Mitomycin C Application After Corneal Cross-linking for Keratoconus Increases Stromal Haze

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ABSTRACT

PURPOSE: To evaluate and compare corneal haze as determined by optical coherence tomography (OCT) after corneal cross-linking (CXL) for the treatment of mild to moderate keratoconus with or without mitomycin C (MMC) application.

METHODS: This was a retrospective analysis of 87 eyes of 72 patients with mild to moderate keratoconus. The first group (n = 44 eyes) underwent CXL between June 2013 and January 2015 and the second group (n = 43 eyes) underwent CXL with MMC (CXL+MMC) between February and December 2015, both following the Dresden protocol. Patients were evaluated preoperatively and at 1, 3, 6, and 12 months postoperatively. Main outcome measures were corneal reflectivity and haze reflectivity measured by a specially developed OCT image analysis software.

RESULTS: Anterior corneal reflectivity at 1 month and 1 year postoperatively was 14.79 ± 4.68 and 25.97 ± 15.01 (P < .001), and 13.88 ± 4.39 and 18.41 ± 9.25 (P = .025) for the CXL and CXL+MMC groups, respectively. The reflectivity of the anterior stromal haze region at 1 month and 1 year postoperatively was 23.15 ± 5.91 and 33.14 ± 16.58 (P = .005), and 20.58 ± 7.88 and 27.14 ± 12.80 (P = .049) for both groups, respectively. The changes in simulated keratometry from preoperatively to postoperatively were similar in both groups. The CXL+MMC group showed larger maximum keratometry flattening: 53.41 ± 6.88 diopters (D) preoperatively and 49.44 ± 5.66 D 1 year postoperatively versus 52.27 ± 5.78 and 50.91 ± 4.25 D for CXL alone (P = .008).

CONCLUSIONS: MMC application following CXL significantly increases corneal haze. Similar studies need to be performed on simultaneous CXL and photorefractive keratectomy to evaluate the role of MMC in haze formation in such procedures.

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Corneal cross-linking (CXL) is a widely performed therapeutic technique for the treatment of ectasias such as keratoconus and postoperative ectasia that has been proven to successfully halt its progression and potentially improve topographic and visual outcomes.[1,2] A common complication after CXL is corneal haze development,[3] which can affect postoperative visual acuity. Corneal haze after CXL in patients with keratoconus usually develops in the first 3 months postoperatively, peaking at 1 month and clearing between 6 and 12 months.[4-6] Eyes with more advanced disease tend to have a higher likelihood of developing more severe and permanent haze.[1,7]

It is believed that CXL-induced keratocyte apoptosis leads to gradual repopulation by unaffected keratocytes between 2 and 3 months postoperatively, returning to baseline by 12 months postoperatively.[6-8]

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TABLE 1
CXL Methods

Parameter	Conventional CXL	CXL+MMC
Treatment target	Keratoconus	Keratoconus
Fluence (total) (J/cm^2)	5.4	5.4
Soak time and interval (minutes)	30(q2)	30(q2)
Intensity (mW)	3	3
Treatment time (minutes)	30	30
Epithelium status	Off	Off
Chromophore	Riboflavin (IROC Innocross AG)	Riboflavin (IROC Innocross AG)
Chromophore carrier	Dextran	Dextran
Chromophore osmolarity	Iso-osmolar	Iso-osmolar
Chromophore concentration	0.1%	0.1%
Light source	UV-X (IROC AG)	UV-X (IROC AG)
Irradiation mode (interval)	Continuous	Continuous
Protocol modifications	None	0.02% MMC applied to stromal bed, soaking time 45 seconds after CXL
Protocol abbreviation in manuscript	CXL	CXL+MMC

CXL = corneal cross-linking; MMC = mitomycin C

These keratocytes repopulate the corneal stroma in an activated state such as myofibroblasts, and proceed with increased and disorganized collagen deposition, which manifests as haze.[9] Mitomycin C (MMC) is an alkylating antibiotic that blocks DNA and RNA replication and protein synthesis.[10,11] It was shown to have both antiproliferative and cytotoxic effects on human keratocytes, as well as time- and dose-related inhibitory effects on human keratocyte proliferation.[12]

The main purpose of this study was to evaluate the benefit of using 0.02% MMC at the end of the CXL procedure to decrease the incidence and severity of postoperative corneal stromal haze by inhibiting the activation of incoming keratocytes.

PATIENTS AND METHODS

This was a retrospective study that included 87 myopic eyes of 72 patients who underwent CXL using the standard Dresden protocol between June 2013 and January 2015 at the American University of Beirut Medical Center in Lebanon. MMC was applied at the end of each CXL procedure as a routine protocol from February to December 2015. The group of patients that underwent CXL alone was compared to the group that underwent CXL with MMC. This study (BIO-2017-0280) was approved by the Institutional Review Board at the American University of Beirut and adhered to the principles of the Declaration of Helsinki.

PATIENT SELECTION

All patients underwent a complete ophthalmic examination as part of their routine work-up before CXL, including Placido-Scheimpflug imaging and corneal optical coherence tomography (OCT). All patients included in the study completed all follow-up examinations through the first postoperative year. Inclusion criteria were patients aged 18 years and older who underwent CXL at the American University of Beirut Medical Center for keratoconus progression. Keratoconus progression was defined as three consecutive tomographic measurements demonstrating an increase of at least 1.00 diopter (D) in the steepest anterior keratometric value (Kmax) in 1 year, and/or a 5% or greater decrease in mean central corneal thickness in 6 months. Exclusion criteria were corneal thickness values less than 400 µm at the thinnest point, intraocular pressure of greater than 21 mm Hg, advanced keratoconus necessitating corneal transplant, active ocular pathology, history of intraocular and/or corneal surgeries, history of herpetic keratitis, and autoimmune and/or connective tissue disease. Other exclusion criteria were preexisting corneal opacification/scars, severe dry eyes, and peripheral marginal degeneration.

CXL TECHNIQUE

All CXL procedures (**Table 1**) were performed according to the Dresden protocol.[13] The eye to be treated was anesthetized by applying proparacaine hydrochloride 0.5% drops on two occasions at 5-minute intervals. An

eyelid speculum was inserted between the eyelids and the central 9-mm corneal epithelium was removed with a blunt spatula. For corneal soaking, a solution of 0.1% riboflavin and 20% dextran (IROC Innocross AG) was instilled every 2 minutes for 30 minutes. An ultraviolet-A lamp with irradiance of 3 mW/cm^2 (UV-X; IROC AG), calibrated between each treatment, was focused on the corneal apex at a distance of 5 cm for 30 minutes (total energy of 5.4 J/cm^2). Meanwhile, during that time, the riboflavin drops were applied to the cornea every 2 minutes. In one of the groups, 0.02% MMC was applied to the stromal bed and left for 45 seconds. At the end of the procedure, in both groups, the eye was copiously irrigated with a balanced salt solution and a drop of 0.3% gatifloxacin followed by placement of a bandage soft contact lens, which was kept for at least 4 days until complete epithelialization ensued as judged by slit-lamp microscopy.

Postoperatively, patients were instructed to instill one drop of 0.3% gatifloxacin four times daily for 2 weeks with one drop of tobramycin–dexamethasone 0.1% four times daily for 1 week, and then one drop of 0.1% fluorometholone four times daily, tapered over 6 weeks.

OCT Measurements and Software Analysis

Using Cirrus high-definition optical coherence tomography (Cirrus HD-OCT; Carl Zeiss Meditec AG) on anterior segment cube 512 × 128 mode,[14] tomographic images and measurements were taken at baseline and at 1, 3, 6, and 12 months after CXL. All images were then evaluated by a dedicated corneal OCT image analysis software, with pending patent, which was developed in conjunction with the computer science department at the American University of Beirut and used by the authors in previous publications.[4,15,16] The software allows the automated and objective detection and classification of corneal haze and demarcation line on OCT images using machine learning (**Figure 1**).

The software measures cross-sectional haze surface area, corneal reflectivity, and haze reflectivity of the anterior, middle, and posterior stroma and total cornea. Corneal haze area is calculated as the percentage of pixels in the haze area of a particular region over the total number of pixels in that region. Haze reflectivity is calculated as the intensity of each pixel in a particular corneal region over the total number of pixels in the region, divided over 255 to obtain the grayscale and then converted into percentage. Gamma decoding was then applied to the reflectivity of the OCT images to restore the initial reflectivity parameters.

Placido and Scheimpflug Measurements

Topographic and tomographic measurements were obtained preoperatively, and at 1, 3, 6, and 12 months postoperatively, using a dual Scheimpflug and Placido system (Galilei; Ziemer). Accordingly, corneal thickness and preoperative and postoperative Kmax and mean keratometry values were extracted.

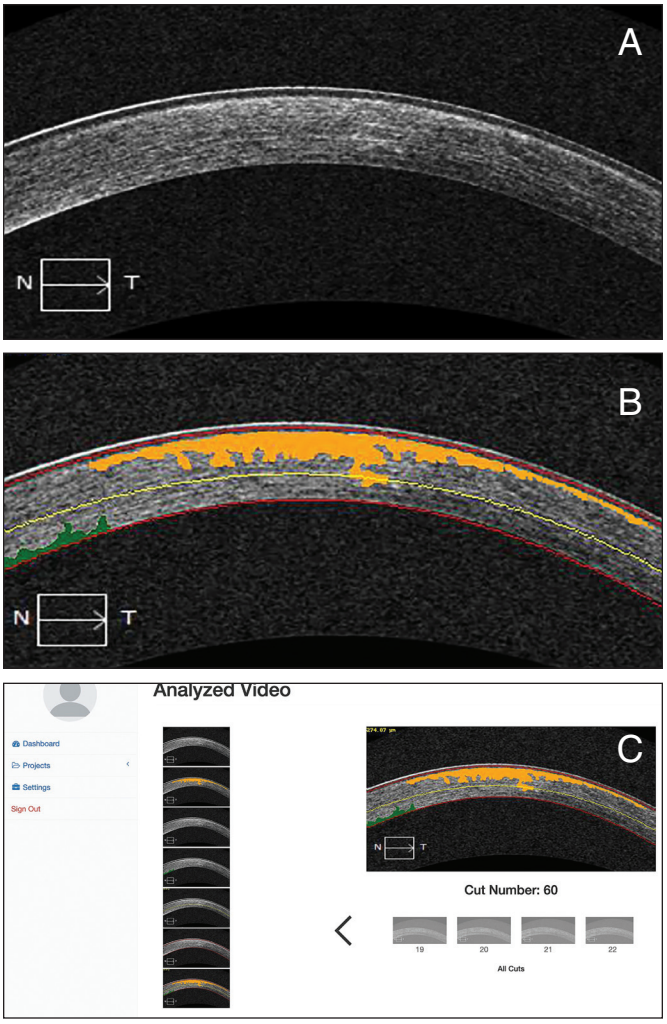

Figure 1. (A) Corneal optical coherence tomography (OCT) section of an eye 6 months after corneal cross-linking and mitomycin C application. (B) The software automatically detects and classifies the stromal haze on OCT images based on location, size, and reflectivity. Additionally, it identifies the demarcation line (in yellow) and its depth. (C) Screenshot of the software display.

Visual Measurements

Visual acuity testing was performed 4 m from the visual acuity chart. Uncorrected and corrected distance visual acuity were measured preoperatively and at 1, 3, 6, and 12 months postoperatively.

Statistical Analysis

Using a 95% confidence interval and a margin of error of 1.25 grayscale units (GSU), the minimum calculated sample size was 40 eyes based on a previously

TABLE 2

Visual and Tomographic Results of Eyes That Underwent CXL or CXL+MMC[a]

	CXL				CXL+MMC			
Parameter	UDVA (logMAR)	CDVA (logMAR)	SimK	Kmax	UDVA (logMAR)	CDVA (logMAR)	SimK	Kmax
Preoperative	0.35 ± 0.31	0.17 ± 0.20	46.6 ± 3.55	52.27 ± 5.78	0.37 ± 0.34	0.20 ± 0.19	47.49 ± 5.12	53.41 ± 6.88
Last follow-up	0.27 ± 0.18	0.14 ± 0.16	45.92 ± 3.71	50.91 ± 4.25	0.39 ± 0.37	0.20 ± 0.20	46.41 ± 4.60	49.44 ± 5.66
Within-group *P* value	.08	.18	< .001[b]	.01[b]	.67	.89	< .001[b]	< .001[b]

CXL = corneal cross-linking; MMC = mitomycin C; UDVA = uncorrected distance visual acuity; CDVA = corrected distance visual acuity; SimK = simulated keratometry; Kmax = steepest anterior keratometric value
[a]*Values are presented as mean ± standard deviation.*
[b]*Statistically significant.*

TABLE 3

Percentage of Corneal Reflectivity in Each Corneal Region After Conventional CXL and CXL+MMC[a]

	Anterior Stroma		Middle Stroma		Posterior Stroma	
Time	CXL	CXL+MMC	CXL	CXL+MMC	CXL	CXL+MMC
Baseline	11.91 ± 2.75 (6.71 to 22.76)	11.56 ± 2.21 (6.71 to 15.48)	9.85 ± 3.31 (3.39 to 19.39)	9.51 ± 2.94 (3.80 to 19.39)	7.76 ± 3.51 (1.93 to 16.90)	7.09 ± 2.69 (2.32 to 15.30)
P	.547		.636		.351	
1 month	14.79 ± 4.68 (0.99 to 24.21)	25.97 ± 15.01 (4.33 to 68.51)	14.65 ± 5.48 (6.13 to 31.58)	17.67 ± 11.54 (2.15 to 48.29)	10.59 ± 4.01 (4.24 to 19.13)	10.87 ± 7.57 (2.33 to 34.81)
P	< .001[b]		.219		.864	
3 months	16.14 ± 4.99 (8.18 to 27.99)	24.76 ± 16.47 (5.55 to 53.01)	13.30 ± 5.86 (5.24 to 27.04)	18.47 ± 14.62 (4.68 to 55.95)	9.41 ± 4.62 (3.39 to 20.92)	11.41 ± 9.94 (2.33 to 40.11)
P	.015[b]		.109		.372	
6 months	13.95 ± 3.77 (7.60 to 23.79)	21.83 ± 11.04 (3.87 to 38.39)	11.04 ± 3.53 (4.41 to 19.08)	14.68 ± 10.13 (2.84 to 38.05)	8.38 ± 3.00 (2.69 to 14.17)	11.35 ± 9.98 (0.95 to 41.70)
P	.002[b]		.113		.182	
12 months	13.88 ± 4.39 (1.58 to 23.78)	18.41 ± 9.25 (4.56 to 44.56)	10.60 ± 3.85 (1.47 to 21.25)	15.46 ± 10.26 (3.36 to 43.61)	8.19 ± 3.55 (1.98 to 18.12)	10.39 ± 8.38 (1.43 to 35.52)
P	.025[b]		.024[b]		.212	

CXL = corneal cross-linking; MMC = mitomycin C
[a]*Values are presented as mean ± standard deviation (range).*
[b]*Statistically significant.*

calculated standard deviation of 4.03 GSU for central corneal haze after CXL using Scheimpflug tomography.[17] SPSS software version 21.0 (SPSS, Inc) was used to perform statistical analysis, whereas data management and analysis were performed by Microsoft Office Excel version 16.16.6 (Microsoft Corporation). Descriptive statistics were reported as mean and standard deviations for continuous variables. Haze area and intensity at different time points were compared using the paired *t* test. Two-way repeated-measures analysis of variance with the Bonferroni correction for post-hoc analysis was used to compare the change in haze after CXL. A *P* value less than .05 was considered statistically significant unless stated otherwise.

RESULTS

DEMOGRAPHICS

A total of 84 myopic eyes of 70 patients were analyzed by OCT. A total of 44 eyes underwent CXL alone (26 males and 13 females, mean age: 22 years), whereas 40 eyes had CXL with MMC (26 males and 14 females, mean age: 26 years).

According to the Amsler-Krumeich classification, 86.4% of eyes had grade 1 or grade 2 keratoconus and 13.6% had grade 3 keratoconus in the CXL group, whereas 85% of eyes had grade 1 or 2 keratoconus and 15% had grade 3 keratoconus in the CXL+MMC group. The recruited eyes had average preoperative Kmax values of 52.27 ± 5.78 and 53.71 ± 7.00 D (*P* =

TABLE 4

Percentage of Haze Reflectivity in Each Corneal Region After Conventional CXL and CXL+MMC[a]

	Anterior Stroma		Middle Stroma		Posterior Stroma	
Time	**CXL**	**CXL+MMC**	**CXL**	**CXL+MMC**	**CXL**	**CXL+MMC**
Baseline	16.66 ± 5.59 (7.78 to 27.68)	16.30 ± 5.12 (7.78 to 27.65)	18.82 ± 6.68 (7.01 to 35.89)	18.61 ± 6.85 (7.01 to 35.89)	19.18 ± 7.60 (9.30 to 42.40)	18.06 ± 6.00 (9.30 to 27.65)
P	.852		.916		.595	
1 month	23.15 ± 5.91 (13.97 to 42.27)	33.14 ± 16.58 (6.54 to 69.73)	21.51 ± 6.09 (11.42 to 39.88)	28.45 ± 14.09 (5.54 to 59.45)	20.60 ± 7.54 (8.14 to 41.06)	27.07 ± 16.92 (0.00 to 58.51)
P	.005[b]		.021[b]		.105	
3 months	24.18 ± 6.66 (14.97 to 37.31)	34.09 ± 19.30 (13.02 to 64.18)	20.67 ± 7.85 (9.53 to 39.69)	30.05 ± 17.47 (9.58 to 68.62)	20.37 ± 7.77 (4.47 to 34.50)	24.55 ± 16.69 (10.60 to 58.51)
P	.014[b]		.020[b]		.316	
6 months	22.56 ± 6.16 (10.79 to 35.56)	28.13 ± 12.52 (6.63 to 48.14)	20.92 ± 6.48 (9.57 to 38.10)	25.55 ± 12.96 (5.69 to 48.18)	21.03 ± 7.11 (7.82 to 38.12)	22.93 ± 11.55 (6.18 to 43.39)
P	.074		.141		.592	
12 months	20.58 ± 7.88 (2.90 to 38.22)	27.14 ± 12.80 (7.09 to 49.28)	22.93 ± 12.16 (2.87 to 50.30)	24.95 ± 12.72 (7.00 to 51.04)	22.31 ± 12.88 (3.14 to 47.99)	25.17 ± 12.61 (6.70 to 45.15)
P	.049[b]		.562		.492	

CXL = corneal cross-linking; MMC = mitomycin C
[a]Values are presented as mean ± standard deviation (range).
[b]Statistically significant.

.47), simulated keratometry values of 46.33 ± 3.15 and 47.49 ± 5.12 D (P = .29), and a central corneal thickness of 477.02 ± 40.84 and 485.85 ± 42.25 µm (P = .36) in the CXL and CXL+MMC groups, respectively.

Demarcation line depth between 1 and 3 months postoperatively was 337.15 ± 95.62 and 329.96 ± 71.09 µm for the CXL and CXL+MMC groups, respectively (P = .75).

Visual and Tomographic Results

The visual and topographic results are summarized in **Table 2**. Simulated keratometry and Kmax values decreased postoperatively in both groups (P < .01), with the decrease in Kmax being more significant in the CXL+MMC group (P < .001).

Refractive Results

The manifest refraction spherical equivalent was -3.34 ± 3.13 preoperatively and -2.97 ± 2.74 postoperatively (P = .72) in CXL and -2.65 ± 3.26 preoperatively and -2.89 ± 4.23 postoperatively (P = .84) in the CXL+MMC group. The change in manifest refraction spherical equivalent was not statistically significant between the two groups (P = .70).

Corneal Haze Measurements

Eyes undergoing CXL with MMC showed higher stromal reflectivity compared to CXL alone (Dresden protocol), especially in the anterior stroma at 1 and 6 months (**Table 3**). Eyes that had CXL and MMC showed more haze area reflectivity than CXL alone in all regions of the cornea throughout the follow-up period, but mainly in the anterior stroma at 1 and 3 months (**Table 4**). The percentage of haze area represents the ratio of cross-sectional haze surface area over the rest of the stromal surface area in a given corneal region (anterior, middle, or posterior), multiplied by 100. The anterior stromal haze area was 48.24 ± 12.65 and 61.46 ± 17.80 at 1 month postoperatively for the CXL and CXL+MMC groups, respectively (P = .013), 46.03 ± 11.13 and 54.41 ± 12.94 at 3 months postoperatively (P = .027), 42.53 ± 11.27 and 48.98 ± 10.37 at 6 months postoperatively (P = .038), and 47.58 ± 12.00 and 42.00 ± 11.14 at 12 months postoperatively (P = .048). The middle and posterior stromal haze areas were also measured at 1, 3, 6, and 12 months postoperatively with a significant value only in the middle stromal haze area at 1 month; 26.21 ± 6.10 and 32.41 ± 6.19 for the CXL and CXL+MMC groups, respectively (P = .045).

DISCUSSION

The safety and efficacy of corneal CXL in the treatment and long-term stabilization of progressive keratoconus has been well documented.[18-20] A recognized complication of CXL is the development of postopera-

tive haze,[3,8] especially affecting eyes with advanced keratoconus and steeper corneas.[1] Postoperative haze peaks at 1 month and usually stabilizes between 3 and 6 months,[5,6,15] with subsequent improvement in corneal transparency between 6 and 12 months after surgery.[4] Corneal transparency is attributed to the regular spacing and diameter of collagen fibrils,[21] as well as the structure and organization of stationary keratocytes.[22] The new covalent bonds established between the collagen lamellae after CXL[18,23] may affect the organization of the stromal structure responsible for corneal transparency.[3]

CXL-induced keratocyte apoptosis is typically restricted to a corneal stromal depth of approximately 350 µm, followed by repopulation of the anterior stroma by activated keratocytes between 1 and 3 months postoperatively.[6-8] The activation and repopulation of keratocytes belongs to a complex process of corneal wound healing intended to recover normal tissue function.

Understanding the corneal wound healing response after photorefractive keratectomy (PRK) may provide a clearer understanding of the process that occurs after CXL. Several studies have been performed on the corneal wound healing process after PRK, showing that PRK greatly diminishes the distribution and quantity of keratocytes in the anterior stroma by inducing apoptosis.[24] Also, the cytokines released by the upper, injured cell layers activate the viable, quiescent keratocytes along the wound borders.[25] The activated keratocytes repopulate the anterior stroma and generate myofibroblast-precursor cells.[26] These myofibroblasts are slightly opaque due to reduced crystallin protein production and they deposit disorganized extracellular matrix,[25] resulting in increased scattering of light and peaked haze formation in the first 3 months postoperatively.[22] Cytokines are necessary for the development and persistence of myofibroblast.[27] The decline in cytokine levels and the myofibroblast apoptosis, both brought about by the regeneration of the epithelial membrane, and the removal of the disorganized collagen by the repopulating keratocytes, are responsible for the late haze regression occurring between 3 and 6 months postoperatively.[25,28]

Interestingly, haze induced by PRK is different from that induced by CXL: corneal hyperreflectivity is more restricted to subepithelial areas following PRK, whereas in CXL areas of opacity can reach deeper layers of the corneal stoma.[3] As mentioned above, keratocytes have a central role in the cellular wound healing response. Remaining keratocytes adjacent to wound borders are activated by cytokines, and under particular mediation of the growth factor beta, there is a transformation of active keratocytes into myofibroblasts.[24] However, unlike PRK, the keratocyte apoptosis following CXL occurs in a much deeper extension of the corneal stroma, and therefore much of the inflammatory response that would be mediated by keratocytes, paradoxically, would not take place due such cellular shortages.

MMC, an antineoplastic alkylating agent,[29] has been successfully used in the prevention of subepithelial haze after PRK.[30] Although there was an early concern about the safety of MMC in PRK, studies have shown that it is relatively safe even when evaluated in the long term.[31] MMC is associated with the diminished presence of haze-related myofibroblasts within the wound.[32] It has been shown that the application of MMC after PRK on rabbit corneas triggers keratocyte and myofibroblast apoptosis.[10] Secondary to this observation, we wanted to test the role of MMC application in haze reduction after CXL. Surprisingly, the results of our study have shown a clinically significant increase in haze after the use of MMC as opposed to the CXL alone group.

Studies have shown that for months after the use of MMC, fewer keratocytes undergo mitosis in the anterior stroma.[10,28,32] This is attributable to MMC's prolonged apoptotic effect[29] and MMC-induced DNA damage in resident keratocytes that inhibits their entry into the cell cycle.[10,32] It has also been shown that MMC-induced DNA damage prevents keratocytes from responding to cytokines, hindering their repopulation of the anterior corneal stroma.[32] We believe that MMC's apoptotic effect on the resident quiescent keratocyte population, synergistic with that of CXL, results in a major cell drop-out in the treated area. The reduced density and mitotic activity of keratocytes interferes with their reparatory role, resulting in a diminished capacity to repopulate the injured area and a diminished removal of the unorganized collagen laid down by the myofibroblasts.[33] We also postulate that the larger magnitude of apoptosis induced by the concomitant use of MMC with CXL may result in a larger amount of cytokine release. These cytokines play an important role in the differentiation and maturation of myofibroblasts.[25] Apart from being opaque themselves, the increased quantity of myofibroblasts will lead to a greater deposition of disorganized collagen that can no longer be cleared out by the apoptotic keratocytes.[25] This can account for the clinical observation of increased haze peaking between 1 and 3 months postoperatively in our series.

The relationship between increased haze after CXL and flattening of the anterior cornea is well known: Hafezi et al[34] were the first to describe massive flattening in patients with marked permanent stromal haze

following standard CXL. Interestingly, the flattening effect of the haze may lead to an improvement in visual acuity in certain patients, where the positive effect of flattening on visual acuity might outweigh the negative effect of loss of transparency. Currently, it is not possible to predict the amount of induced haze and flattening after CXL. If it were possible to actively control the amount of haze, then a planned induction of such haze and flattening might be of benefit in selected cases of keratoconus, especially in cases with a myopic refraction.

This study has some limitations, including the retrospective character of the study. A further limitation is that the study results may not apply equally in all regions of the world. The Middle East, where this study was performed, not only has one of the highest prevalences of keratoconus,[35] but also, according to the observation of the authors, has a higher incidence of clinical haze after CXL than what has been reported in European and North American patients. It has been shown that corneal haze after PRK is more common in patients with darker skin, darker irides,[36] and higher exposure to environmental ultraviolet light.[37] Whether these risk factors apply to CXL still needs to be further elucidated. Finally, our study evaluated mild to moderate forms of keratoconus. The effect of MMC on more advanced forms of the disease, which have been associated with more severe haze, is yet to be evaluated.[1]

Instead of reducing postoperative haze as in its use after PRK, the application of MMC following CXL induced more postoperative haze, and therefore should be avoided. Based on the results of this study, we also recommend caution in using MMC in combined PRK and CXL procedures.

AUTHOR CONTRIBUTIONS

Study concept and design (STA, EAT-N, FH); data collection (LMC, CH, ARD, TT, JT, MAF); analysis and interpretation of data (STA, LMC, CH, ARD, MAF, RS); writing the manuscript (STA, LMC, TT, JT, EAT-N, FH); critical revision of the manuscript (STA, LMC, CH, ARD, MAF, EAT-N, FH, RS); statistical expertise (LMC, ARD, MAF); administrative, technical, or material support (LMC, CH, ARD); supervision (STA)

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