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Compaction of very thin corneas from ultraviolet A riboflavin-vitamin E transepithelial cross-linking

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ABSTRACT

The purpose of the study was to determine the decrease in pachymetry of very thin corneas with advanced keratoconus due to corneal compaction from the ultraviolet-A (UV-A) irradiation phase of transepithelial (epi-on) cross-linking.

Twenty removed corneal buttons were obtained from patients who underwent penetrating keratoplasty for advanced keratoconus. Removed corneal buttons selected from among the post-surgical specimens for this study had intact epithelium, no scarring or surgical cautery, endothelial cell density >2500 cells/mm^2, and average pachymetry over the measured points of below 400 μm. Corneas were mounted in a Franz chamber. Each epithelial surface was soaked in isotonic riboflavin and D-alpha-tocopheryl polyethylene glycol 1000 succinate (Ribocross® IROMED Group, Italy) for 15 min. Pachymetry was measured at three points over both the shielded and unshielded corneal halves for each corneal button. Surfaces were then washed in saline to remove the Ribocross®. Shields from UV-A irradiation over half of each cornea were then fixed to stand 5 mm above the test corneas. UV-A irradiation using the custom fast cross-linking (CF-CXL) protocol was then performed for the typical 10 ± 1.5 min, for a total energy of 1.08 ± 0.6 J/cm^2 after which pachymetry was re-measured.

The average percent change in pachymetry was −0.43% ± 0.38% (maximum −1.06%) in the shielded half. Pachymetry change was −6.2% ± 2.2% (maximum 12%) in the cross-linked halves.

In conclusion, we estimate that the change in corneal thickness from corneal compaction due to the cross-linking reaction itself was −5.8% ± 2.2%. Scanning electron microscopy of cross-linked corneal segments showed stromal fiber contraction.

German researchers clinically demonstrated successful stabilization of keratoconus by riboflavin-ultraviolet-A corneal cross-linking (Spoerl et al., 1998), and a version of the technique known as the Dresden protocol is the world standard treatment of ectatic corneal disease. But riboflavin only poorly penetrates the cornea, surface debridement is necessary, and postoperative recovery can be painful and with some risk of infection and corneal scarring.

A newer protocol of UV-A corneal cross-linking called CF-CXL (custom-fast corneal cross-linking) that uses an isotonic solution of riboflavin and D-alpha-tocopheryl poly (ethylene glycol) 1000 succinate (Ribocross® IROMED Group, Italy), has been described and studied with laboratory and clinical results published (Spadea and Mencucci, 2012; Ostacolo et al., 2013; Caruso et al., 2016; Caruso et al., 2017). The CF-CXL technique is performed without epithelium removal (epi-on procedure), and the UV-A fluence and duration are customized dependent on topography and pachymetry. A 7-year study has documented long-term keratoconus stabilization with CF-CXL (Caruso et al., 2020). With UV-A fluence and irradiation duration in the epi-on CF-CXL protocol depending in part on corneal pachymetry, the CF-CXL can be applied to more advanced cases that are unsafe with the Dresden protocol. Knowing how much the cornea thickness reduces due to the CF-CXL cross-linking itself could be a step on the way to eliminating the need for checking pachymetry during UV-A irradiation.

How much the cornea thins due to the cross-linking reaction itself during treatment is an important parameter that can influence cross-linking safety and effectiveness. The Dresden cross-linking protocol

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originators recommended a safety limit of 400μ minimal thickness of the de-epithelialized cornea to avoid damage to the corneal endothelium (Wollensak et al., 2003a; Wollensak et al., 2003b) that was verified by others (Kymionis et al., 2012).

Indeed, the corneal thickness, already low in keratoconus-affected corneas, is further reduced in the epi-off cross-linking procedure due to the elimination of the epithelium. That is in addition to evaporation and to the corneal compaction from the cross-linking process on the corneal stroma. The safety limit during the Dresden protocol can be slightly extended by using hypotonic riboflavin (Hafezi et al., 2009), using other hypotonic solutions such as cooled sterile water, or stopping the procedure and closing the patient's eyes for several minutes during corneal soaking, all to temporarily swell the treated cornea to 400 μm. Nevertheless, this 400-μm limit excludes a significant group of patients from having cross-linking with the Dresden protocol.

The Dresden protocol limit can be overcome with the CF-CXL procedure, which lowers UV-A beam fluence and duration so as not to exceed the radiation safety limit of 0.35 mW/cm2 of the corneal endothelium (Caruso et al., 2016; Caruso et al., 2017; DiNezza et al., 2020). Corneas undergoing CF-CXL have the added thickness of the undisturbed corneal epithelium that holds the Ribocross® solution that also diffuses into the corneal stroma and shields the endothelium during treatment.

Although the CF-CXL protocol is briefer than the Dresden protocol, there is still some compaction of the cornea due to evaporation and due to the direct effect of the cross-linking reaction. Determining the degree of compaction from the cross-linking reaction itself apart from evaporation helps in optimizing UV-A fluence and duration of CF-CXL within the radiation limits of endothelial safety.

Here we report the results of our *ex vivo* study about CF-CXL performed in corneas with pachymetry lower than 400μ. This study separates the effect of compaction from the cross-linking chemical reaction itself as contrasted with the corneal thinning due to simple evaporation. Knowing the compaction effect of the cross-linking reaction is a help on the way to optimizing settings of programmable pachymetry-dependent time-varying UV-A cross-linkers to stay within endothelial radiation safety limits.

Corneal buttons were obtained during penetrating keratoplasty for advanced keratoconus performed at our institute. Our research adhered to the tenets of the Declaration of Helsinki. An Institutional Review Board/Ethics Committee approval was obtained (authorization number 1269). Informed consent was obtained for the use of each corneal button.

Corneal transplant cases were all in Krumeich stage III characterized by myopia and/or induced astigmatism between 8 and 10 diopters and maximum K readings exceeding 53D. and with inadequate correctable vision with either spectacles or contact lenses. Prior to corneal surgery, patients were informed of risks and benefits of the surgical choices and participated in the choice of type of surgery, principally between penetrating keratoplasty and deep anterior lamellar keratoplasty. The corneal buttons obtained were placed in Optisol and stored at our eye bank for an average of 14 days. The corneal buttons were placed in medium (Carry-C, Alchimia) for corneal deturgescence 24 h before the testing was performed. Before being used, the specimens were inspected with an optical microscope on a slide and only those with intact epithelium were chosen for the tests.

During penetrating keratoplasty the apex of each cornea was marked. Corneal buttons were 8.0 mm–8.5 mm in diameter and with minimum pachymetry between 300 and 400 μm. We examined corneal buttons with light microscopy, and chose only corneas with good transparency, normal endothelial mosaic, and endothelial count more than 2500/mm^2 for use in the cross-linking compaction study. None of the selected corneal buttons had undergone diathermic shrinking of the corneal apex during surgery. Corneas were stored in Optisol for 14 days.

Corneas not showing any significant morphologic change were mounted using a modified Franz-type diffusion cell for the experiment.

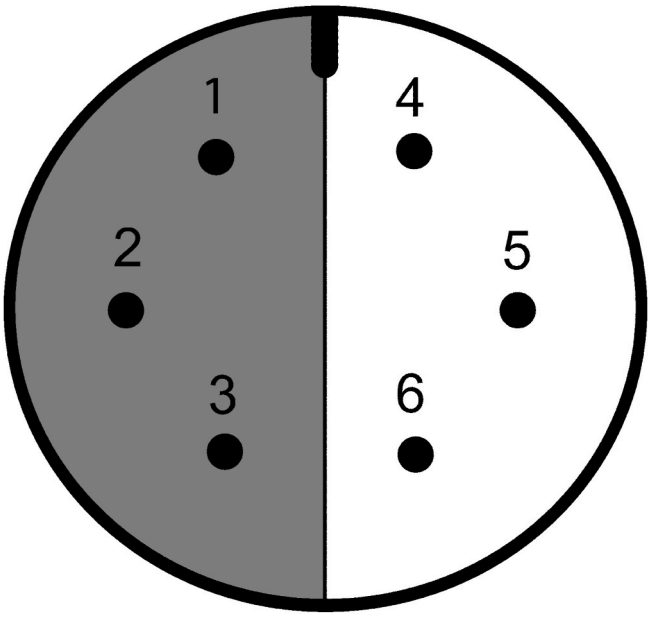

Fig. 1. Diagram showing the shielding and marking of each cornea.

Table 1
Compaction of corneal buttons in cross-linking.

Number of Corneal Buttons Studied			20
Number of Measurement Points			120
Pachymetry (3-point average in microns) on cross-linked side			
Pre Treatment		Avg± SD	328.6± 23.3
Post Treatment		Avg± SD	308.1± 21.2
Sample by sample pachymetry change (percent)			−6.19% ±2.24%
Pachymetry (3-point average in microns) on shielded side			
Pre Treatment		Avg± SD	329.1±22.8
Post Treatment		Avg± SD	327.7± 22.9
Sample by sample pachymetry change (percent)			−0.43% ±0.39%
Maximal change in 3-point average pachymetry among all samples			
Shielded side			−3.3 (−1.06%)
Cross-linked side			−40.6 (−11.7%)
Percent pachymetry change by position			
	Shielded side		Cross-linked side
1	−0.51% ±0.46%	4	−5.35% ±4.28%
2	−0.45% ±0.57%	5	−5.96% ±2.91%
3	−0.33%+/− ±0.53%	6	−7.26% ±3.73%

They were oriented with the inferior, thinner side (nearest to the marked apex of the corneal cone) corresponding to the bottom.

After assuring that the corneal epithelium was intact, six points were marked with blue ink on the surface at a radius of 3 mm from the center of the button equidistantly, three on the left side (numbers 1,2,3) and three on the right side (numbers 4,5,6) (Fig. 1). We performed ultrasonic pachymetry at every point using a 5-μm resolution pachymetry. From among the corneal buttons we obtained, we chose the 20 samples with the most homogenous thickness measuring between 300 and 400 μm for the study.

With corneal epithelium intact, we soaked the anterior surface of each corneal button with a solution of Ribocross®. One drop was delivered to the corneal epithelial surface every 15 s for 15 min. The corneal surface was then rinsed with balanced salt solution to eliminate the pre-corneal Ribocross® film as per the CF-CXL protocol. Corneal pachymetry was measured at all six points just prior to surface rinsing. We shielded half of the corneal surface with an opaque shield to cover three measurement points (1,2,3) and then exposed the buttons to UV-A radiation. The shields covered but did not touch the corneal epithelium so as not to block corneal evaporation but did give a sharp delineation between the irradiated and shielded corneal areas. The UV-A exposure

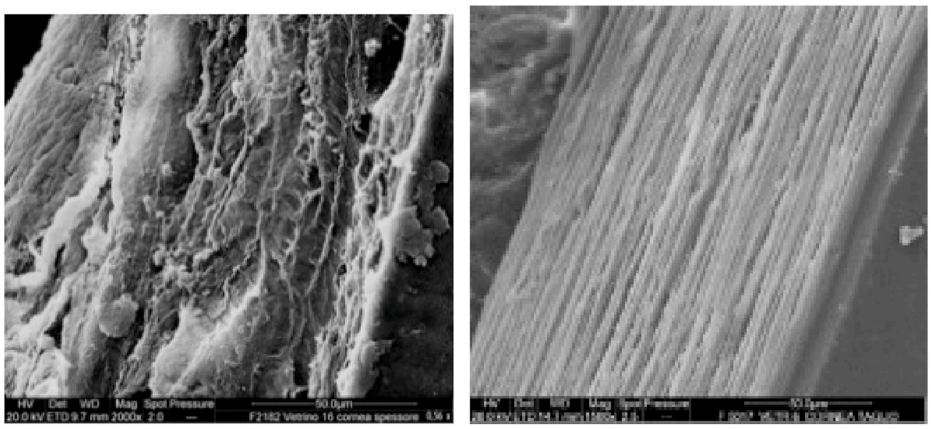

Fig. 2. Scanning electron microscopic examination reveals a compaction and increased organization of collagen fibers at right (post cross-linking) as compared to the appearance of the non-irradiated areas of the same corneal buttons shown at left.

time and intensity were calculated automatically by the CF-X Linker (IROMED Group S. r.l., Rome, Italy) based on the pre-treatment minimal pachymetry of the corneal button. For the experiment, the beam width was set to cover the entire corneal button. Exposure had an average intensity of 1.8 ± 0.9 mW/cm2 for 10 ± 1.5 min (total energy 1.08 ± 0.6 J/cm2. Because of the shielding, only half of each cornea, that involving marked areas 4,5, and 6, was cross-linked. Fig. 1 demonstrates the configuration of the marks on the cornea. No fluid was placed upon the corneas during UV irradiation. After irradiation, the thickness of all six corneal points were measured again with ultrasonic pachymetry.

The percent changes in pachymetry in both the shielded side of the corneas and the UV-irradiated side as well as at each of the six points are shown in Table 1. We found no significant relationship ($P = 0.25$) between pre-treatment corneal thickness within the 300 μm to 400 μm range pachymetry of the selected corneal buttons and the percent compaction during the period of UV-A irradiation.

The UV-A exposed areas showed a significant reduction in thickness compared to the same pre-treatment areas (average thickness change of -20.5 ± 7.9μ or $-6.2 \pm 2.2\%$, $P = 0.006$). During the 10 min of UV-A irradiation the UV-A unexposed areas showed a minimal reduction in thickness compared to the same pre-treatment areas (average pachymetry change -1.4 ± 1.12μ, $-0.43\% \pm 0.38\%$ $P = 0.10$). There were a total of 120 test spots among the corneal buttons.

It is reasonable to believe that this extra compaction of the irradiated corneas was due to the cross-linking reaction. The average percentage change in pachymetry of the irradiated side was −6.2% with 95% of the samples decreasing less than 10% in thickness (100% within 12%). With the experiments having taken place at room temperature and the surface irrigation of the corneal surfaces having taken place after post-Ribocross® soaking pachymetry, the net corneal evaporation was minimal. The change in thickness on the irradiated side we believe was nearly all due to the chemical reaction of cross-linking. Based on the current *ex vivo* study we estimate that the chemical reaction of corneal cross-linking produced an average compaction of 5.8% (a change of −5.8% in thickness).

We also studied in all samples the scanning electron microscopy of corneal epithelium, stroma and endothelium and found stromal fiber contraction and an increase in fiber diameter in the stroma. Photos appear in Fig. 2. Also, there was slight damage to the cell nuclei and inter-cellular tight junctions in the UV-exposed epithelium. There was normal appearance of the endothelium with no cell loss.

The CF-CXL protocol involves UV-A fluence and duration calculated based on corneal pachymetry. It is safe to cross-link corneas using the CF-CXL protocol in cases where minimal pachymetry is below the 400-μm Dresden protocol limit. The revised safety limit of UV-A fluence and duration of UV-A irradiation for thin corneas has already been studied for CF-CXL[5]. The graph in that article shows that the prescribed UV-A fluence for those very thin corneas is less than, and the duration is actually shorter than, for thicker corneas.

The epithelium-on technique of CF-CXL adds the thickness of the epithelium to the treated cornea as compared to having the epithelium removed in the Dresden protocol. The epithelium absorbs and temporarily holds riboflavin that usefully and predictably attenuates the UV-A radiation and tends to protect the corneal endothelium.

Washing away the pre-corneal film of riboflavin-vitamin E TPGS before UV-A irradiation is an important step. Washing the surface eliminates the problem in the Dresden protocol of variable stromal irradiation penetration due to variations in the thickness of the riboflavin film on the corneal surface. For Dresden protocol cross-linking, there is the need for continuous monitoring of pachymetry and sometimes stopping to add a second solution to thicken the cornea.

Our *ex vivo* study helps to inform an improvement in the CF-CXL algorithm. With data from our *ex vivo* study combined with a separate study on evaporation thinning and then clinical validation we hope to eliminate the need for the cumbersome step during cross-linking of intraoperative ultrasonic pachymetry during UV-A irradiation.

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