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Effects of Corneal Collagen Crosslinking on Confocal Microscopic Findings and Tear Indices in Patients with Progressive Keratoconus

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ABSTRACT

Background: To evaluate any change in tear indices and confocal microscopic findings after corneal collagen crosslinking (CXL) in patients with progressive keratoconus.

Methods: Thirty-two consecutive eyes from 23 patients having progressive keratoconus were enrolled in this prospective, interventional cohort study. The standard crosslinking surgery was performed for all patients. Visual, refractive, and topographic evaluations were done before and at 6 months after surgery. Tear function tests and confocal microscopic examination were performed before and at 1 month and 6 months after the procedure.

Results: There was no significant change in Schirmer-1 test results and tear osmolarity at 1 month and 6 months after CXL. Using confocal microscopy, all eyes showed reduced or absent subepithelial nerve plexus. Differences in basal epithelial cell density, epithelial mean cell area, and keratocyte density in anterior and middle stroma and endothelial cell pleomorphism were all significant at 1 month and 6 months after CXL ($P < 0.05$). No significant change was noted in endothelial cell count and their polymegathism at 6 months follow-up. Significant improvement was noted in uncorrected visual acuity, best corrected visual acuity, flattest corneal meridian (K_2), and maximum keratometry in Pentacam (K_{max}) after 6 months of the procedure.

Conclusions: While CXL would have no effect on tear indices and endothelial cell count, it can cause a significant reduction in subepithelial nerve plexus and significant alterations in epithelial cell density in the anterior and middle stroma.

Keywords: Confocal microscopy, corneal collagen crosslinking, keratoconus

INTRODUCTION

Keratoconus is an ectasia of the cornea causing progressive thinning, bulging, and distortion.[1] The disease causes biomechanical weakening of the cornea by alterations in collagen arrangement and changes of extracellular matrix, and keratocyte apoptosis that take place through the stroma and Bowman's layer.[2] Excessive levels of proteolytic enzymes along lack of protease inhibitors are known to occur during the disease course and decrease the stromal thickness and modify its collagen configuration.[3]

Keratoplasty has long been a traditional recourse for advanced keratoconus, and the disease has been the main indication of corneal graft surgery. Riboflavin corneal collagen crosslinking (CXL) by ultraviolet A (UVA) has been the only option that aimed to improve corneal biomechanics and stop the ectatic progression through stiffening the anterior corneal stroma.[4,5] CXL can help maintain the current visual status; it is meant to prevent vision from worsening. Of note, previous studies investigating the role of CXL in the management of progressive keratoconus have also shown an improvement in visual, refractive, and topographic outcomes, as well as optical higher order aberrations.[6] Few confocal microscopic studies are available investigating the effect of CXL on modifications in corneal layers such as apoptosis of keratocytes in the anterior and intermediate stroma and keratocytes repopulation.[7-10] In the present study, we aimed to comprehensively evaluate all confocal microscopic measures in progressive keratoconic patients. In addition, for the first time in Iran, we tried to assess the effect of CXL on tear indices in such cases.

METHODS

Design and ethics

From December 2012 to August 2013, 32 eyes from 23 Iranian patients who met the inclusion criteria were consecutively enrolled in this prospective, interventional cohort study. The protocol was primarily reviewed and approved by the Institutional Ethics Committee of Tehran University of Medical Sciences and all patients gave signed informed consent before inclusion.

Participation criteria

Inclusion criteria were as follows: (i) age ≥13 years; (ii) progressive keratoconus defined as the presence of at least one of the following changes over a year: complain of decreased visual acuity except what originated from noncorneal conditions, an increase of ≥1.00 diopter (D) in the steepest K/manifest cylinder, and an increase of ≥0.50 D in the manifest refraction spherical equivalent; (iii) thickness of the cornea ≥400 μm;

and (iv) maximum keratometry (K_{max}) <60 D as measured by Pentacam Scheimpflug imaging.

Exclusion criteria were as follows: other corneal diseases (e.g., herpetic keratitis and corneal opacities), other ophthalmic problems (e.g., ocular autoimmune disease and previous ocular surgery), and problematic conditions (e.g., diabetes mellitus, pregnancy, and breastfeeding period).

Examinations and pre-/post-operative care

All patients in the study underwent full ophthalmic examination including assessment of uncorrected visual acuity (UCVA) and best spectacle-corrected visual acuity (BSCVA) using the logarithm of the minimum angle of resolution (logMAR) chart, slit-lamp microscopic examination, Schirmer-1 test without anesthetic drop, break-up time (BUT) test, rotating Scheimpflug topography (Pentacam HR, Oculus 70900, Germany), tear osmolarity (TO) (TearLab Corporation, San Diego, CA, USA), and confocal microscopy (Confoscan 3; NIDEK Technology, Padova, Italy).

Study visits were undertaken at baseline (visit 1; preoperative), day 3-5 (visit 2; complete re-epithelialization), and months 1 and 6 (visits 3 and 4), postoperatively.

In cases with rigid contact lens use, patients were instructed not to use them for at least 2 weeks before inclusion in the study and each follow-up examination and also, for 1 month postoperatively.

Procedure

CXL was conducted under sterile conditions in the operating room according to methods described elsewhere in detail.[11] After instilling topical anesthesia (tetracaine 0.5%, SinaDarou, Iran), the central 9 mm of the corneal epithelium were removed by mechanical debridement. The riboflavin solution (0.1% in 20% dextran T500 solution; SinaDarou, Iran) was then administered topically every 3 min for 30 min. Following riboflavin administration, its thorough absorption in the stroma and anterior chamber was confirmed by slit-lamp examination. UVA 365 nm light (UV-X system, IROC AG, Zurich, Switzerland) was used for 30 min at an irradiance of 3.0 mW/cm². UVA exposure was with the continuance of riboflavin instillation every 3 min. After treatment, patients were medicated with topical antibiotic for 2 weeks and corticosteroid for 4 weeks. Soft therapeutic lens (PureVision, Bausch & Lomb Inc., Rochester, NY) were applied until complete re-epithelialization (typically 3–5 days, postoperatively).

Confocal scan methodology

After anesthetizing the eye with topical anesthetic eye drop (tetracaine 0.5%) and applying an immersion gel (Viscotears, Novartis Pharma AG, Switzerland) onto

the tip of the front lens (Achroplan 40×/0.75 W ∞/0), confocal scanning was done using ConfoScan 3 (Nidek Technologies, Padova, Italy). Manual scan mode was used to capture the images of different corneal layers. Endothelial cell count was done by automatic mode using NAVIS software (Nidek, Padua, Italy) and followed by manual editing of automatically recognized cells. The density of stromal keratocytes at different layers and basal epithelial cells were measured by manual mode. An equal "region of interest" was selected for all patients. Stromal images just anterior to endothelium were used to measure keratocyte density of "posterior stroma" (PSKD), and those stromal images just posterior to basal epithelium were considered as "anterior stroma" (ASKD). The middle stromal nerves as a marker were used in combination with the Z-scan curve to detect the level of middle stroma.

Statistics

Data were analyzed using the SPSS software (version 18 for Windows; SPSS Inc., Chicago, IL, USA). Variables are expressed as a mean ± standard deviation. A $P \leq 0.05$ was taken as the significance threshold. Based on the number of cases (>30 cases) and normality analysis based on Shapiro–Wilk ($P = 0.73$), comparative analysis (baseline, 1 and 6 months) was performed using repeated measures ANOVA.

RESULTS

Thirty-two eyes (16 right eyes and 16 left eyes) of 23 patients (18 men and 5 women) were enrolled. The mean age of the patients was 21.57 ± 6.23 years (range: 14–30 years).

Table 1 summarizes the visual, refractive, topographic, and pachymetric results of the patients before CXL and at 6 months after the procedure.

Tear indices

The mean Tear BUT before CXL and at 1 month and 6 months after the procedure were 14.06 ± 2.22 s, 13.19 ± 1.67 s, and 13.2 ± 1.48 s, respectively. The change in BUT was significant 1 month after CXL ($P < 0.05$), but it was not significant after 6 months of CXL. The mean

Schirmer's test result before CXL and after 1 month and 6 months of CXL were 15.69 ± 4.06 mm, 15.41 ± 3.74 mm, and 14.91 ± 2.49 mm, respectively. There was no significant difference between pre- and post-CXL values ($P > 0.1$). Mean TO before CXL and at 1 month and 6 months of procedure were 300.7 ± 16.5 mOsm/L, 300.3 ± 12.9 mOsm/L, and 302.09 ± 10.8 mOsm/L, respectively. The differences between baseline and postoperative values were not significant ($P > 0.05$) [Table 2].

Confocal microscopic indices

Mean corneal basal epithelial cell density before CXL and after 1 month and 6 months of CXL were 6006.3 ± 413.8 cell/mm², 5460.5 ± 703.6 cell/mm², and 5295.3 ± 690.1 cell/mm², respectively. The difference between preoperative and postoperative values was significant ($P < 0.05$), but no significant change was noted between 1 month and 6 months after the procedure ($P > 0.05$) [Table 3]. Mean corneal epithelial cell area before CXL and after 1 and 6 months were 171.5 ± 15.2 μm², 185.4 ± 27.8 μm², and 192.2 ± 25.8 μm², respectively. The difference between baseline and postoperative values was significant ($P < 0.05$), but no change was observed between 1 and 6 months ($P > 0.05$) [Table 3]. Mean ASKD before CXL and after 1 and 6 months were 880.8 ± 85.4 cell/mm², 6.53 ± 22.1 cell/mm², and 45.5 ± 14.35 cell/mm², respectively. The difference between baseline and postoperative values was significant ($P < 0.05$), but no significant difference was noted between 1 and 6 months ($P > 0.05$) [Table 3]. Mean keratocyte density in the corneal middle stroma (MSKD) before CXL and after 1 and 6 months were 644.6 ± 118.8 cell/mm², 139.4 ± 127.2 cell/mm², and 139.1 ± 96.5 cell/mm², respectively. The difference between baseline and postoperative values was significant ($P < 0.05$), but no significant change was detected between 1 and 6 months ($P > 0.05$) [Table 3]. Mean PSKD before CXL and after 1 month and 6 months of procedure were 602.13 ± 97.8 cell/mm², 709.3 ± 203.9 cell/mm², and 659.6 ± 220.2 cell/mm, respectively. The difference between baseline and postoperative values was not significant ($P > 0.05$) [Table 3]. Mean corneal

Table 1: Visual, refractive, topographic, and pachymetric measures before and 6 months after corneal collagen crosslinking

	UCVA	BSCVA	Sub Ref SE	Cycl Ref SE	K_1	K_2	K_{max}	Thin
Before CXL	0.77±0.18	0.71±0.20	−3.68±2.26	−4.78±2.81	50.09±4.11	47.8±4.47	54.02±4.70	450.2±26.9
6 months post-CXL	0.49±0.21	0.41±0.21	−3.40±2.42	−4.46±2.68	49.8±4.09	46.9±4.20	53.07±4.50	427.3±36.3
P	0.012	0.010	0.140	0.101	0.691	0.041	0.044	0.039

CXL=Corneal collagen crosslinking, UCVA=Uncorrected distance visual acuity (LogMAR), BSCVA=Best spectacle-corrected distance visual acuity (LogMAR), Sub Ref SE=Subjective refraction spherical equivalent, Cycl Ref SE=Cyclorefraction spherical equivalent, K_1=Corneal steepest meridian in Pentacam, K_2=Corneal flattest meridian in Pentacam, K_{max}=Maximum K in Pentacam, Thin=Thinnest point pachymetry in Pentacam, LogMAR=Logarithm of the minimum angle of resolution

endothelial cell density before CXL and after 1 and 6 months of procedure were 3005.8 ± 304.4 cell/mm^2, 2922.3 ± 316.4 cell/mm^2, and 2911.3 ± 279.4 cell/mm^2, respectively. The difference between baseline and postoperative values was not significant ($P > 0.05$) [Table 3]. Mean corneal endothelial polymegathism before CXL and after 1 and 6 months of procedure were 27.3 ± 3.3, 27.06 ± 3.6, and 27.2 ± 4.7, respectively. The difference between baseline and postoperative values was not significant ($P > 0.05$) [Table 3]. Mean corneal endothelial pleomorphism before CXL and after 1 and 6 months of procedure were 31.5 ± 8.5, 36.5 ± 9.7, and 37.8 ± 8.7, respectively. The difference between baseline and postoperative values was significant ($P < 0.05$), but no significant difference was noted between 1 and 6 months after CXL ($P > 0.05$) [Table 3].

After 1 month of follow-up, subepithelial nerve plexus was absent in 25 eyes (78.12%) and was reduced in 7 eyes (21.87%). At 6 months follow-up time, the plexus was absent in 22 eyes (68.75%) and was reduced in 10 eyes (31.25%). All these eyes showed complete resolution of inflammation after 6 months of procedure.

No significant correlation was noted between tear indices and confocal microscopic findings except between decreased or absent subepithelial nerve plexus and increased TO at 1 month of follow-up time ($r = 0.63$,

$P < 0.05$). No ocular or systemic adverse event was observed, and no significant intraocular pressure change was seen during 6 months of follow-up.

DISCUSSION

CXL technique is indeed photopolymerization of stromal collagen fibers by the combination of a photosensitizing substance and UVA. The procedure is planned to stiffen the cornea by increasing the number of intrafibrillar and interfibrillar covalent bonds and corneal collagen resistance against enzymatic degradation.[12] One of the earliest long-term studies in 2004 revealed that CXL can delay keratoconus progression and decrease the demand for penetrating keratoplasty.[11,13] Moreover, CXL is reported to improve patients' visual, refractive, and topographic outcomes in some cases.[14,15] However, alterations in tear indices after any cornea procedure are important subjects of concern to the refractive surgeon. To better evaluate the correlation of these indices with corneal structural changes, we preferred to evaluate such indices with subepithelial nerve plexus morphology, simultaneously.

Tear evaluation tests in our study only showed a significant decrease in BUT for 1 month; however, BUT returned to near-normal values over the ensuing 6 months of follow-up. Other tear indices including Schirmer-1 test and TO did not show any significant difference after 1 and 6 months of follow-up. Such a finding may not be explainable by the absence of subepithelial nerve plexus that we found in about 78% and 68% over 1 and 6 months of follow-up, respectively. Such an absence and damage of nerve fibers through anterior stroma has also been reported in two previous works.[16,17] Moreover, one study found near-normal tear indices and amazingly significant central hypoesthesia after 9 months of CXL, like our experience. They attributed this finding to the intact sensitivity of the peripheral cornea. They concluded that these intact nerve fibers in the periphery may be sufficient to maintain the basic secretion.[18]

Overall, BUT of more than 10 s, Schirmer-1 test of more than 10 mm[19] and TO in the range of

Table 2: Tear indices of the patients at three-time points: Before corneal collagen crosslinking, 1 month after corneal collagen crosslinking and 6 months after corneal collagen crosslinking

	Schirmer (mm)	BUT (s)	TO (mOsm/L)
Before CXL	15.69±4.06	14.06±2.22	300.7±16.5
1 months after CXL	15.41±3.74	13.19±1.67	300.3±12.9
6 months after CXL	14.91±2.49	13.20±1.48	302.1±10.8
P (before to 1 month)	0.943	0.046	0.971
P (before to 6 months)	0.711	0.054	0.789
P (1-6 months)	0.821	0.989	0.813

CXL=Corneal collagen crosslinking, BUT=Break up time, TO=Tear osmolarity

Table 3: Confocal microscopic findings at three-time points: Before corneal collagen crosslinking, after 1 month, and after 6 months of corneal collagen crosslinking

	Mean±SD							
	BECD	MCA	ASKD	MSKD	PSKD	ECD	Poly	Pleo
Before CXL	6006.3±413.8	171.5±15.2	880.1±85.1	644.6±118.8	602.1±97.8	3005.8±304.4	27.3±3.3	31.5±8.5
1 months post-CXL	5460.5±703.6	185.4±27.8	6.5±22.1	139.4±127.2	709.3±203.9	2922.3±316.4	27.1±3.6	36.5±9.7
6 months post-CXL	5295.1±690.1	192.2±25.8	45.5±14.3	139.4±96.5	659.6±220.2	2911.3±279.4	27.2±4.7	37.8±8.7
P (before to 1 month)	0.011	0.041	<0.001	<0.001	0.521	0.703	0.888	0.031
P (before to 6 months)	0.006	0.032	<0.001	<0.001	0.837	0.674	0.932	0.022
P (1-6 months)	0.345	0.601	0.455	0.913	0.454	0.451	0.955	0.049

BECD=Basal epithelial cell density, MCA=Epithelial mean cell area, ASKD=Anterior stromal keratocyte density, MSKD=Middle stromal keratocyte density, PSKD=Posterior stromal keratocyte density, ECD=Endothelial cell density, Poly=Polymegathism, Pleo=Pleomorphism, SD=Standard deviation, CXL=Corneal collagen crosslinking

303.52 ± 12.92 mOsm/L[20,21] is known to be normal, and mean of all of these parameters were within normal ranges in our patients before CXL and 1 and 6 months thereafter. Therefore, it seems that dry eye after CXL should not be a concern for the refractive surgeon.

In an *in vivo* confocal study on humans, Mazzotta *et al.* studied the early/late modification of corneal microstructure after CXL treatment. Disconnected nerves during the first 6 months and formation of interconnected fibers after 6 months implied a primary regeneration process. This process and the following interconnection of secondary nerves could partially restore corneal sensitivity and lacrimal reflex.[22] This study demonstrated the disappearance of subepithelial and stromal nerve fibers in the central irradiated area after treatment and gradual microscopic reinnervation at 6 months follow-up. Such a finding was in concordance with previous studies.[7,8,22,23] Similar microstructural evaluation of nerve fibers by Mazzotta *et al.*[8] revealed that the regeneration process origins faster from the subepithelial nerve fibers and grows through the nonirradiated area as soon as 1 month postoperatively; whereas, the regeneration process of anterior stroma fibers toward the deep nerve plexus occurs not sooner than the second and third months. Our results indicate that this regeneration process gradually progresses; however, the structure of the nerve plexus will not be well defined even at 6 months, postoperatively.

In this study, in line with the Touboul *et al.* study,[9] we demonstrated that ASKD and MSKD, but not that in the posterior stroma, severely decreases after CXL and keratocyte repopulation does not complete after 6 months of the procedure. This decrease occurres more at the first month, with a relative recovery over the ensuing 6 months. We found no significant difference in corneal endothelial cell counts and their polymegathism at our 1 and 6 months follow-up times, a finding supported by previous studies.[9-11,14] However, a significant increase in endothelial cell pleomorphism was seen after 1 month which persisted still significantly after 6 months. The lack of evidence for endothelial cell loss is an important safety consideration in assessing CXL.

In our study, CXL led to corneal flattening with a significant UCVA and BSCVA improvement of 0.28 logMAR and 0.30 logMAR, respectively; this was similar to some previous studies.[11,13,14] The reduction of K-readings in our study could explain the improvement in the postoperative visual acuity confirmed in the studies performed by Wollensak *et al.* and Koller *et al.*[11,24] In our study, mean improvements in the SE at 6 months were 0.28 D and 0.32 D with subjective and cyclorefraction, respectively. However, these changes were statistically insignificant; probably, due to poor reproducibility of subjective refraction in cases of irregular corneal topographies. K_{max}, somewhat, evaluates the severity of the keratoconic cone; hence, this variable is indeed a key topographic indicator of CXL success. Although about 1 D reduction in the K_{max} reading in our study may not be clinically enough for visual rehabilitation, such a continuous effect for several years may do so.[24] Hence, longer follow-up period is warranted to determine whether K_{max} will continuously decrease in this setting. Previous studies have reported a decrease in K_{max} of 2.01 D,[11] 1.90 D,[13] 1.46 D,[25] and 1.42 D[26] in keratoconic patients.

In the present study, we found a significant reduction in pachymetric thinnest point. However, this finding could negatively bias by epithelial thinning and keratocyte loss in the anterior-mid stroma.[7] Such a pseudo-reduction may be due to two main reasons: A measurement underestimation and changes in stromal reflectivity after CXL. The treated/untreated stroma demarcation line could have been misread as a pseudoposterior corneal surface. In this regard, ultrasound pachymetry, confocal microscopy, and Visante optical coherence tomography may be less biased by the stromal microstructural alterations following CXL.

The main limitation of our study was the relatively short duration of follow-up. However, our findings are promising and imply that it would be rewarding to further investigate the long-term outcome of this procedure in keratoconus.

CONCLUSIONS

While CXL would have no effect on tear indices and endothelial cell count, it can cause a significant reduction in subepithelial nerve plexus and significant alterations in epithelial cell density in the anterior and middle stroma.

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Conflicts of interest
There are no conflicts of interest.

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