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# Qualitative Investigation of Corneal Changes after Accelerated Corneal Collagen Cross-linking (A-CXL) by *In vivo* Confocal Microscopy and Corneal OCT

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## Abstract

**Purpose:** To assess qualitative micro-morphological corneal changes by confocal microscopy and corneal OCT after accelerated corneal crosslinking (A-CXL) in keratoconic patients.

**Study design:** Prospective non-randomized pilot study.

**Methods:** 20 eyes of 20 patients, aged between 13 and 26 years (mean 22.6 years) underwent A-CXL by the KXL UV-A source (Avedro Inc. Waltham MA, USA). Patients were divided into 4 groups according to different riboflavin solutions and UV A powers.15 patients underwent epithelium-off A-CXL: 5 (Group 1) by riboflavin 0.1% plus dextran 20% at 12 mW/cm$^2$ for 10 min; 5 (Group 2) at 30 mW/cm$^2$ for 4 min; 5 (Group 3) by dextran-free riboflavin 0.1% plus HPMC at 30 mW/cm$^2$ for 4 min and 5 (Group 4) by riboflavin 0.25% plus EDTA, BAK, TRIS epithelium-on A-CXL for 2 min and 40 sec. Micro-morphological analysis was assessed by *in vivo* HRT II confocal microscopy and corneal OCT.

**Results:** Epithelium regenerated into 3 days. Sub-epithelial nerves disappeared after treatment regenerating into 6 months. Epithelium off A-CXL penetration, measured evaluating keratocytes loss at confocal microscopy and demarcation lines at corneal OCT, resulted at 180 µm on average in the Group 1, 160 µm in the Group 2, 150 µm in the Group 3. Epithelium-on A-CXL (Group 4) revealed a penetration at 80 µm on average. No endothelial damage was recorded in all groups.

**Conclusion:** A-CXL shortened conventional CXL procedure under 20 minutes, being well tolerated. Its clinical efficacy needs to be determined in the mid-long term follow-up and in a large cohort of patients.

**Keywords:** Accelerated cross-linking; A-CXL; Keratoconus; Confocal microscopy; Demarcation line

## Introduction

Riboflavin UV-A induced corneal collagen crosslinking (CXL) represents a relatively new procedure available for the conservative treatment of progressive keratoconus [1,2] and secondary corneal ectasia [3] due to its capacity in increasing biomechanical corneal resistance [4,5] and intrinsic anti-collagenase activity [6].

The physiochemical basis of crosslinking lies in the photo-dynamic type I-II reactions [7] induced by the interaction between 0.1% riboflavin molecules absorbed in corneal tissue and UV-A rays delivered at 3 mW/cm2 for 30 minutes (5.4 J/cm2 energy dose) releasing reactive oxygen species (ROS) that mediated cross-links formation between and within collagen fibers [8,9].

The conventional epithelium-off cross-linking procedure (CXL) demonstrated its safety and long-term efficacy stabilizing progressive keratoconus and secondary ectasia in different clinical trials [10-14]. On the other hand the procedure is time consuming lasting from 40 minutes to 1 hour [15] with patient's discomfort.

The physical concept of photochemical reactions stated in the Bunsen-Roscoe's law of reciprocity [16-18] theoretically demonstrated that the photochemical process behind cross-linking depends on the absorbed UV-A energy and its biological effect is proportional to the total energy dose delivered in the tissue [16-18].

According to this physical theory it is theoretically possible to deliver the same energy dose ensuring a proportional biological effect by setting different UV-A powers and exposure times in order to accelerate and shorten the crosslinking procedure in the so called Accelerated Cross-Linking (A-CXL) [17-19].

According to "*equal-dose*" principle 10 mW/cm$^2$ for 9 min, 30 mW/cm$^2$ for 3 min, 18 mW/cm$^2$ for 5 min, 45 mW/cm$^2$ for 2 min at constant energy dose of 5.4 J/cm$^2$ are the same as the standard 3 mW/cm$^2$ for 30 min, a basic concept leading to A-CXL [18,19].

An energy dose of 7.2 J/cm$^2$ was demonstrated to be effective both in terms of corneal strengthening and anti-enzyme activity compared with the standard dose of 5.4 J/cm$^2$, respectively tested by biaxial corneal extensiometry and papain digestion (Avedro's laboratory unpublished data, presented by M. D. Friedman, Ph.D at 8$^{th}$ International CXL Congress, Geneva 8 December 2012).

In this pilot study we report a qualitative analysis of the cornea after A-CXL assessed by means of *in vivo* HRT II scanning laser confocal microscopy (Heidelberg, Germany) and by Visante time domain

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corneal OCT (Zeiss Meditec, Jena, Germany), in a series of 20 patients with progressive keratoconus investigating the induced corneal changes and the penetration of A-CXL.

## Methods

After unanimous approval of the local ethics committee under the principles of the Helsinki declaration and signing of specific informed consent 20 eyes of 20 patients affected from keratoconus, age between 13 and 26 years (mean 22.6 years), underwent Accelerated CXL by the KXL UV-A source (Avedro Inc. Waltham MS, USA) at the Ophthalmology Unit of Siena University Hospital. All patients included in the treatment protocol were affected by progressive keratoconus with a documented clinical and instrumental worsening at least in the last three months of observation.

### Inclusion criteria

The parameters considered to establish keratoconus progression and inclusion criteria for each group were: worsening of UCVA/BSCVA>0.50 Snellen lines, increase of SPH/CYL>0.50 D, increase of topographic symmetry index SAI/SI>0.50 D, increase of maximum K reading>1 D, reduction of the thinnest point at AC OCT optical pachymetry ≥ 10 μm, clear cornea at bio-microscopic examination, absence of reticular dark striations at confocal laser microscopy *in vivo*. We considered "significant" for the inclusion in the study the variation of at least 3 of the parameters listed above (one clinical plus two instrumental). The following examinations were performed before and after the operation: *in vivo* scanning laser confocal microscopy (HRT II, Rostock Cornea Module, Heidelberg, Germany) and anterior segment OCT analysis (Visante OCT, Zeiss Meditec, Jena, Germany) to assess qualitative A-CXL induced corneal changes and treatment penetration.

Patients were divided into 4 groups matched according to age and keratoconus stage as different A-CXL protocols showed in Table 1.

**Group 1 epithelium-off A-CXL:** 5 eyes, age 16-23 y (mean age 19 years) Riboflavin 0.1% plus Dextran 20% (VibeX), 15 minutes of corneal soaking after mechanical epithelial debridement (blunt metal spatula), UV-A power at 12 mW/cm$^2$ (Energy dose: 7.2 J/cm$^2$), 10 min exposure time.

**Group 2 epithelium-off A-CXL:** 5 eyes, age 13-26 y (mean age 19.5 years) Riboflavin 0.1% plus Dextran 20% (VibeX), 20 minutes of corneal soaking after mechanical epithelial debridement (blunt metal spatula), UV-A power at 30 mW/cm$^2$ (Energy dose: 7.2 J/cm$^2$), 4 min exposure time.

**Group 3 epithelium-off A-CXL:** 5 eyes, age 14-24 y (mean age 20.5 years) Riboflavin 0.1% (dextran free) plus HPMC (VibeX Rapid), 10 minutes of corneal soaking after mechanical epithelial debridement (blunt metal spatula), UV-A power at 30 mW/cm$^2$ (Energy dose: 7.2 J/cm$^2$), 4 min exposure time.

**Group 4 epithelium-on A-CXL:** 5 eyes, age 21-26 y (mean age 23.5 years) Riboflavin 0.25% plus EDTA, BAK, TRIS (Paracel) tapered every 90 seconds for 4 minutes of soaking, followed by corneal rinsing with Riboflavin 0.25% saline solution (Vibex X-tra) administered every 90 seconds for 6 min (total epithelium-on soaking time 10 minutes), UV-A power at 45 mW/cm$^2$ (Energy dose: 7.2 J/cm$^2$), 2 min. and 40 sec exposure time.

## Postoperative protocol

All patients underwent a postoperative soft contact lens bandage for 3 days, cyclopentolate eye drops twice for 3 days, ciprofloxacin eye drops four times/day for 3 days, diclofenac eye drops four times/day for 3 days and eye lubricants four times/day and on demand. After therapeutic contact lens removal all patients were medicated by dexamethasone eye drops and sodium hyaluronic acid 0.2% eye lubricants 4 times/day for 15 days and 2 times/day for 15 days.

Treatment penetration (keratocytes loss, cornea edema) was compared by a descriptive point of view with literature data, coming out from our research team on conventional [20-22] and trans-epithelial CXL [23].

## Results

A comprehensive review of treatment groups and results are summarized in Table 1.

### Group 1 epithelium-off A-CXL

Riboflavin 0.1% plus Dextran 20% (VibeX), 15 minutes of corneal soaking, UV-A power at 12 mW/cm$^2$ (Energy dose: 7.2 J/cm$^2$), 10 min exposure time.

All eyes re-epithelialized by 3 days of therapeutic soft contact lens bandage. Epithelial stratification improved in time, being complete at 3$^{rd}$ month. Sub-epithelial and anterior stromal nerves disappeared immediately after treatment. Nerves regeneration started one month after treatment being complete after 6 months. Anterior stromal tissue presented a high reflectivity after A-CXL with keratocytes loss (apoptosis hence photo-necrosis) until 200 μm of depth and classical spongy or lacunar edema as previously demonstrated by us [20-22] in standard epithelium-off CXL was evident until 3$^{rd}$ month, gradually disappearing thereafter. Keratocytes repopulation started one month after treatment increasing at 3$^{rd}$ month and being complete at 6$^{th}$ post-operative month. An uneven demarcation line was determined at a mean depth of 180 μm (range 160-200 μm) measured from epithelial surface (Figure 1). Confocal data of increased stromal reflectivity and

| Group 1 Epithelium-Off A-CXL | Group 2 Epithelium-Off A-CXL | Group 3 Epithelium-Off A-CXL | Group 4 Epithelium-On A-CXL |
|---|---|---|---|
| 5 eyes | 5 eyes | 5 eyes | 5 eyes |
| 16-23 | 13-26 | 14-24 | 21-26 |
| Riboflavin 0.1% plus Dextran 20% (VibeX) | Riboflavin 0.1% plus Dextran 20% (VibeX) | Riboflavin 0.1% plus HPMC (VibeX Rapid) | Riboflavin 0.25% plus EDTA, BAK, TRIS (Paracel) |
| 15 minutes | 20 minutes | 10 minutes | 10 minutes |
| 12 mW/cm$^2$ | 30 mW/cm$^2$ | 30 mW/cm$^2$ | 45 mW/cm$^2$ |
| 10 min | 4 min | 4 min | 2 min. and 40 sec |
| 7.2 J/cm$^2$ | 7.2 J/cm$^2$ | 7.2 J/cm$^2$ | 7.2 J/cm$^2$ |
| 180 μm (range 160-200 μm) | 160 μm (range 150-180 μm) | 155 μm (range 140-180 μm) | 80 μm (range 50-120 μm) |
| 180 μm (range 150-200 μm) | 160 μm (range 150-180 μm) | 150 μm (range 140-180 μm) | noevidentdemarcation line |

**Table 1:** Overview of treatment parameters and respective results according to demarcation lines measurements after *in vivo* scanning laser confocal microscopy and corneal OCT.

demarcation line (defined by corneal edema and keratocytes apoptosis with changes in stromal reflectivity) was established by anterior chamber OCT at a mean depth of 180 µm (range 150-200 µm). The demarcation line was also clinically well evident after treatment at slit lamp examination (Figure 2). No endothelial damage was observed in terms of morphology and cell count after A-CXL.

## Group 2 epithelium-off A-CXL

Riboflavin 0.1% plus Dextran 20% (VibeX), 20 minutes of corneal soaking, UV-A power at 30 mW/cm$^2$ (Energy dose: 7.2 J/cm$^2$), 4 min exposure time.

The results in this group are the same of *Group 1* concerning epithelium and nerves regeneration. Some differences were found in the stromal analysis that showed a higher reflectivity of the anterior stromal tissue combined with keratocytes apoptosis and typical lacunar edema in the first three postoperative months followed by gradual cells repopulation. The demarcation line (defined by corneal edema and keratocytes apoptosis with changes in stromal reflectivity) at confocal scans was unevenly distributed in a mean depth of 160 µm (range 150-180 µm) (Figure 3). No morphological changes in endothelial cells were observed. OCT imaging confirmed a mean depth of the demarcation line (defined by the higher reflectivity of cross-linked tissue) at 160 µm (range 150-180 µm), moreover a demarcation line is clinically evident at bio-microscopy (Figure 4).

## Group 3 epithelium-off A-CXL

Riboflavin 0.1% (dextran free) plus HPMC (VibeX Rapid), 10 minutes of corneal soaking, UV-A power at 30 mW/cm$^2$ (Energy dose: 7.2 J/cm$^2$), 4 min exposure time.

The results in this group are superimposable with those observed in previous epithelium off treatment groups both concerning epithelial regrowth and nerves regeneration. While epithelium regenerates rapidly into 3 days, neural flocculation is detectable one month after treatment. The main differences were recorded in stromal healing where reflectivity was increased compared to preoperative scans but concentrated in the anterior 150 µm of the stroma (Figure 5). In this case the riboflavin solution used for corneal soaking is dextran free, containing the hydroxyl-propyl-methyl-cellulose (HPMC) as riboflavin vehicle [24]. An uneven demarcation line is detectable at mean depth of 155 µm (range 140-180 µm) and the reflectivity of extracellular matrix is relatively lower than those observed in group 1 and 2 patients. Demarcation line depth is confirmed by corneal OCT at a mean depth of 150 µm (range 140-180 µm) (Figure 6). No endothelial damage is detectable in the postoperative period.

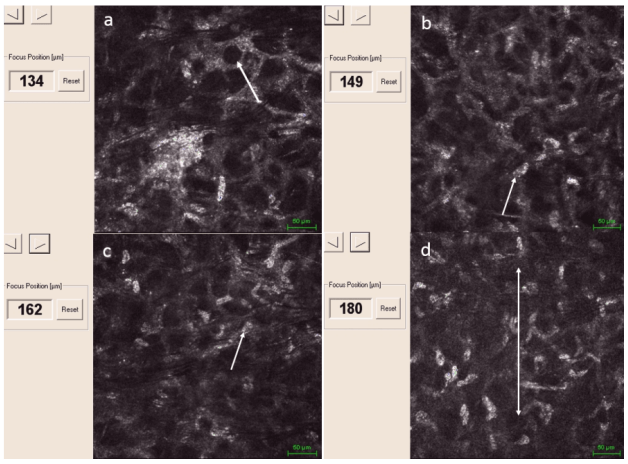


**Figure 1:** Accelerated corneal collagen crosslinking Group 1 (epithelium-off): Riboflavin 0.1% plus Dextran 20%, 15 min of corneal soaking, UV-A power at 12 mW/cm$^2$, Energy dose at 7.2 J/cm$^2$), 10 min exposure time. Left (a) white arrow: biomicroscopic picture of demarcation line after treatment. Right (b) white arrows: corneal OCT imaging revealing the demarcation line after A-CXL at about 180 µm of depth (range 150-200 µm).

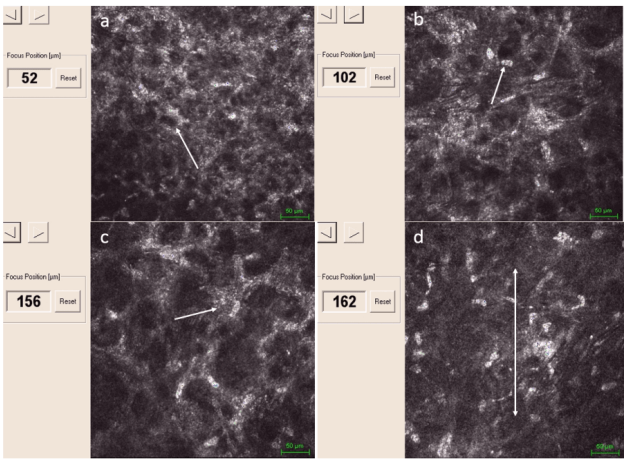


**Figure 3:** Accelerated corneal collagen crosslinking Group 2 (epithelium-off): Riboflavin 0.1% plus Dextran 20%, 20 min of corneal soaking, UV-A power at 30 mW/cm$^2$, Energy dose at 7.2 J/cm$^2$), 4 min exposure time. Left (a) white arrow: biomicroscopic picture of demarcation line after treatment. Right (b) white arrows: corneal OCT imaging revealing the demarcation line after A-CXL at about 160 µm depth (range 150-180 µm).

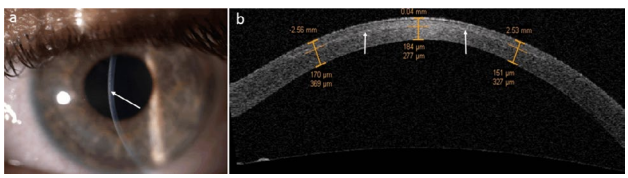


**Figure 2:** Accelerated corneal collagen crosslinking Group 1 (epithelium-off): Riboflavin 0.1% plus Dextran 20%, 15 min of corneal soaking, UV-A power at 12 mW/cm$^2$, Energy dose at 7.2 J/cm$^2$), 10 min exposure time. Postoperative confocal scans revealed lacunar edema (upper left white arrow-a) with keratocytes apoptosis (upper right white arrow - b; bottom left white arrow-c) and increased density of extracellular matrix surrounding edema. A transition between hypo-cellular stroma and stroma unreached by the treatment (vertical transition area) is showed in bottom left - d scan with an estimated penetration depth of 180 µm (range 160-200 µm).

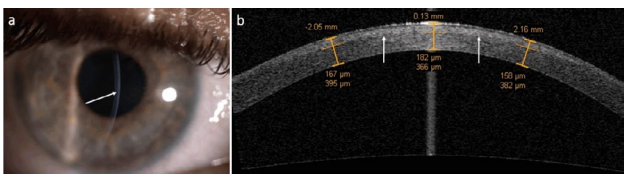


**Figure 4:** Accelerated corneal collagen crosslinking Group 2 (epithelium-off): Riboflavin 0.1% plus Dextran 20%, 20 min of corneal soaking, UV-A power at 30 mW/cm$^2$, Energy dose at 7.2 J/cm$^2$), 4 min exposure time. Postoperative confocal scans revealed lacunar edema (upper left white arro -a) with keratocytes apoptosis (upper right white arrow-b) and increased density of extracellular matrix (bottom left white arrow-c). A transition between hypo-cellular stroma and stroma unreached by the treatment (vertical transition area) is showed in bottom left - d scan with an estimated penetration depth of 160 µm (range 150-180 µm).

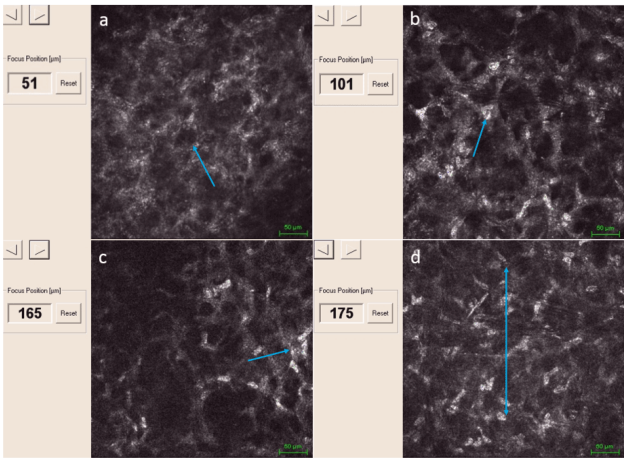


**Figure 5:** Accelerated corneal collagen crosslinking Group 3 (epithelium-off): Riboflavin 0.1% (dextran free) plus HPMC, 10 minutes of corneal soaking, UV-A power at 30 mW/cm² (Energy dose: 7.2 J/cm²), 4 min exposure time. Left (a) light blue arrow: biomicroscopic picture of demarcation line after treatment. Right (b) light blue arrows: corneal OCT imaging revealing the demarcation line after A-CXL at about 150 µm depth (range 140-180 µm).

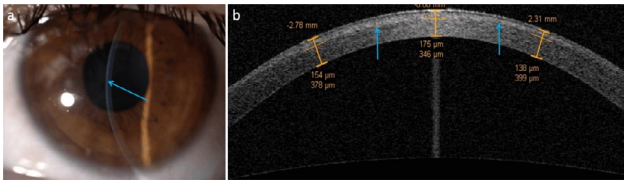


**Figure 6:** Accelerated corneal collagen crosslinking Group 3 (epithelium-off): Riboflavin 0.1% (dextran free) plus HPMC, 10 minutes of corneal soaking, UV-A power at 30 mW/cm² (Energy dose: 7.2 J/cm²), 4 min exposure time. Postoperative confocal scans revealed lacunar edema (upper left light blue arrow-a) with keratocytes apoptosis (upper right light blue arrow-b) and increased density of extracellular matrix (bottom left light blue arrow-c). A transition between the hypo-cellular stroma and stromal tissue unreached by the treatment (vertical transition area) is showed in the bottom left - d scan, with an estimated penetration of 155 µm (range 140-180 µm).

## Group 4 epithelium-on A-CXL

Riboflavin 0.25% plus EDTA, BAK,TRIS (Paracel) for 4 min, Riboflavin 0.25% saline solution (VibeX X-tra) for 6 min, UV-A power at 45 mW/cm² (Energy dose: 7.2 J/cm²), 2 min. 40 sec exposure time.

Epithelium-on treatment showed an acute actinic-like diffuse punctate epitheliopathy that was recovered following 3 days of soft contact lens bandage and sodium hyaluronate lubricants. Sub-epithelial nerves were damaged and partially disappeared after this high UV power setting, even if delivered with epithelium *in situ* . In any case stromal healing demonstrated poor apoptosis and sub-edema more diffuse than lacunar. A limited and uneven apoptotic affect is detectable after epithelium-on A-CXL in the anterior stroma at a mean depth of 80 µm (range 50-120 µm) (Figure 7). No endothelial damage was observed. OCT corneal scans confirmed a slightly increased reflectivity under the Bowman's lamina without an evident demarcation line. Demarcation line is not clinically visible after epithelium on A-CXL also at bio microscopic examination (Figure 8).

## Conventional epithelium-off CXL

Compared with epithelium-off ACXL groups, after epithelium-off standard CXL treatment (3 mW/cm² for 30 minutes of UV-A exposure),

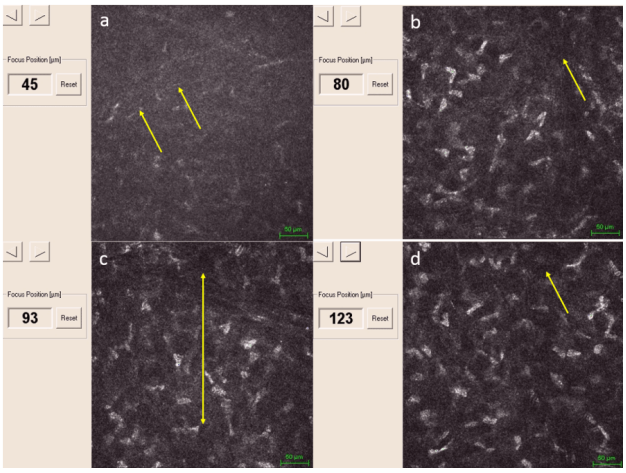


**Figure 7:** Accelerated corneal collagen crosslinking Group 4 (epithelium-on): Riboflavin 0.25% plus EDTA, BAK,TRIS for 4 min, Riboflavin 0.25% saline solution for 6 min, UV-A power at 45 mW/cm² (Energy dose: 7.2 J/cm²), 2 min. 40 sec exposure time. Left (a) yellow arrow: biomicroscopic picture after epithelium-on accelerated cross-linking showed a slight increased density of sub-Bowman stroma without a visible demarcation line. Right (b) yellow arrows: OCT corneal scans confirmed a slightly increased reflectivity under of corneal stroma under the Bowman's lamina without evidence of demarcation line.

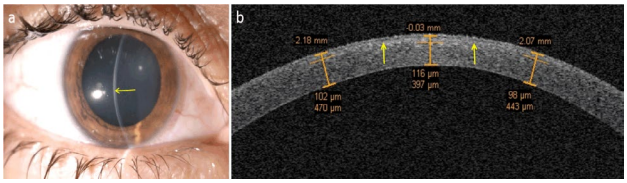


**Figure 8:** Accelerated corneal collagen crosslinking Group 4 (epithelium-on): Riboflavin 0.25% plus EDTA, BAK,TRIS for 4 min, Riboflavin 0.25% saline solution for 6 min, UV-A power at 45 mW/cm² (Energy dose: 7.2 J/cm²), 2 min. 40 sec exposure time. Postoperative confocal scans revealed a superficial diffuse edema under the Bowman's lamina with rarefaction and damage of sub-epithelial nerves fibers (upper left yellow arrows - a); an uneven keratocytes apoptosis is detectable in the anterior stroma (upper right and bottom right yellow arrows - b and d) with a vertical transition area (bottom left yellow arrow - c) at 80-90 µm of depth on average (range 50-120 µm).

epithelial healing and nerves regeneration were superimposable, being completed respectively after 3-4 days and 3-6 months. In A-CXL, keratocytes apoptosis reached the anterior-mid corneal stroma until 150-200 µm instead of 250-300 microns of the classic CXL treatment. Cell apoptosis after CXL was more evident after soft contact lens removal and along the first postoperative month. The apoptotic process after CXL treatment required at least 48-72 hours, becoming well evident at *in vivo* confocal scans (apoptotic bodies) along the first post-operative month. In the first week after treatment it was masked by the presence of marked honeycomb-like stromal edema. Reflectivity of extracellular matrix in the first 3 months was slightly higher in the epithelium-off A-CXL patients compared with standard epithelium-off CXL. No endothelial damage was observed in both treatments modalities.

## Standard epithelium-on (TE-CXL)

Compared with epithelium-on treatment performed by us with Riboflavin 0.1% plus Dextran 15%, EDTA and Trometamol solution, at 3 mw/cm² for 30 minutes, after A-CXL we recorded the same superficial,

diffuse and irregular epithelial photo-chemical damage. Sub-epithelial nerves plexus was present after classic epithelium on treatment, while nerve fibers disappearance was evident after A-CXL. The timing of nerves fibers regeneration after Epi-on ACXL was similar to standard epithelium-off CXL (3-6 months). Keratocytes apoptosis was uneven and confined under 80 microns of depth and stromal edema was unevenly distributed under the Bowman lamina in the anterior stroma. No endothelial damage was observed in both techniques.

## Discussion

*Epithelium-off A-CXL* demonstrated in the first 3 groups morphological changes and treatment penetration defined by stromal edema and keratocytes loss at *in vivo* confocal microscopy and by increased stromal reflectivity at AC OCT, comprised between 150 and 180 µm on average (range140-200 µm).

As reported in literature [25] *in vivo* UV-A induced oxidative damage (apoptotic effect and cell viability) depends on the energy, riboflavin concentration and mode of exposure. In this context, the exposure time together with riboflavin concentration become very important in cross-linking treatment (interactions between UV-A photons, riboflavin and collagen).

Even if UV-A intensity is increased while maintaining a constant energy dose (5.4 or 7.2 J/cm$^2$), a prolonged exposure time influenced a deeper penetration of oxidative damage [25], increasing treatment volume, like demonstrated in our first protocol al 12 mW/cm$^2$ for 10 minutes of exposure time (Group 1) that reported a mean penetration of 180 µm (Figures 1 and 2). The A-CXL protocol at 30 mW/cm2 for 4 minutes of exposure time (Group 2) revealed a mean penetration of 160 µm both at confocal analysis and corneal OCT (Figures 3 and 4), slightly inferior to Group 1.

This result is slightly better with those recently reported in literature by Colin research group [26] probably due to high energy dose that we used in our treatments according to Avedro's laboratory data (7.2 J/cm$^2$ instead of 5.4 J/cm$^2$).

The clinical aspect of the corneas after A-CXL was good after therapeutic soft contact lens removal and in the first postoperative month without any complication such as persistent epithelial defects or haze. A demarcation line was clearly visible in all epithelium-off A-CXL treatments at slit lamp examination just after therapeutic soft contact lens removal (Figures 2,4 and 6).

Keratocytes apoptosis correlating with treatment penetration [23] was limited to the anterior-mid stroma until a maximum depth of 200 µm if compared with conventional Dresden protocol that reached 300 µm without epithelium as well demonstrated by our first confocal studies *in vivo* in humans [20-22]. On the other hand, the intensity of extracellular matrix after epithelium-off A-CXL resulted higher in the anterior 150 µm of stroma suggesting a good collagen compaction and corneal stiffening with reduced corneal edema and less cell toxicity (Figure 3). The higher reflectivity recorded in group 1 and 2 may be explained by the higher tissue dehydration after A-CXL by using riboflavin 0.1% plus Dextran 20% (VibeX) solution.

As reported in literature the most important biomechanical effect related to crosslinking is concentrated in the anterior 200 µm of the cornea [27], in the so called stiff cornea, so the impact of A-CXL may be sufficient in terms of biomechanical and biochemical effect.

A relatively low reflectivity of extracellular matrix was observed in the Group 3 protocol (Figures 5 and 6), compared with Group 1 and 2 patients (Figures 1 and 3), that may be explained by the different (dextran free VibeX Rapid) riboflavin solution used for corneal soaking, containing the hydroxyl-propyl-methyl-cellulose (HPMC) as riboflavin vehicle, reducing intraoperative corneal dehydration [28].

Epithelium on A-CXL demonstrated a powerful toxic effect on epithelium related to enhanced riboflavin solutions containing ethylene-diamine-tetra-acetic acid (EDTA), benzalkonium chloride (BAK), trometamol (TRIS) and also to high UV-A intensity at 45 mW/cm$^2$ delivered in a very short time (2 min and 40 sec) on epithelial cells producing an immediate, even short, postoperative patient discomfort. Moreover higher UV-A intensity, even if delivered with epithelium *in situ* , induced a slight damage of sub-epithelial plexus nerves probably due to altered condition of epithelial surface itself. In any case the stromal healing after epithelium-on A-CXL demonstrated poor cells apoptosis and sub-edema, more diffuse than lacunar. A limited and uneven apoptotic effect is detectable after epithelium-on A-CXL in the anterior stroma (Figure 7), and a demarcation line is not visible (Figure 8), confirming that epithelium leaved *in situ* and the high intraepithelial riboflavin concentration represent a barrier for the UV-A diffusion into the stroma that is essential for cross-linking penetration, inducing a superficial oxidative damage (surface CXL). Also in epithelium-on A-CXL, like in the previous trans-epithelial CXL procedures [29-31], the first analysis *in vivo* in humans by using confocal microscopy [23], demonstrated that the presence of corneal epithelium *in situ* constitutes a physical barrier to UV-A radiation reducing its penetration into the corneal stroma and the results of present qualitative analysis confirm this finding. The low penetration of the epithelium on Accelerated CXL could not stabilize biomechanically the keratoconic cornea in the mid-long term follow-up as demonstrated in literature [32] by us after standard trans-epithelial procedure (TE-CXL).

To date we don't know exactly the optimal interactions between UV-A energy, riboflavin concentration and exposure time in order to obtain the maximum cross-linking effect ensuring a long-lasting (possibly a long-life) keratoconus stability and the better functional outcome, even if the necessity to improve the procedure and shorten the CXL treatment time are highly desirable.

In any case, the conventional epithelium-off CXL procedure (Riboflavin 0.1% plus dextran 20%, UV-A 3 mW/cm$^2$=5.4 J/cm$^2$ for 30 minutes) remains the gold standard in the conservative treatment of early stages progressive keratoconus. On the other hand the rule of Bunsen-Roscoe's law of reciprocity, established for photo-chemical reactions, cannot be directly transferred in terms of photo-biological effect to complex biological systems as the living cornea [25].

Accelerated cross-linking with epithelium removal demonstrated its safety for endothelium and posterior ocular structures. Treatment penetration achieve the anterior part of the stromal tissue stiffening the cornea in the first 160 µm on average (range 140-200 µm) with relative differences between the different protocols we used.

In our experience, A-CXL shortened CXL procedure under 20 minutes being well tolerated by patients. However its clinical efficacy, both in terms of keratoconus stabilization and functional impact, must be determined in the mid-long term follow-up and in a large cohort of patients according to different patient's age, keratoconus stage and progression rate.

## Financial Disclosure

The authors declare that they have no financial interest in the manuscript.

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