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# Assessment of the Effect of Corneal Collagen Cross-Linking on Corneal Endothelium in Patients with Keratoconus

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## ABSTRACT

**Background:** Keratoconus, was a non-inflammatory, conical ectasia of cornea that often exhibits bilateral asymmetric progression over time. **Objective:** Evaluation of corneal endothelium alterations subsequent to Corneal Collagen Cross-linking (CXL) for management of progressive keratoconus. **Patients and methods:** Included 30 eyes from twenty-four individuals diagnosed with keratoconus. Endothelial cell density (ECD), coefficient of variation (CV) and percentage of hexagonal cells (HEX) evaluated by specular microscopy. Together with central corneal thickness (CCT) recorded by using Pentacam. All were evaluated prior to CXL and one month subsequent.

**Results:** One month after CXL, the mean ECD increased from 2740.87 ± 202.56 cells/mm² to 2751.20 ± 226.35 cells/mm². The mean HEX also increased from 66.23 ± 7.54% preoperatively to 66.70 ± 7.979% postoperatively. Additionally, the mean CV decreased from 29.35 ± 4.306% preoperatively to 28.95 ± 4.495% postoperatively. However, changes in ECD, HEX, CV weren`t statistically significant (p=.281, p=.229, p=.052 respectively). CCT decreased from a mean of 476.01 ± 44.03 μm pre-CXL to 471.95 ± 43.33 μm one-month post-CXL, but again this difference was not statistically significant (p=0.06). No statistically significant relationships among duration of procedure, amount of energy and changes in CCT, ECD & HEX or CV (P > .05). Furthermore, the correlation between keratoconus stage and changes in CCT, HEX, and CV didn`t reveal any statistically significant relationships (P > .05).

**Conclusions:** No substantial alterations were seen in corneal endothelial characteristics, as assessed by specular microscopy, after cross-linking in keratoconic eyes.

**Keywords:** Keratoconus, Specular microscopy, CXL, UVA irradiation.

## INTRODUCTION

Keratoconus, was a non-inflammatory, conical ectasia of cornea that often exhibits bilateral asymmetric progression over time [1]. Keratoconus was a significant condition commonly observed in our nation, predominantly impacting younger populations. A condition that has a progressive trajectory, if ignored, may lead to substantial corneal problems, possible vision loss, attributable to irregular astigmatism and myopia, as well as corneal scarring that can impede functionality [2]. Notwithstanding comprehensive study endeavors, exact etiology of keratoconus remains inadequately elucidated. Current hypotheses propose that elevated levels of lysosomal proteolytic enzymes, lower quantities of protease inhibitors, lower CXL and increased pepsin activity may collectively lead to structural weaknesses observed in corneal tissues affected by keratoconus [3].

In initial phases of keratoconus, CXL is a secure, efficacious intervention aimed at enhancing corneal stiffness. This was accomplished by promoting supplementary cross-links within or among collagen fibers with application of ultraviolet A light, a photomediator, riboflavin. Principal objective of CXL was to decelerate, potentially stabilize, in certain instances, reverse advancement of corneal ectasia in individuals with keratoconus [4]. Early implementation of CXL may postpone or may obviate necessity for keratoplasty. Nonetheless, CXL was not devoid of problems. UVA radiation employed in process can potentially harm corneal endothelium, resulting in corneal edema. In infrequent instances, this condition may require keratoplasty [5]. CXL was performed when corneal thickness surpasses 400 microns after de-epithelization to reduce risk of corneal endothelial cell damage from UV radiation. Certain studies suggest that corneal thickness may significantly diminish during operation due to corneal dryness, potentially resulting in endothelial cell injury [6].

This study aimed to document temporary alterations in corneal endothelium subsequent to CXL for keratoconus management. The study aimed to compare corneal parameters, including CCT, CV, ECD, percentage of HEX, before and one month after corneal cross-linking (CXL) using specular microscopy to evaluate procedure's effect on corneal endothelium.

## MATERIALS & METHODS

This non-randomized study was conducted in Ophthalmology Department, Faculty of Medicine, Suez Canal University, Egypt. A total of thirty eyes from twenty-four individuals were assessed for corneal thickness, endothelial cell alterations prior to and one month following CXL via specular microscopy.

**Inclusion criteria:** Cases with keratoconus categorized as grade I & grade II according to Amsler–Krumeich classification, who exhibited proven clinical deterioration, instrumental progression. Diagnosis of keratoconus progression was determined according to criteria set out by global agreement on keratoconus ectatic disorders [7]. All cases exhibited a minimum corneal thickness surpassing 400 μm following epithelium excision, with completely transparent corneas, no ocular or systemic diseases present. Age range of cases was eighteen to forty years.



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**Exclusion criteria:** Cases where corneal thickness at narrowest point was less than 400 µm. A history of herpetic keratitis, concurrent corneal infections, preexisting autoimmune disorders, acute hydrops, severe allergic conjunctivitis, keratoconuscataracts, glaucoma, diffuse central corneal opacity, significant dry eye, advanced and vitreoretinal conditions. Also, people with mental instability, those who recently used contact lenses and those who were pregnant or nursing.

**Before surgery, all cases underwent:** (a) A thorough history assessment that included personal demographic information, presenting complaints, both current and past medical conditions to exclude individuals with collagen disorders & diabetes mellitus, or hormonal changes such as pregnancy. (b) Ophthalmic history [Prior ocular injuries, surgical procedures, allergies and a history of wearing glasses or contact lenses (all individuals with a history of contact lens use were advised to discontinue lens usage for at least two weeks before examination)]. (c) Comprehensive ophthalmic examination included: External assessment for identification of pathologies such as ptosis, lid hemangioma, UCVA, BCVA, IOP measurement via applanation tonometry, posterior segment evaluation for abnormalities using indirect ophthalmoscopy and slit lamp biomicroscopy with an auxiliary contact lens. (d) Corneal assessment employing corneal tomography (Pentacam) to evaluate CCT, corneal endothelial cell analysis through noncontact specular microscopy, concentrating on following parameters: Coefficient of variation (CV percent) of cell size, corneal endothelial cell density (ECD), percentage of hexagonal cells (HEX) prior to and one month following CXL.

**Surgical technique:** A 0.1% riboflavin solution was produced by diluting a 0.5% solution of vitamin B2 riboflavin-5 phosphate with dextran T500. Solution was protected from light & employed within twelve hours. A topical anaesthetic was applied before treatment with benoxinate eye drops. Corneal epithelium was manually dissected over an eight-millimeter diameter area utilizing a blunt tool (Hokey knife), a 0.1% riboflavin solution was intermittently supplied for roughly thirty minutes. Corneal penetration, presence of riboflavin in anterior chamber (riboflavin shielding) were assessed using slit-lamp examination with a blue filter. UVA irradiation was conducted utilizing an optical system (Kohler illumination) comprising a light source consisting of seven UV diodes (365 nm; Nichia, Nuernberg, Germany) and a series potentiometer for voltage adjustment.

Prior to treatment, a target irradiance of three mW/cm² (5.4 J/cm² surface dosage) was calibrated with a UVA meter (LaserMate-Q; LASER 2000, Wessling, Germany) at a distance of one centimeter. Irradiance was administered for thirty minutes at a power density of three mW/cm², resulting in a total dosage of 5.4 J/cm². Throughout treatment, riboflavin solution was administered every three minutes to ensure full saturation of cornea with riboflavin.

Following therapy, a bandage contact lens was employed until corneal epithelium is fully regenerated, often within three days. Postoperatively, cases received topical antibiotics along with artificial tear substitutes.

**Postoperative therapy protocol:** Subsequent to surgery, moxifloxacin hydrochloride 0.5% (Vigamox, Alcon, Texas, USA) eye drops were supplied hourly for a duration of five days. Instill Nevanac nonsteroidal anti-inflammatory drops five times daily for a period of five days. It was recommended to deliver preservative-free artificial tears 4 times daily for 1 month. Bandage contact lens was extracted upon completion of epithelialization, often after four days. After one month, we re-examined all cases utilizing same instruments by specular microscopy. Following removal of contact lens, cases were prescribed a topical mixture of steroid and antibiotic to be administered six times daily, with a gradual reduction over subsequent six weeks, in conjunction with a topical lubricant substitute.

**Ethical considerations: The data that were collected from participants were confidential. The research participants weren't identified by name in any publication or report that addressed this research. The nature and goal of the research, as well as the risk-benefit evaluation have been explained to the participants prior to their admission to this study. Informed consent has been obtained from each participant. Approval of Ethics committees of Ophthalmology Department in Faculty of Medicine, Suez Canal University, was acquired. The study adhered to Helsinki Declaration throughout its execution.**

***Statistical analysis***

SPSS (Statistical Package for Social Sciences) version 20 for Windows® (IBM SPSS Inc., Chicago, IL, USA) was used to encode, process and analyze collected data. Shapiro-Wilk test was used to determine whether data distribution was normal. Quantitative information was displayed as mean ± SD. Samples in pairs of two dependent groups of regularly distributed variables (parametric data) were compared using t-test, two dependent groups of non-normally distributed variables (non-parametric data) were compared using Wilcoxon signed-rank test. To examine link between two variables, Pearson correlation coefficient was used. A P-value ≤ 0.05 was considered significant for every test on list.

## RESULTS

Demographic data of analyzed population comprised thirty eyes from twenty-four cases (thirteen men & eleven women), with a mean age of 29.67 ± 6.1 years (range: eighteen to thirty-eight years).

No problems were noted during follow-up period. In terms of keratoconus staging, thirteen eyes (43.3%) were categorized as stage I, while seventeen eyes (56.7%) were categorized as stage II (Table 1).

**Table (1):** Demographic characteristics & BCVA studied cases

| All patients (n= 24) | | Mean & SD | Median | Range | IQR |
|---|---|---|---|---|---|
| Age (years) | | 29.67 ± 6.112 | 31.50 | 18.00, 38.00 | 26.25, 34.00 |
| Pre-operative BCVA | | 0.63 ± 0.127 | 0.67 | 0.33, 0.83 | 0.50, 0.67 |
| Gender | Male | 41.7% (10) | | | |
| | Female | 58.3% (14) | | | |

**Specular microscopy data** illustrated relationship between pre-CXL and one-month post-CXL specular microscopy measurements concerning CCT, ECD and CV. Study indicated no statistically significant differences among these factors (P > 0.05).

**Table (2):** Preoperative, one-month postoperative specular microscopy measurements of studied cases

| All eyes (n= 30) | Preoperative | One month Postoperative | 95% CI | P |
|---|---|---|---|---|
| CCT | 476.01 ± 44.037 | 471.95 ± 43.330 | -0.28, 8.40 | 0.066 |
| Endothelial cell density | 2740.87 ± 202.56 | 2751.20 ± 226.35 | -29.59, 8.92 | 0.281 |
| Hexagonal cells | 66.23 ± 7.546 | 66.70 ± 7.979 | -1.24, 0.31 | 0.229 |
| CV | 29.35 ± 4.306 | 28.95 ± 4.495 | -0.01, 0.8 | 0.052 |

Relationship among process length, energy consumption and concerning variations in CCT, ECD, HEX & CV in examined eyes was outlined in tables (3 & 4) respectively. Research revealed no statistically significant correlations among operation length, energy levels and alterations in CCT, ECD, HEX, or CV (P > 0.05).

**Table (3):** Correlation among duration of procedure and changes in studied parameters

| All eyes (n= 30) | Correlation coefficient | P |
|---|---|---|
| CCT change | 0.043 | 0.823 |
| Endothelial cell count change | 0.117 | 0.539 |
| Hexagonal cells change | 0.072 | 0.706 |
| CV change | 0.272 | 0.146 |
| P is significant when < 0.05. | | |

Table (4) shows the correlation between amount of energy and changes in the studied eyes regarding CCT changes, Endothelial cell count changes, Hexagonal cells changes and CV changes and it does not show any statistically significant relation between the amount of energy and those parameters (P>0.05).

**Table (4):** Correlation among amount of energy and changes in studied parameters

| All eyes (n= 30) | Correlation coefficient | P |
|---|---|---|
| CCT change | -0.090 | 0.635 |
| Endothelial cell count change | -0.056 | 0.767 |
| Hexagonal cells change | -0.071 | 0.709 |
| CV change | -0.271 | 0.147 |
| P is significant when < 0.05. | | |

Related among keratoconus stages, alterations in CCT, HEX and CV did not demonstrate any statistically significant associations with keratoconus stage (P > 0.05). Alterations in ECD were statistically substantially correlated with stage of keratoconus (r = 0.491, P < 0.05) (Table 5).

**Table (5):** Correlation between keratoconus stage and change in the studied parameters

| All eyes (n= 30) | Correlation coefficient | p |
|---|---|---|
| CCT change | -0.043 | 0.821 |
| Endothelial cell count change | **0.491** | **0.006** |
| Hexagonal cells change | 0.035 | 0.854 |
| CV change | 0.114 | 0.548 |
| P is significant when < 0.05. | | |

## DISCUSSION

This prospective study encompassed thirty eyes from twenty-four cases diagnosed with keratoconus and aimed to assess alterations in corneal endothelium subsequent to corneal cross-linking (CXL) for management of progressive keratoconus. Effects of CXL on corneal endothelium have been thoroughly examined. This process involved a photochemical reaction in which UVA light activates riboflavin. Activation of riboflavin enhances integrity of corneal collagen fibers arresting advancement of keratoconus.

This study revealed that CCT decreased postoperatively compared to preoperative values, this shift lacked statistical significance. These results align with another previous research [8, 9]. A recent meta-analysis encompassing eleven trials yielded analogous findings [10]. A prospective non-randomized investigation involving sixty-eight eyes from forty two cases with keratoconus reported no statistically significant difference in central corneal thickness (CCT) postoperatively, with preoperative, postoperative CCT values of 470 ± 40 µm, 469.8 ± 42 µm, respectively ($p = 0.591$) [11]. Two prospective investigations with cohorts of twenty-eight eyes, thirty eyes, respectively, reported a substantial reduction in CCT over one year of follow-up, yielding $p < 0.05$, 0.001, respectively [12, 13]. Inconsistencies may stem from differences in sample sizes and follow-up periods between studies. Abbreviated follow-up duration in our investigation may have affected results, as shown by **Salman *et al*.** [14] who noted a substantial decrease in CCT after 1 month, succeeded by a non-significant alteration at twelve months. This pattern may suggest initial apoptosis after treatment, succeeded by gradual repopulation of corneal cells over time.

Mechanism responsible for postoperative corneal thinning may be complex, encompassing anatomical, structural alterations [15]. Contributing factors may encompass changes in corneal moisture, reorganization of stromal collagen bundles, keratocyte apoptosis [16]. Ocular ischemia has been recognized as a possible contributing component [17].

Present investigation revealed no statistically significant difference among mean preoperative and postoperative endothelial cell density (ECD). This finding aligns with a study of thirty keratoconic eyes from twenty-three cases, which documented mean preoperative and postoperative endothelial cell counts of 3221.40 ± 243.16 cells/mm² and 3200.42 ± 247 cells/mm² respectively, revealing no statistically significant difference among these values [18]. Similarly, **Arora *et al*.** [13] did research on thirty eyes with a one-year follow-up and observed no statistically significant variation in endothelial cell counts following cross-linking technique. Our findings align with previous studies indicating that mean preoperative ECD was 2963.1 ± 364.1 cells/mm², whereas mean ECD at one month postoperatively was 2956.96 ± 363 cells/mm² demonstrating a statistically non-significant difference ($P = 0.70$).

A study of twenty-three juvenile eyes with progressive keratoconus, monitored over twelve months indicated a substantial reduction in endothelial cell density after corneal cross-linking. This study reported a preoperative ECD of 3212 ± 331.1 cells/mm² , a postoperative ECD of 3188 ± 147.1 cells/mm² ($p < 0.05$) [20]. Other researchers have shown a substantial decrease in ECD after corneal CXL, with a p-value of 0.004. Minimal reduction in cell density occurred in cases with corneal thickness below 450 µm, who, significantly, possessed thinnest corneas [11].

Present investigation found no statistically significant difference among mean preoperative, postoperative HEX. This discovery aligns with research conducted by **Razmjoo *et al*.** [11], which examined endothelial specular microscopy results in sixty eight keratoconic eyes prior to and 1 year subsequent to CXL. Preoperative HEX percentage was reported as 54.14%, which rose to 54.55% postoperatively ($P = 0.517$), signifying no significant alteration in cell morphology.

A prospective, non-randomized controlled interventional investigation revealed that mean percentage of hexagonal cells in eyes subjected to CXL was 65.95 ± 8.07% preoperatively and 67.23 ± 9.11% 1 month postoperatively, with no significant difference among these values ($p = 0.475$) [21].

**Elgazzar *et al*.** [19] found analogous findings, with a mean preoperative HEX percentage of 49.93 ± 7.9% and a mean postoperative value of 48.5 ± 7.3% at 1 month, indicating no statistically significant difference ($p = 0.20$) [19].

Present investigation revealed no statistically significant variation among mean preoperative and postoperative hexagonal cell counts. In accordance with these findings, **Razmjoo *et al*.** [11] analyzed endothelium specular microscopy results in sixty-eight keratoconic eyes prior to and one year after to CXL. Preoperative HEX, indicative of pleomorphism, was recorded at 54.14 ± 6%, which rose to 54.55% postoperatively ($P = 0.517$), signifying no substantial alteration in cell morphology. A prospective, non-randomized controlled interventional investigation indicated that mean percentage of hexagonal cells in eyes subjected to CXL was 65.95 ± 8.07% preoperatively and 67.23 ± 9.11% 1 month postoperatively, with no statistically significant difference among 2 measurements ($P = 0.475$) [21]. **Elgazzar *et al*.** [19] reported similar findings, revealing a mean preoperative HEX of 49.93 ± 7.9% & a mean postoperative value of 48.5 ± 7.3% at 1 month with no statistically significant change ($P = 0.20$). In contrast to current investigation, an analysis of endothelial specular microscopy results in thirty keratoconic eyes prior to and six months subsequent to standard CXL demonstrated a significant reduction in hexagonal cell percentage (HEX), with mean preoperative and postoperative HEX values of 41.73 ± 5.08% & 38.13 ± 5.24% respectively ($P < 0.05$) [18].

Present investigation found no statistically significant difference among mean preoperative and postoperative CV%. This is consistent with a prospective, nonrandomized study involving thirty eyes with keratoconus that indicated a mean preoperative CV% of 36.3 ± 5.9% and 36.2 ± 5.6% 1 month postoperatively with a statistically insignificant difference ($p = 0.69$) [19]. Also, results align with another study that reported a mean CV% of 29.43 ± 5.35% preoperatively & 30.03 ± 6.01% 1 month postoperatively with no significant difference ($p = 0.38$) [21]. In contrast, a prospective non-randomized investigation demonstrated that cell size variability (polymegathism), shown by CV% dramatically rose from 32.72 ± 10.14% preoperatively to 40.21 ± 9.7% postoperatively ($p = 0.021$) [11]. Other investigators who assessed thirty keratoconic eyes from twenty-three cases regarding corneal thickness & endothelial cell alterations before and 6 months subsequent to CXL via specular microscopy observed a notable rise in CV% from 38 ± 3.70 preoperatively to 41.71 ± 2.74% postoperatively ($p < 0.05$) [18].

Conflicting outcomes were noted in previously stated investigations, perhaps attributable to differing protocols of UVA energies employed. At present, no unified defined methodology for CXL was available. A larger quantity of randomized clinical trials was necessary to thoroughly assess each strategy. Variations in software utilized for assessing corneal endothelial cells, viewing angle and cell enumeration with reproducibility of data may account for inconsistencies reported, especially in CV & HEX metrics.

This investigation revealed a negative relation among UVA energy levels and alterations in CCT, ECD, HEX & CV in examined eyes. These relationships lacked statistical significance ($p > 0.05$). Increased UVA radiation intensity may cause injury to nerve plexus, thereby hindering endothelial pump function. Sub-basal nerve plexus secretes neuropeptides, including calcitonin gene-related peptide and substance P, which enhance signal transmission via Na/K-ATPase pumps in corneal endothelium [22]. Presence of corneal endothelial alterations indicates potential damage to endothelium, while it was not completely gone or necrotic. Recovery occurs when intact endothelium glides in and compensates for function in area of injured endothelium. Duration of post-CXL recovery phase seems to be defined and may resemble healing reported following photorefractive keratectomy [23].

This study found no statistically significant correlation among keratoconus stage & changes in CCT, HEX and CV. Alterations in ECD exhibited a statistically significant correlation with stage of keratoconus. Likewise, a prior research has identified a notable decrease in ECD during advanced stages in comparison to mild & moderate stages of keratoconus [24, 25]. This may elucidate why extent of postoperative alteration in ECD could be greater in instances with stage II keratoconus, suggesting a positive relation.

This study's primary merits were its prospective design, inclusion of a single eye per cases, & application of epi-off approach. Study possesses drawbacks. Limitations encompass a limited sample size, sourcing of cases from a singular location and lack of a control group. An extended follow-up duration was crucial to validate stability of reported results. Study was limited by unavailability of most recent technology for CXL and thereby affecting generalizability of results.

## CONCLUSION

CXL significantly impacted corneal endothelium. Specular microscopy was definitive method for assessing corneal endothelial integrity following CXL. To enhance assessment of corneal volume as a marker of endothelial dysfunction post-CXL, extended follow-up durations and bigger sample sizes were necessary.


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