ORIGINAL ARTICLE

In Vitro Histological Analysis of the Human Cornea Undergoing Conventional and Accelerated Cross-linking Protocol: From the Microscope to Clinical Application

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Received on: 25 May 2024; *Accepted on:* 22 June 2024; *Published on:* XX XXXX XX

Abstract

Purpose: *In vitro* assessment of the photo-oxidative effects induced by conventional cross-linking (CXL) and accelerated cross-linking (ACXL) at 5.4 J/cm^2 energy dose (fluence).

Methods: A total of 20 eye-bank human corneas were treated with different epithelium-off (EPI-OFF) CXL UV-A protocols: five EPI-OFF at 3 mW/5.4 J/cm^2 for 30 minutes (Dresden protocol), five at 15 mW/5.4 J/cm^2 for 12 minutes pulsed light (Siena ACXL protocol), and five at 30 mW/5.4 J/cm^2 pulsed light for 6 minutes. Five corneas were used as control group. Semi-thin and ultrathin sections were examined by a Philips transmission electron microscope at the Department of Human Pathology of Siena University. Histology overview included Bowman's lamina thickness measurement, CXL depth, collagen fibers density, and CXL-induced photo-oxidative damage.

Results: The study provides descriptive histological evidences of *in vitro* corneal changes induced by ACXL, identifying the differences between treatment depths based on different UV-A power settings and exposure times. The higher UV-A power with the shorter exposure time induced a lower photo-oxidative CXL penetration, while the longer exposure time increased CXL treatment photo-oxidative depth. Increased fiber density induced by higher UV-A power was observed only in the anterior 50 μm of corneal stroma in all cases.

Conclusion: Cross-linking photo-oxidative damage is fluence-dependent. Keeping a constant fluence, the longer exposure time increased the treatment depth due to more oxygen diffusion, while the higher UV-A power with shorter exposure time without increasing the fluence resulted in reduced treatment depth due to less oxygen diffusion. The higher reduction of collagen fibrils distance in the anterior 50 μm stroma appears to be independent from UV-A power and exposure time.

Keywords: Accelerated cross-linking, Cross-linking, Fluence, Histology, Keratoconus.

International Journal of Keratoconus and Ectatic Corneal Diseases (2024): 10.5005/jp-journals-10025-1204

Introduction

Keratoconus is an ectatic disease of the cornea characterized by biochemical and biomechanical instability of stromal collagen, leading to reduction of corneal thickness, variation in posterior and anterior corneal curvatures, and progressive deterioration of visual acuity due to irregular astigmatism.[1,2] The recent advent of corneal collagen cross-linking (CXL) in the ophthalmology panorama transformed the conventional therapy of keratoconus based at best, on a lifetime of rigid contact lens wearing or, at worst, on corneal transplant, improving its conservative treatment, thus reducing the necessity of lamellar and penetrating corneal graft.[3] Riboflavin UV-A induced corneal collagen CXL demonstrated its efficacy in the conservative treatment of progressive keratoconus and secondary corneal ectasia due to its capability of increasing biomechanical corneal resistance and intrinsic anticollagenase activity.[4–7] The physiochemical basis of CXL lies in the photodynamic type I–II mixed reactions induced by the interaction between 0.1% riboflavin molecules absorbed in corneal tissue and UV-A rays delivered at 3 mW/cm^2 for 30 minutes (5.4 J/cm^2 energy dose), releasing reactive oxygen species that mediate crosslinks formation between adjacent collagen fibers.[8–10] The conventional epithelium-off (EPI-OFF) CXL procedure demonstrated its long-term efficacy in stabilizing progressive keratoconus and secondary ectasia in different clinical trials.[11–15] Conventional CXL requires a long treatment time (1 hour).[16] Recently, in order to shorten the treatment time while maintaining the same efficacy, a novel approach called accelerated

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How to cite this article: Mazzotta C, Baiocchi S, De Santi MM, *et al. In Vitro* Histological Analysis of the Human Cornea Undergoing Conventional and Accelerated Cross-linking Protocol: From the Microscope to Clinical Application. Int J Kerat Ect Cor Dis 2024;xx(x):xx–xx.

Source of support: Nil

Conflict of interest: Dr Cosimo Mazzotta and Dr Adel Barbara are associated as the Editorial board members of this journal and this manuscript was subjected to this journal's standard review procedures, with this peer review handled independently of these Editorial board members and their research group.

Table 1: Treatment groups and protocols

Groups	*Epithelium status*	*Solution*	*UV-A power (mW/cm²)*	*Fluence (J/cm²)*	*Soaking time (min)*	*Irradiation time (min)*	*Total time (min)*
Group A (control)	None	None	0	0	0	0	0
Group B (CXL)	OFF	Riboflavin 0.1%, HPMC 1%	3	5.4	10	30	40
Group C (ACXL)	OFF	Riboflavin 0.1%, HPMC 1%	15	5.4	10	12 pulsed	22
Group D (ACXL)	OFF	Riboflavin 0.1%, HPMC 1%	30	5.4	10	6 pulsed	16

HPMC, hydroxypropylmethylcellulose

cross-linking (ACXL) based on the physical concept of photochemical reactions stated in the Bunsen–Roscoe's law of reciprocity, has been proposed.[17–19] This theory demonstrated that the photochemical process behind CXL, which depends on the absorbed UV-A energy and its biological effect, is proportional to the total energy dose delivered in the tissue.[17–20] Indeed, according to "equal-dose" physical principle, 3 mW/cm^2 for 30 minutes, 9 mW/cm^2 for 10 minutes, 15 mW/cm^2 for 6 minutes (12 minutes with pulsed light irradiation), and 30 mW/cm^2 for 3 minutes (6 minutes with pulsed light irradiation), at a constant energy dose of 5.4 J/cm^2, have the same photochemical impact of conventional 3 mW/cm^2 for 30 minutes.[19,20]

Previous histological reports in literature on conventional Dresden protocol and corneal ACX demonstrated keratocyte photo-oxidative damage and gradual repopulation of the corneal stroma by proliferating cells and an increase in collagen fibers diameter.[21,22] These modifications are the morphological explanation of the process that leads to an increased corneal biomechanical stability conferred by CXL to ectatic corneas. These observations confirmed the previous *in vivo* studies in humans provided by Mazzotta et al. with scanning laser confocal microscopy that demonstrated the reduction in anterior and intermediate stromal keratocytes nuclei followed by gradual repopulation.[23,24] The long-term keratoconus stability after conventional CXL treatment was attributed to the *in vivo* evidence of increased crosslinks formation, synthesis of restructured collagen, new lamellar interconnections, and inhibition of collagenase activity, and hence, enzymatic collagen degradation.[25–27]

In order to reduce the time of Dresden original protocol, thus allowing a major boost in patient's comfort, reducing treatment burden, and optimizing clinical workflow, the technique of CXL has evolved according to recent demonstrations, with accelerated procedures based on delivering the same energy dose to corneal tissue while shortening treatment time by setting different UV-A powers, from the conventional 3 mW/cm^2 up to 30 mW/cm^2 (ACXL).[18,27–33] Our aim was to evaluate the photochemical effects induced by conventional CXL and ACXL in *ex vivo* eye-bank human corneas unsuitable for corneal transplant by means of light microscopy (LM) and transmission electron microscopy (TEM).

Methods

Tissue Samples

After Siena University Hospital's Institutional Review Board ethical approval, 20 eye-bank human corneas unsuitable for transplant (provided by Tuscany Eye Bank of Lucca, Italy) were selected as samples and subdivided as reported in Table 1.

Group A cornea specimens were used as untreated controls; group B corneas underwent a conventional EPI-OFF CXL treatment at 3 mW/cm^2 by the CBM (Caporossi, Baiocchi, Mazzotta) Vega X-Linker UV-A emitter (CSO Florence, Italy); group C and group D corneas underwent the accelerated EPI-OFF ACXL treatment by the KXL I UV-A emitter (Avedro Inc., Waltham, MS, USA) at 15 and 30 mW/cm^2, respectively.

LM and TEM

After UV-A irradiation, samples were immediately fixed in 2.5% cacodylate-buffered glutaraldehyde pH 7.3 for 6 hours at 4°C. The specimens were washed overnight in the same buffer, postfixed in buffered 1% osmium tetroxide for 2 hours, washed, dehydrated through a graded series of ethanol, cleared in propylene oxide, and embedded in epoxy resin (Araldite).

For each sample, semi-thin sections of full-thickness corneas were cut with glass knives on an LKB V Ultratome (Leica, Wetzlar, Germany), stained with toluidine blue, and evaluated by LM to select the appropriate areas. The blocks were trimmed around areas of interest, and ultrathin sections were cut with a diamond knife using the same ultramicrotome, retrieved onto copper grids, double-stained with uranyl acetate and lead citrate, and examined at 100 kV with a Philips 208 S transmission electron microscope (FEI Company, Eindhoven, The Netherlands) at 100 kV, at magnification ranging from 5,600× to 89,000×. Digital electron micrographs were acquired with a Mega-View III CCD (Olympus, Tokyo, Japan), and image analysis and measurements were performed using the Soft Imaging System software, which is embedded in the TEM computer.

For each group, semi-thin sections evaluation included the full-thickness cornea measurement (409–561 μm); the Bowman's layer thickness measurement (8–15 μm) and the CXL depth estimation (38–290 μm) related to different CXL protocols. The number of the keratocytes nuclei was determined by counting the cell nuclei in six fields at 250× magnification. For each specimen, ultrastructural characteristics, including qualitative findings and quantitative measurements, were examined at specific depths (50, 100, 150, 200, 300, and 400 μm and pre-Descemetic stroma) on a field column through the entire thickness of the corneas. The measurements were calculated by fitting the ultrathin corneal sections on micrometric close-mesh net reticulum at 94,000× magnification, measuring fibrils number in each examined micrometric close-mesh field.

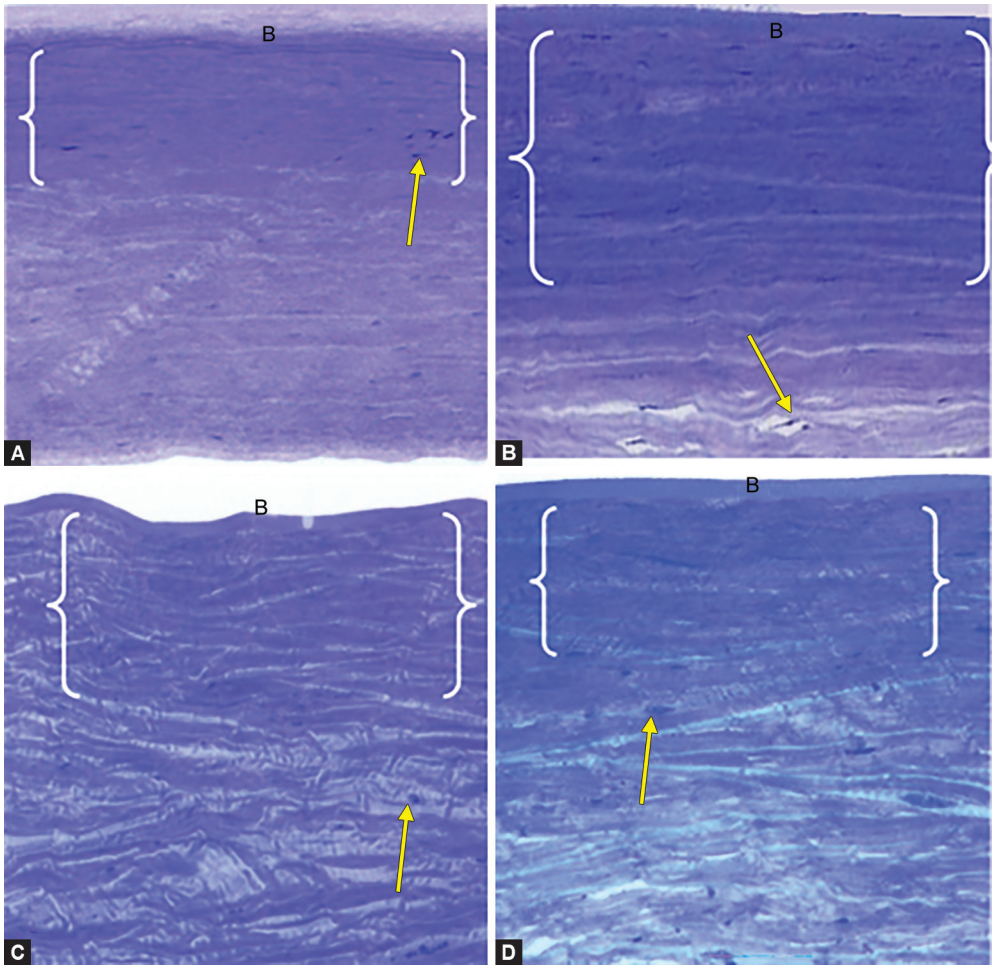
Results

Treatment Penetration (Keratocyte Apoptosis and Lamellar Compaction)

Group B corneas (conventional Dresden CXL, 3 mW/cm^2 for 30 minutes) showed the higher stromal penetration at 287 μm on average without epithelium (range 274–300 μm); group C corneas (ACXL, 15 mW/cm^2 for 12 minutes) showed an average depth of

Table 2: Overall study results

EPI-OFF	*Epithelium status*	*Cross-linked stroma (μm)*	*Bowman's layer thickness (μm)*	*No. of fibrils at 50 μm depth*	*No. of fibrils at 100 μm depth*	*No. of fibrils at 200 μm depth*
Group A (control)	None	0	11.40	44.10	42.20	30.80
Group B (CXL 3 mW/cm^2)	OFF	287	10.94	55.80	52.40	39.30
Group C (ACXL 15 mW/cm^2)	OFF	158	10.90	56.40	51.60	36.80
Group D (ACXL 30 mW/cm^2)	OFF	90	9.10	52.20	43.60	30.40



Figs 1A to D: Semi-thin sections: Yellow arrows indicate keratocytes nuclei; curly brackets indicate the compacted area devoid of keratocytes and with apoptotic keratocytes nuclei; uppercase B indicates Bowman's lamina. (A) Group A (untreated control) showing the compact "stiff cornea" in the first 150 μm; (B) Group B after Dresden conventional CXL at 3 mW/cm^2 showing the deep keratocytes apoptosis associated with lamellar compaction approximately reaching 300 μm of depth approximately duplicating the stiff cornea; (C) Group C (EPI-OFF ACXL at 15 mW/cm^2) showing keratocytes apoptosis with collagen compaction approximately at 200 μm; (D) Group D (EPI-OFF ACXL at 30 mW/cm^2) showing apoptosis and collagen compaction approximately at 100 μm

158 μm without epithelium (range 145–171 μm); group D corneas (ACXL, 30 mW/cm^2 for 6 minutes) reached an average depth of 90 μm (range 76–98 μm) without epithelium (Table 2 and Fig. 1).

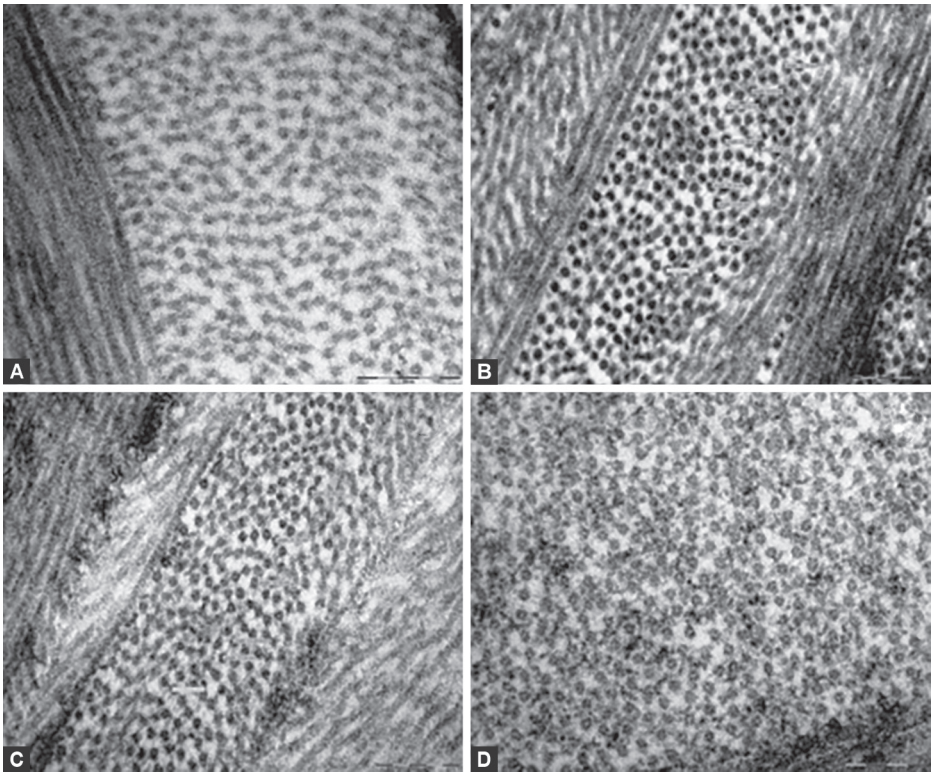
Bowman's Layer Thickness

The Bowman's layer thickness measured demonstrated a slight decrease after EPI-OFF ACXL at 30 mW/cm^2 for 6 minutes (group D) without substantial differences with the other protocols as reported in Table 2 and Figure 1.

Semi-thin Sections

Semi-thin sections of control corneas showed the transition between the stiff cornea and the deeper corneal layers. The stiff cornea in control samples was estimated at 160 micron ± 11 micron (range 149–171) depth on average and showed a denser and compacted collagen lamellae compared with less-packaged deeper layers (Fig. 1A).

Group B corneas (conventional CXL treatment, 3 mW/cm^2 for 30 minutes) showed the deeper collagen compaction and keratocytes



Figs 2A to D: Transmission electron microscopy photomicrograph. Collagen fibrils density at 50 μm depth for (A) group A (control); (B) Group B (conventional CXL at 3 mW/cm^2); (C) Group C (EPI-OFF ACXL pulsed light 12 minutes at 15 mW/cm^2); and (D) Group D (EPI-OFF ACXL pulsed light at 30 mW/cm^2). At this depth, all CXL protocols as shown in B (3 mW), C (15 mW), and D (30 mW) provide a good efficacy in terms of penetration and interfibrillar spacing (corneal stiffening) compared with normal cornea control (A). Uranyl acetate–lead citrate staining at 89,000×

apoptosis approximately at 287 μm (274–300 μm), duplicating the appearance of the stiff cornea as clearly showed in Figure 1B.

Group C corneas (ACXL, 15 mW/cm^2 pulsed light 1 on: 1 off second for 12 minutes) showed a collagen compaction and keratocytes apoptosis approximately at 158 μm (145–171 μm) of corneal stroma (without epithelium) (Fig. 1C).

Group D corneas (ACXL, 30 mW/cm^2 pulsed light 1 on: 1 off second for 6 minutes) showed a stromal collagen compaction and keratocytes apoptosis at 90 μm (76–98 μm) (Fig. 1D).

Number of Collagen Fibrils Estimated at TEM Ultrathin Sections

The average number of collagen fibrils estimated at TEM ultrathin sections was assumed as indicative parameter of CXL-induced corneal collagen compaction at different stromal depths measured without epithelium (50–100–200 μm) compared with control corneas.

At 50 μm depth, the number of collagen fibrils was 44.1 in the control group (Fig. 2A), 55.8 on average (±8) for conventional CXL (Fig. 2B), 56.4 (±9) for ACXL at 15 mW/cm^2 (Fig. 2C), and 52.2 (±6) for ACXL at 30 mW/cm^2 (Fig. 2D).

At 100 μm depth, the number of collagen fibrils was 42.2 (±6) in the control group (Fig. 3A), 52.4 (±7) on average for conventional CXL (Fig. 3B), 51.6 (±9) for ACXL at 15 mW/cm^2 (Fig. 3C), and 43.6 (±8) for ACXL at 30 mW/cm^2 (Fig. 3D).

At 200 μm depth, the number of fibrils was 30.8 (±5) per area in the control group (Fig. 4A), 39.3 (±3) on average per mesh-area for conventional CXL (Fig. 4B), 36.8 (±4) for ACXL at 15 mW/cm^2 (Fig. 4C), and 30.4 (±5) for ACXL at 30 mW/cm^2 (Fig. 4D).

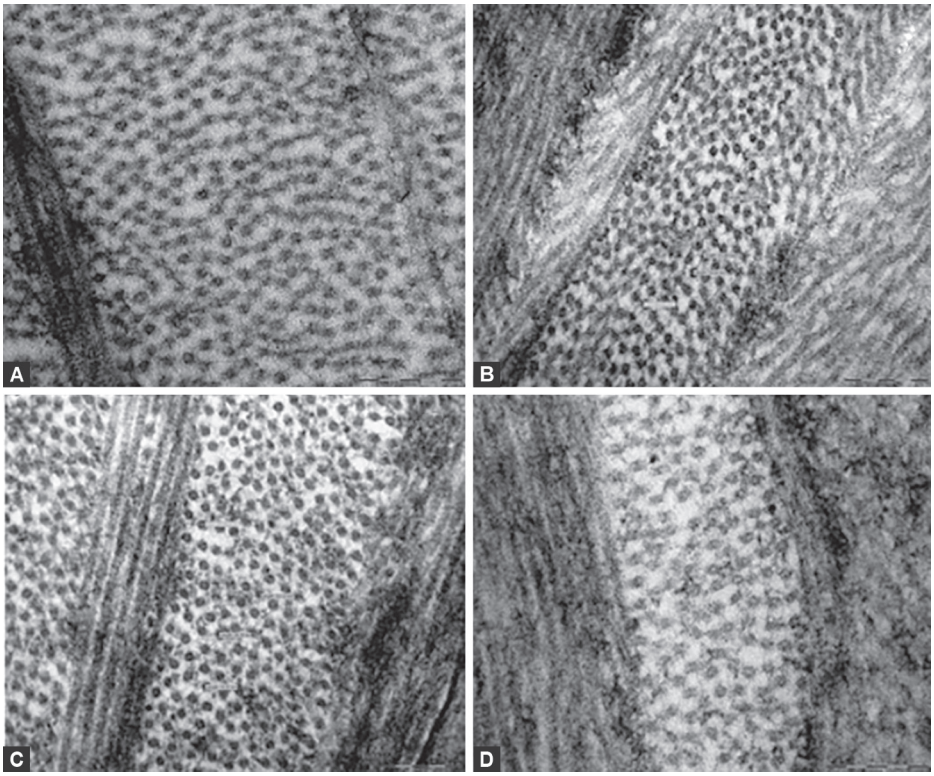
Accelerated CXL induced a diffuse keratocytes apoptosis in the anterior corneal stroma reached by the treatment that was well evident at TEM as shown in Figure 5.

Discussion

After 20 years from CXL introduction, our study provided an *in vitro* insight of conventional and accelerated 5.4 J/cm^2 dose CXL in a series of 20 *ex vivo* eye-bank human corneas, identifying the differences between treatment depths based on different protocols.[34]

Accelerated CXL treatment depth, estimated in terms of CXL-induced keratocytes apoptosis and collagen fibers density, was inversely related to UV-A power intensity (the higher UV-A power = the lower treatment depth), but was positively related to exposure time (the longer exposure time = the deeper cell viability) in EPI-OFF CXL. However, a higher UV-A power does not seem to increase the number of fibrils per analyzed area at 50 and 100 μm depth. The group D ACXL (30 mW/cm^2) showed only a slight superiority in fibril density at 100 μm depth compared with the control group, and a slight inferiority compared with groups B [3 mW standard cross-linking (SCXL) and C (15 mW ACXL].

No substantial differences were found in term of fibrils density between control corneas and the group D at 30 mW/cm^2 at the shorter exposure time (6 minutes of pulsed-light UV-A irradiation) at 200 μm depth. These findings suggested that ACXL at higher UV-A power or intensity (higher-irradiance) with shorter exposure time induced an efficient CXL of the corneal stroma until 100 μm of depth (150 μm with epithelium), thus confirming *in vivo* confocal microscopy review studies.[35]



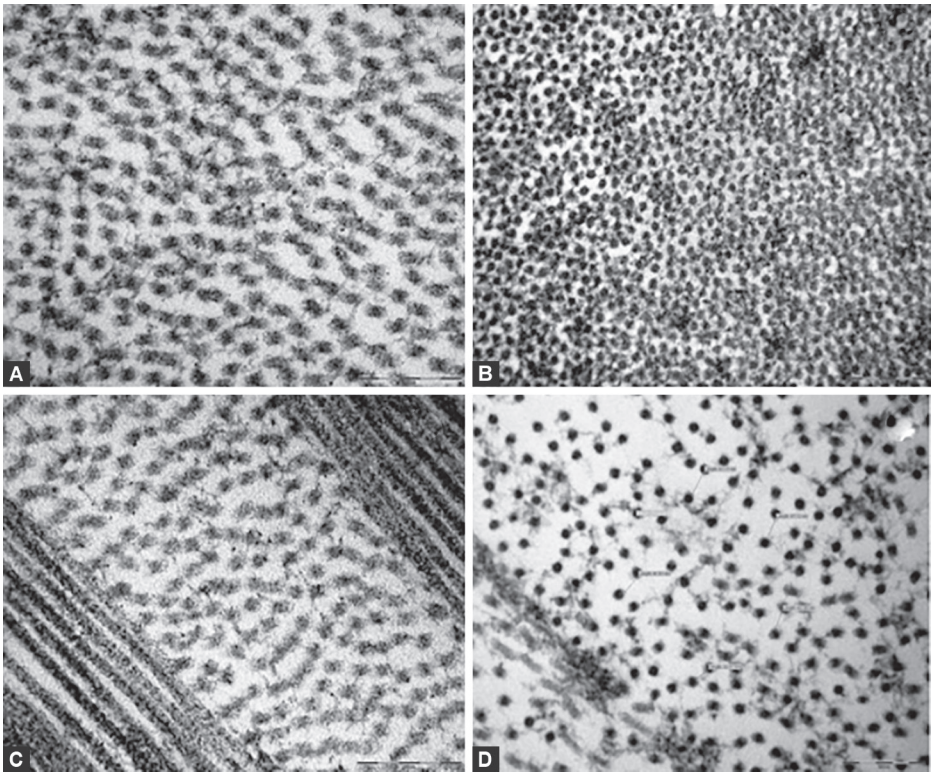
Figs 3A to D: Transmission electron microscopy photomicrograph. Collagen fibrils density at 100 μm depth for (A) group A (control); (B) Group B (conventional CXL at 3 mW/cm^2); (C) Group C (EPI-OFF ACXL pulsed light for 12 minutes at 15 mW/cm^2); and (D) Group D (EPI-OFF ACXL pulsed light for 6 minutes at 30 mW/cm^2). At 100 μm, the efficacy of ACXL is superimposable to conventional CXL, if the UV-A power is calibrated in a range between 9 and 15 mW/cm^2 as evident by the interfibrils distance. There is no substantial difference in penetration and interfibrils distance among (A) normal cornea, (B) 3 mW SCXL, and (C) 15 mW ACXL. There is a consistent lack of efficiency in the 30 mW ACXL (D), with an evident increase of collagen interfibrils distance. Uranyl acetate–lead citrate staining at 89,000×

Based on preclinical biomechanical studies by Krueger et al. showing a substantial equivalence in the stress–strain behavior between SCXL and ACXL at 9 and 15 mw, the Siena ACXL protocol at 15 mW/cm^2 and 12 minutes exposure time and pulsed light was effective as the standard Dresden protocol in terms of fibril density in a 200 μm stromal depth (250 μm with epithelium).[27,28]

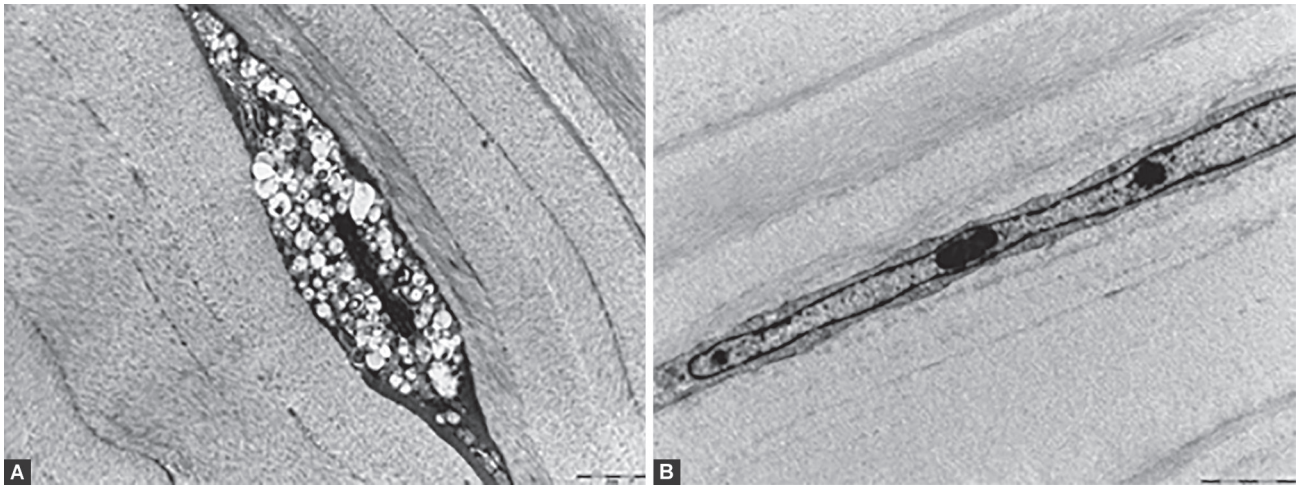
Since biomechanical studies proved that successful CXL treatment in terms of long-term ectasia stabilization should cover at least 200 μm of corneal stroma, the UV-A power and exposure time should be targeted to reach this depth; the protocol of 15 mW ACXL with pulsed light and constant fluence at 5.4 J/cm^2 meets these criteria in our histological observations.[28] Accelerated CXL protocols with epithelium removal, setting the UV-A power between 9 and 15 mW/cm^2 at 5.4 J/cm^2 fluence, and exposure times between 10 and 12 minutes showed a comparable efficacy with the standard 3 mW/cm^2 Dresden benchmark for 30 minutes of continuous light irradiation.[28,29,31,32] Mazzotta et al. documented a demarcation line at a depth of 280 ± 32 μm on average after 15 mW ACXL Siena protocol with the advantage of a considerably less exposure time, better patient's compliance, and no persistent haze development.[28] As a practical consideration, we may affirm that the rapidity of high-irradiance ACXL offers great advantages in clinical applications, optimizing the clinical workflow, reducing adverse events, and increasing patient's compliance.[31] TEM analysis demonstrated that the depth of keratocytes apoptosis, while maintaining an equal energy dose (fluence) depends on UV-A power and exposure time. Our results suggested that keratocytes apoptosis very well complies with treatment depth, as demonstrated by previous histological studies performed by Mencucci et al. and *in vivo* confocal analysis performed by Mazzotta et al. after conventional and ACXL.[22,35]

Conventional CXL treatment demonstrated the higher penetration depth with an appreciable fibril compaction until 200 μm of corneal stroma, allowing a deeper CXL impact (the stiff cornea at semi-thin sections seemed duplicated as shown in Figure 2B). On the contrary, ACXL at 30 mW for 6 minutes, although reducing the treatment time by 20 minutes, induced a superficial CXL impact (keratocytes apoptosis and fibrils compaction were found under 100 μm of stromal depth), thus demonstrating that the higher UV-A power induced a lower treatment penetration and subsequent strengthening in a 400-μm-thick cornea.[21,24,25,36] However, this study demonstrates that what can be considered insufficient in a thick cornea can be a good solution that can be adopted in thin corneas, as often happens in secondary or iatrogenic ectasias. This observation together with confocal microscopy and OCT studies paved the way to the application of 30 mW protocol. This protocol in thin corneas between 300 and 350 μm of minimum corneal thickness as showed in the M nomogram, while keeping a constant fluence of 5.4 J/cm^2, offers a standardized treatment of thin corneas without reducing the fluence.[33,37]

In the present study, a substantially equivalent fiber density was recorded in the anterior 50 μm in all the protocols, while the differences begin just at 100 μm where the 30 mW/cm^2 for 6 minutes of exposure time showed an evident inferiority compared with the Dresden 3 mW SCXL and with the 15 mW Siena ACXL



Figs 4A to D: Transmission electron microscopy photomicrograph. Collagen fibrils density at 200 μm depth for (A) Group A (control); (B) Group B (conventional CXL at 3 mW/cm^2); (C) Group C (EPI-OFF ACXL pulsed light for 12 minutes at 15 mW/cm^2); and (D) Group D (EPI-OFF ACXL pulsed light for 6 minutes at 30 mW/cm^2). Uranyl acetate–lead citrate staining at 89,000×



Figs 5A and B: (A) A stromal keratocyte during "apoptosis," with vacuolization of cytoplasm and nucleus fragmentation; (B) A "normal" stromal keratocyte (uranyl acetate–lead citrate staining at 5,600×)

protocol. Indeed, at 200 μm there were no differences between the 30 mW/cm^2 for 6 minutes and the control corneas. A recently published laboratory study in porcine corneas reported a decreased biomechanical effect of ACXL using high UV-A irradiance with short irradiation time settings and concluded that intrastromal oxygen diffusion capacity and increased oxygen consumption associated with higher irradiances ACXL may be a limiting factor leading to reduced treatment efficiency.[38]

On the contrary, the data reported in the laboratory study by Krueger et al. showed that high-irradiance ACXL with continuous or pulsed UV-A light exposures at 9 and 15 mW/cm^2 with constant fluence of 5.4 J/cm^2 had comparable biomechanical equivalence with the conventional Dresden protocol 3 mW/cm^2 were clinically proven by Mazzotta et al. adopting the 9 mW/5.4 J/cm^2 ACXL protocol and the 15 mW/5.4 J/cm^2 ACXL protocol with pulsed light with 10 minutes of continuous light and 12 minutes of pulsed light (1 second on and 1 second off) UV-A exposure, respectively, confirming that calibrating ACXL UV-A irradiance between 9 and 15 mW is a safe and effective CXL therapy for progressive corneal ectasia.[27,28,30,31]

Accelerated protocols have changed the history of modern CXL, significantly improving the clinical workflow and patient's compliance and significantly reducing the rate of adverse events, with a considerably less exposure time under 20–25 minutes. Moreover, ACXL have created the possibility of developing individualized pachymetry-based protocols for treating thin corneas.[37,39]

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