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Conjunctivo corneal impression cytology changes after corneal collagen cross linking in progressive keratoconus

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Short title: Conjunctivo corneal impression cytology changes after CCXL in progressive keratoconus

ABSTRACT

Purpose: Keratoconus (KC) is defined as a progressive, non-inflammatory corneal ectasia of unknown origin. Corneal collagen cross-linking (CXL) is a well-known therapeutic modality of KC. Impression cytology (IC) is a minimally invasive approach of assessing human ocular surface cells and is considered as the 'gold standard' approach in terms of morphological assessment of cells.

Patients and methods: This study was a prospective study conducted on 21 patients with progressive keratoconus attending to ophthalmic center, Mansoura University. The cases were subjected to history taking and comprehensive ocular examination. CXL was done for all patients using riboflavin and ultraviolet –A irradiation. All patients were followed up (one month, three months, six months) after CXL. Conjunctival-corneal impression cytology was done by cellulose filter paper before the surgery and during follow up.

Results: Regarding the cohesion power follow-up, it was found that baseline and immediately after CXL, 6 cases (28.6%) showed group of coherent cells and 15 cases (71.4%) showed separate individual cells. At one month follow up, 1case (4.8%) showed one large sheet of coherent cells, 19 cases (90.5%) showed group of cells and 1case (4.8%) showed separate individual cells, with statistically significant difference compared to the baseline values. At six months follow up, 16 cases (76.2%) showed one large sheet of coherent cells and 5 cases (23.8%) showed group of cells, with statistically significant difference compared to the baseline values.

Conclusion: The current study concluded that CXL decreases keratoconus progression. postsurgical parameters tracked a reproducible trend over 6 months.

Keywords: Keratoconus, CXL, Impression cytology, Goblet cells, cohesion power.

INTRODUCTION

Keratoconus (KC) is a progressive, non-inflammatory primary corneal ectasia which has been demonstrated to be accompanied by reduction in visual acuity (VA)[1]. The primary cause of corneal ectasia is the reduction of corneal stability due to worsening of collagen structure of the cornea**.** Incidence of this disease about 1 per 2000 in general population which occurs between 2nd and 3rd decade of life[2].

In early stages of keratoconus, glasses can correct refractive errors, but when the disease becomes more severe, rigid contact lenses are needed to achieve acceptable VA. However; glasses or contact lenses correct only the refractive errors but not affect the keratoconus progression. By progression of the keratoconus, cornea becomes more thin, irregular and steep, rigid contact lenses may have no role , the only option left is surgical intervention as corneal transplantation[3].

Now corneal collagen crosslinking is used as a primary treatment in mild and moderate cases to stop the collagen instability which is the primary cause of the disease and also to strengthen the corneal collagen structure[4]. In CXL, riboflavin is used as a photosensitizer in the presence of ultraviolet A (UVA) irradiation to stiffen the cornea by improving biomechanical strength and increase resistance to enzymatic digestion[5].

Keratoconus is usually accompanied by tear film (TF) dysfunction and ocular surface disease, which can be associated with increased visual distortion as well as optical aberration. CXL effect on ocular surface is essential for visual function, so it is important to study the CXL effect on ocular surface[6].

In 1977 Egbert et al. reported method termed (cellulose acetate impression)[7]. Then termed by Nelson et al., conjunctival impression cytology (IC)[8]. It is done by application of a cellulose acetate filter to the ocular surface to remove the superficial layers of the ocular surface epithelium. These cells could then be subjected to histologic, molecular, or immunohistological analysis. This method is simple, non-invasive and non-traumatic, so it is useful in predicting the effect of CXL on conjunctiva and corneal surface[9].

PATIENTS AND METHODS

This was a prospective interventional study that was conducted within the period from January 2021 to February 2022 in Mansoura ophthalmic center, Mansoura University.

This study included 20 patients with progressive Keratoconus. We included the cases from both genders with age less than 20 years, with maximum K increase more than 1 diopter /year and corneal thickness decrease by 2% or more/year. The cases with other comorbidities were ruled out; connective tissue disorders, patients with central corneal opacity, immune-compromised patients, history of herpetic keratitis, pregnancy, ocular surface disorders and corneal thickness <400 µm.

After approval from the IRB of Mansoura Faculty of Medicine (code number: MS/17.3.73) and obtaining an informed written consent from the participants, full and detailed history was obtained for all the cases.

Complete ophthalmic examination was done for all the cases including evaluation of the VA using Landolt's VA chart and after that transferred for statistical analysis to Log MAR.

Slitlamp biomicroscopy (SLB) (Haag-Streit, Switzerland) was utilized to evaluate cornea clarity, anterior chamber for depth and regularity, pupillary shape, size, regularity and reactivity and state of the lens. Cycloplegic refraction was assessed by utilizing automated refractometer following instillation of Swixolate (Cyclopentolate Hydrochloride 10mg/ml CHEMIPHARM) eye drops three times within 30 minutes.

Posterior segment examination was performed using indirect ophthalmoscope and SLB with auxiliary contact lens. Corneal topography analysis was performed by automatically rotating Scheimpflug camera.

Impression cytology (IC)

Impression cytology samples were taken using a cellulose acetate filter paper. It was done before CXL and then immediately after it then after one month, three months, six months.

Impression technique

1. After application of topical anesthesia, drops of isotonic saline were instilled on the cornea to avoid its impairment due to dryness, and the excess saline was carefully dried up from above the conjunctival surface using a piece of sterile gauze.
2. The eye was kept open by means of a speculum and mobilized using a surgical hook. It was moved into the direction that most exposes the temporal bulbar conjunctiva.
3. A smooth, serrated, nontoothed forceps was used to grasp the pointed tip of the filter strip and apply the rough surface onto the conjunctiva; the smooth side of the forceps was used to gently press the filter material onto the bulbar conjunctiva to ensure close contact of the strip and the ocular surface.
4. The filter paper was positioned cautiously on the temporal area of the bulbar conjunctiva, left in place for

three seconds, and after that slightly removed with peeling motion, evading shearing and twisting forces which may distort the specimen then on the nasal, upper and lower bulbar conjunctiva.

5. The filter paper was then peeled off the conjunctival surface and dropped directly into a clean small glass container filled more than half with the fixative solution (97% ethyl alcohol). The container was then shaken to make sure that the fixative solution completely covered the sample. Another method is to directly impress the filter paper on the glass slide then fixed with 97% ethylalcohol.
6. The samples to be stained were passed into ethyl alcohol of decreasing concentrations (90, 70, followed by 50%) and finally into running water for hydration. A metal plate made from aluminum alloy was used.

Microscopic examination

1. This was conducted using a light microscope (ERMA model KD) with ×50 magnification to assess the overall relation of the cells and with × 250 magnifications to study individual cells. Any slide that showed poor cell pickup was discarded as unreliable.
2. The cytological characteristics of the samples were then assessed, including cohesion power, cell size, goblet cell distribution, and degree of keratinization.

Interpretation of Impression Cytology:

1. Cohesion power was graded as follows: grade I, a single large sheet of coherent cells; grade II, a collection of cells; grade III, separate individual cells. (Each grade was given an additional half point to reflect intermediate form)
2. Cell size was graded as follows: average size, 0; enlarged, 1; and shrunken −1.
3. Distribution of goblet cells was as follows; present (I); absent (zero).
4. The four grades of keratinization were as follows: grade zero, which was normal (deep cytoplasm staining); grade I, which was mild (average cytoplasm staining); grade 2, which was moderate (pale cytoplasm); and grade III, which was severe (very faint cytoplasm). (Each grade was given an additional half point to reflect intermediate form)

Surgical technique:

Topical anaesthesia was applied prior to the approach using benoxinate eye drops. The corneal epithelial lining was automatically removed over an area of eight mm in diameter by utilizing a blunt instrument (Hokey knife), and riboflavin 0.1% solution was instilled frequently for about 30 min.

UVA irradiation was conducted by utilizing an optical system (Kohler illumination) with a light source composed of seven UV diodes (365nm; Nuernberg, Germany) and a potentiometer in series to permit the voltage regulation.

During treatment, riboflavin solution was applied every three min for corneal saturation with the riboflavin.

Then impression cytology was conducted as the same technique immediately after CXL.

After the treatment, a bandage contact lens was applied till full regeneration of the corneal epithelial lining which was usually accomplished completely after three days. Postoperatively patients were given topical antibiotic with artificial tear substitutes.

Post-operative care:

After removal of contact lens, the patient was prescribed to take topical combination of steroid and antibiotic 5 times daily, with a gradual decrease over the following 6 weeks together with topical lubricant substitute. After surgery, follow-ups were done at one, three, and six months.

Statistical analysis

The collected data were analyzed by using SPSS (27.0, IBM/SPSS Inc., Chicago, IL) software. Data were defined as frequencies and percentages (%) for Categorical data while data were expressed as mean ± SD / median and range.

McNamara's test or marginal homogeneity tests were used to compare categorical data at three or more time points. P values <0.05 are considered significant.

RESULTS

Table 1 shows that the mean age was (17.43 ± 1.165 years) ranged from 16 to 20 years.

Table 1: Demographic data of the studied sample:

All patients (n= 21)	Mean & SD	Median	Range	IQR
Age (years)	17.43 ± 1.165	17.00	16, 20	16.50, 18.00

Data were expressed as mean and SD, median, range and IQR.

Table 2 shows that regarding the cohesion power follow-up, at the baseline and immediately after CXL, 6 cases (28.6%) showed group of coherent cells and 15 cases (71.4%) showed separate individual cells. It was found that at one month follow up, 1 case (4.8%) showed one large sheet of coherent cells, 19 cases (90.5%) showed group of cells and 1case (4.8%) showed separate individual cells, with statistically significant difference in comparison with the basal values ($p < 0.001$).

At three months follow up, 5 cases (23.8%) showed one large sheet of coherent cells and 16 cases (76.2%) showed group of cells, with statistically significant difference compared to the baseline values.($p < 0.001$).

At six months follow up, 16 cases (76.2%) showed one large sheet of coherent cells and 5 cases (23.8%) showed group of cells, with statistically significant difference compared to the baseline values. ($p < 0.001$).

Table 2: Cohesion power follow-up in the current study:

Cohesion power		Frequency	Percentage	P
Baseline	Groups of cells	6	28.6	-
	Separate individual cells	15	71.4	
Immediately after CXL	Groups of cells	6	28.6	1
	Separate individual cells	15	71.4	
One month	Single large sheet of coherent cells	1	4.8	
	Groups of cells	19	90.5	< 0.001
	Separate individual cells	1	4.8	
Three months	Single large sheet of coherent cells	5	23.8	< 0.001
	Groups of cells	16	76.2	
Six months	Single large sheet of coherent cells	16	76.2	< 0.001
	Groups of cells	5	23.8	

Data were expressed as percentage and frequency. P is generated by comparing each reading to the baseline value.

Table 3 shows that regarding the cell size follow-up, at the baseline 18 cases (85.7 %) showed average size while only 3 cases (14.3%) showed enlarged cells.

Immediately after CXL , 12 cases (57.1%) showed average size,7 cases(33.3%) showed shrunken cells and 2 cases (9.5%) showed enlarged cells, without statistically significant difference in comparison with basal values.($p < 0.057$).

At both one-month and three months follow up, 12 cases (57.1%) showed average size, 5 cases (23.8%) demonstrated enlarged cells, and 4 cases (19 %) showed shrunken cells, without statistically significant difference compared to basal values. ($p < 0.564$).

At six months follow up, 17 cases (81%) showed average size, and 4 cases (19 %) showed enlarged cells, without statistically significant difference compared to baseline values. ($p < 0.655$).

Table 3: Cell size follow-up in the current study:

Cell size		Frequency	Percentage	P
Baseline	**Average size**	18	85.7	-
	Enlarged	3	14.3	
After treatment	**Shrunken**	7	33.3	0.057
	Average size	12	57.1	
	Enlarged	2	9.5	
One month	**Shrunken**	4	19.0	0.564
	Average size	12	57.1	
	Enlarged	5	23.8	
Three months	**Shrunken**	4	19.0	0.564
	Average size	12	57.1	
	Enlarged	5	23.8	
Six months	**Average size**	17	81.0	0.655
	Enlarged	4	19.0	

Data were expressed as percentage and frequency. P is generated by comparing each reading to the baseline value.

Table 4 showed at baseline goblet cells were demonstrated in 19 cases (90.5%), immediately after CXL goblet cells were demonstrated in 2 cases (9.5%)($p< 0.001$), one month after CXL goblet cells were demonstrated in 2 cases(9.5%) ($p < 0.001$), three months after CXL goblet cells were demonstrated in 7 cases(33.3%) ($p < 0.001$), six months after CXL goblet cells were demonstrated in 15 cases (71.4%) ($p=0.262$).

Table 4: Goblet cells follow-up in the current study:

Goblet cells	Frequency	Percentage	P
Baseline	19	90.5	-
After treatment	2	9.5	**< 0.001**
One month	2	9.5	**< 0.001**
Three months	7	33.3	**< 0.001**
Six months	15	71.4	**0.262**

Data were expressed as percentage and frequency. P is generated by comparing each reading to the baseline value.

Table 5 showed that at baseline there were 11 (52.4%) moderate keratinization and 10 (47.6%) severe keratinization, while after treatment 21 (100%) of cases had mild keratinization ($p < 0.001$).

At both one month and three months follow up, 13 (61.9%) of cases had mild keratinization, while 8 (38.1%) had moderate keratinization ($p < 0.001$).

At six months follow up, 14 (66.7%) of cases had moderate keratinization, 6 (28.6%) had mild keratinization, and only one case had severe keratinization ($p = 0.002$).

Table 5: Keratinization follow-up in the current study:

Keratinization		Frequency	Percentage	P
Baseline	**Moderate**	11	52.4	-
	Severe	10	47.6	
After treatment	**Mild**	21	100.0	**< 0.001**
One month	**Mild**	13	61.9	**< 0.001**
	Moderate	8	38.1	
Three months	**Mild**	13	61.9	**< 0.001**
	Moderate	8	38.1	
Six months	**Mild**	6	28.6	**0.002**
	Moderate	14	66.7	
	Severe	1	4.8	

Data were expressed as percentage and frequency. P id significant when < 0.05. P is generated by comparing each reading to the baseline value.

Table 6 showed that the mean baseline K1 in the included cases was 46.01 ± 2.44, at 1 month postoperative, there was a non-statistically significant increase followed by a further, but a statistically significant increase at 3 months postoperative. At 6 months postoperative, the mean K1 showed a statistically significant decrease compared to the basal reading**.**

Table 6: K1 follow-up in the current study:

Items	Before crosslinking	At 1 month postoperative	At 3 months postoperative	At 6 months postoperative	P value
Mean ± SD	46.01 ± 2.44	46.16 ± 2.45	46.30 ± 2.48	45.90 ± 2.46	
P1		0.214	**< 0.001 ***	**0.017 ***	F= 30.118
P2			0.171	**0.043 ***	P < 0.001 *
P3				**< 0.001 ***	

P: probability.

Quantitative data expressed as mean ± SD F: Repeated measures ANOVA

*: Statistically significant (p<0.05)

P1: Significance compared to preoperative data

P2: Significance compared to 1 month

P3: Significance compared to 3 months

Table 7 showed that the mean baseline K2 in the included cases was 49.50 ± 3.08, at 1 month postoperative, there was a non-statistically significant increase followed by a non-statistically significant decrease at 3 months postoperative and further statistically significant decrease at 6 months postoperative compared to the basal reading.

Table 7: K2 follow-up in the current study

Items	Before crosslinking	At 1 month postoperative	At 3 months postoperative	At 6 months postoperative	P value
Mean ± SD	49.50 ± 3.08	49.51 ± 2.74	49.40 ± 2.69	49.20 ± 2.91	
P1		0.868	0.352	**< 0.001 ***	F= 18.389
P2			0.072	**0.001 ***	P < 0.001 *
P3				**0.008 ***	

P: probability.

Quantitative data expressed as mean ± SD F: Repeated measures ANOVA

*: Statistically significant (p<0.05)

P1: Significance compared to presurgical data

P2: Significance compared to 1 month

P3: Significance compared to 3 months

DISCUSSION

Keratoconus (KC) is a progressive ectatic corneal disease, which leads to vision distortion due to the irregular corneal shape. Novel researches revealed that KC could occasionally be accompanied by ocular surface disorder and decrease in tearfilm quality, participating in the visual affection[10].

It has been demonstrated that the traditional CXL approach has become the primary therapeutic approach for KC progression. Many research demonstrated that CXL is a safe and efficient approach with regard to the prevention of KC advancement[11]. An accelerated CXL (A-CXL) protocol is emerged according to the traditional CXL protocol, as a result the duration of therapy could be diminished. Higher ultraviolet-A (UVA) irradiation, which is equivalent to the cumulative therapeutic dose (5.4 J/cm2) in the case of the A-CXL, penetrates the cornea faster. Multiple records demonstrated a reduction in keratometric indices following standard and A-CXL, resulting in an irregular corneal surface. With regard to CXL approach, removal of the corneal epithelium and UVA exposure causes injury to the subepithelial nerve plexus which has been demonstrated to be accompanied by diminished corneal sensitivity. Absence of corneal sensation is thought to negatively affect the reflex which triggers blinking and tear formation[12].

The cytological assessment of the conjunctival impression helps us properly evaluate the ocular surface condition. It has been demonstrated that impression cytology is a non-invasive approach used to diagnose dry eye disease by conjunctival evaluation. It assesses the goblet cell density (GCD) for mucus formation and the structure of the epithelial cells for ocular surface integrity.

A lot of diagnostic approaches demonstrated that cases with KC associated with diminished TF quality have worsening in the conjunctival epithelial structure and GCD. Of note, there is limited data as regards the cytological assessment of the ocular surface and tear functions following CXL in keratoconic cases. Our study aimed to assess whether CXL could improve the ocular surface and TF in keratoconic eyes over time and whether the worsening of the ocular surface persists or deteriorates in KC following CXL[13].

Thus, we assessed the effect of CXL on conjunctiva and corneal surface in progressive keratoconic patients.

This study was a prospective follow up study carried out on 20 patients with progressive keratoconus attending to

ophthalmic center, Mansoura university during the year 2021-2022.

In the current study, the mean age was (17.43 ± 1.165 years) ranged from 16 to 20 years.

In the current study, goblet cells were demonstrated to be decreased immediately and one month after CXL then start to increase at three months after CXL then more increased at six months after CXL.

In the same line, Uysal et al. (2020) revealed that at the three-month follow-up impression cytology displayed an initial worsening with regard to the GCD and the degree of squamous metaplasia in the superior and temporal bulbar conjunctival areas (P<0.001). The impression cytology score of the superior area improved and returned to presurgical level by eighteen-month follow-up (P>0.05). There was a significant improvement in temporal region at month eighteen compared to basal value (P<0.05)[14].

Comparable ouctomes were reported by Renesto et al., 2010, who demonstrated that cases in CXL group revealed a reduction in GCD on the superior conjunctiva following CXL (P<0.05) but no change on the temporal conjunctiva or in the cornea[15].

Akçay et al. (2017) displayed worsening in the temporal and superior bulbar conjunctival impression cytology three months following the acceleration of CXL without any damaging effects on the TF functions[16].

It was previously found that in patients with keratoconus there is a decreased of goblet cells in these patients and this is corrected after CXL[17], which has been shown in the current study.

Our study revealed that there was a significant improvement in the cohesion power along the duration of follow up. At the baseline, there were no cells grouped as a single sheet, however at six month of follow up, about 76.2% of the cells grouped as one large sheet of coherent cells.

This agreed with *Yuksel et al.* who reported that the CXL had higher stiffening effects on the cornea[18].

Of note, the anterior stroma is associated with a considerable increase in interlamellar cohesive strength (ICS) compared to the middle and posterior corneal stroma, and the CXL of anterior stroma could be sufficient for corneal strengthening.

Similar results were reported by *Jabbarvand et al.* where corneal biomechanical evaluation using the Corvis ST denoted significant changes six months after CXL in multiple parameters. These alterations reveal a stiffer cornea following CXL. Rise of corneal stiffness was revealed by a significant increase of SP-A1 and a significant reduction of integrated radius (IR) and deformation amplitude ratio[19].

Also, Hashemi and his colleagues assessed the parameters of corneal stiffness following A-CXL; their outcomes indicated significant rise in SP-A1 and decrease in IR and deformation amplitude ratio following two years[20]. In addition, Sedaghat and his colleagues observed changes in the new stiffness parameters of Corvis ST after four years, although only significant for IR values[21].

In a similar study to detect the ICS of porcine cornea before and after CXL using riboflavin and UVA irradiation by Tao et al., 2013, the mean maximum flap-stroma ICS was 0.088±0.046 N/mm in the experimental group and 0.012±0.004 N/mm in the control group (P=0.009). In experiment 2, incomplete (2 mm long) corneal flaps were utilized and the average stromal ICS was 0.750±0.077 N/mm in the experimental group and 0.338±0.046 N/mm in the control group (P<0.05). Histolopathological examination revealed an irregular ragged separation in the stromal corneal margin with CXL, but a smooth separation corneal surface without CXL[22].

In the current study, the mean baseline K1 in the included cases was 46.01 ± 2.44, at 1 month postsurgical, there was a non-statistically significant increase followed by a further, but a statistically significant increase at 3 months postsurgical. At 6 months postsurgical, the mean K1 showed a statistically significant decrease compared to the basal reading.

This agreed with *Abo Al Majd et al.* who performed a prospective study comprised 20 eyes of 18 cases with mild and moderate degree KC. CXL was done for all the cases. They showed that the results of Mean K1 diminished by 0.16 D in the 1st month, 0.17D in the second month and 0.19D in the 3rd

month; six eyes out of twenty (30%) demonstrated a drop in mean K1 compared to presurgical mean K1 reading. In eight eye (40%) mean K1 remained stable, and six eyes (30%) mean K1 increased by 0.20D compared to presurgical mean k1.[23].

In the current study, the mean baseline K2 in the included cases was 49.50±3.08, at one month postsurgical, there was a non-statistically significant increase followed by a non-statistically significant decrease at 3 months postsurgical and further statistically significant decrease at 6 months postsurgical compared to the basal reading.

In agreement, *Abo Al Majd et al.* showed that mean K2 diminished by 0.03 D in the 1st month, diminished in the 2nd month by 0.12D, and diminished in the 3rd month by 0.15D; mean K2 diminished in eleven eyes out of 20 (55%). The mean K2 is still stable in seven eyes (35%) and only in two eyes (10%) the mean K2 reading increased in comparison with the mean presurgical K2 reading by 0.25D.

On the contrary, *Caporossi et al.* and *Raiskup-Wolf et al.* demonstrated that there was an extensive drop in K values post the one-year follow-up. The maximal K-readings significantly diminished by 2.68D in the 1st year, 2.2D in the 2nd year, and 4.84D in the 3rd year[23].

In addition, this result was studied by Caporossi et al, who recorded topographic mean drop in dioptric power of approximately 2D, on the other hand, they reported initial deteriorating of keratometric readings in the first month which may be owing to transient haze and corneal oedema[24].

Saleem *et al*. showed similar results with statistically significant improvement in postsurgical K readings in both flat and steep meridian (K1 and K2) after 3 years of follow-up[25].

Uysal et al. showed that after initial deterioration at 3 months postsurgical, the median *K*-mean diminished significantly eighteen months following CXL in comparison with basal value (P<0.001). The median *K*-max value demonstrated a significant improvement throughout the eighteen-month period of follow-up (P<0.05)[26].

CONCLUSION

In conclusion, CXL seems to be efficient in reducing KC progression, with improved optical measurements in several cases. The postsurgical parameters evaluated in the current study followed a seemingly reproducible trend in their natural course over six months. In general, the trend noticed was immediate worsening between baseline and one month, resolution at about three months, and improvement thereafter. With regard to predicting outcomes following CXL, no patient characteristics demonstrated relationships with negative therapeutic outcomes, which include visual loss or continual topographic steepening. On the other hand, our study didn't include visual acuity. Such outcome predictors have to be considered when offering CXL to cases with KC or postsurgical corneal ectasia.

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