Prevention of Corneal Limbal Epithelial Cell Death during Collagen Crosslinking and the Pathogenic Mechanisms Involved in Progression of Keratoconus.

THESIS

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by

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CERTIFICATE

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A C K N O W L E D G E M E N T S

There shall be showers of blessing; this is the promise of love; There shall be seasons refreshing, Sent from the Savior above.

There shall be showers of blessing, if we but trust and obey; There shall be seasons refreshing, If we let God have His way.

- Daniel W. Whittle, 1883

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I thank God almighty !!!!!

His trials are not the suffix of your life but the prefix.

God created Heaven and earth, day and night, Land and sea·
Creatures with the breath of life·

He has set trials,
Not to forage your skills·
But to eject your dexterity

He has set trials, neither that you should
Be a ***broken box*** *nor* ***a wrecked ship·***
But to carve yourself as a blissful diamond·

He has set trials,
Not to sit and weep·
But to grow your faith in deep·

-M· Vimalin Jeyalatha

ABSTRACT

Keratoconus is a degenerative ocular disorder where in the corneal curvature becomes cone shaped rather than regular corneal curvature. Keratoconus is commonly reported in the young adult group (19- 25 years) of a population. Hence, the contribution of research in the field of treatment and detecting the exact etiology of keratoconus will aid in designing better management modalities for keratoconus.

Collagen crosslinking (CXL) procedure a treatment modality followed in the management of keratoconus uses UV A radiation. The present study was designed to evaluate the damage of limabal stem cells due to UVA and the ability of polymethylmethacrylate (PMMA) ring in protecting corneal limbal stem cells during CXL. Ten freshly enucleated human cadaveric eyeballs were subjected to the corneal CXL procedure. The cadaveric eye ball was divided into 2 sectors: A and B. Sector A was left unprotected, while sector B was covered by a PMMA shield. Limbal biopsies from both sectors before and after the procedure were analyzed. Each limbal tissue was placed on human amniotic membrane (HAM) to check the cultivability and was subjected to marker studies using reverse transcriptase polymerase chain reaction. Biopsies collected from both sectors before CXL showed outgrowth of cells on human amniotic membrane. Biopsies collected after exposure from sector A showed no growth on HAM while 2 out of the 10 from sector B covered with the PMMA ring did show outgrowth of cells on HAM. The putative stem-cell marker ATP-binding cassette sub-family G member 2 (ABCG2) was negative in all the samples from sector A after CXL and was positive in 2 out of the 10 samples from sector B. The study showed that collagen crosslinking treatment can result in damage to limbal epithelial cells, particularly the stem cells. Covering the limbal region with PMMA ring offered only partial protection to the limbus against the UV rays during the CXL procedure. Experiments were repeated with metal ring in the place of PMMA ring to compare and to determine their protective effect on the corneal limbal stem cells during CXL procedure. Thirty freshly enucleated human cadaveric eyeballs were subjected to a CXL procedure, mimicking the clinical protocol. Limbal biopsies from sectors A and sector B before and after the procedure were analyzed. Each strip of tissue was divided into 3 segments, for cell count of viable cells, for cultivation on human amniotic membrane, and for stem cell and differentiated corneal epithelial

cell marker studies using reverse transcriptase– polymerase chain reaction. There was a statistically significant drop in the mean number of viable cells after CXL in sector A but not in sector B. Biopsies from both sectors before CXL and from sector B after CXL showed good growth on human amniotic membrane. Biopsies from sector A after CXL showed no growth on human amniotic membrane. The putative stem cell marker ABCG2 was absent in all samples and p63 was absent in 3 of 10 samples taken from sector A after CXL. The study showed that the metal ring conferred complete protection to the limbal region.

Apoptosis of stromal cells are considered to play a main role in the progression of keratoconus. A few studies are available on the mediators of apoptosis in keratoconus. Insight into the mediators of apoptosis will result in the development of therapeutic targets that can control the progression of keratoconus. The present study shows two novel mediators of apoptosis involved in the pathogenisis of keratoconus i.e. C- terminal telopeptides and Tumor necrosis factor alpha related apoptosis inducing ligand (TRAIL). Uncontrolled collagen degradation is the characteristics of keratoconus. Degradation of type 1 collagen releases telopeptides. The physiological concentration was obtained by performing ELISA on the tear samples of keratoconus patients. Human primary corneal stromal cells were cultivated and treated with varying concentrations of synthetic telopeptides (3.012µg, 6.125µg, 12.25µg, 12.25µg, 23.5µg, 47µg and 94µg) and incubated for 24 hours, 48 hours and 72 hours. MTT and TUNEL assays were performed following incubation. The difference between the number of viable cells present in the treated and untreated cells were considered for the analysis. Primary corneal stromal cells treated with varying concentrations of synthetic telopeptide at 24 hours and 48 hours had no morphological or apoptotic changes, the viability remained 100%. The percentage viability was altered after 72 hours of incubation with the synthetic telopeptide. Higher concentration (47µg/ml, 94µg/ml) of telopeptide showed considerable decrease in the cell viability ($p<0.05$, *t test*). The results of the study revealed that the synthetic telopeptide does have an apoptotic effect on stromal cells. Similar phenomenon can be observed in keratoconus stromal cells leading to the progression of keratoconus.

To correlate TRAIL mediated apoptosis with the pathogenesis of keratoconus, forty samples of corneal epithelial cells were collected among which 20 were from keratoconus patient and 20 from myopic patients undergoing Epi LASIK treatment (control group). Reverse transcriptase

PCR and Real time PCR was performed to evaluate the expression of TRAIL. The presence of death receptors DR4 and DR5 was demonstrated by indirect immunofluorescence technique on cytospinned corneal epithelial cells and cryostat sections of corneal button from keratoconus patient and a donor. Real time PCR for caspase-8 expression was performed to prove the activated caspase cascade. Statistical analysis of the Real time data was performed using ANOVA. Based on the keratometry readings and corneal thickness, keratoconus eyes were classified as mild, moderate, advanced stages. Reverse transcriptase PCR showed the expression of TRAIL in all the 20 keratoconus epithelial cells whereas the control group did not show the expression of TRAIL gene. Fold change of TRAIL expression was higher in case of patients who were classified under advanced category ($p<0.03$). Keratoconus corneal epithelial cells and the keratocytes showed the expression of DR4, DR5 receptors and TRAIL. But the donor cornea was positive only for the DR4 and negative for DR5 receptor. As the disease progressed the caspase expression was also increased. The level of TRAIL gene expression was found to be increasing as the keratoconus disorder progressed. Hence it is proved that TRAIL can effectively mediate apoptosis in keratoconus corneal epithelial cells through the death receptors and lead to the pathogenic progression of keratoconus.

To conclude, metal ring should be included in the routine CXL procedure to protect the limbal region. Degraded collagen products and over expression of TRAIL has a major role in progression of keratoconus.

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ABCG2	ATP-binding cassette sub-family G member 2
BSA	Bovine serum albumin
CXL	Collagen crosslinking
KC	keratoconus
TAC	Transient amplifying cells
TDC	Terminally differentiated cells
D	Diopters
ECM	Extracellular matrix
HAM	Human amniotic membrane
MMPs	Matrix metallo protinases
RGP	Rigid Gas permeable lenses
INTACS	Intrastromal corneal ring segments
TRAIL	Tumor necrosis factor related apoptosis inducing ligand
TRA	TRAIL receptor agonist
DR	Death receptors
PMMA	Polymethacraylate
UVA	Ultra violet radiation A
DMEM	Dulbecco’s minimal essential media
PBS	Phosphate buffered saline
MEM	Minimal essential media
WBC	White blood cells
RT-PCR	Reverse transcriptase polymerase chain reaction
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
DMEM:F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
DAPI	4',6-diamidino-2-phenylindole

CHAPTER - 1

1. INTRODUCTION:

Keratoconus (KC) is a progressive degenerative disorder of the cornea resulting in corneal thinning. Worsened stromal thinning and weak corneal stroma disturbs the normal corneal curvature leading to a cone shaped cornea. It starts at adolescence and progresses in varying manner [1]. Keratoconus was first described as non- inflammatory disorder and at present described as inflammatory disorder based on their pathophysiology. The condition is usually bilateral. Various etiologies of keratoconus have been described but the exact mechanism of pathogenesis is still under quest.

1.1 History of Keratoconus:

Burchard David Mauchart professor at University of Tübingen, Germany in 1748 was the first to write a report on keratoconus and documented the disorder as **'styphyloma diaphanum'** meaning bulging of the cornea (Figure 1.1). John Nottingham in 1854 differentiated Keratoconus from other corneal ectasias by demonstrating the common signs and symptoms of keratoconus. After the invention of opthalmoscope, in 1859 William Bowman was successful in assessment and in the management of keratoconus. In 1869 Johann Horner named the disorder as **"Keratoconus"** meaning "horn shaped cornea" after which the clinicians tried to remodel the cone shaped cornea to normal corneal curvature [2].

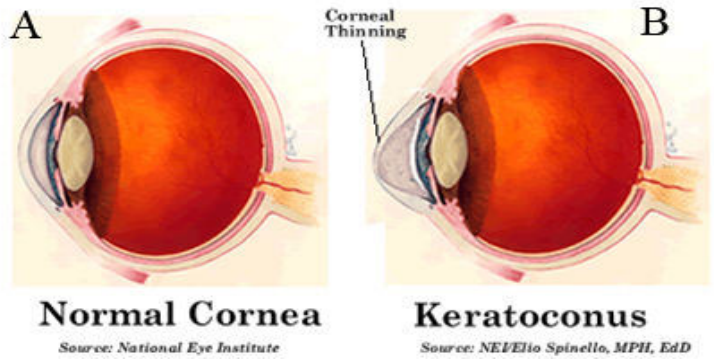


Figure 1.1: Difference between the normal cornea and keratoconus cornea.
A. Normal cornea B. Cone shaped keratoconus cornea due to corneal thinning.

1.2 Epidemiology of Keratoconus:

Keratoconus is found to be prevalent worldwide, affecting both males and females in all ethnic groups. Keratoconus is always bilateral affecting both the eyes and rarely unilateral [3]. The prevalence reports show that 50 – 230 per 100,000 in the general population are affected with keratoconus [4]. Overall distribution of keratoconus is stated to be 1 in 2000 worldwide [5]. The incidence of keratoconus in the Asian population is reported to be high compared to the Caucasian population [6].

1.3 Clinical Presentation of Keratoconus:

Progression of keratoconus results in loss of visual acuity which cannot be corrected like other refractive errors (eg: myopia can be corrected by glasses). Patients present with keratoconus during teenage years with complaint of sudden or gradual visual blur, astigmatism, monocular diplopia and photophobia. Iron deposists called the Fleischer ring or hemosiderin arc is seen within the epithelium near the base of the cone [5]. The stroma of keratoconus patients may present with parallel striations called the Vogt' striae. Munson's sign is one of the key features in advanced keratoconus where in the lower eyelid deforms to V- shape when the eye is positioned downwards. Breakage of the Desemet's membrane leads to oedematous stroma called as Hydrops (Figure 1.2). Corneal thinning and prominent corneal nerves are also observed in advanced cases [7].

1.4 DIAGNOSTIC EVALUATION:

1.4.1 Imaging Studies:

1.4.1.1 Keratometry:

Corneal topography images aids in the grading of keratoconus and helps in differential diagnosis from other corneal disorders. The corneal topography provides an overview of the cornea and the skewness of the cornea (Figure1.3). An uneven separation of the spherical rings inferiorly is observed in the keratoconus corneal topography. The characteristic central tear drop shaped topography called as "keratokyphosis" is also observed [8].

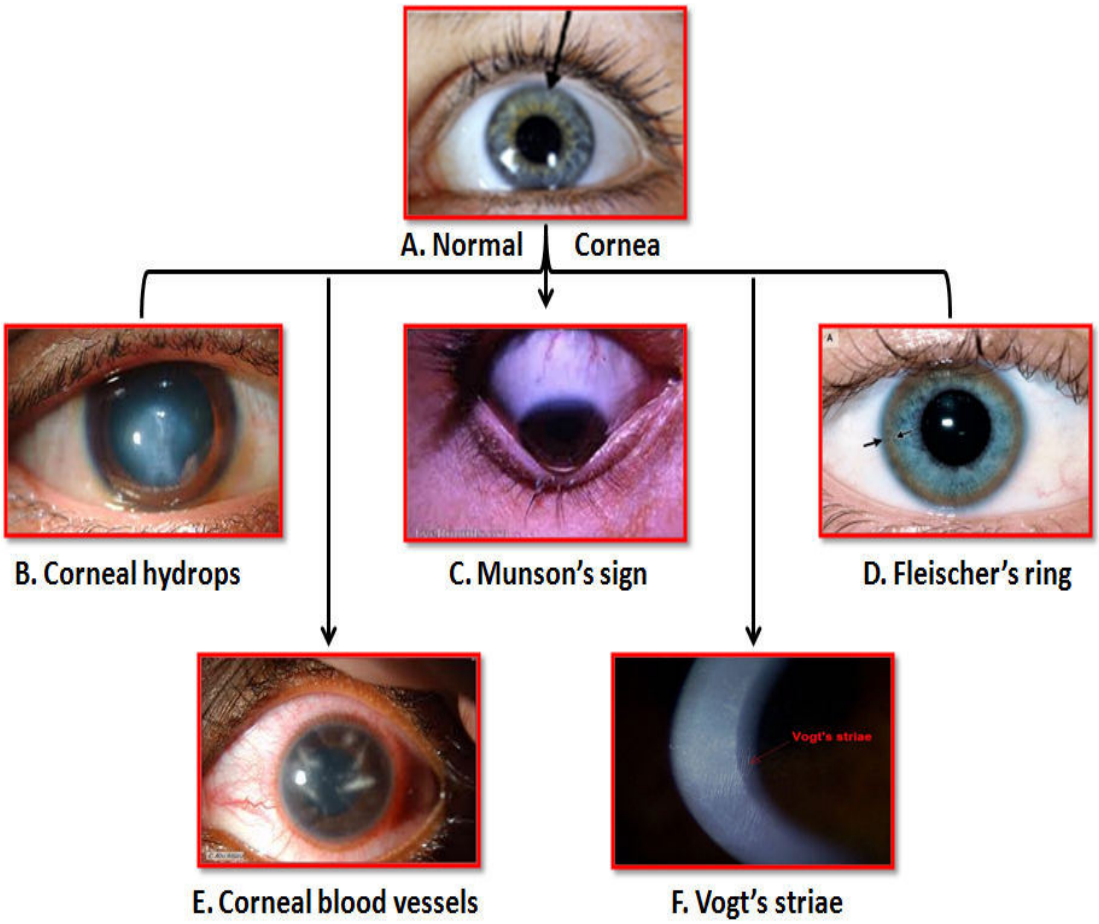


Figure 1.2: Eyes showing the distinct characteristic features of keratoconus. A. Normal cornea, B. Visible white spots in the cornea called hydrops, C. V- shaped lower eyelid called the Munson's sign, D. Iron deposits called the Fleicher's ring, E. prominent corneal blood vessels, F. Parallel striations called the Vogt' striae, (Image courtesy: http://dro.hs.columbia.edu/fleschers.html,http://www.isec.my/pinfo_keratoconus,http://www.clinicavalle.com)

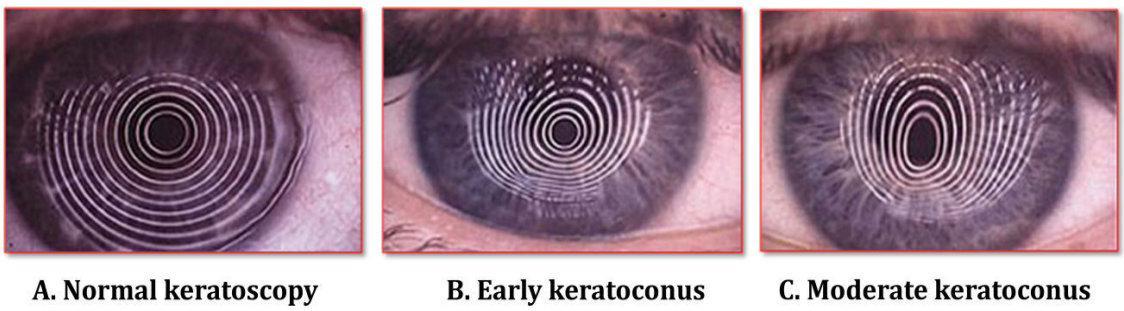


Figure 1.3: Corneal topographic image of normal cornea and keratoconus cornea A. normal cornea showing even separation of concentric rings, B & C Uneven spacing of concentric rings inferiorly showing the steepness of the cornea. (Image courtesy: http://www.yamout.com).

1.4.1.2 Pentacam:

Pentacam is a device which has the capability of imaging the tomography of the anterior segment and the posterior segment of the cornea. Pentacam is more advanced than a keratometer as it images using the pachymetry mapping [9].

1.4.2 Histopathology:

The marked change seen in the keratoconus cornea is the corneal thinning. Histopathological abnormalities are studied in all the layers of the keratoconus cornea. Histopathological studies reveal that the central epithelial thinning is significantly higher in corneas which showed breaks in the Bowman's layer. Discontinuous Bowman's layer and less number of keratocytes beneath the broken Bowman's layer are characteristic features of a keratoconus cornea. The corneal thinning and discontinuous Bowman's layer can be demonstrated using the Haematoxylin eosin staining of the sectioned keratoconus corneal buttons. The iron deposits can be demonstrated using the Prussian blue staining/ Perls test (Figure 1.4) [10].

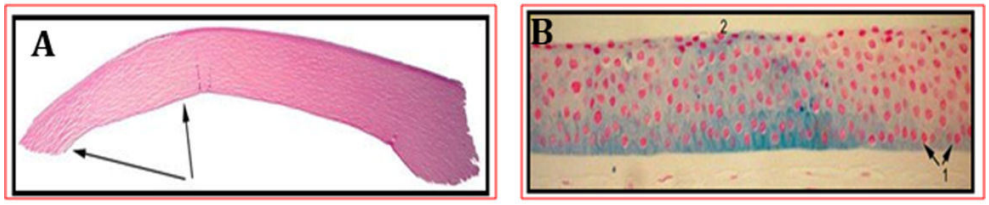


Figure 1.4: **Bisected keratoconus cornea**. A. Haematoxylin eosin staining showing the thinning of the keratoconus cornea. B. Prussian blue staining, the brown oxidised iron deposits within the epithelium. (Image courtesy: www.images.missionforvision.org)

Classification of Keratoconus:

1.4.2 Amsler- Krumeich Classification:

Amsler was the first to classify keratoconus using hand held keratoscope. He classified stages of keratoconus based on the mean K- reading, thickness of the cornea and the refractive error values of the patients (Table 1) [11]

Stage of keratoconus	Parameter for classification
STAGE 1 **MILD**	Eccentric steepening Myopia, induced astigmatism, or both **<5.00 D** Mean central K readings **<48 D**
STAGE 2 **MODERATE**	Myopia, induced astigmatism, or both from **5.00 to 8.00 D** Mean central K readings **<53.00 D**, Absence of scarring Corneal thickness **>400 micron**
STAGE 3 **ADVANCED**	Myopia, induced astigmatism, or both from **8.00 to 10.00 D** Mean central K readings **>53.00 D**, Absence of scarring Corneal thickness **300 – 400 micron**
STAGE 4 **SEVERE**	Refraction not measurable Mean central K readings **>55.00 D**, Central corneal scarring Corneal thickness **< 200 micron**

Table 1.1: Amsler- Krumeich classification of keratoconus.

1.4.3 Morphological patterns of the cone:

Based on the shape of the cone keratoconus can be classified into three types as listed bellow (Figure1.5) [12].

1.4.3.1 Nipple cones:

The nipple cone is 5mm with steep curvature. The cone lies paracentrally and is displaced inferonasally. Nipple cones are easiest to treat with contact lenses.

1.4.3.2 Oval cones:

The diameter of the oval cone is 5-6 mm ellipsoidal. It is called as sagging cone and the apex is displaced inferotemporally. This form of keratoconus is difficult to treat with contact lenses.

1.4.3.3 Globus cones:

The diameter of the globus cone is the largest >6 mm and covers 75 % of the cornea resulting in a generalized corneal thinning is observed. Globus cone are difficult to treat with contact lenses.

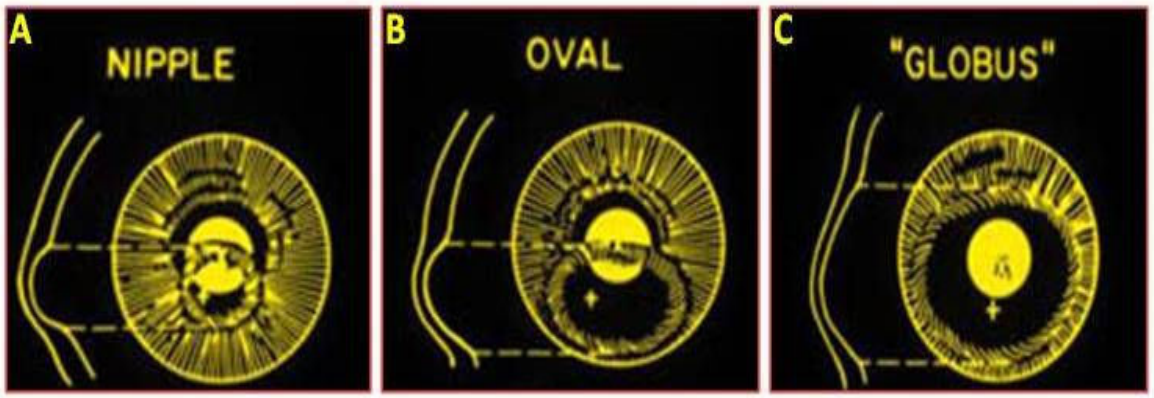


Figure 1.5: Classification of keratoconus based on the shape of the cone.

(Courtesy: http://www.2020mag.com/NYSSO/ViewTest.aspx/LessonId/tHY6RWBXtaU).

1.5 Etiology of Keratoconus:

The etiology of keratoconus is heterogeneous. Various mode of pathogenesis of keratoconus have been studied namely increased levels of enzyme activities, decreased levels of enzyme inhibitor, reactive oxygen species, genetic predisposition, magnesium deficiency, keratocyte apoptosis, eye rubbing and use of poorly fitted contact lenses.

1.5.1 Mechanical Factors:

1.5.1.1 Eye Rubbing:

The incidence of keratoconus due to eye rubbing was found to be 70% and studies show that irreversible damage to the stromal cells is caused by rubbing of the eye and may lead to keratoconus [13]. Keratoconus was also proved mainly associated with mental retardation or allergies. Eye rubbing traumatise the epithelial cells resulting in the release of cytokines. The over expressed cytokines kill the keratocytes leading to corneal thinning. Gritz *etal* has reported the case of a patient who had daily massage of left eye as a ritual which ended up in keratoconus [14]. Thus eye rubbing plays a major role in the etiology of keratoconus.

1.5.1.2 Floppy eyelid syndrome:

Floppy eyelid syndrome, an under diagnosed entity is associated with bilateral keratoconus. The syndrome is characterized by decrease in the elastin content of the eyelid leading to lid laxicity. Ezra *et al* in their case control study showed that floppy eye syndrome is strongly associated with keratoconus [15].

1.5.2 Connective tissue disorders:

Ehlers Danlos syndrome, Marfan syndrome, Lobstin's disease, Mitral valve prolapse are also associated with the etiology of keratoconus [16].

1.5.3 Role of Genetic inheritance:

Genetic inheritance of keratoconus was observed among 6 -18% of patients. Family studies, twin studies and genetic studies were conducted to demonstrate the genetic feature of keratoconus. Single nucleotide polymorphisms are reported in the Genome-

wide association study on case control study using DNA genotyping technology. SOD1 (superoxide dismutase 1), DOCK9 (Dedicator of Cytokinesis 9), VSX1 (Visual System Homeobox 1) are the reported candidate genes associated with keratoconus [17].

1.5.4 Role of inflammatory cytokines and proteolytic enzymes:

Keratoconus was first defined as a non-inflammatory disorder yet studies prove the presence of inflammatory cytokines in the tear film of keratoconus patients [18]. Studies show that the loss of stromal keratocytes occur due to apoptosis induced by interleukein-1, overexpressed in keratoconus cornea. The expression of pro-inflammatory markers interleukin-6, ICAM-1, VCAM-1 are increased 2-40 folds in keratoconus. Decrease in the levels of antioxidants like SOD, glutathione, lactoferrin, IgA, and anti-inflammatory molecule IL-10 are observed in the tears of keratoconus patients. This suggests that the cytokine expression aids in the pathogenic progressive events of the disease (Figure 1.6) [19].

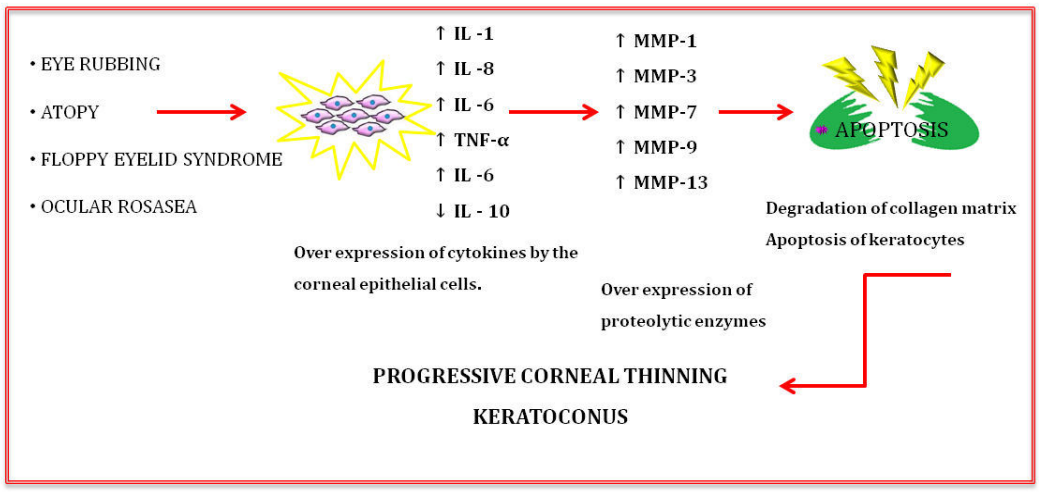


Figure 1.6: Allergic conditions, eye rubbing and ocular surface disorders causes microtrauma in the cornea leading to inflammatory response and there by activates the proteolytic enzymes which degrades the corneal matrix resulting in apoptosis of keratocytes and keratoconus.

1.5.5 Role of Reactive Oxygen Species:

Reactive oxygen species (free radicals) can be produced in the cornea when they are exposed to factors like ultraviolet radiation, poorly fitted contact lenses, smoking and

environmental factors. When the cornea is unable to process the free radicals they accumulate. Thus the toxic byproducts such as peroxynitrite and malondialdehyde leads to the damage of proteins and distrupts the corneal structure and function. Nitrotyrosine a precursor of peroxynitrite was found to be elevated in keratoconus corneas (Figure1.7). Nitrotyrosine elevation can induce the activation of apoptotic cascade. Keratoconus corneas also have elevated levels of toxic aldehydes like malondialdehyde which affects the lysosomal membrane which in turn releases the proteolytic enzymes for the cleavage of collagen matrix [20]. Down regulation of the free radical combating antioxidant enzymes like superoxide dismutase and aldehyde dehydrogenase class 3 also aids in the pathogenesis of keratoconus. Overexpression of cathepsins V/L2 is been observed in the keratoconus corneas. Cathepsins V/L2 is capable of stimulating hydrogen peroxide production. Excessive hydrogen peroxide production enters the lipid peroxidation pathway leading to lysosomal membrane damage and excessive release of cathepsin, thereby the cycle of excessive production of hydrogen peroxide and apoptosis continues. [21].

Various pathogenic mechanisms have been related with the pathogenesis of keratoconus but the exact mechanism behind the progression of keratoconus is yet to be explored.

1.6 Management of Keratoconus:

Management of keratoconus focuses on the correction of visual acuity and to reduce the progression of the disease. Based on the stages of keratoconus the treatment modalities differ.

1.6.1 Contact Lenses:

Spectacles are prescribed when the visual acuity is minimal and manageable. Contact lenses are the immediate option for vision correction when there is moderate irregularity of the cornea. There are different types of lenses to mask the keratoconus cornea. Soft contact lenses, soft-toric contact lenses are advised for the patients with mild or initial stage of keratoconus. Rigid gas permeable (RGP) contact lenses are used for severe cases of keratoconus. Super cone and Rose k are RGP contact lenses which are specially devised for steeper cornea. Newer version of keratoconus contact lenses are Hybrid

lenses which have a RGP centre and a soft periphery which is comfortable over the cornea. Piggy bag contact lenses are used when the RGP lens fitting is not proper. The piggy bag lens has two contact lenses, a RGP lenses is placed over a soft contact lens. Scleral lenses have gained importance in management of keratoconus as they are placed on the sclera and do not touch the cornea and the limbus [22].

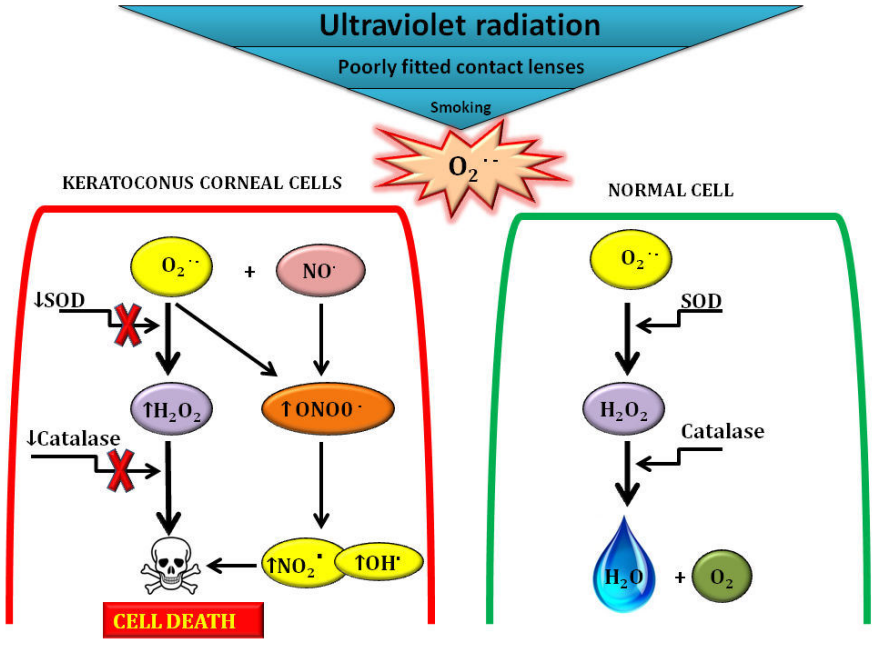


Figure 1.7: Schematic representation of the role of free radicals in the pathogenesis of keratoconus. In the normal cell the reactive oxygen species ($O_2^{\cdot -}$) is converted to hydrogen peroxide (H_2O_2) in the presence of superoxide dismutase (SOD) and H_2O_2 is eliminated as water (H_2O) by the activity of catalase. In case of keratoconus the antioxidant enzymes are downregulated and the oxidants accumulate causing damage to the cells. Reactive oxygen species ($O_2^{\cdot -}$) reacts with nitric oxide ($NO^{\cdot}$) to form peroxynitrite ($ONOO^-$) which accumulates causing cellular damage. Peroxynitrite is unstable and is responsible for the production of hydroxyl radicals ($OH^{\cdot}$) and is potentially toxic to the cells.

1.6.2 Penetrating keratoplasty:

Since many years corneal transplant is the treatment procedure followed for the management of keratoconus. Yet the surgery has limitations of graft rejection, residual

corneal astigmatism and rarely recurrence of keratoconus. Cornea is an avascular structure and is not in communication with the lymphocytes. But corneal graft rejection occurs in 5- 30% of transplant cases. The corneal graft rejection occurs due to various factors like postoperative inflammation and high intraocular pressure.The main reson behind corneal graft rejection is corneal neovascularisation (cornea oppressed by excessive growth of new blood vessels -angiogenesis) [23].

1.6.3 Deep Anterior Lamellar Keratoplasty (DALK):

Lamellar keratoplasty is performed on keratoconus cornea without scarring. In DALK the patient's endothelium is retained and the corneal tissue is substituted with normal donor cornea. Descemet's membrane and the endothelium of the patient is retained thus reduces the risk of graft rejection in DALK [2].

1.6.4 Intrastromal Corneal ring segments (Intacs):

Intrastromal corneal rings are two crescent shaped rings made of polymethacrylate which are used in patients who are uncomfortable with contact lens. Originally Intacs were used for the correction of myopia and was approved by FDA in 1999. In 2004 Intacs were given a Humanitarian Device exemption to be used in reshaping the cornea of keratoconus patients. In 2006 Intacs were approved as management device of keratoconus by FDA. The segments are placed inside the cornea by mechanical and femtolaser assisted tunnels in the cornea. The segments provide mechanical support to the cornea by steepening the periphery and flattening the center cornea. Intacs are considered to be safe in the management of keratoconus. Studies show that visual acuity was improved, the keratometry reading reduced on placement of intacs over the keratoconus cornea. Different modifications of intacs are available namely, Intac SK for treating advanced cases of keratoconus and Ferrara ring reduced astigmatism [23–26].

1.6.5 Collagen cross-linking (CXL):

Collagen crosslinking is the major breakthrough in the management of keratoconus. All the procedures followed in the management of keratoconus do not concentrate in reducing the progression of keratoconus. Collagen crosslinking is the only procedure which targets

the pathophysiology of the disorder. The progression of collagen degradation is studied to play a major role in the progression of the pathogenesis of keratoconus.

The idea of using singlet oxygen for the stromal collagen crosslinking was conceived by Khadem *et al* in 1994 when they worked on activating biological glues using heat and light [27]. In 1991 Kligman *et al* reported pepsin resistant collagen from hairless mice exposed to Ultraviolet-A (UV-A). [28] The procedure was introduced by Professor Theo Seiler and Spörl in 1997 for treating keratoconus. Riboflavin (vitamin B2) is a naturally occurring essential nutrient and a photosensitizer. UVA is capable of inducing crosslinks in collagen but takes a long time hence riboflavin is used as photosensitizer which absorbs UVA and releases singlet oxygen inducing collagen crosslinking. Yet there exist controversies on the exact mechanism involved in collagen crosslinking. A study by Kato *et al* showed that singlet oxygen does not aid in crosslinking instead, sensitised riboflavin induces a photodyanamic modification of collagen leading to aggregation of collagen [29]. Yet there are studies which insist on the role of photo oxidised histidine and lysine in collagen crosslinking [30].

Cornea is composed of 70% of collagen, the balance between the production of proteolytic enzymes and the protease inhibitors are important in maintaining the extracellular matrix of the cornea. But the imunohistochemical studies on keratoconus cornea has demonstrated that there is a considerable decrease in corneal thickness due to the overexpression of proteolytic enzymes and decreased protease inhibitors. Protease inhibitors like α2 macroglobulins, tissue inhibitors of metalloprotinases (TIMP-1) play amajore role in inhibiting the destructive role of the proteolytic enzymes. With the aid of photooxidative crosslinking the loss collagen of the keratoconus cornea can be reverted by inducing covalent bonds between the collagen molecules.

Many studies support the efficacy and safety of collagen crosslinking procedure on keratoconus cornea as the treatment improved visual acuity and improved kertometry reading [31,32]. Recently collagen crosslinking has been used as adjunct therapy with LASIK and keratoplasty. Thus collagen crosslinking of the cornea is considered to be the superior procedure in the management of keratoconus.

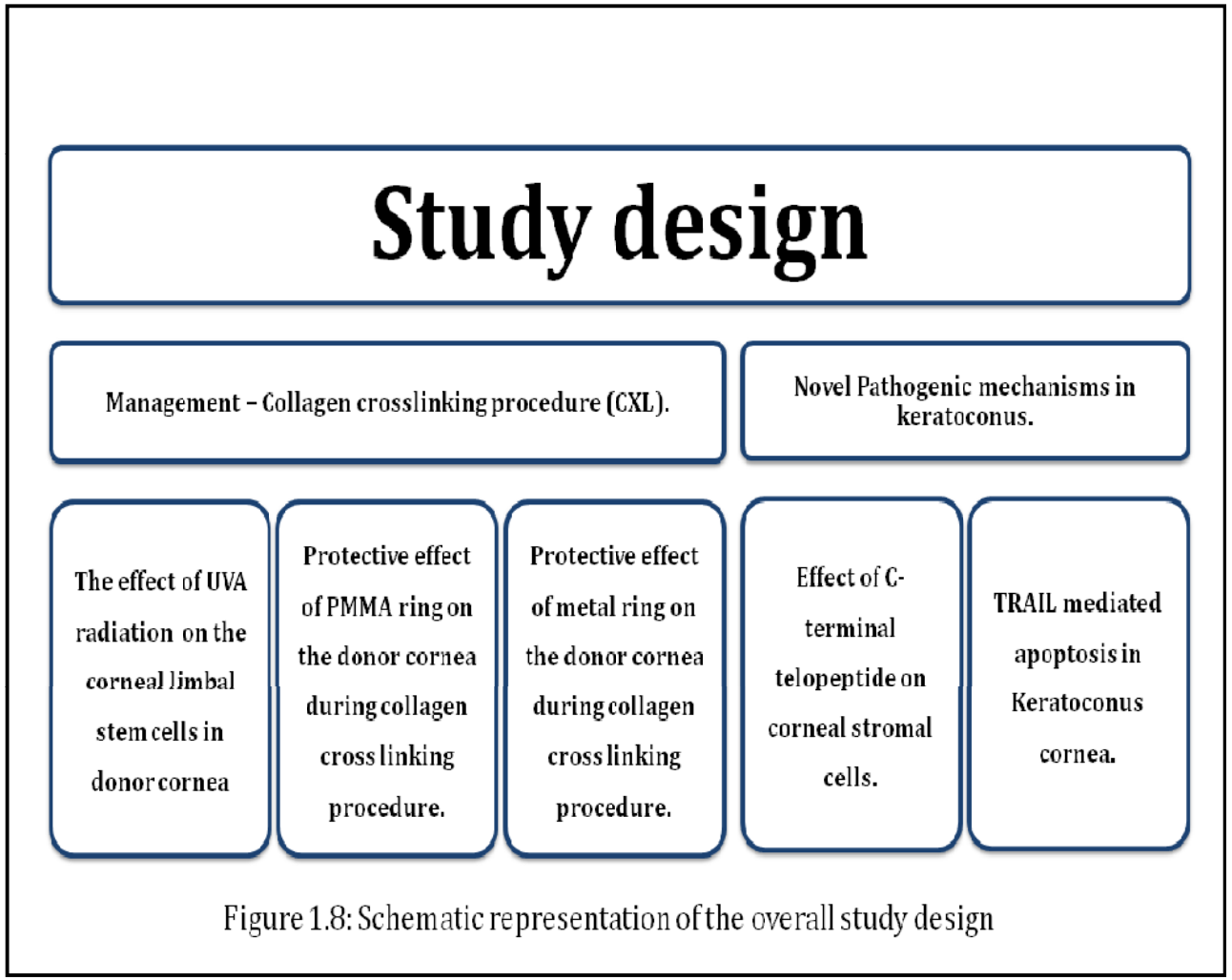


Figure 1.8: Schematic representation of the overall study design

CHAPTER - 2

REVIEW OF LITERATURE

2.1 Collagen cross-linking (CXL) for Keratoconus:

Collagen cross-linking (CXL) is a newer treatment which aims to halt the progression of keratoconus, before it reaches the stage where a graft is required. Over the past decade, corneal collagen crosslinking (CXL) has gained widespread popularity as a technique to confer biomechanical stability to the cornea in eyes with progressive ectatic disorders, such as keratoconus. This procedure is based on increasing the tensile strength of the cornea by cross-linking. Until recently cross-linking was not widely known in ophthalmology, but it is a standard technique used in polymer science, for increasing the mechanical strength of a material. In 1998 Professor Theo Seiler from the Institute for Refractive and Ophthalmic Surgery, Zurich, Switzerland, first introduced the method of cross-linking using UV/riboflavin in Ophthalmology. Laboratory research has not only proved the biomechanical and biochemical benefits of corneal CXL,[33–38] but has also defined the boundaries of safety when it is applied to the human cornea in the clinical setting [39].

The wavelength of UV light, its irradiance and irradiation time, also the concentration of the Riboflavin and the duration for its diffusion into the corneal stroma, were all chosen to ensure that neither the UV irradiation nor the photochemically induced free radicals would damage the corneal endothelium, lens or retina [39]. Based on these requirements, which met the ICNIRP guidelines (International Commission on Non-Ionizing Radiation Protection) [40]. Spoerl *et al* laid down the protocol for the UVA-Riboflavin CXL procedure, often referred to as the Dresden protocol [38]. In brief, it is as follows: the central 9 mm of cornea is debrided of its epithelium; drops of 0.1% Riboflavin in 20% Dextran are instilled onto the cornea every 3 minutes for 30 minutes. The surgeon uses the blue light of a slit lamp to ensure that riboflavin has percolated into the anterior chamber before exposing the cornea to UV irradiation. Using two UVA diodes, a homogenous beam of UVA light with 370$\pm$5nm wavelength, and an irradiance of 3mW/cm^2 is used to irradiate the de-epithelialised 9 mm diameter of cornea for 30minutes. This corresponds to a total dose of 3.4J or a total radiant exposure of

5.4J/cm^2. During the procedure, riboflavin solution is periodically instilled. After the treatment, a bandage contact lens is applied until the epithelial defect has healed. Topical antibiotics and corticosteroid drops are used postoperatively, their dosage and duration generally decided by the clinician. Adopting the prescribed protocol, numerous clinical studies have confirmed not only the efficacy but also the safety of the CXL procedure [41–46].

The ocular tissues vulnerable to the damage of UV radiation are the cornea, lens and retina. The cytotoxicity of the riboflavin-UVA treatment on keratocytes and endothelial cells has been studied [47–49]. Although keratocyte apoptosis has been described in animal and clinical studies, the temporary loss of these cells, before their repopulation which occurs in six months, is not believed to result in any serious consequences. While damage to the corneal endothelium would have far more serious consequences, Wollensak *et al* [49] determined that the clinically applied UV irradiance at the endothelial level was at least a factor of two times smaller than the damage threshold.

The shielding effect of the riboflavin ensures that all structures behind the corneal stroma, including the corneal endothelium, anterior chamber, iris, lens and retina are exposed to a UV radiation far less than the stipulated safety thresholds [39]. The only tissue that might have been at risk of damage is the corneal epithelium. Photokeratitis is a well known consequence of UV exposure, although it is more likely to occur from UVB rather than UVA radiation [50]. Since CXL requires removal of the corneal epithelium, photokeratitis was not considered relevant in the context of a clinical application of the procedure. However, just peripheral to the margin of the denuded cornea, lays the corneo-scleral limbus. The limbal basal epithelial cells are believed to house the progenitor stem cells, which play a crucial role in cellular replacement and corneal epithelial regeneration. Any damage to the function of these cells could result in a plethora of problems ranging from recurrent epithelial erosions to persistent epithelial defects and, in severe stem cell deficiency, to chronic ocular surface inflammation and conjunctivalization of the cornea [51]. In the procedure of CXL, the epithelium overlying the corneo-scleral limbus remains intact. Although, it is the central 7-9mm of the cornea that is directly exposed to the UV radiation, it is quite possible that the limbal epithelium also gets exposed to the UV-A radiation. Eye movements during the 30 minutes of UV-A

exposure, deliberate decentration of the CXL procedure to target the peripheral cornea, lateral diffusion of the superoxide free radicals from the exposed cornea and the lack of a compact collimated UVA beam, are all possible situations which could render the limbal epithelium vulnerable to the possible effects of exposure. The effects of UV radiation on corneal epithelial cells are well documented. Oxidation of riboflavin by UV A radiation synthesises the free radicals which causes many deleterious effects to the cells [52]. UV exposure of 200nm-400nm on rat corneas showed that the UV rays induced inflammatory response, angiogenic vessels and necrosis of the corneal cells [53]. Polymethylmethacrylate (PMMA) is a synthetic polymer extensively used in optical applications. PMMA is highly biocompatible**,** reliable, UV resistance and can be easily manipulated; hence PMMA is widely used for the manufacturing of Intra ocular lenses, contact lenses, bone cements and membrane dialysis [54]. A study to evaluate the ability of PMMA ring and metal ring to protect corneal limbal stem cells during collagen cross-linking can aid in better management of keratoconus.

2.2 Telopeptides in the Pathogenisis of Keratoocnus:

Chronic keratocyte apoptosis is associated with pathogenesis of keratoconus. Alteration of keratocyte morphology and reduction of keratocyte density is evident in keratoconus corneas [55,56]. The loss of keratocytes may be due to the degradation of the extracellular matrix (ECM) or by anoikis mediated cell death in which cells lose their anchorage due to ECM degradation [57]. The effect of degradation and the mediators of ECM degradation is being studied in detail [58] but the effect of the degradation products have not been studied.

The specialized tissue cornea constitutes three functionally distinct layers. The epithelium, endothelium and the collagenous stroma. The cornea is morphologically supported by the collagen composition of the stroma. ECM of the corneal stroma is composed of collagen with proteoglycans. Different layers of cornea constitute different types of collagen -Types I, III, IV, V, VI, VII, and VIII. The distribution of collagen types are not homogenous The main fibril-forming collagens of the adult corneal stromal ECM are types I and V [59]. The collagen fibrils of the corneal stroma are heterotypic fibrils. Type V collagen is involved in initiating fibril assembly [60]. Various factors

contribute to the collagen degradation in keratoconus. Keratoconus mainly involves the uncontrolled degradation of the corneal extracellular matrix which is enhanced by the proteolytic and collagenolytic enzymes [61]. Corneas from patients with keratoconus showed alterations of the extracellular matrix and basement membrane. Keratoconus corneas were characterized by a decrease in type I and IV collagen concentrations and an increase in expression of collagen IX. Studies proved that uncontrolled processes of degradation result in collagenolysis leading to stromal thinning [62]. Stromal fibroblasts are prone to apoptosis via interleukin-1 leading to stromal thinning and keratoconus. Inflammatory cytokines IL-6 and TNF-α are found to be elevated in keratoconus corneas. Eye rubbing is suggested to be the main cause for the activatioin of these inflammatory mediators. TNF- α contributes to the elevation of matrix metalloproteinases (MMPs) thereby resulting in the degradation of the stromal collagen. Chronic inflammatory events mediate the upregulation of MMP-9, which causes ECM degradation and this process is strongly suspected to be the reason for the progression of keratoconus [63]. Cathepsins are cysteine proteases found in the corneal epithelial basement membrane [64]. Since keratoconus shows a characteristic digested basement membrane and Bowman's layer cathepsins are considered to be involved in the progression of the ectasia.

The homogeneity of the stromal collagen type varies for each corneal layer and 75% of the cornea is composed of type 1 collagen. Type 1 collagen is composed of two α1 chains and one α2 chain, forming a continuous triple helical structure with non- helical telopeptides at both the N-terminal and the C-terminal. C-telopeptides corresponds to the C-terminus region of the alpha-1 chain of type 1 collagen [65]. Collagenolytic enzymes act on the native collagen to release the terminal telopeptides (Figure 2.1). The terminal telopeptides are involved in the formation of covalent crosslinks between the monomeric collagen molecules. The removal of telopeptides during collagen degradation reduces the strength of the collagen. MMPs and cathepsin K are capable of degrading the triple helix of the collagen but cathepsins L and B are capable of cleaving the telopeptide region of the collagen [66]. Degradation of collagen during physiological turnover or during excess proteolytic activity leads to the release of telopeptides into the plasma [58,67]. Presence of C-telopeptide indicates the collagen degradation and it is also used as biomarker in heart diseases.

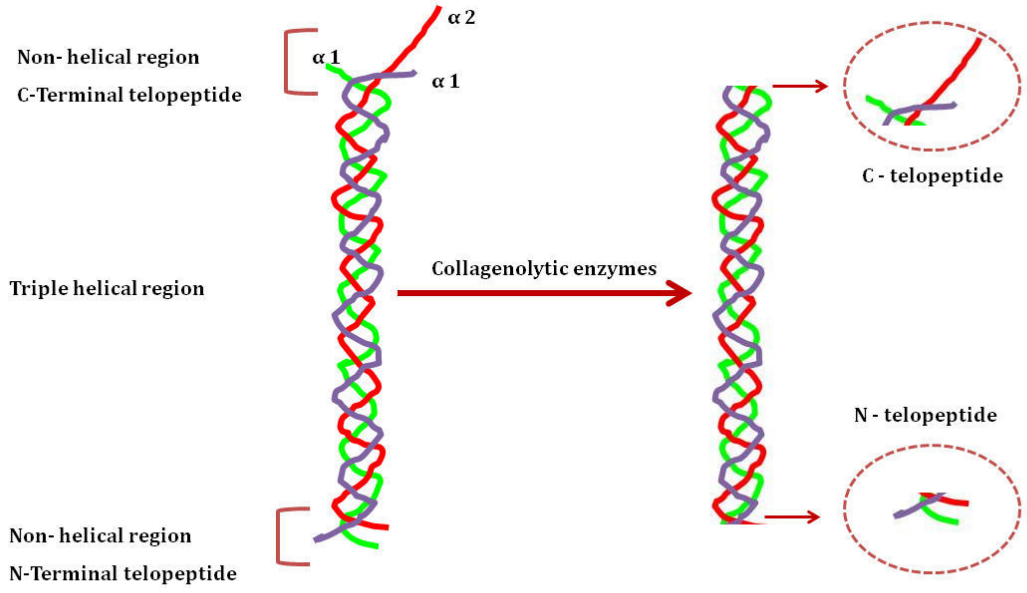


Figure 2.1: Telopeptide formation during collagen degradation by collagenase.

Only few studies are available discussing the role of collagen degradation products *in vivo*. Studies show that the collagen degradation products capable of activating calpain mediated apoptosis in the human vascular smooth muscles [68]. According to Abalain *et al* C- terminal telopeptides in tear of keratoconus patients are considered as the biomarker for the follow up of keratoconus patients [69]. The pathogenic role of these telopeptide on the stromal keratocytes is still unknown.

2.3 Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL) mediated apoptosis:

As discussed previously (Section 1.5), extensive clinical studies have been undertaken to explore the pathogenesis of keratoconus and have shown that misfit contact lens and eye rubbing are the main environmental factors [5, 70]. Keratoconus is also related to the expression levels of genes like VSX1, SOD1, TGFB1, COL4A3/COL4A4, HGF, LOX, and DOCK9 [71]. All the layers of corneal, epithelium stroma, endothelium undergo apoptosis and among these three layers epithelial layer is demonstrated to have more apoptotic cells [72]. Recent studies by You *etal* showed the association of secreted frizzled-related protein (SFRP1) in the keratoconic corneal epithelium and stated its

significant association with the pathogenic progression of keratoconus as SFRP1 is involved in the apoptotic pathway [73]. Progressive Epithelial damage leads to destruction of the underlying stromal cells as they have strong interaction between them [74].Thus it is necessary to study the important mediators of apoptosis which will aid in better understanding of the pathogenesis involved in keratoconus. The present study was proposed to elucidate the expression of TRAIL in keratoconus corneal epithelial cells and its role in the progressive pathogenesis of keratoconus through TRAIL mediated apoptosis.

The apoptotic pathway is composed of extrinsic and intrinsic pathway. The extrinsic pathway is through the death receptors. TRAIL or apo-2 ligand is a member of TNF family. TRAIL acts as mediator in the apoptosis of cancer cells. TRAIL is a type –II transmembrane protein released by the immune cells. Removal of the transmembrane and intracellular domain results in the release of soluble active TRAIL. TRAIL mediates apoptosis through the Death receptors DR4, DR5 [75]. TRAIL mediates apoptosis by binding to the DR4, DR5 resulting in oligomerisation and activation of Fas ligand which recruits Fas-Associated protein with Death Domain (FADD) (Figure 2.2). The caspase cascade is triggered and thereby leading to apoptosis of the cells [76]. The patterns of expression of TRAIL and the TRAIL receptors vary among the different tissues [77]. Normal tissues of the cerebellum, spine and liver were found to show strong positivity of the death receptors and weak positivity of the decoy receptors [78]. Up regulation of TRAIL receptors DR4, DR5 and the downregulation of decoy receptors lead to death of post ischemic brain cells and the vice versa lead to the survival of cells in the preconditioned brain [79]. When TRAIL binds to death receptors of the normal cells the activation of caspase cascade is inhibited by the increased expression of FLICE- like inhibitory protein (FLIP) which interrupts the recruitment and the cleavage of the initiator caspase. (Figure 2.2) Thus the normal cell acquire resistance to the TRAIL mediated apoptosis [80]. In 1975 tumor necrosis factor (TNF) was considered as target for cancer therapy. But later it was proved that the function of TNF was to aid in the production of proinflammatory factors which would be more deleterious. In later years TRAIL was discovered as it had sequence homology with TNF and effectively caused apoptosis of the cancer cells. TRAIL and TRAIL receptor agonists were used as cancer therapeutic agents.

Different TRAIL receptor agonists (TRA) have been designed and are under different phases of clinical trials. (Figure 2.3) TRAIL and the receptors are used as successful targets for cancer treatment.

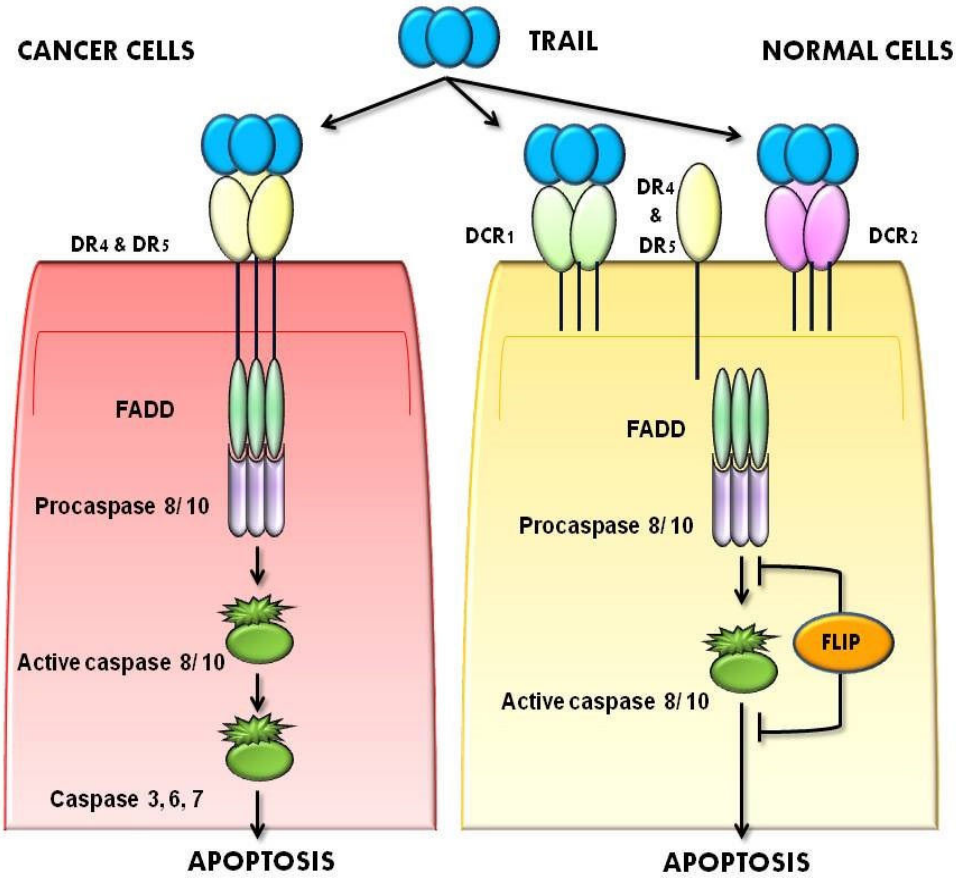


Figure 2.2: Schematic representation of the role of TRAIL and death receptors in the apoptosis of cancer cells. Normal cells do not express the death receptors DR4, DR5 but express the decoy receptors DR1, DR2 which is incapable of inducing apoptosis as they have truncated cytopasmic domain. Cancer cells over express the TRAIL receptors thus when TRAIL binds to these receptors Fas-Associated protein with Death Domain (FADD) mediated caspase cascade is proceed leading to apoptosis.

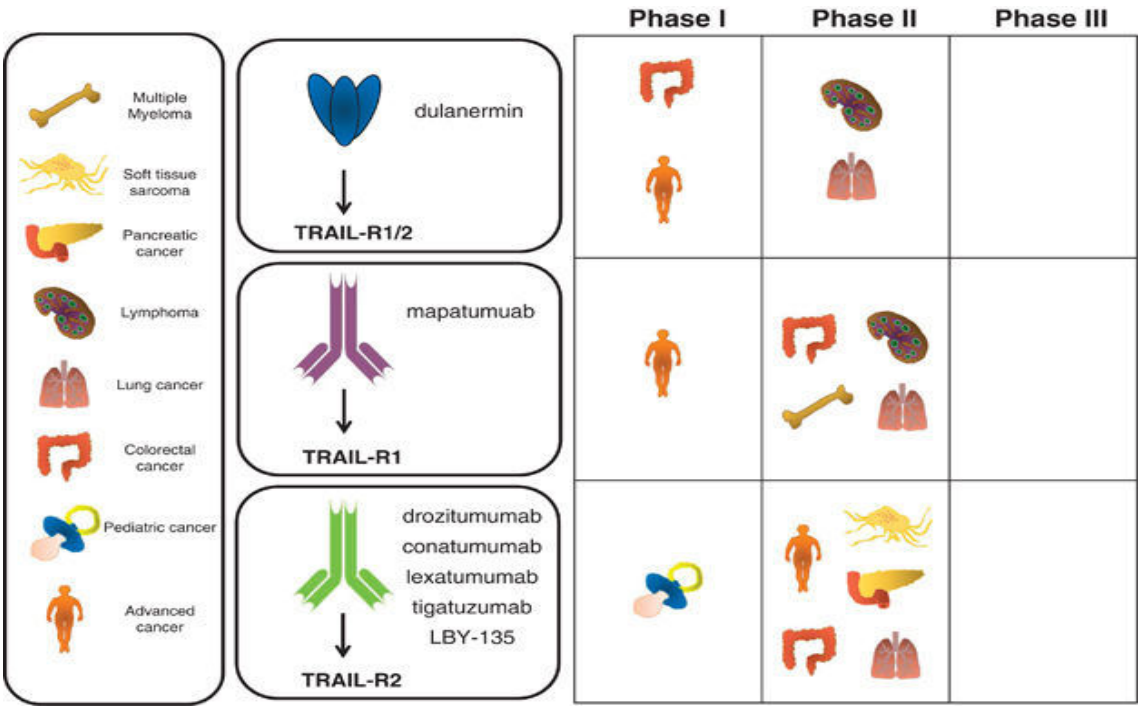


Figure 2.3: Progress of TRA in clinical trials. Schematic representation of the different TRA for which results of clinical trials have been reported. Cancer entities in which the different TRAs have been tested and the respective phase of clinical testing are shown. TRAs have entered succefully into the phase III clinical trial (Lemke *et al*. Getting TRAIL back on track for cancer therapy. Cell Death and Differentiation.2014: 21, 1350–1364).

2.4 GAPS IN EXISTING RESEARCH:

Studies showing the effect of UVA/Riboflavin on keratocytes has been reported in detail but the effect of the same on limbal stem cells are not studied. The study results will help in improving the preventive measure that needs to be taken while performing the procedure in case if the procedure results in limbal stem cell death. PMMA is a material which is widely used to manufacture intraocular lenses. If PMMA ring or the metal can prevent the UVA damage of limbal stem cell then this can result in better management of keratoconus. To the best of our knowledge, there has been no published study that investigates or reports the use of PMMA ring or the metal ring during collagen cross linking procedure.

Till date studies stress only on the over expression of cytokines, MMPs, cathepsins and the degradation of the stromal collagen by these proteolytic enzymes in the pathogenesis of keratoconus. The effect of the collagen degradation products on the stromal cells are not yet explored.

Though the TRAIL death receptors are associated with apoptosis, their distributions in the normal cornea are not reported. The expression levels of TRAIL in normal cornea or the keratoconus cornea and their link with the progression of keratoconus have not been studied yet. Exploring pathogenic mechanisms in keratoconus will lead to newer therapeutic approaches towards keratoconus.

2.5 HYPOTHESIS:

- The CXL treatment involving riboflavin may lead to the loss of limbal stem cells resulting in limbal stem cell deficiency. On covering the limabal region either with a PMMA or a metal ring during collagen crosslinking procedure may protect the limbal stem cell.

- C- terminal telopeptides released due to excessive collagen degradation can lead to apoptosis of corneal stromal cells in keratoconus cornea.

- Over expression of TRAIL and the presence of death receptors in the keratoconus cornea lead to caspase mediated apoptosis of stromal cells leading to the progression of keratoconus.

2.6 OBJECTIVES:

2.6.1 The effect of Collagen crosslinking on the corneal limbal stem cells in donor cornea before and after exposure to UVA radiation and on covering with PMMA and metal ring

- To determine the cultivability of corneal limbal stem cells of the riboflavin treated donor cornea before and after exposure to UVA radiation and on covering with PMMA ring and metal ring.
- To demonstrate the expression of presumed corneal Limbal stem cell markers by Reverse transcriptase-Polymerase chain reaction on Riboflavin treated donor cornea before and after exposure to UVA radiation and on covering with PMMA ring and metal ring.
- To quantify the percentage of viable corneal limbal cells by trypan blue exclusion method on riboflavin treated donor cornea before and after exposure to UVA radiation and on covering with metal ring.

2.6.2 Apoptosis of corneal stromal cells induced by telopeptides: an in *vitro* study

1. To determine the concentration of telopeptides in tears of keratoconus patients.
    - Collection of tears from keratoconus patients and normal subjects.
    - To perform ELISA for detection of telopeptides on tear samples collected from keratoconus patients.
    - Comparative analysis and correlation of telopeptide concentration with clinical condition of the patients.
2. To determine the effect of synthetic telopeptide peptides on the corneal stromal cells *in vitro.*
    - To establish primary cultures of corneal stromal cells from donor cornea.
    - To assess the percentage cell viability of primary stromal cells treated with synthetic telopeptides by performing MTT assay.

- To determine the apoptotic effect of telopeptides in primary stromal cells by calorimetric and fluorescence TUNEL assay.

2.6.3 TRAIL expression in Keratoconus Corneal Epithelium – A novel mechanism in the pathogenesis of keratoconus:

- To demonstrate the expression levels of TRAIL in Keratoconus corneal epithelial cells and control corneal epithelial cells by Reverse transcriptase PCR and Real time PCR.
- To demonstrate the expression of Death receptors (DR4, DR5) by immunofluorescence staining.
- To correlate the expression levels of TRAIL in keratoconus corneal epithelial cells with the keratometry reading and the corneal thickness of keratoconus patients.
- To demonstrate the expression levels of caspase -8 by in Keratoconus corneal epithelial cells and control corneal epithelial cells by Real time PCR.

CHAPTER - 3

MATERIALS AND METHODS

The studies undertaken where approved by the Institutes Review Board in accordance with the declaration of Helsinki. Informed consent was obtained from each person before performing the related procedures.

3.1 The effect of UVA radiation (370nm) on the corneal limbal stem cells in donor cornea before and after exposure to UVA radiation and on covering with PMMA and metal ring.

A total of forty cadaveric eyes were used for the study. Ten cadaveric eye balls were used for the study with PMMA ring (Figure 3.1) and 30 cadaveric eyes were used for the study with metal ring.

3.1.1 Collection of the Limbal biopsy:

The eyeballs were transported from the Eye Bank in moist chambers maintained at $4^{o}C$. A marking pen was used to divide the cornea into 2 halves- sector A and sector B (Figure 3.2). Under the operating microscope, limbal biopsies extending across one O' clock hour meridian were taken from each hemisphere and immediately transferred into labelled vials containing 3% Dulbecco's Minimum Essential Eagle's medium (DMEM) with 10 microgram concentration of ciprofloxacin and gentamycin.

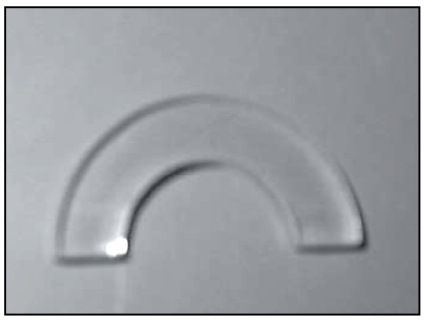


Figure 3.1 Polymethylmethacrylate (PMMA) hemi annulus ring.

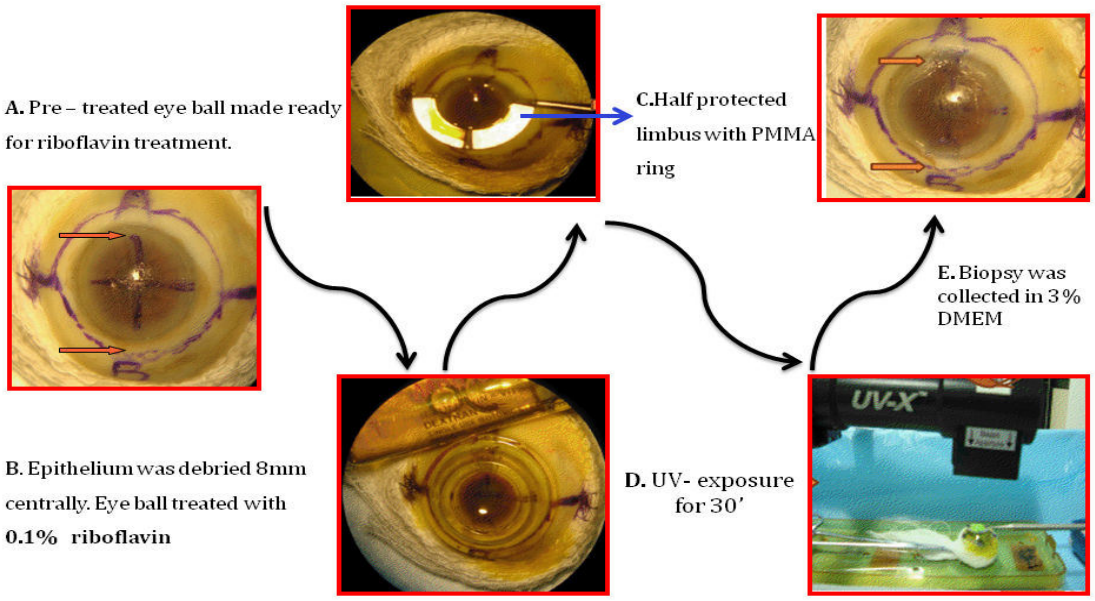


Figure 3.2 : Exposure of cadaveric eye ball to Dresden protocol and collection of limbal biopsy.

The cornea was then subjected to the UVA-Riboflavin treatment, following the Dresden protocol exactly as it would be done in clinical setting for treatment. In brief, after the central 8 mm of corneal epithelial was mechanically scraped off, 0.1% Riboflavin in 20% Dextran was instilled every 2-3 minutes for 30 minutes followed by UVA exposure at an irradiance of 3mW/cm^2 for 30 minutes. During the UVA exposure, however, the limbus of side B was covered by a metal hemi-annulus PMMA/ metal ring that was wide enough to cover the corneo-scleral limbus but not to obstruct the UV radiation to the de-epithelialized cornea (Figure 3.2). At the end of 30 minutes, limbal biopsies were taken from diametrically opposite sites from sectors A & B, extending across one clock meridian each, transferred into separately labelled vials of 3% DMEM with antibiotics and transported to the laboratory. The biopsies were processed further to assess the cultivability and detection of presumed stem cell markers by reverse transcriptase PCR.

3.1.2 Viable cell count estimation:

The tissue was digested with 1% trypsin solution (Hi-Media, Mumbai, India) for 20 minutes. The cells were washed in MEM and centrifuged at 3000rpm for 5 minutes. The cell pellet was reconstituted in 1ml of phosphate buffered saline (PBS). Hundred microliters of the cell pellet was mixed with 900 µls of 1% trypan blue solution. The mixture was incubated at room temperature for 30 minutes. At the end of incubation period, 10µl of the cells were loaded onto Neubaur chamber. Cells that had taken up the trypan blue staining and the unstained cells in the 4 squares were counted (Figure 3.3). Total number of cells was estimated by applying the formula given below.

1. Total number of viable (unstained) cells = Average number of unstained cells counted in four WBC Squares **X** dilution factor **X** depth of the chamber.

2. Total number of non-viable (stained) cells = Average number of stained cells counted in four WBC squares **X** dilution factor **X** depth of the chamber.

The viable and non-viable cells were expressed as a percentage of the total number of cells counted.

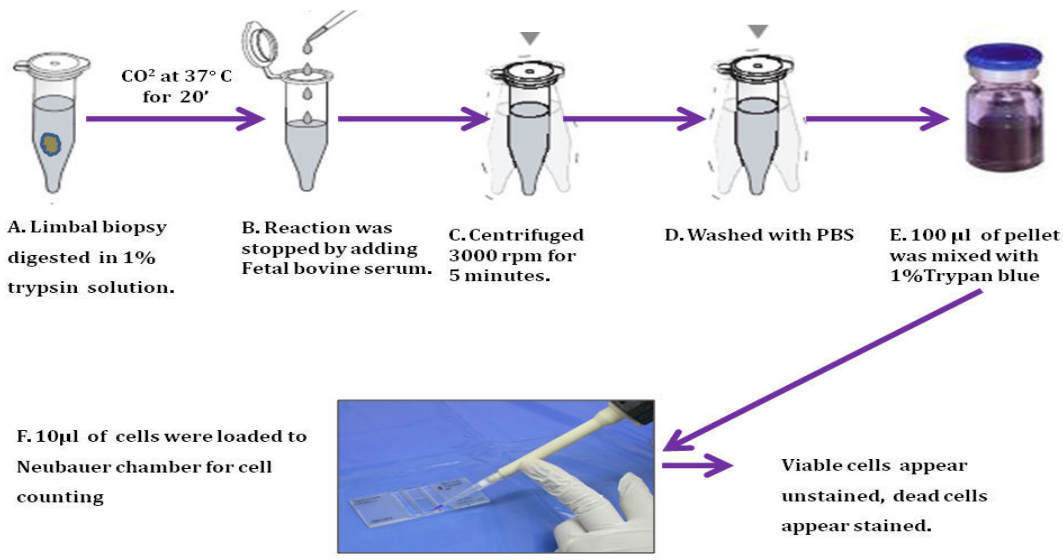


Figure 3.3: Schematic representation of trypan blue exclusion method.

3.1.3 Cultivation of the Limbal Epithelial Cells:

3.1.3.1 Preparation of the denuded amniotic membrane (AM):

- The amniotic membrane (AM) was received after obtaining the consent of the donor.
- The AM was obtained under sterile conditions following elective caesarian section.
- The collected placenta was placed in a sterile stainless steel 12-inch diameter basin, covered with a sterile lid.
- The AM was dissected out from the placenta. The chorion was completely peeled out and was transported in a wide mouth 125 ml screw-capped reagent bottle containing 50 ml transport medium.
- Commercially available Eagles' minimum essential medium (EMEM) supplemented with 3.3% L-glutamine and antibiotics (50 µg/ml gentamicin, 100 units/ml penicillin, 200 µg/ml ciprofloxacin and 1 mg/ml Amphotericin B) was used as transport medium.
- The membrane was immediately transported to the laboratory.
- Under the biosafety cabinet the blood clots and the remaining chorion were removed from the AM.
- The epithelial cells were removed by placing the AM in 1% trypsin solution for 45 minutes.
- With a cell scraper the epithelial cells were scraped out.
- The AM was washed simultaneously while scraping the epithelial cells.
- Then the denuded AM was then placed over individually sterilized 0.22 µm nitrocellulose membranes of 25 mm size without fold or tears.
- AM adhered to the filter membrane was transferred to 50-ml wide mouthed screw-capped irradiated transparent plastic bottles filled with presevarive medium.
- The preservative medium consist of 1:1 (vol/vol) ratio of sterile glycerol (sterilized by autoclave) and Eagles' minimum essential medium (EMEM) with 3.3% L-glutamine, 25 µg/ml gentamicin, 50 units / ml penicillin, 100 µg/ml ciprofloxacin and 0.5 mg/ ml Amphotericin B.
- The AMs in the preservative medium was stored at -80°C for 6 months.

- The AM was thawed by keeping the bottle either at 4°C for 30 minutes or at room temperature for 10 minutes prior use(cultivation limbal stem cells).

3.1.3.2 Explant culture:

Plastic rings of 20mm diameter were individually packed and were sterilized by ethylene trioxide sterilization. The AM (stored at -80°C was thawed and then used) was separated from the nitrocellulose filter membranes and was washed three times with sterile PBS. The washed amniotic membrane was spread out neatly over the sterile rings (Figure 3.4). Limbal biopsies taken before and after UV treatment were grown over denuded AM in tissue culture plate. GIBCO® Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) with Penicillin (150units/ml), Streptomycin(0.75%), Ciprofloxacin (0.1%).

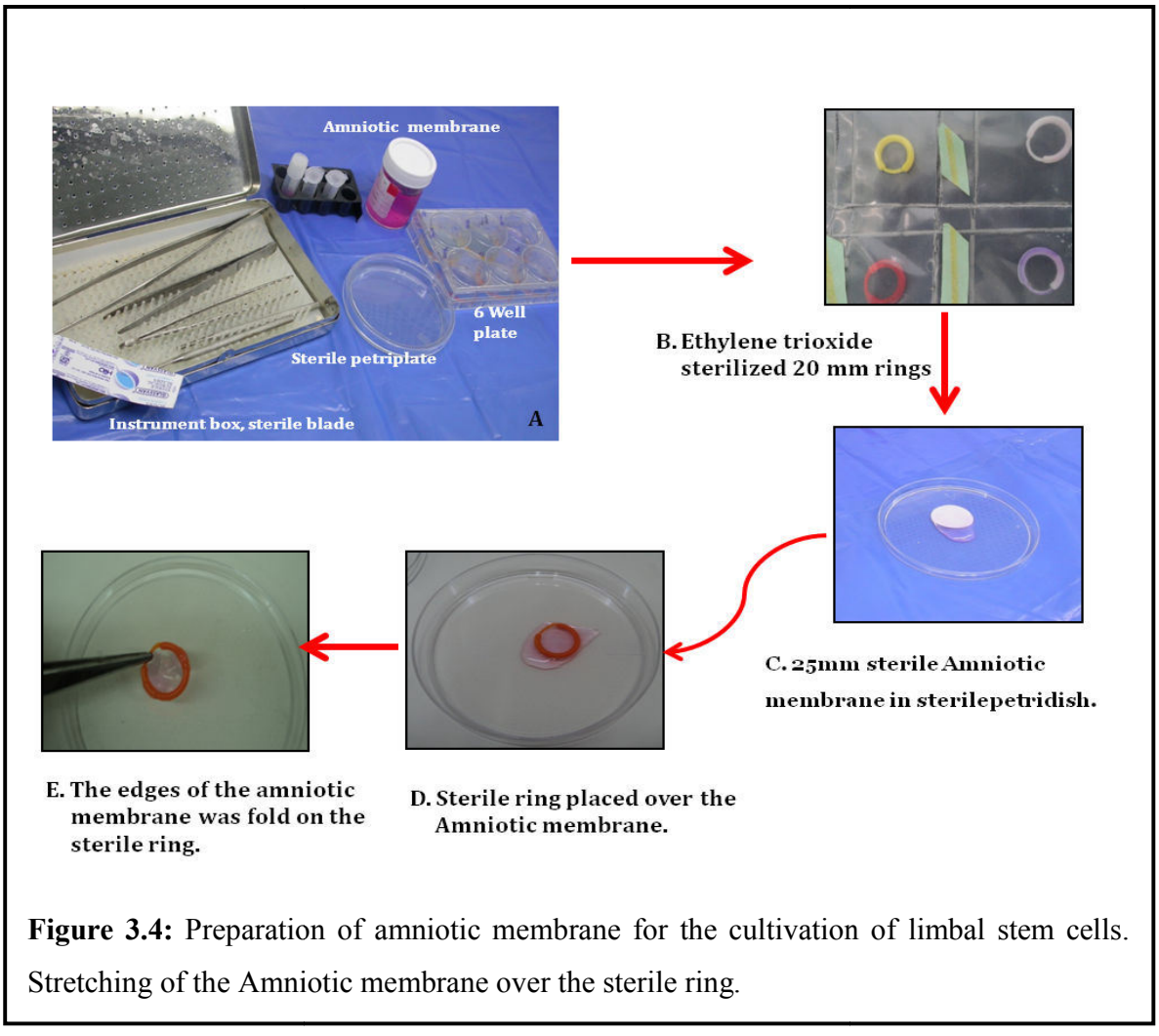


Figure 3.4: Preparation of amniotic membrane for the cultivation of limbal stem cells. Stretching of the Amniotic membrane over the sterile ring.

The limbal biopsy was cut into small bits with sterile blades and then placed on the denuded HAM. The HAM with the tissue bits was placed in the incubator for 10 minutes so that the biopsy bits adhere firmly to the amniotic membrane. About 0.5 ml GIBCO® Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) was added slowly along the sides of the well (so that the tissue bits were not disturbed) followed by 50 µl of heat inactivated FBS (10% FBS).The culture plate was kept for incubation at 37ºC in CO_2 incubator. (Figure 3.5) About 0.5 ml of fresh DMEM- F12 mix along with FBS was added the next day. The medium was changed every third day and the growth of cells was monitored daily under the phase contrast microscope for 21 days.

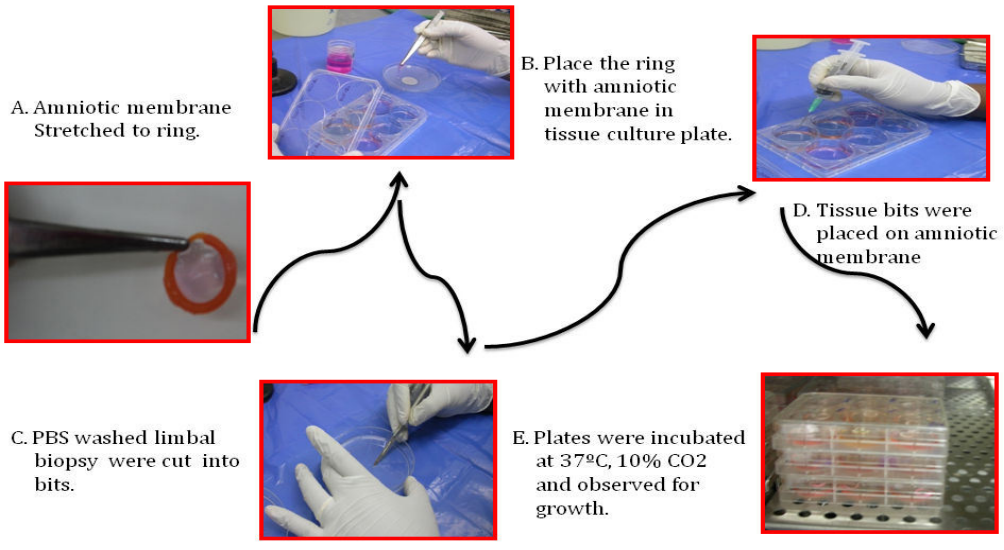


Figure 3.5: Schematic representation of the explant culture of limbal biopsy.

3.1.4 Reverse transcription-polymerase chain reactions (RT-PCR) for limbal stem cell markers:

Total RNA was isolated from all the biopsy tissues to study the expression of various presumed corneal Limbal stem cell markers and corneal epithelial differentiation markers by RT-PCR. The biopsies were collected in TRIZOL reagent (Ambion, Applied Bio-systems, USA). Total RNA was extracted by QIAGEN® RNeasy® Kits (Hilden, Germany) according to the manufacturer's recommended protocol and stored at -80^{o}C until use. Reverse transcription was performed using sensiscript reverse transcriptase

(Qiagen, Hilden, Germany), PCR amplifications of the synthesized cDNAs were performed using specific primer sets with Housekeeping gene, glyceraldehyde-3 phosphate dehydrogenase (GAPDH) as internal control. Following were the markers studied: ΔNp63, ABCG2, Connexin 43, K3 and K12, vimentin, involucrin (Table 4.1). PCR products were fractionated by electrophoresis using 2% agarose gel containing 0.5% ethidium bromide.

Gene	**Primer sequences 5'-3'**	**Annealing temp. ^{0}C**	**PCR product size**
ΔNp63	FP: CAGACTCAATTTAGTGAG RP: AGCTCATGGTTGGGGCAC	54^{0}C	440 bp
ABCG-2	FP: AGTTCCATGGCACTGGCCATA RP: TCAGGTAGGCAATTGTGAAGG	62^{0}C	379 bp
Connexin 43	FP: CCTTCTTGCTGATCCAGTGGTAC RP: ACCAAGGACACCACCAGCAT	61^{0}C	154 bp
K3	FP: GGCAGAGATCGAGGGTCTC RP: GTCATCCTTCGCCTGCTGTAG	47^{0}C	145 bp
K12	FP:CATGAAGAAGAACCACGAGGATG RP: TCTGCTCAGCGATGGTTTCA	61^{0}C	150bp
GAPDH	FP: GCCAAGGTCATCCATGACAAC RP: GTCCACCACCCTGTTGCTGTA	63^{0}C	498 bp
Vimentin	FP: CCCTCACCTGTGAAGTGGAT RP: TCCAGCAGCTTCCTGTAGGT	57^{0}C	349bp
Involucrin	FP: GGACTGCCTGAGCAAGAATGTG RP: TAAGCTGCTGCTCTGGGTTT	61^{0}C	248bp

Table 3.1: Primer sequence and reaction condition for the reverse transcription PCR. [82]

3.2 Apoptosis of Corneal Stromal Cells Induced by Telopeptides: an *invitro* Study

3.2.1 Subjects recruited for the study:

Tear samples were collected from 20 patients with keratoconus (mean age, 20 ± 4 years; range, 11–70) and from 20 human control subjects (mean age, 25.0 ± 3 years; range, 25 - 28) keratoconus patients with a defined clinical diagnosis of keratoconus based on slit-lamp examination, keratometry readings and videotopographic changes were included in the study. The control group included the normal sujects. Patients with active inflammation, other corneal disorders and patients with history of ocular surgery were excluded.

3.2.2 Collection of tear samples:

The tear samples were collected from the inferior tear lake using graduated sterile schirmer strips (Madhu Instruments, India) at the lower lid margin before the administering any topical drops. The collection was performed with as little stimulation as possible. Tear collection was stopped when the Schirmer was wetted till 15 mm which is approximately 10 µl. The tear strips were placed into a sterile 2-ml centrifuge tube, stored on ice for 20 minutes to 1 hour, and then stored at −20 °C until further processed.

3.2.3 Quantification of collagen degradation product:

The concentration of the C- terminal teloptide was analysed by Enzyme Linked Imunosorbent assay using Human Crosslaps (Cr) ELISA kit obtained from Cusabio® (China). The stored Schirmer strips were thawed, 200 µl of PBS with 1%BSA was added to each 2ml centrifuge tube and incubated for 4 hours in room temperature. The strips were removed from the tubes and the samples were processed further following the manufacturer's instructions.

3.2.3.1 Assay procedure:

- All the reagents and the assay plates were placed in room temperature for an hour. 50µl of Standards (known standards provided in the kit ranging from 125ng –

2000ng/ml) and Samples were added per well. The tests were performed in duplicates. A blank well was set without any solution.

- 50µl of HRP-conjugate was added to each well (not to blank well) and was mixed well and incubated for 60 minutes at 37°C.
- The wells were washed twice with 200 µl wash buffer using an ELISA washer and the plate was blot dried using paper towel.
- 50µl of Substrate A and 50µl of Substrate B was added to each well and was incubated at 37°C for 15 minutes in dark.
- The reaction was stopped by adding stop Solution to each well.
- The plate was read at 450 nm within 10 minutes.
- The standard curve was plotted using the OD values obtained for the standards provided in the kit.
- The concentrations of the test samples were obtained by plotting OD values in the standard curve.

3.2.4 Establisment of Primary corneal stromal cells:

Primary culture of stromal cells were established from donor corneas obtained from CU Shah Eye Bank (Sankara Nethralaya, India) and managed according to the guidelines in the Declaration of Helsinki for research involving human tissue. In brief, a whole globe was obtained from 27 years male expired of road accident. The cornea was removed from the whole globe and quatered and rinsed with Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco, Grand Island, NY) containing antibiotics. Manual scraping of epithelium was avoided to reduce the chance of apoptosis of the keratocytes. The central cornea was digested with 3.3 mg/ml Collagenase Type 1 at 37 °C for 45 min to remove the epithelium and the endothelium. Then the stromal explants were placed on to the 6 well plate supplemented with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) with Penicillin (150units/ml), Streptomycin (0.75%), Ciprofloxacin (0.1%) in 5% -10% CO_2, at 37°C. The media was replaced twice weekly.

3.2.5 Synthetic telopeptide:

The sequence of C- terminal telopeptides was obtained from protein database UniProt. The synthetic octapeptide EKAHDGGR *(*Accession No: P02452) was synthesized by

GeneScript, USA.

3.2.6 Induction of Apoptosis by synthesized C-terminal telopeptide:

The established primary cultures of stromal cells were seeded into 96-well plates in DMEM/F12 for 24 hours. After the removal of medium, cells were washed with phosphate-buffered saline (PBS). The synthetic peptide was reconstituted in water at a stock concentration of 9.4 mg/ml. The cells were treated with diluted synthetic peptide at varying concentrations 3.012 µg, 6.125 µg, 12.25 µg, 12.25 µg, 23.5 µg, 47 µg and 94µg respectively. Control assays were performed with untreated cells. The percentage of viable cells were determined using MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The percentage of cells undergoing apoptosis was determined by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay TiterTACS™ Colorimetric Apoptosis Detection Kit (Trevigen Inc. Gaithersburg, Catalog No**:** 4822-96-K) and TACS® 2 TdT Fluorescein Kit (Trevigen Inc. Gaithersburg Catalog No: 4812-30-K). TUNEL assay was performed as per the manufactures instructions.

3.2.7 MTT Tetrazolium assay:

- After incubating the cells with desired concentrations of telopeptide, the media was removed.
- Fresh media (100 µl) containing 20 µl of MTT (5mg/ml) was added and incubated for 4 hours.
- To the crystals 150 µl of DMSO was added and dissolved.
- The result was read at 570 nm.
- The percentage viability was calculated comparing the OD of the untreated controls.

3.2.8 Statistical analysis of the Data:

All experimental data are expressed as mean with +/- standard error of the mean (SEM). The tear samples were processed in duplicates for ELISA. All the *invitro* experiments

were performed three times. Experimental and control conditions significance was determined with Student's *t* test.

3.3 TRAIL expression in Keratoconus Corneal Epithelium – A novel mechanism in the pathogenesis of keratoconus.

3.3.1 Subjects recruited for the study:

Patients diagnosed with specific signs of keratoconus and recommended for collagen cross linking treatment were included in the study and patients diagnosed to have myopia referred for EPI lasik treatment were recruited as controls. The study was performed on 40 corneal epithelial cells. The distributions of samples are shown in table 3.2.

<table>
<tr><td rowspan="4">Total No Of Samples

N= 40</td><td rowspan="2">CASES
Keratoconus corneal epithelial cells
N = 20 (Mean age 18 ± 4 years)</td><td>Male
N=6</td></tr>
<tr><td>Female
N=14</td></tr>
<tr><td rowspan="2">CONTROLS
Myopic corneal epithelial cells
N = 20 (Mean age: 22 ± 2 years)</td><td>Male
N=10</td></tr>
<tr><td>Female
N=10</td></tr>
</table>

Table 3.2: Distribution of case and control samples included in the study.

3.3.2 Expression of TRAIL gene:

The corneal epithelial cells debrided by the clinician before collagen crosslinking treatment and EpiLasik treatment were collected in nuclease free vials filled with RNAlater reagent. Procedure of Reverse trancriptase PCR explain in The details of primer sequence used in the study are stated in table 3.2. [82]

GENE	PRIMER SEQUENCE 5'-3'	BASE PAIR
TRAIL	FP 5' CCC CTG CTG GCA AGT CAA 3' RP 5' CCT CAG AGG TTC TCA AAA TCA TCT T 3'	**68 bp**
GAPDH	FP 5'-GCCAAGGTCATCCATGACAAC-3' RP 5'-GTCCACCACCCTGTTGCTGTA-3'	**498 bp**

Table 3.3: Primers used for amplification of TRAIL gene [82]

3.3.3 Real time PCR for quantification of TRAIL expression:

Real time PCR was performed using Quantitect syber green master mix (Qiagen, Hilden, Germany). The cDNA was initially quantified using Nanodrop and the concentrations of cDNAs were diluted to final concentration of 100ng for a 10µl real time reaction. The specific amplification of the target genes was ascertained by the melt curve analysis. The cycle threshold (Ct) values of the test samples were normalised using GAPDH as the endogenous control.

The results of the real time runs were compiled and the fold changes were calculated compared to the controls (EpiLasik epithelium). The fold cange was calculated using the following formula:

ΔCt = Ct of test sample - Ct of GAPH gene.

$\Delta\Delta Ct = \Delta Ct$ of the test – ΔCt of the control

Fold change = $2^{-\Delta\Delta Ct}$

3.3.4 Cytospin enhanced smearing of corneal epithelial cells for Immunofluorescence staining:

A method was standardized to smear the scraped corneal epithelial cells for immunofluorescence staining. (Figure 3.6) The protocol is as follows.

- Corneal scrapings were collected in RNAlater and stored in the solution till use as the solution maintains the integrity of the cells.

- Note: Cells can be maintained at -80°C in RNAlater for months.

- On the day of use the cells were washed in 1% phosphate buffered saline (PBS) twice at 2000 rpm for 5 minutes in a cooling centrifuge..
- The cells were treated with 0.05% trypsin solution at 37°C for 5 minutes. Trypsin and E.D.T.A solution dissociates the cells from the extracellular matrix.
  - Caution: Do not expose the cells to trypsin for more than 5 minutes.
- The action of trypsin was stopped by adding equal volume of fetal bovine serum.
- The dissociated cells were washed twice with Phosphate buffered saline at 2000rpm for 5 minutes.
- The pellet was suspended in 50µl of Phosphate buffered saline.
  - Notes: The suspended cells can be fixed with ethanol and stored at 4°C for future use.
- The cytocentrifuge chamber was mounted with a clean grease free glass slide and 10µl of cell suspension was place inside.
- The cytocentrifuge chamber was spun at 1000rpm for 5 minutes.
- The smeared area was marked and fixed with acetone. The slides were air dried and used for immunofluorescence staining.

3.3.5 Immunofluorescence staining for DR4, DR5 on corneal epithelial cells:

The primary antibodies antihuman DR4 (Catalogue No: 307202), antihuman DR5 (Catalogue No:307408) were obtained from Biolegend, San Diego, California. The corneal epithelial cells were washed with 1% phosphate buffered saline (PBS) twice and was subjected to trypsinisation (0.05% trypsin). Trypsinisation was stopped by adding equal volume of FBS. Dissociated cells were washed with PBS twice and 10 µl of the cells were loaded on to the cytospin chamber and spun at 1000rpm for 5 minutes. The slides were marked and fixed in acetone and air dried. The cells were blocked with 5%BSA for an hour washed with PBS and was coated with primary antibodies (1:50 dilution) and incubated at 4°C overnight. The unbound primary antibody washed with PBS containing 1.25% BSA. The cells were coated with secondary antibody conjugated with FITC for 1 hour finally DAPI was added as counter stain and the slides were washed well to remove background staining. The slides were mounted with glycerol and the fluorescence was observed using Carl Zeiss, Axio viewer microscope.

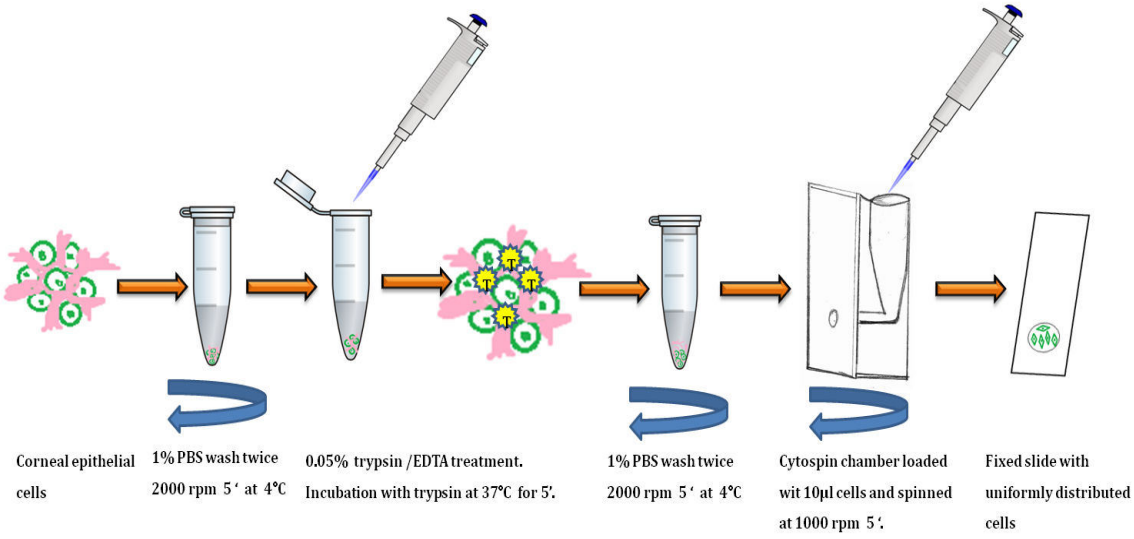


Figure 3.6: Step wise standardized protocol for smearing of corneal epithelial cells. Dissociation of corneal epithelial cells with trypsin (T) and Cytospinning of suspended corneal epithelial cells.

3.3.6 Immunofluorescence staining for TRAIL and DR4, DR5 on corneal buttons:

Keratoconus corneal button was obtained during penetrating keratoplasty performed in a 20 years old male with severe bilateral keratoconus. Control cornea was obtained from a 65 year old donor and the cause of death was cardiac arrest. Quarter of the corneas was snap frozen in Jung Tissue Freezing Medium™ (Leica Microsystems, Germany). Frozen sections of 6 µm thickness were taken using a LeicaCM1510S cryostat. The sections were fixed in acetone for 10 minutes. The sections were stained following the similar procedure used for staining corneal epithelial cells. Negative controls were also processed simultaneously. The slides were mounted with glycerol and the fluorescence was observed using Carl Zeiss, Axio viewer microscope.

3.3.7 Statistical analysis of the data:

Statistical significance of the fold changes obtained within the keratoconus samples were analyzed using one way ANOVA test.

CHAPTER - 4

RESULTS

4.1 Determination of the effect of UV radiation (370nm) on the corneal limbal stem cells in donor cornea before and after exposure to UVA radiation and on covering with PMMA ring.

4.1.1 Cultivability of corneal limbal stem cells of the riboflavin treated donor cornea before and after exposure to UVA radiation and on covering with PMMA ring:

A total of 10 eye balls were processed for the study. Limbal biopsies taken from sectors A & B before UV exposure showed outgrowth in 48 hours. None of the biopsies collected after exposure, from sector A showed outgrowth. Two biopsies obtained from sector B (covered with PMMA ring) out of the ten showed outgrowth of cells, other 8 biopsies obtained from the same site did not show any growth (Table 4.1).

Eye No.	Before exposure	After exposure	
	Sectors A and B	Sector A	Sector B (PMMA protected)
1	+	-	+
2	+	-	-
3	+	-	+
4	+	-	-
5	+	-	-
6	+	-	-
7	+	-	-
8	+	-	-
9	+	-	-
10	+	-	-

Table 4.1 Results of cell growth observed from biopsies taken before and after the exposure to UVA/riboflavin. (+ out growth of limbal setm cell seen, - no out growth)

4.1.2 Expression of presumed corneal Limbal stem cell markers by Reverse transcriptase-Polymerase chain reaction on Riboflavin treated donor cornea before and after exposure to UVA radiation and on covering with PMMA ring:

In 7 biopsies from 10 eyes taken from both sectors A & B before exposure to UV rays and in the two biopsies after exposure to UV rays from sector B (with the limbal area protected with the PMMA ring) the presumed stem cell specific marker ABCG2 expression was detected. On the other hand, all the tissue biopsies from sector A which were exposed to UV rays, failed to express ABCG2. Differentiation markers namely K3, K12, Vimentin, were found reduced significantly after exposure to UV rays and also the sector B protected with PMMA ring. The marker study results are summarized in table 4.2.

Markers	**Before exposure Sector A& B (n = 10)**	**After exposure**	
		Sector A (n = 10)	**Sector B (n = 10)**
CX43	10	6	6
K3	10	5	6
K12	6	5	6
ABCG2	7	0	2
Involucrin	7	0	1
Vimentin	10	5	6

Table 4.2 Summarized results of the reverse-transcriptase PCR marker study performed with limbal biopsies obtained before exposure to UVA/riboflavin and after exposure (covered with PMMA ring). Limbal stem cell marker ABCG2 was absent in the limbal biopsies exposed to the UVA radiation. (Sector A- unprotected, Sector B- covered with PMMA ring. Terminally differentiated cells (TDC) are positive for K_3/K_{12}, transient amplifying cells (TAC) are positive for Cnx_{43}, Vimentin, Involucrine) and stem cells are $ABCG_2$ positive).

4.1.3 Determination of the effect of UV radiation (370nm) on the corneal limbal stem cells in donor cornea before and after exposure to UVA radiation and on covering with metal ring.

A total of 30 eye balls were processed for the study. Each strip of limbal biopsy (one pre-CXL and one post CXL each from sector A and sector B) was divided into 3 segments – (i) for viable cell count, (ii) for culture on human amniotic membrane (HAM) and (iii) subjected to RNA extraction for marker studies by reverse transcriptase PCR (RT- PCR).

4.1.4 Results of viable cells counts by trypan blue exclusion method on riboflavin treated donor cornea before and after exposure to UVA radiation and on covering with metal ring:

Limbal biopsies were taken from diametrically opposite sites from each half of the cornea, marked sector A and sector B. The results are tabulated in Table 4.3. The mean cell count, before commencement of the CXL procedure was 2743.48 $\pm$ 748.83 from sector A and 2791.3 $\pm$ 876.4 from sector B. The difference between them was not statistically significant ($p = 0.698$, Wilcoxan's test). Of the total cells, the mean number of viable cells (unstained by Trypan blue) was 1726.09 $\pm$ 886.37, amounting to 59% of the total cells from sector A and 1782.61 $\pm$ 891.2, amounting to 60.7% of total cells from sector B. The difference between them was also not statistically significant ($p=0.47$).

The CXL procedure was performed, as described earlier, with the limbal area of sector B covered by metallic shield when the cornea was exposed to UVA radiation. Following the procedure, limbal biopsies were again taken from diametrically opposite sites from sectors A & B. The mean cell count was 2991 $\pm$ 807.02 from sector A and 3047.83 $\pm$ 943.5 from sector B. The difference between them was not statistically significant ($p=0.626$). However, the mean viable cells (unstained by Trypan blue) was 1495.65 $\pm$ 565.27 amounting to 49.7% of the total from sector A and 1936.96 $\pm$ 911.94, amounting to 61.23% of the total from sector B. This difference was statistically significant ($p=0.008$).

	Before Exposure			After Exposure		
	Sector A	**Sector B**	**p**	**Sector A**	**Sector B[@]**	**p value**
Mean total cell count	2743.48 ± 748.83	2791.3 ± 876.4	0.698[#]	2991 ± 807.02	3047.83 ± 943.5	0.626[#]
Mean viable cell count	1726.09 ± 886.37	1782.61 ± 891.2	0.47[#]	1495.65 ± 565.27	1936.96 ± 911.94	**0.008***
Viable cells as % of total cells	59%	60.7%		49.7%	61.23%	

Table 4.3: Results of total and viable cell count enumerated by Trypan blue exclusion method. Viable cell count was significantly reduced in the limbal biopsy exposed to UVA radiation. # Statistically insignificant, *Statistically significant (Wilcoxan's t test), [@]sector covered with metal ring.

4.1.5 Cultivability of corneal limbal stem cells of the riboflavin treated donor cornea before and after exposure to UVA radiation and on covering with metal ring:

All the biopsies (30 eyes) obtained from sector A, B before and after exposure where checked for cultivability of corneal limabal stem cell. Limbal biopsies taken from Sectors A & B before UV exposure, and those taken after exposure from Sector B (limbus protected with metal ring), showed outgrowth in an average of 48 hours and reached confluence at an average of 11 days. None of the biopsies after exposure, from Sector A showed outgrowth. The outgrowth of cells from tissue biopsy after exposure to UV, from Sectors A &B are shown in figure 4.1.

4.1.6 Expression of presumed corneal Limbal stem cell markers by Reverse transcriptase-Polymerase chain reaction on Riboflavin treated donor cornea before and after exposure to UVA radiation and on covering with metal ring:

Among 30 donor eyes 20 eyes were analyzed for varied stem cell, transient amplifying cells and differentiated corneal epithelial cell specific markers by Reverse Transcriptase

PCR. Table 4.4 summarizes the results of the RT PCR studies. In all the 40 biopsies from 20 eyes taken from both sectors A & B before exposure to UV rays and in the 20 biopsies after exposure to UV rays from Sector B (with the limbal protection with the metal shield) the presumed stem cell specific markers ABCG2 was expressed. On the contrary, except 4 biopsies out of the 20 from sector A which were exposed to UV rays, failed to express ABCG2. Differentiation markers like k3, k12, vimentin, were positive for all the tissues irrespective of exposure to UV rays.

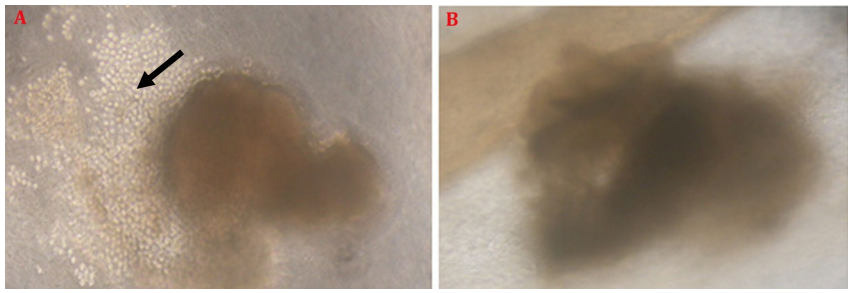


Figure 4.1 Limbal stem cells cultivated on human amniotic membrane. A. Tissue biopsy obtained from sector B of Eye no: 12 after exposure to UVA protecting the limbal area with a metal shield. Outgrowths of cells were seen 48 hours of incubation (20X magnification), B. Tissue biopsy obtained from sector A of Eye no: 12 after exposure to UVA. No outgrowths of cells were seen even after 12 days of incubation.

Targeted Markers	**Before Exposure N=20**		**After Exposure N=20**	
	Sector A	**Sector B**	**Sector A**	**Sector B** (Covered with metal ring)
$ABCG_2$	20	20	4	19
p63	15	15	8	15
Cx43	19	19	16	19
Vimentin	20	20	19	20
Involucrin	20	20	10	20
K_3	20	20	20	20
K_{12}	20	20	20	20

Table 4.4: Results of RT-PCR performed with RNA extracts of 20 limbal biopsies taken from sector A and B before after UV exposure. (Sector A- unprotected, Sector B- covered with metal ring. Terminally differentiated cells (TDC) are positive for K_3/K_{12}, transient amplifying cells (TAC) are positive for Cnx_{43}, Vimentin, Involucrine) and stem cells are p63, $ABCG_2$ positive).

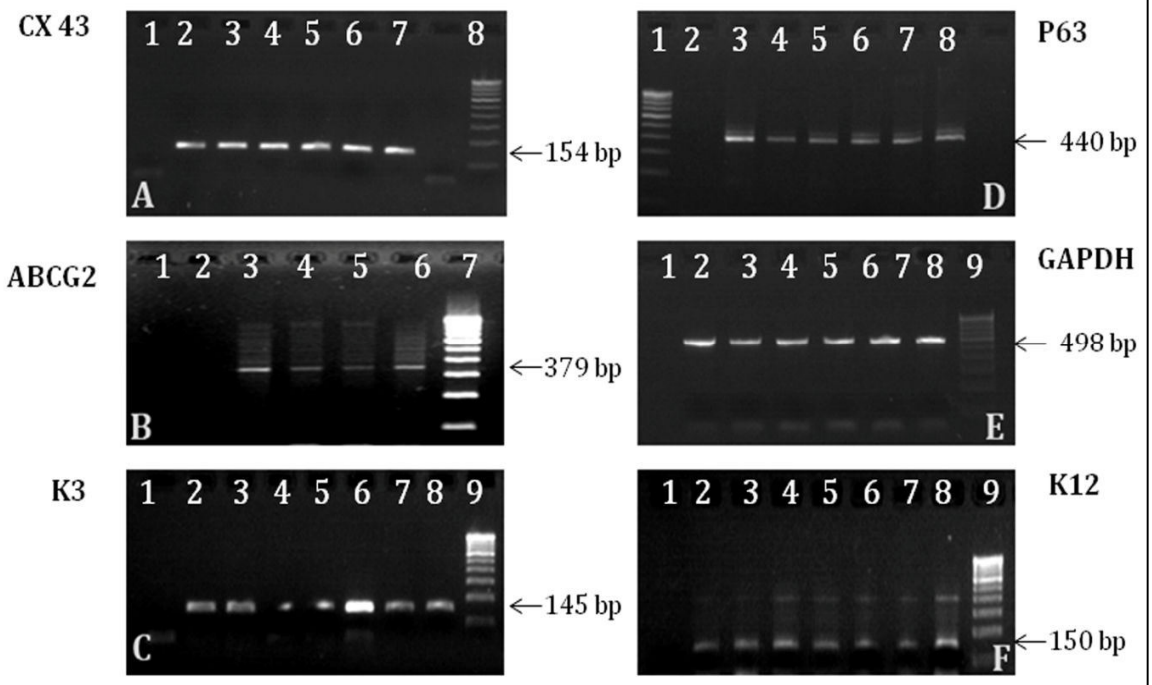


Figure 4.2: Representative Gel image of the markers studied. A: Amplified products of CX43 Lane1: Negative control (NC), 2,3: before exposure Sector A,B, 4,5: After exposure unprotected, 6,7: After exposure covered with metal ring, 8: 100bp molecular weight marker. B: Amplified products of ABCG2 Lane1, 2: After exposure Sector A,B unprotected, 3,4: before exposure, 5,6,: After exposure covered with metal ring, 7: 100bp molecular weight marker. C: Amplified products of K3 Lane1: NC, 2,3: before exposure Sector A,B, 4,5: After exposure unprotected, 6,7: After exposure covered with metal ring, 9: 100bp molecular weight marker. D: Amplified products of P63 Lane1: 100bp molecular weight marker, 2: NC, 3,4: before exposure Sector A,B, 5,6: After exposure unprotected, 7,8: After exposure covered with metal ring. E: Amplified products of GAPDH Lane1: Negative control (NC), 2,3: before exposure Sector A,B, 4,5: After exposure unprotected, 6,7: After exposure covered with metal ring, 9: 100bp molecular weight marker. F Amplified products of K12 Lane1: Negative control (NC), 2,3: before exposure Sector A,B, 4,5: After exposure unprotected, 6,7: After exposure covered with metal ring, 9: 100bp molecular weight marker.

4.2 Apoptosis of Corneal Stromal Cells Induced by Telopeptides: an in *vitro* Study

4.2.1 Quantification of collagen degradation product:

The concentration of telopeptide was assessed in the tear films of control and in the keratoconus group. Telopeptide was not detected in the control tears whereas the tears from keratoconus patients showed variable levels of telopeptides (Table 4.5). In four of the keratoconus tear samples the telopeptides were not detected. There was no correlation between the stage of keratoconus and the concentration of telopeptides. Gender predilections were not analyzed as the sample population was 20 and among them only 5 were female. The determined values were used to derive the concentration of telopeptides to be used to treat the stromal fibroblast *in vitro.* With the concentrations obtained from the ELISA readings a range of 3 – 90 µg concentration was set to assess the effect of telopeptides on the corneal stromal cells *in vitro.*

4.2.2 Establishment of primary stromal cells:

Primary stromal cell culture established from the whole globe obtained from a single donor cornea (Figure 4.3) was subjected to the experiment. Telopeptide treatment was performed on early passages (P1). Percentage viability of telopeptide treated cells was detected by MTT assay.

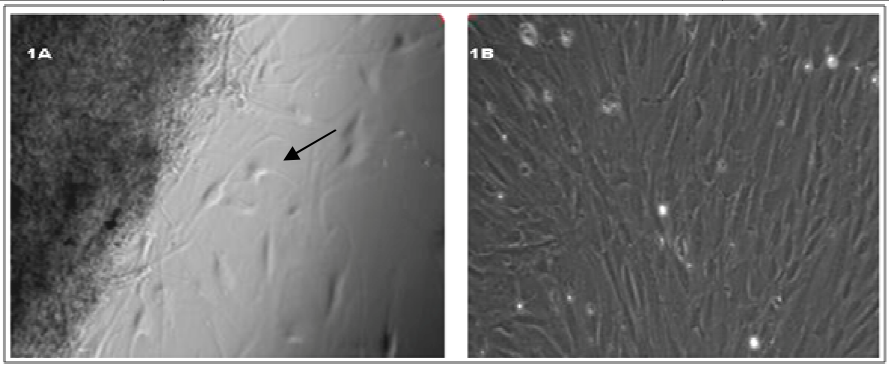


Figure 4.3: Phase contrast image of primary corneal stromal cells (20X magnification). A. Out growth of stromal cells from the explant after 48 hours, B. Confluent monolayer of primary corneal stromal cells after 14 days of incubation.

S.No	Age	OD / OS	Duration	Keratometry readings (D)		Corneal Thickness (µm)	Stage of Keratoconus	Telopeptide concentration (µg /ml)
				K1	K2			
1	15/M	OD	1 Year	71.44D@77	64.79D@167	350	Stage IV (severe)	114.125
2	27/M	OS	1 Year	46.35D@118	44.11D@28	392	Stage II (Moderate)	110.9375
3	16/M	OS	4 Year	61.87D@165	56.62 D@20	417	Stage IV (Advanced)	127.5
4	20/M	OS	3 Year	51.50@103	45.50 D@13	260	Stage II (Moderate)	3.4375
5	24/F	OD	1 Year	54.00D@20	58.75 D@110	402	Stage III (Advanced)	2.5
6	13/M	OD	3 Months	55.18 D@111	48.65 D@21	432	Stage II (Moderate)	113.75
7	17/M	OD	3 Years	55.91 D@135	51.54 D@45	422	Stage II (Moderate)	128.75
8	25/M	OS	1 Year	56.48 D@ 39	51.54 D@ 129	397	Stage IV (Advanced)	129.375
9	20/M	OS	6 Months	44.50 D @ 109	45.25 D @	513	Stage 1 (Mild)	108.125
10	22/M	OD	2 Year	49.50 D @ --	N/A	413	Stage II (Moderate)	17.1875
11	30/M	OS	5 Days	42.75 D @ 116	48.75 D @ --	396	Stage II (Moderate)	102.5
12	22/M	OD	6 Months	45.00 D @55	47.00 D @145	450	Stage II (Moderate)	104.375
13	17/M	OS	6 Months	N/A	N/A	417	Stage III (Advanced)	0.9375
14	14/M	OS	6 Months	44.00D @154	48.25D @68	395	Stage II (Moderate)	58.4375
15	24/F	OD	8 Years	57.00D @110	65.00D @35	392	Stage III (Advanced)	52.1875
16	19/F	OS	5 Years	N/A	N/A	395	Stage III (Advanced)	ND
17	18/M	OD	2 Years	44.39 D@17	47.84 D @103	414	Stage II (Moderate)	ND
18	21/F	OD	2 Years	47.50D@15	55.00D@--	411	Stage II (Moderate)	ND
19	14/F	OD	5 Years	71.21D @ 96	57.65D@6	381	Stage III (Advanced)	140.625
20	16Y/M	OD	5 Years	42.37D @ 10	51.25 D@ 103	427	Stage II (Moderate)	ND

Table 4.5: Telopeptide concentrations and the respective clinical details. (OD – right eye, OS-left eye, K1,K2- keratometry reading at 90° and 180°, ND: not detected)

4.2.3 Determination of percentage cell viability by performing MTT assay on primary stromal cells treated with synthetic telopeptides.

Telopeptide of varying concentrations namely 3.012 µg/ml, 6.125 µg/ml, 12.25 µg/ml, 23.5 µg/ml, 47 µg/ml, 94µg/ml in 1% FBS was used to treat the primary corneal stromal cells at three time points. No significant effect of telopeptide was observed at 24 hours and 48 hours of incubation. The percentage of cell viability decreased considerably at 72hours of incubation with 94µg/ml, 47 µg/ml of telopeptide (Figure 4.4) concentrations. To validate the results obtained from the MTT assay fluorescent TUNEL assay and microscopy based TUNEL assay was performed.

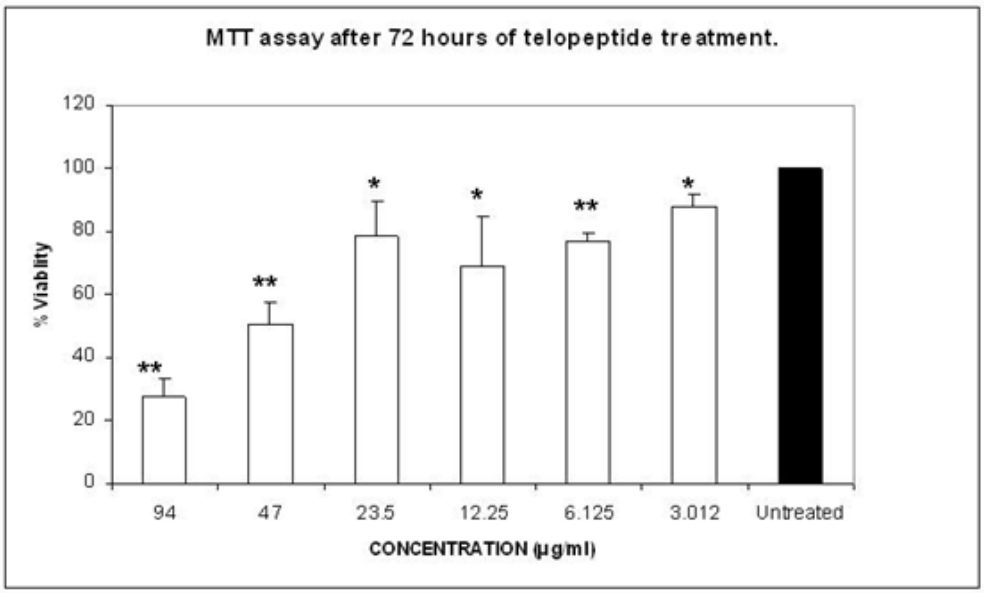


Figure 4.4: Dose dependent effect of telopeptide on primary corneal stromal cells viability. Human primary corneal stromal cells were incubated with telopeptides for 72 hours with varying concentrations of telopeptide. Shown are the percentages of viable cells determined by MTT assay. *p<0.05, **p<0.005. All experimental data are expressed as mean with +/-standard error of the mean (SEM). Experiments were performed in triplicates.

4.2.4 Demonstration of apoptosis on primary stromal cells treated with synthetic telopeptides.

In fluorescent based TUNEL assay the untreated primary corneal stromal cells served as negative control. Untreated monolayer of cells treated with nuclease provided in the TUNEL assay kit served as the positive control. Apoptotic nuclei were observed among the cells treated with 94µg/ml, 47 µg/ml of telopeptide after 72 hours (Figure 4.5). Colorimetric TUNEL assay quantified the apoptotic cells on telopeptide treatment. Concordant results were observed in the colorimetric TUNEL assay. A dose-effect relationship could be obtained between the telopeptide concentration and the apoptotic cells (Figure 4.6). Apoptosis was observed among the cells treated with 94µg/ml, 47 µg/ml of telopeptide after 72 hours. Apoptosis was not observed among the cells treated with 3.012 µg/ml, 6.125 µg/ml, 12.25 µg/ml, 23.5 µg/ml showing that the telopetide concentrations above 47 µg/ml is lethal to the stromal fibroblast.

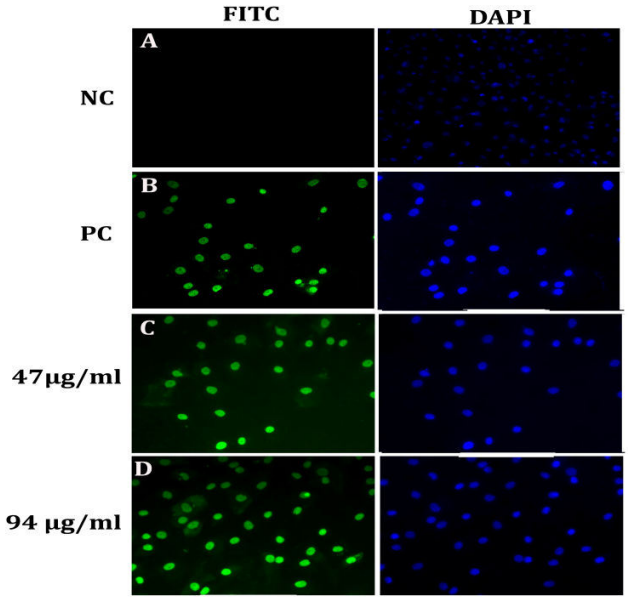


Figure 4.5: TUNEL assay performed after 72 hours on treatment with telopeptides. A.Untreated- negative control (NC), B. Nuclease treated positive control (PC) C. Apoptotic cells on treatment with 47 µg/ml telopeptide. D. Apoptotic cells on treatment with 94 µg/ml telopeptide. (Carl Zeiss Axio vison Magnification 10X)

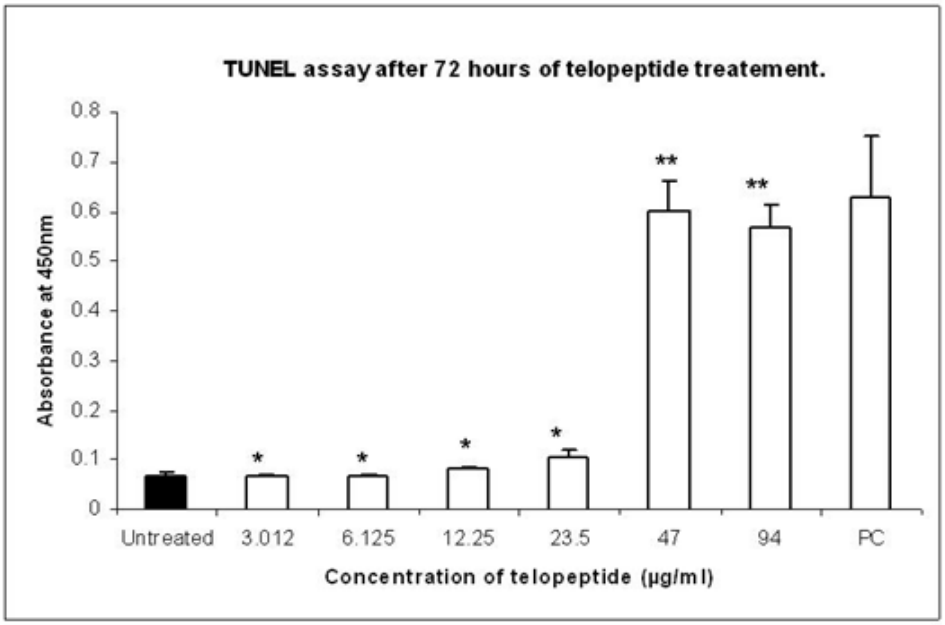


Figure 4.6: Dose dependent effect of telopeptide on primary corneal stromal cells viability using TUNEL assay. Human primary corneal stromal cells were incubated with telopeptide 72 hours with varying concentrations of telopeptide. *p<0.05, **p<0.005. All experimental data are expressed as mean with +/-standard error of the mean (SEM). Experiments were performed in triplicates.

4.3 TRAIL expression in Keratoconus Corneal Epithelium – A novel mechanism in the pathogenesis of keratoconus

4.3.1 Reverse transcriptase PCR for TRAIL expression:

Reverse transcriptase PCR was performed on cDNA obtained from keratoconus corneal epithelial cells and the control epithelial cells. All the keratoconus samples showed the expression of TRAIL gene and none of the controls showed the expression of TRAIL gene (Figure 4.7).

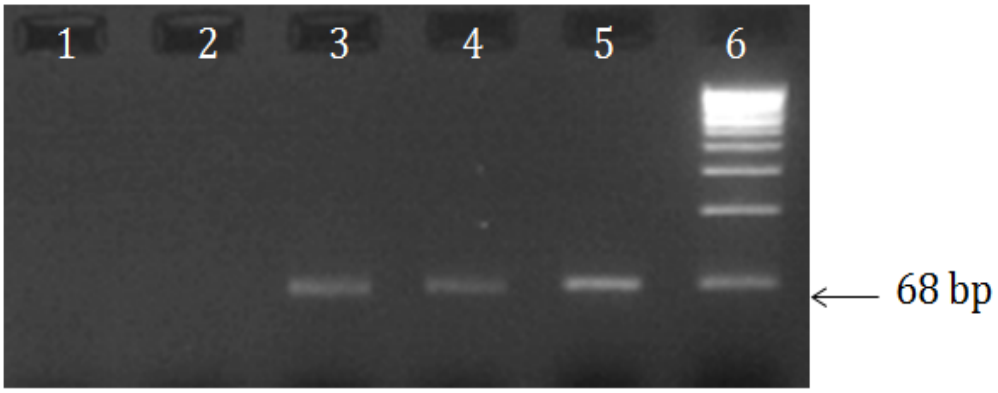


Figure 4.7: Representative gel image showing the expression of TRAIL gene. Lane 1- NC, lane 2- no amplification in the control sample, lane 3-KC 4, lane 4- KC5, lane 5- PC (HUVEC cDNA), lane 8- 100 bp molecular weight marker.

4.3.2 Real time PCR for TRAIL gene expression:

Real time PCR was performed for all the 40 keratoconus corneal epithelial cells. The fold change was calculated using the cycle threshold values.(explained in section 3.3.3) Based on Amsler-Krumeich keratoconus classification [83,84] the patients were classified as mild, moderate and average. All the Keratoconus patients had prominent signs of corneal thinning, prominent corneal nerves, Fleischers Ring, and one case KC-5 with Munsons sign. Among the twenty keratoconus patients nineteen had bilateral keratoconus except one case KC-14 in whom only the right eye was affected. The fold change values were related to the stage of keratoconus (Figure 4.8). As the disorder progressed the TRAIL expression was upregulated and the fold change was found to be statistically significant between the categorized groups of keratoconus (Figure 4.9). The fold change and the respective keratometry readings are given in table 4.6.

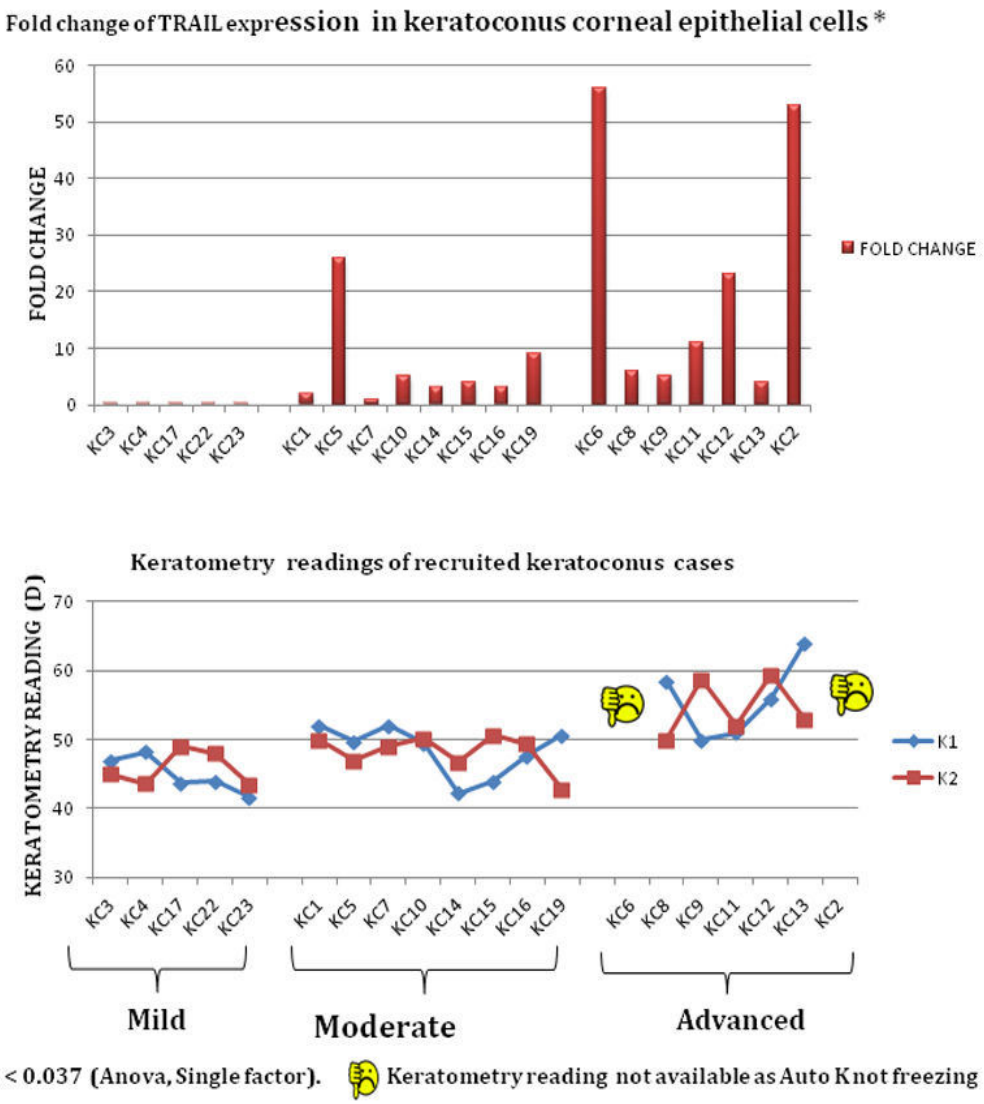


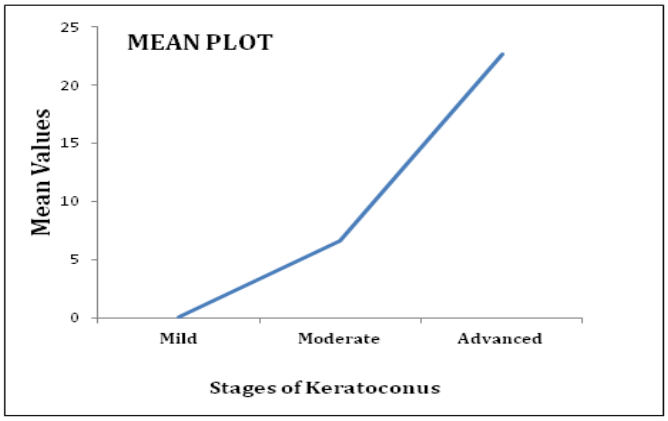
Figure 4.8: Comparison of the expression of TRAIL and the keratometry values of the individual keratoconus patients. Progression of keratoconus positively correlated with the expression of TRAIL. The fold change denoted are in reference to the control group (KC1-KC19: samples from keratoconus patients)

Table 4.6: Grouping of keratoconus patients recruited separately for TRAIL expresion based on Amsler-Krumeich keratoconus classification and the respective real time fold changes calculated using the Ct values.

KC	Stage of keratoconus	Age/ Gender	Fold change Of TRAIL	OD/OS	Keratomerty reading (D)		Corneal Thickness (µm)
					K1	K2	
KC1	**Stage I (Moderate)**	25/F	2	OD	52D@179	50D@40	395
KC2	**Stage III (Advanced)**	19/M	53	OD	NA	NA	455 *
KC3	**Stage I (Mild)**	25/M	0.05	OD	47.00D@145	45.00D@55	403
KC4	**Stage I (Mild)**	16/M	0.004	OD	48.25D@	43.62@ 35	399
KC5	**Stage II (Moderate)**	27/F	26	OD	49.75D@150	47.00D@60	490
KC6	**Stage III (Advanced)**	14/M	56	OD	NA	NA	381
KC7	**Stage II (Moderate)**	14/M	1	OD	52.00D@-- 170	49.00D@20	446
KC8	**Stage III (Advanced)**	31/F	6	OD	58.50D@-- 140	50.00D@30	365
KC9	**Stage III (Advanced)**	18/F	5	OS	50D@165	58.75D@75	385
KC10	**Stage II (Moderate)**	9/M	5	OS	49.50D@14	50.25D@149	427
KC11	**Stage III (Advanced)**	19/M	11	OD	51.0D@25	52D@149	412
KC12	**Stage III (Advanced)**	16/F	22	OD	56.00D@64	59.50D@	398
KC13	**Stage III (Advanced)**	15/M	4	OS	64D@90	53D@180	448
KC14	**Stage II (Moderate)**	24/M	3	OS	48.25D@135	48.75D@--	437
KC15	**Stage II (Moderate)**	11/M	4	OD	44D@26	50.75D@122	418
KC16	**Stage II (Moderate)**	19/M	3	OS	47.67D@120	49.41D@79	476
KC17	**Stage I (Mild)**	17/M	0.005	OD	43.75D@175	49D@30	505
KC19	**Stage II(Moderate)**	16/M	9	OS	50.62D@105	42.75@30	474
KC22	**Stage I (Mild)**	18/F	0.1	OS	44D@134	48D@50	503
KC23	**Stage I (Mild)**	18/M	0.04	OD	41.63D@44	43.58D@40	409

*** Thickness of the cornea due to scarring, NA- kertometry reading not available due to highly irregular cornea, D- Diopters, OD- right eye, OS- left eye.**

A.



B.

Groups	Count	Sum	Average	Variance
Mild	5	0.235826	**0.047165**	0.002392
Moderate	8	52.79791	**6.599739**	68.03799
Advanced	7	158.5421	**22.64887**	522.5831

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1701.046	2	850.5232	4.003267	0.037615	3.591531
Within Groups	3611.774	17	212.4573			
Total	5312.82	19				

Figure 4.9: One way ANOVA analysis to show the significant difference in fold change of TRAIL expression between the mild, moderate, advanced cases of keratoconus.

A. The mean plot depicting the significant correlation between the fold change of TRAIL expression and the stage of keratoconus. B. Summary of the variation between the groups (severe, moderate, mild) of keratoconus and within the groups (severe, moderate, mild) of keratoconus patients. (Count- no of patients in each stage of keratoconus, sum- sum of the fold change for each group).

4.3.3 Real time PCR for caspase expression:

In order to support the TRAIL gene expression and apoptosis, real time PCR analysis was performed to check the expression of caspase 8 gene as caspase 8 protein is one of the major contributors in apoptosis pathway inside the cell. Capase 8 real time was performed for six randomly chosen keratoconus corneal epithelial cells. The six samples included four moderate cases, two advanced cases and one mild case of keratoconus. The

expression of caspase-8 was found to be increased as TRAIL expression increased irrespective of the classification (Figure 4.10). Positive correlation was observed between the caspase expression and TRAIL gene expression using Pearson correlation analysis.

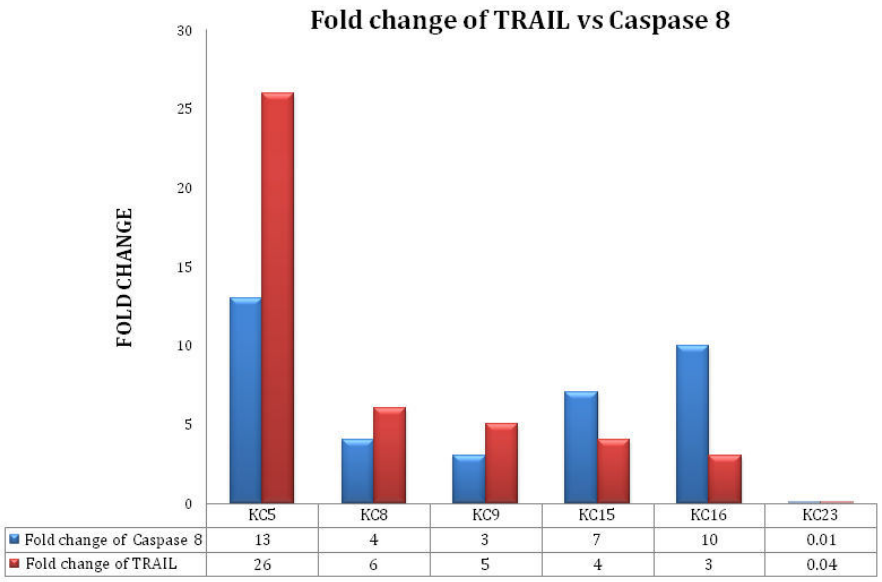


Figure 4.10: Fold change of caspase-8 expression vs the fold changes of TRAIL expression in keratoconus patients. (KC5, 8, 9, 15, 16, 23- samples from keratoconus patients)

4.3.3 Immunofluorescence staining of corneal epithelial cells:

Corneal epithelial cells of KC6 patient classified as advanced and KC16 classified as moderate stage of keratoconus were used for the immunofluorescence staining of the Death receptors DR5, DR4. Controls 5 and 10 were also stained simultaneously. The Death receptors are transmembrane proteins and are located in the cytoplasm when stained. The fluorescence was specific for the receptors and was observed only in the cytoplasm of the keratoconus corneal epithelial cells and the nucleus remained unstained. Cytoplasm of the control epithelial cells did not show any fluorescence, however the nuclear staining of DAPI was observed in the control cells. Immunofluorescence staining revealed that the death receptors were present in the keratoconus corneal epithelial cells in detectable levels and the myopic corneal epithelial cells did not show positivity of death receptors (Figure 4.11, 4.12).

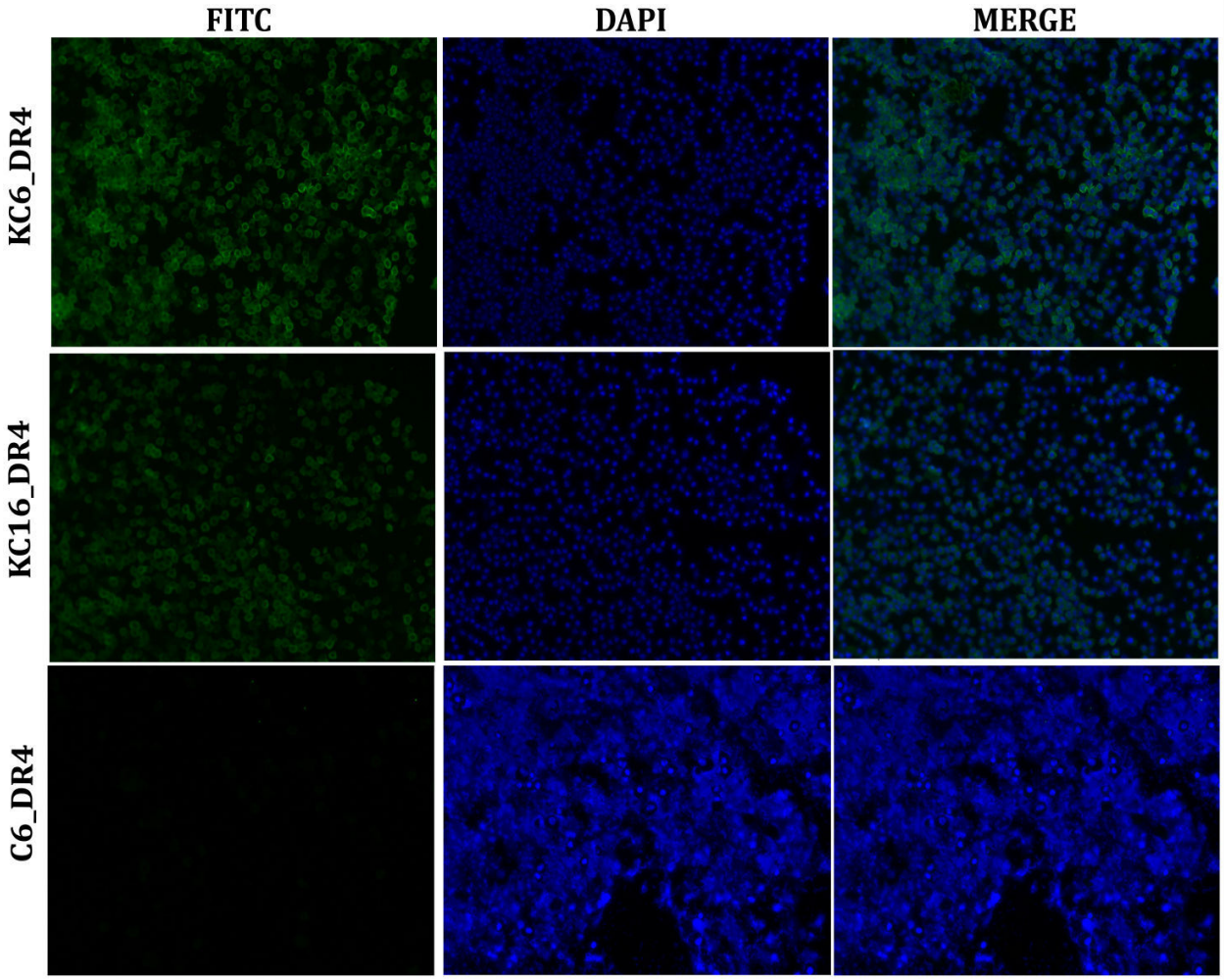


Figure 4.11:

Indirect immunofluorescence staining demonstrating the expression of Transmembrane death receptor DR4 on keratoconus corneal epithelial 6 and 16. C6 the control corneal epithelial cells did not show any expression of DR4 receptors. (Carl Zeiss Axio vison Magnification 10X)

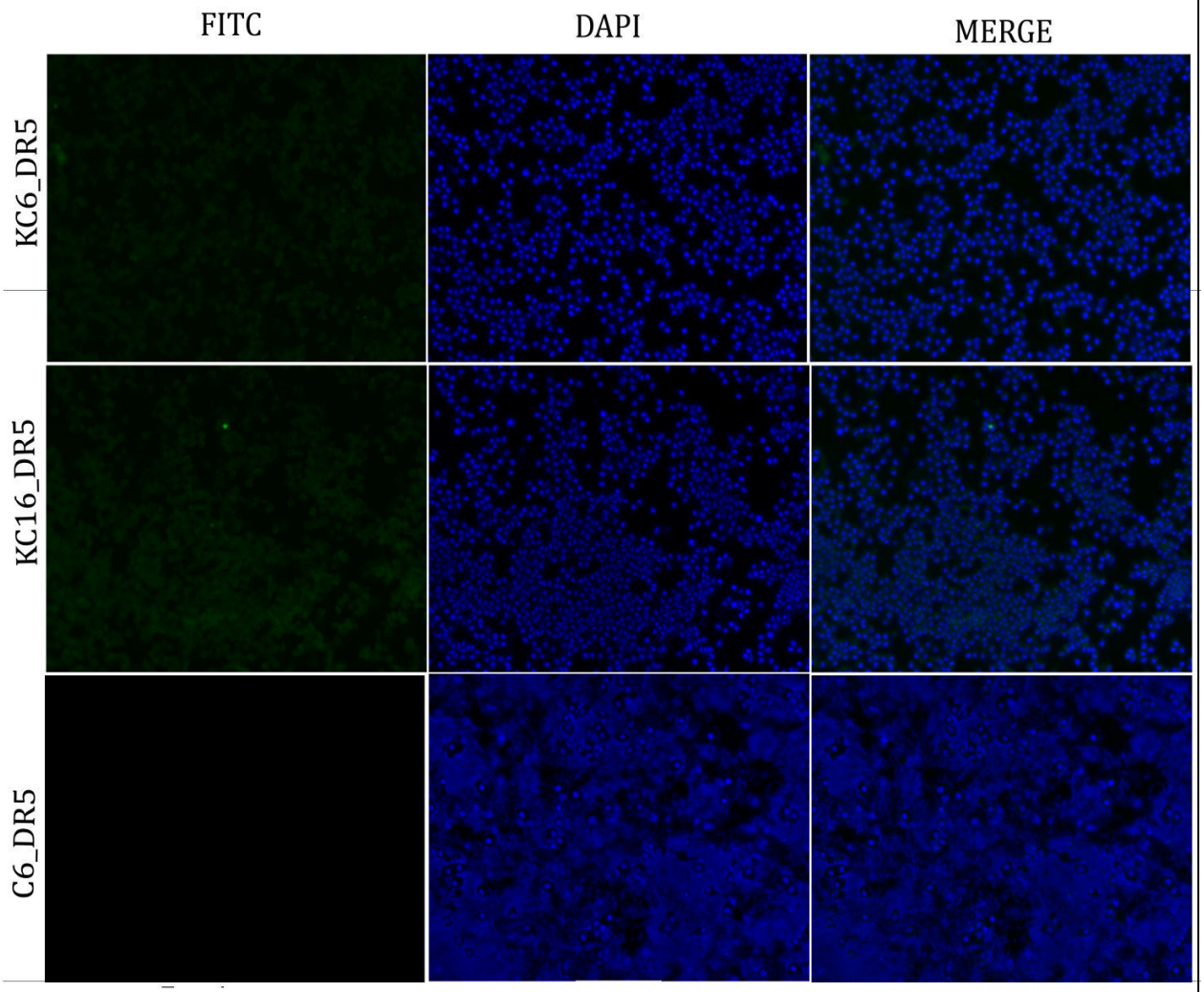


Figure 4.12: Indirect immunofluorescence staining demonstrating the expression of Transmembrane death receptor DR5 on keratoconus corneal epithelial 6 and 16. C6 the control corneal epithelial cells did not show positivity of expression of DR5 receptors**.** (Carl Zeiss Axio vison Magnification 10X)

4.2.4 Immunofluorescence staining of corneal buttons:

The donor cornea used as control and the keratoconus corneal button showed strong positivity for TRAIL death receptor DR4 in the corneal epithelial cells, keratocytes and stromal region. DR5 strong positivity was observed only in the epithelial and stromal layers of keratoconus cornea and not in the control donor. Immunofluorescence staining of TRAIL was observed in the epithelial and the stromal layers of the keratoconus cornea and faint positivity was observed in the stromal region of donor cornea (Figure 4.13, 4.14)

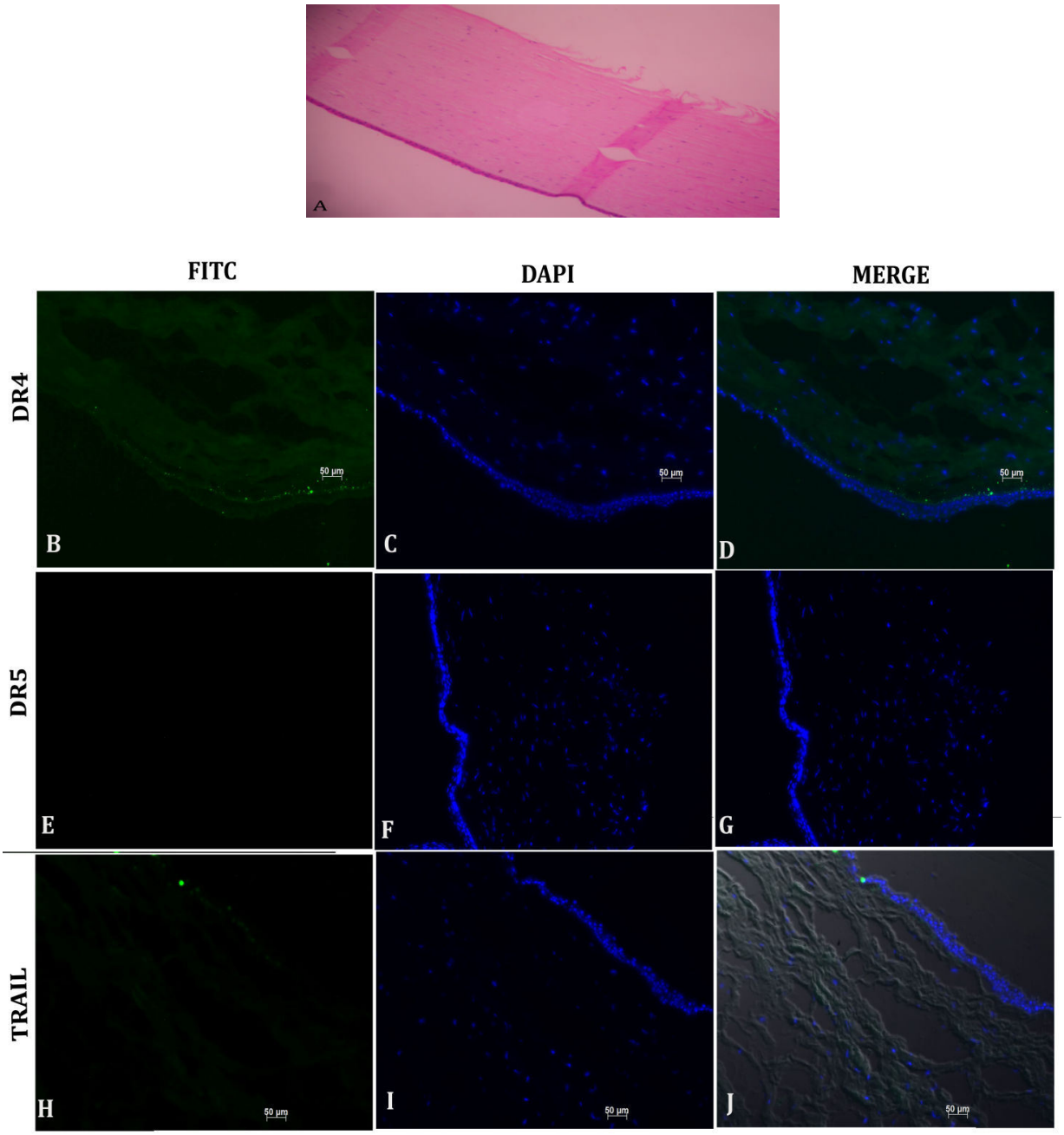


Figure 4.12: Indirect immunofluorescence staining demonstrating the expression of Transmembrane death receptor DR4,DR5, TRAIL on donor cornea. A- Hematoxylin eosin staining on donor cornea showing the intact corneal epithelial cells and the stroma (Nikon light microscope, 20 X magnification). B-D: DR4 positivity of the corneal and the stromal region, E-G: DR5 negative donor cornea, H-J: Faint TRAIL positivity in the stromal region and negative epithelial layer. (Scale bar- 50 µm, Carl Zeiss Axio vison Magnification 10X)

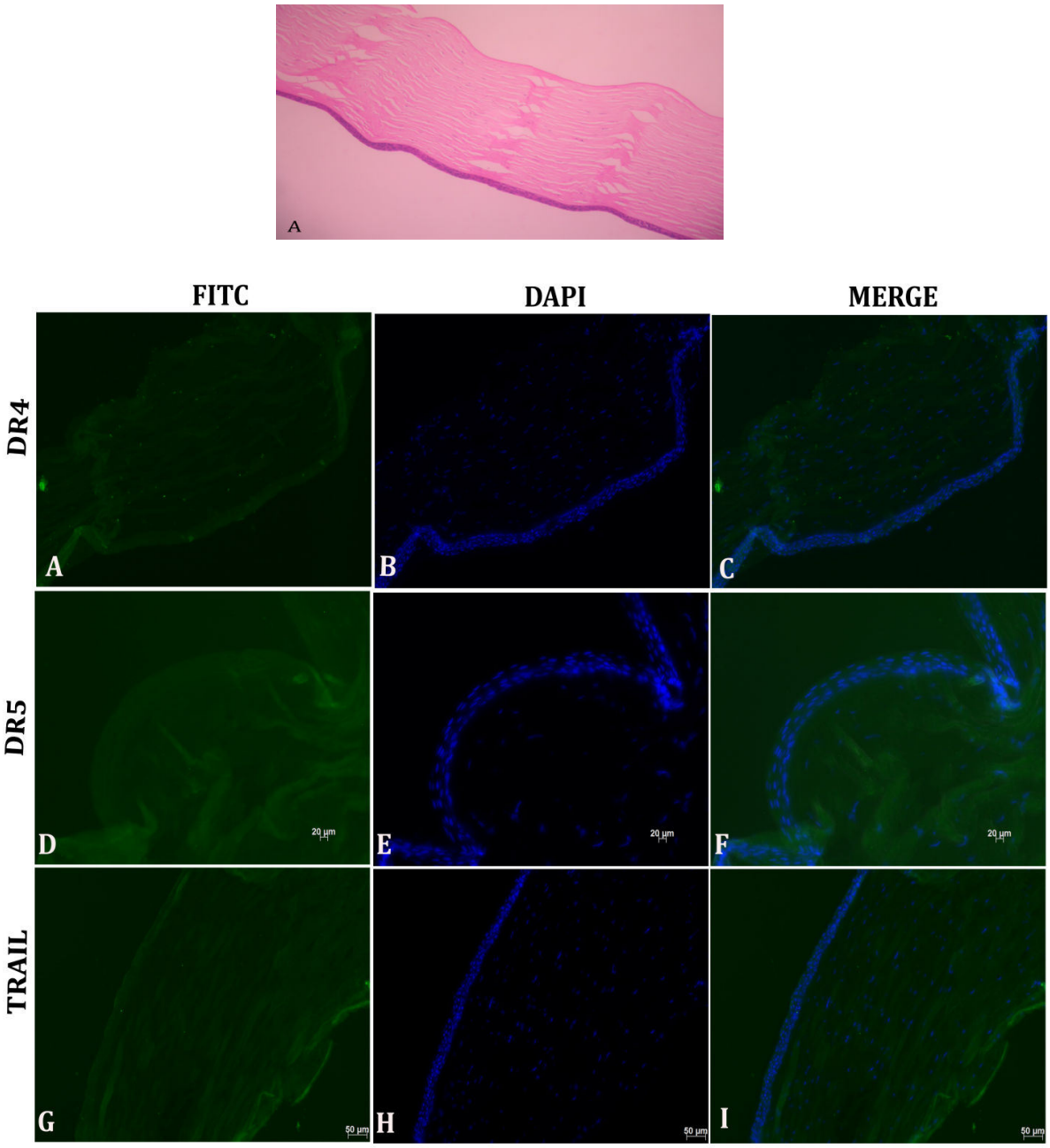


Figure 4.13: Indirect immunofluorescence staining demonstrating the expression of Transmembrane death receptor DR4, DR5, TRAIL on Keratoconus cornea. A-Hematoxylin eosin staining on donor cornea showing the loose stroma(Nikon light microscope, 20X magnification). B-D: DR4 positivity of the corneal and the stromal region, E-G: DR5 positivity of the corneal and the stromal region , H-J: TRAIL positivity the corneal and the stromal region. (Scale bar- 50 µm, Carl Zeiss Axio vison Magnification 10X).

CHAPTER – 5

DISCUSSION

Extensive laboratory and pre-clinical animal research, predominantly from the university of Dresden, led to the introduction of the UVA-Riboflavin CXL as a promising method of treatment for progressive keratoconus [43]. The aim of this treatment was to create additional chemical bonds inside corneal stroma by means of a polymerization in the anterior stroma while minimizing exposure to the surrounding structures of the eye [85]. The riboflavin acts as a photosensitizer, and enhances UVA absorption by the corneal stroma when the overlying corneal epithelium has been removed. Riboflavin also impedes the transmission of UV rays to the deeper tissues of the eye. UVA absorption was shown by Wollensak *et al* to be increased to 95% in the cornea after riboflavin treatment compared to 25-35% without riboflavin [34].

The hazards of UV exposure to the eye are well documented [86]. The extent of damage depends on its wavelength, its irradiance and the irradiation time. Spoerl et al spelt out the guidelines for the safety of UVA-riboflavin CXL procedure in the human eye and prescribed four important pre-requisities to prevent UV damage to the ocular tissues: (1) corneal epithelium should be removed to facilitate diffusion of riboflavin into the corneal stroma, (2) 0.1% concentration of riboflavin solution should be instilled for 30 minutes before UVA exposure, (3) UV irradiance of 3mw /cm^2 and wavelength of 370nm should be homogenous and (4) cornea should be minimum thickness of 400 μm [39]. When these criteria are carefully followed, it is believed that the structure behind the corneal stroma, including the corneal endothelium, anterior chamber, iris, lens and retina.

The cytotoxic effect of UVA-riboflavin on corneal keratocytes has been studied invitro [87] and *in vivo* [88]. It has been shown that UV radiation in a potent modulator of cytokine regulated ICAM-I gene transcription with the capacity to induce both inhibitory as well as enhancing effects on the keratocytes [86]. Confocal microscopic studies have shown a reduction in keratocyte density, keratocyte apoptosis and a lack of nerve fibres in the anterior stroma 1 month after CXL. However, 3-6 months after treatment, an increased stromal density and progressive repopulation of the stroma with activated keratocytes were documented [89]. The transient damage to keratocytes, which is also

seen after Penetrating keratoplasty, LASIK or a long standing epithelial injury, is not believed to pose a serious threat to the cornea. UV radiation is also toxic to corneal epithelial cells. This was however, considered irrelevant because the clinical protocol of CXL requires the debridement of the corneal epithelium in the area exposed to UVA. However, this assumption of safety is only justifiable under very stringent conditions which may not always apply. The epithelium adjacent to the denuded cornea and the epithelium overlying the limbus are vulnerable to UV damage under some realistic circumstances as follows.

(1) It is not unreasonable to expect eye movements during 30 minutes duration of UV exposure.

(2) With growing evidence of its efficacy, the indications for UVA-riboflavin treatment are gradually expanding. Peripheral corneal pathologies like iatrogenic post-Lasik keratectasia, Pellucid marginal degeneration, peripheral ulcerative keratitis, immune-mediated scleral melts may benefit from CXL but may need a deliberate decentration of the UVA exposure to the affected region which may then expose the limbus to UV radiation. Cross Linking of the peripheral cornea prior to penetrating keratoplasty for advanced keratoconus is being considered as a possible approach to stabilize the disease in the peripheral host tissue and potentially reduce post-keratoplasty astigmatism [90].

(3) If the UVA beam is not collimated, the area beyond the intended diameter of treatment may also be exposed to radiation

(4) Lateral diffusion of singlet oxygen and superoxide radicals from the treated area of the cornea to the adjacent limbal basal epithelium may have a cytotoxic effect on those cells.

UV radiation and the free radicals it generates are known to cause lipid peroxidation of cellular membranes, decrease mitochondrial function and indue apopotosis [91]. The limbal basal epithelium may thus suffer damage from the direct effect of UVA rays or from the free radicals generated during the riboflavin-UV interaction. This could have significant clinical implications because the limbal basal epithelium is believed to contain the ‘niche’ that houses the corneal stem cells. Limbal epithelial cells have been shown to have the greatest proliferative potential *in vitro* compared to any other part of the cornea.

Limbal epithelial crypt is located between the ridges of palisades of Vogt. These crypts are believed to have features consistent with a stem cell 'niche' [92].

Youn HY *et al* have demonstrated in their *invitro* studies, that UV radiation decreased ocular cell viability and caused DNA and mitochondrial degradation in human corneal epithelial cell lines. They also demonstrated an increase in the number of apoptotic cells. The use of appropriate UV filters was shown to protect the cells from UV damage [93].

Studies show that limbal stem cells grown on denuded amniotic membrane retain their stemness, express the presumed stem cell markers such as ABCG2, p63 and also do not express the gap junction marker, Cnx43. As the human amniotic membrane (HAM) provides a favourable environment for the growth of limbal stem cells, HAM was chosen to expand the cells in this study (95).

The cultivability of these cells on denuded HAM also seemed to be inhibited when the limbus was unprotected from the UVA. Studies suggest that UVA sensitizes intracellular chromophores, thereby generating reactive oxygen species, which in turn may cause promutagenic DNA lesions either in stem cells or cells in varying stages of differentiation.

Many different materials are known to prevent the penetration of UV rays, yet according to the feasibility, availability and advantage an appropriate material should be selected. Thus in this study we choose PMMA which was hemi-annular with a thickness of 0.5cm. PMMA was chosen because it is low weight, being easy to handle, rust free and not brittle. The results of the experiments on protecting with PMMA ring showed that after exposure to riboflavin/ UVA, the ABCG2 expression was positive in only 2 out of 10 biopsies when the limbus was covered with the PMMA ring; this suggests partial protection of the limbus. The partial protection would not be sufficient for the limbal stem cell, hence metal ring with a thickness of 0.5cm was chosen as an alternative material in the study.

On analysing the results of viable cells counts by trypan blue exclusion method on riboflavin treated donor cornea before and after exposure to UVA radiation and on covering with metal ring, there was no significant difference in the mean cell count between sector A and sector B before or after riboflavin-UVA exposure. The mean

number of viable cells in both sectors before treatment was also comparable. However, after the procedure, the unprotected limbus (sector A) had significantly less viable cells than the protected limbus (sector B).

Some unique characteristics of the limbal epithelial cells make their identification possible in the laboratory. These cells lack the epithleial cells differentiation cytokeratin K_3/K_{12}. Cnx43 a gap junction protein, has been noted in human corneal but not limbal basal epithelium. Vimentin and CK_{19} are also demonstrable in the basement membrane. A transcription factor p63 involved in morphogenesis and an ATP-binding cassette transporter protein $ABCG_2$ have been reported to be expressed in the mammalian corneal epithelial stem cells [95]. The RT-PCR technique was chosen, in our study rather than the immunofluorescent or immunohistochemistry techniques because of its proven superiority and higher specificity.

The lack of protection to the limbus must have exposed the cells to the cytotoxic effect of UVA, even though the UVA beam was focussed only on the central 7mm of the cornea. In the clinical scenario, eye movements can cause this type of damage more significantly.

The result of our studies showed that cells at all levels of differentiation could be identified in the limbal biopsies taken before the riboflavin-UVA exposure – post mitotic and terminally differentiated cells (TDC) (CK_3/K_{12}- positive), transient amplifying cells (TAC) (Cnx_{43} and Vimentin positive, Involucrine and Integrin 9 positive) and stem cells (p63 and $ABCG_2$ positive). However, after exposure to riboflavin UVA, the $ABCG_2$ expression was negative in 16 biopsies taken from sector A but 19 remained positive in the biopsies taken from Sector B – suggesting a depletion of stem cells in the area which was unprotected by the metal shield. The p63 expression was positive in 8 of 20 biopsies from sector A and in all 15 biopsies from Sector B (Table 4.4). The TAC and TDC cell were less affected, in sector A, where the limbus was unprotected. However, in sector B, where the limbus was protected by the metal shield, all the epithelial cells markers remained positive.

Thus the results from the molecular marker studies and the number and growth potential of viable cells suggests that the metallic shield rather than a PMMA over the limbal area could render protection to the limbal epithelial cells to an extent from direct exposure to

the cytotoxic effect of UV-A rays. Although this would not prevent lateral diffusion of free radicals from neighboring irradiated corneal tissues, the short half-life of these radicals may reduce their impact. This study could not investigate if the observed effect of UV on the limbal epithelial cells and stem cells was permanent or transient. Irrespective of this, prevention of this complication is simple but important. In our study, the use of a metal shield has been shown to protect the limbus and this may be useful during the treatment procedure.

Pathogenicity of keratoconus is of unknown etiology hence, is widely studied and has gained importance among ocular researchers for the reason that the progression of the disease is uncontrollable if left untreated. The stroma is mainly composed of keratocytes and these cells manufature and maintain the protein and the collagen content of the corneal stroma. Recent studies have suggested that the proinflammatory marker like IL-6, TNF-α and the matrix metalloproteinase (MMP)-9 play a major role in the pathogenicity of keratoconus [96]. Apoptosis of keratocytes contributes to the alteration in the density of stroma [97,98]. Studies on keratoconus cornea emphasize the uncontrolled process of collagen degradation by the collagenolytic enzymes leading to stromal thinning (100). The degradation of collagen results in the production of telopeptides. C-telopeptides are carboxy terminal peptides of type 1 collagen. Telopeptides are used as biomarker to measure the rate of bone turnover. Study by Abalain *et al* has quantified the amount of telopeptides present in the tear of keratoconus and normal eyes. The tear film of keratoconus eye showed 2.5 fold increase of telopeptide concentration when compared to the tear film of normal eyes [69].

The effect of telopeptides on the organ system are not studied. Yet the effect of collagen degradation has been studied by von Wnuck *et al* where in the degraded collagen rapidly activates and leads to the apoptosis of human vascular smooth muscle cells [68].

In the current study C- terminal telopeptide was detected in the keratoconus tear film and was absent in the normal tear film. Primary Corneal stroma cells were established and were treated with various concentrations of C-telopeptides for 24hours, 48 hours, and 72 hours. Morphological changes or apoptosis was not observed in the primary corneal stromal cells when treated with the synthetic telopeptide for 24 hours and 48 hours. Apoptosis of primary corneal stromal cells were observed at 72 hours of telopeptide

treatment, at concentrations 94 µg, 47 µg. The data showed that a concentration greater than 47 µg causes apoptotic damage to the stromal fibroblasts. The same phenomenon of cell death can occur in keratoconus patients. The excessive collagen degradation products trigger the cells to enter the apoptotic pathway.

The clinical picture of the patients recruited in the study were analyzed further after the collagen crosslinking procedure. In majority of the patients the progression was worsening even after the CXL procedure. The observation showed that the patients with telopeptide concentrations of 50 -100 µg/ml in the tear, had sever progression of the ectasia. But in case of patients with undetectable telopeptide in tear and patients with lesser concentration of telopeptides, the progression was intermediate or constant. Hence, the results from the *in vitro* study and the concentration of telopeptide suggests that the degradation product of collagen could be one of the reason for the progression of keratoconus.

Though the quantification of telopeptides in the stroma of keratoconus patients will be a supporting data to the current study this was not included due to the non-availability of corneal buttons as the treatment modalities like Deep anterior lamellar keratoplasty (DALK) and penetrating keratoplasty are replaced by collagen crosslinking procedure

Initially keratoconus was defined as non-inflammatory corneal ectasia but studies have proven the overexpression of IL-6, TNF-α, and MMP-9 compared to the controls [72,75]. The expressions of these cytokines were considered as mediators of inflammation. Researches also proved that over expression of proteolytic enzymes in the keratoconus corneal epithelial cells leads to the damage of extra cellular matrix causing cell death and corneal thinning. Etiology of keratoconus is also related to the release of reactive oxygen species due to the increased levels of catalase and decreased levels of TIMP-1 [100,101].

Apoptosis is a mode of programmed cell death with two pathways the extrinsic and intrinsic pathway. The extrinsic pathway is death receptor mediated and is activated when specific ligand binds to the receptors. In case of TRAIL mediated apoptosis, TRAIL binds to the death receptors to initiate the cleavage of procaspase -8 to caspase-8 and thereby the caspase cascade extends leading to apoptosis [102]. TUNEL assays performed by Kaldawy *et al* on the keratoconus corneal sections revealed the presence of apoptotic

epithelial cells when compared to the normal corneas indicating that apoptosis could be a mechanism causing corneal thinning and keratoconus [72].

The results of the present study suggest that the apoptosis of corneal epithelial cells is aided by the expression of TRAIL and TRAIL death receptors DR4, DR5. The analysis of the fold change explained the relation between the expression of TRAIL and the progression of keratoconus. In most of the cases the onset was sudden and the progression was worsening. This further supports the finding that TRAIL gene expression is leading to apoptosis of the corneal cells leading to corneal thinning. The tear proteomics study on keratoconus patients by Pannebaker *et al* [103] showed that there were increased DR4 death receptors and proposed that the receptors are released into the tear due to the damage of the corneal epithelial cells as the epithelium is sloughed off in the tear film.

In our study the patients were classified based on the keratometry reading and the corneal thickness but clinically the patients were variable. On analysing the moderate KC group, KC-5 was the outlier with a high fold change of 26. KC-5 grouped under moderate category based on the keratometry readings, the clinical history showed that the onset was sudden with a prominent Munson's sign which could be the reason of elevated TRAIL expression. In other moderate cases the patients had a gradual onset and the duration was 2-3 years.

In advanced group KC-8, KC-9, KC-13 were the outliers and others with fold change ranging from 11-56. The three outliers in advanced group had a common history of sudden onset, gradual progression but the progression worsened when they initially presented to the clinic. During collection of the epithelium the progression of the same cases remained constant and they were using RGP contact lenses for more than a year.

The immunofluorescence data revealed the presence of DR4 and DR5 death receptors on the surface of the keratoconus corneal epithelial cells and the keratocytes. In donor cornea considered as normal controls DR4 was only expressed and not the DR5 death receptor. The myopic corneal epithelial cells treated as controls did not show positivity to both the death receptors. The fact that this excessive TRAIL gene expression leads to caspase pathway was further supported by experiments to show that Caspase 8, an apoptotic pathway regulating protein was also expressed significantly. Caspase 8 was expressed

more as the keratoconus disorder progressed. Increase in the caspase-8 shows that there is excessive procaspase-8 cleavage and activation of the caspase pathway leading to apoptosis.

It is a known fact that keratoconus is initially an anterior corneal degenerative disorder characterized with disarranged epithelium and anterior stromal degeneration (105). The increased expression of TRAIL gene in the keratoconus condition may be the major cause of epithelial damage leading to the death of anterior stomal keratocytes. The mediators of apoptosis in keratoconus cornea are less studied. You *et al* in their extensive study on the SFRP proteins, have shown that the epithelium in the cone region of the keratoconus corneas had abnormally enlarged columnar epithelium which overexpresssed SFRP protein [73]. Corneal epithelial cells are considered to play the key role in maintaining the corneal integrity and are proved to secrete the collagen for the formation of basement membrane [105]. Impairment in corneal epithelial cell maintenance due to apoptosis induced by TRAIL may be the key reason for epithelial thinning in keratoconus cornea.

Apoptosis of stromal keratocytes are the consequence of corneal epithelial damage as the epithelium and the stroma have strong interaction [106]. Interestingly investigations by Woo *et al* proved the significant alteration in the thickness and morphology of the cornea when the corneal epithelium of rabbits were chronically injured [107]. Studies show that injury to the epithelial cells influence the production of extracellular matrix of the stroma. Literature states that death of corneal epithelium induces IL-1 which binds to the IL-1 receptor expessed by the stromal keratocytes leading to apoptotic pathway [108]. To date, expression of TRAIL has not been studied on kertoconus cornea. Our observations suggest that TRAIL and the death receptor recruiting caspase cascade expression are closely associated with keratoconus. Thus the results of our study highlights that corneal epithelial cells can be the seat and site of apoptotic mediators in keratoconus which can eventually affect the stromal keratocytes.

6. OVERALL CONCLUSIONS:

The management of keratoconus has gained importance as the disorder commonly affects the productive age groups (19- 25 Years) of a population. Collagen cross linking treatment is considered as a ray of hope in the management of keratoconus. Series of studies state the safety of collagen cross linking procedure on the corneal layers. The present study stresses on the effect of collagen cross linking (UVA/ Riboflavin) procedure on the corneal limbal epithelial cells. Protecting the limbal region with a metal ring during the treatment procedure can to an extent avoid the damage of the limbal stem cells.

Various pathogenic mechanisms have been proposed as the etiology of keratoconus. But the exact mechanism involved in the progression of keratoconus is unknown. The present study highlights two novel pathogenic mechanisms involved in the apoptosis of the stromal keratocytes, the telopeptides and the TRAIL ligand respectively.

The concentrations of the telopeptides though it did not correlate with the severity of keratoconus it was useful in ascertaining the concentration of synthetic telopeptides for the *invitro* study. Stromal cell viability was decreased with increasing concentrations of telopeptides. Hence the C- terminal telopeptides released as a result of collagen degradation can potentially induce apoptosis of the corneal stromal cells contributing in the progression of keratoconus *in vivo*.

The present study on TRAIL and TRAIL receptors revealed that TRAIL is over expressed in Keratoconus cornea. The death receptors DR4 and DR5 are expressed in keratoconus cornea whereas in the normal corneas only DR4 receptor was expressed. TRAIL and TRAIL receptors are involved in the extrinsic pathway of apoptosis and is used in cancer therapeutics. Over expression of TRAIL and the presence of TRAIL death receptors in the keratoconus cornea shows that TRAIL is involved in the pathogenic progression of keratoconus. Thus a modification in the routine treatment modality will protect the limbal stem cell to an extent from damage. Targeting the detected novel pathogenic mechanisms will contribute to effective therapeutic agents in treating progressive keratoconus.

7. SPECIFIC CONTRIBUTIONS:

- The present study has initiated a spark that the limbal stem cells are affected during the collagen crosslinking procedure. Previous literatures had only focused on the safety of corneal keratocytes and endothelium.
- A simple modification in the collagen cross linking procedure i.e. covering the limbal area with metal ring can effectively protect the limbal stem cells from UVA damage.
- The metal ring is now being routinely used in Sankara Nethralaya during the collagen crosslinking procedure for the keratoconus patients.
- Collagen degradation products are considered as diagnostic tool for the detection of keratoconus. But the apoptotic role of telopepteides has not been assessed. The study has assessed the effect of the C- terminal telopepide on the stromal cells, highlighting their apoptotic role.
- TRAIL is widely studied in cancer treatment; this is the first study to correlate the expression of TRAIL with the progression of keratoconus. The present study has shown the distribution of the death receptors in the normal cornea which will aid as reference for future studies. Novel therapeutic ideas can be initiated from the present study on the expression of TRAIL in keratoconus corneas.

8. FUTURE PROSPECTS:

- Metal ring can be replaced by disposable materials such as modified PMMA. PLEXIGLAS® UV 100 (Darmstadt, Germany) is considered to be 99.7% UV resistant and can be used for protecting the limbus during the collagen cross linking procedure.

- An *in vitro* 3D model mimicking the keratoconus cornea can be created such that the effects of the collagen degraded products can be effectively studied.

- Designing Small interfering RNAs (siRNAs) and microRNAs (miRNAs) against the TRAIL and TRAIL death receptors can result in effective therapeutic agent for Keratoconus.

- Inducing the production of decoy receptors can be studied as these decoy receptors can compete with the death receptors in TRAIL binding resulting in cell survival instead of apoptosis.

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10. APPENDICES

APPENDIX - I

1. **Gel Loading Dye:**

   Solution A - 0.1% Bromophenol blue in 1x TBE buffer

   Solution B - 40% sucrose in 1x TBE buffer

   Prepare solution A and B separately and equal volume was mixed and used for gel loading.

2. **Preparation of Gel and Visualization of PCR Products:**

   Agarose- 0.5 g was weighed and dissolved in 25ml of 1X TBE buffer, by heating in microwave oven until no granules were seen. Then added 8 µl (12.5 µg/ml) of ethidium bromide mixed well and poured to the gel tray with appropriate combs and allowed to solidify. The gel was then placed in the tank with 1X TBE buffer. 10 µl amplified products were mixed with 2 – 3 µl loading gel and loaded onto wells along with a molecular weight marker in each run. The electrophoresis was run at 100 Volts for 30 minutes, and the results were read and documented in the gel documentation system (Vilber Lourmat, Marne La Valle, Cedex, France).

APPENDIX - II

Preparation of media necessary for cell culture maintenance:

1. Antibiotics stock preparation:

Pencillin: 1 vial (RM 132, HiMedia, 1,000,000 units / vial) is reconstituted with 10 ml of Milli Q water, filter sterilized and kept at 2-8^{0}C. Add 0.1 ml for 100 ml of growth medium.

Gentamicin: (1 gm, RM 461- HiMedia,) 1 vial is reconstituted with 10 ml of Milli Q water, filter sterilized and kept at 2-8^{0}C. Add 0.05 ml for 100 ml of growth medium.

Streptomycin: 0.75g of the powder is dissolved in 10ml of MilliQ water, filter sterilized and kept at 2-8^{0}C. Add 0.05 ml for 100 ml of growth medium.

Ciprofloxacin: 10mg of the powder is dissolved in 10ml of MilliQ water, filter sterilized and kept at room temperature. Add 0.05 ml for 100 ml of growth medium.

2. Preparation of Growth medium:

For preparation of growth medium, 10 ml of fetal calf serum for 90ml of basal medium (DMEM :F12, Gibco, Invitrogen), Antibiotic solutions- (Penicillin 0.1 ml, Streptomycin 0.05 ml, Gentamicin 0.05ml & Ciprofloxacin 0.05ml)

3. Maintenance of Primary cell lines:

Trypsin EDTA solution:

Trypsin - 0.02 g

EDTA - 0.03g

Dextrose - 0.5g

Sterile PBS - 100ml

Filter to sterilize

Subculture of primary cell lines:

1. Pipette out the culture medium from the tissue culture flask and rinse the cell line with trypsin-EDTA solution.
2. Add a sufficient amount of trypsin -EDTA solution to cover the cell layer.
3. Remove trypsin-EDTA solution.
4. Incubate the flask at RT/37^{0}C for 2 minutes till the morphology of individual cells are changed.
5. Cells will detach from the culture vessel surface. Tap the side of the flask to dislodge any remaining cells.
6. Add a small amount of growth medium and aspirate several times with a pipette to suspend and separate the cells.
7. Dilute a small sample of the cell suspension with additional growth medium and dispense directly into new flask.

E. Preparation of Preservation medium:

Media A:

Serum free DMEM: F12 media - 7 ml

DMSO (Dimethyl sulphoxide, Cell culture grade, Himedia) - 3 ml

Sterilize by filtration.

Media B:

10% DMEM:F12 – 8ml

Fetal calf serum – 2ml

Preservation of primary cells:

1. Mix equal volume of Media A and media B and aliquot 0.5 ml to labeled cryovials.
3. The monolayer of primary culture was trypsinised
4. Preservation medium (1ml) was added and mix with a pipette to suspend and separate the cells.
5. 1-2 ml of cell suspension was in to cryopreservation vials using a pipette.
6. 8. Place cryotubes in thermo cole box.
7. 9. Cells should be cooled from room temperature to -75^{0}C to -85^{0}C freezer.
8. 10. The cells were then placed in 1° coolant for 18 hours and then stored in liquid nitrogen for long -term storage.

Recovery of frozen cells:

1. Remove the stored cryovials from liquid nitrogen and immediately thaw contents by placing it in a 37^{0}C water bath.
2. Withdraw contents with sterile Pasteur pipette, place in tissue culture flask, add fresh growth medium and incubate at 37^{0}C. Replace media after the cells have attached, or after 24 hours.

APPENDIX-III

LIST OF CONSUMABLES

Agarose SRL, India

Boric acid, Molecular Grade SRL, India

Bromophenol blue SRL, India

RNA extraction kit, Qiagen, Hilden, Germany

cDNA conversion kit, Qiagen, Hilden, Germany

Desalted Oligonucleotide bases Bangalore Genei Pvt, Ld, India

dNTPs Bangalore genei Pvt, Ltd, India.

EDTA, Molecular grade SRL,India

Ethanol SD fine chemicals Pvt Ltd, India

Ethidium Bromide Hi Media, Mumbai, India

Isopropanol (molecular grade) Hi Media, Mumbai, India

Molecular weight marker- 100 bp, 25 bp ladder Bangalore genei Pvt, Ltd, India.

Taq DNA Polymerase Bangalore genei Pvt, Ltd, India.

Turbo DNase kit, (Qiagen, Hilden, Germany)

Tris-HCl, Molecular biology grade SRL, India

DMEM:F12, Gibco, Invitrogen

Fetal bovine serum, Gibco, Invitrogen

Primary antibody, Biolegend, San Diego, California

MTT, Sigma,USA.

LIST OF PUBLICATIONS:

ARTICLES PUBLISHED:

1. Vimalin Jeyalatha, Nidhi Gupta, Malathi Jambulingam, Prema Padmanabhan, Madhavan N Hajib, The effect of Riboflavin-UVA treatment on Corneal Limbal epithelial cells - a study on human cadaver eyes. *Cornea*. 2012; 31:1052-1059.

2. Vimalin Jeyalatha, Malathi Jambulingam, Nidhi Gupta, Prema Padmanabhan, Hajib N. Madhavan. Study on Polymethylmethacrylate Ring in Protecting Limbal Stem Cells during Collagen Cross-Linking. *Ophthalmic Res* 2013;50:113–116

3. Vimalin Jeyalatha, Malathi Jambulingam, Prema Padmanabhan, Madhavan N Hajib. Apoptosis of corneal stromal cells induced by telopeptides: An *in vitro* study.*Ophthalmology Rresearch*. *An International Journal. 2014:*2(6): 40-47

4. Shylaja R, Vimalin Jeyalatha M, Malathi J, Madhavan HN and Biswas J "Standardization and Application of Polymerase Chain Reaction for LruA and LruB gene of Leptospira interrogans in Aqueous humour of Uveitic patient" *Journal of Ocular Immunology and Inflammation* 19(5), 363-366, 2011.

5. Shylaja Ram, Jeyalatha Mani Vimalin, Malathi Jambulingam, Vaidehi Tiru, Ravi Kumar Gopalakrishnan & Madhavan Hajib Naraharirao "Application of PCR-based DNA sequencing technique for the detection of Leptospira in peripheral blood of septicemia patients" *Malaysian Journal of Microbiology* 2012 8:26-33.

ARTICLE ACCEPTED FOR PUBLICATION:

1. Vimalin Jeyalatha, Malathi Jambulingam, Madhavan N Hajib. Stepwise protocol for cytospin enhanced smearing for scraped corneal epithelial cells. Applied Immunohistochemistry & Molecular Morphology.

LIST OF PRESENTATIONS:

NATIONAL-ORAL PRESENTATION:

- Presented paper entitled "Response Of Retinal Pigment Epithelial Cells Towards Leptospiral Recurrent Uveitis Antigens" for Sengupta Immunology award at 38th MICROCON 2014.

NATIONAL POSTER PRESENTATIONS:

- Presented poster entitled "Study to Evaluate the Effect of UV Rays on Riboflavin Treated Cadaveric Corneal Limbal Stem Cells" in IERG conference held on 30th - 31st July 2011 at Hyderabad.
- Presented poster entitled "Apoptostic effect of telopeptides on Corneal Stromal Cells: an in *vitro* Study" in Asia-ARVO 2013 from 28 – 31 October 2013 in New Delhi, India.

AWARDS WON:

- Ranbaxy Laboratories Ltd. Immuno Diagnostic Division Endowment award for the Best Performance in Clinical Immunology.
- ARVO National Travel Grant, to attend Asia-ARVO 2013 from 28 – 31 October 2013 in New Delhi, India.

BIOGRAPHY OF THE CANDIDATE:

Ms. M. Vimalin Jeyalatha completed under graduation in B.Sc Microbiology from Valliammal College of Arts & Science, Anna nagar, Chennai in the year 2007. She joined MS Medical Laboratory Technology conducted by BITS, Pilani and graduated in the year 2010. She worked as a Junior Research Fellow for 1year in the project entitled "A study on the effect of UV rays on riboflavin treated corneal limbal stem cells during the process of collagen cross linking" funded by DST and Senior Research fellow in DBT funded project "An *in vitro* study on the signaling cascade occurring in human corneal epithelial cell line targeted with Hepatitis C virus (HCV) and a clinico molecular microbiological study on HCV associated with keratoconjunctivitis sicca and Sjogren's syndrome". Currently she is working as a Junior scientist in the L&T Microbiology Research Centre. She registered for PhD in BITS, Pilani in August 2010 under the guidance Dr. J. Malathi. She has made 1 oral and 2 poster presentations in National conferences. She has 6 publications. She had conducted practical and theory classes in microbiology for the under graduate (BS Optometry) and post graduate (MSMLT) students registered under off campus courses of BITS Pilani. Mentored 3 students from various colleges for 3-4 months short term research projects and 2 students from BITS, Pilani for Practice School I. Trained more than 30 candidates (National and International) in Molecular Microbiology Techniques (PCR, Real Time PCR, DNA sequencing, cell culture techniques, virology etc) in the summer training programme organized by Vidyasagar Institute of Bio-Medical Sciences (VIBS), a unit of Medical Research Foundation. Involved in conducting and training Antimicrobial surveillance program conducted by WHO-WHONET 2011 & 2013 at L&T Microbiology research center, Vision Research Foundation. She was awarded Ranbaxy Laboratories Ltd. Immuno Diagnostic Division Endowment award for the Best Performance in Clinical Immunology.

BRIEF BIOGRAPHY OF THE SUPERVISOR

Dr. J. Malathi completed her PhD from Birla Institute of Technology and Science in 2003. At present she is the Reader in the Department of Microbiology, L & T Microbiology Research Centre, Vision Research Foundation, Chennai. She has 15 years of rich experience in Medical Microbiology with 14 years postgraduate teaching in Medical microbiology with 60 publications in national and international journals. She is a recognized Ph. D guide in BITS-Pilani, and SASTRA. She had been involved in the initiation of Reverse Transcriptase PCR and Real Time PCR development of nucleic acid–based molecular biological methods in L & T Microbiology Research Centre, Vision Research Foundation, Chennai. She is Principal Investigator of 2 projects and Co-Principal Investigator of 4 Research projects sanctioned by ICMR and DBT. Her areas of special interest are development of rapid diagnostic tests based on molecular techniques for rapid detection of infectious agents and diagnostic DNA chip for infectious diseases and to study the Molecular epidemiology of specific infectious diseases particularly related to ophthalmic infections, studying bacterial pathogenic mechanism and drug resistance. Two patents have been applied. She had done 25 presentations in National & International conferences, Invited academic lectures, National & International Symposia; CME programmes. She is a Life Member of Indian Association of Medical Microbiologists.

BRIEF BIOGRAPHY OF THE CO-SUPERVISOR

Dr. Suman Kapur joined BITS, Pilani as Professor in the Centre for Biotechnology, Biological Sciences Group. She has worked in the capacity of Unit Chief, Community Welfare and International Relations since 1st January 2007. From 16th April 2010 she has taken charge as Dean, Research & Consultancy at the Hyderabad Campus of BITS. With her team of a dozen research scholars has been instrumental in building a state of the art Human Genomics laboratory from funds received as Principal and/or Co-Investigator of now more than eighteen grants awarded since her joining BITS in 2004. As a mentor she has been able to motivate younger faculty to submit and execute independent grants in the form of Women scientist (DST), Research Associate and senior research fellows (ICMR & CSIR). She has published more than 80 research articles in International and journals. Her research interests lie in identifying biomarkers for unraveling the genetic basis of human diseases such as psychianic disorders like depression, schizophrenia, addiction and alzheimeres disease and metabolic disorders such as diabetes (T2DM), obesity, cataract and metabolic syndrome. The group is specifically studying several genes,viz., APOE, CAPN, PPARi5, it-4C ALDH2, ADM.% ADH1C, OPRM1, OB, TPH, CRVGA, CRVGB, D2, D5, ADCV4, ADCV3, CCKAR, CCKBR, cm, CF508, SPNK-1, PS-1, CVP2E1, CTSB, HSP70, TNFii, IC PRSS-1 and several micro-satellite markers on chromosome segments 2, 6 and 10. Chronic diseases have a long latency period and genetic markers can be effectively used for identifying individuals at an increased risk for developing these diseases and advocating appropriate lifestyle measures to delay the onset and progression of such diseases. Dr. Suman Kapur has ably conducted the day to day activities of this unit and was instrumental in orchestrating several student exchanges introduction of new fellowships and opportunities for both students and faculty at BITS campuses.