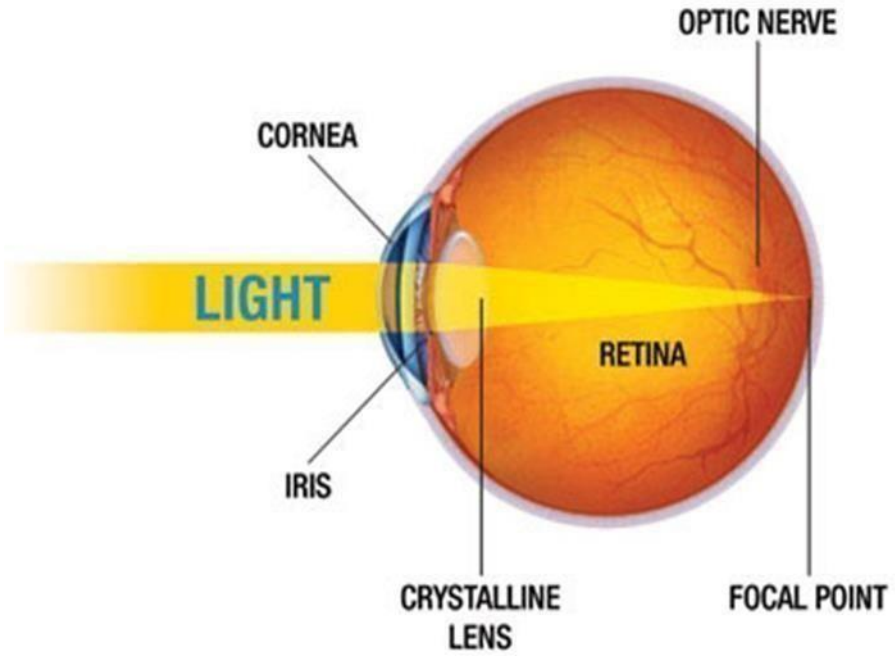

Figure 2.1 Entry of light through the cornea is transmitted to the focal point on retina. (https://nkcf.org/about-keratoconus/how-the-human-eye-works/).

Cornea is essential for clarity of vision providing 40 - 44 Diopter which is approximately two-third of the refractive power of the eye (Meek and Knupp, 2015). There is a variation in cornealthickness between centralcornea and its periphery (Fares et al., 2012). The cornea is flat at the periphery and steep at the center (Doughtyand Zaman, 2000). This is due to the high amount of collagen in peripheral

stroma; while the central cornea is thinner to facilitate better entrance of light into the eye. The thickness of central cornea for the normal eye is 551 to 565 µm while the peripheral cornea is 612 to 640 µm in thickness (Feizi et al., 2014). The average diameter of cornea varies between males and females, in males it is 11.04-12.50 mm while it measures 10.7-12.58 mm in females (Gharaee et al., 2014).

Cornea is an avascular structure with two main components as represented in Figure 2.2; cellular and non-cellular. Cellular components comprise of five layers: corneal epithelium, Bowman's layer, corneal stroma, Descemet's layer, and corneal endothelium (Doughty and Jonuscheit, 2019). Non-cellular components mainly consist of collagen and glycosaminoglycan. Three of the cellular layers are superficialwhich are: epithelium, stroma, and endothelium, while the rest are interface layers (DelMonte and Kim, 2011).

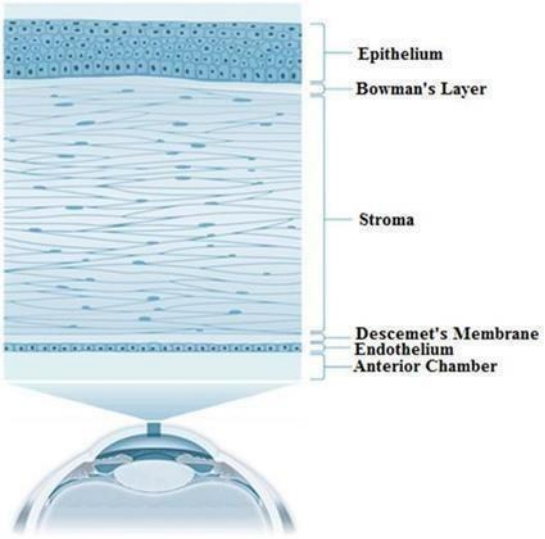

Figure 2.2 Corneal layers. Cornea has five layers consisting of cellular and non-cellular components: epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium.

2.1.1 Corneal epithelium

Corneal epithelium is the outermost layer of the cornea with 5-6 layers of non-keratinized stratified squamous epithelium with a highly regenerative cell. It plays a role in heat and gas exchange (Mglinets, 2015) as represented in Figure 2.3. Corneal epithelium comprises approximately 10% of the general corneal thickness and its function is to protect the eye and absorb oxygen and nutrients. It composed ofthree types of cells: basal cells, squamous cells, and wing cells (Y. Zhang et al., 2021). Cornealepithelium plays a crucial and essential role in the refractive power ofthe eye.

The epithelial layer is sheltered by the tear film (Hiraishi et al., 2020). Tear film is composed of many layers like mucinous, aqueous, and lipid layer. Tear film contains many antimicrobial factors, among them lysozymes are very important for the hydrolysis process (Burcel et al., 2020). Instability of the tear film will lead to severe consequences as the tear film is first barrier against microbial invasion and against the damage occurs from chemicals and toxins. It is also the primary source of growth factors and immunological factors which play an essential role in epithelial repair and health as well as proliferation (Pflugfelder and Stern, 2020) .

To re-populate the cornea, pre-limbal basal epithelial cells first differentiate and then migrate anteriorly. During this process, the surface microvilli started to appear in a gradual manner. Hemidesmosomes adhere to the basement membrane andstroma by the utilization of basal cells. The link between the intracellular skeleton of basal cells and stroma is through the anchoring complex which is composed of the hemidesmosome, anchoring fibril, and anchoring filament complex

(Espana and Birk, 2020). The epithelial layer performs the essential role in the vision.

The life span of the corneal epithelial cells is 7 to 10 days. After that it starts the apoptosis process and desquamation (Hanna et al., 1961). The basal epithelial cells are found between pre-limbal location and the central cornea (Zhou et al.,2006). Basal cells in the corneal epithelium are stained specifically for keratin 3 (K3) and keratin 12 (K12), Connexin 43 (CX43), involucrin, P-cadherin, nestin, andintegrin- alpha 2 (ITGA2), integrin-alpha 6 (ITGA6), and integrin-beta 4 (ITGB4) (Schlötzer-Schrehardt and Kruse, 2005). Epithelial cells can secrete some pro- inflammatory cytokines such as IL-1β, Interleukin-6 (IL-6), Interleukin-8 (IL-8), and tumour necrosis factor-alpha (TNF-α) (Kumar et al., 2006).

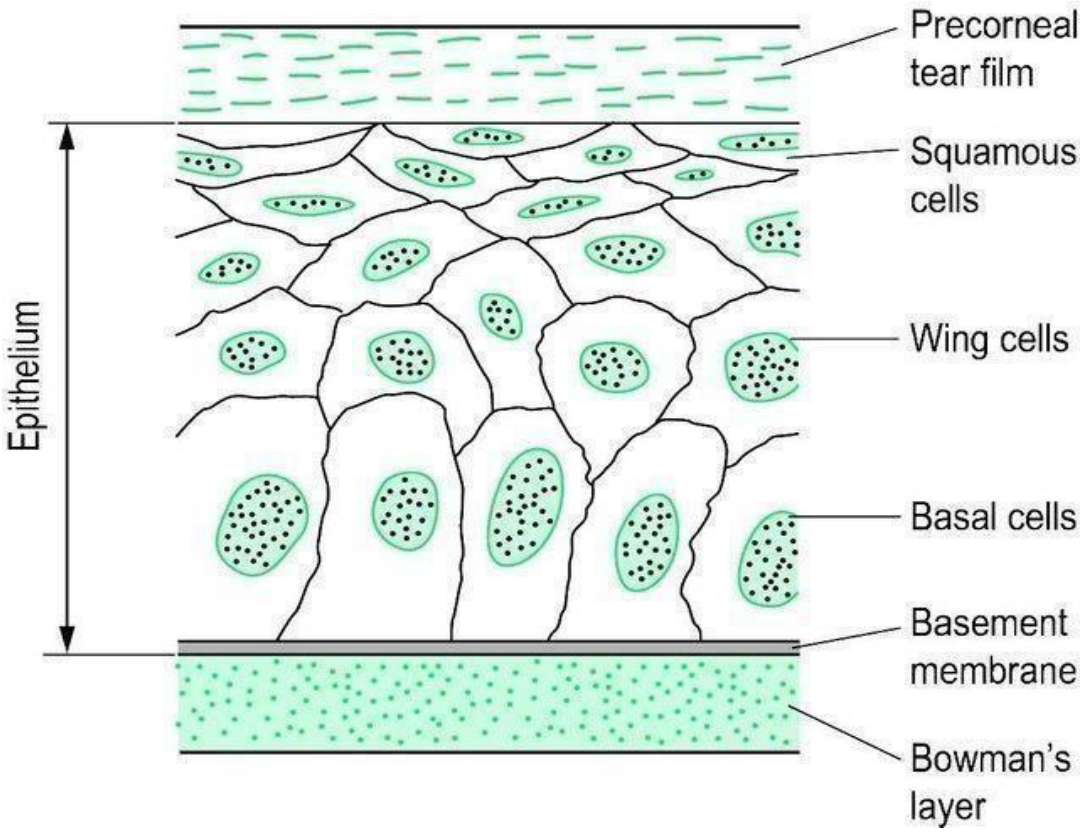

Figure 2.3 Cornea epithelium comprises 5 to 6 layers of non-keratinized stratified squamous epithelium and sheltered by an anterior pre-corneal tear film structure.

Cornea epithelial basement membrane is located between stroma and basal epithelial cells and laid down by the basal epithelial cells. It is an extracellular matrix that anchors epithelial cells to the stroma, and it serves as a scaffold for embryonic development. It is composed of collagen, laminin, heparan sulphate proteoglycans, nidogens, thrombospondin-1 (THBS1), matrilin-2 (MATN2), and matrilin-4 (MATN4) and fibronectin. Vinculin is located in focal adhesion sites together with integrin β1 subunits which is essential for cellular adhesion. Vinculin is a major factor in corneal wound healing (Onochie et al., 2019). F-actin which always binds to phalloidin, is found in the corneal epithelial cells, mainly in the lateral cell membrane (Y. Liet al., 2021).

The basement membrane of the cornea is different from other basement membranes in the human body because of the nature of the cornea, as the corneal stroma is typically avascular (André A.M. Torricelli et al., 2013). It is composed of two major areas: lamina lucid, which is a structure mainly composed of laminin, and lamina densa which is composed of collagen, laminin, heparan sulfate proteoglycans and nidogens (Tuori et al., 1996). Its role is to modulate the effects of the factors on the keratocyte function (Pal-Ghosh et al., 2011). It also releases factors which play an essential role in modulating apoptosis and differentiation processes like platelet-derived growth factor (PDGF) and TGF-β1.

2.1.2 Bowman's layer

It is a non- regenerating layer which is 8-12 μm. Bowman's layer is a specific layer in primates (Eghrari et al., 2015). Over time, it decreases in thickness. It is acellular. Light microscopy examination shows a homogenous layer, while it looks

like an unorganized fibril when scanned by electron microscopy. Fibrils consist mainly of collagen (Seng et al., 2022).

2.1.3 Stroma

The stromal layer provides the strength through its collagen-fibre plates (Mglinets, 2015). It contains collagen fibres type I and IV, ground substances, and an extracellular matrix (Andre A.M. Torricelli and Wilson, 2014). Major cells of the stroma are keratocytes which are the source of production of collagen, matrix Metalloproteinases and glycosaminoglycans (DelMonte and Kim, 2011). Stroma is the main structure in the corneal thickness (80- 85%). Passage of light through this organized networkof fibrils allow the light to pass without been scattering.

2.1.4 Descemet membrane

It is located just under the stroma, composed of collagen-like fibrils (Mglinets, 2015). The Descemet membrane comprises two layers: the anterior banded layer and the posterior non-banded layer (Eghrari et al., 2015). Anterior banded layer developed and started to be detected in the cornea of a fetus after three months of gestation. The posterior non-banded layer usually gets thick over time, andit laid down by endothelial cells (de Oliveira and Wilson, 2020). Descemet membrane assists in corneal dehydration maintenance which is essential to keep and maintain the clarity.

2.1.5 Corneal endothelium

The endothelial layer is also considered as the inner corneal, composed of one layer of hexagonal shaped cells. It plays a role in transporting necessary substances from intraocular fluid to the cornea (Mglinets, 2015). It maintains the stroma dehydrated through an ionic pump located in the plasma membrane. It contains Actin filaments located as dense peripheral bands, and its function is to facilitate the cell migration process (Sie et al., 2020). Corneal endothelial also contains gap junction and play a role in the electrical coupling of the cells of the endothelial layer (Joyce, 2012). The main protein expressed in gap junction is Connexin 43 (Kotini et al., 2018).

2.2 Corneal development

The origin of the eye is the somatic ectoderm, neural tube, and chordamesoderm induction. Chordamesoderm causes the induction of the anterior neural tube, leading to the development of the diencephalon. It expands till it meets the head ectoderm making the ectoderm thicker, inducing thickening of the ectoderm,and leading to the formation of lens placode and leads to lens development(Eghrari etal., 2015). All these events lead to protrusion of the optic nerve. This process facilitatedby the expression of three transcription factors: paired box 6 (PAX6) which is the main transcription factor for eye development and it plays a role in lens differentiation, especially lens specification which is entirely dependent on PAX6 activity, and maintaining multipotency and proliferation of progenitor cells in the retina (Ashery-Padan and Gruss, 2001), and Six homeobox 3 (SIX3) which is the most crucial factor for thickening of surface ectoderm (Liu et al., 2006).

Corneal cells contain stratified squamous epithelium cells that are self-renewing, which are maintained by stem cells. These stem cells are located at the limbus area. After stimulation, it will proliferate (Sacchetti et al., 2018). The cells started to divide asymmetrically when activated into two cells. One will be stored in the stem cell pool and the other will continue to differentiate and migrate to replace the cells shed from the ocular surface due to ageing or injury. If the limbal stem cells population is depleted, the corneal area will be re-epithelized but by conjunctival cells this time, and this will lead to poor vision and pain, and it may lead to blindness(Deng et al., 2020).

The cornea in humans started to differentiate after six weeks of gestation. This process involves an interaction between lens vesicle and surface ectodermoverlying the lens. The lens, cornea and conjunctival epithelium, lachrymal gland, andeyelids' epidermis are formed by the multipotent region of the head of ectoderm (Shalom-Feuerstein et al., 2012). At day 11 or 12 of embryogenesis P63 gene controlled of ectoderm into the epithelial lineages like epidermal, oral, and corneal epithelium. Also, cytokeratin of epithelial progenitors (K4/K5) replaced K8/K18. The corneal specific cytokeratins are keratin 12 (K12) which could be detected at day
11.5 and K3 which appears at day 15.5 (Wolosin et al., 2004). Determination of cornea will happen on the stroma formation stage by maintaining Pax6 signaling (Mglinets, 2015). Gap junctions is made by connexins (26, 30, 31, and 43) are thekey factors in the cellular communication on the corneal epithelium (Yuan et al., 2009).

There are no specific markers for limbal stem cells although tumor protein 63 (*P63*), ATP-binding cassette subfamily G member 2 (*ABCG2*), N-cadherin, NGF receptors tropomyocin receptor kinase A (*TrkA*), integrin alpha 6 (*ITGA6*), and ATP

Binding Cassette Subfamily B member 5(*ABCB5*) expressed (Oie and Nishida, 2016).

2.3 Corneal regeneration

2.3.1 Corneal epithelial wound healing

Corneal epithelium is the eye protection shield against external insult by pathogens. It is hazardous to leave the injured cornea without healing as it will lead to severe consequences and blindness. Wound healing involves three main steps: migration, proliferation, and differentiation (Eslani et al., 2014). Corneal injury may be due to physical or chemical and sometimes pathological damages (Ljubimov and Saghizadeh, 2015). Following an injury, for about 6 hours, latent phase continues with no reduction in the size of the wound.

There is a synthesis of structural proteins like vinculin, talin, and alphaactinin, and F-actin which is usually linked to phalloidin accompanied by actin filaments polymerization. Then the epithelial cells flatten and cover the woundedarea (Ljubimove and Saghizadeh, 2016). So, when the wound occurs there is a formation of focal contact complexes. Then the epithelial cells will flatten and spreadand then migrate to the wounded area. It will be in the form of sheet which is remarkably intact to be able to cover the wound.

To fully cover the wound, the cells started to proliferate, the cells also differentiate. Finally, there will be a reformation of hemidesmosomes followed by formation of the extracellular matrix (Figure 2.4) (Liu and Kao, 2015). The proliferation and differentiation of the cells follow the same steps that occur during normal homeostasis (XYZ theory) (Liu and Kao, 2015; Thoft et al., 1983).

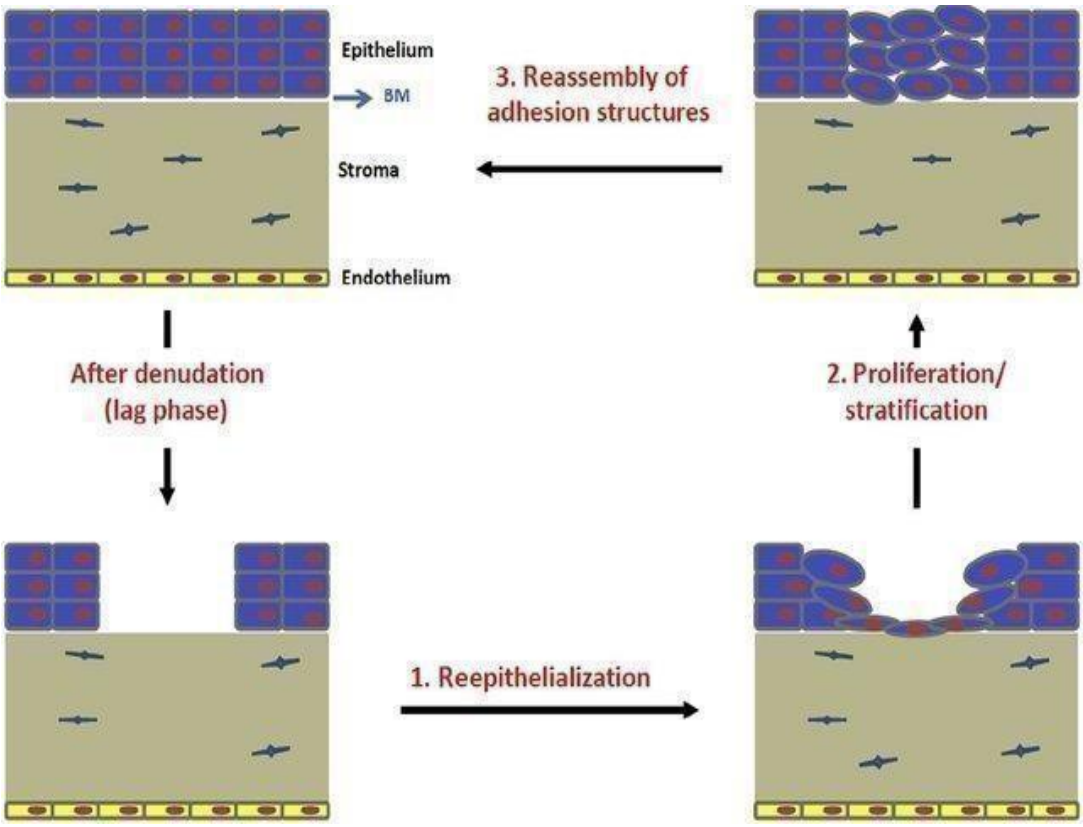

Figure 2.4 Corneal epithelium wound healing steps. The wound healing process starts with the reepithelization, followed by stratification and proliferation, then the adhesion structures reassembled, and finally the lag phase is the last step (Liu and Kao, 2015).

2.3.2 Corneal epithelial maintenance (XYZ theory)

In normal situation, there will be no change in the cornea epithelium mass. Corneal epithelial mass depends on three factors working on the XYZ axis; where X is the proliferation of the basal epithelial cells, Y is the movement of the peripheral cells and its contribution to the cell mass, and Z is the loss of the epithelial cells from the surface. Corneal epithelial maintenance is represented as (X+Y=Z). This equation means that cell loss should the same as cell replacement to maintain the corneal epithelium (Thoft et al., 1983). Shedding of cells from the cornea happens on daily basis. Division of basal cells will replace the lost cells. The mean generation time for this process is estimated to be 4 days (Painter, 2018).

2.4 Corneal transplantation strategies

Ocular surface abnormalities can result from many congenital or acquired pathologies such as trauma, chemical insult, inflammation, infection, and others causing a state of limbal stem cells deficiency (LSCD). LSCD management includes medical therapies such as anti-inflammatory medication, antibiotics, surface lubricants; and also surgical approach. Conventional surgical interventions such as amniotic membrane (AM) patch is performed to facilitate regeneration of epithelial cells. The removal of the fibrovascular pannus followed by either direct kerato- limbal autograft or from contralateral eye is an optional surgical therapy. Donor variation to recipients, risk of infection and post-surgical complication such as impeded epithelialization are some of the downsides of AM applications, prompting the need for alternatives to achieve successful ocular surface transplantation (Le and Deng, 2019; J. Liu et al., 2010).

Corneal transplantation is a definitive treatment for corneal replacement (Gain et al., 2016). Whole corneal transplantation also known as penetrating keratoplasty (PK) is considered as the most frequent transplantation type worldwide. This procedure involves use of cadaveric human donor cornea for allogeneic transplantation(Simpson et al., 2019). It is more successful in the developed countries and less successful in developing countries (Joshi et al., 2012).

A limitation of using autologous transplantation is that the tissues taken from the patient will not be enough and the main advantage is that using of autologous transplantation will lower the rejection risk. In a study by Campbell and colleagues, limbal cells were taken from the healthy eye of the patient, cultured and expanded the cells in the lab and transplanted the cells back to patients damaged eye using an

amniotic membrane. This study was the first randomized control trial in using allogenic corneal epithelial stem cells in the treatment of severe bilateral LSCD and it showed that this approach is feasible and safe (Campbell et al., 2019). Using the human-induced pluripotent stem cells (iPSCs) might be a stem cell source and it is capable of induce epithelial, keratocyte and endothelial phenotype (Chakrabarty et al., 2018).

An alternative of cornea transplant is the keratoprosthesis (KPros) which is the replacing of the cornea with a transparent polymer which will restore the vision partially or fully (Ahearne et al., 2020). KPros are intended for patients who are not prioritized for the conventional donor transplantation and as an alternative to failed grafts. Two recommended devices for Kpros are Boston Type-1 KPro (synthetic) and osteo-odonto-KPro [semi-biological] (Avadhanam et al., 2015). As a corneal substitute, recombinant human collagen type III (RHCIII) hydrogels are used as a first cell free method for corneal regeneration by using a biosynthetic mimic of the corneal extracellular matrix for replacement of affected anterior cornea throughimplantation, to regenerate tissues (Fagerholm et al., 2014). It is successful in improving the conditions for many patients, but it also have a severe complication to some patients like increasing the risk of glaucoma (Crnej et al., 2014), endophthalmitis (Dohlman et al., 2014) and corneal melt (Balasopoulou et al., 2017).

2.5 Corneal epithelial regenerative medicine

The primary purpose of corneal epithelial regenerative strategies is to provide a successful outcome of cellular or tissue transplant with a minimum risk of immune system rejection. Many tissue engineering techniques and strategies are utilized to reach this goal.

Ex vivo cultivated cornea epithelial cells is an option to regenerate corneal epithelium for tissue transplantation (Cabral et al., 2020). Donor limbal tissues (2 X2 mm strip) obtained from the healthy eye were expanded and evenly distributed on an amniotic membrane as a cell carrier and placed over the cornea. After 6 weeks, corneal surface was found to be epithelialized completely, stable, and avascular. Thereis a vast improvement in the visual acuity of the patients from 20/200 to 20/60,or betterwith no complications among all the patients participated in the study. It hasalso beeneffective with and suitable for long lasting vision restoration and corneal regeneration(Basu et al., 2016; Sangwan et al., 2012). The *ex vivo* cultivationmethod can be improved by optimising a method to keep the contact between the cells and the nativeniches as much as possible during the expansion step as the native niches are much better than feeder layer that usually use *ex vivo* (Hynds et al., 2018).

2.6 Principle of tissue engineering

The tissue engineering field involved many factors and make use of it to regenerate the damaged tissues and organs and maintain them by biological substitutes. Tissue engineering uses the cells, scaffolds, and signals to restore and improve the damaged area (Figure 2.5) (Mhanna and Hasan, 2017). The basic idea in this field is to fabricate the cells with biomaterials to reproduce the affectedtissues. This field was introduced for the first time in 1990's (Stock and Vacanti, 2001). The most critical factors in the tissue engineering are type of materials usedfor scaffold fabrication and the method ofscaffold manufacturing (Ahearne et al., 2020).

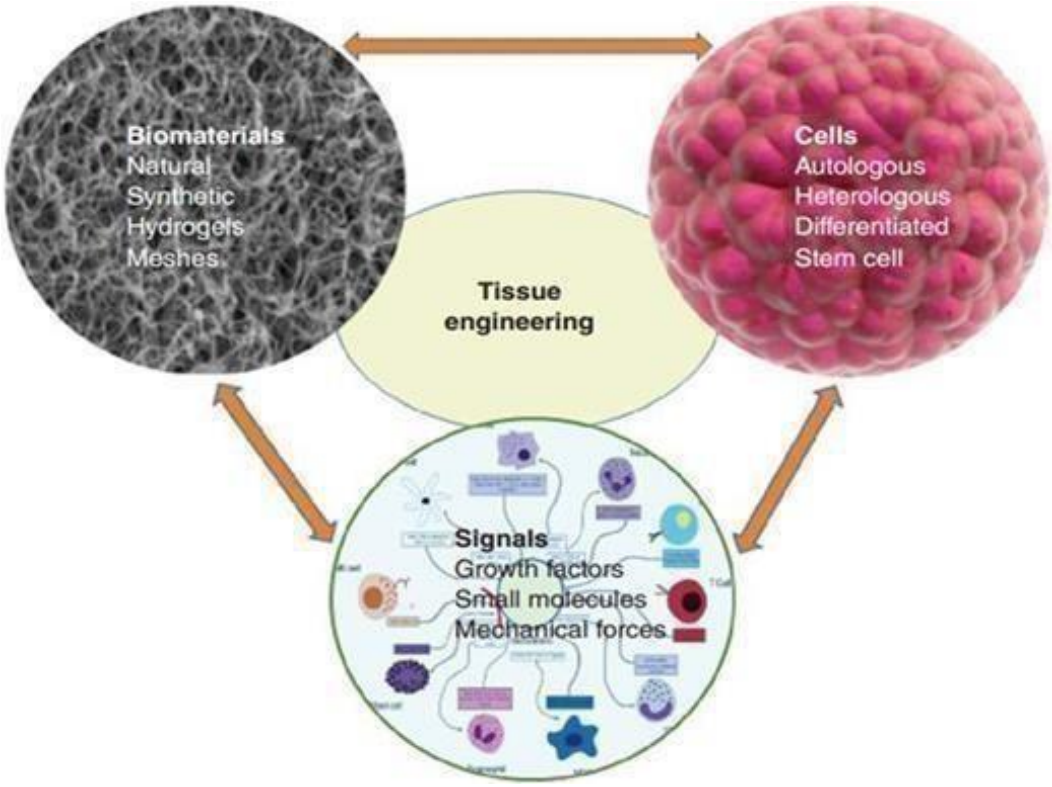

Figure 2.5 Triad of tissue engineering. A field that makes use of the cells, biomaterials, and signals to regenerate, maintain, and improve the damaged cells and tissues (Mhanna and Hasan, 2017).

2.7 Corneal tissue engineering

Corneal blindness is a severe condition affecting millions of people worldwide. Many scaffolds have been designed and tested in animal models both cellular and cell-free strategies. Corneal transplant is a successful strategy and corneal transplant success rate is higher than other body organs since the chance of immune rejection is low because cornea is avascular (Taylor, 2016), however, some limitations can affect this process like risk of viral transmission, scarcity of donors, and small cell yield. Only one cornea was available for every 70 patients needing it (Gain et al., 2016). Corneal tissue engineering regeneration and regenerative membrane aims towards finding a creative and novel plans for patients.

2.7.1 Biomaterials

Tissue-engineered corneal replacement often has a need for biomaterial scaffold system. The basic thing in any scaffold is how this scaffold will be applied to the eye. For limbal stem cell transplantation, a scaffold which can support the cellsis valuable, so the preferable type of scaffold is permeable, thin, and flexible one. The scaffold should also be degradable and when it degrades it should not releaseany cytotoxic or inflammatory materials (Ahearne et al., 2020).

It is essential to decide whether to implant the scaffold or the biomaterial with cells pre-cultured on it or just implant it and give the chance for the patient's cells to repopulate the scaffold. The practical use of the acellular biomaterials is to deliver specific biomolecules which will be helpful in tissue regeneration and repair. For example, the use of amniotic membrane to deliver biomolecules which will be used for inflammation, scarring, and angiogenesis inhibition (Liu et al., 2010).

The physical and chemical properties of the scaffold should not harm thecells. These properties will influence the cell adhesion, migration, proliferation,production of the extracellular matrix (Ahearne et al., 2020). A study by Masterson and Ahearne in 2019 showed that the stiffness of the scaffold is affecting the corneal epithelial cells in relation to the response of corneal epithelial cells to polydimethylsiloxane (PDMS) substrate. Cells on the substrate were shown to adopt the same cobblestone morphology of the epithelial cells (Masterton and Ahearne, 2018).

Cells on the stiffer substrate have higher expression of cytokeratin gene(CK3) which is a marker for mature epithelial cells, the cells on softer substrate expressed cytokeratin 14 (CK14) gene which is a basal epithelial cell marker. The

mechanical properties such as Young's modulus and tensile strength and permeability of the scaffold should be close to the native cornea as much as possible. The modulus of cornea is approximately 100 KPa to 57 MPa. The tensile strength of the cornea is 3-6 MPa (Ahearne et al., 2020).

Real Architecture for 3D tissues (RAFT) is a new technology used to treat corneal disorders (Szebeni et al., 2017). At first there was a use for cellular type I collagen hydrogels as a 3D substrate to create the corneal models, but it is fragile fundamentally because they contain a high proportion of water content. They overcame this problem by blending it with other polymers (Chernikova and Kudryavtsev, 2022), hence a collagen composite was created by blending the collagen hydrogels with polymers. This solution will affect cell seeding ability on topof the scaffold. RAFT process was developed, and it introduced an optimum biomimetic endothelial and epithelial tissue for epithelial and endothelial cells transplantation, andsecondly - to work as a model to study in vitro interaction between cells (Levis et al., 2015). RAFT also has more advantages, co-culture of human limbal epithelial cells (HLE) and corneal stromal stem cells (CSSC) on RAFTis successful with a minimal need for feeder cells derived from animals. RAFT may be a handy tool for transplantation of more than one cell type in a case of ocular diseases to restore the limbal niche (Kureshi et al., 2015) . One of the drawbacks in RAFT is the risk of damaging during movement between vessels. RAFT is commercially available for tissue engineering field with a fast process which takes90 minutes between the setup till generating the RAFT tissue engineering (Levis et al., 2015).

2.7.2 Polymers

Many biomaterials, both natural and synthetic playing a great role recently in the field oftissue regeneration and regenerative medicine by being a part ofthe process of regeneration of the affected body organs by both cells and cell-free therapy via biomolecules transport. Polymers have been used widely in regenerative medicine and tissue engineering due to a large access and availability of polymer selection, and the relatively easyproduction or synthesis of polymers. In addition, different properties of its monomers will give an advantage in the usage of the polymer for various applications. Polymers used in this field are either naturally occurring or synthetic polymers. The naturally occurring polymers are well known as a good biocompatiblematerial and are categorized into protein, hyaluronic acid products, polynucleotides, and polysaccharides. Because of its natural origin there is a risk of initiating an immune response if the material is not pure and if gained any foreign material during the processing stage, due to this reason the use of medical grade natural polymers is essential (Ozdil and Aydin, 2014). Currently, the most well-known and frequently used polymers in many fields are polylactide (PLA), polyglycolide (PGA), polycaprolactone (PCL) and PHA polymers.

2.7.2(a) Poly$_{D,L}$-lactide-co-glycolide (PLGA)

PLGA has been used successfully in the cornea tissue engineering. It is a biodegradable membrane of lactide and glycolide (50:50). Electrospinning technique was used in preparation a PLGA membrane, then an *in vitro* cell culture for the limbal epithelial cells was established. PLGA degrades ex vivo after one to one and half month. The tissue engineered cornea transplanted into the rabbit cornea (Alkali burn rabbit model) and it maintains the regenerative capacity and migration ability

after one year of drying and keeping in low temperature (-20 ºC) (Deshpande, Ramachandran, Sangwan, et al., 2013).

PLGA was used to replace the amniotic membrane due to its low cost and biodegradability (Deshpande, Ramachandran, Sefat, et al., 2013). Using of electrospinning technique is to control the fibre diameter and the thickness of the polymer as well as it is a simple technique. PLGA was tested on rabbit cornea model, and it showed good results, and it will minimize the need of using amniotic membrane. (Deshpande, Ramachandran, Sefat, et al., 2013).

2.7.2(b) Polyhydroxyalkanoate (PHA)

Tissue engineering researchers make use of the characteristics of the nanofibrous scaffolds prepared by electrospinning technique which is very good in stimulation of the protein fibers in the extracellular matrix. PHA used replacement of the damaged corneal tissues. PHA was blended with gelatin to make a nanofibrous scaffold, it increased the adhesion, attachment, proliferation, and increasing the transparency of the cornea. Creation of the corneal epithelium was observed (Datta and Menon, 2019; Kong and Mi, 2016).

PHA was discovered in 1925 by the French scientist Lemoigne who discovered it in a bacterium called Bacillus megaterium in the form of poly (3- hydroxybutyrate) (PHB) (Suzuki et al., 2021). The first described member of this family is Poly-R-3-hydroxybutyrate (PHB) followed by the introduction of many monomers like 3-hydroxyhexanoate (HHx) (McAdam et al., 2020). There are about
150 different PHA monomers which privileged PHA with many different characteristics and extended the applications that PHA could be used in (Lee et al., 2019).

PHA criteria is that it is a nontoxic polymer, biocompatible, biodegradable, elastomeric and thermoplastic (Pulingam et al., 2022). The first reported PHA is P([3HB])- Poly (3-hydroxybutyric). In the 1960s, the hydroxyalkanoates (HA) reported by Rohwedder and Wallen, they reported the 3-hydroxy-valerate and 3-hydroxyhexanoates (3HV and 3HHx) (Koller and Rodríguez-Contreras, 2015).

PHA is classified into three main classes based on the number of the carbon atoms in its side chain; short chain length or scl-PHAs which contains less than 5 carbon atoms, medium chain length or mcl-PHAs and this type contains 5-14 caron atoms and discovered in *P.oleovarans* in 1983; and long chain length or lcl-PHAs which is very rare and contains more 14 carbon atoms (Reddy et al., 2022). PHA is soluble in chloroform, water insoluble and resistant to ultraviolet. Meltingtemperature of PHA is 40 ºC to 180 ºC (Jiang et al., 2018).

PHA is a biopolymer from a family of bio polyesters synthesized from a microbial origin. It is synthesized as an energy and carbon reserve in bacteria and considered as an intracellular carbon and energy storage materials. The main enzyme in the biosynthesis is the PHA synthase. The PHA biosynthesis occurs through three different stages. The initial stage involves a carbon source entering the cell from the surrounding environment, which is conducive for the synthesis of PHA. The routes of carbon entryto the cells are two: either the component diffused into the cell or through the transport system in the cytoplasmic membrane.

Secondly, the conversion of the carbon source into hydroxyacyl coenzyme A which will serve as a substrate for the PHA synthase enzyme. Hydroxyl coenzyme A is a thioester by nature. The last step occurs when the PHA synthase catalyzes the ester bond and the coenzyme A released (Li et al., 2019). Nowadays PHA can be