Guest Editorial

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Corneal Cross linkage – An Optical Marvel

Keratoconus is an inflammatory related progressive ectatic condition of the cornea due to focal biomechanical decompensation.[1,2] This focal biomechanical decompensation leads to progressive stromal thinning and ectasia with resulting topographic and visual abnormalities. Collagen crosslinking (CXL) is the only safe, effective procedure to halt the progression of keratoconus and other ectatic disorders. In this review, we discuss the principles and clinical applications of CXL.

Patho-mechanisms behind ectatic disorders

The biomechanical stiffness of corneas with keratoconus is shown to be decreased by a factor of 0.7 (70 % lesser strength). The total content of collagen in ectatic corneas is similar to healthy corneas but the arrangement of collagen fibrils and lamellae is widely different. The collagen fibrils at the apex of a keratoconus cornea form a wide layer, show no delimitation of the lamellae and almost absent interlacing among the fibrils. This disorganized arrangement and poor interlacing of the collagen lamellae reduces the biomechanical strength of the corneas with keratoconus.

Basics of CXL

The word crosslinking means the formation of chemical bonds or bridges between proteins and other large molecules. These crosslinks increase the tissue strength, stiffness, and resistance to degeneration.

Crosslinking is employed in multiple industries and manufacturing practices. It is utilized to harden materials in polymer industry, to strengthen filling materials in dentistry and to stabilize tissues in bio-engineering.[3] Among many applications in medical industry, crosslinking is used to polymerize intra-ocular lens materials, and to manufacture vascular graft materials.[4]

Natural cross-linking of collagen in cornea

Enzymatic cross-linking is a natural post-translational modification of collagen by lysyl oxidase enzyme. Lysyl oxidase creates covalent cross links between the collagen fibrils through oxidative deamination of the lysine and hydroxylysine residues.[5] Non enzymatic crosslinking occurs through glycation in diabetics as a natural ageing process.[6]

Cross-linking as a chemical process

CXL is a photochemical reaction similar to photosynthesis where light energy (derived from UV-A) is converted into chemical bonds. As riboflavin absorbs UV-A light, it excites and transforms into singlet and then triple excited states. In the presence of oxygen (type-2, aerobic reaction), the excited riboflavin reacts with the triplet oxygen, and generates singlet oxygen radical. This highly reactive oxygen free radical reacts with the carbonyl groups on the amino-acids in the collagen peptides and forms crosslinks. In the absence of oxygen, (type-1, anaerobic reaction), the riboflavin free radicals interact with the collagen peptides and form the crosslinks. The type-1 crosslinking is less efficacious than type-2 crosslinking.

Components of CXL

In the following section, we discuss the three major components of this photochemical reaction – ultraviolet light, riboflavin and oxygen. The discussion focuses on the mechanisms of involvement of these factors, the proposed modifications in their application to improve the technique.

Ultra-violet (UV) light

UV light is the source of the energy for the crosslinking process. The absorption peak of riboflavin is at 370 nm, providing protection to the endothelium and internal ocular structures at this wavelength. In the absence of a photosensitizer, cornea absorbs 35% of the incident UV A irradiation. In the presence of 0.1% riboflavin, corneal stroma of 400 microns thickness, absorbs 90% of the UV-A irradiation, thus less than 10% of the UV-A energy reaches the intraocular structures, which is absorbed by the lens. The resulting endothelial exposure is 0.18mW/cm^2, which is lesser than the safety threshold of 0.35 mW/cm^2.

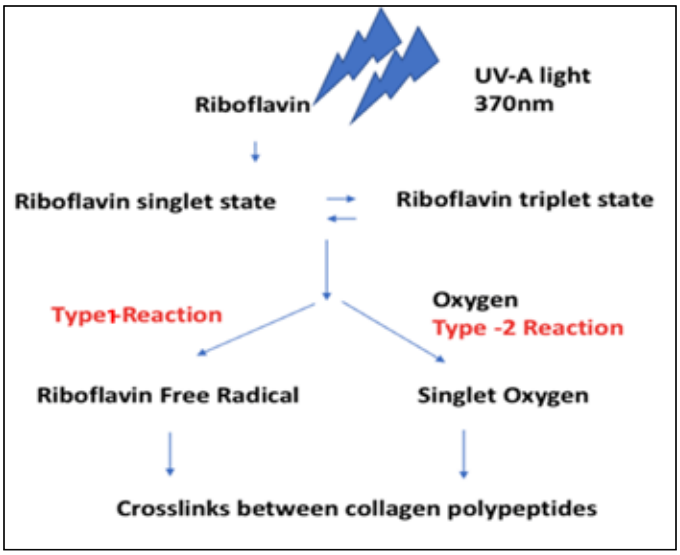

Figure 1: *Mechanism of crosslinking reaction*

Another important parameter that determines the intra- ocular toxicity of UV-A irradiation is the vergence of the beam. Divergent beam from shorter distances such as given by UV devices used during cross-linking has lesser energy density and is less deleterious to ocular structures.

Riboflavin

Riboflavin acts as a photosensitizer in the process of CXL, and increases the stromal absorption of UV- A irradiation. As mentioned earlier, riboflavin has peak absorption at UV-A wavelength, hence protects the intraocular structures from the radiation toxicity. Riboflavin is a micronutrient, used as a food coloring agent and is safe in the event of systemic absorption. Riboflavin has poor permeability through epithelial tight junctions, hence the necessity for epithelial removal during CXL. To improve the permeability, chemicals like Benzalkonium chloride (BAK),[7] Ethylene-diamine-tetraacetic acid (EDTA), were added to riboflavin solution. Trans-epithelial CXL irrespective of the modifications, was inferior to the standard epithelium-off CXL in terms of biomechanical efficacy.[8,9]

Physical and Biomechanical Effects of Collagen Crosslinking

The structural effects of CXL are mentioned in (table1).

Table:1 Physical and Biomechanical Effects of Collagen Crosslinking:

Effects of CXL	Reason / Clinical Significance
Increase in stiffness (Young's modulus)	Halts progressive biomechanical weakening in ectatic disorders
Increase in shrinkage temperature	Indirect evidence of strength of cross-linking
Increase in resistance to enzymatic digestion	Increased resistance to collagenases
Decrease in the swelling pressure	Influx of water is prevented due to interfibrillar crosslinks Can be explored in the treatment of Bullous Keratopathy
Increase in the thickness of collagen fibril diameter	Induced crosslinks may have pushed the collagen polypeptide chains apart, thereby increasing the interpeptide spacing.

Cellular and Extra-cellular effects of CXL:

The effects of cellular and extra-cellular ocular structures are mentioned in the (table 2).

Cellular and Extracellular Effects of CXL on Ocular Structures

Structure	Effect	Recovery
Epithelium	Removal during CXL procedure No damage to limbal cells[10]	Re-epithelialization within 3 days[10]
Keratocytes	Apoptosis of keratocytes in anterior stroma[11,12] Lacunar edema around the apoptotic keratocytes	Repopulation of the anterior stroma by peripheral activated keratocytes by 4-6 weeks
Stromal extracellular matrix	Increased density due to collagen compaction by the induced crosslinks	Long term sustenance of the induced collagen crosslinks explains the long-term stability of ectasia.
Nerves	Disappearance of sub-basal nerve plexus in the crosslinked stroma	Regeneration starts in a week after CXL
Endothelial cells	No significant effect on the density, morphology of endothelial cells[13] (Figure 2)	

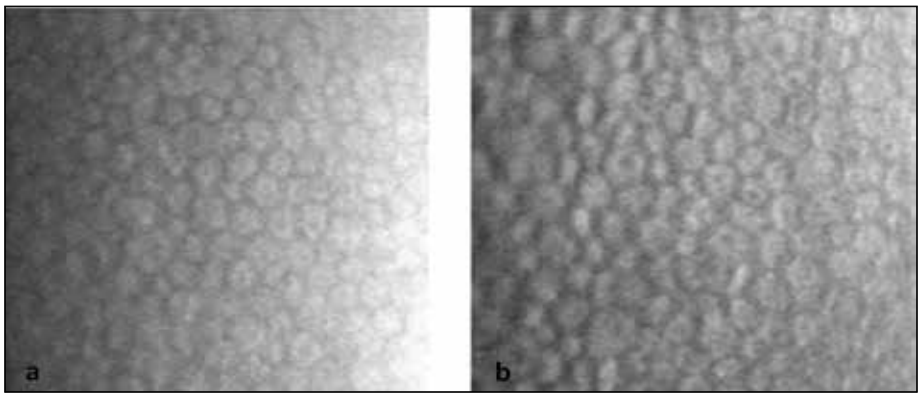

Figure 2: (a)*- Pre-CXL specular microscopy of a cornea – The endothelial density was 2882*
(b)*- Post CXL specular microscopy of a cornea with thickness 390µ that underwent accelerated CXL with UV irradiation dose calculated by NXT-UVA calculator – The endothelial density was 2865*

Dresden Protocol

Wollensak et al reported the first in vivo use of UV-A irradiation with riboflavin in humans.14The protocol named after the place of study (Dresden, Germany) involves debridement of central 7 mm of the epithelium, application of 0.1% riboflavin solution 30 minutes before the procedure and every 5 minutes during the UV-A irradiation at a dose 3mW/cm2 for 30 minutes. The standard CXL has been demonstrated to effectively stabilize keratoconus and improve the topographic and biomechanical outcomes.[15–17]

Accelerated Cross-linking (ACXL)

Technique

The Bunsen-Roscoe law of reciprocity states that the photochemical effect is directly proportional to the total dose of the irradiation(W/cm2) irrespective of the dose and duration of the exposure over certain range.[18] The law has been named after R. Bunsen and H.E. Roscoe for their pioneering work in photochemistry.[19] The Dresden protocol involves the irradiation time of 30 min with 3mW/cm2 dose amounting to a total UV-A energy of 5.4J/cm2. To reduce the irradiation time, multiple combinations of irradiation dose and time, 9mW/cm2 for 15 min, 10 mW/cm2 for 9 min, 18 mW/cm2 for 5min , 30 mW/cm2 for 3 min, and 45 mW/cm2 for 2 min have been studied with the total UV-A energy of 5.4J/cm2.[20–22] These techniques of CXL with shorter irradiation time are termed as accelerated CXL (ACXL).[23] The accelerated protocols are introduced to clinical practice due to the observation that the corneal stiffening effect of the higher UV-A fluences over shorter durations were comparable to the original 3mW/cm2 irradiation. However, it is essential to understand that the Bunsen- Roscoe law of reciprocity is applicable only till certain irradiation intensities. Any accelerated CXL protocol should strike a balance between the irradiation intensity, exposure duration, the biomechanical strengthening effect and the safety profile.

ACXL- Outcomes (Figure 3)

A meta-analysis of eleven trials comparing ACXL with SCXL reported that SCXL resulted in greater reduction in steep keratometry (Kmax) compared to ACXL.[24] However, there are reports of ACXL showing equally good topographic outcomes as SCXL.[25,26] The variance between the topography outcomes can be explained by the different exposure times followed by the authors.[20] Improvement in visual acuity is reported to be similar between ACXL and SCXL.[24] Among the different ACXL protocols, lower irradiance and shorter exposure time protocols may result in better visual outcomes.[27] The depth and latency of onset of the demarcation line is shown to be shallow and delayed in eyes treated with ACXL protocols than SCXL protocol.

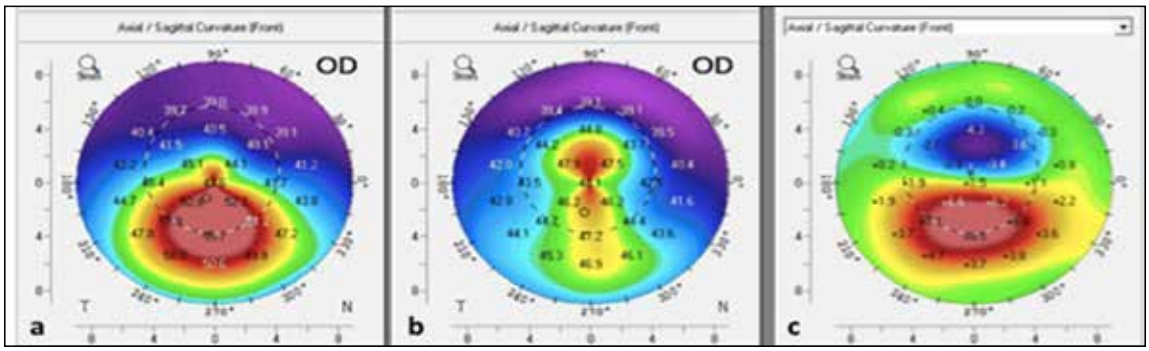

Figure 3: (a)*- Preoperative Sagittal curvature map of an eye with keratoconus, BCVA was 6/12 with the refractive error of +2.25DS/-6.50DC@80°*
(b) *–Sagittal curvature map of the same eye after topography guided trans-epithelial PRK and accelerated crosslinking, BCVA was 6/9 with refractive error of +0.25DS/-2.75DC@110 °*
Note*- the magnitude of keratometric flattening (depicted in c) and the relative surface regularization following the procedure*

Cross-linking in thin cornea

Safe and effective cross-linking in eyes with thin corneas faces two challenges. First is the safety threshold of UVA energy at the endothelium. Wollensak et al, in their original article on collagen cross-linking, reported that in a 500 µ thick cornea with the 3mW/cm2 irradiance at the surface and 0.1% riboflavin, the UVA energy reduces by 95% and the energy at the endothelial level is 0.27J/cm2, leaving a twofold margin for toxic irradiance(0.65J/cm2).[14] However, the total fluence employed in classic Dresden protocol, if used in corneas thinner than 400µ (after de-epithelialization), the toxicity threshold of 0.65J/cm2 could be reached at the endothelial level. Hence, the authors cautioned the use of Dresden protocol in corneas thinner than 400µ. Second is post collagen cross-linking stromal haze, which is seen more often in eyes with advanced keratoconus, and thin corneas.[28] Since significant proportion of patients with keratoconus have eyes with thinnest pachymetry < 400µ, modifications in the technique of collagen cross-linking to make it a safer tool in these corneas is necessary. To ensure safety of cross-linking in thinner corneas, one has to ensure shallow depth of UVA treatment so that the endothelium is not exposed to the UV-A energy beyond the safe threshold level. To achieve this, one can increase the thickness of the cornea, or place a layer of biological or synthetic origin above the cornea. Apart from this, the total fluence of the UVA irradiation can be reduced by reducing the irradiation dose, exposure time or customize the irradiation dose as per the thickness parameters. The newer techniques are mentioned in the (table 3).

*NXT-UVA calculator is a freely available online calculator, that helps customize the UV-A 'on' time to the thickness of the treated cornea.**(Figure 4)** The UV-A 'on' time was calculated based on Lambert- Beer equation. Since, the total irradiation dose is within the limits of endothelial toxic exposure, there is no risk of endothelial toxicity or decompensation.[29,30]*

Figure 4: *NXT -UVA calculator screen. The clinician can enter the thickness of the cornea, the calculator gives the time duration for exposure of UV -A irradiation for 3mW/cm2 and 9mW/cm2 and for the riboflavin concentration of 0.1% and 0.2%. The clinician can follow the UV-A exposure time based on the UV-A irradiation dose and riboflavin concentration*

Table: 3 Modifications of CXL in corneas thinner than 400µ

Modification of CXL	Technique	Outcomes	Limitations
Hypo-osmolar Riboflavin[31] Hafezi et al (2009)	Iso-osmolar 0.1% riboflavin applied every 3 minutes x 30 minutes followed by hypo-osmolar riboflavin- 0.1% in riboflavin in 0.9% Sodium chloride (NaCl) every 20 sec x 5 min/till the pachymetry becomes 400µ	Equal to eyes treated with Iso-osmolar riboflavin[32]	Failure in extremely thin corneas[33]
Contact lens assisted - CXL[34] Jacob et al 2014	Iso-osmolar 0.1% riboflavin applied every 3 minutes x 30 minutes, UV-A barrier free contact lens (Soflens, B&L) immersed in isoosmolar riboflavin x 30 minutes placed over the cornea	Stable keratoconus with no progression in 80% eyes, Keratometric flattening in 40% eyes[35]	Oxygen permeability of the contact lens, UV-A absorption by the contact lens and pre-corneal riboflavin film are limiting factors
Customized epithelial debridement[36] Kymionis et al 2009	Central 8mm of epithelial debridement leaving an island of epithelium over inferior area of steepening and thinning followed by 0.1% riboflavin every 3 minutes for 15 minutes	Stability of keratoconus upto 9 months follow up[36]	Unpredictable riboflavin penetration through intact epithelium over the cone
Stromal Lenticule assisted CXL[37] Sachdev et al 2015	Stromal lenticule (6.2mm diameter) was placed over the apex of the cone of the cornea 0.1% riboflavin every 5 minutes for 30 minutes followed by every 1 minute during UV-A irradiation	Stability of keratoconus demonstrated in 3 patients over 6 months[37]	Limited evidence on the efficacy
Individualized Fluence CXL Hafezi et al NXT UVA calculator[30] Shetty et al (2020)	UV- A irradiation "on" time individualized to the thickness of the treated cornea A simple calculator to decide the UV-A on time in thin corneas	Stability demonstrated at 12 months following CXL	Needs to be validated in large sample size studies

Laser Based Treatment Protocols in KC

Keratoconus and Ocular Aberrations

Keratoconus (KC), being a progressive ectatic condition with asymmetric corneal steepening, causes corneal surface (anterior and posterior) irregularities and induces both lower and higher order aberrations (HOA) in many magnitudes higher than a normal eye.[38] The higher order aberrations are shown to be approximately 5.5 times higher in eyes with KC than in normal eyes.[38] Coma like aberrations including vertical coma are the dominant HOA in these eyes[38,39]

Surface Normalization and the Visual Effects in KC

Before discussing the laser based treatment approaches in KC, we need to understand the concept of surface normalization. The laser based ablation in eyes with KC is not aimed at refractive correction rather to regularize the corneal surface, to create better aspheric profile of the cornea, and to reduce the higher order aberrations. The location of the ectatic cornea affects the corneal asphericity and the magnitude and pattern of higher order aberrations.

Concept of Topography guided surface normalization

The topography guided surface normalization usually follows a specific ablation profile. The ablation pattern is planned in such a way that simultaneously flattens the ectatic cone area and an arcuate area of cornea in the periphery away from the cone usually in the superior nasal location. This peripheral flattening induces steepening adjacent to the cone similar to a hyperopic treatment. The combination of flattening in cone area and adjacent steepening regularizes the corneal surface thereby reducing the HOA.[40]

Let us discuss the planning technique in the two popular laser based platforms employed globally in keratoconus eyes

Parameters

Maximum Ablation depth

Multiple authors have used different permissible upper limits for the stromal ablation in topography guided PRK in ectatic eyes. The maximal permissible ablation depths by various authors are : Kymionis et al – 50 μ[41], kanellopoulos et al – 50 μ[42], Camellin et al – 55μ[43], Shetty et al- 40μ[40].

WaveLight Allegretto Wave™ Excimer Laser System (Wave Light Laser Technologie AG)

For this platform, the topography examinations are done by the ALLEGRETO WAVE Topolyzer Vario, with the T-CAT (Topography guided custom ablation treatment) software for treatment. The platform also enables the clinician to choose the post ablation aspheric profile of the treated cornea.__[42]

Protocol followed by the authors[40]

The asphericity of the cornea, location of the cone (centered vs decentered) and the refractive error is taken into account while planning the topography guided custom ablation in keratoconic eyes.

Eyes with Centered Cone

The ectatic cone area is considered central if more than 50% cone area is with in the central 3mm zone on posterior elevation map. In these eyes, cornea has more negative asphericity (high negative Q value) and a myopic refractive error due to central steepening. We target either the reduction of Q by 20-30% or partial refractive correction, where both approaches would induce central flattening and regularization making the cornea achieve a more physiological aspheric profile. To achieve this, the authors optimize the Zernike polynomials to achieve the equivalency between the defocus (C4) and the spherical aberration (C12) by a specific spherical error input in the targeted refraction tab. The choice to partially correct the refractive error is taken based on the baseline refractive error (<6D) and thinnest pachymetry (>45μ).

Eyes with Decentered Cone

Eyes with decentered cone, have a less negative Q value but other dominant higher order aberrations, including coma and trefoil. During the attempted surface regularization and reduction of HOA, the Q value may reduce significantly to a more negative values. If no refractive correction is attempted, the preoperative Q can be selected as zero. Partial refractive correction can be targeted without breaching the 40μ stromal ablation thickness rule.

We initially assess the plano treatment ablation profile planned by the software, then apply Zernike polynomial optimization (to target C4 (Defocus) and C12 (Spherical Aberration) equivalency, and then finally attempt partial refractive correction based on the baseline refractive error and thinnest pachymetry.

AMARIS (SCHWIND eye- tech- solutions) (Figure 5)

The AMARIS is a flying spot laser platform that performs reversed single step wave-front based customized Trans epithelial PRK. The ablation profile is planned using ORK-CAM software. The step wise planning of topography guided trans PRK is mentioned in detail in ***(figure 5)****.*

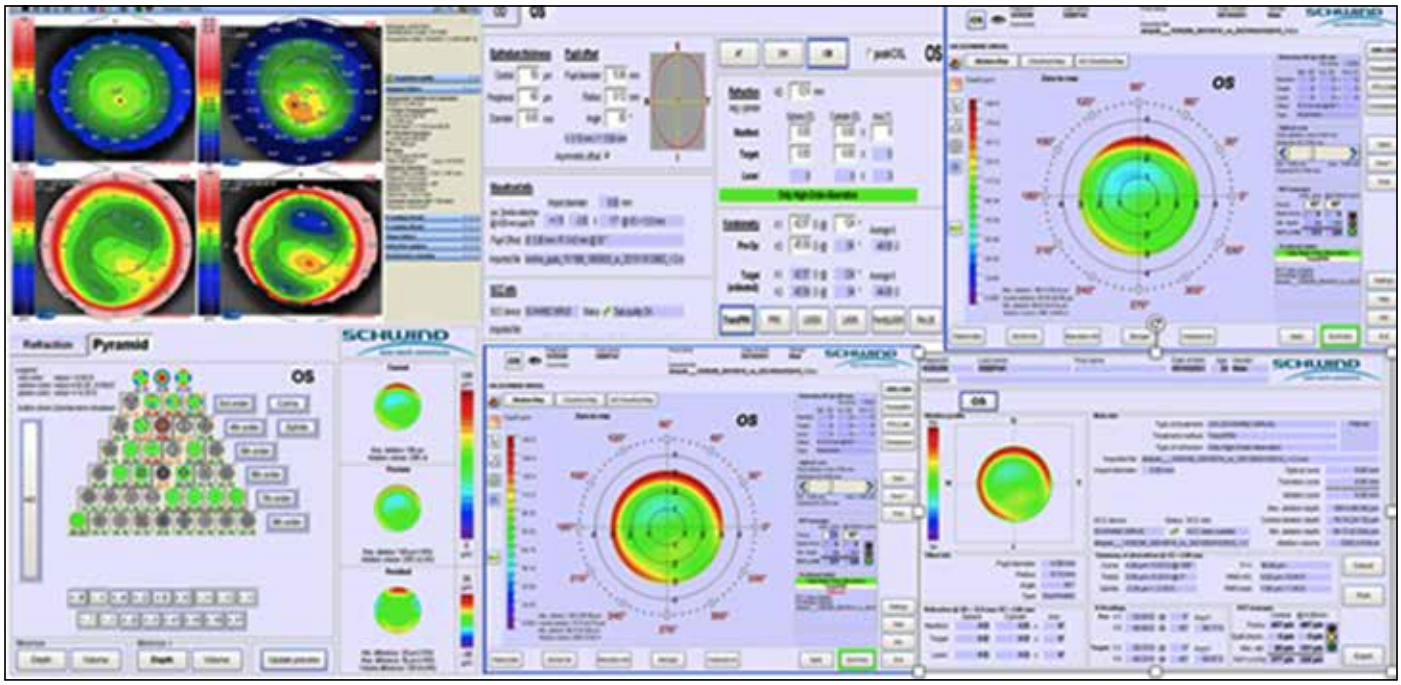

Figure 5: (a)*- The scans captured by the topographer prior to export to planning software*
(b)- *Initially enter zero refractive error*
(c) – *Assess the ablation depth while treating higher order aberration only applying the zero rule*
(d)- Minimize *depth of ablation by correcting only significant higher order aberrations, through selecting the "depth minimization" option. With this input, the software corrects only significant HOA, further reducing the depth of ablation.*
(e)- Assess *the summary of HOA corrected through the ablation (highlighted in red square)*
(f)- Enter *partial refractive error to be corrected keeping the depth of ablation lesser than 50µ*

Outcomes of CXL (Figure 6)

Demarcation line is a transition zone, between the crosslinked anterior stroma and untreated posterior stroma.[44] ***(Figure 7)*** *Ultrastructurally , the treated stroma shows keratocyte apoptosis, increased density of the extracellular matrix and collagen fibre shrinkage. It is still debatable whether the depth of demarcation line is a valid structural marker of efficacy of CXL.*

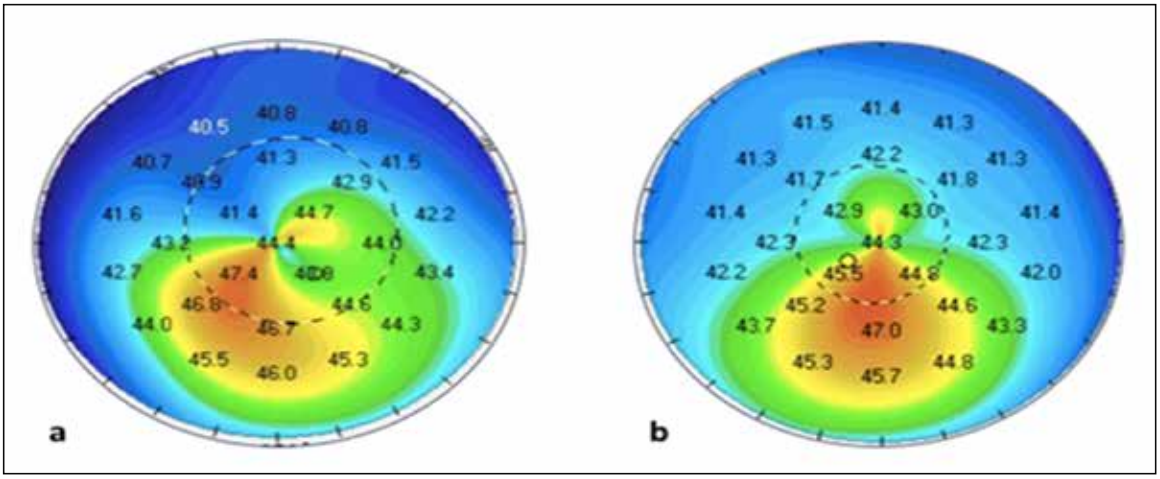

Figure 6: (a and b) *– Two post CXL axial curvature maps of an eye that underwent crosslinking 10 years ago*
Note- *the stability in the keratometry 10 years (b) following crosslinking*

Table 4: Outcomes of CXL

Outcome	Reason
Topographic Flattening	Crosslinking induced compaction of stroma
Demarcation line	Visible transition between crosslinked dense anterior stroma and non/ less crosslinked posterior stroma.
Arrest of progression	Improved biomechanical strength
Improvement in Visual Acuity	Regularization of the corneal surface

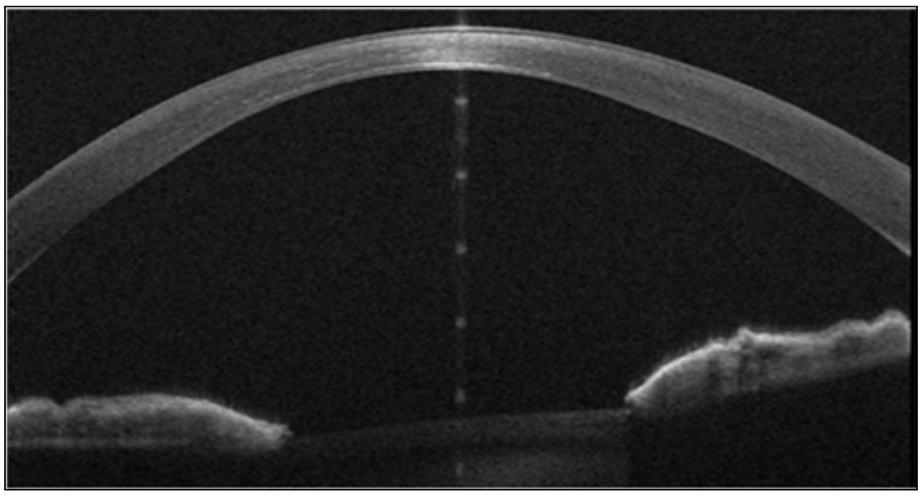

Figure 7: *AS-OCT picture demonstrating the demarcation line*

Conclusion

Newer advances in CXL have reduced the procedural duration, improved the patient comfort without significant reduction in the efficacy outcomes. The techniques of crosslinking in thin corneas have improved the safety profile of crosslinking in thin and ultra-thin corneas where standard CXL could lead to endothelial complications. Individualized CXL is a newer modality with the novel approach of modifying the irradiation times and total UV-A energy based on the pachymetry profile. Topography guided CXL shows superior visual outcomes compared to other forms of CXL.

Future directions in the research on CXL should focus on improving predictable refractive outcomes of the procedure. Customized crosslinking with/without laser ablation protocols will make CXL a truly therapeutic and refractive procedure.

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