Original article

Early confocal microscopy findings after cross-linking treatment

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A B S T R A C T

Objective: To determine the effects of in vivo cross-linking treatment of the cornea.
Methods: Eighteen eyes of eighteen keratoconus patients underwent cross-linking treatment using a 0.1% riboflavin solution and ultraviolet A radiation at 370 nm at 3 mW/cm² for 30 min. In vivo confocal microscopy was performed before, and at 1 week and 1 month after treatment.
Results: At 1 week after treatment, keratocyte activation and collagen fiber organization showed as hyper-reflective structures and were observed from the first sub-epithelial image to a corneal stromal depth of 275.1 ± 85.9 μm. At 1 month after treatment, activated keratocytes and fiber organization were also observed from the first sub-epithelial image to a corneal stromal depth of 324.9 ± 66.0 μm. The deepest hyper-reflective structures at 1 month showed as thick, linear-shaped hyper-reflective structures.
Conclusion: In vivo confocal microscopy in humans showed corneal stromal changes at 1 week and 1 month after cross-linking treatment, in some cases at depths in excess of 300 μm.

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Hallazgos tempranos por microscopia confocal en cross-linking

R E S U M E N

Objetivo: Valorar in vivo los efectos en la córnea del tratamiento con cross-linking.
Métodos: Diecinueve ojos de 18 pacientes con queratocono fueron tratados con cross-linking mediante el uso de solución de riboflavina al 0,1% y radiados con luz ultravioleta A radiados a 370 nm, 3 mW/cm² por 30 min. Se realizó microscopia confocal in vivo previo al tratamiento y a la semana y al mes después de la aplicación de cross-linking.
Resultados: A la semana del tratamiento se encontró activación de queratocitos y organización de las fibras de colágena, observándose como estructuras hiperreflejantes desde la primera imagen subepitelial, hasta una profundidad del estroma corneal de

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275.1 ± 85.9 μm. Al mes del tratamiento se observaron queratocitos activados, así como organización de las fibras de colágeno desde la primera imagen subepitelial, hasta una profundidad del estroma corneal de 324.9 ± 66.0 μm. Al mes del tratamiento, las estructuras hiperreflectivas más profundas se mostraron en forma de líneas gruesas hiperreflectivas.

Conclusions: La microscopia confocal in vivo en humanos tratados con cross-linking mostró cambios estromales a la semana y al mes del tratamiento, excediendo la profundidad de 300 μm en algunos casos.

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**Introduction**

Recent studies have reported successful reticulation through establishing bridges in collagen fibers (cross-links) in the stromal tissue of the cornea to produce hardening able to improve stability in corneal ectatic disorders such as keratoconus or refractive post-surgery ectasia. The cross-linking procedure is carried out applying rivoflavin drops in the corneal stroma, producing a photo stimulation effect in response to subsequent ultraviolet A light radiation directly over the corneal stromal tissue.

This study assessed the in vivo effect of cross-linking treatment in the corneal stroma of patients with keratoconus by means of confocal microscope.

**Materials and methods**

The inclusion criteria were: keratoconus diagnostic with keratometry exceeding 45 D and/or difference between the inferior and superior curvature above 1 D and documented progression of keratoconus covering at least 6 months, pachymetry of at least 400 μm at the thinnest point and age between 18 and 60. Pregnant patients and those having a viral keratitis history due to herpes simplex, severe dry eye, concurrent ocular infection, self-immune disease and any ocular surgery were excluded. Overall, the study comprised 18 eyes of 18 patients with a mean age of 28.8 ± 5.4 (range 21–38), all with keratocone diagnostic based on the Amsler-Krumeich keratocone severity classification, 12 eyes in grades 2 and 6 eyes in grades 3. Patients in grade 1 or 4 were not included in the study. The study patients underwent cross-linking treatment with previous topical anesthesia, removing 10 mm of the central corneal epithelium in the shape of a disk. Photo stimulation was performed applying 0.1% rivoflavin solution with 20% dextran (Medio-cross; Kronen-Apotheke, Germany) on the corneal stroma surface during 15 min. prior to exposure to A ultraviolet light lamp (UV-X Illumination System 1000; IROC, Zürich, Switzerland) and every 15 min during the ultraviolet radiation which was performed with an energy of 3 mW/cm² and at a distance of 1 cm of the corneal stromal surface during 30 min. A therapeutic contact lens was placed (Pure Vision; Bausch & Lomb, Rochester, Nueva York, USA) at the end of the procedure, which was withdrawn at day 5. Treatment was carried out with 0.3% topical tobramycin (Tobrex; Alcon Laboratories, Fort Worth, USA) at the rate of 3 times a day during one week. After re-epithelization of the cornea, 0.1% topical fluoromethalone was prescribed (Flarex; Alcon Laboratories, Fort Worth, USA) 4 times a day during one week and twice a day the following week.

In what concerns in vivo confocal microscopy, all the corneas treated with cross-linking were assessed prior to the procedure and one week and one month post-surgery utilizing a slit confocal microscope (Confoscan 4; Fortune Technologies, Vigonza, Italy). In each confocal microscopy assessment a sequence of digital images (JPEG) was obtained, comprising 2 consecutive scans of the corneal thickness total depth, with a scan being equivalent to obtaining images from endothelium to epithelium and back to endothelium, i.e., from posterior to anterior and back to posterior, in order to allow the displacement of the central corneal thickness “Z” axis. The Z ring device was utilized (Confoscan, Fortune Technologies, Vigonza, Italy), which maintains contact with the cornea in order to obtain reliable thickness measurements free of ocular globe anterior–posterior movements.

Said images were captured automatically in the hard disk (CPU) of a computer for subsequent analysis utilizing the NIDEK v. 3.5.0 software (NIDEK, Multi-Instrument Diagnostic System, Gamagori, Japan).

**Results**

In all patients, corneal epithelium regeneration was completed under therapeutic contact lenses 4 days after the procedure. Presurgery confocal microscopy images revealed normal stromal structures in all patients. The corneal epithelium was normal in all patients both after one week and one month after the procedure. The subepithelial nerves plexus was absent in all the studied corneas one week and one month post-surgery. One week after the procedure, all patients exhibited keratocyte activation and collagen fiber organization, shown as hyper-reflecting structures (Fig. 1), from the first subepithelial image up to a mean depth of 275.1 ± 85.9 μm, representing 64.6% (range of 35.5–96.5%) of the overall stromal thickness of the cornea. One month after the procedure, keratocyte activation and collagen fibers organization continued to be observed in all patients from the first subepithelial image (Fig. 2) up to a mean depth of 324.9 ± 66.0 μm, representing 77.0% (range of 55.1–95.3%) of the overall stromal thickness of the cornea. The deepest stromal effect of the cross-linking treatment one month after surgery (at a
stromal depth of 261 ± 50.0 μm) was observed in the form of hyper-reflecting thick lines in all cases (Fig. 3). Below these hyper-reflecting structures the cellular arrangement of the corneal stroma was normal as well as that of the corneal endothelium in all cases after the cross-linking treatment (Figs. 4 and 5).

Discussion

The possibility of using rivoflavin and ultraviolet radiation to induce bridges between collagen fibers (cross-links) and thus increase rigidity to regulate the progression of corneal ectasia has been recently demonstrated.

Corneal epithelium regeneration 4–5 days after surgery, as is the case of photorefractive keratectomy (PRK), is already abundantly documented and has been confirmed by means of confocal microscopy 5 days after a cross-linking procedure. The absence of subepithelial nerves plexus after corneal surgery has been reported in photorefractive surgery (PRK) as well as in lamellar procedures such as LASIK; in both cases, said nerves plexus recovers fully between 4 and 6 months after the surgical procedure.

Stromal changes such as keratocyte apoptosis become evident in the first 24 h after the cross-linking procedure and histological studies have shown significant increases in collagen fiber diameters as well as marked organization thereof in the anterior half of the corneal stroma.

After a cross-linking procedure on human corneas, confocal microscopy has been utilized to report reduction of stromal keratocytes in the anterior and intermediate portion of the cornea, followed by gradual repopulation up to

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**Fig. 1** – Confocal microscopy image of the first subepithelial image of the corneal stroma one week after cross-linking treatment (340 μm x 255 μm).

**Fig. 2** – Confocal microscopy image of the first subepithelial image of the corneal stroma one month after cross-linking treatment (340 μm x 255 μm).

**Fig. 3** – Confocal microscopy image of the corneal stroma at a depth of 290 μm one month after cross-linking treatment (340 μm x 255 μm).

**Fig. 4** – Confocal microscopy image of the corneal stroma at a depth of 380 μm one month after cross-linking treatment (340 μm x 255 μm).
6 months after the procedure. In a similar study, Dhaliwal and Kaufman also applied confocal microscopy and reported the appearance of hyper-reflective structures at a depth exceeding 300 μm. This study also found hyper-reflective structures in the anterior stroma also at a depth exceeding 300 microns, representing 64.6% (range of 35.5–96.5%) and 77.0% (range of 55.1–95.3%) of the overall corneal stromal thickness one week and one month, respectively, after surgery. We observed that the cross-linking effect on the cornea can be evident at a depth exceeding 300 μm in some cases, which could be noteworthy due to the effects it could have on the underlying corneal endothelium. One month after the procedure, hyper-reflective structures were observed in the form of thick lines at a depth of 261 ± 50 μm. Mazzotta et al. also reported extracellular matrix density increases in the late post-surgery as well as cross-linking effects at the depth of 340 μm, observed a separating line in the posterior stroma. Possibly, the thick hyper-reflective linear structures reported in the study are at the same depth as those reported by Mazzotta et al. and therefore these linear structures represent an increase in the density of extracellular matter which has also been reported by means of confocal microscopy.

At depths below said hyper-reflective structures the stromal cellular morphology was normal in all the studied corneas. Similarly, corneal endothelium appeared without changes when compared against presurgery images.

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**Conflict of interests**

No conflict of interests has been declared by the authors.

**References**

with riboflavin and ultraviolet A. J Refract Surg.