“Epithelium-on” corneal collagen cross-linking in treatment of keratoconus – non randomized retrospective study

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Abstract

Purpose

To evaluate the clinical effect of Riboflavin/UVA, "epithelium-on" corneal cross-linking (CXL) on keratoconic eyes using a novel 0.5 % hypotonic Riboflavin solution.

Patients and methods

The study is designed as a retrospective study of consecutive case series of 100 eyes of 79 patients. A modified CXL-protocol without epithelial removal was applied to enhance Riboflavin penetration to stroma by use of 0.5% hypotonic- instead of "standard" 0.1% isotonic-Riboflavin solution in addition to use of tensioactive penetration enhancers and epithelium scarification.

Results

At the final examination ≥ 12 months after surgery mean uncorrected (UDVA) and corrected (CDVA) distant visual acuity increased significantly from 20/143 and 20/36 to 20/95 and 20/27 respectively, with $P < 0.01$ for both. *Safety index* (ratio between postoperative and preoperative CDVA) was 1.35. Mean manifest cylinder (astigmatism) decreased from -4.11±2.44D (range -9.0 to -0.25) to -3.54±2.39D (range -8.75 to +2.5) ($P< 0.02$). Mean posterior corneal elevation over floating-best-fit-sphere (protrusion) and mean corneal surface irregularity index decreased from 82.88±43.08 µm (range 21.0 to 209.0) to 71.18±34.63µm (range 15.0 to 159.0) ($P<0.01$) and from 49.74±4.49 µm (range 19.0 to 174.0) to 44.97±30.41µm (range 9.0 to 174.0) ($P<0.02$) respectively. Mean baseline endothelial cell count decreased insignificantly to 2598±*374 (P=0.31)*

Conclusion

The modified "epithelium-on" CXL- protocol was safe and effective in treatment of keratoconus and the results were comparable to the results with CXL using standard "epithelium-off" protocol.

Introduction

Optics of vision

The human eye is equipped with several optical elements including the cornea, the crystalline lens, and the retina. Together, these elements work to form vision. When an object is observed, light rays will first pass through the cornea and lens and then onto the retina. For the eye to function correctly, light passing the cornea has to focus on the retina. Bending of light rays at the cornea (refraction) is a function of the corneal shape. The steeper the cornea, the more the cornea bends the light rays and the greater is its "refractive power".(1) Refractive power is measured in diopters (D). The other refractive components of the eye are the aqueous humor, the crystalline lens, and the vitreous humor. The cumulative refractive power of the eye equals 59 D. About two thirds of the 59 D of refractive power of the eye is provided by the anterior surface of the cornea,(1) making its normal physiological shape and curvature essential for light to focus on the retina and to result in sharp vision. Any minor morphological irregularity of the cornea surface will lead to optical distortion and will affect the vision.

Cornea

The cornea is made of a transparent, avascular tissue, and consists of five layers: epithelium, bowman's membrane, stroma, decemets membrane and endothelium. The corneal epithelium is composed of stratified squamous epithelial cells and makes up about 10 % (0,05 mm) of the total corneal thickness.(2) Tight junction proteins between superficial epithelial cells prevent penetration of tear fluid (2) and other macromolecules into the stroma. The epithelium rests on a thin basal lamina supported by a thick specialized layer of corneal stroma known as the Bowman's membrane,(3) which is a smooth layer composed of collagen fibers. Its main function is to help maintain the corneal shape. Beneath the acellular Bowman´s layer, the corneal stroma is composed of an extracellular matrix formed of collagens and proteoglycans.(2) These molecules are produced by keratocytes found as flattened fibroblasts between the collagen lamellae. The vast majority of the corneal stroma consists of 200 to 500 layers of flattened collagenous lamellae extending from limbus to limbus. (4)The lamellar arrangements of collagen fibrils in the stroma have been clearly demonstrated by electron microscopy.(5-9) The collagen structure in the stroma provides the cornea with biomechanical strength, and thus is responsible for curvature and shape of the cornea.

Decemets' membrane is located directly behind the stroma. It serves as a barrier to infectious organisms while allowing water and nutrients to pass through.(10) The fifth and innermost layer of the cornea is the endothelium, which is a one-celled-thick layer responsible for regulating corneal hydration.

Keratoconus

Keratoconus is a common noninflammatory, degenerative disorder of the cornea, characterized by stromal thinning and conical ectasia that results in irregular astigmatism and associated visual loss.(11) The molecular cause of keratoconus is still uncertain, although it mainly seems to be reduced number of collagen cross-links and higher pepsin digestion.(12, 13) Since the early 1980s, metabolic and chemical changes in the keratoconic corneal tissue have been well documented.(14-20)

Andreassen and colleges found that the stiffness of a keratoconic cornea is only 60% that of the normal cornea and that the conical shape assumed by keratoconic cornea is a result of decreased mechanical stability.(12) Using high resolution 3-dimentional imaging, Morishige and colleges (24) were able to show the presence of special, highly interwoven ”sutural” collagen lamellae that in normal corneas insert to the Bowman layer from the stroma. In keratoconus these ”sutural” lamellae are lost.
Taking these findings together it seems plausible that keratoconus may be caused by some primary structural abnormalities and interaction in collagen fibrils and proteoglycans, coupled with an increased level of degenerative enzymes, weakening the biomechanical strength of the cornea. Why these changes occur is still unknown, however, between 6%-18% of patients have a family history of keratoconus. (25) Clinical observations, topographic studies, and segregation analyses of families of patients with keratoconus suggest that genes play a major role in the etiology of keratoconus. (23)

Reported numbers on the prevalence of this corneal disorder vary from 1 in 420 to 1 in 2000. (11, 26) Typically, the disease has an onset in young adulthood and tends to progress during the adolescent years and into the mid-20s and 30s, although progression can occur at any time. (27) As the progression occurs, the thinning of the central cornea worsens, and extreme degrees of irregular astigmatism may develop. (27) Upon examination, keratoconus patients will likely have increased regular and irregular

corneal astigmatism and one or more of the following diagnostic signs: coned appearance of the cornea seen on external examination, iron line surrounding the base of the cone, vertical stress lines in deep corneal stroma, central and inferior stromal thinning. (28) With disease progression, Descemet´s membrane can rupture and aqueous can expand into the corneal stroma, causing significant corneal edema, or hydrops. (28) Several devices are currently available for detecting early keratoconus by assessing anterior corneal topography. These range from simple inexpensive devices, such as handheld keratoscopes (placido disks), to expensive sophisticated devices, such as computer-assisted videokeratographers. The latter generates color-coded maps and topographic indices and is currently the most sensitive and sophisticated devices for confirming the diagnosis of keratoconus. (29)

Treatments for keratoconus have, until now, been limited to treatment of the consequences of progressive weakness of the cornea with hard contact lenses and ultimately corneal grafting (deep anterior lamellar or penetrating keratoplasty).(30) A special feature of the hard contact lens is that it nullifies almost entirely the refraction that normally occurs at the anterior surface of the cornea so that it no longer plays a significant role in the eye's optical system. Instead, the outer surface of the contact lens plays that role,(1) and thus it can greatly improve the vision in patients with keratoconic corneas. However, it does not address the basic defect within the cornea and the progressive collagen weakening will not be affected.(30) In 20 % of the patients corneal transplantation is inevitable.(30) This is a major ophthalmic surgical procedure with a risk of blindness of 1 in 500 and where 30 % of normal vision is considered a good postoperative result.

CXL - Corneal Cross-Linking

Recently, a new technique of corneal crosslinking (CXL) was devised that directly improves the mechanical and biochemical stability of the corneal stroma. This new approach consists of saturating the cornea with Riboflavin (vitamin B2) as a photosensitizer and then exposing the surface of the stroma to UV-A ligth.(31-37) The aim of this treatment is to strengthen the cornea by creating additional chemical cross-links inside the stroma by means of a localized photopolymerization in the anterior stroma, while minimizing UVA exposure to the surrounding structures of the eye. (38) In contrast to other therapeutic measures for treating keratoconus, such as thermal

keratoplasty or intracorneal rings, the new minimally invasive CXL method is the first approach to stop or even reduce the progression of keratoconus.(32) Until now, individuals with progressive forms of keratoconus could only look forward to increasing visual incapacity, corneal transplantation or, at best, a lifetime of rigid contact lens wear.(39)

Intermolecular cross-linking to enhance the rigidity of materials is a well-established method used in synthetic polymer chemistry. In the world of medicine chemical cross-linking with glutaraldehyde is used in the preparation of prosthetic heart valves,(40) and UV cross-linking is used to harden dentistry fillings.(41)

Inducing cross-links between neighboring collagen fibers is achieved by activating the photosensitizer Riboflavin with an initiating UVA beam. Following exposure to the UVA, Riboflavin is excited into a triplet state, thereby generating reactive oxygen species. These then act to induce the formation of new covalent bonds between the amino acids of neighboring collagen molecules among themselves(31, 33, 42) and between proteoglycan (PG) core proteins among themselves, together with some linkages between collagen and PG core proteins.(43) Production of reactive oxygen species only happens when UVA is absorbed by Riboflavin, it is therefore desirable that as much UVA as possible is absorbed. This is achieved by the selection of wavelength that corresponds to Riboflavin's absorption maxima at 370 nm.(44)
In short the standard CXL procedure consists of removing the corneal epithelium, then applying drops of 0.1% isotonic Riboflavin solution every 3 minutes for 30 minutes. The cornea is then exposed to UV-A light for a total time of 30 minutes. During irradiation the cornea is replenished with Riboflavin and a topical anesthetic every 5 minutes.
Since the current study involves introduction of a new protocol, CXL-safety issues are considered in detail.

CXL - Safety

Applied Riboflavin must diffuse into the cornea stroma and this process requires a certain amount of time. The corneal epithelium with its tight junctions and hydrophobic character is considered to be the most important barrier to permeability, making penetration of hydrophilic macromolecules like Riboflavin slow and incomplete(45). For that reason, debridement of the epithelium have traditionally been recommended.

Riboflavin has dual function of acting as a photosensitizer for the production of oxygen free radicals, which induce physical crosslinking of collagen,(46) and absorbing the UVA-irradiation and preventing damage to posterior ocular structures such as the corneal endothelium, the lens, and the retina.(47)

According to the ”guidelines on Limits of Exposure to Ultraviolet Radiation of Wavelengths Between 180 nm and 400 nm” the limiting radiant exposure of 1 J/cm^2 for longer UV irradiation times should not be exceeded.(48) This level is recommended for chronic exposures and is considerably lower than the radiant exposure of 5.4 J/cm^2 applied during CXL. However, when taking the Riboflavin shielding effect into account, this guideline is met regarding the corneal endothelium and deeper structures.(44) The Riboflavin shielding effect is described by the Lambert-Beer law, which is a mathematical means of expressing how light, is absorbed by matter.

In the 400µm thick layer of Riboflavin saturated cornea, the Lambert-Beer law yields a significant reduction of the UVA irradiance caused by absorption. (44) Therefore, because of the riboflavin shielding, all structures behind the corneal stroma are exposed to a residual UV radiant exposure that is less than 1 J/cm^2. (44) These calculations have been confirmed in studies by Spoerl et al, (44) measuring the UV irradiance through a 400µm thick stroma to 0.32 J/cm^2 (0.18 mW/cm^2 for 30 minutes) at the endothelial level.

The cytotoxicity of the Riboflavin-UVA treatment on keratocytes and endothelial cells has been studied by Wollensak.(49-51) His studies showed an abrupt threshold-like cytotoxic irradiance level of combined Riboflavin/UVA treatment at 0.5 mW/cm^2 for keratocytes. Using the Lambert-beer equation it was calculated that in human corneas the cytotoxic keratocyte UVA-irradiance of 0.5 mW/cm^2 is reached down to a stromal depth of 300µm.(52) Accordingly, massive keratocyte damage was observed down to

this stromal depth, but 6 months after CXL treatment, a repopulation of the whole stroma with a normal keratocyte density had taken place.(33, 53) Such cell damages may be tolerable in the keratocyte population but not in the corneal endothelium. Since endothelial cells do not regenerate, any damage to the endothelium would be irreversible. Therefore, preservation of the endothelium is crucial for every treatment involving the cornea; 400- to 800-endothelial cells/mm^2 is the minimum endothelial cell count for a clear cornea.(54) Wollensak (51) showed a specific threshold-like cytotoxic effect of combined Riboflavin-UVA treatment on corneal endothelium starting at an endothelial UVA dose of 0.65 J/cm^2 (0.36 mW/cm^2 for 30 minutes). Using the Lambert-Beer equation it was calculated that in human corneas thinner than 400µm, the cytotoxic endothelial UVA irradiance of 0.36 mW/cm^2 is reached using the standard surface irradiance of 3,0 mW/cm^2. Fortunately, the cytotoxic threshold is not reached in most keratoconus patients (with a corneal thickness of 410 to 470 um).(55) In corneas thinner then 400 um, riboflavin-UVA treatment should be avoided. Therefore, pachymetry measurements are performed routinely before CXL treatment to identify unsuitable cases.

CXL - Complications

Although CXL is considered to be a safe and minimally invasive method, some reports indicate possible adverse effects.(56-62)

The epithelium plays an important role in corneal immunology. After epithelium removal during standard CXL procedure, the cornea is left vulnerable to infection. Several researchers have published case reports of infectious keratitis after CXL. (57-61) Contact with the infectious agent likely occurred during the early postoperative period rather then during surgery because CXL not only damages keratocytes, it also kills bacteria and fungi.(30)

CXL-Surgical Techniques

The “standard” CXL-treatment-protocol described by Wollensak (31) is still most widely used. It involves mechanical debridement of the central 9 mm of the corneal epithelium and subsequent application of Riboflavin solution (0.1%) drops every 3 min for 30 min before the initiation of UVA irradiation (370 nm; 3mW/cm^2).

Applied Riboflavin must diffuse into the corneal stroma. The corneal epithelium with its tight junctions and hydrophobic character is considered to be the most important barrier to permeability, making penetration of hydrophilic macromolecules like Riboflavin slow and incomplete.(45) Mechanical removal of the intact corneal epithelium before the application of riboflavin is therefore by some surgeons considered a must to enable sufficient intrastromal diffusion of Riboflavin.(38, 44, 63)

The safety and efficacy of this method has been confirmed by numerous studies.(31, 32, 44, 62, 64, 65) However, there are several adverse effects associated with this technique. According to Pinelli, the epithelial removal performed in the standard method is responsible for most of the complications reported to date with the CXL procedure; infections, slow healing, subepithelial haze, as well as the discomfort and pain experienced by the patient.(66) To avoid these complications, Boxer-Wachler and Pinelli suggested a modification of the technique where to keep the epithelium intact. They postulated that topical anesthetic drops containing benzalkonium chloride (BAC) can loosen the epithelial tight junctions, allowing entry of Riboflavin into the stroma.(66, 67) According to a nonrandomized comparative study, Pinelli reported no significant difference in the analyzed parameters between the deepithelialized group and the standard one.(66) Meanwhile, other clinicians doubted the efficacy of this "epithelium-on" crosslinking method. Based on basic in vitro, ex vivo and in vivo studies in animal models, it was found that a limited stromal riboflavin concentration was 40-fold lower in epithelium-on corneas compared with epithelium-off corneas.(45, 68) In addition to this the increase of biomechanical strength in corneas without epithelium debridement was only one-fifth of that of corneas with epithelium removal prior to Riboflavin instillation.(69) However, it is still unclear whether the full effect of CXL with epithelial debridement is needed to stop the progression of keratoconus.

The current study

In order to refine the CXL by potentially reducing its complication rate and the patient's postoperative discomfort, while keeping the clinical efficacy at the level comparable to the current "standard procedure", a novel protocol without deepithelialization and with the use of a hypotonic 0.5% instead of isotonic 0.1% Riboflavin solution is proposed. Increased epithelial Riboflavin permeability with use of the hypotonic compared to the standard isotonic Riboflavin solution, was recently reported by Raiskup and Spoerl.(70)

With the concurrent use of tensioactive substances and partial scarifying of the epithelial surface, it offered the theoretical basis for further enhancement of the "epithelium-on" technique and for the current protocol.

The use of chemical and/or mechanical means to enhance the Riboflavin penetration through the epithelium and their influence on the efficacy of the "epithelium-on" CXL has not yet been sufficiently clinically evaluated. Although it has been demonstrated that BAC and mechanical damage to the epithelium increase the Riboflavin penetration,(66) the use of 0.5% hypotonic Riboflavin solution has not yet been studied.

The aim of the study is to evaluate the efficacy and safety of the novel protocol in a retrospective clinical study of 100 eyes of 79 patients treated with the novel "epithelium-on protocol".

Patients and methods

The study was designed as a non-randomized, retrospective study of a consecutive case series of 100 eyes of 79 patients with progressive keratoconus. All patients were referred to CXL treatment to the Department of Ophthalmology at the University Hospital of Northern Norway by practicing ophthalmologists or other eye departments from Norway.

Inclusion criteria were documented progressive keratoconus during the last 12 months before treatment, minimum corneal thickness of no less than 400 μm at the thinnest point measured by ultrasound pachymetry, age ranging from 15 to 55 and lastly a Amsler-Krumeich keratoconus classification graded stage I to III. Exclusion criteria were history of herpes virus keratitis, severe dry eye, concurrent corneal infections, previous ocular surgery and hard contact lens wear for ≤4 weeks before the baseline examination.

Keratoconus was diagnosed by the combination of videokeratography and ultrasound pachymetry as described by Leccisotti et al (71) and verified by Scheimpflug topo/tomography mapping. Progression of keratoconus was diagnosed when in the past 12 months either myopia or astigmatism increased by 1.00 diopter (D), or average SimK increased by 1.50 D.(72)

Pre- and postoperative assessments consisted of UCDVA, CDVA, slit-lamp biomicroscopy (epithelial integrity, corneal edema, corneal haze, lens opacity, CXL-demarcation-line), BUT test (sec), intra ocular pressure (mmHg), ultrasound pachymetry (μm), wavefront aberrometry and Placido-based corneal topography-based measurements ((Sim-K (D), optical asymmetry within the central 3 mm (D) and Klyce keratoconus-indices)). Scheimpflug based corneal topography and tomography ((maximum posterior elevation (μm), minimum corneal thickness (μm)). Endothelial cell count was determined by specular microscopy (cells/mm^2).

The CXL procedure was carried out with the epithelium intact and was conducted under sterile operating room conditions as follows:

1. Two drops of Pilocarpine 2% (Pilokarpin, Ophtha AS, Norway) were applied (to constrict the pupil and minimize the UVA exposure to the crystalline lens and the posterior segments), followed by two drops of local anesthetic Proparacaine 0.5%, (Alcaine, Alcon Norge AS), and two drops of local antibiotic Gentamycin 0.3% (Garamycin, Schering-Plough AS, Norway), all preserved by Benzalkone chloride.
2. One drop of Proparacaine was instilled every minute for 5 minutes (to increase the epithelial permeability by disrupting the epithelial tight junction proteins).
3. A round, 0.5 mm diameter Merocel sponge was inserted into the conjunctival sac (to increase Riboflavin eye exposure and ensure its constant diffusion into the stroma, as well as to produce micro-erosions of the superficial epithelial layer).
4. Two drops of Proparacaine and two drops of 0.5% aqueous Riboflavin solution (Vitamin B2; Streuli, Uznach, Switzerland) *without Dextran* are applied, alternating every 30 seconds 10-20 times until saturation.
5. To confirm corneal saturation, the presence of Riboflavin in the anterior chamber was evaluated by slit-lamp examination. If the “Riboflavin flare” was absent, step 4 was repeated.
6. The Merocel sponge was then removed.
7. In cases where pachymetry exceeded 450 μm, irrigation with isotonic basic salt solution was performed.
8. An eyelid speculum was inserted and a ring-shaped Merocel shield (k20-5021, Katena, Switzerland) was applied to cover the limbus (to protect corneal stem cells from UVA-radiation).

9. UVA-irradiation was then performed for 30 minutes with a wavelength of 365 nm at a working distance of 5 cm with an irradiance of 3 mW/cm^2 with an UV-X lamp (IROC AG, Switzerland).
10. During the irradiation Riboflavin was applied every 3 minutes in eyes with residual stromal pachymetry below 400 μm.
11. Proparacaine drops were added as needed.
12. After irradiation, two drops of Atropine 1% (Atropin minims, Chauvin, England) and two drops of Gentamycin were applied, followed by application of a soft bandage contact lens for 12-18 hours.
13. At the end of the procedure, the patient was instructed to apply a mixture of 0.1% Dexamethasone and 0.5% Chloromycetin (Spersadex med Kloramfenikol, Novartis, Norway) 4 times daily for 7 days, as well as to use artificial tears as needed.

Results

Hundred keratoconic eyes of 79 patients treated with "epi-on" CXL that had observation time ≥ 12 months were analyzed. Mean patient's age was 31.2 ±10.4 (standard deviation) (15-54 years) (range). Twenty one percent of eyes belonged to female and 79% to male patients.

Baseline measurements:

Mean UCDVA and CDVA were 20/143 and 20/36 respectively.
Mean manifest spherical equivalent and cylinder were -2.02±2.92D (-10.25 to +2.75) and -4.11±2.44D (-9.00 to -0.25) respectively.
Mean maximum SimK was 48.63±4.49D (39.5 to 60.0), mean irregularity index 49.74±32.79 (19.0 to 174.0), mean posterior elevation above the best-fit-sphere was 82.88±43.80µm (21.0 to 209.0) and mean minimum pachymetry was 460.4±54.52µm (330 to 622). Mean endothelial cell count was 2632± 321 cell/mm2.

Postoperative measurements at last follow-up examination (≥ 12 months after CXL):

Mean UCDVA and CDVA increased significantly to 20/95 and 20/27 respectively, ($P <$ 0.01 for both). Ten percent of the eyes lost lines of UCDVA, 23% kept the preoperative UCDVA, while 67% gained lines of UCDVA (fig. 1). No eyes lost lines of CDVA 26%

kept the preoperative level and 74% gained lines of CDVA, with a *safety index* (ratio between postoperative and preoperative CDVA) of 1,35. Figure 2 shows loss and gain of lines of CDVA at 1,3,6 and 12 months postoperatively.
Mean manifest spherical equivalent and cylinder decreased to -1.84±3.08D (-16.5 to +5.0) and -3.54±2.39D (-8.75 to +2.5), (P< 0.48 and 0.02 respectively). The stability of postoperative spherical equivalent and cylinder is shown on figures 3 and 4.
Mean maximum SimK decreased to 48.20±4.70D (40.0 to 67.3), mean irregularity index to 44.97±30.41µm (9.0 to 174.0), mean posterior elevation over floating-best-fit-sphere to 71.18±34.63µm (15.0 to 159.0) and mean minimum pachymetry to 450.4±47.82µm (310 to 569), (P<0.08, 0.02, 0.01 and 0.03 respectively) (fig. 5).
Mean baseline endothelial cell count insignificantly decreased to 2598±*374 (P=0.31)*

Discussion

Ever since its proposal "epithelium-on" method has been somewhat controversial. The main complaint was, and is that the method does not allow sufficient Riboflavin penetration into the stroma to secure effective collagen cross-linking.(45, 68, 69) However, the quoted studies used a non-adjusted "standard" protocol except for the intact epithelium. Hence the studies did not bring any conclusive evidence concerning the "epithelium-on" protocol as used in practice. Recently clinical studies by Leccisotti have shown favorable results with the "epithelium-on" method.(72) In addition, laboratory studies on rabbit eyes performed by Kissner and colleagues have shown that "epithelium-on" crosslinking as performed by Pinelli(73) induces sufficient epithelial permeability for the passage of Riboflavin and results in increased corneal stiffening.(74)

Increased epithelial Riboflavin permeability with the use of the hypotonic compared to the standard isotonic Riboflavin solution was the theoretical basis for the current protocol. Although the clinical safety of CXL with hypotonic Riboflavin solution has been shown, (75) we considered the issues of the endothelial cell toxicity because of the decreased UV-absorption coefficient of the hypotonic Riboflavin reported by Wollensak in 2010 (absorption coefficient α for 0.1 % isotonic Riboflavin solution ≈ 53 cm^{-1}, while for 0.1 % hypotonic Riboflavin solution ≈ 42 cm^{-1}).(76) By applying the Lambert-Beer law a curve (figure 6) was constructed, showing how the UV-irradiance changes

for the two solutions as the irradiation gets deeper in the cornea due to the difference in their Riboflavin UV-absorption coefficients. According to the curve, the irradiance level at a given depth is higher with the hypotonic solution (figure 6). Moreover, the endothelium is placed at risk since a cytotoxic irradiance level of 0.36 mW/cm2 (49) is maintained up to the corneal depth of 500 μm (when the standard condition with surface irradiance of 3.0 mW/cm^2 for 30 minutes is applied).
Nevertheless, according to the Lambert-Beer law the absorption coefficient increases with increased riboflavin concentration and the absorption coefficient of 0.5% hypotonic Riboflavin solution (used in the current study) is quite similar to that of the standard 0.1% isotonic solution (figure 7), as is the irradiance level at 400 μm (figure 8), which alleviates the endothelial toxicity issue. However, these calculations assume that the whole of the cornea is saturated with 0.5% Riboflavin with no concentration gradient, which may not be the case after only 30 minutes diffusion time. By Applying Ficks law and the one dimensional time dependent diffusion equation we calculated that after 30 minutes diffusion, Riboflavin concentration gradient from the corneal surface to the endothelial level (figure 9), ranges from 0.5% at the corneal surface to 0.175% at 400 μm. A hypotonic 0.175% Riboflavin solution has an absorption coefficient of 54 cm^{-1} (figure 10), meaning that for the whole of the cornea the absorption coefficient must be between 54 and 57 cm^{-1} (figure 11). This results in a demarcation line (transition zone between effective and ineffective cross-linking)(77) at around 330 μm (figure 11), which coincides with our clinical findings on optical coherence tomography (figure 12).

Comparison of the results from the current study with the published CXL results by Vinciguerra,(78) using standard “epithelium-on” protocol and by Leccisotti,(70) using “epithelim-on” protocol with 0.1% isotonic Riboflavin solution is presented in table 1. It shows that our results concerning the safety index and change in CDVA are closer to Vinciguerra’s than Leccisotti’s results, which may justify our protocol modification with an aim to achieve the efficacy of “epithelial-on” , comparable to “epithelium-off” CXL.

Conclusion

The current results using our modified “epithelium-on” CXL- protocol in treatment of 100 keratoconic eyes show statistically significant improvement in UCDVA, CDVA and the amount of manifest astigmatism, safety index (1,35), as well as the improvements in corneal topography measurements of surface regularity and posterior surface elevation which is comparable to the outcomes of CXL with use of standard “epithelium-off” protocol.

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	Leccisotti	Vinciguerra	Stojanovic
Number of eyes	64	28	100
Safety index	1.05	1.38	1.38
Mean SE change	- 0.35 D ($P <$ 0.05)	- 0.36 D ($P <$ 0.05)	- 0.18 D ($P < 0.05$)
Surface irregularity* change	$P < 0.05$	$P < 0.05$	$P < 0.05$
Mean CDVA change	-0.04 logmar, $P < 0.05$	-0.14 logmar, $P < 0.05$	-0.12 logmar, $P < 0.05$

Table 1: Comparison of the outcomes 12 months or later after CXL in treatment of keratoconus.(SE: Spherical Equivalent, CDVA: Corrected Distance Visual Acuity)

*Surface irregularity index in the three studies was measured by use of different methods, therefore only the P-values and not the measurements themselves were compared.

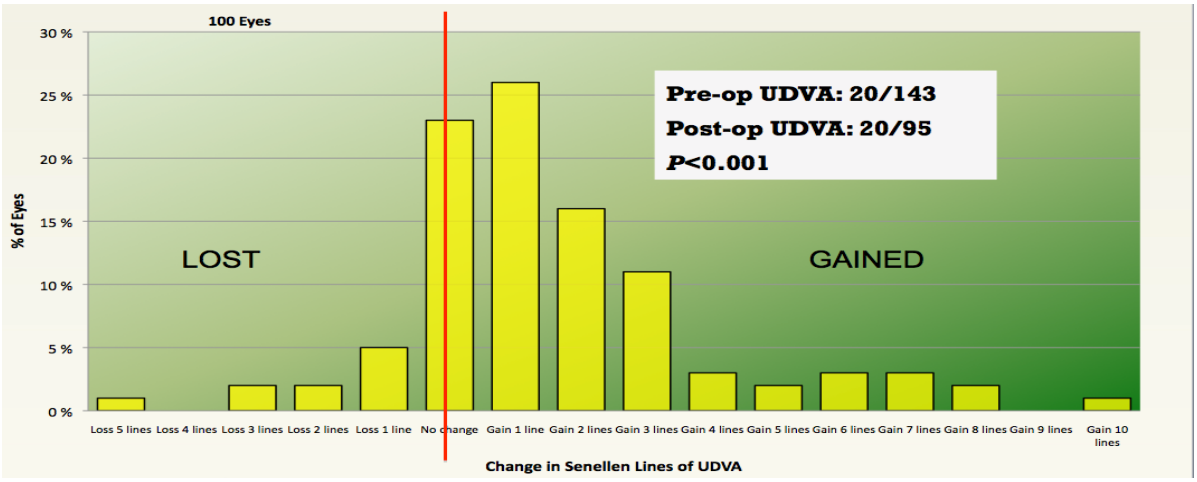

Figure 1: Change in Snellen's lines of UDVA ≥ 12 months after CXL (UDVA: Uncorrected Distance Visual Acuity).

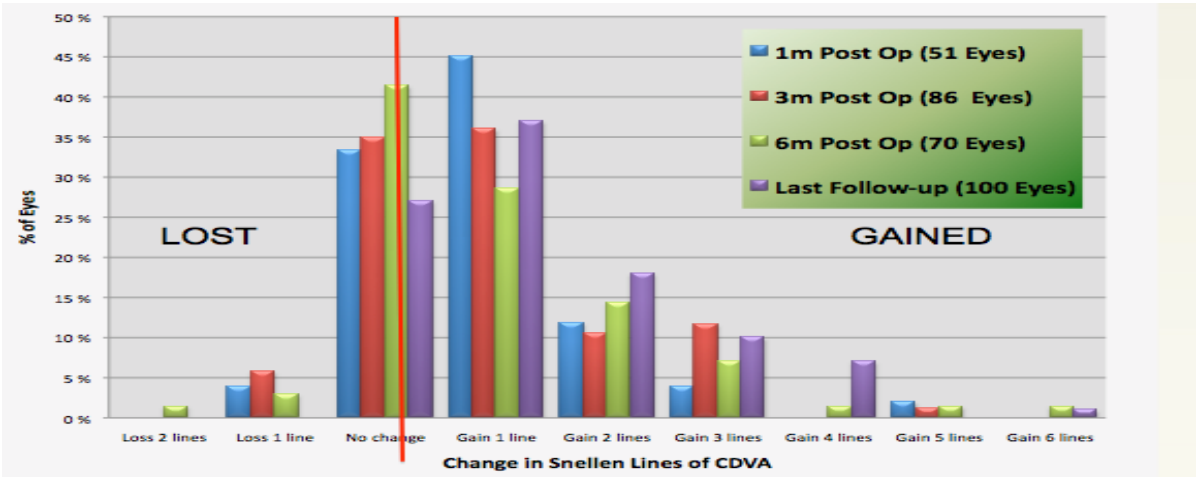

Figure 2: Change in Snellen's lines of CDVA 1, 3, 6 and ≥ 12 months after CXL (CDVA: Corrected Distance Visual Acuity)

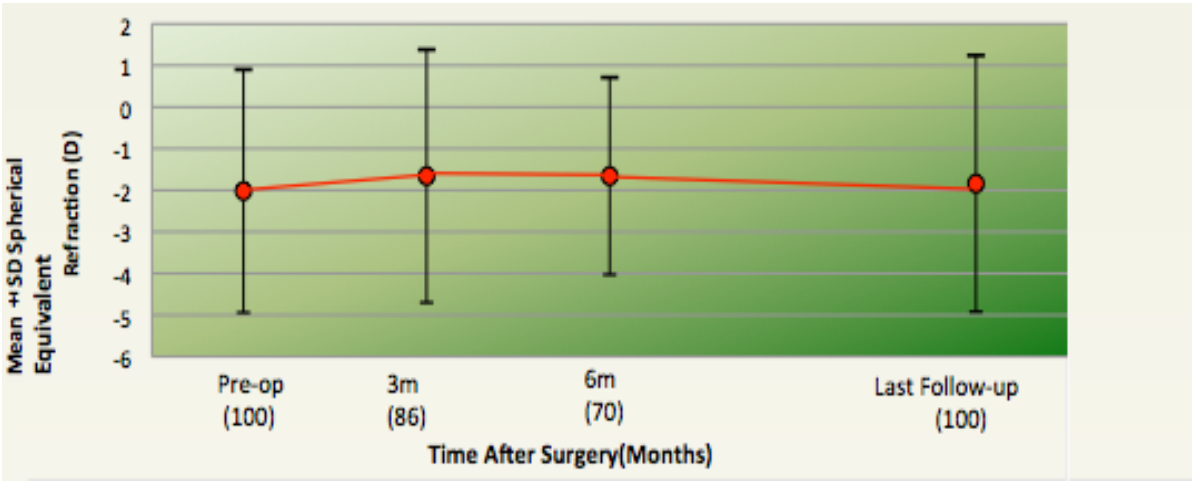

Figure 3: Stability of mean manifest spherical equivalent after CXL

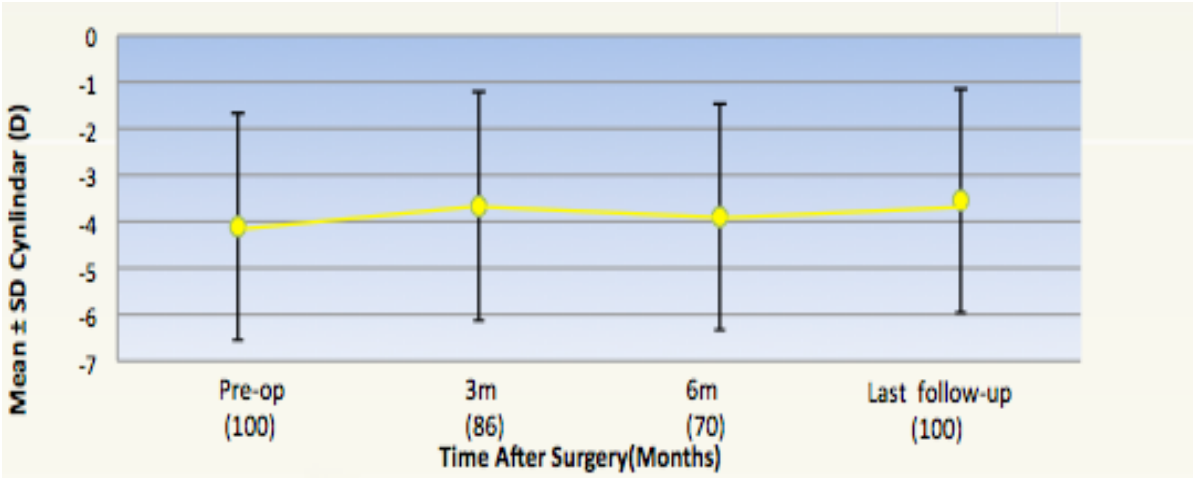

Figure 4: Stability of mean manifest cylinder after CXL

Figure 5: Posterior floating elevation, maximum SimK, irregularity index and pachymetry before and after CXL

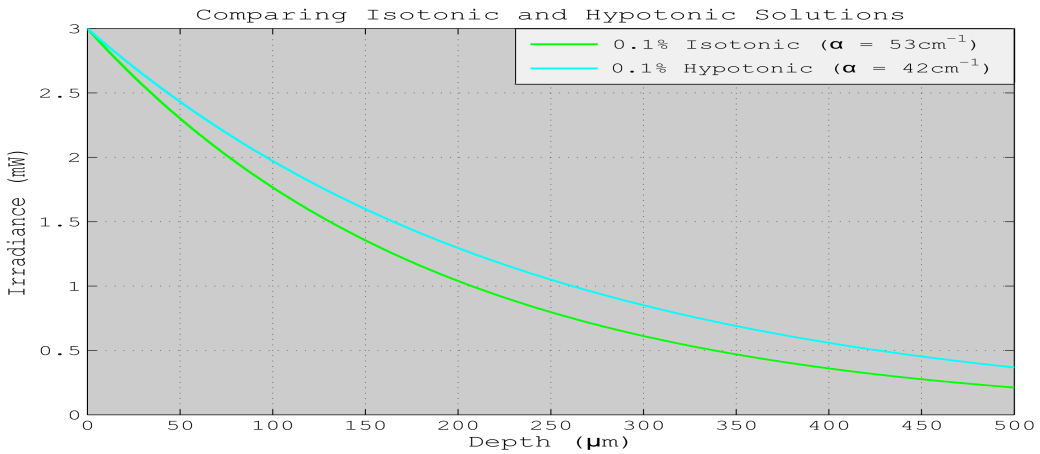

Figure 6: Irradiance vs. corneal depth for 0.1% isotonic and 0.1% hypotonic Riboflavin solution, expressed in mW (based on irradiation time of 30 minutes with 3 mW source)

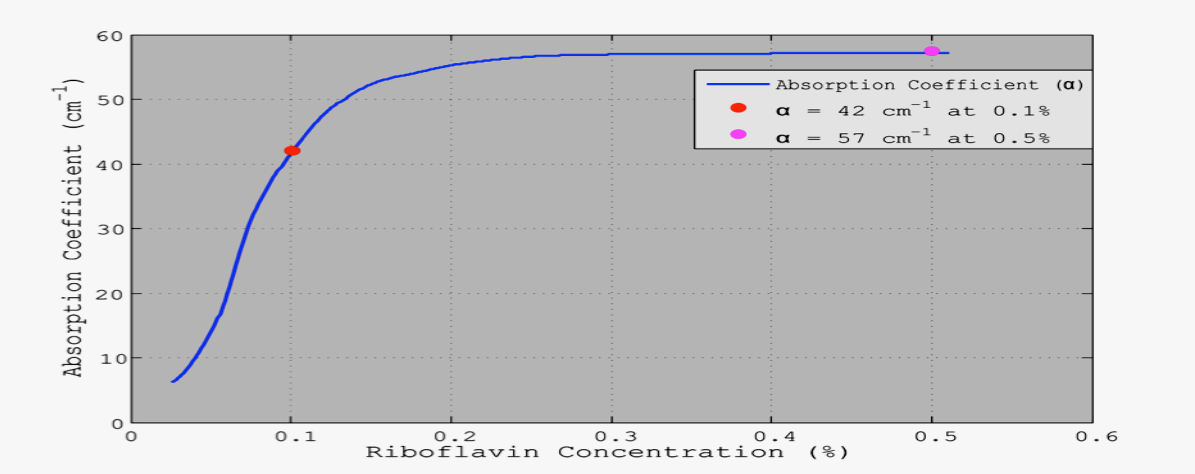

Figure 7: UV-absorption coefficient (α) for hypotonic Riboflavin solutions. The α of the 0.5 % hypotonic Riboflavin solution ($\approx 57\ cm^{-1}$) is quite similar to the α of 0.1 % isotonic Riboflavin solution ($\approx 53\ cm^{-1}$)

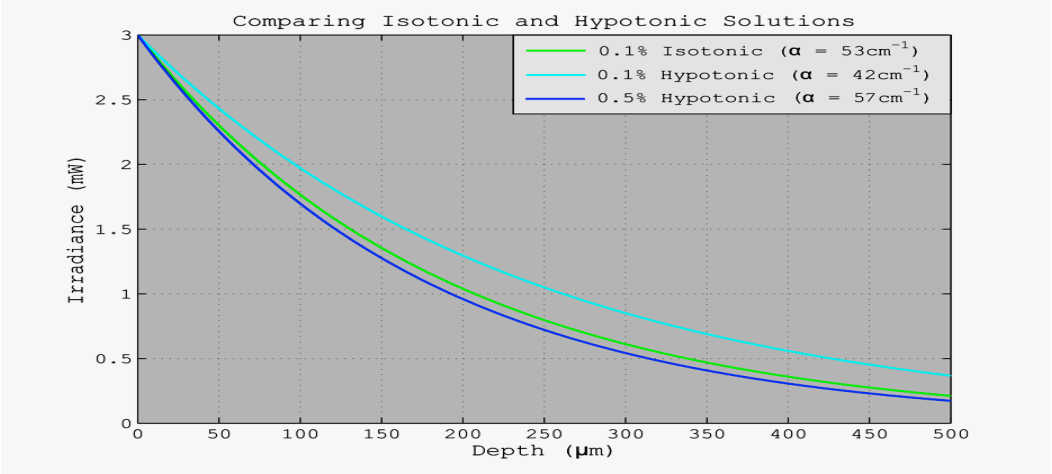

Figure 8: Irradiance vs. corneal depth for a. 0.1% isotonic, b. 0.1% hypotonic and c. 0.5% hypotonic Riboflavin solution

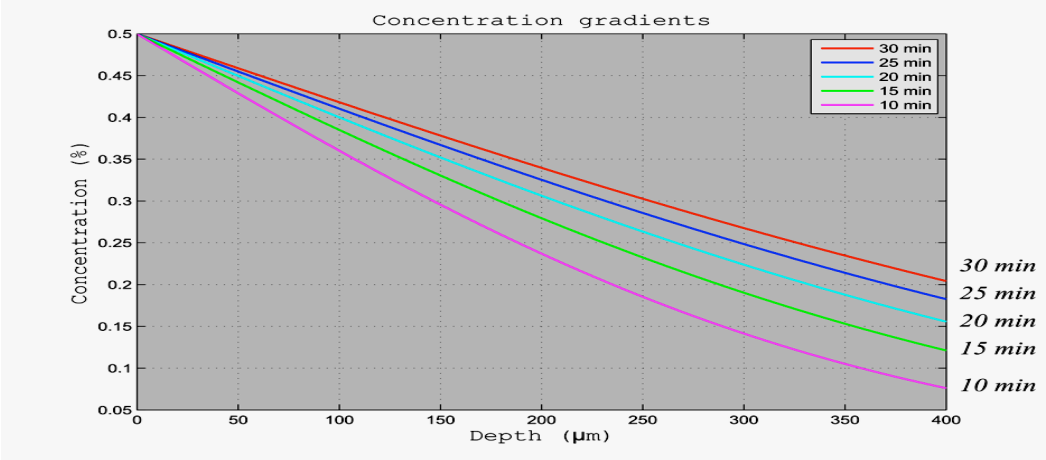

Figure 9: Riboflavin corneal concentration gradient after 10 min, 15 min, 20 min, 25 min and 30 min application time.

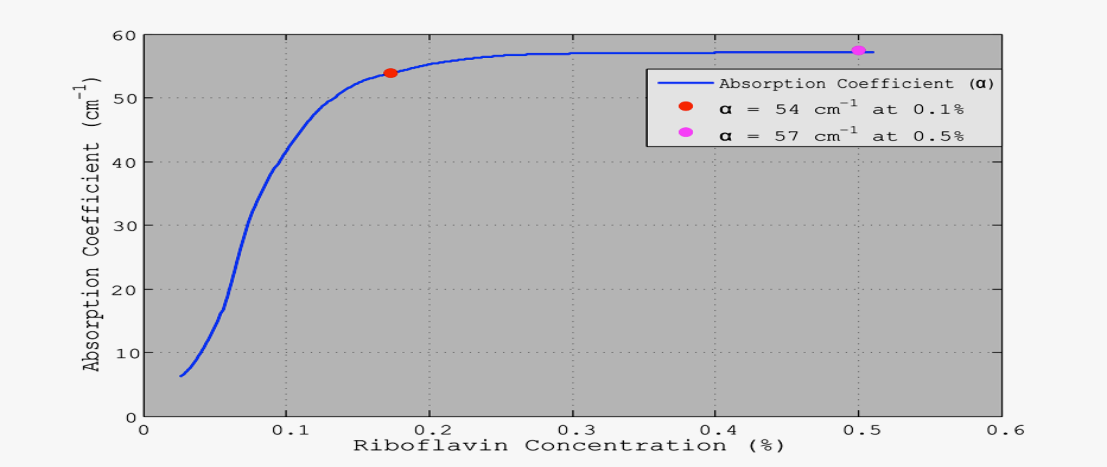

Figure 10: UV-absorption coefficient (α) for hypotonic Riboflavin solutions.

Hypotonic 0,175% Riboflavin solution has an absorption coefficient of 54 cm^{-1}

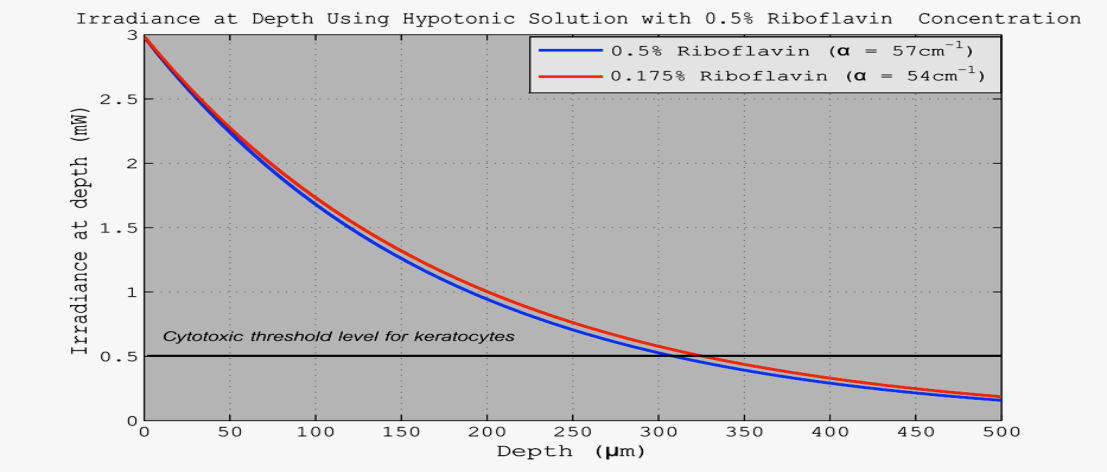

Figure 11: Irradiance vs. corneal depth for 0.175% hypotonic and 0.5% hypotonic Riboflavin solution, expressed in mW (based on irradiation time of 30 minutes with 3 mW source).

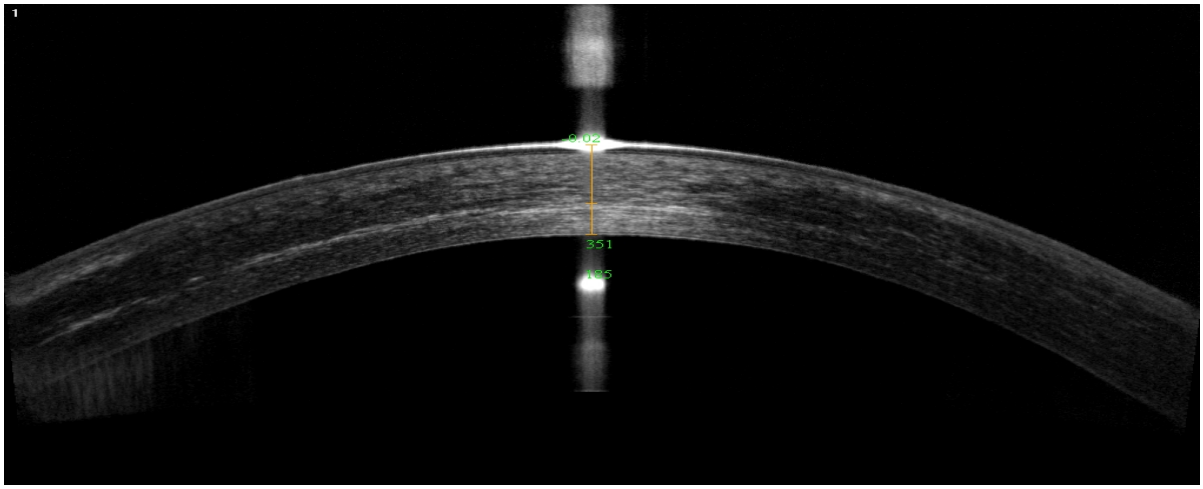

Figure 12: Postoperative optical coherence tomography (OCT) of a case treated with the current protocol showing demarcation line at 351µm.