

BioMed Research International

Cross-Linking and Corneal Imaging Advances

Guest Editors: A. John Kanellopoulos, Ronald R. Krueger,
and George Asimellis





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Editorial

Cross-Linking and Corneal Imaging Advances

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Received 22 December 2014; Accepted 22 December 2014

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1. Introduction

Corneal cross-linking, a technique employing UV-A illumination and a photomediator to induce corneal rigidity, is a widely recognized procedure for the stabilization or even possibly reversal of corneal ectasia progression in patients with keratoconus and post-LASIK ectasia. A rapidly growing number of clinical reports suggest a consistent stabilizing effect of cross-linking along with a variable improvement in corneal shape and visual function in some patients. In the past ten years there has been a continuous effort into understanding, ensuring safety and efficacy, and further expanding its applications, as well as exploring modifications aiming to optimize the technique. Research in the field of CXL is highly dynamic; techniques, concepts, and indications are constantly evolving. Recent advances in corneal cross-linking include applications for infections treatment that do not respond to topical medications; accelerated, high-fluence applications; prophylactic application in refractive surgery; modified beam profiles for selective treatments; fully customized induction of refractive changes in nonectatic eyes. We welcome in this special issue several papers on this subject covering topics such as the issue of epithelial removal with hypotonic riboflavin solution, as well as a contralateral study on this subject; study investigating rate of corneal collagen cross-linking redo, investigating risk factors and safety, including a study investigating the profile of microbial keratitis following CXL; long-term investigation of safety and visual outcome of Visian toric ICL implantation after CXL in keratoconus; long-term investigation of accelerated CXL in

paediatric patients; biomechanical effects investigation of the correlation between tomographic and biomechanical severity of keratoconic corneas; and a novel application of intraoperative optical coherence tomography in CXL.

Keratoconus is considered an unpredictably progressive eye disease that “softens” the cornea. The progressive thinning and “bulging” of the cornea may distort or even significantly reduce vision. In advanced cases, one or more corneal transplant procedures and possibly additional eye surgeries may be required for visual rehabilitation. As it mainly affects younger people, it has severe consequences in their quality of life and their ability to contribute to the active workforce during their most productive years. In our experience within our ophthalmology center in Greece, through extensive studies conducted the last 10 years, we have found that in unpublished data possibly more than 1 out of 35 patients display some form of keratoconus in modern cornea diagnostics, compared to 1 out of 1,000–2,000 reported in Northern Europe and the United States. In addition, we have noted a higher degree of familial correlation of keratoconus reaching 90% topographic or tomographic suspicion in one of the two parents of a known young adult with keratoconus, a marked difference compared to the 10% genetic correlation that has been previously reported.

Over the last decade a new treatment, collagen cross-linking (CXL), has been introduced. In this treatment, vitamin B2 and ultraviolet light (UV-A) are applied to the cornea in a short procedure that “stiffens” the cornea and stops disease progression.

2. Current Treatment Options for Keratoconus Management

Keratoconus progression was traditionally observed. Visual rehabilitation was managed with spectacle correction and/or soft contact lenses, until irregular astigmatism necessitated application of rigid gas permeable (RGP) contact lenses.

In cases when this was not possible or there was RGP intolerance (estimated up to 21% of cases [1]), traditionally, a penetrating keratoplasty (PK) in which the patient's cornea is discarded and replaced with a fresh donor cornea was employed. This procedure is associated with significant morbidity [2], as usually it takes about a week for the patient to return to normal everyday life and months, if not years, before that eye can be adequately visually rehabilitated. It is noted that, despite the use of this drastic procedure, visual rehabilitation may still necessitate additional repair and/or refractive procedures in order to reduce the very common irregular astigmatism and high postoperative anisometropia associated with penetrating keratoplasty.

Even in cases where PK generally achieved acceptable visual outcomes, long-term graft survival in keratoconic eyes declined rapidly after the second decade because the endothelial cells of the donor cornea tend to be slowly rejected by the host. Primary graft survival rates have been reported to 50% at 20 years [3], falling even further with repeat grafts.

An alternative to PK is deep anterior lamellar keratoplasty (DALK) which does not have the disadvantage of short lifespan and associated complications. In DALK this risk is possibly lower as the endothelial cell layer of the host is preserved: a median graft survival of 49 years for DALK versus 17 years for PK has been reported. It is noted, however, that DALK techniques are technically challenging.

Other introduced treatment options for keratoconus are the insertion of intracorneal ring segments (ICRS). These inserts appear to significantly shift the shape of the cornea and may provide significant visual rehabilitation. Although, in clinical use for several years, there is no unison assessment of their stability and safety, we have reported along with other clinicians a number of significant short- and long-term complications associated with the ICRS.

Collagen cross-linking, on the other hand, has proven that it can effectively arrest the progression of keratoconus and corneal ectasia. The standard, epithelium-off Dresden protocol has been proven to be effective in arresting keratoconus progression.

Despite substantiated safety, we have reported, along with other clinicians, a range of complications associated with CXL. In addition to the standard CXL, other protocol variations introduced include alternative levels and amounts of energy, pulsing, oxygen supplementation, riboflavin solution concentrations, and route of administration within the cornea of the riboflavin solution. The underlying premise of these alternatives is that delivering a similar effect over a shorter period of time will not compromise safety in comparison with the standard protocol.

Our team has contributed many of the evolutionary steps of the initially introduced CXL technique:

- (1) higher fluence,
- (2) use of dextran-free riboflavin solution,
- (3) combination of CXL with topography-guided excimer normalization of ectatic corneas (the Athens Protocol),
- (4) prophylactic CXL in routine myopic and hyperopic LASIK,
- (5) in situ CXL through a femtosecond laser created corneal pocket,
- (6) photorefractive CXL.

Specifically, we have introduced the concept of accelerated, high-fluence collagen cross-linking (CXL) in post-LASIK ectasia, as well as the utilization of prophylactic CXL in routine LASIK, and in situ, femtosecond laser-assisted treatment of corneal ectasia, in attempting corneal deturgescence in bullous keratopathy, and as a prophylactic intervention adjunct to Boston keratoprosthesis surgery.

3. The Need for Comparative Evaluation of CXL Protocols

Over the last ten years CXL has evolved to be a valid treatment for the arrest of the progression of keratoconus. Since the original Dresden protocol (3 mW/cm² for 30 minutes), several treatment CXL protocol variations have been introduced, most of them by our team [4]. These, however, have not been fully compared as far as their correlating effect. These variations involve higher fluence, such as the use of 6, 10, 18, and 30 mW/cm², and correspondingly shorter UV-A exposure time, aiming to deliver the same (5.4 J/cm²) or more total amount of energy, and presumably adequate stiffening effect [5]. Besides the original CXL protocol parameters evaluated more extensively, all newer-introduced CXL protocols have not been evaluated and correlated as extensively either clinically or ex vivo. In order to correlate the efficacy of the standard to newer CXL protocols the following prospective studies must be conducted, both clinically and ex vivo with the following parameters:

- (i) ectasia stabilization (topographic and anterior elevation stability and/or improvement),
- (ii) safety in regard to visual acuity loss, corneal clarity, corneal inflammation, and endothelial cell loss,
- (iii) biomechanical/biochemical response parameters.

To the best of our knowledge, so far no direct and thorough comparative study of these CXL protocols has been conducted. The lack of CXL-techniques comparison is a noteworthy shortcoming. In a recent example, in a smaller-scale precursory prospective randomised trial carried by our team, contralateral eyes of 21 patients with progressive keratoconus were randomised to either conventional or high-fluence CXL (7 mW/cm² for 15 min).

Several assessment modalities for the evaluation of CXL efficacy exist. They include ex vivo biomechanical (tensile strength), biochemical (enzymatic digestion) [6], and in vivo

methods, for example, via OCT imaging demarcation line [7], corneal hysteresis (CH), and corneal resistance factor (CRF). CH is considered indications of corneal viscous damping, reflecting the capacity of corneal tissue to absorb and dissipate energy; CRF is considered an indicator of the overall corneal resistance.

The latter may be evaluated by dynamic tonometry (visualization of fast deformation of the cornea), employing the Corvis ST (Oculus Optikgerate GmbH, Wetzlar, Germany) and the Ocular Response Analyzer (Reichert, Buffalo, NY). The Corvis ST is a functional in vivo corneal biomechanics analyzer employing a noncontact tonometer and enabling recording the corneal reaction to an air impulse. An incorporated high-speed Scheimpflug camera (4,330 frames/sec) records still frames of the oscillating cornea. The device enables assessment of corneal biomechanics for various applications of refractive surgery, keratoconus screening, and cross-linking assessment.

Several studies have evaluated the reduction in corneal biomechanical strength following refractive surgeries such as LASIK. However, there is inconclusive evidence in the peer-review literature on the specificity of these techniques in the evaluation of the effect of corneal cross-linking [8].

Ex vivo corneal biomechanical evaluation may be conducted with biaxial stress-strain measurements. The BioTester 5000 (Cell Scale, Waterloo, Ontario, Canada) is a specifically developed biomaterials biaxial strength analyzer applicable to ex vivo corneal rigidity (Young's modulus) measurements within a temperature-controlled media bath. Two high-performance actuators (two per axis) are capable of μm positional resolution for accurate test motion, with inline overload-protected load cell on each axis. The device captures and graphically displays live time, force, and synchronized video images for results analysis and verification. Data are easily exported to standard spreadsheet programs. Future promising diagnostics may include devices that are based on phonon spectroscopy as demonstrated already in studies on the Brillouin-based investigative devices.

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Research Article

Understanding the Correlation between Tomographic and Biomechanical Severity of Keratoconic Corneas

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Received 31 May 2014; Revised 14 October 2014; Accepted 15 October 2014

Academic Editor: George Asimellis

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Purpose. To evaluate correlation between tomographic gradation of keratoconus (KC) and its corresponding air-puff induced biomechanical response. **Methods.** Corneal tomography and biomechanics were measured with Scheimpflug imaging in 44 normal and 92 KC corneas. Deformation waveform was also analyzed with Fourier series. A custom KC severity scale was used from 1 to 3 with 3 as the most severe grade. Tomographic and biomechanical variables were assessed among the grades. Sensitivity and specificity of the variables were assessed using receiver operating characteristics (ROC). **Results.** Curvature variables were significantly different between normal and disease ($P < 0.05$) and among grades ($P < 0.05$). Biomechanical variables were significantly different between normal and disease ($P < 0.05$) but similar among grades 1 and 2 ($P > 0.05$). All variables had an area under the ROC curve greater than 0.5. The root mean square of the Fourier cosine coefficients had the best ROC (0.92, cut-off: 0.027, sensitivity: 83%, specificity: 88.6%). Spearman correlation coefficient was significant between most variables ($P < 0.05$). However, tomographic segregation of keratoconus did not result in concomitant biomechanical segregation of the grades. **Conclusions.** There was lack of significant biomechanical difference between mild disease grades, despite progressive corneal thinning. Mathematical models that estimate corneal modulus from air-puff deformation may be more useful.

1. Introduction

The hypothesis that the biomechanical strength of the cornea needs to be restored forms the basis of various treatment modalities used in the management of keratoconus [1]. Corneal transplantation using normal corneal tissue was one of the obvious options to restore vision in affected patients. In recent times, ultraviolet (UV-A) collagen crosslinking with a photosensitive crosslinking agent was used widely to restore the biomechanical strength of the cornea [1, 2]. This treatment resulted in flattening of the zone of focal biomechanical weakening [1] and a concomitant reduction in corneal wavefront aberrations [3, 4]. To treat keratoconus, an alternate procedure combined topography guided photorefractive keratectomy and UV-A crosslinking [5]. The combined treatment resulted in better visual outcomes than crosslinking alone

though no information was available on the postoperative biomechanical status of the keratoconic cornea [6].

Prior to selecting the appropriate treatment, it was important to evaluate the preoperative biomechanical status of the cornea [2]. There was evidence that crosslinking alone provided better outcomes in early and mild keratoconus but not necessarily in advanced cases [7]. Additionally, most studies used a definition of “progression” that was based on documented topographic steepening over a period of six months to a year [5]. This progression of the disease may also be considered to be an indicator of progressive biomechanical weakening. However, this hypothesis remained untested in KC patients. Recently, a high-speed Scheimpflug imaging device, the Corvis-ST (Oculus Optikgerate GmbH, Germany), was used to detect corneal deformation in response to an air-puff incident on the anterior corneal surface [7].

Corneal deformation (or displacement) was a quantifier of the biomechanical stiffness of the cornea [7] and it was intuitive that biomechanically weaker corneas would deform more. In this study, the biomechanical status of different grades of keratoconus, segregated based on a custom severity scale, was evaluated using the Corvis-ST. The custom severity scale was designed based on the anterior surface mean keratometry value such that it stratified the severity of the KC disease as a linear function of the grade. Further, the study evaluated the relative impact of thinning and decrease in stiffness of the cornea on the progression of the disease.

2. Methods

The study was a retrospective, observational study in a tertiary eye care center in southern India. The study protocol was approved by the institutional review board of the center and followed the tenets of the Declaration of Helsinki. The study included 44 (44 subjects) normal and 92 (92 subjects) keratoconic eyes. The diagnosis of keratoconus was based on evidence of stromal thinning on slit-lamp, focal protrusion or increase in corneal curvature, Fleischer's ring, Vogt's striae, scissoring of the red reflex, an abnormal retinoscopy, and curvature asymmetry leading to abnormal corneal astigmatism. Further, the classification of the severity of the keratoconus was performed using corneal tomography. Based on the anterior surface mean keratometry value (Kmean), three keratoconus grades and a normal grade classified as grade 0 were established: grade 1—Kmean < 48 D; grade 2—48 D ≤ Kmean < 52 D; grade 3—Kmean ≥ 52 D [8]. The number of subjects in grades 1, 2, and 3 was 36, 29, and 27 eyes, respectively. The exclusion criteria were glaucoma, a history of eye surgery, or current topical medication use. For “normal” eyes, manifest spherical error and astigmatism were limited to ±2 D.

Corneal tomography was evaluated with the Pentacam (Oculus Optikgerate GmbH, Germany). The tomography variables that were selected for analyses were steep (K2) and flat (K1) axis keratometry, mean keratometry (Kmean), maximum axial curvature (Kmax), central corneal thickness (CCT), the thickness of the thinnest point of the cornea (TPT), and the location of the cone. The location of the cone was assessed as the distance between the location of peak tangential curvature and the geometric center of tangential curvature map. Kmean was the average of K1 and K2. Biomechanics of the cornea was evaluated with the Corvis-ST (Oculus Optikgerate GmbH, Germany). The Corvis-ST also had an ultra-high-speed Scheimpflug imaging system that captured 140 frames of a cross-section (along the horizontal meridian) of the deforming cornea over a time period of 30 milliseconds. Advanced edge detection algorithm was used to measure the displacement of the anterior and posterior edge of the deforming cornea. The device reported the displacement of the anterior corneal apex as a function of the application time of air-puff. There were several variables reported by the device based on the measured displacement of the cornea apex. In this study, the following variables were used for analyses of the biomechanical status of corneas: A1—time of first applanation, A2—time of second applanation,

Time—time of peak displacement of the corneal apex, DA—deformation amplitude (or magnitude of peak displacement of the corneal apex), IOP—intraocular pressure measured by Corvis-ST.

Further, a Fourier series fit to the displacement of the corneal apex was performed [9]. The Fourier series fit was simply a nonlinear regression of DA versus time [9]. Three variables were defined based on the Fourier coefficients of the regression: AUDA—area under the deformation amplitude curve, “an” RMS—root mean square of cosine Fourier coefficients, “bn” RMS—root mean square of Fourier sine coefficients [9]. Fourier coefficients up to order 31 were used for the Fourier series fit. With AUDA being a measure of biomechanical status of the cornea, a larger AUDA implied a biomechanically weaker cornea and vice versa [9]. Similarly, an RMS and bn RMS were expected to be greater in weak corneas compared to normal corneas [9].

2.1. Statistical Analyses. The variables were tested for normality of distribution. Since variables were observed to be nonparametrically distributed, all continuous variables were reported as median ±95% confidence interval (CI). Difference between the grades was assessed with Kruskal-Wallis test followed by post hoc analyses. Correlation between all the variables was assessed with the Spearman correlation coefficient. The sensitivity and specificity of each variable to detect keratoconus were analyzed with receiver operating characteristic (ROC). A *P* value less than 0.05 was considered to be statistically significant. MedCalc v12.5.0 (MedCalc Inc., Belgium) was used for statistical analyses.

3. Results

Table 1 lists the median and 95% CI for all the variables. All variables increased in magnitude with increasing severity of keratoconus (Table 1). Figures 1(a) and 1(b) show the median curvature and thickness of the different grades. The severity scale graded the curvature and thickness of keratoconic corneas as a linear function of grade number (Figures 1(a) and 1(b)). Statistical analyses of curvature and thickness yielded a statistically significant difference between the grades. Kmax of grades 0, 1, 2, and 3 were significantly different from each other (*P* < 0.0001). Also, Kmean, K1, and K2 differed significantly among the grades (*P* < 0.0001). Both CCT and TPT differed significantly among the grades as well (*P* < 0.0001). The location of the cone was similar among all keratoconus grades (*P* = 0.25).

The biomechanical parameters were also evaluated. Figures 2(a) and 2(b) show the median values of A1, Time, A2, and deformation amplitude (DA) of the grades. While A1 decreased, A2 and Time increased with increasing severity of keratoconus (Table 1). A1 of grade 0 was significantly different from other grades (*P* < 0.0001). A1 of grade 1 was similar to grade 2 (*P* > 0.05) but not to grade 3 (*P* < 0.05). Time did not differ significantly among the grades (*P* = 0.83). A2 of grades 0 and 1 were similar (*P* > 0.05). A2 of grades 2 and 3 were similar (*P* > 0.05) but were different from grades 0 and 1 (*P* < 0.05). DA also differed among the groups. DA of grade 0 differed from all other grades (*P* < 0.001).

TABLE 1: Median ± 95% CI of the variables evaluated in normal corneas and diseases grades.

	Normal Grade 0		Grade 1		KC Grade 2		Grade 3	
	Median	95% CI	Median	95% CI	Median	95% CI	Median	95% CI
K1 (D)	42.7	42.4–43.2	44	43.3–44.5	47.7	47.2–49.0	54	52.2–58.0
K2 (D)	43.7	43.2–44.2	46.8	45.8–47.8	53.4	52.4–53.8	60.9	57.5–65.5
Kmax (D)	44.2	43.6–44.6	50.6	48.9–52.5	59.2	58.4–60.6	69.6	65.4–75.2
Kmean (D)	43.3	42.7–43.6	45.8	44.5–46.1	50.4	50.0–51.3	56.9	54.9–61.3
Location of cone (mm)	—	—	0.28	0.12–1.53	0.25	0.16–1.48	0.25	0.12–1.15
CCT (micron)	526.5	521.0–536.9	488	466.6–505.0	454	446.7–464.7	421	391.4–440.3
TPT (micron)	522	517.0–528.9	475	459.3–494.7	443	435.5–448.1	403	376.0–432.6
IOP (mmHg)	16.3	15.5–17.0	14	13.5–14.7	13.5	12.0–14.0	12.5	11.2–13.0
A1 (msec)	7.48	7.41–7.55	7.15	7.08–7.26	7.06	6.88–7.15	6.92	6.74–7.03
Time (msec)	15.02	14.78–15.25	14.78	14.78–15.10	15.02	14.73–15.53	15.02	14.78–15.40
A2 (msec)	21.41	21.26–21.57	21.56	21.42–21.74	21.63	21.51–21.81	21.85	21.73–22.05
DA (mm)	1.10	1.06–1.13	1.22	1.15–1.26	1.23	1.21–1.30	1.39	1.34–1.47
AUDA (mmHg·msec)	13.01	12.09–13.84	18.5	15.72–19.41	18.42	16.70–20.99	23.84	21.13–28.64
an RMS (mm)	0.095	0.091–0.099	0.111	0.107–0.118	0.113	0.109–0.124	0.132	0.125–0.142
bn RMS (mm)	0.034	0.033–0.037	0.026	0.023–0.029	0.028	0.023–0.032	0.030	0.025–0.039

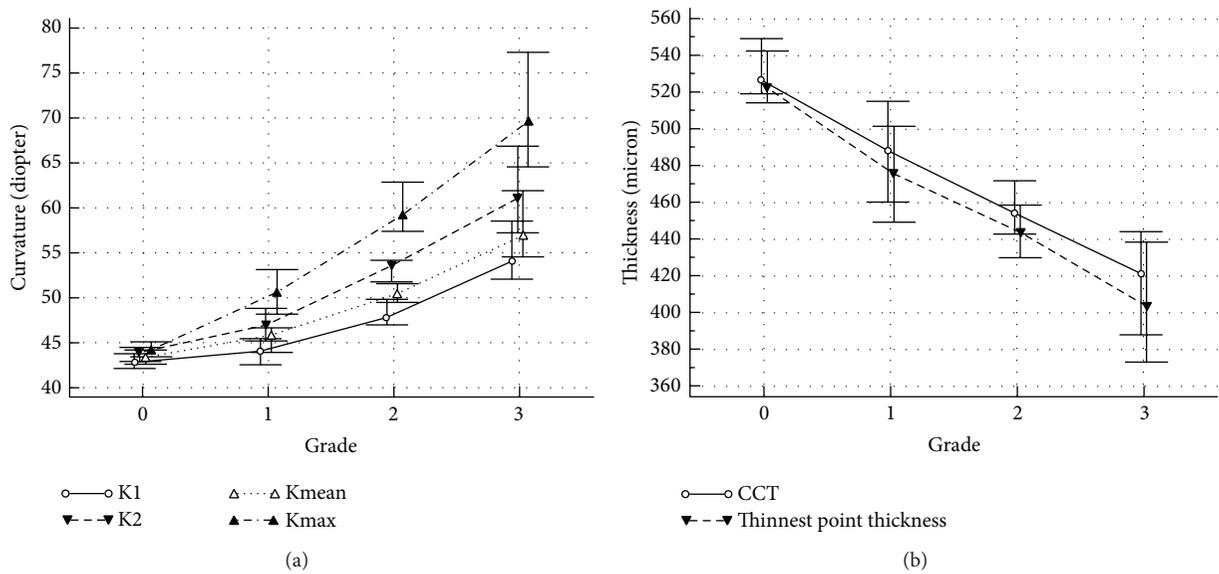


FIGURE 1: (a) Median curvature in diopters (simulated keratometry (K1, K2), mean curvature (Kmean), and maximum curvature (Kmax) as a function of KC grade. Grade 0 implies unaffected eyes; (b) median central corneal thickness (CCT) and thickness of thinnest point as a function of grade.

While DA of grades 1 and 2 were similar ($P > 0.05$), DA of grade 4 differed significantly from other grades ($P < 0.05$). Figures 3(a) and 3(b) show the median values ±95% CI of AUDA and an RMS of all grades. AUDA of grades 0 and 4 differed significantly from other grades and from each other ($P < 0.05$). However, AUDA of grades 1 and 2 were similar to each other ($P > 0.05$) and differed from grades 0 and 4 ($P < 0.05$). Similar trends were observed with an RMS but bn RMS of all grades were similar to each other ($P > 0.05$). IOP of grade 0 was significantly greater than all keratoconus

grades ($P < 0.001$). However, IOP of grades 1, 2, and 3 were similar ($P > 0.05$).

The correlation between all variables was assessed with the Spearman correlation coefficient (Table 2). Most of the correlations were statistically significant ($P < 0.05$). Keratometry correlated well with all biomechanical variables ($P < 0.001$). Interestingly, AUDA and an RMS had a very high correlation (0.945 and 0.919) with DA. As an example, Figures 4(a), 4(b), 4(c), and 4(d) show the linear regression of AUDA and DA with Kmean and TPT. Both AUDA and

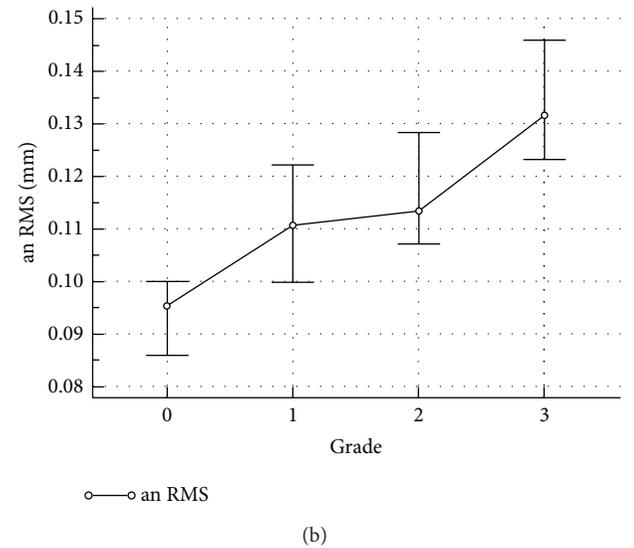
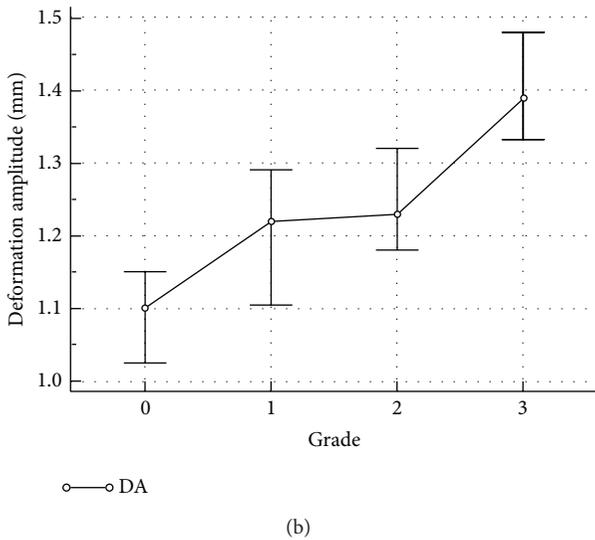
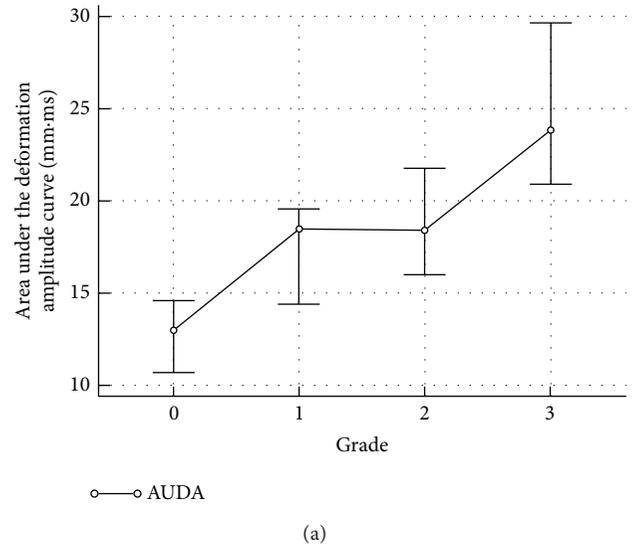
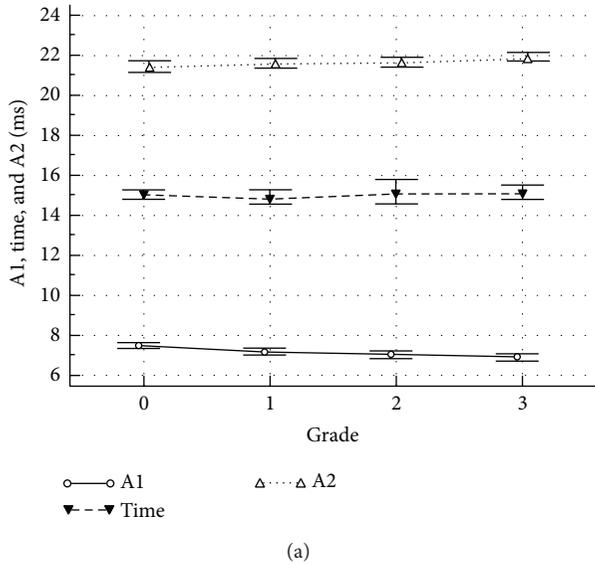


FIGURE 2: (a) Time of first appplanation (A1), peak appplanation (Time), and second appplanation (A2) as a function of KC grade. Grade 0 implies unaffected eyes. All units are msec; (b) deformation amplitude in mm as a function of KC grade. All values are the medians ± 95 CI.

FIGURE 3: (a) Area under the deformation amplitude curve in mm·msec as a function of KC grade. Grade 0 implies unaffected eyes; (b) an RMS, the root mean square of Fourier cosine coefficients, as a function of grade. All values are the medians ± 95 CI.

DA had a significantly negative correlation with Kmean and TPT. Figure 5 shows the correlation between AUDA and DA using all the grades. Table 3 lists the results from the ROC analyses. Time had the least area under the ROC curve equal to 0.511 with a sensitivity and specificity of 70.5% and 62.8%, respectively. Since keratometry was used for gradation, it had the highest area under the ROC curves among all variables (greater than 0.9). Among the biomechanical variables, an RMS had the best area under the ROC curve equal to 0.915 with sensitivity and specificity of 83% and 88.6%, respectively. AUDA was a close second (area under the ROC curve = 0.886, sensitivity = 73.9%, specificity = 93.2%). Figure 6 shows an overlay of DA of four corneas, one from each grade, with CCT and IOP reported next to the grade label. From Figure 6,

salient observations relative to grade 0 were as follows: (a) quicker increase in DA in the first half of the appplanation test in higher disease grades; (b) DA was greater in higher disease grades; (c) slower decrease in DA in the second half of the appplanation test in higher disease grades; (d) globe deformation was similar in all the corneas.

4. Discussion

Corneal tomography attracted a lot of attention as the primary diagnostic tool for keratoconus [10–14]. Steepening of the cornea coupled with thinning of the stroma and epithelium [15] contributed to the worsening vision in keratoconus patients. The steepening of the cornea was an end result of both biomechanical weakening and thinning of the cornea

TABLE 2: Spearman correlation coefficients to compare correlation between the variables. The *P* values are listed for each correlation and represent the statistical significance of the correlation.

	A1	Tune	A2	DA	IOP	AUDA	an RMS	bn RMS	TPT	CCT	K1	K2	Kmean	Kmax
A1		-0.244 0.0048	-0.741 <0.0001	-0.793 <0.0001	0.959 <0.0001	-0.843 <0.0001	-0.818 <0.0001	0.104 0.2367	0.56 <0.0001	0.566 <0.0001	-0.475 <0.0001	-0.568 <0.0001	-0.605 <0.0001	-0.547 <0.0001
Time			0.289 0.0008	0.198 0.0228	-0.25 0.0038	0.217 0.0125	0.169 0.0531	-0.31 0.0003	0.04 0.6465	0.029 0.7394	0.004 0.967	0.06 0.4943	0.038 0.6669	0.03 0.7352
A2				0.756 <0.0001	-0.72 <0.0001	0.754 <0.0001	0.745 <0.0001	0.078 0.3728	-0.335 0.0001	-0.364 <0.0001	0.345 0.0001	0.388 <0.0001	0.372 <0.0001	0.376 <0.0001
DA					-0.753 <0.0001	0.957 <0.0001	0.954 <0.0001	0.083 0.3464	-0.648 <0.0001	-0.659 <0.0001	0.541 <0.0001	0.662 <0.0001	0.671 <0.0001	0.63 <0.0001
IOP						-0.806 <0.0001	-0.785 <0.0001	0.098 0.2638	0.529 <0.0001	0.535 <0.0001	-0.452 <0.0001	-0.545 <0.0001	-0.576 <0.0001	-0.523 <0.0001
AUDA							0.949 <0.0001	0.027 0.7596	-0.66 <0.0001	-0.667 <0.0001	0.577 <0.0001	0.685 <0.0001	0.693 <0.0001	0.659 <0.0001
an RMS								0.04 0.6516	-0.702 <0.0001	-0.718 <0.0001	0.581 <0.0001	0.705 <0.0001	0.716 <0.0001	0.67 <0.0001
bn RMS									0.047 0.5932	0.012 0.8942	-0.065 0.4565	-0.139 0.1123	-0.165 0.0593	-0.119 0.1757
TPT										0.976 <0.0001	-0.714 <0.0001	-0.804 <0.0001	-0.793 <0.0001	-0.785 <0.0001
CCT											-0.726 <0.0001	-0.805 <0.0001	-0.788 <0.0001	-0.79 <0.0001
K1												0.876 <0.0001	0.85 <0.0001	0.955 <0.0001
K2													0.939 <0.0001	0.974 <0.0001
Kmean														0.929 <0.0001
Kmax														

[1]. Several new tomographic indices were postulated for improved detection of keratoconus, with higher sensitivity and specificity [10–14]. A recent study in a large cohort of keratoconus subjects demonstrated that anterior surface irregularity indices were better in diagnosing the disease in early stages than visual acuity and pachymetry [16]. Thus further studies are needed to assess the sensitivity and specificity of these new tomographic variables in other centers. To assess the biomechanical weakening of the cornea, the ocular response analyzer (ORA) was the first device available commercially. It was extensively used to study the biomechanical status of keratoconus [17–22]. The ORA did not report corneal deformation. However, the reflected beam intensity from the anterior surface of the cornea was considered to be representative of corneal deformation. Based on the ORA waveform, corneal hysteresis (CH) and corneal resistance factor (CRF) were lower in KC eyes, with a sensitivity and specificity below 80% [17], and insensitive to corneal stiffening caused by collagen crosslinking [18]. Thus, the true relationship of CH and CRF to the biomechanical status of the cornea, such as Young’s modulus, was not understood.

The grading system used in this study was based on the data reported in a past study on keratoconus using the ORA [8]. In that study, CH and CRF did not produce statistically significant biomechanical difference between the grades [8]. Even though CH and CRF were insensitive to collagen crosslinking, each ORA waveform had characteristic features that may reveal the biomechanical status of keratoconic corneas [19–22]. These studies introduced new variables that had better sensitivity and specificity compared to CH and CRF in the detection of keratoconus [19–22], like the hysteresis loop area [22], which was similar by definition to AUDA. Some of the common conclusions from ORA studies on keratoconus included an earlier appplanation, lower pressure required to cause appplanation, lower signal peak, and delayed recovery of the ORA signal after minimum concavity was attained in keratoconic corneas. However similar to CH and CRF, these new variables did not report any significant biomechanical differences between grades of keratoconus [19–22]. Thus, the physical meaning of these new ORA variables was also undetermined.

Similar to the ORA, the Corvis-ST showed earlier appplanation (A1 decreased), greater deformation (DA increased),

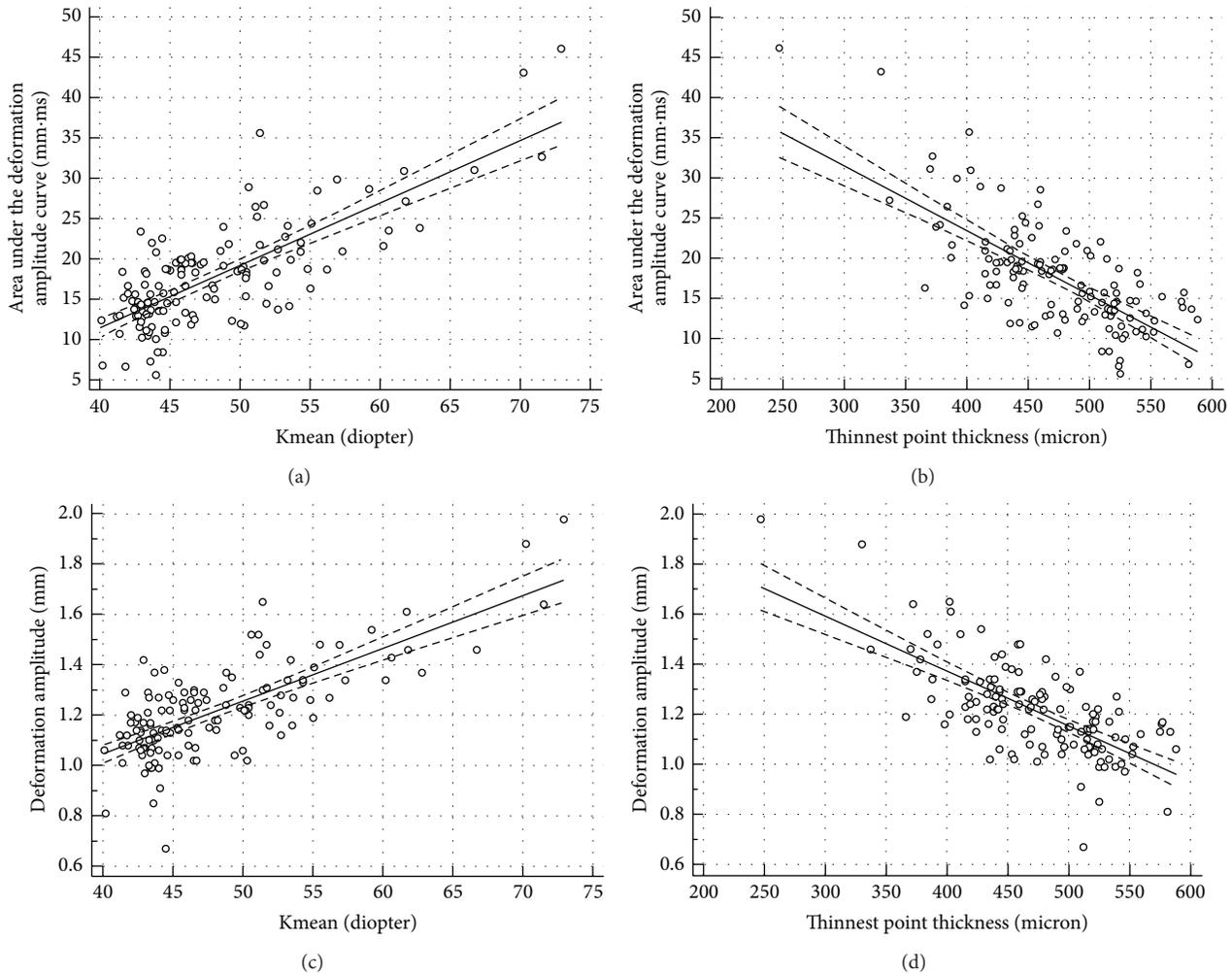


FIGURE 4: (a) Area under the deformation amplitude curve (AUDA) versus mean keratometry (Kmean); (b) area under the deformation amplitude curve (AUDA) versus thinnest point thickness (TPT); (c) deformation amplitude curve (DA) versus mean keratometry (Kmean); (d) deformation amplitude curve (DA) versus thinnest point thickness (TPT). The solid line is the linear regression of the data. The dotted lines are 95% CI limits of the regressed line.

delayed recovery of the cornea (A2 increased), and lower biomechanical response (DA and AUDA increased) with increasing disease severity (Figure 6). The root mean square variables, which were a measure of the undulations or noise in the signal [9], also increased with increasing disease severity. The correlation between the variables was also significant (Table 2). Interestingly, the correlation between AUDA and DA was very high, indicating that if DA was known a priori, AUDA may be estimated with more than 90% accuracy in keratoconus. The sensitivity and specificity of the biomechanical variables were in excess of 80% but lower than the topographic variables in this study and those reported in other studies [10–14]. In this study, IOP was similar among all keratoconus grades but was significantly different from normal corneas. In biomechanically normal corneas, decrease in IOP from 36 to 15 mmHg resulted in a keratometric decrease of 1 D [23]. In keratoconic corneas, increase in IOP from 16 to 36 mmHg resulted in a keratometric increase of 4.1 D [24]. By

linear interpolation, this may imply that keratometry would change by 0.8-0.9 D in keratoconus eyes for a 4-5 mmHg change in IOP in the physiological range. However, IOP was similar among all disease grades in this study ($P > 0.05$). Since IOP was not responsible for increase in keratometry between the grades (1, 2, and 3), it may be concluded that tomographic and biomechanical changes were responsible for disease progression. Since the area under the curve from the ROC analyses of the biomechanical variables was lower than tomography, the biomechanical variables in this study may not be sensitive to early changes in the biomechanical status of the KC cornea compared to tomography [16].

Since deformation amplitude of grades 0 and 3 was significantly different from grades 1 and 2, it may be concluded that corneal deformation reported by Corvis-ST may be more representative of the biomechanical state of keratoconic corneas than the ORA variables. Further, DA, AUDA, and an RMS demonstrated similar biomechanical response of grades 1 and

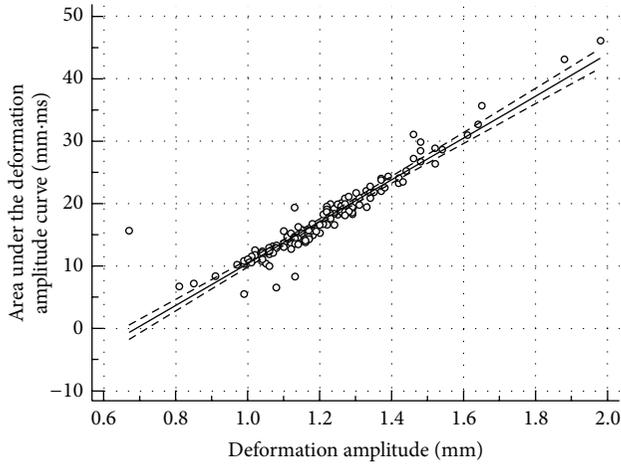


FIGURE 5: Area under the deformation amplitude curve (AUDA) versus deformation amplitude (DA).

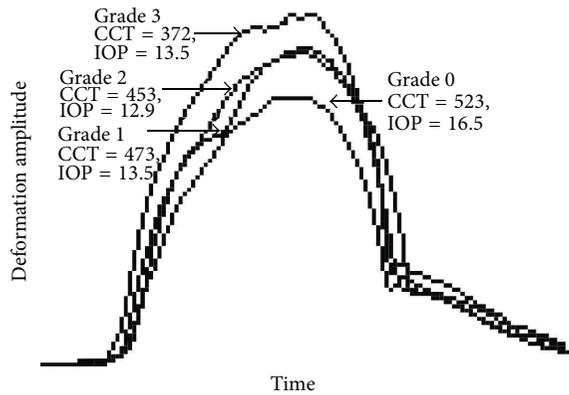


FIGURE 6: Overlay of deformation amplitude of four corneas, one from each grade. The corneal thickness (CCT) and intraocular pressure (IOP) are reported next to the grade label.

2, which may explain why crosslinking halts or delays the progression of disease in early and mild keratoconic corneas but not in advanced cases (grade 3) [7]. In advanced cases, the biomechanical stiffness may be too low to be compensated by the magnitude of stiffening caused by crosslinking. While tomography worsened nearly linearly from grade 1 to grade 3, grades 1 and 2 had similar AUDA, DA, and an RMS but the same differed from grade 3. Thus, thinning of the cornea may be one of the drivers of disease progression from grade 1 to grade 3, while the viscoelastic properties of the cornea may have remained similar across grades 1 and 2. Another study on keratoconic corneas using the Corvis-ST also reported greater DA in keratoconus but the sensitivity of DA to detect the disease was 0.77 [25].

Deformation of the corneal apex is a sum of both the corneal and globe deformation. The globe deformation was only about 1/10th of the measured deformation amplitude at its peak value [26]. Therefore, globe deformation was unlikely to influence the outcomes of this study using the investigated variables. Refined mathematical models based on continuum

TABLE 3: Receiver operator characteristics (ROC) curve for each variable. The area under the ROC curve with 95% CI in brackets, cut-off, sensitivity, and specificity are listed for each column.

	Area under the ROC curve	Cut-off	Sensitivity	Specificity
A1	0.87 (95% CI: 0.80, 0.92)	<21.48	68.2	88.6
Time	0.51 (95% CI: 0.42, 0.60)	>15.71	70.5	62.8
A2	0.69 (95% CI: 0.42, 0.60)	>1.17	19.3	97.7
DA	0.86 (95% CI: 0.79, 0.91)	>16.59	76.1	86.4
AUDA	0.89 (95% CI: 0.82, 0.94)	>0.104	73.9	93.2
an RMS	0.92 (95% CI: 0.85, 0.96)	<0.027	83.0	88.6
bn RMS	0.69 (95% CI: 0.61, 0.77)	<0.027	53.4	88.6
K1	0.88 (95% CI: 0.80, 0.92)	>44.3	77.3	93.2
K2	0.95 (95% CI: 0.89, 0.98)	>45.3	88.2	95.5
Kmean	0.93 (95% CI: 0.88, 0.97)	>45.4	80.7	100.0
Kmax	0.99 (95% CI: 0.97, 1.0)	>46.5	95.5	100.0
CCT	0.93 (95% CI: 0.87, 0.96)	<516	92.0	84.1
TPT	0.86 (95% CI: 0.84, 0.97)	<509	83.3	84.1

soft tissue mechanics may be required such that a measure of Young’s modulus or nonlinear modulus could be defined [26–28]. The device has a limited depth resolution and finer biomechanical abnormalities in the corneal stroma cannot be measured. Thus, anisotropy of the cornea may not be measured accurately, for example, depth variation in mechanical strain due to crosslinks between collagen lamellas [29]. Techniques to resolve the depth dependent differences in the biomechanical strength of the cornea are in development [30–32]. The air-puff caused deformation of the cornea up to a radius of 3 mm from its geometric center [26, 27]. Hence keratoconic cones beyond the 3 mm radius central cornea may not undergo any deformation. A recent study showed that both ORA and Corvis-ST may be required to differentiate between pellucid marginal degeneration (PMD) and normal corneas [33]. However, the devices were unable to distinguish between keratoconus and PMD [33]. In practice, both devices assessed the central cornea and the outcomes of the recent study [33] were confusing. This highlighted the need of advanced analysis methods [26–28] or measurement tool [30–32] that may perform cone location specific measurements. In this study, cone location was unlikely to influence the study outcomes as there was no significant difference ($P > 0.05$) in cone location among the grades of keratoconus. In conclusion, corneal deformation

by Corvis-ST was a direct measure of the biomechanical status of the cornea and may aid to accurately quantify the grades of keratoconus. Separate biomechanical grading scale of keratoconus severity is the need of the hour in addition to traditional tomographic grading.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Intraoperative Optical Coherence Tomography Using the RESCAN 700: Preliminary Results in Collagen Crosslinking

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Received 13 June 2014; Revised 20 October 2014; Accepted 30 October 2014

Academic Editor: George Asimellis

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Purpose. To compare the penetration of riboflavin using a microscope-integrated real time spectral domain optical coherence tomography (ZEISS OPMI LUMERA 700 and ZEISS RESCAN 700) in keratoconus patients undergoing accelerated collagen crosslinking (ACXL) between epithelium on (epi-on) and epithelium off (epi-off). **Methods.** Intraoperative images were obtained during each of the procedures. Seven keratoconus patients underwent epi-on ACXL and four underwent epi-off ACXL. A software tool was developed using Microsoft.NET and Open Computer Vision (OpenCV) libraries for image analysis. Pre- and postprocedure images were analyzed for changes in the corneal hyperreflectance pattern as a measure of the depth of riboflavin penetration. **Results.** The mean corneal hyperreflectance in the epi-on group was 12.97 ± 1.49 gray scale units (GSU) before instillation of riboflavin and 14.46 ± 2.09 GSU after AXCL ($P = 0.019$) while in the epi-off group it was 11.43 ± 2.68 GSU and 16.98 ± 8.49 GSU, respectively ($P = 0.002$). The average depth of the band of hyperreflectance in the epi-on group was 149.39 ± 15.63 microns and in the epi-off group it was 191.04 ± 32.18 microns. **Conclusion.** This novel *in vivo*, real time imaging study demonstrates riboflavin penetration during epi-on and epi-off ACXL.

1. Introduction

Keratoconus is a bilateral, asymmetric, degenerative disorder of the cornea that is characterized by progressive thinning of the stroma, leading to significant visual morbidity. Though the disease has been studied extensively in the past, its etiology is still unclear [1–3]. Of the various treatment options the most effective in delaying its progression is corneal collagen crosslinking (CXL) [4, 5]. The surgical technique of standard crosslinking involves removal of the epithelium to allow penetration of riboflavin into the stroma and then irradiation with ultraviolet A (UVA) light. There is still no consensus on how long the stroma should be imbued with the solution (riboflavin 0.1% and dextran T 500 20%) in order to ensure a sufficient intrastromal concentration of vitamin

B2. While some groups have studied the depth of riboflavin penetration [6, 7], others have deliberated on the importance of removal of corneal epithelium during CXL [8, 9]. Use of imaging modalities like the optical coherence tomography (OCT), Brillouin microscopy, and second harmonic imaging has allowed better understanding of changes occurring in the cornea and thereby helps in predicting treatment outcomes [10–13]. These methods, however, do not allow the assessment of tissues *in vivo*. We have previously compared the extent of penetration of riboflavin in epi-on and epi-off procedures with a hand held spectral domain OCT (SD-OCT, Bioptigen, Inc.) with few limitations [7].

The microscope integrated intraoperative SD-OCT is a technology that offers continuous real time visualization of the corneal changes *in vivo* during surgical procedures.

TABLE 1: This table shows the mean keratometry (Km), mean thinnest cornea thickness (TCT), mean refractive spherical equivalent (MRSE), and grade of keratoconus as per the Amsler-Krumeich classification grade of patients enrolled in the study.

	Km (D)	TCT (micron)	MRSE	Grade of keratoconus
Patient 1 (epi-on)	54	429	-4.5	2
Patient 2 (epi-on)	48.6	408	-6.25	2
Patient 3 (epi-on)	62.1	417	-16	4
Patient 4 (epi-on)	54.9	415	-13	4
Patient 5 (epi-on)	46.7	420	-2.5	1
Patient 6 (epi-on)	48.1	411	-5	2
Patient 7 (epi-on)	54.3	390	-4	3
Patient 1 (epi-off)	47.1	496	-4.5	1
Patient 2 (epi-off)	43.5	490	-2.5	1
Patient 3 (epi-off)	47.9	485	-2	1
Patient 4 (epi-off)	48.2	472	-1	2

The surgical microscope (OPMI LUMERA 700, Zeiss) is combined with an SD OCT having a wavelength of 840 nm and scanning speed of 27000 A-scans per second (RESCAN 700 from ZEISS). We used this system to observe the depth of penetration of riboflavin as well as to compare the difference in the reflectance pattern of the cornea in real time between accelerated collagen crosslinking (ACXL) without debridement of epithelium (epi-on) and ACXL with epithelium debrided (epi-off).

2. Materials and Methods

This observation study was conducted at a tertiary care center in Bangalore, India. The study protocol was approved by the institute's ethics committee and was conducted with strict adherence to the guidelines laid down by the declaration of Helsinki. The study comprised 11 eyes of 11 consecutive patients: eight males and three females. The mean age was 20 years (14 to 26 years). Details of all patients are given in Table 1.

All patients underwent preoperative visual acuity testing and complete slit lamp examination. Progression of keratoconus was defined as an increase of 0.5 diopter (D) or more in two or more keratometry values in the steep meridian between two sagittal curve maps or a decrease in corneal thickness of 10% or more at the thinnest point between two pachymetry maps on Pentacam (Oculus, Wetzlar, Germany) in the preceding 6 months [1, 2]. Seven patients who had a corneal thickness of $<420 \mu\text{m}$ underwent epi-on ACXL. However, in patient 1 (age 14 years) and patient 5 (age 15 years), due to their younger age group, epi-on ACXL was preferred even with a pachymetry $\geq 420 \mu\text{m}$ for better post-operative comfort. After anesthetizing the eye with topical proparacaine 0.5% (Paracaine, Sunways, (India) Pvt. Ltd), 0.25% riboflavin solution containing hydroxypropyl methylcellulose and benzalkonium chloride (ParaCel, Avedro Inc. USA) was applied every 90 seconds for a total of 4 minutes followed by an application of 0.22% riboflavin isotonic

solution every 90 seconds for a total of six minutes (VibeX Xtra, Avedro Inc. USA). After rinsing the cornea with balanced salt solution (BSS), ultraviolet A (UV-A) irradiation was initiated using 45 mW/cm^2 for 2 minutes and 40 seconds for a surface dose of 7.2 J [14].

Four patients with adequate corneal thickness underwent ACXL after corneal epithelial debridement. The central 7–9 mm of the epithelium was removed with a mechanical epithelium scraper followed by application of 0.1% riboflavin 5-phosphate and 20% dextran solution once every 2 minutes for 20 minutes. Exposure to UV-A (30 mW/cm^2) radiation for 4 minutes was done in all these. A soft bandage contact lens (BCL) with good oxygen permeability was placed at the end of the procedure. The patients were reviewed till the removal of the BCL.

For intraoperative imaging we used the RESCAN 700 (Zeiss), which is a real time intraoperative SDOCT integrated with the operating microscope. This system is based on the fiber optics Michelson interferometer configuration that allows noninvasive tissue observation through sectional cuts of the ocular structure in real time. The scan depth is 2 mm and scan length can vary from 3 to 16 mm. The axial and transverse resolution of the anterior segment OCT in tissue are $5 \mu\text{m}$ and $15.5 \mu\text{m}$, respectively. The microscope provides uninterrupted OCT imaging and video recording during the entire length of the surgery in a small window adjacent to the operating field. The OCT images are recorded in a horizontal and vertical orientation. The RESCAN 700 includes Z-tracking and focus control for image stabilization and quality control. Due to the possibility of inactivation of riboflavin due to light, only intermittent OCT scanning was done during the procedure. The video output was stored and later reviewed for critical steps of the surgery. A single observer performed all the image acquisitions with enhanced image quality. A cube (512×128) was used to image the required area. The size of the cube was adjusted in order to achieve optimal coverage.

A software tool was developed separately using Microsoft.NET and Open Computer Vision (OpenCV) libraries for image analysis. Images were first extracted from the video sequence. The region of interest was automatically marked using polynomial regression (curve fitting, 5th order) with an option for fine adjustment of the selected region. The boundary of the selected region could be altered using a tool similar to the pen tool of Adobe Photoshop. Once the region of interest (ROI) was marked, a sliding window approach was used to measure the parameters within the ROI. A window of dimension $10 \times$ was used and the average intensity was calculated by summing up the intensity values and dividing it by the number of pixels ($10 \times$ ROI height). A histogram was generated on the active window region and based on the mode (most repeated value, peak of the histogram) a threshold was determined. When this threshold was applied to the windowed region, pixels with less gray values were removed and only the hyperluminescent (reflective) ones were retained. To identify the presence of hyperluminescence, the image of the corneal cross section was divided into the anterior two-thirds and posterior one-third. The ratios of average intensities were calculated

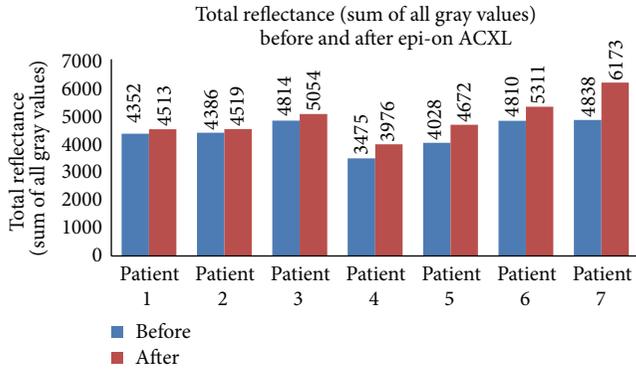


FIGURE 1: Total reflectance (sum of all gray values) seen in patients undergoing epi-on ACXL before and after the procedure.

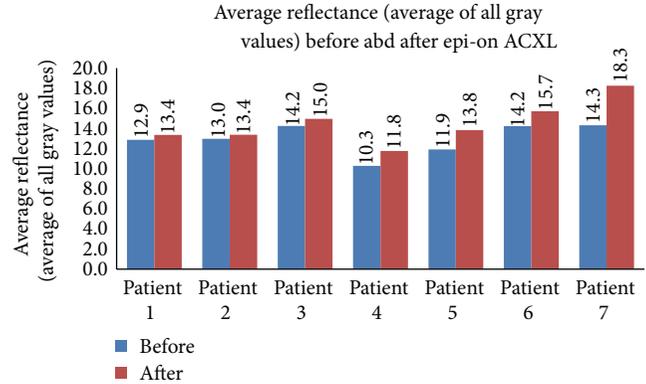


FIGURE 2: Average reflectance (average of all gray values) seen in patients undergoing epi-on ACXL before and after the procedure.

for images before instillation of riboflavin and at the end of the procedure. The images were enhanced using contrast stretching and the software measured the dimensions (depth) of hyperluminescence/reflectance. The first image taken prior to starting instillation of riboflavin served as a control with any prior hyperreflectivity inherent to the corneal stroma taken as baseline. The second image was taken at the end of the entire CXL procedure. The band of increased reflectivity seen in the corneal stroma after the procedure was taken as a representation of the penetration of riboflavin. The extracted data were correlated and analyzed. Finally the scale was converted from pixels to microns using the data sheet provided by the manufacturer.

2.1. Statistical Analysis. The OCT images were analyzed and the data thus obtained was entered into an Excel spreadsheet (Microsoft Corp.). Statistical analysis of the results was performed using the SPSS software (version 22, IBM SPSS Statistics). Normality of the distribution of all measurements was confirmed using the Shapiro-Wilk test, which is more appropriate for small sample sizes than the Kolmogorov-Smirnov test. Student's *t*-test for paired data was used; a *P* value of less than 0.05 was considered statistically significant. The mean of the corneal stromal hyperreflectance and ratio of reflectance of the anterior two-thirds and posterior one-third of the cornea in each group was used for the statistical analysis.

3. Results

The total reflectance (sum of all gray values) in the epi-on group (Figure 1) was 4386.13 ± 497.38 gray scale units (GSU) before ACXL and 4888.03 ± 387.62 GSU after ACXL and the average reflectance (Figure 2) was 12.97 ± 1.49 GSU before starting the procedure and 14.46 ± 2.09 GSU after ACXL ($P = 0.019$). The total reflectance in the epi-off group (Figure 3) was 4664.0 ± 1094.88 GSU and 6767.0 ± 805.74 GSU before and after the procedure, respectively. The mean corneal hyperreflectance (Figure 4) in the same group was 11.43 ± 2.68 GSU and 16.98 ± 8.49 GSU before and after ACXL, respectively ($P = 0.002$). The mean of the ratio of the anterior two-thirds and posterior one-third of cornea in epi-on group

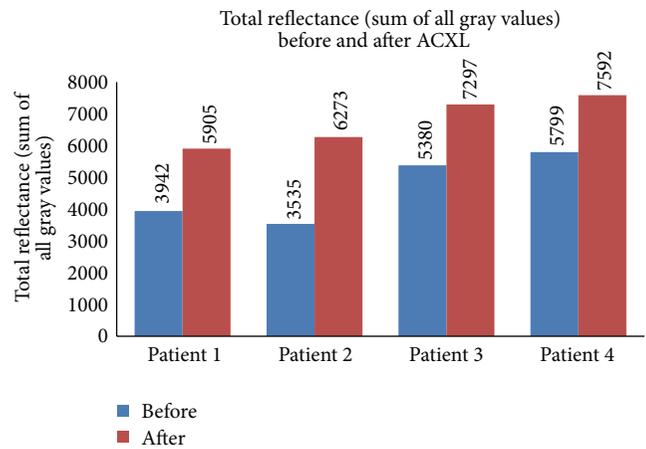


FIGURE 3: Total reflectance (sum of all gray values) seen in patients undergoing epi-off ACXL before and after the procedure.

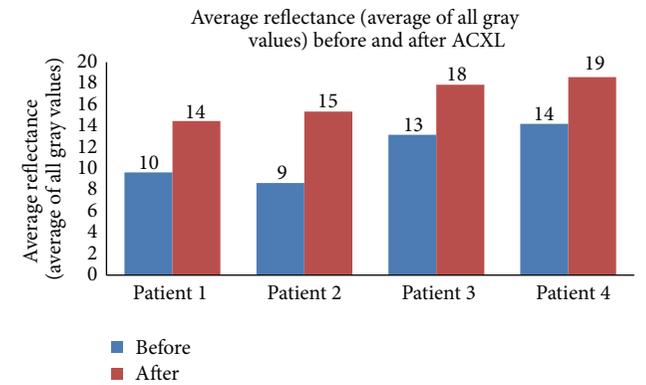


FIGURE 4: Average reflectance (average of all gray values) seen in patients undergoing epi-off ACXL before and after the procedure.

before starting of the procedure was 51.95 ± 10.22 GSU and after ACXL was 57.34 ± 10.23 GSU ($P = 0.035$) and in the epi-off group it was 44.63 ± 16.98 GSU and 64.63 ± 7.21 GSU before and at the end ACXL, respectively ($P = 0.034$).

The average depth of the hyperreflective band in the epi-on group was 149.39 ± 15.63 microns (Figure 5) and in the epi-off group was 191.04 ± 32.18 microns (Figure 6).

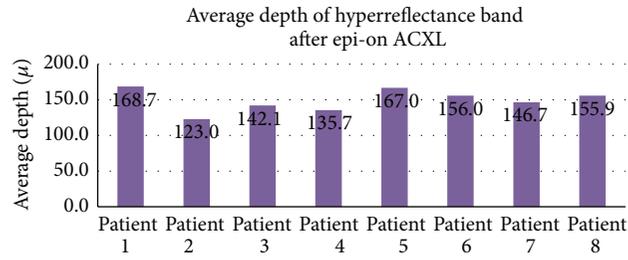


FIGURE 5: Average depth of hyperreflectance band seen in patients after epi-on ACXL.

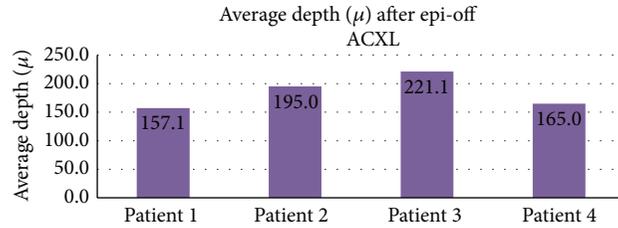


FIGURE 6: Average depth of hyperreflectance band seen in patients after epi-off ACXL.

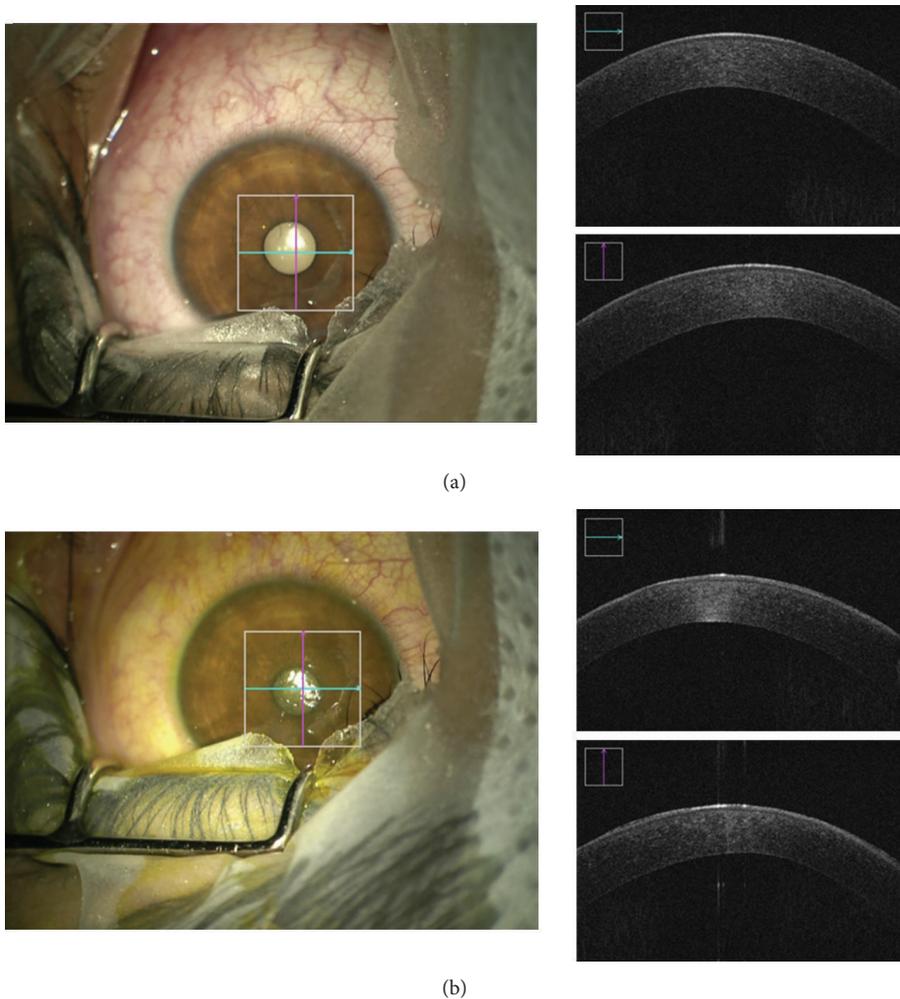


FIGURE 7: Epi-on ACXL being performed under ZEISS OPMI LUMERA 700. (a) Preprocedure picture with intraoperative OCT (top right); (b) postprocedure picture with intraoperative OCT (bottom right).

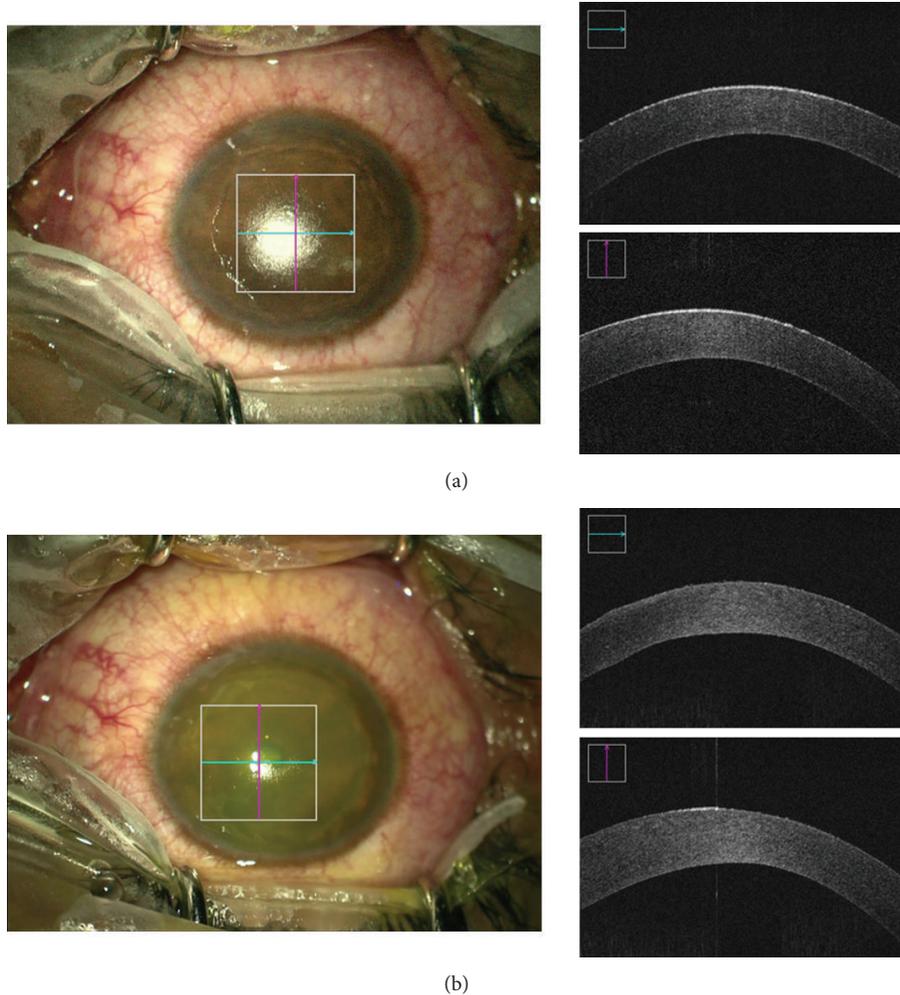


FIGURE 8: Epi-off ACXL being performed under ZEISS OPMI LUMERA 700. (a) Preprocedure picture with intraoperative OCT (top right); (b) postprocedure picture with intraoperative OCT (bottom right).

4. Discussion

Corneal collagen crosslinking is currently the most preferred treatment option for the management of progressive keratoconus and other corneal ectasias [3, 4] and works by increasing the biomechanical stability of the cornea [6, 15]. There are several CXL protocols using different energy levels with and without epithelial debridement [14, 16–18]. Without epithelial debridement the major theoretical challenge is in the penetration of the hydrophilic macromolecule of riboflavin across the hydrophobic corneal epithelial barrier. With continued research on the safety and efficacy of transepithelial CXL (TECXL) [14] and use of drug delivery systems like iontophoresis to achieve riboflavin delivery into the cornea [19], TECXL is fast emerging as a treatment option for patients with thin corneas giving encouraging results [17].

It is therefore interesting to know the depth of riboflavin in the various protocols of CXL. Researchers have used keratocyte apoptosis after corneal collagen crosslinking as an indirect measure of depth and effectiveness of this procedure [20]. Seiler and Hafezi have used SD-OCT as an effective biomicroscopic tool to determine the depth of riboflavin

penetration [21]. They have assessed the effectiveness of the procedure with the help of a demarcation line noticed two weeks after CXL. Caporossi et al. performed confocal microscopy analyses in humans after crosslinking and detected *in vivo* the effective depth of treatment by identifying distinct vertical and lateral transition areas at a depth of 270 to 330 μm [22].

John Kanellopoulos and Asimellis introduced a novel, noninvasive, quantitative technique utilizing anterior segment OCT images to quantitatively assess the depth and cross-sectional area of CXL in the corneal stroma based on digital image analysis [23]. Our study is comparable in that the SDOCT was used to image the depth, but we used microscope integrated real time *in vivo* imaging. We used the change in reflectance pattern depth as a measure of riboflavin penetration by taking images before and after the procedure. We also compared the difference in riboflavin penetration between epithelium debrided and transepithelial crosslinking. Hence in this study, using the intraoperative SD-OCT, we describe a zone of hyperreflectance as a measure of the depth of penetration of riboflavin (Figures 7 and 8).

We have previously used the hand held SD-OCT to show the penetration of riboflavin for both epithelium-on and epithelium-off procedures [7]. However, since it was not integrated into the operative microscope, image acquisition was cumbersome. Also due to the lack of continuous video capturing mode, serial image capture throughout the procedure was not possible. This was overcome with the use of an intraoperative real time OCT, with serial image acquisition. In the previous study we found that the mean depth of the hyperreflective band after epi-off CXL was $54.2 \pm 5.2 \mu\text{m}$ and $72.4 \pm 7.1 \mu\text{m}$ at 30 and 60 minutes, respectively. In this study, the average depth of the hyperreflective band in the epi-on group was 149.39 ± 15.63 microns and in the epi-off group was 191.04 ± 32.18 microns. This difference could be attributed to the different devices used for imaging, the method of image analysis, and the different CXL protocols used.

There was an increase in the depth of reflectance in both crosslinking groups after the procedure suggestive of penetration of the drug into the anterior corneal stroma. Since the second image was acquired after CXL, its influence on the reflectance pattern is a strong possibility. Nevertheless, this imaging technology can be used to possibly compare the effect of crosslinking in various protocols, in riboflavin concentrations, in dyes and energy settings, in real time, intraoperatively, and *in vivo* and hence help refining protocols for CXL. To the best of our knowledge, this study is the first to assess quantitatively the penetration of riboflavin during CXL *in vivo*, in both epithelium-on and epithelium-off procedures using the microscope integrated intraoperative SD OCT.

5. Conclusion

This novel *in vivo* imaging study demonstrates the penetration of riboflavin during ACXL in both epithelium-on and epithelium-off procedures. The hyperreflectance noted in the images shows a penetration of approximately 149.39 ± 15.63 microns and 191.04 ± 32.18 microns in epi-on and epi-off groups, respectively. Long-term postoperative evaluation of changes in corneal biomechanics will further help validate our findings.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Profile of Microbial Keratitis after Corneal Collagen Cross-Linking

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Received 7 June 2014; Accepted 25 August 2014; Published 11 September 2014

Academic Editor: George Asimellis

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Purpose. To report the profile of microbial keratitis occurring after corneal collagen cross-linking (CXL) in keratoconus patients. **Methods.** A retrospective analysis of 2350 patients (1715 conventional CXL, 310 transepithelial CXL, and 325 accelerated CXL) over 7 years (from January 2007 to January 2014) of progressive keratoconus, who underwent CXL at a tertiary eye care centre, was performed. Clinical findings, treatment, and course of disease of four eyes that developed postprocedural moxifloxacin resistant *Staphylococcus aureus* (MXRSA) infectious keratitis are highlighted. **Results.** Four eyes that underwent CXL (0.0017%) had corneal infiltrates. All eyes that developed keratitis had conventional CXL. Corneal infiltrates were noted on the third postoperative day. Gram's stain as well as culture reported MXRSA as the causative agent in all cases. Polymerase chain reaction (PCR) in each case was positive for eubacterial genome. All patients were treated with fortified antibiotic eye drops, following which keratitis resolved over a 6-week period with scarring. All these patients were on long-term preoperative oral/topical steroids for chronic disorders (chronic vernal keratoconjunctivitis, bronchial asthma, and chronic eczema). **Conclusion.** The incidence of infectious keratitis after CXL is a rare complication (0.0017%). MXRSA is a potential organism for causing post-CXL keratitis and should be identified early and treated aggressively with fortified antibiotics.

1. Introduction

The treatment of keratoconus has been revolutionized with the introduction of newer treatment modalities like corneal collagen cross-linking. Corneal collagen cross-linking with riboflavin (CXL) has been reported to increase the mechanical rigidity of cornea and thus delay or even halt the progression of keratoconus [1, 2]. CXL is a relatively safe surgery without sight-threatening complications [1]. It does not alter the ocular surface as evidenced by no significant changes in objective dry eye parameters after CXL [3]. Its long term stability, safety, and efficacy coupled with other procedures have been reported [4, 5]. Customization of CXL is in vogue [6]. However, microbial keratitis due to varied etiology including herpetic, bacterial (*Escherichia coli*, *Pseudomonas*, *Staphylococcus* and *Streptococcus*), and

Acanthamoeba has been reported after CXL [7–12]. We have evaluated the incidence of post-CXL infectious keratitis among keratoconus patients in our centre and reported a series of patients who developed moxifloxacin resistant *Staphylococcus aureus* (MXRSA) keratitis following CXL.

2. Materials and Methods

In this retrospective analysis, records of 2350 patients undergoing CXL (1715 conventional CXL, 310 transepithelial CXL, and 325 accelerated CXL) in the last 7 years at a tertiary eye care centre in southern India were reviewed. All patients were diagnosed with progressive keratoconus (an increase of 0.5 diopter (D) or more in two or more keratometric values in the steep meridian between two sagittal curve maps or a decrease in corneal thickness of 10% or more at the thinnest

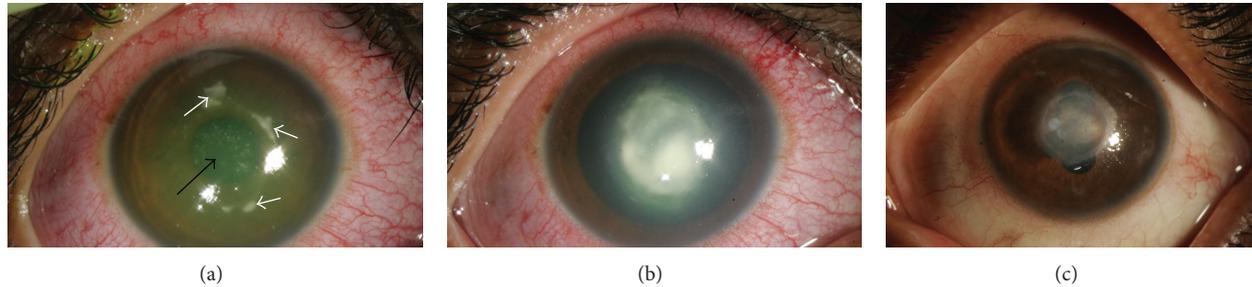


FIGURE 1: Slit lamp photographs showing multifocal anterior stromal infiltrates (arrows) with overlying epithelial defect (3rd postoperative day) (a), coalescence of the infiltrates (6th postoperative day) (b), and anterior stromal scars with no active infiltrate (four weeks after procedure) (c).

point between two pachymetry maps on Pentacam (Oculus, Wetzlar, Germany) in the preceding six months) and required surgical intervention [13]. Protocol of CXL used was based on the thinnest pachymetry values. Those with thinnest pachymetry more than 450 microns underwent conventional or accelerated CXL. Patients with pachymetry less than 450 microns were chosen for transepithelial CXL.

All four patients who developed postprocedure keratitis underwent conventional CXL at our hospital on different days. CXL being a safe procedure, sending conjunctival swab preoperatively, is not routinely practised. Exceptions to this are one eyed patients. Prophylactic antibiotics, moxifloxacin hydrochloride 0.5% (Vigamox, Alcon, USA) three times a day, were started 3 days prior to the surgery. Conventional CXL was performed using the standard protocol advised by Spoerl et al. [14]. Local anaesthesia consisting of proparacaine hydrochloride 0.5% (Paracain, Sunways Pvt. Ltd., India) eye drop was instilled in the treated eye under aseptic conditions. A lid speculum was placed in the fornix. The central 8.0 mm of the corneal epithelium was debrided using an epithelial scraper. Thirty minutes prior to the actual irradiation, 1 drop of riboflavin 0.1% photosensitizer solution containing 10 mg riboflavin-5-phosphate (in 10 mL 20% wt/vol dextran 500) was instilled onto the debrided central cornea every 5 minutes. The corneas were then irradiated with UVA for 30 minutes (irradiance 3 mW/cm²; dose 5.4 J/cm²) using a 370 nm UVA double-diode light source. Following CXL a BCL (bandage contact lens, Ciba Vision, CIBA Vision Corp, Duluth, GA) was inserted; the BCL was removed by the surgeon once the epithelial defect had healed. Transepithelial CXL involved soaking of nondebrided corneas with 0.25% riboflavin every 2 minutes for a period of 30 minutes. This was followed by irradiation for a period of 2 minutes 40 seconds (irradiance 45 mW/cm²; dose 7.2 J/cm²). In accelerated CXL, corneal epithelium was debrided either manually using a scraper or with an excimer laser (phototherapeutic keratectomy). After a soaking time of twenty minutes with riboflavin 0.1%, an irradiation of 5.4 J/cm² (30 mW/cm² for 3 minutes; 18 mW/cm² for 5 minutes; and 9 mW/cm² for 10 minutes) was delivered. Postoperative treatment for all patients included topical antibiotics, moxifloxacin hydrochloride 0.5%, 3 times a day for 1 week. We did not start steroids in the immediate postoperative period as our protocol is to defer steroids till

the epithelial defect has healed completely. As per protocol, all patients were followed up on days 1 and 3, and then at 1 week, 1 month, 3 months, and 6 months after procedure.

3. Results

There were no intraoperative complications with any of the patients. Patients were seen on the third postoperative day for BCL removal. All 4 patients reported here had complaints of severe pain, watering, and photophobia. Table 1 highlights the clinical profile of these patients. On examination, the lids were edematous and conjunctiva showed diffuse congestion. There were multifocal anterior stromal infiltrates with well circumscribed margins on the cornea associated with edema around the infiltrates in the central 4 mm and an overlying epithelial defect; the anterior chamber was quiet (Figure 1(a)); BCL was present in situ. A complete microbiological workup was ordered for all cases; the BCL was also sent for culture. Gram's stain showed gram positive cocci, while potassium hydroxide (KOH) wet mount did not show any fungi. All patients were advised to use moxifloxacin eye drops hourly till culture and sensitivity test reports were received; they were reviewed daily. Steroids were not started in these cases. All eyes showed an increase in both symptoms (worsening of pain and photophobia) and signs (increase in the infiltrate size and coalescing) over the next couple of days with an active anterior chamber reaction (Figure 1(b)). Polymerase chain reaction (PCR) was positive for eubacterial genome. The bacterial culture (including the one from the BCL) showed significant number of *Staphylococcus aureus* which on antibiogram were resistant to moxifloxacin while being sensitive to gatifloxacin, tobramycin, and cefazoline. Topical medications were changed immediately to hourly fortified cefazoline 5% and tobramycin 1.3%. Patients were reviewed daily for 3 days and then on alternate days. Over the next week they showed a decrease in infiltrate size. After six weeks none of the patients had any active infiltrate; only anterior stromal scars were visible (Figure 1(c)). Fluorometholone acetonide 0.1% (FML, Allergan Ltd.) was added to reduce the scarring and tapered over three weeks. None of the patients had any vitritis during the entire course; all patients categorically stated that they had not handled their contact lens after insertion by the ophthalmologist.

TABLE 1: Clinical profile of patients who developed keratitis after collagen cross-linking.

Clinical profile (Age/Sex)	Associated conditions and treatment for the same duration	Preoperative BCVA	Procedure	Day of presentation of symptoms from day of surgery	Treatment	Time for resolution of symptoms	Rehabilitation	Final BCVA
Patient 1 (27/F)	Bronchial asthma (12 years) Oral/Inhalational steroids (12 years)	20/30	Conventional CXL	3	Fortified antibiotics	6 weeks	FEK	20/20
Patient 2 (18/M)	Vernal catarrh (10 years) Topical steroids (10 years)	20/20	Conventional CXL	3	Fortified antibiotics	4 weeks	RGP	20/30
Patient 3 (25/M)	Eczema (5 years) Oral cyclophosphamide (5 years)	20/20	Conventional CXL	3	Fortified antibiotics	5 weeks	Advised PKP	20/120
Patient 4 (16/M)	Vernal catarrh (6 years) Topical steroids (6 years)	20/20	Conventional CXL	3	Fortified antibiotics	5 weeks	AMG, under follow-up	20/200

M: male, F: female, BCVA: best corrected visual acuity, FEK: femtosecond enabled penetrating keratoplasty, PKP: penetrating keratoplasty, RGP: rigid gas permeable lens, and AMG: amniotic membrane grafting.

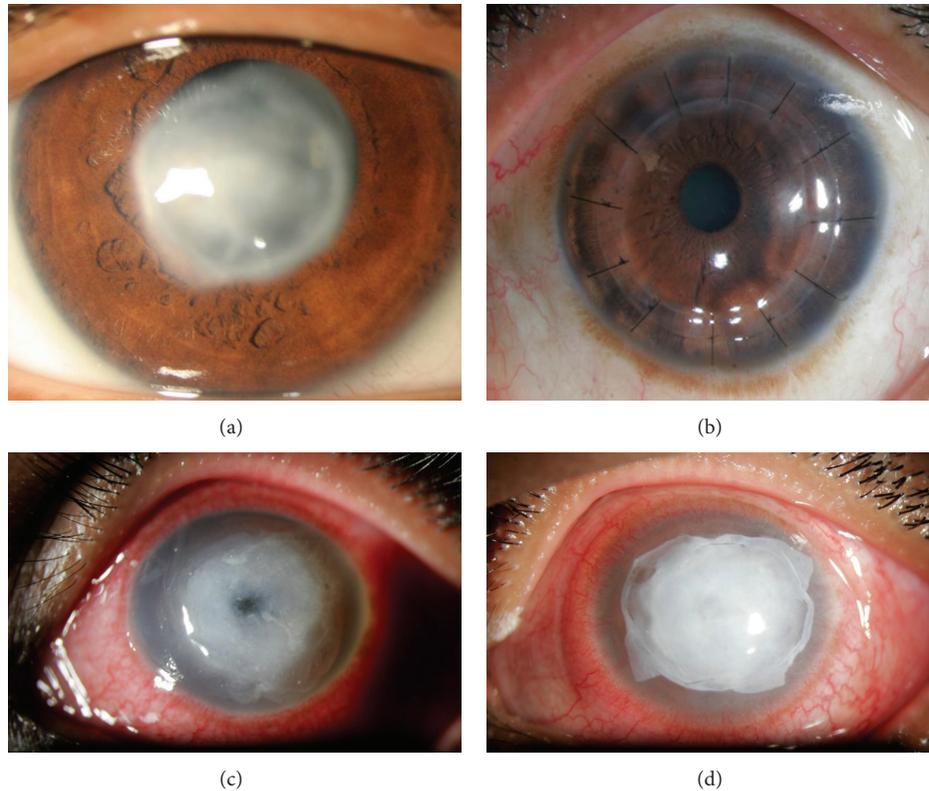


FIGURE 2: Slit lamp photographs of patient 1 who had dense stromal scarring (a) 2 months after resolution of keratitis; the patient underwent femtosecond enabled keratoplasty (FEK) (b); patient 4 developed stromal melt a week after resolution of keratitis (c) and underwent successful amniotic membrane grafting (d).

Two months after resolution of keratitis, patient 1 underwent femtosecond enabled full thickness penetrating keratoplasty with good clinical outcome (Figures 2(a) and 2(b)) (Table 1). Patient 2 was given rigid gas permeable lenses. Patient 3 has been advised to undergo penetrating keratoplasty. Since patient 4 had stromal melt, amniotic membrane grafting was done, which stopped further lysis and healed with scarring (Figures 2(c) and 2(d)) (Table 1).

4. Discussion

Cross-linking is currently one of the most widely used treatment strategies for keratoconus. Despite the well-established safety profile of the procedure, there have been reports with regard to postoperative infections following CXL with riboflavin and UVA (Table 2). Kymionis et al. described a patient who developed epithelial herpetic keratitis and iritis after CXL treatment and hypothesised that UVA light could be a potent stimulus to induce reactivation of latent HSV infections even in patients with no history of clinical herpes virus ocular infections [7]. They also postulated that corneal epithelial/stromal trauma or actual damage of the corneal nerves could be the mechanism of HSV reactivation and also the use of topical corticosteroids may be additional risk factors. Another group reported a case of post-CXL corneal melt wherein corneal scraping was positive for *Acanthamoeba*;

the patient had to undergo therapeutic keratoplasty; their patient washed eyelids and face with tap water with the BCL in situ which was a potential risk factor [9]. Zamora and Males reported that their patient, who presented with culture proven polymicrobial keratitis 3 days following CXL, had a history of handling the BCL in the immediate postoperative period; they postulated that this could have been a risk factor for keratitis [11]. Though none of the patients claimed to have handled the BCL in our series, it is difficult to ascertain whether the use of BCL is in itself a potential risk factor for the development of keratitis post-CXL.

The original treatment protocol (Wollensak et al.) proposed the use of antibiotic ointments in the postoperative period after CXL [1]. Various other studies have highlighted the use of postoperative steroids and/or nonsteroidal anti-inflammatory drugs (NSAIDs) along with an antibiotic agent [7, 8, 10]. However, it is also known that the use of topical corticosteroids and/or NSAIDs has the potential to exacerbate an infection [15]. Hence, in our practice, we do not use topical steroids till the epithelium has healed. In the reported cases, steroids were not started as infection was noticed on the third postoperative day. We have also modified our protocol to follow up our patients every day till the epithelium is healed completely. We also recommend the use of BCL after procedure as it enhances epithelial healing and decreases discomfort [16].

TABLE 2: Reports of keratitis after collagen cross-linking in literature.

Author	Number of cases reported	CDVA	Time to presentation (Days)	Organisms	Final CDVA
Pollhammer and Cursiefen [8]	1	20/400	3	<i>Escherichia coli</i>	20/63
Rama et al. [9]	1	—	5	<i>Acanthamoeba</i>	20/40
Pérez-Santonja et al. [10]	1	20/20	2	<i>Staphylococcus epidermidis</i>	20/22
Zamora and Males [11]	1	CF	3	<i>Streptococcus salivarius</i> , <i>Streptococcus oralis</i> , and coagulase-negative <i>Staphylococcus</i> species	20/50
Sharma et al. [12]	1	HMNF	3	<i>Pseudomonas aeruginosa</i>	20/200

CF: counting fingers, HMNF: hand movements near to face, and CDVA: corrected distance visual acuity.

Unlike other drugs like ofloxacin [17], voriconazole [17], pilocarpine [18] and fluorescein [19] which have reduced penetration through cross-linked cornea, penetration of moxifloxacin into the anterior chamber has been proven to be unaltered by CXL [20]. Moxifloxacin has enhanced potency against *S. aureus* and higher bactericidal activity against highly resistant strains than ciprofloxacin [21]. When keratitis is noted, the accepted practice is to perform a culture and sensitivity tests to determine the appropriate antibiotic that would be ideal for the organism and then change the treatment regimen. In our series, since Gram's stain showed *S. aureus* as the causative organism and MXRSA keratitis has not been reported to date, we did not change the drug, but rather increased the frequency.

Moxifloxacin, a topical fourth generation quinolone is four- to eight-fold more potent against *S. aureus* than ciprofloxacin, despite similar inhibition of topoisomerase IV and DNA gyrase by both the drugs. Moxifloxacin, similar to other 8-methoxyquinolones, has been shown to preferentially target topoisomerase IV in vivo in *S. aureus* [21]. Thus, even a single mutation in topoisomerase IV could contribute to moxifloxacin resistance. It might be possible that mutations may have been caused in the *S. aureus* species in our series due to the UVA radiation used during CXL, similar to events like activation of latent herpes virus [7]. We understand that this is currently a hypothesis at best; a more detailed research and understanding of the mechanisms involved is needed.

Our group has already reported the effectiveness of CXL in treating nonresolving microbial keratitis with superficial stromal involvement [22]. However it is interesting that CXL itself might be a precipitating factor in causing keratitis. The common link between all four patients in our series could potentially have been the long-term use of preoperative steroids (topical/systemic) (Table 1). This might have led to an immunocompromised status. Previous studies have shown that there are changes in ocular flora due to the chronic use of topical steroids in keratoconus patients with VKC. This could possibly lead to an increased risk of postoperative keratitis [23]. Hence it might be important to monitor the use of topical/oral steroids in these patients and inform the patient and treating physician of the potential risks.

Both epithelial debridement and CXL have been shown to cause damage to stromal keratocytes which have a role in corneal immune response [24–26]. This might suggest that all high risk patients like those on oral or topical steroids or other immunosuppressive drugs should be counselled prior to surgery and followed up more carefully. Simultaneous bilateral surgeries should be avoided in such patients. Drug resistant infections should also be kept in mind. It is interesting to note that in all the reported cases published and also in our centre, the infections occurred after conventional CXL. There are no reports of infections after accelerated (KXL) or transepithelial cross-linking (TEKXL). We could hypothesize that the longer exposure to UV-A and duration of the conventional CXL might be a potential precipitating factor. There might be a role for TEKXL or KXL as an alternative to CXL, as the total procedure time is significantly lesser in these procedures; further studies to validate this are needed.

Moxifloxacin is preferred by most surgeons for surgical prophylaxis [27]. It has proven advantageous over older fluoroquinolones as well as other topically available antimicrobials, has a broader spectrum of action and excellent penetration into eye tissues, and is able to deliver a concentration thousands of times the minimum inhibitory concentration [28–31]. To the best of our knowledge, this is the first report of MXRSA keratitis following CXL. Culture and PCR allowed timely intervention. If keratitis develops in spite of antibiotic coverage, a high level of suspicion of drug resistance should be present. While treating postprocedure keratitis, the proper course of action might be to use alternate or fortified antibiotics rather than increasing the frequency of the fluoroquinolones in the interim.

The infection might reduce, but the visual morbidity may still be high. A larger multicentric cohort is needed to validate these conclusions.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Accelerated Corneal Collagen Cross-Linking in Pediatric Patients: Two-Year Follow-Up Results

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Received 29 May 2014; Revised 11 August 2014; Accepted 25 August 2014; Published 11 September 2014

Academic Editor: George Asimellis

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Purpose. To evaluate the effectiveness and safety of accelerated corneal collagen cross-linking (ACXL) in patients below 14 years of age with progressive keratoconus. **Materials and Methods.** Thirty eyes of 18 patients with established progressive keratoconus underwent preoperative and postoperative visual acuity assessment, topography, and specular microscopy prior to ACXL and were followed up for 24 months. **Results.** Mean age of the patients was 12.7 years with ten males and eight females. There was an improvement in the mean postoperative uncorrected distant visual acuity (from 0.76 ± 0.26 to 0.61 ± 0.25 ; $P = 0.005$), mean corrected distant visual acuity (from 0.24 ± 0.19 to 0.12 ± 0.12 ; $P < 0.001$), mean spherical refraction (from $-3.04 \text{ DS} \pm 3.60$ to $-2.38 \text{ DS} \pm 3.37$; $P = 0.28$), mean cylinder (from $-3.63 \text{ DC} \pm 1.82$ to $-2.80 \text{ DC} \pm 1.48$; $P = 0.008$), and spherical equivalent (from $-4.70 \text{ D} \pm 3.86$ to $-3.75 \text{ D} \pm 3.49$; $P = 0.15$). Three eyes of two patients with vernal keratoconjunctivitis (VKC) showed progression. There were no intra- or postoperative complications. **Conclusion.** In pediatric patients ACXL is an effective and safe procedure for the management of keratoconus. Optimal management of VKC is important to arrest the progression of keratoconus.

1. Introduction

Keratoconus is characterized by progressive corneal protrusion and thinning, leading to irregular astigmatism and impairment in visual function, secondary to changes in the structure and organization of corneal collagen [1, 2]. The ectasia progresses at a variable rate but may be more rapid in pediatric patients afflicted with vernal keratoconjunctivitis (VKC) [3]. Reeves et al. found that keratoconus progression was more frequent and faster in patients under 18 years of age, with a seven-fold higher risk of requiring corneal transplantation [4]. There have been some studies that have used corneal collagen cross-linking (CXL) in the management of young patients with progressive keratoconus and found it to be effective [5]. Corneal collagen cross-linking causes photopolymerization of the stromal collagen fibers by using the combined action of a photosensitizing substance (riboflavin or vitamin B2) and ultraviolet- (UV-) A irradiation. This results in corneal stiffening due to an increase in the number of intrafibrillar and interfibrillar

covalent bonds with heightened corneal collagen resistance against enzymatic degradation [6, 7]. A shorter duration of treatment may offer some advantage to the pediatric age group. Hence we undertook a pilot study to analyze the safety and effectiveness of “accelerated” collagen cross-linking (ACXL) in patients under 14 years of age.

2. Materials and Methods

This was a prospective interventional study of 30 eyes of 18 patients. The inclusion criteria for the study were eyes with progressive keratoconus documented by serial topography for at least six months, corneal thickness >400 microns at the thinnest location, and children in the age group of 11–14 years. An increase in the steep K-value by more than 1.0 to 1.5 D and a corresponding change (form >1.0 to 1.5 D) in the subjective refraction in the last six months or a 5% or more decrease in the thinnest pachymetry in the preceding six months was defined as “progression.”

Eyes with corneal thickness <400 microns at the thinnest point, concurrent corneal infections, central or paracentral scarring, and those who had a history of herpetic keratitis were excluded.

Written informed consent was obtained from parents of all patients undergoing the procedure, and the study protocol was approved by the hospital's ethics committee and was performed according to the tenets of the Declaration of Helsinki. All patients underwent a detailed ophthalmic examination including assessment of the uncorrected distant visual acuity (UDVA) and corrected distant visual acuity (CDVA), subjective acceptance, slit lamp, specular microscopy, and dilated fundus examination. Both UDVA and CDVA were recorded using Snellen's chart and later converted to logMAR values. All patients underwent corneal topography using the Scheimpflug camera Pentacam (Oculus, Wetzlar, Germany). Keratometric values (K1 and K2) and minimum pachymetry values were derived from the Pentacam and the pachymetry was confirmed with an ultrasound pachymetry. All patients underwent the above tests at baseline and at all subsequent visits.

2.1. Surgical Technique. Corneal collagen cross-linking was performed under sterile conditions in the operating room. Topical proparacaine hydrochloride 0.5% eye drops were instilled preoperatively. The central 8 mm of the corneal epithelium was removed using an epithelial scraper. Riboflavin 0.1% solution (10 mg riboflavin-5-phosphate in 10 mL dextran-T-500 20% solution) was applied as a photosensitizer every 2 minutes for 30 minutes. After confirming permeation of riboflavin through the cornea using a PSL Portable Slit Lamp (Reichert, Depew, NY), UV-A irradiation of 9 mW/cm² with a wavelength of 365 nm was initiated using the Avedro KXL system (Waltham, MA) for 10 minutes. During irradiation, drops of riboflavin solution were reapplied to the cornea every two minutes to sustain the necessary concentration and to prevent desiccation of the cornea. A 9 mm sized beam was used with care taken to avoid any damage to the limbus by using a limbal guard made of polymethyl methacrylate. A silicone hydrogel bandage contact lens (Pure Vision, Bausch and Lomb) was applied following the treatment and was removed on the 3rd postoperative day or once the epithelium healed.

Postoperative treatment included prednisolone acetate 1% eye drops in tapering dosage for three weeks, moxifloxacin hydrochloride 0.5% eye drops for one week, nepafenac 0.1% eye drops three times a day for three days, and topical artificial tears supplements for three months. Patients with associated VKC were treated with topical antiallergic medication and cyclosporine as prophylaxis to prevent acute exacerbations.

For residual refractive errors three months after ACXL, patients were prescribed contact lenses (rigid gas permeable, Rose-K, or hybrid lenses). Patients who had allergic eye disease or were intolerant to contact lenses were given spectacle correction.

2.2. Statistical Analyses. The raw data was entered on excel sheets (Microsoft Corp.) and imported to the Statistical

TABLE 1: Table showing the mean UDVA (uncorrected distant visual acuity), CDVA (best corrected visual acuity), spherical and cylindrical refraction, SE (spherical equivalent), and K1/K2 (flat keratometry/steep keratometry) preoperatively and at 2-year post-operative period.

	Pre-op	2 years	P value
UDVA (in log MAR)	0.76 (±0.26)	0.61 (±0.25)	0.005
CDVA (in log MAR)	0.24 (±0.19)	0.12 (±0.12)	0.001
Spherical refraction (D)	-3.04 (±3.6)	-2.38 (±3.37)	0.28
Cylindrical refraction (D)	-3.63 (±1.82)	-2.80 (±1.48)	0.008
SE (D)	-4.7 (±3.86)	-3.75 (±3.49)	0.15
K1 (D)	48.53 (±3.57)	46.49 (±4.21)	0.001
K2 (D)	53.77 (±4.82)	51.70 (±5.41)	0.007

Package for Social Sciences (SPSS Inc., Chicago, IL, version 17.0) for analysis. We used both eyes of patients when eligible. As the outcomes are likely to be correlated between the two eyes of a patient, we used generalized estimating equations to adjust for the same during all statistical comparisons. The significance level was set at <0.05.

3. Results

Thirty eyes of 18 patients were included in the study with ten males and eight females; mean age of the patients was 12.7 years (range: 11–14 years). Seventeen eyes (56.7%) of 10 patients (55.5%) had an associated vernal keratoconjunctivitis (VKC). The mean pachymetry as measured by the Pentacam was 453.14 microns (range: 432–510 microns).

Table 1 shows the changes in UDVA, CDVA, spherical equivalent (SE), K1 (flat K), and K2 (steep K) preoperatively and 24 months postoperatively.

3.1. UDVA and CDVA (in LogMAR). The mean preoperative UDVA and CDVA were 0.76 (±0.26) and 0.24 (±0.19), respectively. At the end of two years, there was a statistically significant change in the mean UDVA (0.61±0.25; $P < 0.001$) and mean CDVA (0.12 ± 0.12; $P < 0.001$).

3.2. Spherical, Cylinder, and Spherical Equivalent. At the end of two years, there was an improvement in the mean preoperative sphere, cylinder, and spherical equivalent from -3.04 DS (±3.60), -3.63 DC (±1.82), and -4.70 D (±3.86) to -2.38 DS (±3.37), -2.80 DC (±1.48), and -3.75 D (±3.49), respectively. Statistically significant improvement ($P < 0.001$) was seen only in the cylindrical error.

3.3. Keratometry: Figure 1. There was a flattening of 2.04 D in the mean K1 and 2.07 D in mean K2 at the end of the 2-year follow-up, which was statistically significant ($P < 0.001$).

Three eyes of two patients with VKC and a history of eye rubbing showed progression at the end of two years. Hence, keratoconus patients with VKC had higher chances of failure of ACXL (3 out of 17 eyes with VKC, 17.65%) as compared to those without VKC who were stable two years after ACXL.

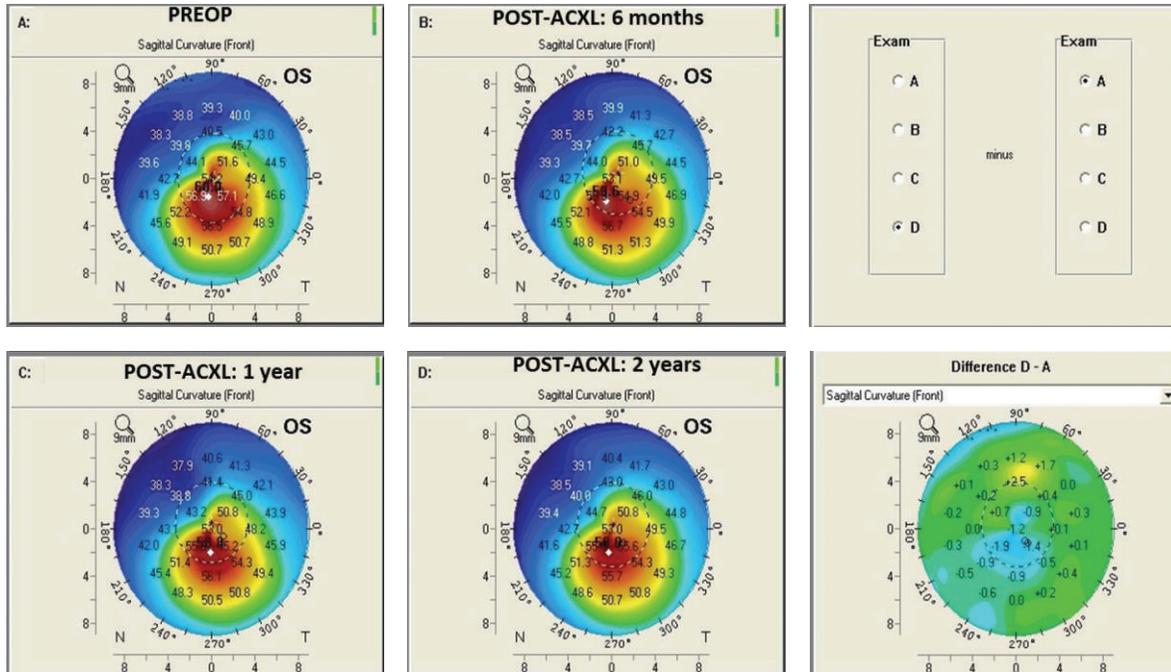


FIGURE 1: Pentacam difference map showing serial changes in sagittal curvature in an 11-year-old patient at preaccelerated corneal collagen cross-linking and postaccelerated corneal collagen cross-linking (6 months, 1 year, and 2 years).

There were no complications noted after ACXL in any of the patients. Postoperatively, the mean time for epithelial healing was 3.32 ± 1.15 days. Mild haze was noticed in a majority of subjects on slit-lamp examination but did not have any effect on the visual acuity and subsided completely by eight weeks after surgery. The mean preoperative endothelial cell count was 2732.5 ± 174.08 cells/mm³ and showed no significant change at any of the postoperative visits up to two years (2689 ± 192.4 ; $P = 0.36$). There was also no alteration in the endothelial cell polymegathism (coefficient of variance; preoperative— 36.12 ± 6.07 ; 2 years post-op— 37.31 ± 6.12 ; $P = 0.45$) on specular microscopy. There was no evidence of delayed wound healing, ocular surface damage, or uveitis after ACXL in any of the patients.

4. Discussion

Corneal collagen cross-linking is an emerging treatment option for pediatric patients with keratoconus [8, 9]. The Siena CXL Pediatrics trial, the largest prospective study report involving 152 eyes of 77 patients (from 10 to 18 years) with the longest follow-up of 3 years demonstrated that, after CXL, keratoconus stabilized and demonstrated rapid and significant visual function improvement in pediatric patients [10]. They found an improvement in both UDVA and CDVA in patients under 18 years of age when compared to those in the 19–26 years age group, but it was not statistically significant. In patients over 27 years of age, there was a poorer functional response when compared with other age groups.

There have been various modifications to the original Dresden protocol when treating children [11]. It has been

suggested that transepithelial CXL (epi-on) may be a better option in them as it is associated with lesser pain and provides a similar efficacy with fewer complications [12]. However, Malhotra et al. in their in vivo study on riboflavin penetration have shown that an intact epithelium blocks adequate penetration of riboflavin into the corneal stroma and hence reduces the effectiveness of CXL [13]. Additionally, a three-year follow-up study in corneas after epi-OFF or standard CXL compared with epi-ON CXL found better reduction in the steepest keratometry after the epi-OFF procedure [14]. There have also been changes in the protocols of the energy used and UV-A irradiation exposure time during CXL to shorten the duration of the procedure. Mrochen in his ex vivo experiments on “accelerated” cross-linking (AXCL) has shown that the biomechanical stiffening effect on corneal tissue using energies up to 10 mw/cm² is similar to that with the standard protocol [15]. Cinar et al. compared conventional CXL with ACXL and found that at six months the change in UDVA and CDVA was statistically significant in the accelerated CXL group but did not reach statistical significance in the conventional CXL group [16].

Encouraged with the positive results of ACXL, we used it in the treatment of children less than 14 years of age with progressive keratoconus and found at the 2-year follow-up that there was a statistically significant improvement in the mean UDVA, CDVA, cylindrical refraction, and keratometry (K1 and K2). However, the improvement in the spherical refraction and SE was not statistically significant which may be explained by the small sample size of our study.

Vinciguerra et al. [17] demonstrated that the endothelial cell density did not alter after CXL in 40 eyes of 40 pediatric (from 9 to 18 years) after a two-year follow-up.

In our study with ACXL, we found similar results of no significant reduction in the endothelial cell count at the end of two years. Failure of CXL to arrest progression of keratoconus is attributed to different genetic patterns, relative biomechanical modifications potentially occurring in the corneal stroma and the negative influences of other conditions such as allergy and atopy [18–21]. Ghosh et al. studied reviewed various proteomic and genomic expressions and discovered molecules that can serve as biomarkers which may have potential role in the management of keratoconus [20]. They also found that a history of allergy, atopy (eczema, asthma, and hayfever), corneal injury, eye rubbing, and rigid contact lens usage has been shown to be associated with the development of keratoconus. In our study we noticed a higher incidence of VKC in children with progressive keratoconus (55.5%). There was also a failure to stabilize keratoconus with ACXL in these patients (17.65%). Hence, management of the underlying cause in such children is of prime importance as persistent eye rubbing may “nullify” the effect of CXL. Topical treatment with steroids, mast cell stabilizers, and also the use of immunomodulators such as cyclosporine eye drops may help in alleviating allergy.

Other factors that may influence treatment are limbal stem cell (LSC) damage caused by chronic VKC [22] and UV-A damage [23]. The change in ocular flora due to chronic use of topical steroids in keratoconus patients with VKC can lead to an increased risk of postoperative keratitis [24]. Hence, while using ACXL to treat children, we recommend the usage of a limbal guard to protect the LSCs from damage and stoppage of the use of topical steroids (once the VKC is controlled) for at least two weeks prior to the procedure.

5. Conclusion

Very few studies have been published about the effectiveness of CXL in the younger age group. To the best of our knowledge this is the first study to evaluate the effectiveness of ACXL in children less than 14 years of age. The higher energy and shorter duration of treatment (9 mW/cm² for 10 mins) of ACXL may prove to be a good option in children. It can potentially prevent amblyopia, improve the fit of contact lenses, and deter an early penetrating keratoplasty. For children with keratoconus and chronic VKC undergoing CXL, it would be prudent to treat the allergy aggressively with topical steroids during the active phase and with topical antiallergics and topical cyclosporine in chronic cases to reduce the chances of failure of CXL.

Conflict of Interests

The authors have no proprietary or commercial interests in any concept or product discussed in this paper.

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Clinical Study

Corneal Collagen Cross-Linking with Hypoosmolar Riboflavin Solution in Keratoconic Corneas

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Received 24 March 2014; Revised 13 June 2014; Accepted 22 July 2014; Published 14 August 2014

Academic Editor: George Asimellis

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Purpose. To report the 12-month outcomes of corneal collagen cross-linking (CXL) with a hypoosmolar riboflavin and ultraviolet-A (UVA) irradiation in thin corneas. **Methods.** Eight eyes underwent CXL using a hypoosmolar riboflavin solution after epithelial removal. The corrected distance visual acuity (CDVA), manifest refraction, the mean thinnest corneal thickness (MTCT), and the endothelial cell density (ECD) were evaluated before and 6 and 12 months after CXL. **Results.** The MTCT was $413.9 \pm 12.4 \mu\text{m}$ before treatment and reduced to $381.1 \pm 7.3 \mu\text{m}$ after the removal of the epithelium. After CXL, the thickness decreased to $410.3 \pm 14.5 \mu\text{m}$ at the last follow-up. Before treatment, the mean *K*-value of the apex of the keratoconus corneas was 58.7 ± 3.5 diopters and slightly decreased (57.7 ± 4.9 diopters) at 12 months. The mean CDVA was 0.54 ± 0.23 logarithm of the minimal angle of resolution before treatment and increased to 0.51 ± 0.21 logarithm at the last follow-up. The ECD was 2731.4 ± 191.8 cells/mm² before treatment and was 2733.4 ± 222.6 cells/mm² at 12 months after treatment. **Conclusions.** CXL with a hypoosmolar riboflavin in thin corneas seems to be a promising method for keratoconic eyes with the mean thinnest corneal thickness less than $400 \mu\text{m}$ without epithelium.

1. Introduction

Keratoconus is a common disease of the cornea; the incidence in the general population is about 1/2000 [1]. It is characterized by progressive thinning and ectasia of the cornea. Corneal transplantation is inevitable leading to severe visual deterioration and corneal scarring in 20% of patients [2]. CXL is considered a promising and less invasive technique. Most studies suggest that CXL treatment improves the corneal rigidity [3, 4] and increases the corneal resistance to enzymatic digestion [5]. With UVA irradiation (365 nm) and riboflavin (as photosensitizer), new covalent bonds are induced between collagen molecules, fibers, and microfibrils by photosensitized oxidation [6]. The collagen fibrils diameter was increased and the proteoglycan area was reduced in the human keratoconus cornea after CXL treatment [7].

In 2003, Wollensak et al. pioneered CXL treatment to stop progression of keratoconus [8]. After that, a number of studies have demonstrated efficacy of arresting the progression of keratoconus by using CXL “standard” protocol [9–11]. According to “standard” protocol (epithelium removal, using

isoosmolar 0.1% riboflavin solution, and UVA irradiation for 30 minutes), a minimal stromal thickness (without the corneal epithelium) of at least $400 \mu\text{m}$ was required for safety [12, 13]. Unfortunately, in many cases of advanced progressive keratectasia, patients are often excluded from the CXL treatment because their corneal thickness is less than $400 \mu\text{m}$. To solve this problem, various modifications of the “standard” protocol have been attempted. Some studies found stabilization of the corneal ectasia by leaving the epithelium over the thinnest area intact, but the effect was mild [14, 15]. Hafezi and associates proposed an alternative treatment protocol by using hypoosmolar riboflavin solution (0.1% riboflavin in 0.9% saline instead of dextran) to swell the corneal stroma [16]. The results showed stabilization of keratometry and no complications by using hypoosmolar riboflavin solution [16, 17]. However, little has been known about the safety and efficacy of this treatment, and a failure case was reported by using hypoosmolar riboflavin solution in an extremely thin cornea [18].

In this study, we investigated the effectiveness and safety of CXL using hypoosmolar riboflavin solution and UVA

for the treatment of keratoconus with the thinnest corneal thickness less than 400 μm without epithelium.

2. Material and Methods

The study was approved by the ethics committee of the Shandong Provincial Hospital affiliated to Shandong University under the principles of the Helsinki Declaration. Informed consent was obtained from all study participants before the initiation of CXL treatment.

Patients with keratoconus were prospectively recruited from the Cornea Outpatient Clinic of Shandong Provincial Hospital affiliated to Shandong University. The inclusion criteria were progressive keratoconus (stages 1 to 3 keratoconus, according to the Krumeich classification [19]) documented within the past 12 months as evidenced by astigmatic refraction and/or topography, no previous ocular surgery, thinnest corneal thickness of less than 400 μm (without epithelium), and no wearing of contact lenses before initial evaluation and treatment. Progression was considered if at least one or more of the following criteria were met: an increase of at least 1.00 diopter (D) in the steepest simulated keratometry reading (K_{max}) derived from computerized videokeratography; an increase in astigmatism as determined by manifest subjective refraction of at least 1.00 D; an increase of 0.50 D in manifest refraction spherical equivalent. Exclusion criteria were a minimum corneal thickness $>400 \mu\text{m}$, previous refractive surgery or other corneal surgery, a history of severe infections, or other corneal or ocular surface disease, and pregnancy or lactation (female patients).

Hypoosmolar riboflavin solution (0.1%) was generated by diluting vitamin B2-riboflavin-5-phosphate 0.5% (Shandong Fangming Pharmaceutical Limited by Share Ltd., Shandong, China) with physiological salt solution (sodium chloride 0.9% solution; 310 mOsmol/L; Sichuan Kelun Pharmaceutical Limited by Share Ltd., Sichuan, China). The procedure was performed under sterile conditions. After topical anesthesia using proparacaine hydrochloride 0.5% (Alcaïne; Alcon Laboratories, Inc., Fort Worth, Texas, USA) eye drops, a lid speculum was inserted. The central cornea was contacted with a filter paper soaked with 20% alcohol for 60 seconds (the diameter was 9 mm), then the central 9 mm of cornea epithelium was removed with a blunt spatula (Asico AE2766). Deepithelialization was followed by measuring the corneal thickness with OCT (Cirrus HD-OCT 4000; Carl Zeiss Meditec Inc., Hacienda Drive, Dublin, USA) to validate that the thinnest thickness was less than 400 μm . Hypoosmolar riboflavin solution (0.1%) was applied to the cornea every 3 minutes for 30 minutes. The corneal thickness was checked by OCT and hypoosmolar riboflavin solution was again administered until corneal thickness was more than 400 μm at the thinnest point. A digital slit-lamp photograph (True Digital Slit Lamp SL DC-3; Topcon Corporation, Hasunuma-cho, Itabashi-Ku, Tokyo, Japan) was performed to ensure the appearance of riboflavin in the anterior chamber.

Then the eye was irradiated with UVA of 370 nm wavelength (UV-X illumination system version 1000, UVXTM, IROC AG, Zurich, Switzerland) at a working distance of 5 cm. An area with 9 mm diameter in the center of the cornea was

irradiated with an irradiance of 3.0 mW/cm². During the 30 minutes of irradiation, hypoosmolar riboflavin solution was applied every 3 minutes to maintain the riboflavin saturation in cornea stroma. At the end of the procedure, a combination of dexamethasone 0.1% and tobramycin 0.3% (Tobradex, Alcon Co. Ltd., USA) was administered 4 times daily in all patients and a bandage soft contact lens was applied until healing of the corneal epithelium was completed.

The MTCT was examined before and after removal of epithelium, after swelling, and 6 and 12 months after CXL by OCT device. The CDVA with glasses or contact lenses, manifest refraction (diopters; D), and corneal topography (Orbscan II; Bausch & Lomb Incorporated, Rochester, New York, United States) were assessed before and 6 and 12 months after the procedure. The ECD was acquired using a Specular Microscope (Specular Microscope SP-3000P; Topcon Corporation, Hasunuma-cho, Itabashi-Ku, Tokyo, Japan) before and 6 and 12 months after CXL.

Statistical evaluation of values before and 6 and 12 months after CXL was performed using the nonparametric test (Wilcoxon test) with SPSS software version 17 (SPSS GmbH Software, Munich, Germany). A P value below 0.05 was considered statistically significant.

3. Results

The analysis included 8 eyes of 8 patients (5 males and 3 females) with a mean age of 27.4 ± 3.6 years. All eyes had transparent corneas before the procedure.

Before treatment, the MTCT was $413.9 \pm 12.4 \mu\text{m}$ and $381.1 \pm 7.3 \mu\text{m}$ with and without epithelium. After swelling by the hypoosmolar riboflavin solution, the cornea thickness increased to $443.8 \pm 23.9 \mu\text{m}$. The MTCT decreased at 6 months ($411.5 \pm 15.2 \mu\text{m}$) and remained stable at 12 months ($410.3 \pm 14.5 \mu\text{m}$) after treatment (Figure 1). Before and after operation the MTCT differences were not significant at 6 months ($P = 0.4$) and 12 months ($P = 0.233$).

The mean K -value from the apex of the keratoconus was 58.7 ± 3.5 diopters before treatment. Six months after treatment, this value was maintained at 58.5 ± 4.8 ($P = 0.674$) and reduced to 57.7 ± 4.9 at 12 months ($P = 0.611$) (Figure 2). The differences between pre- and postoperative mean K_{max} values were all not significant (all with $P > 0.05$).

The mean CDVA at the time of the treatment was 0.54 ± 0.23 logarithm of the minimal angle of resolution and increased to 0.52 ± 0.13 ($P = 1$) at 6 months and 0.51 ± 0.21 ($P = 0.285$) at 12 months after treatment (Figure 3). The mean CDVA showed no significant change at these follow-up visits compared to pre-CXL values (all with $P > 0.05$). At the 12-month follow-up, 25% (2 of 8 eyes) gained at least 1 Snellen line and 62.5% (5 of 8 eyes) showed a stable CDVA.

The mean ECD was 2731.4 ± 191.8 cells/mm² before treatment and decreased to 2722.5 ± 211.5 cells/mm² ($P = 0.208$) at 6 months after treatment and returned to 2733.4 ± 222.6 cells/mm² ($P = 0.327$) at 12 months (Figure 4). There was no significant change in the mean ECD counts between values before and 6- and 12-month values after treatment (all with $P > 0.05$).

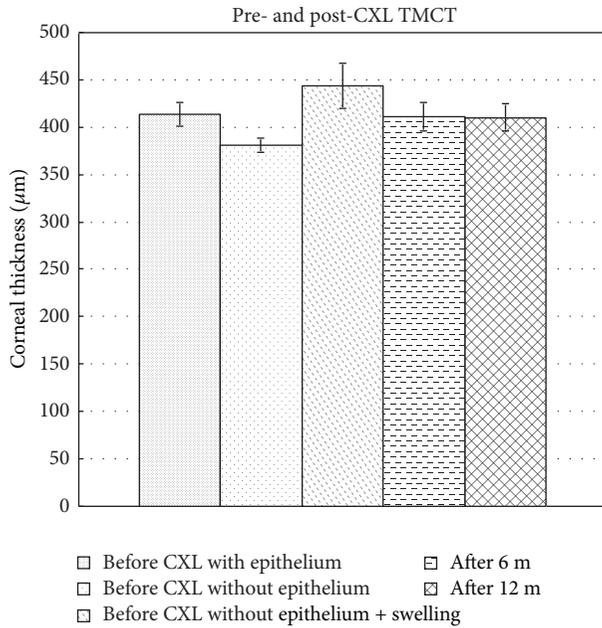


FIGURE 1: Bar graph showing the mean thinnest corneal thickness (MTCT) of patients with/without epithelium, after swelling by the hypoosmolar riboflavin solution and 6 months and 12 months after CXL.

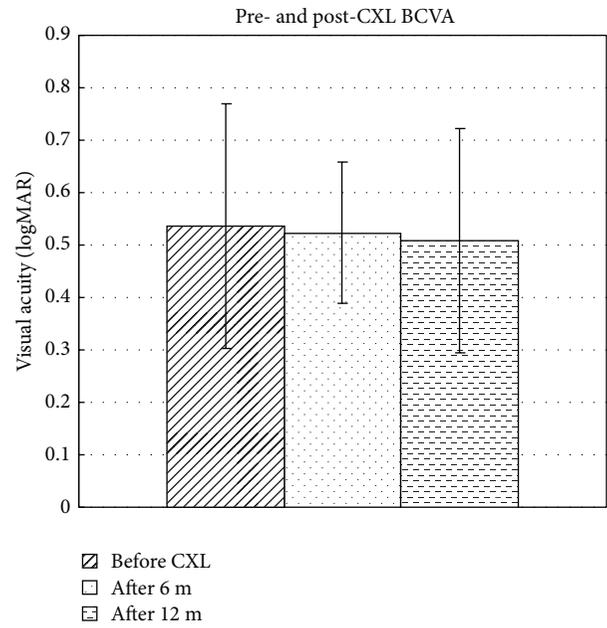


FIGURE 3: Bar graph showing the mean best corrected visual acuity (BCVA) of patients before CXL and 6 months and 12 months after CXL with hypoosmolar riboflavin solution. logMAR: logarithm of the minimal angle of resolution.

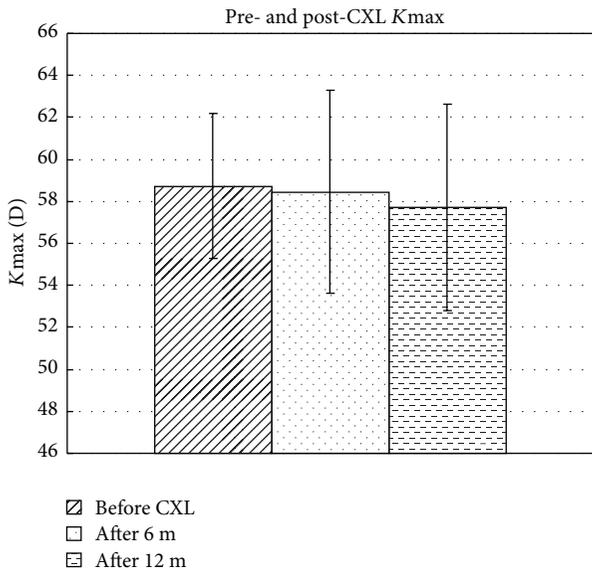


FIGURE 2: Bar graph showing the mean K-value at the apex of keratoconus (Kmax) before CXL and 6 months and 12 months after CXL with hypoosmolar riboflavin solution.

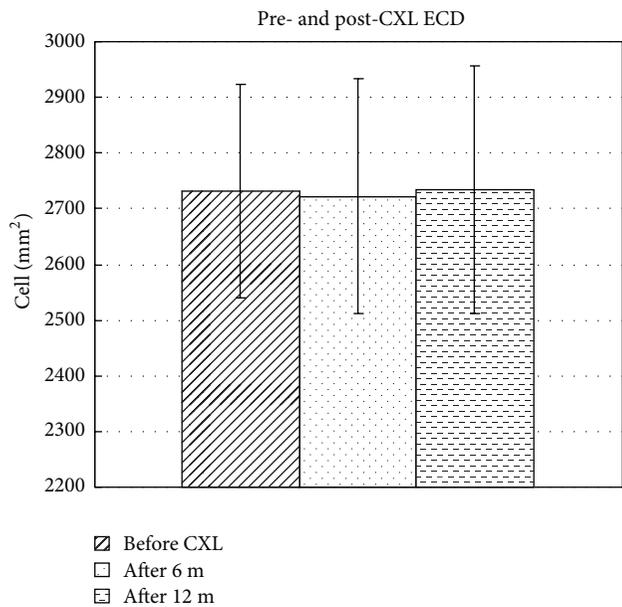


FIGURE 4: Bar graph showing the mean endothelial cell density (ECD) of patients before CXL and 6 months and 12 months after CXL with hypoosmolar riboflavin solution.

All corneal epithelial defects were closed 1 week after treatment. No corneal stroma infections were observed after treatment.

4. Discussion

CXL is a minimally invasive surgical technique, which stabilizes the progression of corneal ectasia and postpones the

need of lamellar or penetrating keratoplasty [20–23]. Studies showed that CXL increase the diameter of the collagen fibers with most changes occurring in the anterior 300 μm in the anterior stroma [22, 23]. As collagen bonds are established at a depth of 300 μm in the anterior stroma, a minimum of 400 μm stromal thickness is suggested for the safety of

the endothelium [12, 13]. According to the criteria, patients with corneal stromal thickness less than $400\ \mu\text{m}$ would be excluded from treatment. In order to overcome this limitation, hypotonic riboflavin was used to increase corneal stromal thickness in CXL treatment for the safety [16].

In our study, we used this modified technique in 8 patients with thin corneas. Before treatment, the MTCT was $413.9 \pm 12.4\ \mu\text{m}$ with epithelium and seemingly did not clearly fall under the thin category. After removal of epithelium, however, the MTCT reduced to $381.1 \pm 7.3\ \mu\text{m}$ and fulfilled the inclusion criteria of our study. Results showed an improvement in the mean CDVA and a decrease in keratometry readings (the mean K_{max} values) during the first year after treatment. The results of these parameters were similar to the previous studies [24–27]. We considered that these effects may be related to the corneal remodeling process after CXL. Studies found that CXL changed the abnormal keratoconic collagen fibrils distribution into normal fibrils distribution [7]. After CXL, the keratoconus corneal structure showed a modification in the collagen fibrils diameter, interfibrillar spacing, and the proteoglycan area. These modifications of the cornea stroma might result in a stable or decreased maximal keratometry readings and an improvement of CDVA. However, the K -value reduction achieved was rather small (-1.0 D) and not statistically significant. At our last follow-up, 25% (2 of 8 eyes) gained at least 1 Snellen line and 62.5% (5 of 8 eyes) showed a stable CDVA.

The mean ECD in our study remained stable at 6- and 12-month follow-up points. No adverse endothelial reaction and endothelial cell-related complications such as corneal edema were observed. These results were not consistent with the published literature following the CXL standard protocol in thin corneas, which resulted in a significant endothelial cell count loss postoperatively [28].

We observed that the MTCT was increased after swelling but decreased during the follow-up examination. A number of studies reported changes in corneal thickness after CXL treatment. Some studies showed that corneal thickness gradually increased after treatment and this increasing value did not reach preoperative reading at last follow-up [29–33]. Kanellopoulos and Asimellis reported the corneal thickness rebounding at three months [34]. In our study, however, we observed a decrease of corneal thickness after CXL, in agreement with a recent publication [35]. We thought the corneal deturgescence following treatment may be the reason for this decrease. It is well known that CXL could influence the swelling behavior of tissue [36, 37]. Wollensak et al. showed that the swelling behavior was dependent on the degree of CXL: the higher the CXL, the lower the corneal swelling behavior [38]. Alternatively, the reduced corneal thickness may be explained by the increase in endothelial pump activity or density induced by the treatment [33]. The reason and mechanism for these different results remained unexplained. Despite the decrease in the corneal thickness, we did not find a difference at each follow-up examination after CXL. We suggested that this decrease in corneal thickness did not imply a negative effect of CXL.

A study has shown that the epithelium was significantly thinner over areas of the corneal protrusion. Kanellopoulos

et al. showed that the epithelium over an ectatic area was approximately $35\ \mu\text{m}$ in one example [39]. In our study, we found that the MTCT reduced to $381.1 \pm 7.3\ \mu\text{m}$ after removal of epithelium and the decreased value (the thickness of the epithelium) was approximately $33\ \mu\text{m}$, which was similar to Kanellopoulos' study. Kanellopoulos et al. also showed that the patients treated with CXL had epithelium thickness distributions that were similar to the normal group. They put forward a novel theory of “reactive” epithelial hyperplasia in biomechanically unstable corneas [39, 40].

Some limitations of our study included the limitation of the measurement method and a relatively small number of patients. Ultrasound pachymetry (UP) has been considered the gold standard for measurement of corneal thickness. In contrast to ultrasound pachymetry, OCT is a noninvasive, noncontact method. Publication suggested that corneal thickness measured with OCT may not be reliable because of the increased light scattering and absorption in swollen corneas [41]. However, studies have shown a high correlation between measurements of corneal thickness using both instruments [42, 43]. Kanellopoulos and Asimellis have investigated the application of anterior-segment OCT in various states of corneal transparency and found that at least compared to the Scheimpflug-principle devices (i.e., Pentacam) the OCT may be superior to Pentacam. The use of UP may be challenging due to the coupling required, although it is mostly “popular” in many countries [44]. In swollen cornea, the OCT has underestimated pachymetric measurements in some cases and overestimated ones in the others compared with the UP [45]. The reasons for these differences were not clear. On the other hand, our results may also be influenced by the small sample size. The number of eyes included in our study is small and this small sample size has less power to reach a stronger conclusion. More cases and long-term studies should address this finding in the future. Besides, further studies of corneal thickness changes by modalities, such as confocal microscopy and ultrasound pachymetry, may be warranted.

5. Conclusions

In essence, our results showed that CXL with a hypotonic riboflavin solution seemed to be a promising method for thinner corneas. A longer follow-up and larger patient series would be needed to evaluate the long-term effect and safety of the method in thin corneas.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors thank Fengjiao Li, Haiqun Yu, Hui Li, Zhiwei Li, Chunqin Wang, and Ya Wang for their help.

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Clinical Study

Corneal Collagen Cross-Linking with and without Epithelial Removal: A Contralateral Study with 0.5% Hypotonic Riboflavin Solution

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Received 13 March 2014; Accepted 27 May 2014; Published 22 June 2014

Academic Editor: George Asimellis

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Purpose. Our main purpose was to compare safety and efficacy in the treatment of progressive keratoconus with “epithelium-on” and “epithelium-off” corneal collagen cross-linking (CXL). Our secondary purpose was to evaluate efficacy of CXL when hypotonic 0.5% riboflavin is used as photosensitizer. **Methods.** One eye of 20 patients with bilateral progressive keratoconus was randomly treated for “epithelium-on” CXL (group 1) while the fellow eye underwent “epithelium-off” CXL (group 2). Hypotonic 0.5% riboflavin was used in both groups. Visual acuity, refraction, corneal topography, and wavefront aberrometry were evaluated at baseline and after 1, 6, and 12 months. Specular microscopy was performed on 10 patients preoperatively and after 12 months. Postoperative pain was evaluated using a patient questionnaire. **Results.** Uncorrected and corrected distance visual acuity improved significantly in both groups. Refraction, topography, and aberrometry showed nonsignificant changes from the preoperative status throughout the 12-month follow-up in both groups. Moreover, the outcomes between the groups were comparable at all follow-up points. Endothelial cell-count was stable. Postoperative pain length was shorter in group 1 ($P < 0.001$). **Conclusion.** “Epithelium-on” and “epithelium-off” CXL using hypotonic 0.5% riboflavin were equally safe and effective in stabilization of keratoconus. Topography and aberrometry outcomes in both groups failed to show any significant improvements. This study is registered at ClinicalTrials.gov: NCT01181219.

1. Introduction

Reaction between riboflavin (vitamin B2), oxygen, and UV-A light is utilized in corneal collagen cross-linking (CXL) for creation of additional covalent bonds between the corneal stromal collagen fibers [1, 2]. Proper penetration of riboflavin within the stroma is essential for CXL because the riboflavin acts both as a photosensitizer leading to collagen cross-linking and as a protective agent shielding the deeper ocular structures from UV-A irradiation [3, 4]. However, the corneal epithelium is impermeable to compounds such as riboflavin, whose molecular weight is greater than 100 Da [5]. Therefore, epithelial debridement is normally performed in standard

“epithelium-off” CXL to allow a dextran-based 0.1% isotonic riboflavin solution to penetrate the corneal stroma [3].

Several laboratory and clinical studies indicate that standard CXL is effective in increasing the biomechanical rigidity of the cornea and stopping the progression of keratoconus [2, 6–11]. Standard CXL, however, may lead to serious complications like postoperative infection [12], stromal haze [13], and corneal melting [14]. Hence, a CXL technique that does not require epithelial removal may be preferable to increase the safety of the procedure. For this reason, and for the assumed increase in patient comfort, transepithelial CXL (“epithelium-on” CXL) was proposed [15]. Thereafter, several approaches have been pursued to solve the major limitation

of the “epithelium-on” CXL—an inadequate and inhomogeneous riboflavin penetration [5]. These include partial grid-like pattern deepithelialization [16], excimer laser superficial epithelial removal [17], the replacement of the isotonic by hypotonic riboflavin solution [18], and chemical enhancers such as benzalkonium chloride (BAC) [19], trometamol and ethylenediaminetetraacetic acid (EDTA) [20], tetracaine [21], and ethanol [16].

The current study utilizes a multifactorial approach to enhance the riboflavin penetration by employing: (1) BAC-containing local preoperative medication; (2) hypotonic riboflavin solution without dextran; (3) increased concentration of riboflavin (0.5%); and (4) prolongation of the riboflavin-induction time until objective verification of the stromal saturation is confirmed. This protocol has proved to be safe and effective in treatment of progressive keratoconus in a retrospective study by Stojanovic et al. [22]. The main aim of the current study was to evaluate “epithelium-on” and “epithelium-off” corneal CXL by comparing their safety and efficacy in eyes with progressive keratoconus using a randomized, contralateral, prospective design with the proposed treatment protocol. The secondary aim was to evaluate the efficacy of a hypotonic 0.5% riboflavin solution, which has only been reported in one previous study [22].

2. Patients and Methods

The treatments were performed at The Eye Department of the University Hospital North Norway, Tromsø, Norway. Inclusion criteria were as follows: (1) patients with documented progression of keratoconus during the last 12 months before treatment (increase of astigmatism or myopia by 1.00 D or increase in average Sim K by 1.50 D); (2) minimum corneal thickness of no less than 400 μm at the thinnest point measured by Scheimpflug-based corneal topo-/tomography (Precisio, iVIS Technology, Taranto, Italy); and (3) Amsler-Krumeich keratoconus classification stages II to III. Exclusion criteria were as follows: (1) history of herpes virus keratitis; (2) severe dry eye; (3) concurrent corneal infections; (4) previous ocular surgery; and (5) hard contact lens wear ≤ 4 weeks before the baseline examination.

2.1. Pre- and Postoperative Examinations. Pre- and postoperative examinations consisted of slit lamp biomicroscopy, tonometry (Icare tonometer, Revenio Group Corporation, Helsinki, Finland), Precisio topo-/tomography, Placido disk-based topography and wavefront aberrometry (OPD-Scan II, Nidek Co., Ltd. Aichi, Japan), uncorrected (UDVA) and corrected (CDVA) distance visual acuity measurement, and manifest refraction measurement (Nidek RT 2100 system, Nidek Co. Ltd., Aichi, Japan). The patients were examined preoperatively and 1, 6, and 12 months postoperatively. The verbal rating scale [23] was used in patients' evaluation of pain. The patients were asked to denote the postoperative pain intensity with a list of adjectives; these adjectives were assigned numbers from 0 to 5 (Table 1). The patients were also questioned on how many hours after the surgery the pain

occurred, when it was most intense, and which of the two eyes was considered the most comfortable.

The regional ethics committee (REK Nord) approved the study. The research complied with the tenets of the Declaration of Helsinki. Written informed consent was obtained from each participant before examination.

2.2. Surgical Technique. One eye of the patient was randomly chosen to be treated with “epithelium-on” CXL and the fellow eye was treated with “epithelium-off” CXL. For each patient, the eye with the best CDVA was determined as the “best eye.” Blocked randomization was used to ensure that each group had an equal number of “best eyes.” The treatments of two eyes were performed 1 to 6 months apart. To reduce the risk for UV-exposure of the retroiridal eye structures, miosis was induced by application of two drops of pilocarpine 2% (Pilocarpin, Ophtha AS, Norway) in both groups.

The “epithelium-on” CXL was performed without epithelial debridement. Two drops of local anesthetic proparacaine 0.5% (Alcaine, Alcon Norway AS), preserved by 0.001% BAC, and two drops of local antibiotic gentamycin 0.3% (Garamycin, Schering-Plough AS, Norway), preserved by 0.005% BAC, were applied to the cornea, one drop every minute for the initial five minutes. Thereafter, two drops of proparacaine and two drops of hypotonic 0.5% aqueous riboflavin solution without dextran (Vitamin B2; Streuli, Uznach, Switzerland) were applied alternating every 30 seconds until the riboflavin saturation was verified by the slit-lamp inspection of the cornea and by determination of presence of riboflavin flare in the anterior chamber.

In the “epithelium-off” group, the epithelium was removed in a diameter of 8 mm with an Amoils-epithelial scrubber (Innovative Excimer Solutions Inc, Toronto, Canada). Two drops of proparacaine and two drops of hypotonic 0.5% aqueous riboflavin solution without dextran were applied alternating every 30 seconds until the riboflavin saturation was verified in the same way as group 1.

For both groups, the initial slit-lamp saturation evaluation was performed 15 minutes after the first application of riboflavin and repeatedly every five minutes until the saturation was confirmed. During the premedication and riboflavin induction, the patient was in supine position with eye speculum inserted. In group 1, irrigation with isotonic balanced salt solution (BSS) was performed before the UVA irradiation in order to avoid UVA-attenuation by the shielding effect of riboflavin covering the epithelium. Ring-shaped Merocel shield k20-5021 (Katena Products, Inc. Denville, NJ) was used to protect the limbal region and its stem cells from UVA radiation.

The cornea was subjected to UVA radiation with a wavelength of 365 nm at a working distance of 5 cm for 30 minutes. The UV-X lamp (IROC AG, Zürich, Switzerland) provided an irradiance of 3 mW/cm within a circular diameter of 9 mm. During the irradiation, BSS was applied every three minutes and proparacaine drops were added as needed.

After the UVA irradiation, two drops of atropine 1% (Atropine minims, Chauvin, England) and 2 drops of gentamycin were applied. The cornea was protected with a soft

TABLE 1: Verbal rating scale for pain after CXL.

Assigned number	Intensity of the pain	Description of the pain
0	No pain	No pain or discomfort
1	Very little	Mild discomfort (foreign body sensation, dry eye...), no pain
2	Little	Mild pain
3	Intermediate	Moderate pain, relieved by closing the eyes or by artificial tears
4	Much	Severe pain (relieved by use of oral analgetics)
5	Very much	Unbearable pain (must use oral analgetics and local anesthetic drops)

TABLE 2: Pain evaluation.

	Pain score	Time point of the most intense pain (hour)	Pain length (hour)	Preference
“Epithelium-on” CXL	3.03 ± 0.73	3.78 ± 1.67	11.63 ± 5.89	13
“Epithelium-off” CXL	3.33 ± 0.38	6.25 ± 3.38	33.90 ± 23.76	5
<i>P</i> value	0.3765	0.002	0.000	—

bandage contact lens for 12–18 hours for group 1 and for one week for group 2. Instructions were given to apply a mixture of 0.1% dexamethasone and 0.5% chloramphenicol (Spersadex med Kloramfenikol, Novartis, Norway) eye drops four times daily for one week, as well as to use artificial tears as needed.

In a subgroup of 10 patients (20 eyes), specular microscopy was performed with Konan CellCheck XL (Konan Medical, Irvine, CA) preoperatively and 12 months postoperatively to obtain the endothelial cell count.

2.3. *Statistical Analysis.* All visual acuity values were recorded as Snellen values and converted to LogMAR for statistical analyses. Statistical analysis was performed using SPSS 17.0 software. The difference in patients’ subjective pain score between groups was analyzed by Wilcoxon signed ranks test. The distribution of all the other data was tested by Kolmogorov-Smirnov test. The paired *t*-test was used to assess differences at each follow-up point between the two groups as well as the changes of pre- and postoperative data within the groups. A *P* < 0.05 was considered statistically significant.

3. Results

All of the 20 patients recruited in the study were available for evaluation. The mean age of the 17 men and 3 women was 29.5 years (range 19 to 51 years). All measured data had normal distribution.

3.1. *Pain Evaluation.* Table 2 shows patients’ subjective pain evaluation. There was no significant difference in the pain score; however, the most intense pain occurred earlier and had a shorter duration in group 1. Thirteen of 20 patients stated preference for “epithelium-on” CXL, 5 patients preferred “epithelium-off” CXL, and 2 patients evaluated the two techniques as equally uncomfortable.

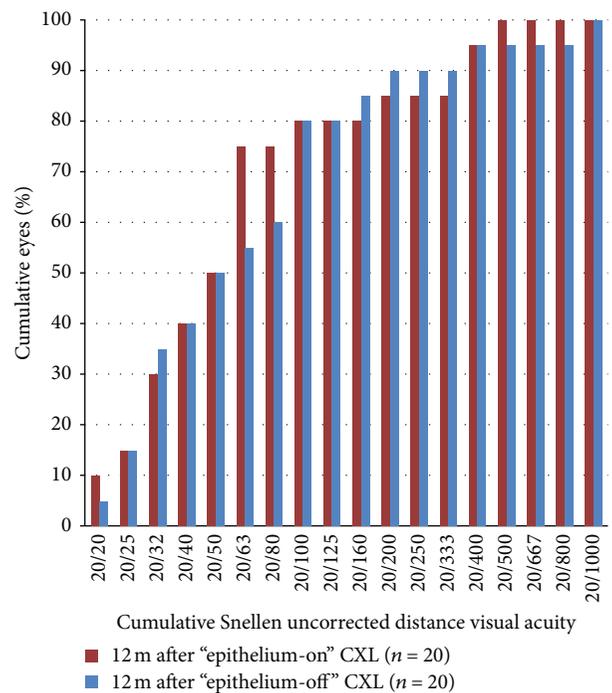


FIGURE 1: UDVA 12 months after “epithelium-on” and “epithelium-off” CXL.

3.2. *Visual Acuity and Refraction.* Figures 1, 2, 3, 4, 5, 6, and 7 and Table 3 show the visual acuity and refraction pre- and postoperatively for both groups. None of the eyes lost lines of CDVA, while 65% of the eyes in group 1 and 25% of the eyes in group 2 gained 2 or more lines. Safety index for group 1 and group 2 was 1.50 and 1.29, respectively. Mean spherical equivalent refraction and refractive cylinder remained stable compared to preoperative status throughout the 12-month follow-up in both groups. No significant difference was found

TABLE 3: Changes in visual acuity and refraction during 12-month follow-up.

Parameter	CXL	Preoperative	1 m	6 m	12 m
UDVA (lgMAR)	Epithelium-on	0.77 ± 0.39	0.62 ± 0.36*	0.54 ± 0.37*	0.62 ± 0.37*
	Epithelium-off	0.67 ± 0.44	0.62 ± 0.45	0.54 ± 0.43*	0.50 ± 0.44*
	<i>P</i> value	0.394	0.962	0.991	0.289
CDVA (lgMAR)	Epithelium-on	0.20 ± 0.19	0.11 ± 0.14*	0.06 ± 0.12*	0.02 ± 0.89*
	Epithelium-off	0.16 ± 0.13	0.13 ± 0.18	0.09 ± 0.15*	0.05 ± 0.12*
	<i>P</i> value	0.402	0.633	0.424	0.239
SE (D)	Epithelium-on	-1.58 ± 3.00	-1.32 ± 2.97	-1.97 ± 3.10	-1.73 ± 2.66
	Epithelium-off	-1.81 ± 2.48	-2.06 ± 3.39	-1.61 ± 2.24	-1.68 ± 2.32
	<i>P</i> value	0.746	0.436	0.619	0.945
Cylinder (D)	Epithelium-on	-3.19 ± 2.49	-2.58 ± 2.11	-2.64 ± 2.35	-2.66 ± 2.34
	Epithelium-off	-3.54 ± 2.10	-3.26 ± 2.07	-3.09 ± 2.33	-2.79 ± 2.25
	<i>P</i> value	0.543	0.167	0.453	0.852

*The difference between pre- and postoperative data was statistically significant ($P < 0.05$).

TABLE 4: Changes in topography features and wavefront aberrations during 12-month follow-up.

Parameter	CXL	Preoperative	1 m	6 m	12 m
Pachymetry (μm)	Epithelium-on	459.50 ± 39.24	445.50 ± 48.92*	453.50 ± 47.02	458.25 ± 41.09
	Epithelium-off	463.05 ± 31.34	434.35 ± 33.90*	434.65 ± 31.85*	450.55 ± 32.14*
	<i>P</i> value	0.625	0.286	0.051	0.273
IRI (μm)	Epithelium-on	34.25 ± 18.57	34.35 ± 18.66	33.65 ± 20.16	35.30 ± 20.09
	Epithelium-off	36.40 ± 13.10	38.05 ± 14.41	34.30 ± 13.91	33.15 ± 13.75
	<i>P</i> value	0.658	0.400	0.890	0.618
PE (μm)	Epithelium-on	61.80 ± 24.90	62.25 ± 28.22	60.80 ± 24.11	63.00 ± 31.67
	Epithelium-off	60.65 ± 27.01	59.55 ± 27.30	63.90 ± 25.57	67.35 ± 28.60
	<i>P</i> value	0.865	0.717	0.611	0.486
Sim K1 (D)	Epithelium-on	47.89 ± 4.46	47.75 ± 4.05	47.83 ± 4.59	47.82 ± 4.10
	Epithelium-off	47.51 ± 2.98	47.52 ± 4.29	47.74 ± 4.43	47.25 ± 3.91
	<i>P</i> value	0.695	0.758	0.923	0.544
Sim K2 (D)	Epithelium-on	44.29 ± 2.77	44.34 ± 2.77	44.34 ± 2.77	44.47 ± 2.80
	Epithelium-off	44.71 ± 2.98	44.17 ± 3.25	44.17 ± 33.14	44.01 ± 2.97
	<i>P</i> value	0.614	0.996	0.776	0.452
K max (D)	Epithelium-on	52.68 ± 5.35	52.95 ± 5.38	52.40 ± 5.74	52.78 ± 5.55
	Epithelium-off	53.59 ± 4.72	53.40 ± 5.03	53.58 ± 5.59	53.28 ± 5.18
	<i>P</i> value	0.525	0.555	0.620	0.755
RMS: HOAs	Epithelium-on	1.18 ± 0.67	1.20 ± 0.60	1.20 ± 0.71	1.20 ± 0.77
	Epithelium-off	1.15 ± 0.55	1.15 ± 0.51	1.12 ± 0.57	1.07 ± 0.58
	<i>P</i> value	0.980	0.496	0.714	0.458
RMS: S3 + 5 + 7	Epithelium-on	1.15 ± 0.65	1.16 ± 0.59	1.12 ± 0.71	1.16 ± 0.76
	Epithelium-off	1.11 ± 0.55	1.03 ± 49	0.97 ± 0.43	0.99 ± 0.52
	<i>P</i> value	0.953	0.545	0.769	0.344

*The difference between pre- and postoperative data was statistically significant ($P < 0.05$).

in visual acuity or refraction measurements between the two groups at any follow-up point.

3.3. Corneal Topography and Wavefront Aberrations. Table 4 shows pre- and postoperative topography features and wavefront aberrations. Scheimpflug topography data showed non-significant change in irregularity index (IRI) throughout

the 12-month follow-up in both groups. Posterior corneal elevation increased nonsignificantly throughout the 12-month follow-up in both groups.

Scheimpflug tomography-measured pachymetry in Figure 8 shows a decrease in thickness at the 1-month follow-up and a gradual increase in both groups thereafter. However, the thickness of corneas from group 1 increased to

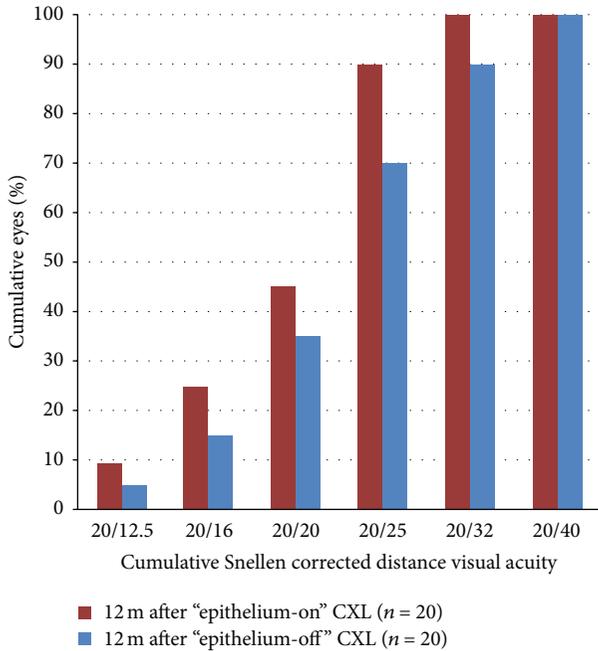


FIGURE 2: CDVA 12 months after "epithelium-on" and "epithelium-off" CXL.

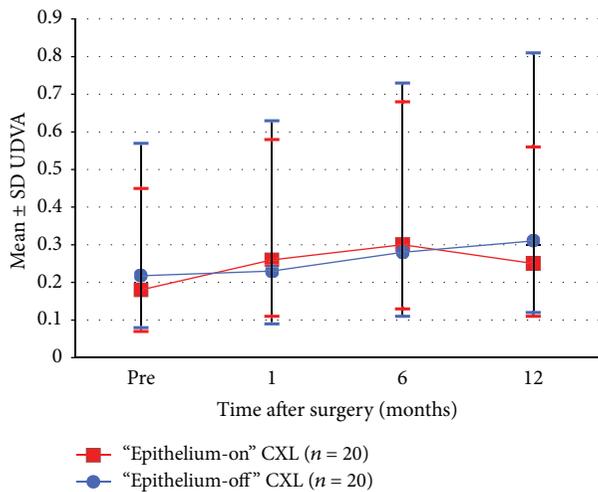


FIGURE 3: Development in UDVA pre- and 1-12 months postoperative.

the preoperative level by the 12-month follow-up ($P = 0.273$), while the thickness of corneas from group 2 remained thinner than that measured preoperatively ($P = 0.019$).

The Placido-topography data regarding curvature along the steepest meridian (sim-K1), the flattest meridian (sim-K2) (both measured within the central 3 mm zone), and at the location of the cone (max-K) did not show significant changes in either of the two groups throughout the 12-month follow-up.

Higher order aberrations measured within the central 5 mm zone did not show significant changes either in RMS

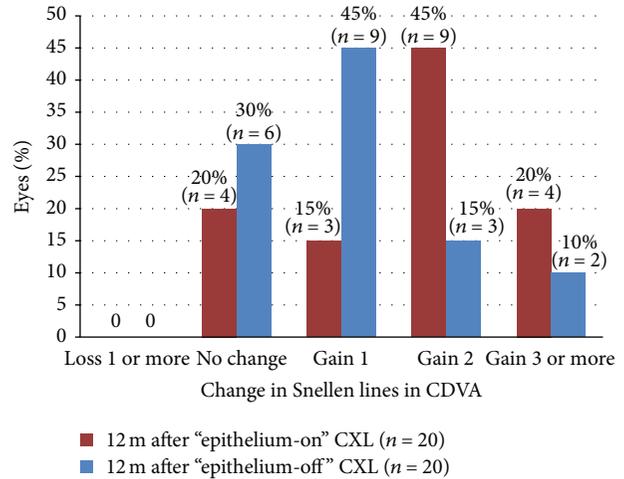


FIGURE 4: Change in Snellen lines in CDVA 12 months after "epithelium-on" and "epithelium-off" CXL.

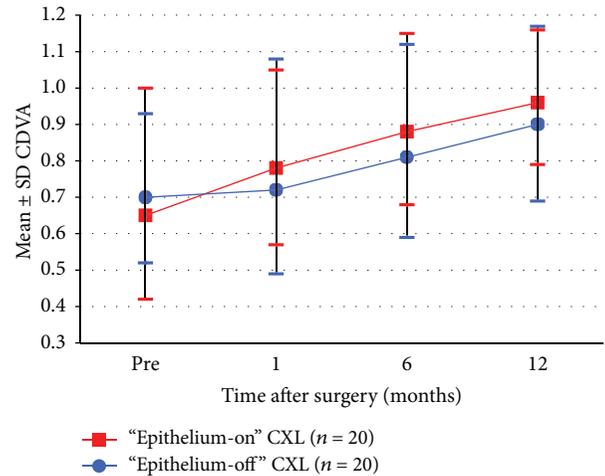


FIGURE 5: Development in CDVA pre- and 1-12 months postoperative.

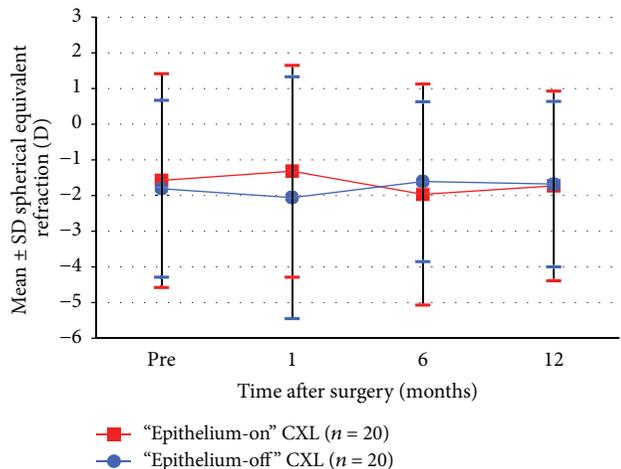


FIGURE 6: Stability of SE 1-12 months after "epithelium-on" and "epithelium-off" CXL.

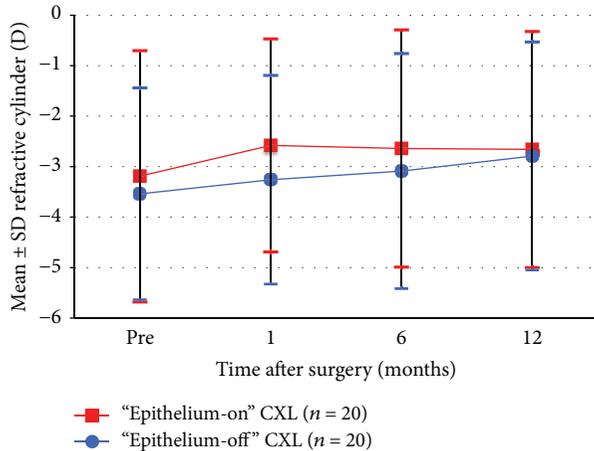


FIGURE 7: Stability of refractive cylinder 1–12 months after “epithelium-on” and epithelium-off” CXL.

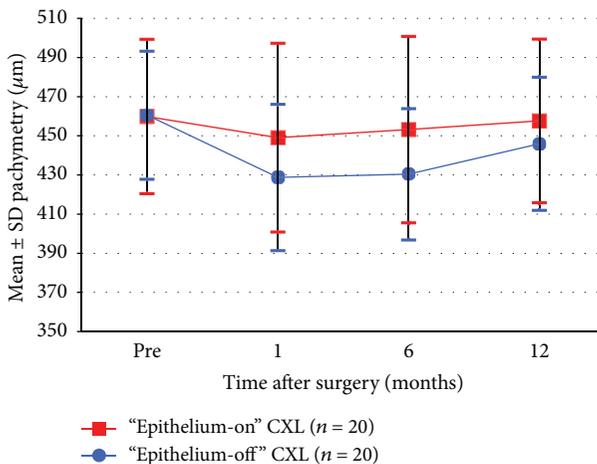


FIGURE 8: Change in pachymetry 1–12 months after “epithelium-on” and “epithelium-off” CXL.

or in odd-order (S3 + 5 + 7) throughout the 12-month follow-up in both groups.

No significant differences were found in the measurements of topography features or wavefront aberrations between the two groups at any follow-up point.

In the subgroup of 20 eyes of 10 patients where specular microscopy was available, endothelial cell-count changed from 2544.7 ± 330.3 and 2577.6 ± 265.7 cells/mm² to 2532.4 ± 325.3 and 2580.6 ± 261.1 cells/mm² for groups 1 and 2, respectively. The change within each group was neither statistically significant ($P = 0.101$ and 0.725 for groups 1 and 2, resp.) nor was the difference between the groups ($P = 0.499$ and 0.349 for the preoperative difference between groups and 12-month postoperative difference between groups, resp.).

No postoperative complications were recorded in the current population.

4. Discussion

The “standard” CXL-protocol described by Wollensak and colleagues includes mechanical removal of the corneal epithelium in a diameter of 7 mm and use of 0.1% isotonic riboflavin solution in 20% dextran as a photosensitizer [3]. This protocol proved to be effective in the stabilization of keratoconus and improved the refractive and topographic features in most cases. Henriquez and coworkers [11] showed that the “standard” CXL method reduced spherical equivalent (by 2.25 D), maximum and minimum keratometry values (by 2.66 D and 1.61 D, resp.), and anterior and posterior elevation, which was assumed to be the cause of UDVA improvement. Vinciguerra et al. reported the use of “standard” CXL-protocol in stage III keratoconus [9]. The procedure led to UDVA and CDVA improvement and a decrease of the steep Sim K from 50.37 D to 44.21 D, as well as reduction of corneal asymmetry and spherical aberration. Epithelial removal has also been attempted by use of excimer laser; either via lamellar phototherapeutic keratectomy (PTK) [24], topography guided [25], or noncustomized PRK [26]. In addition to gentle epithelial removal, all three options showed stabilizing CXL effect and certain visual improvements due to better corneal optics.

“Epithelium-on” CXL was introduced [15] in 2010 with the rationale of eliminating the complications ascribed to deepithelialization; such as decreasing postoperative pain, making CXL possible on thin corneas and in less cooperative patients, increasing vision during the initial postoperative period, and lowering requirements for a sterile environment. Wollensak and Iomdina found a significant increase in corneal rigidity after “epithelium-on” CXL. However, the effect was estimated to be only one fifth of “epithelium-off” CXL [27]. Using 0.1% riboflavin solution containing trometamol (Tris-hydroxymethyl aminomethane) and sodium EDTA, Filippello et al. [20] showed stabilization of keratoconus with improvement of all visual, topographic (steep Sim K decreased from 51.02 D to 48.05 D; apical K from 59.12 D to 57.95 D), and aberrometric parameters (RMS higher order aberrations decreased from $4.68 \mu\text{m}$ to $3.93 \mu\text{m}$, coma form $2.21 \mu\text{m}$ to $2.11 \mu\text{m}$, and spherical aberration form $0.98 \mu\text{m}$ to $0.73 \mu\text{m}$). The efficacy of the “epithelium-on” CXL has also been assessed *in vivo* in rabbits by comparing hypoosmolar 0.1% riboflavin solution containing BAC 0.02% and BAC 0.04% with isotonic unpreserved 0.1% riboflavin in dextran solution [19]. Stress-strain measurements showed that treatment with hypoosmolar riboflavin induced a sufficient increase in epithelial permeability to allow passage of riboflavin, resulting in increased corneal stiffening similar to that with “epithelium-off” CXL using isotonic solution. However, Koppen et al. [28] showed a statistically significant progressive increase of Scheimpflug-derived maximum K and decreased pachymetry throughout their clinical study, concluding that the “epithelium-on” CXL was less efficient than the “epithelium-off” CXL, despite the stabilized Placido-ring derived topographic features. Similar results were found in another clinical study [29] on “epithelium-on” CXL that used a modified protocol with a prolonged riboflavin-induction time of 4 hours and eye

drops containing gentamicin, EDTA, and BAC. Caporossi and colleagues [30] demonstrated that keratoconus in young patients with rapid progression was relatively stable only in the first 12 months after “epithelium-on” CXL and returned to preoperative status by 24 months. They concluded that “epithelium-on” CXL can be recommended for patients with thin corneas (with the thinnest point less than 400 μm) and in patients older than 26 years with slowly progressive keratoconus; but not for pediatric patients 18 years or younger whose cornea was 400 μm or higher at the thinnest point. A retrospective study using a similar protocol to that employed here, with a wide range in population, concluded that the “epithelium-on” CXL was effective and safe in halting progressive keratoconus and to some extent in improving the corneal shape [22].

Baiocchi et al. [5] used 0.1% riboflavin-dextran 20% solution to soak the human corneas *in vitro*. The authors demonstrated that stromal concentrations of riboflavin increased with exposure only if the epithelium was removed, which was ascribed to the impermeability of the corneal epithelium for substances with molecular weight greater than 100 Da (riboflavin has a molecular weight of 338 Da). Consequently, the clinical studies applying the same or only slightly modified “epithelium-off” CXL protocol without removal of the epithelium showed very limited or no efficacy due to the insufficient and inhomogeneous transepithelial riboflavin diffusion into the corneal stroma. Hence, the use of the “epithelium-on” CXL requires a protocol modification to assure that the proper amount of riboflavin reaches the stroma before UVA-irradiation commences. Reported clinical studies with “epithelium-on” CXL, where efficacy in treatment of keratoconus was confirmed [20, 28, 29], all used modified versions of the standard “epithelium-off” CXL-protocol.

In the current study, a multifactorial approach described earlier was applied [22]. First, chemical disruption of the epithelial tight junctions was attempted by application of several tensio-active substances including BAC, tetracaine, and gentamicin [31]. Secondly, hypotonic riboflavin solution without use of dextran was used to increase the permeability of the corneal epithelium [32]. Wollensak et al. [33] found that hypotonic riboflavin solution also helps increase riboflavin penetration into the stroma. Thirdly, the concentration of riboflavin in our hypotonic solution was increased from 0.1% to 0.5% to increase the concentration gradient across the epithelium, with an aim to enhance its penetration and achieve higher UVA absorption [33]. The absorption coefficient of riboflavin has been found to linearly correlate with concentration up to 0.5% [34, 35]. Moreover, Bottos et al. found improved penetration (i.e., after 30 minutes) through intact epithelium in rabbit corneas with 1% compared to 0.1% riboflavin [36], yielding a stromal riboflavin concentration (expressed as UV-absorption coefficient) of 20.27/cm and 18.50/cm, respectively. In the current study, we confirmed riboflavin saturation by slit-lamp inspection of the cornea and by the presence of riboflavin flare in the anterior chamber before UVA-irradiation was attempted, without any time limits on the induction time (riboflavin saturation time was 38.2 minutes for group 1 and 19.8 minutes for group 2).

Investigation of potential differences in CXL-efficacy in treatment of keratoconus with different protocols is a formidable task, since the baseline of the treated population can vary significantly according to the severity of disease. Furthermore, it is hampered by differences in cone localization and shape as well as variations in population age. We used a contralateral design with blocked randomization to minimize the bias of the inhomogeneous baseline when comparing the “epithelium-on” and the “epithelium-off” CXL. To minimize possible confounding factors, we used hypotonic 0.5% riboflavin solution in both “epithelium-off” and “epithelium-on” CXL groups, although its efficacy in the “epithelium-off” protocol has not been shown elsewhere.

The pain score reported by the two groups in the current study showed no significant difference. The results suggest that even without deepithelialization, pain or discomfort may be attributed to apoptosis of keratocytes and damage of anterior stromal nerve fibers caused by toxic effects of CXL, as reported in several studies [6, 30, 37]. Corneal denervation could theoretically generate dry eye-related problems due to the decreased blinking rate and increased tear evaporation and exposure of corneal surface. However, Kontadakis et al. [37] reported no significant change in Schirmer's I test and tear film break-up time after 1 month post CXL, and Taneri et al. [38] found pathologic staining with fluorescein and Rose Bengal, as well as tear film height at 3 and 6 months after CXL, to be comparable to preoperative measurements. These findings revealed that dry eye does not seem to be a significant complication after CXL in patients with keratoconus. This is in accordance with the current study, where no patient complained about symptoms of dry eye after 1 month postoperatively.

The results of the current study showed visual improvements and no progression of keratoconus after the treatment and throughout the 12-month follow-up period in both groups. No significant difference between the groups was observed at any point. However, the effect of CXL, as normally estimated by corneal topography- and wavefront aberrometry-changes, was lower than previously reported, for both groups. Neither Sim K-values, maximum K-values, nor higher order aberrations were reduced as previously shown for both “epithelium-off” CXL [9, 11] and “epithelium-on” CXL [20]. As all previous reports applied 0.1% riboflavin, we speculate that use of 0.5% riboflavin solution may have caused the decrease in effect of CXL in the current study. One possible rationale for this is the increased use of oxygen by riboflavin due to its higher concentration in the stroma. Richoz et al. [39] recently revealed that the removal of oxygen after 30 seconds of irradiation essentially halts the photopolymerization process in CXL. They concluded that CXL requires oxygen in sufficient quantities to participate in the reaction. It is conceivable that when CXL is performed with 0.1% riboflavin, the supply of oxygen balances its consumption and allows the CXL process to continue. In contrast, the increased riboflavin concentration in the stroma, due to the use of hypotonic 0.5% solution, may have led to quicker oxygen consumption and therefore a reduction in the efficiency of CXL. Additionally, according to Larrea and associates [40], the diffusion factor for oxygen in

human corneal stroma is calculated to be $2.81 \times 10^{-5} \text{ cm}^2/\text{s}$, while in water it is $2.10 \times 10^{-5} \text{ cm}^2/\text{s}$, implying that corneal hydration caused by the hypotonic solution may have led to slower oxygen transportation. This may have contributed to a prolonged state of corneal hypoxia, bringing the process of CXL to an early halt. Finally, it is possible that the retrospective study, where 0.5% riboflavin was used in a similar protocol, showed a somewhat better effect “improving the corneal shape to some extent” [22] due to the difference in the position of the patient during the riboflavin induction. Riboflavin induction performed with the patient in a supine position in the current study, as opposed to a sitting position in the retrospective study, may have led to a higher riboflavin concentration in the corneal stroma in the latter, resulting in higher consumption of oxygen and halting of the CXL reaction. This may explain the somewhat better effect in terms of corneal shape in the retrospective case study.

Epithelial absorption/filtering of UVA radiation that could potentially lead to less energy delivery to riboflavin saturated stroma has been stated as an argument against the use of “epithelium-on” CXL. However, previous studies draw different conclusions. A study by Baiocchi et al. [5] claims that human corneal epithelium and the underlying basement membrane naturally absorb 30% to 33% of UVA radiation (400 to 350 nm), while other studies showed that the epithelial UV absorption occurs only with wavelengths lower than 310 nm [41]. Bottós et al. [42] showed that the porcine corneal epithelium reduces the effectiveness of CXL by preventing the penetration of the drug, not by limiting the UVA transmittance. We assumed UV absorption of riboflavin within the epithelium to be low for two reasons. First, the epithelial cells are hydrophobic and do not absorb riboflavin and, second, the epithelial interstitial space is of negligible volume. To minimize the potential attenuation of UV radiation, we washed the corneas in the “epithelium-on” CXL group with BSS before the procedure and did not add any riboflavin to the cornea during the treatment.

5. Conclusion

The current study showed no difference in safety and efficacy between the “epithelium-on” and the “epithelium-off” CXL using a protocol that ensures corneal saturation with 0.5% hypotonic riboflavin solution. However, efficacy in reversing topography features of keratoconus with use of 0.5% hypotonic riboflavin seems to be lower compared to the reported results with “standard” 0.1% riboflavin.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors have no financial, professional, or personal competing interests in connection with any of the materials mentioned in this paper. The SynsLaser Surgery AS and the

Norwegian Research Council supported this research. No additional external funding was received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

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