

## EFFECT OF ACCELERATED CORNEAL COLLAGEN CROSS-LINKING ON KERATOCONUS ENDOTHELIUM CELLS

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### ABSTRACT

**Purpose:** Evaluating the safety of Accelerated Corneal Collagen Cross-Linking on Keratoconus Endothelium cells. **Methods:** In this prospective study, (50) eyes of (27) progressive Keratoconus patients underwent Accelerated Corneal Collagen Cross-Linking (ACXL), (15) Epithelium-off (Epi-off) and (12) Epithelium-on (Epi-on) in the Ophthalmology Department in Tishreen University Hospital. Endothelial specular microscopy was performed on (50) eyes before, after 1, 3, and 6 months of ACXL. Studied parameters included endothelial cell density (ECD), coefficient of variation (CV) in cell area, percentage of hexagonality (EX) and corneal thickness. **Results:** Mean endothelial cell densities before and after six months of ACXL were  $2702.6 \pm 242.2$  and  $2697.8 \pm 280.2$ , respectively ( $p = 0.4$ ). Mean coefficient of variation before and after ACXL were  $29.3 \pm 4.3$  and  $28.8 \pm 4.08$  respectively ( $p = 0.09$ ). Mean percentage of hexagonal cells before and after ACXL were  $59.9 \pm 7.06$  and  $58.5 \pm 7.6$  ( $p = 0.09$ ), respectively. By comparing the changes of the endothelial cell parameters between the two ACXL groups, the intensity at the end of the follow-up in the Epi-off and Epi-on groups were ( $2781.5 \pm 250.8$  and  $2702.8 \pm 317.2$ ), respectively ( $p = 0.3$ ). The coefficient of cell volume at the end of follow-up in the Epi-off and Epi-on groups, was ( $29.4 \pm 4.1$  and  $28.4 \pm 4.2$ ), respectively ( $p = 0.4$ ). Average percentage of hexagonal cells at the end of follow-up in the Epi-off and Epi-on groups ( $57.08 \pm 9.4$  and  $61.6 \pm 5.5$ ), respectively ( $p = 0.6$ ). Corneal thickness at the end of follow-up in the Epi-off and Epi-on groups ( $462.2 \pm 46.5$  and  $454.9 \pm 41.7$ ) respectively ( $p = 0.2$ ). **Conclusion:** Accelerated Corneal Collagen Cross-Linking, including Epi-off and Epi-on, doesn't have a significant effect on corneal endothelial cells density or morphology in this study. Transient changes, that are statistically insignificant in both types, are less valuable in Epi-on ACXL.

**KEYWORDS:** Keratoconous, Accelerated Corneal Collagen Cross-Linking, Corneal Endothelial Cells.

### INTRODUCTION

Mature Corneal Endothelial Cells (CEC) are thought to have no proliferative capacity.<sup>[1]</sup> The underlying reasons to believe that mature CECs are not regenerated are cell-cell contact inhibition, and negative cell life cycle modifiers Like cyclin-dependent kinase inhibitors, p27kip1 and p15INK4b. Moreover, there are growth inhibitors in the aqueous humor such as Transforming Growth Factor B (TGFB). The last reason is, early aging induced by stress.<sup>[2,3,4]</sup> On the other hand, the study of Yam (Yam et al., 2019) demonstrates the existence of a new internal transition zone containing progenitor-like cells that could provide the regenerative capacity of the corneal endothelium.<sup>[5]</sup> Lately Sie study (Sie et al., 2020) states that there is evidence which indicates that the endothelium periphery contains less differentiated cells. Sie study also signals some authors who suggest that

these cells are precursors of endothelial cells and precursors of cells of the trabecular network. The migration of these cells may occur after the injury; and this migration may be age dependent.<sup>[1]</sup> Therefore, we conclude that the presence of endothelial progenitor-like cells in the peripheral endothelium and the adjacent transitional zone near the posterior limbus supports the hypothesis that the corneal endothelium is somehow able to repair the deficiency of subsequent lesions.

Although the combined use of riboflavin with UVA reduces the toxic effect of radiation by about 10 times,<sup>[6]</sup> the keratoconus endothelium remains vulnerable, as studies of the endothelial cells of keratoconus patients have shown that it is unstable and predisposed to damage,<sup>[7]</sup> represented by the oxidative stress associated with CXL. The effect of localized oxidative stress is not

limited to the stroma, oxidative radicals may reach the posterior surface of the cornea, and thus the structures such as the endothelium become vulnerable to necrosis or apoptosis. Note that the effect of this oxidative stress does not reach the endothelium except to a small percentage.<sup>[8]</sup>

## PATIENTS AND METHODS

- **Study design:** a descriptive prospective study.
- **Place and time of study:** Tishreen University Hospital in Lattakia, between 2019-2020.
- **Sample size:** 27 patients (50 eyes) of progressive keratoconus (PKC) patients attending the refractive specialist ophthalmic clinic at Tishreen University Hospital in Lattakia.

### Entry criteria

1. Keratoconus topography confirmed (grade I and grade II of the Amsler-Krumeich classification), provided that contact lenses should be stopped before the topography by two weeks for the soft lenses and one month for the rigid lenses.
2. The progression is documented by an increase of more than 1 diopter in keratometry readings and a reduced central corneal thickness of 10  $\mu\text{m}$  during  $\geq$  3- 6 months of follow-up.
3. Minimum corneal thickness > 400 microns.
4. A completely transparent cornea without any accompanying eye disease.
5. Age between 12-34 years old.

### Exclusion criteria

1. Previous corneal diseases: herpes simplex keratitis, active corneal infection.
2. Autoimmune diseases: hypothyroidism and others.
3. Associated ophthalmic diseases: severe dry eyes, severe allergic conjunctivitis, central corneal densities.
4. Advanced Keratoconus (Grade III and Grade IV of the Amsler-Krumeich Classification).
5. Glaucoma, cataract and vitreoretinal diseases.
6. Pregnancy and breast-feeding.
7. The presence of ailments in the lining of the cornea.

### Eye examinations performed before Corneal Cross-Linking

1. Detailed clinical story.
2. Measurement of uncorrected visual acuity and optimal eyeglass correction using the Snellen chart.
3. Measurement of the refractive errors using an Autorefractometer (Grand Seiko GR-3500KA, Japan).
4. Slit lamp examination of the anterior and posterior sections of the eye.
5. A Sirius corneal topography.
6. Conducting a study of corneal endothelium cells with a non-contact, Perseus, Specular microscope.

### The Corneal Cross-Linking protocol used in this study

1. A local anesthetic is used, followed by 4% povidone sterilization, and a sterile eyelid speculum.
2. Riboflavin is distilled:
  - In Epi-on patients: instillation is made every 30 seconds for 15 minutes with a fast-acting ophthalmic solution of high-soluble riboflavin phosphate with 10% dextran with a diffusion promoter vitamin E TPGS.
  - In Epi-off patients: First 20% diluted alcohol is applied to the central 8 mm, then the epithelium is scraped with a micro-dryer or a spatula. Then dripping every 3 minutes for 30 minutes of a solution of Dextran-riboflavin; It is 0.2 ml of riboflavin-5-phosphate at a concentration of 0.5%, with dextran T-500 20% (0.2 g), two solutions in 0.8 ml of Saline physiological solution 0.9%.
3. Then, UVA rays are applied to all patients with an exposure diameter of 8 mm, at a height of 5 cm from the cornea, and (9 mw /cm<sup>2</sup>) for 10 minutes (Accelerated Corneal Cross-Linking) with the PXL device SAPPHER 318.

### Management after Corneal Cross-Linking

- For Epi-off patients, a bandage contact lens will be placed for 4 days.
- All patients will be provided with the following supplies:
  - Antibiotic drop.
  - Steroid drop.
  - Artificial tear drop.
  - Pain reliever if needed.

**Studied parameters** endothelial cell density (ECD), coefficient of variation (CV) in cell area, percentage of hexagonality (EX) and corneal thickness are taken (preoperatively, one month, 3 months, 6 Months postoperatively).

**Statistical analysis:** The analysis was performed using the Statistical Package for Social Sciences (SPSS) (version 20) (IBM Corporation, Armonk, New York, USA) and Excel 2010 program. A predictive value less than 0.05 was considered statistically significant. Basic Descriptive statistics included means, standard deviations (SD), Frequency and percentages. Statistical analyses were performed using the Friedman Test, and Independent T student.

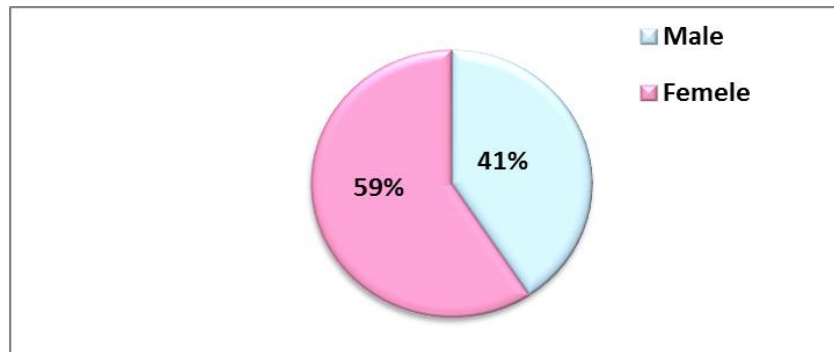
## RESULTS

### 1. Sample distribution

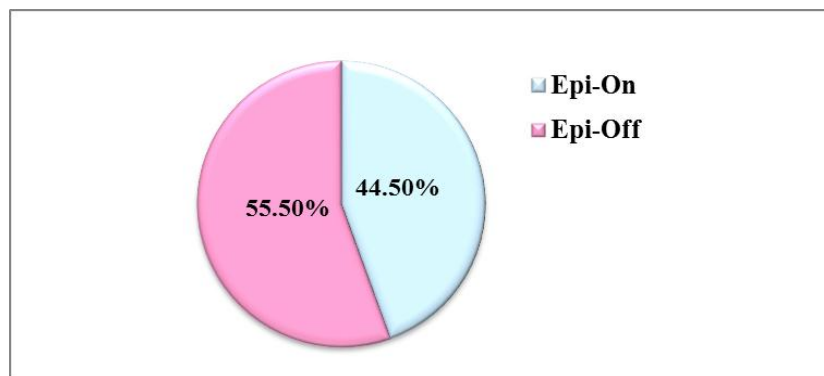
27 patients (50 eyes) of progressive keratoconus participated in this study. They had visited the specialist ophthalmic clinic at Tishreen University Hospital in Lattakia during 2019-2020. These patients met the inclusion criteria of the study. The number of females is 16 patients (59.3%), and the number of males 11 patients

(40.7%). See Graph 1. The ages ranged from 12 to 34 years, and the average age was  $21.2 \pm 5.5$  years. The

sample is divided into two groups: Epi-on (12 patients) and Epi-off (15 patients) graph2.



Graph 1: Distribution of the sample by gender: A sample of 27 keratoconus patients who underwent ACXL was distributed according to gender with a ratio of Sex Ratio (F: M) = 1.4: 1.



Graph 2: Sample distribution according to the ACXL type applied.

2. Mean differences in the Endothelial Cells parameters in the study sample

Table 1: The mean differences in the endothelial cells density preoperative and postoperatively (1- 3-6 months) in progressive keratoconus patients undergoing ACXL at Tishreen University Hospital in Lattakia 2019-2020.

Time	Density(Mean ± SD)	Range	P-value
Preoperative	2702.6±242.2	2167-3218	0.4
1st month	2654.1±422.7	1990-3100	
3rd month	2684.2±305.4	2054-3160	
6th month	2697.8±280.2	2081-3290	

Table 2: The mean differences in coefficient of variation (CV) preoperative and postoperatively (1- 3-6 months) in progressive keratoconus patients undergoing ACXL at Tishreen University Hospital in Lattakia 2019-2020.

Time	CV (Mean ± SD)	Range	P-value
Preoperative	29.3±4.3	22-39	0.09
1st month	31.2±5.3	22-52	
3rd month	29.3±4.4	23-41	
6th month	28.8±4.08	23-38	

Table 3: The mean differences in percentage of hexagonality (EX) preoperative and postoperatively (1- 3-6 months) in progressive keratoconus patients undergoing ACXL at Tishreen University Hospital in Lattakia 2019-2020.

Times	EX (Mean ± SD)	Range	P-value
Postoperative	59.9±7.06	43-71	0.7
1st month	56.7±9.7	36-73	
3rd month	57.08±9.6	32-71	
6th month	58.5±7.6	41-72	

**Table 4: The mean differences in corneal thickness preoperative and postoperatively (1- 3-6 months) in progressive keratoconus patients undergoing ACXL at Tishreen University Hospital in Lattakia 2019-2020.**

Times	Corneal thickness (Mean ± SD )	Range	P-value
Postoperative	475±40.3	401-594	0.06
1st month	461.2±40.9	389-540	
3rd month	458.2±46.3	358-562	
6th month	460.2±41.9	399-564	

### 3. Comparison of changes in endothelial cell parameters between the two types of ACXL

**Table 5: Comparison of changes in corneal endothelial cell density before and after ACXL, according to the ACXL type applied to progressive keratoconus patients at Tishreen University Hospital in Lattakia 2019-2020.**

Times	ACXL Type		P-value
	Epi-off	Epi-on	
Preoperative	2781.7±193.3	2711.6±332.2	0.5
1st month	2621.8±526.3	2648.6±348.9	0.8
3rd month	2659.7±387.5	2661.6±345.9	0.5
6th month	2781.5±250.8	2702.8±317.2	0.3
P-value	0.9	0.4	

**Table 6: Comparison of changes in CV before and after ACXL, according to the ACXL type applied to progressive keratoconus patients at Tishreen University Hospital in Lattakia 2019-2020.**

Times	ACXL Type		P-value
	Epi-off	Epi-on	
Preoperative	30.1±4.2	29.7±4.8	0.8
1st month	32.9±5.8	30.3±5.5	0.06
3rd month	30.2±4.9	28.6±4.06	0.2
6th month	29.4±4.1	28.4±4.2	0.4
P-value	0.2	0.8	

**Table 7: Comparison of changes in EX before and after ACXL, according to the ACXL type applied to progressive keratoconus patients at Tishreen University Hospital in Lattakia 2019-2020.**

Times	ACXL Type		P-value
	Epi-off	Epi-on	
Preoperative	58.7±5.6	62.2±5.4	0.9
1st month	52.08±11.5	60.5±6.1	0.07
3rd month	54.1±11.9	60.6±6.9	0.2
6th month	57.08±9.4	61.6±5.5	0.6
P-value	0.9	0.6	

**Table 8: Comparison of changes in corneal thickness before and after ACXL, according to the ACXL type applied to progressive keratoconus patients at Tishreen University Hospital in Lattakia 2019-2020.**

Times	ACXL Type		P-value
	Epi-off	Epi-on	
Preoperative	481.5±41.7	456.06±41.6	0.07
1st month	458.6±44.7	454.5±42.4	0.6
3rd month	458.7±53.1	453.2±39.9	0.9
6th month	462.2±46.5	454.9±41.7	0.2
P-value	0.06	0.2	

## DISCUSSION

Throughout this study, we have found that Accelerated Corneal Cross-Linking (ACXL) causes a transient and statistically insignificant change in the evaluated criteria of endothelial cells. We haven't notice any difference in the results between the two types of (ACXL), but these transient changes are of less value in the Epithelium-on

(Epi-on) type. As mentioned previously, the corneal endothelium of keratoconus patients is unstable and susceptible to damage,<sup>[7]</sup> by ultraviolet rays type (A) (UVA) and the resulting oxidative stress. Despite the beneficial and desirable effect of Corneal Cross-Linking (CXL) in increasing the stiffness of the cornea and inhibiting further progression of keratoconus, the

oxidative stress associated with this photochemical reaction has highlighted the importance of conducting further studies to investigate the integrity of the endothelium after (CXL). Nevertheless this oxidative stress effect does not reach the endothelium but in a small percentage.<sup>[8]</sup> The results of this study come in accordance with some studies, but differ with some others, taking into consideration the difference in the cross-linking protocol, follow-up periods, and sample size.

Our results are consistent with some of the results reached by (Cingü et al., 2013),<sup>[9]</sup> and (Badawi et al., 2016),<sup>[10]</sup> and (Razmjoo et al., 2015).<sup>[11]</sup>

Cingü study includes (36) patients with progressive keratoconus, who have underwent Epithelium-off (Epi-off) ACXL. This procedure includes irradiating for 5 minutes with UVA 18 mW / cm<sup>2</sup>. During the irradiation time, riboflavin is applied every 2 minutes. The age range is 11 to 32 years. preoperatively endothelial cell counts are performed on the treated eye and the fellow eye for each patient, at the first week, the first month, third and sixth months after the procedur. Cingü has found a significant change in the endothelial cell density (ECD), coefficient of variation (CV) in cell area, percentage of hexagonality (EX) in the first week and first month after the procedure in the treated eye compared to preoperative values, and the values of the fellow untreated eye. This conforms with the results of this study, which also finds that this change occurs in the first month after the (ACXL), but statistically insignificant values. According to Cingü, The corneal endothelial cell count returns to preoperative values in the sixth month. whereas the EX and the CV go back to preoperative values in the third month. In this study, the gradual change of all variables starts from the third month fulfilling returning to close values of those preoperative ones in the sixth month. considering the corneal thickness values, Cingü refers to its significant increase after one month of (ACXL). these values decrease gradually to reach values close to the preoperative values. however, this study implies the occurrence of 2.9% decrease in corneal thickness after the first month of the (ACXL) compared with the value before the procedure. afterwards, there has been a return to a value closer to the one before the procedure after only 6 months, with no statistic significant differences. This divergence in results between us may be due to the applied energy differences between the two studies. it is (9 mw/cm<sup>2</sup>)in this study but (18 mw/cm<sup>2</sup>)in Cingü's study.

On the other hand, Badawi found a significant decrease in the number of endothelial cells, especially in the third and sixth months after the procedure. In addition to that, She found a statistic significant increase in CV in the third and sixth months postoperation compared to the values preoperation, with a slight change in EX. All the previous mentioned changes decreased in patients'

sample at the end of the follow-up year. Our study agreed with Badawi's the negative effect of the ACXL on the density and morphology of the endothelium cells. the values returned to numbers close to the baseline of the variables after 6 months in our study but one year in Badawi's. Badawi noticed a statistic significant decrease in thickness after one year of follow-up. In our study, we observed a clinical similar decline in the Epi-off group, but it wasn't statistically significant at the end of the sixth month follow-up period; acknowledging that our study did not include monitoring changes after one year.

It is common knowledge in ophthalmology that the corneal endothelial cells are not renewed; Due to its lack of ability to divide. Hence, Badawi attributes her results, which comply clinically with ours, to measurement errors or to cell rearrangements that may mask the actual damage of the endothelial cells. However, we justify our findings depending on the latest promising studies that mentions the existence of "peripheral endothelial Progenitor-like cells".<sup>[1,5]</sup> Hopefully, we believe these results stem from the presence of some induction of these Progenitor-like cells to differentiate into mature endothelial cells to compensate this slight subsequent loss after CXL. We think that this does not contradict with the already known mechanism of endothelial healing by sliding and enlarging the healthy cells to replace the function in the damaged endothelium area.<sup>[12]</sup> In other words, the peripheral endothelial Progenitor-like cells generate mature cells which will locate peripherally, pushing the adjacent cells of the lesion to perform that well-known mechanism.

Moreover, there is a study conducted by Razmjoo. He has observed a slight decrease in the corneal endothelium cell density ( $p = 0.004$ ), and an increase in the coefficient of cell volume (CV) ( $P = 0.021$ ) after one year of follow-up, without noticing a significant change in corneal thickness ( $P = 0.591$ ) and the proportion of hexagonal cells (EX)( $P = 0.517$ ). We have found similar results in the Epi-off group and in the whole study sample in terms of no significant change in thickness and EX, but we differ in the endothelial cell density decrease. This decrease is statistically significant after a year of follow-up in the Razmjoo sample. while the decrease in our study sample happens during the first month, then gradually starts to increase in the third and sixth months of follow-up, without being statistically significant. Endoscopic microscopy measurements may be affected by mild corneal edema and the epithelium healing process occurring within Epi-off group. Considering the coefficient of cell volume change, we agree with the increase in the early follow-up period and differ in the final outcome at the end of the follow-up. That means, the increase declines in our sample but continues in Razmjoo's. The previous mentioned differences may be due to the difference in the length of the follow-up period, as well as that the changes occurred after the sixth month in our sample have not been followed up. In



addition to that, there is a difference in the used CXL protocol.

On the other side, the results of our study differ with the results of ElSayed study (ElSayed et al., 2019).<sup>[13]</sup> No significant difference has been found in the values of central thickness and endothelial cell density before and after CXL. That is, ElSayed has not noticed a transient change as we have found in our study. The difference may be due to the different CXL protocol used. Despite applying the same total energy dose, the irradiation intensity per the unit of time is higher in the accelerated protocol.<sup>[11]</sup> However, the CV and the EX have decreased significantly. That is similar to the findings of this study in the Epi-off group and in the total sample at the first month of follow-up, but it is statistically insignificant. The difference in statistical significance may be due to the small sample size of the ElSayed study or the fact that the parameter values of the patients of our study are centered around the mean values.

The difference in results in all these studies can be attributed to several reasons such as the differences in race, the age groups included, the devices used in the study, and the sample size.

One of the limitations of our study and the other reference studies is that they assess the changes of only the central part of corneal endothelium. However, (Goukon et al., 2020).<sup>[14]</sup> studies the effect of CXL on the periphery of the cornea, and has found no effect on the density and shape of the surrounding corneal cells. This supports our view of the safety of CXL, based on the possibility of the integrity of endothelial Progenitor-like cells located in the peripheral cornea, which are supposed to support endothelial healing as mentioned in the context of the theoretical section of this study.

## CONCLUSION

Accelerated Corneal Collagen Cross-Linking, including Epi-off and Epi-on, doesn't have a significant effect on corneal endothelial cells density or morphology in this study. Transient changes, that are statistically insignificant in both types, are less valuable in Epi-on ACXL.

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